Degradation of the Twin-Arginine Translocation Substrate YwbN by Extracytoplasmic Proteases of *Bacillus subtilis*

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Bacterial twin-arginine translocases can export fully folded proteins from the cytoplasm. Such proteins are usually resistant to proteolysis. Here we show that multiple extracellular proteases degrade the *B. subtilis* Tat substrate YwbN. This suggests either that secreted YwbN is not fully folded or that folded YwbN exposes protease cleavage sites.

Protein folding is a specificity determinant in the transport of proteins across bacterial membranes. Major protein transport machineries, such as the Sec translocase, translocate only unfolded proteins (6). In contrast, the twin-arginine (RR) translocase (Tat) is known to translocate fully folded proteins that may even contain cofactors (1, 22, 23, 28, 35). Importantly, the folding state of a protein is also critical for its biological activity and stability. Therefore, proteins have to fold efficiently either before or after membrane translocation, depending on their translocation via the Tat or Sec pathways (4, 9, 16, 27, 28, 30, 31).

Tat translocases are present in Gram-negative and Gram-positive bacteria (1, 5, 13, 22, 29, 31, 35). Proteins are specifically targeted to these Tat translocases by signal peptides that possess a twin-arginine (RR) motif in their N terminus (1, 8, 11, 22, 23, 31, 35). In *Escherichia coli*, it was shown that cofactor-containing Tat substrates require dedicated proofreading chaperones such as TorD, DmsD, HyaW, or NapD for folding and cofactor insertion prior to translocation (8, 23, 25, 29). These chaperones (known as redox enzyme maturation proteins, or REMPs) sequester the RR-signal peptides of their substrates until these are properly assembled. Only then do the REMPs dissociate from the RR-signal peptide, thereby allowing Tat-dependent translocation of the cofactor-containing folded protein (8, 29). This REMP activity has not yet been demonstrated in all bacteria, and, especially in Gram-positive bacteria such as *Bacillus subtilis*, the mechanisms for folding and quality control of Tat substrates have remained enigmatic (14, 31, 33). Notably, proteins that are not properly folded are rejected by the Tat translocase and degraded. It seems that this quality control process relies on general proteolytic systems that are responsible for the turnover of misfolded proteins (1, 4, 7, 18, 27).

Proteolysis is an important theme in the physiology of *B. subtilis*, which is appreciated both as a model for fundamental scientific research on Gram-positive bacteria and as a workhorse in the biotechnological production of enzymes and vitamins (9, 31). Proteases produced by *B. subtilis* can be distinguished into the “quality control proteases” and “feeding proteases” categories (19, 24, 31). Quality control proteases are generally responsible for degradation of misfolded proteins in the cytoplasm, membrane, and cell wall, whereas feeding proteases are secreted to degrade extracellular proteins for the provision of nutrients (3, 9, 24, 31). Extracellular proteases represent major bottlenecks in the production of heterologous proteins that fold inefficiently or expose protease cleavage sites (9, 10). Accordingly, it was proposed that it might be beneficial to export heterologous proteins with RR-sig-

nal peptides via Tat, thereby allowing them to fold in the cytoplasm prior to export and exposure to the feeding proteases (34). In addition, the deletion of multiple genes for feeding proteases would preclude the degradation of the folded secreted proteins (9, 24, 31).

B. subtilis possesses two Tat translocases called TatAdCd and TatAyCy that operate in parallel (12, 14). The TatAdCd translocase is expressed only under conditions of phosphate limitation, as is the case for its specific substrate, PhoD (13, 26). In contrast, the TatAyCy translocase is constitutively produced (21). To date, the putative Dyp-type peroxidase YwbN is the only protein known to be specifically secreted via TatAyCy (12). Based on its secretion via Tat, it was generally assumed that YwbN is translocated across the membrane in a folded state (14, 31). This would confer resistance to the HtrA and HtrB quality control proteases in the membrane and to the wall-bound and secreted quality control protease WprA, as well as to the extracellular feeding proteases AprE, Bpr, Epr, Mpr, NprB, NprE, and Vpr (9, 30, 31). The present studies were aimed at testing this assumption using a collection of protease mutant strains that lacked increasing numbers of extracellular proteases and quality control proteases (Table 1), all of which are expressed under the tested conditions (21).

To assess the impact of proteases on the extracellular levels of YwbN, cells were grown in lysogeny broth (LB). Next, cells were separated from the growth medium by centrifugation and the amounts of YwbN in both fractions were assessed by Western blotting using specific antibodies. Unexpectedly, compared to the 168 parental strain, a major increase in the levels of extracellular YwbN was observed upon the consecutive deletion of genes for feeding and quality control proteases (Fig. 1). In contrast, only a relatively minor change in the levels of the secreted LipA control protein was observed. The increasing levels of YwbN did not relate to cell lysis, as evidenced by the extracellular levels of the cytoplasmic protein TrxA, which was previously established as a lysis marker (15). Consistent with this view, the cellular levels of YwbN and TrxA were by and large the same in all tested strains (Fig. 2).

