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Quantitation of the Capacity of the Secretion Apparatus and Requirement for PrsA in Growth and Secretion of α-Amylase in *Bacillus subtilis*

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Regulated expression of AmyQ α-amylase of *Bacillus amyloliquefaciens* was used to examine the capacity of the protein secretion apparatus of *B. subtilis*. One *B. subtilis* cell was found to secrete maximally 10 fg of AmyQ per h. The signal peptidase SipT limits the rate of processing of the signal peptide. Another limit is set by PrsA lipoprotein. The wild-type level of PrsA was found to be 2×10^4 molecules per cell. Decreasing the cellular level of PrsA did not decrease the capacity of the protein translocation or signal peptide processing steps but dramatically affected secretion in a posttranslocational step. There was a linear correlation between the number of cellular PrsA molecules and the number of secreted AmyQ molecules over a wide range of *prsA* and *amyQ* expression levels. Significantly, even when *amyQ* was expressed at low levels, overproduction of PrsA enhanced its secretion. The finding is consistent with a reversible interaction between PrsA and AmyQ. The high cellular level of PrsA suggests a chaperone-like function. PrsA was also found to be essential for the viability of *B. subtilis*. Drastic depletion of PrsA resulted in altered cellular morphology and ultimately in cell death.

Proteins synthesized with a signal peptide are secreted from bacterial cells by the action of the protein secretion apparatus, which consists of several components involved in protein targeting, translocation, signal peptide processing, and posttranslocational folding (4, 7). Extensive studies of *Escherichia coli* and *Bacillus subtilis* have identified and characterized to a substantial extent the components of the apparatus that translocates secretory proteins across the cytoplasmic membrane. In *B. subtilis*, they include the SecY, SecE, and SecG proteins, which form the core of the translocation channel or translocator (30, 47) and are associated in the membrane with the SecDF protein (3). Furthermore, there are several signal peptidases (43, 45). The role of SecA ATPase on the *cis* side of the membrane in targeting and coupling the energy required for translocation has been well established (15, 48). Many components of the secretion apparatus are known to be under temporal control; their maximal level of expression parallels the onset of protein secretion in the early stationary growth phase (15, 43).

The stages of protein secretion that take place outside the cytoplasmic membrane are less well understood. A central feature of secretion is posttranslocational folding. The correct folding of many secreted proteins is not spontaneous but dependent on assisting folding factors. In *E. coli* they include protein-specific chaperones, periplasmic peptidyl-prolyl *cis/trans* isomerases, and enzymes (Dsb proteins) involved in the formation and rearrangement of disulfide bonds (8, 16, 19, 31, 32). The depletion of foldases such as SurA and PpiD causes misfolding stress that activates the σ^E- and *cpx*-dependent stress response (5, 6, 31). This results in the induction of expression of the periplasmic protease and foldases (6, 37), often resulting in the degradation of misfolded and other abnormal proteins in the extracytoplasmic compartment of the cell.

In the gram-positive bacterium *B. subtilis*, only one protein outside the cytoplasmic membrane, PrsA, is known to be involved in protein secretion. PrsA is a lipoprotein that consists of a 33-kDa lysine-rich protein part and the N-terminal cysteine with a thiol-linked diacylglycerol anchoring the protein to the outer leaflet of the cytoplasmic membrane (21, 23, 28). The PrsA protein is crucial for efficient secretion of a number of exoproteins. In *prsA* mutants, the secretion and stability of some model proteins is decreased, while overproduction of PrsA enhances the secretion of exoproteins engineered to be expressed at a high level (18, 23, 28). Although the nature of the PrsA protein hints at an activity outside the cytoplasmic membrane, its mode of action and interaction with other components of the secretion apparatus and the specific steps(s) of secretion in which it is involved remain to be elucidated.

High-level expression of secretory proteins can saturate the secretion apparatus in *B. subtilis*. In strains overexpressing the α-amylase of *B. amyloliquefaciens* (AmyQ) or of *B. licheniformis* (AmyL), there is cell-associated accumulation of precursors with uncleaved signal peptide (pre-AmyQ and pre-AmyL), either associated with the cytoplasmic membrane or in the cytoplasm (15, 22). There were no cell-associated precursors of overexpressed levansucrase or α-amylase (AmyE) of *B. subtilis*, but processed, mature protein accumulated with slow release from the cell, indicating a rate-limiting step for these proteins after translocation and cleavage of the signal peptide (26).

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TABLE 1. Plasmids used in this study

Plasmid	Characteristics	Reference or source
pDG148	Ap^r Km^r, P_{spac}	42
pGDL100	Km^r, contains replication functions of the *Lactococcus lactis* WG2 plasmid pWVO1, encodes pre-(A13i)-β-lactamase and *sipT*	43
pKTH10	Km^r, pUB110 derivative carrying the α-amylase gene (*amyQ*) of *B. amyloliquefaciens*	35
pKTH277	Cm^r Em^r, pHP13 derivative carrying the *prsA* gene	21
pKTH1601	Ap^r Tet^r, Cm^r, pJH101 derivative carrying the *amyQ* gene and a 0.9-kb fragment of the *ywlG* region of the *B. subtilis* chromosome	20
pKTH3326	Em^r Cm^r, pE194 derivative carrying a *prsA* deletion cassette	This study
pKTH3327	pDG148 derivative with the *prsA* gene placed under P_{spac} control	This study
pKTH3339	pSX50 derivative for expression of xylose-inducible *amyQ*	This study
pKTH3362	pKTH1601 derivative carrying the *prsA* gene	This study
pKTH3384	pMUTIN4 derivative with a 0.3-kb 5′ fragment of the *prsA* gene	This study
pMUTIN4	Ap^r Em^r, integrable vector	46
pSX50	Cm^r, P_{xyn}	14

High-level expression of AmyQ also resulted in cell-associated accumulation of mature α-amylase in addition to the precursor (22). However, quantitative studies on the capacity of the bacterial protein secretion apparatus are few (2), and there are no published data on *B. subtilis*. Furthermore, it is not known how the translocator complex interacts with the components involved in the later stages of secretion, including posttranslocational folding; the contribution of these two stages to the overall secretion capacity remains unclear.

In this work, we first determined the maximal capacity of the wild-type secretion apparatus of *B. subtilis* in terms of translocation and the processing of pre-AmyQ. The PrsA protein was found to act independently of the translocator complex, its activity being confined to the cellular compartment outside the cell membrane. The rate of AmyQ secretion was dependent on the cellular level of PrsA in a manner consistent with a mechanism affecting folding of AmyQ. The necessity of PrsA for viability was established, suggesting that the role of PrsA in the folding of extracytoplasmic proteins is not restricted to exoproteins.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. Plasmids and *B. subtilis* strains are listed in Tables 1 and 2, respectively. Bacteria were grown in L broth, modified 2× L broth (2% tryptone, 1% yeast extract, 1% NaCl), and Spizizen's minimal salts medium. Modified 2× L broth was supplemented with 2% starch when AmyQ secretion was studied. For plasmid maintenance, chloramphenicol (5 μg/ml), kanamycin (10 μg/ml), erythromycin (1 or 100 μg/ml), or ampicillin (100 μg/ml) was added to the culture media.

