Differential Dependence of Levansucrase and α-Amylase Secretion on SecA (Div) during the Exponential Phase of Growth of *Bacillus subtilis*

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SecA, the translocation ATPase of the preprotein translocase, accounts for 0.25% of the total protein in a *degU32*(Hy) *Bacillus subtilis* strain in logarithmic phase. The SecA level remained constant irrespective of the demand for exoprotein production but dropped about 12-fold during the late stationary phase. Modulation of the level of functional SecA during the exponential phase of growth affected differently the secretion of levansucrase and α-amylase overexpressed under the control of the *sacB* leader region. The level of SecA was reduced in the presence of sodium azide and in the *div341* thermosensitive mutant at nonpermissive temperatures. Overproduction of SecA was obtained with a multicopy plasmid bearing *secA*. The gradual decrease of the SecA level reduced the yield of secreted levansucrase with a concomitant accumulation of unprocessed precursor in the cells, while an increase in the SecA level resulted in an elevation of the production of exocellular levansucrase. In contrast, α-amylase secretion was almost unaffected by high concentrations of sodium azide or by very low levels of SecA. Secretion defects were apparent only under conditions of strong SecA deprivation of the cell. These data demonstrate that the α-amylase and levansucrase precursors markedly differ in their dependency on SecA for secretion. It is suggested that these precursors differ in their binding affinities for SecA.

The protein SecA is an essential component of the general translocation pathway in bacteria. It is the peripheral subunit of the preprotein translocase, a multisubunit integral protein complex (see recent reviews in references 7 and 10). SecA is present in many gram-negative and gram-positive bacteria (2, 15, 18, 35), in primitive algae (47), and in cyanobacteria and the chloroplasts of higher plants (1, 14, 50–52). Studies with *Escherichia coli* have shown that the ATPase activity of SecA is essential for protein translocation (23, 33). SecA is the only ATPase involved in protein translocation, and its activity is stimulated by high-affinity interactions with preproteins (6, 8, 16, 24, 28).

The *Bacillus subtilis secA* homologue gene, *div⁺*, has been identified, cloned, and sequenced (39, 40). The deduced amino acid sequence is very similar to that of the *E. coli* SecA protein (31, 41), with 50% sequence identity. Like its *E. coli* SecA counterpart, Div is a homodimer possessing translocation ATPase activity (19, 45, 48). Nevertheless, Div complements *secA* mutants of *E. coli* only when it is expressed at a very low level (19, 49). The amino-terminal ATP binding domain of Div can functionally replace the corresponding region of SecA, and this region is thought to regulate the integration of SecA into the cytoplasmic membrane (25, 36).

The *div-341* mutation was initially identified during screening for septum initiation mutants (38). This temperature-sensitive mutation affects not only cell division but also the initiation of sporulation and competence at nonpermissive temperatures, and it reduces protease production (39). This

parallels the first *secA* mutation isolated in *E. coli*, which is also a temperature-sensitive mutation located within or near a cluster of genes responsible for cell division and septation (30). The protein translocation defect in the *div-341* mutant is believed to be caused by the rapid degradation of the SecA variant (46).

The dependence of native *B. subtilis* proteins expressed from their chromosomal genes on SecA has never been investigated in detail. We have, therefore, compared the effects of the modulation of the SecA level on the secretion of levansucrase and α-amylase during the exponential phase of growth when these two proteins are expressed in the same genetic context and under the same regulated control. The results indicate a vast difference in SecA dependency for the secretion of levansucrase and α-amylase. While the secretion of levansucrase gradually varies with the SecA level in the cell, the secretion of α-amylase is affected only under conditions of strong SecA depletion. These results are discussed in terms of differences in affinities of the precursors for SecA.

