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In Vivo and In Vitro Characterization of the *secA* Gene Product of *Bacillus subtilis*

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The putative amino acid sequence from the wild-type *Bacillus subtilis div*$^+$ gene, which complements the temperature-sensitive *div-341* mutation, shares a 50% identity with the sequence from *Escherichia coli secA* (Y. Sadaie, H. Takamatsu, K. Nakamura, and K. Yamane, Gene 98:101–105, 1991). The *B. subtilis div-341* mutant accumulated the precursor proteins of α-amylase and β-lactamase at 45°C as in the case of *sec* mutants of *E. coli*. The *div-341* mutation is a transition mutation causing an amino acid replacement from Pro to Leu at residue 431 of the putative amino acid sequence. The *B. subtilis div*$^+$ gene was overexpressed in *E. coli* under the control of the *tac* promoter, and its product was purified to homogeneity. The Div protein consists of a homodimer of 94-kDa subunits which possesses ATPase activity, and the first 7 amino acids of the putative Div protein were found to be subjected to limited proteolysis in the purified protein. The antiserum against *B. subtilis* Div weakly cross-reacted with *E. coli* SecA. On the other hand, *B. subtilis* Div could not replace *E. coli* SecA in an *E. coli* in vitro protein translocation system. The temperature-sensitive growth of the *E. coli secA* mutant could not be restored by the introduction of *B. subtilis div*$^+$, which is expressed under the control of the *spac-1* promoter, and vice versa. The *B. subtilis div*$^+$ gene is the *B. subtilis* counterpart of *E. coli secA*, and we propose that the *div*$^+$ gene be referred to as *B. subtilis secA*, although Div did not function in the protein translocation system of *E. coli*.

Bacillus subtilis has a strong ability to secrete extracellular enzymes into the culture medium. The secretion mechanism of these enzymes, however, has not been clarified yet in this organism. We have cloned the *B. subtilis div*$^+$ gene in a temperate *B. subtilis* phage, ρ11, which can complement the growth of the temperature-sensitive *div-341* mutant at 42°C (23). The mutant was identified as a cell division-deficient mutant under nonpermissive temperature conditions (16). At the same time, the mutation caused a deficiency in the production of extracellular enzymes, autolysis, development of competence, sporulation, and spore outgrowth. *div*$^+$ is located at map position 84° in the *B. subtilis* chromosome (24). The analysis of the cloned gene revealed that the predicted amino acid sequence from the *B. subtilis div*$^+$ gene shared a 50% identity with that of the *Escherichia coli* SecA protein (25, 28), which is one of the constituents of the protein secretion apparatus in *E. coli* (3, 21). Furthermore, highly homologous regions were observed in the NH_2-terminal quarters of the two proteins (25), in a region of *E. coli* SecA which plays important roles in protein translocation (8). Therefore, we suggested that the *B. subtilis div*$^+$ gene was the *B. subtilis* counterpart of *E. coli secA* (25).

The *E. coli secA* gene, which is located at 2.5 min on its genetic map, has been cloned by Oliver and Beckwith (21), and the gene product was found to be involved in protein translocation and cell separation (7, 21). The biochemical characteristics and the role of the *E. coli* SecA protein in the molecular mechanism of protein translocation have been studied in detail in vivo and in vitro by many investigators (3, 9, 11, 21, 30). It was demonstrated that the SecA protein plays an indispensable role in the translocation of exported proteins across the cytoplasmic membrane of *E. coli* (3, 7, 21). In order to analyze the functional similarity between the *B. subtilis* Div and *E. coli* SecA proteins in vivo and in vitro, we characterized the *B. subtilis div*$^+$ gene and its product.

In this paper, we show that the *B. subtilis div-341* mutant is defective in the secretion of extracellular α-amylase and β-lactamase. The properties of a purified *B. subtilis* Div protein were similar to those of *E. coli* SecA, but the former did not function in *E. coli* cells.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. The bacterial strains, phage, and plasmids used in this study are listed in Table 1.

Construction of plasmids pTUE855, pTUBE861, and pTUBE863. To express the *B. subtilis div*$^+$ gene in *E. coli* under the control of the *tac* promoter, a *Spe*I-*Pst*I 2.6-kb DNA fragment containing the wild-type *div*$^+$ gene was prepared from ρ11-*div*$^+$ DNA, and the fragment was inserted into the *Xba*I-*Pst*I site of an *E. coli* vector, pUC18. Then a *Bam*HI-*Pvu*II 2.7-kb fragment containing the *div*$^+$ gene was obtained from the constructed plasmid. The 2.7-kb fragment replaced with a *Bam*HI-*Sma*I 3.0-kb fragment containing *E. coli secA* in pMAN400, in which *E. coli secA* had been inserted behind the *tac* promoter. The constructed plasmid was designated pTUE855 (7.9 kb). To express *B. subtilis div*$^+$ and *E. coli secA* under the control of the *spac-1* promoter in both *E. coli* and *B. subtilis* cells, an *Eco*RI-*Bam*HI 1.7-kb fragment containing the *spac-1* promoter, multicloning sites, and *lacI* was obtained from pDH88 and was inserted into an *E. coli-B. subtilis* shuttle plasmid,

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TABLE 1. Bacterial strains, phage, and plasmids used in this experiment