DNA manipulation and strain constructions. To construct plasmid pKTH3327, in which the expression of *prsA* is under the control of the *spac* promoter (P_{spac}), a fragment containing the ribosome-binding site, the coding region, and the transcription termination site of the *prsA* gene was amplified by PCR and then cloned into the *Hin*dIII site of pDG148 (42). The PCR primers were 5′-CTCC ACAAGCTTGGAATGATTAGGAGTGTT-3′ and 5′-CTCCACAAGCTTG CAGTTCTCAGCAGCATG-3′, and the template was the DNA of pKTH277 (21). The chromosomal *prsA* gene was made controllable by using pMUTIN4 (46). A 0.3-kb fragment of *prsA* containing the ribosome-binding site and part of the coding region was amplified with primers 5′-CTCCACAAGCTTGGAA TGATTAGGAGTGTT-3′ and 5′-GCGGATCCCGAGGGCAGTATATTG-3′, and the obtained fragment was cloned between *Hin*dIII and *Bam*HI sites of pMUTIN4. One plasmid with the *prsA* fragment, pKTH3384, was transformed into *B. subtilis* strain 168. The plasmid was integrated into the *prsA* locus by a Campbell-type mechanism, and the expected construct, which placed the *prsA* gene under P_{spac}, was confirmed by Southern blotting. To disrupt the chromosomal *prsA* gene, we constructed a DNA cassette containing a fragment from the 5′ flanking region of *prsA*, the chloramphenicol acetyltransferase gene (*cat*) of pC194, and a fragment from the 3′ flanking region of *prsA*. The fragments of the 5′ and 3′ regions were 524 and 500 bp and were 154 nucleotides upstream and 76 nucleotides downstream from the coding region of the *prsA* gene, respectively. Plasmid pKTH3326, which carries the cassette, was linearized with *Bcl*I and transformed into strain IH6973 by selecting for chloramphenicol resistance. One transformant, which was Cm^r but Em^s and shown by PCR and Southern hybridization to have the *prsA* gene replaced with the *cat* gene, was named IH7075.

Strain IH7163 was constructed as follows. The 1.7-kb *Eco*RI-*Bam*HI fragment of pKTH3327 containing the P_{spac}-controlled *prsA* (P_{spac}-*prsA*) and P_{penP}-*lacI* genes were inserted between the respective sites of pKTH1601 to construct pKTH3362. pKTH3362 also carries a 0.9-kb fragment of the *ywlG* region of the *B. subtilis* chromosome (24), enabling integration of the plasmid into the chromosome by a Campbell-type mechanism (10, 11, 20). A *prsA3* mutant, IH6525, was transformed with pKTH3362 for Cm^r to obtain IH7118, and isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent expression of P_{spac}-*prsA* and complementation of the *prsA3* mutation was confirmed. Then a *glyB* auxotrophic strain, IH6538, was transformed with the chromosome of IH7118 to introduce the IPTG-inducible *prsA* gene into this strain. The strain obtained, IH7120, was made AmyQ secreting by transformation with pKTH10. Finally, the native *prsA* gene was disrupted by transformation with the chromosome of IH7075 (*prsA*::*cat*) and selecting for *glyB*$^+$. The *prsA*::*cat* marker of IH7075 is cotransformed with the *glyB* marker at a high frequency (about 40%). IPTG dependency of AmyQ secretion revealed recombinants of *prsA*::*cat*, one of which was named IH7163. Strain IH7136 harbors plasmid pKTH3327 (P_{spac}-*prsA*) and one copy of the *amyQ* gene integrated in the chromosome (*ywlG* locus). To construct IH7162, the chromosomal *prsA* gene of IH7136 was disrupted by transformation with the DNA of IH7075 as above.

In pKTH3339, the *amyQ* gene was placed under P_{xyn} control by inserting a PCR fragment (template pKTH10) containing the ribosome-binding site, the coding region, and the transcription termination site of *amyQ* between *Cla*I and *Sal*I sites of the expression vector pSX50 (14).

Quantitative immunoblotting. Cells were harvested from 1 ml of culture by centrifugation and resuspended in 100 μl of protoplast buffer (20 mM potassium phosphate [pH 7.5], 15 mM $MgCl_2$, 20% sucrose, 1 mg of lysozyme/ml). The cell suspension was incubated for 15 min at 37°C; 25 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (63 mM Tris-HCl [pH 6.5], 2% SDS, 5% β-mercaptoethanol, 9% glycerol) was added to the cell suspension and to 0.1 ml of culture medium prior to boiling for 10 min and the separation of proteins by SDS-PAGE using the Mini-Protean II electro-

TABLE 2. *B. subtilis* strains used in this study

Strain	Characteristics	Reference or source
IH6525	*prsA3 hisA1 trpC2* (pKTH10)	22
IH6538	*glyB133 hisA1 trpC2*	21
IH6961	*glyB133* (::pKTH1601)	S. Leskelä[a]
IH6973	Low protease (total) *sacA321* (pKTH3327)	This study
IH7075	IH6973 *prsA*::*cat* (pKTH3327)	This study
IH7118	IH6525(::pKTH3362)	This study
IH7120	IH6538(::pKTH3362)	This study
IH7136	IH6538(::pKTH1601, pKTH3327)	This study
IH7138	IH6538(::pKTH3362, pKTH10)	This study
IH7171	*prsA3 glyB133 hisA1*	H.-L. Hyyryläinen[a]
IH7162	IH7136 *prsA*::*cat*	This study
IH7163	IH7138 *prsA*::*cat*	This study
IH7211	168(::pKTH3384)	This study
IH7441	IH6538(pKTH3339)	This study
IH7558	IH7171(pKTH3339)	This study
IH7673	IH6538(::pKTH3384, pKTH3339)	This study
IH7983	IH7441(pGDL100)	This study
168	*trpC2*	1A1 in BGSC[b]

[a] National Public Health Institute, Helsinki, Finland.
[b] BGSC, Bacillus Genetic Stock Center, Columbus, Ohio.

phoresis system (Bio-Rad). Proteins were transferred to an Immobilon-P membrane (Millipore) in a Mini Trans-Blot cell (Bio-Rad) and visualized by specific antisera, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin IgG (Bio-Rad), and ECL (enhanced chemiluminescence) immunoblotting detection reagents (Amersham). The membrane was placed on Hyperfilm-βmax (Amersham), and densities of protein bands were measured quantitatively by optical scanning with Bio Image (Milligen/Biosearch).

