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Azaserine Resistance in *Escherichia coli*: Chromosomal Location of Multiple Genes

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Resistance to azaserine in *Escherichia coli* is the result of mutations in at least three different loci. All spontaneously arising azaserine-resistant mutants harbor a lesion in the *aroP* gene. However, a lesion in this gene is not solely responsible for resistance. All spontaneously arising intermediate-level azaserine-resistant mutants also harbor a lesion in a gene designated *azaA*, which lies near min 43 on the chromosome. High-level resistant mutants harbor lesions in the *aroP* and *azaA* genes and in a third gene designated *azaB*, which lies near min 69 on the chromosome. Transport studies demonstrate that mutants harboring lesions in the *azaA* gene are not defective in the transport of the aromatic amino acids, but that mutants which harbor lesions in the *azaB* gene are defective in phenylalanine transport but not in tyrosine or tryptophan transport.

Several procaryotic organisms have been reported to be resistant to the glutamine analog azaserine (*O*-diazoacetyl-L-serine) (1, 17). Resistance to the antibiotic is thought to be the result of a decreased rate of uptake of azaserine by the cells (1, 2, 16). In *Salmonella typhimurium*, resistance to azaserine has been reported to be the result of a mutation in the *aroP* gene, whose product is involved in the general aromatic amino acid transport system (1). However, a mutation in the *dhuA* gene, whose product is involved in the regulation of histidine transport, results in the organism acquiring sensitivity to the antibiotic even when the organism harbors a lesion in the *aroP* gene (2). This implies that the antibiotic may be transported by multiple transport systems.

Previously we reported that there are at least three levels of resistance to azaserine in *Escherichia coli* K-12 (17). Transductional analyses indicate that all spontaneously arising azaserine-resistant mutants harbor a lesion in the *aroP* gene, but that this lesion is not solely responsible for resistance to the antibiotic in *E. coli* (16).

This report describes the mapping of a gene (*azaA*) which is involved with intermediate and high-level resistance to azaserine, and the mapping of a second gene (*azaB*) which is involved in high level resistance to the antibiotic. Transport studies demonstrate that mutants which harbor a lesion in the *azaA* gene are not defective in the transport of the aromatic amino acids, but mutants with lesions in the *azaB* gene are defective in the transport of phenylalanine.

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MATERIALS AND METHODS

Organisms. *E. coli* K-12 strains used in this study are listed in Table 1. The genotypes *aroP azaA* and *aroP azaB* denote organisms which phenotypically exhibit an intermediate level of resistance to azaserine (minimal inhibitory concentration of 4×10^{-4} M), and the genotype *aroP azaA azaB* denotes organisms which phenotypically exhibit a high level of resistance to azaserine (minimal inhibitory concentration of $>1 \times 10^{-2}$ M). Only organisms that possess these genotypes exhibit an intermediate or high level of resistance to azaserine; all other organisms used in this study exhibit a low level of resistance to the antibiotic (minimal inhibitory concentration of 7×10^{-5} M). The methods employed for the isolation of azaserine-resistant mutants have been described previously (17). These methods consist of isolating resistant organisms from the zones of inhibition caused by azaserine, or passaging organisms in media which contain successively higher concentrations of azaserine.

Chemicals. Azaserine was purchased from Calbiochem. 5-Methyltryptophan and β-2-thienylalanine were obtained from Sigma Chemical Co. All chemicals were of the highest purity available. L-[*U*-^{14}C]tyrosine (405 mCi/mmol) was purchased from ICN. L-[*U*-^{14}C]-phenylalanine (514 mCi/mmol) and L-[2,3-^{3}H-side chain]tryptophan (20 mCi/mol) were purchased from New England Nuclear Corp.

