Acriflavine-binding Capacity Controlled by the *acrA* Gene of *Escherichia coli*

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The *acrA* mutation in *Escherichia coli* led to a substantial increase of the acriflavine-binding capacity of the cell, whereas the related mutations *acrB* (*gyrB*) and *acrC* did not. Metal ions such as Na⁺, K⁺, Mg²⁺, Ca²⁺ and Al³⁺ effectively released the bound acriflavine, in proportion to their ionic strengths. The presence of cations, in fact, increased the survival fraction of the cells in the acriflavine-containing medium. Polymyxin B, an antibiotic which binds to membrane phospholipid, competed with acriflavine for binding sites. Cell wall digestion by treatment with lysozyme and EDTA slightly decreased the acriflavine-binding capacity. Almost no difference was observed in acriflavine-binding capacity between intact cells and cells from which lipopolysaccharide has been extracted (46.9% removed from the *acrA* cells and 47.4% from the *acrA*⁺ cells). Acriflavine bound to the cells was most effectively extracted by ethanol containing 1% HCl or by 2% (w/v) SDS. The difference in the acriflavine-binding capacity between the *acrA* and *acrA*⁺ cells was also observed in the spheroplasts. These facts indicate a relationship between the *acrA* gene product and the acriflavine-binding capacity of the cells.

INTRODUCTION

Sensitivity of *Escherichia coli* to acriflavine (AF) and other basic dyes is controlled by the genetic loci *acrA*, *acrB* (*gyrB*), *acrC* and *acrD* located respectively at mins 10.6, 83, 4 and near 94 on the chromosome (Nakamura, 1965, 1979*a*; Nakamura *et al.*, 1978; Bachmann, 1983). The *acrA* mutation leads to sensitivity not only to acridine and basic dyes but also to SDS, phenethyl alcohol (Nakamura, 1965, 1967, 1968), mitomycin C (Otsuji, 1968; Otsuji *et al.*, 1972, 1978), erythromycin, lincomycin (Henson *et al.*, 1982; Henson & Walker, 1982), novobiocin (Coleman & Leive, 1979) and salt hypertonicity (Nakamura, 1977; 1979*b*). Analysis of the composition of membrane proteins showed that the *acrA* mutant is deficient in a membrane protein with a molecular weight of about 60 000 (referred to an *acrA*⁺ protein), as compared with the wild-type (*acrA*⁺) membrane. Furthermore, observation in the electron microscope of a freeze-fracture face of the plasma membrane has demonstrated that intramembranous particles (probably proteinous complexes) of the largest class on the plasma face are lost in *acrA* mutants (Nakamura & Sukanuma, 1972; Nakamura *et al.*, 1981). These results suggest that the *acrA*⁺ membrane protein is responsible for cellular sensitivity to AF. The *acrA*⁺ protein has also been implicated in the stability of F and R plasmids in the cell (Nakamura, 1974, 1976).

The purpose of the present study was to define the AF-binding sites determined by the *acrA* gene. It is concluded that the main sites responsible for AF binding on the cell surface are phospholipids of the plasma membrane; the presence of the *acrA*⁺ protein decreases the binding of AF on the outer side of the phospholipid bilayer.

Abbreviation: AF, acriflavine.

Table 1. *Bacterial strains used*

All strains used were derivatives of *E. coli* K12.

Strain	Genotype
W4573	Wild-type (<i>acrA</i> ⁺)
N43	<i>acrA1</i>
N90	<i>acrA</i> ⁺ recombinant
N2310	<i>acrA</i> ⁺ revertant
N2616	<i>acrCI</i> ^{ts}
N2879	<i>acrB</i>
N2926	<i>acrA acrB</i>
N43(pAF1)	Containing <i>acrA</i> ⁺ plasmid
N90(pAF1)	Containing <i>acrA</i> ⁺ plasmid

METHODS

Bacterial strains. These are listed in Table 1. Plasmid pAF1 was constructed by annealing a 2 kb DNA fragment containing the *acrA*⁺ gene with the *E. coli* vector pBR322. Strains N2616, an *acrCI*^{ts} mutant, was resistant to AF at 30 °C but was sensitive to it at 43 °C (Nakamura, 1979a). It did not divide at 43 °C even in the absence of AF.

