Acquisition of azide-resistance by elevated SecA ATPase activity confers azide-resistance upon cell growth and protein translocation in *Bacillus subtilis*

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We isolated four azide-resistant *secA* mutants of *Bacillus subtilis* and found that all of them were the result of a single amino acid replacement of threonine 128 of SecA by alanine or isoleucine. In the presence of 1.5 mM sodium azide, cell growth and protein translocation of the wild-type strain were completely inhibited, but those of the azide-resistant mutant strains were not. Wild-type and two mutant SecA proteins were purified. Both the basal level and the elevated ATPase activity of the mutant SecA proteins were threefold higher than those of the wild-type SecA. The elevated ATPase activity of the SecA mutants was reduced upon the addition of 1.5 mM sodium azide by only 5–10% as compared with 40% for that of the wild-type. These results indicate that the elevated ATPase activity of the SecA mutants is resistant to sodium azide and that it is also required for the protein translocation process of *B. subtilis*.

Keywords: *Bacillus subtilis*, SecA, azide resistance, *secA* mutant, ATPase activity

INTRODUCTION

The Gram-positive bacterium *Bacillus subtilis* has a strong ability to secrete extracellular enzymes into the culture medium, but little is known about its protein translocation machinery in comparison with *Escherichia coli*. Recently, *B. subtilis* genes which code for proteins homologous to *E. coli* SecA (Sadaie *et al.*, 1991; Overhoff *et al.*, 1991), SecY (Nakamura *et al.*, 1990; Suh *et al.*, 1990) and SecE (Jeong *et al.*, 1993) have been identified, suggesting similarities between the protein translocation machinery of *E. coli* and *B. subtilis*.

The *B. subtilis* SecA protein, which consists of 841 amino acid residues, has 50% sequence identity with the *E. coli* SecA protein and, like *E. coli* SecA, has an ATPase activity (Takamatsu *et al.*, 1992). *E. coli* SecA is a peripheral membrane protein which plays a crucial role as a translocase in protein translocation (Oliver *et al.*, 1990a; Wickner *et al.*, 1991) interacting with SecY and SecE (Brundage *et al.*, 1990; Hendrick & Wickner, 1991). It interacts with the signal peptide and the mature part of the protein to be secreted (Cunningham & Wickner, 1989; Lill *et al.*, 1990), and its ATPase activity is elevated in the presence of a precursor protein, SecY/E, and phospho-

lipids, under which conditions it is called 'translocation ATPase' (Lill *et al.*, 1989). SecA couples ATP hydrolysis and protein translocation, and the translocation ATPase activity is known to be necessary for protein translocation in *E. coli* (Mizushima *et al.*, 1991). Recently, van der Wolk *et al.* (1993) and Klose *et al.* (1993) have reported that lysine 106 of *B. subtilis* SecA is important for SecA ATPase activity through their studies using mutant SecA proteins. However the role of ATPase activity in *B. subtilis* SecA is still unclear.

Sodium azide is known to inhibit the translocation ATPase activity of SecA protein and protein translocation in *E. coli* (Oliver *et al.*, 1990b). Knott & Robinson (1994) recently reported that sodium azide inhibited protein translocation across the chloroplast thylakoid membrane. Therefore, sodium azide is thought to be an inhibitor of the Sec-dependent protein translocation pathway. It does not inhibit free-state SecA and has no effect on the basal level SecA ATPase activity. In *E. coli*, mutations that confer azide-resistance are located at various positions in the *secA* gene (Oliver *et al.*, 1990b; Fortin *et al.*, 1990), and in *B. subtilis*, a gene which confers azide-resistance is known to be located near the *secA* gene on the genetic map (Henner & Hoch, 1982). Meens *et al.* (1993) have

