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Evaluation of Bottlenecks in the Late Stages of Protein Secretion in *Bacillus subtilis*

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Despite a high capacity for secretion of homologous proteins, the secretion of heterologous proteins by *Bacillus subtilis* is frequently inefficient. In the present studies, we have investigated and compared bottlenecks in the secretion of four heterologous proteins: *Bacillus lichenifomis* α-amylase (AmyL), *Escherichia coli* TEM β-lactamase (Bla), human pancreatic α-amylase (HPA), and a lysozyme-specific single-chain antibody. The same expression and secretion signals were used for all four of these proteins. Notably, all identified bottlenecks relate to late stages in secretion, following translocation of the preproteins across the cytoplasmic membrane. These bottlenecks include processing by signal peptidase, passage through the cell wall, and degradation in the wall and growth medium. Strikingly, all translocated HPA was misfolded, its stability depending on the formation of disulfide bonds. This suggests that the disulfide bond oxidoreductases of *B. subtilis* cannot form the disulfide bonds in HPA correctly. As the secretion bottlenecks differed for each heterologous protein tested, it is anticipated that the efficient secretion of particular groups of heterologous proteins with the same secretion bottlenecks will require the engineering of specifically optimized host strains.

Bacillus subtilis and related bacilli are attractive hosts for the production and secretion of heterologous proteins. First, these gram-positive eubacteria secrete proteins directly into the growth medium, which greatly facilitates their downstream processing. Second, these organisms have a huge capacity for protein secretion. For example, *B. subtilis* has been reported to secrete the *Bacillus amyloliquefaciens* α-amylase (23) or the *Staphylococcus aureus* protein A (9) to gram-per-liter concentrations in the growth medium. Third, *B. subtilis* is a genetically highly amenable host organisms for which a large variety of genetic tools have been developed (11) and which is well known with respect to fermentation technology. Fourth, *B. subtilis* has a transparent genome, because its complete sequence is known (18). Finally, *B. subtilis* is nonpathogenic and free of endotoxins. Notwithstanding these advantages, the secretion of various heterologous proteins by bacilli, in particular proteins of eukaryotic origin, is frequently inefficient, which limits the application potential of these organisms (for reviews, see references 22 and 30).

Various bottlenecks for protein secretion in *B. subtilis* have been identified in recent years. Such bottlenecks are related to both the properties of the secreted protein and the machinery for protein secretion. Most secreted proteins are synthesized as precursors with an amino-terminal signal peptide, which is required for their targeting to the preprotein translocase in the cytoplasmic membrane (7, 26, 41). During or shortly after translocation of the preprotein across the membrane, the signal peptide is removed by signal peptidases (SPases), which is a prerequisite for release of the mature protein from the membrane (for a recent review, see reference 5). Five paralogous chromosomally encoded type I SPases have been identified in *B. subtilis*, two of which, designated SipS and SipT, are of major importance for the processing of secretory preproteins (35, 36).

Thus far, five potential bottlenecks in the secretion pathway of *B. subtilis* have been documented. First, heterologous proteins may form insoluble aggregates in the cytoplasm due to limited activity of chaperones (43). Second, the SPase SipS can be a limiting factor in preprotein processing (2, 40). Third, it has been shown that the folding catalyst PrsA, which is attached to the extracytoplasmic side of the membrane by lipid modification, sets a limit to the high-level secretion of certain secretory proteins (17). Fourth, it has been suggested that the cell wall forms a barrier for at least one secreted heterologous protein, human serum albumin (28). Fifth, it has been known for a long time that *B. subtilis* secretes large amounts of proteases into the medium, which can degrade secreted heterologous proteins (22, 30). Recent studies suggest that not only the secreted proteases but also cell-associated proteases are responsible for the degradation of secreted heterologous proteins (21, 34).

Secretion bottlenecks relating to the secreted protein are presently poorly defined. Therefore, in the present studies, we have compared secretion bottlenecks of four different heterologous reporter proteins from eubacteria and eukaryotes (*Bacillus licheniformis* α-amylase [AmyL], *Escherichia coli* TEM β-lactamase [Bla], human pancreatic α-amylase [HPA], and a single-chain antibody against lysozyme [SCA-Lys]), using the same expression and secretion signals. The results show that different stages in secretion, following translocation across the membrane, determine the secretion efficiency of each of these reporter proteins.

MATERIALS AND METHODS

Plasmids, bacterial strains, and media. Table 1 lists the plasmids and bacterial strains used. Tryptone-yeast extract medium contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). Minimal medium for *B. subtilis* was prepared as previously described (36). S7 media 1 and 3, used for labeling of *B. subtilis* proteins with [^{35}S]methionine (Amersham), were prepared as described

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TABLE 1. Plasmids and bacterial strains

