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The Absence of FtsH Metalloprotease Activity Causes Overexpression of the σ^W-Controlled *pbpE* Gene, Resulting in Filamentous Growth of *Bacillus subtilis*

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FtsH is a membrane-bound and energy-dependent metalloprotease in bacteria which is involved in the posttranslational control of the activity of a variety of important transcription factors and in the degradation of uncomplexed integral membrane proteins. For *Bacillus subtilis*, little is known about the target proteins of FtsH protease. Its gene is not essential, but knockout strains display a pleiotropic phenotype including sensitivity toward salt and heat stress, defects in sporulation and competence, and largely filamentous growth. Comparison of the intracellular proteomes of wild-type and *ftsH* knockout strains revealed that at least nine proteins accumulated in the absence of *ftsH*, four of which could be identified. Two of these proteins turned out to be members of the σ^W regulon. Accumulation of one of these σ^W-controlled proteins, the penicillin-binding protein PBP4*, was analyzed in more detail. We could show that PBP4* is not a proteolytic substrate of FtsH and that its overproduction is due to the enhanced transcription of its gene (*pbpE*) in *ftsH* null mutants. The filamentous growth phenotype of Δ*ftsH* strains was abolished in a Δ*ftsH* Δ*pbpE* double knockout. In *ftsH* wild-type strains with the *pbpE* gene under regulatable control, *pbpE* overexpression caused filamentation of the cells. DNA macroarray analysis revealed that most genes of the σ^W regulon are transcribed at elevated levels in an *ftsH* mutant. The influence of FtsH on σ^W-controlled genes is discussed.

FtsH belongs to the AAA (ATPases associated with diverse cellular activities) protein superfamily, and members of this family are involved in a wide variety of cellular processes, such as vesicle-mediated protein transport, cell cycle control, control of gene expression, and proteolysis (for recent reviews, see references 19 and 35). The AAA proteins constitute a subfamily of the Walker-type nucleoside triphosphatases and contain conserved ATPase domains, typically spanning 200 to 250 residues, referred to as AAA modules (6, 20). FtsH is a member of the AAA proteases, which occur in eubacteria and eukaryotic organelles such as mitochondria and chloroplasts, but not in archaea, and contain an AAA module and a zinc metalloprotease domain in a single polypeptide chain. Bacterial FtsH proteins are anchored via two transmembrane segments in the cytoplasmic membrane, with the short N- and the long C-terminal parts facing the cytoplasm. These metalloproteases are involved in the quality control of membrane-bound proteins and in the degradation of short-lived cytoplasmic regulatory proteins (for recent reviews, see references 16 and 26).

While several target proteins of the *Escherichia coli* FtsH metalloprotease have already been identified, they remained elusive in *Bacillus subtilis*. Therefore, we reasoned that comparing the proteomes of *B. subtilis* wild-type and *ftsH* knockout cells should reveal target proteins accumulating in the absence of the protease. It should be mentioned that, in contrast to that of *E. coli*, the *B. subtilis ftsH* gene is dispensable, though its absence results in a pleiotropic phenotype (9). Indeed, this proteomic approach led to the discovery of at least nine proteins present in elevated amounts in the *ftsH* null mutant, four of which could be identified by matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectrometry. Interestingly, two of these proteins belong to the σ^W regulon, and further experiments revealed that most mRNAs of this regulon are present at elevated levels in the *ftsH* knockout, suggesting that this membrane-anchored metalloprotease exerts a direct or indirect effect on the regulation of the σ^W regulon.

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MATERIALS AND METHODS

Growth conditions, bacterial strains, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown aerobically at 37°C in Luria broth (LB). Ampicillin was included for all plasmid-bearing *E. coli* strains. Chloramphenicol, neomycin, tetracycline, and erythromycin were added at concentrations of 5, 10, 15, and 1 μg ml^{-1}, respectively.

DNA manipulations and analysis. Plasmid DNA was purified on columns (Qiagen, Hilden, Germany), and PCR products were generated with *DeepVent* DNA polymerase as specified by the manufacturer (New England Biolabs). Cloning was carried out by standard methods (24).

Proteomics. Protein extracts were prepared, and proteins were separated by two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE), as previously described (4). Individual protein spots were analyzed by MALDI-TOF mass spectrometry for identification.

Preparation of polyclonal antibodies against PBP4*. The coding region of *pbpE* was amplified by PCR using appropriate primers, and the product was integrated into the vector plasmid pQE30, thereby fusing six histidine residues to the N terminus of PBP4*. The recombinant plasmid pQE30-*pbpE* was transformed into *E. coli* strain XL-1 Blue, and overexpression of the His-tagged protein was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After His-PBP4* was purified, it was sent to the "Elevage Scientifique des Dombes" company to be used for raising antibodies in rabbits. Polyclonal antibodies were used in Western blot experiments at the following dilutions: αHtpG, 1:10,000; αPBP4*, 1:10,000; αYdjF (a gift of A. Atalla), 1:10,000; and αAbrB, 1:2,000.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Escherichia coli		
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *lacZ*M15 Tn*10*]	Stratagene
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.
Bacillus subtilis		
1012	*leuA8 metB5 trpC2 hsrM1*	23
WW01	*ftsH*::*erm* derivative of 1012	36
ED04	*ftsH*::*tet* in 1012	9
MP01	*ftsH*::*cat* in 1012	9
sigW	*sigW*::*neo* allele in 1012	37
SWV119	*abrB*::*tet*	30
Δ*pbpE*	Δ*pbpE*::*erm* allele isolated from PS1805 and transformed into 1012	21
BFS233	pMUTIN inserted into *yuaF*; promoter of *yuaF* fused to *lacZ*	27
MSW01	pX2-*ftsH*	This work
SZ01	Δ*pbpE*::*erm ftsH*::*cat*	This work
SZ06	Δ*pbpE*::*erm amyE*::P_{xylA}-*pbpE ftsH*$^+$ in 1012	This work
SZ08	Δ*sigW*::*neo* pX2-*ftsH*	This work
SZ09	Δ*pbpE*::*erm amyE*::P_{xylA}-*pbpE ftsH*::*tet* in 1012	This work
TW51	*amyE*::P_{yuaF}-*lacZ*; derived from pP_{yuaF}-*bgaB*, with *bgaB* replaced by *lacZ*	This work; P_{yuaF}-*bgaB* from reference 37
TW52	*amyE*::P_{yuaF}-*lacZ ftsH*::*cat*	This work
Plasmids		
pQE30	3.5-kb vector allowing overproduction of proteins with six N-terminal histidine residues	32
pX	Carries xylose-regulatable expression cassette for insertion at the *amyE* locus	14
pX2	Carries xylose-regulatable expression cassette to be fused in front of the gene of interest	18