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
<i>E. coli</i> strains		
JM109	<i>relA1 supE44 endA1 hsdR17 gyrA96 mcrA mcrB⁺ thi</i> $\Delta(lac-proAB)/F'[\textit{traD36 proAB}^+ \textit{lacI}^q \textit{lacZ}\Delta M15]$	Sambrook <i>et al.</i> (1989)
MM52	$F^- \textit{araD136} \Delta(lac)U169 \textit{relA rpsL thi secA51}^{ts}$	Oliver & Beckwith (1981)
BA13	$F^- \textit{araD136} \Delta(lac)U169 \textit{relA rpsL thi secA13}^{am} \textit{supF}^{ts}$ $\textit{xgb}::\text{Tn10}$	Cabelli <i>et al.</i> (1988)
K003	$\text{Lpp}^- \Delta\textit{uncBC}::\text{Tn10}$	Yamane <i>et al.</i> (1987)
<i>B. subtilis</i> strains		
NIG1121	<i>met his</i>	Sadaie & Kada (1985)
NIG1152	<i>met his secA341</i> ^{ts}	Sadaie & Kada (1985)
168	<i>trpC2</i>	Laboratory stock
AZ1	<i>trpC2 secAT128A</i>	This study
AZ2	<i>trpC2 secAT128 I</i>	This study
<i>E. coli</i> plasmids		
pUC18	Amp ^r , <i>lac</i> promoter	Sambrook <i>et al.</i> (1989)
pUC18H6	Amp ^r , <i>lac</i> promoter, 6xHis	H. Takamatsu and others (unpublished)
pTUE855	Amp ^r , <i>lacI</i> , <i>tac</i> promoter, <i>B. subtilis secA</i> ⁺	Takamatsu <i>et al.</i> (1992)
pTUE2004	Amp ^r , <i>lacI</i> , <i>tac</i> promoter, <i>B. subtilis secAT128A</i>	This study
pTUE2006	Amp ^r , <i>lacI</i> , <i>tac</i> promoter, <i>B. subtilis secAT128 I</i>	This study
pTUE1121	Amp ^r , 6xHis	This study
pTUE1122	Amp ^r , <i>lacI</i> , <i>tac</i> promoter, 6xHis	This study
pSI053	Amp ^r , <i>E. coli ompA</i> ⁺ , SP6 promoter	S. Matsuyama and others (unpublished)
pTUE1133	Amp ^r , <i>lacI</i> , <i>tac</i> promoter, <i>E. coli ompA245H6</i>	This study
pUSI2	Amp ^r , <i>lacI</i> , <i>tac</i> promoter	Shibui <i>et al.</i> (1988)
<i>B. subtilis</i> plasmids		
pTUB256	Km ^r , <i>bla</i> from pBR322, expressed by <i>amyE</i> promoter and signal peptide	Ohmura <i>et al.</i> (1984)
pTUB101	Km ^r , <i>B. subtilis amyE</i> ⁺	Yamane <i>et al.</i> (1984)

recently shown that translocation of OmpA in *B. subtilis* is also inhibited by sodium azide, as in *E. coli*, and suggested that it is carried out by a protein translocation machinery similar to that of *E. coli* which consists of SecA, SecE and SecY.

In this study we examined the effect of sodium azide on the ATPase activity of SecA in the protein translocation system of *B. subtilis*. We isolated and analysed four azide-resistant *secA* mutants of *B. subtilis*. All of the mutants have one amino acid replacement at threonine 128 of SecA, and their elevated ATPase activities were resistant to sodium azide.

METHODS

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

Mutagenesis of *B. subtilis*. The azide-resistant mutants of *B. subtilis* were isolated by the procedure of Adelberg *et al.* (1965). *B. subtilis* NIG1121 strain (wild-type) was cultured in penassay broth at 37 °C and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) at 50 µg ml⁻¹ at 37 °C for 1 h; 10¹⁰ cells were then spread onto 100 L-agar plates (10⁸ cells on each plate) and incubated at 37 °C overnight.

Synthetic oligonucleotides and PCR procedures. Three over-

lapping DNA regions [A: -75 to +882; B: +734 to +1644; C: +1558 to +2582 (Sadaie *et al.*, 1991)] of the *B. subtilis secA* gene of the azide-resistant mutants were prepared by PCR (Sambrook *et al.*, 1989) using the appropriate chromosomal DNA as the templates and the six DNA primers as follows: BSA-1, 5'-AGAGGTATACATGGACTAGT-3'; BSA-2, 5'-TAAGGCCTGGTTGATATGGT-3'; BSA-3, 5'-AAGCGG-AGAAGGATTACACG-3'; BSA-4, 5'-TCTGCGCATCAA-TTCATCTT-3'; BSA-5, 5'-AATCAGCTTCGAGGTCGTT-C-3'; and BSA-6, 5'-CTCATTAAGATCGCAGCC-3'. These oligonucleotides were purchased from Sawady Technology (Tokyo, Japan). The reaction mixtures contained 660 ng of each forward and reverse primer, 1 µg genomic DNA, and 2.5 U *Taq* polymerase (Promega) in a final volume of 100 µl. Amplification proceeded as follows: 2 min at 94 °C, then 1 min at 55 °C and 2 min at 72 °C for 50 cycles. The amplified products (A, 957 bp; B, 911 bp; C, 1025 bp) were purified by agarose gel electrophoresis.