Strain, phage, or plasmid	Relevant genotype or phenotype	Reference
B. subtilis		
NIG1121	*met his*	24
NIG1152	*met his div-341*	24
E. coli		
MC4100	F⁻ *araD136* Δ(*lac*)*U169 relA rpsL thi*	6
MM52	F⁻ *araD136* Δ(*lac*)*U169 relA rpsL thi secA51*(Ts)	20
K003	Lpp⁻ Δ*uncBC*-Tn*10*	32
JM109	*relA1 supE44 endA1 hsdR17 gyrA96 thi mcrA mcrB*⁺ Δ(*lac-proAB*)/F′ [*traD36 proAB*⁺ *lacI*^{q} *lacZ*ΔM15]	27
B. subtilis phage ρ11-*div*⁺	*div*⁺	23
B. subtilis plasmids		
pTUB101	Km^r, *B. subtilis amyE*⁺	31
pTUB256	Km^r, *bla* from pBR322, expressed by *amyE* promoter and signal peptide	19
E. coli plasmids		
pUC18	Amp^r	27
pDH88	Amp^r *cat*, *spac-1* promoter, *lacI*	4
pMAN400	Amp^r, *tac* promoter, *E. coli secA*⁺	7
pTUE855	Amp^r, *tac* promoter, *B. subtilis div*⁺	This work
E. coli-B. subtilis shuttle plasmids		
pHY300PLK	Amp^r and Tet^r in *E. coli*, Tet^r in *B. subtilis*	5
pTUBE1500	pHY300PLK with the DNA region for the *spac-1* promoter and *lacI* of pDH88	This work
pTUBE861	pTUBE1500 with *E. coli secA*⁺	This work
pTUBE863	pTUBE1500 with *B. subtilis div*⁺	This work

pHY300PLK. The constructed plasmid was designated pTUBE1500 (6.6 kb). Then the *Bam*HI-*Bgl*II 2.7-kb fragment containing *B. subtilis div*⁺ from pTUE855 was inserted into the *Bgl*II site of pTUBE1500, and pTUBE863 (9.3 kb) was obtained. The *Bam*HI-*Bam*HI 3.0-kb fragment containing *E. coli secA* from pMAN400 was inserted into the *Bgl*II site of pTUBE1500, and the plasmid obtained was designated pTUBE861 (9.6 kb).

Purification of the *B. subtilis* Div protein from an *E. coli* transformant. The Div protein was purified through procedures similar to those used for the purification of *E. coli* SecA (1). An *E. coli* MM52 transformant carrying pTUE855 was grown at 30°C for 6 h (reading of 400 in a Klett colorimeter) in 2.5 liters of L broth containing 50 μg of ampicillin per ml. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h, the cells were harvested by centrifugation, washed once with 10 mM K-phosphate buffer (pH 7.2), and suspended in the same buffer (80 ml). The cells were disrupted 10 times by sonic oscillations at 20 kC for 2 min in ice water, and the cell debris was removed by centrifugation (10,000 × *g*, 20 min). The sample was then centrifuged at 50,000 × *g* for 3 h, and ammonium sulfate was added to the resulting supernatant. The precipitate with $(NH_4)_2SO_4$ (40 to 50% saturation) was collected and dialyzed against 10 mM K-phosphate buffer (pH 7.2). Then it was applied to a hydroxyapatite column (2.5 by 12 cm) which had been equilibrated with the same buffer. Div was eluted with a linear gradient of 10 to 250 mM K-phosphate buffer (pH 7.2). Ten-microliter aliquots of the collected fractions were electrophoresed in the sodium dodecyl sulfate (SDS)-polyacrylamide (9%) gels, and the fractions containing Div were pooled. This sample, which was approximately 90% pure, was used in the in vitro protein translocation assay.

To determine the ATPase activity of Div, the sample was further purified by gel filtration with a TSK gel G3000 SW column (Toso Ltd., Tokyo, Japan) for high-pressure liquid column chromatography (HPLC). To prepare the rabbit antiserum against *B. subtilis* Div as shown later, the sample purified through the hydroxyapatite column was applied to a preparative SDS-polyacrylamide gel for electrophoresis and the band corresponding to Div was electroeluted (Bio-Rad electroeluter model 422).

E. coli SecA was purified from *E. coli*(pMAN400) by the same strategy as that adopted for the purification of *B. subtilis* SecA.

Preparation of rabbit antisera. Rabbit antiserum against *B. subtilis* Div was prepared in this experiment by the method described previously (14) using the purified *B. subtilis* Div preparation. Preparation of rabbit antisera against *E. coli* SecA and *B. subtilis* α-amylase was as previously described (14, 34). Rabbit antiserum against β-lactamase was kindly provided by R. H. Doi.

Pulse-chase experiments to analyze the formation of mature α-amylase and β-lactamase in *B. subtilis* cells. *B. subtilis* NIG1121 and NIG1152 harboring pTUB101 or pTUB256 were first grown at 30°C in the medium S7 (17), and when the cultures reached a reading of 80 in a Klett colorimeter, they were shifted to 45°C. After 210 min of incubation at 45°C, the synthesis and formation of the precursor and mature forms of α-amylase and β-lactamase were examined by pulse-labeling of the cells with 20 μCi of [^{35}S]methionine (Amersham Plc.) per ml for 60 s followed by chase using 200 μg of unlabeled L-methionine per ml. The labeled cultures were

