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Acriflavine-binding Capacity of *Escherichia coli* in Relation to Acriflavine Sensitivity and Metabolic Activity

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ABSTRACT

NAKAMURA, HAKOBU (Konan University, Kobe, Japan). Acriflavine-binding capacity of *Escherichia coli* in relation to acriflavine sensitivity and metabolic activity. J. Bacteriol. **92**:1447–1452. 1966.—Inheritance of the acriflavine resistance gene by an acriflavine-sensitive strain of *Escherichia coli* K-12 resulted in a reduction in the cellular accumulation of acriflavine and other basic dyes, but had no effect on the accumulation of acid dyes. In both acriflavine-resistant and -sensitive strains, the cooling of cells from 37 to 0 C increased acriflavine accumulation. This increased accumulation was released from the cells after restoration to 37 C. Acriflavine accumulation was increased by carbon shortage and by metabolic disturbance caused by potassium cyanide, arsenite, puromycin, chloramphenicol, 2-thiouracil, and 8-azaguanine. The functional relation of acriflavine accumulation to the acriflavine concentration of the medium suggests that adsorption is involved in the accumulation.

Resistance of *Escherichia coli* to acriflavine (AF) and other basic dyes is controlled by a gene located near the determinant for lactose utilization. Considerable time is needed for AF-sensitive cells to express full resistance after the introduction of the resistance gene by mating, as compared with the expression of enzyme synthesis (4).

The present paper reports that the AF resistance gene appears to control some mechanism which lowers the capacity of cells to bind AF and other basic dyes, and that the AF binding of cells is affected by a metabolic activity which probably involves macromolecular synthesis.

MATERIALS AND METHODS

Strains. An Hfr strain of *E. coli* K-12, W1895, which is methionineless and is resistant to AF and other basic dyes, was used as the donor of the AF resistance gene.

A female strain, 18/1042, which is sensitive to AF and negative for lactose and arabinose utilization, was used as the recipient of the AF resistance gene and as the standard of AF sensitivity. This strain was a spontaneous mutant from a wild-type female strain originally resistant to AF. Another female strain, N90, a hybrid obtained by recombination between W1895 and 18/1042, was used as a standard AF-resistant strain. The characteristics of these strains were previously described in more detail (4).

Media. The strains were maintained on Brain Heart Infusion (Difco). Cells to be used for experiments were cultured overnight at 37 C in a broth medium containing 1 g of glucose, 5 g of sodium chloride, and 8 g of Difco Nutrient Broth per liter. A minimal medium, S_1 (4), was supplemented with 0.4% glucose, 1% lactose, or 1% arabinose as sole carbon source; the media thus obtained are referred to as glucose-S_1, lactose-S_1, and arabinose-S_1, respectively. Medium S_3 was composed of 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.6 g of Na_2SO_4, and 0.4 g of $MgCl_2$ per liter. Initial *p*H of glucose-, lactose-, and arabinose-S_1 was 7.2; S_3 and the broth medium were adjusted to *p*H 7.2 and 7.4, respectively, with NaOH.

For preparing dye-containing media, dye solutions were sterilized separately at 100 C for 20 min and added to nutrient media after cooling. The dyes used were AF, methylene blue (MB), toluidine blue (TB), crystal violet (CV), methyl green (MG), pyronin B, eosin Y, and erythrosine.

Solid media were prepared by adding 1.5% agar. Dye-containing broth-agar was prepared by adding a dye solution after the broth-agar had cooled to 60 to 70 C.

Mating and isolation of AF-sensitive and AF-resistant recombinants. W1895 and 18/1042 from overnight cultures were inoculated in Brain Heart Infusion and shaken for 2 hr at 37 C. These log-phase cultures were

mixed together so as to obtain a final concentration of about 10^7 W1895 cells and 2×10^8 18/1042 cells. A thin layer of the mating mixture was incubated at 37 C without agitation to allow zygote formation. After 60 min, the mating mixture was diluted by a factor of 10^4 with saline and spread on lactose-S_1-agar and arabinose-S_1-agar.

Colonies of *lac*$^+$ *met*$^+$ and *ara*$^+$ *met*$^+$ recombinants were isolated after 48 hr of incubation, and cells from each colony were spread again on S_1-agar containing the respective sugar in order to purify the strains. Each recombinant clone was cultured in broth and tested for AF sensitivity by use of an AF-gradient plate. The AF-gradient plate was prepared in a $10 \times 30 \times 2.5$ cm rectangular dish, with normal broth-agar for the sloping lower layer and broth-agar containing 30 μg/ml of AF for the sloping upper layer. After being kept overnight at 37 C, the plates were streaked, in duplicate, with a loopful of each of the recombinant cultures along the concentration gradient of AF (10-cm long streak). After overnight incubation, the length of the growth streak on the plate was determined.