DNA sequencing. DNA sequencing was performed by the dideoxy chain-termination method of Sanger *et al.* (Sambrook *et al.*, 1989). The double-stranded pUC18 plasmid DNAs, in which PCR products were inserted, were used as templates for the *Taq* Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The same oligonucleotides used for the PCR reaction were used as specific primers for the sequence reaction. Readout of DNA sequence was performed with an automatic DNA sequencer (Applied Biosystems, model 373A).

Fig. 1. Structure of plasmid pTUE1133.

Construction of plasmids. To express the *secA* genes of *B. subtilis* azide-resistant mutant strains in *E. coli* under the control of the *tac* promoter, *Cla*I–*Hpa*I 0.5 kbp DNA fragments containing azide-resistant mutations were prepared from the PCR products of two azide-resistant strains (Azi-1 and Azi-14) and inserted into the *Cla*I–*Hpa*I site of plasmid pTUE855. The constructed plasmids were designated as pTUE2004 (*secAT128A*) or pTUE2006 (*secAT128I*) (7.9 kbp).

Plasmid pUC18H6 was digested by *Eco*RI, treated with T4 DNA polymerase, and then digested by *Ssp*I. The resulting 0.7 kbp DNA fragment was inserted into the *Pvu*II site of plasmid pUC18 to obtain plasmid pTUE1121 (3.1 kbp). Plasmid pTUE1121 was digested by *Eco*RI, treated with T4 DNA polymerase, and then digested by *Sca*I. The resulting 0.8 kbp DNA fragment was religated with the *Bam*HI (treated with T4 DNA polymerase)–*Sca*I fragment of plasmid pUSI2. The constructed plasmid was designated as pTUE1122 (4.5 kbp).

To express the *E. coli ompA* gene under the control of the *tac* promoter in *E. coli*, the *Pvu*II–*Ssp*I 0.8 kbp DNA fragment containing the truncated *ompA* gene from plasmid pSI053 was inserted into the *Sma*I site of plasmid pTUE1122. The constructed plasmid was designated as pTUE1133 (5.3 kbp). Plasmid pTUE1133 contains the β -lactamase and *lacI* genes, and the *ompA* gene which expresses under the control of the *tac* promoter (Fig. 1). The OmpA protein produced has six histidine residues in its carboxyl terminal and it was purified using affinity binding with Ni-NTA resin (QIAGEN).

Radiolabelling, immunoprecipitation and electrophoresis of α -amylase and β -lactamase. Processing of the signal peptide of β -lactamase and α -amylase in the transformants of the wild-type (*B. subtilis* 168) and azide-resistant strains (AZ1 and AZ2) carrying plasmids pTUB101 or pTUB256 was analysed by pulse-chase. Cells were grown at 37 °C in M9 medium containing 0.4% (v/v) glycerol and 18 amino acids (20 μ g ml⁻¹) excluding methionine and cysteine, in the presence of 10 μ g kanamycin ml⁻¹. After 3 h (OD₆₆₀ approx. 0.20), 1.5 mM sodium azide was added. After appropriate periods, the cells were pulse-labelled with 20 μ Ci [³⁵S]methionine ml⁻¹ (740 kBq ml⁻¹) (NEN Research Products) for 1 min and chased with 200 μ g unlabelled methionine ml⁻¹. The cells were withdrawn at periods of 0, 1, 2 and 4 min. They were incubated with 10 mg egg-white lysozyme ml⁻¹ at 37 °C for 10 min, boiled for 3 min in the presence of 0.1% SDS, and used for immunoprecipitation with the antiserum against α -amylase or β -lactamase. The

precipitates were then separated by SDS-PAGE and autoradiograms were obtained. The intensity of the bands for precursor and mature proteins in the autoradiograms were quantified using a densitometer (Bio Image, Millipore), and the processing rates of α -amylase and β -lactamase were calculated.

