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

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In the absence of the ATP-dependent metalloprotease FtsH, the sporulation frequency of *Bacillus subtilis* cells is reduced by several orders of magnitude. This indicates that FtsH has to degrade or to regulate the steady-state level of one or more proteins that interfere with successful sporulation. Here, we show that the amount of the master regulator protein Spo0A is reduced in an *ftsH* knockout and the small amounts of Spo0A protein present are inactive. Phosphorylation of Spo0A occurs through a phosphorelay. Four negative regulators have been identified here which directly interfere with the phosphorelay through *ftsH*, 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 %. When purified Spo0E was incubated with FtsH, partial degradation of the phosphatase was observed. In contrast, two mutant versions of Spo0E with truncated C-termini remained stable. Transfer of the C-terminal 25 aa of Spo0E to a shorter homologue of Spo0E, YnzD, which is not a substrate of FtsH, conferred instability. When a mutant Spo0A was produced that was active in the absence of phosphorylation, spores were formed at a normal rate in an *ftsH* knockout, indicating that *ftsH* is needed only during phase 0.

INTRODUCTION

Upon depletion of nutrients, species of the genera *Bacillus* and *Clostridium* have the ability to undergo a cellular differentiation process leading to the formation of a dormant spore; the sporulation process is triggered by starvation, and the population density has to be high (for recent reviews, see Phillips & Strauch, 2002; Errington, 2003). *Bacillus subtilis* cells code for an extremely complex and sophisticated decision-making apparatus, which monitors a huge range of internal and external signals. This information is 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 phosphoryl group 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 phosphoryl group is transferred by two

intermediates, Spo0F and Spo0B, and finally to Spo0A (Burbulys *et al.*, 1991). Two of the three phosphoproteins are subject to regulation by phosphatases, and one group, the Rap phosphatases, are regulated by pentapeptides (Perego, 1998). The Rap phosphatases specifically dephosphorylate Spo0F~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 & Wilkinson, 2001). The FtsH protein and its biochemical and biological functions have been studied in detail in *Escherichia coli*. It carries two transmembrane segments close to its N-terminus which anchor the 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 Zn²⁺. 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; Lysenko *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. The reduced sporulation

Abbreviation: GST, glutathione S-transferase.

Supplementary tables of strains and primers are available with the online version of this paper.

frequency seems to interfere with the synthesis or/and activity of Spo0A since an *abrB-lacZ* transcriptional fusion fails to be turned off during stage 0 (Deuerling *et al.*, 1997). Based on this result we assume 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 its degradation. Furthermore, we show that FtsH is needed only during stage 0.

METHODS

Bacterial strains, plasmids, media and growth conditions. All strains and plasmids used in this study are listed in Supplementary Table S1, available with the online version of this paper. *E. coli* DH10B was used for plasmid construction and propagation. *E. coli* A8926 is a derivative of W3110 used for the expression of glutathione S-transferase (GST)-tagged proteins. *B. subtilis* strain 1012 was used in most of the experiments. All strains were grown either in Luria-Bertani medium (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 promoterless *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 Supplementary Table S2) preceding the *skf* operon, which is activated by a low amount of active Spo0A (Fujita *et al.*, 2005), and 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). P_{skf} was inserted between the *EcoRI* and *HindIII* sites of pDG1728; P_v and P_s were ligated into the *EcoRI* and *BamHI* sites.

Inactivation of the four genes *rapA*, *rapB*, *rapE* and *spo0E* was achieved by the replacement method as follows. First, the two flanking regions of each gene (about 300 bp) were amplified (see Supplementary Table S2 for the primer sequences) and inserted into pBluescript SKII⁺. Next, the chloramphenicol-resistance cassette was amplified using pDG1662 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 from pBluescript SKII⁺-phleo was ligated between the two flanking regions. 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 & 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, the coding region for the last 25 aa of *spo0E* was 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 *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. Expression was induced by adding 1 mM IPTG (final concentration) to the cell cultures at an OD₆₀₀ of 0.8 followed by transfer of the cultures to 25 °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 has been published (Teff *et al.*, 2000).