Determination of dye content of cells. Samples were taken at intervals from dye-containing cultures. They were centrifuged ($14{,}500 \times g$) for 5 min at 5 C, and the dye concentration of the supernatant fluid was determined by spectrophotometry. The amount of the dye lost from the medium will be referred to as the dye content of cells and will be considered as an index of the dye-binding capacity of the cells. The absorption maximum used for the spectrophotometric determination of AF was at 450 mμ, both in glucose-S_1 and in the broth medium. The absorption maxima for MB, TB, CV, MG, and pyronin B were at 665, 635, 590, 630, and 552 mμ, respectively, in glucose-S_1, and at 667, 640, 594, 632, and 555 mμ, respectively, in the broth. The absorption maxima of the acidic dyes, eosin Y and erythrosine, were at 518 and 526 mμ, respectively, in both glucose-S_1 and broth (Hitachi EPU-2A spectrophotometer). Although the broth itself became yellowish, the absorption spectra of dyes could be differentiated when the medium was diluted two to four times and the broth without dye was used in the reference cell.

RESULTS

Genetic control of AF binding of cells. Thirty-four *ara*$^+$ *met*$^+$ recombinant clones and 38 *lac*$^+$ *met*$^+$ recombinant clones obtained by crossing strains W1895 and 18/1042 were subjected to duplicate tests for their AF sensitivity by use of AF-gradient plates. The solid growth on the gradient plate was shorter than 0.5 cm with the AF-sensitive recombinants and was longer than 10 cm with the AF-resistant recombinants. All the recombinants could thus be distinctly classified into the two classes (4). The AF-sensitive and AF-resistant clones thus classified were cultured separately overnight in broth at 37 C and washed three times with saline. Cells were suspended in broth medium containing AF (20 μg/ml), to give the same cell concentration as in the stationary growth phase, and the AF content of cells was determined after 20 min. Figure 1 shows that the AF-sensitive and the AF-resistant recombinants were distinctly differentiated into two classes of AF content. Hence, there is little doubt that the AF resistance gene controls the AF-binding capacity of cells.

Binding of other basic dyes and acid dyes. It was reported previously that the AF resistance gene appears to control resistance to MB, TB, CV, MG, and pyronin B, but not that to eosin Y and erythrosine. Hence, an AF-sensitive strain, 18/1042, and an AF-resistant strain, N90, were compared with each other for their dye binding. Cells from overnight cultures of the two strains were washed three times and suspended in broth and glucose-S_1 media containing a dye at the concentration shown in Table 1, the cell concentration being made the same as in the stationary growth phase. After 10 min at 37 C, the dye content of cells was determined. Table 1 presents the ratio of dye content of the AF-sensitive strain to that of the AF-resistant strain. With the synthetic medium, the binding of basic dyes was higher in the AF-sensitive strain than in the AF-resistant strain, whereas there was no difference in the binding of acidic dyes. The same tendency was also observed with broth.

Temperature shift. Cells of N90 and 18/1042 grown overnight in broth medium were washed, suspended in broth containing 5 μg/ml of AF, and incubated in a water bath at 37 C. After 60 min, a portion of each culture was transferred to an ice bath. Time courses of the AF content of cells are shown in Fig. 2. The AF binding of cells increased rapidly at the low temperature, and the increase was more marked in the AF-sensitive strain than in the other. According to experi-

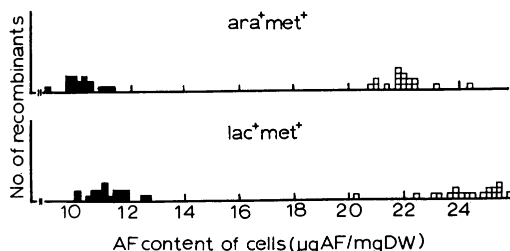

FIG. 1. *Frequency distribution of ara*$^+$ *met*$^+$ *and lac*$^+$ *met*$^+$ *recombinants with different AF contents. Recombinants obtained by crossing W1895 and 18/1042 were incubated for 20 min in broth containing 20 μg/ml of AF. Solid symbols, AF-resistant recombinant; open symbols, AF-sensitive recombinant (one square for one recombinant clone).*

