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Genetic Determination of Resistance to Acriflavine, Phenethyl Alcohol, and Sodium Dodecyl Sulfate in *Escherichia coli*

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Wild-type strains of *Escherichia coli* K-12 are resistant to acriflavine. Gene *acrA*$^+$ which determines resistance to acriflavine is located near the *lac* region of the chromosome. This gene determines not only resistance to basic dyes but also resistance to phenethyl alcohol. Acriflavine resistance was transmitted, together with phenethyl alcohol resistance, from a resistant Hfr strain to a sensitive recipient by mating. Reversion of the mutant gene *acrA1* (phenotypically acriflavine-sensitive) to acriflavine resistance was accompanied by a change from phenethyl alcohol sensitivity to resistance, and conversely the revertants selected for phenethyl alcohol resistance were resistant to acriflavine. A suppressor mutation, *sup-100*, closely linked to the *acr* locus, suppresses the *acrA1* gene (phenotypically acriflavine-resistant), but does not determine resistance to phenethyl alcohol and basic dyes other than acriflavine. The genetic change in the locus *acrA1* to types resistant to basic dyes and phenethyl alcohol was accompanied by an increase in resistance to sodium dodecyl sulfate, a potent solvent of lipopolysaccharide and lipoprotein. It is suggested that gene *acrA* determines synthesis of a membrane substance. The system seemed to be affected strongly by the presence of inorganic phosphate.

Phenethyl alcohol (PEA) has been reported to have a bacteriostatic rather than a bactericidal effect on *Escherichia coli* (2, 15). However, previous experiments (19) have shown that PEA is not necessarily bacteriostatic; its effect on *E. coli* is influenced by factors such as the *p*H of the medium and, undoubtedly, the allelic forms of certain genes. It has been suggested that the gene determining response to PEA is also the gene (designated *acrA*) determining response to acriflavine (AF); this gene is located near the marker for lactose utilization. Gene *acrA1* may be located in the same operon as Sugino's gene *Mb* which is responsible for sensitivity to methylene blue and acridine (cf. 16, 28).

In the present paper, we report further studies on the genetic determination of sensitivity to AF and PEA. By genetic crosses and mutations, it was found that the gene (*acrA*$^+$) determining AF resistance also determines PEA resistance, but this cross-resistance was not observed in some mutants which had become resistant to AF through the action of a suppressor mutation.

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When cells were treated with the detergent sodium dodecyl sulfate (SDS), which is a potent solvent for lipopolysaccharide and lipoprotein, strains resistant both to PEA and AF showed higher viability than sensitive strains and the suppressed mutant. On the basis of present and previous results, we suggest that the cell membrane plays an important role in the resistance mechanism. [An abstract of a part of the present work has appeared elsewhere (Japan. J. Genet. **42**:428, 1967).]

MATERIALS AND METHODS

Strains used. The bacterial strains used are all derivatives of *E. coli* K-12. These strains and their genetic characters are listed in Table 1. [The genetic nomenclature and strain designations used have appeared in previous publications and conform to the recommendations of Demerec et al. (5).] Derivatives of these strains are described in the following tables. The *acrA1* mutant (phenotype AF-sensitive, strain N43) was originally isolated as a spontaneous mutant derived from the parental, wild-type AF-r (phenotype AF-resistant) strain W4573 carrying *acrA*$^+$. The other *acr* mutants (strains N427, N447, and N467) were also derived from clones of strain W4573, but these clones seem to differ from the original strain W4573 in stability of the gene *acr*$^+$.

TABLE 1. *Partial list of bacterial strains and their genetic markers*[a]

Strain	Genetic character	Origin	Reference
W1895	Hfr: K-12 *met*		HfrC; transmitted with an order of O-*acrA-lac-ara-*
N558	Hfr: K-12 *thi*	CA161[b]	HfrH; transmitted with an order of O-*thr-lac-acrA-gal-*
W4573	F⁻: K-12 *lac ara mal xyl mtl gal str*		
W4573a, W4573b, W4573c	Same as in W4573 but variable in response to acridine		
N43	F⁻: *acrA1*; other markers same as in W4573	W4573	Formerly designated 18/1042
N90	F⁻· same as in W4573	W1895 × N43[c]	
N427	F⁻; *acr-3*; other markers same as in W4573	W4573a	
N447	F⁻: *acr-4*; other markers same as in W4573	W4573b	
N467	F⁻: *acr-5*; other markers same as in W4573	W4573c	
N545	F⁻: same as in W4573	N43	Revertant (selected in PEA)
N559	F⁻: *acrA1 sup-100*; other markers same as in W4573	N43	Revertant (selected in AF)
N562	F⁻: same as in W4573	N43	Revertant (selected in AF)

[a] Genes determining biosynthesis: *met*, methionine; *thi*, thiamine; *thr*, threonine. Genes determining sugar utilization: *lac*, lactose; *ara*, arabinose; *mal*, maltose; *xyl*, xylose; *mtl*, mannitol; *gal*, galactose. Genes determining response to drug: *acrA1*, an AF-sensitivity mutation (*see* Fig. 4); *acr-3*, *-4*, and *-5*, other AF-sensitivity mutations; *str*, streptomycin. Gene suppressible to *acrA1*: *sup-100*. Origin of genetic transfer: O.