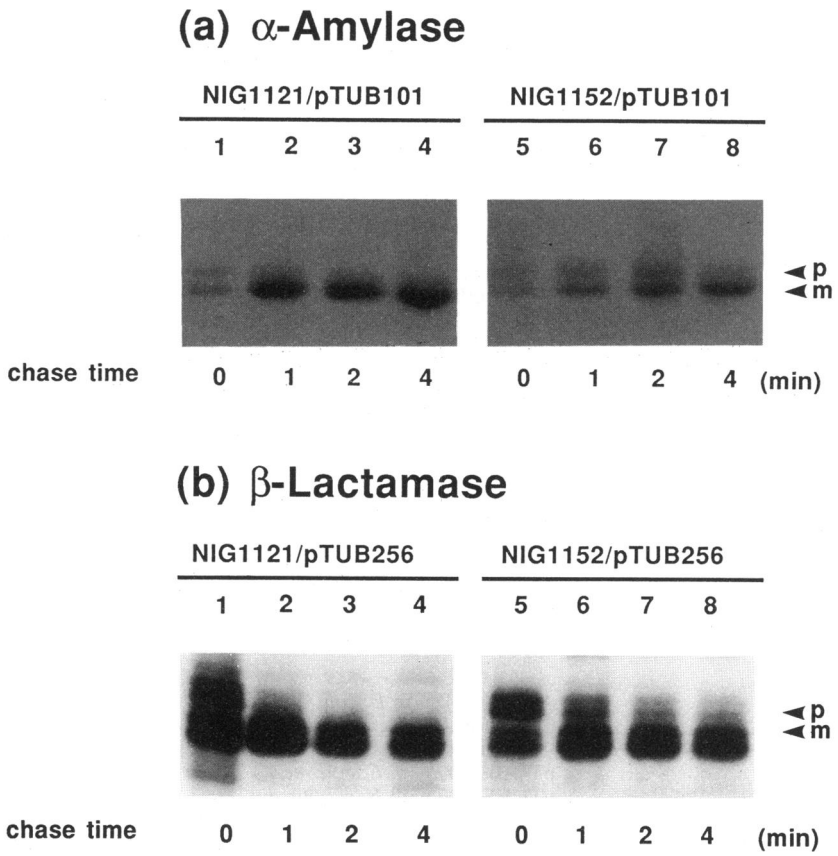

FIG. 1. Autoradiographs of SDS-polyacrylamide gels resolving immunoprecipitates by rabbit anti-α-amylase (a) and anti-β-lactamase (b) antisera from the transformants of *B. subtilis* NIG1121 and NIG1152. *B. subtilis* NIG1121 containing pTUB101, *B. subtilis* NIG1152 containing pTUB101, *B. subtilis* NIG1121 containing pTUB256, and *B. subtilis* NIG1152 containing pTUB256 were pulse-labeled with [^{35}S]methionine for 60 s and chased for 0, 1, 2, and 4 min. Samples were immunoprecipitated with the indicated antiserum and analyzed. p, precursor proteins of the two extracellular enzymes; m, mature forms.

incubated with 10 mg of egg white lysozyme per ml at 37°C for 10 min and boiled for 1 min in the presence of 0.1% SDS. The two enzymes were then precipitated with each antiserum. The immunoprecipitated materials were washed, boiled for 3 min in the presence of 1% SDS and 1% mercaptoethanol, and applied to an SDS-polyacrylamide (7.5%) gel for electrophoresis, and autoradiographs of the dried gels were made.

Assay of ATPase activity. *E. coli* SecA and *B. subtilis* Div were incubated at 37°C in 100 μl of 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM ATP, 30 mM KCl, 30 mM NH_4Cl, 5 mM $Mg(OAc)_2$, and 1 mM dithiothreitol. After incubation for appropriate periods of time, the amount of released P_i was measured by the photomeric method of Lowry and Lopez (12).

In vitro protein translocation assay system of *E. coli*. To prepare the [^{35}S]methionine-labeled OmpF-Lpp hybrid protein (33) possessing an uncleavable signal peptide as the marker of an exported protein in the *E. coli* system, mRNA for the hybrid protein was synthesized with SP6 RNA polymerase (15) and was translated by the *E. coli* system in the presence of [^{35}S]methionine as described previously (33). The synthesized ^{35}S-labeled OmpF-Lpp was purified by immunoaffinity chromatography (33). The inverted membrane vesicles were prepared from *E. coli* K003 as described previously (32).

General DNA techniques. DNA manipulations and transformation of *E. coli* were accomplished by the methods outlined by Sambrook et al. (27).

Protein concentration. Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.) with bovine serum albumin as the standard.

RESULTS

Characterization of the *B. subtilis div-341* strain as a *sec* mutant. The *div-341* mutant showed temperature-sensitive excretion of α-amylase and protease (23, 24), indicating that protein translocation may be defective in the strain. To determine whether the *div-341* mutation caused the presumed defect, the accumulation of the precursor proteins of α-amylase and β-lactamase was analyzed by pulse-chase experiments in the mutant cells at 45°C. As shown in Fig. 1a, in the wild-type strain NIG1121 containing pTUB101, the [^{35}S]methionine-labeled precursor protein of α-amylase was completely converted into its mature form during 1 min of chase, whereas the α-amylase precursor protein accumulated in the mutant strain NIG1152 containing pTUB101 and approximately 20% of the protein still remained as the precursor after 4 min of chase. The accumulation of the precursor protein was also detected for the extracellular β-lactamase in the mutant strain (Fig. 1b). This accumulation of the precursor proteins of these two extracellular enzymes