Purification of *B. subtilis* wild-type and azide-resistant SecA proteins from *E. coli* transformants. *B. subtilis* SecA proteins of azide-resistant and wild-type strains were expressed and purified from *E. coli* MM52 transformants carrying plasmid pTUE855, pTUE2004 or pTUE2006, under the conditions described previously (Takamatsu *et al.*, 1992). The purity of the SecA proteins exceeded 90%, based upon SDS-PAGE followed by staining with Coomassie Brilliant Blue (data not shown).

Purification of proOmpA connecting with six histidine residues on Ni-NTA resin. An *E. coli* BA13 transformant carrying pTUE1133 was cultured in L-broth supplemented by 50 μ g ampicillin ml⁻¹ at 30 °C for 2 h, and 2 mM IPTG was added for a further 1 h. The culture was then shifted to 42 °C for 3 h. The cells were harvested by centrifugation and suspended in sonication buffer (50 mM Na₂HPO₄/NaH₂PO₄, pH 7.8; 300 mM NaCl). Cells were disrupted by sonic oscillation (20 Kc, 4 min) using a Kubota Isonator Model 200M (Kubota Medical Appliance Supply, Tokyo) in iced water, and the disrupted cells were collected by centrifugation at 10000 *g* for 20 min at 4 °C. The pellet was suspended in buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH adjusted to 8.0 with NaOH), and the cell debris was removed by centrifugation at 20000 *g* for 10 min. The supernatant was gently mixed with Ni-NTA resin (QIAGEN) at 4 °C for 1 h. The mixture was centrifuged and the pellet was washed three times with buffer C' (50 mM Tris/HCl, pH 6.5, 10 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 8 M urea). The pellets were then mixed with buffer C' containing 200 mM imidazole for 2 min at room temperature and centrifuged to obtain a supernatant containing the purified proOmpA protein having six histidine residues. The supernatant was used as the proOmpA preparation.

Assay of ATPase activity of SecA protein. Purified *B. subtilis* SecA (1 μ g) was dissolved in 100 μ l reaction buffer [50 mM Tris/HCl, pH 7.0, 30 mM KCl, 30 mM NH₄Cl, 5 mM (CH₃COO)₂Mg, 1 mM DTT] and reacted with 5 mM ATP at 37 °C to evaluate the basal level ATPase activity.

Assays for the elevated ATPase activity were according to the method of van der Wolk *et al.* (1993). To remove membrane-bound *E. coli* SecA, inverted membrane vesicles prepared from *E. coli* strain K003 by the procedure of Yamada *et al.* (1989) were treated with 4 M urea in TKMD buffer [10 mM Tris/acetate, pH 7.0, 50 mM KCl, 10 mM (CH₃COO)₂Mg, 1 mM DTT] at 0 °C for 45 min and reprecipitated by centrifugation for 30 min at 90000 r.p.m. in a TLA-100.2 rotor at 4 °C. The resulting pellet was resuspended in a buffer [50 mM Tris/HCl, pH 7.0, 30 mM KCl, 30 mM NH₄Cl, 5 mM (CH₃COO)₂Mg] and used as urea-treated inverted membrane vesicles. The purified proOmpA (1 mg ml⁻¹ in buffer C') was diluted 50-fold into reaction mixtures (100 μ l) containing the urea-treated inverted membrane vesicles (15 μ g proteins in 100 μ l), *B. subtilis* SecA protein (1 μ g), and 5 mM ATP. This was incubated at 37 °C, arrested on ice, and the amount of released phosphate was measured as the elevated ATPase activity.

Measurements of released phosphate were performed as previously described (Takamatsu *et al.*, 1992).

General DNA manipulations. DNA manipulation and *E. coli* transformation were accomplished by the methods outlined by Sambrook *et al.* (1989), and *B. subtilis* was transformed by the method of Wilson & Bott (1968).