Plasmid or strain	Properties or genotype	Source or reference
Plasmids		
pGA14	pWVO1 replicon; contains SPO2 promoter; encodes *B. licheniformis* α-amylase (AmyL) without signal peptide; Em^r	24
pS-A2	As pGA14; encodes pre-A2-AmyL; Em^r	This work
pSB-A2	As pGA14; encodes pre-A2-Bla; Em^r	This work
pSB-A2d	As pGA14; encodes pre-A2d-Bla; Em^r	This work
pGDL51	As pGA14; encodes pre-A2d-Bla; carries the *sipS* gene of *B. subtilis* and the kanamycin resistance marker from pKM1; Em^s Km^r	This work
pGDL52	As pGDL51, lacks the *sipS* gene; Em^r Km^r	This work
pKM1	pUC7 derivative; Ap^r Km^r	14
M13mp10HPA	Phage M13mp10 carrying HPA gene	42
pSH-A2	As pGA14; encodes A2-HPA; Em^r	This work
pSH-A13	As pGA14; encodes A13-HPA; Em^r	This work
pUR4129	Plasmid containing SCA-Lys gene	Unilever Research Laboratories
pGA2Lys	As pGA14; encodes A2-SCA-Lys; Em^r	This work
pGA13Lys	As pGA14; encodes A13-SCA-Lys; Em^r	This work
pKA13lys	As pGA13Lys; carries additional kanamycin resistance gene; Em^r Km^r	This work
pGLys	As pGA2Lys; lacks signal sequence A2; Em^r	This work
Strains		
8G5	*trpC2 tyr his nic ura rib met ade*	3
DB104	*his nprE aprE*	13
DB430	*his nprE aprE bpf isp1*	6
WB600	*trpC2 nprE nprB apr epr mpr bpr* Em^R	44

by van Dijl et al. (38). When required, media for *E. coli* were supplemented with ampicillin (100 μg/ml), kanamycin (20 μg/ml), or erythromycin (100 μg/ml); media for *B. subtilis* were supplemented with erythromycin (1 μg/ml) or kanamycin (10 μg/ml).

DNA techniques. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Sambrook et al. (27). Enzymes were from Boehringer Mannheim. *B. subtilis* was transformed as described by Tjalsma et al. (36). Residues 28 to 64 of signal sequence A2 were deleted by the gapped duplex DNA method for site-directed mutagenesis with the pMa/c phasmid vectors developed by Stanssens et al. (33) and the mutagenic primer IA2 (5′-TGCCGCCGCTGCGGTCTGACT CAGTTTTACTTGTAAATGGGA-3′). This resulted in signal sequence A2d. To obtain an HPA reporter gene cassette flanked by *Sma*I and *Sal*I restriction sites, a *Sma*I site was introduced at the 3′ end of the HPA signal sequence on M13mp10HPA by using the pMa/c phasmid vectors and the mutagenic primer HAI (5′-CCCTTATGACCCGGGTCGTCT-3′). A SCA-Lys reporter gene cassette, flanked by *Sal*I and *Hin*dIII restriction sites, was obtained by PCR with the oligonucleotides HT1 (5′-CTAGAGTCGACCGCCCAAGCCCAGGTGCAGC TGC-3′) and HT2 (5′-ATTGATAAGCTTACCGTTTGA TCTCGAGC-3′), using plasmid pUR4129 as a template.

Protein labeling, immunoprecipitation, SDS-PAGE, and fluorography. Pulse-chase labeling of *B. subtilis*, immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography were performed as described previously (38). To measure the kinetics of protein release into the medium, samples withdrawn at various intervals after the chase were immediately diluted in ice-cold medium. Next, cells and growth medium were separated by centrifugation. To inhibit the translocation ATPase activity of SecA, sodium azide (1.5 mM) was added to the cells 5 min prior to labeling (15). Experiments were repeated at least two times, with the variation in the observed percentages of preprotein processing or release being in the 10% range.

Western blot analysis. Western blotting was performed as described by Towbin et al. (37). Samples for SDS-PAGE were prepared as described by van Dijl et al. (38). After separation by SDS-PAGE, proteins were transferred to Immobilon polyvinylidene difluoride (Millipore Corporation) or nitrocellulose (BA 85; Schleicher and Schuell) membranes. The presence of SCA-Lys in the media of overnight cultures was monitored with antibodies raised against Fv-antilysozyme (Unilever), and subsequent visualization of the bound antibodies was achieved with anti-rabbit immunoglobulin G conjugates (Promega).

Enzyme activity assays. α-Amylase activity in culture supernatant was measured by using Starch Azure (Sigma) as described by Smith et al. (32). The presence of biologically active SCA-Lys in supernatants of overnight cultures was measured with a pin-enzyme-linked immunosorbent assay as recommended by the supplier (Collworth). Before the assay, 2 mM phenylmethylsulfonyl fluoride and 10 mM EDTA were added to the culture supernatants.

RESULTS

Processing, release into the medium, and degradation of YvcE fusion proteins. Signal sequence A2, which was randomly selected from the *B. subtilis* genome with a signal sequence selection vector based on AmyL (Fig. 1A) (31), was previously shown to direct the efficient secretion of AmyL and Bla by *B. subtilis* (32). With the availability of the complete sequence of the *B. subtilis* genome (18), signal sequence A2 was shown to correspond to the 5′ region of the *yvcE* gene, which is located at 305.3° on the genetic map (11a). *yvcE* is predicted to encode a protein of 473 residues that shows a high degree of similarity to the cell wall-attached protein P54 of *Enterococcus faecium*, a protein with unknown function that is very rich in serine and asparagine residues (10). Signal sequence A2 specifies the first 60 residues of YvcE (Fig. 1B), including a typical tripartite signal peptide, with a putative type I SPase cleavage site between residues 30 and 31 (Fig. 1B). SPase cleavage at this site was confirmed by amino-terminal sequencing of mature Bla and AmyL that were exported to the periplasm of *E. coli* or the growth medium of *B. subtilis*, respectively, by using signal sequence A2 (data not shown). Consequently, the mature parts of secretory proteins fused to signal peptide A2 contain an amino-terminal extension which corresponds to the first 30 residues of mature YvcE. Below we refer to the latter mature fusion proteins as A2-Bla and A2-AmyL.

