Influence of a Cell-Wall-Associated Protease on Production of α-Amylase by Bacillus subtilis

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AmyL, an extracellular α-amylase from Bacillus licheniformis, is resistant to extracellular proteases secreted by Bacillus subtilis during growth. Nevertheless, when AmyL is produced and secreted by B. subtilis, it is subject to considerable cell-associated proteolysis. Cell-wall-bound proteins CWBP52 and CWBP23 are the processed products of the B. subtilis wprA gene. Although no activity has been ascribed to CWBP23, CWBP52 exhibits serine protease activity. Using a strain encoding an inducible wprA gene, we show that a product of wprA, most likely CWBP52, is involved in the posttranslational stability of AmyL. A construct in which wprA is not expressed exhibits an increased yield of α-amylase. The potential role of wprA in protein secretion is discussed, together with implications for the use of B. subtilis and related bacteria as hosts for the secretion of heterologous proteins.

The cell envelope of the gram-positive bacterium Bacillus subtilis consists of a single (cytoplasmic) membrane surrounded by a relatively thick cell wall consisting of similar proportions of peptidoglycan and covalently attached anionic polymers. The absence of an outer membrane means that there is no equivalent of the membrane-enclosed periplasm found in gram-negative bacteria. However, by virtue of its thickness and high density of negative charge, the cell wall may perform some of the roles of the periplasm in gram-positive bacteria.

The absence of an outer membrane in gram-positive bacteria also simplifies the secretion pathway, and, consequently, B. subtilis and its close relatives have the potential to secrete proteins directly into the growth medium, at concentrations in excess of 5 grams per liter (4). Despite its extensive use in the production of commercially important Bacillus enzymes (e.g., α-amylases and alkaline proteases), attempts to exploit B. subtilis for the production of heterologous proteins at high concentrations have proved disappointing (8). One reason for this failure is the production and release into the culture medium of several extracellular proteases (24, 28, 37). Although native Bacillus proteins are generally resistant to these proteases, heterologous proteins are often rapidly degraded in their presence. As a result, strains of B. subtilis that are multiply deficient in extracellular proteases have been developed (11, 37). The more developed of these strains have less than 1% of the proteolytic activity of the wild type (37). To date, efforts have concentrated mainly on the proteases which reside in a truly extracellular location, while those which remain cell associated have been largely overlooked.

Although strains deficient in extracellular proteases have improved the productivity of B. subtilis for the production of heterologous proteins, they have only partially overcome problems of unexpectedly low yields. We and others have recently shown (22, 31) that significant amounts of secretory protein are degraded within minutes of being synthesized. This degradation is observed even for Bacillus proteins that are highly resistant to proteases released into the culture medium, suggesting that a component of this degradation is cell associated. Margot and Karamata recently reported the identification of a cell-wall-associated protease encoded by the wprA gene (21). The primary product of this gene is a 96-kDa polypeptide that is processed into two previously identified cell wall proteins, namely, CWBP2 and CWBP23. The processing of the WprA precursor during secretion accompanies the targeting of CWBP2 and CWBP23 to the cell wall and is analogous to the processing of another B. subtilis cell-wall-bound protein, namely, WapA (5). The amino acid sequence of CWBP52 shows a high degree of similarity with serine proteases of the subtilisin family, and phenylmethylsulfonil fluoride (PMFs)-sensitive protease activity was detected in proteins extracted from the cell wall of a wprA− strain, but not in one of this gene that had been insertionally inactivated (21). In the absence of homology to proteins in the databases, the N-terminal CWBP23 moiety was presumed to function as a chaperone-like propeptide that is proteolytically processed on the trans side of the membrane in this paper, we report on a potential role of products of wprA in the integrity of secretory proteins during late stages in the secretion pathway. We also discuss the potential of wprA mutants to increase the productivity of B. subtilis for secretory proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. In general, the bacterial strains were grown in 2xYT medium (1.6% [wt/vol] tryptone, 1% [wt/vol] yeast extract, 0.5% NaCl), which was supplemented as required with chloramphenicol (6 μg/ml), erythromycin (1 μg/ml), lincomycin (25 μg/ml), or ampicillin (100 μg/ml). Spizizen’s minimal salts (SMS) (29) with 1% (wt/vol) ribose as a non-catabolite-repressing carbon source was used for pulse-chase experiments. Cultures were grown at 37°C in an orbital incubator, with shaking at 180 rpm. The α-amylase from Bacillus licheniformis (AmyL) was expressed in B. subtilis during exponential phase with a xylose-inducible promoter system developed by Novo Nordisk A/S (9, 10, 31), and xylose (1% [wt/vol]) was included in the growth medium to induce the synthesis of α-amylase when required. To avoid problems associated with plasmid instability (2, 7), the xylose-inducible amyL cassette was integrated into the chromosome of the α-amylase (AmyE)-negative B. subtilis strain DN1885 by a Campbell-type recombination between plasmid and chromosomally encoded xylR genes (31).