Fig. 2. Determination of the mutation site on *secA* responsible for azide-resistance. Three overlapping DNA regions (A: -75 to +882; B: +734 to +1644; C: +1558 to +2582) of the *B. subtilis* *secA* gene of azide-resistant mutants were prepared by PCR using the appropriate chromosomal DNA as the templates and six DNA primers. Their location and direction on the *B. subtilis* *secA* gene are shown by arrows. The amplified products (A, 957 bp; B, 911 bp; C, 1025 bp) were purified by agarose gel electrophoresis, and their ability to transform to an azide-resistant phenotype was tested using *B. subtilis* NIG1121 as the recipient. The arrow head (▼) indicates the mutation site of *secA341*(*secA*^{ts}).

RESULTS

Isolation of *B. subtilis* azide-resistant *secA* mutants

To evaluate the sensitivity of *B. subtilis* to sodium azide, the wild-type strain NIG1121 was cultured at 37 °C on L-

agar plates containing 0.15, 0.3, 0.75, 1.5, or 3 mM sodium azide. This strain barely formed any colonies on the plate containing 0.75 mM sodium azide, and no colonies were found when the concentration of the reagent was increased to 1.5 mM.

We then tried to isolate azide-resistant mutants of *B. subtilis* which have a mutation in the *secA* gene. *B. subtilis* NIG1121 strain (wild-type *secA*) was treated with NTG and 10^{10} cells were then spread onto L-agar plates containing 1.5 mM sodium azide and incubated at 37 °C overnight. Twenty bacterial colonies were initially isolated as possible azide-resistant mutants and were designated as Azi-1 to Azi-20. They showed almost the same colony morphology on L-agar plates containing 1.5 mM sodium azide compared with a wild-type colony in the absence of sodium azide. The chromosomal DNA of each mutant was extracted and introduced into the *B. subtilis* NIG1152 (*secA341*^{ts}) strain which has a transition mutation resulting in an amino acid replacement from proline to leucine at residue 431 in SecA (Takamatsu *et al.*, 1992). The transformants were spread onto L-agar plates

Fig. 3. Determination of the mutation sites in the *secA* gene that confer azide-resistance and the deduced amino acid replacement at the amino acid position threonine 128. Shaded letters indicate the replaced nucleotides in azide-resistant mutants.

Fig. 4. Effect of sodium azide on cell growth of the wild-type and azide-resistant strains of *B. subtilis*. Strains AZ1 (Δ), AZ2 (□) and 168 (○) were cultured in M9 medium at 37 °C. After 3 h cultivation, 1.5 mM sodium azide was added (a), or not added (b).

Fig. 5. Effect of sodium azide on the translocation of β -lactamase (a), and α -amylase (b). *B. subtilis* strains 168, AZ1, and AZ2, harbouring plasmid pTUB256 or pTUB101, were cultured at 37 °C in M9 medium. When cultures reached an OD₆₆₀ of 0.20, 1.5 mM sodium azide was added. After 30 min, the cells were pulse-labelled with [³⁵S]methionine for 1 min and chased with non-radiolabelled methionine for 0 (lane 0), 1 (lane 1), 2 (lane 2), and 4 min (lane 4). Cells cultured in the presence or absence of sodium azide were immunoprecipitated with anti- β -lactamase or anti- α -amylase antiserum, separated by electrophoresis on SDS-polyacrylamide gels, and visualized by autoradiography. p, Precursor of α -amylase or β -lactamase; m, mature form.

containing 1.5 mM sodium azide and incubated at 42 °C. Azide-resistant mutants whose genomic DNA enabled the *secA341^{ts}* mutant strain to grow both at the non-permissive temperature (42 °C) and on azide-containing plates were considered as possessing azide-resistance mutations at or near the *secA* gene. Four of the twenty original mutant strains, Azi-1, Azi-14, Azi-15 and Azi-16, were thus selected as azide-resistant *secA* mutants. The other 16 original mutants were not because their ability to transform to an azide-resistant phenotype was incomplete compared with that of the former strains.

Determination of the mutational sites of azide-resistant mutants

To determine the mutational sites in the four azide-resistant mutants, three DNA fragments (A, B and C, shown in Fig. 2) of the *secA* gene were prepared by PCR. Their chromosomal DNA was used as template and six synthetic DNA fragments were used as primers (Fig. 2).

