

Novel Acriflavine Resistance Genes, *acrC* and *acrD*, in *Escherichia coli* K-12

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Acriflavine-resistant mutants were isolated from an acriflavine-sensitive (*acrA*) strain of *Escherichia coli* K-12 and then tested for temperature sensitivity of cell division. Genetic analysis characterized two new genetic loci, *acrC* and *acrD*. The former was mapped between *tonA* and *proA*, and the latter between the origin of genetic transfer of *HfrH* and *aerB*. *acrC* and *acrD* mutants could divide but did not initiate a new round of deoxyribonucleic acid (DNA) replication at 43°C. DNA synthesis of the *acrC* mutant cells ceased after a period of time following temperature shift-up, and thereafter DNA degradation occurred. However, cell mass continued to increase for a long time at the nonpermissive temperature. On the other hand, DNA synthesis of the *acrD* mutant cells ceased soon after the shift-up, and the cell mass did not appreciably increase during the prolonged incubation.

Acridine dyes specifically inhibit the replication of plasmids rather than that of the chromosome (16, 17, 20). They have a mutagenic effect on microbial cells and an antitumor effect on higher organisms (6). Lerman (11) and other investigators (24, 25) established that acridines are intercalated between adjacent base pairs, particularly adenine and thymine, of DNA. The intercalation model has been cited to explain frame-shift mutations and inhibition of DNA and RNA synthesis (9). However, we have implicated plasma membrane as an important site of action of acridines. (i) Acridines are impermeable to the plasma membrane (13, 14, 22); (ii) they are lipophilic, which suggests the membrane as a possible site of action (6); (iii) sensitivity to the acridines is associated with a membrane protein, determined by the gene *acrA* (15, 19). Recently, we have described another gene, *acrB*, which also determines sensitivity to acriflavine (Fig. 1; 18).

In the present study, we undertook to isolate and characterize temperature-sensitive mutants accompanied by acriflavine resistance. We found two genes involved, *acrC* and *acrD*.

MATERIALS AND METHODS

Bacterial strains. All the strains used are characterized in Table 1. Strain N43 was an acriflavine (AF)-sensitive (*acrA*) strain, and the temperature-sensitive mutants were derived from this strain after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1). For transduction experiments, phage P1vir was used (5).

Media. Broth media PGY L and L, and synthetic

medium D, were prepared as described by Nakamura (12), Lennox (10), and Davis (4), respectively. The D medium was supplemented with either 0.1% glucose or 0.5% of another sugar. For amino acid-requiring mutants the medium was supplemented with 20 µg of each amino acid per ml. Streptomycin sulfate was added to 200 µg/ml to select *rpsL* (Str^r) recombinants. pH of the broth media was adjusted to 7.4 with 1 N NaOH, unless otherwise stated.

For AF-containing medium, PGY was modified as follows: 5 g of polypeptone, 1 g of yeast extract, 3 g of NaCl, 1 g of glucose, and 1,000 ml of deionized water, pH adjusted to 7.8.

Bacterial crosses. General procedures for bacterial crosses and P1 phage transduction were described previously (5, 12). The matings were performed by mixing donor and recipient cells at a ratio of 1:20. The recombination frequencies in the representative crosses, W1895 × N2616 and W1895 × N2624, selecting for *lac*⁺ *met*⁺ *rpsL*, were 2.5×10^{-3} and 1.4×10^{-3} per Hfr, respectively, under these conditions. These values were lower than that in a W1895 × N43 cross (6.0×10^{-3} per Hfr) as control.

Growth experiments. For all the experiments, overnight 30°C-grown cells were used. Cell mass was measured by optical absorption of culture at 660 nm in a spectrophotometer.

DNA synthesis of the cells was measured with incorporation of [³H]thymidine. Freshly grown cells (about 10⁸ cells per ml) were inoculated into the PGY medium containing 10 of µCi [³H]thymidine per ml. Samples of 0.1 ml were pipetted into 5 ml of cold 5% trichloroacetic acid, and, after 30 min, the precipitates were filtered onto a membrane filter (0.45 µm in pore size). The precipitate was washed with 20 ml of the acid solution, and, after drying, the radioactivity was measured in a Packard liquid scintillation counter.