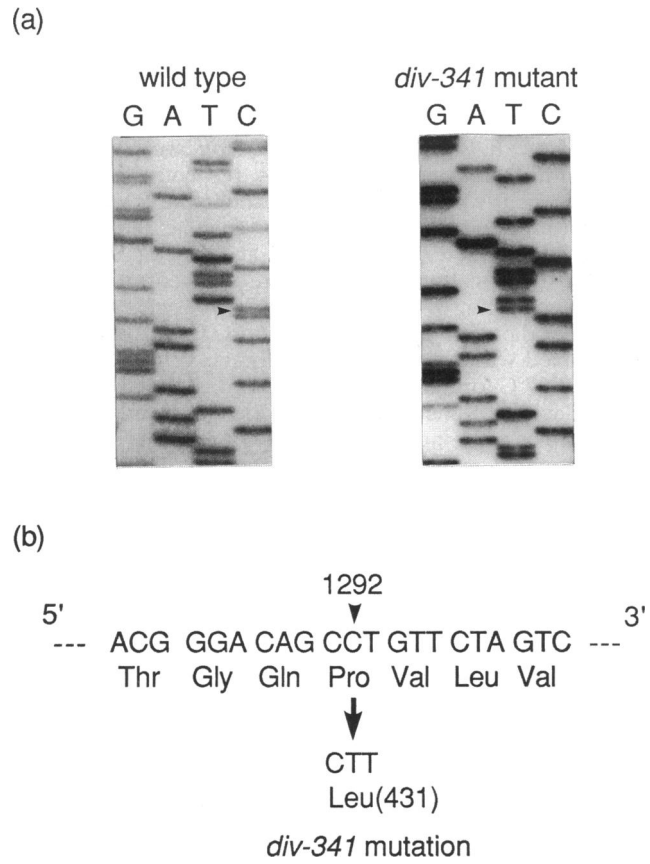

FIG. 2. Determination of the nucleotide substitution at the *div-341* mutational site. (a) Autoradiographs of DNA sequence analyses showing the different nucleotide residues (►) in the wild-type *div*$^+$ gene and the *div-341* mutant gene; (b) predicted amino acid replacement at the mutational site.

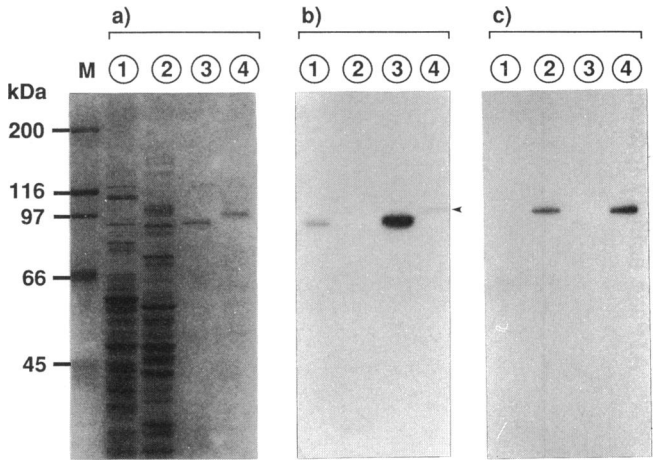

FIG. 3. Molecular masses and Western blot analyses of the *B. subtilis* Div and *E. coli* SecA proteins. After SDS-polyacrylamide (9%) gel electrophoresis, the proteins were stained with Coomassie brilliant blue (a) and blotted onto polyvinylidene difluoride membranes, and *B. subtilis* Div and *E. coli* SecA were detected with rabbit antisera against *B. subtilis* Div (b) and *E. coli* SecA (c). Lanes 1, total-protein preparation (10 μg) of *B. subtilis* NIG1121; lanes 2, total-protein preparation (10 μg) of *E. coli* MC4100; lanes 3, 0.1 μg of the *B. subtilis* Div preparation after hydroxyapatite chromatography; lanes 4, 0.1 μg of the *E. coli* SecA preparation after hydroxyapatite chromatography. Myosin (molecular mass, 200 kDa), β-galactosidase (116 kDa), phospholipase b (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa) were used as molecular mass markers. ◄ , *E. coli* SecA band weakly cross-reacted with anti-*B. subtilis* Div antiserum.

in the *B. subtilis* mutant corresponds to the characteristic phenotype of the *sec* mutants in *E. coli*. The phenotype of the *div-341* defect is some what leaky at the nonpermissive temperature, as in the cases of *E. coli sec* mutants.

Mutational site of *div-341* gene of *B. subtilis*. We isolated the *B. subtilis div*$^+$ gene by cloning a chromosomal DNA fragment which contained the transformation activity of temperature-sensitive *div-341* to temperature resistance. A *Cla*I-*Sma*I 0.8-kb fragment of the isolated gene could also transform the mutant strain as the ρ11-*div*$^+$ DNA did. However, the other parts of the gene did not. To identify the mutational site of *div-341*, the *Cla*I-*Sma*I 0.8-kb fragment containing the mutational site was synthesized from the chromosomal DNA of strain NIG1152 by the polymerase chain reaction method (26). On the basis of DNA sequencing of five independent clones of the synthesized fragment, the C residue at position 1292 from the A residue of the translation initiation site was replaced by the T residue in the five clones (Fig. 2). Therefore, it is suggested that the *div-341* mutation is caused by the replacement of a Pro (CCT) residue at amino acid position 431 for Leu (CTT) in the Div protein. The mutation sites in the temperature-sensitive *secA* mutants so far isolated in *E. coli* and *div-341* of *B. subtilis* were observed in the regions conserved in both organisms.

Characterization of *B. subtilis* Div produced by *E. coli*. To analyze the properties and functions of Div protein, the *B. subtilis div*$^+$ gene was expressed in *E. coli* under the control of the *tac* promoter. The *B. subtilis* Div protein was extracted and purified, and its properties were compared with those of *E. coli* SecA. Total proteins of *B. subtilis* and *E. coli* and the purified *B. subtilis* Div and *E. coli* SecA proteins were analyzed by SDS-polyacrylamide (9%) gel electrophoresis followed by staining with Coomassie brilliant blue (Fig. 3a) and by Western blotting (immunoblotting) (29) using antisera against *B. subtilis* Div (Fig. 3b) and *E. coli* SecA (Fig. 3c). As shown in Fig. 3a, the purity of the Div preparation after hydroxyapatite chromatography exceeded 90%, and the molecular mass was estimated to be 94,000 Da. The molecular mass was approximately 8,000 Da smaller than that of *E. coli* SecA, consistent with the predicted amino acid sequences of the two proteins. By Western blot analysis of the total proteins of the wild-type *B. subtilis* NIG1121 cells with anti-*B. subtilis* Div antiserum (Fig. 3b), we detected a band whose molecular mass was the same as that of the band for the purified Div preparation from *E. coli* (lanes 1 and 3, Fig. 3b). This observation suggested that the same Div protein molecule as that found in *B. subtilis* was synthesized in the *E. coli* cells. A band corresponding to Div was also detected in *B. subtilis* NIG1152, the *div-341* mutant (data not shown). The anti-*B. subtilis* Div antiserum weakly cross-reacted with *E. coli* SecA (Fig. 3b, lane 4). Anti-*E. coli* SecA antiserum cross-reacted very weakly with *B. subtilis* SecA, and a weak band was detected when 1 μg of Div was applied to the gel for electrophoresis (data not shown).