Proteolysis experiments. Degradation reactions were performed as described by 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 BSA ml⁻¹, 100 µg ml⁻¹ of the target GST-tagged protein (or 100 µg ml⁻¹ of β-casein, as a positive control to verify the proteolytic activity of purified GST-FtsH), 50 µg ml⁻¹ 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 water. Reactions were performed at 40 °C for the time points indicated. Aliquots of the reaction mixtures were analysed by 15 % SDS-PAGE followed by staining with Coomassie blue.

β-Galactosidase assay. Cells were grown in DSM at 37 °C and samples were collected at the indicated time points. β-Galactosidase assays were performed in triplicate on soluble extracts using o-nitrophenyl-β-D-galactoside as substrate (Miller, 1972) and yielded comparable results. The activities of one representative experiment are presented. β-Galactosidase activities are given in units, where one unit is defined as ΔA₄₀₅ min⁻¹ × OD₅₇₈⁻¹ × 10⁻³, in which OD₅₇₈ is the optical density of the growth culture.

Western blots. Western blotting was carried out as described by 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 antibodies were used for the detection of Spo0A (Fujita *et al.*, 2005).

Determination of sporulation frequency. Sporulation frequencies were determined by the heat-resistance assay (Harwood & Cutting, 1990). Briefly, the strains were inoculated in 50 ml DSM and incubated at 37 °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 MgSO₄), and 100 µl samples of appropriate dilutions were plated on DSM agar to determine the number of vegetative cells. Cells were challenged at 80 °C for 20 min, and then 100 µl samples were plated on DSM agar to determine the number of heat-resistant spores.

RESULTS

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

Based on the analysis of transcriptional fusions, we concluded that *ftsH* interferes with the synthesis or/and

activity of Spo0A (Deuerling *et al.*, 1997). To confirm this assumption, we visualized the amount of Spo0A produced in the presence and absence of *ftsH* by Western blotting. Cells were grown in DSM and aliquots removed at t_0 (corresponding to the end of exponential growth and entry into transition phase) to t_3 . As can be seen from Fig. 1, Spo0A started to be present from stage 0 on in the wild-type strain and continued to be produced to at least stage 3. In contrast, in the *ftsH* knockout, Spo0A was present in greatly reduced amounts (Fig. 1).

Are the small amounts of Spo0A present in the *ftsH* knockout active, i.e. 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 analysed in the wild-type background, the β -galactosidase activity started to increase from stage 0 onwards (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). These results confirm that *ftsH* does indeed interfere with the synthesis or/and activation of Spo0A, where both regulatory circuits are interwoven (Strauch *et al.*, 1992).

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 μ g). First row, wild-type (WT) 1012 and WW01 (Δ *ftsH*); second row, AL31 (Δ *rapA*) and AL32 (Δ *rapA* Δ *ftsH*); third row, AL33 (Δ *rapB*) and AL34 (Δ *rapB* Δ *ftsH*); fourth row, AL35 (Δ *rapE*) and AL36 (Δ *rapE* Δ *ftsH*); fifth row, AB07 (Δ *spo0E*) and AB08 (Δ *spo0E* Δ *ftsH*).

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 Methods. Then, these null alleles were each combined with an *ftsH* knockout, and all six strains were analysed for their sporulation frequencies, for the amount of Spo0A present and for its activity status. While about 59% of the cells in our wild-type strain were able to form heat-resistant spores (Table 1), the sporulation frequency dropped by five orders of magnitude in the absence of the FtsH protein, confirming earlier data (Deuerling *et al.*, 1997). The sporulation frequencies in all three *rap* knockouts were higher than that of the wild-type strain and ranged from 67% to 72%. A similar observation has been published for RapA and RapE (Jiang *et al.*, 2000a). The sporulation frequencies in the double knockouts were 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 analysed 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 either *rapA* or *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 into 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 was somewhat different among 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). These results suggest that both the *rapA* and the *rapE* genes could be involved in shutting off *P_{skf}-lacZ* at t_2 .