RESULTS AND DISCUSSION

Isolation of temperature-sensitive AF-resistant mutants of strain N43. Nine cultures from single colonies of strain N43 were separately treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and AF-resistant mutants were isolated from each of the cultures at 30°C. As shown in Table 2, they could be divided into three classes: (i) resistant to AF at both 30 and 43°C, (ii) resistant to AF at 30°C but sensitive at 43°C, and (iii) resistant to AF at 30°C but not viable at 43°C even in the absence of the drug. The present study deals exclusively with the third class of mutations.

AF sensitivity of the temperature-sensitive mutants. Freshly grown cells of the mutants N2630 from class I, N2598 from II, N2616 and N2624 from III, and N43 (as control), after proper dilution, were plated on prewarmed media (pH 7.8) containing varied concentrations of AF. As shown in Fig. 2, bacterial strains of each class were resistant to AF at 30°C as compared with the parental strain N43. However, at higher temperatures (37 or 43°C), strains N2598 (class II) and N2616 and N2624 (class III) were as sensitive to AF as N43, but strain N2630 (class I) remained AF resistant.

Of 25 temperature-resistant revertants of N2616, 22 were AF sensitive and the other 3 were resistant. On the other hand, when gene *acrC*⁺ (temperature resistance) of strain N2700 was transduced by P1 to N2616, all of 122 transductants tested were sensitive to AF as compared with the recipient. Both temperature-resistant revertants of N2624 isolated were sensitive to AF.

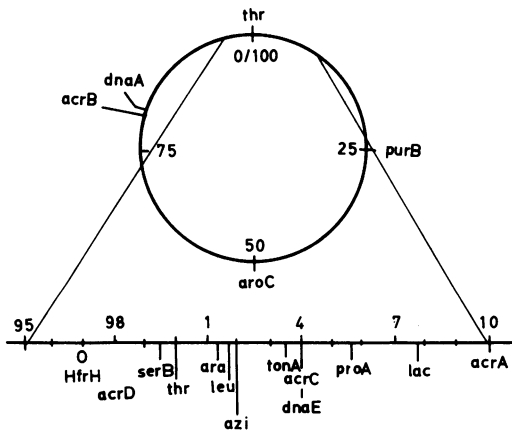
Genetic analysis: classification of class

FIG. 1. Chromosome map of *E. coli* showing relative positions of the relevant genes. Genetic symbols are those described by Bachmann et al. (2).

TABLE 1. Bacterial strains^a

Strain	Sex	Relevant character	Source or reference
W1895	Hfr	<i>met</i> (O- <i>acrA</i> ⁺ - <i>lac</i> ⁻ - <i>ara</i> ⁺ -)	(12)
N2734	Hfr	<i>met</i> (O- <i>gal</i> ⁺ - <i>lac</i> ⁺ -)	F8/N2616
N2700	Hfr	<i>met azi tonA</i>	<i>azi</i> and <i>tonA</i> mutant of W1895
N2109	Hfr	<i>thi metB</i> (O- <i>thr</i> ⁺ - <i>leu</i> ⁺ -)	Nitrosoguanidine mutagenesis of HfrH (7)
RB30	Hfr	<i>thi serB</i>	(3)
AB313	Hfr	<i>thr leu</i>	(23)
N43	F ⁻	<i>acrA1 lac ara xyl rpsL</i>	(12)
N2776	F ⁻	<i>tonA azi acrC</i>	Derived from N2700 × N2616
N2752	F ⁻	<i>acrC ara rpsL</i>	Derived from W1895 × N2616
N2802	F ⁻	<i>thr leu ara acrD rpsL</i>	Derived from AB313 × N2624
AB1157	F ⁻	<i>proA leu</i>	Howard-Flanders
N2631	F ⁻	class I mutant of N43	This paper
N2630	F ⁻	class I mutant of N43	This paper
N2598	F ⁻	class II mutant of N43	This paper
N2616	F ⁻	class III (<i>acrC</i>) mutant of N43	This paper
N2619	F ⁻	class III mutant of N43	This paper
N2620	F ⁻	class III mutant of N43	This paper
N2622	F ⁻	class III mutant of N43	This paper
N2624	F ⁻	class III (<i>acrD</i>) mutant of N43	This paper
N2628	F ⁻	class III mutant of N43	This paper

^a Genetic symbols are those described by Bachmann et al. (2).

