Function of the ATP-dependent metalloprotease FtsH during sporulation in *Bacillus subtilis*

Dissertation

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

-Dr. rer. nat.-

der Fakultät für Biologie, Chemie und

Geowissenschaften der Universität Bayreuth

vorgelegt von

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Bayreuth 2008

Die vorliegende Arbeit wurde in der Zeit von Januar 2005 bis Oktober 2007 an der Universität Bayreuth am Lehrstuhl für Genetik unter der Betreuung von Prof. Dr. Wolfgang Schumann angefertigt.

Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.).

Promotionsgesuch eingereicht am: 09.01.2008
Tag des wissenschaftlichen Kolloquiums: 14.03.2008

1. Gutachter: Prof. Dr. Wolfgang Schumann
2. Gutachter: Prof. Dr. Franz X. Schmid

Acknowledgements

This thesis was carried out in the group of Prof. Dr. Wolfgang Schumann at the Department of Genetics, University of Bayreuth.

I would like to express my special gratitude to my supervisor Prof. Dr. Wolfgang Schumann who shared with me a lot of his expertise and research insight. I appreciate his excellent direction and useful comments on this work.

I would like to thank PD. Dr. Thomas Wiegert for his helpful comments throughout this process.

Thanks to Karin Angermann for the technical discussions and her kindness.

I wish to thank all my friends and my country-mates in Bayreuth for inestimable friendship. Special thanks to Hue Nguyen, one of my great friends, for her assistance on the ministerial computer.

My most sincere thanks for the financial support of the Deutsche Forschungs-gemeinschaft (Schu 414/20-2) and the EU (LSGH-CT2004-503468).

Last but not least, I am forever indebted to my parents, my husband and my daughter for their understanding, endless patience and encouragement when it was most required.

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I. Summary

The analysis of the function of the *ftsH* gene of *Bacillus subtilis* started about ten years ago. It was shown at that time that an *ftsH* knockout was viable, but exhibited a pleiotropic phenotype. Cells are sensitive to salt and heat shock, exhibit filamentous growth, are difficult to be transformed and are almost unable to sporulate. Despite the severe phenotype caused by the absence of the *ftsH* gene, the precise functions of this protein remained unclear. This PhD thesis presents data to elucidate the function of *ftsH* during sporulation. Furthermore, it describes the construction of a cold-inducible expression system.

The major finding of this thesis is that the FtsH protease interferes with the synthesis and/or phosphorylation of Spo0A, the master regulator during initiation of sporulation called phase 0. In the *ftsH* knockout, the amount of Spo0A is greatly reduced, and the small amounts present are inactive. When the wild-type *spo0A* allele was replaced by an IPTG-inducible allele coding for mutant Spo0A protein being fully active in the absence of phosphorylation (Spo0A-Sad67), spores were formed at a normal rate in an *ftsH* knockout. Again, this result indicates that FtsH is clearly involved in the formation of active Spo0A and that this protease is only essential during stage 0 of sporulation. To become active, Spo0A needs to be phosphorylated by the multi-component system called phosphorelay. Since no active Spo0A is present in an *ftsH* knockout, it was hypothesized that FtsH has to degrade one or more negative regulator(s) either preventing the phosphorylation of Spo0A or/and being involving in its rapid dephosphorylation. The further analysis focused on four antagonists of the phosphorelay, three Rap phosphatases being involved in the dephosphorylation of Spo0F~P, and Spo0E which targets Spo0A~P. When a null allele in any one of them was combined with the *ftsH* knockout, the wild-type amount of Spo0A was restored only in the case of the *ftsH spo0E* knockout and the sporulation frequency was increased by two to three orders of magnitude in all double knockouts, but remained below 1%. Since overexpression of Spo0E reduces the sporulation frequency and removal of the gene from the genome has an opposite effect, a direct interaction between FtsH and Spo0E was envisaged. *In vitro* proteolysis assays with purified GST-FtsH and GST-Spo0E showed that Spo0E is indeed a target of FtsH. In contrast, the two homologs of Spo0E, YisI and YnzD, remained stable upon incubation with FtsH. Since all three proteins are distinguished by a C-terminal extension of about 25 amino acids present in Spo0E, but not in the two other phosphatases, these additional amino acids could serve as a target for FtsH. When two mutant versions of Spo0E, Spo0E94 and Spo0E11, with truncated C-terminal ends were

analyzed, they turned out to be stable in the presence of FtsH. When the C-terminal 25 amino acids was transferred to YnzD, this fusion protein became unstable when incubated with FtsH. In conclusion, the C-terminal end of Spo0E confers instability to this enzyme. Since a *spo0E* knockout in a wild-type background does not result in a sporulation frequency close to 100% and a combination of a *spo0E* and an *ftsH* knockout raises the sporulation frequency only close to 1%, it can be concluded that there are additional targets for FtsH interfering with the synthesis of active Spo0A.

Moreover, it is likely that FtsH also exerts a function late during sporulation. It could be shown that SpoVM, a small peptide essential for spore morphogenesis, inhibits the proteolytic activity of the *B. subtilis* FtsH protease *in vitro*. It can be inferred that SpoVM also inhibits activity of FtsH during sporulation, and in the absence of SpoVM, FtsH will degrade at least one protein essential for successful completion of sporulation. When the intracellular proteomes of *spoVM*$^+$ and *spoVM*$^-$ cells were compared, a total of 83 proteins were identified being either completely absent or present in reduced amounts in the absence of the peptide.

Analysis of the expression of the *spoVM* gene revealed that cells started to synthesize the *spoVM* transcript at stage 2 while the SpoVM peptide accumulated at stage 4. The 5´ untranslated region of the *spoVM* transcript has been identified to act as a negative regulator of its own transcription or translation.

Furthermore, a cold-inducible expression system has been constructed allowing intra- and extracellular production of recombinant proteins. This expression system makes use of a two-component signal transduction system, which senses changes in the fluidity of the cytoplasmic membrane.

I. Zusammenfassung

Die Analyse der Funktion des *ftsH*-Gens von *Bacillus subtilis* begann vor etwa 10 Jahren. Damals konnte gezeigt werden, dass eine *ftsH* Knockout-Mutante lebensfähig ist, aber über einen pleiotropen Phänotyp verfügt. Die Zellen sind Salz- und Hitze-sensitiv, wachsen filamentös, sind schwierig zu transformieren und zeigen eine stark reduzierte Sporulationsfrequenz. Trotz dieser gravierenden Phänotypen blieb die Funktion von *ftsH* bislang im Dunkeln. Diese Doktorarbeit präsentiert Daten, die einige Funktionen von *ftsH* während der Sporulation aufdecken. Außerdem wird die Konstruktion eines Kälte-induzierbaren Expressionssystems beschrieben.

Das besondere Ergebnis dieser Dissertation ist der Befund, dass die FtsH Protease mit der Synthese und/oder der Phosphorylierung von Spo0A, dem Master-Regulator während der Initiation, der Phase 0, interferiert. In einer *ftsH*-Knockout ist die Menge an Spo0A signifikant reduziert und die geringen Mengen sind inaktiv. Wenn das Wildtyp-Allel von *spo0A* durch ein IPTG-induzierbares Allel ersetzt wurde, welches für ein mutantes Protein codiert, das auch in Abwesenheit von Phosphorylierung voll aktiv ist (Spo0A-Sad67), dann wurde eine Sporulationsfrequenz gemessen, die der von Wildtyp-Zellen entsprach. Aus diesem Ergebnis ist zu folgern, dass *ftsH* nur während der Phase 0 essentiell ist. Um Aktivität zu erlangen, muss Spo0A phosphoryliert werde, und dies geschieht durch ein Phosphorelay. Da in einer *ftsH*-Mutante kein aktives Spo0A nachweisbar ist, ist zu vermuten, dass FtsH einen oder mehrere negative Regulatoren abbauen muss, die entweder die Phosphorylierung von Spo0A verhindern oder an einer schnellen Dephosphorylierung beteiligt sind. Die weitere Analyse konzentrierte sich auf vier verschiedene Antagonisten des Phosphorelays, drei Rap Phosphatasen, die Spo0F~P dephosphorylieren und Spo0E, welche Spo0A~P dephosphoryliert. Wenn Null-Allele der vier Phosphatasen mit einer *ftsH*-Knockout kombiniert wurden, dann wurde nur im Fall von Δ*spo0E* Wildtyp-Mengen an Spo0A detektiert. Die Sporulationsfrequenz wurde in allen vier Stämmen um 2-3 Größenordnungen erhöht gegenüber der *ftsH*-Knockout, blieb aber in allen Fällen unter 1%. Da eine Überexpression von Spo0E die Sporulationsfrequenz reduziert und ein *spo0E*-Knockout den gegenteiligen Effekt hat, wurde eine direkte Interaktion zwischen FtsH und Spo0E in Betracht gezogen. *In vitro* Proteolysetests mit gereinigtem GST-FtsH und GST-Spo0E ergaben, dass Spo0E abgebaut wird. Im Gegensatz dazu erwiesen sich zwei Homologe von Spo0E, YisI und YnzD, als stabil. Da alle drei Phosphatasen sich nur in ihren N-Termini unterscheiden und nur Spo0E einen um etwa 25 Aminosäurereste verlängerten C-Terminus enthält, war zu vermuten, dass dieser Anhang von FtsH als Target erkannt wird. Wenn zwei

mutante Versionen von Spo0E, SpoE94 und Spo0E11, mit FtsH inkubiert wurden, erwiesen sie sich als stabil. Beide Versionen haben einen verkürzten C-Terminus. Nach Transfer des C-Terminus von Spo0E auf YnzD erwies sich dieses Fusionsprotein als instabil. Zusammengefasst ist festzustellen, dass der C-terminus von Spo0E für die Instabilität von Spo0E gegenüber FtsH verantwortlich ist. Da die Sporulationsfrequenz in einer *spo0E*-Knockout nicht 100% beträgt, und sie in einer *spo0E ftsH* Doppelknockout nur bei knapp 1% liegt, muss FtsH zusätzliche Targets erkennen, die die Synthese von aktivem Spo0A negativ beeinflussen.

Darüber hinaus übt FtsH einen Einfluss in einer späten Phase der Sporulation aus. Es konnte im verlauf der Dissertation gezeigt werden, dass SpoVM, eine kleines essentielles Peptid für die Sporen-Morphogenese, die proteolytische Aktivität der FtsH-Protease *in vitro* inhibiert. Daher kann davon ausgegangen werden, dass SpoVM diese Protease auch während der Sporulation hemmt und in seiner Abwesenheit FtsH eines oder mehrere Proteine abbaut, die für einen erfolgreichen Abschluss der Sporulation notwendig sind. Wenn die intrazellulären Proteome von *spoVM*$^{+}$ und *spoVM*$^{-}$-Zellen verglichen wurden, dann konnten insgesamt 83 Proteine identifiziert werden, die in der *spoVM*-Knockout entweder völlig fehlten oder in reduzierten Mengen synthetisiert wurden.

Eine Analyse der Expression des *spoVM*-Gens ergab, dass das *spoVM*-Transkript bereits in der Phase 2 nachzuweisen war, während das Peptid erst in der Phase 4 erschien. Es konnte eine 5' nicht-translatierte Region identifiziert werden, die als negativer Regulator der eigenen Transkription oder Translation fungiert.

Außerdem wurde ein Kälte-induzierbares Expressionssystem konstruiert, welches die intra- und extrazelluläre Produktion rekombinanter Proteine erlaubt. Dieses Expressionssystem basiert auf einem 2-Komponenten Signaltransduktionssystem, welches Änderungen in der Fluidität der cytoplasmatischen Membran wahrnimmt.

2. Introduction

Throughout evolution, cells have employed a class of proteins, the ATP-dependent proteases, to control a variety of critical cellular functions. These intracellular proteases govern such diverse cellular processes as cell division, replication, DNA repair, signal transduction and stress responses. Remarkably, a single protease can be responsible for controlling several of these disparate processes, pointing out the importance of regulated proteolysis which requires a protease to degrade specific targets at an appropriate time.

2.1. FtsH - an ATP-dependent membrane protease

2.1.1. Structure and function

FtsH is a membrane-anchored metallo-protease that functions as a master regulator in *Escherichia coli* (Ito *et al.,* 2005). In addition to its two transmembrane segments, FtsH has a large cytoplasmic region that includes two functional domains (Fig. 1) (Tomoyasu *et al.*, 1993a). The first is a peptidase domain, which includes a zinc-binding motif, HEXXH, which is important for catalyzing the cleavage of bound polypeptides (Tomoyasu *et al.*, 1993b). The second is an ATPase domain, also termed the AAA module that is found in a family of well-conserved proteins, called the “AAA+” family (ATPases associated with diverse cellular activities) (Tomoyasu *et al.*, 1993b). The characteristics of this AAA module are the presence of a segment called SRH (Second Region of Homology) and the Walker A and B motifs, which are responsible for nucleotide binding and hydrolysis (Ogura and Wilkinson, 2001) and might be involved in the dissociation or assembly of protein complex (Confalonieri and Duguet, 1995). The

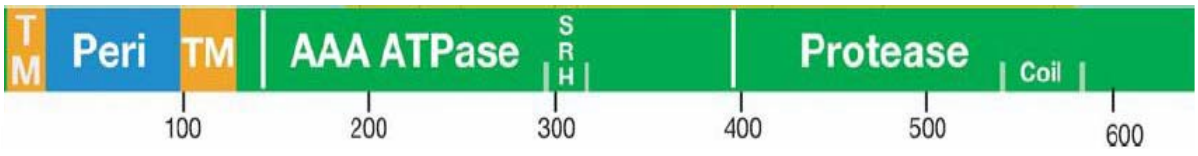

FIG. 1. Sequence characteristics of FtsH. The cytoplasmic, transmembrane (TM), and periplasmic regions of FtsH are colored in green, brown and blue, respectively. The main cytoplasmic region consists of the AAA-ATPase and the Zn^{2+}-metalloprotease domains as indicated, which include the second region of homology (SRH) and an α-helical coiled-coil region (Coil), respectively **(Ito and Akiyama, 2005)**.

AAA+ family of proteins is implicated in a number of essential biological processes including secretion, vesicle-mediated transport, replication, cell division, respiration, and proteolysis (Ogura and Wilkinson, 2001). The mechanism of action of the AAA module is still subject to speculation, and elucidating its role might provide insights into what common themes are shared by these diverse biological processes.

In prokaryotes, *ftsH* gene was first discovered in *E. coli* called *hflB* (*hfl* stand for high frequency of lysogenization) (Belfort and Wulff, 1974) and one year later as *ftsH* (filamentation temperature-sensitive) (Santos and Almeida, 1975). Subsequently, genes that could encode homologous proteins have been identified in *Lactococcus lactis* (Nilsson *et al*., 1994), in *B. subtilis* through an insertion mutant unable to grow under hyperosmotic condition (Geisler and Schumann, 1993) and within *Haemophilus influenzae* and *Mycoplasma genitalium* as part of their genome sequencing projects (Fleischmann *et al.,* 1995; Fraser *et al.,* 1995). The homology shared between the five prokaryotic FtsH proteins is that all contain two hydrophobic segments at the N-terminus and a conserved C-terminal domain which is similar to the active site motif of zinc-metalloproteases (Lysenko *et al*., 1997).

Crystal structures of the AAA module FtsH homologs from *E. coli* and *Thermus thermophilus* have been resolved at the atomic level (Niwa *et al.,* 2002; Krzywda *et al.,* 2002). Modeling of these structures suggests that FtsH forms a hexameric ring structure **(Fig. 2)** similar to those of the NSF (N-ethyl maleimide-sensitive factor) and p97 (valosin-containing protein) proteins, which are involved in membrane fusion and membrane protein degradation in eukaryotes, respectively (Jarosch *et al*., 2002; Woodman *et al*., 2003). The conserved features of AAA modules imply a common mechanism for operation. Topological modeling predicts that FtsH forms a barrel-like structure in which the proteolytic active site is buried inside a cavity (Niwa *et al.,* 2002). Substrates are predicted to access the proteolytic chamber through a narrow pore of insufficient width to allow the passage of globular proteins. Therefore, only unfolded substrate proteins can enter the proteolytic chamber. The pore, located at the apical domain of the barrel, is proposed to face the membrane.

2.1.2. Alteration of FtsH has pleiotropic effects on cell physiology

FtsH function is crucial in organisms as diverse as bacteria, chloroplasts and human. Mutations that impair the function of the human mitochondrial FtsH orthologue result in increased cellular sensitivity to oxidative stress (Atorino *et al*., 2003; Ito *et al*., 2005). Complete inactivation of this human orthologue causes neuro degeneration in hereditary spastic paraplegia, a disease characterized by a slow paralysis of the lower limbs (Casari *et al*., 1998) and alteration of the FtsH protease in plants results in leaf

variegation and perturbation of photosynthesis. These mutant phenotypes indicate the important physiological role of FtsH orthologues in higher organisms.

Among bacteria, FtsH is the only universally conserved ATP-dependent protease (Karzai *et al*., 2000). It is also the only ATP-dependent protease that is essential for growth in *E. coli*. However, the presence of a second site suppressor that permits

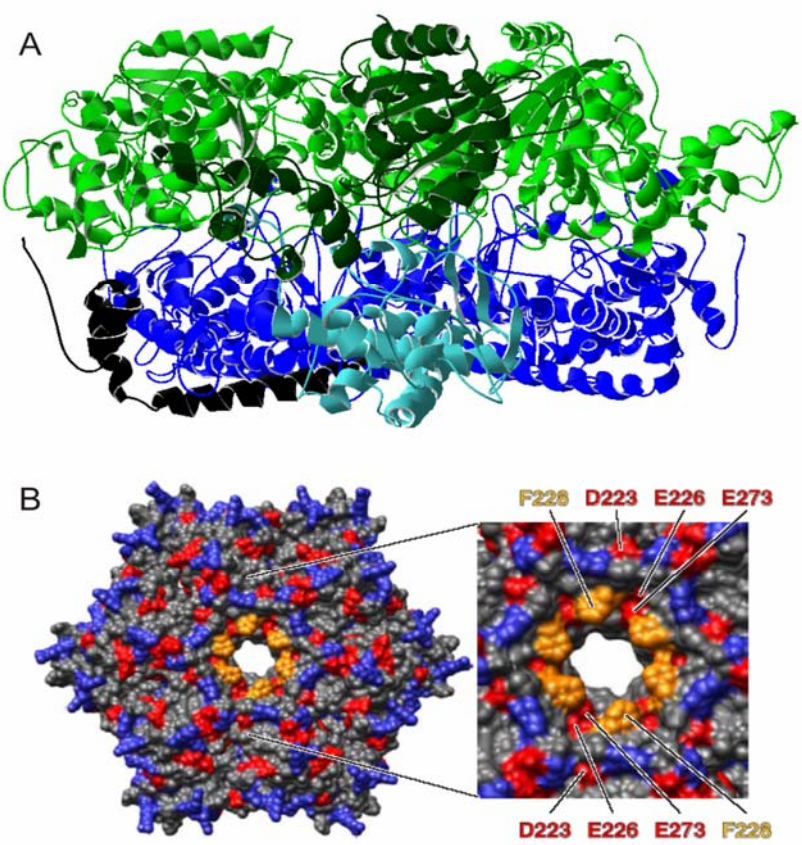

FIG. 2. Structure of the FtsH protease. (A) Hexameric ribbon cartoon of the soluble part of FtsH from *Thermus thermophilus*. The ATPase domain is shown in green with one subunit highlighted in dark green. The protease domain is shown in blue with one subunit highlighted in pale blue to point out the hexameric structure of FtsH. The coiled-coil region of one FtsH subunit is shown in black. The model was created with the Swiss-PdbViewer3.7 (Guex and Peitsch, 1997). (B) Top view on the surface of the ATPase domain of *E. coli* FtsH. Phe228 is colored orange, acidic residues are colored red, basic residues are colored blue, and others are colored gray. The detailed view highlights the entrance gate of the pore, Phe228 and three acidic residues important for proteolysis of RpoH and LpxC are indicated **(Okuno *et al*., 2006)**.

viability has allowed an *E. coli* strain carrying a chromosomal deletion of *ftsH* to be characterized. Inactivation of FtsH results in the lethal accumulation of LPS (lipopolysaccharide) due to stabilization of an enzyme, LpxC, which diverts a precursor of phospholipids into the LPS pathway (Ogura *et al*., 1999). In the absence of FtsH, the synthesis of phospholipids is diminished, resulting in cell death. The suppressor mutation of Δ*ftsH* (*sfhC*21), which localizes to the *fabZ* gene, counterbalances LPS accumulation by restoring a normal proportion to the synthesis rates of phospholipids and LPS. Thus, FtsH plays a critical role in membrane biogenesis by maintaining the proper ratio of LPS/phospholipids. Interestingly, the *E. coli* Δ*ftsH* strain shows increased sensitivity to a variety of stresses (Qu *et al*., 1996). Mutations that result in partially diminished FtsH function are associated with a variety of phenotypes (Ito *et al*., 2005). These include resistance to colicin (Qu *et al*., 1996; Teff *et al*., 2000; Makino *et al*., 1997), stabilization of mRNA (Wang *et al*., 1998), mis-insertion of membrane proteins (Akiyama *et al*., 1994ab), defect in cell division (Tomoyasu *et al*., 1993a) and alteration of phage developmental decisions (Herman *et al*., 1993). The wide-ranging effects of FtsH mutants are attributable to its unique role as a protease with both housekeeping and regulatory functions employed in degrading both cytosolic and integral membrane proteins **(Fig. 3)**. The crucial housekeeping function of FtsH involves degrading unassembled membrane proteins and incompletely translated nascent cytoplasmic proteins that emerge from stalled ribosomes carrying a ubiquitin-like SsrA degradation tag (Akiyama *et al*., 1996; Herman *et al*., 1998). In conclusion, the pleiotropy associated with *ftsH* mutants has led to the suggestion that FtsH could function in some way as a chaperone, facilitating protein secretion and proteolysis.

In *B. subtilis,* initially *ftsH* mutant have been described to be sensitive to heat and salt stress. Under conditions of temperature or osmotic upshift, the *ftsH* mutant was unable to recover and maintain cell growth suggesting that FtsH may also be involved in the cellular response to stress (Geisler and Schumann, 1993; Nilsson *et al*., 1994; Deuerling *et al*., 1995). To determine general functions of FtsH, an *ftsH* mutant was constructed encoding for a truncated protein with a modified activity; this mutant protein accumulated in reduced amounts in the cytoplasmic membrane. Cells expressing the mutant protein were exhibited significant cell growth defects, failure to grow in minimal media, defective incorporation of some PBPs (Penicillin Binding Protein) into the cytoplasmic membrane and failure to differentiate under conditions of nutrient depletion (Lysenko *et al.,* 1997). The relative abundance of three PBPs was altered in the *ftsH* mutant: there were an accumulation of PBP2A and a reduction in the amounts of PBP2B and PBP4. The function of PBP4 is least understood in *E. coli* as transpeptidase cleaving peptidoglycan peptide cross-links (Korat *et al.,* 1991), but PBP2A has been implicated in the synthesis

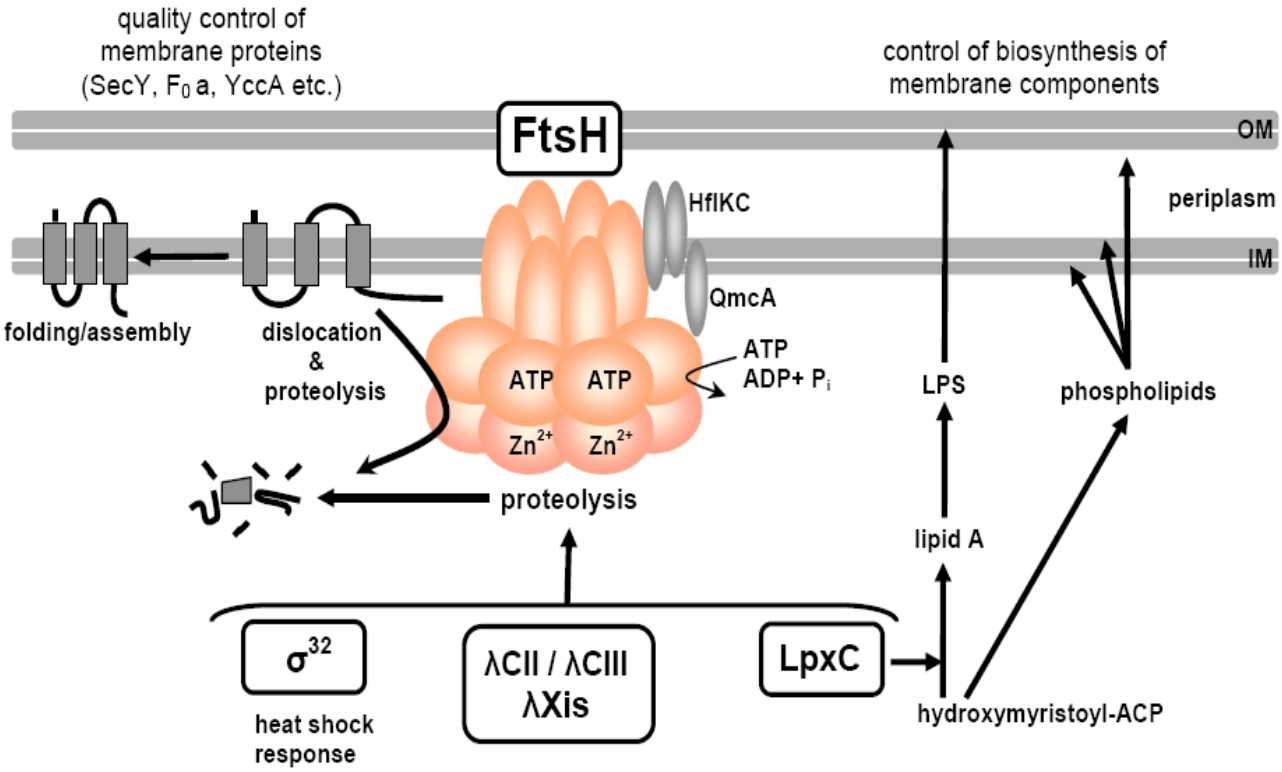

FIG. 3.The cellular functions of the protease FtsH in *E. coli.* FtsH is drawn as a hexameric ring (in red). The best studied membrane and cytosolic substrates are indicated **(Krzywda *et al.*, 2002)**.

of the cell's sidewalls (Sowell and Buchanan, 1983) and the presence of this protein in membranes declines rapidly as cells enter sporulation. In contrast, PBP2B is required for the formation of the septum during vegetative growth (Yanouri *et al*., 1993) and is also required for sporulation, where it is used for construction of the asymmetric spore septum, and synthesis of this polypeptide increases dramatically during development. One explanation for the defective PBP assembly is that FtsH is involved in the membrane incorporation of these proteins, perhaps as a chaperone. Alternatively, FtsH could play a more direct role in the translocation of the PBPs into the plasma membrane.

During sporulation, mutations in *ftsH* delayed and reduced expression of several sporulation specific developmental genes (*spoIIA*, *spoIIE*, *spoIID*, *spoIVF*, *sspE* and *gerE*) (Lysenko *et al.,* 1997; Deuerling, *et al.,* 1997) in which *spoIIA* and *spoIIE* are expressed very early during sporulation (stage 0), prior to formation of the two cell types (Beall and Lutkenhaus, 1991) and are controlled by the master regulator Spo0A. A more important observation was that expression of *spo0A* is greatly reduced in a *ftsH* mutant. It seems likely that the absence of sufficient amounts of the transcriptional regulator

Spo0A is responsible for the failure to sporulate in a *ftsH* null mutant strain (Deuerling, *et al.,* 1997). Hence, the first function of FtsH proposed is related to the initiation stages of sporulation. Another study could show that a *spoVM* mutant is blocked in expression of sigmaK-dependent genes (as expected for a mutant blocked at stage II-III). Extragenic suppressors of *spoVM* mutants mapped in the *ftsH* gene and it was evident that the 26-amino-acid SpoVM peptide could inhibit the proteolytic activity of FtsH (Cutting *et al.,* 1997).

2.1.3. Mechanism of substrate degradation by FtsH

All characterized ATP-dependent proteases exhibit multimeric ring-like structures in which the proteolytic active site is buried within a central cavity. A consequence of this configuration is that the active site is accessible only to unfolded and extended polypeptides. According to the prevailing model, proteases utilize specific degradation signals to recognize and bind substrates. Then, powered by ATP hydrolysis, they unfold these substrates and translocate them into the proteolytic chamber (Sauer *et al.,* 2004). Within this chamber, the protein is hydrolyzed to small peptides, which are released into the cytoplasm. The evidence supporting this model is derived primarily from studies of the bi-partite cytoplasmic ClpAP and ClpXP proteases (Weber-Ban *et al*., 1999; Kim *et al*., 2000; Hoskins *et al*., 2000). These Clp-family proteases exhibit robust unfoldase activity, enabling them to degrade substrate proteins with intrinsic thermostabilities (Kenniston *et al*., 2003).

A recent work indicates that FtsH operates through a different mode of degradation than these cytoplasmic proteases (Herman *et al*., 2003). In contrast to the Clp-family proteases, FtsH lacks a robust unfoldase activity which would be necessary to thread a substrate into the proteolytic cavity. It was proposed that the weak unfoldase activity allows the cell to control the rate at which FtsH degrades regulatory targets like σ^{32} and LpxC (Herman *et al*., 2003). Under appropriate conditions, it is likely that FtsH employs co-factors that accelerate the unfolding and the degradation of targets. Thus, FtsH can sense the folding state of regulatory substrates within the physiological range of the cell.

Apart from its role in regulated proteolysis, it was speculated that the weak unfoldase activity employed by FtsH may be a central feature of membrane protein degradation in general (Herman *et al*., 2003). FtsH might be able to sense the folded state of proteins, and degrade only those that exhibit low intrinsic thermostability (e.g. unassembled or misfolded membrane proteins), independently of a specific degradation signal.

2.1.4. Cytoplasmic protein degradation by FtsH

Most of the FtsH substrates thus far characterized are from *E. coli* in which soluble substrates are the most part naturally short-lived. The bacteriophage lambda protein CII is short-lived transcription factor for genes required for the establishment of lysogenization. It is rapidly degraded by FtsH *in vivo* and *in vitro* (Kihara *et al.,* 1997; Shotland *et al.,* 1997; Shotland *et al.,* 2000). The *cIII* gene product, a small membrane-interacting protein with an amphiphilic *α*-helix region, is slowly degraded by FtsH *in vivo* (Herman *et al.,* 1997), and it antagonizes the FtsH-catalyzed *in vitro* proteolysis of CII (Shotland *et al.,* 2000). Thus, the balance among these gene products is an important factor for the decision between the lytic growth and lysogenization/integration of the infecting *λ* genome (Hoyt *et al.,* 1982).

The heat shock sigma factor σ^{32} is rapidly degraded in the absence of a heat shock or other stress, with an half-life that is affected by the level of FtsH (Herman *et al.,* 1995b; Tomoyasu *et al.,* 1995) as well as by other ATP-dependent proteases (Kanemori *et al.,* 1997). In a purified reaction system, FtsH degrades σ^{32} in Zn^{2+}- and ATP-dependent manners (Okuno *et al.,* 2004, Toyomasu *et al.,* 1995). FtsH may also contribute to the degradation of another short-lived transcription factor, SoxS (Griffith *et al.,* 2004).

LpxC is the most important FtsH substrate because degradation of LpxC renders FtsH essential. Since the same reaction precursor (R-3-hydroxymyristoyl-ACP) is used by the *lpxC* (*envA*) gene-encoded deacetylase for the biosynthesis of lipid A, a LPS component, and by the *fabA* gene-encoded dehydrase for fatty acid biosynthesis, the balance of these enzymes is important to maintain a proper LPS/phospholipids ratio in *E. coli* cells. The LpxC deacetylase is short-lived (half-life of 4 min) owing to FtsH-catalyzed degradation, dysfunction of which results in the lethal over-accumulation of LPS (Ogura *et al.*, 1999). LpxC is a globular protein. Basing on the structure of LpxC from *Aquifex aeolicus* has been solved (Coggins *et al.*, 2003; Whittington *et al.*, 2003) suggesting that the final 14 residues are not structured. As the *E. coli* protein contains an extension of eleven residues, an unstructured C-terminus of 25 amino acids was predicted. *In vivo* degradation LpxC by FtsH was evident and a detailed mutational analysis revealed six non-polar residues in the C-terminus of LpxC that are critical for degradation (Führer *et al.,* 2007).

The SsrA-tag is a short sequence that is appended to the C-terminus of truncated proteins on stalled ribosomes to promote their proteolysis (Keiler *et al*., 1996). In *E. coli,* degradation occurs mainly through the ClpAP/XP proteases (Keiler *et al.*, 1996; Gottesman *et al.*, 1998), which recognize specific residues within the SsrA-tag (Flynn *et*

al., 2001). In addition, FtsH also recognizes the SsrA-tag and efficiently degrades the λCI-SsrA model substrate (Herman *et al.*, 1998) though the λCI N-terminal domain is a stable cytosolic protein. It appears that the SsrA-tag confers a relatively unspecific degradation signal to the protein at which it is attached (Herman *et al.*, 1998). It remains to be elucidated how the different proteases, ClpAP/XP and FtsH, recognize the same degradation tag.

2.1.5. Membrane protein degradation by FtsH

The major housekeeping function proposed for FtsH is the rapid removal of harmful membrane protein subunits when they fail to integrate into functional complexes (Ito *et al.*, 2005). A study of membrane degradation has been carried out with two membrane substrates, the translocase subunit SecY, and YccA, a membrane protein of unknown function. SecY is one of the major components of the translocation apparatus. It becomes a substrate for FtsH mediated degradation when it fails to assemble into a complex with its partner, SecE and SecG (Kihara *et al.*, 1995). This can occur either when SecY is overexpressed, or when the SecY-SecE interaction is weakened through a mutation (Chiba *et al.*, 2000). YccA is inherently unstable, and it is unknown whether YccA forms any higher order complexes (Kihara *et al.*, 1998). YccA is degraded by FtsH with protease recognition through its cytoplasmic tail. However, YccA associates with FtsH even when its degradation signal is absent (Kihara *et al.*, 1998). FtsH can also degrade integral membrane proteins starting from their cytoplasmic ends, provided that there is an unfolded cytoplasmic tail, of any sequence composition, that is at least 20 amino acids in length (Chiba *et al.*, 2002). Such degradation can begin at the N- or C-terminus and proceeds sequentially to the other end, apparently pulling the protein through the membrane, as the periplasmic domains are degraded in an FtsH-dependent manner (Chiba *et al.*, 2002).

Because the proteolytic active site of FtsH resides in the cytoplasm, substrates must be extracted from the lipid bilayer before entering the proteolytic cavity, a process called dislocation (Kihara *et al.*, 1999). Dislocation of membrane proteins raises the intriguing question of how hydrophilic domains traverse the plasma membrane. Numerous studies on protein transport across cellular membranes have demonstrated the requirement of proteinaceous hydrophilic pores for the translocation process (Prakash *et al.*, 2004). More specifically, the turnover of membrane proteins in the yeast endoplasmic reticulum, mediated by the 26S proteosome, involves the retrograde translocation of substrates into the cytosol via the Sec61 translocase machinery (Stirling *et al.*, 2006; Groll *et al.*, 2005). Sec61 is the yeast homolog of *E. coli* SecY. A recent *in vitro* study, using purified FtsH and YccA in reconstituted proteoliposomes, showed that

FtsH is able to dislocate and degrade YccA in the absence of other components (Akiyama *et al.*, 2003).