Western and Northern blotting and DNA macroarray analysis. Samples were prepared for sodium dodecyl sulfate (SDS)-PAGE and immunoblot analysis as described previously (11). Preparation of high quality RNA, Northern blotting, and DNA macroarray analysis have been described previously (37). In summary, the analysis was carried out by using Panorama *B. subtilis* Gene Arrays and cDNA labeling primers (optimized for *B. subtilis*), all from Sigma Genosys. Two micrograms of total RNA was hybridized to 4 μl of cDNA labeling mix. Hybridization signal intensities were quantified with ArrayVision software (version 5.1; Imaging Research, St. Catherine's, Ontario, Canada). For normalization and background subtraction, the overall spot normalization function of ArrayVision was used. Each analysis was carried out using two independently isolated RNA preparations and different array batches. Means and deviations of expression level ratios from these different experiments were calculated using Microsoft Excel. Ratios were calculated only from spots with a signal-to-noise ratio larger than 3 in the Δ*ftsH* experiments.

β-Galactosidase assays. β-Galactosidase activities were assayed at 28°C as described previously (37) by using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. All assays were repeated at least three times, and the replicates yielded comparable results.

RESULTS

At least nine proteins accumulate in an *ftsH* knockout. In contrast to *E. coli*, where several proteins have been identified as substrates for the FtsH protease, no target proteins for the *B. subtilis* integral membrane protease have been demonstrated so far. We reasoned that in the absence of FtsH, potential substrate proteins could accumulate. To detect these proteins, cellular extracts prepared from an *ftsH* wild-type strain and its isogenic *ftsH*::*cat* knockout strain were resolved by the 2-D gel electrophoresis technique. Careful comparison of the two patterns revealed at least nine proteins which accumulated in the absence of the proteolytic activity (Fig. 1). By use of MALDI-TOF mass spectrometry, four of these proteins present in elevated amounts in the Δ*ftsH* strain were identified as PBP4*, RocF, YvlB, and YfmJ. While PBP4* is encoded by the *pbpE* gene and belongs to the group of penicillin-binding proteins (21), RocF is an arginase (10). The *yvlB* gene codes for a protein of unknown function, and *yfmJ* codes for a protein similar to quinone oxidoreductase. Most interestingly, the *pbpE* and *yvlB* genes belong to the σ^W regulon (12). These data suggest that the FtsH protease either affects the stability of one or more of these four proteins directly or affects their synthesis indirectly. To distinguish between these two possibilities, we have chosen PBP4* for more-detailed analysis of protein accumulation in *ftsH* knockout strains with respect to synthesis and stability.

Accumulation of PBP4* in *B. subtilis* is due to enhanced transcription of the *pbpE* gene. To elucidate whether the accumulation of PBP4* in the Δ*ftsH*::*cat* strain is the result of enhanced stability or of increased synthesis, several experiments were carried out. First, the amounts of PBP4* in wild-type and *ftsH* knockout strains were determined by Western blot experiments. When cell extracts prepared from identical numbers of cells were probed with polyclonal antibodies raised against purified PBP4*, significantly greater amounts of the penicillin-binding protein were detected in the *ftsH*::*cat* and *ftsH*::*erm* strains (Fig. 2), corroborating the results obtained by 2-D gel electrophoresis. As expected, no PBP4* protein could be demonstrated in the *pbpE* knockout strain (Fig. 2). In in vitro experiments with purified FtsH protein incubated with purified His_6-Pbp4*, no degradation of the penicillin-binding

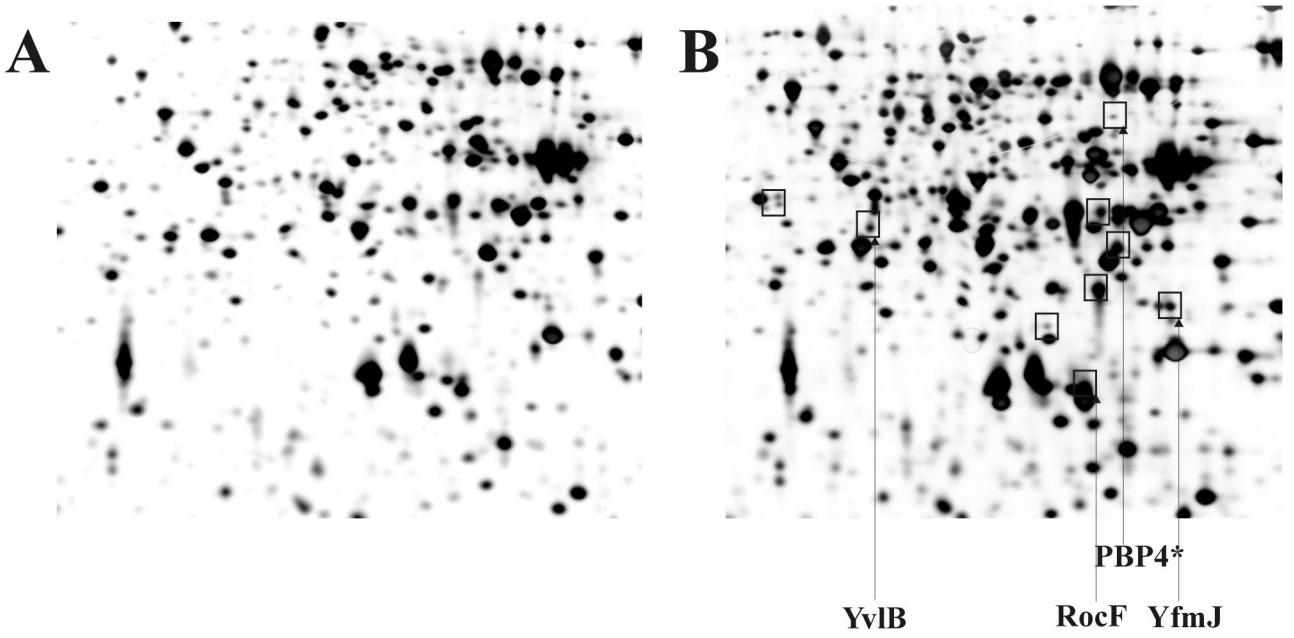


FIG. 1. Comparative proteomics of the *B. subtilis* wild-type strain 1012 (A) and its isogenic *ftsH*::*cat* knockout (B). Intracellular proteins of *B. subtilis* 1012 and MP01 (*ftsH*::*cat*) exponentially grown in minimal medium at 37°C were separated in a pH gradient of 4 to 7 in the first dimension. Gels were stained with silver nitrate. Proteins present in elevated amounts in the *ftsH*::*cat* strain are boxed.