[b] Kindly supplied by A. Matsushiro.

[c] Interrupted at 5 min after onset of the mating and selected for phenotype AF-r.

Hence, the unstable (parental) clones were tentatively designated W4573a, W4573b, and W4573c.

Phenotype with regard to resistance and sensitivity to a drug is designated by an abbreviation (capital letters) of the drug's name and −r or −s; for example, MG-r represents methyl green resistance and PEA-s represents PEA sensitivity.

Media. Brain Heart Infusion (BHI; Difco) was used for bacterial mating and for maintaining the stocks. Thiamine was added at a final concentration of 2 μg/ml for culturing and mating of the Hfr strain N558. The normal broth for liquid culture consisted of 8 g of Nutrient Broth (Difco), 5 g of sodium chloride, and 1 g of glucose per liter, and was adjusted to *p*H 7.4 with 1 N NaOH. Phosphate-buffered broth was prepared by mixing equal portions of double-strength broth and double-strength phosphate buffer after sterilizing each separately; the final concentration of phosphates was 0.067 M, and the *p*H was 6.2 and 7.4.

For the solid medium, another broth, referred to as PG, was used; it consisted of 10 g of polypeptone (Daigo-Eiyo, Ltd., Osaka, Japan), 3 g of sodium chloride, and 1 g of glucose per liter. Powdered agar was added to a final concentration of 1.5%. The initial *p*H of the agar medium was adjusted to 7.4 with 1 N NaOH for cell counting and to 8.2 for assaying the response of cells to dyes.

Several basic dyes, including methylene blue (MB), toluidine blue (TB), crystal violet (CV), methyl green (MG), and pyronine B (PY), were used, as well as AF. The dye-containing medium was prepared by mixing the molten agar medium and a given volume of dye solution after sterilizing each separately. The solution of SDS to be added to broth medium was sterilized at 100 C for 30 min.

Gradient plates were prepared in rectangular dishes (10 by 30 by 2.5 cm), with the dye-containing agar forming the upper sloping layer as described previously (18). The plates were dried for about 2 hr at 37 C before use.

The mineral medium, S_1, used to select against the parental strains in the bacterial crosses was the same as described in a previous report (16). S_1-lactose, S_1-arabinose, and S_1-glucose indicate that the medium was supplemented with 1% lactose, 1% arabinose, and 0.4% glucose, respectively.

Mating. General procedures for bacterial matings have been described previously (16, 17). The donors used were W1895 and N558, and the recipients were N43 and its AF-r derivatives. The mating period for zygote formation and the dilution factor of the mating mixture for plating onto selective medium differed from experiment to experiment. Recombinant colonies on the selective medium were picked and were purified by respreading on the same medium used for selection; each of them was maintained in the normal liquid broth (*p*H 7.4) for experimental use.

No significant difference among N43 and its derivatives was found in recombination frequency (about 5% for the W1895 donor) or in rate of genetic transfer (introduction of *acrA*$^+$ from W1895 to the recipients occurred about 2.5 min after onset of mating).

Determination of the level of drug resistance. For distinguishing between resistance and sensitivity to basic dyes, one loopful of cell suspension (about 10^9 cells/ml) was point-inoculated on PG agar (*p*H 8.2) containing 50 μg of AF per ml and 250 μg of MG per ml. Growth was observed after overnight incubation at 37 C. An isolate was judged as a resistant strain when confluent growth occurred on the inoculation point (about 0.3 cm in diameter) and as a sensitive strain when no growth occurred or when only a few (usually one to two) isolated colonies grew at the inoculation point.

Resistance to AF and other basic dyes was determined by measuring the length of confluent growth along the streak of inoculation on gradient plates. For determining resistance to PEA, which is a volatile substance, a different method was used. Cells cultured overnight in normal liquid broth were inoculated into two test tubes each containing 2 ml of broth adjusted to *p*H 6.2 by the addition of phosphate buffer at a final concentration of 0.067 M. After the tubes had been shaken (usually for 30 min), PEA was added to one of the tubes to give the desired concentration, and the tubes were shaken for 2 to 4 hr. Samples from each tube were mixed with diluted agar solution and were layered on PG agar plates. Colonies were counted after overnight incubation. The viable count of the culture exposed to PEA was expressed as a percentage of that of the unexposed culture.

To compare AF sensitivity with PEA sensitivity, a similar type of experiment was run with AF, using the basal medium buffered at *p*H 7.4 with phosphate buffer at a final concentration of 0.067 M. On the basis of the relation to *p*H of PEA (19) and AF (20) sensitivities, and taking into consideration the fact that PEA is volatile, the *p*H values adopted and the method used seemed to be most suitable.