Media. Broth medium PGY was composed of 10 g polypeptone (Daigo-eiyo Co., Osaka, Japan), 3 g yeast extract (Oriental Yeast Co., Osaka, Japan), 3 g NaCl and 1 g glucose in 1 l deionized water. The initial pH of the medium was adjusted to pH 7.4 with 1 M-NaOH. The bacteria were grown in this medium for 18 h at 37 °C, except for the *acrCI*^{ts} mutant, which was grown at 30 °C.

Determination of dye-binding capacity of the cells. Freshly grown bacteria were washed once with saline (0.85% NaCl solution) and then twice with distilled water. A sample of this bacterial suspension was used for determination of the dry weight, and the remainder was centrifuged and suspended in fresh PGY (pH 7.4). To samples (5 ml) of the cell suspension AF was added to obtain a final concentration of 4 µg ml⁻¹, and the tubes were shaken for 20 min at 37 °C. The cells were then separated by centrifugation (12500 r.p.m., 13000 g, for 10 min at 5 °C), and the AF contents of the supernatants were determined with a spectrophotometer (Hitachi, model 124) at 450 nm. The AF-binding capacity of the cells relative to the dry weight was calculated from the AF content of the supernatants. The control did not contain cells. The centrifugation temperature was maintained carefully since it had been shown previously that cooling of the culture to 0 °C rapidly increased the AF-binding capacity of cells (Nakamura, 1966).

Extraction of lipopolysaccharide from cells by EDTA treatment. The procedure for lipopolysaccharide (LPS) extraction from the cells was basically as described by Leive (1968). Freshly grown bacteria were washed once with saline and once with distilled water, and were resuspended in distilled water. Tris/HCl buffer (pH 8.0) was added to a final concentration of 0.12 M and the suspension was centrifuged (6000 r.p.m., 3000 g, for 10 min at 5 °C). The cells were suspended in 0.12 M-Tris/HCl buffer (pH 8.0) prewarmed at 37 °C, and an EDTA solution (pH 8.0) was added to give a final concentration of 15 mM. After stirring gently for 5 min at 37 °C, a CaCl₂ stock solution was added to a final concentration of 40 mM to stop the effect of EDTA. After extraction of LPS the cells were centrifuged at 10000 r.p.m. (8500 g) for 20 min at 5 °C and washed once with saline for use in the AF binding experiment.

Determination of LPS content. The LPS content of the EDTA-extracted and the control (not extracted) cells was determined by the method of Westphal & Jann (1955). Cells of strains N43 and N90 were each suspended in 4 ml distilled water prewarmed at 70 °C, and 5 ml 90% (v/v) phenol solution at 70 °C was added. After 5 min, they were cooled to 10 °C on ice and centrifuged at 3000 r.p.m. (750 g) for 10 min at 5 °C. The resulting aqueous layer was carefully removed with an L-form pipette, and the remaining fraction was again extracted with 5 ml of water at 70 °C. The aqueous extracts were combined and centrifuged again. The supernatant was then dialysed against distilled water until the smell of phenol had disappeared. The dialysed sample was centrifuged to 40000 r.p.m. (140000 g) for 120 min at 2 °C. The pellet was washed with a small amount of distilled water, and was analysed as the LPS fraction.

The LPS content was estimated as 2-keto-3-deoxyocturonic acid (KDO), a component of LPS (Osborn, 1963), with deoxyribose as standard. Protein was estimated by the method of Lowry.