The kinetics of secretion of A2-Bla were determined by pulse-chase labeling, sampling at various intervals after the chase, and subsequent separation of cells and growth medium. As shown in Fig. 2A and B, processing of pre-A2-Bla was very fast when cells were labeled at 37°C. Directly after a 1-min pulse with [^{35}S]methionine (time zero), ≈92% of the total amount of labeled A2-Bla was present in the mature form. Interestingly, compared to the fast processing of the precursor, the release of mature A2-Bla into the medium was delayed, as only ≈61% of the mature protein was detected in the medium

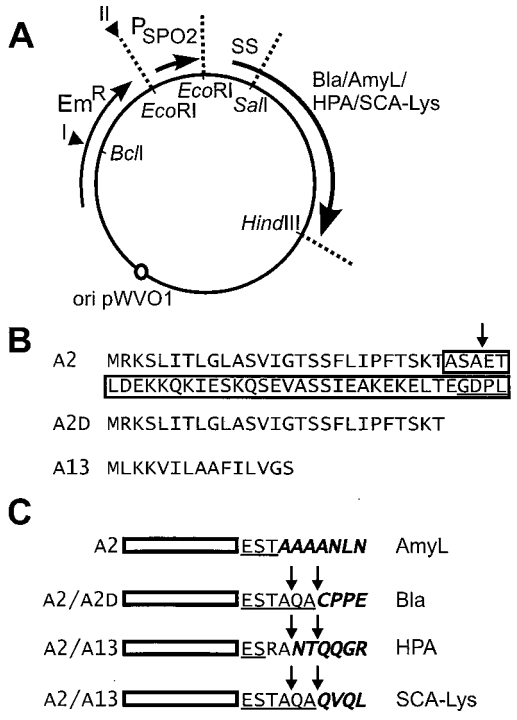

FIG. 1. Plasmids encoding hybrid precursors. (A) The plasmids used in this study contain four modules: (i) the replication functions of the broad-host-range lactococcal vector pWVO1 with an erythromycin resistance gene (Em^r), as described for plasmid pGA14 by Perez-Martinez et al. (24); (ii) the *B. subtilis* bacteriophage SPO2 promoter; (iii) a signal sequence (SS); and (iv) the coding sequences for mature TEM β-lactamase (Bla), *B. amyloliquefaciens* α-amylase (AmyL), HPA, or SCA-Lys. The restriction sites used for the constructions are indicated. Plasmid pGDL52 is based on the same modular structure, but it contains the *sipS* gene inserted at position I and a kanamycin resistance marker at position II. Similarly, plasmid pKA13Lys contains a kanamycin resistance marker at position II. (B) Sequences of the signal peptides A2, A2d (a deletion variant of A2), and A13. The residues that were deleted from signal peptide A2, resulting in signal peptide A2d, are boxed. The SPase cleavage site of signal peptide A2, as determined by amino-terminal sequencing of purified A2-Bla (from *E. coli*) and A2-AmyL (from *B. subtilis*), is indicated with an arrow. The residues at the point of fusion between the signal peptide and reporter protein are underlined. (C) Amino acid sequences at the fusion points (underlined) between the various signal peptides and secretory proteins. Residues of AmyL, Bla, HPA, and SCA-Lys are indicated in boldface italics. Putative SPase cleavage sites are indicated with arrows. Pre-A2-Bla and pre-A2-AmyL were processed at the site indicated in panel B.

at time zero. As the rate of preprotein processing is temperature dependent, A2-Bla processing and release were also studied at 25°C. Consistent with the results obtained at 37°C, the delay between processing and release was even more pronounced when cells were incubated at 25°C; after 1 min of chase, ≈58% of the labeled A2-Bla was in the mature form, whereas only ≈8% of the mature A2-Bla was released into the growth medium (Fig. 2C and D). Interestingly, a difference of about 3 min was observed between the time point at which 50% of the pre-A2-Bla molecules were processed and the time point at which 50% of the mature molecules were released into the medium. This suggests that cell wall passage of a mature A2-Bla molecule takes about 3 min. Qualitatively similar results were obtained for A2-AmyL. Also in this case, processing of the precursor to the mature form was fast. In fact, no pre-A2-AmyL could be detected in pulse-chase experiments performed at 37°C (Fig. 2E and F). Furthermore, the release of mature A2-AmyL was slow compared to the processing of pre-A2-AmyL, in particular when cells were labeled at 25°C (Fig. 2G and H); after 5 min of chase, only ≈10% of the total amount of labeled A2-AmyL was detected in the medium, whereas ≈85% was in the mature form. Interestingly, at 25°C, A2-AmyL was released into the medium at a much lower rate than A2-Bla, whereas pre-A2-AmyL was processed much faster than pre-A2-Bla.

For both A2-Bla and A2-AmyL, degradation products with increased mobility on SDS-PAGE were observed. Notably, degradation products of A2-Bla were observed exclusively in the growth medium (Fig. 2A and C), whereas degradation products of A2-AmyL were observed both in the cellular fraction and in the medium (Fig. 2E and G). These observations indicate that A2-Bla is subject to degradation by proteases in the growth medium, whereas A2-AmyL is degraded both by cell-associated and secreted proteases. This degradation is not due to the major secreted proteases of *B. subtilis*, subtilisin (AprE) and neutral protease (NprE), as shown with the *aprE nprE* double mutant strain DB104 (only the results for A2-Bla are documented in Fig. 2A to D). Strikingly, the site of A2-AmyL degradation strongly depended on the growth temperature. At 37°C, A2-AmyL was prone to degradation in the growth medium. In contrast, at 25°C, A2-AmyL was degraded while it was still cell associated.

