Degradation of Extracytoplasmic Catalysts for Protein Folding in *Bacillus subtilis*

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The general protein secretion pathway of *Bacillus subtilis* has a high capacity for protein export from the cytoplasm, which is exploited in the biotechnological production of a wide range of enzymes. These exported proteins pass the membrane in an unfolded state, and accordingly, they have to fold into their active and protease-resistant conformations once membrane passage is completed. The lipoprotein PrsA and the membrane proteins HtrA and HtrB facilitate the extracytoplasmatic folding and quality control of exported proteins. Among the native exported proteins of *B. subtilis* are at least 10 proteases that have previously been implicated in the degradation of heterologous secreted proteins. Recently, we have shown that these proteases also degrade many native membrane proteins, lipoproteins, and secreted proteins. The present studies were therefore aimed at assessing to what extent these proteases also degrade extracytoplasmic catalysts for protein folding. To this end, we employed a collection of markerless protease mutant strains that lack up to 10 different extracytoplasmic proteases. The results show that PrsA, HtrA, and HtrB are indeed substrates of multiple extracytoplasmic proteases. Thus, improved protein secretion by multiple-protease-mutant strains may be related to both reduced proteolysis and improved posttranslational protein folding and quality control.

The bacterial Sec pathway for general protein secretion transports proteins in an unfolded state from the cytoplasm to extracytoplasmatic locations within the cell or the extracellular milieu. Accordingly, these proteins need to fold efficiently into their active and protease-resistant conformations once membrane translocation is completed (1–5). In the Gram-positive bacterium and biotechnological workhorse *Bacillus subtilis*, which is especially appreciated for its high protein secretion capacity, the folding of secreted proteins can be facilitated by at least four different extracytoplasmic chaperones and quality control factors, namely, PrsA, BdbD, HtrA, and HtrB.

PrsA is an essential lipoprotein that is attached to the outer surface of the cytoplasmic membrane through a diacyl-glycerol modification (6). In *B. subtilis*, PrsA is known to influence the folding of penicillin-binding proteins, such as PBP2a, PBP2b, PBP3, and PBP4 (6, 7). Overexpression of PrsA is known to increase the yields of secreted heterologous proteins, α-amylases in particular (6, 8, 9). The BdbD protein is a thiol-disulfide oxidoreductase that is localized to the outer side of the cytoplasmic membrane by its N-terminal membrane anchor (10, 11). In conjunction with the quinone reductase BdbC, BdbD facilitates the formation of disulfide bonds in competence proteins needed for DNA uptake, such as ComEC and ComGC (12–15). BdbD also catalyzes oxidative folding of secreted heterologous proteins, such as the alkaline phosphatase PhoA of *Escherichia coli* (10, 16, 17). HtrA is known to combine a chaperone function with a protease function, allowing it to degrade translocated proteins that cannot be productively folded (18). In *B. subtilis* HtrA appears to be needed for folding of the secreted protein YqXL and various other extracellular proteins (19, 20). Likewise, the HtrA parologue HtrB seems to have combined chaperone and protease functions (20, 21). Both HtrA and HtrB are bound to the membrane by an N-terminal membrane anchor, but it must be noted that HtrA is also detectable in the growth medium of the sequenced *B. subtilis* prototype strain 168 (19, 22). Under conditions of protein secretion stress, for example, due to high-level α-amylase production, the synthesis of HtrA and HtrB is strongly enhanced, which is related to tight regulation by the CssRS two-component regulatory system (22–25). Furthermore, CssRS mediates the cross-regulation of the *htrA* and *htrB* genes, the expression of one going up if the other is inactivated (23, 25). Both HtrA and HtrB are needed for cell survival under conditions of severe protein secretion stress (22, 24, 25).

*B. subtilis* produces at least eight extracellular proteases known as AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr, and WprA, which are usually secreted during the stationary growth phase (3, 26, 27). Importantly, the WprA protease is also localized to the cell wall (28–31). These proteases perform a variety of functions, including the degradation of proteins in organic matter for nutrient provision and the proteolytic processing of other proteins (32, 33). Although it was believed that, upon correct folding, the native exported proteins of *B. subtilis* would be largely resistant to the extracytoplasmic proteases of the organism, our recent studies have shown that many extracytoplasmic proteins of the bacterium are in fact degraded (2, 20). On the other hand, it has been known for a long time that the extracellular proteases have a high propensity for degrading heterologous secreted proteins, which often fold slowly upon membrane translocation (34, 35). Though this may ensure product quality control in some cases, it mostly leads to huge commercial losses. Multiple-protease-mutant strains of *B. subtilis* were therefore constructed, including the frequently used strains WB600, WB700, and WB800 (19, 36, 37). Indeed, these strains displayed increased levels of heterologous protein secretion, which was attributed to reduced proteolysis. However, to date, it has never been assessed whether the absence of multiple extracytoplasmic proteases might also have a positive impact on
TABLE 1 B. subtilis strains used in the study