ATPase activity of Div. Lill et al. (9, 10) reported that *E. coli* SecA carries ATP-hydrolyzing activity. If *B. subtilis* Div corresponds to *E. coli* SecA, Div may also have ATP-hydrolyzing activity. ATPase activity was detected in the

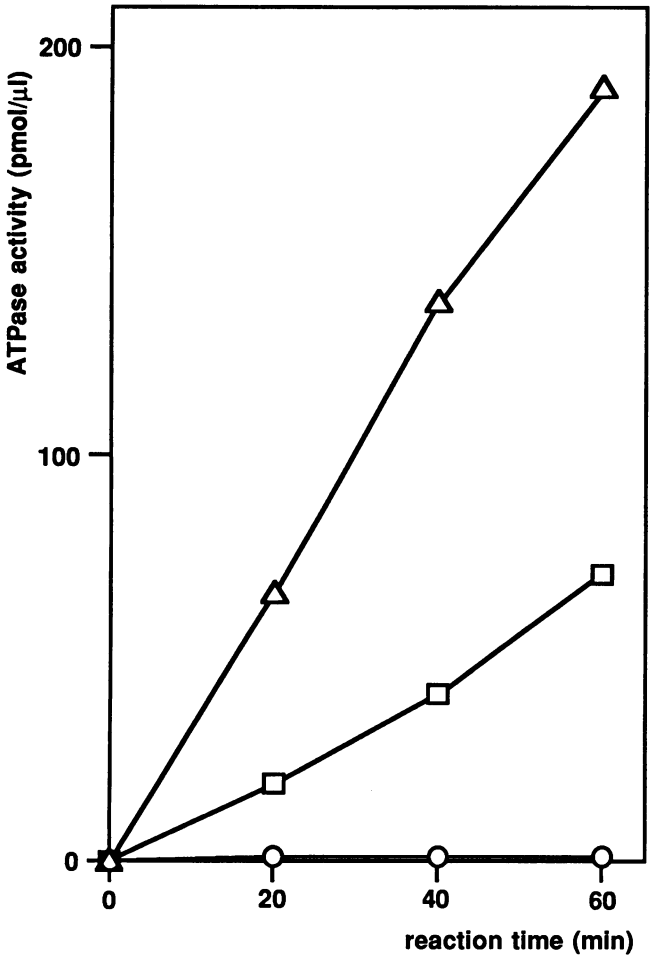

FIG. 4. ATP-hydrolyzing activity of *B. subtilis* Div and *E. coli* SecA. Aliquots containing 1 μg of *B. subtilis* Div (△) and *E. coli* SecA (□) in the major protein peaks of the gel filtration columns were incubated for the indicated periods at 37°C, and the amounts of released P_i were measured. ○, no addition.

Div preparation after HPLC, and the specific activity (picomoles of P_i liberated per microgram of each protein) was compared with that of *E. coli* SecA (Fig. 4). The specific activity of ATPase of Div was three times higher than that of *E. coli* SecA. The peaks of ATPase activity in the eluates from gel filtration by HPLC coincided with the protein peaks (Fig. 5). The molecular mass of the major peak was estimated to be approximately 200,000 Da. The difference in the molecular mass of Div estimated by gel filtration and SDS-polyacrylamide gel electrophoresis suggested that the major part of the Div protein consisted of a homodimer with a 94,000-Da subunit, as in the case of *E. coli* SecA. A minor peak of ATPase activity corresponded to a tetramer with 94,000-Da subunits (2).

NH_2-terminal amino acid sequences of *B. subtilis* Div and *E. coli* SecA proteins prepared from *E. coli* cells. The NH_2-terminal amino acid sequence of Div was analyzed with an automatic Edman degradation sequencer (Applied Biosystems model 470A) after the purified protein was blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) according to the method of Matsudaira (13). The sequence of Div was MFDPTKRTLNRY. The NH_2-terminal amino acid sequence of *E. coli* SecA was VFGSRNDRT LRR, and the SecA protein was functional in the in vitro protein translocation system as well as the nondegraded SecA was (16a). These sequences indicated that 7 and 8 amino acid residues in the predicted translation initiation sites of the *B. subtilis div*$^+$ and *E. coli secA* genes were omitted in the two purified proteins, respectively.

