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The extracellular proteome of *Bacillus subtilis* under secretion stress conditions

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Summary

The accumulation of malfolded proteins in the cell envelope of the Gram-positive eubacterium *Bacillus subtilis* was previously shown to provoke a so-called secretion stress response. In the present studies, proteomic approaches were employed to identify changes in the extracellular proteome of *B. subtilis* in response to secretion stress. The data shows that, irrespective of the way in which secretion stress is imposed on the cells, the levels of only two extracellular proteins, HtrA and YqxI, display major variations in a parallel manner. Whereas the extracellular level of the HtrA protease is determined through transcriptional regulation, the level of YqxI in the growth medium is determined post-transcriptionally in an HtrA-dependent manner. In the absence of secretion stress, the extracellular levels of HtrA and YqxI are low because of extracytoplasmic proteolysis. Finally, the protease active site of HtrA is dispensable for post-transcriptional YqxI regulation. It is known that *Escherichia coli* HtrA has combined protease and chaperone-like activities. As this protein shares a high degree of similarity with *B. subtilis* HtrA, it can be hypothesized that both activities are conserved in *B. subtilis* HtrA. Thus, a chaperone-like activity of *B. subtilis* HtrA could be involved in the appearance of Yqxl on the extracellular proteome.

Introduction

Proteins must be correctly folded in order to perform their biological function. Consequently, unfolded or malfolded proteins are generally useless or even detrimental for the cell. The misfolding of proteins can be due to events that affect either the folding process per se, or the conformation of fully folded proteins. Subsequently, misfolded proteins can be completely degraded or repaired (Wickner *et al.*, 1999). This involves two types of cellular strategies: proteases can degrade the malfolded proteins (Gottesman, 1996), whereas chaperones can refold them and prevent their aggregation (Wickner *et al.*, 1999). Notably, degradation and refolding can be carried out by the same catalysts (Gottesman *et al.*, 1997; Suzuki *et al.*, 1997). In the Gram-negative eubacterium *Escherichia coli*, the HtrA (DegP) serine protease combats the stress caused by malfolded proteins in the periplasm employing its molecular chaperone or proteolytic activities as a function of the temperature (Spiess *et al.*, 1999). At low temperature, HtrA is able to refold chemically denatured substrates, like a chaperone. In contrast, HtrA is predominantly acting as a protease at high temperature.

In the Gram-positive eubacterium *Bacillus subtilis*, three genes encoding HtrA-like proteases have been identified: *htrA* (or *ykdA*), *htrB* (or *yytA*) and *yyxA* (or *yycK*) (Kunst *et al.*, 1997; Tjalsma *et al.*, 2000; Darmon *et al.*, 2002). Each HtrA-like protease of *B. subtilis* has an N-terminal domain with a predicted membrane-spanning segment (*N<sub>in</sub>–C<sub>out</sub>* orientation), a catalytic protease domain and a unique C-terminal PDZ domain. In contrast, HtrA of *E. coli* has two PDZ domains. The latter two PDZ domains form the mobile sidewalls of a central cavity in HtrA hexamers in which the proteolytic sites and potential docking sites for unfolded proteins are located (Krojer *et al.*, 2002). Interestingly, the known human HtrA orthologues have only one PDZ domain (Clausen *et al.*, 2002). In contrast to HtrA of *E. coli*, the human HtrA2/Omi protein appears to be active as a homotrimer (Li *et al.*, 2002; Maurizi, 2002). The structure of HtrA-like proteases in *B. subtilis* is presently not known. Another difference between HtrA-like proteases of *E. coli* and *B. subtilis* concerns the local-
ization of these proteins. Whereas HtrA of *E. coli* is a soluble periplasmic protein, HtrA, HtrB and YyxA of *B. subtilis* are predicted to be membrane-bound proteins (Noone et al., 2000; Tjalsma et al., 2000). Interestingly, htrA, htrB or yyxA single mutant strains of *B. subtilis* display a normal growth phenotype, whereas the growth of an *htrA* htrB double mutant is significantly reduced (Noone et al., 2001). Colonies of this double mutant are round and have a mucoid appearance. Moreover, the htrA htrB mutant cells accumulate suppressor mutations very rapidly (Noone et al., 2001). Remarkably, a strain lacking HtrB and mutated in the protease active site of HtrA displays the same growth phenotype as the *htrA* htrB double mutant, but does not seem to accumulate suppressor mutations (Noone et al., 2001). These observations, although demonstrating the importance of the protease activity for HtrA biological function, also suggest that this protein has an additional activity. By analogy with HtrA of *E. coli*, this could be a chaperone-like activity.