MATERIALS AND METHODS

Strains and media. The strains and plasmids used are listed in Table 1. *B. subtilis* GM96104, containing a *sacR-amyE* fusion, was constructed as described for strain GM96101 (21). Plasmid pGMS57 was inserted by double crossing over into the chromosome of strain NIG1156 (39). The resulting strain, GM96200, was transformed with pGMK58 (21) by a Campbell-like mechanism. Transformants were selected on Luria broth plates containing the appropriate antibiotic. One of the transformants (strain GM96104) containing the fusion *sacR-amyE* and having sucrose-inducible α-amylase production was used. Strain GM9801 overproducing SecA was obtained by transforming strain QB112 with pWMKL1 (17). Plasmid pWMKL1, derived from pWH1520 (37), contained the cloned *secA* wild-type gene of *B. subtilis* 168 under the inducible control of xylose.

B. subtilis QB112, GM96101, and GM96801 were each grown at 37°C in minimal medium (4) supplemented with 1% (wt/vol) glucose or 1% glucitol, 0.25% Casamino Acids, and 15 μg of tetracycline ml⁻¹, respectively. CaCl₂ was added at 0.5 mM to the culture medium of strains GM96101 and GM96104.

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TABLE 1. Strains of *B. subtilis* and plasmids

Strain or plasmid	Relevant genotype or characteristics	Source or reference
Strains		
QB112	*degU32*(Hy) *sacA321*	22
GM96101	*degU32*(Hy) *sacA321* Δ*(sacR-sacB)* *sacR-amyE*	21
NIG1156	*degU32*(Hy) *secA*(Ts) *(div-341)*	39
GM96200	NIG1156 Δ*(sacR-sacB)*	This study
GM96104	*degU32*(Hy) *secA*(Ts) Δ*(sacR-sacB)* *sacR-amyE*	This study
GM9801	*degU32*(Hy) *sacA321*; pWMKL1	This study
Plasmids		
pGMS57	pGM56 Sp^r	21
pGMK58	pGMK50 with the *Bam*HI-*Eco*RV fragment deleted and the *sacR-amyE* fusion ligated at these sites	21
pWH1520	Ap^r Tc^r	37
pWMKL1	pWH1520 with *secA* under the control of the *xylR* promoter	19

Strains NIG1156 and GM96104 were grown in the same medium at 30°C (permissive temperature) or at nonpermissive temperatures as indicated. Levansucrase and α-amylase expression was induced by sucrose at the concentrations indicated in the figure legends. SecA synthesis by the strain containing pWKLM1 was induced by 0.5% xylose.

Enzyme assays. Levansucrase activity was assayed in an acetone-water mixture (vol/vol), pH 6, containing 50 mM sucrose (5). Under these conditions, one enzyme unit corresponds to 6 mg of pure protein. α-Amylase activity was assayed at 37°C, with *p*-nitrophenyl-maltotrioside as the substrate (bioMerieux) at pH 6.3 in 0.1 M potassium phosphate or potassium acetate. One enzyme unit corresponds to 25 mg of pure protein. Differential rates of synthesis were evaluated by measuring enzyme production as a function of growth (4).

Quantification of proteins in cell extracts. Samples of 2-ml culture were centrifuged, and the pellets were resuspended in electrophoresis sample buffer and sonicated (three 30-s pulses). Aliquots of the extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantitative immunoblotting by using a standard calibration curve of the pure protein. Radioactive bands were quantified with a PhosphorImager (Molecular Dynamics). The precursors of levansucrase and α-amylase and the SecA protein were analyzed by immunoblotting (34). Proteins were measured by the Bradford assay (3).

Pulse-labelling and chase experiments. Culture samples (0.5 ml) were pulse-labelled with [^{35}S]methionine for 3 min, and reactions were stopped in ice-cold stopping buffer (0.1 M sodium phosphate, pH 7, containing 2.4 M KCl, 200 μg of chloramphenicol ml^{-1}, and 0.2 mM phenylmethylsulfonyl fluoride). Cell suspensions were centrifuged, and the supernatants were dialyzed against 1 mM sodium phosphate at pH 6 for 150 min at 4°C and then lyophilized. The dry samples were resuspended in electrophoresis sample buffer, boiled for 3 min, and analyzed by SDS-PAGE.

