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Temporal Expression of the \textit{Bacillus subtilis} \textit{secA} Gene, Encoding a Central Component of the Preprotein Translocase

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In \textit{Bacillus subtilis}, the secretion of extracellular proteins strongly increases upon transition from exponential growth to the stationary growth phase. It is not known whether the amounts of some or all components of the protein translocation apparatus are concomitantly increased in relation to the increased export activity. In this study, we analyzed the transcriptional organization and temporal expression of the \textit{secA} gene, encoding a central component of the \textit{B. subtilis} preprotein translocase. We found that \textit{secA} and the downstream gene (\textit{prfB}) constitute an operon that is transcribed from a vegetative (\sigma\text{-dependent}) promoter located upstream of \textit{secA}. Furthermore, using different independent methods, we found that \textit{secA} expression occurred mainly in the exponential growth phase, reaching a maximal value almost precisely at the transition from exponential growth to the stationary growth phase. Following to this maximum, the \textit{de novo} transcription of \textit{secA} sharply decreased to a low basal level. Since at the time of maximal \textit{secA} transcription the secretion activity of \textit{B. subtilis} strongly increases, our results clearly demonstrate that the expression of at least one of the central components of the \textit{B. subtilis} protein export apparatus is adapted to the increased demand for protein secretion. Possible mechanistic consequences are discussed.

In the work described here, we analyzed the transcriptional organization and temporal expression of the \textit{B. subtilis} \textit{secA} gene, encoding the translocation ATPase subunit of the preprotein translocase. We show that \textit{secA} is the first gene in an operon and that transcription of this operon is initiated from a promoter which is highly similar to \sigma\text{-dependent} promoters. Furthermore, we found by different independent criteria that expression of \textit{secA} is maximal at the end of the exponential growth phase, after which a decrease in transcription can be observed. The results indicate that, like the synthesis of secreted proteins, the synthesis of at least one of the central components of the \textit{B. subtilis} preprotein translocase is temporally controlled.

MATERIALS AND METHODS

Bacterial strains and growth conditions. \textit{E. coli} JM109 (53) was grown at 37°C in Luria-Bertani (LB) medium (25) containing ampicillin (100 \mu g mL\textsuperscript{-1}) as required. \textit{B. subtilis} DB104, a strain that secretes only low levels of proteases into the culture supernatant (16), and its derivatives DB104-4 and DB104-4T were grown at 37°C in LB, 2xYT (39), or minimal (41) medium containing chloramphenicol (5 \mu g mL\textsuperscript{-1}) or kanamycin (20 \mu g mL\textsuperscript{-1}) as required. \textit{B. subtilis} DB104-4, containing a transcriptional \textit{secA-lacZ} fusion integrated into the chromosomal \textit{amyE} gene, was constructed by transforming DB104 with linearized plasmid pDG268-4 (see below), selecting for chloramphenicol resistance and screening for an \alpha-amylase-negative phenotype on LB plates containing 0.6% (w/v) amylopectin-azure (Sigma, Deisenhofen, Germany). DB104-4T, containing a translational \textit{secA-lacZ} fusion integrated into the \textit{amyE} gene, was constructed by the same procedure except that plasmid pDG268-4T (see below) was used.

DNA techniques and plasmid constructions. Standard procedures (36) were used for preparation of plasmid DNA, isolation of DNA fragments, restriction, ligation, and other DNA techniques. pDG268 is a transcriptional \textit{spoVG-lacZ} fusion vector that allows the subsequent integration of the respective \textit{lacZ} reporter fusions in single copy into the chromosomal \textit{amyE} gene of \textit{B. subtilis} via a double-crossover event (1). A transcriptional \textit{secA-lacZ} fusion was constructed by ligating a 456-bp \textit{MscI} fragment, containing the \textit{orf189-secA} intergenic region and the 5' end of the \textit{secA} gene, from plasmid pBO1 (31) into pDG268 which had been digested with EcoRI, filled in with Klenow DNA polymerase, and then redigested with HinclIII. In the resulting plasmid, pDG268-4, transcription of the \textit{spoVG-lacZ} reporter gene is under the control of the \textit{secA} promoter. For construction of a \textit{secA-lacZ} fusion, the DNA fragment containing the \textit{secA} gene (529 bp), from plasmid pDG268-4, was inserted into the HindIII site of pDG268.
total RNA was quantified by measuring the optical density at 260 nm (OD260) of (Qiagen, Hilden, Germany) as instructed by the manufacturer. The amount of derived solely from the secA-prfB secA of secA 9 pMHP1. For probe 3, covering the 3'-initiated, the resulting plasmid pMHP3 was linearized at a plasmid pMKL4 (17), encompassing the entire secA gene, was ligated into PstI-digested pGEM3Z, resulting in plasmid pMHP1. For probe 3, covering the 3' end of secA, a 2.63-kb PstI fragment from plasmid pMKL4 (17), encompassing the entire secA gene, was ligated into PstI-digested pGEM3Z. Before in vitro transcription from the SP6 promoter was initiated, the resulting plasmid pMHP3 was linearized at a Clal site, located within the secA coding region, thereby resulting in a DIG-labeled RNA probe derived solely from the 3' end of the secA gene. For probe 2, covering the 5' end of secA, a 488-bp BamHI/HpaI fragment from pMH3 was ligated into BamHI/HindIII-digested pGEM3Z, resulting in plasmid pMHP2. For probe 4, covering part of the intergenic secA-prfB region and the 5' end of the prfB gene, a 488-bp Asp718/PstI fragment from pMKL4 was ligated into Asp718/PstI-digested pGEM3Z, resulting in plasmid pMHP4.