protein could be detected (data not shown). Therefore, to test whether Pbp4* accumulation was due to overexpression of *pbpE* at the transcriptional level, *pbpE* without its indigenous σ^W-controlled promoter was fused to the xylose-regulatable promoter P_{xylA} (14) and ectopically integrated at the *amyE* locus (Fig. 3A). The *pbpE* gene at its chromosomal location was inactivated by insertion of an erythromycin resistance cassette. Two versions of that strain were constructed, one with *ftsH* (strain SZ06) and one without *ftsH* (strain SZ09). Both strains were grown to the mid-exponential-growth phase, and *pbpE* expression was induced by addition of 1% xylose. Extracts were prepared before and 90 min after addition of xylose and were probed with different antibodies. While FtsH is clearly absent from the *ftsH*::*tet* strain SZ09, comparable amounts of PBP4* are present in the presence and absence of the FtsH protease (Fig. 3B). As a control, we checked for HtpG, a heat-inducible protein (25); the amount of this protein was not significantly changed (Fig. 3B). These results suggested that PBP4* is not a substrate of FtsH.

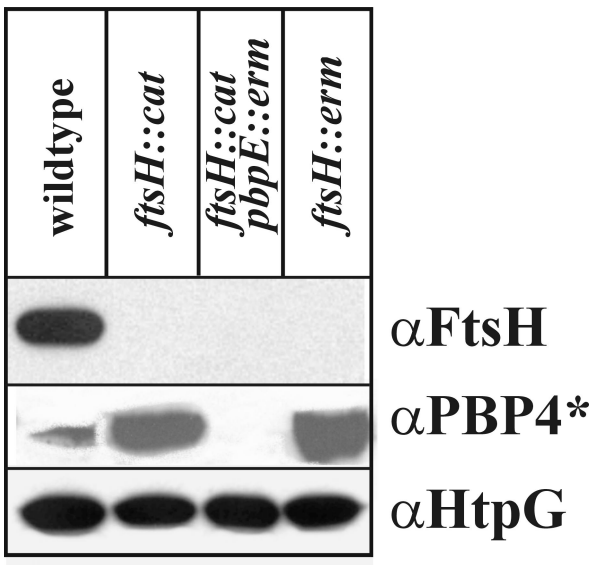


FIG. 2. Pbp4* accumulates in *ftsH* knockout strains. Immunoblot analysis detects increased amounts of PBP4* in *ftsH* null mutants. The different *B. subtilis* strains indicated were grown to the mid-exponential-growth phase in LB at 37°C. Proteins present in cell lysates were separated by SDS-PAGE, blotted, and developed with polyclonal antibodies against PBP4*, FtsH, or (as an internal control) HtpG.

If PBP4* does not represent a target protein for FtsH, the protease must influence its synthesis, most probably at the transcriptional level. This conjecture was tested by Northern blot analysis. Total RNAs were prepared at three different time points during the growth curves of the *ftsH* wild-type and *ftsH*::*erm* knockout strains and were probed with *pbpE* antisense RNA. While *pbpE* mRNA was not detectable in the presence of FtsH, significant amounts, increasing over time, were present in the *ftsH* null mutant (Fig. 3C). This result unequivocally demonstrates that FtsH influences the transcription of *pbpE* rather than the stability of PBP4*.

Overexpression of *pbpE* in an Δ*ftsH* strain causes filamentous growth. An imbalance in the synthesis of penicillin-binding proteins is reported to cause filamentous growth of bacteria (2). Therefore, we asked whether in *B. subtilis* the overproduction of PBP4* observed in the *ftsH* knockout might cause the filamentous-growth phenotype. First, we analyzed the phenotype of *B. subtilis* cells in the presence and absence of the wild-type *ftsH* and *pbpE* alleles. While wild-type cells were rod shaped (Fig. 4A), inactivation of *ftsH* resulted in filamentous growth (Fig. 4B). The absence of both *ftsH* and *pbpE* restored