2.1.6. Recognition strategy for FtsH

The ability of FtsH to discriminate between correct and incorrect protein substrates is critical to both its housekeeping and regulatory functions. FtsH recognizes and degrades cytoplasmic proteins that contain C-terminal non-polar tails (Herman *et al.*, 1998). This tail-specific recognition is physiologically relevant, and is used for degrading the λCII activator, SsrA-tagged proteins and the LpxC deacetylase (Herman *et al.*, 1998; Kobiler *et al.*, 2002). The SsrA-tag consists of 11 residues that is appended to the C-terminus of truncated proteins on stalled ribosomes to promote their proteolysis (Keiler *et al.*, 1996). The aforementioned tail-specific recognition, however, the C-terminal region is not important for degradation in case of the heat shock factor σ^{32} (Toyomasu *et al.*, 2001); Instead, an internal region may be important for the FtsH-mediated degradation of this protein (Bertani *et al.*, 2001). A mode of σ^{32} proteolysis is also described, in which it is degraded by FtsH in the N- to C- terminus direction (Okuno *et al.*, 2004; Obrist *et al.*, 2007). It is clear that FtsH readily degrades functional native protein substrates that are susceptible to the proteolytic reaction of FtsH in initiation-signal-dependent manners. This mode of proteolysis likely involves sequential substrate unfolding that could be initiated at an initiation signal and then propagated along the polypeptide chain (Ito *et al*., 2005).

In addition to specific sequence recognition, the substrate protein thermostability plays an important role in the decision to degrade it; the more thermostable the protein, the less likely that FtsH will degrade it, even when it carries a good recognition tag. This is due to the fact that FtsH does not possess a robust unfoldase activity, which would be necessary to thread a thermostable substrate into the proteolytic cavity. Lacking of a robust unfoldase allows FtsH to carry out a second regulatory step in the decision to degrade a protein by enabling FtsH to sense the folding state of proteins within the physiological range of the cell, and degrade only those that display low thermostability (Herman *et al.*, 2003). This second regulatory step in substrate selection may be crucial for the degradation of regulatory proteins and membrane proteins. σ^{32}, a natural substrate of FtsH, indeed contains region of low thermodynamic stability (Ito *et al*., 2005).

2.2. The sporulation in *Bacillus subtilis*

Under conditions of nutrient deprivation, cells of *B. subtilis* can undergo a process of development that leads to the formation of dormant, environmentally resistant spores. Sporulation takes approximately six to eight hours and involves extensive changes in

gene expression and morphology (Errington, 2003; Hilbert *et al.,* 2004; Piggot *et al.,* 2004). The hallmark of endospore formation is an asymmetric cell division that produces two cell types: a larger cell called the mother cell, and a smaller cell called the forespore.

2.2.1. Morphological stages

The series of complex morphological changes that occur during the sporulation process in *B. subtilis* have been extensively studied **(Fig. 4).** Entry into sporulation is characterized by the formation of a so-called axial filament in which two chromosomes from the last round of DNA replication become aligned across the long axis of the cell. Next, a septum is formed at an extreme polar position. This partitions the developing cell (hereafter referred to as the sporangium) into large and small compartments known as the mother cell and the forespore (or prespore), respectively, where each receives a chromosome (Stragier and Losick, 1996).

Initially, the large and the small compartments lie side-by-side, but in the next stage of development the forespore becomes engulfed by the mother cell. During engulfment, the membrane on the mother cell face of the polar septum migrates around the membrane surrounding the forespore and eventually the forespore is completely pinched off as a free protoplast within the mother cell, such that the sporangium becomes a cell-within-a-cell (for this reason spores of *Bacillus* and related genera are more properly known as endospores) (Stragier and Losick, 1996).

In subsequent morphogenesis, the forespore produces large amounts of a family of small acid-soluble proteins known as SASP. Some of these proteins bind to and coat the forespore chromosome, packaging it into a doughnut-like structure and conferring on it resistance to ultraviolet radiation. Meanwhile, in the intermembrane space between the forespore and mother cell, a thin layer of peptidoglycan known as the germ cell wall is produced on the surface of the forespore membrane. This is followed by the synthesis of a thick layer of peptidoglycan known as the cortex, which is thought to be involved in attaining or maintaining the dehydrated and heat-resistant state of the spore. The mother cell produces a proteinaceous coat that assembles on the outside surface of the mother-cell membrane around the forespore. The coat consists of a lamellar inner layer and an electron-dense outer layer and provides a thick, protective barrier that encases the mature spore. Eventually, after about 6–8 hours of development, when maturation is complete, the fully ripened spore is liberated by lysis of the mother cell. Thus, the mother cell is mortal in that it undergoes programmed cell death, whereas the forespore is immortal in that it becomes the spore and gives rise to subsequent progeny (Stragier and Losick, 1996).

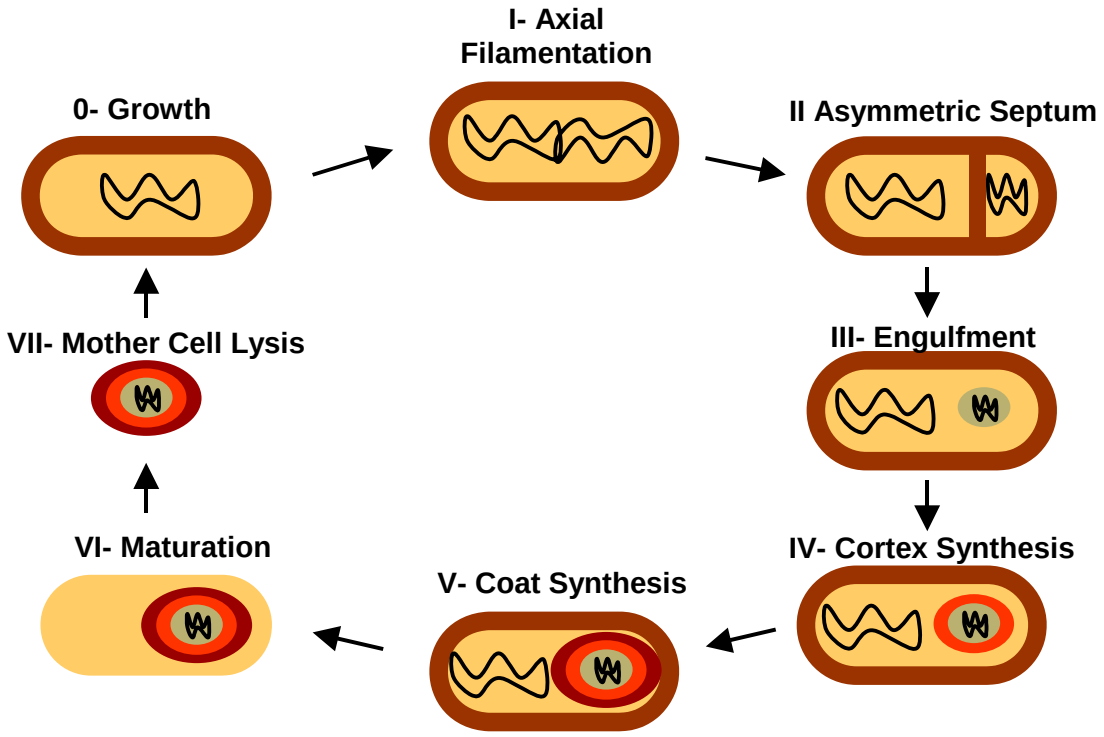

FIG. 4. Schematic representation of the stages of sporulation in *Bacillus subtilis* This sequence of morphological events is divided into different stages: Stage 0 represents cells that have not entered the sporulation pathway. Stage I represents cells that have entered the pathway and have formed an axial filament; Stage II and III refer to sporangia that have reached the stages of polar septation and engulfment, respectively. Synthesis of a distinctive form of peptidoglycan between the membranes surrounding the prespore is defined as stage IV. Deposition of spore coat around the prespore is defined as stage V. Stage VI is maturation, when the spore acquires its full resistant properties spore. Stage VII represents lysis of the mother cell and release of the mature spore **(Hilbert and Piggot, 2004)**

2.2.2. Spo0A

2.2.2.1. Spo0A is a master regulator of sporulation initiation

The master regulator for entry into sporulation in *B. subtilis* is the DNA-binding protein Spo0A, which is a member of the response regulator family of transcription factors (Perego and Hoch, 2002). The activation of this key transcriptional regulatory protein occurs through environmental and physiological signals, triggered by nutrient depletion and cell density. Activation of Spo0A proceeds through several phases. Initial activation at the end of exponential growth leads to the 'transition state', which is associated with such phenomena as protease production, motility, competence for transformation (Sonenshein, 2000), biofilm formation (Branda *et al.,* 2001; Hamon and

Lazazzera, 2001) and even cannibalism (Gonzalez-Pastor *et al.,* 2003) and spore formation which is thought to require increased the phosphorylation of Spo0A.

The molecular details of the interaction of Spo0A with its target DNA, the 'Spo0A box', a consensus 7-bp sequence (5′-TGNCGAA-3′, with a preference for N = T), have now been analyzed with a crystal structure (Zhao *et al.,* 2002). Spo0A has been found to influence, directly or indirectly, the expression of over 500 genes during the early stages of development. An approach in combination with transcriptional profiling using gene microarrays, gel electrophoretic mobility shift assays, using the DNA-binding domain of Spo0A, and bioinformatics enabled to assign a total 121 genes, which are organized as 30 single-gene units and 24 operons, are likely to be under the direct control of Spo0A. About one-third of these genes are activated and the remainder are repressed (Molle *et al.*, 2003). Among the identified members of the regulon where transcription was stimulated by Spo0A are genes for metabolic enzymes and genes for efflux pumps (Molle *et al.*, 2003)**.** Among the members where transcription that was inhibited by Spo0A are genes encoding components of the DNA replication machinery and genes that govern flagellum biosynthesis and chemotaxis.

During stage 0 of sporulation, the active form of Spo0A (Spo0A~P) acts as a repressor of certain vegetatively expressed genes (e.g. *abrB*) (Perego *et al*., 1988; Strauch *et al*., 1989; Strauch and Hoch, 1993; Fujita and Sadaie, 1998) and an activator of genes directly involved in sporulation (Piggot and Losick, 2002). So far, a total of 10 transcription units which are organized in six single-gene units (*abrB*, *kinA*, *kinC*, *spo0A*, *spo0F* and *spoIIE*) and four operons (*dlt*, *sin*, *spoIIA* and *spoIIG)* are controlled by Spo0A~P. Among the genes activated by Spo0A~P are those involved in remodeling the sister chromosomes of the sporulating cell into an 'axial filament' (Pogliano *et al*., 2002; Ben-Yehuda *et al*., 2003) and in the formation of a polar septum that divides the developing cell into a small forespore compartment and a large mother cell compartment (Levin and Losick, 1996; Ben-Yehuda and Losick, 2002). Spo0A~P is also responsible for activating genes that lead to the appearance of the cell-specific regulatory proteins σ^F and σ^E which act in the forespore and the mother cell, respectively (Stragier and Losick, 1996; Piggot and Losick, 2002). Recent work indicates that Spo0A~P continues to function after the polar septum is formed, when it accumulates to high levels and directs transcription in the mother cell (Fujita and Losick, 2003). Clearly, Spo0A has a profound effect on the global pattern of gene expression (Molle *et al.*, 2003).

Importantly, cells require a high threshold of active Spo0A to initiate sporulation (Fujita *et al*., 2005). Mutations within the phosphorelay, leading to lower concentrations of intracellular Spo0A~P, caused a smaller population of cells initiating sporulation

(Molle, *et al*., 2003*)*. There are four categories of genes within the Spo0A regulon that respond to different thresholds of Spo0A as follows: (i) those that require a high level of Spo0A to become activated, (ii) those that required a high level of Spo0A to be repressed, (iii) those that were activated at a low level of the regulator, and (iv) those that were repressed at a low dose of the regulator. Genes that required a high dose of Spo0A to be activated were found to have low binding constants for Spo0A~P. Some genes that were turned on at a low dose of Spo0A either had a high binding constant for the regulatory protein or were activated by an indirect mechanism involving Spo0A-mediated relief of repression by repressor protein AbrB (Fujita *et al.,* 2005).

Moreover, Spo0A~P also sets in motion several positive and negative regulatory loops that govern the rate of expression of *spo0A* and other relay and phosphatase genes. Detailed accounts of the functioning of the phosphorelay, the nature of these regulatory loops, and the modulation of phosphate flow by specific phosphatase are indicated below.

2.2.2.2. Activation of Spo0A - The phosphorelay

The activity of Spo0A is governed by a multicomponent phosphorelay - an extended version of the typical two-component system, which consists of five histidine autokinases (KinA, KinB, KinC, KinD and KinE) and two phosphorelay proteins (Spo0F and Spo0B) (Jiang *et al*., 2000) **(Fig. 5)**. Differential signals activate multiple histidine kinases to autophosphorylate and then transfer their phosphoryl group to the intermediate response regulator Spo0F (Burbulys *et al*., 1991; Jiang *et al*., 2000). Spo0F~P is the substrate for a phosphotransferase, Spo0B, which transfers the phosphoryl group to the Spo0A response regulator and transcription factor (Burbulys *et al*., 1991).

Signal integration is the responsibility of the phosphorelay, whose structural complexity reflects the requirement for precise coordination of numerous cellular events. The multicomponent structure of the phosphorelay provides multiple entries for regulatory signals affecting the final goal of producing the appropriate level of Spo0A~P. These regulatory mechanisms are exerted both on the level of transcription of the phosphorelay components and on their enzymatic activity.

KinA is the primary kinase in the phosphorelay and it has the major role at the onset of spore formation (Stephenson and Hoch, 2001). It has been demonstrated that the fraction of cells that initiate sporulation is decreased in a *kinA* mutant background (Chung *et al*., 1994). The most amino-terminal end of the domains in KinA is important for spore formation and has been shown to bind ATP, but is unlikely to be regulated directly by ATP levels (Stephenson and Hoch, 2001).

2.2.2.3. The auto-stimulation of Spo0A

The activity of Spo0A is subject to several auto-stimulatory loops (Strauch *et al*., 1992; Strauch *et al*., 1993; Fujita and Sadaie, 1998). These loops involve transcription of *spo0A* and phosphorylation of Spo0A. Transcription of *spo0A* is directly activated by Spo0A~P (Strauch *et al*., 1992) and indirectly activated by induced expression of *spo0H*. First of all, Spo0A~P represses the expression of *abrB*, a gene encoding a transcriptional

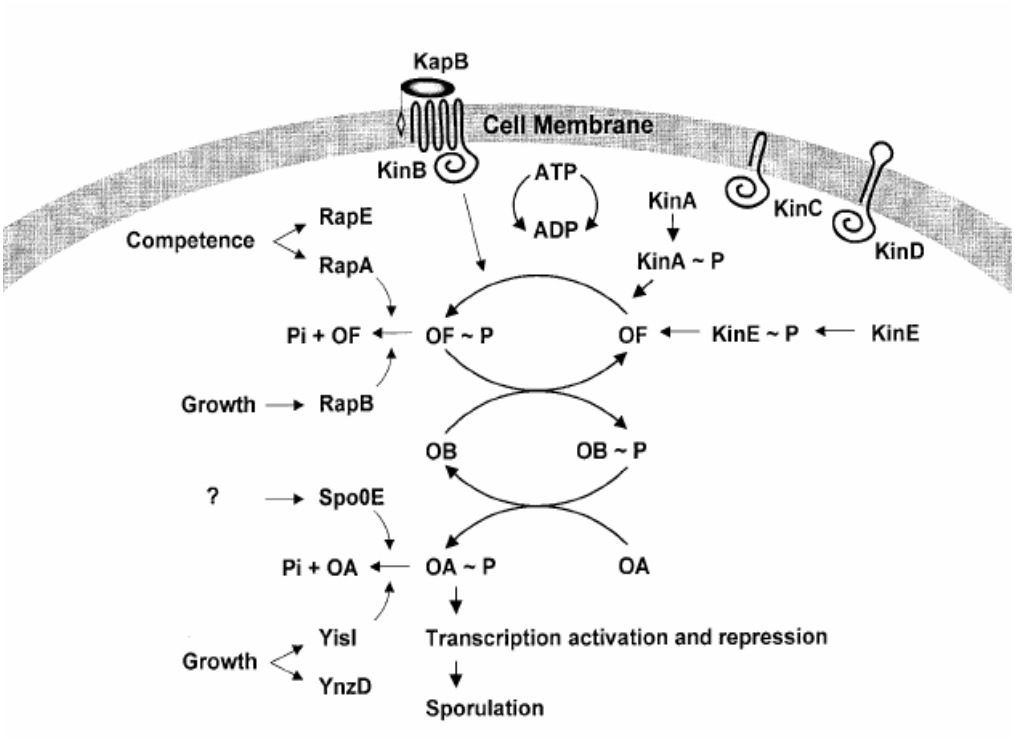

FIG. 5. The phosphorelay signal transduction system for sporulation initiation. In the phosphorelay, two cytoplasmic kinases (KinA and KinE) and three membrane-bound kinases (KinB, KinC, KinD) phosphorylate the Spo0F (0F) response regulator in response to differential signals. Spo0F~P transfers the phosphoryl group to the Spo0B (0B) phosphotransferase that, in turn, transfers it to the Spo0A (0A) response regulator and transcription factor for sporulation initiation. KapB is a lipoprotein essential for KinB activity. The Rap phosphatases dephosphorylate the Spo0F~P intermediate while Spo0E, YisI and YnzD dephosphorylate Spo0A~P. Transcription of the phosphatase coding genes is activated by physiological conditions antithetical to sporulation such as growth and competence to DNA transformation **(Perego, 2001).**

regulator that inhibits various stationary phase processes (Robertson *et al*., 1989). This results in an indirect autostimulatory loop actived *via* the transcriptional regulator AbrB. During exponential growth, AbrB represses various stationary phase processes, including the transcription of genes required for sporulation (e.g. *kinA*) (Strauch *et al*., 1989). Importantly, AbrB represses gene expression of the alternative RNA polymerase sigma factor σ^H that recognizes an alternative promoter upstream of *spo0A*, and in addition, activates genes required for phosphorylation of Spo0A such as *kinA* and *spo0F* (Predich *et al*., 1992). Thus, when Spo0A is phosphorylated, alleviation of AbrB repression by Spo0A~P stimulates both transcription of *spo0A* and indirectly phosphorylation of Spo0A. A simplified scheme of the autostimulation of Spo0A is described in **Fig. 6**.

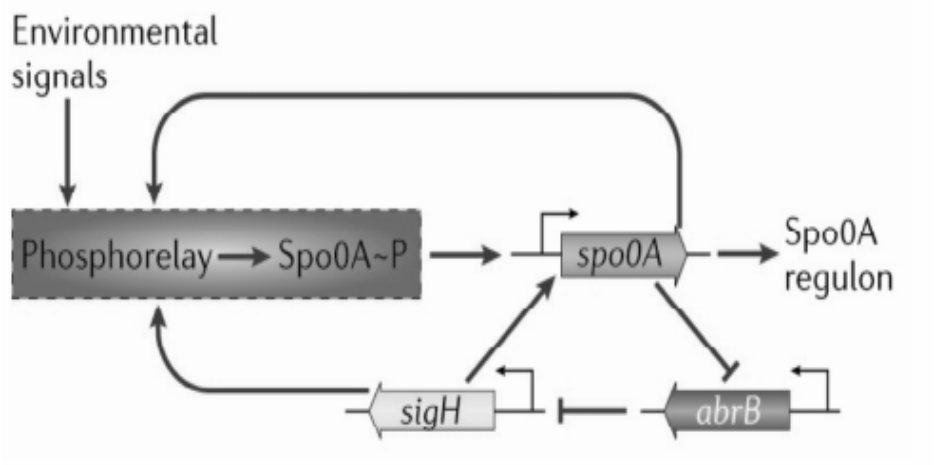

FIG. 6. Simplified schematic representation of the autostimulatory loop involving transcription and activation of Spo0A. Perpendiculars and arrows represent the negative and positive regulations, respectively **(Smits *et al*., 2006).**

2.2.3. Regulation of the phosphate flow

2.2.3.1. Regulation of kinase

The discovery that the level of Spo0A~P is crucial in determining the cell fate led to the discovery of a series of mechanisms that modulate the flux of phosphate in the phosphorelay in response to specific signals. The first level of control is on the histidine kinases, KinA and KinB. Although mechanisms of activation of these kinases are predictable, a negative regulator of phosphate input has been described as an inhibitor of the kinase activity of KinA. KipI is a potent inhibitor of the autophosphorylation reaction of kinase A but does not inhibit phosphate transfer to the Spo0F response regulator once

kinase A is phosphorylated. KipI inhibits of the catalytic domain of kinase A affecting the ATP/ADP reactions but not the phosphotransferase functions of this domain (Wang *et al.,* 1997). The activity of the kinase inhibitor protein KipI, is counteracted by an anti-inhibitor protein, KipA, the gene of which belongs to the same operon as the *kipI* gene (Perego, 1998). This operon is responsive to the availability of glucose and fixed nitrogen, therefore providing these metabolites a regulatory entry in the signal transduction pathway leading to sporulation.

KipI may be the first representative of a new class of signal transduction inhibitors that function by direct interaction with the catalytic domain of histidine kinases to counteract signals influencing the "sensor" domain of such kinases. Such an inhibitor may be a paradigm for new mechanisms of signal transduction control.

2.2.3.2. Regulation of response regulators

The modulation of phosphate output has been elucidated by the discovery of response regulator aspartyl-phosphate phosphatases (Perego *et al.,* 1996). These phosphatases were originally identified as negative regulators of sporulation because their overproduction inhibits the process whereas their absence results in increased sporulation. The first phosphatase identified, Spo0E, acts specifically on the Spo0A~P protein and can be seen as the ultimate checkpoint in the regulation of phosphate flux in the phosphorelay pathway (Ohlsen *et al.,*1994). Unfortunately, the mechanisms that regulate the activity or expression of Spo0E are not well understood. Regulated dephosphorylation occurs upstream in the pathway, at the level of the Spo0F response regulator, by means of the RapA, RapB and RapE members of the Rap family of aspartyl phosphate phosphatases (Perego *et al.,* 1994). RapA, RapB and RapE specifically dephosphorylate Spo0F~P. Because Spo0F~P and Spo0A~P are connected via the Spo0B phosphotransferase, whose activity is freely reversible, dephosphorylation of one component rapidly results in lowered phosphate levels in the other component, thus preventing accumulation of the threshold level of Spo0A~P necessary for sporulation initiation.

2.2.3.2.1. The Rap family of phosphatases

The *Bacillus subtilis* family of Rap phosphatases comprises 11 members but only three of them, RapA, RapB and RapE are known to target the sporulation phosphorelay by dephosphorylating Spo0F~P (Perego, 1998). These proteins are approximately 44.5 kDa and have in common six 34-residue (tetratricopeptide) repeats, which are associated with protein-protein interaction. Thus, RapA, RapB and RapE may cause dephosphorylation of Spo0F~P by binding to it and stimulating its autophosphatase

activity, rather than by functioning directly as phosphatases; the binding activities of the Rap phosphatase are regulated by pentapeptides (Core *et al.,* 2003). Consistent with this interpretation, RapA is displaced from a stable complex with Spo0F~P by addition of its cognate pentapeptide. The Rap phosphatases are known to be differentially activated by physiological processes alternative to sporulation, e.g, competence development induces RapA and RapE, while vegetative growth conditions induce RapB (Perego *et al*., 1994; Jiang *et al*., 2000).

2.2.3.2.2. The Spo0E family of phosphatases

The phosphorylated active form of Spo0A is subject to deactivation by small protein Spo0E (9.6 kDa) and two homologs YisI (6.5 kDa) and YnzD (6.5 kDa) (Perego, 2001). These proteins identify a new family of phosphatases – named Spo0E family and reveal additional regulatory elements. Amino acid sequence analyses show a significant level of homology between Spo0E and YisI or YnzD **(Fig. 7).** The homology extends over the first 54 amino terminal residues of Spo0E as YisI and YnzD are of a smaller size (56 and 57 amino acids, respectively, compared with the 85 amino acids of Spo0E). Transcription of these genes is quite distinct in the timing and in the underlying controlling regulatory mechanisms. *spo0E* is induced at the end of the exponential phase as repression by AbrB is relieved owing to accumulation of Spo0A~P and its transcription continues for 2 h into sporulation (Perego and Hoch, 1991). The YisI protein is expressed mainly during the transition phase, its induction is dependent upon the presence of an intact *spo0A* gene and is not affected by AbrB repression. A direct interaction of Spo0A with the *yisI* promoter is suggested by the presence of a Spo0A box

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YisI   ---------MNSKIEEMRITLIETAQKYGMNSKETIQCSQELDILLNTRIKEEMIFGRYLENSRM------------------- 56
YnzD   ----MIREHLLKEIEKKRAELLQIVMANGMTSHITIELSQELDHLLIQYQKQRLRAVAGDE---------------------- 57
Spo0E  MGGSSEQERLLVSIDEKRKLMIDAARKQGFTGHDTIRHSQELDCLINEYHQLMQENEHSQGIQGLVKKLGLWPRRDVMPAYDANK 85
                .   *:. *  .:: . ..* . ..**  ***** *:...
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FIG. 7. Amino acid sequence alignment of the YisI, YnzD and Spo0E phosphatases. Identical and conserved residues are indicated by asterisks and colons, respectively. The dots indicate residues identical or conserved between Spo0E and either YisI or YnzD. The underlined Q and W residues in the Spo0E sequence are the sites of the spo0E94 and spo0E11 mutations, respectively, resulting in Spo0E C-terminal truncated proteins with hyperphosphatase activity **(Perego, 2001).**

overlapping the putative -35 consensus sequence. Transcription of the *ynzD* gene in sporulation growth conditions is limited to the early exponential phase and is not affected by *spo0A* mutation. Therefore, Spo0E appears to be the major negative regulator acting on Spo0A~P during sporulation initiation.

Still, there is no evidence for auxiliary proteins involved in modulating the activity of Spo0E, YnzD and YisI. It is, however, intriguing that the carboxy-terminal truncated forms of Spo0E found in the Spo0E11 and Spo0E94 mutants are hyperactive in dephosphorylating Spo0A~P. This suggested that the C-terminal 25 residues of Spo0E might have an inhibitory role. This could be achieved by means of an intramolecular mechanism, by interaction with an unknown auxiliary protein acting as regulator or by targeted proteolysis. The YnzD and YisI proteins lack the C-terminal inhibitory region, suggesting that perhaps their presence in the cell is mainly controlled by transcriptional mechanisms that do not necessitate additional regulatory elements (Perego, 2001).

There are many similarities between the enzymatic activities and roles of Rap phosphatases and Spo0E, despite having no significant homology in the primary amino acid sequence. Purified proteins specifically induce dephosphorylation of their targets, Spo0F~P or Spo0A~P, with no apparent cross-reactivity and no direct effect on the remaining members of the phosphorelay, kinases and Spo0B. Deletion of either *spo0E* or *rapA* causes increased sporulation efficiency and can at least partly suppress mutations that decrease production of Spo0A~P (Perego and Hoch, 1991; Mueller *et al.,* 1992; Perego *et al.,* 1994). Some mutant alleles of *spo0E* and *rapA* cause a decrease in sporulation (Perego *et al.,* 1994) and these hypermorphs appear to encode more active phosphatases (Ohlsen *et al.,* 1994; Perego *et al.,* 1994). A similar phenotype is observed when the wild-type genes are overexpressed (Perego and Hoch, 1991; Perego *et al.,* 1994). Although the physiological role of these phosphatases in controlling sporulation is not known, they could help to prolong the transition state before cells could activate a sufficient amount of Spo0A to stimulate transcription of the *spo* loci. Therefore, these phosphatases act as negative regulators of the developmental process and provide a means for additional signals to have an impact on the phosphorylation level of the phosphorelay pathway.

2.2.3.3. Control of the Rap phosphatases

Although the transcriptional control of the Rap phosphatases depicts a complex regulatory network, an even more sophisticated mechanism has been evolved in the cell to modulate the phosphatase activity of Rap. The phosphatase activity of the Rap family is negatively modulated by small proteins called Phr, which are encoded by the adjacent phosphatase regulator *phr* gene located on the same transcript immediately downstream

of the *rap* genes. The Phr peptides are exported by the SecA-dependent pathway and subsequently processed to the carboxyl-terminal five-amino acid active inhibitor**.** The pentapeptide has been shown to be the active agent that directly or specifically inhibits the Rap phosphatase activity. The journey of the *phr* gene product to its final pentapeptide form, involving export from the cytoplasm and subsequent importation back into the cell, can be seen as a control circuit: the time it takes to export the pro-inhibitor, process it to the inhibitor pentapeptide and re-import it through the oligopeptide transport system (Opp) (Perego and Hoch, 2002).

2.2.4. Bistable outcoming in sporulation

Within an isogenic culture of sporulating *B. subtilis* cells, some initiate the developmental program of sporulation, whereas others do not (Chung *et al*., 1994). Therefore, initiation of sporulation appears to be a regulatory process with a bistable outcome. This phenotypic variation could contribute to the fitness of the species, because the heterogeneous population is able to quickly react to changing environments (Balaban *et al*., 2004). As sporulation is an energy-intensive process, and irreversible after its earliest stage (Parker *et al*., 1996), cells that are delayed to commit to sporulate could have an advantage over sporulating cells if food resources were to become plentiful again (Grossman, 1995)

Theoretical modeling and experiments in both prokaryotic and eukaryotic model systems have demonstrated that positive feedback of a transcriptional regulator can lead to a bimodal probability distribution in expression (Hasty *et al.*, 2000; Becskei *et al.*, 2001). Here it is believed that Spo0A autostimulation is responsible for bistability based on two analytical approaches: First, using flow cytometry to monitor single cells expressing GFP under control of a Spo0A~P dependent promoter (P*spoIIA*), it was observed that there are two distinct subpopulations which have low and high expression state (Veening *et al.,* 2005); Second, using an inducible and constitutively active variant of Spo0A, it was demonstrated that sporulation bistability is abolished when the Spo0A autostimulation is replaced by a graded induction (Veening *et al.,* 2005; Fujita *et al*., 2005). Furthermore, the activation of Spo0A by the phosphorelay is also subject to autostimulatory influences (Strauch *et al*., 1992; Strauch *et al*., 1993; Fujita and Sadaie, 1998). Mutations within the phosphorelay, leading to lower concentrations of intracellular Spo0A~P, caused a change in the outcome of the bistable response (Chung *et al*., 1994). Thus, the activation of Spo0A is subject to a bistable switch and that the switch is mediated by a positive-feedback loop involving the gene for Spo0A and genes for proteins that govern its phosphorylation (Veening *et al.,* 2005). Moreover, the most likely mode of action is that Spo0A autoactivation is already triggered at an early stage,

generating a subpopulation of cells in the Spo0A-ON state (containing high amount of active Spo0A) and a subpopulation in the Spo0A-OFF state (containing low amount of active Spo0A).

Another mechanism that could act as a sporulation delay system was previously described (Gonzalez-Pastor *et al*., 2003). The authors showed that, within a sporulating culture, sporulating cells are able to kill their siblings that have not yet initiated this process. This phenomenon is responsible for the reduction in cell density of a sporulating culture. The operon responsible for producing the sporulation killing factor (*skf*) is activated by Spo0A~P. The Spo0A-inactive subpopulation is sensitive to the sporulation-killing factor secreted by the Spo0A-active cells, and will subsequently lyse and release nutrients available for the remainder of the population.

Phosphatases modulate sporulation bistability

Importantly, the phosphorelay phosphatases have a modulating action on the bistable expression pattern of a sporulating culture by altering the threshold-level of Spo0A~P. Monitoring the expression of active Spo0A dependent gene - *spoIIA* in a *spo0E* mutant, and in strains containing artificially inducible Rap phosphatase - Rap60 show that the final bistable distribution of sporulation gene expression was almost abolished (Veening *et al.*, 2005).

rapA, also known as *gsiAA* (*g*lucose *s*tarvation *i*nduced protein A), was activated under glucose limiting conditions and high cell densities (Mueller *et al*., 1992). When looking at expression levels in individual cells, some cells initiate sporulation and repress *rapA* gene expression while the non-sporulating cells continue to accumulate RapA (Veening *et al.*, 2005). Artificial induction of a heterologous Rap phosphatase restored heterogeneity in a *rapA* or *spo0E* mutant (Veening *et al.*, 2005). This implied that the main function of RapA is to maintain the bistable gene expression that originates from the autostimulatory *spo0A* activation and with external phosphatases, *B. subtilis* can use the phosphorelay as a tuner to modulate the bistable outcome of the sporulating culture.

The exact function of Spo0E in sporulation regulation is unclear. Transcriptional regulation of *spo0E* involves a feedback loop and a connection between competence and sporulation. Transcription of *spo0E* is repressed by AbrB (Strauch *et al*., 1989; Perego and Hoch, 1991). Thus, the small amounts of Spo0A~P needed to repress transcription of *abrB* cause an increase in expression of *spo0E,* which should inhibit overstimulation of Spo0A~P. It is possible that this negative feedback loop contributes to the maintenance of the stable subpopulation of cells that do not sporulate in a culture subjected to sporulation conditions (Chung *et al.,* 1994).

2.2.5. SpoVM - an essential morphogenetic protein

spoVM is a developmental gene which encodes a small polypeptide of less than 3 kDa with an open reading frame of just 26 codons (Levin *et al.,* 1993). The expression of this gene occurs in the mother cell chamber and is controlled by the mother cell-specific transcription factor σ^E and the DNA-binding protein SpoIIID. A *spoVM* mutant arrests sporulation at stage IV-V and allows the formation of the forespore but impairs synthesis and assembly of the spore cortex (Levin *et al.,* 1993). An extragenic suppressor mutant was identified as restoring a Spo^+ phenotype to *spoVM* transposon insertional mutant as being allelic to *ftsH*. However, the suppressor mutations in *ftsH* restore sporulation only to the *spoVM* transposon insertional mutant not in the *spoVM* deletion. Thus, it is believed that FtsH and SpoVM interact functionally (Cutting *et al.,* 1997). Using FtsH purified from *E. coli*, the authors demonstrated that *in vitro* SpoVM inhibits FtsH protease activity and SpoVM is a substrate for the FtsH protease. An examination of the secondary structure of a synthetic SpoVM suggested that this protein is amphipathic and its alpha-helical conformation is favoured in a lipid environment that enabling SpoVM target to the membranes and interact with FtsH and antagonizes the action of FtsH (Cutting *et al.,* 1997). The interaction of FtsH and SpoVM also was suggested involving in prespore engulfment and forespore synthesis. As expected for a mutant blocked at stage II-III, expression of sigmaK-dependent genes was undetectable in the *spoVM ftsH* mutation, but complete blocking of sigmaK production not observed in either the single mutant (Cutting *et al.,* 1997).

Investigation of the subcellular localization of SpoVM, by using a fusion SpoVM-GFP demonstrated that SpoVM is recruited to the polar septum shortly after the sporangium undergoes asymmetric division and the fusion protein localizes to the mother cell membrane that surrounds the forespore during the subsequent process of engulfment. Evidently, the function of this small protein is strongly dependent upon its ability to localize to the mother cell membrane that surrounds the forespore. A patch of amino acids near the N-terminus of the protein is proposed to have an alpha-helical conformation, that is needed both for tightly restricting SpoVM-GFP to the outer forespore membrane and for the normal function of SpoVM in sporulation. Furthermore, substitution of three N-terminal proximal amino acids that mediate localization of SpoVM also produces heat-sensitive spores, suggesting that spore morphogenesis is dependent on proper localization of SpoVM (van Ooij and Losick, 2003).

A recent work had shown that the proper localization of SpoVM is dependent on SpoIVA, and conversely, that proper localization of SpoIVA is dependent on SpoVM. SpoIVA is the morphogenetic protein needed for cortex formation and for proper

assembly of the coat around the forespore. GFP-SpoIVA has been shown to surround the forespore in a shell-like structure that is believed to serve as a basement layer for the coat (van Ooij and Losick, 2003; Ramamurthi *et al.,* 2006). Genetic, biochemical and cytological evidence indicates that this mutual dependence is mediated in part by contact between an amino acid side-chain located near the extreme C-terminus of SpoIVA and an amino acid side-chain on the hydrophilic face of the SpoVM helix. SpoVM serving as a membrane anchor, it adheres to the outer forespore membrane via the hydrophobic face of the helix first and then tethers SpoIVA (Ramamurthi *et al.,* 2006).