RNA techniques. To isolate total RNA, we used an RNeasy total RNA kit (Qiagen, Hilden, Germany) as instructed by the manufacturer. The amount of total RNA was quantified by measuring the optical density at 260 nm (OD260) of each preparation. Furthermore, the RNA samples used in the Northern blotting and the primer extension experiments were additionally examined with respect to the amount and intactness of the RNA preparations by formaldehyde agarose gel electrophoresis and staining with ethidium bromide (49). Northern blot analyses were performed essentially as described by Börmann et al. (4). To obtain DIG-labeled RNA probes 1 to 4 (locations in the ori189-secA-prfB region are shown in Fig. 1), plasmids pMHP1 to 4 were used as templates for in vitro transcription using a DIG RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany) as instructed by the manufacturer. For detection of hybridization signals, a Boehringer nonradioactive nucleic acid labeling and detection kit was used. Primer extension experiments were performed as described by Sambrook et al. (36). The synthetic oligonucleotide OMKL48 (GTATCTATTGACCCGTTAG), which is complementary to the noncoding strand of the 5' end of the secA gene (corresponding to nucleotides 39 to 57 of the secA structural gene), was used as the primer in the primer extension experiments. Dideoxynucleotide chain termination sequencing reactions (37), using the same primer and an appropriate plasmid DNA (pB01) as the template, were run in parallel on the gel to allow determination of the endpoints of the extension products.

Other techniques. β-Galactosidase activities of B. subtilis cells containing transcriptional or translational secA-locZ fusions were determined by the procedure of Nicholson and Setlow (29), using α-nitrophenyl-β-D-galactopyranoside as the substrate. The measured β-galactosidase activity was normalized by the method of Miller (25). α-Amylase secreted into the culture supernatant of B. subtilis DB104 was assayed by the method of Berndorf (2), and the resulting activities (units) were expressed in terms of micromoles of maltose liberated in 60 min at 37°C by 1 ml of culture supernatant. Total amounts of secreted proteins in the culture supernatant of DB104 were determined by the bicinchoninic acid method of Walker (51) after precipitation of the proteins with trichloroacetic acid (10%, final concentration). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using anti-B. subtilis SecA antibodies were performed as described previously (24).

RESULTS

The B. subtilis secA gene is the first gene in a bicistronic operon. The chromosomal organization of the B. subtilis secA region is shown in Fig. 1. The secA gene is located downstream of the fliD operon, whose gene products are involved in the formation of flagella, and a gene (orf189) of unknown function, which is followed by a putative rho-independent terminator (7). Downstream of secA, we identified a gene (prfB) which is preceded by a putative ribosome-binding site and which encodes a homologue of the E. coli protein release factor 2 (32). No putative promoter or terminator sequences are found in the secA-prfB intergenic region, suggesting that secA and prfB may be located in an operon which is transcribed by a promoter located upstream of secA. In fact, a promoter sequence (TGTGGA-16 bp-TATGAT) showing good agreement to the consensus promoter sequence recognized by the major vegetative sigma factor (σE) (12) was identified 80 to 107 bp upstream of the secA start codon (31, 34). In addition, an operon structure consisting of secA and prfB was also suggested from computer analyses of the complete nucleotide sequence of the B. subtilis chromosome (18).