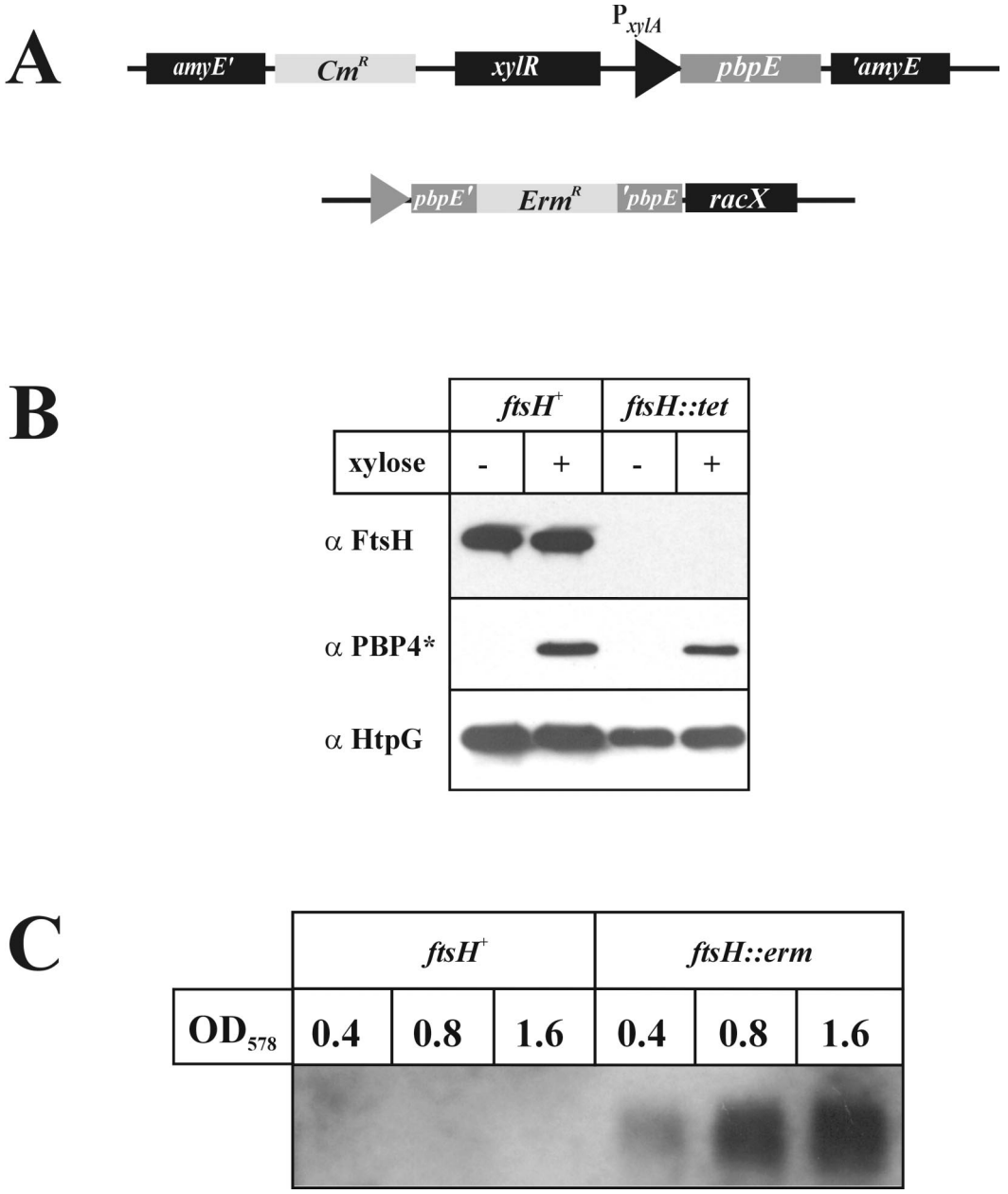


FIG. 3. Accumulation of Pbp4* in *ftsH* knockout strains is due to enhanced transcription of *pbpE*. (A) Schematic drawing showing fusion of the *pbpE* gene to the xylose-regulatable promoter P_{xylA}. Comparable amounts of PBP4* are present in the presence and absence of *ftsH*. (B) Proteins from cells grown as described above were separated by SDS-PAGE and probed with three different antibodies as indicated. +, presence of xylose; −, absence of xylose. (C) Northern blot analysis reveals increased amounts of *pbpE* transcript in the *ftsH* knockout mutant. Total RNAs were prepared from *B. subtilis* 1012 and its *ftsH*::*erm* derivative at the OD_{578} indicated and were hybridized with digoxigenin-labeled *pbpE* antisense RNA.

the wild-type cell morphology (Fig. 4C), indicating that enhanced expression of the *pbpE* gene is indeed responsible for the filamentous phenotype. If this conclusion is true, overexpression of *pbpE* in the presence of an intact *ftsH* allele should also result in filamentous growth. To test this idea, strain SZ06, allowing induction of *pbpE* by xylose (see Fig. 3A), was grown in the presence and absence of the inducer. In the absence of xylose, no Pbp4* was detectable in a Western blot and the cells exhibited a normal phenotype (Fig. 5A and B). Upon addition of xylose to the growth medium and Pbp4* overproduction (Fig. 5A), the cells grew largely in filaments (Fig. 5C). We conclude from these results that increased expression of *pbpE* due to either the absence of *ftsH* or artificial overproduction of PBP4* causes filamentous growth.

The concentration of AbrB is not altered in a *B. subtilis ftsH* knockout. It has been reported that the *pbpE* gene is under the negative control of the transcriptional repressor AbrB (31). To analyze whether the influence of FtsH on *pbpE* transcription is indirect, due to an influence of FtsH on AbrB, cellular extracts of wild-type and isogenic *ftsH* and *abrB* knockout strains were

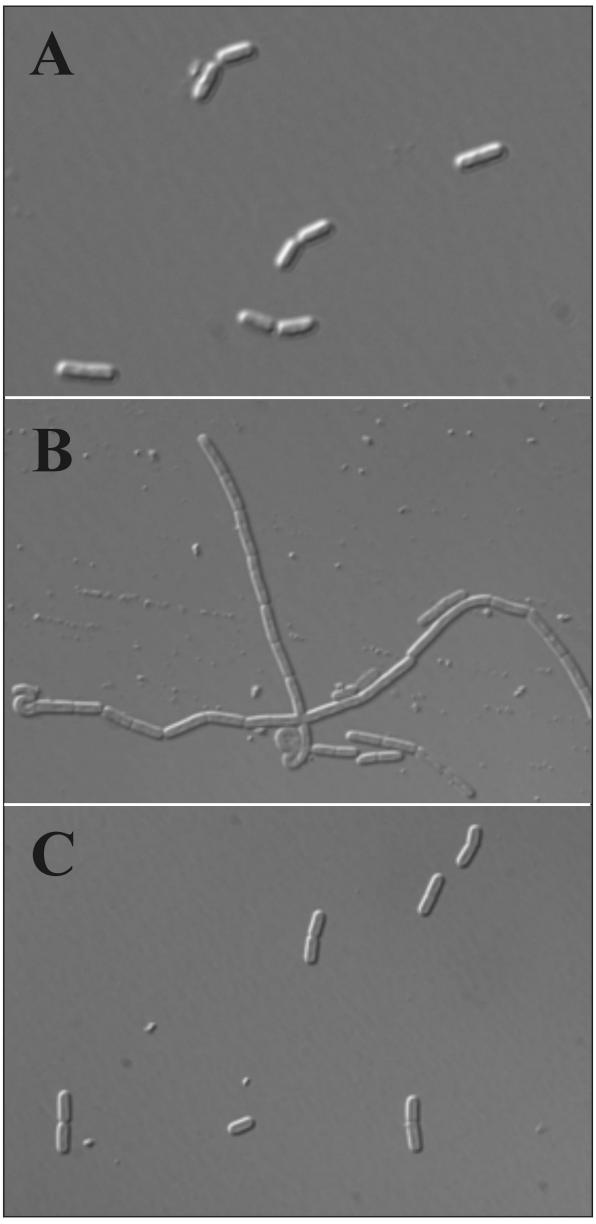


FIG. 4. Accumulation of Pbp4* causes filamentous growth of *B. subtilis* cells. *B. subtilis* strains 1012 (wild type) (A), MP01 (*ftsH*::*cat*) (B), and SZ01 (*ftsH*::*cat pbpE*::*erm*) (C) were grown in LB medium to the mid-logarithmic-growth phase. Cells were analyzed by phase-contrast microscopy.

tested for their amounts of AbrB protein by Western blotting. As can be seen in Fig. 6, the amounts of the repressor AbrB are identical in the absence and presence of FtsH. As an internal control, we also determined the amounts of the heat shock protein HtpG, which are identical in the extracts prepared from all four strains. We conclude from these results that FtsH does not influence the cellular level of AbrB and that *pbpE* induction in *ftsH* knockout strains is not due to altered amounts of AbrB.