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"BRB, Bacillus RecipharmCobra Biologics.

the levels of extracytoplasmic chaperones and quality control factors, which could be beneficial for the quality and yields of secreted heterologous proteins. The present studies were therefore aimed at determining the impact of extracytoplasmic proteases on the levels of PrsA, BdbD, HtrA, and HtrB. In brief, these analyses show that PrsA, HtrA, and HtrB are substrates of multiple extracytoplasmic proteases.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The strains were grown under vigorous agitation at 37°C in lysogeny broth (LB) (also known as Luria broth or Luria-Bertani medium), which consisted of 1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.4.

Sample preparation. Cells of B. subtilis 168 and the protease mutants BRB02 to -14 were grown overnight in LB medium at 37°C. The overnight cultures were diluted in fresh LB medium, and growth continued until 2 h after entry into the stationary phase. The cells were then separated from the growth medium by centrifugation (4,000 × g; 10 min; 4°C) and disrupted by bead beating as previously described (38).

Western blotting. For Western blot analyses, cellular proteins were separated on precast 10% Bis-Tris NuPAGE gels (Invitrogen) and semidry blotted (75 min at 1 mA/cm²) onto a nitrocellulose membrane. Prior to gel loading, all protein samples were corrected for optical density at 600 nm (OD600). Specific polyclonal antibodies raised in rabbits were used to detect the blotted BdBD (11), HtrA (19), HtrB (36), FeuA (39), PrsA (6–8), thioredoxin A (TrxA) (11), and YclQ (20) proteins. Bound antibodies were visualized using IRDye 800CW goat anti-rabbit secondary antibodies in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). Fluorescence was recorded at 800 nm.

RESULTS AND DISCUSSION

To investigate the effects of extracytoplasmic proteases on the levels of PrsA, BdbD, HtrA, and HtrB, a series of strains (BRB02 to -14) with multiple markerless protease gene deletions were used (Table 1). In particular, the strains lacking seven or more protease genes (i.e., BRB07, -08, -11, -12, and -14) were recently shown to be suitable for industrial-scale fermentations to produce the protective antigen of Bacillus anthracis (40). This secreted protein and potential vaccine candidate is highly susceptible to proteolytic degradation and cannot be produced in the parental strain 168. Remarkably, only one significant pleiotropic effect was detected for the 10-fold protease mutant BRB14, which involved the induction of a cell wall stress regulon that encodes the quality control protease HtrC (YyxA/YyCk). HtrC is a homolog of HtrA and HtrB, and therefore, the htrC induction most likely reflects an attempt of the cells to compensate for the absence of HtrA and HtrB (40).

Upon growth of the different protease mutant strains in LB, the cells were separated from the growth medium by centrifugation. To assess the PrsA levels, both fractions were analyzed by Western blotting using PrsA-specific antibodies. As controls, we monitored the levels of the lipoproteins FeuA and YclQ, which are both involved in iron acquisition and have no roles in extracytoplasmic protein folding or quality control. This analysis revealed a cell-association degradation product of PrsA (denoted PrsAΔ) that was absent from the multiple-protease-mutant strains BRB08, -11, -12, and -14 (Fig. 1). These four strains lack the WprA protease, which indicates that the cell wall-bound form of WprA is involved in PrsA cleavage. In contrast, the control lipoproteins FeuA and YclQ were apparently not degraded in cells of the tested strains.