2.3. Construction of a cold-inducible expression system for recombinant proteins in *B. subtilis*

One of the major drawbacks during high-level production of recombinant proteins in bacteria is the inability of many proteins to reach their native conformation. Under conditions of overproduction, proteins tend to accumulate within refractile aggregates designated inclusion bodies (Mogk *et al.*, 2002). Several strategies have been described to reduce the formation of inclusion bodies including cultivation of the cells at low temperatures (Thomas and Baneyx, 1996). Besides reducing formation of inclusion bodies, low-temperature expression lowers the degradation of proteolytically sensitive proteins (Emerick *et al.*, 1984;Chesshyre and Hipkiss, 1989). To ensure high level production of recombinant proteins at low temperature, two different strategies can be used: (i) Fusion of the coding region of the protein of interest to an inducible promoter followed by growth at a low temperature, e.g. 20°C. (ii) Fusion of the gene of interest to a cold-inducible promoter, growth of the expression strain at the physiological temperature first followed by induction at the appropriate low temperature. Such cold-inducible expression systems have already been developed for *E. coli* (Mujacic *et al.*, 1999;Qing *et al.*, 2004). Here, the promoter region of the cold-inducible *cspA* (for cold-shock proteins A) gene has been used. This gene is expressed at all temperatures, but the transcript is extremely unstable at physiological temperatures and greatly stabilized after a temperature downshift to 20°C (Fang *et al.*, 1997).

A sudden decrease in temperature affects membrane fluidity, and to restore its fluidity *B. subtilis* cells increase the level of a membrane-bound desaturase (Aguilar *et al.*, 1998). This enzyme (called Δ5-Des) is encoded by the *des* gene and catalyzes the introduction of a *cis* double bond at the Δ5 position of a wide variety of fatty acids (Aguilar *et al.*, 1998). While the *des* transcript is barely detectable at 37°C, its synthesis is transiently induced upon a temperature downshift (Aguilar *et al.*, 1999). Expression of the *des* gene does not depend on *de novo* protein synthesis, but on a two-component signal transduction system which consists of the sensor kinase DesK and the response

regulator DesR (Aguilar *et al.*, 2001). It is assumed that the transmembrane domain of the kinase senses a temperature downshift through changes in the physical state of the cytoplasmic membrane (Hunger *et al.*, 2004). The C-terminal kinase domain of DesK undergoes autophosphorylation, and the phosphoryl group is then transferred to the response regulator DesR. Phosphorylated DesR binds to two adjacent DNA-binding sites leading to the recruitment of RNA polymerase to the *des* promoter and activation of transcription (Cybulski *et al.*, 2004). The Δ5-desaturase directly introduces double bonds into membrane lipids leading to a return to the original fluidity of the membrane. This is sensed by DesK which changes from a kinase to phosphatase activity leading to a dephosphorylation of DesR with a concomitant turn off of the *des* gene (Mansilla and De Mendoza, 2005). Based on these data, a cold-inducible expression system for *B. subtilis* was developed.

3. Results and Discussion

Part A: Function of FtsH during initiation of sporulation

3.1. Influence of FtsH on the synthesis and activation of the master regulator Spo0A

3.1.1. Synthesis of Spo0A is partly impaired in the *ftsH* knockout

Based on previous studies, *ftsH* insertion mutants were completely deficient in sporulation where the sporulation frequency of the mutant was less than 10^{-8} as compared to the wild-type strain (Deuerling *et al.*, 1997). The stage of the sporulation program, which was impaired in the *ftsH* mutants had been determined based on transcriptional fusions of the *lacZ* reporter to genes expressed during two different sporulation stages: *spoIIA-lacZ* and *spoIID-lacZ* representing stage 0 and stage II, respectively. Both transcriptional fusions were induced during the transition phase in the wild-type strain, but their induction was completely abolished in the *ftsH* mutants (Deuerling *et al.*, 1997). These results clearly demonstrated that *ftsH* is required at an early stage of the sporulation process.

Spo0A is a master regulator of the initiation of sporulation (Errington, 1993). Here, I asked whether *ftsH* interferes with the synthesis or activity of Spo0A. An *ftsH* null mutant, in which the *ftsH* gene was completely replaced by an *erm*-cassette (Wehrl *et al.*, 2000) was used throughout this study. The amount of Spo0A was measured by Western blotting (Fig. 1, Part A). In the wild-type strain, Spo0A started to be present from stage 0 on and continued to be produced to at least stage 3. On the contrary, **Spo0A was present in greatly reduced amounts in the *ftsH* knockout**.

The *spo0A* gene is transcribed from the two promoters P*v* (for vegetative) and P*s* (for stationary). During exponential growth, the transcription of *spo0A* is under the σ^A-dependent P*v* promoter and occurs at a low level. Under sporulation conditions, so far unknown metabolic signals trigger the phosphorylation of Spo0A through the phosphorelay. The phosphorylated Spo0A (Spo0A~P) directly activates the P*s* promoter through binding to a Spo0A box located adjacent to this promoter which is recognized by σ^H. It indirectly stimulates its own transcription by inhibiting the synthesis of AbrB, which is a repressor of the gene coding for σ^H (Hoch, 1991). Furthermore, *kinA* (gene coding for the sensor kinase A of the phosphorelay*)* and *spo0A* belong to the category of low-threshold activated Spo0A genes, and both genes are transcribed by the σ^H-containing RNA polymerase holoenzyme (Predich *et al.,* 1992; Fujita and Sadaie, 1998*)*. Low-

threshold activation of *kinA* and *spo0A* is likely to be mediated by the positive feedback loop as indicated above (see Introduction - 2.2.3) involving synthesis and activation of Spo0A. Thus, the transcription of *spo0A* during stationary phase is stimulated by a certain amount of phosphorylated Spo0A (Spo0A~P), and in turn, the phosphorylation of Spo0A is accelerated through the Spo0A auto-stimulatory loop.

3.1.2. Activation of Spo0A does not occur in the *ftsH* knockout

There is likely to be at least a partial correlation between the level of Spo0A and the level of its phosphorylation as Spo0A is part of a positive feedback loop, in which the response regulator directly and indirectly stimulates the expression of genes involved its phosphorylation (Hoch, 1991). We asked whether the small amount of Spo0A in the *ftsH* knockout is present in its active or inactive form. Recently, it was reported that many of the genes of the Spo0A regulon respond to the transcription factor in a dose-dependent manner. Four different categories of responses to active Spo0A were distinguished: (i) genes that require a high level of Spo0A~P to be activated (e.g. *spoIIA*), (ii) those that require a high level of Spo0A~P to be repressed (e.g. *rapA*), (iii) those that are activated at a low level of the regulator (e.g. *skf*), and (iv) those that are repressed at a low dose of the regulator (e.g. *abrB*) (Fujita *et al.*, 2005). Indeed, I could show progressive increases in the level of Spo0A~P in the wild-type strain leading to the transcription of *skf-lacZ* and *spoIIA-lacZ* from the onset of the transition phase on defined as t_0. While further accumulation of *abrB-lacZ was* successfully repressed in the wild-type strain, the *ftsH* knockout failed to activate transcription of *skf-lacZ* and *spoIIA-lacZ* or to repress expression of *abrB-lacZ* **(Fig. 8)**. These results agree with previous findings that expression of *spoIIE-lacZ* was prevented in the *ftsH*::*spc* mutant (Lysenko *et al.,* 1997) and suggest that **Spo0A is present in its inactive form in the *ftsH* mutant**.

The absence of sufficient amounts of active Spo0A are responsible for the failure to sporulate. It can be hypothesized that either Spo0A is not phosphorylated at all or immediately dephosphorylated after phosphorylation. Therefore, it has to be assumed that FtsH has to degrade one or more proteins which act, directly or indirectly, as an negative regulators of the synthesis or/and activation of Spo0A.

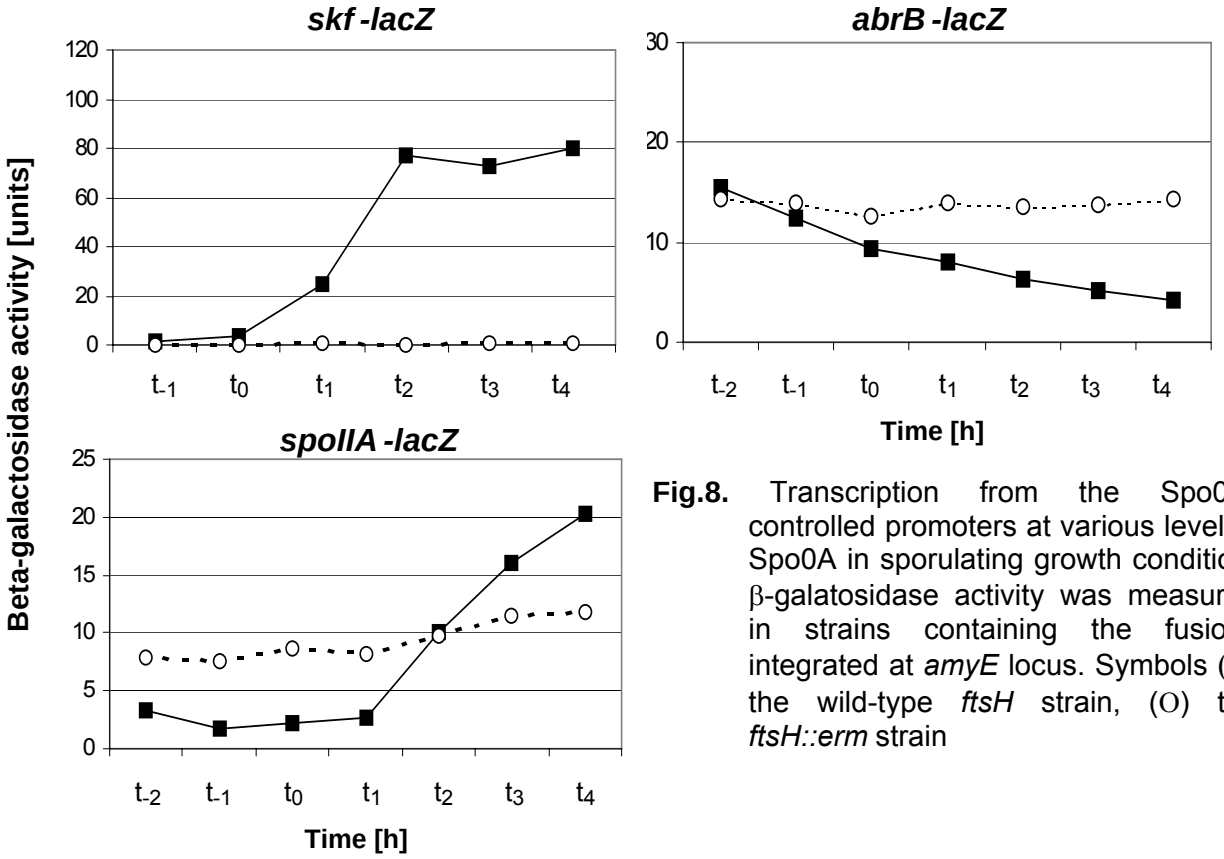

Fig.8. Transcription from the Spo0A-controlled promoters at various level of Spo0A in sporulating growth condition. β-galatosidase activity was measured in strains containing the fusions integrated at *amyE* locus. Symbols (■) the wild-type *ftsH* strain, (O) the *ftsH::erm* strain

3.1.3. After artificial induction of active Spo0A, cells are able to sporulate in the absence of FtsH

To examine whether FtsH is essential for sporulation only because of its affect on the synthesis or/and phosphorylation of Spo0A, the wild-type *spo0A* allele was replaced by an IPTG-inducible variant of Spo0A (*spo0A-sad67D56N*), which is active in the complete absence of phosphorylation (Ireton *et al*., 1993). The sporulation frequencies upon induction of the constitutively active form of Spo0A in the presence or absence of *ftsH* were comparable and reached 37.2% and 33.5%, respectively (Table 2, Part A), while the sporulation frequency was low in the absence of IPTG. These results strongly suggest that ***ftsH* is only essential during stage 0 of sporulation.**

3.2. The level of Spo0A is restored in the *ftsH spo0E* knockout, but not in the *ftsH rap* knockout

Since FtsH has been clearly shown to be involved in the synthesis and activation of Spo0A, it can be assumed that FtsH can influence one of the different components of the phosphorelay, thereby preventing phosphorylation of Spo0A. I focused on a series of antagonists of the phosphorelay, each of which responds to a particular environmental

signal thereby altering the rate of accumulation of Spo0A~P. Of particularly interest are the phosphorelay-associated phosphatases Rap (RapA, RapB and RapE) and the Spo0E family (Spo0E, YisI and YnzD), which dephosphorylate Spo0F~P and Spo0A~P, respectively. They were first examined whether they are involved in the production of active Spo0A in the presence and absence of FtsH.

Eight strains were analysed for the production of Spo0A by Western blotting. While in the absence of *rapA*, *rapB* and *spo0E* the amount of Spo0A was increased at t_0 as compared to the wild-type situation, its amount was reduced at both stage 0 and stage 1 in the *rapE* knockout (Fig. 1, Part A). When the *ftsH* null allele was added, the amount of Spo0A dropped in all strains carrying *rap* disruptant alleles as already observed for the wild-type strain in the absence of *ftsH*, but not in the case of the ∆*spo0E*. In the ∆*spo0E* ∆*ftsH* strain, the amount of Spo0A was reduced at stage 0 and further increased to levels comparable to those present in the *ftsH*$^+$ strain. This result suggests **an interaction between FtsH and Spo0E, either directly or indirectly, thereby influencing on the expression of *spo0A*.**

3.3.The activation of Spo0A in the phosphorelay phosphatase deletion strains

The transcriptional *skf-lacZ* fusion, which responds to a low threshold of Spo0A~P for activation, was used as a reporter system to study the activity of Spo0A in strains with mutant *rap* and *spo0E* alleles. While the expression started in all strains at stage 0 and reached its plateau value at stage 2 in the wild-type and in the ∆*rapB* strain, it accelerated in the ∆*spo0E* earlier and further increased in the ∆*rapA* and the Δ*rapE* strains (Fig. 2, Part A). However, in the absence *ftsH*, the failure to activate the *skf* promoter was observed in all strains. In conclusion, the absence of *rap* or *spo0E* alleles in an *ftsH* knockout leads to an expression of the *spo0A* gene, but the protein remained inactive. These data indicate that ***ftsH* influences production of active Spo0A by either allowing its phosphorylation or preventing its rapid dephosphorylation.**

3.4. Sporulation frequency increasing in the phosphorelay phosphatase deletion strains

In order to assess the relative contribution of each Rap and of the Spo0E phosphatase in modulating the activity of Spo0A, the sporulation efficiency in each phosphatase mutant in the presence or absence of FtsH was measured. The sporulation frequencies in all *rap* and s*po0E* knockouts were higher than that of the wild-type strain and ranged from 67% to 75%, where the sporulation frequency of wild-type cells was determined to be 58%. A similar observation has been published for RapA, RapE (Jiang *et al.*, 2000a) and Spo0E (Perego and Hoch, 1991). If a null allele in any one of them

was combined with an *ftsH* knockout, the sporulation frequency was increased by about two to three orders of magnitude as compared to a single *ftsH* mutant, but always remained below 1% (Table 1, Part A). The sporulation frequency in the phosphatase and *ftsH* double knockouts was only partly restored and still about 100-fold lower than the wild-type level. It can be concluded that *ftsH* is somehow involved in the synthesis of active Spo0A.

This result agrees with the previous studies that deletion of the *rap* genes or *spo0E* gene results in increased sporulation frequency (Jiang *et al.*, 2000a; Perego, 2001), while over-production of these genes results in inhibition of sporulation. It is likely that the phosphatases act as negative regulators of the developmental process and respond to in- and external signals to influence the amount of active Spo0A. Members of the Rap family of phosphatases are known to be differentially activated by physiological processes alternative to sporulation, e.g. competence development induces RapA and RapE, while vegetative growth conditions induce RapB (Perego *et al*., 1994; Jiang *et al.,* 2000). Since vegetative growth and competence are processes that cannot occur in a sporulating cell, the induction of *rap* phosphatases prevents sporulation from interfering with these processes.

Transcription of the Spo0E phosphatase is affected by signals that are still unknown (Perego and Hoch, 1991; Ohlsen *et al.,* 1994), but it is induced at the end of the exponential growth phase as repression by AbrB is relieved owing to accumulation of Spo0A~P (Strauch *et al*., 1989; Perego, 2001). Apparently, cells that initiate *spo0A* auto-activation also induce the Spo0E levels. This co-expression suggests that Spo0E serves as some kind of a ‘safety lid’ to prevent over-stimulation of *spo0A* autoactivation.

3.5. Spo0E is a target of FtsH *in vitro*

The observation that FtsH interferes with the synthesis and phosphorylation of Spo0A resulted in the hypothesis that FtsH has to degrade one or more negative regulators either preventing the phosphorylation of Spo0A or being involved in its rapid dephosphorylation. Since Spo0E directly targets Spo0A~P and represses sporulation when overproduced and increases sporulation as its absence, one possibility to explain these data is a direct interaction between FtsH and Spo0E resulting in its degradation.

To test this hypothesis, FtsH was purified with a GST-tag as reported before (Kotschwar *et al.*, 2005). This purification tag keeps the protein soluble in the absence of any added detergent. Furthermore, GST has a low thermodynamic stability, which is efficiently degraded by FtsH (Okuno *et al*., 2003). The C-terminus of GST contains an alpha-helix as a last structured element (Andujar-Sanchez *et al.,* 2005), which is followed by an unstructured spacer; this tag was expected to facilitate exposure of fused

polypeptides. Indeed, GST is generally stable in *E. coli* but when the proteolytic SsrA-tag is added, the fusion protein is rapidly degraded (Okuno *et al*., 2004). From our data, GST-FtsH is stable in *B. subtilis* (data not shown).

In the *in vitro* degradation assay with both purified GST-FtsH and GST-Spo0E, it could be shown that Spo0E was degraded by FtsH (Fig 3, Part A). Since no antibodies against Spo0E were available, antibodies against GST were used to confirm the instability of Spo0E.

As mentioned above, two homologs of Spo0E, YisI and YnzD, are also able to dephosphorylate Spo0A *in vitro* (Perego, 2001). These two phosphatases are distinguished from Spo0E by two characteristics: First, their genes are expressed during the vegetative growth phase and second, they lack a C-terminal extension of about 25 amino acid residues. It could be shown that both GST-YisI and GST-YnzD remained stable upon incubation with FtsH up to at least 5 h (Fig. 4, Part A). These data clearly demonstrated that neither YisI nor YnzD are substrates of FtsH. They further suggest that the C-terminal extension of Spo0E renders this phosphatase unstable.

Does FtsH fully degrade Spo0E in all cells or does it modulate its steady-state level? Based on my data, I would like to suggest that FtsH regulates the steady-state level of Spo0E rather than completely degrading it. Alternatively, though less likely, it might fully degrade Spo0E is some cells and not attack it at all in others. The first assumption is based on two observations:(i) a *spo0E* knockout leads to an increase in the sporulation frequency, which never reaches 100%; (ii) the cellular amount of Spo0E is low as the protein is barely detectable in cellular extracts (data not shown). A similar observation has been published for LpxC of *E. coli*, where only a few hundred molecules per cell are present (Führer *et al*., 2006). Here, a tight control of the amount of this enzyme by FtsH is essential to prevent the accumulation of abnormal membranes in the periplasm (Ogura *et al.*, 1999) leading to cell death (Sullivan and Donachie, 1984). This finding further indicates that FtsH has to degrade or regulate the steady-state level of one or more proteins interfering negatively with successful sporulation.

3.6. The C-terminal end of Spo0E is responsible for degradation by FtsH

A remarkable feature of the FtsH protease is its specificity for target recognition. To initiate cytoplasmic protein degradation, FtsH recognizes a tail, normally located at C-terminus, which contains a critical signal for degradation initiation. The published observation that the two Spo0E homologs, the proteins YisI and YnzD, lack about 25 amino acid residues at their C-terminus and that the two truncated versions of Spo0E, Spo0E94 and Spo0E11, are still able to dephosphorylate Spo0A~P strongly suggest that

the C-terminus of Spo0E may have an inhibitory role rather than being required for its enzymatic activity.

Spo0E11 and Spo0E94 were tagged with GST, overproduced in *E. coli* and purified. When these two purified proteins were incubated with FtsH, both remained stable for at least 5 h, while β-casein as a control was degraded under these conditions (Fig. 4, Part A). Since the full-length Spo0E protein is unstable when incubated with FtsH, I infer that the 25 C-terminal amino acid residues is responsible for this instability.

3.7. The 25 C-terminal amino acids of Spo0E is considered as the proteolytic tag for FtsH-mediated degradation

Since Spo0E serves as a target for FtsH, but two its homologs YisI and YnzD not, I fused the coding region for the Spo0E C-terminal 25 amino acids to *ynzD* designated YnzD-0E. The GST-tagged hybrid protein was overproduced in *E. coli*, purified by affinity chromatography and incubated with GST-FtsH. As can be seen from Fig. 6, Part A (lane 6), the YnzD-0E is largely degraded over time. In conclusion, the C-terminal 25 amino acids of Spo0E contain the residues recognized by FtsH and it was considered as proteolytic tag for *B. subtilis* FtsH-mediated degradation. Similar observations were made with two different *E. coli* FtsH substrates, the phage λ CII protein, whose C-terminus is required for degradation by FtsH (Kobiler *et al*., 2002), and the SsrA-degradation-tag (Keiler *et al*., 1996), in which the λ CI protein has been converted into an FtsH substrate by attachment of the SsrA-tag to its C-terminus (Herman *et al*., 1998).

Which amino acids are recognized by the FtsH protease? It has been suggested that the FtsH protease recognizes flexible tail specificity or even the length of the target protein (Herman *et al*., 1998; Führer *et al.,* 2007). The tail-specific recognition is physiologically relevant, and is used for degrading the λ CII activator, SsrA-tagged proteins (Herman *et al*., 1998; Kobiler *et al*., 2002) and LpxC**,** the key enzyme in lipopolysacharide formation by controlling the ratio between LPS and phospholipids (Sorensen *et al.*, 1996)**.** These sequences are enriched in non-polar amino acids at their very C-terminus. Comparison of the C-termini from LpxC and λ CII revealed no similarities. In contrast, the SsrA-tag exhibits a remarkable similarity to the final eleven residues of LpxC. The exact LpxC degradation-tag was determined by mutational analysis. Six non-polar amino acids within the C-terminal eleven residues of LpxC turned out to be required for degradation (Führer *et al*., 2007). From several studies with FtsH and its eukaryotic homologs, it was concluded that the protease prefers hydrophobic and non-polar residues at cleavage sites whereas acidic residues abolish degradation (Ito and Akiyama, 2005; Koppen and Langer, 2007). In the case of Spo0E, there is no similarity to the *B. subtilis* SsrA-tag (Wiegert and Schumann, 2001). Therefore, the amino

acid sequence recognized by the FtsH protease is different from that of the SsrA-tag. Experiments are in progress to identify the amino acid residues of Spo0E recognized by FtsH.

Part B: Function of FtsH late during sporulation

3.1. Assumption that FtsH interacts with the small sporulation peptide SpoVM

spoVM is a developmental gene essential for sporulation. Some *spoVM* mutants arrest sporulation at stage IV-V and allow the formation of the forespore but impair synthesis and assembly of the spore cortex (Levin *et al.,* 1993). SpoVM was considered as a morphogenetic protein since it is synthesized in the mother cell compartment and almost quantitatively localized to the engulfing membrane (Levin *et al*., 1993; van Ooij and Losick, 2003). Furthermore, spore morphogenesis is dependent on the proper localization of SpoVM (van Ooij and Losick, 2003).

A transposon insertion within *spoVM* leading to sporulation-deficient cells was used to select for extragenic suppressors; such extragenic suppressors were mapped within *ftsH* (Cutting *et al*., 1997). Furthermore, it could be shown that chemically synthesized SpoVM was able to inhibit degradation of σ^{32} by purified *E. coli* FtsH (Cutting *et al.,* 1997). I could show in an *in vitro* degradation assay with purified *B. subtilis* FtsH that β-casein, an unstructured protein strongly degraded by FtsH, turned out to be stabilized in the presence of SpoVM (Fig. 1, Part B).

These findings strongly suggest that, first, FtsH and SpoVM interact functionally and that, second, SpoVM inhibits the *B. subtilis* FtsH protease late during sporulation. This assumption is sustained by two observations: First, FtsH-GFP has been shown to accumulate within the asymmetric septum (Wehrl *et al*., 2000) and, second, SpoVM-GFP colocalized with the polar septum, too (van Ooij and Losick, 2003). It can be inferred that, in the absence of SpoVM, FtsH will degrade at least one protein essential to complete successful sporulation or, alternatively, regulate the steady-state level of SpoVM; both possibilities are not mutually exclusive. In some degradation assays, the partial disappearance of SpoVM was observed (data not shown) as described for the bacteriophage λ CIII peptide, which is known to inhibit λ CII protein degradation by FtsH, but being unstable when FtsH is overproduced (Herman *et al.,* 1997). A short domain (residues 16-37) of CIII may form an amphipathic α-helix which is essential for its activity (Kornitzer *et al.,* 1991). Interestingly, SpoVM was also predicted to form such an amphipathic α-helix, though it displays no sequence similarity with λ CIII (Prajapati, *et al.,* 2000; Ramamurthi *et al.,* 2006). We infer from these data that the essential SpoVM

peptide, by interaction with FtsH, either prevents degradation of one or more proteins essential for successful spore formation or that FtsH influences the steady-state level of SpoVM.

3.2. Screening potential substrates for FtsH late during sporulation

Since SpoVM interferes with the proteolytic activity of FtsH, We examined the possibility that FtsH will degrade one or more proteins essential to complete spore formation. The experiment is based on the rational that the substrate protein(s) should be degraded in a *spoVM*-defective mutant. To identify such proteins, the cytoplasmic proteomes of a wild-type and a *spoVM* knockout were compared using the technique of two-dimensional gel electrophoresis followed by identification of the proteins by MALDI-TOF MS. A total of 83 protein spots were present in the wild-type (data not shown), but completely absent or present in significantly reduced amounts in the *spoVM* null mutant. This dramatic change in the protein pattern suggests an additional role for SpoVM besides acting as a target for the FtsH protease. Interestingly, one of these proteins turned out to be SpoIVA, which seems to be completely absent in the *spoVM* knockout (Fig. 2, Part B). SpoIVA is a morphogenetic protein, is synthesized in the mother cell compartment and almost quantitatively localized to the engulfing membrane (Levin *et al*., 1993; van Ooij and Losick, 2003). The proper localization of SpoVM and SpoIVA in the outer membrane of the forespore is mutually dependent (Ramamurthi *et al.,* 2006). This raises the question whether SpoIVA is unstable in the absence of SpoVM. If SpoIVA is a substrate for FtsH, it will be interesting to find out whether tethering of SpoIVA to membrane-bound SpoVM will be sufficient to prevent its degradation or whether SpoVM has to directly interact with the protease acting as an anti-protease as described for the phage λ CIII protein (Halder *et al.,* 2007). This work is being continued to clarify the mutual effect of these three proteins.

3.3. Transcription of *spoVM* during sporulation

The *spoVM* gene forms a monocistronic operon and the transcription of this gene is controlled by the sporulation-specific sigma factor σ^E acting in conjunction with the DNA-binding protein SpoIIID, a 93-amino-acid protein (Levin *et al.,* 1993). Analysis of the expression of the *spoVM* gene revealed that wild-type cells started to synthesize a *spoVM*-specific transcript at stage 2 and continued produce it in large quantities at least until stage 5 (Fig. 3, Part B). A similar expression pattern was described in a previous study when using a *spoVM-lacZ* transcriptional fusion (Levin *et al*., 1993).

Western blot analysis pointed out that the SpoVM peptide can be detected in sporulating *B. subtilis* cells. As shown in Fig. 4 of Part B, the SpoVM peptide started to

accumulate at stage 4 and further increased during the next 2 h. In summary, these results clearly demonstrate that while the *spoVM* transcript is present about 3 h after entry into the transition phase, synthesis of the SpoVM peptide is delayed by 2 h suggesting posttranscriptional regulation of *SpoVM*.

3.4. The 5' untranslated region of *spoVM* acts as negative regulator of its own translation

The lag-phase of about 2 h between the onset of *spoVM* transcription and translation suggests a so far unknown mechanism that prevents early appearance of the peptide. This could involve stabilization of the transcript, delayed translation initiation or stability of the peptide. A visual inspection of the *spoVM* transcript revealed an 87 nucleotide untranslated region (5' UTR) preceding the coding region (Fig. 5A, Part B). I asked whether this 5' UTR is involved in expression of *spoVM*. Two different translational fusions using *lacZ* as a reporter gene were constructed, one with and the other without the untranslated region. To discriminate between *cis*- and *trans*-acting factors, I first analyzed the two fusions in *E. coli*. This decision was based on the assumption that *trans*-acting factors should be completely absent from this host. There is no difference in the β-galactosidase activities between these two fusions (Fig. 5B, Part B). This result indicates that the 5' UTR does not act as a *cis*-acting factor in *E. coli*, e.g. by influence on the stability of the transcript.

In *B. subtilis*, expression of *spoVM-lacZ* without the 5' UTR occurs at a significantly higher level (about 5-fold) as compared to the transcript containing the 5' UTR, and further increased at later sporulation times (Fig. 5C, Part B). In summary, the 5' UTR of *spoVM* negatively influences its own transcription or translation in *B. subtilis*. Since this effect was not observed in *E. coli*, I would like to conclude that a *trans*-active regulator is involved in transcription or translation of *spoVM* which is not present in *E. coli*. Yet another possibility is that the 5' UTR affects the stability of the transcript. I regard this mechanism as rather unlikely as such an effect could not be observed in *E. coli*. The factor influencing regulation at the 5' UTR could be a translational repressor protein binding within the untranslated region, e.g. at the inverted repeat (Fig. 5A, Part B). Alternatively, a non-coding (nc) RNA may interact with the *spoVM* transcript thereby reducing its translation. Recently, several ncRNAs have been described which are under sporulation control (Silvaggi *et al*., 2006).

Part C: The cold-inducible expression system

Two different vectors have been constructed designated pAL10 and pAL12 (Fig. 1, Part C). They allow the cold-inducible production of intracellular (pAL10) and extracellular (pAL12) recombinant proteins. When *lacZ* was fused to the *des* promoter, its expression turned out to become cold-inducible and its activity declined after about 90 min in the wild-type strain due to the turn off as to be expected (Fig. 2a, Part C). In contrast, this turn off was not observed in a *des* knockout and the β-galactosidase activity further increased up to about 12 h after the temperature downshock (Fig. 2b, Part C). In another experiment, it could be shown that the intracellular protein HtpG (Fig. 3, Part C) and the membrane-associated protein Pbp4* could be overproduced where a higher amount of the latter remained soluble when compared to its overexpression at 37°C (Fig. 4, Part C). Using pAL12, it was possible to obtain increased secretion of the α -amylase encoded by the *amyQ* gene (Fig. 5, Part C).

In summary, this plasmid-based cold-inducible expression system can be used as an alternative to keep the recombinant proteins soluble and to prevent formation of inclusion bodies.

4. References

Aguilar,P.S., Cronan,J.E., Jr., and De Mendoza,D. (1998) A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. *J Bacteriol* **180:** 2194-2200.

Aguilar,P.S., Hernandez-Arriaga,A.M., Cybulski,L.E., Erazo,A.C., and De Mendoza,D. (2001) Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis. EMBO J* **20:** 1681-1691.

Aguilar,P.S., Lopez,P., and De Mendoza,D. (1999) Transcriptional control of the low-temperature-inducible *des* gene, encoding the Delta5 desaturase of *Bacillus subtilis. J Bacteriol* **181:** 7028-7033.

Akiyama,Y., and Ito,K. (2003) Reconstitution of membrane proteolysis by FtsH. *J Biol Chem* **278:** 18146-18153.

Akiyama,Y., Kihara,A., Tokuda,H., and Ito,K. (1996) FtsH (HflB) is an ATP-dependent protease selectively acting on SecY and some other membrane proteins. *J Biol Chem* **271**: 31196-31201.

Akiyama,Y., Ogura,T., and Ito,K. (1994a) Involvement of FtsH in protein assembly into and through the membrane. I. Mutations that reduce retention efficiency of a cytoplasmic reporter. *J Biol Chem* **269**: 5218-5224.

Akiyama,Y., Shirai,Y., and Ito,K. (1994b) Involvement of FtsH in protein assembly into and through the membrane. II. Dominant mutations affecting FtsH functions. *J Biol Chem* **269**: 5225-5229.

Atorino,L., Silvestri,L., Koppen,M., Cassina,L., Ballabio,A., Marconi,R., Langer,T., and Casari.G. (2003) Loss of m-AAA protease in mitochondria causes complex I deficiency and increased sensitivity to oxidative stress in hereditary spastic paraplegia. *J Cell Biol* **163**: 777-787.

Balaban,N.Q., Merrin,J., Chait,R., Kowalik,L., and Leibler,S. (2004) Bacterial persistence as a phenotypic switch. *Science* **305**: 1622-1625.

Beall,B., and Lutkenhaus,J. (1991) FtsZ in *Bacillus subtilis* is required for vegetative septation and for asymmetric septation during sporulation. *Genes Dev* **5**: 447-455.

Becskei,A., Seraphin,B., and Serrano,L. (2001) Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *EMBO J* **20**:2528-2535.

Belfort,M., and Wulff,D.L. (1974) The roles of the lambda *cIII* gene and the *Escherichia coli* catabolite gene activation system in the establishment of lysogeny by bacteriophage lambda. *Proc Natl Acad Sci USA* **71**: 779-782.

Ben-Yehuda,S., and Losick,R. (2002) Asymmetric cell division in *B-subtilis* involves a spiral-like intermediate of the cytokinetic protein FtsZ. *Cell* **109**: 257-266.

Ben-Yehuda,S., Rudner,D.Z., and Losick,R. (2003) RacA, a bacterial protein that anchors chromosomes to the cell poles. *Science* **299**: 532-536.

Bertani,D., Oppenheim,A.B., and Narberhaus,F. (2001) An internal region of the RpoH heat shock transcription factor is critical for rapid degradation by the FtsH protease. *FEBS Lett* **493**: 17-20.

Branda,S.S., González-Pastor,J.E., Ben Yehuda,S., Losick,R., and Kolter,R. (2001) Fruiting body formation by *Bacillus subtilis*. *Proc Natl Acad Sci USA* **98**: 11621-11626.

Burbulys,D., Trach,K.A., and Hoch,J.A. (1991) Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell* **64**: 545-552.

Casari,G., De Fusco,M., Ciarmatori,S., Zeviani,M., Mora,M., Fernandez,P., De Michele,G., Filla, A., Cocozza,S., Marconi,R., Durr,A., Fontaine,B., and Ballabio,A., (1998) *Spastic paraplegia* and OXPHOS impairment caused by mutations in paraplegin, a nuclear-encoded mitochondrial metalloprotease. *Cell* **93:** 973-983.

Cervin,M.A., Spiegelman,G.B., Raether,B., Ohlsen,K., Perego,M., and Hoch,J.A. (1998) A negative regulator linking chromosome segregation to developmental transcription in *Bacillus subtilis*. *Mol Microbiol* **29**: 85-95.

Chesshyre,J.A., and Hipkiss,A.R. (1989) Low temperatures stabilize interferon σ32 against proteolysis in *Methylophilus methylotrophus* and *Escherichia coli*. *Appl Microbiol Biotechnol* **31**: 158-162.

Chiba,S., Akiyama,Y., and Ito,K. (2002) Membrane protein degradation,by FtsH can be initiated from either end. *J Bacteriol* **184**: 4775-4782.

Chiba,S., Akiyama,Y., Mori,H., Matsuo,E., and Ito,K. (2000) Length recognition at the N-terminal tail for the initiation of FtsH-mediated proteolysis. *EMBO reports* **1**: 47-52.

Chung,J.D., Stephanopoulos,G., Ireton,K., and Grossman,A.D. (1994) Gene expression in single cells of Bacillus subtilis: evidence that a threshold mechanism controls the initiation of sporulation. *J Bacteriol* **176**: 1977-1984.

Coggins,B.E., Li, X., McClerren,A.L., Hindsgaul,O., Raetz,C.R.H., and Zhou.P., (2003) Structure of the LpxC deacetylase with a bound substrate-analog inhibitor. *Nat Struct Biol* **10**: 645-651.