Cells were pulse-labelled at an optical density at 600 nm (OD$_{600}$) of 2 by adding 0.25 mCi (9 mBq) of [^{35}S]methionine (800 mCi mmol^{-1}) to a 1-ml culture suspension maintained at 37°C for 45 s. Nonradioactive methionine (4 mM final concentration) was then added. Samples (0.2 ml) were withdrawn at intervals, and all reactions were immediately stopped by diluting the samples threefold with ice-cold stopping buffer. Cell suspensions and bacterial pellets were treated as described previously (21). The samples were finally analyzed by SDS-PAGE, and the bands were quantified with a PhosphorImager.

RESULTS

SecA level in a *degU32*(Hy) strain of *B. subtilis*. *B. subtilis* produced exoproteins during the exponential and stationary phases of growth. During the logarithmic phase, SecA accounts for approximately 0.25% of the total cellular protein (225 ng per optical density unit), but the level decreases after the cells enter the stationary phase, reaching a basal level about 12-fold
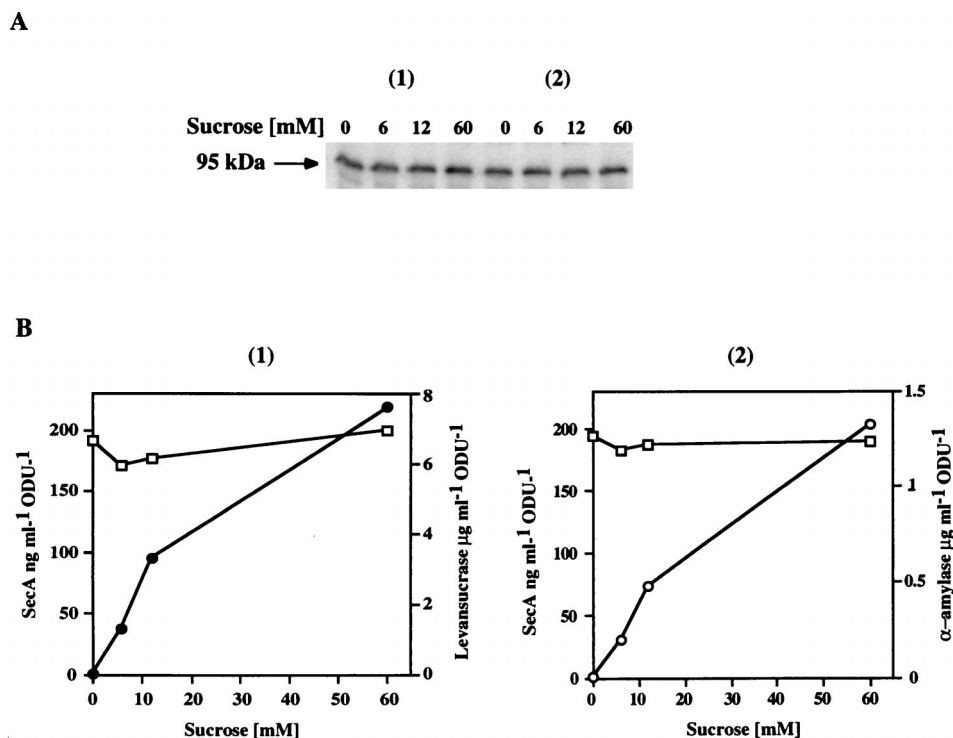

