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Gene-Controlled Resistance to Acriflavine and Other Basic Dyes in *Escherichia coli*

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ABSTRACT

NAKAMURA, HAKOBU (Konan University, Kobe, Japan). Gene-controlled resistance to acriflavine and other basic dyes in *Escherichia coli*. J. Bacteriol. **90**:8-14. 1965.—The genetic determinant controlling the sensitivity of *Escherichia coli* K-12 W1895 to the basic dyes acriflavine, methylene blue, toluidine blue, crystal violet, methyl green, and pyronine B appears, from results of mating experiments, to be located between the marker governing the utilization of lactose and the origin of genetic transfer. The determinant controlling this resistance to basic dyes does not control resistance to acid dyes. After the introduction of the resistance gene into merozygotes, acriflavine resistance is not established immediately but develops slowly.

Under precisely controlled conditions, certain of the acridine dyes eliminate genetic factors from microorganisms as follows: the sex factor, F, from male cells of *Escherichia coli* (Hirota, 1960; Hirota and Iijima, 1957); several of the factors controlling antibiotic resistance in various members of the Enterobacteriaceae and in certain strains of staphylococci (Mitsuhashi, Harada, and Kameda, 1961; Mitsuhashi et al., 1963; Hashimoto, Kono, and Mitsuhashi, 1964; Watanabe and Fukasawa, 1961); the donor genetic element introduced into *Salmonella typhimurium* by transducing phage P22 (Hubaček, 1963); and cytoplasmic factor(s) affecting the respiratory competence of yeast (Ephrussi, 1952). These dyes also inhibit the maturation of bacteriophage (Foster, 1948; DeMars, 1955) and they are mutagenic for bacteria (Witkin, 1947) and phage (DeMars, 1953; Brenner, Benzer, and Barnet, 1958).

During a study of acriflavine resistance in *E. coli*, cross-resistance to a number of basic dyes was noticed. Data from the present study show that the resistance to these basic dyes is probably determined by a single gene, and that considerable time is needed for the phenotypic expression of acriflavine resistance after the introduction of the gene into the recipient cell.

MATERIALS AND METHODS

Strains. An Hfr strain of *E. coli* K-12, W1895, kindly supplied by Y. Hirota, was resistant to acriflavine (AF). An AF-sensitive F^- strain, 18/1042, was obtained as follows. A culture of an AF-resistant F^- strain, W4573, also supplied by Y. Hirota, was spread, after dilution, on an agar plate; more than 100 colonies were isolated from the plate, and cultures of them were tested for their AF sensitivity; an AF-sensitive strain, 18/1042, was found.

The strains chiefly used in the present study are listed in Table 1.

Culturing. Brain Heart Infusion (BHI) was employed for maintaining stocks, for growth, and for mating experiments. Broth agar (BA), used for some experiments, consisted of 8 g of nutrient broth (Difco), 5 g of sodium chloride, 1 g of glucose, and 15 g of powdered agar per liter.

A minimal medium, S_1, was composed of 3.5 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 1 g of NH_4Cl, 1 ml of 1 M $MgSO_4$, and 1 ml of 10^{-3} M $FeCl_3$ per liter. It was made up by mixing stock solutions which had been sterilized separately. Another minimal medium, S_2, contained less phosphate to avoid precipitation of Mg; it consisted of 2 g of K_2HPO_4, 1 g of $(NH_4)_2SO_4$, 1 ml of 1 M $MgSO_4$, and 1 ml of 10^{-3} M $FeCl_3$ per liter. The media were supplemented with either glucose or another carbohydrate (at concentrations of 0.4 and 0.5%, respectively), previously sterilized separately from the minerals. The initial *p*H was 7.2 in S_1 and 7.8 in S_2. AF was more toxic to bacteria with glucose-S_2 agar than with glucose-S_1 agar, presumably owing to the alkalinity of the former.

For testing sugar utilization, S_1 agar in which the test sugar was the sole carbon source was used. Eosin-methylene blue (EM) agar without succinate (as modified by Hirota, (1960), plus a given sugar, was sometimes used in diagnosing sugar utilization for basic dye-resistant strains; the growth of sensitive cells was inhibited by this medium, although such strains could utilize the sugar in it.

For solid media, powdered agar, previously

washed several times with deionized water, was used: 1.5% for harder media and 0.4% for soft agar media.