Previous work has shown that the HtrA and HtrB proteins are more similar to each other than either is to YyxA (Noone et al., 2001). Furthermore, the transcription of *htrA* and *htrB* is inducible by both heat and the high-level production of secretory proteins, whereas *yyxA* transcription is insensitive to these stimuli. Finally, the transcription of *htrA* and *htrB* is negatively auto- and cross-regulated (Noone et al., 2000; 2001). All these transcriptional responses of *htrA* and *htrB* are modulated by theCssRS two-component system (Hyrylääinen et al., 2001; Kobayashi et al., 2001; Darmon et al., 2002). This system consists of a sensor histidine kinase, CssS, and a cognate response regulator, CssR. CssS has two potential membrane spanning domains with an N\textsubscript{N} and C\textsubscript{N} orientation and is proposed to sense secretion and heat stress by the detection of unfolded protein accumulation at the membrane–cell wall interface (Hyrylääinen et al., 2001). Similar to other two-component systems (Stock et al., 1989; Parkinson and Kofoid, 1992), information is most likely transferred from CssS to CssR through autophosphorylation of CssS and subsequent phosphotransfer to CssR. When CssR is phosphorylated, it regulates the transcription of specific genes thereby provoking an appropriate cellular response to the original stimuli. Interestingly, not only the *htrA* and *htrB* genes, but also the cssR and cssS genes seem to be members of the CssRS regulon (Kobayashi et al., 2001; Darmon et al., 2002). These findings support the view that HtrA, HtrB and CssRS play important roles in the combat against misfolded proteins resulting from both heat stress and the high-level production of secretory proteins (Noone et al., 2001). In this respect, it is important to note that most secretory proteins of *B. subtilis* appear to be exported from the cytoplasm in an unfolded state via the Sec translocon. Upon their translocation across the membrane, the folding of these proteins is facilitated by a variety of catalysts, which may become limiting when secretory proteins are overproduced (Tjalsma et al., 2000; van Dijl et al., 2001).

The present studies were aimed at determining the effects of protein secretion stress on the composition of the extracellular proteome of *B. subtilis*. The analysis of this type of stress by proteomics is, so far, unprecedented. By definition, secretion stress in *B. subtilis* includes all stimuli that trigger a CssRS-dependent cellular response. Thus, secretion stress was induced either by removing proteins, which are required to combat secretion stress (HtrA or HtrB), or by high-level production of the α-amylase AmyQ of *Bacillus amyloliquefaciens*. Strikingly, the levels of only two extracellular proteins, HtrA and Yqxl, were significantly affected by secretion stress, irrespective of the way in which this stress was induced. Changes in the extracellular HtrA level can be attributed to the effects of secretion stress on *htrA* transcription. In contrast, variation in the extracellular Yqxl level is determined by post-transcriptional events. The present proteomic studies show that the level of Yqxl in the growth medium is modulated through extracytoplasmic proteases, possibly involving a conserved chaperone-like activity of HtrA.

**Results**

**Dual localization of HtrA**

In order to investigate whether the HtrA and HtrB proteases influence the composition of the extracellular proteome of *B. subtilis* during the stationary growth phase, the proteins in the growth media of *htrA* and *htrB* single and double mutants were studied by two-dimensional (2D) gel electrophoresis (Fig. 1). Surprisingly, the subsequent mass spectrometric analysis revealed that one of the proteins that disappeared from the medium of the *htrA* mutant was the HtrA protein itself (Fig. 1A and B). The presence of HtrA in the medium of the parental strain 168 was unexpected as this protein was predicted to be membrane-bound with an N-terminal membrane anchor (N\textsubscript{N}–C\textsubscript{out} orientation). As demonstrated by N-terminal sequencing, the extracellular HtrA starts with Sec\textsubscript{for}, showing that the predicted transmembrane region is cleaved off. Thus, the soluble HtrA has a calculated molecular mass of about 37 kDa and starts with a stretch of serine residues, a feature absent from HtrB and YyxA (data not shown).

To determine whether HtrA also exists in a cell-associated form, Western blotting experiments were performed. As shown in Fig. 2, a cell-associated form of HtrA, with an apparent molecular mass of 62 kDa, was present in *B. subtilis* 168 (C). In addition, a sec-
ond cell-associated form of HtrA (apparent molecular mass of 47.5 kDa) was detectable, which co-migrated with the HtrA in the growth medium (M). Both immunoreactive bands are HtrA-specific as they were absent from the sample of the htrA mutant (DN26). Two additional immunoreactive bands were detectable in cell extracts of B. subtilis 168 (C), but these bands are not related to HtrA as they were also detectable in the cell extracts of the ΔhtrA strain. The immunostained protein profiles of strain 168 (C and M) and the htrB mutant strain (DN111; C and M) were very similar demonstrating that the HtrA antibody does not cross-react with HtrB.