RESULTS

Genetic transfer of AF and PEA resistance. It was shown previously that the genetic transfer of AF resistance from W1895 to N43 begins about 2.5 min after the onset of mating when the mating mixture is incubated at 37 C (16). An attempt was made to see whether the AF-r recombinants were resistant to PEA also, even if the mating was interrupted at different times. The experimental procedures for mating, phenotypic expression of AF resistance, and dilution were the same as described in the previous report (16) except that mating was interrupted by treatment in a Waring Blendor at 4, 6, and 30 min after the onset of mating. Selection for PEA-resistant (PEA-r) recombinants was impossible because no PEA concentration was found which killed (i.e., suppressed colony formation of) the sensitive (PEA-s) cells but allowed PEA-r cells to grow.

Twenty AF r recombinants were isolated from each blended sample of the mating mixture and their PEA sensitivity was tested. As a control, 20 clones from each of the parent strains were tested in the same manner. The distribution of viable counts of PEA-poisoned cultures (expressed as the percentage of the unpoisoned control) is shown in Fig. 1. Clones of the parent strains were differentiated clearly by their PEA sensitivity, and the AF-r recombinants were on the whole as resistant as, or more resistant than, the PEA-r parent.

A question arose as to whether or not the PEA resistance of AF-r recombinants was a result of exposure of the recombinants to AF in the selection medium, because it was observed that when an AF-r strain is precultured in an AF-containing medium the cells become slightly more resistant to AF than when they are precultured in an AF-free medium (*unpublished data*). Thus, an attempt was made to see whether recombinants inheriting AF resistance as an unselected trait were more resistant to PEA than AF-s recombinants.

Forty Ara$^+$ Met$^+$ recombinants and 40 Lac$^+$ Met$^+$ recombinants were obtained by crossing W1895 and N43. These clones were purified by

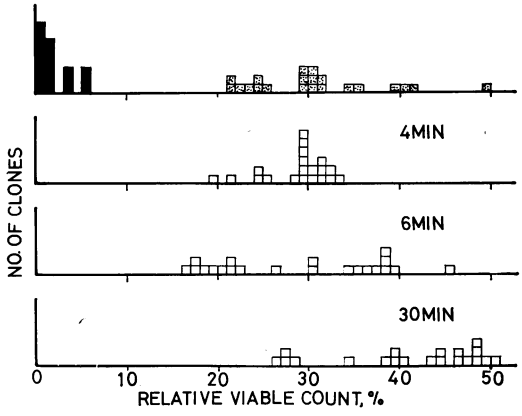

FIG. 1. *Concomitant transfer of PEA resistance with AF resistance from the resistant donor W1895 to the sensitive recipient N43. Mating was carried out for 4, 6, and 30 min (by blending the mating mixture at each time), and recombinants were selected for phenotype AF-r. Each of 20 recombinants isolated and purified was inoculated into a pair of tubes containing phosphate-buffered broth (pH 6.2). Shaking for 30 min was followed by addition of 0.08% PEA to one of the paired tubes. After further incubation for 2 hr, cells were plated onto PG agar to count viable cells. Abscissa represents viable count in percentage of control unexposed to PEA. Dotted squares, W1895; solid squares, N43; open squares, AF-r recombinant.*

streaking on S_1-arabinose and S_1-lactose agar media, but the purified strains were maintained in liquid medium. AF sensitivity was assayed by observing the growth on the gradient plate containing 30 μg of AF per ml in the sloping upper layer. After overnight incubation, confluent growth occurred on the plate in streaks less than 0.5 cm long with N43 and AF-s recombinants, whereas W1895 and AF-r recombinants grew confluently along the whole length of the gradient plate (10 cm). All of the recombinants could be distinctly classified into two classes. Each strain of AF-s and AF-r recombinants was tested to determine its PEA sensitivity, as described under Materials and Methods. Figure 2 shows that 1% or less of the cells of N43 and AF-s recombinants remained viable after 2 hr of exposure to 0.2% PEA, whereas 13 to 27% of the cells of W1895 and AF-r recombinants were viable after the same exposure. Hence, it can be concluded that the PEA resistance of the recombinants selected for their AF resistance is not due to the previous exposure to AF. It most probably is due to the presence of wild-type *acrA* allele.

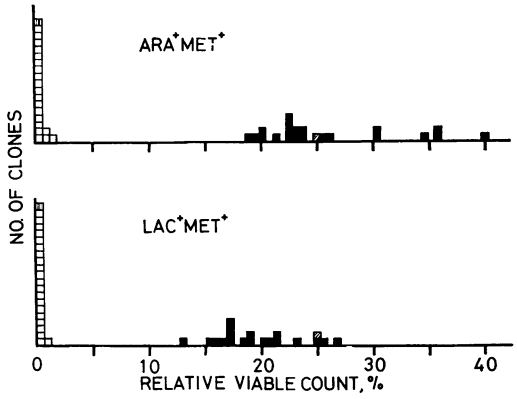

FIG. 2. *PEA resistance of recombinants carrying an unselected marker responsible for phenotype AF-r. Mating was carried out between strains W1895 and N43 by allowing them to stand for 2 hr at 37 C. Selection was made for Lac+ Met+ and Ara+ Met+ recombinants. After purification, they were tested for AF sensitivity by streaking the broth culture on the gradient plate containing AF (30 μg/ml) in the sloping upper layer and were divided into two classes of phenotypes, AF-r and AF-s. Twenty clones of each phenotype class were inoculated separately into paired tubes containing broth of pH 6.2. After the tubes had been shaken for 30 min., PEA was added to one of the paired tubes to make 0.2% and a further incubation for 2 hr was followed by plating for determination of viable count. Abscissa represents relative viable count (%) after 2 hr of exposure to PEA. Open squares, AF-s recombinant; solid squares, AF-r recombinant; vertically ruled squares, N43; diagonally ruled squares, W1895.*