SPase limits the release of A2d-Bla into the medium. As a first approach to study the relationships between processing, release into the medium, and degradation of hybrid preproteins containing signal peptide A2, residues 28 to 64 of signal peptide A2 were deleted. The resulting signal peptide, designated A2d, lacks the original SPase cleavage site of YvcE plus the highly charged amino-terminal region of YvcE, which contains 10 acidic and 6 basic residues (Fig. 1B). Alternative SPase cleavage sites were specified by the sequences at the point of fusion between signal sequence A2d and the *bla* gene (Fig. 1C). As determined by β-lactamase activity assays, the deletion in signal peptide A2 had no effect on the total amount of β-lactamase secreted into the medium (data not shown). Next, the kinetics of processing of pre-A2d-Bla by SPase and release of the mature form into the medium were tested by pulse-chase labeling at 37 or 25°C. Irrespective of the temperature, processing of pre-A2d-Bla was slower than that of pre-A2-Bla (Fig. 2 and 3), but, interestingly, mature A2d-Bla was hardly detectable in the cells (Fig. 3). Similarly, mature A2d-AmyL was detectable only in the medium (data not shown). Thus, it seemed that translocation across the membrane or processing by SPase was the bottleneck for the secretion of A2d-Bla and A2d-AmyL. To discriminate between these possibilities, the SPase SipS was overproduced at least 20-fold (data not shown) by using plasmid pGDL51, which specifies both SipS and A2d-Bla. As shown in Fig. 4, the rate of processing of pre-A2d-Bla was indeed significantly increased by SipS overproduction; after 2 min of chase, ≈65% of the labeled A2d-Bla was mature in cells overproducing SipS, whereas only ≈35% of the labeled A2d-Bla was mature in cells producing wild-type levels of SipS. Even though the rate of processing was greatly increased by SipS overproduction, hardly any mature A2-Bla accumulated in the cell fraction (Fig. 4). Thus, by deletion of the original SPase cleavage site and the 34 amino-terminal residues of the mature YvcE from signal peptide A2, the bottleneck in the secretion of A2-Bla was shifted from release into the growth medium to processing by SPase.

The cell wall is a barrier for secretion of HPA. Secretion of HPA in an *aprE nprE* mutant *B. subtilis* strain was tested with signal peptide A2 or A13. Like A2, signal sequence A13 was randomly selected from the *B. subtilis* genome (31) and was shown to direct the efficient secretion of Bla and AmyL into the growth medium (32, 39). Genome sequencing has revealed