FIG. 1. The cellular SecA level is not affected by the high-level production of levansucrase and α-amylase. Strains QB112 and GM96101 were induced for synthesis of levansucrase or α-amylase with various concentrations of sucrose inducer at an OD$_{600}$ of 0.25. Samples of 2-ml cell suspensions were withdrawn after three generations (final OD$_{600}$ of 2) and centrifuged. Levansucrase or α-amylase activities in the supernatants were assayed. The cell pellets were treated as described in Materials and Methods and analyzed by immunoblotting with SecA antibodies. Known quantities of pure SecA were used as standards for quantitative immunoblotting and quantified with a PhosphorImager. (A) SecA immunoblotting from strains QB112 (panel 1) and GM96101 (panel 2) induced with the sucrose concentrations indicated; (B) levansucrase (●) and α-amylase (○) activities assayed in QB112 and GM96101 supernatants, respectively, and SecA in cells (□) as a function of sucrose concentration. ODU, optical density unit.
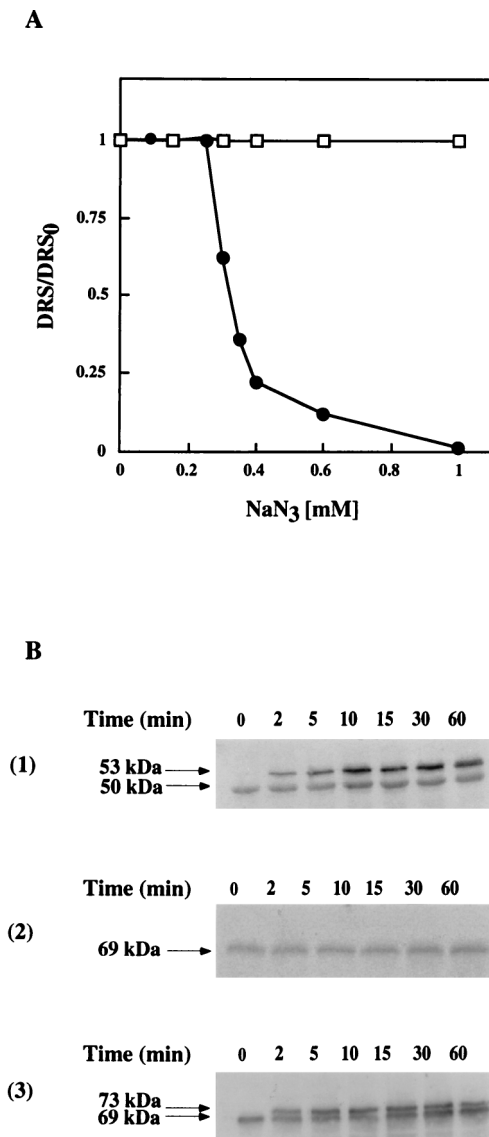
A

B

FIG. 2. (A) Effect of sodium azide on the production of exocellular levansucrase and α-amylase by strains QB112 and GM96101, respectively. Cells at an OD$_{600}$ of 0.2 were induced with 8 mM sucrose for levansucrase synthesis and 60 mM sucrose for α-amylase synthesis. After two generation times (90 min), the cell suspensions were divided and supplemented with various concentrations of sodium azide. Levansucrase (●) and α-amylase (□) were assayed in the supernatants of samples taken from cultures grown in the presence of sodium azide concentrations up to 1 mM. DRS and DRS$_0$, differential rates of the enzyme released in supernatant after the addition of sodium azide and in the absence of azide, respectively. (B) Processing of the precursors of levansucrase and α-amylase in strains QB112 and GM96101 grown in the presence of sodium azide was analyzed by immunoblotting of cell extracts. Strains QB112 (panel 1) and GM96101 (panels 2 and 3) were induced with 8 and 60 mM sucrose, respectively, at an OD$_{600}$ of 0.5. After two generation times, sodium azide was added (panels 1 and 2, 0.3 mM final concentration; panel 3, 3 mM), and at the times indicated, samples of 5 ml were withdrawn, centrifuged, and analyzed by immunoblotting as described in Materials and Methods.

lower. To determine the influence of exocellular protein production on the level of SecA in the cell during exponential growth, the production of levansucrase or α-amylase was examined as a function of the SecA level in strain QB112 or GM96101, respectively (Fig. 1A and B). In these strains, the synthesis of levansucrase and α-amylase is under the control of the sucrose-inducible *sacB* promoter, and production of SecA

increases almost linearly with the sucrose concentration (4, 21). The level of intracellular SecA remained unchanged (about 1.8 μM) regardless of the yield of levansucrase or α-amylase produced. The induction of α-amylase expression at 60 mM sucrose in GM96101 cells and of levansucrase at 8 mM sucrose in QB112 cells resulted in similar yields of the two gene products. This permits modulation of the level of SecA under conditions in which both exoproteins are secreted at the same rate.