AF-r and PEA-r revertants of N43. The relationship between AF resistance and PEA resistance was studied by use of back mutants from the sensitive strain N43. To obtain independent mutants to AF resistance, an overnight culture of strain N43 was used to inoculate 20 tubes containing 5 ml of normal broth with about 100 cells each. These cultures were grown to stationary phase (for about 30 hr). The cells of cultures were centrifuged, washed once, and suspended in 0.3 ml of saline. Half of each suspension was spread separately on S_1-glucose agar plates containing 30 μg of AF per ml. The remaining half of each was stored in a refrigerator for later use. After 48 hr of incubation, two or more resistant colonies were formed on each of five plates and one colony was formed on each of four plates; the remaining 11 plates showed no visible colonies. When 3×10^8 cells of the overnight culture of the sensitive strain used to inoculate the 20 tubes were seeded on the AF-containing agar plates, no viable colonies were formed on any of the plates. Hence, the inoculum for the above-mentioned 20 tubes must have contained very few, if any, resistant cells. The AF-r clones found on the plates were isolated and purified. These revertants were numbered from N559 to N567. The sensitivity of each AF-r revertant to 0.1 and 0.125% PEA and to 5 μg of AF per ml was determined on the basis of the viable count in the presence of PEA or AF

TABLE 2. *Cross-resistance between PEA and AF in AF-r revertants of N43*[a]

Strain	AF (5 μg/ml)	PEA	
		0.1%	0.125%
N559	93.0[b]	1	0
N560	71.5	1	0
N561	64.3	0.7	0
N562	95.2	16.2	13.7
N563	98.9	0	0
N564	86.0	0	0
N565	88.5	0	0
N566	126	0	0
N567	65.4	0	0
N43	0	0	0

[a] Each of the revertants was inoculated into three tubes containing broth at *p*H 6.2 and into two tubes containing broth at *p*H 7.4. After 30 min, PEA was added to two tubes of the former class to give 0.1 and 0.125%, and AF was added to one of the latter class to give 5 μg/ml. Viable counts in the presence and absence of the drugs were determined after a further incubation for 3 hr.

[b] Viable count after exposure to AF or PEA as a percentage of that of the unexposed control.

as a percentage of the control value after incubation for 3 hr. Table 2 indicates that only N562 was resistant to PEA; the other strains were sensitive at nearly the same level as their parent, N43.

Next, to obtain PEA-r revertants of N43, 20 independent cultures were prepared as indicated above, and 0.5-ml portions of each of the cultures were inoculated into 5 ml of broth containing 0.2% PEA. After incubation for 72 hr at 37 C, eight tubes were found to be turbid. One loopful of each of these cultures was inoculated into fresh broth containing 0.2% PEA, and after 24 hr samples from each culture were streaked onto PG agar. These two-step selections for PEA-r revertants were performed with the view that mutants carrying a low level of PEA resistance may have predominated in the first step of selection since PEA concentration in the medium gradually decreases during the incubation for 72 hr. Hence, there was the possibility that the PEA-r revertants obtained might be multiple mutants selected during a 96-hr exposure to PEA.

Three types of colonies, as judged by their morphological characteristics, developed on each plate. However, no significant difference in their PEA resistance was found. The PEA-r clones thus obtained were numbered from N544 to N551.

Freshly grown cells of the revertants were tested for their sensitivity to 0.125% PEA and 5 μg of AF per ml during 2 hr of incubation. As represented in Table 3, seven of eight revertants were resistant to AF as well as to PEA; only strain N544 was sensitive to AF but resistant to PEA. It is interesting that strain N544 was resistant to AF if inorganic phosphate was absent. The other PEA-r strains were AF-resistant in the presence and in the absence of inorganic phosphate. Examination of the phosphate-elicited repression of expression of AF resistance in the conditionally AF-r mutant strain will be described elsewhere.

TABLE 3. *Cross-resistance between PEA and AF in PEA-r revertants of N43*[a]

Strain	PEA (0.125%)	AF (5 μg/ml)
N544	4.9[b]	0
N545	7.2	11.8
N546	4.6	10.5
N547	7.1	9.2
N548	7.2	12.0
N549	5.1	11.0
N550	8.4	8.5
N551	9.3	16.6
N43	0	0

[a] Each strain was inoculated into a pair of tubes containing broth of *p*H 6.2 and into another pair of tubes containing the broth at *p*H 7.4. After 40 min, PEA was added to one of the former pair to give 0.125% and AF was added to one of the latter pair to give 5 μg/ml. After a further incubation for 2 hr, cells were plated to determine viability.

[b] See footnote *b* of Table 2.

