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Published in:
Molecular Microbiology

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 268, No. 6, Issue of February 25, pp. 4504–4510, 1993
Printed in U.S.A.

Lysine 106 of the Putative Catalytic ATP-binding Site of the *Bacillus subtilis* SecA Protein Is Required for Functional Complementation of *Escherichia coli secA* Mutants *in Vivo**

(Received for publication, July 31, 1992)

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The SecA protein is a major component of the cellular machinery that mediates the translocation of proteins across the *Escherichia coli* plasma membrane. The *secA* gene from *Bacillus subtilis* was cloned and expressed in *E. coli* under the control of the *lac* or *trc* promoter. The temperature-sensitive growth and secretion defects of various *E. coli secA* mutants were complemented by the *B. subtilis* SecA protein, provided the protein was expressed at moderate levels. Under overproduction conditions, no complementation was observed. One of the main features of the SecA protein is the translocation ATPase activity which, together with the protonmotive force, drives the movement of proteins across the plasma membrane. A putative ATP-binding motif can be identified in the SecA protein resembling the consensus Walker A type motif. Replacement of a lysine residue at position 106, which corresponds to an invariable amino acid residue, in the consensus motif by asparagine (K106N) resulted in the loss of the ability of the *B. subtilis* SecA protein to complement the growth and secretion defects of *E. coli secA* mutants. In addition, the presence of the K106N SecA protein interfered with protein translocation, most likely at an ATP-requiring step. We conclude that lysine 106 is part of the catalytic ATP-binding site of the *B. subtilis* SecA protein, which is required for protein translocation *in vivo*.

The SecA protein, a peripheral protein of the cytoplasmic membrane, plays a crucial role in *Escherichia coli* protein export (Oliver *et al.*, 1990; Wickner *et al.*, 1991). SecA is required for the productive binding of precursor proteins to the translocation sites in the plasma membrane (Cunningham *et al.*, 1989). In addition, the SecA protein has an ATPase activity (Lill *et al.*, 1989), which mediates the initial channeling of precursor proteins into the export pathway. Binding of ATP to SecA allows limited translocation of approximately 20 amino acid residues of the precursor protein, which, upon hydrolysis of the bound ATP, is released from the SecA protein (Schiebel *et al.*, 1991). Translocation is completed with the protonmotive force as the main driving force (Schiebel *et al.*, 1991; Driessen, 1992a, 1992b) and requires SecY (Ito, 1984), SecE (Schatz *et al.*, 1989), SecD, and SecF (Gardel *et al.*, 1990), which are thought to constitute the integral membrane part of the preprotein translocase (Wickner *et al.*, 1991).

Compared with the situation in *E. coli*, much less is known about the mechanism of protein secretion in Gram-positive bacteria (Freudl, 1992), although these organisms (*e.g. Bacillus* species) are widely used for the production of extracellular enzymes (Priest, 1977). The recent identification of *Bacillus subtilis* genes that code for homologues to the *E. coli* SecA (Overhoff *et al.*, 1991; Sadaie *et al.*, 1991) and SecY (Suh *et al.*, 1990; Nakamura *et al.*, 1990) proteins strongly suggested that the export machinery of *B. subtilis* might involve components that are structurally and functionally related to the *E. coli* Sec proteins.

The *B. subtilis secA* gene (also termed *div*) encodes an 841-amino acid residue polypeptide, which is 53% identical to the *E. coli* SecA protein (Sadaie *et al.*, 1991). Among the most conserved regions, amino acid sequences can be identified in both of the SecA proteins corresponding to the consensus motifs A and B, which can be found in ATP-binding proteins and which are thought to constitute the nucleotide-binding fold (Walker *et al.*, 1982; Gill *et al.*, 1986). The regions that correspond to motif A (^{102}MKTGEGKT^{109} in *E. coli* SecA; ^{100}MKTGEGKT^{107} in *B. subtilis* SecA) and motif B (^{201}RKLHYALVDEVDSILID^{217} in *E. coli* SecA; ^{199}RPLHFAVIDEVDSILID^{215} in *B. subtilis* SecA) are distinct from other regions that have also been suggested to be involved in ATP binding by *E. coli* SecA (Oliver *et al.*, 1990) but which show only limited homologies to the consensus sequences proposed by Walker *et al.* (1982).

In this study, we show that the *B. subtilis* SecA protein can complement the growth defects of various temperature-sensitive *E. coli secA* mutants. In addition, translocation of proOmpA at the nonpermissive temperature could be restored in these mutants by the *B. subtilis* SecA polypeptide. Characterization of *B. subtilis* SecA mutants, which are altered in the region corresponding to the ATP-binding site consensus motif A (Walker *et al.*, 1982; Gill *et al.*, 1986), strongly suggests that this region (amino acid residues 100–107) is part of the catalytic ATP-binding site required for the function of the *B. subtilis* SecA protein *in vivo*.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Phages, and Growth Media—Unless indicated otherwise, strains were grown in L-broth or on L-agar (Miller, 1972) supplemented with 50 μg of ampicillin/ml, 15 μg of tetracycline/ml,