Protoplasts of B. subtilis 168 were prepared and examined for the presence of HtrA. It is noticeable that the 62 kDa form of HtrA predominated in the protoplast-associated fraction (Fig. 2, lane P). This form disappeared when protoplasts were treated with trypsin (Fig. 2, lane PT), indicating that it is exposed on the protoplast surface. Thus it seems that the large cellular form of HtrA represents the protein with its membrane anchor, which is exposed at the extracytoplasmic side of the membrane. The smaller cellular form may represent the cleaved HtrA before its release from the membrane and secretion into the medium.

Fig. 1. The extracellular proteomes of htrA and htrB mutant B. subtilis strains. The extracellular proteomes of B. subtilis 168 (A), and its derivatives ΔhtrA (DN26; B), ΔhtrB (DN111; C), ΔhtrA ΔhtrB sup (DN115, suppressed; D) and htrA1 ΔhtrB (DN200; E) were analysed by 2D gel electrophoresis. Cells were grown in LB until one hour after entry into the stationary growth phase. Only the acidic pH range (pH 3–6) is shown. Protein spots identified by mass spectrometry and/or N-terminal sequencing are indicated. The HtrA and YqxI protein spots are highlighted with a circle. Notably, the residual spot at the position of HtrA in B cannot relate to HtrA (see Fig. 2).

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Fig. 2. Localization of HtrA. Cells of B. subtilis 168, ΔhtrA (DN26) and ΔhtrB (DN111) were grown in LB medium until an OD at 550 nm of about 1. Next, cells (C) of all three strains were separated from the growth medium (M) and samples for SDS-PAGE were prepared using 20 µg of total cell protein and 300 µl of medium respectively. Furthermore, 20 µg of protoplasts were prepared of B. subtilis 168, which were incubated for 10 min in the absence (P) or presence (PT) of 1 mg ml⁻¹ trypsin. As a positive control, 20 ng of pure HtrAecd was loaded. Immunodetection after Western blotting was performed with antibodies raised against HtrAecd.
Absence of YqxI from the medium of the ∆htrA mutant strain

The comparison of the extracellular proteomes of the parental strain 168 and the htrA mutant (DN26; Fig. 1A and B) displayed a second major difference: the YqxI protein was absent from the medium of the htrA mutant. Interestingly, the yqxI gene (also known as yqdE) is located on the SKIN prophage (map position at 227.5° on the chromosome of B. subtilis 168). It encodes a non-essential protein of 159 amino acids with a predicted molecular mass of 17 kDa and a calculated isoelectric point of 5.44. So far, the function of YqxI is unknown and it shows no significant amino acid sequence similarity to any other protein. Consistent with its extracellular localization, YqxI contains a potential signal peptide with a signal peptidase I cleavage site between amino acids 28 and 29 (AKA-QE; Tjalsma et al., 2000; van Dijl et al., 2001).

Response of HtrA and YqxI to secretion stress

To further investigate the effects of secretion stress on the extracellular proteome, plasmid pKTH10L, which directs the overproduction of the α-amylase AmyQ of B. amylo liquefaciens, was introduced in B. subtilis 168. Next, the protein composition of the growth medium of this strain was determined by proteomics. As expected, AmyQ was the most abundant extracellular protein of B. subtilis 168 containing pKTH10L (Fig. 3B). Notably, the levels of the HtrA and YqxI proteins were increased in response to the high-level production of AmyQ (Fig. 3A and B).

Previous studies have shown that AmyQ-induced secretion stress results in the transcription of htrA at elevated levels (Noone et al., 2000; Hyryläinen et al., 2001). The present observations indicate that the extracellular levels of the HtrA protein can be correlated with the transcriptional levels of the corresponding gene. To determine whether the increase of the extracellular amount of YqxI in response to AmyQ overproduction is also effected at the transcriptional level, the transcription of htrA and yqxI in strains with and without pKTH10L was studied by Northern blotting. The transcription of htrA was increased because of the overexpression of AmyQ, especially during the mid-exponential growth phase (Fig. 4A and data not shown). The level of yqxI transcription in the absence of AmyQ production was highest around the transition point between the exponential and stationary growth phases.
that the increase of the amounts of extracellular HtrA upon the high-level production of AmyQ is determined at the transcriptional level, whereas the increase of the extracellular amounts of Yqxl under the same conditions is determined post-transcriptionally.