Determination of the specific secretion rate and number of AmyQ and PrsA molecules. In all gels subjected to quantitative immunoblotting, a series of dilutions of purified AmyQ or PrsA protein with a known amount was applied in addition to the cell samples to be analyzed. Integrated optical densities of protein bands of AmyQ and PrsA visualized by ECL immunodetection were obtained by optical scanning. Amounts of the proteins in the cell samples were calculated from a standard curve drawn with data for the standards. The specific secretion rates of AmyQ were obtained by first plotting α-amylase concentrations in the medium versus time of incubation. Tangents of the resulting curves yielded the secretion rates, which were then divided by cell densities to obtain the specific secretion rates. Cell densities were determined by microscopically counting cells with a Petroff-Hausser counting chamber. The specific secretion rates were expressed as femtograms per hour per cell. The number of AmyQ molecules in the culture medium and PrsA molecules in the cell were calculated using the molecular weights 54,820 and 30,640 for AmyQ and PrsA, respectively.

Protease accessibility of pre-AmyQ in protoplasts. The expression of P_{xyn}-*amyQ* in IH7441(pKTH3339) and IH7558(*prsA3*, pKTH3339) was induced at a cell density of 100 Klett units (Klett 100) with 0.2% xylose. Cells were collected after 1 h of induction by centrifugation (2,500 × *g*, 10 min) and resuspended in 1 ml of protoplast buffer (20 mM potassium phosphate [pH 7.5], 15 mM $MgCl_2$, 20% sucrose, 1 mg of lysozyme/ml) (fivefold concentrated). After incubation at 37°C for 30 min, the formed protoplasts were centrifuged at 2,500 × *g* for 5 min, and the pellet was resuspended in 1 ml of protoplast buffer. The resuspended protoplasts were then divided into three portions. One portion was diluted by adding only protoplast buffer (twofold-concentrated preparation). Another was similarly diluted with protoplast buffer containing 1 mg of trypsin/ml. The protoplasts in the third portion were solubilized in 2% Triton X-100 and then diluted with trypsin-containing protoplast buffer. These three protoplast preparations were incubated at 37°C for 30 min, after which trypsin inhibitor (final concentration, 1.2 mg/ml) was added. The trypsin-treated and nontreated protoplasts were then centrifuged at 5,000 × *g* for 2 min. The pelleted protoplasts were solubilized in SDS-PAGE sample buffer and then boiled for 10 min. The contents of AmyQ, PrsA, and GroEL (a cytoplasmic protein marker) in the samples (and in the trypsin-treated Triton X-100-solubilized sample) were analyzed by immunoblotting with specific antibodies.

α-Amylase assay. A sample of culture medium was appropriately diluted and pipetted into 1 ml of α-amylase assay buffer (50 mM morpholineethanesulfonic acid [MES; pH 6.0], 50 mM NaCl, 0.1 mM $CaCl_2$), one-fourth of a Phadebas tablet (Pharmacia) was dispersed in the buffer, and the mixture was incubated for 1 h at 37°C. The reaction was stopped by adding 30 μl of 10 M NaOH. The mixture was then filtered through Whatman no. 1 filter paper. The intensity of the blue color in the filtrate released from the Phadebas tablet by the action of α-amylase was measured at 616 to 624 nm, using 800 to 804 nm as a reference wavelength range. Enzyme concentrations (in micrograms per milliliter) were calculated from a standard curve using purified α-amylase from *B. amyloliquefaciens* (Sigma).

Microscopy of cells depleted of PrsA. IH7211 was grown in modified 2× L broth supplemented with 1 mM IPTG to a cell density of Klett 100. Cells were harvested and stored in 10% glycerol at −70°C. For microscopy of cells with low levels of PrsA, the frozen bacteria were thawed, washed, and used to inoculate 10 ml of the above medium (1/2,000 dilution) containing different concentrations of IPTG. The cultures were incubated with shaking for 7 h, and then a drop was spread on a slide, air dried, and fixed by heating. Gram staining of the cells was done by the Hucker method as described in reference 9. Cells were photographed through an Axiophot photomicroscope (Zeiss).

RESULTS

Capacity of the secretion apparatus of *B. subtilis*. Expression of a secretory protein at a high level causes saturation of the protein secretion apparatus (27). By using a xylose-inducible expression system, we determined the threshold of saturation of the secretion apparatus for AmyQ α-amylase. In strain IH7441, *amyQ* has been placed under P_{xyn} control in plasmid pKTH3339. P_{xyn}-*amyQ* was induced in rich medium at the exponential growth phase. Steady-state levels of secreted AmyQ, cell-associated pre-AmyQ, and mature AmyQ were measured by quantitative immunoblotting. The amount of AmyQ in the culture medium increased in a xylose concentration-dependent manner but only up to 0.04%, at which point it leveled off (Fig. 1A and B). However, the total amount of AmyQ increased up to the highest xylose concentration (0.2%); high xylose concentrations caused concentration-dependent accumulation of pre-AmyQ in the cells (Fig. 1A and B). At 0.2% xylose, the precursor constituted approximately 74% of the total amount

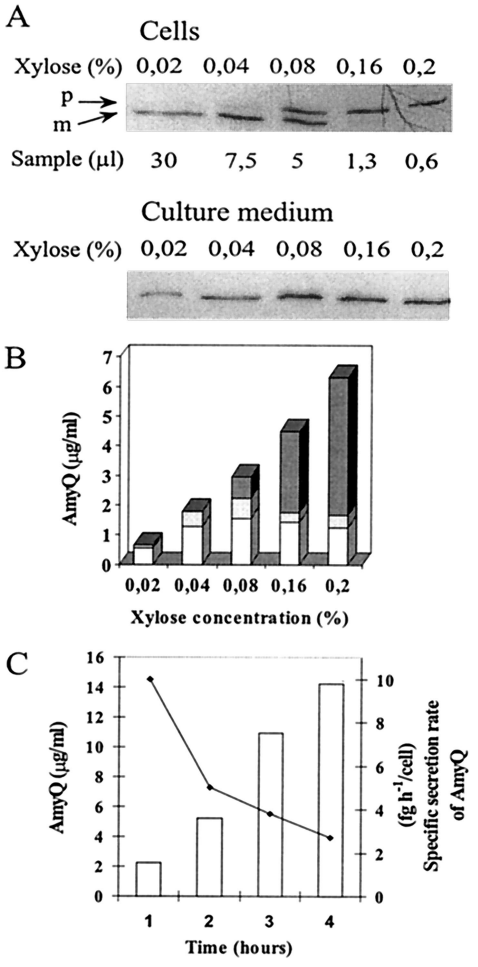

FIG. 1. Saturation of the secretion apparatus of *B. subtilis* by overexpressing AmyQ. (A) IH7441 (P_{xyn}-*amyQ*) was grown in modified 2× L broth up to a cell density of Klett 100, at which point *amyQ* gene expression was induced by adding xylose (concentrations were as indicated). After 1 h of induction, cell and medium fractions were separated and AmyQ in the fractions was analyzed by ECL immunoblotting. Cell samples corresponding to the indicated volumes of culture and medium samples corresponding to 10 μl of culture were immunoblotted. Precursor (p) and mature (m) forms of AmyQ are indicated by arrows. (B) The protein bands in panel A were quantified by optical scanning. For quantitation of the cell-associated mature AmyQ in cultures containing 0.16 or 0.2% xylose, another immunoblot with higher sample volumes was used. Columns show the content of secreted (white), cell-associated mature (light grey), and precursor (dark grey) forms of AmyQ in the cultures. (C) Accumulation of AmyQ in the culture medium of IH7441 (bars) induced with 0.04% xylose and specific secretion rate (line). The horizontal axis shows time after Klett 100.

of AmyQ synthesized (Fig. 1B), while at 0.04% there was hardly any pre-AmyQ despite a peak level of secretion (Fig. 1A and B). The results indicate a clearcut threshold level of AmyQ synthesis above which the secretion apparatus is saturated.