Confalonieri,F., and Duguet,M. (1995) A 200-amino acid ATPase module in search of a basic function. *BioEssays* **17**: 639-650.

Core,L., and Perego,M. (2003) TPR-mediated interaction of RapC with ComA inhibits response regulator-DNA binding for competence development in *Bacillus subtilis*. *Mol Microbiol* **49**: 1509-1522.

Cutting,S., Anderson,M., Lysenko,E., Page,A., Tomoyasu,T., Tatematsu,K. *et al.* (1997) SpoVM, a small protein essential to development in *Bacillus subtilis*, interacts with the ATP-dependent protease FtsH. *J Bacteriol* **179**: 5534-5542.

Cybulski,L.E., Del Solar,G., Craig,P.O., Espinosa,M., and De Mendoza,D. (2004) *Bacillus subtilis* DesR functions as a phosphorylation-activated switch to control membrane lipid fluidity. *J Biol Chem* **279**: 39340-39347.

Deuerling,E., Mogk,A., Richter,C., Purucker,M., and Schumann,W. (1997) The *ftsH* gene of *Bacillus subtilis* is involved in major cellular processes such as sporulation, stress adaptation and secretion. *Mol Microbiol* **23**: 921-933.

Deuerling,E., Paeslack,B., and Schumann,W. (1995) The *ftsH* gene of *Bacillus subtilis* is transiently induced after osmotic and temperature upshock. *J Bacteriol* **177**: 4105-4112.

Emerick,A.W., Bertolani,B.L., Ben-Bassat,A., White,T.J., and Konrad,M.W. (1984) Expression of a β-lactamase preproinsulin fusion protein in *Escherichia coli*. *Bio/Technology* **2**: 165-168.

Errington,J. (1993) *Bacillus subtilis* sporulation: Regulation of gene expression and control of morphogenesis. *Microbiol Rev* **57**: 1-33.

Errington,J. (2003) Regulation of endospore formation in *Bacillus subtilis*. *Nat Rev Microbiol* **1**: 117-126.

Fang,L., Jiang,W., Bae,W., and Inouye,M. (1997) Promoter-independent cold-shock induction of *cspA* and its derepression at 37°C by mRNA stabilization. *Mol Microbiol* **23**: 355-364.

Fleischmann,R.D., Adams,M.D., White,O., Clayton,R.A., Kirkness,E.F., Kerlavage,A.R. *et al.* (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**: 496-512.

Flynn,J.M., Levchenko,I., Seidel,M., Wickner,S.H., Sauer,R.T., and Baker,T.A. (2001) Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. *Proc Natl Acad Sci USA* **98**: 10584-10589.

Fraser,C.M., Gocayne,J.D., White,O., Adams,M.D., Clayton,R.A., Fleischmann,R.D. *et al.* (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**: 397-403.

Führer,F., Langklotz,S., and Narberhaus,F. (2006) The C-terminal end of LpxC is required for degradation by the FtsH protease. *Mol Microbiol* **59**: 1025-1036.

Führer,F., Müller,A., Baumann,H, Langklotz,S., Kutscher,B., and Narberhaus,F. (2007) Sequence and length recognition of the C-terminal turnover element of LpxC, a soluble substrate of the membrane-bound FtsH protease. *J Mol Biol* **372:** 485-496

Fujita,M., Amemura,A., and Aramaki,H. (1998) Transcription of the *groESL* operon in *Pseudomonas aeruginosa* PAO1. *FEMS Microbiol Lett* **163**: 237-242.

Fujita,M., and Losick,R. (2003) The master regulator for entry into sporulation in *Bacillus subtilis* becomes a cell-specific transcription factor after asymmetric division. *Genes Dev* **17**: 1166-1174.

Fujita,M., and Sadaie,Y. (1998) Feedback loops involving Spo0A and AbrB in *in vivo* transcription of the genes involved in the initiation of sporulation in *Bacillus subtilis*. *J Biochem* **124**: 98-104.

Fujita,M., Gonzalez-Pastor,J.E., and Losick,R. (2005) High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J Bacteriol* **187**: 1357-1368.

Geisler,U., and Schumann,W. (1993) Isolation of stress mutants of *Bacillus subtilis* by a novel genetic method. *FEMS Microbiol Lett* **108**: 251-254.

Gonzalez-Pastor,J.E., Hobbs,E.C., and Losick,R. (2003) Cannibalism by sporulating bacteria. *Science* **301**: 510-513.

Gottesman,S., Roche,E., Zhou,Y.N., and Sauer,R.T. (1998) The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev* **12**: 1338-1347.

Griffith,K.L., Shah,I.M., and Wolf,R.E. (2004) Proteolytic degradation of *Escherichia coli* transcription activators SoxS and MarA as the mechanism for reversing the

induction of the superoxide (SoxRS) and multiple antibiotic resistance (Mar) regulons. *Mol Microbiol* **51**: 1801-1816.

Groll,M., Bochtler,M., Brandstetter,H., Clausen,T., and Huber.R. (2005) Molecular machines for protein degradation. *Chembiochem* **6:** 222-256.

Grossman,A.D. (1995) Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. *Annu Rev Genet* **29**: 477-508.

Guex,N., and Peitsch,M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18: 2714-2723.

Halder,S., Datta,A.B., and Parrack,P. (2007) Probing the antiprotease activity of λCIII, an inhibitor of the *Escherichia coli* metalloprotease HflB (FtsH). *J Bacteriol* **189**: 8130-8138.

Hamon,M.A., and Lazazzera,B.A. (2001) The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol Microbiol* **42**: 1199-1209.

Hasty,J., Pradines,J., Dolnik,M., and Collins.J.J. (2000) Noise-based switches and amplifiers for gene expression. PNAS 9: 2075-2080.

Herman,C., and D'Ari,R. (1998) Proteolysis and chaperones: The destruction/reconstruction dilemma. *Curr Opin Microbiol* **1**: 204-209.

Herman,C., Ogura,T., Tomoyasu,T., Hiraga,S., Akiyama,Y., Ito,K. *et al.* (1993) Cell growth and lambda phage development controlled by the same essential *Escherichia coli* gene, *ftsH/hflB*. *Proc Natl Acad Sci USA* **90**: 10861-10865.

Herman,C., Prakash,S., Lu,C.Z., Matouschek,A., and Gross,C.A. (2003) Lack of a robust unfoldase activity confers a unique level of substrate specificity to the universal AAA protease FtsH. *Mol Cell* **11**: 659-669.

Herman,C., Thévenet,D., Bouloc,P., Walker,G.C., and D'Ari,R. (1998) Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Genes Dev* **12**: 1348-1355.

Herman,C., Thévenet,D., D'Ari,R., and Bouloc,P. (1995) Degradation of σ^{32}, the heat shock regulator in *Escherichia coli*, is governed by HflB. *Proc Natl Acad Sci USA* **92**: 3516-3520.

Herman,C., Thévenet,D., D'Ari,R., and Bouloc,P. (1997) The HflB protease of *Escherichia coli* degrades its inhibitor λCIII. *J Bacteriol* **179**: 358-363.

Hilbert,D.W., and Piggot,P.J. (2004) Compartmentalization of gene expression during Bacillus subtilis spore formation. *Microbiol Mol Biol Rev* **68**: 234-262.

Hoskins,J.R., Kim,S.Y., and Wickner,S. (2000) Substrate recognition by the ClpA chaperone component of ClpAP protease. *J Biol Chem* **275**: 35361-35367.

Hoyt,M.A., Knight,D.M., Das,A., Miller,H.I., and Echols,H. (1982) Control of phage λ development by stability and synthesis of cII protein: Role of the viral cIII and host *hflA*, *himA*, and *himD* genes. *Cell* **31**: 565-573.

Hunger,K., Beckering,C.L., and Marahiel,M.A. (2004) Genetic evidence for the temperature-sensing ability of the membrane domain of the *Bacillus subtilis* histidine kinase DesK. *FEMS Microbiol Lett* **230**: 41-46.

Ireton,K., Rudner,D.Z., Siranosian,K.J., and Grossman,A.D. (1993) Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. *Genes Dev* **7**: 283-294.

Isaacs,F.J., Hasty,J., Cantor,C.R., and Collins,J.J. (2003) Prediction and measurement of an autoregulatory genetic module. *Proc Natl Acad Sci USA* **100**: 7714-7719.

Ito,K., and Akiyama,Y. (2005) Cellular functions, mechanism of action, and regulation of *ftsH* protease. *Annu Rev Microbiol* **59**: 211-231.

Jarosch,E., Taxis,C., Volkwein,C., Bordallo,J., Finley,D., Wolf,D.H., and Sommer,T. (2002) Protein dislocation from the ER requires polyubiquitination and the AAAATPase Cdc48. *Nat Cell Biol* **4**:134-139.

Jiang,M., Grau,R., and Perego,M. (2000a) Differential processing of propeptide inhibitors of Rap phosphatases in *Bacillus subtilis*. *J Bacteriol* **182**: 303-310.

Jiang,M., Shao,W., Perego,M., and Hoch,J.A. (2000b) Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Mol Microbiol* **38**: 535-542.

Kanemori,M., Nishihara,K., Yanagi,H., and Yura,T. (1997) Synergistic roles of Hs1VU and other ATP-dependent proteases in controlling in vivo turnover of σ^{32} and abnormal proteins in *Escherichia coli*. *J Bacteriol* **179**: 7219-7225.

Karzai,A.W., Roche,E.D., and Sauer,R.T. (2000) The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat Struct Biol* **7**: 449-455.

Keiler,K.C., and Sauer,R.T. (1996) Sequence determinants of C-terminal substrate recognition by the Tsp protease. *J Biol Chem* **271**: 2589-2593.

Kenniston,J.A., Baker,T.A., Fernandez,J.M., and Sauer,R.T. (2003) Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA^+ degradation machine. *Cell* **114**: 511-520.

Kihara,A., Akiyama,Y., and Ito,K. (1995) FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. *Proc Natl Acad Sci USA* **92**: 4532-4536.

Kihara,A., Akiyama,Y., and Ito,K. (1997) Host regulation of lysogenic decision in bacteriophage lambda: Transmembrane modulation of FtsH (HflB), the cII degrading protease, by HflKC (HflA). *Proc Natl Acad Sci USA* **94**: 5544-5549.

Kihara,A., Akiyama,Y., and Ito,K. (1998) Different pathways for protein degradation by the FtsH/HflKC membrane-embedded protease complex: An implication from the interference by a mutant form of a new substrate protein, YccA. *J Mol Biol* **279**: 175-188.

Kihara,A., Akiyama,Y., and Ito,K. (1999) Dislocation of membrane proteins in FtsH-mediated proteolysis. *EMBO J* **18**: 2970-2981.

Kim,Y.I., Burton,R.E., Burton,B.M., Sauer,R.T., and Baker.T.A. (2000) Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. *Mol Cell* **5:** 639-648.

Kim,K.I., Cheong,G.W., Park,S.C., Ha,J.S., Woo,K.M., Choi,S.J., and Chung,C.H. (2000) Heptameric ring structure of the heat-shock protein ClpB, a protein-activated ATPase in *Escherichia coli*. *J Mol Biol* **303**: 655-666.

Kobiler,O., Koby,S., Teff,D., Court,D., and Oppenheim,A.B. (2002) The phage lambda CII transcriptional activator carries a C-terminal domain signaling for rapid proteolysis. *Proc Natl Acad Sci USA* **99**: 14964-14969.

Korat,B., Mottl,H., and Keck,W. (1991) Penicillin-binding protein 4 of *Escherichia coli*: molecular cloning of the *dacB* gene, controlled overexpression, and alterations in murein composition. *Mol Microbiol* **5**: 675-684.

Kornitzer,D., Altuvia,S., and Oppenheim,A.B. (1991) The activity of the CIII regulator of lambdoid bacteriophages resides within a 24-amino acid protein domain. *Proc Natl Acad Sci USA* **88**: 5217-5221.

Kotschwar,M., Harfts,E., Ohanjan,T., and Schumann,W. (2005) Construction and analyses of mutant *ftsH* alleles of *Bacillus subtilis* involving the ATPase- and Zn-binding domains. *Curr Microbiol* **49**: 180-185.

Krzywda,S., Brzozowski,A.M., Verma,C., Karata,K., Ogura,T., and Wilkinson,A.J. (2002) The crystal structure of the AAA domain of the ATP-dependent protease FtsH of *Escherichia coli* at 1.5 Å resolution. *Structure* **10**: 1073-1083.

Levin,M.E., Hendrix,R.W., and Casjens,S.R. (1993a) A programmed translational frameshift is required for the synthesis of a bacteriophage lambda tail assembly protein. *J Mol Biol* **234**: 124-139.

Levin,P.A., and Losick,R. (1996) Transcription factor Spo0A switches the localization of the cell division protein FtsZ from a medial to a bipolar pattern in *Bacillus subtilis*. *Genes Dev* **10**: 478-488.

Levin,P.A., Fan,N., Ricca,E., Driks,A., Losick,R., and Cutting,S. (1993b) An unusually small gene required for sporulation by *Bacillus subtilis*. *Mol Microbiol* **9**: 761-771.

Lysenko,E., Ogura,T., and Cutting,S.M. (1997) Characterization of the *ftsH* gene of *Bacillus subtilis*. *Microbiology* **143**: 971-978.

Obrist,M., Milek,S., Klauck,E., Hengge,R., and Narberhaus,F. (2007) Region 2.1 of the *Escherichia coli* heat-shock sigma factor RpoH (sigma32) is necessary but not sufficient for degradation by the FtsH protease. *Microbiology* **153**: 2560-2571

Makino,S., Qu,J.N., Uemori,K., Ichikawa,H., Ogura,T., and Matsuzawa,H. (1997) A silent mutation in the ftsH gene of *Escherichia coli* that affects FtsH protein production and colicin tolerance. *Mol Gen Genet* **254**: 578-583.

Mansilla,M.C., and De Mendoza,D. (2005) The *Bacillus subtilis* desaturase: a model to understand phospholipid modification and temperature sensing. *Arch Microbiol* **183**: 229-235.

Michaels,M.L., Cruz,C., Grollman,A.P., and Miller,J.H. (1992) Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. *Proc Natl Acad Sci USA* **89**: 7022-7025.

Mogk,A., Mayer,M.P., and Deuerling,E. (2002) Mechanisms of protein folding: molecular chaperones and their application in biotechnology. *Chembiochem* **3**: 807-814.

Molle,V., Fujita,M., Jensen,S.T., Eichenberger,P., Gonzalez-Pastor,J.E., Liu,J.S., and Losick,R. (2003) The Spo0A regulon of *Bacillus subtilis*. *Mol Microbiol* **50**: 1683-1701.

Mueller,J.P., and Sonenshein,A.L. (1992) Role of the *Bacillus subtilis gsiA* gene in regulation of early sporulation gene expression. *J Bacteriol* **174**: 4374-4383.

Mueller,J.P., Bukusoglu,G., and Sonenshein,A.L. (1992) Transcriptional regulation of *Bacillus subtilis* glucose starvation-inducible genes: Control of *gsiA* by the ComP-ComA signal transduction system. *J Bacteriol* **174**: 4361-4373.

Mujacic,M., Cooper,K.W., and Baneyx,F. (1999) Cold-inducible cloning vectors for low-temperature protein expression in *Escherichia coli*: application to the production of a toxic and proteolytically sensitive fusion protein. *Gene* **238**: 325-332.

Nilsson,D., Lauridsen,A.A., Tomoyasu,T., and Ogura,T. (1994) A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli ftsH* gene product. *Microbiology* **140**: 2601-2610.

Niwa,H., Tsuchiya,D., Makyio,H., Yoshida,M., and Morikawa,K. (2002) Hexameric ring structure of the ATPase domain of the membrane-integrated metalloprotease FtsH from *Thermus thermophilus* HB8. *Structure* **10**: 1415-1423.

Ogura,M., Hirao,S., Ohshiro,Y., and Tanaka,T. (1999a) Positive regulation of *Bacillus subtilis sigD* by C-terminal truncated LacR at translational level. *FEBS Lett* **457**: 112-116.

Ogura,M., Liu,L., Lacelle,M., Nakano,M., and Zuber,P. (1999b) Mutational analysis of ComS: evidence for the interaction of ComS and MecA in the regulation of competence development in *Bacillus subtilis*. *Mol Microbiol* **32**: 799-812.

Ogura,T., and Wilkinson,A.J. (2001) AAA^+ superfamily ATPases: common structure-diverse function. *Genes to Cells* **6**: 575-597.

Ohlsen,K.L., Grimsley,J.K., and Hoch,J.A. (1994) Deactivation of the sporulation transcription factor Spo0A by the Spo0E protein phosphatase. *Proc Natl Acad Sci USA* **91**: 1756-1760.

Okuno,T., Yamada-Inagawa,T., Karata,K., Yamanaka,K., and Ogura,T. (2004) Spectrometric analysis of degradation of a physiological substrate σ^{32} by *Escherichia coli* AAA protease FtsH. *J Struct Biol* **146**: 148-154.

Okuno,T., Yamanaka,K., and Ogura,T. (2006) An AAA protease FtsH can initiate proteolysis from internal sites of a model substrate, apo-flavodoxin. *Genes Cells* **11**: 261-268.

Parker,G.F., Daniel,R.A., and Errington,J. (1996) Timing and genetic regulation of commitment to sporulation in *Bacillus subtilis*. *Microbiology* **142**: 3445-3452.

Perego,M. and HochJ.A. (2002) Two-component systems, phosphorelays and regulation of their activities by phosphatases, pp.473-481. *In* A.L. Sonenshein, et.

al. (ed.) *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells. ASM Press, Washington, D.C.

Perego,M. (2001) A new family of aspartyl phosphate phosphatases targeting the sporulation transcription factor Spo0A of *Bacillus subtilis*. *Mol Microbiol* **42**: 133-143.

Perego,M., and Hoch,J.A. (1991) Negative regulation of *Bacillus subtilis* sporulation by the *spo0E* gene product. *J Bacteriol* **173**: 2514-2520.

Perego,M., and Hoch,J.A. (1996) Cell-cell communication regulates the effects of protein aspartate phosphatases on the phosphorelay controlling development in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **93**: 1549-1553.

Perego,M., Hanstein,C., Welsh,K.M., Djavakhishvili,T., Glaser,P., and Hoch,J.A. (1994) Multiple protein-aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in *B. subtilis*. *Cell* **79**: 1047-1055.

Perego,M., Spiegelman,G.B., and Hoch,J.A. (1988) Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. *Mol Microbiol* **2**: 689-699

Piggot,P.J., and Losick,R. (2002) Sporulation genes and intercompartmental regulation. In *Bacillus subtilis* and its Closest Relatives: from Genes to Cells. Sonenshein, A.L., Hoch, J.A., and Losick, R. (eds). Washington, DC: American Society for Microbiology Press: 483-517.

Piggot,P.J., and Hilbert,D.W. (2004) Sporulation of *Bacillus subtilis*. *Curr Opin Microbiol* **7**: 579-586.

Pogliano,J., Sharp,M.D, and Pogliano,K. (2002) Partitioning of chromosomal DNA during establishment of cellular asymmetry in *Bacillus subtilis*. *J Bacteriol* **184**: 1743-1749.

Prajapati,R.S., Ogura,T., and Cutting,S.M. (2000) Structural and functional studies on an FtsH inhibitor from *Bacillus subtilis*. *Biochim Biophys Acta Gen Subj* **1475**: 353-359.

Prakash, S. and Matouschek,A. (2004) Protein unfolding in the cell. *Trends Biochem Sci* **29**: 593-600.

Predich,M., Nair,G., and Smith,I. (1992) *Bacillus subtilis* early sporulation genes *kinA, spo0F*, and *spo0A* are transcribed by the RNA polymerase containing σ^H. *J Bacteriol* **174**: 2771-2778.

Qing,G.L., Ma,L.C., Khorchid,A., Swapna,G.V.T., Mal,T.K., Takayama,M.M. *et al.* (2004) Cold-shock induced high-yield protein production in *Escherichia coli*. *Nat Biotechnol* **22**: 877-882.

Qu,J.N., Makino,S., Adachi,H., Koyama,Y., Akiyama,Y., Ito,K. *et al.* (1996) The *tolZ* gene of *Escherichia coli* is identified as the *ftsH* gene. *J Bacteriol* **178**: 3457-3461.

Ramamurthi,K.S., Clapham,K.R., and Losick,R. (2006) Peptide anchoring spore coat assembly to the outer forespore membrane in *Bacillus subtilis*. *Mol Microbiol* **62**: 1547-1557.

Robertson,J.B., Gocht,M., Marahiel,M.A., and Zuber,P. (1989) AbrB, a regulator of gene expression in *Bacillus subtilis*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthesis gene. *Proc Natl Acad Sci USA* **86**: 8457-8461.

Santos,D., and Almeida,D.F. (1975) Isolation and characterization of a new temperature-sensitive cell division mutant of *Escherichia coli* K-12. *J Bacteriol* **124**: 1502-1507.

Sauer,R.T., Bolon,D.N., Burton,B.M., Burton,R.E., Flynn,J.M., Grant,R.A. *et al.* (2004) Sculpting the proteome with AAA plus proteases and disassembly machines. *Cell* **119**: 9-18.

Shotland,Y., Koby,S., Teff,D., Mansur,N., Oren,D.A., Tatematsu,K. *et al.* (1997) Proteolysis of the phage lambda CII regulatory protein by FtsH (HflB) of *Escherichia coli*. *Mol Microbiol* **24**: 1303-1310.

Shotland,Y., Shifrin,A., Ziv,T., Teff,D., Koby,S., Kobiler,O., and Oppenheim,A.B. (2000a) Proteolysis of bacteriophage lambda CII by *Escherichia coli* FtsH (HflB). *J Bacteriol* **182**: 3111-3116.

Shotland,Y., Teff,D., Koby,S., Kobiler,O., and Oppenheim,A.B. (2000b) Characterization of a conserved λ-helical, coiled-coil motif at the C-terminal domain of the ATP-dependent FtsH (HflB) protease of *Escherichia coli*. *J Mol Biol* **299**: 953-964.

Silvaggi,J.M., Perkins,J.B., and Losick,R. (2006) Genes for small, noncoding RNAs under sporulation control in *Bacillus subtilis*. *J Bacteriol* **188**: 532-541.

Smits,W.K., Kuipers,O.P., and Veening,J.W. (2006) Phenotypic variation in bacteria: the role of feedback regulation. *Nat Rev Microbiol* **4**: 259-271.

Sonenshein,A.L. (2000) Control of sporulation initiation in *Bacillus subtilis*. *Curr Opin Microbiol* **3**: 561-566.

Sorensen,P.G., Lutkenhaus,J., Young,K., Eveland,S.S., Anderson,M.S., and Raetz,C.R. (1996) Regulation of UDP-3-O-[R-3-hydroxymyristoyl]-N-acetylglucosamine deacetylase in *Escherichia coli*. The second enzymatic step of lipid a biosynthesis. *J Biol Chem* **271**: 25898-25905.

Sowell,M.O., and Buchanan,C.E. (1983) Changes in the penicillin binding proteins during sporulation of *Bacillus subtilis. J Bacteriol* **153:** 1331-1337.

Stephenson,K., and Hoch,J.A. (2001) PAS-A domain of phosphorelay sensor kinase A: A catalytic ATP-binding domain involved in the initiation of development in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **98**: 15251-15256.

Stirling,C.J. and Lord,J.M. (2006) Quality control: linking retrotranslocation and degradation. *Curr Biol* **16:** 1035-1047.

Stragier,P., and Losick,R. (1996) Molecular genetics of sporulation in *Bacillus subtilis*. *Annu Rev Genet* **30**: 297-341.

Strauch,M.A., and Hoch,J.A. (1993a) Signal transduction in *Bacillus subtilis* sporulation. *Curr Opin Genet Dev* **3:** 203-212.

Strauch,M.A., and Hoch,J.A. (1993) Transition-state regulators: sentinals of *Bacillus subtilis* post-exponential gene expression. *Mol Microbiol* **7**: 337-342.

Strauch,M.A., Spiegelman,G.B., Perego,M., Johnson,W.C., Burbulys,D., and Hoch,J.A. (1989) The transition state transcription regulator *abrB* of *Bacillus subtilis* is a DNA binding protein. *EMBO J* **8**: 1615-1621.

Strauch,M.A., Trach,K.A., and Hoch,J.A. (1992) Spo0A activates and represses its own synthesis by binding at its dual promoters. *Biochimie* **74**: 619-626.

Strauch,M.A., Wu,J.-J., Jonas,R.H., and Hoch,J.A. (1993) A positive loop controls transcription of the *spo0F* gene, a component of the sporulation phosphorelay in *Bacillus subtilis*. *Mol Microbiol* **7**: 967-974.

Sullivan,N.F., and Donachie,W.D. (1984) Transcriptional organization within an *Escherichia coli* cell division gene cluster: direction of transcription of the cell separation gene *envA*. *J Bacteriol* **160**: 724-732.

Teff,D., Koby,S., Shotland,Y., Ogura,T., and Oppenheim,A.B. (2000) A colicin-tolerant *Escherichia coli* mutant that confers Hfl phenotype carries two mutations in the region coding for the C-terminal domain of FtsH (HflB). *FEMS Microbiol Lett* **183**: 115-117.

Thomas,J.G., and Baneyx,F. (1996) Protein misfolding and inclusion body formation in recombinant *Escherichia coli* cells overexpressing heat-shock proteins. *J Biol Chem* **271**: 11141-11147.

Tomoyasu,T., Arsène,F., Ogura,T., and Bukau,B. (2001) The C terminus of σ^{32} is not essential for degradation by FtsH. *J Bacteriol* **183**: 5911-5917.

Tomoyasu,T., Gamer,J., Bukau,B., Kanemori,M., Mori,H., Rutman,A.J. *et al.* (1995) *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor σ^{32}. *EMBO J* **14**: 2551-2560.

Tomoyasu,T., Yamanaka,K., Murata,K., Suzaki,T., Bouloc,P., Kato,A. *et al.* (1993a) Topology and subcellular localization of FtsH protein in *Escherichia coli*. *J Bacteriol* **175**: 1352-1357.

Tomoyasu,T., Yura,T., Morimura,S., Mori,H., Yamanaka,K., Niki,H. *et al.* (1993b) The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J Bacteriol* **175**: 1344-1351.

Van Ooij,C., and Losick,R. (2003) Subcellular localization of a small sporulation protein in *Bacillus subtilis*. *J Bacteriol* **185**: 1391-1398.

Veening,J.-W., Hamoen,L.W., and Kuipers,O.P. (2005) Phosphatases modulate the bistable sporulation gene expression pattern in *Bacillus subtilis*. *Mol Microbiol* **56**: 1481-1494.

Wang,C.C., and Tsou,C.L. (1998) Enzymes as chaperones and chaperones as enzymes. *FEBS Lett* **425**: 382-384.

Weber-Ban,E.U., Reid,B.G., Miranker,A.D., and Horwich,A.L. (1999) Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. *Nature* **401**: 90-93.

Wehrl,W., Niederweis,M., and Schumann,W. (2000) The FtsH protein accumulates at the septum of *Bacillus subtilis* during cell division and sporulation. *J Bacteriol* **182**: 3870-3873.

Whittington,D.A., Rusche,K.M., Shin,H., Fierke,C.A., and Christianson,D.W. (2003) Crystal structure of LpxC, a zinc-dependent deacetylase essential for endotoxin biosynthesis. *Proc. Natl. Acad. Sci. USA* **100**: 8146-8150.

Wiegert,T., and Schumann,W. (2001) SsrA-mediated tagging in *Bacillus subtilis*. *J Bacteriol* **183**: 3885-3889.

Woodman,P.G. (2003) A protein coping with multiple identities. *J Cell Sci* **116**: 4283-4290.

Yanouri,A., Daniel,R.A., Errington,J., and Buchanan,C.E. (1993) Cloning and sequencing of the cell division gene *pbpB*, which encodes penicillin-binding protein 2B in *Bacillus subtilis*. *J Bacteriol* **175**: 7604-7616.

Zhao,H., Msadek,T., Zapf,J., Madhusudan, Hoch,J.A., and Varughese,K.I. (2002) DNA complexed structure of the key transcription factor initiating development in sporulating bacteria. *Structure* **10**: 1041-1050

5. Own Contribution

Part A

The Spo0E phosphatase of *Bacillus subtilis* is a substrate of the FtsH metalloprotease

Ai Thi Thuy Le and Wolfgang Schumann

Submitted to Molecular Microbiology

Contribution to part A:

The study was designed by the last author and myself. All the results of this part are from my work. This part was written by the last author with contribution by myself (about 20%).

Part B

Function and Regulation of the *spoVM* Gene of *Bacillus subtilis*

Ai Thi Thuy Le and Wolfgang Schumann

Submitted to Journal of Bacteriology

Contribution to part B:

The study was designed by the last author and myself. All the results of this part are from my work. This part was written by the last author with contribution by myself (about 20%).

Part C

A novel cold-inducible expression system for *Bacillus subtilis*

Ai Thi Thuy Le and Wolfgang Schumann

Published on Protein Expression and Purification 53 (2007): 264-269

Contribution to part C:

The study was designed by the last author and myself. All the results of this part are from my work. This part was written by the last author with contribution by myself (about 20%).

Appendix : Own publications

Part A

The Spo0E phosphatase of *Bacillus subtilis* is a substrate of the FtsH metalloprotease

Ai Thi Thuy Le and Wolfgang Schumann

Submitted to Molecular Microbiology

The Spo0E phosphatase of *Bacillus subtilis* is a substrate of the FtsH metalloprotease

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Summary

In the absence of the ATP-dependent metalloprotease FtsH, the sporulation frequency of *Bacillus subtilis* cells is significantly reduced. This finding indicates that FtsH has to degrade or to regulate the steady state level of one or more proteins interfering negatively with successful sporulation. Here, we show that in the *ftsH* knockout the amount of the master regulator protein Spo0A is significantly reduced and the small amounts of Spo0A protein present are inactive. Active Spo0A is phosphorylated through a phosphorelay. Four negative regulators have been identified here which directly interfere with the phosphorelay namely the phosphatases RapA, RapB, RapE and Spo0E. If a null allele in any one of them was combined with an *ftsH* knockout, the sporulation frequency was increased by two to three orders of magnitude, but remained below 1%. Since the highest sporulation frequency was measured in the *spo0E ftsH* double knockout, we asked whether the Spo0E phosphatase acts as a substrate for FtsH. When purified Spo0E was incubated with FtsH, degradation of the phosphatase was observed. In contrast, two mutant versions of Spo0E with truncated C-terminal ends remained stable. Transfer of the 25 C-terminal amino acids of Spo0E to a shorter homolog of Spo0E, YnzD, which is not a substrate of FtsH, conferred instability. When a mutant Spo0A was produced which is active in the absence of phosphorylation, spores are formed at a normal rate in an *ftsH* knockout indicating that *ftsH* is only needed only during phase 0.

Introduction

Upon depletion of nutrients, species in the genera *Bacillus* and *Clostridium* have the ability to undergo a cellular differentiation process leading to the formation of a dormant spore (for recent reviews, see (Phillips and Strauch, 2002;Errington, 2003;Piggot and Hilbert, 2004)). Complex regulatory circuits govern the alterations in gene expression that occur upon entry into the sporulation program. The sporulation process is triggered by starvation, and the population density has to be high. *B. subtilis* cells code for an extremely complex and sophisticated decision-making apparatus, which monitors a huge range of internal and external signals. These informations are channelled through several separate regulatory systems, of which the most prominent component is the master regulator Spo0A, a DNA-binding protein which directly affects transcription of a total of 121 genes, either negatively or positively (Molle *et al.*, 2003). The synthesis of Spo0A is controlled at the transcriptional level, and the activity of the

protein is regulated by phosphorylation through the phosphorelay signal transduction system (Burbulys *et al.*, 1991;Hoch, 1993). The transfer of the phosphate to Spo0A involves a complex network consisting of several kinases (KinA, KinB, KinC, KinD and KinE), where each probably responds to a different stimulus (Jiang *et al.*, 2000b). Upon autophosphorylation, the phosphate is transferred by two intermediates, Spo0F and Spo0B and finally to Spo0A (Burbulys *et al.*, 1991). The phosphotransfer reactions or the phosphoproteins are subject to regulation by phosphatases, where one group, the Rap phosphatases, are regulated by pentapeptides (Perego, 1998). The Rap phosphatases specifically dephosphorylate Spo0A~P, while another three phosphatases (Spo0E, YisI and YnzD) attack Spo0A~P.

The *ftsH* gene coding for a membrane-anchored metalloprotease is present in most if not all bacterial species (Schumann, 1999;Ogura and Wilkinson, 2001). The FtsH protein and its biochemical and biological functions have been studied in detail in *E. coli*. It carries two transmembrane segments close to its N-terminal end which anchor this protein into the cytoplasmic membrane in such a way that both its short N- and its long C- terminus are exposed into the cytoplasm (Tomoyasu *et al.*, 1993a). The C-terminal part contains a Walker A and B box, involved in binding and hydrolysis of ATP (Tomoyasu *et al.*, 1993b), and a binding site for Zn^{2+}. The *Aquifex aeolicus* FtsH protein devoid of its transmembrane segments has been crystallized and shown to form a ring-like hexameric structure (Suno *et al.*, 2006). While the *ftsH* gene in *E. coli* is essential (Ogura *et al.*, 1999), a *B. subtilis ftsH* knockout is viable, but displays a pleiotropic phenotype (Deuerling *et al.*, 1997). Cells with an *ftsH* null allele are sensitive to heat- and osmotic stress, grow largely as filaments and last, but not least, exhibit a significantly reduced sporulation frequency. Here, we started to analyze the role of *ftsH* during sporulation. So far, we could show that *ftsH* interferes with the synthesis or/and phosphorylation of Spo0A. Based on this result we hypothesize that the FtsH protease has to degrade one or more proteins involved, directly or indirectly, in the production of a sufficient amount of active Spo0A. To this end, we have identified the Spo0E phosphatase as one of the targets of FtsH, and our observations indicate that the C-terminus of Spo0E is necessary for degradation. Furthermore, we show that FtsH is needed only during stage 0.

Results

In the absence of the FtsH metalloprotease only small amounts of inactive Spo0A are present during the onset of sporulation

Based on the analysis of transcriptional fusions, we concluded that *ftsH* interferes with the synthesis or activity of Spo0A (Deuerling *et al.*, 1997). Next, we attempted to

identify the gene(s) responsible for this effect. We first measured the sporulation frequencies in the wild-type and the *ftsH* knockout strain (Table 1). While about 59% of the cells in our wild-type strain were able to form heat-resistant spores, the sporulation frequency dropped by five orders of magnitude in the absence of the *ftsH* allele confirming earlier data (Deuerling *et al.*, 1997) and further underlining the importance of the metalloprotease for the sporulation process. Next, we measured the amount of Spo0A in both strains by Western blotting. As can be seen from Fig. 1, Spo0A started to be present from stage 0 on and continued to be produced to at least stage 3. On the contrary, Spo0A is present in greatly reduced amounts in this knockout (Fig. 1). Are the small amounts of Spo0A present in the *ftsH* knockout active that means present in the phosphorylated form? To answer this question, we constructed a transcriptional fusion between the promoter of the *skf* operon and the *lacZ* reporter gene and integrated this fusion ectopically at the *amyE* locus. It has been reported that small amounts of active Spo0A (Spo0A~P) are sufficient to activate the *skf* operon (Fujita *et al.*, 2005). When this fusion was analyzed in the wild-type background, the β-galactosidase activity started to increase from stage 0 on (Fig. 2A). When the same operon fusion was tested in the *ftsH* knockout, only a very low background activity was measured without any increase at least up to t_3 (Fig. 2A). We conclude from these results that *ftsH* interferes with the synthesis or/and activation of Spo0A, where both are interwoven (Strauch *et al.*, 1992). We further assume that FtsH has to degrade one or more proteins which act, directly or indirectly, as negative regulators of the synthesis or/and activation of Spo0A.