Response of HtrA and Yqxl to the absence of the sensor CssS

The CssRS two-component system mediates the appropriate cellular response to combat secretion stress (Hyryrläinen et al., 2001; Darmon et al., 2002). To identify possible changes in the extracellular proteome of cssS mutant cells subject to secretion stress, the protein composition of the medium of the B. subtilis 168 cssS::Sp (BV2001) strain containing pKTH10L was investigated by proteomics (Fig. 3C). The only two major changes in the extracellular proteome resulting from the absence of CssS were a decreased HtrA level and a disappearance of Yqxl (Fig. 3B and C). To investigate the cause of these effects, the transcription of htrA and yqxl were studied by Northern blotting using RNA extracted from cells that were grown under the same conditions (Fig. 6). The htrA transcription decreased dramatically in cssS mutant cells, even when secretion stress was imposed on these cells (Fig. 6A). In contrast, the transcription of yqxl did not change in the absence of CssS (Fig. 6B). The yqxl level of transcription was also studied using a transcriptional yqxl-lacZ fusion (Fig. 5A) in strains with either an intact or a mutated cssS gene (strains BV2028 and BV2029, respectively). As shown in Fig. 6C, the disruption of cssS did not result in a change in yqxl-lacZ expression as a function of growth, which confirms the results obtained by Northern blotting. These observations show that the absence of CssS leads to a reduction of htrA transcription, and this is reflected by the lower HtrA protein level on the extracellular proteome. In contrast, the disappearance of Yqxl under the same conditions is not correlated with the transcription of the yqxl gene.

Responses of HtrA and Yqxl to the absence of HtrB

HtrA and HtrB act together to combat secretion stress and mutation of either htrA or htrB affects expression of both genes (Noone et al., 2001). To investigate how HtrB contributes to the composition of the extracellular proteome and, in particular, to the levels of HtrA and Yqxl, the proteins in the growth medium of B. subtilis 168 htrB::Km strain (DN111) were analysed by proteomics (Fig. 1C). The only major changes in the extracellular proteome of the htrB mutant were the increased amounts of the HtrA and Yqxl proteins. To assess whether these changes occurred at the transcriptional level, Northern blotting experiments were performed using htrA- or yqxl-specific

Surprisingly, the level of yqxl transcription did not increase when AmyQ was overexpressed (Fig. 4B; for unknown reasons, two yqxl-specific transcripts of ~850 and ~750–800 nucleotides, respectively, are detectable). The transcription of yqxl was also studied as a function of growth using a transcriptional yqxl-lacZ fusion (BV2028; Fig. 5A). As shown by the β-galactosidase activities, the level of yqxl-lacZ transcription did not increase in response to AmyQ-induced secretion stress in the strain containing pKTH10L (Fig. 4C). All together, these data demonstrate...
 probes and RNA extracted from strains with an intact or disrupted htrB gene (Fig. 7). The level of htrA transcription rose significantly upon disruption of the htrB gene (Fig. 7A). In contrast, transcription of yqxl was not increased by the mutation in htrB (Fig. 7B). This result was confirmed by β-galactosidase measurements in an htrB mutant strain containing a transcriptional yqxl-lacZ fusion (data not shown). Thus, the increase in the amount of extracellular HtrA, caused by an htrB mutation, can be explained by an effect at the transcriptional level. On the other hand, the similar behaviour of the Yqxl protein is apparently caused by a post-transcriptional event.

Response of Yqxl to HtrA overexpression

The data presented above suggest that the Yqxl protein is subject to a post-transcriptional regulation in a manner that correlates with extracellular HtrA levels. To further investigate whether the presence of HtrA influences the level of Yqxl, a xylose-inducible (his)_6-htrA gene was introduced in the amyE locus of a strain lacking a major part of the htrA gene (DN26). This resulted in B. subtilis 168 htrA::amyE (Fig. 5B). Importantly, the (His)_6-htrA fusion protein complemented the htrA null mutation (data not shown). The extracellular proteome of the (His)_6-HtrA inducible strain, grown in the absence or presence of 1% xylose, was studied by 2D gel electrophoresis (Fig. 8A). Because HtrA is processed at the N-terminus during secretion from the cell, the presence of the His-tag did not affect the electrophoretic properties of the extracellular form of HtrA. As shown in Fig. 8A (panel b), cells grown in the presence of xylose produced a significant quantity of HtrA. Strikingly, a concomitant increase in the level of Yqxl was observed. To assess the effect of (His)_6-HtrA induction with xylose on the transcription of yqxl, a transcriptional yqxl-lacZ fusion was introduced into this strain (BV2026). The expression profiles show that the transcription of yqxl-lacZ remains unaffected by xylose addition. A similar yqxl-lacZ transcription profile was observed in a control strain containing the native htrA gene (data not shown). Furthermore, parallel experiments with a xylose-inducible (His)_6-HtrB fusion instead of the (His)_6-HtrA fusion demonstrated that induction of (His)_6-HtrB did not lead to an increase of the extracellular Yqxl level (data not shown). These observations imply that the amount of Yqxl on the extracellular proteome is determined post-transcriptionally by the level of HtrA. In fact, the observed correlation between the extracellular levels of HtrA and Yqxl suggests that HtrA promotes, directly or indirectly, the folding of Yqxl into a protease-resistant conformation after Yqxl translocation across the membrane. This effect is specific for HtrA as the overexpression of HtrB, a paralog of HtrA, does not have any effect on Yqxl levels.

