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Mutations Conferring Resistance to Azide in *Escherichia coli* Occur Primarily in the *secA* Gene

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Mutant strains of *Escherichia coli* were screened for the ability to grow on L agar plates containing 3.4 or 4.6 mM sodium azide. Most mutants had mutations located in the leucine region, presumably at the *azi* locus. Two of these mutants were found to have a mutation in the *secA* gene, but expression of the resistance phenotype also required the presence of upstream gene *X*. While a plasmid carrying the *X-secA* mutant gene pair was able to confer azide resistance to a sensitive host, a similar plasmid harboring the wild-type *secA* allele rendered a resistant strain sensitive to azide, indicating codominance of the two alleles. That azide inhibits SecA is consistent with the fact that SecA has ATPase activity, an activity that is often prone to inhibition by azide.

Bacteria resistant to sodium azide were among the first mutants to be isolated (6). Resistance to this inhibitor has been studied to a considerable extent in both *Escherichia coli* and *Salmonella typhimurium*, but neither the gene responsible for conferring this phenotype nor its encoded product has been clearly defined.

Azide is a well-known inhibitor of the terminal segment of the electron transport chain, but it has also been reported to have several effects on the growth of bacterial cells. For example, it was shown to interfere with DNA and RNA synthesis (4, 18) and to induce mutations (reviewed in reference 11). In addition, azide-resistant mutant cells are often defective in their control of cell division (3, 4, 18). Whether all these different phenotypes represent specific cellular responses or are manifestations of a more general effect of azide is uncertain. The proton-translocating ATPase complex (F_0F_1) which catalyzes the terminal step of oxidative or photophosphorylation in chloroplasts, mitochondria, and bacterial membranes is particularly prone to inhibition by inorganic azide (8).

The ATPase complex of *E. coli* can be mutationally altered to give an enzyme which is more resistant to azide than its wild-type counterpart (17). However, this latter observation is not consistent with the fact that the *azi* locus is located near *leu*, in a region which contains none of the genes encoding the various ATPase subunits (1). Therefore, the leucine-linked alleles responsible for the azide resistance phenotype define an as yet uncharacterized function. The present study reports on the cloning and identification of these alleles. It will be shown that the primary site of the inhibitory action by azide in *E. coli* is SecA, an essential component of the protein export system which exhibits ATPase activity (7, 10).

E. coli AB1157 was the basic strain used in the present study. The low-copy-number plasmid vectors used were pLG339 or pAD400 derivatives (12, 16). pAD400 is pLG339 but with the multiple cloning site of pUC18 inserted in the *Pvu*II site. The procedure of Mosesson et al. for in vivo cloning with the mini-Mu replicon pRRA101 was described previously (9). The construction of the plasmids used during the present study is summarized in Fig. 1. Complex medium was L broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) adjusted to pH 7.2 with NaOH. The concentration of sodium azide used in the medium was 3.4 mM unless otherwise specified, and the concentrations of antibiotics used were (in micrograms per milliliter): kanamycin, 100; tetracycline, 15; and ampicillin, 30. Resistance to sodium azide was routinely determined by streaking for single colonies on sections of L agar plates containing sodium azide. A mutant was considered azide resistant if growth occurred uniformly throughout the streaked section, whereas cells were considered sensitive if growth was only observed at the beginning of the streaked region.

The azide-resistant mutants were obtained after mugagenesis with nitrosoguanidine or spontaneously on L agar plates containing either 3.4 or 4.6 mM sodium azide. Twelve different mutations were transduced into a *leuA* auxotroph with selection for leucine independence, and all were found to be linked to *leu*, presumably at the *azi* locus. Two mutants, YF201 and YF248, were chosen as donors in an in vivo cloning experiment. Mutant YF201 was mutagenically induced and selected on 3.4 mM azide, while YF248 was obtained spontaneously with 4.6 mM azide. First, they were made lysogenic with Mu *cts*62 and then transformed with the mini-Mu replicon pRRA101. Lysis was induced by incubation at 44°C for 30 min, and the lysate obtained was used to infect cells of AB1157 lysogenic for Mu^+. Selection was made on L agar plates containing azide and kanamycin.