Preparation of spheroplasts. Fresh PGY culture (80 ml) was centrifuged. The cells were washed twice with 10 mM-Tris/HCl (pH 8.0) and were resuspended in 30 ml of ice-cold 0.3 M-sucrose/10 mM-Tris/HCl (pH 8.0). EDTA and egg-white lysozyme (Sigma) were each added to a final concentration of 100 µg ml⁻¹. The suspension was kept on ice for 15 min and centrifuged at 12000 r.p.m. (12000 g) for 15 min at 5 °C, and the sediment was washed once with 0.3 M-sucrose/10 mM-Tris/HCl (pH 8.0) to remove the reagents. Observation under the light microscope showed that the morphology of the cells was changed completely to spheroplasts (a round shape) by this procedure. The colony-forming ratio of the spheroplast fraction was less than 10⁻⁴.

RESULTS

Genetic control of the AF-binding capacity of the cells

When an AF-resistant (*acrA*⁺) Hfr C strain was crossed with the AF-sensitive (*acrA*) strain N43 and selected for *lac*⁺ *met*⁺ and *ara*⁺ *met*⁺, the AF-sensitive recombinants among them bound AF [22–24 µg (mg dry wt)⁻¹] twice as much as the AF-resistant recombinants (Nakamura, 1966). The purpose of the experiments described in this paper was to investigate the effect of the other AF sensitivity mutations, *acrB* and *acrC*, on the AF-binding capacity of the cells.

First, the AF-binding capacity was compared among strains N43, N90, N2310, N43(pAF1) (*acrA*⁺/*acrA*) and N90(pAF1) (*acrA*⁺/*acrA*⁺). The introduction of *acrA*⁺ gene(s) into N43 by conjugation (N90), plasmid transformation [N43(pAF1) and N90(pAF1)] and true reversion of *acrA* to *acrA*⁺ (N2310) decreases the AF-binding capacity of cells to the level of the wild-type strain (W4573; Fig. 1.). Interestingly, an increase of the *acrA*⁺ gene dose seems to diminish the AF-binding capacity of the host cell, as shown in strains N43(pAF1) and N90(pAF1). The cloned *acrA*⁺ plasmid and the F' (F₁₃) plasmid containing the *acrA*⁺ gene significantly increased the AF resistance of their host cells (data not shown).

Secondly, the effect of the *acrB* mutation on the AF-binding capacity was investigated. The *acrB* mutant (N2879) was less sensitive to AF than the *acrA* mutant (N43). The *acrA acrB* double mutant (N2926) was much more sensitive to AF than either of the single mutants (Nakamura *et al.*, 1978). Though the *acrB* mutant had increased AF-binding capacity, the level was 65% of that of the *acrA* mutant. Furthermore, the *acrA acrB* double mutant bound 81% AF when compared with the *acrA* strain. Thus the amount bound was intermediate between the amounts for the *acrA* and *acrB* single mutants.

Finally, the AF-binding capacity of the *acrC* mutant (N2616) was investigated. Freshly grown cells of strains N2616 and N43, after washing the saline and distilled water, were suspended in PGY medium (pH 7.4), containing 4 µg AF ml⁻¹, at 30 °C and 43 °C. After 20 min incubation, the AF-binding capacity of cells was determined. No difference was found in the AF-binding capacity of the strains or between the incubation temperatures.

Effect of various salts on the AF binding of cells

AF taken up by *E. coli* cells is not incorporated in the cytoplasm but is adsorbed on the cell surface (Nakamura, 1966; Silver *et al.*, 1968). Therefore the position of the AF-binding site(s) on the envelope structure of the cell was investigated.

First, the effect of washing *acrA* and *acrA*⁺ cells with saline (0.85% NaCl) and distilled water (control) was studied. The cells were incubated in PGY medium containing 4 µg AF ml⁻¹ for 20 min at 37 °C, and were washed one to five times with 5 ml of either the saline or distilled water. The bound AF was released markedly (50%) by several washes with saline, but not with water (Fig. 2). However, when the NaCl concentration of the washes was increased to 500 mM, the AF bound to cells decreased by 66% (per dry wt) for strain N43 and 72% for strain N90.