The ftsH *interferes with the expression or activity of three Rap phosphatases*

Expression and activation of Spo0A is embedded in a sophisticated network involving a plethora of regulators among them three phosphatases termed RapA, RapB and RapE which specifically dephosphorylate Spo0F~P, the second component of the phosphorelay (Perego, 1998). First, we asked whether *ftsH* influences the sporulation frequencies in the presence or absence of one of the three phosphatases. We constructed knockouts in all three genes as described in the Experimental procedure section. Then, these null alleles were combined with an *ftsH* knockout each, and all six strains were analyzed for their sporulation frequencies, for the amount of Spo0A present and for its activity status. As can be seen from Table 1, the sporulation frequencies in all three *rap* knockouts are higher than that of the wild-type strain and range from 67% to 72%, where the sporulation frequency of wild-type cells was determined to be 59%. A similar observation has been published for RapA and RapE (Jiang *et al.*, 2000a). When the sporulation frequencies in the double knockouts were measured, it turned out to be increased by two to three orders of magnitude as compared to a single *ftsH* null mutant,

but remained below 1% (Table 1). These data clearly indicate an influence of the *ftsH* allele on all three Rap phosphatases.

Next, we analyzed all six strains for the production of Spo0A by Western blotting. Fig. 1 shows the results from a representative assay. The absence of any of the three phosphatases in the otherwise wild-type background exhibited a different outcome. While in the absence of both *rapA* and *rapB* the amount of Spo0A was increased at t_0 as compared to the wild-type situation, its amount was reduced at both t_0 and t_1 in the *rapE* knockout (Fig. 1). When the *ftsH* null allele was added, the amount of Spo0A dropped as already observed for the wild-type strain in the absence of *ftsH* (Fig. 1). Is the Spo0A protein present in the double knockouts active? To answer this question, the P_{skf}-*lacZ* fusion was introduced in all six strains followed by measurement of the β-galactosidase activities of the strains grown in sporulation medium. Expression of the P_{skf}-*lacZ* fusions is somewhat different within the six strains. While expression started in all strains at t_0 and reached its plateau value at t_2 in the wild-type and in the Δ*rapB* strains, it further increased in the Δ*rapA* and the Δ*rapE* strains (Fig. 2). In the presence of Δ*ftsH*, the expression of the operon fusion did not increase over the basal level with the exception of Δ*rapE* where a slight increase to about 20 units was observed (Fig. 2D). We conclude from these results that both the *rapA* and the *rapE* genes are involved in shutting off P_{skf}-*lacZ* at t_2.

The ftsH *gene interferes with the phosphorylation status of Spo0A through Spo0E*

Besides the Rap phosphatases, another set of three phosphates is involved in the specific dephosphorylation of Spo0A~P designated Spo0E, Yis and YnzD, where only the first is active during sporulation (Perego, 2001). While overproduction of Spo0E reduced the sporulation frequency, deletion of *spo0E* resulted in an increase (Perego and Hoch, 1991). We constructed a *spo0E* knockout, combined it with the *ftsH* null allele and measured the sporulation frequencies in both strains. As to be expected the sporulation frequency raised in the absence of the *spo0E* gene above the level observed in the wild-type strain (Table 1). If combined with an *ftsH* knockout, the sporulation frequency was increased 1000-fold over the level measured in the Δ*ftsH* strain, but was still about 100-fold lower than the wild-type level (Table 1). Next, we analyzed for the production of Spo0A in both mutant strains. The Western-blot analysis revealed that Spo0A is present in large amounts already at t_0 in the Δ*spo0E* strain followed by no significant further increase when cells entered the sporulation pathway (Fig. 1). When the Δ*ftsH* allele was added, the amount of Spo0A was reduced at t_0 and further increased to levels comparable to those present in the *ftsH*$^+$ strain (Fig. 1). This result suggests an

interaction between both proteins, either directly or indirectly, thereby influencing expression of *spo0A*. When we tested for the activity of Spo0A in both mutant strains, it turned out to result in a higher activation of the *skf* promoter, but completely failed to activate this promoter in the absence of *ftsH* (Fig. 2E). In conclusion, the absence of an active *spo0E* allele in an *ftsH* knockout leads to an expression of the *spo0A* gene, but the protein remained inactive. These data indicate that *ftsH* influences production of active Spo0A by either allowing its phosphorylation or preventing for its rapid dephosphorylation.

Spo0E is a target protein for FtsH

One possibility to explain the interaction between FtsH and Spo0E is a direct one whereby FtsH degrades Spo0E. To test that possibility, we decided to purify both proteins and to incubate them under conditions where FtsH is able to degrade β-casein (Kotschwar *et al.*, 2005). FtsH was purified with a GST-tag as reported before where the purification tag keeps the protein soluble in the absence of any detergent (Kotschwar *et al.*, 2005). Since we failed to overproduce Spo0E equipped with a His-tag (unpublished data), we decided to add the GST-tag as well. Next, both proteins were incubated in the presence and absence of ATP. While in the absence of ATP, the GST-Spo0E remained stable during a 4 h incubation time, it was largely degraded in the presence of the nucleotide (Fig. 3A). This could be verified by probing some lanes with αGST (Fig. 3B). To rule out the possibility that cleavage occurs at or within the GST tag rather than within Spo0E, this tag was purified and incubated with GST-FtsH. It could be shown that GST remained stable for at least 5 h (data not shown).

As mentioned above, two homologues of Spo0E, YisI and YnzD, are also able to phosphorylate Spo0E (Perego, 2001). Are these two phosphatases also a substrate of FtsH? While β-casein was completely degraded within 5 h of incubation, both GST-YisI and GST-YnzD remained stable under these conditions (Fig. 4). These data clearly demonstrate that neither YisI nor YnzD are substrates of FtsH. It further confirms that the GST-tag is not recognized by FtsH.

The mutant proteins Spo0E11 and Spo0E94 are not degraded by FtsH

The *spo0E11* and *spo0E94* gain-of-function mutations encode overactive phosphatases that inhibit sporulation by specifically dephosphorylating Spo0A~P (Perego and Hoch, 1991;Ohlsen *et al.*, 1994). Both mutations resulted in a stop codon reducing the length of the proteins from 85 to 71 (*spo0E11*) and 59 amino acids (*spo0E94*) (Perego and Hoch, 1987;Ohlsen *et al.*, 1994). To find out whether these two shortened versions of the Spo0E protein are still a target for FtsH, both were tagged with

GST, overproduced in *E. coli* and purified. When these two purified proteins were incubated with FtsH, both remained stable for at least 5 h, while β-casein as a control was degraded under these conditions (Fig. 4). Since the full-length Spo0E protein is unstable when incubated with FtsH, we infer that the C-terminal 25 amino acid residues are responsible for this instability.

The C-terminal end of Spo0E confer target specificity to FtsH

As already mentioned the Spo0E phosphatase is distinguished from the YisI and YnzD phosphatases by a C-terminal extension of about 25 amino acid residues (Perego, 2001). Since Spo0E serves as a target for FtsH, but YisI and YnzD not, we asked whether the C-terminal extension of Spo0E is responsible for recognition by FtsH. To answer this question, we fused the coding region for the C-terminal 25 amino acids to *ynzD* (YnzD-0E). The GST-tagged hybrid protein was overproduced in *E. coli*, purified by affinity chromatography and incubated with GST-FtsH. As can be seen from Fig. 6 (lane 6), the YnzD-0E is largely degraded over time. We conclude from this experiment that indeed the C-terminal end of Spo0E contains the recognition sequence for the FtsH protease.

Does the absence of spo0E influence expression of yisI or/and ynzD?

The *yisI* and *ynzD* genes have been reported to be expressed during the vegetative growth phase while *spo0E* is induced at around t_0 (Perego and Hoch, 1987). We asked whether there is a crosstalk between these genes concerning their expression level. Is there increased expression of either *yisI* or/and *ynzD* in a *spo0E* knockout? First, we fused the promoters of the two genes to *lacZ* and integrated both transcriptional fusions at the *amyE* locus. Next, the *spo0E* knockout was introduced into both strains. Then, all four strains (see Table 3) were grown in DSM, samples were taken from t_0 up to t_3, and the β-galactosidase activity was determined. While no difference was measured for the *ynzD* promoter independent of the presence or absence of the *spo0E* allele (Fig. 7B), there was a slight increase in the transcription of the *yisI* gene (Fig. 7A) in the absence of *spo0E*. To conclude removal of the *spo0E* gene did not influence expression of the two other genes significantly excluding a crosstalk at the transcriptional level.

Does the spo0A-sad67 allele allow successful sporulation in the ftsH knockout?

Several *spo0A* mutations have been isolated and analyzed among them those which are active in the absence of phosphorylation. One of these mutations, *spo0A-sad67D56N*, carries an internal in-frame deletion removing amino acids 63 through 81 and a point mutation exchanging the aspartate to an asparagine (Ireton *et al.*, 1993). The

aspartate residue at position 56 of Spo0A acts as the phosphorylation site (Burbulys *et al.*, 1991) and is dispensable in the *spo0A-sad67* allele (Ireton *et al.*, 1993). We asked whether an *ftsH* knockout strain is able to form spores in the presence of the *spo0A-sad67D56N* allele. We measured the sporulation frequencies in strain SIK190 which carries the *spo0A-sad67D56N* allele fused to an IPTG-inducible promoter. While a sporulation frequency of 0.06% was measured in the absence of IPTG (Table 2), induction of the mutant allele at t_0 resulted in 37% heat-resistant cells. Then, the *ftsH*::*tet* knockout was introduced into SIK190 (SIK190F) and the sporulation frequency was determined. While the sporulation frequency was low in the absence of IPTG, it was high after IPTG-induction and both values were comparable to those measured in the *ftsH* wild-type strain (Table 2). These results strongly suggest that *ftsH* is needed only during phase 0 and that the *spo0A-sad67D56N* allele can be expressed in the absence of *ftsH*.

Measure activation of the two promoters (Ps and Pv) preceding the spo0A *gene*

Transcription of the *spo0A* gene is initiated at two different promoters termed P*v* and P*s* (Ferrari *et al.*, 1985;Kudoh *et al.*, 1985). While P*v* is recognized by the housekeeping sigma factor σ^A and functions as a low-level promoter to produce a maintenance level of the Spo0A protein during exponential growth (Yamashita *et al.*, 1989), the second promoter, P*s*, is recognized by the stationary sigma factor σ^H. This promoter is required for induction of the protein at the end of exponential growth and during stage 0 (Ferrari *et al.*, 1985;Yamashita *et al.*, 1989) and is further activated indirectly by Spo0A~P, which represses expression of *abrB*, a negative regulator of *sigH* (Perego *et al.*, 1988). Therefore, phosphorylation of Spo0A at the onset of sporulation activates an autoregulatory loop leading to an increase in activated Spo0A. We asked whether *ftsH* or/and *spo0E* influences transcription at either promoter. Both promoters were separately fused to *lacZ* and the transcriptional fusions were ectopically integrated at the *amyE* locus. Then, either the *ftsH* or the *spo0E* knockouts or both were added and the β-galactosidase activities were determined in all eight strains. While the β-galactosidase activity initiated at P*v* increased slightly up to t_1 followed by a modest decrease in the wild-type strain, its activity was reduced to about 50% in the *ftsH* knockout (Fig. 8A). While the enzymatic activity in the *spo0E* null mutant was comparable to that measured in the wild-type strain, addition of the *spo0E* null allele to that of *ftsH* resulted in a slight increase in the β-galactosidase activity (Fig. 8A). In summary, the influence of both *spo0E* and *ftsH* on the P*v* promoter is minor.

Next, we measured the β-galactosidase activity of *lacZ* fused to the P*s* promoter. In the wild-type background, this promoter is induced about 7-fold between t_{-1} and t_1 (Fig. 8B). In the absence of *spo0E*, it is induced about 10-fold, while an only 3-fold

induction was measured in the *ftsH* knockout which was not increased in the double knockout (Fig. 8B). To conclude transcription at P*s* is strongly reduced in Δ*ftsH* which is not compensated by Δ*spo0E*. In total, *ftsH* influences only transcription at P*s* most probably through the strongly reduced level of active Spo0A which is needed as part of the autoregulatory loop.

Discussion

When *B. subtilis* cells enter the transition phase, several different genetic programs are activated including the production of extracellular enzymes and peptide antibiotics, cells become motile and competent, and, as the response of last resort, initiate the process of spore formation. It has been shown that cells either become competent or sporulate, never both together (Errington, 1993). It is also known that never 100% of the cells sporulate. The decision to sporulate or not to sporulate is dependent on the amount of active Spo0A at the end of stage 0 which takes about 2 h. Sporulating cells sense a multitude of mostly unknown signals including the metabolic state, the fate of the chromosomes, the cell density and others, integrate and process these signals by the phosphorelay which controls the level of phosphorylated Spo0A. Cells able to synthesize active Spo0A above a threshold value have been called Spo0A-ON and those which fail to do so Spo0A-OFF (Chung *et al.*, 1994). The formation of two subpopulations of otherwise isogenic cells is designated bistability (Smits *et al.*, 2006;Dubnau and Losick, 2006). But it has to be questioned whether cells in the transition phase exhibit multi- rather than bistability based on the observation that so different genetic programs are activated. This can be tested by double and triple labelling using transcriptional fusions between program-specific promoters and *gfp* and its derivatives (Margolin, 2000).

Several years ago, we discovered that the sporulation frequency in an *ftsH* knockout is reduced by about five orders of magnitude (Deuerling *et al.*, 1997). The *ftsH* gene codes for a membrane-anchored ATP-dependent metalloprotease which seems to be present in all bacterial species (Schumann, 1999;Ogura and Wilkinson, 2001). The objective of this ongoing research project is to elucidate the role of the FtsH protease during sporulation of *B. subtilis* cells. It is based on the assumption that FtsH has to degrade or to regulate the steady-state level of one or more proteins negatively interfering with the sporulation program. To this end we could show that the activity of FtsH is needed only during phase 0 which culminates in the Spo0A-ON status. Cells carrying an *ftsH* knockout synthesize a significantly reduced amount of Spo0A which, based on genetic data, is inactive. This observation explains why *ftsH* null mutants exhibit a dramatically reduced sporulation frequency. But *ftsH* could also play a role

during subsequent sporulation stages. This possibility could be ruled out by introduction of an *ftsH* null allele in a strain carrying a mutant *spo0A* allele which is active in the absence of phosphorylation. Upon expression of the *spo0A-sad67D56N* allele, cells exhibited a normal sporulation frequency. Therefore, the role of *ftsH* is exclusively confined to the synthesis or/and activation of wild-type Spo0A.

The next question to be raised is why the amount of Spo0A is significantly reduced and why this reduced amount is inactive? So far, we used the candidate approach to identify putative substrate proteins of FtsH. First, we constructed knockouts for four different phosphatases where it has already been published that the sporulation frequencies is slightly, but reproducibly enhanced in their absence of two of them (Jiang *et al.*, 2000a); these results could be confirmed and extended for two additional phosphatases. Upon introduction of an *ftsH* null allele into these four mutant strains, the sporulation frequencies were increased by two to three orders of magnitude, but still remained very low. Not surprisingly, no increase in the amount of active Spo0A could be measured. We would like to conclude that *ftsH* interferes directly or indirectly with expression or activity of these four phosphatases. To explain the increases in the sporulation frequencies, we further suggest that a few cells within the whole population are in the Spo0A-ON state. To investigate this possibility, we will fuse the promoter of the *skf* and the *spoIIA* operons to *gfp* and analyze single cells under the fluorescence microscope. Whereas the *skfA* promoter needs a low amount of active Spo0A, the *spoIIA* one requires a high amount to become activated (Fujita *et al.*, 2005).

M. Perego identified two homologs of the Spo0E phosphatase, YisI and YnzD (Perego, 2001). She could show that both phosphatases are able to dephosphorylate Spo0A~P *in vitro*. These two phosphatases are distinguished from Spo0E by two characteristics: First, their genes are expressed during the vegetative growth phase and second, they lack a C-terminal extension of about 25 amino acid residues. She suggested that this C-terminal extension could be recognized by a protease. Based on her assumption and our finding that an *ftsH spo0E* double knockout exhibited the highest sporulation frequency of all tested phosphatase null alleles, we asked the question whether the Spo0E protein is a substrate for FtsH. Both proteins were overproduced in *E. coli* with a GST immobilization tag and incubated under appropriate conditions. We could show that indeed Spo0E is degraded by FtsH. When GST-tagged YisI or YnzD were incubated with FtsH, these proteins turned out to be stable under the same conditions. In the last experiment, we fused the C-terminal extension of Spo0E to YnzD. This fusion protein was shown to be unstable in the presence of FtsH. To conclude, the C-terminal 25 amino acids of Spo0E contain the residues recognized by FtsH.

Which amino acids are recognized by the FtsH protease? One of the substrate proteins of the *E. coli* FtsH protease is LpxC enzyme (Ogura *et al.*, 1999). This enzyme represents the key enzyme in lipopolysacharide (LPS) formation and controls the ration between LPS and phospholipids (Sorensen *et al.*, 1996). Since overproduction of LpxC causes accumulation of abnormal membranes in the periplasm (Ogura *et al.*, 1999) leading to cell death (Sullivan and Donachie, 1984), the amount of LpxC must be carefully regulated which is done by FtsH. Here, the C-terminus has been identified, too, to be responsible for being recognized and degraded by FtsH (Führer *et al.*, 2006). The authors pointed to the about ten amino acids being present at the immediate C-terminus which resemble the SsrA-tag. In the case of Spo0E, there is no similarity to the *B. subtilis* SsrA-tag (Wiegert and Schumann, 2001). Therefore, the amino acid sequence recognized by the FtsH protease is different from that of the SsrA-tag. Experiments are in progress to identify the amino acid residues of Spo0E recognized by FtsH.

Does FtsH fully degrade Spo0E in all cells or does it modulate its steady-state level? Base on our data, we would like to suggest that FtsH regulates the steady-state level of Spo0E rather than completely degrading it. This assumption is based on the observation that a knockout of *spo0E* leads to in increase in the sporulation frequency which should not occur when Spo0E is completely degraded by FtsH.

In conclusion, our results strongly suggest that regulation of stability of several proteins involved directly or indirectly in the synthesis of active Spo0A exerts a new level of posttranslational regulation through the FtsH protease. Another protease has been identified yielding a comparable phenotype. Inactivation of *clpP* resulted in cells deficient in sporulation initiation and in competence and in a highly filamentous morphology (Msadek *et al.*, 1998;Gerth *et al.*, 1998). In such a mutant, the expression of *spo0A* and *spo0H*, coding for the stationary sigma factor σ^H, was significantly decreased (Nanamiya *et al.*, 2000). Introduction of a mutant *spo0E* allele into the *clpP* knockout restored the expression of *spo0A*, but not sporulation. Based on our results, additional genes have to be identified to influence the synthesis of active Spo0A. These genes will be identified with three different experimental strategies: First, the candidate strategy; second, saturated transposon mutagenesis using pMarA (Le Breton Y. *et al.*, 2006); and third, construction of an *ftsH* trap mutant (Flynn *et al.*, 2003). Identification of these additional targets might shed some light on the molecular mechanism of bistability.

Experimental procedures

Bacterial strains, plasmids, media and growth conditions

All strains used in this study are listed in Table 3. *E. coli* DH10B was used for plasmid construction and propagation. *E. coli* A8926 is a derivative of W3110 used for the expression of GST-tagged proteins. The *B. subtilis* strain 1012 was used in most of the experiments. All strains were either grown in Luria-Bertani (LB) or in Difco Sporulation medium (DSM). Antibiotics were added when appropriate at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}; erythromycin, 50 μg ml^{-1}; neomycin, 10 μg ml^{-1}; kanamycin, 20 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}.

Construction of plasmids and recombinant strains

All transcriptional fusions were constructed using the integration vector pDG1728 (Guérout-Fleury *et al.*, 1996). This vector contains a promoter-less *lacZ* and allows insertion of the operon fusions ectopically at the *amyE* locus. Three different promoters were fused to *lacZ* generated by PCR using chromosomal DNA of strain 1012 DNA as template. These promoters are P_{skf} (amplified by primers ON1 and ON2; see Table 4) preceding the *skf* operon which is activated by a low amount of active Spo0A (Fujita *et al.*, 2005), the vegetative and the stationary phase induced promoters P_v (ON3/ON4) and P_s (ON5/ON6), respectively, of the *spo0A* gene (Chibazakura *et al.*, 1991). While P_{skf} was inserted between the *Eco*RI and *Hin*dIII sites of pDG1728, P*v* and P*s* were ligated into the *Eco*RI and *Bam*HI sites.

Knockouts in the four genes *rapA*, *rapB*, *rapE* and *spo0E* were constructed as follows. First, the two flanking regions of each gene (about 300 bp) were amplified (see Table 4 for the primer sequences) and inserted into pBluescript $SKII^+$. Next, the chloramphenicol resistance cassette was amplified using pDG364 as template and inserted between the flanking regions of the three *rap* genes. In the case of the *spo0E* gene, a phleomycin resistance marker generated plasmid pBlue$SKII^+$-phleo was ligated between the two flanking regions. In the last steps, PCR fragments containing the resistance marker and the flanking regions were transformed into *B. subtilis* 1012 followed by selection on LB plates containing either chloramphenicol or phleomycin. Chromosomal DNA was prepared from several transformants each and checked by Southern blotting for replacement of the wild-type alleles. One knockout mutant each was kept for further studies.

Recombinant vectors allowing overexpression and purification of GST-tagged proteins were prepared using pGEX-2T. The genes *ftsH* (ON21/22), *spo0E* (ON23/ON24), *yisI* (ON25/26) and *ynzD* (ON29/ON30) were amplified using chromosomal DNA of strain 1012. The mutants *spo0E11* and *spo0E94* carry stop codons at positions 72 and 60, respectively (Perego and Hoch, 1987). The two truncated versions were generated by amplification of the appropriate coding region (*spo0E11*:

ON23/28; *spo0E94*: ON23/27) followed by a stop codon. To construct a *ynzD-spo0E* fusion gene where the coding region for the last 25 amino acid residues of *spo0E* were fused to *ynzD* using ON31/ON32. All amplicons were fused in-frame to the coding region of GST.

Expression and purification of GST tagged proteins

The recombinant pGEX-2T plasmids were transformed into the *E. coli* strain A8296 (Tatsuta *et al.*, 1998). This strain carries an *ftsH* knockout to avoid production of two types of FtsH proteins, one encoded by the *E. coli* and the other by the *B. subtilis* gene, Expressions were induced by adding 1 mM IPTG (final concentration) to the cell cultures at an OD_{600} of 0.8 followed by were transfer of the cultures to 25^{o}C and further growth overnight. GST-tagged proteins were purified by binding to glutathione (GSH)-agarose beads followed by elution with GSH. A detailed description of the purification procedure was published (Teff *et al.*, 2000).

Proteolysis experiments

Degradation reactions were performed as previously described (Tomoyasu *et al.*, 1995). The complete reaction mixture (30 µl) consisted of the following components: 50 mM Tris-acetate (pH 8.0), 5 mM magnesium acetate, 12.5 µM zinc acetate, 80 mM NaCl, 1.4 mM β-mercaptoethanol, 5 mM ATP, 50 µg/ml bovine serum albumin (BSA), 100 µg/ml of the target GST-tagged protein (or 100 µg/ml β-casein serving as a positive control to verify the proteolytic activity of purified GST-FtsH), 50 µg/ml of purified *B. subtilis* GST-FtsH and 1 µl EDTA-free "Complete" inhibitor mix (Roche Diagnostics) solution. The solution was prepared from one Inhibitor tablet dissolved in 1 ml of water. Reactions were performed at 40°C for the time points indicated. Aliquots of the reaction mixtures were analyzed by 15% SDS-PAGE followed by staining with Coomassie blue.

β-galactosidase assay

Cells were grown in DSM medium at 37P^{o}C and samples were collected at the indicated time points. β-galactosidase assays were performed in triplicate on soluble extracts using substrate *o*-nitrophenyl-β-D-galactosidase as described (Miller, 1972) and yielded comparable results. The activities of one representative experiment are presented each. The β-galactosidase activities are given in units where one unit was defined as ΔE_{405}*min^{-1} *OD_{578}^{-1}*10^{-3}, in which OD_{578} is the optical density of the growth culture.

Western blots

Western blotting was carried out as described previously (Towbin *et al.*, 1979) except that immunoblots were developed by an ECL Western blotting detection kit (Amersham) according to the manufacturer's instructions. Polyclonal anti-Spo0A and anti-HtpG antibodies were used for the detection of Spo0A and HtpG, respectively.

Determination of the sporulation frequencies

Sporulation frequencies were determined by the heat resistance assay as described (Harwood and Cutting, 1990). Briefly, the strains were inoculated in 50 ml DSM and incubated at 37^{o}C for 36 h. Cells were serially diluted in potassium phosphate buffer (10 mM potassium phosphate buffer, pH 7.4 supplemented with 50 mM KCl and 1 mM $MgSO_4$), and 100 μl samples of appropriate dilutions were plated on DSM agar to calculate the amount of vegetative cells. Furthermore, cells were challenged to 80^{o}C for 20 min, and then 100 μl samples were plated on DSM agar.

Acknowledgements

We would like to thank Dr. M. Fujita and Dr. I. Barak for their generous gift of α Spo0A antibodies, Andreas Brandl for the construction of the *spo0E* knockout and the Deutsche Forschungsgemeinschaft (Schu 414/20-2) and the EU (LSGH-CT2004-503468) for financial support.

References

Burbulys,D., Trach,K.A., and Hoch,J.A. (1991) Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell* **64**: 545-552.

Chibazakura,T., Kawamura,F., and Takahashi,H. (1991) Differential regulation of *spoOA* transcription in *Bacillus subtilis*: Glucose represses promoter switching at the initiation of sporulation. *J Bacteriol* **173**: 2625-2632.

Chung,J.D., Stephanopoulos,G., Ireton,K., and Grossman,A.D. (1994) Gene expression in single cells of Bacillus subtilis: evidence that a threshold mechanism controls the initiation of sporulation. *J Bacteriol* **176**: 1977-1984.

Deuerling,E., Mogk,A., Richter,C., Purucker,M., and Schumann,W. (1997) The *ftsH* gene of *Bacillus subtilis* is involved in major cellular processes such as sporulation, stress adaptation and secretion. *Mol Microbiol* **23**: 921-933.

Dubnau,D., and Losick,R. (2006) Bistability in bacteria. *Mol Microbiol* **61**: 564-572.

Errington,J. (2003) Regulation of endospore formation in *Bacillus subtilis*. *Nat Rev Microbiol* **1**: 117-126.

Errington,J. (1993) *Bacillus subtilis* sporulation: Regulation of gene expression and control of morphogenesis. *Microbiol Rev* **57**: 1-33.

Ferrari,F.A., Trach,K., LeCoq,D., Spence,J., Ferrari,E., and Hoch,J.A. (1985) Characterization of the *spo0A* locus and its deduced product. *Proc Natl Acad Sci USA* **82**: 2647-2651.

Flynn,J.M., Neher,S.B., Kim,Y.I., Sauer,R.T., and Baker,T.A. (2003) Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. *Mol Cell* **11**: 671-683.

Fuhrer,F., Langklotz,S., and Narberhaus,F. (2006) The C-terminal end of LpxC is required for degradation by the FtsH protease. *Mol Microbiol* **59**: 1025-1036.

Fujita,M., Gonzalez-Pastor,J.E., and Losick,R. (2005) High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J Bacteriol* **187**: 1357-1368.

Gerth,U., Krüger,E., Derré,I., Msadek,T., and Hecker,M. (1998) Stress induction of the *Bacillus subtilis clpP* gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance. *Mol Microbiol* **28**: 787-802.

Guérout-Fleury,A.M., Frandsen,N., and Stragier,P. (1996) Plasmids for ectopic integration in *Bacillus subtilis*. *Gene* **180**: 57-61.

Harwood,C.R., and Cutting,S. (1990) Molecular biological methods for *Bacillus* Chichester, England: John Wiley & Sons.

Hoch,J.A. (1993) Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. *Annu Rev Microbiol* **47**: 441-465.

Ireton,K., Rudner,D.Z., Siranosian,K.J., and Grossman,A.D. (1993) Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. *Genes Dev* **7**: 283-294.

Jiang,M., Grau,R., and Perego,M. (2000a) Differential processing of propeptide inhibitors of Rap phosphatases in *Bacillus subtilis*. *J Bacteriol* **182**: 303-310.

Jiang,M., Shao,W., Perego,M., and Hoch,J.A. (2000b) Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Mol Microbiol* **38**: 535-542.

Kotschwar,M., Harfts,E., Ohanjan,T., and Schumann,W. (2005) Construction and analyses of mutant *ftsH* alleles of *Bacillus subtilis* involving the ATPase- and Zn-binding domains. *Curr Microbiol* **49**: 180-185.

Kudoh,J., Ikeuchi,T., and Kurahashi,K. (1985) Nucleotide sequences of the sporulation gene spo0A and its mutant genes of *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **82**: 2665-2668.

Le Breton Y., Mohapatra,N.P., and Haldenwang,W.G. (2006) In Vivo Random Mutagenesis of Bacillus subtilis by Use of TnYLB-1, a mariner-Based Transposon. *Appl Environ Microbiol* **72**: 327-333.

Margolin,W. (2000) Green fluorescent protein as a reporter for macromolecular localization in bacterial cells. *Methods* **20**: 62-72.

Miller,J.H. (1972) Experiments in Molecular Genetics Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Molle,V., Fujita,M., Jensen,S.T., Eichenberger,P., Gonzalez-Pastor,J.E., Liu,J.S., and Losick,R. (2003) The Spo0A regulon of *Bacillus subtilis*. *Mol Microbiol* **50**: 1683-1701.

Msadek,T., Dartois,V., Kunst,F., Herbaud,M.-L., Denizot,F., and Rapoport,G. (1998) ClpP of *Bacillus subtilis* is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation. *Mol Microbiol* **27**: 899-914.

Nanamiya,H., Takahashi,K., Fujita,M., and Kawamura,F. (2000) Deficiency of the initiation events of sporulation in *Bacillus subtilis clpP* mutant can be suppressed by a lack of the Spo0E protein phosphatase. *Biochem Biophys Res Commun* **279**: 229-233.

Ogura,T., Inoue,K., Tatsuta,T., Suzaki,T., Karata,K., Young,K. *et al.* (1999) Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in *Escherichia coli*. *Mol Microbiol* **31**: 833-844.

Ogura,T., and Wilkinson,A.J. (2001) AAA^+ superfamily ATPases: common structure-diverse function. *Genes to Cells* **6**: 575-597.

Ohlsen,K.L., Grimsley,J.K., and Hoch,J.A. (1994) Deactivation of the sporulation transcription factor Spo0A by the Spo0E protein phosphatase. *Proc Natl Acad Sci USA* **91**: 1756-1760.

Perego,M. (2001) A new family of aspartyl phosphate phosphatases targeting the sporulation transcription factor Spo0A of *Bacillus subtilis*. *Mol Microbiol* **42**: 133-143.

Perego,M. (1998) Kinase-phosphatase competition regulates *Bacillus subtilis* development. *Trends Microbiol* **6**: 366-370.

Perego,M., and Hoch,J.A. (1987) Isolation and sequence of the *spo0E* gene: its role in initiation of sporulation in *Bacillus subtilis*. *Mol Microbiol* **1**: 125-132.

Perego,M., and Hoch,J.A. (1991) Negative regulation of *Bacillus subtilis* sporulation by the *spoOE* gene product. *J Bacteriol* **173**: 2514-2520.

Perego,M., Spiegelman,G.B., and Hoch,J.A. (1988) Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spoOA* sporulation gene in *Bacillus subtilis*. *Mol Microbiol* **2**: 689-699.

Phillips,Z.E.V., and Strauch,M.A. (2002) *Bacillus subtilis* sporulation and stationary phase gene expression. *Cell Mol Life Sci* **59**: 392-402.

Piggot,P.J., and Hilbert,D.W. (2004) Sporulation of *Bacillus subtilis*. *Curr Opin Microbiol* **7**: 579-586.

Saito,H., Shibata,T., and Ando,T. (1979) Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg. *Mol Gen Genet* **170**: 117-122.

Schumann,W. (1999) FtsH - a single-chain charonin? *FEMS Microbiol Rev* **23**: 1-11.

Smits,W.K., Kuipers,O.P., and Veening,J.W. (2006) Phenotypic variation in bacteria: the role of feedback regulation. *Nat Rev Microbiol* **4**: 259-271.

Sorensen,P.G., Lutkenhaus,J., Young,K., Eveland,S.S., Anderson,M.S., and Raetz,C.R. (1996) Regulation of UDP-3-O-[R-3-hydroxymyristoyl]-N-acetylglucosamine deacetylase in Escherichia coli. The second enzymatic step of lipid a biosynthesis. *J Biol Chem* **271**: 25898-25905.

Strauch,M.A., Trach,K.A., and Hoch,J.A. (1992) Spo0A activates and represses its own synthesis by binding at its dual promoters. *Biochimie* **74**: 619-626.

Sullivan,N.F., and Donachie,W.D. (1984) Transcriptional organization within an Escherichia coli cell division gene cluster: direction of transcription of the cell separation gene envA. *J Bacteriol* **160**: 724-732.

Suno,R., Niwa,H., Tsuchiya,D., Zhang,X., Yoshida,M., and Morikawa,K. (2006) Structure of the whole cytosolic region of ATP-dependent protease FtsH. *Mol Cell* **22**: 575-585.

Tatsuta,T., Tomoyasu,T., Bukau,B., Kitagawa,M., Mori,H., Karata,K., and Ogura,T. (1998) Heat shock regulation in the *ftsH* null mutant of *Escherichia coli*: dissection of stability and activity control mechanisms of s^{32} *in vivo*. *Mol Microbiol* **30**: 583-594.

Teff,D., Koby,S., Shotland,Y., Ogura,T., and Oppenheim,A.B. (2000) A colicin-tolerant *Escherichia coli* mutant that confers Hfl phenotype carries two mutations in the region coding for the C-terminal domain of FtsH (HflB). *FEMS Microbiol Lett* **183**: 115-117.

Tomoyasu,T., Gamer,J., Bukau,B., Kanemori,M., Mori,H., Rutman,A.J. *et al.* (1995) *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor s^{32}. *EMBO J* **14**: 2551-2560.

Tomoyasu,T., Yamanaka,K., Murata,K., Suzaki,T., Bouloc,P., Kato,A. *et al.* (1993a) Topology and subcellular localization of FtsH protein in *Escherichia coli*. *J Bacteriol* **175**: 1352-1357.

Tomoyasu,T., Yura,T., Morimura,S., Mori,H., Yamanaka,K., Niki,H. *et al.* (1993b) The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J Bacteriol* **175**: 1344-1351.

Towbin,H., Staehelin,T., and Gordon,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350-4354.

Wehrl,W., Niederweis,M., and Schumann,W. (2000) The FtsH protein accumulates at the septum of *Bacillus subtilis* during cell division and sporulation. *J Bacteriol* **182**: 3870-3873.

Wiegert,T., and Schumann,W. (2001) SsrA-mediated tagging in *Bacillus subtilis*. *J Bacteriol* **183**: 3885-3889.

Yamashita,S., Kawamura,A., Yoshikawa,H., Takahashi,H., and Kobayashi,Y. (1989) Dissection of the expression signals of the *spo0A* gene of *Bacillus subtilis*: glucose represses sporulation-specific expression. *J Gen Microbiol* **135**: 1335-1345.

Table 1. Effect of different mutations on the sporulation frequency[a]

Strain	Relevant phenotype	Cells/ml	Spores/ml	% Spores
1012	WT	5.8×10^9	3.4×10^9	58.6
WW01	*ftsH*::*erm*	2.5×10^7	1.5×10^2	0.0006
AL31	*rapA*::*cat*	1.4×10^9	1.0×10^9	71.4
AL32	*rapA*::*cat ftsH*::*erm*	5.5×10^8	2.9×10^5	0.053
AL33	*rapB*::*cat*	6.5×10^8	4.7×10^8	72.3
AL34	*rapB*::*cat ftsH*::*erm*	6.7×10^8	5.0×10^6	0.74
AL35	*rapE*::*cat*	1.4×10^9	9.4×10^8	67.1
AL36	*rapE*::*cat ftsH*::*erm*	1.9×10^7	1.6×10^4	0.084
AB07	*spo0E*::*bleo*	1.1×10^8	8.3×10^7	75.5
AB08	*spo0E*::*bleo ftsH*::*erm*	7.6×10^7	6.5×10^5	0.85

Cells were grown in DSM at 37°C, and after 36 h, dilutions of the cell cultures were made and aliquots were plated on LB medium. This gave total viable cell counts (cells per ml). The diluted samples were heated then heated at 80°C for 20 min, and aliquots were plated. This gave heat-resistant spores. The percentage of heat-resistant spores was calculated.