Recombinant plasmid DNA from several transductants was isolated and digested with *Eco*RI. All plasmids were found to possess at least three *Eco*RI fragments of 0.8, 2.5, and 2.6 kb, fragments shown to belong to the 2-min region and to contain the *envA*, *X*, and *secA* genes (Fig. 1). Plasmid DNA from pYFB4, the mini-Mu replicon that carries the *aziR201* mutation, was digested with *Eco*RI, and the mixture was ligated in the presence of *Eco*RI-restricted pLG339. The ligated DNA was used to transform cells of AB1157, and transformants were selected on L agar plates containing azide and tetracycline, a resistance marker present in the pLG339 vector but not in the mini-Mu replicon. Thirty transformants were analyzed and all were found to carry the same three *Eco*RI fragments of 0.8, 2.5, and 2.6 kb, indicating that azide resistance was conferred by at least one of the genes carried by these fragments (Fig. 1).

One of the plasmids thus obtained, pYFR6, was partially

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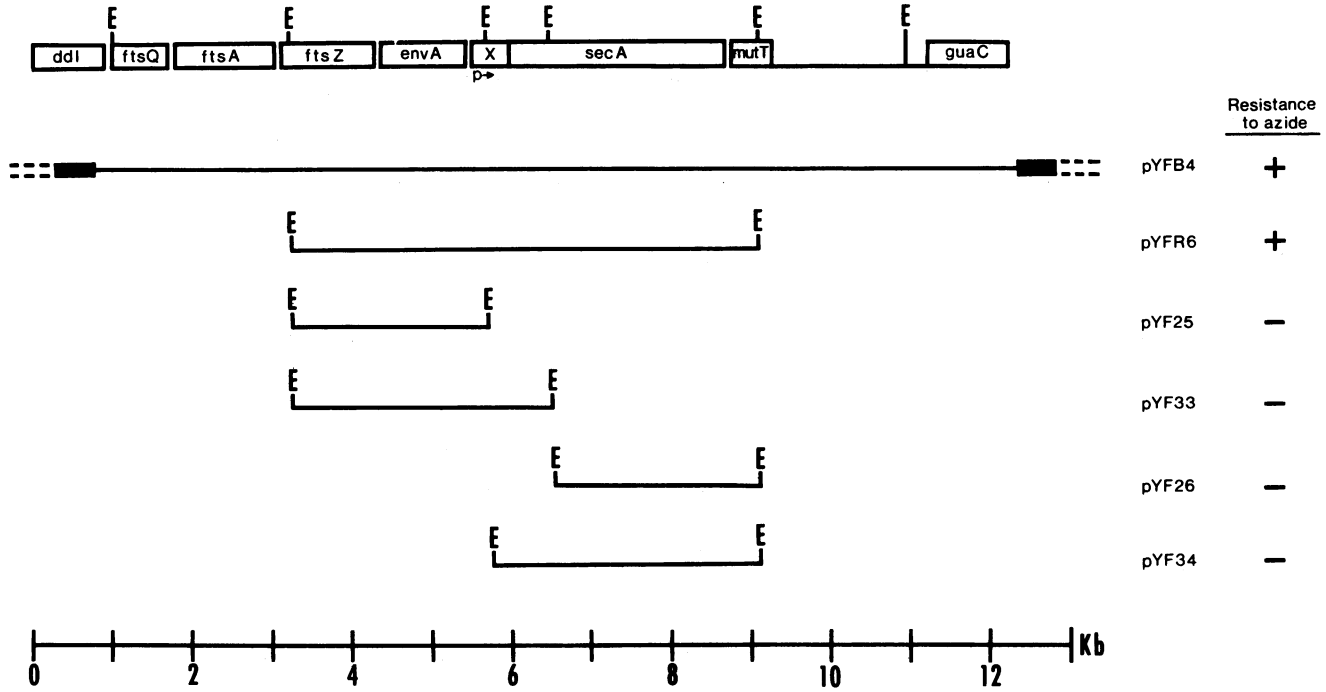

FIG. 1. Organization and partial restriction map of a number of genes which have been identified within the 2-min region of the *E. coli* chromosome. Only the *Eco*RI restriction sites (E) are indicated. Data were collected from references 2, 12, and 15. pYFB4 was a mini-Mu replicon carrying chromosomal DNA and is represented by the thin line. Other plasmids were derivatives of pGL339 harboring different *Eco*RI fragments of pYFB4. Solid boxes and dashes indicate portions of the mini-Mu replicon DNA; p→ indicates promoter and direction of transcription.