We determined the specific secretion rate of AmyQ in the exponential growth phase under conditions in which the secretion apparatus was already saturated but no precursor had yet accumulated. At a cell density of Klett 100, 0.04% xylose was added and samples were taken at hourly intervals for measurements of α-amylase activity and determination of cell densities by microscopy. The specific secretion rate was 10 fg h^{-1}/cell 1 h after the addition of xylose and decreased to 2.5 fg h^{-1}/cell during the following 3 h. The concentration of AmyQ in the culture medium increased from 2 to 14 μg/ml during the same time period (Fig. 1C).

PrsA deficiency does not affect the cell-associated accumulation of precursors of a secretory protein. Our previous studies showed that PrsA is involved in a late stage of protein secretion (18, 21, 23, 28) but did not pinpoint the stage in the context of translocation. We have now addressed this by studying the nature and location of precursors of a secretory protein accumulating in the cell when PrsA is depleted. The model protein was again the α-amylase expressed by P_{xyn}-*amyQ* (pKTH3339). To control the expression of *prsA*, it was placed under the P_{spac} control by integrating the plasmid pMUTIN into the chromosome. The strain containing P_{spac}-*prsA* and pKTH3339 was designated IH7673. The amounts of PrsA and the cell-associated and secreted AmyQ were assayed by immunoblotting. The growth rates of this strain were similar at IPTG concentrations of between 24 μM and 1 mM (see below) and thus independent of the level of PrsA protein expressed in this range of induction.

Maximal induction of the P_{spac}-*prsA* gene was achieved in the presence of 1 mM IPTG. At this inducer concentration, an abundant band of PrsA protein was detected by immunoblotting; the PrsA level was close to that in the wild-type cells (data not shown), and was not affected by the expression level of P_{xyn}-*amyQ* (from 0.02 to 0.2% xylose [Fig. 2, top right]). Similar amounts of secreted AmyQ were found in the culture medium at inducer levels of 0.02% and higher (Fig. 2, middle), indicating that under these growth conditions the secretion apparatus was already saturated at the lowest *amyQ* expression level studied. Again pre-AmyQ clearly accumulated in the cells in the same xylose-dependent manner (Fig. 2, bottom) as observed in $prsA^+$ cells (Fig. 1). A small amount of pre-AmyQ and a putative degradation product were also detected in culture media that were supplemented with high concentrations of the inducer (0.08 to 0.2% xylose), presumably due to secretion stress and cell lysis (Fig. 2, middle). Thus, consistent with the results obtained when PrsA was expressed from the native gene, there was a block in the secretion and expression level-dependent accumulation of pre-AmyQ.

When expression of the P_{spac}-*prsA* gene was induced with 24 μM IPTG, a low level of PrsA protein was detectable in an immunoblot assay. Again, the protein level was hardly dependent on the expression of P_{xyn}-*amyQ* (Fig. 2A, top left). Although the growth rate was not affected, the cells formed long filaments (see below). Even at this low level of PrsA, the amount and pattern of cell-associated AmyQ proteins were quite similar to those detected in the cells containing a high

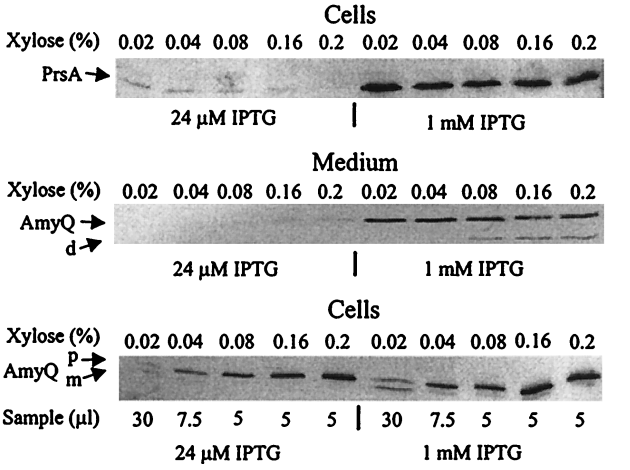

FIG. 2. Decreased levels of PrsA protein affect only a late stage of secretion. Saturation of the secretion apparatus by overexpressed AmyQ in cells with a low level of PrsA was determined. IH7673 (P_{spac}-*prsA* P_{xyn}-*amyQ*) was grown in modified 2× L broth supplemented either with 24 μM or 1 mM IPTG. At a cell density of Klett 100, expression of P_{xyn}-*amyQ* was induced as described in the legend to Fig. 1. Cell and medium fractions were prepared, and PrsA and AmyQ content in the fractions was analyzed by ECL. The analyzed samples were from 40 μl (PrsA; top) or 7.5 μl (secreted AmyQ; middle) of culture, or as indicated (cell-associated AmyQ; bottom). Precursor (p) and mature (m) forms and degradation product (d) are indicated by arrows.

level of PrsA (Fig. 2, bottom). In particular, ratios of cell-associated pre-AmyQ to processed AmyQ were similar in both cases; the drastic accumulation of pre-AmyQ began at the level of AmyQ expression obtained at 0.04% xylose. However, there was strikingly little accumulation of AmyQ in culture medium when the PrsA level was low (Fig. 2, middle).

The accumulated pre-AmyQ is primarily exposed on the outer surface of protoplasts. To identify the rate-limiting step in the secretion of overexpressed AmyQ, we used protease accessibility in protoplasts to determine the cellular location of the accumulated precursor. We also compared the protease accessibility of pre-AmyQ in protoplasts of a *prsA* mutant (*prsA3* [23]) and its wild-type parent to investigate whether PrsA deficiency increases the proportion of intracellular pre-AmyQ, although it did not affect the pre-AmyQ/AmyQ ratio or the threshold of saturation. The *prsA3* mutation causes the change of Asp_{268} to Asn_{268} near the C terminus of PrsA.

IH7441(pKTH3339) and IH7558(*prsA3*, pKTH3339) were grown in modified 2× L broth, and P_{xyn}-*amyQ* of pKTH3339 was induced at the cell density of Klett 100 with 0.2% xylose to express pre-AmyQ at a level sufficient to saturate the secretion apparatus. After 1 h of induction, cells were harvested and protoplasts were prepared (see Materials and Methods). The protoplasts were either not treated or treated with trypsin (1 mg/ml) or with trypsin and Triton X-100 (2%), followed by measurement of the levels of pre-AmyQ, GroEL, and PrsA by immunoblotting. The level of GroEL, a trypsin-sensitive cytoplasmic protein, was determined to monitor protoplast lysis during trypsin treatment. The presence of inverted membrane vesicles in the protoplast preparations was estimated by the degree of degradation of PrsA, which is a lipoprotein and thus is located on the outer surface of the cell membrane.