Different dose-dependent effects of sodium azide on the processing and production of levansucrase and α-amylase. The effect of low levels of functional SecA on levansucrase and α-amylase secretion was studied by using sodium azide to specifically inhibit SecA ATPase activity (11, 32). The growth of QB112 and GM96101 was similarly affected in the presence of sodium azide. It remained unmodified up to 0.4 mM NaN$_3$, was slightly reduced by higher concentrations, and was completely blocked by 3 mM; cells started to lyse at 10 mM (data not shown). The production of exocellular α-amylase was not affected when cells were grown in the presence of 0 to 1 mM sodium azide, while the levansucrase secretion decreased rapidly at NaN$_3$ concentrations above 0.25 mM (Fig. 2A). To determine if α-amylase secretion is totally insensitive to sodium azide, the effect of 3 mM NaN$_3$ was tested. Under these drastic conditions, cells no longer divided and α-amylase was not secreted (data not shown). Next, the effect of sodium azide on the fate of the precursors of each protein after the addition of 0.3 mM sodium azide to culture was monitored. This concentration inhibited levansucrase production by about 50% but had no effect on α-amylase (Fig. 2A). Unprocessed levansucrase precursor rapidly accumulated in the cells (Fig. 2B, panel 1), but there was no detectable unprocessed precursor of α-amylase (Fig. 2B, panel 2). The unprocessed precursor of α-amylase accumulated only when azide was added at a 10-fold-higher azide concentration (3 mM) (Fig. 2B, panel 3). This suggests that different levels of functional SecA are required for the efficient processing of levansucrase and α-amylase precursors. However, this suggestion is based on a specific effect of sodium azide on the SecA ATPase activity (11, 32), leaving open the possibility that this metabolic poison has other effects on cell physiology, which could complicate the interpretation.

The yield of exocellular levansucrase production is proportional to the amount of SecA. To correlate the production of exoproteins with the amount of SecA in the cells, further experiments were performed with the *secA* thermosensitive mutant (the *div-341* mutant). The residual SecA was estimated by quantitative immunoblotting, and the yield of exocellular α-amylase and levansucrase was determined by pulse experiments at various nonpermissive temperatures. For this purpose, strain GM96104 was derived from strain NIG1156 (39) by first deleting the chromosomal region *sacB*, yielding strain GM96200, and then introducing the *sacR-amyE* fusion by Campbell-like integration of the plasmid pGMK58 (Table 1) (21). Strain GM96200 was used as a control. Growth of double-mutant strains which were *secA*(Ts) *degU32*(Hy) and had been transferred from 30°C (permissive temperature) to nonpermissive temperatures was affected after one generation at 40 and 43°C and more rapidly at 46°C (Fig. 3A). The levels of SecA in cell extracts of both strains were identical, and they decreased sharply at temperatures above 30°C (Fig. 3B), in agreement with results reported by Nakane et al. (29). The production of exocellular levansucrase and α-amylase after incubation for 8 min at each temperature was measured by means of a 3-min pulse experiment with radioactive methionine. α-Amylase production remained constant up to 40°C, while levansucrase pro-
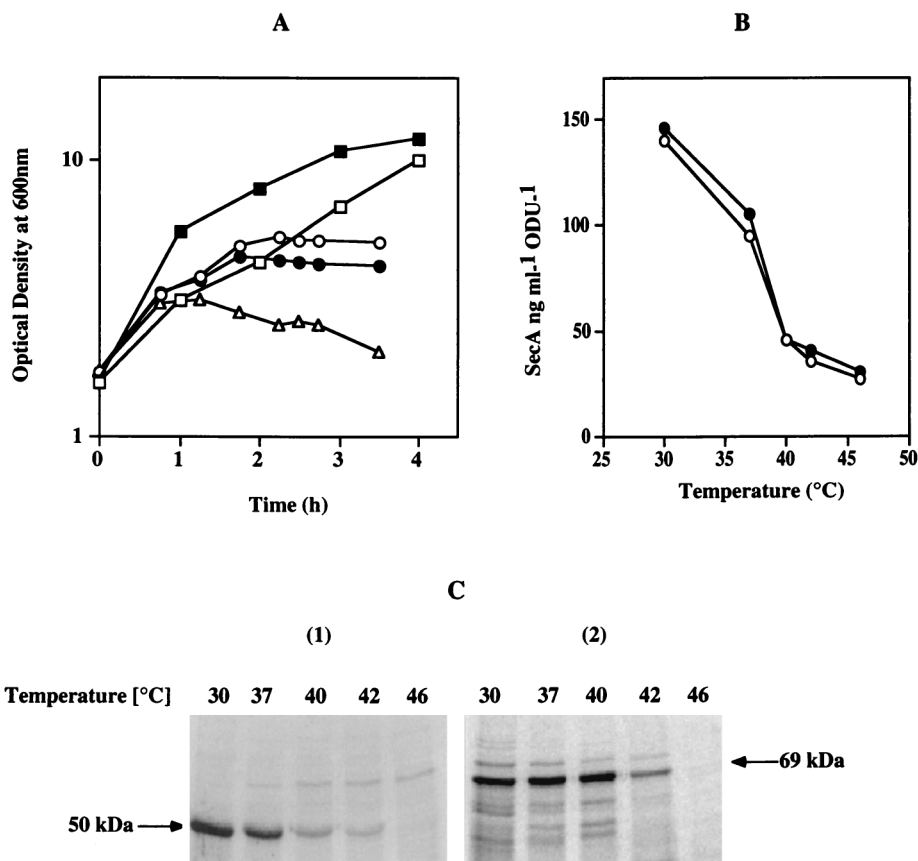
A