***B. subtilis* Div protein cannot function in the in vitro protein translocation system of *E. coli*.** To analyze the functional similarity between *B. subtilis* Div and *E. coli* SecA, their protein translocation activities were assayed in an in vitro system consisting of inverted membrane vesicles and OmpF-Lpp hybrid protein of *E. coli*. *B. subtilis* Div or *E. coli* SecA was mixed with the inverted membrane vesicles and incubated at 37°C for 5 min. Then the OmpF-Lpp hybrid protein labeled with [^{35}S]methionine was added to the mixtures, and the samples were incubated at 37°C for a further 10 min and treated with 1 mg of proteinase K per ml. The OmpF-Lpp hybrid protein incorporated into the membrane vesicles was analyzed by SDS-polyacrylamide (12.5%) gel electrophoresis. An autoradiograph of the gel is shown in Fig. 6. The presence of *E. coli* SecA stimulated the translocation of the OmpF-Lpp hybrid protein, whereas the Div protein could not stimulate the translocation. Furthermore, Div does not compete with the function of *E. coli* SecA, because the presence of an eightfold-larger amount of Div did not inhibit the protein translocation activity of *E. coli* SecA. Therefore, these results indicated that the *B. subtilis* Div protein cannot function as a substitute for *E. coli* SecA in the in vitro protein translocation system.

Introduction of the wild-type *B. subtilis div*$^+$ gene cannot complement the *E. coli secA* mutation in vivo. To analyze the functional relationships between *B. subtilis div*$^+$ and *E. coli secA* in vivo, *E. coli-B. subtilis* shuttle plasmids, pTUBE861 and pTUBE863, were constructed. The two plasmids contained the *B. subtilis div*$^+$ or *E. coli secA* gene under the control of the IPTG-inducible *spac-1* promoter (35). *B. subtilis* NIG1121 and NIG1152 and *E. coli* MC4100 and MM52 which harbored plasmid pTUBE861, pTUBE863, or pTUBE1500 were grown at 30°C for 6 h and for a further 2 h in the presence of 2 mM IPTG. Cells were harvested by centrifugation (6,000 × *g* for 10 min), and the amount of SecA proteins expressed in each strain was measured by Western blotting (Fig. 7). In the *E. coli* cells (Fig. 7a), the *B. subtilis div*$^+$ gene was highly expressed, and the content of the protein reached 7 to 10% of that of the total proteins. In the *B. subtilis* cells (Fig. 7b), the *E. coli secA* gene was also highly expressed (2 to 4% of total protein). On the contrary, the expression of the *B. subtilis div*$^+$ gene was restricted in *B. subtilis* cells. The amounts of Div protein were only two to three times higher in the *B. subtilis* transformants with pTUBE863 than those in the strains with pTUBE1500.

To determine whether introduction of the cloned *div*$^+$ and *secA* genes in the temperature-sensitive *E. coli secA51* and *B. subtilis div-341* mutants could compensate for their defective growth under nonpermissive temperature conditions, the *B. subtilis* and *E. coli* strains harboring each plasmid were grown on agar plates at 30 and 42°C in the presence of 1 mM IPTG. The growth of the *E. coli secA51* and *B. subtilis div-341* mutants resumed with the presence of their own *secA* and *div*$^+$ genes after 24 h incubation. However, the growth of the *B. subtilis div-341* mutant did not resume with the presence of the *E. coli secA* gene, and the presence of the *B. subtilis div*$^+$ gene could not complement the *E. coli secA51* mutant after 48 h of cultivation (Table 2). The

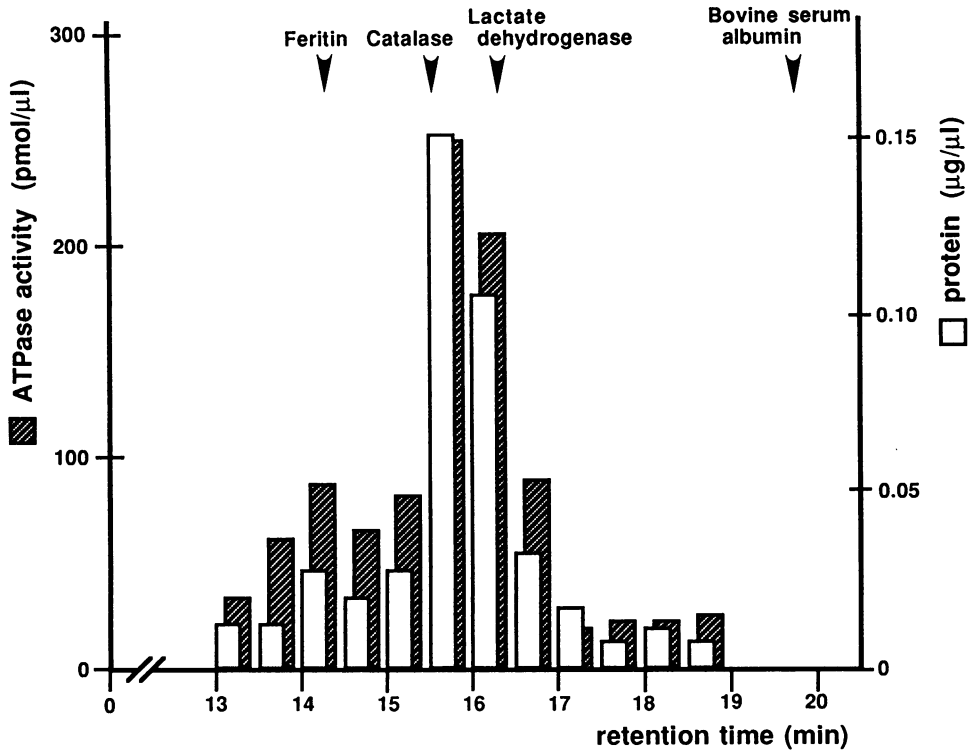

FIG. 5. Coelution of *B. subtilis* Div protein and ATP-hydrolyzing activity from gel filtration on a TSK gel G3000 SW column. The *B. subtilis* Div preparation after hydroxyapatite chromatography was dialyzed against 50 mM Tris-hydrochloride buffer (pH 7.0) containing 50 mM NaCl, 50 mM KCl, and 5 mM $MgCl_2$ and applied to the gel filtration column which had been equilibrated with the same buffer. Fractions were collected and analyzed for the determination of the total protein content and ATPase activity. The elution positions of ferritin (450 kDa), catalase (240 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (66 kDa) are indicated.