GroEL was almost completely protected from degradation

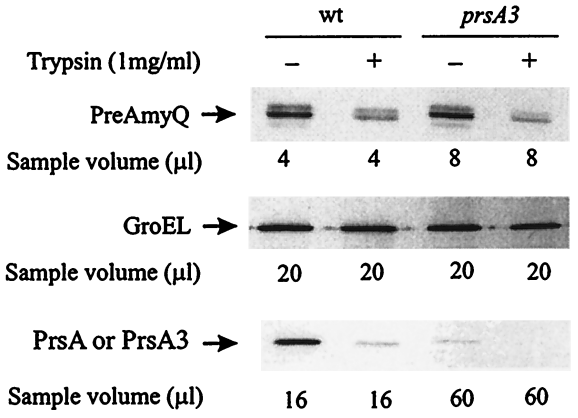

FIG. 3. Accessibility of pre-AmyQ in protoplasts to external trypsin. IH7441(pKTH3339) and IH7558(*prsA3*, pKTH3339) were induced with 0.2% xylose to express P_{xyn}-*amyQ* at a level that saturates the secretion apparatus. Protoplasts were prepared under osmotic protection and treated with trypsin. The level of pre-AmyQ was analyzed by quantitative immunoblotting. The levels of GroEL and PrsA were also analyzed to assess the degree of protoplast lysis and the presence of inverted membrane vesicles, respectively. The analyzed samples correspond to the indicated volumes of original cultures. wt, wild type.

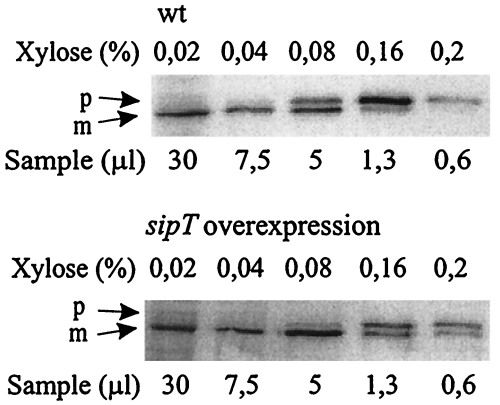

FIG. 4. Effect of *sipT* overexpression on the threshold of saturation of the secretion apparatus. The wild-type (wt) strain IH7441(pKTH3339) and its derivative strain harboring plasmid pGDL100 with the *sipT* gene were cultivated as described in the legend to Fig. 1A, and the expression of P_{xyn}-*amyQ* was induced with different concentrations of xylose as indicated. The levels of cellular pre-AmyQ and AmyQ were analyzed by immunoblotting. Precursor (p) and mature (m) forms of AmyQ are indicated by arrows.

in both strains, indicating that the protoplasts were intact, as shown in Fig. 3. The protoplast preparation of wild-type cells was also nearly devoid of inverted membrane vesicles, as indicated by almost complete degradation of PrsA (Fig. 3). The level of PrsA3 protein in the *prsA3* mutant is less than 10% of that in the wild type (Fig. 3) (23), due to proteolytic degradation during posttranslocational folding by some unknown membrane- or cell wall-associated protease(s). Interestingly, approximately 70% of the pre-AmyQ was degraded after trypsin treatment of protoplasts of both strains (Fig. 3). No traces of pre-AmyQ or GroEL were detected when protoplasts were lysed with Triton X-100 (not shown). These findings suggest that the major rate-limiting step is in a posttranslocational phase of secretion and that PrsA deficiency does not increase the level of intracellular pre-AmyQ.

The rate of signal peptide processing limits the secretion of overexpressed AmyQ. The above result suggested that a posttranslocational step is rate limiting in the secretion of overexpressed AmyQ. To test if this step is the processing of the signal peptide, we overexpressed *sipT*, encoding the main signal peptidase responsible for the processing of pre-AmyQ (44), in IH7983(pGDL100, pKTH3339) and carried out the saturation analysis as for Fig. 1.

The results (Fig. 4) showed that in IH7983, the threshold of saturation of the secretion apparatus clearly was at a higher expression level of P_{xyn}-*amyQ* (0.16% xylose) than in the strain with a wild-type level of SipT (0.08% xylose). At the highest expression levels (0.16 and 0.2% xylose), the proportion of mature AmyQ in cells of the SipT overproducer was significantly higher than in those of the wild type. Thus, SipT indeed is one rate-limiting factor in the secretion of AmyQ.

Stoichiometric requirement of PrsA protein for extracellular accumulation of overexpressed α-amylase. To relate the rate of α-amylase secretion to the number of PrsA molecules in the cell, we constructed strain IH7163. It secretes constitutively α-amylase at a level saturating the secretion machinery, and its *prsA* has been placed under the P_{spac} control (see Table 1 and Materials and Methods). IH7163 was cultivated in the presence of different concentrations of IPTG (0 to 1,000 μM); samples were withdrawn in late exponential growth phase (3 h after the culture had reached the turbidity of Klett 100). This growth phase was chosen in order to obtain high concentrations of AmyQ in the medium while avoiding the accumulation of extracellular proteases (22). The cell density was determined by a direct cell count. Quantitation of PrsA and secreted AmyQ was performed by immunoblotting. PrsA protein was barely detectable in uninduced cultures (Fig. 5), whereas full induction of the P_{spac}-*prsA* gene (100 μM IPTG) resulted in a

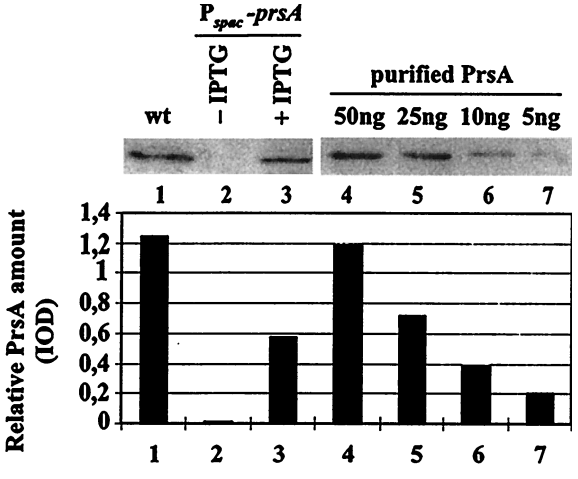

FIG. 5. Quantitation of expression of P_{spac}-*prsA*. Whole-cell samples were prepared from late-exponential-phase cultures of IH7163 (P_{spac}-*prsA*) grown in the presence or absence of 100 μM IPTG and of IH6538, an isogenic wild-type (wt) strain. Samples corresponding to 30 μl of the cultures were subjected to SDS-PAGE (12% gel), and PrsA was analyzed by ECL immunoblotting with anti-PrsA serum. PrsA was quantitated by optical scanning using purified PrsA as a standard. The lower panel shows the integrated optical densities (IOD) of the bands in the upper panel.