Incubation temperature was 37 C unless otherwise indicated.

Mating. W1895 and 18/1042 were separately inoculated in BHI. The cultures were in the late logarithmic phase of growth after 2 hr of shaking at 37 C. The two cultures were mixed together so as to obtain a final concentration of ca. 10^8 donor cells and ca. 2×10^9 recipient cells per ml. The mating mixture, 10.3 ml in volume, was kept in a 500-ml Erlenmeyer flask standing in a water bath at 37 C to allow zygote formation. The cell concentration used in the present experiments was higher than that usually used, as it was hoped that division of zygotes would be suppressed, allowing observation of the phenotypic expression of AF resistance by zygotes themselves. The condition of the mating mixture was presumed to be aerobic, because the ratio of surface to volume was high. At various times, samples were withdrawn from the mating mixture, diluted in phosphate buffer solution, and plated onto selective media. Recombinants were scored after 2 days of incubation on the selective media.

RESULTS

Gene locus controlling AF sensitivity. The mating mixture of W1895 and 18/1042 was incubated for 3 hr, diluted by a factor of 10^4 with phosphate buffer, mixed in soft agar, and plated on the following media: (i) 20 μg/ml of AF plus glucose in S_1, (ii) lactose in S_1, or (iii) L(+)arabinose in S_1. At this concentration of AF, strain 18/1042 was not viable, whereas AF-resistant recombinants were fully viable. The Hfr strain did not grow on S_1 because of its requirement for methionine. For control purposes, the parent strains were plated in a similar manner.

About 100 colonies each of *lac*$^+$, *ara*$^+$, and AF-resistant recombinants were sampled. The *lac*$^+$ and *ara*$^+$ recombinants were further purified by respreading on S_1 agar plates containing the respective sugars. Each isolate was tested for AF resistance and for ability to utilize lactose, arabinose, and mannitol (Table 2). By use of the model of partial and oriented transfer of the *E. coli* "chromosome" (Jacob and Wollman, 1961; Hayes, 1953), it was concluded that the genetic determinant for AF resistance is a chromosomal gene, located not far from *lac*$^+$. The fact that all the clones selected by EM medium are AF-resistant suggests that the resistance to methylene blue (or eosin) is closely related to AF resistance.

To confirm the position of the locus governing AF resistance, the kinetics of genetic transfer during conjugation were studied. Samples were withdrawn from the mating mixture at various intervals, diluted 1:400, and treated at low temperature for 30 sec in a Waring Blendor. This treatment caused complete separation of mating pairs without reducing the viability of the culture. The treated cells were suspended in BHI which contained 200 μg/ml of dihydrostreptomycin, and, after incubation for 60 min at 37 C, cells were suspended in soft agar and plated on glucose-S_1 agar containing 20 μg/ml of AF and on lactose-S_1 agar not containing AF. A period of incubation preceding the plating was necessary for the phenotypic expression of AF resistance in zygotes, as will be explained below.

The results presented in Fig. 1 show that the transfer of the AF-resistance gene occurs earlier than that of the *lac*$^+$ gene. Hence, it is assumed that the AF-resistance gene is located nearer to the origin than *lac*$^+$ in the donor strain.

An experiment was designed to determine whether the AF-sensitive strain 18/1042 becomes fully AF-resistant after recombination with the resistant Hfr, W1895. Twenty-nine *lac*$^+$ colonies selected from lactose-S_1 agar plates seeded from a mating mixture of 18/1042 × W1895 were isolated and treated as follows. Each clone was cultured overnight in broth and plated on BA medium with and without 300 μg/ml of AF to estimate AF resistance. Clones derived from the parent strains were treated in the same way. Figure 2 represents the frequency

TABLE 1. *Characteristics of the strains used**

Strain	Sex	Auxotrophic character, *met*	Carbon-source utilization						Drug resistance	
			lac	gal	ara	xyl	mtl	mal	Streptomycin	Basic dyes
W1895	Hfr	−	+	+	+	+	+	+	S	R
18/1042	F$^-$	+	−	−	−	−	−	−	R	S
W4573	F$^-$	+	−	−	−	−	−	−	R	R
N90†	F$^-$	+	−	−	−	−	−	−	R	R

* Abbreviations: *met*, requirement for methionine; lac, lactose, gal, galactose; ara, arabinose; xyl, xylose; mtl, mannitol; mal, maltose; S, sensitive; R, resistant.