Most genes controlled by the extracytoplasmic function sigma factor σ^W are overexpressed in an *ftsH* null mutant. Since two of the four genes identified by the proteomic approach (*pbpE* and *yvlB*) are members of the σ^W regulon, we investigated whether additional genes of that regulon exhibit increased expression in the absence of *ftsH*. First, *B. subtilis ftsH* null and *ftsH*$^+$ strains expressing a transcriptional fusion of the *yuaF* promoter to *lacZ* were tested for β-galactosidase activities at different growth stages. The *yuaF* promoter is one of the strongest σ^W-controlled promoters (37). It turned out

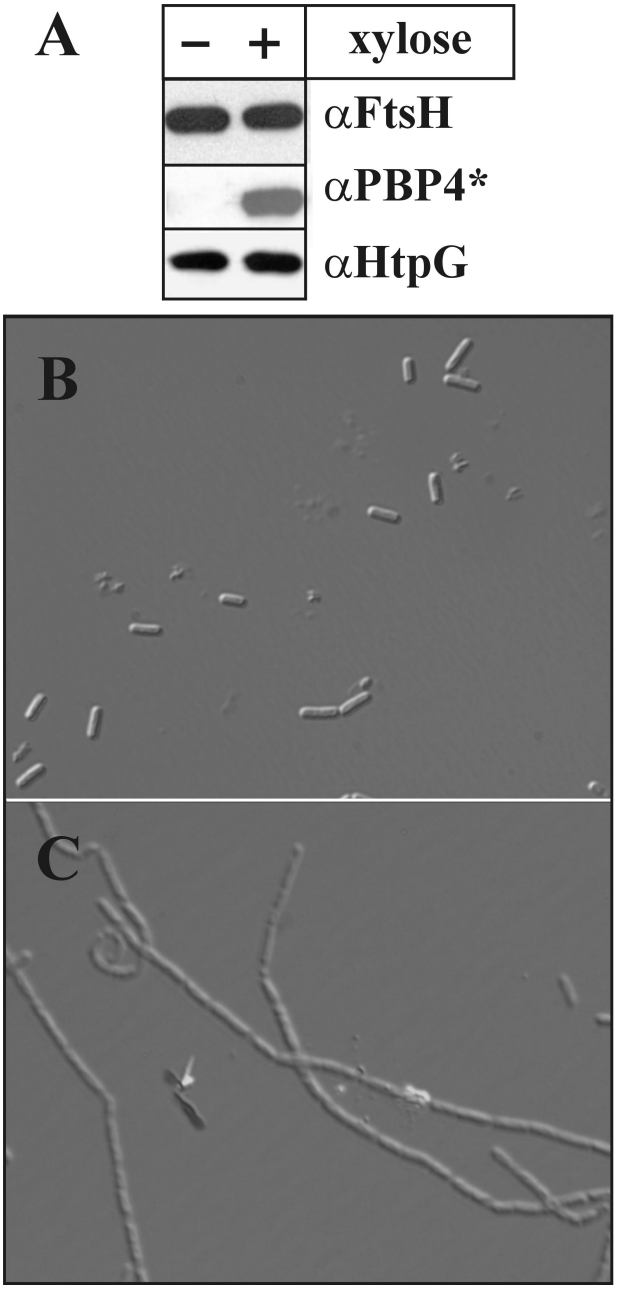


FIG. 5. Overexpression of *pbpE* in the presence of the *ftsH* wild-type allele causes filamentous growth. (A) *B. subtilis* strain SZ06 (*ftsH*$^+$ P_{xylA}-*pbpE*) was grown either with or without addition of xylose to the medium to mid-logarithmic phase and was checked for the presence of FtsH, Pbp4*, and HtpG (internal control) by Western blotting. (B and C) Cells grown without (B) and with (C) xylose were then analyzed by phase-contrast microscopy.

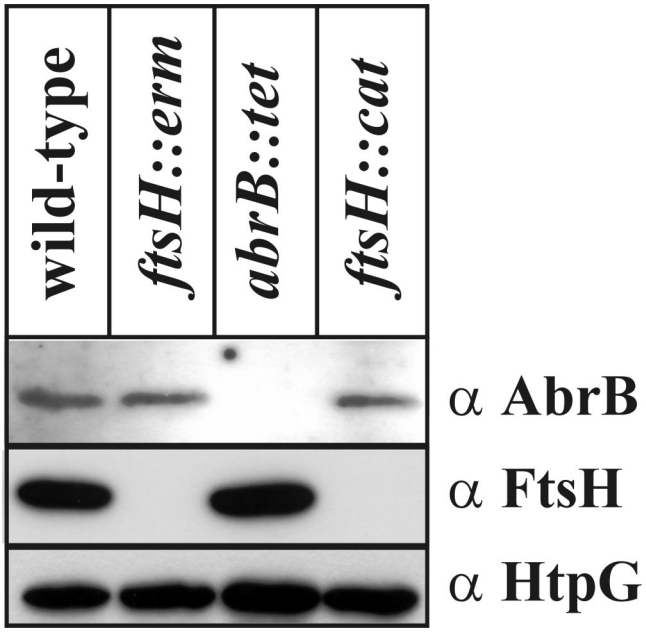


FIG. 6. Cellular AbrB levels are not altered in *B. subtilis ftsH* knockout strains. *B. subtilis* 1012 (wild type), WW01 (*ftsH*::*erm*), SWV119 (*abrB*::*tet*), and MP01 (*ftsH*::*cat*) were grown to mid-logarithmic phase and submitted to Western blot analysis as described above.

that both the basal level of β-galactosidase activity and the level during stationary phase were enhanced in the *ftsH* null mutant over those in the isogenic wild-type strain (Fig. 7A). Second, using specific antibodies against the YdjF protein, we analyzed the expression of the σ^W-dependent gene *ydjF* in a strain where the promoter of *ftsH* has been replaced by the xylose-controllable promoter P_{xylA} (Fig. 7B). In the absence of xylose in the growth medium, no FtsH protein could be visualized in cellular lysates and large amounts of YdjF protein were detected (Fig. 7B, lane 1). Upon xylose-induced *ftsH* expression or at wild-type levels of FtsH, the amount of YdjF protein was significantly reduced (Fig. 7B, lanes 2 and 3).

Both experiments suggest that in the absence of the FtsH metalloprotease, there is an upregulation of the whole σ^W regulon. Therefore, the global transcription pattern of a *B. subtilis* wild-type strain compared to that of the *ftsH*::*erm* knockout (WW01) at an optical density at 578 nm (OD_{578}) of 0.8 was analyzed by the DNA macroarray technique. Indeed, it turned out that most genes of the σ^W regulon exhibited 1.5- to 3-fold-enhanced expression in the *ftsH* null mutant, while some were expressed up to 9-fold in the absence of the FtsH protein (Fig. 7C). It should be noted that σ^W-controlled genes such as *xpaC*, *yvlC*, and *yxzE*, which are obviously not induced in Δ*ftsH* mutants, also respond poorly to the strong inducing conditions of an alkaline shock when assayed in DNA microarrays (37). The σ^W-controlled genes were reproducibly induced, as was also seen when the global transcriptional pattern was analyzed at ODs of 0.4 and 1.2 (data not shown). Probably because of the various phenotypic defects of Δ*ftsH* strains, the expression pattern of various other genes altered to different extents, and without further experiments it is not possible to present a conclusive overall transcriptional pattern of Δ*ftsH* strains.