* This work was supported by a grant from the Bundesministerium für Forschung und Technologie (to M. K., K.-L. S., and R. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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0.5% (w/v) glucose, 1 mM ONPF,[1] or 1 mM IPTG, as required. For the labeling experiments (see below), minimal salts medium (Vogel and Bonner, 1956) supplemented with 1.5% methionine assay medium (Difco) and 1 μg of thiamine/ml was used. Chromosomal DNA was isolated from *B. subtilis* ATCC6057. Cloning, plasmid constructions, and SecA overproduction were done in *E. coli* JM109 (*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* Δ(*lac-proAB*) [F′, *traD36, proAB, lacI*^{q}Δ*M15*]) (Yanish-Perron *et al.*, 1985). Phages of the M13 series (Messing and Vieira, 1982; Yanish-Perron *et al.*, 1985), propagated on JM109, were used for DNA sequencing. Uracil-containing single-stranded template DNA for oligonucleotide-directed mutagenesis was obtained by growing the corresponding M13 phages on *E. coli* BW313 (*dut, ung, thi, relA, spoT* [F′, *lysA*]) (Kunkel, 1985) in YT medium (Miller, 1972) supplemented with 0.3 μg of uridine/ml. SecA complementation experiments were done in *E. coli* strains MM52 (MC4100, *secA51*^{ts}) (Oliver and Beckwith, 1981), BA13 (MC4100, *secA13*^{am}, *supF*^{ts}, *zch::Tn10*) (Cabelli *et al.*, 1988), MM66 (geneX^{am}, *supF*^{ts}) (Oliver and Beckwith, 1982), and JM105.1 (*secA51*^{ts}, *leu::Tn10, thi, rpsL, endA, sbcB15, hspR4,* Δ(*lac-proAB*), [F′, *traD36, proAB, lacI*^{q}Δ*M15*]). JM105.1 was constructed in the following way. The *secA51*^{ts} allele from *E. coli* MM54 (MC4100, *secA51*^{ts}, *phoR, leu::Tn10*) (Oliver and Beckwith, 1981) was transferred into JM105 (Yanish-Perron *et al.*, 1985) by transduction with P1vir (Miller, 1972) and selected for tetracycline resistance. JM105.1 was obtained by screening the transductants for lack of growth and for defective proOmpA translocation at 42 °C.

DNA Techniques—PCR amplification was essentially done as described (Erlich, 1989). The PCR reactions contained 20 pmol of each of the PCR primers, 10 ng of linearized plasmid, 1 × PCR buffer (10 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 5% dimethyl sulfoxide) (Chamberlain *et al.*, 1990), and 2.5 units of Taq DNA polymerase (Boehringer Mannheim). Amplification was performed for 30 cycles using a programmable thermal cycler (Perkin-Elmer Cetus Instruments). Cycles consisted of 2 min at 94 °C, 2 min at 55 °C, and 1 min at 72 °C, respectively. Isolation of chromosomal DNA, preparation of plasmid and phage M13 DNA, Southern blotting, and other DNA techniques were according to standard procedures (Maniatis *et al.*, 1982). For DNA sequencing, the dideoxy chain termination method (Sanger *et al.*, 1977) was used.

Molecular Cloning and Plasmid Constructions—Plasmid pBO1 (see Fig. 1), which contains a DNA fragment encoding the 364 amino-terminal amino acid residues of the *B. subtilis* SecA protein, has been described previously (Overhoff *et al.*, 1991). In order to obtain an overlapping DNA fragment encoding the carboxyl-terminal part of the *B. subtilis* SecA protein, chromosomal DNA from *B. subtilis* ATCC6057 was digested with *Acc*I and a 2.66-kb fragment that hybridized to the digoxigenin-labeled 1.2-kb *Spe*I-*Asp*718 fragment from pBO1 was cloned in the *Sma*I site of plasmid pUC18 after filling in with Klenow polymerase. The resulting plasmid was designated pMKL0 (see Fig. 1). For the construction of a *B. subtilis secA* gene which can be expressed in *E. coli*, the gene was placed under the control of the *lac* regulatory elements. The 1.2 kb *Spe*I/*Xba*I fragment from pBO1 was ligated into the *Xba*I site of plasmid pUC18, resulting in pSecA3 (see Fig. 1). From pSecA3, a 1.2-kb *Sph*I-*Asp*718 fragment was isolated and cloned into pUC19, which had been linearized with the same restriction enzymes. The resulting plasmid, pMKL1, carries a *B. subtilis secA* fragment encoding the 364 amino-terminal amino acid residues under the control of the *lac* promoter/operator. To complete the *secA* gene, a 2.3-kb *Cla*I/*Asp*718 fragment from pMKL0, encoding amino acid residues 275–841 of the *B. subtilis* SecA protein (Sadaie *et al.*, 1991), was ligated into pMKL1, from which the small *Cla*I/*Asp*718 fragment had been deleted. The resulting plasmid, pMKL4 (see Fig. 1) harbors the completed *B. subtilis secA* gene under the regulatory control of the *lac* promoter/operator. The *B. subtilis secA* gene was also subcloned into the expression vector pTRC99A (Amann *et al.*, 1988), which, in addition to the *trc* promoter/*lac* operator regulatory elements, contains the constitutively expressed gene (*lacI*^{q}) encoding the *lac* repressor. A 2.63-kb *Pst*I fragment from pMKL4 was ligated into *Pst*I-digested pTRC99A, resulting in plasmid pMKL40 (see Fig. 1). Plasmid pMF8 contains most of the geneX-*secA-mutT* operon of *E. coli* (Schmidt and Oliver, 1989). In order to place the *E. coli secA* gene under the control of the *lac* regulatory elements, two overlapping PCR fragments were prepared using pMF8 as template. The sequences of the corresponding PCR primers are shown in Table I. Primers OMKL13 and OMKL25 possess *Eco*RI or *Xba*I restriction sites at their respective 5′-ends (Table I and see Fig. 2). The primers OMKL10 and OMKL12 hybridize to a region of the *E. coli secA* gene, which contains a unique *Asp*718 restriction site encompassing the codons for amino acid residues Gly^{557} and Thr^{558} (Table I and see Fig. 2). Using OMKL12 and OMKL13 as primers, a 1.06-kb DNA fragment was amplified from pMF8. After purification, this 1.06-kb fragment was digested with *Asp*718 and *Eco*RI and ligated into *Asp*718/*Eco*RI-digested pUC19, yielding pMKL16. The 1.68-kb DNA fragment, which was obtained by using OMKL25 and OMKL10 as primers, was cleaved with *Asp*718 and *Xba*I and ligated into Asp^{718}/*Xba*I-digested pMKL16. The resulting plasmid (pMKL18) harbors the *E. coli secA* gene under the regulatory control of the *lac* promoter/operator (see Fig. 2). All DNA manipulations were confirmed by DNA sequencing (Sanger *et al.*, 1977).