Media and growth conditions. The minimal medium of Davis and Mingioli (8) was supplemented as required with a 10-μg/ml concentration of the appropriate amino acids, vitamins, purines, and pyrimidine, except for histidine, which was added to a final concentration of 50 μg/ml. All media contained thiamine at a concentration of 10 μg/ml. When required, streptomycin sulfate was added to a final concentration of 120 μg/ml. The minimal medium used for the selection of *aroP* mutants and genetic recombinants contained 100 μM β-2-thienylalanine and 200 μM 5-methyltryptophan in addition to the growth requirements. Penassay medium contained 17.5 g of Difco antibiotic

TABLE 1. *Properties of E. coli K-12 strains*

Strain	Sex	Mutation present	Source
UTH4	F^-	*hisG4*	Univ. of Texas at Houston Stock Collection
UTH7036	F^-	*hisG4 aroP1 azaA1*	(16)
UTH7055	F^-	*aroP7 azaA7 azaB5*	(16)
UTH7074	HfrH	*aroP10 azaA10 azaB13*	Derivative of UTH7073
UTH7076	HfrH	*aroP9 azaA9*	Derivative of UTH7073
UTH7087	Hfr (AB311)	*thr leu aroP12 azaA12*	Derivative of UTH14
UTH7110	F^-	*hisG4 aroP31*	Derivative of UTH4
MW1015	HfrH	*met-82 relA1 rpsL mtr-24*	KY4124[a]
MW1017	F^-	*thr leu pro hisG arg aroP31 uvrC*	Derivative of UTH4550
MW1018	F^-	*aroP31 azaB5*	Derivative of UTH7109
MW1030	F^-	*aspB argG aroP25*	Derivative of UTH6674
MW1035	HfrH	*met-82 relA1 rpsL mtr-24*	Derivative of MW1015
MW1052	F^-	*hisG4 mtr-24*	Derivative of UTH4
MW1055	F^-	*hisG4 aroP1 azaA1 mtr-24*	Derivative of UTH7036
MW1056	F^-	*aroP7 azaA7 azaB5 mtr-24*	Derivative of UTH7055
MW1057	F^-	*hisG4 aroP31 mtr-24*	Derivative of UTH7110
MW1064	F^-	*hisG4 azaA1*	Derivative of UTH7036
MW1065	F^-	*azaA31 azaB5*	Derivative of UTH7055

[a] Obtained from Barbara Bachmann, Coli Genetic Stock Center (CGSC #4919).

medium no. 3 per liter of water. Solid medium contained 15 g of agar per liter, and soft medium contained 6 g of agar per liter. All cultures were grown at 37°C.

Mating procedure. Conjugation experiments were performed using the membrane filter method of Matney and Achenback (10), with the exception that the membranes which supported the mating cells were incubated in soft Penassay medium rather than soft minimal medium. After incubation, the mating pairs were separated by vigorous mixing in minimal medium containing streptomycin sulfate and the nutrient requirements of the recipient. To allow for phenotypic expression of azaserine resistance, recombinants were incubated with shaking for 3 h. Samples were then plated onto minimal agar containing 0.1 mM azaserine or onto minimal agar containing 0.1 mM β-2-thienylalanine and 0.2 mM 5-methyltryptophan, which is selective for *aroP* recombinants.

Transductional procedure. All transductions were performed using modifications of the procedure described by Miller (11) for P1 transduction. The modifications consisted of growing the cells in minimal medium rather than L-broth and then plating the phage-bacterial suspensions directly onto minimal agar instead of a soft minimal overlay.

Transport procedure. The transport of the aromatic amino acids was determined by using the procedure of Whipp and Pittard (15). Cells were grown in minimal medium containing the necessary growth requirements for at least two doublings. Turbidity was monitored by using a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 560 nm. Cells were harvested in mid-exponential phase of growth by centrifugation, washed twice in minimal medium, and suspended in this medium containing chloramphenicol (80 μg/ml) and the specific growth requirements. The cells were incubated at 37°C for 10 min and then stored at 4°C until used.

Cells were brought to 30°C, and the assay was initiated by adding the radioactively labeled amino acid. Samples (0.1 ml) were withdrawn from the reaction mixture at various times after initiation of the assay, passed through membrane filters of pore size 0.45 μm, and washed with two 2-ml volumes of minimal medium at 30°C. The filters were dried, 7.5 ml of scintillation solution [600 ml of toluene, 400 ml of 95% ethanol, 5 g of 2.5 diphenyloxazole, and 0.3 g of 1,4 bis-2-(5-phenyloxazolyl) benzene per liter] was added, and radioactivity was determined using a Beckman liquid scintillation counter, model LS 100.