B

C

FIG. 3. Effect of SecA depletion on the secretion of levansucrase or α-amylase by the *secA*(Ts) strains NIG1156 and GM96104. (A) Growth of *secA*(Ts) strains at permissive and nonpermissive temperatures. Cells of strains NIG1156 and GM96104 were grown at 30°C (□) in minimal medium supplemented with 1% glucose, and levansucrase or α-amylase synthesis was induced with 8 or 60 mM sucrose, respectively. At an OD_{600} of 2, the initial cultures were divided into parts, and each part was transferred at the following temperatures: 37°C (■), 40°C (○), 43°C (●), and 46°C (△). (B) SecA levels in cell extracts of *secA*(Ts) strains NIG1156 (●) and GM96104 (○) at various temperatures. Samples were processed as described above, analyzed for SecA by immunoblotting, and quantified with a PhosphorImager. (C) Production of exocellular levansucrase and α-amylase in *secA*(Ts) strains NIG1156 (panel 1) and GM96104 (panel 2) at various temperatures. Cells were grown to an OD_{600} of 2 as described above, shifted to the temperature indicated, and incubated for 8 min. Then 2 ml of each was pulse-labelled for 3 min with 0.15 mCi of [^{35}S]methionine as described in Materials and Methods. The supernatants were analyzed by SDS-PAGE.

duction decreased at temperatures above 30°C (Fig. 3C). The yield of secreted levansucrase and α-amylase plotted against the residual level of SecA (Fig. 4) again reveals the differential requirements of SecA for levansucrase and α-amylase secretion.

To determine at which step secretion was affected after depletion of the SecA function, pulse-chase experiments were performed at nonpermissive temperatures. At 37°C, processing remained too fast to detect α-amylase precursor (results not shown), whereas at the same temperature, the rate of levansucrase precursor processing decreased dramatically from a half-life of 5 s for the wild type (34) to 60 s for the thermosensitive mutant (Fig. 5A). Pulse-chase experiments at 42°C with the thermosensitive mutant producing α-amylase indicate that a large decrease in SecA also results in the blockage of the processing of α-amylase precursor (Fig. 5B). These results further support our notion that α-amylase secretion and levansucrase secretion have different SecA dependencies in vivo.