To summarize, these experiments show that the FtsH protease exerts an influence on the expression of the σ^W regulon which is either direct or indirect.

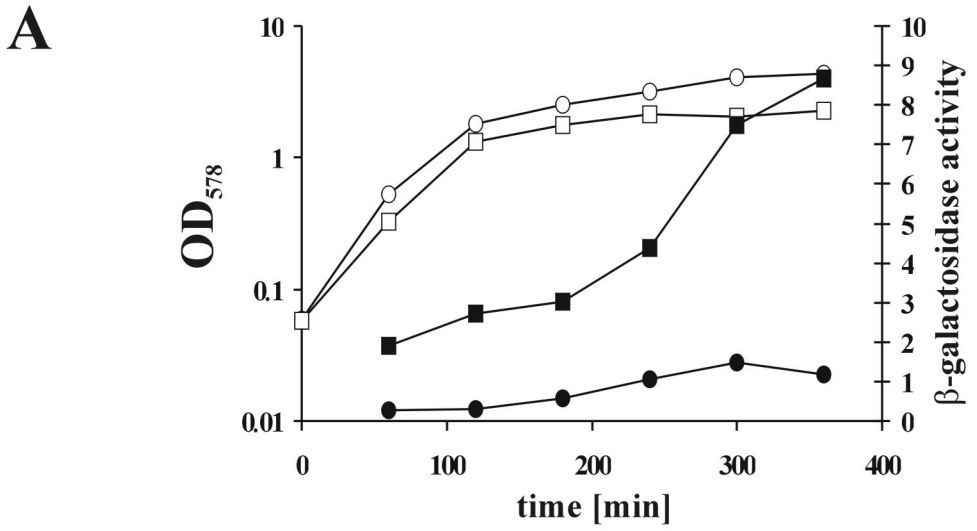
Cells carrying a *sigW ftsH* double knockout display a growth defect. It is possible that the absence of FtsH activity generates a certain stress for *B. subtilis* cells, which in turn induces the σ^W regulon, helping the cells to overcome the stressful situation. Therefore, we tested whether induction of the σ^W regulon is of crucial importance for the survival of *ftsH* knockout strains. Cells carrying single knockouts in *sigW* or *ftsH* are viable, though the latter exhibit a pleiotropic phenotype as already mentioned. However, when the *ftsH* knockout was recombined into a *sigW* null mutant strain, cell colonies remained small and reached a final OD of only about 0.5 in liquid culture. This suggests that the combination of both null alleles severely affects viability.

To analyze this in more detail, we crossed the *sigW* null allele into strain MSW01, where expression of *ftsH* is xylose dependent. The resulting strain SZ08 was grown overnight in the presence of 1.5% xylose, and cells were washed twice with LB to remove the xylose and were then used to inoculate LB medium without xylose. To fully deplete FtsH, these cells were grown to early-stationary phase and used to inoculate cultures with and without xylose to an OD_{578} of 0.08. As controls, strain MSW01 with the xylose-regulatable *ftsH* and wild-type *sigW* background, the *sigW*::*neo* strain, and the isogenic wild-type *B. subtilis* strain were treated in the same manner. The *B. subtilis* wild-type and *sigW*::*neo* strains grew independently of the addition of xylose (Fig. 8). Strain MSW01 reached about half of the final OD when xylose was omitted, showing that FtsH depletion is harmful to the cells. The growth of strain SZ08 was impaired more severely upon depletion of FtsH, as the strain reached a final OD of about 0.8 (Fig. 8), showing that induction of the σ^W regulon in the absence of FtsH is crucial for survival of the cells under standard laboratory conditions.

DISCUSSION

The ATP- and Zn^{2+}-dependent, membrane-anchored metalloprotease FtsH is not essential in *B. subtilis*, though knock-