Oligonucleotide-directed Mutagenesis—The procedure used for site-directed mutagenesis of the *B. subtilis secA* gene was basically the same as described by Klose *et al.* (1988). The mismatch oligonucleotides, the resulting amino acid substitutions, and the designation of the corresponding *secA* alleles are shown in Table I. The oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (model 380) using phosphoramidite chemistry. The respective mismatch oligonucleotides were annealed to uracil-containing single-stranded template DNA of phage M13mp19 containing the 1.2-kb *Sph*I/*Asp*718 restriction fragment of pMKL1, which codes for the 364 amino-terminal amino acid residues of the *B. subtilis* SecA protein. The complementary strand was synthesized by T4 DNA polymerase using the mismatch oligonucleotide as primer. The resulting double-stranded phage DNA was transfected into JM109, and DNA from the phage clones was screened for the presence of the desired mutations by DNA sequencing (Sanger *et al.*, 1977). The *Sph*I/*Asp*718 restriction fragment, isolated from the replicative form of the phage DNA, was ligated into pMKL4 from which the wild type fragment had been removed. The resulting plasmids pMKL20 and pMKL21 harbor the *secA(K101N)* and *secA(K106N)* alleles, respectively. Plasmids pMKL200 and pMKL210 were constructed by cloning the 2.63-kb *Pst*I fragments of pMKL20 and pMKL21 into the expression vector pTRC99A, respectively. In the final plasmids, the presence of the respective mutations was confirmed again by DNA sequencing (Sanger *et al.*, 1977).

Pulse-Chase Experiments and Immunoprecipitation—For radiolabeling, cells were grown in minimal salts medium containing glucose (0.5%) at 30 °C to approximately 5 × 10^6 cells/ml and divided into two equal portions. One portion was shifted to 42 °C for 2 h, and the other portion was further incubated at 30 °C. Prior to labeling, the cells were washed once with minimal salts medium and resuspended in minimal salts medium supplemented with 0.5% (w/v) glucose, 1 mM ONPF, or 1 mM IPTG, as required. After additional incubation at the respective temperatures for 30 min, the $A_{600\,nm}$ was adjusted to 0.3. The cells (2 ml) were labeled with 15 μCi of [^{35}S]methionine (1130 Ci/mmol; Du Pont-New England Nuclear) for 1 min and chased with an excess of nonradioactive methionine. Samples (500 μl) were withdrawn after various time intervals and immunoprecipitated with anti-OmpA serum as described previously (Overhoff *et al.*, 1991).

Miscellaneous Techniques—The procedure used for the purification of the *B. subtilis* SecA protein will be described elsewhere. Rabbit antiserum against the *B. subtilis* SecA protein was prepared as described (Harlow and Lane, 1988). For the electrophoretic separation of proteins, Laemmli-type polyacrylamide gels were used (Laemmli, 1970). Immunoblotting was performed as outlined previously (Overhoff *et al.*, 1991).

RESULTS

Expression of the B. subtilis secA Gene in E. coli—For the functional expression of the *B. subtilis secA* gene in *E. coli*, the promoterless *B. subtilis secA* gene was placed under the control of the *lac* regulatory elements of the cloning vector pUC19. Cells of *E. coli* JM109, harboring the corresponding plasmid pMKL4 (Fig. 1), were induced with IPTG for 3 h, and total cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis. Compared with the uninduced culture and with control cells, massive overproduction of a protein (approximately 100-fold the amount of SecA protein found in *E. coli* wild type cells; up to 5% of the total cellular protein) that migrates with an apparent molecular weight of 95,000 was

[1] The abbreviations used are: ONPF, *ortho*-nitrophenyl-β-D-fucoside; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PCR, polymerase chain reaction; kb, kilobase.

TABLE I

Mismatch oligonucleotides and PCR amplification primers

Mismatch primers used for oligonucleotide-directed mutagenesis, the resulting amino acid substitutions, and the designation of the corresponding *B. subtilis secA* alleles are shown. The mismatch bases are underlined. Numbers indicate the position in the protein. Primers used for the amplification of the *E. coli secA* gene fragments are shown. Relevant endonuclease restriction sites are indicated below the nucleotide sequence.

		Amino acid substitution	*B. subtilis secA* allele
Mismatch oligonucleotides			
OMKL3	5′-GCG GAA ATG AAA ACA GGG GAA CGC CTT TAC TTG TGT CCC CTT	$Lys^{101} \rightarrow Asn$	*K101N*
OMKL4	5′-GGG GAA GGG AAA ACA TTA ACG CCC CTT CCC TTG TGT AAT TGC	$Lys^{106} \rightarrow Asn$	*K106N*
PCR amplification primers			
OMKL10	5′-AC GCT CGG TAC CGA TGA TAT		
OMKL12	5′-TAC ATC GGT ACC GAG CGT CAC		
OMKL13	5′-ATC GAT GAA TTC GGC GCA GAA TCC TGC EcoRI		
OMKL25	5′-TAC GAA TCT AGA ATT ATG CTA ATC AAA XbaI		