FIG 1 The cell-associated lipoprotein PrsA is a potential WprA substrate. Cells of B. subtilis 168 (wild type [Wt]) and the protease mutants BRB02 to -14 were grown overnight in LB medium at 37°C. The overnight cultures were diluted in fresh LB medium, and growth continued until 2 h after entry into the stationary phase. The cells were then separated from the growth medium by centrifugation and disrupted by bead beating. Cellular proteins were separated on NuPAGE gels and blotted onto a nitrocellulose membrane. Prior to gel loading, all protein samples were corrected for OD600. Specific polyclonal antibodies raised in rabbits were used to detect the blotted PrsA, FeuA, and YclQ proteins. The positions of intact PrsA, a degradation product of PrsA (PrsAΔ), intact FeuA, and intact YclQ are marked with arrows. Note that the absolute amounts of PrsA in WprA-deficient cells were found to vary in different biological replicates, but they were always significantly higher than in WprA-proficient cells. The position of a 37-kDa molecular mass marker is indicated.
Subsequent inspection of the respective growth medium fractions revealed that all protease mutants lacking WprA released massive amounts of PrsA into the growth medium (Fig. 2). This implies that WprA has a major role in the degradation of extracellular PrsA. Notably, the dominant released form of PrsA in the medium of multiple protease mutant strains lacking \( \text{wprA} \) (e.g., BRB08) was barely detectable in the medium of single \( \text{wprA} \) mutant cells (Fig. 3, upper right), showing that one or more of the seven proteases lacking in strain BRB07 are responsible for the degradation of the PrsA that is liberated from the cells when WprA is present. Furthermore, the deletion of only \( \text{wprA} \) from the 168 strain had a mildly positive effect on the cellular levels of PrsA (Fig. 3, upper left). However, this concerned mainly the PrsA\(^{D2} \) degradation product. The effect of the chromosomal \( \text{wprA} \) deletion was reversed upon ectopic expression of \( \text{wprA} \) from a plasmid (Fig. 3).

In the case of FeuA, we observed two released forms, with the lower-\( M_w \) form being dominant in the medium of the parental strain 168 and the higher-\( M_w \) form being dominant in the medium of strain BRB14, which lacks the largest number of extracytoplasmic proteases (Fig. 2). The lower-\( M_w \) form of FeuA in the medium of strain BRB14 is the result of FeuA cleavage by an as-yet-unidentified protease. For example, this could be the HtrC protease, which was shown to be upregulated in the BRB14 strain (40), but it might also be another as-yet-unidentified protease that is still present in the strain (4, 18). Similar to FeuA, two forms of YcQ were released into the medium, but in this case, the lower-\( M_w \) form was completely absent from the medium of strain BRB14 (Fig. 2). This indicates a dominant role of HtrA, HtrB, and WprA in the generation of the low-\( M_w \) form of released YcQ.

In contrast to PrsA, multiple protease gene deletions had no detectable effects on the cellular BdbD levels (Fig. 4). However, substantially increased amounts of full-size BdbD were detectable in the medium fractions of strains BRB11 and -14, both of which lack WprA plus HtrA. Since the media of these strains also con-
was analyzed by Western blotting, as described in the legend to Fig. 1. The growth media of multiple-protease-mutant strains were composed of cytoplasmic proteases, the levels of HtrA and HtrB in the cells and strain BRB11 (Fig. 5). BRB11, -12, and -13 are comparable, while the media of strains since the levels of extracellular TrxA in the growth media of strains 3 and 4). To be

FIG 5 Accumulation of full-size BbdD and degradation products of HtrA and HtrB in the growth media of multiple-protease-mutant strains. The presence of BbdD, HtrA, HtrB, and the cytoplasmic lysis marker TrxA in growth medium fractions of B. subtilis 168 (Wt) and the protease mutants BRB02 to -14 was analyzed by Western blotting, as described in the legend to Fig. 1. The positions of BbdD-, HtrA-, HtrB-, and TrxA-specific bands are marked with arrows. Note that only the major HtrA and HtrB bands are marked but that smaller HtrA and HtrB degradation products were also detected. Furthermore, the anti-HtrB antibodies display some minor cross-reactivity with the major extracellular form of HtrA. The positions of 37-, 24-, and 11-kDa molecular mass markers are indicated. Note that the higher–M_{r} forms of HtrA and HtrB in the growth medium samples of strains BRB12 and BRB11 are the full-size forms of these proteins, which run at ~50 and ~53 kDa, respectively (cf. Fig. 3 and 4).

tained elevated levels of the cytoplasmic marker protein TrxA (Fig. 5), the most likely explanation of this finding is that the full-size BbdD was released into the medium due to elevated levels of cell lysis (20). However, reduced levels of BbdD degradation due to the absence of WprA and HtrA cannot be excluded completely, since the levels of extracellular TrxA in the growth media of strains BRB11, -12, and -13 are comparable, while the media of strains BRB12 and -13 show much less extracellular BbdD than that of strain BRB11 (Fig. 5).