† N90 was isolated from a mating mixture of W1895 × 18/1042.

distributions of clones which showed the percentage of viability indicated on the abscissa. Of the 29 *lac*$^+$ recombinant clones tested, 7 were AF-sensitive and the rest showed the same AF-resistance level as the donor strain. When

TABLE 2. *Genetic constitution of recombinants selected with the various media*

Selective medium	Relative frequency (%) of			
	AF resistance	*lac*$^+$	*ara*$^+$	*mtl*$^+$
Glucose-S_1 plus 20 μg/ml of AF.....	100	77*	30*	0*
Lactose-S_1.....	83	100	34	0
Arabinose-S_1.....	48	55	100	0
Lactose-EM.....	100	100	28	0
Arabinose-EM.....	99	89	100	2

* Percentage of recombinants capable of using lactose (*lac*$^+$), arabinose (*ara*$^+$), and mannitol (*mtl*$^+$).

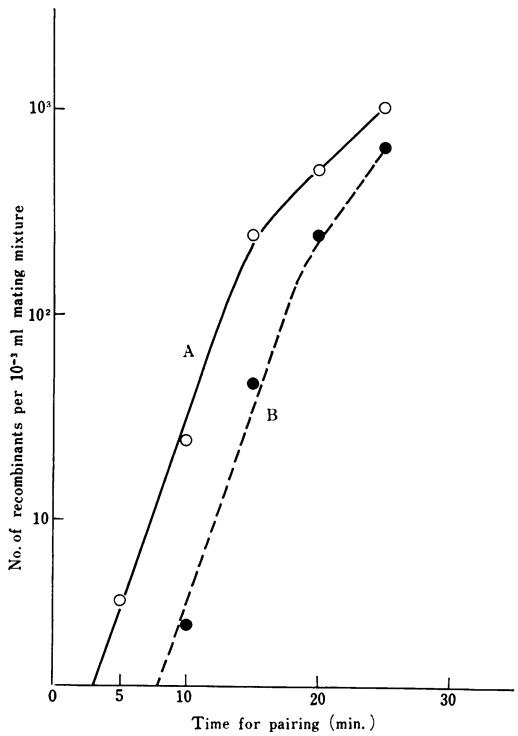

FIG. 1. *Time course of transfer of two genetic determinants from the Hfr donor to the recipient. Mating pairs were interrupted by a Waring Blendor at various times, incubated for 60 min in the complete medium with dihydrostreptomycin, and plated onto selective plates. (A) AF-resistant recombinants; (B) lac*$^+$ *recombinants.*

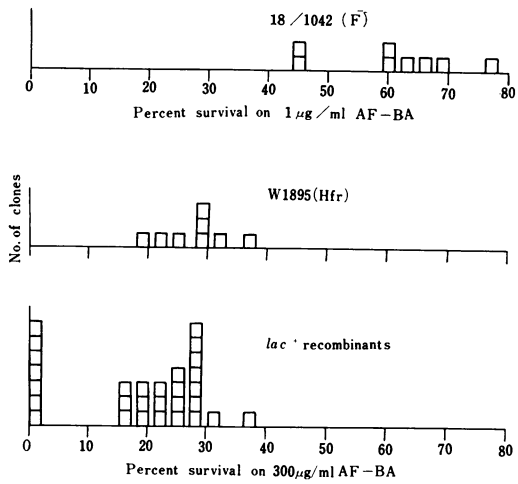

FIG. 2. *AF resistance of Hfr W1895, F*$^-$ *18/1042, and their lac*$^+$ *recombinants, represented by frequency distribution of clones having different survival percentages on AF-containing plates. AF concentrations were 1 μg/ml for F*$^-$ *and 300 μg/ml for Hfr and recombinants.*

plated on agar containing 1 μg/ml of AF, the AF-sensitive recombinants showed roughly the same distribution of per cent survival as strain 18/1042.

Cross-resistance to basic dyes. It was found in the course of these experiments that, although some of *lac*$^+$ and *ara*$^+$ recombinants did not grow on EM test plates, all the clones which grew on EM agar were resistant to AF (Table 2). Sugino (*unpublished data*) also concluded, on the basis of experiments with other strains of *E. coli* K-12, that one gene (Mb$^+$) controls both methylene blue (MB) resistance and AF resistance. He stated that the gene, Mb, is located between that for phage T6 resistance and that for purine synthesis. Therefore, the gene which controls the AF resistance, as found in the present work, seems to be identical with Sugino's Mb$^+$.