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TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Comment</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><em>B. subtilis</em> DN1885</td>
<td>amyE amyR2</td>
<td>(\alpha)-amylase-negative derivative of <em>B. subtilis</em> 168</td>
<td>3; Novo Nordisk A/S</td>
</tr>
<tr>
<td><em>B. subtilis</em> KS408</td>
<td>amyE; xylose-inducible amyL; Cm'</td>
<td>DN1885 with xylose-inducible amyL integrated at xyR</td>
<td>31</td>
</tr>
<tr>
<td><em>B. subtilis</em> KS408 wprA::pMutin2</td>
<td>amyE; xylose-inducible amyL; IPTG-inducible wprA; Cm' Em' Lac'</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> XL1-blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacF73AM15 Tn10 (Tet')]</td>
<td>Stratagene</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td>Integrates into the chromosome of <em>B. subtilis</em> at the wprA locus by homologous recombination</td>
<td>S. D. Ehrlich</td>
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**Medium and reagents.** The medium used in this study was purchased from Difco Laboratories Ltd. (East Molesey, United Kingdom). Other reagents were obtained from Sigma (Poole, United Kingdom).

Quantitation of \(\alpha\)-amylase activity. Overnight cultures were used to inoculate 20 mL of 2xYT-xylose broth, which was incubated at 37°C with shaking. Samples were centrifuged (10,000 \(\times\) g, 5 min, room temperature) to pellet the cells, and the \(\alpha\)-amylase activity in the supernatant was quantified in a scaled-down version of the Phadebas assay (Pharmacia Diagnostics, Uppsala, Sweden), according to the manufacturer's protocol. In all cases, multiple samples were used for the Phadebas assay to calculate a mean value for \(\alpha\)-amylase activity. One unit of \(\alpha\)-amylase activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 \(\mu\)mol of glycosidic linkage per min at 37°C.

**Stability of AmyL in culture supernatants.** Cultures were grown for 48 h in 20 mL of 2xYT-xylose broth to allow the accumulation of \(\alpha\)-amylase and extra-cellular proteases in the medium. The cells were removed by centrifugation (10,000 \(\times\) g, 30 min, 4°C), and the supernatants were filtered (0.45-\(\mu\)m-pore-size Acrodisc filters; Gelman Sciences) to ensure the complete removal of cellular material. Cell-free supernatants were incubated at 4°C in the presence or absence of 10 mM EDTA, and samples were removed at intervals for determinations of \(\alpha\)-amylase activity and Western blotting.

**DNA manipulations and PCR.** Restriction enzymes and Pfu DNA polymerase were obtained from Promega. All DNA manipulations were carried out according to standard protocols (27). DNA fragments were purified from agarose gels with Qiaquick columns (Qiagen Limited, Dorset, United Kingdom), and plasmids were isolated from *Escherichia coli* with Qiaprep-100 columns (Qiagen Limited). *B. subtilis* DN1885 was transformed with integration plasmids after the induction of natural competence (1).

A DNA fragment corresponding to the 5' end of the wprA gene was PCR amplified with oligonucleotide primers WPR-F (5'-GGCCGGCGCGGATCCGG GATAACATGAACGGC-3') and WPR-R (5'-GGCCGGCGCGGATCCCCAT CTCCGGCGTTG-3'). These primers were designed with 5' extensions to introduce BamHI restriction sites into the ends of the PCR product to facilitate cloning.