For identification of secA-specific transcripts, total RNA was extracted from B. subtilis DB104 at various time points during growth (measured by OD600) in sporulation medium (Fig. 2A), and Northern hybridization experiments were performed with DIG-labeled RNA probes, encompassing the 3' end of ori189 (probe 1), the 5' end of secA (probe 2), the 3' end of secA (probe 3), and the 5' end of prfB (probe 4) (Fig. 1). With the 5' end of secA as a probe, two major hybridizing bands, 3.8 and 0.3 kb in size, were identified (Fig. 2B). Furthermore, additional hybridization signals were found associated with the 16S and 23S RNAs; these most likely were caused by hybridization to degradation products which were trapped by the rRNAs (14). The 3.8-kb mRNA was also detectable with probes 3 and 4 but not with probe 1 (data not shown), strongly suggesting that secA and prfB are transcribed together starting from a promoter located in the ori189-secA intergenic region. The 0.3-kb RNA could be detected with probe 2 only (not with probes 1, 3, and 4 [data not shown]), suggesting that this RNA might represent a stable breakdown product from the 5' end of the larger 3.8-kb secA-prfB transcript or, alternatively, might be produced by premature transcription termination early in the secA gene. A further observation was that the amount of de-
detectable secA-specific transcripts in the RNA preparations isolated from cells grown to the end of the exponential (lane 3) or to the post-exponential phase (lane 4) was significantly lower than the corresponding signals which were obtained with RNA isolated from exponentially growing cells (lanes 1 and 2), suggesting that secA transcription is maximal during vegetative growth.

To identify the start point of the secA-prfB-specific transcript, primer extension experiments were performed with primer OMKL48, using RNA isolated at various time points during growth in rich (2xYT) medium. As shown in Fig. 3, a signal corresponding to a transcriptional start site located 73 bp upstream of the secA translational start codon was detected in all samples. Since this transcriptional start site is located immediately downstream to the σA-dependent promoter previously identified by sequence analysis, our data clearly indicate that this promoter is in fact the promoter element involved in secA transcription initiation. We observed that in the primer extension experiments, as in the Northern blotting experiments, the intensities of the obtained signals were significantly higher in RNA preparations extracted from exponentially growing cells (lanes 1 and 2) than in RNA extracted from cells grown to the late exponential (lane 3) or post-exponential (lane 4) phase. Identical results were obtained for RNA isolated at comparable time points during growth from cells that had been grown in sporulation medium (data not shown).

Transcription of the secA gene is temporally regulated. Since the results described above suggested that expression of secA is

FIG. 2. Northern blot analysis. (A) Growth of B. subtilis DB104 in sporulation medium. Samples 1 to 4 were withdrawn for total RNA isolation at the time points indicated. (B) Northern hybridization of equal amounts of total RNA isolated from samples 1 to 4 (lane numbers correspond to sample numbers), using a probe (probe 2; Fig. 1) derived from the 5' end of the secA gene. Positions of the secA-specific RNAs are indicated by arrows. 16S and 23S denote positions of the 16S and 23S rRNAs, respectively.

FIG. 3. Mapping of the 5' end of the secA-prfB mRNA by primer extension. (A) Growth of B. subtilis DB104 in 2xYT medium. Samples 1 to 4 were withdrawn for total RNA isolation at the time points indicated in the growth curve. (B) Primer extension experiment using oligonucleotide OMKL48 as the primer and equal amounts of RNA isolated from samples 1 to 4 (lane numbers correspond to sample numbers). The major extension product is indicated by an arrow. Lanes C, T, A, and G, sequencing ladder obtained with the same primer (OMKL48) and pBO1 (31) as the template. The relevant part of the nucleotide sequence is shown on the left.
temporarily modulated in the cell, quantitative measurements of secA expression were performed with transcriptional and translational fusions of the lacZ reporter gene to 5′ fragments of secA. A 456-bp MsuI/HindIII DNA fragment from pBO1 (31) containing the orf189-secA intergenic region and the 5′ end of the secA gene was cloned into the lacZ fusion vector pDG268 (1) in two different ways. In plasmid pDG268-4, the spoVG-lacZ gene of pDG268 was placed under the regulatory control of the secA promoter, resulting in a transcriptional fusion. In pDG268-4T, the 5′ end of secA was fused in frame to the lacZ reporter gene, resulting in a translational secA-lacZ fusion. Both fusions were introduced by double-crossover recombination in single copy into the amyE locus of the chromosome of B. subtilis DB104, and β-galactosidase activity in the resulting strains (DB104-4 and DB104-4T) was monitored throughout growth (Fig. 4). In both strains, an increase in β-galactosidase activity was observed during exponential growth, reaching a maximal value at the end of the exponential growth phase (which is defined as T0). Upon entering the post-exponential phase, the strains exhibited a relatively sharp decrease of β-galactosidase activity, reaching a low basal level approximately 2 to 3 h after T0. The observed patterns were independent of the growth medium, since similar profiles of β-galactosidase activity were obtained with both strains in sporulation medium (Fig. 4A and C), rich medium (Fig. 4B and D), and minimal medium (data not shown). In addition, these results were in agreement with the temporal effects observed in the Northern blotting and primer extension experiments, which also indicated a maximal expression of secA during exponential growth and a sharp decrease of transcription shortly after T0. Furthermore, the analysis of α-amylase secretion (Fig. 4E) and of protein secretion in general (by measuring the amount of total protein in the supernatant) (Fig. 4F) during growth showed that the time point of maximal secA expression coincides with the beginning of the high-level protein secretion period in B. subtilis.