The FtsH protein interferes with the phosphorylation status of Spo0A through Spo0E

Besides the Rap phosphatases, another set of three phosphatases is involved in the specific dephosphorylation

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 were withdrawn for measurement of β -galactosidase activity. (a) AL37 (*ftsH*⁺) and AL38 (Δ *ftsH*); (b) AL39 (Δ *rapA ftsH*⁺) and AL40 (Δ *rapA ΔftsH*); (c) AL41 (Δ *rapB ftsH*⁺) and AL42 (Δ *rapB ΔftsH*); (d) AL43 (Δ *rapE ftsH*⁺) and AL44 (Δ *rapE ΔftsH*); (e) AL45 (Δ *spo0E ftsH*⁺) and AL46 (Δ *spo0E ΔftsH*). ■, *ftsH*⁺; ○, Δ *ftsH*.

of Spo0A~P, designated Spo0E, YisI and YnzD; 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 & Hoch, 1991). We constructed a *spo0E* knockout, combined it with the *ftsH* null allele and measured the sporulation

Table 1. Effect of different mutations on the sporulation frequency

The results are representative of three different experiments. 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 ml⁻¹). The diluted samples were then heated at 80 °C for 20 min, and aliquots were plated. This gave heat-resistant spores ml⁻¹. The percentage of heat-resistant spores was calculated.

Strain	Relevant genotype	Cells ml ⁻¹	Spores ml ⁻¹	Spores (%)
1012	Wild-type	5.8×10^9	3.4×10^9	58.6
WW01	<i>ftsH::erm</i>	2.5×10^7	1.5×10^2	0.0006
AL31	<i>rapA::cat</i>	1.4×10^9	1.0×10^9	71.4
AL32	<i>rapA::cat ftsH::erm</i>	5.5×10^8	2.9×10^5	0.053
AL33	<i>rapB::cat</i>	6.5×10^8	4.7×10^8	72.3
AL34	<i>rapB::cat ftsH::erm</i>	6.7×10^8	5.0×10^6	0.74
AL35	<i>rapE::cat</i>	1.4×10^9	9.4×10^8	67.1
AL36	<i>rapE::cat ftsH::erm</i>	1.9×10^7	1.6×10^4	0.084
AB07	<i>spo0E::bleo</i>	1.1×10^8	8.3×10^7	75.5
AB08	<i>spo0E::bleo ftsH::erm</i>	7.6×10^7	6.5×10^5	0.85

frequencies in both strains. As to be expected the sporulation frequency rose 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 analysed the production of Spo0A in both mutant strains. Western-blot analysis revealed that Spo0A was present in large amounts already at *t*₀ in the Δ *spo0E* strain and remained at that high level for the next 3 h (Fig. 1). In the Δ *ftsH* strain, 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 the two proteins, either directly or indirectly, thereby influencing expression of *spo0A*. When we tested for the activity of Spo0A in both mutant strains, it was found to result in a higher activation of the *skf* promoter in the *ftsH*⁺ strain, 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 expression of the *spo0A* gene, but the protein remains inactive. These data indicate that *ftsH* influences production of active Spo0A by either promoting its phosphorylation or preventing 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, both proteins were purified and

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

incubated under conditions where FtsH is able to degrade β -casein (Kotschwar *et al.*, 2004). FtsH was purified with a GST-tag as reported before; the purification tag keeps the protein soluble in the absence of any detergent (Kotschwar *et al.*, 2004). 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, it was largely, but not completely, degraded in the presence of the nucleotide (Fig. 3a). This could be verified by probing some lanes with anti-GST antibody (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. 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 dephosphorylate Spo0A (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 is a substrate of FtsH. They further confirm 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 & 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 aa (*spo0E94*) (Perego & Hoch, 1987; Ohlsen *et al.*, 1994). To find out whether these two shortened versions of the Spo0E protein are still a target for

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. Aliquots of the reaction mixtures were separated by SDS-PAGE and stained with Coomassie brilliant blue. β -Casein served as a control.

FtsH, both were tagged with GST, overproduced in *E. coli* and purified. When the 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. 5). Since the full-length Spo0E protein is unstable when incubated with FtsH, we infer that the C-terminal 25 aa are responsible for this instability.

The C-terminal region of Spo0E confers 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 aa (Perego, 2001). Since Spo0E serves as a target for FtsH, but YisI and YnzD do 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 aa 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), YnzD-0E was largely degraded over time. We conclude from this experiment that indeed the C-terminal region 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 & 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 (AL53–AL56; see Supplementary Table S1) 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 in the presence and 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*. We conclude that 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 analysed, 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 63–81 and additionally a point mutation changing 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*.