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TABLE 1 Strains

<i>B. subtilis</i> strain ^a	Relevant property(ies)	Reference or source
168	<i>trpC2</i>	17
BRB02	<i>trpC2; ΔnprB, ΔaprE</i>	Cobra Biologics
BRB03	<i>trpC2; ΔnprB, ΔaprE, Δepr</i>	Cobra Biologics
BRB04	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr</i>	Cobra Biologics
BRB05	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE</i>	Cobra Biologics
BRB06	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr</i>	Cobra Biologics
BRB07	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr</i>	Cobra Biologics
BRB08	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔwprA</i>	Cobra Biologics
BRB09	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔhtrA</i>	Cobra Biologics
BRB10	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔhtrB</i>	Cobra Biologics
BRB11	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrA</i>	Cobra Biologics
BRB12	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrB</i>	Cobra Biologics
BRB13	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔhtrA, ΔhtrB</i>	Cobra Biologics
BRB14	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrA, ΔhtrB</i>	Cobra Biologics
BRB07 AyCy	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, tatAy-tatCy::Sp; Sp^r</i>	This study
BRB07 AdCd	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, tatAd-tatCd::Cm; Cm^r</i>	This study
BRB07 AyCyAdCd	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, tatAd-tatCd::Cm; tatAy-tatCy::Sp; Sp^r, Cm^r</i>	This study
BRB07aprE	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr; pHBAprE; Em^r; Cm^r</i>	This study
BRB07bpr	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr; pHBBpr; Em^r; Cm^r</i>	This study
BRB07epr	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr; pHBEpr; Em^r; Cm^r</i>	This study
BRB07mpr	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr; pHBMpr; Em^r; Cm^r</i>	This study
BRB07nprB	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr; pHBNprB; Em^r; Cm^r</i>	This study
BRB07nprE	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr; pGS1npr; Cm^r</i>	This study
BRB07vpr	<i>trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr; pHBVpr; Em^r; Cm^r</i>	This study

^a BRB, *Bacillus Recipharm*, Cobra Biologics.

FIG 1 Increasing levels of YwbN in the growth media of protease mutants. Cells of *B. subtilis* 168 and BRB02 to BRB14 were grown overnight in LB medium at 37°C. The overnight cultures were diluted in fresh LB medium and grown for 6 h. Cells were then separated from the growth medium by centrifugation. Proteins in the medium fractions were separated using precast 10% Bis-TrisNuPAGE 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). The presence of YwbN, LipA, and TrxA was detected with specific polyclonal antibodies raised in rabbits. Visualization of bound antibodies was performed using IRDye 800 CW goat anti-rabbit secondary antibodies in combination with an Odyssey infrared imaging system (Li-Cor Biosciences). Fluorescence was recorded at 800 nm. Please note that the apparently reduced LipA level in the medium of strain BRB02 is an outlier that is not normally encountered in this strain. This could relate to an incidental fluctuation in the *lipA* expression level or to a technical problem during sample processing.

Interestingly, all *wprA* mutants contained cell-associated degradation products of YwbN, suggesting that these are normally degraded by the WprA quality control protease. These findings indicated that YwbN is subject to extensive proteolysis during cell wall passage and secretion. To verify this idea and to pinpoint proteases involved in YwbN degradation, a complementation analysis was performed using the BRB07 7-fold protease mutant strain. This mutant can be readily transformed by routine methods (17), in contrast to strains lacking additional proteases (not

FIG 2 Detection of intact and proteolyzed YwbN in protease mutant cells. Cells of *B. subtilis* 168 and BRB02 to BRB14 were grown overnight in LB medium at 37°C. The overnight cultures were diluted in fresh LB medium and grown for 6 h. Cells were collected by centrifugation and disrupted by bead-beating as previously described (33). Cellular proteins were analyzed by PAGE and Western blotting as described in the legend of Fig. 1 using specific antibodies against YwbN and TrxA. Please note that cells lacking the *ywbN* gene give barely any signal with the YwbN-specific antibody in Western blotting experiments compared to *ywbN*-proficient cells (20) (data not shown).