The aromatic amino acids ([^{14}C]phenylalanine specific activity, 10 μCi/μmol; [^{14}C]tyrosine specific activity, 10 μCi/μmol; and [^{3}H]tryptophan specific activity, 2.5 μCi/μmol) were used at a final concentration of 10 μM, which is saturating for both the common and specific aromatic amino acid transport systems (6). Control filtrations (without cells) were performed with each experiment to correct for background radiation and nonspecific adsorption of radioactive material to the filters. Corrected values are expressed as nanomoles of amino acid transported per minute per milligram of protein. Protein was determined by using the modified Lowry procedure described by Hartree (9).

RESULTS

Conjugation analyses. When employing spontaneously arising intermediate-resistant males with different origins of chromosomal transfer, conflicting results were obtained in mating experiments. When strain UTH7076, which donates the chromosome clockwise starting at min 99, was the donor, the *aroP* gene entered the recipient approximately 10 min after mating, whereas azaserine resistance was not recovered until 50 min after mating. However, when strain UTH7087, which donates the chromosome counterclockwise starting at min 44, was the donor, the *aroP* gene entered the recip-

ient, and azaserine resistance was recovered 56 min after mating. A similar phenomenon was observed in conjugational analyses employing high-level resistant mutants.

These results indicated that multiple genes were involved in azaserine resistance. To determine more accurately the location of the genes involved with azaserine resistance, various strains harboring a lesion in the *aroP* gene were constructed and employed as recipients in various conjugation experiments. A second gene, designated *azaA*, which is involved with intermediate-level resistance, was shown to map near the *hisG* gene (min 44).

With regard to high resistance, a third gene designated *azaB* was shown to map near *argG* (min 68). However, only recombinants that had a genotype of *aroP azaA azaB* exhibited a high level of resistance to the antibiotic.

Mapping of *azaA* gene by transduction. The *azaA* gene cotransduced with a frequency of 52% (183/351) with the *hisG*$^+$ gene and with a frequency of 47% (167/359) with the *uvrC*$^+$ gene. Using the formula of Wu (18), it was possible to calculate that the *azaA* gene mapped 0.7 min and 0.54 min from the *hisG*$^+$ and *uvrC*$^+$ genes, respectively (Fig. 1). The position of the *azaA* locus relative to the *hisG* and *uvrC* loci was confirmed by three-factor analyses (Table 2). The lowest frequency in the first cross was observed in the transductant having the genotype *uvrC*$^+$ *hisG*$^+$ *azaA*$^+$. Assuming that this genotype is the result of a double crossover within the transducing fragment, then the gene order is *uvrC azaA hisG*. This was confirmed by the data obtained with selected *hisG*$^+$ transductants. Thus the *azaA* gene maps near min 43 on the *E. coli* chromosome.

Mapping of the *azaB* gene by transduction. The *azaB* gene cotransduced with a frequency of 14% (21/140) with the *argG* gene and with a frequency of 71% (100/140) with the *aspB* gene. It was calculated that the *azaB* gene mapped 0.94 and 0.22 min from the *argG* and *aspB* genes, respectively (Fig. 2). The position

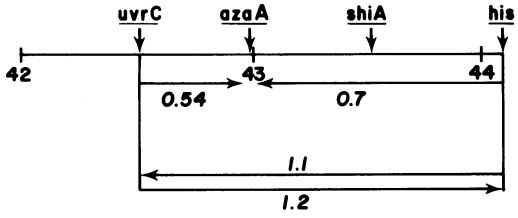

FIG. 1. *The chromosome map position of azaA taken from Bachmann et al. (3). Numbers in minutes taken from cotransduction frequencies calculated according to Wu (18).*

TABLE 2. *Three-factor analyses of the azaA region*[a]

Selected marker	Unselected markers	Total no. of each genotype
uvrC$^+$	*hisG*$^+$ *azaA*$^+$	9
	hisG$^+$ *azaA*	22
	hisG azaA$^+$	183
	hisG azaA	145
hisG$^+$	*azaA uvrC*	162
	azaA uvrC$^+$	21
	azaA$^+$ *uvrC*	165
	azaA$^+$ *uvrC*$^+$	3

[a] Donor, UTH7076 (*hisG*$^+$ *azaA uvrC*$^+$); recipient, MW1017 (*hisG azaA*$^+$ *uvrC*). *uvrC*$^+$ was selected by spotting transductants on Penassay agar and then exposing to UV light from a distance of 23 cm for 30 s.