FIG. 7. Genes controlled by σ^W are overexpressed in *B. subtilis ftsH* knockout strains. (A) Strains with a transcriptional fusion of the σ^W-controlled *yuaF* promoter to *lacZ* were grown at 37°C in LB medium. At different time points, samples were withdrawn and β-galactosidase activities were determined. Circles, *B. subtilis* TW51 (*amyE*::P_{yuaF}-*lacZ*); squares, TW52 (*amyE*::P_{yuaF}-*lacZ ftsH*::*cat*). Open symbols, OD_{578}; solid symbols, β-galactosidase activity. (B) *B. subtilis* strain MSW01 (*sigW*$^+$ pX2-*ftsH*) was grown in the absence (lane 1) or presence (lane 2) of xylose to mid-logarithmic phase and analyzed by Western blotting using antibodies against FtsH and YdjF. *B. subtilis* 1012 (wild type) grown without xylose (lane 3) was used as a control. (C) Schematic representation of DNA macroarray analysis of *B. subtilis* 1012 *ftsH*::*cat* compared to that of the wild type. DNA arrays of the entire *B. subtilis* genes were hybridized with cDNA probes generated from RNA extracted from cells of the two strains at an OD_{578} of 0.8. Mean results and standard deviations from two independent experiments are presented. Spot intensities were quantified as described previously (37), and the quotients of *ftsH*::*cat*/wild-type intensities for σ^W-controlled genes are given.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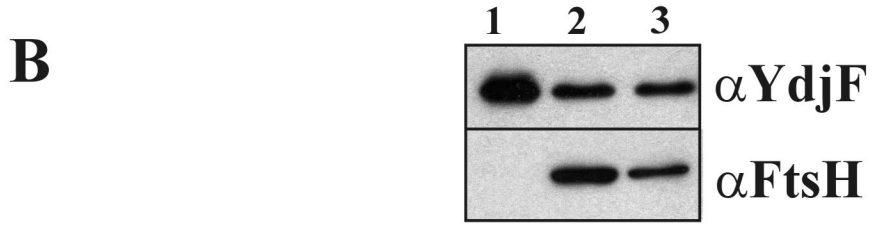
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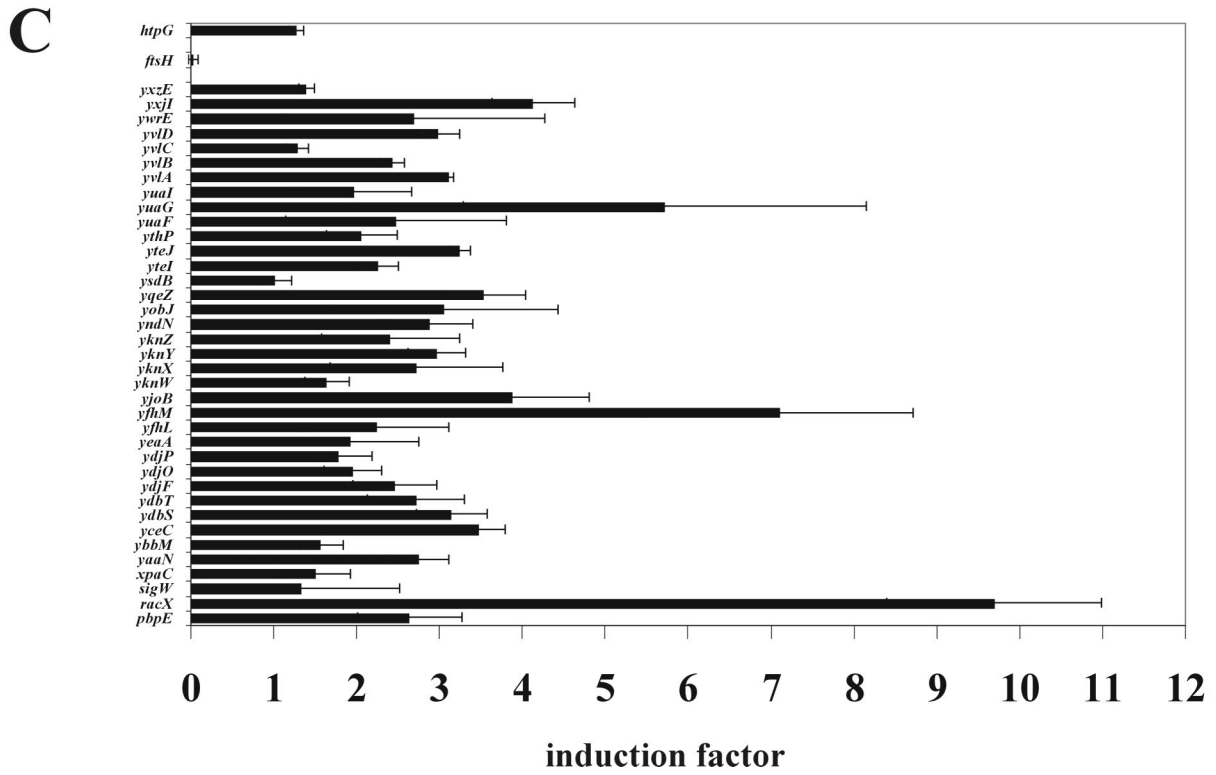
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12
induction factor

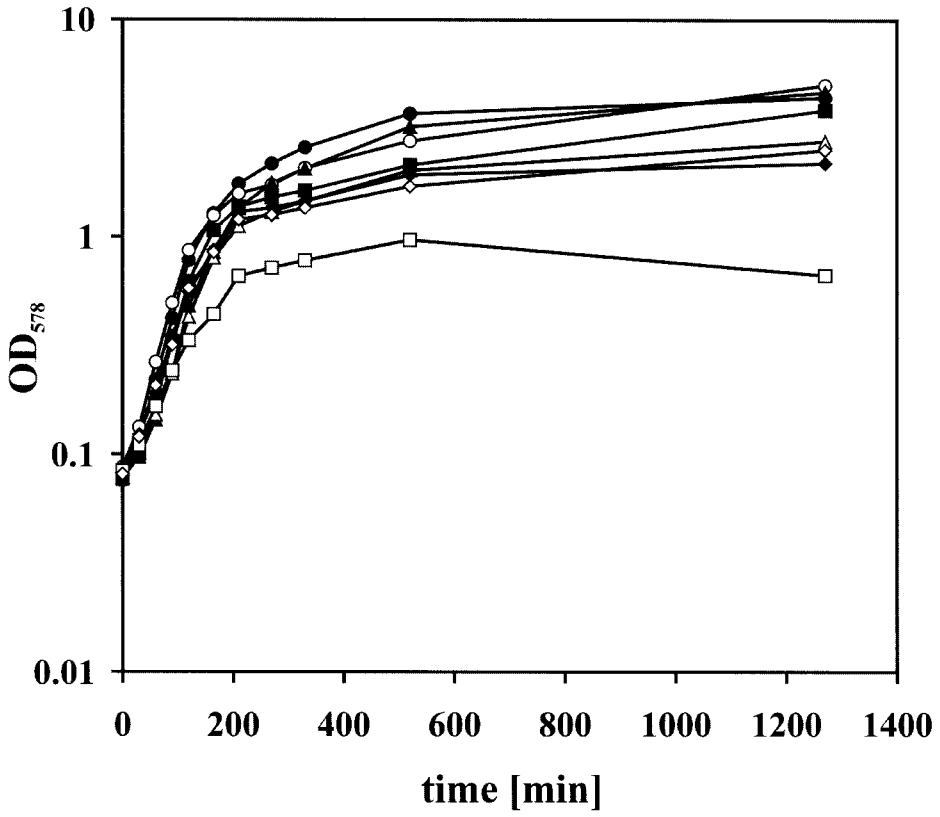

FIG. 8. Cells of a *B. subtilis sigW* knockout display a growth defect upon depletion of FtsH protein. Growth of *B. subtilis* strains 1012 (wild type) (circles), MSW01 (*sigW*$^+$ pX2-*ftsH*) (triangles), 1012 *sigW*::*neo* (*sigW* knockout; *ftsH*$^+$) (diamonds), and SZ08 (*sigW* knockout; pX2-*ftsH*) (squares) was monitored in the presence (solid symbols) and absence (open symbols) of xylose added to the growth medium. Cultures were inoculated from early-stationary-phase cultures without xylose to an OD_{578} of 0.08.

outs display a pleiotropic phenotype including a complete inability to sporulate, severe growth defects under heat and salt stresses, and largely filamentous growth (9). Here, we report a new phenotype, which is upregulation of most genes of the σ^W regulon in *ftsH* null mutants.

The present study was originally aimed at identifying substrate proteins recognized by the FtsH protease. So far, no such proteins have been unambiguously identified in *B. subtilis*, though it has been reported that the *B. subtilis* SpoVM protein is degraded by the *E. coli* FtsH protease and strongly inhibits proteolysis of σ^{32} in vitro (7). This result is reminiscent of those obtained with the λ CIII protein, which also inhibits the proteolytic activity of FtsH and is later degraded (29). It may be assumed that the 26-amino-acid SpoVM peptide will also be degraded by *B. subtilis* FtsH, which has to be proven experimentally.