Stabilization of HtrA and Yqxl in the absence of extracellular proteases

To investigate the possible involvement of extracellular proteases in the turnover of HtrA and Yqxl, the protein composition of the growth medium of B. subtilis WB700 was investigated. This strain lacks the extracellular serine proteases AprE (subtilisin), Bpr, Epr, Mpr and Vpr, as well as the metalloproteases NprB and NprE (Ye et al., 1999).
Interestingly, both HtrA and YqxI were present at significantly increased levels in the medium of strain WB700 (Fig. 8B, panels a and b). In addition, elevated amounts of specific degradation products of the wall protein WapA and an undegraded form of the YvcE protein were detectable, which is in accord with previously documented observations (Antelmann et al., 2002). As shown by Northern blotting, the htrA and yqxI-specific mRNA levels in strain WB700 were comparable to those in the parental strain 168 (data not shown). This indicates that the absence of seven extracellular proteases does not trigger a secretion stress response. The latter view was confirmed by the analysis of the extracellular proteome of strain WB700 cssS, which contained high levels of HtrA and YqxI (Fig. 8B, panel c). Importantly, these levels were comparable to the HtrA and YqxI levels on the extracellular proteome of strain WB700. Taken together, these observations show that both HtrA and YqxI are subject to extracytoplasmic degradation by one or more extracellular proteases of B. subtilis.

Extracellular appearance of YqxI does not require protease activity of HtrA

The HtrA protein of B. subtilis could have both protease and chaperone-like functions as shown for its orthologue in E. coli. In order to investigate whether the protease activity of HtrA is involved in determining the YqxI level, the extracellular proteome of an htrA1 htrB double mutant strain (DN200) was studied by proteomics. Importantly, the htrA1 mutation results in the synthesis of a mutant HtrA protein lacking the protease active site serine residue at position 290. As a control, the extracellular proteome of an htrA htrB double mutant strain (DN115), which contains an uncharacterized suppressor mutation, was studied. Neither HtrA nor YqxI were present on the extracellular proteome of the suppressed cells inactivated for htrA and htrB (Fig. 1D). In contrast, HtrA and YqxI levels were significantly increased in the medium of the strain lacking HtrB and producing the HtrA protein with a
mutation in its protease active site (Fig. 1E). These results show that the protease activity of HtrA is not essential for the extracellular appearance of YqxI. The transcription of htrA and yqxI was analysed by Northern blotting using RNA from the htrA1 htrB double mutant strain (DN200; Fig. 9). Consistent with the absence of HtrB, the level of htrA transcription increased in the htrA1 htrB double mutant strain, but to a higher extent than in the htrB mutant alone (compare Fig. 7A and Fig. 9A). However, the transcription levels of yqxI in the htrA1 htrB double mutant strain and the parental strain 168 were comparable (Fig. 9B).

In conclusion, these data suggest that HtrA has an activity that, directly or indirectly, determines the level of
Yqxl on the extracellular proteome of *B. subtilis* by preventing the proteolysis of this protein either at the membrane–cell wall interface and/or in the growth medium. This post-transcriptional activity does not involve the protease active site of HtrA. It is tempting to suggest by analogy with HtrA of *E. coli* that this is a chaperone-like activity.

**Discussion**

Previous work has shown that 2D gel electrophoresis and mass spectrometry are powerful tools to visualize changes in the extracellular proteome of *B. subtilis* caused by mutations in the protein secretion machinery (Antelmann *et al.*, 2001; 2002). Moreover, these proteomic techniques have been elementary in the description of stress responses in the *B. subtilis* cell (Hecker and Engelmann, 2000). Therefore, proteomics was employed in the present studies to characterize the effects of protein secretion stress on the composition of the extracellular proteome of *B. subtilis*. Such stress conditions were induced by (combinations of) overproduction of the α-amylase AmyQ of *B. amyloliquefaciens*, or absence of HtrA or HtrB. Several noteworthy observations were made. First, the HtrA protein can exist both in membrane-associated and extracellular forms. In this respect, HtrA of *B. subtilis* seems to resemble the human HtrA2/Omi protein, which is first imported into mitochondria as a full-length membrane protein and, subsequently, processed to an active soluble form that is released into the intermembrane space (Clausen *et al.*, 2002). Second, the levels of only two extracellular proteins, HtrA and Yqxl, varied significantly in response to secretion stress, suggesting that similar stimuli were generated under all secretion stress-inducing conditions tested. Third, unlike the changes in the amounts of extracellular HtrA, the changes in the amounts of Yqxl were not determined at the transcriptional level. Instead, the presence of Yqxl in the medium was correlated with the presence of extracellular HtrA under all conditions tested. Finally, the protease activity of HtrA was dispensable for the appearance of Yqxl in the medium. In principle, these findings could be explained with a chaperone-like activity of HtrA that would facilitate the folding of Yqxl. By doing so, membrane-bound and/or extracellular HtrA would protect Yqxl against extracytoplasmic degradation by proteases, such as AprE, Bpr, Epr, Mpr, NprB, NprE and/or Vpr. In fact, HtrA itself seems to be subject to degradation by one or more of these proteases. Even though this is an attractive hypothesis, it is presently not possible to exclude indirect effects of HtrA on Yqxl as chemical cross-linking provided no evidence for direct interactions between these two proteins, neither in whole cells nor medium fractions (data not shown).