**SDS-PAGE and Western blotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (17) with 10% (wt/vol) or 12.5% (wt/vol) gels. For Western blotting, proteins were transferred from SDS-PAGE gels (34) onto nitrocellulose membranes (0.4-\(\mu\)-m pore size; Anderman Ltd., Kingston upon Thames, United Kingdom). Bands corresponding to \(\alpha\)-amylase were detected with rabbit anti-AmyL polyclonal antiserum and anti-rabbit-horseradish peroxidase conjugate (Dako Immunochemicals A/S, Glostrup, Denmark).

**Pulse-chase and immunoprecipitation.** Cultures were grown in SMS-ribose-xylose broth with shaking at 37°C with xylose. Samples were removed for the determination of OD660 and \(\alpha\)-amylase activity (Fig. 1A). Western blotting confirmed that the loss of \(\alpha\)-amylase activity in the presence of EDTA was due to proteolytic degradation, rather than irreversible inactivation of enzyme activity, since the amount of a\(\alpha\)-amylase activity (Fig. 1A).

**RESULTS**

**Stability of AmyL in spent culture medium.** During the transition from exponential to stationary phase, *B. subtilis* secretes a battery of extracellular enzymes into the culture medium, including several proteases (24, 25). Therefore, we determined the stability of mature AmyL in spent culture medium following growth of *B. subtilis* KS408 to stationary phase. In its native conformation, AmyL was stable in the presence of cosecreted proteases over an incubation time of nearly 300 h, as indicated by only a slight reduction in \(\alpha\)-amylase activity (Fig. 1A).

\(\alpha\)-Amylases display a common requirement for Ca\(^{2+}\) ions which are necessary for both enzymatic activity and structural integrity. \(\alpha\)-Amylase preparations from which Ca\(^{2+}\) ions have been removed become highly susceptible to proteolysis (19, 30, 35). When AmyL was incubated in spent culture medium in the presence of the metal ion chelator EDTA, the enzyme became sensitive to proteolysis, as indicated by a marked reduction in \(\alpha\)-amylase activity with time (Fig. 1A). Western blotting confirmed that the loss of \(\alpha\)-amylase activity in the presence of EDTA was due to proteolytic degradation, rather than irreversible inactivation of enzyme activity, since the amount of a cross-reacting band at 56 kDa (AmyL) decreased with time and a number of major putative degradation products were visualized (Fig. 1B). The data presented show the extent of proteolysis at 4°C, since the rate of loss of activity at 37°C in the absence of free Ca\(^{2+}\) ions was too rapid (>95% in 5 min) to be determined with precision.

**Construction of an inducible wprA gene.** Despite its stability in the presence of extracellular proteases (Fig. 1), newly synthesized AmyL is subject to substantial degradation (31). Since AmyL is stable in the growth medium, the protease(s) responsible for this degradation is likely to be cell associated and localized at the cytoplasmic membrane as lipoprotein with its activity on the *trans* side and/or immobilized in the matrix of the cell wall. In either case, the protease(s) would have access to secretory proteins emerging from the translocation complex.
One such protease is CWBP52, a processed product of the \textit{wprA} gene, that has been shown to be bound noncovalently to the cell wall (21).

To determine whether the products of the \textit{wprA} gene are involved in the co- or posttranslocational degradation of AmyL, we constructed a strain of \textit{B. subtilis} in which an intact copy of the gene was under the control of the isopropylthio-
\textit{b}-D-galactoside (IPTG)-inducible \textit{Pspac} promoter (38). The constructs were made with the pMutin2 integration vector (provided by S. D. Ehrlich [see Fig. 2]). A 357-bp DNA fragment corresponding to the 5\(^\text{\textsuperscript{\textprime}}\) end of the \textit{wprA} gene was amplified by PCR from \textit{B. subtilis} KS408 chromosomal DNA with oligonucleotide primers WPR-F and WPR-R. This fragment was cloned into the unique \textit{BamHI} restriction site of pMutin2 with \textit{E. coli} XL1-blue as the host. Recombinant plasmids were screened for the correct orientation of the insert by PCR and then sequenced to verify the fidelity of the PCR and cloning steps (data not shown). The resultant plasmid, pM2\textit{wprA}FP, was used to transform \textit{B. subtilis} KS408 to produce strain KS408 \textit{wprA}::pMutin2 selecting for resistance to erythromycin and lincomycin. Since pM2\textit{wprA}FP does not have a functional \textit{B. subtilis} origin of replication, transformants were selected in which the plasmid had integrated into the chromosome by a homologous single-crossover (Campbell-type) recombination between the plasmid and chromosomal sequences of \textit{wprA} (Fig. 2). The authenticity of the integrants was confirmed by PCR (data not shown). Since the \textit{wprA} gene of KS408 \textit{wprA}::pMutin2 is under the control of the \textit{Pspac} promoter, its expression can be controlled by the presence or absence of IPTG. Additionally, a transcriptional fusion (\textit{wprA}\textendash\textit{lacZ}) between the native \textit{wprA} promoter and \textit{lacZ} was created to allow the expression of \textit{wprA} to be monitored via \textit{\beta}-galactosidase activity (Fig. 2).