[a] Representative of three different experiments. WT, wild-type

Table 2. Sporulation frequencies of strains expressing *spo0A* active in the absence of phosphorylation[a]

Strain	*ftsH* genotype	IPTG[b] added	Viable cell count	Spore count	% of sporulation
SIK190	+	-	6.0×10^6	3.7×10^3	0.06
SIK190	+	+	2.5×10^{10}	9.3×10^9	37.2
SIK190F	-	-	4.8×10^6	2.4×10^3	0.05
SIK190F	-	+	1.7×10^{10}	5.7×10^9	33.5

See legend to Table 1 for technical details.

[a] Representative of three different experiments

[b] IPTG was added at a final concentration of 1 mM

Table 3. Bacterial strains and plasmids used in this study

Plasmid or strain	Relevant genotype	Source
Strains		
E. coli		
DH10B	*mcrA* Δ(*mrr hsdRMS mcrBC*) ϕ80*d lacZ*M15 Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL endA1 nupG*	Bethesda Research Laboratories, Inc.
A8296	*sfhC zad-220*::Tn*10* Δ*ftsH3*::*kan*	(Tatsuta *et al.*, 1998)
B. subtilis		
1012	*leuA8 metB5 trpC2 hsrM1*	(Saito *et al.*, 1979)
WW01	1012 Δ*ftsH*::*erm*	(Wehrl *et al.*, 2000)
ED04	1012 Δ*ftsH*::*tet*	(Deuerling *et al.*, 1997)
AL31	Δ*rapA*::*cat*	This study
AL32	Δ*rapA*::*cat* Δ*ftsH*::*erm*	This study
AL33	Δ*rapB*::*cat*	This study
AL34	Δ*rapB*::*cat* Δ*ftsH*::*erm*	This study
AL35	Δ*rapE*::*cat*	This study
AL36	Δ*rapE*::*cat* Δ*ftsH*::*erm*	This study
AB07	Δ*spo0E*::*bleo*	A. Brandl
AB08	Δ*spo0E*::*bleo* Δ*ftsH*::*erm*	A. Brandl
AL37	*amyE*::P_{skf}–*lacZ spc*	This study
AL38	*amyE*::P_{skf}–*lacZ spc* Δ*ftsH*::*erm*	This study
AL39	*amyE*::P_{skf}–*lacZ spc* Δ*rapA*::*cat*	This study
AL40	*amyE*::P_{skf}–*lacZ spc* Δ*rapA*::*cat* Δ*ftsH*::*erm*	This study
AL41	*amyE*::P_{skf}–*lacZ spc* Δ*rapB*::*cat*	This study
AL42	*amyE*::P_{skf}–*lacZ spc* Δ*rapB*::*cat* Δ*ftsH*::*erm*	This study
AL43	*amyE*::P_{skf}–*lacZ spc* Δ*rapE*::*cat*	This study
AL44	*amyE*::P_{skf}–*lacZ spc* Δ*rapE*::*cat* Δ*ftsH*::*erm*	This study
AL45	*amyE*::P_{skf}–*lacZ spc* Δ*spo0E*::*bleo*	This study
AL46	*amyE*::P_{skf}–*lacZ spc* Δ*spo0E*::*bleo* Δ*ftsH*::*erm*	This study

AL47	*amyE*::$P_{v(spo0A)}$-*lacZ spc*	This study
AL48	*amyE*::$PB_{v(spo0A)}$-*lacZ spc* Δ*ftsH*::*erm*	This study
AL49	*amyE*::$PB_{v(spo0A)}$-*lacZ spc* Δ*spo0E::bleo*	This study
AL50	*amyE*::$P_{s(spo0A)}$-*lacZ spc*	This study
AL51	*amyE::*$P_{s(spo0A)}$-*lacZ spc* Δ*ftsH*::*erm*	This study
AL52	*amyE::*$P_{s(spo0A)}$-*lacZ spc* Δ*spo0E::bleo*	This study
AL53	*amyE::*P_{yisI}-*lacZ spc*	This study
AL54	*amyE::*P_{yisI}-*lacZ* Δ*spo0E::bleo*	This study
AL55	*amyE::*P_{ynzD}-*lacZ spc*	This study
AL56	*amyE::*P_{ynzD}-*lacZ* Δ*spo0E::bleo*	This study
AL57	*amyE*::$P_{v(spo0A)}$-*lacZ spc* Δ*spo0E::bleo* Δ*ftsH*::*erm*	This study
AL58	*amyE::*$P_{s(spo0A)}$-*lacZ spc* Δ*spo0E::bleo* Δ*ftsH*::*erm*	This study
SIK190	*amyE::(*P_{spac}-*spo0A-sad67D56N cat*), *spo0A::erm*, Em^r, Cm^r	(Ireton *et al.*, 1993)
SIK190F	*ftsH::tet* in SIK190	This study
Plasmids		
pDG1728	Permits transcriptional fusion to *lacZ*	(Guérout-Fleury *et al.*, 1996)
p1728-P*skf*	*amyE::*P_{skf}*–lacZ*	This study
p1728-Pv	*amyE::*$P_{v(spo0A)}$-*lacZ*	This study
p1728-Ps	*amyE::*$P_{s(spo0A)}$-*lacZ*	This study
pGex-2t	Expression vector	Pharmacia
pGST-*ftsH*	P*tac*-GST-*ftsH*	This study
pGST-*spo0E*	P*tac*-GST-*spo0E*	This study
pGST-*yisI*	P*tac*-GST-*yisI*	This study
pGST-*ynzD*	P*tac*-GST-*ynzD*	This study
pGST-*spo0E94*	P*tac* GST-*spo0E94*	This study
pGST-*spo0E11*	P*tac*-GST-*spo0E11*	This study
pGST-*ynzD-*C_{0E}	P*tac*-GST-*ynzD-*C_{0E}	This study
pBluecript *SKII*$^+$	Cloning vector	Stratagene
p*rapA-cat*	pBluescript *SKII*$^+$ with 300 bp up- and downstream flanking regions of *rapA* gene and *cat* cassette	This study
p*rapB-cat*	pBluescript *SKII*$^+$ with 300 bp up- and	This study

	downstream flanking regions of *rapB* gene and *cat* cassette	
p*rapE-cat*	pBluescript *SKII*$^{+}$ with 300 bp up- and downstream flanking regions of *rapE* gene and *cat* cassette	This study

Table 4. Oligonucleotides used in this study

0ligonucleotide primer	Sequences[a]	Description
ON1	ggccatGAATTCttacaggagacttcattcatt (*Eco*RI)	5′ *skfA* promoter
ON2	ggccatAAGCTTaagtaaacctcctctcaattttt (*Hind*III)	3′ *skfA* promoter
ON3	ggccatGAATTCgaaaagtgatcggtgctgtcac (*Eco*RI)	5′ *spo0A* vegetative promoter
ON4	ggccatGGATCCatcttcttttgtatattttaccgta (*Bam*HI)	3′ *spo0A* vegetative promoter
ON5	ggccatGAATTCattcacgtttccttgtttgtcaaa (*Eco*RI)	5′ *spo0A* sporulation promoter
ON6	ggccatGGATCCgtttcttcctccccaaatgtagtt (*Bam*HI)	3′ *spo0A* sporulation promoter
ON7	ggccatAAGCTTttgaggatgaagcagacgattccg (*Hind*III)	5′ *rapA* upstream
ON8	ggccatGAATTCttcgagaagccctgtcagcttgta (*Eco*RI)	3′ *rapA* upstream
ON9	ggccatGGATCCcgaagcgcaaaaaaagtatcgtga (*Bam*HI)	5′ *rapA* downstream
ON10	ggccatTCTAGAatttcatataaacaatctcctctc (*Xba*I)	3′ *rapA* downstream
ON11	ggccatAAGCTTatggccgcgtacgagatcccgtca (*Hind*III)	5′ *rapB* upstream
ON12	ggccatGAATTCatactcagataatccggagatgct (*Eco*RI)	3′ *rapB* upstream
ON13	ggccatGGATCCgccgcgatacggtactatgaaaa (*Bam*HI)	5′ *rapB* downstream
ON14	ggccatCCGCGGtacttcatataaacaatcgttcct (*Sac*II)	3′ *rapB* downstream
ON15	ggccatAAGCTTttgatatcaatcacatcagctgaa (*Hind*III)	5′ *rapE* upstream
ON16	ggccatGAATTCgaagttataataatatgcccgcat (*Eco*RI)	3′ *rapE* upstream
ON17	ggccatGGATCCcaagcaatggattgcttccgcaaa (*Bam*HI)	5′ *rapE* downstream
ON18	ggccatTCTAGAgatttcatacatgcattccccttttcg (*Xba*I)	3′ *rapE* downstream
ON19	ggccatGAATTCcggatttttcgctacgctcaaatcc (*Eco*RI)	5′ *cat* cassette
ON20	ggccatGGATCCatcttcaactaacggggcaggtta (*Bam*HI)	3′ *cat* cassette
ON21	ggccatGGATCCaatcgggtcttgcgtaatacc (*Bam*HI)	5′ *ftsH*

ON22	ggccatAGATCTttactctttcgtatcgtctttcttttc (*Bgl*II)	3´ *ftsH*
ON23	ggccatGGATCCggcggttcttctgaacaagaaa (*Bam*HI)	5´ *spo0E*
ON24	ggccatCCCGGGattatttatttgcatcatatgctggc (*Sma*I)	3´ *spo0E*
ON25	ggccatGGATCCaacagtaaaattgaagaaatga (*Bam*HI)	5´ *yisI*
ON26	ggccatCCCGGGattacatacgggagttttcaagat (*Sma*I)	3´ *yisI*
ON27	ggccatCCCGGGattattgggaatgttcgttttcttgcata (*Sma*I)	3´ 75-nucleotides-shorten *spo0E*
ON28	ggccatCCCGGGattaccacaagcctaatttctttacaaggc (*Sma*I)	3´ 39-nucleotides-shorten *spo0E*
ON29	ggccatGGATCCattagagagcatctattaaaag (*Bam*HI)	5´ *ynzD*
ON30	ggccatCCCGGGttcatcacccgctactgctcga (*Sma*I)	3´ *ynzD*
ON31	ggccatGAATTCaattcaaggccttgtaaagaaattag (*Eco*RI)	5´ C- teminus of *spo0E*
ON32	ggccatGAATTCattatttatttgcatcatatgctggc (*Eco*RI)	3´ C- teminus of *spo0E*

[a] restriction endonuclease sites are shown in capital letters

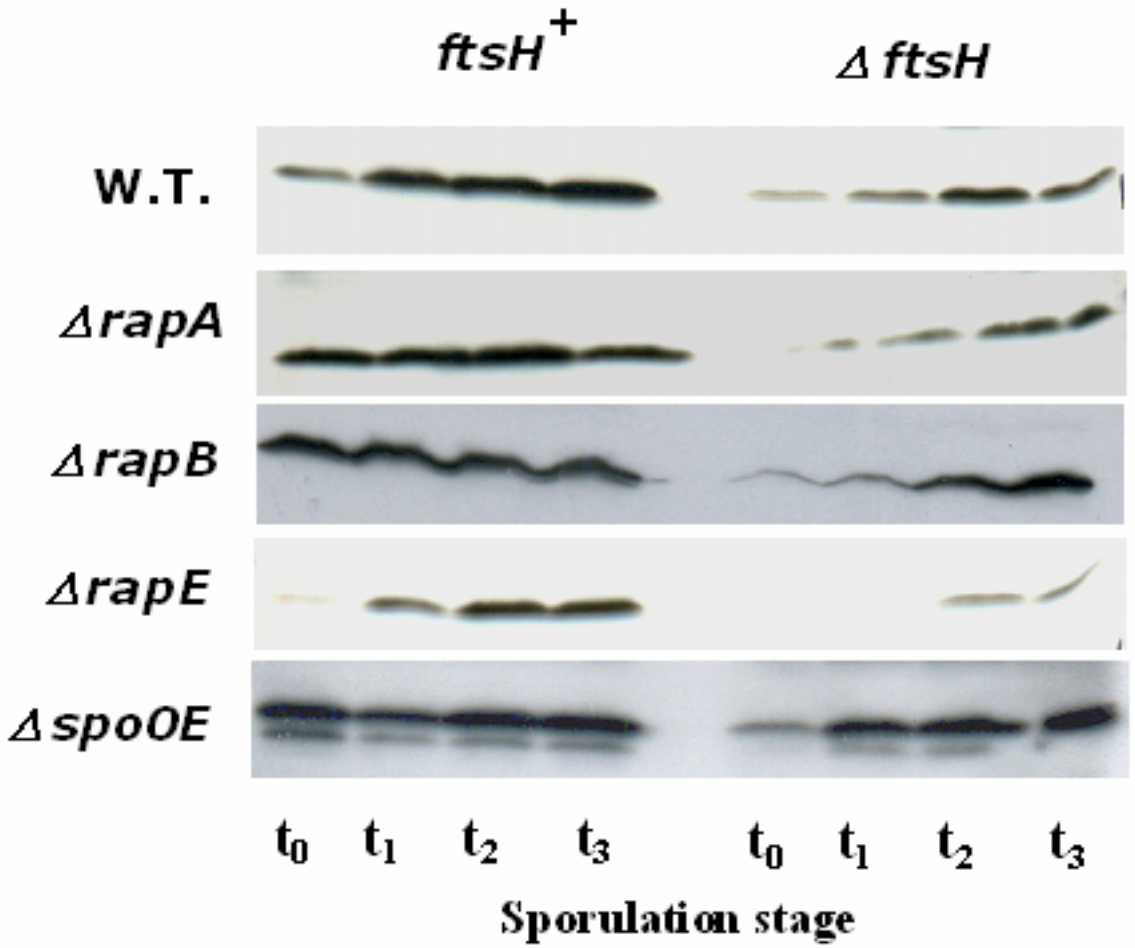

Fig. 1. Amount of Spo0A in different *B. subtilis* strains. The strains were grown in DSM at 37°C, and aliquots were taken after entry into the transition phase (t_0) and up to 3 h later (t_1 to t_3). Cells were lysed by sonication and equal amounts of proteins were applied per lane (5 µg). First line: wild-type 1012 and WW01 (Δ*ftsH*); second line: AL31 (Δ*rapA*) and AL32 (Δ*rapA* Δ*ftsH*); third line: AL33 (Δ*rapB*) and AL34 (Δ*rapB* Δ*ftsH*); fourth line: AL35 (Δ*rapE*) and AL36 (Δ*rapE* Δ*ftsH*); fifth line: AB07 (Δ*spo0E*) and AB08 (Δ*spo0E* Δ*ftsH*).

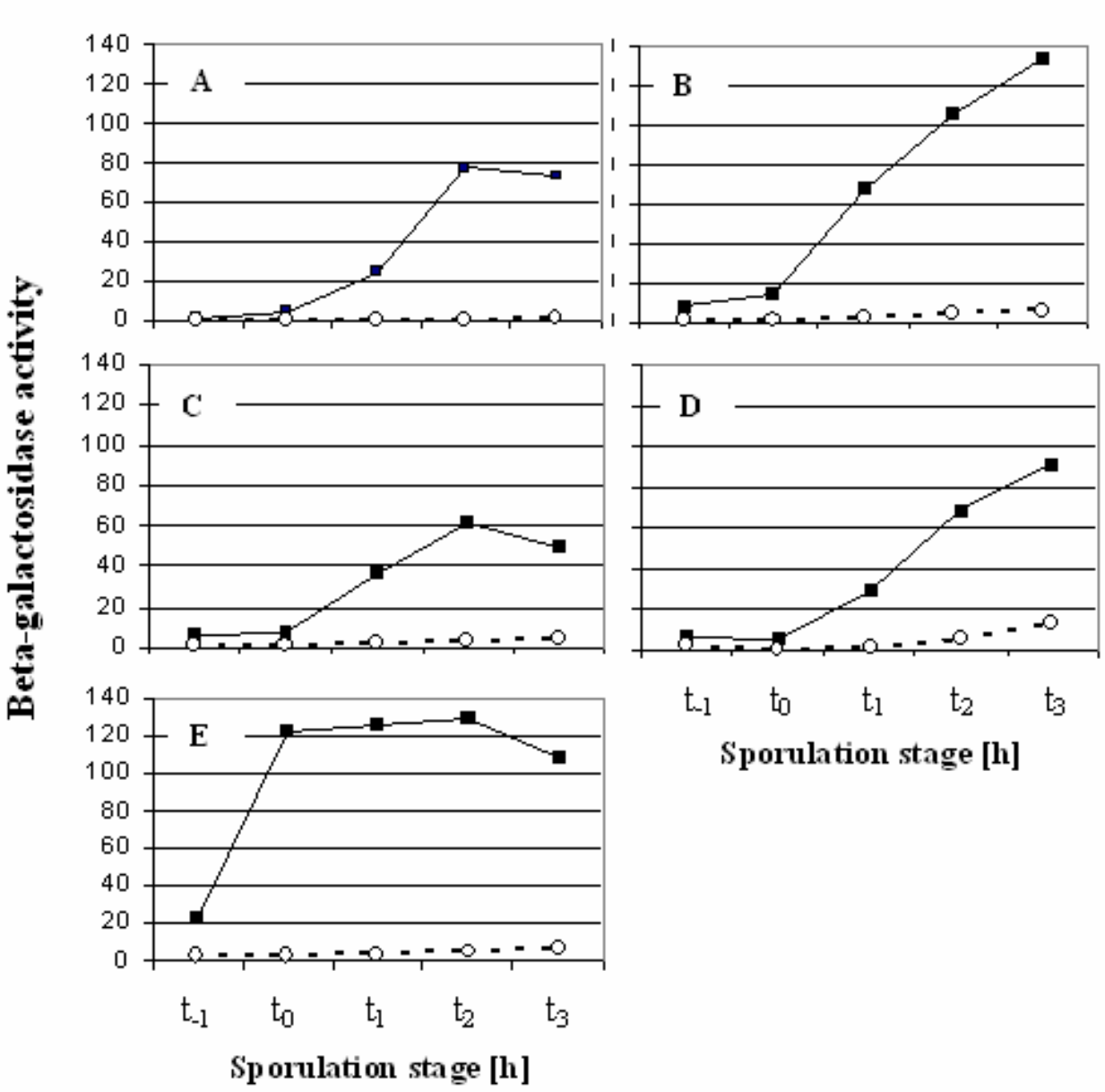

Fig. 2. Transcription from the Spo0A~P-activated promoter *skf*. Cells containing the P*skf-lacZ* fusion integrated ectopically at the *amyE* locus were grown in DSM at 37°C, and aliquots (5 OD_{578} units) were withdrawn for measurement of β-galactosidase activities. (A) AL37 (*ftsH*$^+$) and AL38 (Δ*ftsH*); (B) AL39 (Δ*rapA ftsH*$^+$) and AL40 (Δ*rapA* Δ*ftsH*); (C) AL41 (Δ*rapB ftsH*$^+$) and AL42 (Δ*rapB* Δ*ftsH*); (D) AL43 (Δ*rapE ftsH*$^+$) and AL44 (Δ*rapE* Δ*ftsH*); (E) AL45 (Δ*spo0E ftsH*$^+$) and AL46 (Δ*spo0E* Δ*ftsH*). ■ *ftsH*$^+$, ○ Δ*ftsH*.

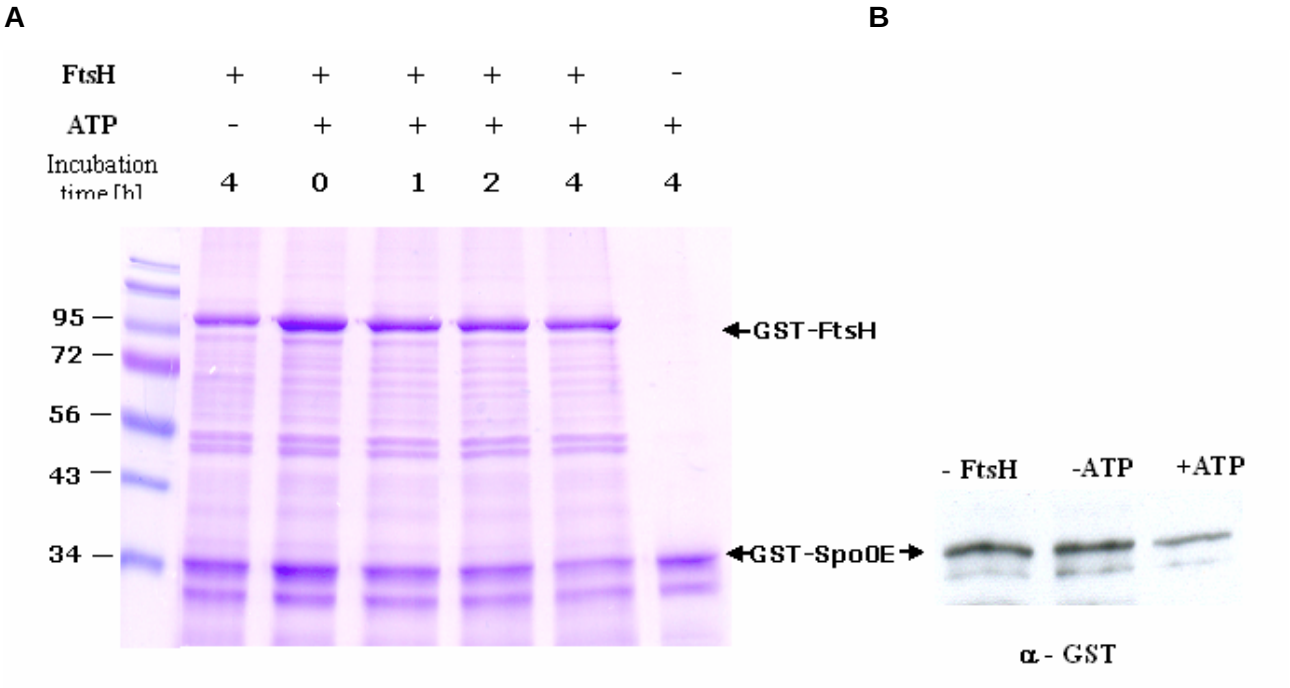

Fig. 3. Spo0E acts as a substrate for FtsH. Purified GST-FtsH was incubated with GST-Spo0E under conditions described in the Experimental procedures. (A) Aliquots of the reaction mixtures were separated by SDS-PAGE and stained with Coomassie brilliant blue. (B) Western blot using αGST and showing GST-Spo0E. The incubation time for all three samples was 4 h.

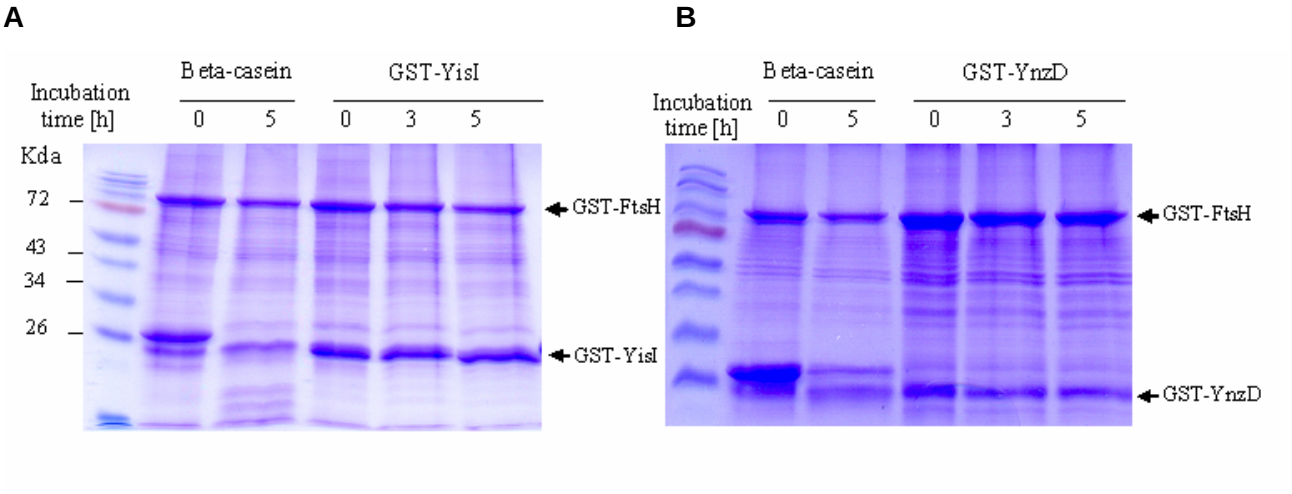

Fig. 4. The phosphatases YisI and YnzD are stable in the presence of FtsH. GST-FtsH was incubated with (A) GST-YisI and (B) GST-YnzD. β-casein served as a control.

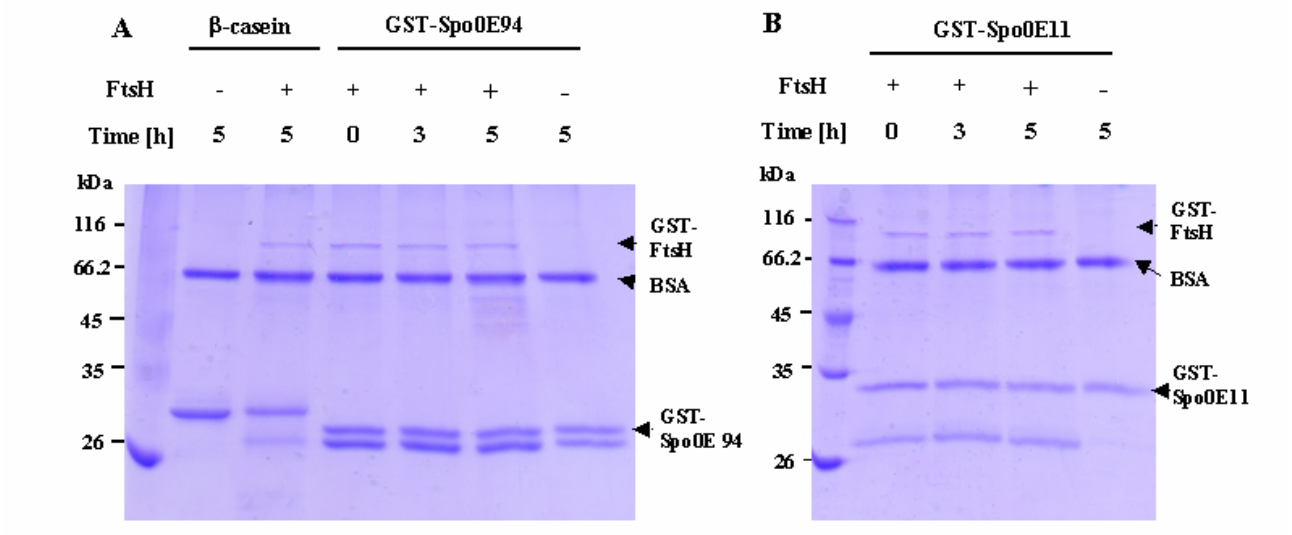

Fig. 5. Two mutant Spo0E proteins are stable in the presence of FtsH. GST-tagged Spo0E94 and Spo0E11 proteins were incubated with FtsH. The reaction products were resolved by SDS-PAGE and stained with Coomassie blue.

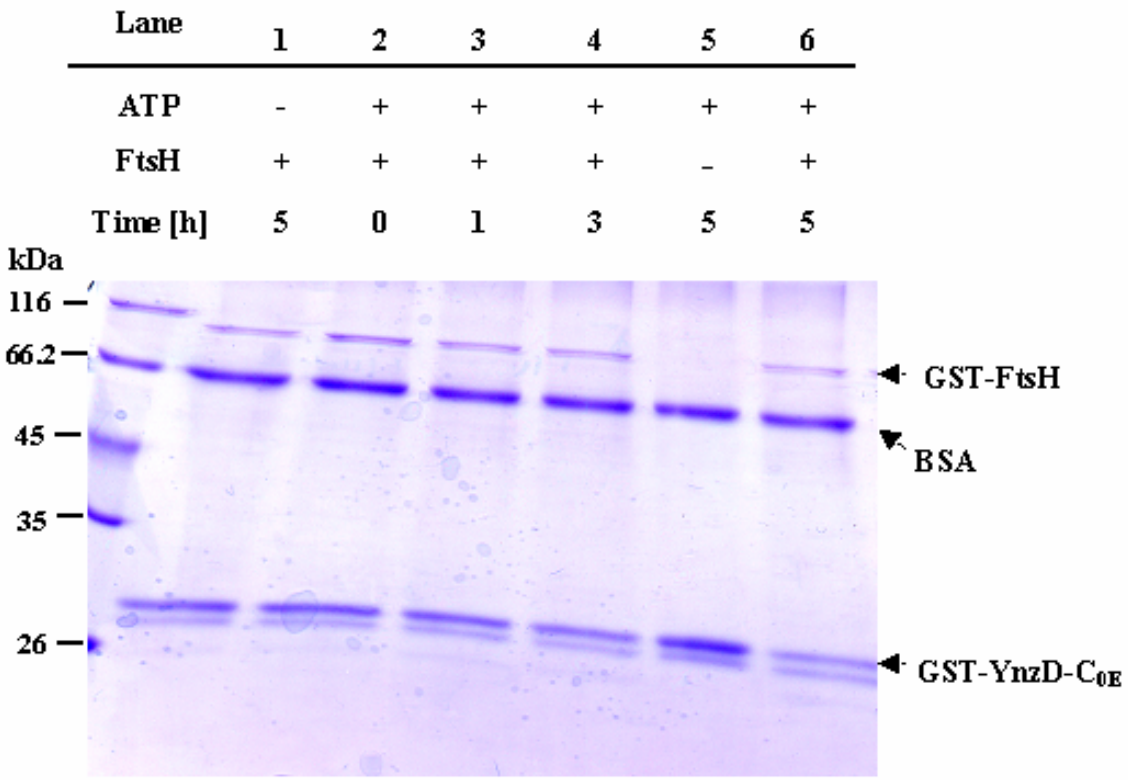

Fig. 6. The C-terminal end of Spo0E confers instability to the YnzD protein. The C-terminal 25 amino acid residues of Spo0E were fused to the C terminus of GST-YnzD. The hybrid protein was overproduced in *E. coli*, purified and incubated with GST-FtsH.

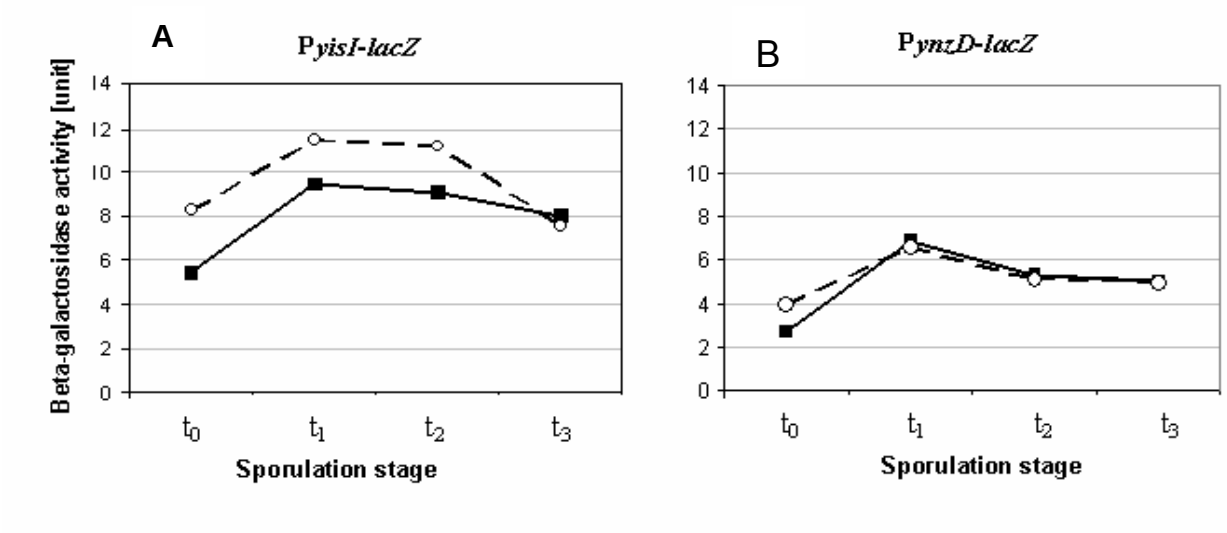

Fig. 7. Transcriptional analysis of the *yisI* and *ynzD* promoters. The *lacZ* reporter gene was transcriptionally fused to the promoter regions of the genes *yisI* and *ynzD* in pDG1728. Strains were grown in DSM and aliquots were taken at the time points indicated for determination of β-galactosidase activity. (A) Strains AL53 and AL54 (P_{yisI}-*lacZ spo0E*$^{+/-}$). (B) Strains AL55 and AL56 (P_{ynzD}-*lacZ spo0E*$^{+/-}$); (■) *spo0E*$^{+}$, (○) *spo0E*$^{-}$. The x-axis is divided into hours preceding or following the end of exponential growth, which is labelled t_0.

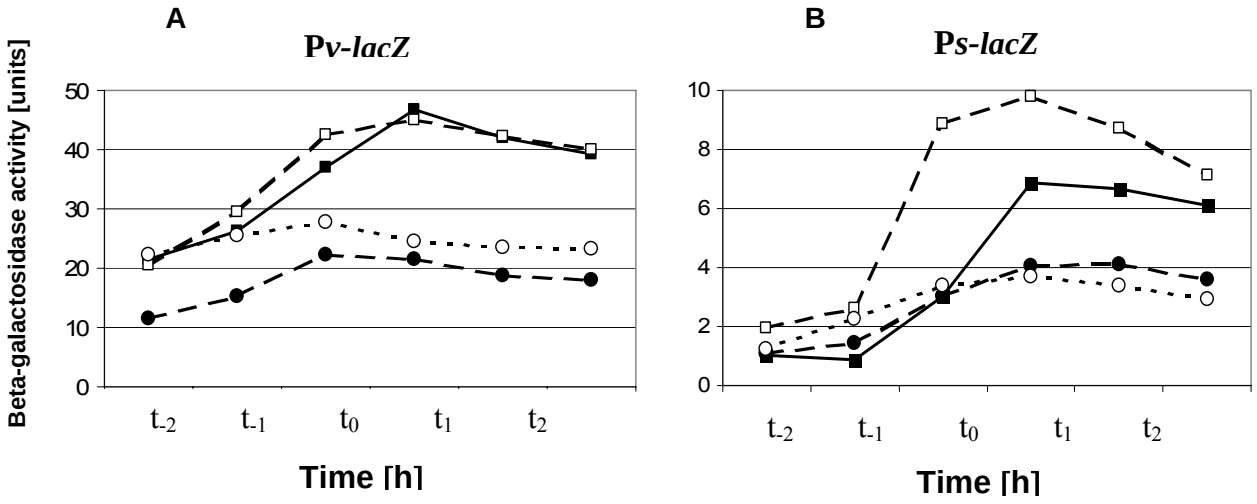

Fig. 8. Transcriptional analysis of the two promoters preceding the *spo0A* gene. Both promoters were separately amplified by PCR and fused transcriptionally to *lacZ* in pDG1728. The x-axis is labelled as in figure 7. (A) Strains AL47 (P*v-lacZ*), AL48 (P*v-lacZ ΔftsH*), AL49 (P*v-lacZ Δspo0E*) and AL57 (P*v-lacZ Δspo0E ΔftsH)*; (B) Strains AL50 (P*s-*

lacZ), AL51 (P*s-lacZ ⊿ftsH*), AL52 (P*s-lacZ ⊿spo0E*) and AL58 (P*s-lacZ ⊿spo0E ⊿ftsH*) (■) WT, (□) *spo0E* $^{-}$, (●) *ftsH* $^{-}$, (○) *spo0E* $^{-}$ *ftsH* $^{-}$.