FIG 4 Tat-dependent secretion of YwbN in a multiple protease mutant. To verify the Tat-dependent secretion of YwbN by strain BRB07, mutant derivatives of this strain were constructed that lacked the *tatAyCy*, *tatAdCd*, or *tatAyCy-tatAdCd* genes. Next, the levels of YwbN in the growth medium and cells were assayed by Western blotting as described in the legends of Fig. 1 and 2.

shown). Briefly, the *nprB*, *aprE*, *epr*, *bpr*, *mpr*, or *vpr* genes were amplified by PCR from the genome of *B. subtilis* 168 and cloned into the pHB201 low-copy-number expression vector (2). The resulting plasmids (or the pGS1npr plasmid for *nprE* expression) (32) were then used to transform *B. subtilis* BRB07. As shown in Fig. 3, the production of AprE, Bpr, NprE, or Vpr resulted in decreased extracellular YwbN levels comparable to that of the 168 parental strain. In contrast, ectopic expression of *nprB*, *epr*, and *mpr* did not result in relatively low extracellular YwbN levels such as were encountered in the parental 168 strain. Most likely, this means that NprB, Epr, and Mpr are not involved in YwbN degradation. However, we cannot exclude the possibility that, upon ectopic expression of the respective genes, the production levels of NprB, Epr, and Mpr are lower than those in the parental strain. In contrast to YwbN, the secreted control protein FeuA was not affected by the ectopic expression of protease genes. Lastly, we showed that YwbN was still TatAyCy-dependently secreted in the BRB07 strain by using *tatAyCy*, *tatAdCd*, or *tatAdCd-tatAyCy* mutant derivatives (Fig. 4). Taken together, our data show that the Tat-dependently secreted protein YwbN is a substrate for multiple extracellular proteases of *B. subtilis*, including both feeding and quality control proteases. Our findings thus challenge the hypothesis that Tat-dependently secreted proteins of *B. subtilis* would be protease resistant. Instead, our observations suggest either that YwbN is not fully folded upon membrane translocation, cell wall passage, and secretion into the growth medium or that the folded

YwbN exposes protease cleavage sites, which would be highly remarkable for a native secretory protein of *B. subtilis*. At present, we do not know whether the posttranslational degradation of YwbN is advantageous for *B. subtilis*. Clearly, the degradation of YwbN is not disadvantageous for the cells under the tested conditions, suggesting that the steady-state production level of this protein is high enough to sustain optimal growth and cell viability.

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REFERENCES

- Berks BC, Sargent F, Palmer T. 2000. The Tat protein export pathway. *Mol. Microbiol.* 35:260–274.
- Bron S, et al. 1998. Protein secretion and possible roles for multiple signal peptidases for precursor processing in bacilli. *J. Biotechnol.* 64:3–13.
- Dalbey RE, Wang P, van Dijk JM. 2012. Membrane proteases in the bacterial protein secretion and quality control pathway. *Microbiol. Mol. Biol. Rev.* 76:311–330.
- DeLisa MP, Tullman D, Georgiou G. 2003. Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. *Proc. Natl. Acad. Sci. U. S. A.* 100:6115–6120.
- Desvaux M, Hébraud M, Talon R, Henderson IR. 2009. Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. *Trends Microbiol.* 17:139–145.
- Driessen AJ, Nouwen N. 2008. Protein translocation across the bacterial cytoplasmic membrane. *Annu. Rev. Biochem.* 77:643–667.
- Fisher AC, DeLisa MP. 2004. A little help from my friends: quality control of presecretory proteins in bacteria. *J. Bacteriol.* 186:7467–7473.
- Fröbel J, Rose P, Müller M. 2012. Twin-arginine-dependent translocation of folded proteins. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367:1029–1046.
- Harwood CR, Cranenburgh R. 2008. *Bacillus* protein secretion: an unfolding story. *Trends Microbiol.* 16:73–79.
- Jensen CL, Stephenson K, Jorgensen ST, Harwood CR. 2000. Cell-associated degradation affects the yield of secreted engineered and heterologous proteins in the *Bacillus subtilis* expression system. *Microbiology* 146:2583–2594.
- Jongbloed JDH, et al. 2002. Selective contribution of the twin-arginine translocation pathway to protein secretion in *Bacillus subtilis*. *J. Biol. Chem.* 277:44068–44078.
- Jongbloed JDH, et al. 2004. Two minimal Tat translocases in *Bacillus*. *Mol. Microbiol.* 54:1319–1325.
- Jongbloed JD, et al. 2000. TatC is a specificity determinant for protein

FIG 3 Identification of proteases that degrade YwbN. To identify proteases responsible for YwbN degradation, strain BRB07 was transformed with plasmid-borne copies of the protease genes that had been deleted from this strain. The *nprB*, *aprE*, *epr*, *bpr*, *mpr*, or *vpr* genes were cloned in the pHB201 expression plasmid (2). For *nprE* expression, the previously constructed plasmid pGS1npr was used (32). The presence of the YwbN and FeuA proteins in growth medium fractions of the transformed strains was detected by PAGE and Western blotting with specific polyclonal rabbit antibodies as described in the legend to Fig. 1.