Next, expression of secA was analyzed by Western blotting using SecA-specific antibodies. Cells of B. subtilis DB104 were grown in sporulation medium (Fig. 5A and C) or LB medium (Fig. 5B and D), and samples were taken at various time points during growth. Equal amounts of proteins were applied to SDS-PAGE followed by Western blotting. In both media, the amount of SecA protein reaches a maximal value around T0, decreasing slowly thereafter to significantly lower levels. These results are consistent with those obtained with the lacZ reporter gene fusions, indicating that secA expression is strongly decreased at T0 and that the SecA protein synthesized up to this point is diluted within the cells by ongoing cell division. Our finding that SecA protein can nevertheless be detected several hours after the end of exponential growth can be explained by the high in vivo stability of the B. subtilis SecA protein (46) and eventually by some residual de novo transcription of the secA gene.

DISCUSSION

The increase in protein secretion activity is, among competence development and sporulation, one of the various possible post-exponential growth means by which B. subtilis and related Bacillus species adapt to unfavorable growth conditions, such as nutrient limitation (42). Synthesis of the precursor forms of the secreted proteins drastically increases at the level of transcription of the corresponding genes upon transition from exponential to stationary growth, and this increase is controlled by complex regulatory networks (11). It remains an open question whether the number of protein export sites normally present in low-level secreting exponentially growing cells is sufficient to allow the effective translocation of the abundance of exoproteins in the postexponential growth phase or whether the amounts of one or several components of the protein secretion apparatus must be adapted to this elevated secretion activity.

In this report, we have shown that the secA gene, encoding one of the central components of the B. subtilis preprotein translocase, is transcribed predominantly in the exponential growth phase. This result is in agreement with our finding that the secA gene, together with the prefB gene which is located downstream of secA, is transcribed from a promoter with a sequence very similar to promoter sequences recognized by σ54, the major vegetative sigma factor of B. subtilis (12). Maximum secA expression is reached at the end of the exponential growth phase, almost precisely at the time point corresponding to T0. Surprisingly, upon transition to the stationary growth phase, de novo transcription of the secA gene sharply decreases to a low basal level. Despite this fact, significant amounts of SecA protein can be detected also at much later time points of growth, probably due to the long half-life of the SecA protein in B. subtilis (46). Upon abrupt shutoff of secA transcription at the transition to stationary growth phase, the slow decrease in the amount of SecA protein that is observed at later time points can be explained by a dilution of the presynthesized SecA protein to the daughter cells during the remainder of cell divisions after T0; possibly some residual de novo synthesis may also occur. With respect to the time period of high-level protein secretion, which extends from approximately T0 to T0/T5 (33, 38), it is worth emphasizing that maximal secA gene expression and, as a consequence, the highest amount of SecA protein is reached at the beginning of the period of elevated secretion activity. This implies that the amount of SecA protein synthesized up to this point is sufficient to ensure effective protein translocation during the entire high-level secretion period. Besides its role as a membrane-associated energy-coupling subunit of preprotein translocase, SecA can most likely in its soluble form (6, 22), interact also with precursor proteins occurring free in the cytosol (45). One might speculate that the high amount of SecA at the beginning of the high-level secretion period can function as a kind of a secretory protein-specific chaperone buffer which helps to maintain the massive amounts of precursor proteins in an export-competent state for their subsequent membrane translocation.