III mutants. To find the loci determining the temperature sensitivity of the mutants N2616, N2619, N2620, N2622, N2624, and N2628, these strains were crossed with Hfr W1895, and *lac*⁺ *met*⁺ *rpsL* clones were selected. Table 3 shows that there are at least two loci for class III mutants: one of the temperature resistance genes of the donor is transferred to the recipient at frequencies of some 30% of *lac*⁺, and the other is transferred at frequencies of several percent. The former gene was referred to as *acrC* and the latter as *acrD*. The following analyses are for these mutations.

***acrC* mutation in strain N2616.** An interrupting conjugation experiment was performed using strains N2700 as donor and N2616 as recipient. The result showed that donor *acrC*⁺ is transmitted to the recipient between genes *lac* and *azi*, i.e., very close to *tonA*. Several types of conjugation experiments were conducted using strains N2700 and N2734 as donors and N2616, AB1157, and N2752 as recipients. The recombi-

TABLE 2. Classification of AF-resistant mutants of strain N43 induced by nitrosoguanidine^a

Nitrosoguanidine-treated culture	No. of clones isolated	Phenotype (%) of clones AF resistant at 30°C		
		I	II	III
A	97	6	18	2
B	82	7	23	13
C	24	8	29	17
D	21	10	33	10
E	37	11	3	3
F	39	10	31	5
G	36	6	19	8
H	72	4	38	4
I	18	11	39	6
Average		7	24	7

^a Class I: AF resistant both at 30 and 43°C; class II: AF resistant at 30°C but AF sensitive at 43°C; class III: AF resistant at 30°C but AF sensitive at higher temperature, and nonviable at 43°C. The AF sensitivity of the cells was determined by the AF (50 µg/ml) gradient plate method.

nant analysis showed that gene *acrC* is located between *tonA* and *proA*.

To strengthen this conclusion, the following transduction experiments were performed. First, strains N2776 and W1895 were used as P1 donor and recipient, respectively, and selection was done for azide resistance (*azi*). Of 430 clones tested, 0.7 and 4.0% of *acrC* and *tonA* alleles, respectively, were cotransduced with *azi*. Second, P1 donor and recipient were N2700 and N2616, respectively, and selection was done for temperature resistance (*acrC*⁺). Of 122 *acrC*⁺ transductants, 67 and 1.6% harbor mutations *tonA* and *azi*, respectively. Another *acrC*⁺ transduction showed 60% cotransduction of *tonA*.

The third transduction was performed using N2776 as P1 donor and W1895 as recipient, and selection was for T1 phage resistance (*tonA*). It is well known that the spontaneous mutation rate to T1 phage resistance is rather high as compared with other types of mutations. Thus, when selected for *tonA*, the cotransduction frequency of *acrC*⁺ will be underestimated. However, the frequency increased from experiment to experiment, because it became possible to select for the true transductants based on colony morphology. The highest value for *acrC*⁺ cotransduction was 65% of the *tonA* transductants (Table 4). This value is consistent with the average of 64% *tonA* cotransduction, when selection was for *acrC*⁺. Control experiments showed that (i) none of 284 spontaneous mutants tested of W1895 resistant to azide was temperature sensitive, and (ii) none of 150 spontaneous mu-

tants tested of W1895 resistant to T1 phage was temperature sensitive.

acrD mutation in strain N2624. The data in Table 3 show that *acrD*⁺ of the donor was transferred at a frequency of 2.2% among the *lac*⁺ recombinants, suggesting that *acrD* is distal to *lac* from the point of entry of the donor. When an interrupted conjugation experiment was conducted using strain N2700 as donor and N2624 as recipient, the donor genes were transferred to the recipient in the order of *lac*⁺-*tonA*⁺-*ara*⁺-*acrD*⁺.