The resulting 12 DNA fragments were separately introduced into wild-type *B. subtilis* strain NIG1121 and tested for the ability to transform the wild-type strain into an azide-resistant form on L-agar plates containing 1.5 mM sodium azide. All the A fragments from the four azide-resistant strains had this transformation ability. They were then inserted into the *Sma*I site of the pUC18 vector and sequenced, revealing that azide-resistance was the result of single mutations in the *secA* gene. The Azi-1 strain had an A to G transition at nucleotide position +382, corresponding to the replacement of threonine at amino acid position 128 of the SecA protein with alanine. Azi-14, Azi-15 and Azi-16 strains had a C to T transition at nucleotide position +383, corresponding to the replacement of threonine 128 with isoleucine (Fig. 3). The other DNA fragments (B and C) from Azi-1 and Azi-14 mutant chromosomal DNA were also sequenced, but no mutation was found. *B. subtilis* 168 strain (wild-type *secA*) was transformed by the A fragments of Azi-1 and Azi-14, and the resulting transformants which have a single mutation in the *secA* gene (in AZ1, threonine 128 of SecA

Table 2. Basal level ATPase activity of SecA proteins

Aliquots containing 1 µg each purified SecA were incubated at 37 °C in the presence of the indicated concentration of sodium azide.

Protein	Activity [10 ² × pmol (μg protein) ⁻¹ min ⁻¹]				
	Azide concn (mM)...	0	1.5	7.5	15
Wild-type SecA		2.5	2.3	2.4	2.4
AZ1 SecA		6.4	6.3	6.4	6.4
AZ2 SecA		5.7	5.7	5.7	5.8

was replaced by alanine; in AZ2, by isoleucine) were selected and used for further studies.

Effect of sodium azide on the growth of *B. subtilis*

The azide-resistant *secA* mutant strains of *B. subtilis*, AZ1 and AZ2, formed colonies on L-agar plates in the presence of 1.5 mM sodium azide but the wild-type strain, 168, did not. Similarly, in L-broth at 37 °C, AZ1 and AZ2 grew well in the presence of 1.5 mM sodium azide but 168 hardly grew at all. These azide-resistant mutants seemed to be cold-sensitive strains: they showed abnormal cell-shape and poor growth at 23 °C in the absence of sodium azide (data not shown).

To examine the most suitable conditions for the pulse-chase experiment, the growth of AZ1, AZ2 and 168 in M9 medium was measured. The wild-type and azide-resistant strains were cultured in L-broth at 37 °C overnight by which time their cell densities had reached an OD₆₆₀ of about 0.85. They were then inoculated into fresh M9 medium and cultured at 37 °C. After 3 h (OD₆₆₀ about 0.20), 1.5 mM sodium azide was added (Fig. 4a). Upon addition of sodium azide, growth of the wild-type strain began to slow after 1 h, but that of the azide-resistant strains did not. In the absence of sodium azide, both the wild-type and azide-resistant strains grew well in M9 medium at about the same rate (Fig. 4b).

Effect of sodium azide on protein translocation in *B. subtilis*

To study the effect of sodium azide on protein translocation in *B. subtilis*, the processing of the signal peptide of α-amylase and β-lactamase was analysed by pulse-chase in the transformants of the wild-type (168) and azide-resistant (AZ1 and AZ2) strains carrying plasmid pTUB101 (α-amylase) or pTUB256 (β-lactamase) (Fig. 5). In the absence of sodium azide, the precursor of both α-amylase and β-lactamase was converted almost in its entirety into its mature form after 1 min chase in all cells. In the presence of 1.5 mM sodium azide, more than 60 % of β-lactamase and 20 % of α-amylase accumulated in their

Fig. 6. Azide sensitivity of wild-type *B. subtilis* 168. *B. subtilis* 168 carrying pTUB256 was grown in M9 medium at 37 °C for 3 h. (a) Ten minutes after the addition of the indicated concentrations of sodium azide, the cultures were pulse-labelled with [³⁵S]methionine for 1 min, chased with non-radiolabelled methionine for 1 min, then analysed for the processing of β-lactamase by gel electrophoresis. (b) The culture was pulse-labelled with [³⁵S]methionine for 1 min and chased with non-radiolabelled methionine for 1 min, after cultivation in 3 mM sodium azide for the indicated lengths of time. p, Precursor of β-lactamase; m, mature form; C, control.

precursor form after 4 min chase in the wild-type, whereas in the azide-resistant strains, most of the two precursors were converted into their mature form after only 1 min chase. Therefore, sodium azide inhibited the translocation of α-amylase and β-lactamase in wild-type *B. subtilis*, but only slightly affected it in the azide-resistant *secA* mutants.