TABLE 1. *Comparison of dye accumulation by an acriflavine (AF)-sensitive strain (18/1042) and an AF-resistant strain (N90)*[a]

Medium	Basic dyes						Acidic dyes	
	AF	MB	TB	CV	MG	Pyronin B	Eosin Y	Erythrosine
Broth	2.0	1.0–1.3	1.0–1.3	1.3	1.2	1.4	1.0	1.0
Glucose-S_1		1.8	1.5	1.5	1.5	1.7	1.0	1.0

[a] Dye content was determined after 10 min of incubation in dye-containing broth and glucose-S_1 at 37 C. The dyes were incorporated into the media at the following levels (micrograms per milliliter): AF, 10; toluidine blue (TB) and methyl green (MG), 64; methylene blue (MB), crystal violet (CV), pyronin B, eosin Y, and erythrosine, 32. Results are expressed as the ratio of accumulation of the sensitive strain versus the resistant strain.

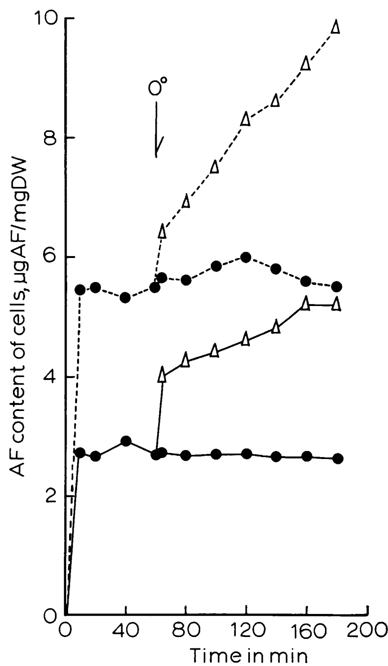

FIG. 2. *Effect of temperature shift-down on the AF binding of cells. N90 (solid lines) and 18/1042 (dashed lines) were incubated at 37 C in broth containing 5 µg/ml of AF. After 60 min, a portion (△) of each culture was chilled in an ice bath.*

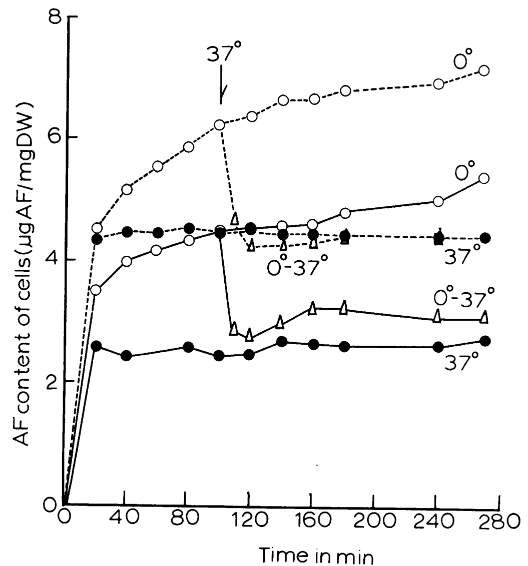

FIG. 3. *AF release from chilled cells by warming to 37 C. Freshly grown N90 (solid lines) and 18/1042 (dashed lines) cells were suspended in broth containing 5 µg/ml of AF and incubated in a water bath at 37 C (●) and in an ice bath (○). Portions (△) of the chilled cultures were transferred to a water bath at 37 C.*

mental results not presented here, the rate of AF binding at 0 C was higher when cells were suspended in glucose-S_1 medium containing AF than when suspended in AF-containing broth.

Cells from overnight cultures were suspended in broth containing AF (5 µg/ml) at 0 C; after 90 min, portions were transferred to a water bath at 37 C. Figure 3 shows that some accumulated AF in the cooled cells was released soon after the rise in temperature. The amount of AF remaining in the cells was roughly at the level of accumulation by cells kept at 37 C from the beginning of incubation.