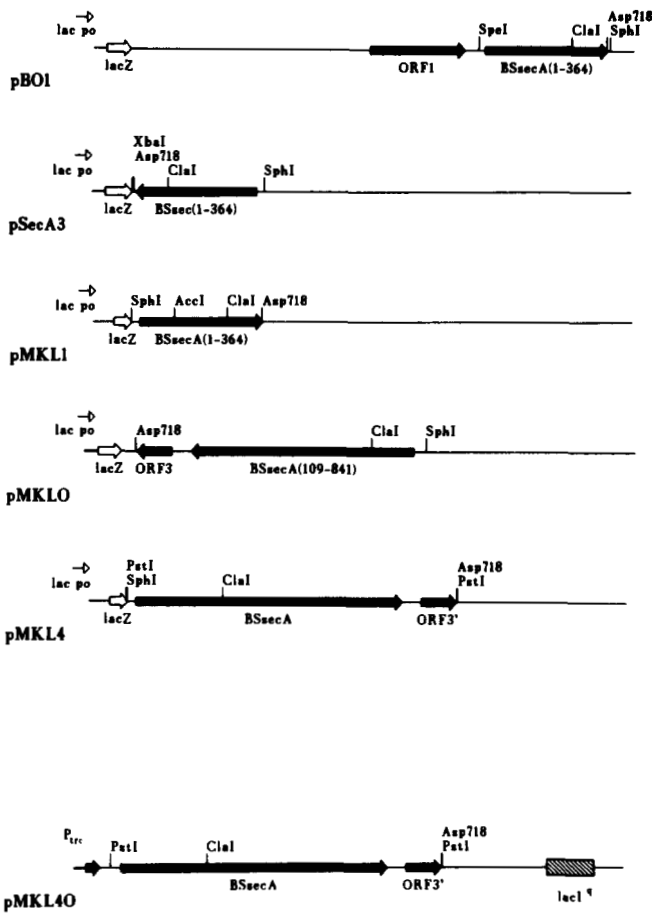

FIG. 1. **Structure of plasmids containing *B. subtilis* secA genes.** *Numbers*, amino acid positions in the respective proteins; *thin line*, vector DNA; *black arrows*, *B. subtilis* genes; *open arrows*, gene encoding the β-galactosidase α-peptide; *hatched box*, gene (*lacI*^{q}) encoding the *lac* repressor; *lac po*, *lac* promoter/operator; P_{trc}, trc promoter/lac operator; *ORF1* and *ORF3*, genes that code for open reading frames located upstream and downstream of the *secA* gene in the *B. subtilis* chromosome (Sadaie *et al.*, 1991); *BSsecA*, *B. subtilis secA* gene.

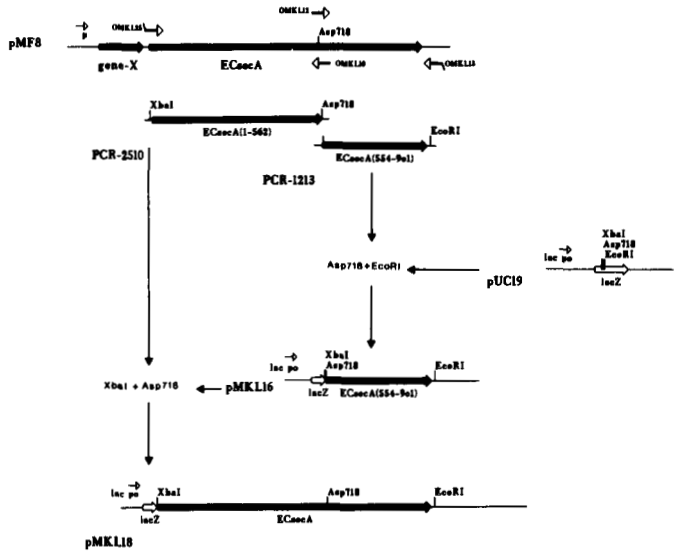

FIG. 2. **Construction of pMKL18.** *Numbers*, amino acid positions in the respective proteins; *thin line*, vector DNA; *black arrows*, genes of the geneX-*secA*-*mutT* operon; *open arrows*, gene encoding the β-galactosidase α-peptide; *lac po*, *lac* promoter/operator; *p*, promoter of the geneX-*secA*-*mutT* operon; *ECsecA*, *E. coli secA* gene. The positions of the PCR primers are indicated by *small arrows*.

observed (see Fig. 3*A*). The size of this polypeptide was in good agreement with the molecular weight calculated from the DNA sequence (Sadaie *et al.*, 1991). Despite this massive overproduction, no adverse effects on cell growth and viability of the respective host bacteria could be detected. The *B. subtilis* SecA protein was recognized by antibodies raised against *E. coli* SecA protein (see Fig. 3*B*). Vice versa, the *E. coli* SecA protein was recognized by antibodies directed against the purified *B. subtilis* polypeptide (see Fig. 3*C*). The *B. subtilis* SecA protein was also detected, although at much lower concentrations (approximately 2–5-fold the amount of SecA protein found in *E. coli* wild type cells), when the pMKL4-containing cells had been grown under noninduced conditions (*i.e.* in the presence of 0.5% glucose) (see Fig. 3). Most likely, this residual expression is due to the leakiness of the *lac* regulatory elements under repressed conditions. Similar results were obtained with the *B. subtilis secA* gene cloned in the expression vector pTRC99A (pMKL40) (see Fig. 3).

To allow a direct comparison, pMKL18, which harbors the *E. coli secA* gene under the control of the *lac* regulatory elements, was constructed (Fig. 2). Under all conditions, the amount of *E. coli* SecA protein produced in pMKL18-containing cells was comparable to the amount of *B. subtilis* SecA found in pMKL4- or pMKL40-containing cells. The *E. coli*

SecA protein is easily distinguished from the *B. subtilis* polypeptide by its slower electrophoretic mobility (Fig. 3).

The B. subtilis SecA Protein Complements the Growth Defects of Temperature-sensitive E. coli secA Mutants—The temperature-sensitive *E. coli secA* mutant strains MM52 (*secA51*^{ts}) (Oliver and Beckwith, 1981), BA13 (*secA*^{am}, *supF*^{ts}) (Cabelli *et al.*, 1988), and MM66 (geneX^{am}, *supF*^{ts}) (Oliver and Beckwith, 1982) were used to test whether the *B. subtilis* SecA protein could functionally replace the *E. coli* SecA polypeptide. After a shift to the nonpermissive temperature (42 °C), full-length, although nonfunctional, SecA protein is synthesized in MM52 due to the presence of the *secA51*^{ts} allele, which codes for a SecA protein in which leucine 43 has been replaced by proline (Oliver and Beckwith, 1982; Schmidt *et al.*, 1988). In BA13, a truncated amino-terminal SecA fragment is synthesized at the nonpermissive temperature due to an amber stop codon early in the *secA* gene (Cabelli *et al.*, 1988). However, this fragment is very unstable and can be detected only in very low amounts (Cabelli *et al.*, 1988, 1991). In contrast, no SecA protein is synthesized in *E. coli* MM66 at 42 °C (Oliver and Beckwith, 1982) due to the strong polar effect caused by an amber stop codon in geneX, the gene preceding *secA* in the geneX-*secA*-*mutT* operon (Schmidt *et al.*, 1988). In all cases, cessation of growth at the nonpermissive temperature is a consequence of the depletion of functional SecA protein, leading to a severe defect in protein translocation.