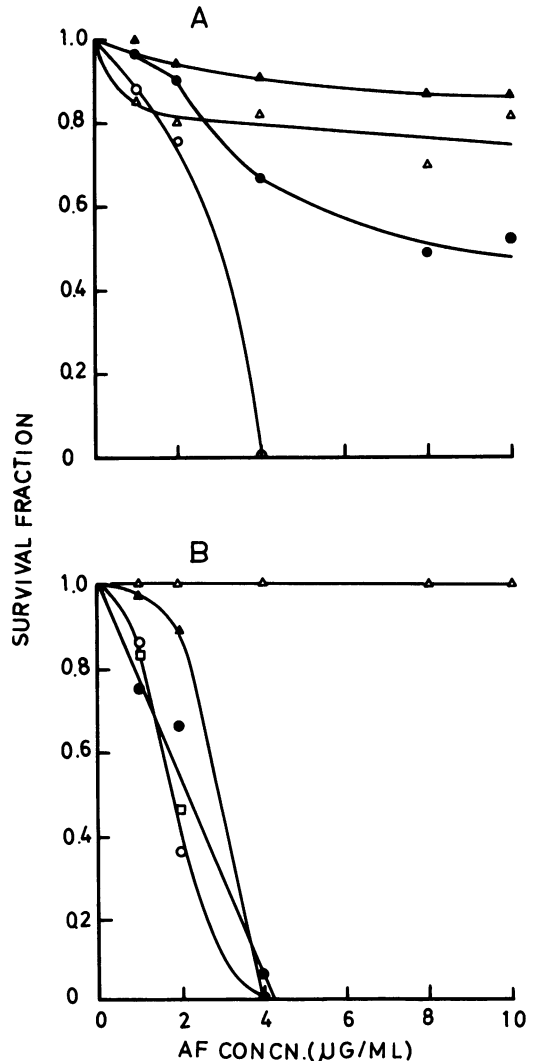


FIG. 2. AF sensitivity of the temperature-sensitive mutants of N43 at 30°C (A) and higher temperatures (B). (A) ○, N43; △, N2630; ▲, N2598; ●, N2616. (B) ○, N43 at 37°C; □, N43 at 43°C; ●, N2616 at 37°C; ▲, N2598 at 43°C; △, N2630 at 43°C.

TABLE 3. Genetic analysis of the AF-resistant mutants

Class	Cross		<i>lac</i> ⁺ <i>met</i> ⁺ <i>rpsL</i> recombinants tested	AF-sensitive recombinants (%)	Temp-resistant ^a recombinants (%)
	Donor	Recipient			
I	W1895	N2630	304	2.0	
		N2631	331	3.0	
II	W1895	N2598	204	0	
III	W1895	N2616	363	27.5	35.0
		N2628	200	16.0	29.0
		N2619	219		6.9
		N2620	173		2.3
		N2622	150		9.4
		N2624	226		2.2

^a Temperature-resistant recombinants capable to form colonies on PGY agar (pH 7.4) at 43°C.

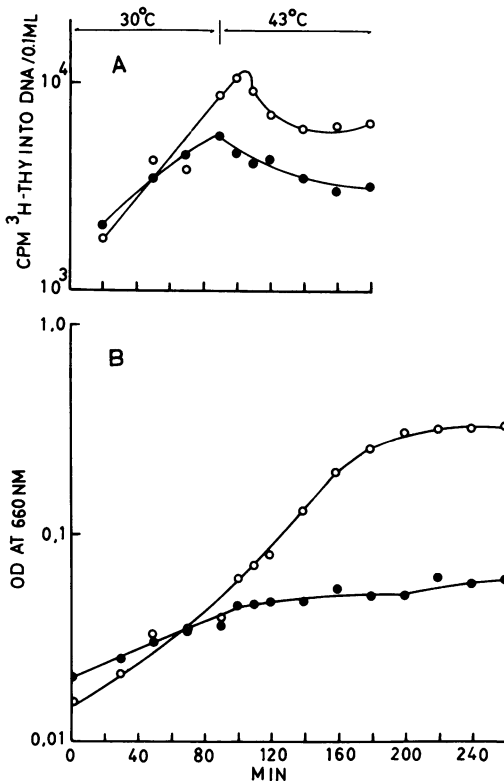


FIG. 3. DNA synthesis (A) and cell growth (B) of strains N2616 (O) and N2624 (●) when temperature was shifted up. DNA synthesis was measured by [³H]thymidine incorporation into acid-insoluble material and cell growth was measured by optical absorption of PGY cultures at 660 nm. At 90 min after inoculation, temperature was shifted from 30 to 43°C.