Effect of metabolic disturbances. Some experiments were conducted to determine the effect of metabolic activity on the AF binding of AF-sensitive and AF-resistant cells. Freshly grown cells of the two strains were washed and suspended in a salt mixture, S_3, with and without addition of glucose (0.4%). After incubation for 3 hr at 37 C, cells from each suspension were washed and transferred to S_3 containing 5 µg/ml of AF, with and without glucose. The AF content of cells was determined after 60 min at 37 C (Fig. 4). The AF binding of cells was increased by carbon

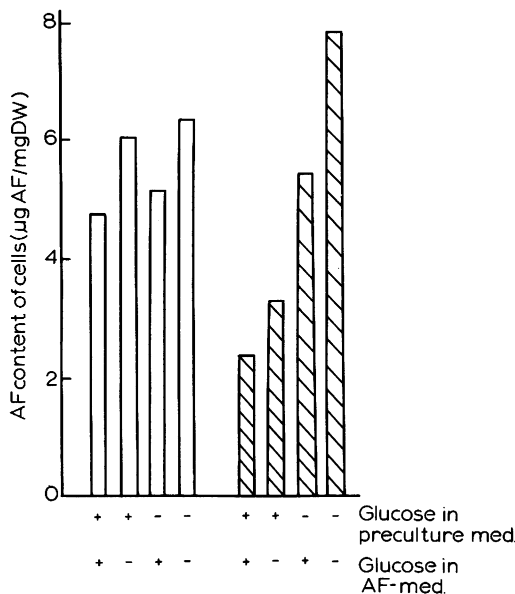

FIG. 4. *Effect of carbon shortage on AF binding of cells. N90 (shaded) and 18/1042 (unshaded) cells were suspended in S_3 with and without glucose for 3 hr. The carbon-starved and nonstarved cells were then transferred to S_3 containing 5 µg/ml of AF with and without glucose, and AF content was determined after 30 min at 37 C.*

deficiency more markedly in the AF-resistant strain, N90. This result differs from those reported for cell permeability to nutritional and non-nutritional substances (1, 2, 3, 7).

The effects of arsenite and cyanide were also determined. Freshly grown cells were washed and suspended in broth containing up to 300 µg/ml of AS_2O_3 and up to 150 µg/ml of KCN. After 30 min of incubation, AF was added to these cultures (final concentration, 5 µg/ml), and the amount of AF removed by the cells from the medium was determined after 5 hr. The AF binding of cells was increased by these inhibitors, the effect being more remarkable in the resistant than in the sensitive strain (Table 2).

The effects of chloramphenicol, puromycin, 8-azaguanine, and 2-thiouracil (all at 80 µg/ml) were observed by incubating cells for 120 min in broth media containing these agents, followed by 4 hr of exposure to AF. AF accumulation was increased by all of these agents (Table 2).

Effect of AF concentration. It is usually considered that an active transport mechanism will be saturated when the external concentration of a substance to be carried into cells is sufficiently high. Hence, the effect of AF concentration on

TABLE 2. *Effect of metabolic inhibitors and base analogues on acriflavine (AF) binding*[a]

Test substance	Concn	AF content[b]	
		Strain N90	Strain 18/1042
	µg/ml		
As_2O_3	0	2.0	3.2
	20	2.9	3.9
	50	3.0	4.1
	100	3.2	4.0
	200	3.2	4.0
	300	3.1	3.9
KCN	0	2.3	3.9
	25	3.6	4.6
	50	3.7	4.5
	100	3.7	4.3
	150	3.8	4.4
Control		2.2	3.5
Puromycin	80	3.2	5.6
Chloramphenicol	80	3.7	5.7
8-Azaguanine	80	3.1	5.4
2-Thiouracil	80	3.3	4.7

[a] Strains N90 and 18/1042 were incubated in broth containing the inhibitors and base analogues and, after 60 min with As_2O_3 and KCN or 120 min with puromycin, chloramphenicol, 8-azaguanine, and 2-thiouracil, AF was added to a concentration of 5 µg/ml. AF content of cells was determined after 5 hr (metabolic inhibitors) or 4 hr (base analogues).

[b] Expressed as micrograms of AF per milligram (dry weight) of cells.

the rate of AF accumulation was studied. Freshly grown cells of N90 and 18/1042 were washed and suspended in broth containing various concentrations of AF. The AF content of the cells was determined after 10 min of incubation at 37 C. Figure 5 shows that, in both strains, there is an approximate proportionality between AF accumulation by cells and AF concentration in the medium.

DISCUSSION

Some experiments were conducted to determine the relationship of AF binding of cells to the AF sensitivity and metabolic activity of cells. The dye may be bound differentially to different constituents of the cells. But even loosely bound and tightly bound forms could not be determined separately. When cells were successively extracted by various solvents, the cells continued to excrete AF, although in decreasing amounts with each extraction. Since AF could not be extracted completely, even with HCl-containing ethyl alcohol and alkaline water, the present method of determining dye accumulation was adopted.