The idea that the combined proteolytic and chaperone activities of *E. coli* HtrA are conserved in HtrA of *B. subtilis* has an interesting implication for the role of PDZ domains in HtrA function. As shown by crystallography, the PDZ domains form flexible sidewalls of a central cavity in *E. coli* HtrA hexamers. Protein folding or degradation seems to take place in this cavity. It has been proposed that the PDZ domains are required for substrate binding and entry into the central cavity (Krojer *et al.*, 2002). The fact that only one of the two PDZ domains of *E. coli* HtrA (i.e. PDZ1) is conserved in *B. subtilis* HtrA, indicates that the presence of this unique PDZ domain is sufficient for the proteolytic function of HtrA and, if conserved, the chaperone function of this protein. In this respect, it is important to note that recent studies on HtrA of *Thermotoga maritima* indicate that PDZ domains are dispensable both for proteolytic and chaperone-like functions of this protein (Kim *et al.*, 2002).

Under all tested conditions of protein secretion stress, the amount of the extracellular HtrA protein follows the transcription of its gene. Furthermore, the Northern blot-
ting experiments using an htrA-specific probe are consistent with the previously reported negative auto- and cross-regulation of htrA and htrB (Noone et al., 2000; 2001) and the CssS-dependence of htrA transcription (Hyryräläinen et al., 2001). This implies that the amount of extracellular HtrA is primarily determined at the transcriptional level. On the other hand, the level of YqxI on the extracellular proteome shows variations that are independent of the transcription of the yqxI gene. Interestingly, the largest of the two yqxI-specific mRNA molecules detected by Northern blotting has a size (∼850 nucleotides) that corresponds to the yqxI gene plus the downstream gene yqxJ, encoding a protein with an unknown function (data not shown). This is consistent with the operon-like organization of these two genes within the SKIN prophage. In contrast to YqxI, YqxJ has a predicted cytoplasmic localization. Thus, the post-transcriptional regulation of extracellular YqxI via HtrA may give the cell, or the SKIN prophage itself, the possibility to modulate the YqxI level independently of the YqxJ level despite the co-transcription of the corresponding genes.

In contrast to HtrA, the paralogous HtrB protein has, so far, not been detected on the extracellular proteome of B. subtilis. It is presently not clear whether this is due to the technical limitations of the 2D gel electrophoresis-based proteomics approach, whether the extracellular protein levels of HtrB are too low to be detectable, or whether HtrB is not secreted into the growth medium at all. Similar explanations can be proposed to explain why merely two significant changes in the composition of the extracellular proteome can be observed under conditions of secretion stress. In view of the fact that substantial amounts of HtrA are localized at the membrane–cell wall interface and that secretion stress is sensed by CssS at the same subcellular location (Hyryräläinen et al., 2001), it is conceivable that secretion stress conditions have a stronger impact on those proteins that are localized close to the extracytoplasmic membrane surface of B. subtilis than on the proteins secreted into the growth medium. In this respect, it is relevant to note that protein secretion stress does not provoke detectable changes in the composition of the cytoplasmic proteome (H. Antelmann, unpubl. obs.).

In conclusion, the present observations show that HtrA of B. subtilis is a protein with a dual localization and, possibly, a dual function. Although a direct interaction between HtrA and YqxI has not been demonstrated yet, it seems as if HtrA promotes, directly or indirectly, the folding of YqxI into a protease-resistant conformation after its translocation across the membrane and/or secretion into the medium. By doing so, HtrA would protect YqxI from degradation by the proteases that reside in the cell envelope and growth medium of B. subtilis (Tjalsma et al., 2000). For a complete understanding of the function of HtrA of B. subtilis, it will be essential to characterize the (putative) chaperone-like and protease activities of the membrane-bound and extracellular forms of this protein in vitro. Furthermore, it will be important to examine the role of HtrA in the biogenesis of membrane and cell wall-associated proteins of B. subtilis.

**Experimental procedures**

**Plasmids, bacterial strains and growth conditions**

The bacterial strains and plasmids used are listed in Table 1. Strains were grown with agitation at 37°C in LB (Luria–Bertani, Difco BRL) or TY (1% tryptone, 0.5% yeast extract and 1.0% NaCl) media. Antibiotics were used in the following concentrations: ampicillin (Ap), 50 μg ml⁻¹; chloramphenicol (Cm), 5 μg ml⁻¹; erythromycin (Em), 1 μg ml⁻¹; kanamycin (Km), 10 μg ml⁻¹; spectinomycin (Sp), 100 μg ml⁻¹. To visualize α-amylase activity, TY plates were supplemented with 1% starch.