Secondly, the dependence of dissociation of AF on charge number and ionic strength of metal ions in the washing solution was determined. The N43 and N90 cells treated with 4 µg AF ml⁻¹, as described above, were resuspended in 5 ml solutions of NaCl, KCl, MgCl₂, CaCl₂ and AlCl₃ at various concentrations. They were immediately centrifuged at 15000 r.p.m. (18000 g) for 5 min at 5 °C. Divalent and trivalent cations dissociated AF from both strains more efficiently than monovalent cations (Fig. 3). AF was released proportionally with rising of the ionic strength for the divalent and trivalent cations, while for the monovalent cations, the release of AF increased rapidly at ionic strengths over 0.05.

Effect of salt on cellular sensitivity to AF

The effect of salts on survival of cells in the presence of AF was investigated. Fresh cultures of strains N43 and N90 were pipetted into test tubes containing 5 ml of media which consisted of 5 g polypeptone and 1 g yeast extract in 1 l deionized water (pH 8.5). The media also contained 5 µg AF ml⁻¹ and various concentrations of NaCl, KCl, MgCl₂ or CaCl₂. Survival curves for

Fig. 1

Fig. 2

Fig. 1. AF-binding capacities of *E. coli* strains N43 (*acrA*), N90 (*acrA*⁺ recombinant), N2310 (*acrA*⁺ true revertant from N43), W4573 (wild-type, *acrA*⁺), N43(pAF1) (*acrA*⁺/*acrA*, containing cloned plasmid) and N90(pAF1) (*acrA*⁺/*acrA*⁺, containing cloned plasmid). The relative AF-binding capacity of the cells was calculated from the AF bound per mg dry weight; the value was 22–24 μg (mg dry wt)⁻¹ for N43. The data in this and following figures are means of three parallel experiments, and the SD was within 30% of the mean.

Fig. 2. AF-binding capacities of *E. coli* strains N43 (open symbols) and N90 (filled symbols) on repeated washing with distilled water (circles) or saline (0.85% NaCl; squares).

Fig. 3. Effect of washing with various ionic strengths of salt solutions on AF release from AF-treated cells of *E. coli* strains (a) N43 and (b) N90. The washing solution contained NaCl (○), KCl (●), MgCl₂ (△), CaCl₂ (▲) or AlCl₃ (□).

the cells after 2 h incubation at 37 °C are shown in Fig. 4. The survival fraction of strain N43 increased rapidly in the presence of more than 350 mM-NaCl and in 860 mM-NaCl reached the value of strain N90 (Fig. 4a). Salt marginally increased the survival of N90 in the AF medium but the effect was not concentration-dependent. For KCl, the survival fraction of strain N43 increased gradually with concentrations of KCl of more than 31 mM (Fig. 4b). Strain N90 became more tolerant to AF in KCl concentrations of over 125 mM. On the other hand, the effect of MgCl₂ was more marked; the survival fraction of strain N43 reached a maximal level of 10⁻¹ in the presence of 31 mM-MgCl₂ (Fig. 4c). Strain N90 was also more tolerant to AF in the presence of MgCl₂, but high concentrations of the salt were toxic to the strain. The same trends were also observed for CaCl₂; survival of strain N43 increased sharply in concentrations greater than 31 mM, and that of strain N90 also increased at levels greater than 63 mM (Fig. 4d).

Fig. 4. Effect of (a) NaCl, (b) KCl, (c) MgCl₂ and (d) CaCl₂ concentrations on the survival of cells in medium containing $5 \mu\text{M}$ AF ml⁻¹. The survival fraction was determined after incubation for 2 h at 37 °C. ●, *E. coli* N90; ○, *E. coli* N43.

However, higher concentrations of CaCl₂ were harmful to both strains. Although the same type of test was done for AlCl₃, precipitation occurred in the media under the conditions used (data not shown).

Competition of AF with polymyxin B

It was concluded from above that AF probably bound to negatively charged site(s) on the cell envelope, and thus the bound AF was exchangeable with metal ions. Other results indicated that an increase of hydrogen ion concentration effectively released AF from the AF-treated cells; complete dissociation of AF was achieved by washing with highly acidic solutions (unpublished). Therefore, the next approach was to examine whether AF competed with polymyxin B. Polymyxin B is a cationic peptide antibiotic possessing a broad range action spectrum against Gram-negative bacteria (Sud & Feingold, 1971), and it binds to phosphoric sites of the phospholipid molecules of the cell wall and plasma membrane to express its antibiotic activity (Imai *et al.*, 1975; Teuber & Bader, 1976*a*, *b*).