digested with *Eco*RI, religated, and used in the transformation of wild-type cells, with selection for tetracycline resistance on a medium containing no azide (Fig. 1). pYF25, pYF33, pYF26, and pYF34, which have been deleted for one or two *Eco*RI fragments, did not confer azide resistance to the host cell (Fig. 1). Interestingly, cells which carried pYF26 or pYF34 but not those harboring pYF25 or pYF33 gave isolated colonies when streaked on azide agar, indicating a marker rescue and suggesting that the mutation conferring the azide resistance phenotype was most likely in *secA*. The fact that pYF34, a plasmid which carried an intact *secA* gene, could not confer azide resistance when transferred into a sensitive host can be explained by the absence of gene *X*, since *secA* is totally dependent on the presence of this gene for its expression (15). However, these results did not exclude the participation of *envA* in the expression of the resistance.

envA participation was examined by deleting the region containing the *envA* gene from plasmid pYFR6. pYFR6 DNA was partially digested with *Pvu*II, religated, and used for the transformation of AB1157 cells, with selection for tetracyline resistance (Fig. 2). Colonies which appeared on the tetracycline-agar medium were replicated on kanamycin-containing plates, and those which could not form colonies on this medium were tested for their resistance to azide. A plasmid, identified as pAZR1, which was deleted for the 4.6-kb *Pvu*II segment, conferred the azide resistance phenotype (results not shown). It can therefore be concluded that *envA* was not required for the expression of the azide resistance phenotype.

Marker rescue experiments were carried out with different fragments of *secA* inserted in pUC19 and introduced into a wild-type cell. The results indicated that for the two alleles, the mutations were present in the *Kpn*I-*Pvu*II fragments located near the 3′ ends of the mutated *secA* genes (results not shown). The nucleotide sequences of these fragments were determined. As shown in Fig. 3, both mutations involved the replacement of a C by a T. This occurred at position 2710 in YF201 and at position 2787 in YF248 (position numbers are from the published sequence of the

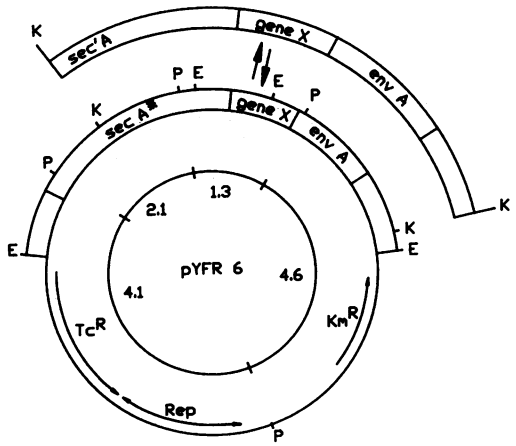

FIG. 2. Circular map of pYFR6. The plasmid, which carries the *secA201* mutation (Fig. 1), was deleted of its *envA* gene by partial cleavage with *Pvu*II to remove the 4.6-kb fragment, yielding pAZR1. pYFR6 was cleaved with *Kpn*I, and the 4.4-kb segment was replaced by the corresponding one from pYF29 (indicated by arrows), a plasmid which is identical to pYFR6 but carries the wild-type *secA* allele. The asterisk identifies the mutated allele. Abbreviations: E, *Eco*RI; K, *Kpn*I; P, *Pvu*II. Numbers represent kilobase pairs.

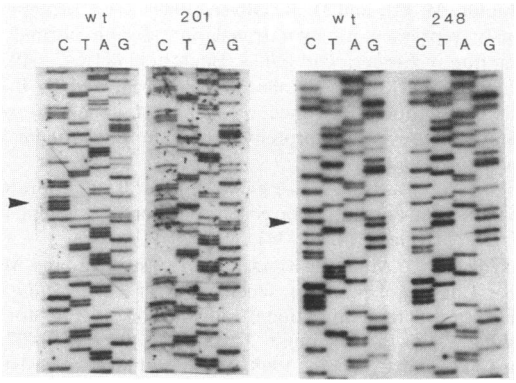

FIG. 3. Portions of DNA sequence gels showing the differences between the wild-type *secA* (wt) and the *secA201* and *secA248* mutations.

secA gene [15]). The amino acid changes identified were valine (alanine in the wild type) and cysteine (arginine in the wild type) for the *secA201* and *secA248* mutations, respectively.