The mating mixture of W1895 and 18/1042 was plated on glucose-S_1 plates which contained AF, MB, toluidine blue (TB), crystal violet (CV), methyl green (MG), or pyronine B (PY) at concentrations shown in Table 3. Sensitive cells (of 18/1042) were not viable on these plates; 100 colonies were isolated from each kind of plate, and their resistance to the dyes and their capacities for utilizing various sugars were tested. All of the colonies capable of growing in the presence of one of the dyes were also resistant to the rest of the dyes used. Hence, the resistance to the six kinds of basic dyes tested seems to be controlled by the same gene. The recombination frequencies

(Table 3) suggest that *lac*$^+$, *ara*$^+$, and *mtl*$^+$ are arranged in this order on the distal side of the AF-resistance gene on the Hfr chromosome.

The gradient-plate method was used to determine whether all dye resistance is controlled entirely by the AF-resistance gene. Gradient plates were prepared in petri dishes 9 cm in diameter; 20 ml of normal BA medium were used for the sloping lower layer, and the same volume of BA (*p*H 8.2) containing AF, MB, TB, CV, MG, or PY was used for the upper sloping layer. After overnight incubation at 37 C, these plates were streaked with cultures (ca. 2×10^9 cells per milliliter) of W1895, 18/1042, and N90 (see Table 1) along the concentration gradient of the dyes. Strain N90 was used as an AF-resistant recombinant. The length of the solid growth on each plate after overnight incubation is shown in Table 4. On the AF and PY gradient plates, the length of solid growth of N90 was the same as that of W1895. Hence, there is little doubt that the resistance of AF and PY can be fully transferred by the AF-resistance gene. On the plates containing CV and MG, on the other hand, the length of solid growth of N90 was shorter than that of W1895. This could result from a diffusion effect, or it may be that some genetic determinant other than the AF-resistance gene contributes to the apparently high CV and MG resistance of W1895. This matter requires further study.

TABLE 3. *Genetic constitution of recombinants selected by glucose-S$_1$ agar containing AF, MB, TB, CV, MG or PY**

Addition to glucose-S$_1$ agar	Fermentation† of		
	Lactose	Arabinose	Mannitol
AF, 20 μg/ml	77	30	0
MB, 20 μg/ml	74	35	2
TB, 10 μg/ml	77	39	1
CV, 4 μg/ml	76	31	0
MG, 80 μg/ml	80	36	0
PY, 5 μg/ml	73	33	2

* AF, acriflavine; MB, methylene blue; TB, toluidine blue; CV, crystal violet; MG, methyl green; PY, pyronine.

† Percentage of clones utilizing indicated sugar when contained in EM agar.

An experiment was conducted to examine the capacity of AF-resistant strains to grow in the presence of acid dyes. Overnight cultures of W1895, 18/1042, and N90 were streaked on gradient plates of BA (*p*H 5.0) with the upper layer containing 1,500 μg/ml of eosin Y or 1,000 μg/ml of erythrosin. Incubation temperature was 30 C, and all the other conditions were the same as in the experiments for basic dyes. The length of solid growth was about 1.5 cm on the eosin plate and about 1.0 cm on the erythrosin plate for 18/1042 and N90, whereas W1895 grew over the whole range on either plate. Hence, it seems that N90 had not received the determinant(s) for resistance to eosin Y and erythrosin from W1895, and that the AF-resistance gene does not control resistance to these acid dyes. Viability of the strains used apparently was not impaired by congo red or trypan blue up to 500 and 250 μg/ml, respectively. Hence, resistance to these dyes was not studied.

Phenotypic expression of AF resistance. It was shown above that the AF-resistance gene begins to be transferred at about 3 min after the onset of conjugation. In an effort to ascertain the length of time required for phenotypic expression of AF resistance, the following experiment was carried out. Samples were taken from the mating mixture at intervals, diluted, and plated on glucose-S$_2$ agar containing 20 μg/ml of AF and on lactose-S$_1$ agar. Since sensitive cells were nonviable at this AF concentration, the progress of phenotypic expression of AF resistance could be traced by scoring the number of colonies on the AF plates inoculated with successive samples. The transfer of *lac*$^+$ was followed with lactose plates.