**DNA techniques**

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and transformation of competent E. coli cells were carried out as described in Sambrook et al., (1989). Bacillus subtilis was transformed as described by Leskela et al. (1996). Enzymes were from Roche Molecular Biochemicals. Polymerase chain reaction was carried out with the Taq (Roche Molecular Biochemicals) or Pwo (New England Biolabs) DNA polymerases, using chromosomal DNA of B. subtilis 168 as a template (van Dijl et al., 1995). The nucleotide sequences of primers used for PCR are listed in Table 1; restriction sites used for cloning are underlined. Constructs were first made in E. coli MC1061 or ER2566 and then, if required, introduced into B. subtilis.

To construct the yqxI::pMutin2 mutation (Fig. 5A) and the transcriptional yqxI-lacZ fusion, an internal fragment (173 nucleotides) of the yqxI gene was amplified by PCR with the oligonucleotides yqxI7 and yqxI8 (Table 1). This fragment was cloned into plasmid pMutin2, using the HindIII and BamHI sites. The B. subtilis 168 yqxI::pMutin2 (BV2028) strain was obtained by Campbell-type integration (single cross-over) of the resulting plasmid into the chromosome of B. subtilis 168. The correct integration was verified by PCR. The B. subtilis 168 cssS::Sp yqxI::pMutin2 (BV2029) strain was constructed by transformation of B. subtilis 168 yqxI::pMutin2 with chromosomal DNA of B. subtilis 168 cssS::Sp (BV2001) and selection for spectinomycin resistance.

To construct a B. subtilis strain with an inducible (his)₆-htrA gene (BV2026; Fig 5B), the htrA gene was amplified by PCR with primers N-HH1 (specifying a hexa-histidine tag) and N-HH2 (See Table 1). The amplified fragment (1400 nucleotides) was cloned into plasmid pX, using the SpeI and BglII sites, resulting in pX(his)₆-htrA. B. subtilis 168 htrAΔ439 amyE(X)(his)₆-htrA (BV2026) was obtained by a double cross-over recombination event between the flanking regions of amyE located on pX(his)₆-htrA and the chromosomal amyE gene of B. subtilis 168 htrAΔ439 (DN26). Correct mutants were chloramphenicol resistant and showed no α-amylase activity on plates containing starch.

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The B. subtilis WB700 cssS strain was constructed by transformation of B. subtilis WB700 with chromosomal DNA of B. subtilis 168 cssS::Sp (BV2001) and selection for specinomycin resistance.

Expression of the extracytoplasmic domain of HtrA

The extracytoplasmic domain of the HtrA protein (HtrAecd) was purified using the IMPACT-CN™ System (New England Biolabs) (Chong et al., 1997). A fragment encoding HtrAecd was amplified by PCR with primers HTRA-AbF and HTRA-AbR. Upon cleavage with Nhel-SapI, this fragment was cloned into the vector pTYB1. Thus a C-terminal protein fusion of HtrAecd (from amino acid Thr67 until the C-terminal Ser290) to the intein domain and chitin binding tag of pTYB1 was created. The resulting plasmid pDN500, was used to transform E. coli ER2566. Production of the fusion protein was induced by the addition of 0.5 mM IPTG and subsequent growth for 4 h at 30°C. The fusion protein was purified and HtrAecd was cleaved from the intein tag according to the IMPACT-CN™ System manual. The N-terminal sequence of the purified HtrAecd protein was determined by N-terminal amino acid sequencing. In accordance with the cloning strategy, HtrAecd was shown to start with Ala and Ser, directly followed by the HtrA protein sequence starting with Thr67.

Production of an HtrA-specific antibody

An HtrA-specific antibody was prepared by injection of 100 μg HtrAecd, emulsified in Freund's complete adjuvant, into New Zealand white rabbits. The initial injection was followed by boosts at 14 and 28 days and a final injection at 49 days. Bleeds were taken before the first injection (preimmune) and 14 days after the final injection. The anti-HtrAecd activity of pre- and postimmune sera was checked by Western blotting using HtrAecd and B. subtilis total cell protein. Anti-HtrAecd antibody was purified by immuno-affinity chromatography on a column of HtrAecd covalently bound to CNBr-activated Sepharose 4B Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's instructions. The anti-HtrAecd antibody used in this study was purified by immuno-affinity chromatography on a column of HtrAecd covalently bound to CNBr-activated Sepharose 4B Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's instructions.
analysis was eluted using 0.2 M glycine pH 2.2 into tubes containing 2 M Tris pH 8.8.