As shown above, cloning of the *secA201* and *secA248* mutations required only a simple selection procedure because their presence on a plasmid conferred an azide-resistant phenotype to the host cell. This was indicative that the mutant phenotype was dominant over the wild-type *secA*. However, this was found not to be exactly the case, because the presence of pYF29, a plasmid identical to pYFR6 but carrying the wild-type *secA* allele, rendered all the resistant strains tested sensitive to azide (Table 1). This suggested a codominance of the wild-type and the mutant allele, a phenomenon which presumably could best be demonstrated when one of the alleles was present in more than one copy per cell, that is, about six in the present experiment, because a pSC101-derived plasmid was used.

Also shown in Table 1 are the colony-forming abilities of the two mutants when they harbored pAZS4, a plasmid like pYF29 carrying the wild-type *X-secA* gene pair but deleted for the 4.6-kb *Pvu*II fragment to remove the *envA* gene (Fig. 2). It can be seen that pAZS4 was less efficient than pYF29 in suppressing the azide resistance phenotype in all mutants. The colony-forming abilities of mutant YF248, the more resistant strain, when harboring pAZS4 were about the same on the azide and the azide-free medium, but the colonies were smaller on azide agar plates, indicating slower growth and hence decreased resistance to the inhibitor (results not shown). That the presence of *envA* on the plasmid assisted the neighboring wild-type *secA* in suppression of resistance to azide is likely the result of increased expression of the *secA* gene in pYF29. In fact, Schmidt and Oliver (14) have reported that there is readthrough of the terminator located at the end of the *envA* gene (2). However, *trans*-acting effects of EnvA on the expression of *secA* or on the stability of the SecA protein are other possibilities that should not be excluded.

TABLE 1. Suppression of the azide resistance phenotype by plasmids pYF29 and pAZS4[a]

Strain	No. of colonies at 24 h with plasmid:					
	None		pYF29 (*envA*$^+$ *X secA*$^+$)		pAZS4 (*X secA*$^+$)	
	L	L + azide	L	L + azide	L	L + azide
YF201	644	776	477	0	445	7
YF248	1,172	1,242	656	0	724	782

[a] Number of colonies was determined after 24 h of incubation. Cultures of strains carrying the indicated plasmids or no plasmid were grown overnight and diluted to 10^{-5}, and 0.1-ml samples were spread on the medium indicated.

In the event that a mutation causing an alteration in another protein would also confer azide resistance, new resistant mutants in genes other than *secA* were isolated. This was done by nitrosoguanidine mutagenesis of cells which harbored a wild-type *secA* on a plasmid because, by virtue of the codominance phenomenon described above, the induction of a mutation in *secA* either on the chromosome or on one of the plasmid copies would not be expected to confer a resistant phenotype. Cells of AB1157 containing pYF29 or the pLG339 vector were mutagenized with nitrosoguanidine and plated on the selective medium described above. The number of colonies on the plates was 100-fold lower when the cells harbored pYF29 than when they harbored pLG339, suggesting that most of the *secA*-associated mutations had been eliminated by this procedure. Several of the resistant mutants examined carried a chromosomal mutation that was unlinked to *leu*. Mapping, cloning, and restriction analyses of the resistance alleles from three mutants revealed in all cases a mutation located in the region adjacent to *alaS* at min 58.0 (5, 13).

The azide-resistant allele was delimited within a 1.2-kb region located immediately at the 3′ side of *alaS*, and for the three mutants examined, the azide resistance was expressed at 37°C but not at 30°C (results not shown). No information about the role of this gene is available. It could encode, for example, a modified tRNA that causes missense suppression of *secA* translation.

In conclusion, the finding that azide resistance is conferred by a mutation in *secA*, combined with the fact that such mutations can be easily obtained, affords a useful tool for dissecting the biochemical functions of SecA.

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