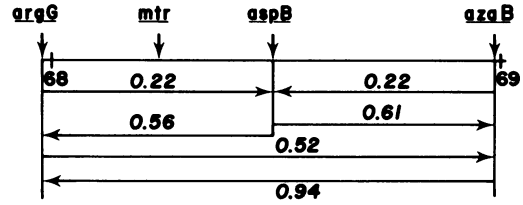

FIG. 2. *The chromosome map position of azaB taken from Bachmann et al. (3). Number in minutes taken from cotransduction frequencies calculated according to Wu (18).*

of the *azaB* gene was confirmed by three-factor analyses (Table 3). The lowest frequency in the first cross was *argG*$^+$ *aspB azaB*. Since the donor carried *argG*$^+$ and *azaB* and the recipient *aspB*, it demonstrates that the *aspB* locus lies between *argG* and *azaB*. The gene order *argG aspB azaB* was confirmed by the additional data listed in Table 3.

Cross-resistance studies. Strains were examined for their resistance to azaserine and 5-methyltryptophan by using a standard disk assay (Table 4). 5-Methyltryptophan is normally transported by the common aromatic permease system. However, by incorporating phenylalanine and tyrosine (10 μM) into the medium, the transport of the analog by the common system is blocked, and the analog is then transported by the tryptophan-specific transport system (12). Also, phenylalanine and tyrosine cause a repression in the synthesis of intermediates in the aromatic amino acid pathways, resulting in an increased sensitivity of the organism to 5-methyltryptophan due to the decreased synthesis of tryptophan by the cell (12).

Strains that are resistant to azaserine, for example UTH7055, were also resistant to 5-methyltryptophan when grown in minimal medium but sensitive to 5-methyltryptophan when

grown in minimal medium containing phenylalanine and tyrosine. Only when azaserine-resistant strains were constructed that harbored a lesion in the *mtr* gene, whose product is involved in the tryptophan-specific transport system, did the azaserine-resistant strains exhibit cross-resistance to 5-methyltryptophan when grown in minimal medium containing phenylalanine and tyrosine. Strains defective in the *mtr* gene were not resistant to azaserine regardless of the medium in which they were grown. The introduction of a defective *aroP* gene into a strain harboring a defective *mtr* gene did not result in the strain becoming azaserine resistant.

Transport studies. Strains that harbored lesions in the *aroP*, *azaA*, and *azaB* genes were examined for their ability to transport the aromatic amino acids (Table 5). A lesion in the *aroP* gene (UTH7110) resulted in an 85% decrease in the transport of the aromatic amino acids. A mutant (UTH7036) which harbored lesions in the *aroP* and *azaA* genes did not differ significantly from organism UTH7110 in its ability to transport the aromatic amino acids. The fact that the *azaA* gene product is not involved in the transport of the aromatic amino acids was verified when the rate of transport of the aromatic amino acids in strain MW1064 (*azaA*) did not differ significantly from that of strain UTH4 (wild type).

Strains UTH7055, MW1018, and MW1065, which harbor a lesion in the *azaB* gene, were defective in their ability to transport L-phenylalanine. There was a decrease in phenylalanine uptake of 97, 93, and 30%, respectively, in these strains as compared to UTH4, but there was no decrease in the transport of tyrosine or tryptophan. The rate of uptake in these strains was also related to the presence of a functional *aroP* gene, but the transport was independent of a functional *azaA* gene.