Part B

Function and Regulation of the *spoVM* Gene of *Bacillus subtilis*

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Submitted to Journal of Bacteriology

Function and Regulation of the *spoVM* Gene of *Bacillus subtilis*

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Keywords: *ftsH*, 5' UTR, *spoIVA*, *spo0A*, sporulation

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The *spoVM* gene of *Bacillus subtilis* codes for a 26-amino-acid peptide which is essential for sporulation. Here, we show that the purified SpoVM peptide inhibits degradation of β-casein by the FtsH protease. Furthermore, SpoIVA, among other proteins, is absent in a *spoVM* knockout. Analysis of the expression of the *spoVM* gene revealed that wild-type cells started to synthesize a *spoVM*-specific transcript at t_2 while the SpoVM peptide accumulated at t_4. Both, the transcript and the peptide were absent from a *spoVM* knockout strain. The 5' untranslated region of the *spoVM* transcript acts as negative regulator of its own translation. The functions of SpoVM during sporulation are discussed.

Development of *Bacillus subtilis* endospores involves a modification of the process of cell division, in which the septum formation switches from the midcell position to an extreme polar position of the developing cell (22). This septum divides the cell into two compartments, designated as forespore and mother cell. Next, a series of proteins produced in the mother cell degrades the asymmetric septum and triggers migration of the membrane around the prespore, a process termed engulfment (22). Again, a large number of sporulation-specific proteins are synthesized in the mother cell and targeted specifically to the mother cell face of the engulfing membrane forming the spore coat later. The coat is a complex macromolecular structure consisting of over 25 different gene products that are packaged in a tight shell around the outside of the spore (6). One of these proteins is the 26-amino-acid peptide SpoVM which is synthesized under control of the mother cell-specific transcription factor σ^E and almost quantitatively localized to the engulfing membrane (14, 26). After completion of engulfment, the forespore is separated from the mother cell by two phospholipid bilayers known as the inner and outer forespore membranes, and SpoVM decorates the outer forespore membrane. Cells carrying a *spoVM* deletion produce a dysfunctional spore that is not heat-resistant (14). The same phenotype is observed when the three N-terminal proximal amino acids that mediate localization of SpoVM were substituted strongly indicating that spore morphogenesis is dependent on proper localization of the peptide (26). Using one of the mislocalization mutants, an extragenic suppressor was isolated and mapped in the *spoIVA* gene which turned out to be allele-specific (17). Evidence has been presented that SpoVM forms an amphipathic α-helix (16) and associates with the membrane via hydrophobic interactions (17). It has been proposed that SpoVM serves as membrane anchor that tethers SpoIVA to the outer forespore membrane. SpoIVA itself creates a basement layer for the entire spore coat (6).

Besides serving as a membrane anchor, a second function has been suggested for SpoVM. When a transposon insertion within *spoVM* which is biologically inactive was used to identify possible interacting proteins, the membrane-bound FtsH metalloprotease was identified (3). Mutations in *ftsH* suppressed the sporulation defect of certain *spoVM* mutants but not others, another case of allele-specific extragenic suppressors. Furthermore, it could be shown that chemically synthesized SpoVM is able to inhibit degradation of σ^{32} by purified *E. coli* FtsH (3). Based on these findings, one can assume that a second function of SpoVM is to inhibit the *B. subtilis* FtsH protease late during sporulation. This assumption is sustained by two observations: First, FtsH-GFP has been shown to accumulate within the asymmetric septum (28) and, second, SpoVM-GFP colocalizes with the polar septum, too (26). It can be inferred that, in the absence of SpoVM, FtsH will degrade at least one protein essential to complete successful sporulation or, alternatively, regulate the steady-state level of SpoVM. The objective of the current work was to analyze whether SpoVM inhibits the protease activity and to study expression of the *spoVM* gene.

Purified GST-FtsH was incubated with or without the SpoVM peptide for up to 4 h (Fig. 1). While the band of β-casein partly disappeared after 3 h of incubation in the presence of GST-FtsH (lane 2), it was almost completely absent 4 h after of incubation (lane 4). Most interestingly, the β-casein was not completed degraded, but converted into distinct degradation products. If the SpoVM peptide was present, the β-casein turned out to be stabilized (lanes 3 and 5). In the absence of GST-FtsH, β-casein remained stable (lane 6). In conclusion, GST-FtsH is able to degrade the substrate protein β-casein into specific fragments, and this proteolytic activity can be inhibited by the SpoVM peptide. In some experiments, we observed the partial disappearance of SpoVM (data not shown) as described for the bacteriophage λ encoded CIII peptide (10, 12), where a short domain (residues 16-37) may form an amphipathic α-helix which is essential for CIII activity (13). Interestingly, SpoVM was also predicted to form such an amphipathic α-helix, though it displays no sequence similarity with CIII (16, 17). We infer from these data that the essential SpoVM peptide, by interaction with FtsH, either prevents degradation of one or more proteins essential for successful spore formation or that FtsH influences the steady-state level of SpoVM. Both possibilities are not mutually exclusive.

To construct a *spoVM* knockout, about 300 bp each of its up- and downstream region were amplified by PCR and ligated into pBR322 (2). The upstream region was generated using the primer pair ON01 and ON02 (Table 1), the downstream region ON03 and ON04 and chromosomal DNA of strain 1012 (18) as template. While the amplicon representing the upstream region was flanked by *Eco*RI and *Sma*I sites, the

downstream amplicon was flanked by *Sma*I and *Hin*dIII sites allowing their insertion into *Eco*RI and *Hin*dIII cleaved pBR322 resulting into pMB02. In the next step, a spectinomycin resistance cassette was amplified using the primer pair ON05 and ON06 and pK2-spec (9) as template and ligated into *Sma*I linearized pMB02 resulting in pMB03. Then, pMB03 was transformed into *B. subtilis* 1012 where the plasmid is unable to replicate. Transformed cells were plated on LB agar plates containing spectinomycin to select for those cells where the *spoVM* wild-type allele has been replaced by the knockout allele. Chromosomal DNA from several candidates was prepared and checked by Southern blotting for successful replacement (data not shown). Strain MB03 was kept for further studies.

Next, we determined the sporulation frequency of the knockout strain by growing cells in DSM, a sporulation medium (19), at 37°C for 36 h, heated them for 20 min to 80°C and then plated for survivors as described (5). Whereas about 82% of the cells of the wild-type strain 1012 were able to sporulate, cells of strain MB03 turned out to be completely deficient in sporulation (less than 10^{-6}). These data are in agreement with previously published results where *B. subtilis* strains with mutant *spoVM* alleles were unable to produce heat-resistant spores (3, 14).

Since SpoVM interferes with the proteolytic activity of FtsH, we examined the possibility that FtsH will degrade one or more proteins essential to complete spore formation. This protein(s) could be located either in the outer spore membrane or in the cytoplasm of the mother cell. In a first attempt to identify this protein(s), strains 1012 and MB03 were grown in DSM to t_5. In one experiment, the membranes of the mother cell and of the prespore were isolated as described (1) and the membrane proteins resolved by SDS-PAGE. A few protein bands could be identified which are either absent or present in the *spoVM* knockout (data not shown). Experiments are in progress to identify these proteins in collaboration with the group of Dr. M. Hecker, University of Greifswald. In a second experiment, we compared the cytoplasmic proteomes of strains 1012 and MB03 using the 2D-gel electrophoresis technique. Here, a total of 83 protein spots were present in the wild-type, but completely absent or present in reduced amounts in the *spoVM* null mutant. One of these proteins turned out to be SpoIVA which seems to be completely absent in the *spoVM* knockout (Fig. 2). This raises the interesting question whether SpoIVA is unstable in the absence of SpoVM and which ATP-dependent protease is responsible for its degradation. If SpoIVA is a substrate for FtsH, it will be interesting to find out whether tethering of SpoIVA to membrane-bound SpoVM will be sufficient to prevent its degradation or whether SpoVM has to directly interact with the protease acting as an antiprotease as described for the phage λ CIII protein (8).

The *spoVM* gene forms a monocistronic operon, and its transcription is controlled by the sporulation-specific sigma factor σ^E which acting in conjunction with the DNA-binding protein SpoIIID, a 93-amino-acid protein (14). To find out when transcription of *spoVM* is initiated, cells of strain 1012 were grown in DSM at 37°C into stationary phase. Aliquots were taken immediately upon entering the stationary phase (t_0) and up to 5 h later (t_1 to t_5). As can be seen from Fig. 3, the *spoVM* transcript (about 200 nucleotides in length) started to appear after t_2 and continued to be produced in large quantities at least until t_5. Using a *spoVM-lacZ* transcriptional fusion, a similar expression pattern was described (14). When total RNA isolated from the *spoVM* knockout at t_5 was analyzed, no signal was obtained as expected (Fig. 3).

Next, we wanted to find out when the SpoVM peptide can be detected in sporulating *B. subtilis* cells. Cells of strain 1012 were grown again as described before and aliquots where prepared for a Western blot. As shown in Fig. 4, the SpoVM peptide started to accumulate at t_4 (lane 4) and further increased during the next 2 h (lane 5 and 6). No SpoVM peptide could be detected in the null mutant strain at t_6 (lane 7). Chemically synthesized SpoVM served as a positive control (lane 8). All aliquots were also checked for the presence of the heat shock protein HtpG (20) which served as a control for a protein not subject to sporulation regulation (Fig. 4). In summary, these results clearly demonstrate that while the *spoVM* transcript is present about 3 h after entry into the transition phase, synthesis of the SpoVM peptide is delayed by 2 h suggesting posttranscriptional regulation of *spoVM* (see below).

The lag-phase of about 2 h between the onset of *spoVM* transcription and translation suggests a so far unknown mechanism that prevents early appearance of the peptide. This could involve stabilization of the transcript, delayed translation initiation or stability of the peptide. A close inspection of the *spoVM* transcript reveals an 87 nucleotide - untranslated region (5' UTR) preceding the coding region (Fig. 5A). We asked whether this 5' UTR is involved in expression of *spoVM*. Two different translational fusions using *lacZ* as a reporter gene were constructed one with and the other without the untranslated region. In both cases, the 9^{th} codon of *spoVM* was fused to the 8^{th} codon of *lacZ*. To discriminate between *cis*- and *trans*-acting factors, we first analyzed the two fusions in *E. coli*. This decision was based on the assumption that *trans*-acting factors are completely absent from this host. When both fusions were analyzed in *E. coli*, between 30 and 50 units of β-galactosidase activity were measured during the exponential growth phase with no difference between the two fusions (Fig. 5B). This result indicates that the 5' UTR does not act as a *cis*-acting factor in *E. coli*, e.g. by influencing the stability of the transcript.

Next, we investigated the influence of the 5' UTR in *B. subtilis* grown in DSM. In the presence of the untranslated region the β-galactosidase activity started to rise at t_2 and increased from about 7 to 20 units at t_5 and dropped thereafter (Fig. 5C). When the translational fusion without the 5' UTR was analyzed, expression of the reporter gene started at t_2, too, but at a significantly higher level (about 5-fold) and further increased at later sporulation times (Fig. 5C). In summary, the 5' UTR of *spoVM* negatively influences its own transcription or translation in *B. subtilis*. Since this effect was not observed in *E. coli*, we conclude that a *trans*-active regulator is involved in transcription or translation of *spoVM* which is not present in *E. coli*. Yet another possibility is that the 5' UTR affects the stability of the transcript. We regard this mechanism as rather unlikely because such an effect could not be observed in *E. coli*. The factor influencing regulation at the 5' UTR could be a translational repressor protein binding within the untranslated region, e.g., at the inverted repeat (Fig. 5A). Alternatively, a non-coding (nc) RNA may interact with the *spoVM* transcript thereby reducing its translation. Recently, several ncRNAs have been described which are under sporulation control (21).

In conclusion, the SpoVM peptide exerts at least two functions. First, as shown by R. Losick and coworkers, it adheres to the outer forespore membrane via hydrophilic amino acid side-chains on the hydrophobic face of the helix (17). Then, it will recruit the SpoIVA protein, a morphogenetic protein that forms the basement layer of the spore coat. Whether really SpoVM first adheres to the membrane and then tethers SpoIVA is not clear. Alternatively, both components could interact in the cytoplasm and then binds to the membrane. Second, SpoVM, at least *in vitro*, inhibits the proteolytic activity of FtsH, and we can assume that it does the same *in vivo*. Since we could show that FtsH is essential for cells to enter the sporulation program (5), an early synthesis of SpoVM could prevent cells go beyond stage 0. Why FtsH has to be inhibited by SpoVM? At least two possibilities can be considered, which are not mutually exclusive. First, FtsH might fine-tune the amount of SpoVM and thereby prevent accumulation of increased amounts which might be deleterious for the cells as has been shown for the LpxC protein (15). Second, by binding to FtsH SpoVM may prevent degradation of at least one protein needed to complete the sporulation program. Since SpoIVA is absent in a *spoVM* knockout as revealed by a 2D-gel analysis, this morphogenetic protein might be a substrate for FtsH and its interaction with SpoVM will protect it from degradation.

We would like to thank Prof. M. Hecker and his group for their generous help with the 2D-gel electrophoresis and the identification of protein spots by mass spectrometry. We also appreciate the help of Monika Batzer with some of the experiments. This project was supported by the Deutsche Forschungsgemeinschaft (Schu 414/20-2).

References

1. **Bagyan, I., M. Noback, S. Bron, M. Paidhungat, and P. Setlow**. 1998. Characterization of *yhcN*, a new forespore-specific gene of *Bacillus subtilis*. Gene **212**:179-188.

2. **Bolivar, F., R. Rodriquez, M. Betlach, and H. Boyer**. 1977. Construction and characterization of new cloning vehicles I: Ampicillin-resistant derivatives of the plasmid pMB9. Gene **2**:75-93.

3. **Cutting, S., M. Anderson, E. Lysenko, A. Page, T. Tomoyasu, K. Tatematsu, T. Tatsuta, L. Kroos, and T. Ogura**. 1997. SpoVM, a small protein essential to development in *Bacillus subtilis*, interacts with the ATP-dependent protease FtsH. J. Bacteriol. **179**:5534-5542.

4. **Cutting, S. M. and P. B. Vander Horn**. 1990. Genetic analysis, p. 27-60. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons, Chichester.

5. **Deuerling, E., A. Mogk, C. Richter, M. Purucker, and W. Schumann**. 1997. The *ftsH* gene of *Bacillus subtilis* is involved in major cellular processes such as sporulation, stress adaptation and secretion. Mol. Microbiol. **23**:921-933.

6. **Driks, A.** 1999. *Bacillus subtilis* spore coat. Microbiol. Mol. Biol. Rev. **63**:1-20.

7. **Eymann, C., A. Dreisbach, D. Albrecht, J. Bernhardt, D. Becher, S. Gentner, l. T. Tam, K. Buttner, G. Buurman, C. Scharf, S. Venz, U. Volker, and M. Hecker**. 2004. A comprehensive proteome map of growing *Bacillus subtilis* cells. Proteomics. **4**:2849-2876.

8. **Halder, S., A. B. Datta, and P. Parrack**. 2007. Probing the antiprotease activity of □CIII, an inhibitor of the *Escherichia coli* metalloprotease HflB (FtsH). J. Bacteriol. **189**:8130-8138.

9. **Härtl, B., W. Wehrl, T. Wiegert, G. Homuth, and W. Schumann**. 2001. Development of a new integration site within the *Bacillus subtilis* chromosome and construction of compatible expression cassettes. J. Bacteriol. **183**:2696-2699.

10. **Herman, C., D. Thévenet, R. D'Ari, and P. Bouloc**. 1997. The HflB protease of *Escherichia coli* degrades its inhibitor lambdacIII. J. Bacteriol. **179**:358-363.

11. **Homuth, G., S. Masuda, A. Mogk, Y. Kobayashi, and W. Schumann**. 1997. The *dnaK* operon of *Bacillus subtilis* is heptacistronic. J. Bacteriol. **179**:1153-1164.

12. **Kobiler, O., S. Koby, D. Teff, D. Court, and A. B. Oppenheim**. 2002. The phage lambda CII transcriptional activator carries a C-terminal domain signaling for rapid proteolysis. Proc. Natl. Acad. Sci. USA **99**:14964-14969.

13. **Kornitzer, D., S. Altuvia, and A. B. Oppenheim**. 1991. The activity of the CIII regulator of lambdoid bacteriophages resides within a 24-amino acid protein domain. Proc. Natl. Acad. Sci. USA **88**:5217-5221.

14. **Levin, P. A., N. Fan, E. Ricca, A. Driks, R. Losick, and S. Cutting**. 1993. An unusually small gene required for sporulation by *Bacillus subtilis*. Mol. Microbiol. **9**:761-771.

15. **Ogura, T., K. Inoue, T. Tatsuta, T. Suzaki, K. Karata, K. Young, L.-H. Su, C. A. Fierke, J. E. Jackman, C. R. H. Reatz, J. Coleman, T. Tomoyasu, and H. Matsuzawa**. 1999. Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in *Escherichia coli*. Mol. Microbiol. **31**:833-844.

16. **Prajapati, R. S., T. Ogura, and S. M. Cutting**. 2000. Structural and functional studies on an FtsH inhibitor from *Bacillus subtilis*. Biochim. Biophys. Acta Gen. Subj. **1475**:353-359.

17. **Ramamurthi, K. S., K. R. Clapham, and R. Losick**. 2006. Peptide anchoring spore coat assembly to the outer forespore membrane in *Bacillus subtilis*. Mol. Microbiol. **62**:1547-1557.

18. **Saito, H., T. Shibata, and T. Ando**. 1979. Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **170**:117-122.

19. **Schaeffer, P., J. Millet, and J. P. Aubert**. 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54**:704-711.

20. **Schulz, A., S. Schwab, S. Versteeg, and W. Schumann**. 1997. The *htpG* gene of *Bacillus subtilis* belongs to class III heat shock genes and is under negative control. J. Bacteriol. **10**:3103-3109.

21. **Silvaggi, J. M., J. B. Perkins, and R. Losick**. 2006. Genes for small, noncoding RNAs under sporulation control in *Bacillus subtilis*. J. Bacteriol. **188**:532-541.

22. **Stragier, P. and R. Losick**. 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu. Rev. Genet. **30**:297-341.

23. **Tam, T., H. Antelmann, C. Eymann, D. Albrecht, J. Bernhardt, and M. Hecker**. 2006. Proteome signatures for stress and starvation in *Bacillus subtilis* as revealed by a 2-D gel image color coding approach. Proteomics. **6**:4565-4585.

24. **Teff, D., S. Koby, Y. Shotland, T. Ogura, and A. B. Oppenheim**. 2000. A colicin-tolerant *Escherichia coli* mutant that confers Hfl phenotype carries two mutations in the region coding for the C-terminal domain of FtsH (HflB). FEMS Microbiol. Lett. **183**:115-117.

25. **Tomoyasu, T., J. Gamer, B. Bukau, M. Kanemori, H. Mori, A. J. Rutman, A. B. Oppenheim, T. Yura, K. Yamanaka, H. Niki, S. Hiraga, and T. Ogura**. 1995. *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor □^{32}. EMBO J. **14**:2551-2560.

26. **Van Ooij, C. and R. Losick**. 2003. Subcellular localization of a small sporulation protein in *Bacillus subtilis*. J. Bacteriol. **185**:1391-1398.

27. **Warrens, A. N., M. D. Jones, and R. I. Lechler**. 1997. Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. Gene **186**:29-35.

28. **Wehrl, W., M. Niederweis, and W. Schumann**. 2000. The FtsH protein accumulates at the septum of *Bacillus subtilis* during cell division and sporulation. J. Bacteriol. **182**:3870-3873.

TABLE 1. Oligonucleotides used

Primer	Sequence (5' to 3')[a]
ON01	**GGCCAT** GAATTC GAGCTGATCATTTTTTAGGAAAC; *EcoR*I
ON02	**GGCCAT** CCCGGG AAACGAAAAAGTACCTCGTGAAT; *Sma*I
ON03	**GGCCAT** CCCGGG TTCAAAGCCCTCTTTCACCACAT; *Sma*I
ON04	**GGCCAT** AAGCTT TGAAAGATGATGAAACAATAGTTGC; *Hin*dIII
ON05	**GGCCAT** CCCGGG CGATTTGACATTTTTCTTGTG, *Sma*I
ON06	**GGCCAT** CCCGGG ATCAATAGTTACAAATTCTTTCA; *Sma*I
ON07	**GGCCAT** GGATCC CTGGCCGTCGTTTTACAACGT; *Bam*HIII
ON08	**GGCCAT** GGATCC TTATTTTTGACACCAGACCAACTGGTAAT; *Bam*HI
ON09	**GGCCAT** GGATCC AATATCCTCTAAATAATTGTCATAT; *Bam*HIII
ON10	**GGCCAT** AAGCTT CGGCAATTTAATGGTGTAAAATTT; *Hin*dIII
ON11	AAAGCCATATTAATAATGATAAGTATAGGAGGGGACAAAAATG
ON12	CTTATCATTATTAATATGCCTTTT

[a] G/C clamps are shown in boldface; restriction sites are underlined

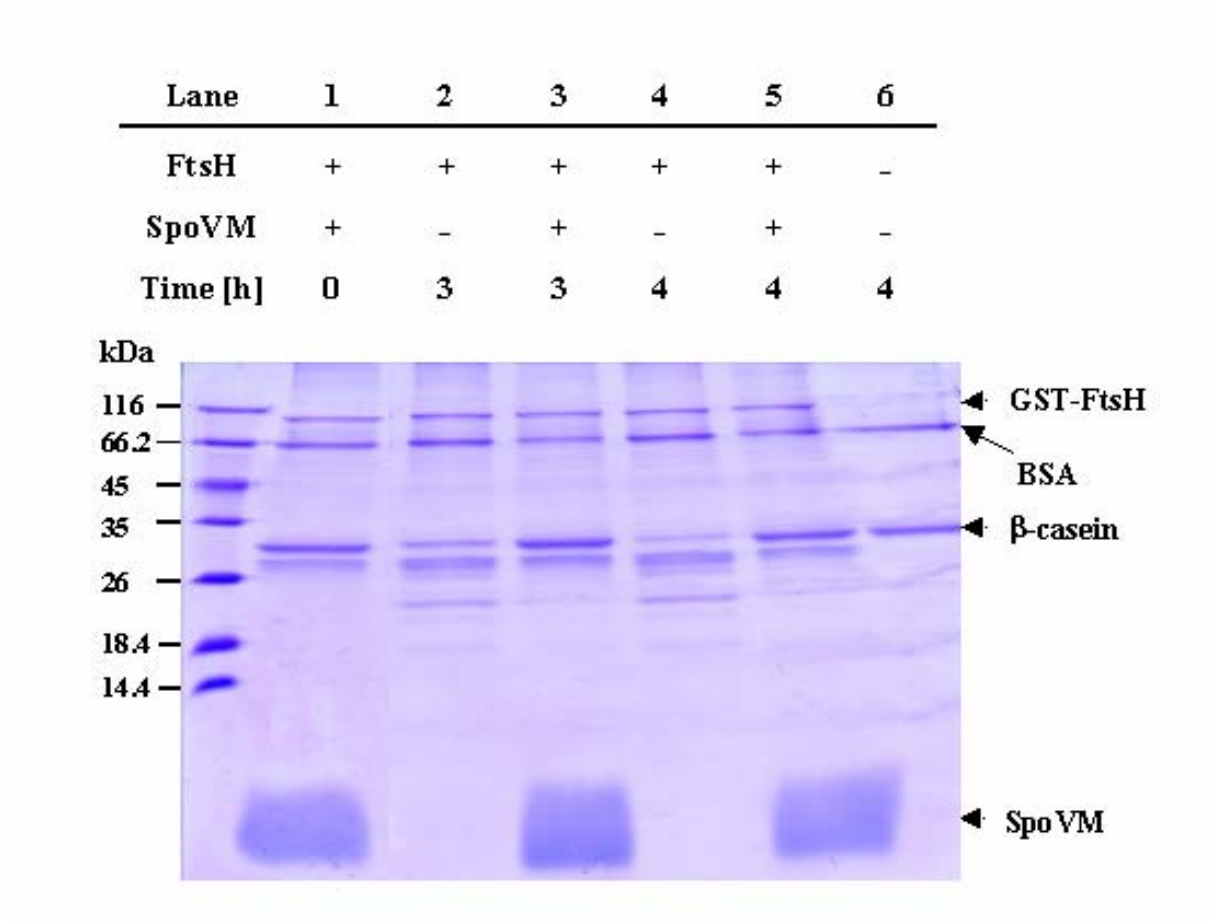


FIG. 1. The SpoVM peptide retards degradation of β-casein by the FtsH protease. The SpoVM peptide has been prepared by chemical synthesis (KLH; Peptide Speciality Laboratories, Heidelberg, Germany) and FtsH has been purified as a GST-tagged protein as described (24). Incubation of the different components followed a published method (25). The complete reaction mixture (30 µl) consisted of the following components: 50 mM Tris-acetate (pH 8.0), 5 mM magnesium acetate, 12.5 µM zinc acetate, 80 mM NaCl, 1.4 mM β-mercaptoethanol, 5 mM ATP, 100 µg/ml bovine serum albumin (BSA), 200 µg/ml of purified SpoVM peptide, 50 µg/ml β-casein and 50 µg/ml of purified *B. subtilis* GST- FtsH. SpoVM peptide, β-casein, and GST-FtsH were present at a molar ratio of 132:4:1. Reactions were performed at 40°C for the time points indicated. Aliquots of the reaction mixtures were analyzed by 15% SDS-PAGE followed by staining with Coomassie blue.

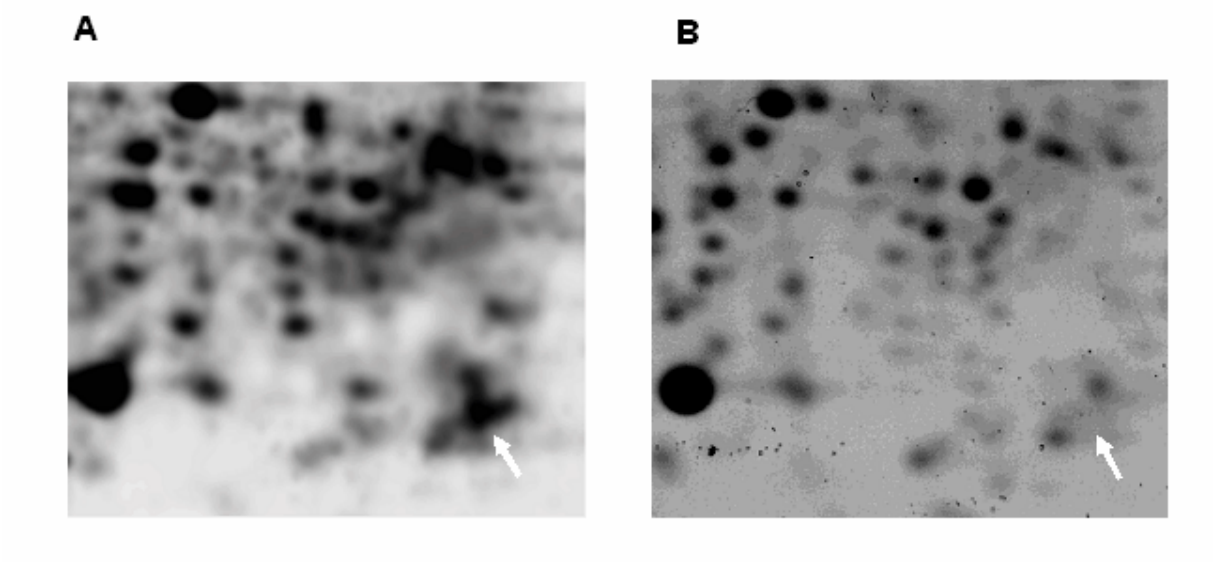


FIG. 2. The cytoplasmic proteomes of *B. subtilis* wild-type (A) and Δ*spoVM*::*spec* (B) strains. Strains 1012 and MB03 were grown in DSM at 37°C into stationary phase. Cytoplasmic proteins were separated by two-dimensional (2D) gel electrophoresis using immobilized pH gradients (IPG) in the range 4-7 as described (23). For identification of the proteins by mass spectrometry, the 2D gels were stained with Colloidal Coomassie brilliant blue (Amersham Biosciences). Spot cutting, tryptic digestion of the proteins and spotting of the resulting peptides onto the MALDI targets were performed as described (7). Here, only a small part of the gel is shown. The white arrow indicates the position of the SpoIVA protein.

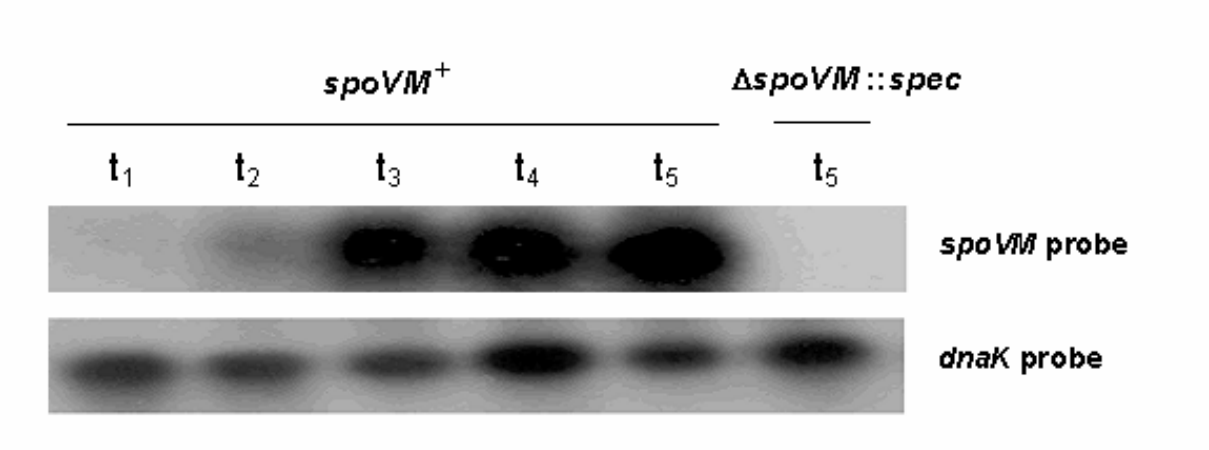


FIG. 3. Transcriptional analysis of *spoVM* by Northern blot. Cells of strains 1012 and MB03 (Δ*spoVM*) were grown in DSM at 37°C into stationary phase and aliquots were taken upon entering (t_0) and from t_1 to t_5. Total RNA was prepared from each aliquot as described (11) and subjected to an RNA blot (10 µg/lane). This blot was hybridized with DIG-labeled *spoVM* and *dnaK* antisense RNA. RNA prepared from the *spoVM* null mutant at t_5 was also applied. Each sample was also analyzed for the *dnaK* transcript, which served as a loading control.

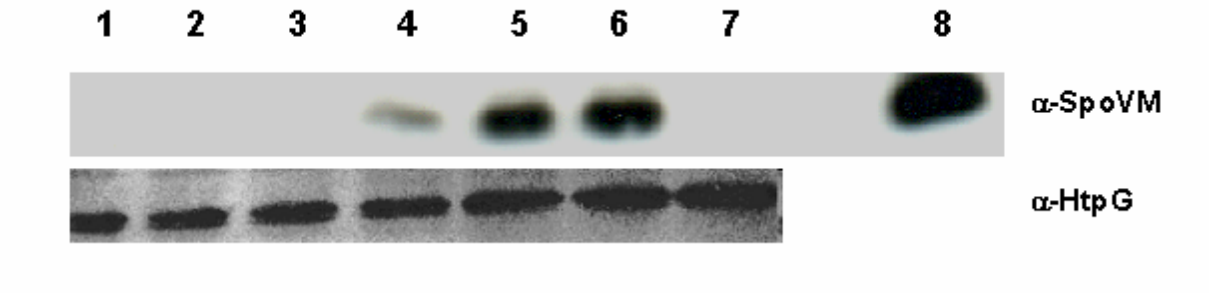


FIG. 4. SpoVM protein starts to accumulate about 5 h after onset of sporulation. Cells of strains 1012 and MB03 were grown as described in the legend to Fig. 3. Protein samples (obtained from equal amounts of cells) were separated by 15% SDS-PAGE, transferred to Nylon filters and treated with polyclonal antibodies raised against SpoVM and HtpG as published elsewhere (11). Protein samples withdrawn from strain 1012 at t_1 to t_6 (lanes 1 to 6) and from strain MB03 at t_6 (lane 7); chemically synthesized SpoVM (lane 8).

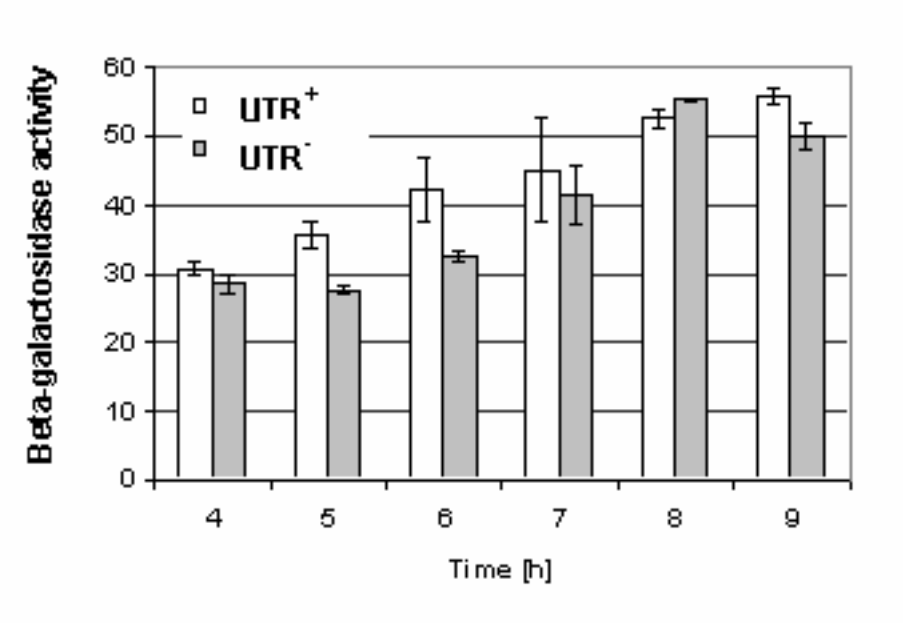
FIG. 5A.

CATTCATTCACGAG**GTACTTTTTC**GTTTGCAAAAGG**CATATTAAT**AATGAT**A**AGaagc acggattaagcatgtgctttcattcaattgctgaaaacaatgaacaaggcggttacgggacgtTATAGGAGGGGAC AAAA - **ATG**

Fig. 5. The 5' untranslated region of *spoVM* negatively influences its own translation.