The influence of \textit{wprA} on the yields of \(\alpha\)-amylase released into the culture medium. \textit{B. subtilis} KS408 was grown in 2xYT-xylose, and KS408 \textit{wprA}::pMutin2 was grown in the same medium in the absence or presence of 10 mM IPTG. The strains grew at identical growth rates (Fig. 3), in agreement with pre-
vious observations (21). However, when the yields of released α-amylase were compared, the strains differed markedly. In the absence of IPTG (wprA uninduced), the yield of α-amylase from KS408 wprA::pMutin2 started to increase above that of KS408 during exponential phase and continued to do so after transition to stationary phase. Approximately 13% more α-amylase activity was detected in the supernatant of KS408 wprA::pMutin2 at the start of stationary phase, and this increased still further to 41% after 38 h (data not shown).

In contrast, the yield of α-amylase from KS408 wprA::pMutin2 in the presence of IPTG (wprA induced) was lower, and upon transition to stationary phase the yield of α-amylase was 95% of that of KS408. The presence of IPTG had no effect on the yield of α-amylase from strain KS408 (data not shown). These data demonstrate that expression of wprA markedly influences the yield of released α-amylase.

Coupled pulse-chase and immunoprecipitation techniques were used to investigate the secretion kinetics of AmyL in KS408 and KS408 wprA::pMutin2. Cultures were grown to exponential phase (OD 660 nm ~ 0.8) and pulse-chased with L-[35S]methionine. Following immunoprecipitation and subsequent SDS-PAGE, both the precursor and mature forms of AmyL were visualized by autoradiography (Fig. 4A). In the case of KS408, the processing of the AmyL precursor to the mature form was rapid; in samples taken immediately following the chase (0 min), only 27% of the total AmyL (precursor plus mature) synthesized during the pulse was in the precursor form (Fig. 4). Processing was completed by 5 min post-chase, when all of the α-amylase was in the mature form. The amount of mature AmyL in the whole-culture sample (cells plus growth medium) peaked at 1 min, after which it declined until it reached a constant level of approximately 25% of the maximum detected, representing a significant loss of newly synthesized α-amylase during or shortly after translocation across the cytoplasmic membrane.

The amount of mature AmyL released into the culture medium was seen to increase with time until it reached a constant level of approximately 25% of the total synthesized, an amount consistent with AmyL remaining in the whole culture samples. The observation that only a proportion of the α-amylase synthesized during the pulse was released into the supernatant in an intact form was in agreement with previous data (31). An indication of the proportion of AmyL that remains cell associated at each time point can be obtained by subtracting the amount of released AmyL from that observed in whole culture samples. Figure 5 shows that following an initial increase, mature AmyL is degraded and by approximately 7 min post-chase no

FIG. 5. Cell-associated degradation of AmyL as measured by subtracting the data for released mature AmyL from those obtained for the whole-culture samples. The amount of AmyL at each interval is expressed as a percentage of the maximum amount of AmyL (precursor plus mature) synthesized during the pulse.
AmyL remains in a cell-associated form. These data suggest that the observed degradation of AmyL occurs during or shortly after translocation across the membrane and in a cell-associated location.