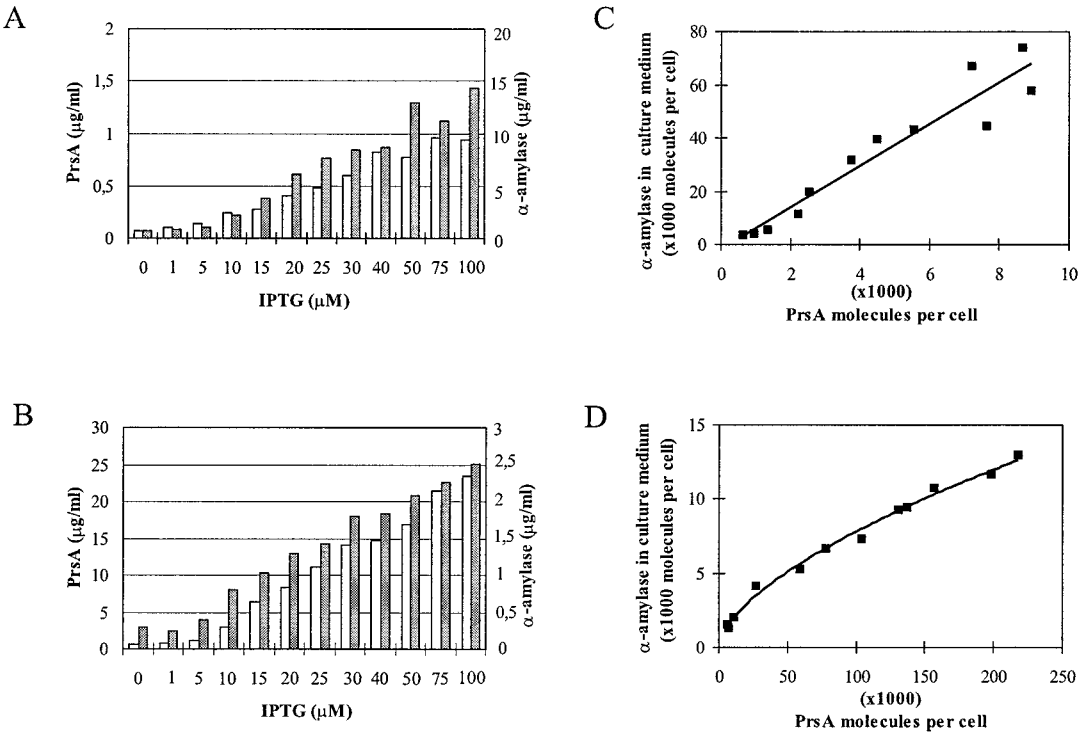

FIG. 6. Dependence of AmyQ secretion on the content of PrsA protein in the cell. Strains IH7163(::pKTH3362, pKTH10) (A and C) and IH7162(::pKTH1601, pKTH3327) (B and D) were grown to late exponential phase in the presence of various concentrations of IPTG. The cellular level of PrsA (white bars) was determined as described in the legend to Fig. 5. Secreted α-amylase (grey bars) was determined enzymatically from the culture medium. Values represent the means of triplicate determinations. Numbers of PrsA and AmyQ molecules (C and D) per cell were calculated using the data in panels A and B, and cell densities of the cultures were determined by counting with a Petroff-Hausser counting chamber.

more than 10-fold enhancement of the PrsA level (Fig. 5). This was about 40% of the level observed in the wild-type cultures (Fig. 5), indicating that P_{spac} is a weaker promoter than the *prsA* gene's own promoter.

In fully induced cultures, there was about 10 μg of secreted α-amylase per ml (Fig. 6A), which is comparable to the level found in cultures with a massive cell-associated accumulation of pre-AmyQ (Fig. 1C). At intermediate concentrations of IPTG, the amylase level in the medium was dependent on the inducer concentration up to 50 μM (Fig. 6A). Under these conditions, there was a linear correlation between the number of PrsA molecules in the cell and the number of AmyQ molecules in the culture medium; a twofold increase in the number of PrsA molecules caused nearly a twofold increase in the number of secreted AmyQ molecules (Fig. 6C).

Molar excess of PrsA stimulates the secretion of α-amylase even when expressed at a low level. The observations described above show that AmyQ secretion is stoichiometrically dependent on PrsA when the cell expresses AmyQ at a high level. To examine whether such dependence was simply due to the limiting threshold level of PrsA, we also studied the secretion of α-amylase in the reverse situation. We constructed strain IH7162, which overexpresses PrsA from the P_{spac}-*prsA* gene in plasmid pKTH3327 and expresses AmyQ at a low level from a single copy of *amyQ* integrated in the chromosome.

In the culture maximally induced with IPTG (100 μM), the level of PrsA protein was about 10-fold higher than in the wild type (data not shown) and thus about 20-fold higher than in IH7163, in which there is only one copy of the P_{spac}-*prsA* gene (Fig. 5 and 6A). Although expression of the chromosomal *amyQ* gene was far below the level required to cause saturation of the secretion apparatus in the experiment illustrated in Fig. 1C (Fig. 6B; compare also Fig. 6A and B), an almost linear dependence of AmyQ secretion on the level of PrsA was seen up to 2.2×10^5 PrsA molecules per cell (Fig. 6D). Furthermore, there was no indication that the AmyQ accumulation was leveling off even at highest amounts of PrsA obtainable under the experimental conditions used.

PrsA is essential for viability of *B. subtilis*. Our previous attempts to disrupt the *prsA* gene by Campbell-type integration were unsuccessful, suggesting that this gene is essential for the viability of *Bacillus* cells (23). In this study, we endeavored to demonstrate directly that the functional *prsA* gene is indispensable. Since the uninduced level of expression from P_{spac} on pMUTIN4 (46) is very low, this plasmid can be used to examine whether or not a gene is essential for growth.

The strain containing P_{spac}-*prsA* (IH7211) was grown on L plates supplemented with 1 mM IPTG. Bacteria from one colony were suspended in 1 ml of sterile water, and the viable count was determined by plating a series of diluted samples onto plates with or without the inducer. Cells that were induced to express the P_{spac}-*prsA* gene formed colonies that appeared to be identical to those of the wild type; the viable count was 3×10^7/ml (Table 3). In the absence of inducer, however, the viable count was only 8×10^3/ml, and the colonies were heterogeneous in size; most were very small. The

TABLE 3. Dependence of growth of *B. subtilis* on expression of *prsA*

Dilution	Colonies/plate[a]	
	+IPTG	−IPTG[b]
10^{-1}	Confluent	27
10^{-2}	Confluent	8
10^{-3}	2×10^3	1
10^{-4}	1.7×10^2	0
10^{-5}	27	0
10^{-6}	3	0

[a] One colony of IH7211 (P_{spac}-*prsA*) from an L plate supplemented with 1 mM IPTG was suspended in 1 ml of water and diluted in water as indicated; 100-μl samples were spread on L plates (no induction of P_{spac}-*prsA*) or L plates supplemented with 1 mM IPTG (P_{spac}-*prsA* induced). Plates were incubated for 20 h at 37°C, and raised colonies were counted.