Do *ftsH* or/and *spo0E* influence expression at the two promoters Ps and Pv preceding the *spo0A* gene?

Transcription of the *spo0A* gene is initiated at two different promoters termed Pv and Ps (Ferrari *et al.*, 1985; Kudoh

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 25 C-terminal aa 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.

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. The two 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). The enzymic activity in the *spo0E* null mutant was comparable to that measured in the wild-type strain, but 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 was induced about sevenfold between t_{-1} and t_1 (Fig. 8b). In the absence of *spo0E*, it was induced about tenfold, while only a threefold induction was measured in the *ftsH* knockout, which was not increased in the double knockout (Fig. 8b). We conclude that transcription at P_s is strongly reduced in Δ *ftsH*, an effect 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.

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*^{+/−}). $\blacksquare$, *spo0E*⁺, $\circ$, *spo0E*⁻.

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

The results are representative of three different experiments. See legend to Table 1 for technical details.

Strain	<i>ftsH</i> genotype	IPTG added*	Viable cell count	Spore count	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

*IPTG was added at a final concentration of 1 mM.

DISCUSSION

When *B. subtilis* cells enter the transition phase, several different genetic programmes 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 do 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 and the cell density, and integrate and process these signals by a phosphorelay which controls the level of phosphorylated Spo0A. It turned out that, while about 60 % of the cells manage to synthesize active Spo0A above a threshold value [these cells have been called Spo0A-ON (Chung *et al.*, 1994)], the remaining 40 % fail to do so (Spo0A-OFF). The formation of two subpopulations of otherwise isogenic cells is designated bistability (Smits *et al.*, 2006; Dubnau & Losick, 2006). The molecular basis for this bistability remains elusive. But it has to be asked whether cells in the transition phase exhibit multi- rather than bistability based on the observation that so many different genetic programmes are activated. This can be tested by double

and triple labelling using transcriptional fusions between programme-specific promoters and *gfp* and its derivatives (Margolin, 2000).

Several years ago, we and others discovered that the sporulation frequency in an *ftsH* knockout is reduced by about five orders of magnitude (Deuerling *et al.*, 1997; Lysenko *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 & Wilkinson, 2001). The objective of this ongoing research project is to elucidate the role of the FtsH protease during sporulation of *B. subtilis*. 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 synthesis of active Spo0A. Cells carrying an *ftsH* knockout synthesize a significantly reduced amount of Spo0A which, based on genetic data, is inactive (Figs 1 and 2). 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 was ruled out by introduction of an *ftsH* null allele into 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 (Table 2). Therefore, the role of *ftsH* is exclusively confined to the synthesis or/and activation of wild-type Spo0A.

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. (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 ΔftsH), AL52 (P_s-lacZ Δspo0E) and AL58 (P_s-lacZ Δspo0E ΔftsH). ■, Wild-type; ●, ΔftsH; □, Δspo0E; ◇, Δspo0E ΔftsH.

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 have used the candidate approach to identify putative substrate proteins of FtsH. First, we constructed knockouts by the gene replacement method in four different genes coding for phosphatases for which it has already been reported that the sporulation frequencies are slightly, but reproducibly enhanced in the absence of two of them (Jiang *et al.*, 2000a; Perego & Hoch, 1987); these results could be confirmed and extended for an additional phosphatase (RapB). 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 (Table 1). Not surprisingly, no increase in the amount of active Spo0A could be measured. On the basis of this analysis, we conclude that *ftsH* interferes directly or indirectly with expression or activity of these four phosphatases. In the double knockout, only about 1% of the cells reach the Spo0A-ON state. This conclusion can be confirmed by fusing the promoter of the *skf* and the *spoIIA* operons to *gfp* and analysing single cells under the fluorescence microscope. Whereas the *skf* promoter needs a low amount of active Spo0A, the *spoIIA* promoter requires a high amount to become activated (Fujita *et al.*, 2005).