Effect of SecA overproduction on levansucrase production. The marked sensitivity of levansucrase production to small changes in functional SecA suggests that the endogenous level of SecA does not saturate one or more steps in the secretion of levansucrase. Hence, overproduction of SecA might increase the levels of levansucrase secretion. The effect of increasing
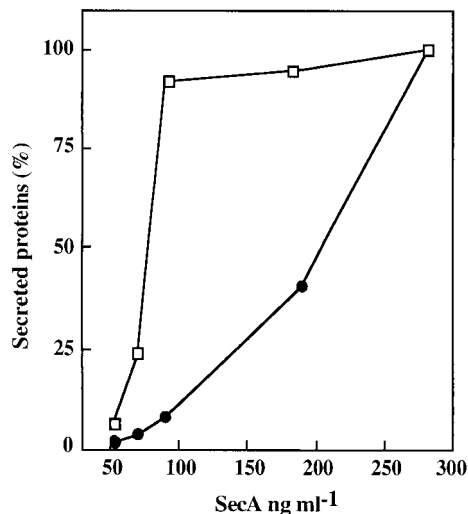

FIG. 4. Production of levansucrase (●) and α-amylase (□) as a function of SecA. Data were taken from Fig. 3C, panels 1 and 2.
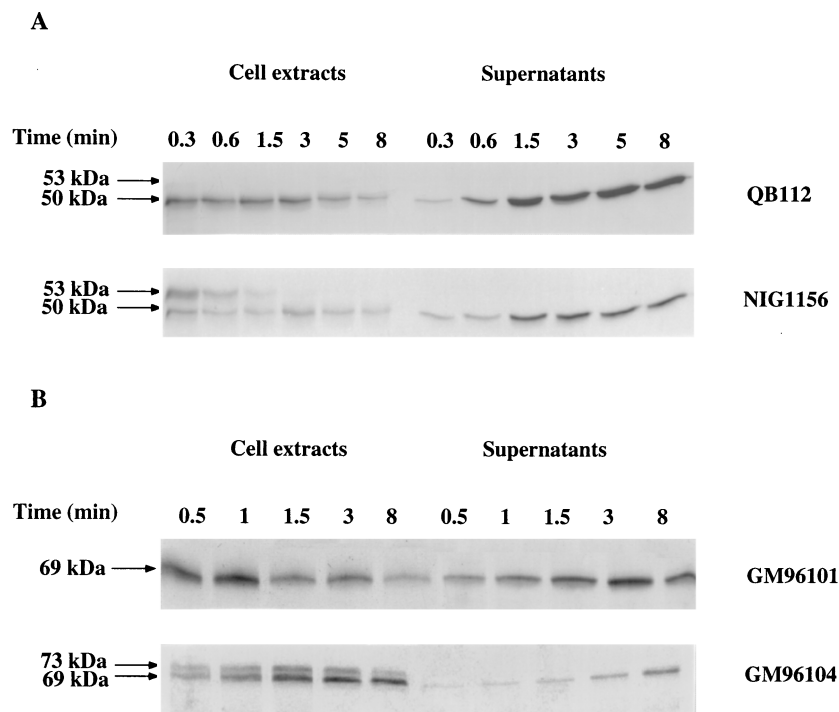
A

B

FIG. 5. Kinetics of levansucrase (A) or α-amylase (B) processing and release by strains NIG1156 and QB112 or GM96104 and GM96101, respectively. Four generations after induction of the strains with sucrose (8 mM for NIG1156 and QB112 or 60 mM for GM96104 and GM96101), cultures were shifted to 37°C (A) or 42°C (B) for 8 min. Samples (2 ml) were labelled with 0.15 mCi of [35S]methionine for 45 s and chased with an excess of nonradioactive methionine. Cell extracts were immunoprecipitated, and supernatants were dialyzed, lyophilized, and finally analyzed by SDS-PAGE.

the SecA concentration on the secretion of levansucrase was examined by using the multicopy plasmid pWKML1, which bears the *secA* gene under the regulated control of *xylR* (17). Strain QB112 was transformed with pWKML1 (strain GM9801), and SecA was overproduced after induction with 0.5% xylose. The presence of xylose did not modify the growth or rate of levansucrase synthesis of cells grown in minimal medium, since xylose is not metabolized by *B. subtilis* (25). SecA overproduction in this strain was blocked by glucose, since *xylR* is controlled by catabolite repression (20). Glucitol was thus used as a carbon source because it does not modify the levansucrase synthesis or secretion in the control strain QB112. Induction of SecA gene expression by pWKML1 led to a sevenfold overproduction of SecA. The rates of levansucrase production by strain GM9801 induced by 60 mM sucrose were determined in the presence and absence of xylose. Measurements were made one generation after sucrose induction to reduce catabolite repression by the glucose generated by levansucrase sucrose hydrolysis. Under these conditions, levansucrase production was about 40% greater in the presence of xylose than in its absence.