Freshly grown cells of strains N43 and N90 were washed once with saline and twice with distilled water, and were resuspended in fresh PGY medium. The culture was divided into six tubes (5 ml in each), and $4 \mu\text{g}$ AF ml⁻¹ and various concentrations (10 – $1000 \mu\text{g}$ ml⁻¹) of polymyxin B were added. After shaking for 20 min at 37 °C and centrifugation at 12500 r.p.m. (13000*g*) for 10 min at 5 °C, the AF contents of the supernatants were determined photometrically. There was competition between AF and polymyxin B molecules for the negatively charged sites. (Fig. 5). The effect of polymyxin B in decreasing bound AF was more pronounced in strain N43 than in strain N90.

Fig. 5

Fig. 5. Competition between AF and polymyxin B for binding sites. Various concentrations of polymyxin B were added to media containing $4 \mu\text{g AF ml}^{-1}$ which was then incubated for 20 min at 37°C . ●, *E. coli* N90; ○, *E. coli* N43.

Fig. 6

Fig. 6. AF-binding capacities of AF-treated spheroplasts of *E. coli* strains N43 (open symbols) and N90 (filled symbols) on repeated washing with 0.3 M -sucrose (circles) or saline containing 0.3 M -sucrose (squares).

Comparison of AF-binding capacity between intact cells and spheroplasts

To determine the distribution of AF-binding sites on the cell wall and plasma membrane, fresh, spheroplasts of strains N43 and N90 and their intact cells were used. The spheroplasts and intact cells were suspended in the hypertonized Tris/HCl solution containing $4 \mu\text{g AF ml}^{-1}$ and after 20 min incubation at 37°C , the AF-binding capacity was determined. The AF bound was compared per unit of protein. For both strains, AF binding capacity of spheroplasts was lower than that of intact cells (59–88%). However, it must be stressed that the major sites for the AF binding seem to be on the plasma membrane, rather than on the cell wall. In a control experiment, no lysis of the spheroplasts was observed even by treatment with $100 \mu\text{g AF ml}^{-1}$ for 90 min.

To examine the possibility of LPS as the AF-binding site, cells of strains N43 and N90 from which LPS had been extracted were prepared as described in Methods and tested for their AF-binding capacity. According to our procedure for LPS extraction, 46.9% and 47.4% LPS were removed from the cells of strains N43 and N90 respectively. However, the extraction did not reduce the AF-binding capacity of either strain (data not shown).

AF release from the AF-treated spheroplasts was compared in 0.3 M -sucrose and in saline containing 0.3 M -sucrose. The bound AF was more effectively released when NaCl was present (Fig. 6). Furthermore, the AF released by the 0.3 M -sucrose saline washing of AF-treated spheroplasts was more pronounced than release of AF by saline in the AF-treated intact cells (Figs 2 and 6).

The role of the plasma membrane as the AF-binding site was also investigated. Cells of strains N43 and N90 were routinely treated with AF and suspended in 0.3 M -sucrose/ 10 mM -Tris/HCl (pH 8.0) containing $100 \mu\text{g EDTA ml}^{-1}$ or $100 \mu\text{g lysozyme ml}^{-1}$, or both. Only small amounts of AF were released by treatment with lysozyme plus EDTA; binding ranged from 77 to 81% of that obtained with the AF-treated bacteria. This is good evidence that digestion of the cell wall of the AF-treated cells has no major effect on AF-binding capacity.