Figure 3 shows that *lac*$^+$ recombinant colonies were increasing during the initial 40 min of mating incubation, finally reaching a constant evel. On the other hand, a much longer period of ncubation was needed for AF resistance to be

TABLE 4. *Length of solid growth of AF-resistant, AF-sensitive, and recombinant strains on gradient plates containing indicated concentrations of dyes in the upper sloping layer*

Strain	Length (cm) of solid growth on					
	AF* (200 μg/ml)	MB (300 μg/ml)	TB (300 μg/ml)	CV (50 μg/ml)	MG (500 μg/ml)	PY (200 μg/ml)
W1895	6.5	Full†	Full	Full	Full	3.5
18/1042	0	3.0	4.0	0	0	0
N90	6.3	Full	Full	2.0	5.5	3.6

* For abbreviations; see Table 3.

† Full means that a streak grew over the whole range, 9 cm, of the concentration gradient plate.

expressed by the recombinants. AF-resistant recombinants began to appear at about 30 min after the onset of conjugation and continued to increase for more than 2 hr.

It is possible that the level of AF resistance increases gradually as the cellular resistance mechanism is being manufactured after the introduction of the AF-resistance gene. To differentiate resistance levels of the recombinants, two levels of AF concentrations were used, namely, 60 μg/ml, which is the highest concentration permitting 100% viability of resistant recombinants, and 4 μg/ml, which is sufficient to inhibit sensitive cells, when the basal medium is glucose-S_2. Samples were withdrawn from a mating mixture at intervals, and viability on the two kinds of AF medium was observed. A longer period of incubation was needed for recombinant cells to become viable on the medium containing the higher AF concentration. This suggests that cells which have received the AF-resistance gene from the donor build up the resistance mechanism rather gradually. It could be, however, that this process was so slow in the present experiment because of the high cell concentration of the mating mixture.

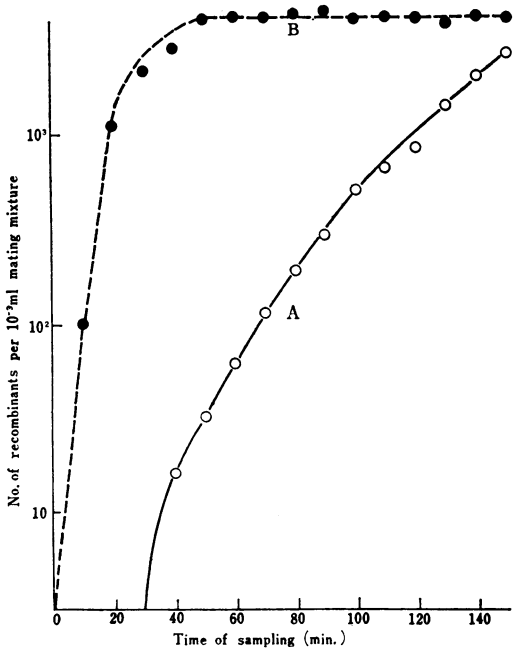

FIG. 3. *Time course of phenotypic expression of AF resistance after introduction of the determinant. Parents were mixed together at zero time. Number of recombinants viable on (A) medium containing 20 μg/ml of AF and (B) lactose medium.*

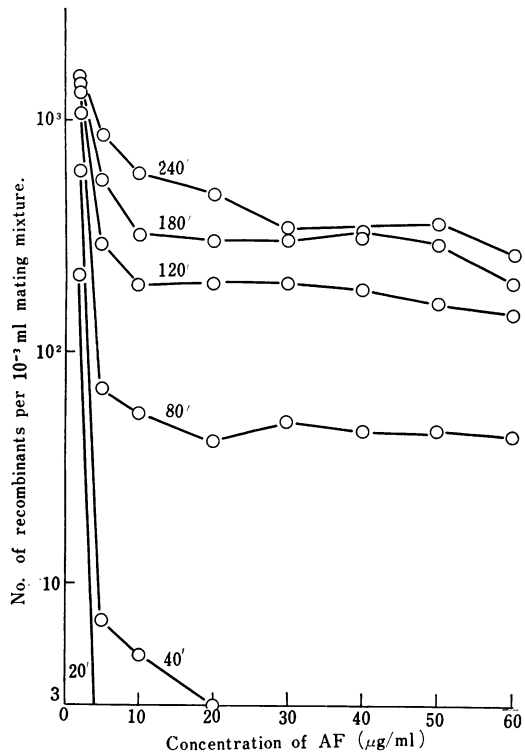

FIG. 4. *Successive stages of phenotypic expression of resistance to various concentrations of AF. Time (minutes) of sampling after mixing of parents indicated on each curve.*