presence of the exogenous *secA* gene in *E. coli* MC4100 or *div*$^+$ in *B. subtilis* NIG1121 did not inhibit their growth at either 30 or 42°C. The growth restoration of NIG1152 by the presence of pTUBE863 was not caused by the integration of the plasmid into the chromosome, because the mutant phenotype remained after curing of pTUBE863. Eighteen of 100 colonies showed temperature-sensitive growth after 24-h cultivation of NIG1152 harboring pTUBE863, which had

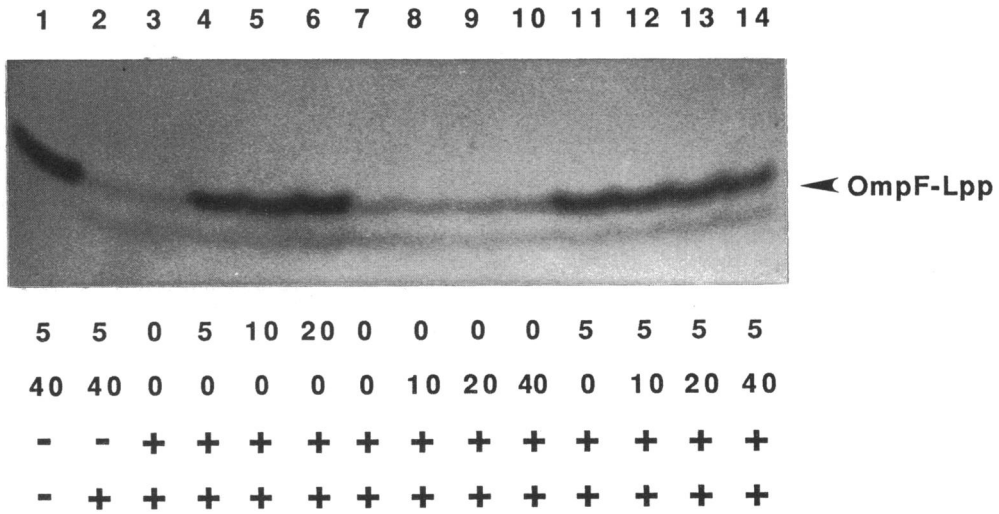

FIG. 6. Accumulation of [^{35}S]methionine-labeled OmpF-Lpp hybrid protein in the inverted membrane vesicles by *E. coli* SecA and *B. subtilis* Div. The indicated amounts of *E. coli* SecA and/or *B. subtilis* Div were incubated in 30 μl of reaction mixtures containing 2.5 μg of the inverted membrane vesicles, 80,000 dpm of [^{35}S]methionine-labeled OmpF-Lpp hybrid protein, 50 mM K-phosphate (pH 7.5), 5 mM $MgSO_4 \cdot 7H_2O$, 5 mM ATP, and 5 mM NADH. After treatment with proteinase K, incorporated ^{35}S-labeled protein was precipitated by 10% trichloroacetic acid, dried, and analyzed by SDS-polyacrylamide (12.5%) gel electrophoresis.

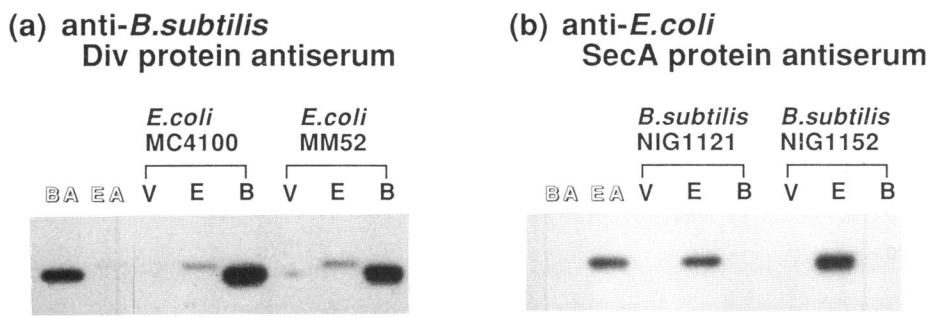

FIG. 7. Hyperexpression of *B. subtilis div*$^+$ in *E. coli* MC4100 and MM52 (a) and of *E. coli secA* in *B. subtilis* NIG1121 and NIG1152 (b). (a) The *E. coli* transformants (lanes V, carrying pTUBE1500; lanes E, carrying pTUBE861; lanes B, carrying pTUBE863) were grown at 30°C for 2 h in the presence of 2 mM IPTG after 6 h of cultivation at 30°C. The cells were harvested, washed, treated with 4 mg of lysozyme per ml for 10 min at room temperature, and boiled for 3 min in the 1% SDS–1% mercaptoethanol solution. The samples were analyzed by Western blotting using anti-*B. subtilis* Div antiserum. (b) The *B. subtilis* transformants were grown under the same conditions as those for the *E. coli* transformants, and expressed *E. coli* SecA was also analyzed by Western blotting using anti-*E. coli* SecA antiserum. Lanes BA, purified *B. subtilis* Div preparation; lanes EA, purified *E. coli* SecA preparation.

been grown at 42°C, in L broth at 30°C. On the basis of these results, we concluded that the *B. subtilis div*$^+$ gene could not complement the *E. coli secA51* mutation and *E. coli secA* could not complement the *B. subtilis div-341* mutation. After the experiments, pTUBE863 extracted from the *E. coli* MM52 transformants can complement the growth of *B. subtilis div-341* at the nonpermissive temperature.