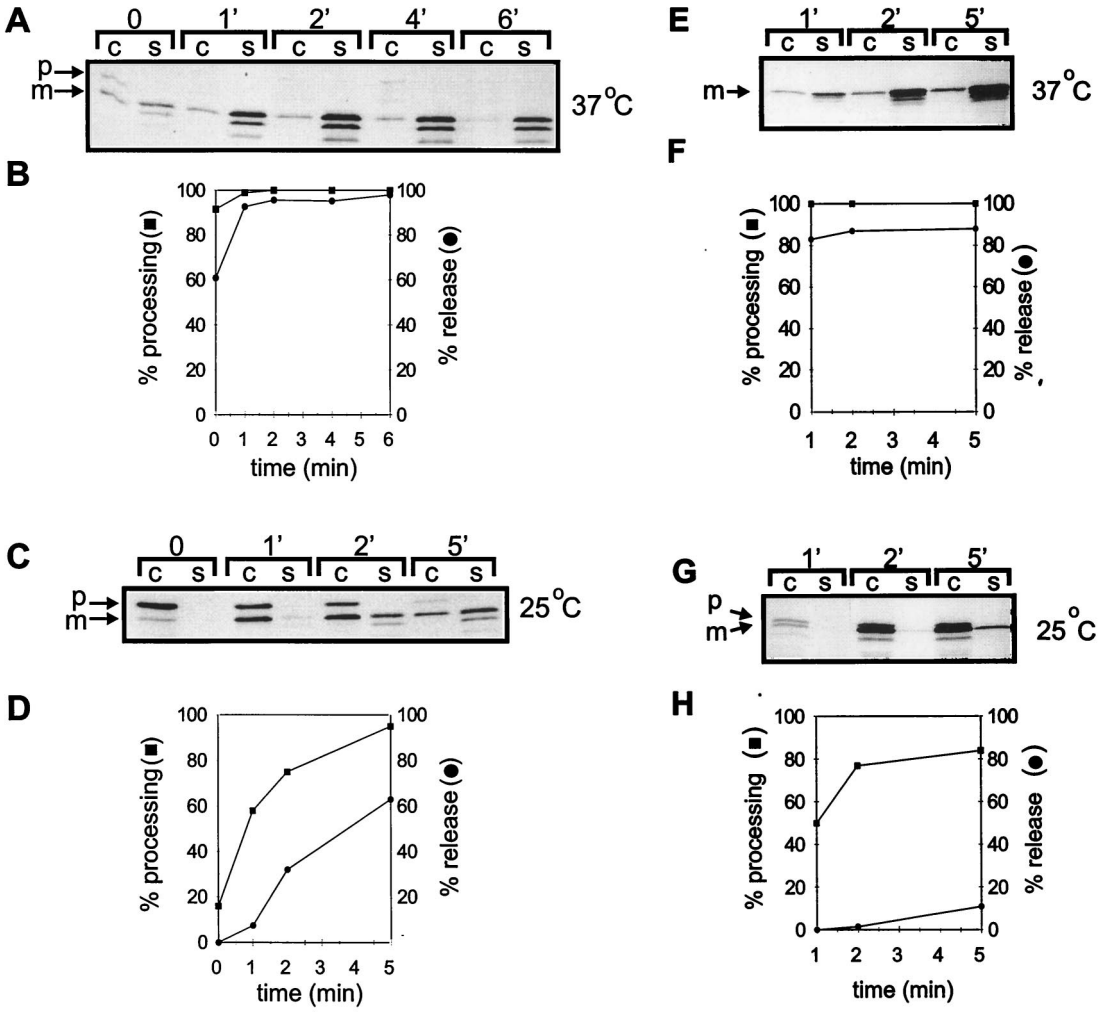

FIG. 2. Processing and release into the medium of A2-Bla and A2-AmyL. (A to D) Processing and release of A2-Bla into the medium by *B. subtilis* DB104(pSB-A2) were analyzed by pulse-chase labeling at 37°C (A and B) and 25°C (C and D) and subsequent immunoprecipitation, SDS-PAGE, and fluorography. Cells were labeled with [^{35}S]methionine for 1 min prior to a chase with excess nonradioactive methionine. Samples were withdrawn at the times indicated, and cells and medium were separated by centrifugation. c, cells; s, supernatant; p, pre-A2-Bla; m, mature A2-Bla. (B and D) Kinetics of precursor processing and release of the mature protein into the medium. Relative amounts of precursor and mature forms of secreted proteins were estimated by scanning of autoradiographs. The percentage of precursor processing (■) at each time point after the chase is calculated as the amount of mature protein in both cellular and supernatant fractions (including degradation products) divided by the total amount of labeled protein (precursor form plus mature form in cell and supernatant fractions). The percentage of release (●) is calculated as the amount of mature protein in the supernatant fraction divided by the total amount of labeled protein. (E to H) Processing and release of A2-AmyL into the medium by *B. subtilis* 8G5(pS-A2) were analyzed by pulse-chase labeling at 37°C (E and F) and 25°C (G and H) as described for A2-Bla. c, cells; s, supernatant; p, pre-A2-AmyL; m, mature A2-AmyL.

that the signal sequence A13 is derived from the *ydjM* gene, which is located at 58.0° on the genetic map (11a). YdjM is predicted to contain a typical signal peptide, with putative type I SPase cleavage sites between residues 21 and 22 or residues 28 and 29. Signal sequence A13 specifies the first 15 residues of YdjM, and alternative SPase cleavage sites are specified by sequences at the fusion point between A13 and the reporter genes used in the present studies (Fig. 1B). When fused to signal peptide A13, both Bla and AmyL were efficiently processed by SPase and secreted into the growth medium (data not shown).

As determined by enzyme activity assays and Western blotting, only very low levels of A2-HPA or A13-HPA accumulated in the growth medium (data not shown), suggesting that these hybrid proteins were poorly secreted and/or degraded in the growth medium. The latter possibility was investigated by expressing A2-HPA or A13-HPA in *B. subtilis* WB600, a strain lacking six extracellular proteases (AprE, Bpr, Epr, Mpr, NprB, and NprE) (44). However, no increase in the amounts of secreted HPA was observed (data not shown), suggesting that degradation in the growth medium by any of these proteases did not represent the major bottleneck in HPA secretion.

Pulse-chase labeling experiments were carried out to examine the synthesis and kinetics of processing of A2-HPA and A13-HPA. Both hybrid proteins were efficiently synthesized and processed to the mature form (Fig. 5A; only the results for A2-HPA are shown); immediately after the pulse (time zero), ≈91% of the labeled A2-HPA was mature. However, even after a chase of 1 h, no mature A2-HPA or A13-HPA was released into the medium (Fig. 5B). To verify that the observed maturation of A2-HPA was due to translocation across the membrane and subsequent processing by SPase, pulse-chase labeling experiments were carried out in which, prior to the labeling with [^{35}S]methionine, sodium azide was added to the

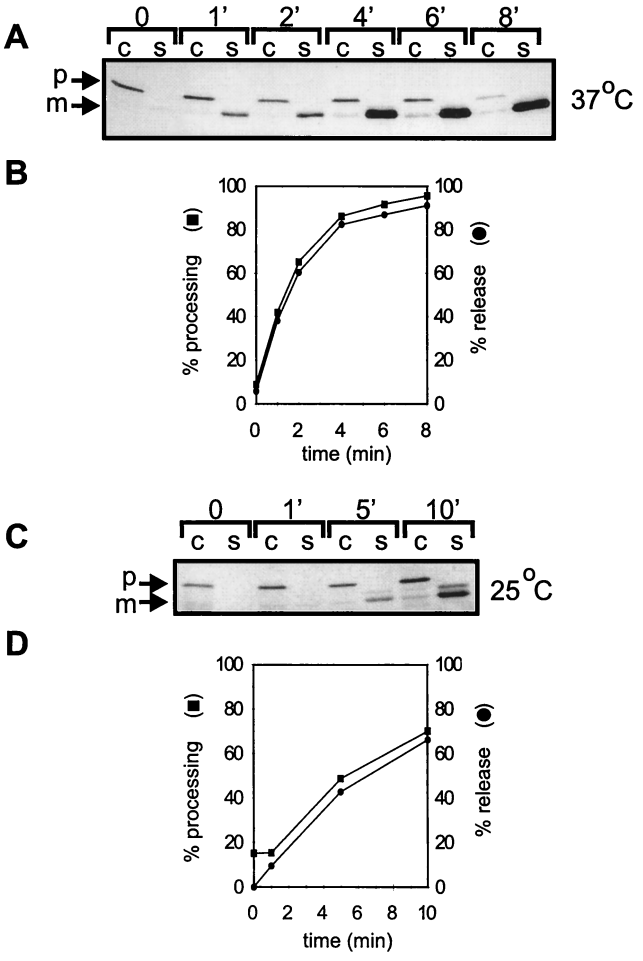

FIG. 3. Processing and release of A2d-Bla into the medium. Processing and release of A2d-Bla by *B. subtilis* DB104(pSB-A2d) were analyzed by pulse-chase labeling at 37°C (A and B) and 25°C (C and D) as described in the legend to Fig. 2. c, cells; s, supernatant; p, pre-A2d-Bla; m, mature A2d-Bla.