Suppressor mutation. It is assumed from the above observations that, in strain N562 (selected in AF) and in all the PEA-r revertants selected in PEA (strains N544 through N551), reversion had occurred at the locus *acrA1* responsible for AF-r phenotype of N43, but that the mutations in other AF-r revertants selected in AF were at other loci and hence would be suppressor mutations. Previous experiments (16, 17) have shown that the gene $acrA^+$ responsible for the AF-r phenotype determines resistance not only to AF but also to other dyes, such as MB, TB, MG, CV, and PY. Reverse mutation at the *acrA1*, therefore, would be expected to produce resistance to all basic dyes.

The revertants obtained above were tested for sensitivity to dyes by use of the gradient plate technique. The original dye concentration in the sloping upper layer of the gradient plates was 300 μg/ml in MB and TB, 50 μg/ml in CV, 500 μg/ml in MG, and 100 μg/ml in PY. As a control, N43 and N90 were tested also. The length of confluent growth on the plate was determined after overnight incubation at 37 C. It is evident from the data in Table 4 that N90, and W4573a, W4573b, and W4573c are resistant to all of the basic dyes, whereas N43, N427, N447, and N467 are sensitive to these dyes. In the revertant strains, only the strains which showed resistance to both PEA and AF are resistant to the basic dyes.

If the AF-r revertants selected in AF (except N562) are suppressed mutants, the locus *acrA1* must remain mutant in them. To examine this, the genetic constitution for MG resistance of Lac^+ Met^+ recombinants which were obtained by crossing W1895 (Hfr, AF-r and MG-r) and the revertants (F^-, AF-r and MG-s) was compared with that of the recombinants obtained by crossing W1895 and N43 (F^-, AF-s and MG-s). Each of the cultures of the AF-r revertants and N43 was mixed with that of W1895 and, after incubation for 15 min, mating pairs were separated with a Waring Blendor, diluted by a factor of 2×10^3, and plated on S_1-lactose agar to select Lac^+ Met^+ recombinants as described in Materials and Methods. Over 100 colonies were isolated after 48-hr incubation, purified, and cultured in broth (*p*H 7.4). The donor marker lac^+ entered the zygote about 7.5 min after onset of the mating under this condition. One loopful of each of the cultures was point-inoculated on the PG agar containing 250 μg of MG per ml, and, after overnight incubation, the MG sensitivity of each clone was determined. The data in Table 5 show that the donor marker $acrA^+$ (phenotypically MG-r in this experiment) was transferred to the Lac^+ Met^+ recombinants derived from the AF-r

TABLE 4. *Resistance of strains carrying phenotypes AF-r and AF-s to basic dyes*[a]

Strain	Dye				
	MB, 300 μg/ml	TB, 300 μg/ml	MG, 500 μg/ml	CV, 50 μg/ml	PY, 100 μg/ml
N43	0[b]	0	0	0	0
N90	>10	>10	>5	>3	>5
N427	<1	<1	0	0	0
N447	<1	<1	0	0	0
N467	<1	<1	0	0	0
W4573a	>10	>10	>5	>3	>5
W4573b	>10	>10	>5	>3	>5
W4573c	>10	>10	>5	>3	>5
N544	>10	>10	>5	>3	>5
N545	>10	>10	>5	>3	>5
N546	>10	>10	>5	>3	>5
N547	>10	>10	>5	>3	>5
N548	>10	>10	>5	>3	>5
N549	>10	>10	>5	>3	>5
N550	>10	>10	>5	>3	>5
N551	>10	>10	>5	>3	>5
N559	<1	<1	0	0	0
N560	<1	<1	0	0	0
N561	<1	<1	0	0	0
N562	>10	>10	>6	>3	>5
N563	<1	<1	0	0	0
N564	<1	<1	0	0	0
N565	<1	<1	0	0	0
N566	<1	<1	0	0	0
N567	<1	<1	0	0	0

[a] Cells were streaked on the gradient plates, each containing a basic dye at the indicated concentration in the sloping upper layer. The basal medium was PG agar adjusted to *p*H 8.2.

[b] Length (cm) of confluent growth on the plate after overnight incubation at 37 C; 10 cm was the maximum.

revertants N559, N560, N561, and N563, at frequencies of 84.8 to 88.9%, comparable to the frequency of 83.0% for those derived from N43. The Lac$^+$ Met$^+$ recombinants from the cross between N562 and W1895, on the other hand, contained no MG sensitivity marker. It is presumed, therefore, that the MG-s property of the four revertants tested is conferred on them by the unchanged *acrA1* mutant allele.

To determine the presence of a suppressor mutation in the AF-r and MG-s revertants of N43, a culture of mutant N559 was mixed with a culture of W1895, blended after 15 min of incubation, and selection was made for Lac$^+$ Met$^+$ as above. Five hundred fifty-nine recombinants were tested for AF sensitivity by point-inoculation on a PG agar plate containing AF. As shown in Table 5, five (0.89%) of them were scored as phenotype AF-s, whereas, in the cross W1895 × N43, 17% of Lac$^+$ Met$^+$ recombinants were of the AF-s phenotype. Hence, it was concluded that strain N559 carries a suppressor mutation (designated *sup-100*) responsible for phenotype AF-r, and that it is located near gene *acrA*. It is possible, however, that such a rare occurrence of AF-s recombinants is due to the suppressor allele being located distal to the *lac* region. If this is the case, the proportion of AF-s recombinants might increase when the mating period is extended.