To determine the concentration of sodium azide required to inhibit protein translocation in *B. subtilis*, β-lactamase processing was analysed by pulse-chase in the presence of various concentrations of sodium azide (Fig. 6a). In the presence of 0.15 mM sodium azide, 30 % of β-lactamase accumulated in the precursor form after 1 min chase. In the presence of 0.75 mM sodium azide, protein translocation was completely inhibited.

In *E. coli*, sodium azide inhibits protein translocation rapidly (Oliver *et al.*, 1990b). We studied how quickly sodium azide inhibited protein translocation in *B. subtilis*. Wild-type *B. subtilis* cultures were pulse-labelled after cultivation in the presence of 3 mM sodium azide for various lengths of time, and processing of β-lactamase was analysed (Fig. 6b). Protein translocation in *B. subtilis* was inhibited completely within 1 min after addition of sodium azide. This result indicates that inhibition of protein translocation by sodium azide in *B. subtilis* is extremely rapid, as in *E. coli*.

Effect of sodium azide on the ATPase activities of *B. subtilis* SecA protein

Sodium azide is known to inhibit the translocation ATPase activity of *E. coli* SecA and block incorporation of proOmpA into the inverted membrane vesicles, but it

Table 3. Elevated ATPase activity of SecA proteins

Aliquots containing 1 µg each purified SecA, 2 µg proOmpA and urea-treated inverted membrane vesicles were incubated at 37 °C in the presence of the indicated concentration of sodium azide.

Protein	Activity [10 ² × pmol (μg protein) ⁻¹ min ⁻¹]				
	Azide concn (mM)...	0	1.5	7.5	15
Wild-type SecA		7.6	4.6	3.8	3.4
AZ1 SecA		21.0	20.0	16.4	15.8
AZ2 SecA		18.6	17.7	14.9	14.5

does not affect the basal level SecA ATPase activity (Oliver *et al.*, 1990b). Van der Wolk *et al.* (1993) have shown that *B. subtilis* SecA does not stimulate proOmpA incorporation into the inverted membrane vesicles of *E. coli*, but its ATPase activity is elevated in the presence of proOmpA and inverted membrane vesicles. We studied the effect of sodium azide on the basal level ATPase activity and the elevated ATPase activity of the purified wild-type and mutant SecA proteins of *B. subtilis*. Basal level ATPase activity of the SecA mutants was about two to three times higher than that of wild-type *B. subtilis* SecA (Table 2). The basal level ATPase activity of the wild-type and mutant SecA proteins of *B. subtilis* was not affected by 1.5–15 mM sodium azide as is the case with the wild-type and azide-resistant SecA mutants of *E. coli*. The elevated ATPase activity of the SecA proteins was measured in the presence of proOmpA and urea-treated inverted membrane vesicles of *E. coli* (Table 3). The elevated ATPase activity of both mutant and wild-type SecA proteins increased almost threefold compared with the basal level ATPase activity. Inhibition of the elevated ATPase activities was then analysed: the elevated ATPase activity of the wild-type SecA in the presence of 1.5 mM sodium azide showed only 50–60 % of its azide-free activity; in contrast, the SecAs of the mutants were much less inhibited in the presence of 1.5 mM sodium azide, retaining 90–95 % of their elevated ATPase activity.

DISCUSSION

Sodium azide is an inhibitor of the Sec-dependent protein translocation pathway in *E. coli*. Oliver *et al.* (1990b) and Fortin *et al.* (1990) analysed two different azide-resistant mutants, and found that the mutations were located in the *secA* gene: an A to T transversion at nucleotide position 1356, corresponding to the replacement of asparagine 179 with tyrosine; a T to A transversion at nucleotide position 2755, corresponding to the replacement of leucine 645 with glutamine (Oliver *et al.*, 1990b); a C to T transition at nucleotide position 2710, corresponding to the re-

placement of alanine 630 with valine; and a C to T transition at nucleotide position 2787, corresponding to the replacement of arginine 656 with cysteine (Fortin *et al.*, 1990). All of these amino acid replacement sites were located within the ATP binding cassettes (ABC) I or II of the *E. coli* SecA (Mitchell & Oliver, 1993).