cells. Thus, the translocation ATPase activity of the SecA protein, which is the force generator for protein translocation across the membrane (8, 29), was blocked. Indeed, the addition of sodium azide strongly inhibited the processing of pre-A2-HPA (Fig. 5C), showing that the observed processing in the absence of sodium azide is translocation specific. Taken together, these findings suggested that translocated mature A2-HPA accumulated in the cell wall. To test this hypothesis, cells were treated with lysozyme, and subsequently the resulting protoplasts were separated from released cell wall components by centrifugation. As shown by Western blotting, ≈60% of the total amount of the A2-HPA was localized in the cell wall fraction (Fig. 5D). In each fraction the presence of GroEL, which was used as a cytoplasmic reporter for protoplast lysis,

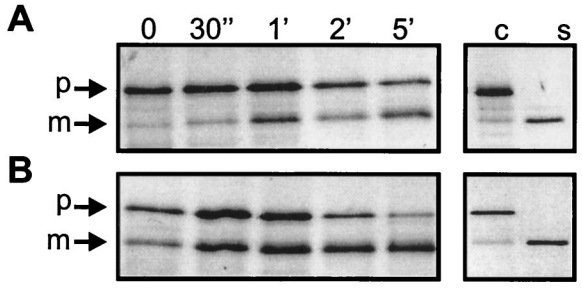

FIG. 4. Improved processing of pre-A2d-Bla by SipS overproduction. Processing and release of A2d-Bla in *B. subtilis* DB104(pGDL52) (no *sipS* gene) (A) and *B. subtilis* DB104(pGDL51) (carries the *sipS* gene) (B) were analyzed by pulse-chase labeling at 37°C. (Left panels) Samples which include cell-associated and secreted A2d-Bla; (right panels) cellular and medium fractions which were separated by centrifugation after a chase of 2 min. c, cells; s, supernatant; p, pre-A2-Bla; m, mature A2-Bla.

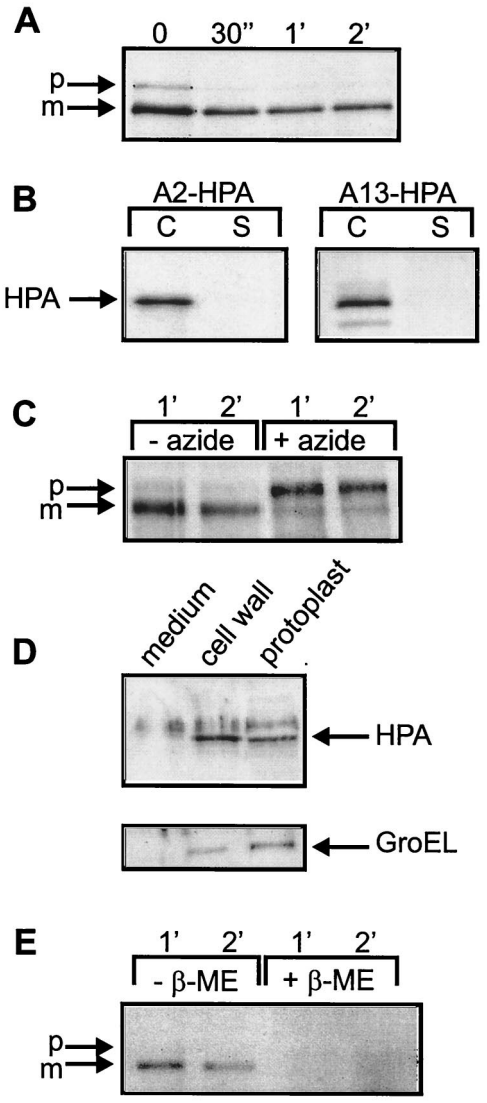

FIG. 5. Accumulation of mature A2-HPA in the cell wall. (A) Processing of pre-A2-HPA in *B. subtilis* DB104(pSH-A2) was analyzed by pulse-chase labeling at 37°C as described in the legend to Fig. 2. p, pre-A2-HPA; m, mature A2-HPA. (B) Cellular and medium fractions were separated by centrifugation after a chase of 1 h. C, cells; S, supernatant. (C) Processing of pre-A2-HPA in *B. subtilis* DB104 was analyzed by pulse-chase labeling at 37°C in the absence or presence of sodium azide (1.5 mM). (D) Cells of *B. subtilis* DB104 producing A2-HPA were grown overnight in TY medium. Cells were collected by centrifugation, resuspended in protoplast buffer (20% sucrose, 50 mM Tris-HCl [pH 7.5], 15 mM $MgCl_2$), and incubated in the presence of lysozyme (0.2 μg/ml) for 30 min at 37°C. Cell wall and protoplast fractions were separated by centrifugation. Samples were analyzed by SDS-PAGE and Western blotting. The samples were used to monitor HPA (upper panel) or GroEL (cytoplasmic control) (lower panel). (E) Pulse-chase labeling of *B. subtilis* DB104(pSH-A2) in the absence or presence of 5 mM β-mercatoethanol (β-ME).