Three types of the three-factor crosses were performed to map detail of the *acrD* locus. First, *acrD*⁺ donor N2109 was crossed with strain N2624, and *ara*⁺ *thi*⁺ *rpsL* recombinants were

scored for unselected markers. Among 191 recombinants tested, *ara*⁺ (donor marker [D]) *thr*⁺ (D) *acrD*⁺ (D) recombinants resulting from double crossing-over were 72.3% and *ara*⁺ (D) *thr* (recipient marker [R]) *acrD*⁺ (D) recombinants resulting from quadruple crossing-over were 4.6%. Therefore, the gene order is *ara-thr-acrD*. Second, strain RB30 as *acrD*⁺ *serB* donor and N2624 were crossed, and *ara*⁺ *thi*⁺ *rpsL* recombinants were selected. Genetic analysis showed that, among 252 recombinants tested, *ara*⁺ (D) *serB* (D) *acrD*⁺ (D) recombinants resulting from double crossing-over were 4.4% and *ara*⁺ (D) *serB*⁺ (R) *acrD*⁺ (D) resulting from quadruple crossing-over were 0.8%; gene order was *ara-serB-acrD*. In the last cross, strain RB30 was crossed with strain N2802, and selection was for *thr*⁺ *leu*⁺ *thi*⁺ recombinants. Of 394 recombinants scored, *thr*⁺ (D) *serB* (D) *acrD*⁺ (D) recombinants resulting from double crossing-over were 42%, and *thr*⁺ (D) *serB*⁺ (R) *acrD*⁺ (D) recombinants resulting from quadruple crossing-over were 1.5%. Therefore, it is concluded that the gene *acrD* is located between origin of genetic transfer of HfrH and *serB*. More detailed mapping of the locus of *acrD* was not performed since we did not have a useful standard marker between the the origin of HfrH and the *serB* gene.

Effect of *acrC* or *acrD* mutation on cell division. When approximately 10⁶ cells of strains N2616, N2624, W1895 (as control), and N43 (as control) per ml were shifted from 30 to 43°C, the *acrC* and *acrD* mutant cells divided once but did not begin a new round of the division. DNA synthesis in the *acrC* mutant cells continued for a while after the temperature shift-up. Thereafter, DNA degradation occurred, although the turbidity increased steadily for a long period (Fig. 3). Filamentous cells increased in frequency and length with incubation at the nonpermissive temperature. On the other hand, DNA synthesis of the *acrD* mutant cells ceased soon after the temperature shift-up; thereafter, DNA degradation occurred as in the *acrC* mutant cells. However, the *acrD* mutant cells did not elongate under these conditions.

Gene *acrC* was shown to be mapped at min 4 on the *E. coli* chromosome. This region contains

TABLE 4. Cotransduction frequency of *acrC* among *tonA* transductants^a

Selected for	No. tested	Frequency of <i>acrC</i> (%)
<i>tonA</i>	300	21
<i>tonA</i> , mucoidy	50	56
<i>tonA</i> , non-mucoidy	100	65

^a Donor strain N2776; recipient W1895.

some of genes relating to cell division: *ftsC* (8, 21) and *dnaE* (2). On the other hand, gene *acrD* was located between the origin of entry of the HfrH chromosome at min 97 and *serB* at min 99 (c.f. Fig. 1). In this region, there are genes *dnaC* (2) and *deo* (2) relating to DNA synthesis. Although the relationship between these genes is still unclear, it is apparent that there is a significant difference in the role played in cell division between *acrC* and *acrD*.

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