In the case of KS408 wprA::pMutin2, the processing of the AmyL precursor occurred at a rate comparable to that observed for strain KS408 (Fig. 4). However, in the absence of IPTG (wprA uninduced), the rate of degradation of mature AmyL in whole-culture samples was reduced. The amount of mature AmyL decreased to approximately 35% (cf. 25% in strain KS408) of the total synthesized. AmyL was released with similar kinetics, but the final yield in the culture medium was increased to approximately 36% (cf. 25% in strain KS408).

Conversely, in the presence of IPTG (wprA induced), the rate of degradation of mature AmyL in whole-culture samples was increased above that of KS408, and only 14% of the amount of AmyL initially synthesized could be detected 30 min post-chase (Fig. 4). In agreement with these observations, the amount of released AmyL was much lower and remained at less than 10% of the total amount of AmyL synthesized during the pulse.

These data show that the extent of cell-associated AmyL degradation is influenced by the products of the wprA gene, in agreement with the data shown in Fig. 3.

Transcriptional activity of the wprA gene. The transcriptional activity of the native wprA promoter in strain KS408 wprA::pMutin2 was monitored by measuring β-galactosidase activity expressed from the transcriptionally fused lacZ gene (Fig. 2). During exponential growth and on transition to stationary phase, the β-galactosidase activity was relatively constant at approximately 40 Miller units/OD₆₅₀ unit (Fig. 6), confirming previous reports that wprA is expressed during exponential growth (21). However, following transition to stationary phase, β-galactosidase activity increased, reaching a peak of 75 Miller units/OD₆₅₀ unit after ~28 h (data not shown). Since β-galactosidase is relatively unstable in B. subtilis (unpublished observations), the maintenance of this level of activity in stationary phase is likely to reflect de novo protein synthesis. The presence of IPTG in the growth medium had no effect on the β-galactosidase activity of KS408 wprA::pMutin2, and the amount of activity detected in strain KS408 was negligible (Fig. 6).

Analysis of cell wall-bound proteins. Proteins were extracted from the cell walls of B. subtilis KS408 and KS408 wprA::pMutin2 and analyzed by SDS-PAGE (Fig. 7). A prominent band corresponding in size to that of the CWBP52 serine protease (52 kDa) was observed in protein extracts from the wall of KS408 (Fig. 7, lane 1). This protein was absent from the wall extracts of KS408 wprA::pMutin2 (Fig. 7, lane 2).

In addition to CWBP52, a prominent band of approximately 96 kDa was observed in the wall extract from KS408. This protein, which was absent from the wall extract of KS408 wprA::pMutin2, is likely to be the WprA precursor that is processed into CWBP23 and CWBP52. However, it was not possible to determine whether this protein was present in the wall prior to cell breakage or whether it was part of the intracellular pool that subsequently contaminated the wall preparation following disruption with the French pressure cell. The latter is possible because the WprA precursor has a calculated pI of 10.1 (21) and would be likely to associate with negatively charged components of the cell wall via electrostatic interactions.

Discussion

Two previously identified cell-wall-bound proteins of B. subtilis, CWBP23 and CWBP52, have recently been identified as the processed products of the wprA gene (21). Although CWBP52 was identified as a wall-associated serine protease, its role in cell growth and physiology was not defined, since its absence has no discernible effect on growth rate, cell morphology, sporulation, or motility.

When the B. licheniformis α-amylase, AmyL, is secreted from B. subtilis, it is subjected to considerable cell-associated proteolytic degradation (31). This proteolysis results in only a proportion of the newly synthesized α-amylase being released into the culture medium. Our data reveal a time window between translocation and release into the growth medium during which AmyL is susceptible to proteolytic degradation since, once released, it is stable for prolonged periods. This observation prompted us to investigate the influence of CWBP52, a product of the wprA gene, on the yield of released AmyL.

We constructed a mutant in which the expression of wprA was controlled by IPTG. In the absence of induction, the amount of AmyL released into the culture medium increased significantly compared with the wild type. Conversely, when wprA was induced, the yield of AmyL was reduced. These data point to a role of the wprA gene product(s) in the stability of AmyL. Pulse-chase studies showed that AmyL was subjected to cell-associated proteolysis and that the extent of this degradation was reduced in the absence of the wprA gene products.