To assess the possible degradation of HtrA and HtrB by extracytoplasmic proteases, the levels of HtrA and HtrB in the cells and growth media of multiple-protease-mutant strains were compared (Fig. 4 and 5). Intriguingly, these two proteins appear also to be substrates of WprA, since the cellular levels of both HtrA and HtrB were elevated in the multiple-protease mutant BRB08 lacking the wprA gene. Additionally, highly elevated levels of HtrA were detected in htrB mutant cells (BRB10 and -12), and highly elevated levels of HtrB were detected in htrA mutant cells (BRB09 and -11). This is most likely due to the CssRS-dependent cross-regulation of htrA and htrB (23, 25). The deletion of only two extracytoplasmic proteases (NprB and AprE) in the BRB02 mutant caused an elevated level of HtrA in the growth medium, which is in line with the previous observation that the extracellular HtrA levels are increased in multiple-protease-mutant cells (19, 34, 36). The increased level of extracellular HtrA in the BRB02 mutant is not related to lysis, as evidenced by blotting for the cytoplasmic marker protein TrxA (Fig. 5). In contrast to HtrA, an almost linear increase in the accumulation of HtrB in the growth medium was observed when increasing numbers of protease genes were deleted (Fig. 5). This cumulative effect of multiple protease mutations shows that HtrB is more sensitive to extracytoplasmic degradation than HtrA. Notably, the highest levels of HtrA and HtrB in the growth medium fractions were observed in strains lacking either htrA or htrB, which most likely reflects the aforementioned cross-regulation between the two genes (23, 25). We also assessed the effects of a single wprA mutation on the cellular and extracellular HtrA and HtrB levels. Remarkably, it turned out that the wprA single-mutant cells contained barely any HtrA and HtrB and that these proteins were mainly detectable in the growth medium of the cells (Fig. 3, middle and bottom). The presence of HtrA and HtrB in the cells was restored when the wprA deletion was complemented with a plasmid-borne copy of wprA (Fig. 3). Altogether, these observations suggest that WprA is needed either for the expression of htrA and htrB or for the stabilization of HtrA and HtrB in a genetic context where other extracytoplasmic proteases are produced. The latter possibility would be in line with a previously suggested chaperone function of WprA in addition to its protease function (3, 31).

Finally, we performed a complementation analysis in which plasmid-borne copies of individual protease genes were reinserted into strain BRB07 and the impacts on the cellular and extracellular levels of PrsA, HtrA, HtrB, and the control lipoprotein FeuA were assessed. In principle, strains with more protease gene deletions than BRB07 would have been preferred for this analysis, but it turned out that these strains were not transformable in our hands. Importantly, the complementation analysis showed that reintroduction of the bpr gene caused a reduction in the cellular levels of PrsA, HtrA, and HtrB and the extracellular levels of HtrA and HtrB (Fig. 6). Furthermore, the reintroduction of nprE in BRB07 caused substantially reduced extracellular levels of HtrB and, to a lesser extent, HtrA (Fig. 6). These findings show that Bpr and NprE have important roles in the degradation of PrsA, HtrA, and HtrB.

Taken together, our present findings show that the cell-associated folding catalyst PrsA is targeted by WprA and that the released PrsA is subject to degradation by other extracellular proteases. Furthermore, HtrA and HtrB are prone to proteolysis by cell-associated WprA and proteases in the medium. Together, these findings suggest that the elevated levels of secreted (heterologous) proteins in multiple protease mutant strains may be due not only to reduced degradation, but also to elevated levels of cell-associated protein-folding catalysts, in particular PrsA, and quality control factors like HtrA and HtrB. Accordingly, it is very conceivable that the improved capabilities for protein secretion of previously constructed multiple-protease-mutant B. subtilis strains (35–37, 41–44), particularly the WB800 strain lacking wprA (44), can be attributed at least in part to elevated levels of PrsA, HtrA, and/or HtrB. On the other hand, presently, we cannot fully exclude the possibility that PrsA, HtrA, and HtrB are subject to proteolytic turnover, for example, upon loss of functionality. In this case, the reduced degradation of these proteins would have little impact on the yield of secreted homologous or heterologous proteins.

Irrespective of such considerations, our present findings imply that the deletion of multiple protease genes is likely to be an
important beneficial element in the construction of next-generation supersecreting Bacillus strains (45).

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REFERENCES

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