The *E. coli secA* mutant strains were transformed with plasmids pUC19 (vector only), pMKL4 (encoding the *B. subtilis* SecA protein), or pMKL18 (encoding the *E. coli* SecA protein) and plated on LB medium containing 0.5% glucose or 1 mM IPTG, respectively. Whereas the presence of pMKL18 allowed the mutants to grow at the nonpermissive temperature on both media, pMKL4 could not support growth at 42 °C irrespective of whether the medium contained glucose or IPTG (Table II). This finding suggested that the Gram-positive SecA homologue either could not substitute for its *E. coli* counterpart or that the level of *B. subtilis* SecA protein synthesized in the *E. coli secA* mutants was not in the correct concentration range that might be required for successful complementation. In fact, the level of expression has been suggested to be an important parameter for the ability of the *B. subtilis* SecY protein to complement the translocation defect of exported proteins in an *E. coli secY* mutant (Suh *et al.*, 1990; Nakamura *et al.*, 1990).

Since *E. coli* strains MM52, BA13, and MM66 do not contain the constitutively expressed *lacI*^{q} repressor gene, it might be possible that too much *B. subtilis* SecA protein is synthesized even in the presence of glucose and in the absence of the inducer. Therefore, the *B. subtilis secA* gene was cloned into the expression vector pTRC99A under the regulatory control of the *trc* promoter/*lac* operator. In addition, pTRC99A contains a copy of *lacI*^{q}, allowing repression of the *B. subtilis secA* gene also in strains lacking *lacI*^{q}. The resulting plasmid (pMKL40; Fig. 1) was transformed into *E. coli secA* mutants MM52, BA13, and MM66, and growth was monitored on 0.5% glucose- or 1 mM IPTG-containing LB plates at 30 or 42 °C, respectively. In agreement with the results obtained with pMKL4, the *E. coli secA* mutants harboring pMKL40 did not form colonies on IPTG-containing plates at 42 °C. In contrast, pMKL40 complemented the growth defects of the *secA* mutants at 42 °C when the cells were plated on medium containing glucose and lacking IPTG (Table II). Similar results were obtained when complementation by plasmid pMKL4 was tested in *E. coli* JM105.1, which harbors a single copy of the *lacI*^{q} gene on the F′ episome (for details, see "Experimental Procedures"). Also in this case, the growth defects at 42 °C could be complemented only in the presence of glucose and in the absence of the inducer (Table II). When transcription of the *secA* gene from pMKL4 was further suppressed by addition of the anti-inducer ONPF (Jayaraman *et al.*, 1966), pMKL4 no longer supported growth of JM105.1 at 42 °C (data not shown).

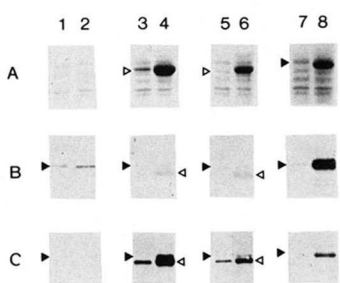

FIG. 3. **Expression of *secA* genes.** Cells of *E. coli* JM109 carrying plasmids pTRC99A (*lanes 1* and *2*), pMKL4 (*lanes 3* and *4*), pMKL40 (*lanes 5* and *6*), or pMKL18 (*lanes 7* and *8*) were pregrown in the presence of 0.5% glucose to an $A_{600\,nm}$ = 0.5. The cells were washed twice, resuspended in fresh medium containing 0.5% glucose (*odd numbered lanes*) or 1 mM IPTG (*even numbered lanes*), and incubated for 3 h at 37 °C. Total cell extracts were applied to SDS-polyacrylamide gel electrophoresis. *A*, Coomassie Brilliant Blue-stained gels; *B*, immunoblots using anti-SecA (*E. coli*) serum; *C*, immunoblots using anti-SecA (*B. subtilis*) serum. *Open arrowheads*, *B. subtilis* SecA protein; *solid arrowheads*, *E. coli* SecA protein.

The results clearly show that the *B. subtilis* SecA protein could overcome the growth defects associated with various *E. coli secA* mutants at the nonpermissive temperature when synthesized in low amounts. In contrast, overexpression of the *B. subtilis* SecA polypeptide, although without effect on cell viability at the permissive temperature, prevents growth complementation at 42 °C.

The B. subtilis SecA Protein Restores the Defective Translocation of proOmpA in E. coli secA Mutant BA13—We have previously shown that a 275-amino acid residue amino-terminal fragment of the *B. subtilis* SecA protein complemented the growth and secretion defects of the *E. coli secA* mutant MM52 (*secA51*^{ts}) (Overhoff *et al.*, 1991). A similar result was obtained by using a 239-residue amino-terminal fragment of *E. coli* SecA for the complementation of the *secA51*^{ts} mutation (Cabelli *et al.*, 1991). Complementation of the SecA51^{ts}-derived growth and secretion defects by the amino-terminal SecA fragments most likely occurred by a mechanism proposed by Matsuyama *et al.* (1990) for the reconstitution of ATP binding activity by mixing overlapping SecA fragments. However, neither the *B. subtilis* (Overhoff *et al.*, 1991) nor the *E. coli* (Cabelli *et al.*, 1991) SecA fragment complemented *E. coli* mutant BA13 (*secA*^{am}, *supF*^{ts}), showing that an amino-terminal fragment of SecA cannot substitute for the full-length protein. In contrast to MM52 (*secA51*^{ts}), no complete SecA protein is synthesized in BA13 after the shift to the nonpermissive temperature (Cabelli *et al.*, 1988).