Strains W1895 and N559 were mated for 23 min (blended at that time and diluted $1:3 \times 10^3$) and for 120 min (without interruption and diluted $1:10^4$), and recombinants were selected for Lac$^+$ Met$^+$ as above. Under the conditions used, the frequency of Lac$^+$ Met$^+$ recombinants increased about 20-fold when the mating time was extended from 15 min to 23 min and about 160-fold when the time was extended from 15 min to 120 min. Seven (0.90%) of 775 Lac$^+$ Met$^+$ recombinants from the 23-min mating and 6 (1.50%) of 387 Lac$^+$ Met$^+$ recombinants from 120-min mating were found to be phenotype AF-s.

Another mapping experiment was made by employing the Hfr strain N558, which transfers its chromosome in the direction opposite to that of W1895 (*see* Table 1). The mating mixture consisting of strains N558 (Hfr *thi*) and N559 (F$^-$, *acrA1*, *sup-100*) was kept standing for 120 min, diluted $1:10^4$, and plated onto S_1-lactose agar to select for Lac$^+$ Thi$^+$ recombinants as described in Materials and Methods. Of 349 Lac$^+$ Thi$^+$ recombinants scored, five (1.40%) had the phenotype AF-s, whereas in the cross N558 × N43 26.7% of Lac$^+$ Thi$^+$ recombinants tested were AF-s (Table 5). Thus, it appears that the possibility of a location of suppressor *sup-100* distal to the *lac* region is eliminated, and that the suppressor must be located close to gene *acrA*. Strains N560, N561, and N563 also, although examined only briefly, were inferred to carry a suppressor.

AF$^-$ and PEA-sensitivity mutation. Strain N43 is a mutant of W4573, a wild-type strain of *E. coli* K-12. It was found, however, that another type of AF-s mutant originates from a strain of W4573. When the culture of W4573 was spread on PG agar, giant colonies appeared at a frequency of 10% or more (over half in a certain strain's culture) among the small, original type colonies, and all of the clones forming the giant colonies were found to be AF-s phenotype. Strains N427 (geno-

TABLE 5. *Genetic constitution of recombinant classes Lac+ Met+ and Lac+ Thi+ obtained from crosses of W1895 and N558 with N43 and its revertants, N559 through N563*[a]

Mating	Time (min)	Selected for	No. tested	No. of clones grown on			
				MG (250 μg/ml)		AF (50 μg/ml)	
				Growth	No growth	Growth	No growth
W1895 × N43	15	Lac+ Met+	100	83 (83.0)[b]	17 (17.0)	83 (83.0)	17 (17.0)
W1895 × N559	15	Lac+ Met+	159	135 (84.4)	24 (15.2)		
			559			555 (99.1)	5 (0.89)
	23	Lac+ Met+	775			768 (99.1)	7 (0.90)
	120 (not blended)	Lac+ Met+	387			381 (98.5)	6 (1.50)
N558 × N43	120 (not blended)	Lac+ Thi+	161	118 (73.3)	43 (26.7)	118 (73.3)	43 (26.7)
N558 × N559	120 (not blended)	Lac+ Thi+	349			344 (98.6)	5 (1.40)
W1895 × N562	15	Lac+ Met+	258	258 (100)	0 (0)		
			1,471			1,470 (99.9)	1 (0.07)
W1895 × N560	15	Lac+ Met+	118	104 (88.0)	14 (12.0)	117 (99.1)	1 (0.86)
W1895 × N561	15	Lac+ Met+	119	106 (88.9)	13 (11.1)	118 (99.2)	1 (0.84)
W1895 × N563	15	Lac+ Met+	117	103 (88.0)	14 (12.0)	116 (99.1)	1 (0.85)

[a] See text for the cross conditions and for the determination of sensitivity of strains to AF and MG.
[b] Numbers in parentheses represent percentage of the total number tested.

type *acr-3*), N447 (*acr-4*), and N467 (*acr-5*) are AF-s and originated by independent mutations from the parental strains (phenotype AF-r) of W4573 (referred tentatively to as W4573a, W4573b, and W4573c, respectively; see Table 1). The levels of sensitivity and resistance of these strains to AF and PEA are shown in Table 6. The strains used show cross-sensitivity to AF and PEA.

Sensitivity to SDS of the mutants. SDS solutions is a potent solvent for lipoprotein and lipopolysaccharide, but is not a specific inhibitor of metabolism. As SDS may injure cells by disorganizing the cell membrane, genetic differences in SDS sensitivity may reflect differences in chemical constitution of the membrane. An attempt was made to see whether or not the strains resistant to AF and PEA are also resistant to SDS.