**Western blotting**

Total protein from *B. subtilis* was prepared by resuspending the cell pellets in Z-buffer excluding β-mercaptoethanol (Miller J.H., 1982) containing lysozyme and DNase (10 µg ml⁻¹ each) and incubation at 37°C for 20 min. Protoplasting (10 min incubation with 1 mg ml⁻¹ lysozyme) and protease accessibility studies were performed as described by Bolhuis et al. (1998). Proteins from the growth medium were collected by precipitation with TCA at a final concentration of 10% w/v. The precipitate was washed with acetone and resuspended in Z-buffer. Protein amounts were determined using the Bio-Rad Protein Assay. Protein samples were separated with 12.5% SDS-polyacrylamide gels according to (Laemmli, 1970) on a Bio-Rad Protean 3 mini-system and transferred to PVDF membranes using the Bio-Rad trans-blot system following the manufacturer's instructions. Membranes were blocked with 2% w/v non-fat milk powder in TBST (10 mM Tris-HCl at pH = 7.5, 150 mM NaCl, 0.5% Tween 20) and incubated with anti-HtrAecd antibody (1:10 000 dilution) for 90 min in the same solution. HtrAecd-antibody complexes were detected by incubation for 60 min with horseradish peroxidase conjugated to goat anti-rabbit secondary antibody (1:30 000 dilution) and visualized by chemiluminescence (Roche Molecular Biochemicals).

**Preparation of extracellular protein fractions for two-dimensional (2D) gel electrophoresis**

*Bacillus subtilis* cells were grown in 500 ml LB and samples of 250 ml were collected at the transition point between the exponential and the stationary growth phases (t₀), and one hour after the beginning of the stationary phase (t₁). Cells were removed from the growth medium by centrifugation for 20 min at 4°C and 10 000 r.p.m. Then, proteins in the medium were precipitated with ice-cold 10% TCA (w/v), and collected by centrifugation (40 000 g, 45 min, 4°C). The resulting protein pellet was scraped from the wall of the centrifuge tube with a spatula, washed three times with 96% ethanol (v/v) and dried.

**2D polyacrylamide gel electrophoresis**

The dried protein pellets were resolved in a solution containing 2 M thiourea and 8 M urea. Subsequently, insoluble material was removed by centrifugation. The protein concentration of the resulting extracellular protein sample was determined according to Bradford (Bradford, 1976), and the volume of an equivalent of 80 µg of this sample was adjusted to 360 µl with the thiourea/urea solution. Next, 40 µl of a 10-fold concentrated reswelling solution was added containing 2 M thiourea, 8 M urea, 10% Nonidet P-40, 200 mM DTT and 5% Pharnkalyte. This sample containing reswelling solution was used for the rehydration of IPG strips in the pH range of 3–10. Isoelectric focusing was performed using the Multiphor II unit (Amersham Pharmacia Biotech) and SDS-PAGE in the second dimension was carried out using the Investigator 2D electrophoresis system (Genomic Solutions, Chelmsford) as described previously (Buttner et al., 2001). Finally, the resulting 2D gels were stained with silver nitrate according to Blum et al. (Blum et al., 1987). All gels will be made available in the Sub2D proteome database (http://microbio2.biologie.uni-greifswald.de:8880/).

**Protein identification**

Protein identification by MALDI-TOF mass spectrometry was performed using a Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City) as described previously (Antelmann et al., 2001). Peptide mass fingerprints were analysed using the ‘MS-Fit’ software as provided by Baker and Clausner through http://prospector.ucsf.edu/.

**Northern blotting**

Total RNA of *B. subtilis* was isolated from cells grown in LB at different time points using the acidic phenol method of Majumdar et al. (1991). Northern blotting was performed as described previously (Wetzstein et al., 1992). Briefly, internal fragments of the yqxI and htrA genes, respectively, were first amplified with the YqxIvor-YqxIrev and HtrAvor-HtrArev primer pairs (Table 1). Note that the 3’ primer for each of the amplified genes contains a T7 promoter sequence. Next, the amplified fragments were used for the T7 RNA polymerase-directed *in vitro* synthesis of digoxigenin-labelled gene-specific RNA probes.

**β-galactosidase activity assay**

To assay β-galactosidase activities, overnight cultures were diluted in fresh TY medium and grown at 37°C. Samples were taken at different intervals for OD readings at 600 nm and β-galactosidase activity determinations. For strains containing a transcriptional lacZ fusion, the β-galactosidase assay and the calculation of β-galactosidase units (Miller units: nmol × OD₆₀₀ × min⁻¹) were performed as described by Hyryläinen et al., (2001). Experiments were repeated at least twice, starting with independently obtained transformants. In all experiments, the relevant controls were performed in parallel. Although some differences were observed in the absolute β-galactosidase activities, the ratios between these activities in the various strains tested were largely constant. A ratio of about 1.5 was generally reproducible.

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