DISCUSSION

In prokaryotic cells, precursor proteins with a typical signal sequence are secreted by a common system, the preprotein translocase. They enter the translocase via the peripheral subunit SecA, which utilizes the energy of ATP binding and hydrolysis to drive the stepwise translocation of the precursor across the membrane (7). In this work, we compared the dependence of levansucrase and α-amylase secretion on SecA. For this purpose, the respective structural genes of these two native *B. subtilis* exoproteins were expressed under the control

of the sucrose-inducible *sacR* promoter. We obtained similar yields of gene expression products by using different concentrations of the inducer, 8 mM for levansucrase and 60 mM for α-amylase. Under such conditions, the effects of SecA expression on secretion are directly comparable. Our study demonstrates that various precursors may exhibit major differences in their dependency on the amount of functional SecA in the cell. The amount of functional SecA was varied in three independent ways: (i) by means of the inhibitory effect of sodium azide, which selectively blocks the preprotein-stimulated ATPase activity of SecA (27, 29, 32); (ii) by the use of the *secA*(Ts) *div-341* mutant at nonpermissive temperatures (39); and (iii) by overproduction of SecA using pWKML1 (17). The data show that the yield of levansucrase secreted by *B. subtilis* cells is proportional to the amount of SecA present, whereas α-amylase secretion is rather insensitive to a large decrease in the SecA level. This main difference in the secretion potential in responses to the modulation of the amount of functional SecA in the cell is most likely due to a difference in the affinity of the precursors for SecA.

In *E. coli*, SecA is involved in the initial steps of the protein secretion, and it mediates the entry of the preprotein into the export pathway (9, 10). SecA directly recognizes the signal sequence and unknown elements of the mature domain of the precursor (6, 16, 24). The levansucrase and α-amylase sequence signals are very different (42). The hydrophobic domain of the α-amylase signal sequence is 1.5 times longer than that of levansucrase, and its overall hydrophobicity is much greater. Also, the net charge of the first two amino acids immediately downstream of the signal sequence is negative for α-amylase and positive for levansucrase. These aspects of the signal sequence can be crucial for binding to SecA (16, 28), and

to a large extent, they explain the difference in SecA dependency.

Cells of *B. subtilis* are straight rods (1.5 μm long and 0.65 μm wide). The SecA concentration is approximately 1.8 μM during the exponential phase and about 0.15 μM during the stationary phase. The levansucrase precursor has an affinity constant of about 1.10^6 M^{-1} ($K_d = 1$ μM), since the secretion efficiency is half maximal at this SecA concentration. The α-amylase precursor affinity is at least 1 order of magnitude greater since α-amylase is normally secreted during the stationary phase (46) and, as shown in this work, is efficiently secreted even when the level of SecA during the exponential phase is very low. It is worthy to note that ProOmpA binds to SecA in vitro with a K_d of 0.06 μM (13). Therefore, it appears that levansucrase precursor is a poor affinity substrate for the translocase.

In *E. coli*, a subset of precursor proteins is stabilized in an unfolded state by the chaperone SecB. SecB targets these precursors to the translocase by direct binding to SecA (13). Through these events, SecB facilitates the proper recognition of the precursor protein by the translocase. So far, no SecB homologue has been identified in *B. subtilis*, despite the availability of the complete genome sequence. One could argue that a chaperone function for stabilizing the partially folded precursors might not be required in this bacterium. This hypothesis is supported by evidence that α-amylase and levansucrase from *B. subtilis* are spontaneously stabilized in an intermediate folding state under cytosolic conditions of pH and calcium concentration (12, 43). This emphasizes the importance of information borne by the signal sequence and mature domains in the secretion of *B. subtilis* proteins.

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