Isolation and characterization of a *Bacillus subtilis* secA mutant allele conferring resistance to sodium azide

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Abstract: A mutation has been isolated in the *Bacillus subtilis* secA gene (secA10) which allows cell growth and residual protein translocation in the presence of 1.5 mM sodium azide. Besides conferring resistance to sodium azide, the corresponding SecA10 mutant protein, in which glutamic acid at position 338 has been changed to glycine, seems to possess a secretion defect even in the absence of azide. In addition, the secA10 mutant protein was found to be recessive to wild-type secA with regard to azide resistance. Our results strongly suggest that, like the situation in *Escherichia coli*, the *B. subtilis* SecA protein is a main target for the lethal action of sodium azide.

Key words: *Bacillus subtilis*; Protein translocation; SecA protein; Translocation ATPase; Azide

Introduction

The SecA protein is a central component of the *Escherichia coli* preprotein translocase and is required for the productive binding of precursor proteins to the export sites in the plasma membrane [1,2]. The catalytic activity of SecA during protein translocation involves the binding and hydrolysis of ATP [3]. ATP binding to SecA leads to a limited translocation of approximately 20 amino acid residues of the precursor protein which, upon ATP hydrolysis, is subsequently released from SecA [4]. This so-called translocation ATPase activity requires the interaction of SecA with precursor proteins, acidic phospholipids and the SecY/E protein of the cytoplasmic membrane [3]. Azide has been found to be a specific inhibitor of protein translocation in *E. coli* and this effect is due to inhibition of SecA translocation ATPase activity [5,6].

In *Bacillus subtilis*, a homologue of SecA has been identified [7,8] which is 53% identical to the *E. coli* SecA protein [8]. We have previously shown that, like the situation in *E. coli*, azide is inhibitory to the translocation of secretory proteins in *B. subtilis* [9], strongly suggesting that SecA might be involved in the corresponding translocation steps. In this report, we describe the isolation and characterization of an azide-resistant mutant allele of the *B. subtilis* secA gene.
The corresponding SecA mutant protein, in which glutamic acid at position 338 has been changed to glycine, confers resistance to the inhibitory action of 1.5 mM azide on growth and protein translocation in B. subtilis.

Materials and Methods

Bacterial strains and growth conditions
B. subtilis NIG1121 [10], NIG1152 [10], and MIK10 (this study) were grown in LB medium [11] or S7 minimal medium [12] supplemented with 15 μg ml⁻¹ tetracycline, 0.5% (w/v) glucose, 1 mM IPTG, or 1.5 mM sodium azide, as required.

DNA techniques
The isolation of chromosomal DNA, preparation of plasmid DNA and other DNA techniques followed standard procedures [13]. Polymerase chain reaction (PCR) was carried out as described previously [14]. The secA genes from NIG1121 and MIK10 were isolated by PCR using the corresponding chromosomal DNA as template and oligonucleotides OMKL23.1 (5'-GGCTCTAGAGATGATAGAGGAGCG) and OMKL24 (5'-TGTGGTACCCATTTCATTCAC-CTC) as primers. The corresponding 2.6-kb PCR DNA fragments were digested with XbaI and Asp718 and ligated into SpeI/Asp718-digested pWH1520 [15], resulting in plasmids pWMKL1 (containing wild-type secA) and pWAZ1 (containing the secAlO gene).

Miscellaneous techniques
Pulse-chase experiments and Western blotting were done as described previously [9]. PBS1-mediated transduction of B. subtilis was carried out as described by O'Kane et al. [16].

Results

Isolation of an azide-resistant B. subtilis secA mutant allele
We have previously shown that 3 mM sodium azide completely prevented the translocation of secretory precursor proteins across the cytoplasmic membrane of B. subtilis [9]. Since, by analogy to the situation in E. coli, the most likely target for azide is the B. subtilis SecA homologue, we attempted to isolate a mutation in the B. subtilis secA gene which allows cell growth and protein translocation in the presence of azide. Cells of B. subtilis NIG1121 [10] were plated onto LB agar plates containing 1.5 mM sodium azide and spontaneously arising azide-resistant mutants were selected at 37°C. The mutant colonies which grew after 24 h of incubation were pooled and this pool was used as a donor for phage PBS1-mediated transduction of B. subtilis NIG1152 which, due to a mutation in secA (div431), is temperature-sensitive for growth [10]. One of the transductants (MIK10) which allowed growth at 42°C in the presence of 1.5 mM sodium azide was analysed further. Although growing normally at 37°C and 42°C, we noticed that MIK10 grew much more slowly at 30°C when compared to NIG1121, even in the absence of sodium azide. In addition, microscopic examination of MIK10 showed that the cells harbored a strong tendency to form long filaments at 30°C (data not shown). Chromosomal DNA was isolated from MIK10 and the corresponding secA gene (secAlO) was amplified by PCR in duplicate. Nucleotide sequencing of the entire gene revealed that a single mutation (A to G transition at position 1013 according to the numbering of Sadaie et al. [8]) had occurred in the secAlO gene, resulting in the replacement of glutamic acid-338 by glycine.