DISCUSSION

In a previous paper (25), we suggested that the *B. subtilis div*$^+$ gene is a homolog of *E. coli secA*, on the basis of the homology in the predicted amino acid sequences from the cloned *B. subtilis div*$^+$ and *E. coli secA* genes and the similarity of their mutant phenotypes, although their map positions and the gene organization around the two genes differed in each chromosome. In this experiment, we showed that the mutational site of *div-341* was located at the position Pro-431 in the *B. subtilis div*$^+$ gene, where Pro (CCT) was replaced by Leu (CTT). The *div-341* mutant strain, *B. subtilis* NIG1152, exhibited a protein transport defect in the pulse-chase experiments of extracellular α-amylase and β-lactamase similar to those for the membrane and periplasmic proteins in the protein secretion-deficient mutants of *E. coli secA* gene (20). On the basis of these results, we concluded that the *div*$^+$ gene of *B. subtilis* is the counterpart of the *E. coli secA* gene. This conclusion was also supported by the fact that the *B. subtilis* Div (SecA) protein could be purified through procedures similar to those for the purification steps of *E. coli* SecA and that the purified *B. subtilis* Div (SecA) protein carried ATPase activity. Furthermore, the anti-*B. subtilis* Div (SecA) antiserum weakly cross-reacted with *E. coli* SecA, and the anti-*E. coli* SecA antiserum cross-reacted with *B. subtilis* Div (SecA), though very weakly. Therefore, we would like to refer to the *B. subtilis div*$^+$ gene as *B. subtilis secA* hereafter and suggest that the name should be changed.

TABLE 2. Effect of expression of *B. subtilis div*$^+$ and *E. coli secA*$^+$ on the growth of *E. coli* MC4100 and MM52 and *B. subtilis* NIG1121 and NIG1152

Strain and genotype	Plasmid carried	Growth at [a]:			
		30°C		42°C	
		+IPTG	−IPTG	+IPTG	−IPTG
E. coli MC4100 (*secA*$^+$)	pTUBE1500	+	+	+	+
	pTUBE861	+	+	+	+
	pTUBE863	+	+	+	+
E. coli MM52 [*secA51*(Ts)]	pTUBE1500	+	+	−	−
	pTUBE861	+	+	+	+
	pTUBE863	+	+	−	−
B. subtilis NIG1121 (*div*$^+$)	pTUBE1500	+	+	+	+
	pTUBE861	+	+	+	+
	pTUBE863	+	+	+	+
B. subtilis NIG1152 [*div-341*(Ts)]	pTUBE1500	+	+	−	−
	pTUBE861	+	+	−	−
	pTUBE863	+	+	+	+

[a] *E. coli* and *B. subtilis* transformants carrying each plasmid were incubated in L-broth agar plates with or without supplementation with 1 mM IPTG. Triplicates were cultured under permissive (30°C) and nonpermissive (42°C) temperature conditions. +, growth scored after 24 h; −, absence of growth scored after 48 h.

On the other hand, the *B. subtilis* SecA protein could not stimulate the incorporation of the OmpF-Lpp hybrid protein into the membrane vesicles in the in vitro protein translocation system of *E. coli*, and the presence of the *B. subtilis secA* gene in *E. coli* MM52 could not complement the growth of the *E. coli secA* mutant under nonpermissive temperature conditions. In the preliminary experiments, the *B. subtilis secA* gene under the control of the *lac* and *tac* promoters in *E. coli* MM52 could not compensate for the protein transport defect (6) that was analyzed by the processing of the precursor protein of OmpA to the mature form. These results indicate that the *B. subtilis* SecA protein did not function in the protein transport system of *E. coli*. At least two reasons for these findings can be suggested. (i) *B. subtilis* SecA could not interact efficiently with the membrane and protein constituents of the translocation system of *E. coli*, and (ii) the protein secretion system of *B. subtilis* may contain some specific constituents in addition to those found in the system of *E. coli*. However, there are other possibilities to explain nonfunction of the *B. subtilis* SecA protein in the *E. coli* in vitro protein translocation system, because the *B. subtilis* SecA was prepared from *E. coli* cells and the protein was subjected to limited proteolysis. These possibilities are less likely because *B. subtilis secA* could not complement the growth of *E. coli* MM52 at the nonpermis-

sive temperature and because the *E. coli* SecA was also subjected to similar proteolysis and functioned well in the in vitro system. We have tried several times to purify the protein from *B. subtilis* cells without success, because of the low content of the protein in the cells.

To analyze differences between *B. subtilis* and *E. coli* SecA proteins, the mutant *B. subtilis secA* genes, in which the COOH-terminal coding region was deleted at various lengths, were introduced into *E. coli* MM52. Some of the resulting transformants could growth under the nonpermissive temperature conditions (28a). Overhoff et al. also reported (22) that the presence of a DNA fragment encoding 275 amino acids of an NH_2-terminal region of a *B. subtilis* homolog of *E. coli secA* restored growth of *E. coli* MM52 at 42°C, and they also observed the recovery of the translocation defect of OmpA by the presence of the DNA fragment. We have already reported that the *B. subtilis* gene homologous to *E. coli secY* was able to complement the protein translocation defect of an *E. coli secY* mutant (*secY24*) on the basis of the formation of mature OmpA (18). Therefore, it is suggested that the major parts of the protein secretion systems in *B. subtilis* and *E. coli* are conserved but that the two systems contain minor differences.

In the in vitro protein translocation experiments, the signal peptide and inverted membrane vesicles used were derived from *E. coli*. Therefore, it is possible that the *B. subtilis* SecA could not function at all in the in vitro system. To analyze the function of the *B. subtilis* SecA protein in the secretion of extracellular enzymes, we have been developing a *B. subtilis* in vitro protein translocation system.

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