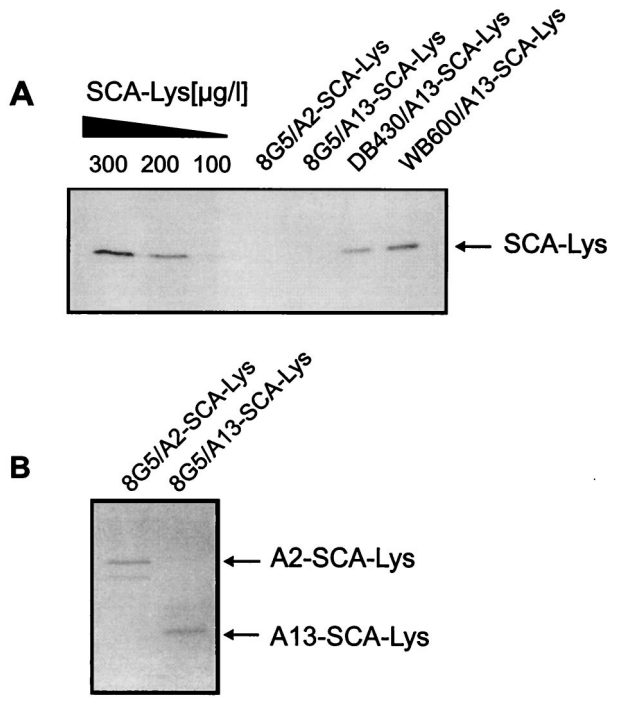

FIG. 6. Secretion of SCA-Lys by *B. subtilis*. (A) Secretion of A2-SCA-Lys and A13-SCA-Lys by cells of *B. subtilis* 8G5(pGA2Lys), 8G5(pGA13Lys), DB430(pGA13Lys), and WB600(pKA13Lys) grown overnight in tryptone-yeast extract medium. Cells were removed from the growth medium by centrifugation, and the medium fractions were analyzed by SDS-PAGE and Western blotting. The amount of secreted SCA-Lys was compared to that in SCA-Lys samples with known concentrations (300, 200, and 100 μg/liter), purified from *E. coli* and applied to the first three lanes, respectively. (B) Secretion of A2-SCA-Lys and A13-SCA-Lys by *B. subtilis* 8G5(pGA2Lys) and 8G5(pGA13Lys) was analyzed by pulse-labeling of exponentially growing cells for 2 min at 37°C. Labeled cells were removed from the growth medium by centrifugation, and secreted proteins were precipitated from the medium fraction with trichloroacetic acid at 0°C. Subsequently, the presence of A2-SCA-Lys or A13-SCA-Lys in the medium was analyzed by immunoprecipitation, SDS-PAGE, and fluorography. The presence of A2-SCA-Lys or A13-SCA-Lys in cell fractions could not be monitored due to high cross-reactivity of the anti-SCA-Lys antibodies with cellular proteins.

was monitored in parallel. From the results we conclude that fewer than 30% of cells had lysed during protoplasting (Fig. 5D), suggesting that a large fraction of translocated mature A2-HPA accumulated in the cell wall. This accumulation seems to depend on the formation of disulfide bonds, as no mature A2-HPA was detectable in cells treated with the reducing agent β-mercaptoethanol (Fig. 5E) at 5 mM, a concentration that is not inhibitory for growth of *B. subtilis* (data not shown).

Degradation of single-chain antibodies in the growth medium. To study the secretion of SCA-Lys by *B. subtilis*, the signal peptides A2 and A13 were used, and the accumulation of A2-SCA-Lys or A13-SCA-Lys in the medium of overnight cultures was monitored by Western blotting. Similar to the case for A2-HPA and A13-HPA, no SCA-Lys could be detected in the growth medium of *B. subtilis* 8G5, which is wild type with respect to the secretion of proteases into the medium (Fig. 6A). However, unlike A2-HPA and A13-HPA, upon pulse-chase labeling, mature A2-SCA-Lys and A13-SCA-Lys could be detected in the medium (Fig. 6B). These observations suggested that SCA-Lys was secreted into the growth medium and subsequently degraded. To test this hypothesis, the secretion of A13-SCA-Lys was monitored in *B. subtilis* DB430, a strain lacking three secreted proteases (AprE, Bpf, and NprE), and in *B. subtilis* WB600, which lacks six secreted proteases (see above). As shown in Fig. 6A, secreted A13-SCA-Lys was detectable in the medium of *B. subtilis* DB430 (≈200 μg/liter), and the amount of this secreted protein was slightly increased in *B. subtilis* WB600 (≈250 μg/liter), showing that A13-SCA-Lys is subject to degradation by secreted proteases of *B. subtilis*. Interestingly, A2-SCA-Lys appeared to be more sensitive to proteolysis than A13-SCA-Lys, as it was not detectable in the medium of *B. subtilis* DB430 (results not shown). To test whether the secreted A2-SCA-Lys or A13-SCA-Lys was biologically active, a pin-enzyme-linked immunosorbent assay against lysozyme was performed. The concentrations of biologically active A13-SCA-Lys matched well with the concentrations of A13-SCA-Lys as determined by Western blotting. For example, the concentrations of active A13-SCA-Lys in the media of *B. subtilis* 8G5, DB430, and WB600 were 89 (± 15), 202 (± 8), and 293 (± 14) μg/liter, respectively. From these observations, we conclude that all secreted A13-SCA-Lys was active.

DISCUSSION

In the present studies, we have investigated bottlenecks in the secretion of four different heterologous proteins, AmyL, Bla, HPA, and SCA-Lys, making use of the same expression and secretion signals. Interestingly, all identified secretion bottlenecks are at late stages in the secretion process, which follow translocation across the cytoplasmic membrane. Furthermore, the nature of the secretion bottleneck was determined largely by the secreted protein and, to a lesser extent, by the combination of the signal peptide and secretory protein.