To test whether the *B. subtilis* SecA protein allows the *in vivo* translocation of secretory proteins in the absence of a full-length *E. coli* SecA protein, pulse-chase experiments were performed in *E. coli* BA13 (*secA*^{am}, *supF*^{ts}). As shown in Fig. 4, the *B. subtilis* SecA protein (encoded by pMKL40; *lanes 11* and *12*), when synthesized under the same conditions used for the growth complementation assay (*i.e.* presence of 0.5% glucose), significantly restored processing of proOmpA in BA13 at 42 °C, although with lower efficiency than the *E. coli* SecA protein (encoded by pMKL18; *lanes 7* and *8*).

From these results and the results of the previous section, we conclude that the *B. subtilis* SecA protein can complement

TABLE II

Growth complementation of E. coli secA mutants

E. coli strains MM52 *secA51*^{ts}, JM105.1 *secA51*^{ts}, BA13 *secA*^{am}, and MM66 geneX^{am} carrying the respective plasmids were grown on LB plates supplemented with 0.5% glucose (G) or 1 mM IPTG (I). The growth temperatures were 30 or 42 °C; +, growth; (+), poor growth; −, no growth.

secA Allele	Plasmid	Strain															
		MM52				JM105.1				BA13				MM66			
		30 °C		42 °C		30 °C		42 °C		30 °C		42 °C		30 °C		42 °C	
		G	I	G	I	G	I	G	I	G	I	G	I	G	I	G	I
	pUC19	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−
	pTRC99A	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−
B. subtilis Wild type																	
	pMKL4	+	+	−	−	+	+	+	−	+	+	−	−	+	+	−	−
	pMKL40	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−
Mutant																	
K101N	pMKL200	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−
K106N	pMKL210	(+)	−	−	−	(+)	−	−	−	(+)	−	−	−	(+)	−	−	−
E. coli Wild type	pMKL18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

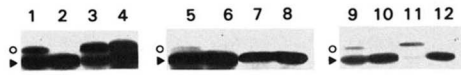

FIG. 4. **Processing of proOmpA.** Cells of BA13 (*secA*^{am}, *supF*^{ts}) containing plasmids pTRC99A (*lanes 1–4*), pMKL18 (*lanes 5–8*), or pMKL40 (*lanes 9–12*) grown in minimal salts medium containing 0.5% glucose at 30 °C (*lanes 1*, *2*, *5*, *6*, *9*, and *10*) or 42 °C (*lanes 3*, *4*, *7*, *8*, *11*, and *12*) were labeled with [^{35}S]methionine for 30 s and subsequently chased with an excess of nonradioactive methionine. Samples were withdrawn 30 s (*odd numbered lanes*) and 3 min (*even numbered lanes*) after the chase and immunoprecipitated with anti-OmpA serum as described under "Experimental Procedures." *Open circle*, OmpA precursor; *arrowhead*, mature OmpA.

FIG. 5. ***B. subtilis* SecA mutant proteins.** Amino acid residues of the SecA proteins from *E. coli* and *B. subtilis* corresponding to the ATP-binding motif A (Walker *et al.*, 1982; Gill *et al.*, 1986) are shown. *Numbers*, amino acid residues in the respective proteins; *arrows*, amino acid substitutions in the *B. subtilis* SecA mutant proteins.

the growth and secretion defects of *E. coli secA* mutant strains *in vivo*.

Alterations in the Putative Catalytic ATP-binding Site of the B. subtilis SecA Protein Result in the Loss of the Complementing Activity—Amino acid sequences that correspond to the consensus motif A, often found in nucleotide-binding proteins and supposed to be involved in ATP binding and hydrolysis (Walker *et al.*, 1982), can be identified in the *B. subtilis* SecA protein at amino acid residues 100–107 (Overhoff *et al.*, 1991; Sadaie *et al.*, 1991). The corresponding region of the *E. coli* SecA protein (Schmidt *et al.*, 1988) is highly conserved and is located between amino acid residues 102 and 109.

Since ATP binding and hydrolysis by the SecA protein has been shown to be a main feature of the SecA catalytic function during initiation of protein translocation in *E. coli* (Schiebel *et al.*, 1991), the complementing activity of the *B. subtilis* SecA protein should be lost if the catalytic ATP-binding site involved in translocation ATPase activity is altered by site-directed mutagenesis. The lysine residue at amino acid position 106 in the *B. subtilis* SecA protein, corresponding to a highly conserved amino acid residue in the G*XXXX*GK(T/S) motif (Walker *et al.*, 1982; Gill *et al.*, 1986), was changed to asparagine (pMKL210; K106N) (Fig. 5). In addition, the same alteration was introduced at an adjacent lysine residue at position 101 (pMKL200; K101N), which does not correspond to a conserved amino acid in the binding motif (Fig. 5). pMKL200 and pMKL210 were transformed into *E. coli* JM109, and expression was monitored under repressed (0.5% glucose) and fully induced (1 mM IPTG) conditions. Under all conditions, the amounts of the *B. subtilis* SecA mutant proteins were indistinguishable from the amount found for the wild type protein (data not shown).

Next, plasmids pMKL200 and pMKL210 were introduced into *E. coli secA* mutants MM52 (*secA51*^{ts}), MM66 (geneX^{am}, *supF*^{ts}), and BA13 (*secA*^{am}, *supF*^{ts}). Whereas the *E. coli secA* mutants harboring pMKL40 or pMKL200 grew normally at the permissive temperature, the pMKL210-containing cells grew poorly at 30 °C even in the presence of 0.5% glucose. Furthermore, full induction of *secA(K106N)* expression with IPTG was lethal to all *E. coli secA* mutant strains. When growth complementation was tested under conditions of low level synthesis (*i.e.* presence of 0.5% glucose), the K101N mutant protein (pMKL200), like the *B. subtilis* wild type SecA protein (pMKL40), restored growth of all *E. coli secA* mutants tested. In contrast, no growth was observed at 42 °C in the *secA* mutants expressing the K106N SecA mutant protein (pMKL210) (Table II). Furthermore, the ability of the *B. subtilis* SecA mutant proteins K106N and K101N to complement the secretion defects of proOmpA in the *E. coli secA* mutants parallels their ability for growth complementation (data not shown).