In E. coli, secA expression is coupled to the secretion status of the cell by an autoregulatory mechanism (30). Under normal protein export conditions, excess SecA binds to its own mRNA, thereby autorepressing its translation. Under conditions of limiting protein export, the mRNA-bound SecA is released (possibly by a titration mechanism) and translation of secA mRNA increases 10- to 20-fold (23, 35, 40). So far, we have found no evidence for a comparable autoregulation of

FIG. 4. Monitoring of secA gene expression during growth of cells containing secA-lacZ fusions and analysis of α-amylase secretion and protein secretion activity in general. Graphs show expression of a transcriptional secA-lacZ fusion during growth of B. subtilis DB104 in sporulation medium (A) or LB medium (B); expression of a translational secA-lacZ fusion during growth of B. subtilis DB104 in sporulation medium (C) or LB medium (D); secretion of α-amylase during growth of B. subtilis DB104 in LB medium (E); and total amount of proteins secreted into the supernatant of B. subtilis DB104 during growth in LB medium (F). Squares, OD600, circles, β-galactosidase (LacZ) specific activity (A to D); α-amylase activity (E), or total amount of protein in the supernatant (F).
secA expression in B. subtilis. High-level expression of a secretory protein (α-amylase AmyL of B. licheniformis) in B. subtilis, resulting in the massive accumulation of AmyL precursor in the cytosol, did not lead to an increase in the amount of SecA protein (13). Furthermore, overexpression of SecA in B. subtilis DB104, containing a translational orf189-secA-lacZ fusion integrated in the amy locus, did not result in a reduced expression of β-galactosidase activity (13). However, since these observations are negative evidence, the existence of an autoregulatory mechanism for secA expression in B. subtilis cannot be totally excluded.

Very little is known about the regulation of other components of the B. subtilis preprotein translocase. Li et al. (19) have shown that the B. subtilis secY gene, which is located within the S10-spc-α region and encodes one of the central integral membrane components of the translocase, is primarily cotranscribed within one large (15-kb) transcriptional unit together with the other (mostly ribosomal) genes of the S10-spc-α region and that this transcription is driven by two σA-dependent promoters located upstream of the S10 gene. In addition, Suh et al. (44) identified two weak promoter-active regions located within the two genes immediately upstream of secY, which are able to stimulate transcription of a lacZ reporter gene mainly in the stationary growth phase. However, it is unclear whether these weak promoter sequences significantly contribute to secY gene expression in the stationary growth phase. Furthermore, since stable integration of SecY into the cytoplasmic membrane, at least in E. coli, requires a concomitant coexpression of SecE (21), it is also unclear whether the weak transcriptional activity of these potential secY promoter sequences in the stationary growth phase would in fact result in a significantly elevated level of SecY protein in the plasma membrane.

Recently, Bolhuis et al. cloned the secDF gene of B. subtilis

FIG. 5. Detection of SecA protein by Western blotting. (A and B) Growth of B. subtilis DB104 in sporulation medium (A) or LB medium (B). Samples 1 to 14 were withdrawn for the preparation of total-cell extracts at the time points indicated, and equal amounts of protein were subsequently applied to SDS-PAGE and Western blotting. (C and D) Western blot analyses using SecA-specific antibodies of total-cell extracts isolated from B. subtilis DB104 grown in sporulation medium (C) or LB medium (D). The lane numbers in panels C and D correspond to sample numbers in panels A and B, respectively.
and analyzed its expression during growth in different media, using a transcriptional secDF-lacZ fusion (3). Whereas secDF expression was more or less constitutive when the cells were grown in minimal medium, a maximum of secDF transcription was found in the early post-exponential growth phase when rich (TY) medium was used for growth. Interestingly, the time point of maximum secDF expression in rich medium is 1 to 2 h later in the growth phase compared to the time point of maximum secA expression.

* B. subtilis* contains at least four closely related type I signal peptidases (SipS, SipT, SipU, and SipV) which are responsible for the removal of the signal peptides from nonlipoprotein precursor proteins (48). Whereas the sipU and sipV genes are transcribed at a more or less constant level during all growth phases, the transcription of sipS and sipT increases upon transition from exponential to stationary growth. Due to their finding that the increase in sipS and sipT transcription is controlled by the DegU-DegS two-component signal transduction system, which is mainly involved in the upregulation of the synthesis of secreted precursor proteins after T₉₀, the authors (48) speculate that SipS and SipT serve to increase the capacity of *B. subtilis* for protein secretion concomitantly with the increasing amounts of secretory precursor proteins synthesized in the post-exponential growth phase. Interestingly, in contrast to secA gene expression, which is maximal at the beginning of the secretion period (approximately T₉₀), sipS and sipT transcription is relatively low at T₉₀ and increases steadily throughout the entire period of high-level protein secretion after T₉₀ (48). These findings taken together suggest that at the post-exponential growth phase, *B. subtilis* adjusts the amount of some of the central components of the protein secretion machinery in relation to the increased demand for protein export. However, differences in the timing of this adjustment seem to exist between different components.

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