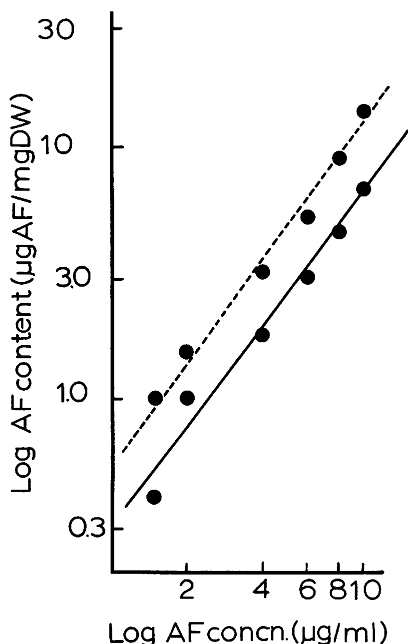

FIG. 5. *Relationship between AF concentration in medium and AF accumulation by cells. Strains N90 (solid line) and 18/1042 (dashed line) were incubated in broth containing various concentrations of AF, and AF content of cells was determined after 10 min.*

All of the AF-resistant recombinants of *lac*$^+$ *met*$^+$ and *ara*$^+$ *met*$^+$ bind smaller amounts of AF than do the AF-sensitive recombinants. It was shown previously that the difference in AF sensitivity between sensitive and resistant recombinants is ascribable to a gene (4). Hence, there is little doubt that this gene controls the AF-binding capacity of cells. Significant differences were found between 18/1042 and N90 strains in the accumulation of a number of basic dyes. This fact also corresponds well with the previous suggestion that the same gene controls the sensitivity to AF and to other basic dyes, but not to acid dyes.

The AF-binding capacity of cells is also influenced by physiological conditions. When the temperature of a cell suspension in an AF-containing medium was changed from 37 to 0 C, the AF content of the cells increased markedly; the added amount of AF was released from the cells when the temperature was raised back to 37 C. Ring (5, 6) found that permeability of *Streptomyces hydrogenans* to an amino acid and thiourea increased considerably (but reversibly) upon cooling to about 0 C. He suggested that the increase in permeability was attributable to the phase transition of membrane lipid and not to metabolic activity of the cells, since respiratory inhibitors did not increase the permeability appreciably. But in the present case, AF accumulation was increased not only by temperature decrease but also by carbon starvation and metabolic inhibitors.

If the enhanced AF accumulation by cooling were due to "opening" of the membrane (10), warming of the cell suspension should shrink the pore; hence, AF already permeated through the membrane would be difficult to release. The accumulated AF, however, was released from the cells very easily when the original temperature was restored. Hence, it is suggested that the AF-binding capacity, rather than AF permeability, is affected by temperature.

Active transport does not seem to limit AF accumulation, since reduction in temperature to 0 C caused significant increase of AF content within 5 min (Fig. 2). Increases in AF content due to carbon starvation (Fig. 4) and addition of arsenite or cyanide (Table 2) also preclude the possibility of active transport. Figure 5 seems to be consistent with an assumption that adsorption is involved in AF accumulation by cells.

Since puromycin, chloramphenicol, 2-thiouracil, and 8-azaguanine increased AF accumulation, it is tempting to assume that binding of AF by cellular substances, which probably possess a negative charge, is interfered with by some macromolecule, the synthesis of which is controlled by the AF resistance gene and is inhibited by puromycin and other inhibitors and by carbon starvation. The interfering effect of this macromolecule may decrease if it is released from cells at 0 C, as suggested by Strange et al. (8, 9), or its synthesis may cease at 0 C.

The higher AF binding of the sensitive strain (18/1042) compared with the resistant strain (N90) does not seem to be the result of cell injury by AF. This is because (i) AF binding of 18/1042 was significantly higher than that of N90, even under conditions where viability of the former strain was not reduced by AF, as, for example, when cells were suspended in broth containing 2 μg/ml of AF (*p*H 7.4) for 20 min, and (ii) the ratio of the AF-binding capacity of 18/1042 to N90 was the same over the AF concentration range from 1 through 10 μg/ml (Fig. 5).

It should be pointed out that N90 is far more resistant to AF than is 18/1042. Hence, the difference in AF sensitivity between the two strains cannot be explained simply by the difference in the AF-binding capacity. A study on this problem is now in progress.

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