Based on the results shown in Table 1, we asked whether the phosphatases are a substrate for FtsH. To this end, we overexpressed and purified the Spo0E phosphatase and incubated it with purified FtsH. Indeed, Spo0E is partially degraded by FtsH (Fig. 3); there are at least three possibilities to explain this result. First, our GST-FtsH protease does not exhibit full activity. Second, part of Spo0E is refractory to digestion by FtsH, and third, the GST-tag provides partial protection. We favour the second possibility based on the following observations. In *E. coli*, two substrate proteins have been identified for which FtsH controls their steady-state level: LpxC and KdtA; both are involved in the LPS biosynthetic pathway (Führer *et al.*, 2006; Katz & Ron, 2008). LpxC is a key enzyme in LPS formation and catalyses the second reaction and the first committed step in the biosynthesis of lipid A (Sorensen *et al.*, 1996). Overproduction of LpxC causes accumulation of abnormal membranes in the periplasm (Ogura *et al.*, 1999), which is toxic for the cell (Sullivan & Donachie, 1984), indicating that the amount of LpxC must be carefully regulated, and this occurs at the protein level via degradation by the protease FtsH (Führer *et al.*, 2006). KdtA is the only essential glycosyltransferase in the core oligosaccharide biosynthesis pathway (Belunis & Raetz, 1992). Here, too, FtsH regulates the concentration of the precursor of the sugar moiety 3-deoxy-D-manno-octulosonate (KDO) via the KdtA enzyme (Katz & Ron, 2008). When both purified enzymes were incubated with purified FtsH protease, no complete degradation was observed, reminiscent of our observation that complete degradation of Spo0E could not be obtained.

Why is FtsH unable to fully degrade these three proteins, at least under *in vitro* conditions? We assume that these

proteins are present in two different conformations, one recognized and the other not recognized by FtsH. An interesting question is whether this ratio can be influenced by a mechanism, e.g. by molecular chaperones.

Perego (2001) identified two homologues of the Spo0E phosphatase, YisI and YnzD. She showed 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 aa. She suggested that this C-terminal extension could be recognized by a protease. When GST-tagged YisI or YnzD was incubated with FtsH, these proteins turned out to be stable under the same conditions (Fig. 4). Two truncated versions of Spo0E also turned out to be stable (Fig. 5). Based on these findings, we asked whether the C-terminal region of Spo0E is responsible for its instability. When the C-terminal extension of Spo0E was fused to YnzD, the fusion protein was susceptible to degradation by FtsH (Fig. 6). We conclude that the C-terminal 25 aa of Spo0E contain the residues recognized by FtsH. Since the *spo0E11* mutation turned out to be insensitive to proteolysis by FtsH, the recognition site can be narrowed down to the last 14 aa.

Which amino acids are recognized by the FtsH protease? One of the substrate proteins of the *E. coli* FtsH protease is the LpxC enzyme (Ogura *et al.*, 1999). This enzyme represents the key enzyme in lipopolysaccharide (LPS) formation and controls the ratio 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 & Donachie, 1984), the amount of LpxC must be carefully regulated; this is done by FtsH. Here, too, the C-terminus has been identified as being responsible for recognition and degradation by FtsH (Führer *et al.*, 2006), specifically a stretch of about ten amino acids at the immediate C-terminus, which resembles the SsrA-tag. In the case of Spo0E, there is no similarity to the *B. subtilis* SsrA-tag (Wiegert & Schumann, 2001). Therefore, the amino acid sequence recognized by the FtsH protease is different from that of the SsrA-tag. We suggest that FtsH regulates the steady-state level of Spo0E in a similar way. This can be analysed by creating a Spo0E-GFP fusion protein followed by single-cell analysis as described above.

The studies reported here strongly suggest that regulation of stability of several proteins involved directly or indirectly in the synthesis of active Spo0A exerts a new level of post-translational 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 influencing the synthesis of active Spo0A need to be identified. This will be accomplished by three different experimental strategies: First, the candidate strategy; second, saturated transposon mutagenesis using pMarA (Le Breton *et al.*, 2006); and third, construction of an *ftsH* trap mutant (Flynn *et al.*, 2003). Identification of these additional targets should shed light on the molecular mechanism of bi- or even multistability.

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