Freshly grown cells of N447 (phenotype AF-s and PEA-s), W4573b (parent of N447; AF-r and PEA-r), N559 (suppressed mutant of N43; AF-r and PEA-s), N562 (selected in AF; AF-r and PEA-r), N545 (selected in PEA; AF-r and PEA-r), N90 (recombinant between W1895 and N43; AF-r and PEA-r), and N43 (AF-s and PEA-s) were inoculated into phosphate-buffered broth (*p*H 7.4) containing varied concentrations of SDS. After shaking for 60 min at 37 C, the cells were plated onto PG agar to determine the viable count. As shown in Fig. 3, strains N562, N545, and N90 which are resistant to PEA and basic dyes are also resistant to SDS, whereas strain N43, which is sensitive to these chemicals, is also sensitive to SDS. On the other hand, the suppressed mutant N559 is as sensitive to SDS as is N43. The SDS sensitivity of N447 and W4573b was intermediate between the two groups, and there was essentially no difference between these

TABLE 6. *Cross-resistance between PEA and AF in mutants carrying phenotype AF-s and their wild type parents*[a]

Strain	PEA		AF	
	0.10%	0.15%	2.0 μg/ml	5.0 μg/ml
W4573a	35.4[b]	19.6	57.9	11.5
W4573b	70.0	28.7	55.1	12.2
W4573c	36.6	15.3	49.0	10.9
N427	6.1	0	3.8	0
N447	4.0	0	3.0	0
N467	5.8	0	2.6	0
N43	0.4	0	0	0

[a] Each strain was inoculated into three tubes containing broth of *p*H 6.2 and three tubes containing broth of *p*H 7.4. Shaking for 30 min was followed by addition of PEA to two tubes of the former class to give 0.1 and 0.15% and AF was added to two tubes of the latter class to give 2.0 and 5.0 μg/ml. Viable-cell count in the presence and absence of the drug was determined by plating a dilution of the culture on PG agar after a further 2-hr incubation.
[b] See footnote *b* of Table 2.

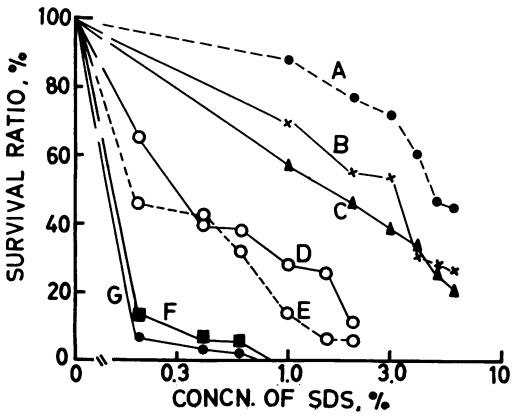

FIG. 3. *SDS sensitivity of strains resistant and sensitive to AF and PEA. Freshly grown cells were suspended in a broth medium (pH 7.4) containing varied concentrations of SDS and, after 60 min of incubation at 37 C, viable cells were counted. Survivors are expressed as a percentage of the control unexposed to SDS. (A) N562 (AF-r and PEA-r revertant of N43, selected in AF); (B) N545 (AF-r and PEA-r revertant of N43, selected in PEA); (C) N90 (AF-r recombinant between W1895 and N43); (D) W4573b (AF-r and PEA-r); (E) N447 (AF-s and PEA-s mutant of W4573b); (F) N43 (AF-s and PEA-s mutant of W4573); (G) N559 (suppressed mutant of N43; phenotype AF-r and PEA-s).*

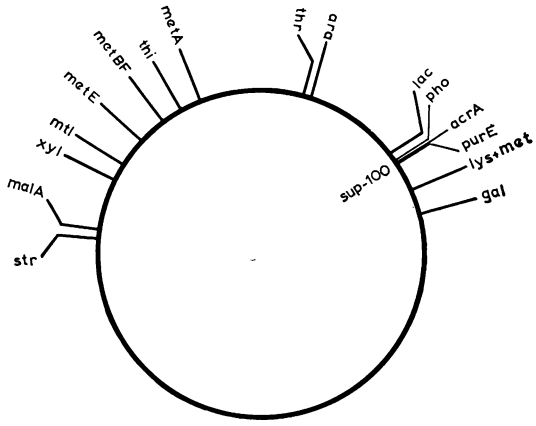

FIG. 4. *Genetic map of E. coli K-12 showing the location of relating loci. Symbols for the genetic markers: pho, alkaline phosphatase; purE, requirement for purines. For symbols of other markers, see footnote a of Table 1.*

strains. This suggests that their SDS sensitivity is independent of sensivitity to PEA and basic dyes.

DISCUSSION

In the following discussion, it is assumed that the drug sensitivity of cells determined by any of the three methods used can be considered mutually comparable. Although this assumption may not be seriously doubted, it should be borne in mind that it has not particularly been confirmed in the present study.

Gene *acrA1*, which is located near the *lac* region, is responsible for AF sensitivity of strain N43 (16, 17; Fig. 4). That this gene is responsible also for PEA sensitivity in *E. coli* K-12 was demonstrated by the following experiments. A strain carrying a wild-type allele $acrA^+$ from which mutants sensitive to both AF and PEA were isolated is resistant also to PEA (Table 6). In a mating between an Hfr strain, W1895 (phenotype AF-r and PEA-r), and an F^- strain, N43 (phenotype AF-s and PEA-s), genetic transfer of AF resistance was always accompanied by the transfer of PEA resistance (Fig. 1). Reverse mutation to PEA-r phenotype (giving rise to strains N544 through N551) from strain N43 accompanies AF-r phenotype and, conversely, the reverse mutation to AF-r phenotype (strain N562) accompanies PEA-r phenotype (Tables 2 and 3).