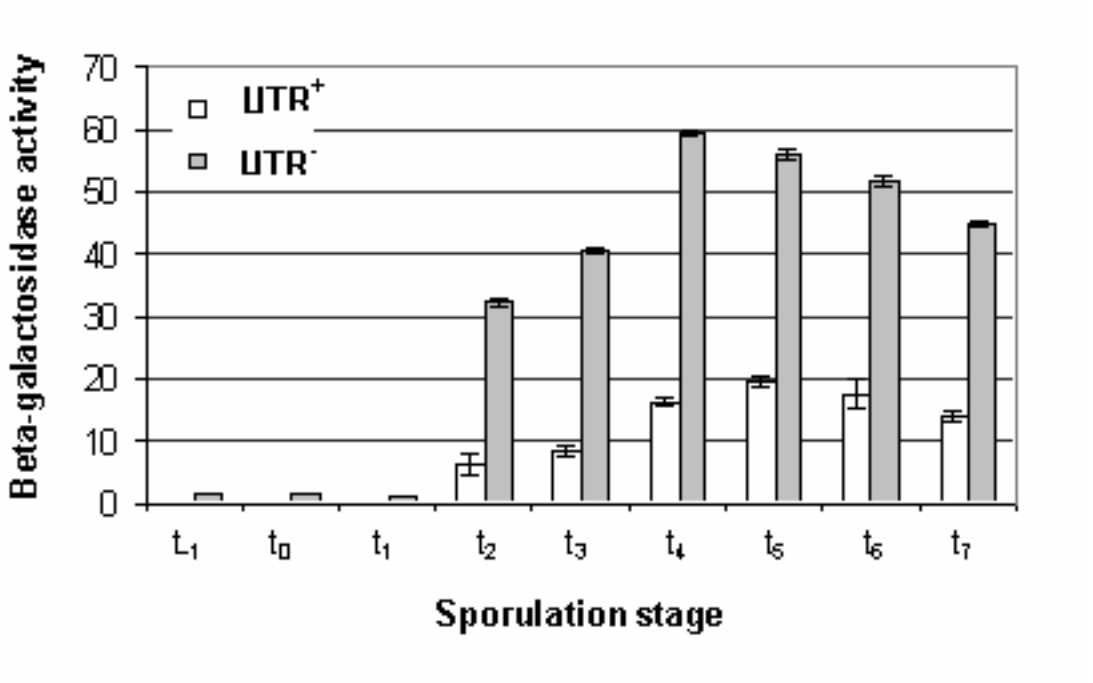
(A) Promoter region of the *spoVM* gene. The σ^E-dependent promoter is shown in bold face and underlined, the transcriptional start site (14) in bold face and double underlined, the start codon of *spoVM* is in bold face and an inverted repeat within the untranslated region is underlined. The internal part of the untranslated region deleted in translational fusions is given in lowercase letters.

FIG. 5B.



(B) The translational fusions between the promoter region of the *spoVM* gene and *lacZ* including either the full-length or the truncated form of the 5' untranslated region exhibit comparable activities in *E. coli*. To construct the full-length fusion, the whole region was amplified using the ON07/ON08 primer pair and chromosomal DNA of strain 1012 as template. The resulting amplicon was cut with *Hin*dIII and *Bam*HI and ligated into pDG364 (4) resulting in pAL15. The same region with an internal deletion removing most part of the untranslated region (see Fig. 5A) was obtained with the primer pairs ON09/ON10 and ON11/ON12 and the splice overlap method (27) resulting in pAL16. The correct DNA sequence of the two inserts was confirmed by DNA sequencing. Next, both plasmids were transformed separately into *E. coli* strain DH5α, and the β-galactosidase activities were measured in both strains during a growth curve starting 4 h after inoculation. The enzymatic activities were determined in three independent experiments and resulted in comparable activities. The data of one representative experiment are given.

FIG. 5C



(C) β-Galactosidase activities. Plasmids pAL15 and pAL16 were separately transformed into *B. subtilis* strain 1012 and integration of the gene fusions at the *amyE* locus by a double crossing over event confirmed by Southern blotting (data not shown) resulting in the strains AL08 and AL09, respectively. Both strains were grown in DSM, and aliquots were withdrawn for determination of β-galactosidase activities at the time points indicated. The β-galactosidase activities are given in units (one unit was defined as ΔE_{405}*min^{-1} *OD_{578}^{-1}*10^{-3}, in which OD_{578} is the optical density of the growth culture) and were measured three times using independent cultures, and the standard deviations are given.

Part C

A novel cold- inducible expression system for *Bacillus subtilis*

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Protein Expression and Purification 53 (2007): 264-269

Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 53 (2007) 264–269
Protein Expression & Purification
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A novel cold-inducible expression system for *Bacillus subtilis*

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Received 17 August 2006, and in revised form 19 December 2006
Available online 9 January 2007

Abstract

Production of recombinant proteins at low temperatures is one strategy to prevent formation of protein aggregates and the use of an expensive inducer such as IPTG. We report on the construction of two expression vectors both containing the cold-inducible *des* promoter of *Bacillus subtilis*, where one allows intra- and the other extracellular synthesis of recombinant proteins. Production of recombinant proteins started within the first 30 min after temperature downshock to 25 °C and continued for about 5 h.

Keywords: Cold shock; *Des* promoter; *lacZ*; *htpG*; α-Amylase; Penicillin-binding protein

One of the major drawbacks during high-level production of recombinant proteins in bacteria is the inability of many proteins to reach their native conformation. Under conditions of overproduction, proteins tend to accumulate within refractile aggregates designated inclusion bodies [1]. Since inclusion body formation is believed to arise from the unproductive association of folding intermediates [2], one experimental approach to prevent formation of these aggregates is to increase the intracellular concentration of molecular chaperones to favour "on-pathway" folding reactions and prevent the accumulation of kinetically trapped folding intermediates [3]. An alternative method to limit the aggregation of recombinant proteins consists in cultivating the cells at low temperatures [4]. Besides reducing formation of inclusion bodies, low-temperature expression lowers the degradation of proteolytically sensitive proteins [5,6].

To ensure high level production of recombinant proteins, two different strategies can be used: fusion of the coding region of the protein of interest to an inducible promoter, e.g., using an IPTG- or xylose-inducible promoter [7], or, alternatively, to make use of a cold-shock inducible promoter. When mid-exponential phase bacterial cells are rapidly transferred from 37 to 25 °C or even a lower temperature, the synthesis of most cellular proteins greatly decreases, while that of cold-shock proteins is transiently upregulated [8]. In *Bacillus subtilis*, one of these cold-shock proteins is a membrane-bound desaturase (Δ5-Des) encoded by the *des* gene [9]. This enzyme catalyzes the introduction of a *cis* double bond at the Δ5 position of a wide variety of saturated fatty acids. It has been shown that the *des* gene is tightly regulated during cold shock. While the *des* mRNA is barely detectable at 37 °C, its synthesis is transiently induced upon a temperature downshift [10]. Expression of the *des* gene does not depend on *de novo* protein synthesis, but on a two-component signal transduction system which consists of the sensor kinase DesK and the response regulator DesR [11]. It is assumed that the kinase senses a temperature downshift through changes in the physical state of the cytoplasmic membrane. The C-terminal kinase domain of DesK undergoes autophosphorylation, and the phosphoryl group is then transferred to the response regulator DesR. Phosphorylated DesR binds to two adjacent DNA-binding sites leading to the recruitment of RNA polymerase to the *des* promoter and activation of transcription [12]. The Δ5-desaturase directly introduces double bonds into membrane lipids leading to a return to the original fluidity of the membrane. This is sensed by DesK which changes from a kinase to phosphatase activity

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doi:10.1016/j.pep.2006.12.023

leading to a dephosphorylation of DesR with a concomitant turn off of the *des* gene [13].

Based on these data, we developed a cold-inducible expression system for *B. subtilis* making use of the *des* promoter. We show here that cold-induction results in a significant induction of reporter genes largely preventing formation of aggregates of an aggregation-prone protein. Cold-inducible expression systems have also been developed for *Escherichia coli* which are based on a different principle [14,15]. Cold-inducible expression systems provide an inexpensive alternative technology especially for industrial production of recombinant proteins complementing the widely used IPTG- and xylose-inducible systems.

Materials and methods

Materials

Bacteria, plasmids and growth conditions

Bacterial strains and plasmids used are listed in Table 1. Cells were grown in Luria Broth (LB) medium at 37 or 25 °C under aeration. Antibiotics were added where appropriate (ampicillin at 100 μg/ml, neomycin at 10 μg/ml and chloramphenicol at 10 μg/ml).

Methods

Construction of a transcriptional fusion between the des promoter and the lacZ reporter gene

The promoter region of the *des* gene was fused to the *lacZ* reporter gene using the integration vector pDG1728 [16]. The resulting transcriptional fusion is sandwiched between *amyE*-front and *amyE*-back allowing its integration into the *B. subtilis* chromosome at the *amyE* locus. The *des* promoter region was amplified using oligonucleotides (ON) ON1 (GGCCATGAATTCTCCGGCATCCCGATCATCGC; restriction site underlined) and ON2 (GGCCATAAGCTTTCTCATTGTGTGTCTCGGTTCAG). The amplicon was cleaved with *Eco*RI and *Hin*dIII and inserted into pDG1728 cut with the same enzymes resulting in pDG1728-*des*. This recombinant plasmid was transformed into strain WW02, and transformants were selected on LB plates containing chloramphenicol and screened for the loss of the neomycin resistance marker, and one positive transformant (AL03) was kept for further studies.

Construction of a des null mutant

To construct a *des* knockout, the gene including flanking regions was amplified using the primer pairs ON3/ON4 (GGCCATGTCGACTGAACCGAGACACACAATG; GGCCATGAGCTCATAGTTGAGCACCTTTGG), and the amplicon was cleaved with *Sal*I and *Sac*I and cloned into pBluescript SKII$^+$ treated with the same enzymes. Next, the recombinant plasmid was treated with *Hin*dIII and *Bcl*I to remove a 61-bp internal fragment of *des* which was replaced by the *neo* marker using pBgaB as template and the primer pair ON5/ON6 (GGCCATAAGCTTAGGTCGAGATCAGGGAATGAGTT; GGCCATTGATCAGATCAATTCTGACAGCCATG). Using the primer pair ON3/ON4, the modified gene was amplified and transformed into *B. subtilis* 1012. Neomycin-resistant transformants were selected and checked by Southern-blot for chromosomal replacement of the *des* by the *neo* gene (data not shown). One strain (AL02) was kept for further studies.

Table 1
Strains and plasmids used in this study

Strains	Genotype	Reference/source
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr hsdRMS mcrBC*) φ80d *lacZ* Δ*M15 deoR recA1 araD139* Δ(ara leu)$_{7697}$ *galU galK* λ$^-$ *rpsL endA1 nupG*	BRL
B. subtilis		
1012	*leuA8 metB5 trpC2 hsrM1*	[26]
WW02	1012 *amyE*::*neo*	[27]
AL02	1012 *des*::*neo*	This work
AL03	1012 *amyE*::P*des-lacZ*	This work
AL04	AL02 *des*::*neo amyE*::P*des-lacZ*	This work
AL05	AL02 *htpG*::*erm*	This work
AL06	AL02 *pbpE*::*erm*	This work
Plasmids		
pBluescript SK$^+$	Cloning vector	Stratagene
pBgaB	Integration vector containing the *bgaB* gene	[28]
pDG1728	Vector allowing integration of DNA sequences at the *amyE* locus	[16]
pHT01	Derivative of pNDH33 without a direct repeat	[22]
pKTH10	Recombinant vector containing the *amyQ* gene	[17]
pAL10	Expression vector allowing cold-inducible intracellular production of recombinant proteins	This work
pAL12	Expression vector allowing cold-inducible secretion of recombinant proteins	This work
pNDH33-*htpG*	*htpG* fused to an IPTG-inducible promoter	[22]

The P*des*–*lacZ* fusion was introduced into AL02 by transformation resulting in AL04.

Construction of the two expression vectors pAL10 and pAL12

We started from the vector pHT01, where *lacI*, P*grac* and *bgaB* were removed by *Sac*I/*Bam*HI digestion followed by religation with the *des* promoter region including the binding sites for DesR generated by PCR using ON7/ON8 (GGCCATGAGCTCTCCGGCATCCCGATCATCGC; GGCCATGGATCCTCTTGATCGCCTCCTCATTGTGTGTCTCGG) and resulting in the new expression vector pAL10 (Fig. 1a). This vector allows intracellular production of recombinant proteins. A second vector allowing secretion of proteins was obtained by fusing the signal sequence of the *amyQ* gene [17] to the *des* promoter (ON9/ON10 (GGGCCCATGGATCCATGATTCAAAAACGAAAGCGGACAG; GGCCATTCTAGATTTTTCTGAACATAAATGGAGACG) and pKTH10 as template) resulting in the expression-secretion vector pAL12 (Fig. 1b). To test suitability and efficacy of the new expression vectors, different genes were fused to the *des* promoter and the synthesis was monitored after temperature downshock to 25 °C. pAL10 was tested by insertion of *htpG*, coding for a heat shock protein of unknown function [18], and *pbpE* encoding the penicillin-binding protein Pbp4* [19]. The *htpG* gene was amplified using ON11/12 (GGCCATGGATCCATGGCGAAAAAAGAGTTTAAAGC; GGCCATTCTAGATTACACCATGACCTTGCAAATATTGTTCG), *pbpE* ON13/14 (GGCCATGGATCCATGAAGCAGAATAAAAGAAAGC; GGCCATGGATCCTTACTACTTCGTACGGACCGCTTCT) and chromosomal DNA of *B. subtilis* 1012 as template. To analyse for the versatility of pAL12, the coding region for *amyQ* [17] was inserted (ON15/16 (GGCCATTCTAGAGTAAATGGCACGCTGATGCAGT; GGCCATCCCGGGTTATTTCTGAACATAAATGGAGACG) and pKTH10 as template).

Determination of enzymatic activities and Western blot analysis

The β-galactosidase activities encoded by *lacZ* was determined as described elsewhere [20], with the exception that LacZ activity was measured kinetically in a microplate reader (VersaMax, Molecular Devices) at 405 nm at 28 °C. One unit was defined as $\Delta E_{405} * \mathrm{min}^{-1} * \mathrm{OD}_{578}^{-1} * 10^{-3}$, in which OD_{578} is the optical density of the growth culture when samples were drawn. Western blot analyses were car-

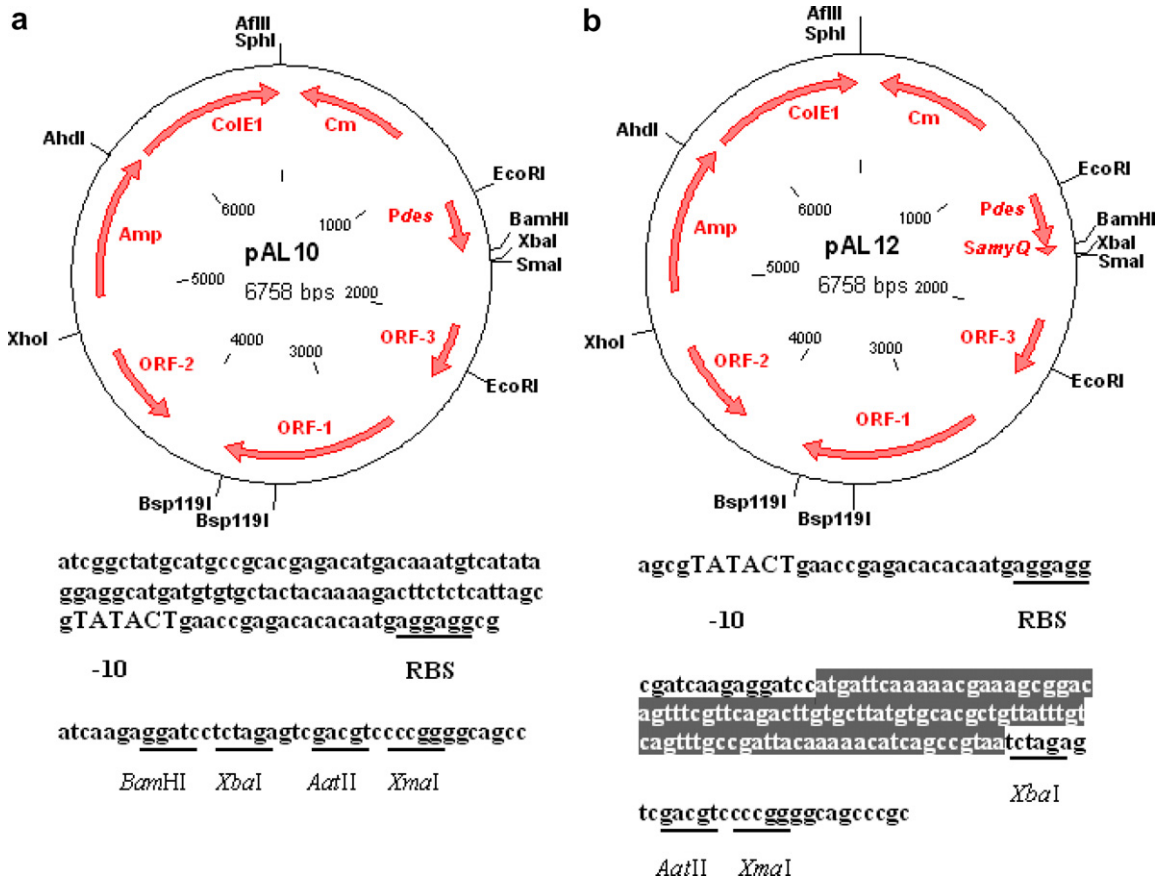

Fig. 1. Genetic and restriction map of the two vectors pAL10 and pAL12 allowing intra- and extracellular expression of recombinant proteins, respectively. (a) pAL10 and the DNA sequence of the P*des* promoter (in capital letters); (b) pAL12 and the DNA sequence of P*des*, the ribosome-binding site (underlined) and the coding region for the signal sequence (highlighted in grey). Unique restriction sites which can be used for insertion of recombinant genes are also presented.

ried out as published [21]. Blots were developed with polyclonal antibodies against HtpG and Pbp4* used at a dilution of 1:10,000.

Results and discussion

Construction and analysis of an operon fusion between the des promoter and lacZ

Based on published data [11], we devised a cold-inducible expression system consisting of the regulatory region of the *des* gene consisting of the *des* promoter and its upstream region serving as binding sites for DesR ~ P. This region was cloned into the integration vector pDG1728 [16] followed by insertion at the *amyE* locus. Strain AL03 was first grown at 37 °C to the early exponential growth phase. Then, the culture was divided into two subcultures where one was further kept at 37 °C while the second was challenged with a cold shock to 25 °C. Aliquots were withdrawn just before dividing the culture ($t = 0$) and up to 12 h post-induction for determination of the β-galactosidase activities. As shown in Fig. 2, the enzymatic activity of the unshocked culture started with about 4 units and increased to about 15 units over time. In contrast, the cold-shocked culture exhibited an increase to about 105 units after 1.5 h followed by a decrease to 50 units 5 h after induction (Fig. 2). The decrease can be explained by induction of the *des* gene from the chromosome restoring the fluidity of the membrane followed by turning off expression of *des* gene [11].

It should be possible to prevent turning off expression of the *des* gene by deleting this gene from the chromosome as published [11]. This has been done as described under Materials and methods, and the operon fusion was integrated at the *amyE* locus of strain AL03 where the *des* gene has been replaced by a neomycin resistance marker. When this strain AL04 was analysed, the β-galactosidase activity was even lower during growth at 37 °C, while the activity increased from 7 to about 100 units within the first 12 h after cold challenge (Fig. 2). This result clearly demonstrates that no turn off of the *lacZ* expression occurs in the absence of the desaturase. Based on this finding we asked whether a further increase in the enzymatic activity can be obtained upon prolonged incubation. As shown in Fig. 3, while the OD_{578} continued to increase steadily for at least 58 h, the β-galactosidase activity increased up to about 12 h and decreased thereafter. This result indicates that either the half-live of the enzyme or/and the synthesis capacity of the cells decrease during prolonged incubation at 25 °C. We also measured the β-galactosidase activity after temperature downshift to 20 and 15 °C. While about 100 units were measured after 5 h of growth at 25 °C (Fig. 2), 60 units and 5 units were determined at 20 and 15 °C, respectively (data not shown). This results suggests that growth at 20 °C reduces the expression level to 60%, while expression of the *lacZ* gene is completely abolished at 15 °C.

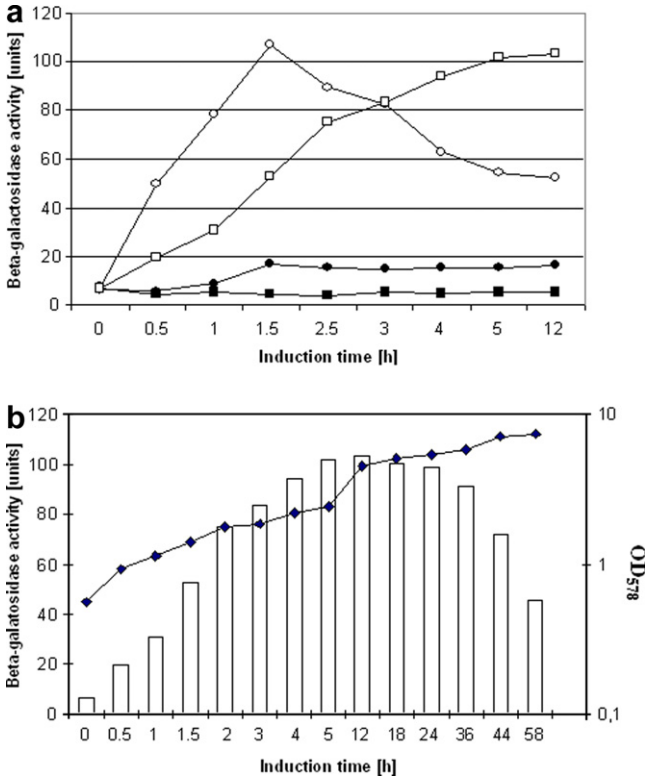

Fig. 2. Induction of β-galactosidase activity in two *B. subtilis* strains grown at two different temperatures. (a) *B. subtilis* strains AL03 and AL04 (Δ*des*::*neo*) were grown in LB medium at 37 °C to the early logarithmic growth phase. Then, the cultures were divided into two subcultures (at $t = 0$) where one was further grown at 37 °C, while the second was challenged with 25 °C. Aliquots were removed for determination of β-galactosidase activities at the time points indicated. The complete experiments were repeated three times and yielded comparable results. Data from one of these experiments are presented. AL03 grown at 37 °C (●) or 25 °C (○); AL04 grown at 37 °C (■) or 25 °C (□). (b) *B. subtilis* strain AL04 was grown up to 58 h after the temperature downshock. The OD_{578} was measured during growth (◆) and the β-galactosidase activities as indicated (white columns).

The expression vector pAL10 allows production of recombinant proteins to a significant level

Next, we attempted to directly visualize the amount of recombinant proteins produced. To accomplish this goal, two different genes were fused to P*des* in the expression vector pAL10, namely the *htpG* and the *pbpE* gene coding for a heat shock protein of unknown function and a penicillin-binding protein, respectively [18,19]. Both strains (the chromosomal copies of *htpG* and *pbpE* have been deleted) were grown in LB medium to the mid-exponential growth phase, divided into two subcultures where one was further incubated at 37 °C, while the second was cold-shocked to 25 °C. Aliquots were withdrawn at different time points for the analysis of the presence of the HtpG or Pbp4* protein as indicated. While no HtpG protein was visible when the strain AL05 containing the plasmid pAL10-*htpG* was incubated at 37 °C, this protein became apparent already after 3 h and increased in its amount up to 9 h (Fig. 3a)

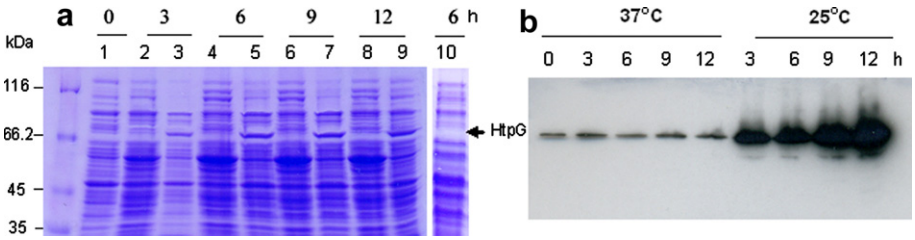

Fig. 3. Identification of the *htpG* gene product. Cells of strain AL05 carrying the plasmid pAL10-*htpG* were grown in LB medium at 37 °C to mid-log ($t = 0$), divided into two cultures, where one was further incubated at 37 °C and the second cold-shocked to 25 °C. As a control, cells of strain 1012 carrying pNDH33-*htpG* were grown at 37 °C to mid-log and then cold-shocked and induced by addition of 1 mM IPTG for 6 h. Cells were lysed by sonification and 0.5 μg of protein was loaded per lane on an 10% SDS–PAGE. (a) After gel electrophoresis, the proteins were stained with Coomassie blue. 37 °C culture: lanes 1, 2, 4, 6 and 8; 25 °C culture: lanes 3, 5, 7 and 9; lane 10, IPTG-treated cells grown at 25 °C. (b) Immunoblot analysis of HtpG. Cells were grown and treated as described. After separation of the proteins and Western blot, HtpG was detected using antibodies raised against this protein. Molecular weight markers are indicated.

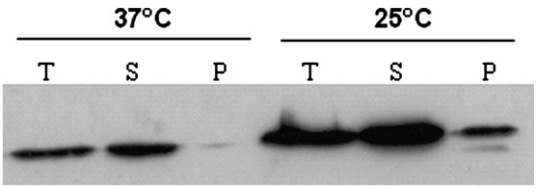

Fig. 4. Immunoblot analysis of Pbp4*. *B. subtilis* strain 1012 carrying pAL10-*pbpE* was grown as described in the legend to Fig. 3. Cells were lysed by sonification and the cellular lysate was applied directly (T) or after a centrifugation step to separate soluble (S) from insoluble (P) protein. 0.3 μg of protein were applied per lane.

where it represented about 10% of the total cellular protein. When the *htpG* gene was expressed from an IPTG-inducible promoter for 6 h at 25 °C, only tiny amounts of the HtpG protein became visible (Fig. 3a, lane 10). We conclude from this result that the expression level is rather low at 25 °C from the IPTG-inducible promoter and can be compensated from a cold-inducible promoter. In parallel, we visualized HtpG by Western blot. While small amounts were present at 37 °C most probably due to the leakiness of the promoter, it increased dramatically up to 9 h after cold-shock (Fig. 3b).

The Pbp4* protein has been reported to be membrane-attached due to one or more hydrophobic patches [19]. We could already show that overproduction of this protein at 37 °C leads mainly to insoluble Pbp4* [22]. Therefore, we wondered whether overproduction at low temperature will influence the folding of Pbp4* leading to mainly soluble protein. We analysed the amount of Pbp4* by Western blot from three different fractions: total cellular content, soluble and insoluble fraction obtained after a centrifugation step. While some Pbp4* protein was present already at 37 °C incubation as reported for HtpG, its amount increased significantly 6 h after incubation of the cells at 25 °C (Fig. 4). As can be seen, most of the recombinant protein stayed soluble indicating that the lower temperature favours formation of folded polypeptides as described for the aggregation-prone fusion protein preS2-S′-β-galactosidase in *E. coli* [23]. A comparable result has been obtained during constitutive high level production of the DnaK and GroE chaperone systems [22]. We conclude that production of aggregation-prone recombinant proteins at low temperatures is alternative way to largely prevent formation of aggregates.

The expression-secretion vector pAL12 allows regulated secretion of exoproteins

To test the secretion capability at low temperature, the *amyQ* gene coding for an α-amylase [17] was inserted into pAL12 resulting in pAL12-*amyQ*. Strain AL02 carrying pAL12-*amyQ* was grown in LB medium at 37 and 25 °C, and aliquots were taken at the time points indicated in Fig. 5. If the amount of α-amylase present at 37 and 25 °C were compared, significantly more enzyme was present at 25 °C as compared to 37 °C (Fig. 5). We also mea-

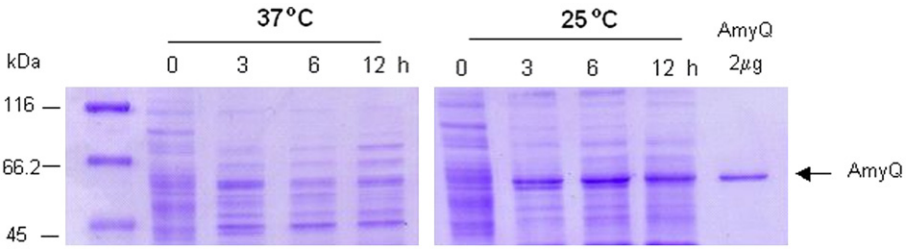

Fig. 5. Detection of extracellular α-amylase by SDS–PAGE. Strain AL02 carrying pAL12-*amyQ* was grown as described in the legend to Fig. 3. Aliquots were taken from the supernatant of both cultures at the time points indicated. Purified α-amylase was added to one lane. 11 μg of protein were applied per lane.

sured the α-amylase activities within supernatant and compared it to those produced at 37 °C. While the activities were comparable during the first 5 h, higher activities were measured at later times in accordance with the results obtained by gel analysis (data not shown). At 20 °C, we have been unable to detect any α-amylase indicating that secretion of this enzyme and most probably many others is severely impaired under these growth conditions. It has been reported that the SecA abundance in *E. coli* was ~3-fold higher at 20 °C than at 37 °C [24], in accordance with the notion that the *E. coli* protein export includes some intrinsically cold-sensitive element [25]. Based on these observations we can only speculate that at least one component of the Sec pathway does not function properly at 20 °C. This could be the SecA motor protein or/and the SecYEG translocons or/and a so far unknown component.

Acknowledgments

This work was financially supported by the Deutsche Forschungsgemeinschaft (Schu 414/20-2). The two expression vectors pAL10 and pAL12 can be ordered from MoBiTec (www.mobitec.com).

References

[1] A. Mogk, M.P. Mayer, E. Deuerling, Mechanisms of protein folding: molecular chaperones and their application in biotechnology, Chembiochem 3 (2002) 807–814.
[2] A. Mitraki, J. King, Protein folding intermediates and inclusion body formation, Bio/Technology 7 (1989) 690–697.
[3] J.G. Thomas, F. Baneyx, Protein misfolding and inclusion body formation in recombinant *Escherichia coli* cells overexpressing heat-shock proteins, J. Biol. Chem. 271 (1996) 11141–11147.
[4] C.H. Schein, M.H.M. Noteborn, Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature, Bio/Technology 6 (1988) 291–294.
[5] A.W. Emerick, B.L. Bertolani, A. Ben-Bassat, T.J. White, M.W. Konrad, Expression of a β-lactamase preproinsulin fusion protein in *Escherichia coli*, Bio/Technology 2 (1984) 165–168.
[6] J.A. Chesshyre, A.R. Hipkiss, Low temperatures stabilize interferon α-2 against proteolysis in *Methylophilus methylotrophus* and *Escherichia coli*, Appl. Microbiol. Biotechnol. 31 (1989) 158–162.
[7] B. Härtl, W. Wehrl, T. Wiegert, G. Homuth, W. Schumann, Development of a new integration site within the *Bacillus subtilis* chromosome and construction of compatible expression cassettes, J. Bacteriol. 183 (2001) 2696–2699.
[8] M.H.W. Weber, M.A. Marahiel, Bacterial cold shock responses, Science Progress 86 (2003) 9–75.
[9] P.S. Aguilar, J.E. Cronan Jr., D. De Mendoza, A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase, J. Bacteriol. 180 (1998) 2194–2200.
[10] P.S. Aguilar, P. Lopez, D. De Mendoza, Transcriptional control of the low-temperature-inducible *des* gene, encoding the Delta5 desaturase of *Bacillus subtilis*, J. Bacteriol. 181 (1999) 7028–7033.
[11] P.S. Aguilar, A.M. Hernandez-Arriaga, L.E. Cybulski, A.C. Erazo, D. De Mendoza, Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*, EMBO J. 20 (2001) 1681–1691.
[12] L.E. Cybulski, G. Del Solar, P.O. Craig, M. Espinosa, D. De Mendoza, *Bacillus subtilis* DesR functions as a phosphorylation-activated switch to control membrane lipid fluidity, J. Biol. Chem. 279 (2004) 39340–39347.
[13] M.C. Mansilla, D. De Mendoza, The *Bacillus subtilis* desaturase: a model to understand phospholipid modification and temperature sensing, Arch. Microbiol. 183 (2005) 229–235.
[14] M. Mujacic, K.W. Cooper, F. Baneyx, Cold-inducible cloning vectors for low-temperature protein expression in *Escherichia coli*: application to the production of a toxic and proteolytically sensitive fusion protein, Gene 238 (1999) 325–332.
[15] G.L. Qing, L.C. Ma, A. Khorchid, G.V.T. Swapna, T.K. Mal, M.M. Takayama, B. Xia, S. Phadtare, H.P. Ke, T. Acton, G.T. Montelione, M. Ikura, M. Inouye, Cold-shock induced high-yield protein production in *Escherichia coli*, Nat. Biotechnol. 22 (2004) 877–882.
[16] A.M. Guérout-Fleury, N. Frandsen, P. Stragier, Plasmids for ectopic integration in *Bacillus subtilis*, Gene 180 (1996) 57–61.
[17] I. Palva, Molecular cloning of alpha-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*, Gene 19 (1982) 81–87.
[18] A. Schulz, S. Schwab, S. Versteeg, W. Schumann, The *htpG* gene of *Bacillus subtilis* belongs to class III heat shock genes and is under negative control, J. Bacteriol. 10 (1997) 3103–3109.
[19] D.L. Popham, P. Setlow, Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis pbpE* operon, which codes for penicillin-binding protein 4* and an apparent amino acid racemase, J. Bacteriol. 175 (1993) 2917–2925.
[20] T. Wiegert, G. Homuth, S. Versteeg, W. Schumann, Alkaline shock induces the *Bacillus subtilis* σ^W regulon, Mol. Microbiol. 41 (2001) 59–71.
[21] G. Homuth, S. Masuda, A. Mogk, Y. Kobayashi, W. Schumann, The *dnaK* operon of *Bacillus subtilis* is heptacistronic, J. Bacteriol. 179 (1997) 1153–1164.
[22] T.T.P. Phan, H.D. Nguyen, W. Schumann, Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in *Bacillus subtilis*, Protein Expr. Purif. 46 (2006) 189–195.
[23] J.A. Vasina, F. Baneyx, Expression of aggregation-prone recombinant proteins at low temperatures: a comparative study of the *Escherichia coli cspA* and *tac* promoter systems, Protein Express. Purif. 9 (1997) 211–218.
[24] A. Murakami, H. Nakatogawa, K. Ito, Translation arrest of SecM is essential for the basal and regulated expression of SecA, Proc. Natl. Acad. Sci. USA 101 (2004) 12330–12335.
[25] K.J. Pogliano, J. Beckwith, The Cs *sec* mutants of *Escherichia coli* reflect the cold sensitivity of protein export itself, Genetics 133 (1993) 763–773.
[26] H. Saito, T. Shibata, T. Ando, Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg, Mol. Gen. Genet. 170 (1979) 117–122.
[27] W. Wehrl, M. Niederweis, W. Schumann, The FtsH protein accumulates at the septum of *Bacillus subtilis* during cell division and sporulation, J. Bacteriol. 182 (2000) 3870–3873.
[28] A. Mogk, R. Hayward, W. Schumann, Integrative vectors for constructing single-copy transcriptional fusions between *Bacillus subtilis* promoters and various reporter genes encoding heat-stable enzymes, Gene 182 (1996) 33–36.

6. Abbreviations

σ	Sigma factor
Δ	deletion
µg	microgram
µl	microliter
^{0}C	degree centigrade
2D-Gel	two-dimensional gel
5' UTR	5' UnTranslated Region
aa	amino acid(s)
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
B. subtilis	*Bacillus subtilis*
bp	base pairs
BSA	Bovine Serum Albumin
cat	gene coding for chloramphenicol-acetytransferase
cfu	colony forming units
DNA	Deoxyribonucleic acid
DSM	Difco Sporulation medium
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
erm	Gene coding for erythromycine resistance
et al.	*et alteri*
G	gram
GFP	Green Fluorescent Protein
GSH	Glutathione
GST	Glutathione-S-transferase
H	hour(s)
IPG	Immobilized pH gradient
IPTG	Isopropyl-β-D-thiogalacto pyranoside

kDa	kilo-Dalton
l	liter
lacZ	beta-galactosidase gene
LB	Luria-Bertani (growth medium)
LPS	Lipopolysacharide
M	Molar
MALDI-TOF	Matrix-assisted Laser Desorption-ionization Time-of-flight
MS	Mass Spectrometry
mg	milligram
min	minute(s)
ml	mililiter
mM	milimole
mRNA	messenger RNA
OD	Optical Density
PBP	Penicillin binding protein
PBS	Phosphate-buffer saline
PCR	Polymerase Chain Reaction
RBS	Ribosome Binding Site
RNA	Ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sec	second
spec	Gene coding for Spectinomycin
t_x	stage x of sporulation program
tet	Gene coding for tetracycline resistance
WT	Wild-type

Erklärung

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

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

Ai Thi Thuy Le

Bayreuth, December 2007