TABLE 3. *Three-factor analyses of the azaB region*[a]

Selected marker	Unselected markers	Total no. of each genotype
argG$^+$	*aspB azaB*$^+$	64
	aspB azaB	3
	aspB$^+$ *azaB*$^+$	80
	aspB$^+$ *azaB*	80
aspB$^+$	*argG*$^+$ *azaB*	62
	argG$^+$ *azaB*$^+$	94
	argG azaB$^+$	73
	argG azaB	19
azaB$^+$	*aspB*$^+$ *argG*$^+$	20
	aspB$^+$ *argG*	80
	aspB argG$^+$	1
	aspB argG	39

[a] Donor, UTH7055 (*aspB*$^+$ *azaB argG*$^+$); recipient, MW1030 (*aspB azaB*$^+$ *argG*).

DISCUSSION

It has been proposed that in procaryotes azaserine is transported by an aromatic amino acid transport system (1, 4, 5). Ames reported that *aroP* mutants of *S. typhimurium* were obtained by isolating spontaneously arising mutants which were resistant to azaserine, and concluded that the antibiotic was transported by the general or common aromatic amino acid transport system but not by the specific aromatic amino transport systems (1). However, additional studies demonstrated that strains which harbor lesions in the *dhuA* and *aroP* genes are sensitive to azaserine (2). The *dhuA* gene product is in-

TABLE 4. *Resistance of mutants to azaserine and 5-methyltryptophan, determined by a disk assay*

Strain	Genotype	Zone of inhibition[a] (mm) on medium:			
		Azaserine minimal[b]	Minimal + phenylalanine and tyrosine[c]	Minimal	5-Methyltryptophan minimal + phenylalanine and tyrosine[c]
UTH4	*hisG4*	29	17	19	29
UTH7110	*aroP31*	29	14	0	14
UTH7036	*aroP1 azaA1*	24	14	0	14
UTH7055	*aroP7 azaA7 azaB5*	0	0	0	19
MW1052	*mtr-24*	24	15	14	0
MW1057	*aroP31 mtr-24*	24	14	0	0
MW1055	*aroP1 azaA1 mtr-24*	19	14	0	0
MW1056	*aroP7 azaA7 azaB5 mtr-24*	0	0	0	0

[a] Values represent the average of three experiments and are expressed as the diameter of the zone of inhibition minus the 6-mm diameter of the disk.

[b] Disks were impregnated with a 10^{-2} M concentration of the analogs.

[c] Minimal medium containing 10 μM phenylalanine and 10 μM tyrosine.

TABLE 5. *Transport of the aromatic amino acids in mutants*

Strain	Genotype	Transport[a]		
		Phenylalanine	Tyrosine	Tryptophan
UTH4	*hisG4*	3.18	2.7	2.8
UTH7110	*aroP31*	0.44	0.40	0.45
UTH7036	*aroP1 azaA1*	0.41	0.38	0.50
MW1064	*azaA1*	2.80	2.53	2.61
UTH7055	*aroP7 azaA7 azaB5*	0.09	0.29	0.43
MW1018	*aroP31 azaB5*	0.23	0.31	0.41
MW1065	*azaA7 azaB5*	2.11	2.63	2.84

[a] Values are expressed as the nanomoles of aromatic amino acid transported per minute per milligram of protein.

volved in the regulation of the high-affinity transport system. This indicates that azaserine may be transported by the general aromatic amino acid transport system and the high-affinity histidine transport system in *S. typhimurium*.

Previously it was reported that azaserine inhibited the growth of *E. coli*, and that of all the aromatic amino acids, only L-phenylalanine could reverse this growth inhibition (5, 17). There was an inverse relationship between the level of azaserine resistance versus the initial rate of uptake of phenylalanine and the efflux of the amino acid from the cell (16). The greater the level of resistance to the antibiotic, the lower the rate of phenylalanine uptake.

Results of this study demonstrated that intermediate resistance to azaserine is the result of a two-step mutation involving both the *aroP* and *azaA* genes. The *azaA* locus mapped near min 43 on the *E. coli* chromosome. A gene (*tyrP*) whose product has been reported to be involved with the tyrosine-specific transport system has been reported to lie near min 44 (15). We do not know whether these loci are identical, and we have been unable to obtain *tyrP* strains. However, since tyrosine has no effect in reversing the growth inhibition in *E. coli* caused by azaserine, and since strains that harbor a defective *azaA* gene are not defective in tyrosine transport, it has been concluded that these mutations represent separate loci.