From these results, we conclude that the presence of lysine 106 is crucial for the complementation of *E. coli secA* mutants by the *B. subtilis* SecA protein. Furthermore, the K106N mutant SecA protein impairs growth of the *E. coli secA* mutant strains even at the permissive condition.

The B. subtilis SecA (K106N) Protein Interferes with the Translocation of proOmpA in E. coli MM52 (secA51^{ts}*)*—Since expression of the *B. subtilis secA* (K106N) gene negatively affects growth of *E. coli secA* mutant strains, it might be possible that the *B. subtilis* K106N SecA protein interferes with the translocation of proteins across the plasma membrane. In fact, pulse-chase experiments (Fig. 6) clearly demonstrated that such an interference by the K106N mutant

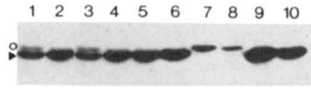

FIG. 6. **Interference by the K106N SecA mutant protein.** Cells of MM52 (secA51^{ts}) containing plasmids pTRC99A (*lanes 1* and *2*), pMKL40 (*lanes 3* and *4*), pMKL200 (*lanes 5* and *6*), pMKL210 (*lanes 7* and *8*), or pMKL18 (*lanes 9* and *10*) grown in the presence of 0.5% glucose at 30 °C were labeled with [^{35}S]methionine for 30 s. Samples were withdrawn 30 s (*odd numbered lanes*) and 3 min (*even numbered lanes*) after the chase with nonradioactive methionine and immunoprecipitated with anti-OmpA serum as described under "Experimental Procedures." *Open circle*, OmpA precursor; *arrowhead*, mature OmpA.

polypeptide can be detected in *E. coli* MM52 (*secA51*^{ts}) at 30 °C. In contrast to MM52 containing plasmids pTRC99A (*lanes 1* and *2*), pMKL40 (*lanes 3* and *4*), pMKL200 (*lanes 5* and *6*), or pMKL18 (*lanes 9* and *10*), massive accumulation of proOmpA, which during the chase period is not converted into mature OmpA, was found in *E. coli* MM52 containing plasmid pMKL210 (*lanes 7* and *8*). These results strongly suggest that the *B. subtilis* K106N SecA protein interferes with protein translocation at a step most likely requiring a functional catalytic ATP-binding site.

DISCUSSION

In *B. subtilis*, a gene has been identified encoding a homologue of the SecA protein (Overhoff *et al.*, 1991; Sadaie *et al.*, 1991), a central component of the *E. coli* preprotein translocase complex (Oliver *et al.*, 1990; Wickner *et al.*, 1991). *B. subtilis* strains, which harbor a temperature-sensitive mutation in the *secA* gene (also called *div*), are defective in protein translocation across the plasma membrane (Takamatsu *et al.*, 1992). In this communication, we have shown that the *B. subtilis* SecA homologue can functionally replace the *E. coli* SecA polypeptide. Furthermore, we have shown that lysine 106, which is most probably part of the high affinity catalytic ATP-binding site, is required for the activity of the *B. subtilis* SecA protein *in vivo*.

High level expression of the *B. subtilis secA* gene in *E. coli*, using the *lac* or *trc* promoter/*lac* operator, resulted in the massive appearance (up to 5% of the total cellular protein) of a 95-kDa polypeptide, which is recognized by anti-SecA (*E. coli*) antibodies. In the presence of glucose and the absence of the inducer IPTG, the *B. subtilis* SecA protein was synthesized at reduced levels comparable to the amount of chromosomally encoded *E. coli* SecA protein. Undoubtedly, both of the promoters allowed residual expression even in the presence of multiple copies of the *lacI*^{q} repressor gene. Similar levels of SecA protein were obtained when the *E. coli secA* gene, which is normally the second gene in the geneX-*secA*-*mutT* operon (Schmidt *et al.*, 1988), was expressed from the *lac* promoter under identical conditions. High level expression of the *B. subtilis secA* gene did not affect the viability of the respective host bacteria, suggesting that overproduction of the *B. subtilis* SecA protein did not interfere with protein translocation in *E. coli* wild type strains.

The *B. subtilis* SecA protein complemented the growth defects of *E. coli secA* mutants MM52, JM105.1, BA13, and MM66 at the nonpermissive temperature. Previously, it has been shown that effects, not directly related to protein translocation (*e.g.* slowing of protein synthesis by the addition of sublethal doses of chloramphenicol), could suppress the growth defect of *E. coli secA* mutant MM52 (*secA51*^{ts}) (Lee and Beckwith, 1986). However, such chloramphenicol treatment did not allow the growth of a *secA13*^{am} mutant at the nonpermissive temperature (Lee and Beckwith, 1986), suggesting that suppression of the growth defect by decreasing protein synthesis requires the presence of a full-length SecA protein. Since the *B. subtilis* SecA protein can complement the growth defects of the BA13 (*secA*^{am}) and MM66 (*geneX*^{am}) mutant strains, it seems unlikely that this complementation is due to effects related to the efficiency of protein synthesis. This is further supported by the fact that expression of the *B. subtilis secA* gene was without noticeable effects on the growth behavior of the respective host bacteria. Likewise, effects influencing the autoregulation of the *E. coli* SecA protein (Oliver *et al.*, 1990) are very unlikely to be responsible for the complementation, since no *E. coli* SecA protein can be detected in MM66 (*geneX*^{am}) at the nonpermissive temperature in the presence of the *B. subtilis* SecA protein.[2]