The first step after preprotein translocation across the membrane is processing by SPases. We have previously shown that the rate of processing of a slowly processed hybrid preprotein (pre-A13i-Bla) could be increased by overproduction of SipS (40). Here we show that this is also true for pre-A2d-Bla. Taken together, these observations suggest that processing by SPase is a secretion bottleneck for those proteins whose mature forms reach the growth medium but whose precursors are slowly processed. Subsequent steps in the secretion process involve folding of mature proteins to their active and protease-resistant conformation. As shown for A2-Bla and A2-AmyL, passage through the cell wall is the major bottleneck in the secretion of proteins which are rapidly processed and folded into an active conformation. For the secretion of these proteins, proteolysis seems to be a major problem. In the case of AmyL, we show that proteolytic degradation takes place not only before its release into the medium, as recently reported by Stephenson and Harwood (34), but also after its release. The latter observation is unprecedented. This also applies to our novel observation that the site of AmyL degradation depended on the temperature, suggesting that the expression of activity of proteases involved in this process is temperature dependent or that reduced rates of translocation and release at 25°C make AmyL more vulnerable to cell-associated proteases. In contrast to the case for AmyL, no cell-associated degradation products of Bla were observed, suggesting that the degradation of this protein takes place largely in the medium. This is apparently also true for SCA-Lys, which confirms earlier observations by Wu et al. (45), who showed that a secreted antidigoxin SCA was sensitive to secreted proteases of *B. subtilis*. The stability of secreted SCA-Lys seems to depend also on the type of fusion protein that was constructed, because A2-SCA-Lys was less stable than A13-SCA-Lys. Finally, some heterologous proteins are unable to pass the cell wall, as examplified by HPA.

There are at least three major reasons why the cell wall of *B. subtilis* can form a bottleneck in the secretion of heterologous

proteins. First, the wall is relatively thick (10 to 50 nm), with a high concentration of immobilized negative charge (e.g., teichoic or teichuronic acids) (1). This negative charge might affect the release of proteins with surface-exposed positively charged residues into the growth medium. Second, translocated proteins have to fold in the wall environment to become active and protease resistant. This requires the presence of folding catalysts, such as PrsA (12, 16, 17), thiol-disulfide oxidoreductases (our unpublished observations), and cations (e.g., Ca^{2+} and Fe^{3+}) (25). Third, the membrane-cell wall interface contains numerous proteases, such as the *wprA*-encoded CWBP52 (20, 34) and homologues of the *E. coli* HtrA and Tsp proteases (our unpublished observations), which appear to play a role in the quality control of secreted proteins.

In the case of A2-Bla, it is not entirely clear why passage through the cell wall is a bottleneck in secretion. Notably, A2-AmyL passes the wall at a much lower rate than A2-Bla, which may be related to differences in the physicochemical properties (e.g., molecular mass, rate of folding, shape, and surface charge) of these proteins. The observed degradation of A2-AmyL in the wall most likely is related to a combination of relatively slow folding of A2-AmyL and the presence of wall-associated protease activity, as shown for AmyL secreted with its authentic signal peptide (34). The reason for the failure of A2-HPA to pass the wall most likely is related to its misfolding. This view is based on the observation that no A2-HPA activity could be demonstrated in wall fractions that contained substantial amounts of the A2-HPA protein as shown by Western blotting (data not shown). As judged from the amounts of A2-HPA in these wall fractions, we should have been able to show its activity if this material has been correctly folded. Thus, it seems that incorrectly folded A2-HPA is unable to pass the wall, which could be due to electrostatic interactions with wall components. This would be consistent with the hypothesis that the rate of cell wall passage is determined by the unfolding-folding transition of proteins during or shortly after their translocation across the cytoplasmic membrane (4, 19, 25). Alternatively, the misfolded A2-HPA could form aggregates in the wall which cannot be released into the growth medium. As all A2-HPA accumulating in the wall was proteolyzed upon the addition of the reducing agent β-mercaptoethanol, even in a strain lacking six secreted proteases (data not shown), it seems that the formation of disulfide bonds is required to stabilize (misfolded) A2-HPA. Notably, these disulfide bonds are probably incorrectly formed, as the A2-HPA accumulating in the wall is not active (see above). The view that disulfide bonds are required to stabilize A2-HPA is consistent with our unpublished observation that at least two of the three putative disulfide bond oxidoreductases of *B. subtilis*, which we have recently identified, are required for the productive secretion of proteins with a disulfide bond required for stability. Taken together, these observations suggest that (correct) formation of disulfide bonds is a major bottleneck for the secretion of heterologous proteins with multiple disulfide bonds in *B. subtilis*.

In conclusion, this comparative analysis has identified various bottlenecks in the late stages of the secretion of heterologous proteins by *B. subtilis*. Some of these bottlenecks, such as SPase limitation and proteolysis of the secreted protein in the wall and growth medium, have been identified previously. A novel bottleneck concerns the catalysis of disulfide bond formation. Most importantly, the present results clearly point out that the optimization of the secretion of individual heterologous proteins is likely to require engineered *B. subtilis* strains in which specific secretion bottlenecks have been removed. We are confident that the engineering of dedicated *B. subtilis* host strains for the secretion of a wide range of heterologous proteins at commercially significant levels will soon be possible, because of our increasing knowledge of the secretion apparatus, the availability of the complete genome sequence, and the genetic amenability of this organism.

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The first two authors contributed equally to this work.

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