The secAlO mutation is recessive to wild-type secA
In E. coli, the azide-resistant secA mutant alleles examined so far were found to be codominant to wild-type secA, i.e. sensitivity or resistance to a certain concentration of azide was dictated by the gene dosage of the respective secA allele [5,6]. In order to test whether the secAlO allele is also co-dominant to B. subtilis wild-type secA, plasmid pWMKL1, which harbors the B. subtilis wild-type secA gene under the regulatory control of the B. megaterium xyl promoter/operator [15], was transformed into MIK10. Under inducing conditions, massive amounts of the corresponding SecA protein were
synthesized when compared to uninduced cells or cells harboring the vector plasmid without insert (Fig. 1). The presence of pWMKL1 in MIK10 rendered the cells sensitive to 1.5 mM sodium azide, regardless of whether or not SecA synthesis was fully induced (Table 1). On the other hand, when NIG1121 was transformed with pWAZ1, which harbors the secA10 gene under xylose-inducible control, no acquisition of resistance to 1.5 mM sodium azide was obtained, even when the SecA10 mutant protein was far in excess of wild-type SecA (Fig. 1 and Table 1). From these results we conclude that the secA10 mutation is recessive to wild-type secA.

**Effect of 1.5 mM sodium azide on pre-OmpA processing in NIG1121 and MIK10**

Since, in *E. coli*, azide interferes with protein translocation by inhibiting the SecA translocation ATPase activity [5,6], the ability of the SecA10 mutant protein to promote the translocation of a model protein (*E. coli* pre-OmpA protein [9]) across the *B. subtilis* plasma membrane in the presence of 1.5 mM sodium azide was analysed by pulse–chase experiments. Plasmid pJM20, harboring the gene for pre-OmpA under IPTG-inducible control [9] was transformed into NIG1121

**Table 1**

<table>
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Cells of *B. subtilis* NIG1121 or MIK10 harboring the indicated plasmids were plated onto LB-plates containing 0.5% glucose (G) or 0.5% xylose (X) in the presence or absence of 1.5 mM sodium azide. (+): growth; (−): no growth.
and MIK10 and processing of pre-OmpA was examined in both strains at 30°C and 37°C in the presence or absence of sodium azide, respectively (Fig. 2). In the absence of azide at 37°C, processing of pre-OmpA was significantly slower in MIK10 (Fig. 2B, lanes 5–8) when compared to NIG1121 (Fig. 2B, lanes 1–4), suggesting that the azi mutation in MIK10 results in a partial defect of the corresponding SecA protein in protein translocation. Since, probably due to this defect, an improvement of pre-OmpA processing in MIK10 in the presence of sodium azide is not clearly visible at 37°C, pulse–chase experiments were performed at 30°C. At this temperature, the inhibitory effect of 1.5 mM sodium azide on pre-OmpA processing is much more pronounced in NIGl121 (Fig. 2A, lanes 9–12) when compared to 37°C (Fig. 2B, lanes 9–12). Furthermore, a significant improvement of pre-OmpA processing is now observed in MIK10 in the presence of azide (Fig. 2A, lanes 13–16) when compared to NIG1121 (Fig. 2A, lanes 9–12).

**Discussion**

Like the situation in *E. coli*, sodium azide has been found to be an inhibitor of protein translocation in *B. subtilis* [9]. In this communication, we describe the isolation and characterization of a *B. subtilis secA* allele which confers resistance to the lethal action of 1.5 mM sodium azide.

In the SecA10 mutant protein, glutamic acid-338 has been altered to glycine. Besides conferring resistance to azide, the corresponding mutation seems to negatively affect the activity of the *B. subtilis SecA* protein during protein translocation, even in the absence of azide. This assumption is based on the following observations. First, cells harboring the *secA10* allele grew poorly at 30°C. Second, in contrast to the azide-resistant *E. coli secA* alleles characterized so far [5,6], the *secA10* allele is recessive to wild-type *secA*. Finally, compared to wild-type cells, processing of pre-OmpA is clearly delayed in *secA10*-containing cells in the absence of sodium azide. However, despite these negative effects, the SecA10 protein allows cell growth and residual protein translocation in the presence of 1.5 mM sodium azide.

From these results we conclude that, similar to the situation in *E. coli*, the *B. subtilis SecA* protein is a main target for the inhibitory action of sodium azide. Most likely, as has been shown for azide-resistant *E. coli SecA* mutant proteins [5,6], the azide resistance of the MIK10 mutant strain might be caused by an altered translocation ATPase activity of the corresponding SecA10 mutant protein.

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