Successful complementation of the growth defects of the *E. coli secA* mutants required that the *B. subtilis* SecA protein was produced at reduced levels (*i.e.* in the absence of inducer). In the presence of IPTG, no complementation was observed. These findings are in agreement with results described by Takamatsu *et al.* (1992), which showed that the *E. coli secA* mutant MM52 could not be complemented by a *B. subtilis secA* gene expressed from the *spac* promoter under inducing conditions. From these experiments it has been concluded that the *B. subtilis* SecA protein could not substitute for the *E. coli* SecA protein *in vivo*. However, the results presented in this paper clearly show that the *B. subtilis* SecA protein can fully replace its *E. coli* counterpart *in vivo*, provided its concentration is below a critical value. We do not exactly know how much *B. subtilis* SecA protein is required for successful complementation. However, inspection of Fig. 3 implies that optimal *B. subtilis* SecA levels resemble the amount of *E. coli* SecA protein normally found in wild type strains. Previously, comparable levels of a 275-amino acid residue amino-terminal fragment of the *B. subtilis* SecA protein have been shown to complement the growth and secretion defects of *E. coli* MM52 (Overhoff *et al.*, 1991). However, this complementation required the presence of the mutationally altered full-length SecA protein, which is synthesized in MM52 at 42 °C in derepressed amounts; no complementation was observed in BA13 by this fragment. In contrast, the completed *B. subtilis* SecA protein complemented the growth defects of BA13 and MM66. In these *E. coli* mutant strains, no full-length SecA protein is synthesized after the shift to the nonpermissive temperature (Oliver and Beckwith, 1981; Cabelli *et al.*, 1991). In addition, the *B. subtilis* SecA protein substantially restored processing of proOmpA in BA13 at 42 °C, although with lower efficiency when compared with the *E. coli* SecA protein. Also in these experiments, overproduction of the *B. subtilis* SecA protein precluded the complementing activity. A possible explanation for the SecA concentration dependence of the complementing activity might be the blocking of a crucial step in protein translocation by high concentrations of *B. subtilis* SecA in the absence of functional *E. coli* SecA protein. Compared with *E. coli* SecA, the purified *B. subtilis* SecA protein has a reduced ability to insert into membranes and, in addition, seems to have a lower affinity for the translocation sites in the plasma membrane.[3] In the absence of functional *E. coli* SecA, the interaction of precursor-bearing *B. subtilis* SecA with the translocation sites might be competitively inhibited by an excess of precursor-free SecA protein. The higher affinity of *E. coli* SecA to the membrane and to the SecY/E protein might be the reason why an excess

[2] M. Klose, K.-L. Schimz, J. van der Wolk, A. J. M. Driessen, and R. Freudl, unpublished results.

[3] J. van der Wolk, M. Klose, E. Breukink, R. A. Demel, B. de Kruijff, R. Freudl, and A. J. M. Driessen, unpublished results.

of *B. subtilis* SecA was found to be without negative effects in *E. coli secA* wild type strains. In addition, the *E. coli* SecA protein might also have a higher affinity for typical Gram-negative signal peptides. However, the exact reason for the lack of complementation activity in the *secA* mutants overproducing *B. subtilis* SecA awaits further studies. Remarkably, all *secA* mutants could be complemented by *E. coli* SecA under overproducing conditions, the reason for which is also unknown.

Together with the protonmotive force, the ATPase activity of the SecA protein is part of the driving force that allows the translocation of proteins across the plasma membrane (Tani *et al.*, 1989; Schiebel *et al.*, 1991; Driessen, 1992a, 1992b). This so-called translocation ATPase activity requires the interaction of SecA with precursor proteins, acidic phospholipids, and the SecY/E protein (Lill *et al.*, 1989; Hendrick and Wickner, 1991). However, although various regions in the *E. coli* SecA protein have been proposed as being involved in ATP binding (Oliver *et al.*, 1990), the exact location of the catalytic ATP-binding site has not yet been determined. A sequence that corresponds to the G*XXXX*GK(T/S) motif, found in many nucleotide-binding proteins (Walker *et al.*, 1982; Gill *et al.*, 1986), can be identified in the *E. coli* and *B. subtilis* SecA proteins. In order to test whether this region is required for SecA function *in vivo*, we replaced the invariable lysine residue at position 106 of the *B. subtilis* SecA protein by asparagine (K106N). Similar alterations have been shown to result in the loss of hydrolyzing activity, although the binding of the nucleotide could still occur (Saraste *et al.*, 1990). In contrast to the wild type and the K101N SecA mutant protein, in which the invariable amino acid residues of this putative ATP-binding site remained intact, the K106N mutant protein lost its ability to complement the growth and the secretion defects of *E. coli secA* mutants. In addition, growth of the *E. coli secA* mutants at the permissive temperature was negatively affected by the K106N mutant protein even under repressed conditions. This behavior strongly suggested that the K106N protein might interfere with protein translocation, probably at an ATP-requiring step. Indeed, such interference could be clearly demonstrated in *E. coli* MM52 at 30 °C; processing of proOmpA was almost completely blocked by the K106N polypeptide. Characterization of the purified mutant protein has shown that the K106N SecA protein has lost its translocation-ATPase activity and the ability to be released from the membrane.[2] Similarly, the secretion defect in MM52 is most likely caused by the aberrant distribution of the SecA51^{ts} protein within the cell; the SecA51^{ts} polypeptide intrinsically has a substantially higher affinity to the plasma membrane than the wild type protein (Cabelli *et al.*, 1991). Therefore, the effects of the *E. coli* SecA51^{ts} protein and the *B. subtilis* K106N mutant polypeptide, both being defective in the release from the plasma membrane, might be additive, leading to a pronounced block in protein translocation.

In summary, our results clearly demonstrate that the *B. subtilis* SecA protein can complement the growth and secretion defects of *E. coli secA* mutants, indicating that the mechanism of protein secretion has been highly conserved in Gram-negative and Gram-positive bacteria. Furthermore, lysine 106 has been shown to be part of the catalytic ATP-binding site of the *B. subtilis* SecA protein, the integrity of which is required to allow effective catalytic cycles of the SecA protein during the transport process *in vivo*.

Acknowledgments—We are very grateful to D. Oliver, J. Beckwith, and B. Wickner for generous gifts of bacterial strains, plasmids, and anti-SecA (*E. coli*) antibodies. Anti-OmpA serum was a kind gift of Y.-D. Stierhof (Tübingen, Germany). We thank H. Sahm for continuous support and valuable discussions, J. Carter-Sigglow for critically reading the manuscript, and M. Spies for expert technical assistance.

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