[b] Colonies on these plates displayed heterogeneous morphology.

particular colonial morphology was maintained in subcultures. Bacteria that were able to grow on the noninducing plates appear to contain spontaneous suppressor mutations, which allow some, albeit impaired, growth, even in the absence of PrsA (17).

To determine the lowest level of PrsA protein able to maintain normal growth, we cultivated IH7211 in L broth supplemented with different concentrations of IPTG and examined growth and PrsA content in the culture with the lowest IPTG concentration that supported normal growth. IPTG concentrations of 16 μM or lower did not induce *prsA* expression sufficient for normal growth (Fig. 7A); at 2 μM or lower, practically no growth was observed. However, growth was not impaired in the presence of 24 μM IPTG (Fig. 7A).

The morphological changes in cells with low levels of PrsA were also studied. When growth was only slightly inhibited (16 μM IPTG), cells formed long filaments (compare Fig. 7B and C) with swelling at the ends of some of the filaments. The growth abnormalities observed at 8 μM IPTG were more severe, the cells becoming enlarged and spherical (Fig. 7D). Such cells appeared to be quite fragile, as indicated by the cell debris seen in Fig. 7D.

DISCUSSION

The objective of this investigation was to characterize the relationship of the PrsA protein with the translocase complex,

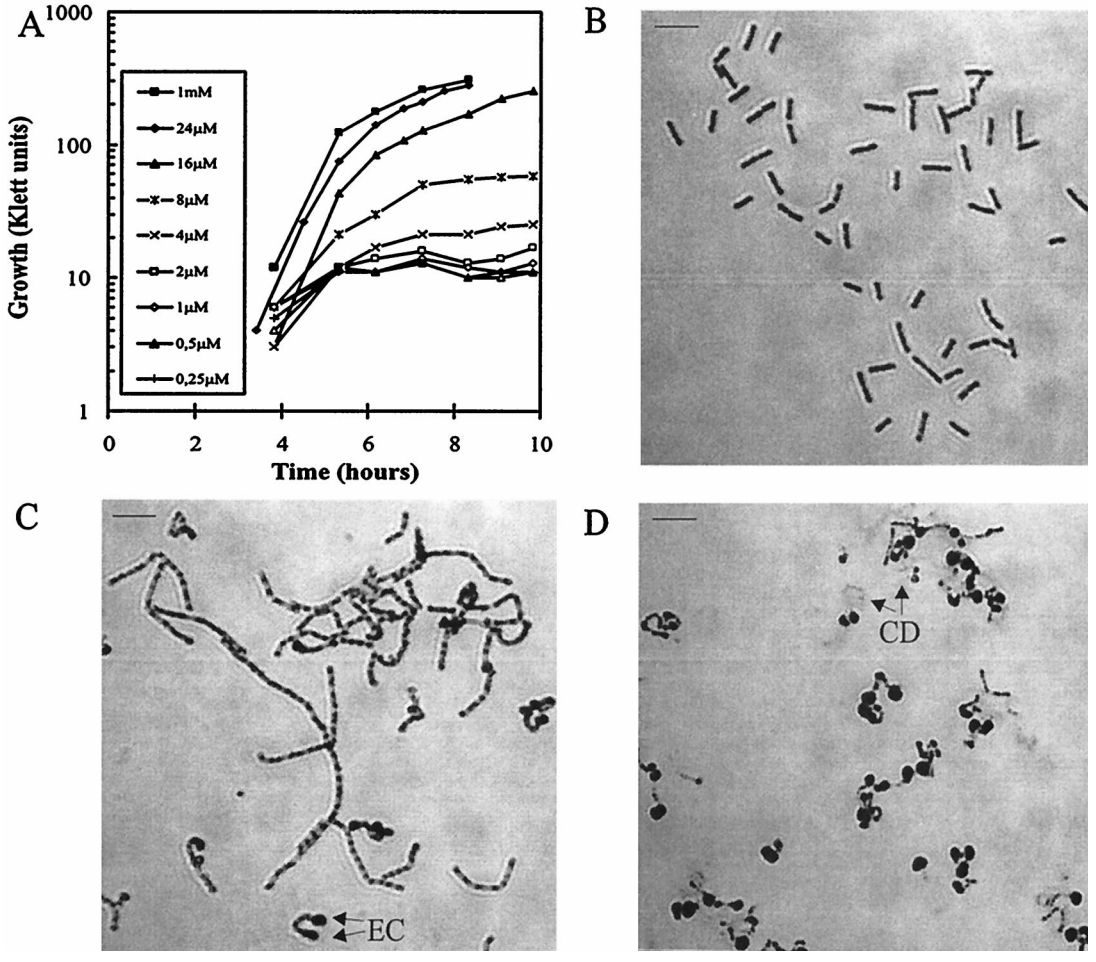

FIG. 7. Morphological changes in cells depleted of PrsA. (A) Growth of IH7211 (P_{spac}-*prsA*) in the presence of different concentrations of IPTG. Photomicrographs show filament formation and cell shape of IH7211 grown in the presence of 32 μM IPTG (B), 16 μM IPTG (C), or 8 μM IPTG (D). Cells were Gram stained for microscopic examination. EC and CD denote enlarged cell and cell debris, respectively. The scale bars represent 8 μm.

its mode of action, its interaction with secreted proteins, and its role in cell viability.

We showed that PrsA is a highly abundant protein in *B. subtilis*. As measured by an immunoblot assay, PrsA protein is present at approximately 2×10^4 molecules per wild-type cell, a level clearly higher than that of most other membrane proteins. Estimates of the levels of different membrane components of the preprotein translocase (Sec) in *E. coli* have given values from fewer than 30 to 900 molecules per cell (33, 36). Similar quantitation has not been carried out with the corresponding Sec components of *B. subtilis*, but if their cellular levels are even approximately similar to those in *E. coli*, PrsA protein is indeed in high molar excess compared to the translocase complex. It thus seems unlikely that the PrsA protein is associated firmly and in a stoichiometric way with the translocase complex. The enhanced secretion in strains overproducing PrsA at up to 2×10^5 molecules per cell also argues against a stoichiometric relationship.

The high number of PrsA molecules, its lipoprotein nature, and possible free lateral movement in the membrane may ensure that enough PrsA molecules are available to interact with translocated secreted protein molecules even without specific association with the translocase complex.