On the other hand, Wada and Yura (30), using a PEA-r strain (C600) of *E. coli*, found that the genetic locus for resistance is located between *lys* (a gene for lysine synthesis) + *met* and *gal*. Hence, the genetic locus for the PEA resistance shown in the present study differs distinctly from that described by Wada and Yura.

It was reported previously (16, 17) that the gene $acrA^+$ was responsible for resistance to both AF and other basic dyes, but not for resistance to acid dyes. However, the locus *sup-100* suppressing to gene *acrA1*, which is assumed to be located with a close linkage with the gene *acrA1* (*see* Table 5), does not determine resistance to PEA and to basic dyes. Hence, among the reverse mutants tested, only those mutants that were resistant to PEA were resistant to AF and other basic dyes.

Rosenkranz et al. (23), using B and B/r strains, showed that there was no cross-resistance relationship between PEA and proflavine, which is a main component of the acriflavine used in the present study. Greenberg and his collaborators (6, 33, 34), on the other hand, have shown with a wide variety of strains of *E. coli* that proflavine is typically radiomimetic in the sensitivity response of bacteria. Hence, the radiomimesis of PEA seems to be dependent upon the genetic locus determining sensitivity to it.

Acridine dyes have been demonstrated (13) to become intercalated between adjacent base pairs in deoxyribonucleic acid (DNA; 13), to lead to deletion or insertion of bases (3, 4), and to inhibit enzymatic synthesis in vitro of DNA and ribonucleic acid (RNA), preferentially the former

(7). The basic dyes MB, TB, CV, and MG are believed to exhibit high degrees of specificity in the affinity for DNA (11, 31) and PY is specific for RNA (11).

PEA reversibly inhibits DNA synthesis (2, 10, 29, 33) and initiation of chromosome replication (12) in *E. coli*, while it allows RNA and protein synthesis to proceed. Under certain conditions, however, RNA synthesis, rather than DNA synthesis, was shown to be attacked primarily by PEA (21–24). PEA and basic dyes are similar in their inhibition of the synthesis of nucleic acids (DNA might be a common target).

Lester (14) showed that PEA alters the permeability to some amino acids in *Neurospora crassa*, and Silver and Wendt (27) also observed that PEA, like toluene (8), causes a breakdown of the permeability barrier to AF and potassium in *E. coli*.

These two aspects of the PEA effect are suggested to be monogenetic if the primary site of PEA action is the cell membrane, because both DNA replication and cell permeability may be under a functional control by the cell membrane (9, 27, 29).

It was shown above that the strains carrying gene *acr* and those carrying suppressor *sup-100* (*see* Fig. 4) lost cell viability more noticeably than those carrying acr^+ when cells were treated with a medium containing SDS. According to Bayer and Anderson (1), Weidel (35), and Salton (25), the cell wall of gram-negative bacteria is organized with layers, the inner layer being composed mainly of lipopolysaccharide and the outer layer mainly of lipoprotein. Current studies have clearly demonstrated that the cytoplasmic membrane (surface membrane complex) is also composed of lipid substances (26). Since SDS is considered a potent solvent for these lipid complexes (1), it is possible that the genetic difference in SDS sensitivity reflects a difference in the membrane structure of cells.

It should be noted here that the AF-s strain N447 is a derivative of strain W4573 from which strain N43 (phenotype AF-s) was isolated. As shown in Fig. 3, strain N447 is more resistant to SDS than strain N43; hence, N447 can be classified as SDS-r (SDS-resistant) while N43 is SDS-s (SDS-sensitive). (This suggests that the *acr* allele responsible for phenotype AF-s and SDS-r in strain N447 may differ from the allele *acrA* responsible for phenotype AF-s and SDS-s in strain N43.) On the other hand, strain N562, a revertant of N43, is much more resistant to SDS than is the wild type W4573 phenotype; phenotypically one could characterize it as SDS-vr, where "vr" represents "very resistant."

In an earlier communication (18), it was reported that the uptake of AF and other basic dyes, but not of acid dyes, is lower in strains carrying gene $acrA^+$. The phenotypic expression of the $acrA^+$ gene following its introduction into AF-s cells by mating is inhibited by a variety of inhibitors of RNA and protein synthesis (*unpublished data*). Hence, it is conceivable that gene $acrA^+$ determines synthesis of a certain membrane substance which protects the DNA (and RNA) synthesis against the inhibiting effect of PEA and the basic dyes, and that the cellular uptake of basic dyes is regulated by the amount of that substance (18). The fact that inorganic phosphate suppresses the phenotype expression of AF resistance in a revertant, N544, and partially in strain N90 (*detailed data unpublished*) might suggest that biosynthesis of the membrane substance is regulated by a specific regulator gene which requires phosphate as a corepressor. Studies on the resistance mechanism and the regulation system for it are now in progress. The problem of the specific control of AF sensitivity by the suppressor *sup-100* also remains to be investigated.

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