Our screen was based on the assumption that FtsH target proteins synthesized under physiological conditions may be present at elevated levels in the absence of the protease. To investigate this possibility, the proteomes of a wild-type and an *ftsH* knockout strain were compared. It turned out that at least nine proteins were present in increased amounts and that four of them could be identified by MALDI-TOF mass spectrometry. These proteins accumulate in the *ftsH* null mutant either because they are true substrate proteins or, alternatively, because *ftsH* is involved in the regulation of their synthesis. Two of these proteins (PBP4* and YvlB) are members of the σ^W regulon (12), and we have chosen PBP4* for further analyses to find out why this protein accumulates in the absence of the protease. PBP4* is similar to *E. coli* PBP4, which has been demonstrated to cleave peptidoglycan peptide cross-links (15). However, *B. subtilis* PBP4* does not have a classical signal peptide and is found mainly in the cytoplasmic fraction.

It has already been reported that *ftsH* influences the level of penicillin-binding proteins. While the amount of PBP2A was increased in an *ftsH* mutant, the amounts of PBP2B and PBP4 were decreased (17). In general, penicillin-binding proteins are involved in peptidoglycan synthesis and cell shape determination during cell division, sporulation, and germination (1). After identifying PBP4* as a protein overproduced in the absence of *ftsH*, we asked whether the increased amount of PBP4* might be responsible for filamentous growth. While cells of a *pbpE ftsH* double-knockout strain indeed failed to form filaments, overexpression of *pbpE* in an *ftsH* wild-type strain led to the same phenotype. Therefore, elevated amounts of PBP4* are the causative agent inducing filamentous growth. How this phenotype is induced by PBP4* is completely unknown, mainly because the function of this protein remains elusive. On the other hand, depletion of PBP2B results in filamentation, too (8).

Western blot experiments using two different *ftsH* knockout strains confirmed the results obtained by the 2-D PAGE anal-

ysis, namely, enhanced levels of PBP4*. To distinguish between degradation of PBP4* and increased overproduction due to the involvement of *ftsH* in the regulation of expression of *pbpE* on the transcriptional level, the *pbpE* gene was fused to a controllable promoter. Upon induction of the ectopically induced *pbpE*, comparable amounts of the penicillin-binding protein were detectable in the presence and absence of *ftsH*. This result strongly argues against PBP4* being a target protein of FtsH. This assumption is confirmed by the finding that purified FtsH was unable to degrade PBP4* in an in vitro assay (data not shown).

Since FtsH is not involved in the degradation of PBP4*, it must influence its expression. This hypothesis could be confirmed by Northern blot analysis. While significant amounts of the *pbpE* transcript could be seen with the *ftsH* null mutant, this transcript was not detectable in the wild-type strain. Therefore, the absence of FtsH induces transcription of *pbpE*. Since, in addition to PBP4*, YvlB, another product of the σ^W regulon, is also present in increased amounts, we asked whether additional σ^W-dependent genes exhibit enhanced expression in an *ftsH* null mutant; this was shown for *ydjF* and *yuaF*. Global analysis of all protein-coding genes of the *B. subtilis* genome by the DNA macroarray technique revealed that most genes of the regulon are indeed transcribed at elevated levels. Taken together, these findings show that *ftsH* somehow influences expression of the σ^W regulon.

Negative regulation of the *pbpE* gene by the global repressor protein AbrB has been described previously (31). In addition, an interaction between the σ^W regulon and AbrB has been revealed recently. It was shown that AbrB binds not only to the promoter region of *pbpE* but also to other genes of the σ^W regulon, including the *sigW* operon itself (22). Since recent work also suggested an influence of FtsH on *abrB* transcription (9), we tested for the level of AbrB protein in *ftsH* knockout strains. In the case of an influence of FtsH on AbrB and therefore on σ^W-controlled genes, reduced levels of AbrB in *ftsH* knockout strains would be expected. By use of specific antibodies against AbrB, it could be shown that its levels are comparable in the presence and absence of the FtsH protease, excluding the possibility that altered levels of AbrB cause overexpression of σ^W-controlled genes in *ftsH* knockout strains.

What could be the role of *ftsH* in expression of the σ^W regulon? σ^W is one of seven extracytoplasmic function sigma factors of *B. subtilis*, and work carried out over recent years has identified a minimum of 30 promoters (controlling about 60 genes) recognized by σ^W (5, 12). The mechanism that induces the σ^W regulon is unknown, but it has been shown that the σ^W regulon is activated early in the stationary phase (13), is strongly induced by a sudden imposition of alkali stress (37), and, most interestingly, is induced by antibiotics such as vancomycin which inhibit peptidoglycan biosynthesis (5). Most probably, the signal that activates σ^W is sensed and transduced by a membrane-bound anti-sigma factor (RsiW) encoded by the *ybbM* gene, which lies downstream of *sigW*, and this signal is linked to cell wall damage or stress induced by antimicrobial compounds.

The role of FtsH in the expression of the σ^W regulon might be direct or indirect. First, *E. coli* FtsH is known to degrade the heat shock sigma factor σ^{32} and is therefore involved in modulating the heat stress response (3, 33). Similarly, it is possible that FtsH degrades σ^W when it is present in an uncomplexed form in the cytoplasm and/or when it is sequestered to the membrane via the RsiW anti-sigma factor. Second, the influence of FtsH might be indirect and comparable to that of the *E. coli* Cpx extracytoplasmic stress response system, which has been described most recently to be induced in the absence of FtsH (28); in this case, the absence of FtsH would result in the accumulation of compounds that in turn would induce the σ^W regulon. The mechanism of resolution of this stress is elusive, because the functions of most of the σ^W-controlled genes are unknown. The importance of an induction of σ^W-controlled genes in *ftsH* knockout is underlined by the fact that a *sigW ftsH* double-knockout strain displays severe growth defects. The compounds accumulated might be either uncomplexed membrane proteins or penicillin-binding proteins that alter cell wall integrity. A screen to isolate mutants with increased σ^W activity had resulted in three different types of mutants, related to (i) genes of transport, (ii) sugar metabolism, and (iii) antibiotic biosynthesis (34). It was speculated that upregulation of sigma factor activity was due to the inability of the cell to export toxic compounds. An influence of *B. subtilis* FtsH on export has never been shown in detail, but it may well be that certain transport processes are impaired because of the accumulation of nonnative proteins in the membrane. The 2-D gel electrophoresis technique used in this work is not able to resolve membrane proteins. Therefore, most of the proteins belonging to the σ^W regulon did not appear, as most of them are membrane bound (12). For the same reason, other true FtsH substrate proteins that might accumulate in the membrane could not be identified.

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