- secretion via the twin-arginine translocation pathway. *J. Biol. Chem.* 275: 41350–41357.
14. Jongbloed JD, van der Ploeg R, van Dijl JM. 2006. Bifunctional TatA subunits in minimal Tat protein translocases. *Trends Microbiol.* 14:2–4.
 15. Kouwen TR, et al. 2007. Thiol-disulphide oxidoreductase modules in the low-GC Gram-positive bacteria. *Mol. Microbiol.* 64:984–999.
 16. Kramer G, Boehringer D, Ban N, Bukau B. 2009. The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nat. Struct. Mol. Biol.* 16:589–597.
 17. Kunst F, Rapoport G. 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.* 177: 2403–2407.
 18. Lindenstrauss U, Matos CFRO, Graubner W, Robinson C, Brüser T. 2010. Malfolded recombinant Tat substrates are Tat-independently degraded in *Escherichia coli*. *FEBS Lett.* 584:3644–3648.
 19. Molière N, Turgay K. 2009. Chaperone-protease systems in regulation and protein quality control in *Bacillus subtilis*. *Res. Microbiol.* 160:637–644.
 20. Monteferrante CG, Miethke M, van der Ploeg R, Glasner C, van Dijl JM. 5 July 2012, posting date. Specific targeting of the metallophosphoesterase YkuE to the *Bacillus* cell wall requires the twin-arginine translocation system. *J. Biol. Chem.* [Epub ahead of print].
 21. Nicolas P, et al. 2012. Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science* 335:1103–1106.
 22. Palmer T, Berks BC. 2012. The twin-arginine translocation (Tat) protein export pathway. *Nat. Rev. Microbiol.* 10:483–496.
 23. Palmer T, Sargent F, Berks BC. 2005. Export of complex cofactor-containing proteins by the bacterial Tat pathway. *Trends Microbiol.* 13: 175–180.
 24. Pohl S, Harwood CR. 2010. Heterologous protein secretion by the *Bacillus* species from cradle to grave. *Adv. Appl. Microbiol.* 73:1–25.
 25. Pommier J, Mejean V, Giordano G, Iobbi-Nivol C. 1998. TorD, a cytoplasmic chaperone that interacts with the unfolded trimethylamine N-oxide reductase enzyme (TorA) in *Escherichia coli*. *J. Biol. Chem.* 273: 16615–16620.
 26. Pop O, Martin U, Abel C, Müller JP. 2002. The twin-arginine signal peptide of PhoD and the TatAd/Cd proteins of *Bacillus subtilis* form an autonomous Tat translocation system. *J. Biol. Chem.* 277:3268–3273.
 27. Robinson C, et al. 2011. Transport and proofreading of proteins by the twin-arginine translocation (Tat) system in bacteria. *Biochim. Biophys. Acta* 1808:876–884.
 28. Sanders C, Wethkamp N, Lill H. 2001. Transport of cytochrome c derivatives by the bacterial Tat protein translocation system. *Mol. Microbiol.* 41:241–246.
 29. Sargent F. 2007. Constructing the wonders of the bacterial world: biosynthesis of complex enzymes. *Microbiology* 153:633–651.
 30. Sarvas M, Harwood CR, Bron S, van Dijl JM. 2004. Post-translocational folding of secretory proteins in Gram-positive bacteria. *Biochim. Biophys. Acta* 1694:311–327.
 31. Tjalsma H, et al. 2004. Proteomics of protein secretion by *Bacillus subtilis*: separating the “secrets” of the secretome. *Microbiol. Mol. Biol. Rev.* 68: 207–233.
 32. Van Den Burg B, Eijssink VG, Stulp BK, Venema G. 1989. One-step affinity purification of *Bacillus* neutral proteases using bacitracin-silica. *J. Biochem. Biophys. Methods* 18:209–219.
 33. van der Ploeg R, et al. 2011. Environmental salinity determines the specificity and need for Tat-dependent secretion of the YwbN protein in *Bacillus subtilis*. *PLoS One* 6:e18140. doi:10.1371/journal.pone.0018140.
 34. van Dijl JM, et al. 2002. Functional genomic analysis of the *Bacillus subtilis* Tat pathway for protein secretion. *J. Biotechnol.* 98:243–254.
 35. Yuan J, Zweers JC, van Dijl JM, Dalbey RE. 2010. Protein transport across and into cell membranes in bacteria and archaea. *Cell. Mol. Life Sci.* 67:179–199.