The results shown above suggest that the major site of AF binding is the plasma membrane, rather than the cell wall. Therefore, we examined an interaction of AF and phosphatidylethanolamine (PE), a main component of membrane phospholipids. PE from sheep brain lipid was dissolved in chloroform in a final concentration of 0.25 mM and warmed to 37°C . An aqueous AF solution (0.01 mM) at 37°C was mixed well with the PE solution. After incubation for 20 min with vigorous agitation, the mixture was centrifuged at $3000 \text{ r.p.m. (750 g)}$ for 10 min. A

Fig. 7. AF-binding capacities of AF-treated cells of *E. coli* strains (a) N43 and (b) N90 after treatment with (A) 1% HCl/ethanol, (B) 2% (w/v) SDS, (C) acetone, (D) saline, (E) boiling saline and (F) toluene or chloroform. The control (X) was not treated.

substantial amount of AF was shown to transfer from the water to the chloroform layer, while a little AF moved into a PE-free chloroform layer (control experiment). This result shows that an AF-PE complex forms which is lipophilic. However, when the PE solution was pretreated with NaCl solution or when the AF solution contained NaCl, much less AF was transferred into the PE layer.

AF extraction by various solvents

Cells of N43 and N90 were freshly grown in 80 ml PGY medium (pH 7.4), and after washing twice with distilled water, were suspended in PGY medium (pH 7.4) containing $4 \mu\text{g AF ml}^{-1}$. After shaking for 20 min at 37°C , the cells were centrifuged and resuspended in 5 ml of the following solvents: (1) ethanol containing 1% HCl, (2) 2% (w/v) SDS, (3) acetone, (4) saline (0.85% NaCl solution), (5) boiling saline and (6) toluene or chloroform. After stirring well, the cell suspensions were centrifuged at 12500 r.p.m. (13000 *g*) for 5 min at 5°C , and the AF contents of the supernatants were determined. Ethanol containing 1% HCl, and 2% SDS were most effective at reducing the AF-binding capacity of cells, as compared with other solvents (Fig. 7). This suggests that compounds which disorganize membrane lipid bilayers are also effective extractants of AF.

DISCUSSION

The *acrA* mutant takes up significantly more AF than the wild-type (*acrA*⁺) cell. However, it has been demonstrated kinetically that the uptake of AF is due to binding on the cell surface and not due to incorporation into cytoplasm (Nakamura, 1966; Silver *et al.*, 1968). The AF binding occurs very rapidly and has no lag time. The present study showed that the AF-binding capacity of the *E. coli* cell is most effectively determined by the *acrA* gene as compared with *acrB* (*gyrB*) and *acrC*.

The present data strongly suggest that the AF-binding sites are on the plasma membrane. Although the phospholipids are also a component constituting the bacterial cell wall, experiments inducing LPS extraction, spheroplast formation, digestion of the walls of the AF-treated cells and some others seem to show that the cell wall only plays a minor role in AF binding. The plasma membrane is primarily composed of a phospholipid bilayer, and the structure presents a barrier to permeant substances. AF is a cationic dye and thus must be unable to pass through the hydrophobic phase. Also, AF does not seem to be incorporated by using a carrier. Therefore, it is reasonable to assume that the AF molecule binds to a negatively charged site, probably phosphate, of the outer side of the phospholipid bilayer.

How does the *acrA* gene control the AF-binding capacity of the cell? We have already shown that the *acrA* mutation leads to a loss of *acrA*⁺ protein (molecular weight 60000) in the plasma membrane (Nakamura *et al.*, 1982). Observation in the electron microscope of a freeze-fracture face of the membrane has also shown that the largest class of the intramembranous particles is lost from the *acrA* mutant membrane, and that the non-particle area of the mutant membrane is increased by 34% as compared to the wild-type membrane (Nakamura *et al.*, 1981). Moreover, in the *acrA* mutant, a considerable lamellar structure is formed in the presence of low concentrations of AF but not in the *acrA*⁺ strain (Nakamura *et al.*, 1982). Furthermore, it appears unlikely that certain membrane protein(s) have a specific affinity to AF, since no difference in the AF-staining intensity is found among the proteins separated by acrylamide gel electrophoresis. Thus, we are led to the conclusion that the area responsible for the AF-binding capacity of the plasma membrane is mainly the phospholipid region, and that the *acrA* mutation leads to an increase in binding through the loss of the *acrA*⁺ protein.

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