In this study, we isolated four azide-resistant *secA* mutants of *B. subtilis*. Sodium azide inhibits not only SecA but also the enzymes related to the respiratory chain (Kobayashi & Anraku, 1972). However, the single mutation occurring in the *secA* gene conferred azide-resistance to *B. subtilis*, so the effect of 1.5 mM sodium azide on the enzymes other than SecA was thought not to be critical. All of the mutants had only one amino acid replacement, occurring at threonine 128 (Fig. 3). In the case of *E. coli*, mutations which confer azide resistance occur at various positions in the *secA* gene. Therefore, it may be possible to isolate other *B. subtilis* azide-resistant mutants in which SecA contains amino acid replacements other than at threonine 128 if we were to isolate more mutants by other procedures. However, this threonine residue is conserved among the SecA proteins of several species and is located in the ATP binding cassette I including the ATP binding motif A (¹⁰⁰MKTGEGKT¹⁰⁷) of *B. subtilis* SecA (Mitchell & Oliver, 1993). Both the basal level and the elevated ATPase activities of azide-resistant SecA proteins were threefold higher than those of the wild-type SecA (Tables 2 and 3). Moreover, the amino acid replacement of threonine 128 of *B. subtilis* SecA by alanine to isoleucine resulted in azide resistance and a defect in growth at low temperature (23 °C). Therefore, this residue seems to have an important role in the proper function of SecA including its ATPase activity.

In this study, the processing of α -amylase and β -lactamase was inhibited in wild-type *B. subtilis* in the presence of 1.5 mM sodium azide. More than 20 % and 60 % of the pre- α -amylase and pre- β -lactamase accumulated after 4 min chase in the wild-type cells, respectively (Fig. 5). Recently, Meens *et al.* (1993) showed that sodium azide inhibited the translocation of proOmpA in *B. subtilis*. In the presence of 0.15 mM sodium azide, 30 % of pre- β -lactamase accumulated after 1 min chase in *B. subtilis* (Fig. 6a). Compared with the fact that the presence of less than 1 mM sodium azide does not inhibit protein translocation in *E. coli* (Oliver *et al.*, 1990b), *B. subtilis* seems to be more sensitive to sodium azide. Furthermore, protein translocation of *B. subtilis* was completely inhibited within 1 min after the addition of 3.0 mM sodium azide. This inhibition is extremely rapid comparable to that of *E. coli* (Fig. 6b). These facts indicate that sodium azide inhibits SecA-dependent protein translocation in *B. subtilis* very effectively.

In the azide-resistant *secA* mutants, processing of α -amylase and β -lactamase was marginally affected by sodium azide. The mutant cells grew normally and their protein translocation systems were not inhibited in the presence of 1.5 mM sodium azide (Figs 4a and 5). The inhibition of protein translocation in *E. coli* by sodium azide is due to inhibition of the translocation ATPase

activity of SecA protein (Oliver *et al.*, 1990b). We examined the effect of sodium azide on the basal level and the elevated ATPase activities of purified *B. subtilis* SecA proteins. The basal level ATPase activity of both the wild-type and azide-resistant SecA proteins was not affected by sodium azide (Table 2). The elevated ATPase activity of wild-type *B. subtilis* SecA in the presence of proOmpA and inverted membrane vesicles was inhibited by sodium azide, as is that of wild-type *E. coli* SecA. In contrast, the elevated ATPase activity of the azide-resistant SecA mutants was less affected by sodium azide than that of the wild-type (Table 3). Therefore, the elevated ATPase activity of *B. subtilis* SecA, analogous to 'translocation ATPase' activity in *E. coli*, is thought to be inhibited by sodium azide and to have an important role in the protein translocation process. We propose that acquisition of azide resistance by the elevated ATPase activity confers azide resistance upon cell growth and protein translocation in *B. subtilis*.

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