High resistance to azaserine is the result of lesions in the *aroP*, *azaA*, and *azaB* loci. Genetic analyses demonstrated that the *azaB* locus lies approximately 0.5 min from a gene (*mtr*) whose product is involved with the tryptophan-specific transport system. The fact that these loci are different is supported by cross-resistance studies which demonstrated that it is possible to phenotypically distinguish azaserine-resistant strains from strains that are defective in the tryptophan-specific transport system, based upon their resistance to azaserine and 5-methyltryptophan. Strains harboring a defective *mtr* locus are sensitive to azaserine when grown in either minimal medium or minimal medium containing phenylalanine and tyrosine. Also, these strains are sensitive to 5-methyltryptophan when grown in minimal medium, demonstrating that they possess a functional *aroP* gene. However, when these strains are grown in minimal medium containing sufficient concentrations of phenylalanine and tyrosine to saturate the common aromatic amino acid transport system, they are resistant to 5-methyltryptophan, demonstrating that they harbor a defective *mtr* gene. The converse is true with azaserine-resistant strains. These strains are resistant to azaserine regardless of the medium on which they are grown, and the diameter of the zone of inhibition correlates to the level of azaserine resistance exhibited by these organisms. Mutants resistant to azaserine were resistant to 5-methyltryptophan when grown on minimal medium, demonstrating that they harbored a lesion in the *aroP* gene, but when these strains were grown on minimal medium containing phenylalanine and tyrosine they were sensitive to 5-methyltryptophan, demonstrating that they possessed a functional tryptophan-specific transport system.

Transport analyses further demonstrated that strains possessing a defective *azaB* gene were not defective in the transport of tryptophan by the tryptophan-specific transport system. However, these strains were defective in the transport of L-phenylalanine. The rate of uptake of phenylalanine in strain MW1065 was 34% of that found in the control. This effect was due to the defective *azaB* gene and not to the *azaA* gene, since it has been demonstrated that strains with a defective *azaA* gene have normal transport rates for the aromatic amino acids.

Whipp et al. (14) have described a gene they designated *pheP*, whose product is involved with the phenylalanine-specific transport system. They state that this gene maps at a different location from the *tyrP* and *aroP* genes, but no mapping data are given. It is possible that the *azaB* and the *pheP* genes are the same; however, we have been unable to obtain these *pheP* strains.

Strains that exhibit an intermediate level of resistance to azaserine can possess the genotype *aroP azaA* or *aroP azaB*, and these strains can be distinguished by genetic or transport analyses. The mechanism of resistance to azaserine is different for these strains, depending on the genotype. Strains possessing the *aroP azaB* genotype are resistant due to a decreased ability to transport the antibiotic, and *aroP azaA* strains are resistant possibly due to a more rapid efflux

of the antibiotic from the cell. Although it is possible to construct strains having the genotype *aroP azaB*, none has been isolated as a spontaneously arising azaserine-resistant mutant. Thus there is some natural selective mechanism that favors the formation of the *aroP azaA* genotype in strains that exhibit an intermediate level of resistance to the antibiotic.

Brown (6, 7) proposed that, by employing different analogs, it might be possible to isolate mutants defective in the aromatic amino acid uptake as a result of the mutational effects on different macromolecular elements of the general or specific aromatic amino acid transport systems. This was verified in that mutants of both *E. coli* (6, 7, 12) and *S. typhimurium* (1, 2, 13) have been isolated using β-2-thienylalanine, indole acrylic acid, and azaserine. These mutants all have defective aromatic amino acid transport systems, and the lesions have been shown to lie at different locations on the chromosome.

The data presented in this report demonstrate that resistance to azaserine in *E. coli* is more complex than originally proposed. Azaserine is transported by both the common aromatic amino acid transport system and the phenylalanine-specific transport system, indicating that the antibiotic affects different macromolecular elements of the aromatic amino acid transport systems. Resistance to azaserine is due in part to a decreased rate of uptake of the antibiotic by the cell. Studies are presently under way to elucidate the role of the *azaA* gene product in azaserine resistance and in aromatic amino acid transport.

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