What could be the mode of action of PrsA? Several lines of consideration point to a role in the posttranslocational folding of secreted proteins. First, PrsA shows high sequence homology with the parvulin (PpiC) family of peptidyl-prolyl *cis*/*trans* isomerases (39, 41). One of them, SurA, is a major periplasmic foldase of *E. coli* assisting in the assembly of outer membrane proteins. Second, there is sequence homology in the whole length of PrsA with the lactococcal PrtM lipoprotein, a dedicated folding factor of the PrtP exoprotease (12, 13, 21). Third, there is extensive cell-associated degradation of some secreted model proteins when the level of functional PrsA is low (18, 28), consistent with the depletion of a folding factor. However, the mode of action of PrsA as well as the steps of the secretion pathway where it interacts with the process of secretion are not known.

Experiments with PrsA depletion now show that the PrsA protein is not involved in translocation or in the processing of secreted proteins. We could demonstrate the saturation of the secretion apparatus in terms of the cell-associated accumulation of pre-AmyQ when a threshold level of expression was increased. Neither the threshold level nor the pattern of cell-associated AmyQ (ratio of pre-AmyQ to processed AmyQ) was affected by the depletion of PrsA. This result is consistent with a similar finding that a defect in the PrsA protein does not disturb the rate of processing of the signal peptide of pre-AmyQ as analyzed by pulse-chase labeling (28). Moreover, we have also now demonstrated that PrsA deficiency does not increase the proportion of intracellular pre-AmyQ. However, there was a decreased amount of AmyQ secreted into the culture medium. This would be consistent with the involvement of the PrsA protein in a posttranslocational stage of secretion and with the degradation of secreted proteins after their translocation when PrsA is deficient. Depletion of the PrsA protein also showed that the secretion of AmyQ was linearly dependent on the cellular level of PrsA under conditions where AmyQ was expressed at a level high enough to saturate the secretion apparatus and the number of PrsA molecules in the cell was below that of the wild type (between 500 and 8,000 molecules per cell). This finding is consistent with our previous studies of *prsA* point mutants and strains that overexpress PrsA, pointing to PrsA as a bottleneck for the secretion of highly expressed exoproteins (23). Strikingly, however, there was also a dependence of AmyQ secretion on the level of PrsA protein when AmyQ was expressed at a low level. This stimulation was not linear, but even at the highest achievable level of PrsA (2.2×10^5 molecules per cell), secretion did not level off. These findings suggest a mode of action for PrsA that involves reversible association and disassociation of PrsA with α-amylase, compatible both with chaperone and foldase-like modes of activity.

The dependence of AmyQ secretion on PrsA, even when there was clearly a huge (about 1,000-fold) excess of the number of PrsA molecules over those of translocase complexes could also mean that only a fraction of PrsA molecules are readily available at the site where posttranslocational folding takes place. Thus, the number of PrsA molecules adjacent to the translocase could be limiting in spite of an apparent large overall excess. Alternatively, such an excess may be advantageous for the cell under some growth conditions, as has been demonstrated in the case of the DivIB cell division protein: much higher levels of DivIB are needed for normal growth at 47°C than at 30°C (40). Our findings also suggest that overexpression of PrsA is beneficial not only in biotechnical applications in which a secreted protein saturates the secretion apparatus (23) but also at lower levels of expression. This might be of special importance for the production of valuable heterologous proteins when the prime goal is to minimize degradation and ensure correct folding of the product. To determine the secretion capacity of *B. subtilis*, we studied the secretion of α-amylase expressed from an inducible promoter. In a cell with wild-type levels of all components of the secretion apparatus, the secretion of α-amylase into the culture medium was limited to 10 fg h^{-1}/cell. At this secretion level, one cell secreted about 30 AmyQ molecules per s. Estimating that there are approximately 300 translocases in one cell, a new AmyQ molecule is released from the secretion pathway every 10 s, in perfect agreement with the estimated translocation rate of individual precursor proteins in *E. coli* (2). However, we found that in *B. subtilis* translocation is not a rate-limiting step in the secretion of AmyQ. Most of the pre-AmyQ which accumulated in the cell when overexpressed was at least partly translocated, as indicated by its accessibility in protoplasts to degradation by external trypsin.

Our results showed that there are in *B. subtilis* at least two factors the amount of which limits the secretion of overexpressed AmyQ, PrsA and SipT. The availability of SipT limits the rate of the signal peptide cleavage. The overexpression of *sipT*, however, does not enhance the accumulation of AmyQ in the growth medium (data not shown), although it enhances the processing. The availability of PrsA most probably limits the secretion rate to 10 fg h^{-1}/cell, since concentrations of PrsA below or above the wild-type level can significantly restrict or enhance, respectively, AmyQ secretion, most probably by affecting posttranslocational folding (above results and reference 23).

To examine the effects of decreased levels of PrsA protein on *B. subtilis* viability, we constructed a controllable *prsA* gene.

Immunoblotting showed that when the level of PrsA protein in the cells was decreased to below 1% (about 200 molecules per cell) of that in the wild type, cells stopped dividing and their morphology changed strikingly. About 200 molecules per cell still supported normal growth, but at this PrsA level, the cells formed long filaments. When the PrsA level was decreased further, cells became distorted (enlarged and spherical) and finally lysed. The morphological changes correlated well with drastically decreased plating efficiency of the strain on media devoid of IPTG.

The morphological changes in cells resulting from PrsA depletion closely resemble those observed when the synthesis of cell wall polymers is deficient. There is both filamental growth and cell lysis in mutants deficient in the synthetic pathway of peptidoglycan (1) or if synthesis is inhibited by β-lactamases (34, 38). Abnormal spherical cells have been observed when the synthesis of wall teichoic acids (polyglycerophosphate) is impaired (25, 29). The synthesis of this cell wall polymer is also essential for cell growth (29). This may indicate a role of PrsA in the folding or activity of enzymes in the synthetic pathway of cell wall polymers or their interaction with their substrates.

Notably, there were also stable revertants that formed colonies of various morphologies when PrsA was depleted; most colonies were pinpoint in size. This raises also the interesting possibility that some of the revertants have been caused by mutations in genes other than *prsA*. Indeed, we have observed that the growth inhibition caused by PrsA depletion can be partially suppressed by targeted mutations in genes affecting the charge of anionic cell wall polymers (17).

PrsA is the only known extracytoplasmic folding factor in *B. subtilis*. The *B. subtilis* genome sequence has revealed another putative member of the parvulin family, YacD, with similarity to PrsA. This protein, however, is nonessential for viability and secretion (O. Tunnela, unpublished result). The absolute requirement for PrsA raises the question of whether PrsA homoloques of pathogenic gram-positive bacteria could be used as new targets for antibiotic design, especially considering its extracytoplasmic location.

Our results, although suggesting interactions between PrsA and secreted proteins, cannot rule out completely the possibility of other effects, such as those mediated by the inhibition of unbalanced and harmful activity of cell wall or membrane-associated proteases by PrsA. To fully understand the function of PrsA, it will be important to develop methods for an in vitro assay of PrsA activity, to examine the structure-function relationships of PrsA, and to identify its putative interactions with proteins of the cytoplasmic membrane or the cell wall and with components of the peptidoglycan-teichoic acid matrix.

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