To be competent for translocation, secretory proteins are prevented from folding into their native conformations in the cytosol by, for example, the action of cytoplasmic molecular chaperones (26). During or shortly after translocation, secre-
tory proteins fold into their native conformations on the trans side of the cytoplasmic membrane and in *B. subtilis* this process appears to be assisted by the putative extracytoplasmic chaperone, PrsA (14, 15). Secretory proteins such as AmyL, when partially or fully unfolded, are susceptible to proteolytic degradation, and the rate at which the fully folded state is reached determines the extent of degradation (31). Our data suggest that the CWBP52 serine protease, by virtue of its cellular location, is able to degrade a significant proportion of newly translocated AmyL before it is able to achieve its fully folded, and therefore protease-resistant, conformation. However, we cannot rule out the possibility that the effect(s) of the *wprA* gene product(s) on AmyL is indirect, for example via its activity on a protein such as PrsA.

Furthermore, it is possible that expression of *wprA* from the inducible promoter (possibly to a level above that of the native promoter) resulted in limited obstruction of the secretion apparatus by WprA, leading to the induction of other cellular proteases which could in turn contribute to the degradation of AmyL. However, this is unlikely since expression of *wapA*, encoding an unrelated cell-wall-associated protein, using the pMM1 system, did not have a significant effect on the yield of released AmyL (data not shown).

This poses the question as to why *B. subtilis* possesses a cell-wall-associated protease that limits the yield of secretory proteins. Protein secretion is an essential cellular process for bacteria, and blockages of the secretion apparatus have potentially lethal consequences (13, 18). One role for a cell-associated protease such as CWBP52 could be to authenticate secreted proteins and to clear slowly folding or misfolded proteins from the vicinity of the translocation complex. It may also be important that highly expressed secretory proteins do not attach inappropriately to the cell wall, restricting the activity of cell wall-active proteins or the passage of other molecules into or out of the cell.

It is likely that native *B. subtilis* secretory proteins have co-evolved with the secretion apparatus to avoid potentially undesirable activities of cell-associated proteases such as CWBP52. AmyL itself is not native to *B. subtilis*, and the extent of its observed degradation (~75% of the total synthesized during the pulse) may reflect this fact. Certain chimeric secretory proteins expressed in *E. coli* are also subject to proteolysis at a late stage in the secretion process (6), and a periplasmic protease, DegP, has been shown to be involved in degradation of abnormal periplasmic proteins in this organism (32, 33). Therefore, it appears that mechanisms operate in both *E. coli* and *B. subtilis* to ensure the fidelity of the secretion process, although in the latter case such proteases would need to be anchored in the cell wall or the membrane as a consequence of the different cell envelope architecture.

We were not able to determine the individual roles of CWBP52 and CWBP23 in the degradation of AmyL. However, previous data suggests that the serine protease activity can be solely attributed to CWBP52 (21). CWBP23 shows some sequence homology to eukaryotic protease modulators, and it is possible that this protein regulates the activity of CWBP52 in a manner analogous to the modifer protein (LytD) of the *B. subtilis* N-acetylglucosaminyl-t-alanine amidase (LytC) (12, 16, 20).

In summary, this study has identified one role for the products of the *B. subtilis* *wprA* gene. Proteolysis of AmyL was still observed in cells not induced for *wprA*, suggesting either that there was a small amount of *wprA* expression from the *P* _spac_ promoter under noninducing conditions or that other, not yet identified proteases are also involved. However, a null *wprA* mutant produces α-amylase at a level comparable to that for the inducible *wprA* strain in the absence of induction (data not shown), suggesting that the level of proteolysis observed is not due to readthrough but to another protease. Although the cellular location of CWBP52 and CWBP23 is within the wall cylinder, it is reasonable to assume that integral membrane or membrane-linked proteases could have similar effects on secretory proteins and contribute to lower yields of released protein. These data have important implications for the use of the *B. subtilis* and other members of the genus as hosts for the secretion of native and nonnative *Bacillus* proteins. Cell-associated and truly extracellular proteases contribute to low yields of secretory proteins, and, for the reasons discussed above, nonnative proteins would be expected to be affected to a greater extent than native proteins which have co-evolved with the *Bacillus* secretion apparatus.

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