The same kind of experiment was conducted for the resistance assay, with the use of a series of AF concentrations ranging from 2 to 60 μg/ml. The results (Fig. 4) showed that most of the cells viable in 10 μg/ml of AF were also viable in 60 μg/ml. The resistance mechanism which makes cells viable in the presence of up to 60 μg/ml of AF seems to be under the control of the AF-resistance gene.

DISCUSSION

Wild-type *E. coli* K-12 is rather resistant to AF, and spontaneously produces sensitive mutants. AF-resistant cells may arise again by spontaneous mutation of the latter (*unpublished data*).

When recombinants of an AF-resistant Hfr and a sensitive F^- were selected for resistance to one of the six kinds of basic dyes, AF, MB, TB, CV, MG and PY, clones of the selected recombinants were resistant to each of the other five dyes. It therefore appears highly probable that a single gene controls the resistance to all of these dyes. On the other hand, resistance to acid dyes,

such as eosin Y and erythrosin, appears not to be controlled by the gene of the basic dye resistance.

Sugino (*unpublished data*) found that there is a cross-resistance between MB and AF. The experiments reported here indicate that the determinant for AF resistance is located on the proximal side of *lac*$^+$ in Hfr W1895.

An AF-resistant recombinant strain (N90) was as resistant to AF and PY as its donor parent, but was less resistant to CV and MG than the donor. Hence, the resistance to the latter two dyes seems to be influenced by some other determinant(s) which is not necessarily transferred from the donor strain to the recipient when AF-resistance gene is transferred.

PY is unique among the basic dyes in its selective affinity for ribonucleic acid (RNA; Kurnick, 1955). MG exhibits a high degree of specificity in the affinity for deoxyribonucleic acid (DNA; Kurnick, 1955). Acridine dyes are said to become intercalated between adjacent base pairs in DNA (Lerman, 1963), and to inhibit enzymatic synthesis in vitro of DNA and RNA (Hurwitz et al., 1962) and incorporation in vitro of amino acid into protein (Soffer and Gros, 1964). CV, MB, and TB are also believed to combine with DNA.

On the other hand, Witkin (1961), using a strain of *E. coli* B/r, found that these basic dyes differentially influence the induction of mutation when ultraviolet-irradiated cells are treated with them during postirradiation incubation. If the resistance to AF, PY, MB, and TB, and the partial resistance to CV and MG are determined by a single gene, the resistance mechanism may be common to all. Brief inspection of their chemical structure shows that a feature common to them all is that they are charged positively. This suggests that some electronegative site is concerned in the resistance mechanism. At this time, it is tempting to assume that the depression of cell permeability to the various basic dyes is responsible for the basic dye resistance.

It was found that the number of AF-resistant cells continued increasing for 3 hr or more after the introduction of the resistance gene (Fig. 3 and 4). Cell division of *lac*$^+$ zygotes seems to begin very gradually at about 200 min after the mixing of the parents (*unpublished data*). Thus, AF resistance seems to be expressed in the partial diploid zygote, which contains both the AF-resistance gene from the donor and the sensitivity gene of the recipient. This is in conformity with the finding of Sugino (*unpublished data*) that MB resistance is dominant to sensitivity in the zygote of the strain of *E. coli* employed by him.

A considerable period was needed for the AF resistance to be expressed. Since the concentration of cells was so high that it inhibited cell multiplication in our experiments, various metabolic activities may have been suppressed to a considerable extent. Jacob (1959) and Pardee (1959), using a cell concentration of about 10^8 F$^-$ cells per milliliter, found that β-galactosidase was fully synthesized within a few minutes after introduction of the *lac*$^+$ gene. In one experiment, in which the cell concentration was lower than that used by Jacob, AF resistance was established more rapidly than in the experiment represented in Fig. 3, but still far more slowly than in the case reported for β-galactosidase. A study of the effect of various conditions, including cell concentration, on the rate of expression of AF resistance is now in progress.

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