Effect of Inorganic Phosphate on Acridine Inhibition and Plasmid Curing in *Escherichia coli*

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SUMMARY

Some mutants and stock strains of *Escherichia coli* K12 were sensitive to acriflavine in the presence of inorganic phosphate but were resistant to acriflavine in its absence. They mutated spontaneously to resistance to acriflavine plus phosphate. The synergistic effect of phosphate on acriflavine sensitivity was increased at high pH values. Genetic analysis suggested that the mutations occurred in the gene *acrA*. Electron microscopic observation suggested that the presence of acriflavine plus phosphate affected the structure of the plasma membrane and the cytoplasm under it. This structural alteration was not caused by acriflavine alone. Acridine orange plus phosphate can more effectively eliminate the plasmid F8-*gal*⁺ than acridine orange alone.

INTRODUCTION

Acridines eliminate some plasmids carried by bacteria (Hirota, 1960; Nakamura, 1974). Their efficiency, however, is determined by alleles of the gene *acrA*, which is located between *proC* and *purE* on the *Escherichia coli* chromosome (Nakamura, 1968, 1974; Nakamura, Tojo & Greenberg, 1975). This gene is involved in the organization of plasma membrane (Nakamura & Suganuma, 1972) by determining biosynthesis of a membrane protein (unpublished data). We presume that the principal point of action of acridines is in the plasma membrane and that it is physically and physiologically associated with the plasmid and chromosome.

During the course of these investigations it was observed that some but not all strains resistant to acriflavine (AF), an acridine dye, were sensitive to AF plus phosphate, and that plasmid elimination by acridines was stimulated by phosphate. The present paper deals with these observations.

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Table 1. *Bacterial strains used*

Strain	Character*	Origin
N43	F ⁻ : AcrA ⁻ , Acp ⁻ , <i>lac</i> , <i>ara</i>	W4573
N544, N701, N705, N707, N709	F ⁻ : AcrA ⁺ , Acp ⁻ , <i>lac</i> , <i>ara</i>	Independent revertants of N43
W6	F ⁺ : AcrA ⁺ , Acp ⁻	Stock strain
AB311	Hfr: AcrA ⁺ , Acp ⁻	Stock strain
AB1899	F ⁻ : AcrA ⁺ , Acp ⁻	Stock strain
N545	F ⁻ : AcrA ⁺ , Acp ⁺	Revertant of N43
W1895	Hfr: AcrA ⁺ , Acp ⁺	Stock strain
HfrH	Hfr: AcrA ⁺ , Acp ⁺	Stock strain
χ478	F ⁻ : AcrA ⁺ , Acp ⁻	Stock strain
N2025	F': F- <i>gal</i> ⁺ / <i>acrA</i>	Nakamura (1974)

* Symbols of phenotypes: AcrA⁻ and AcrA⁺, sensitivity and resistance respectively to AF; Acp⁻ and Acp⁺, sensitivity and resistance respectively to AF + phosphate. Symbols of genotypes are as described by Taylor & Trotter (1972).

METHODS

Bacterial strains. These were derivatives of *Escherichia coli* K12 and are listed in Table 1.

Media. Broth media PGY, L, and BTB-galactose were as described by Nakamura (1965), Lennox (1955), and Nakamura (1971), respectively, and minimal medium S₁-glucose was as described by Nakamura (1968). Medium DK consisted of (g/l H₂O): tryptone, 10; NaCl, 5; glucose, 1.

For phosphate-enriched media, PGY or DK was mixed with an equal volume of 0.2 M-phosphate buffer pH 7.4. As a control medium, 0.2 M-tris-HCl buffer pH 7.4 or distilled water was added instead of the phosphate solution.

Crosses. General procedures for the genetic crosses were as described by Nakamura (1965).

Electron microscopy. The general procedures were as described by Nakamura & Suganuma (1972).

RESULTS

Sensitivity to AF plus phosphate

Thirty independent spontaneous revertants of strain N43 resistant to AF were isolated and tested for sensitivity to AF + phosphate. There was a continuous distribution, rather than a small number of well-defined classes, of sensitivities to AF + phosphate. The mutants N544 (Nakamura, 1968), N701, N705, N707 and N709 were the strains most sensitive to AF + phosphate (Fig. 1, *a* to *e*).

Strains AB1899, χ478, W6, HfrH, W1895 (Fig. 1, *f* to *j*) and AB311 (Fig. 2) were stock cultures in our laboratory. Strains HfrH and W1895 were resistant to AF both in the presence and absence of phosphate. The others were sensitive to AF + phosphate, but were resistant to AF in the absence of phosphate (Figs. 1, 2).

When the strains sensitive to AF + phosphate were plated on to agar medium containing AF (15 µg/ml) and phosphate, stable mutants resistant to AF + phosphate appeared. The effect of AF + phosphate on one such mutant, derived from AB311, is shown in Fig. 2.

Control experiments showed that incubation with DK medium containing phosphate alone did not kill any cells in any strain tested. It was also confirmed that the phosphate effect was not observed when other injurious agents such as streptomycin and u.v. radiation were used, so that it is probably a specific synergistic effect.

It was thought possible that the phosphate effect on sensitivity to AF was due to some

Fig. 1. Survival of the various strains in AF-containing media with and without 0.1 M-phosphate. The bacteria were incubated in DK medium containing AF (○) and AF+phosphate (●) for 60 min and plated on to normal DK agar medium to determine survival. (a) N43; (b) N701; (c) N705; (d) N707; (e) N709; (f) AB1899; (g) $\chi 478$; (h) w6; (i) HfrH; (j) w1895.

impurity in the reagents, even though special-grade reagents were used. To test this, Na_2HPO_4 and KH_2PO_4 (special grade; Wako Pure Co., Osaka, Japan) were recrystallized nine times through redistilled water before use. Since these highly purified reagents gave the same results as the original, less pure, reagents (Fig. 1), it is unlikely that the phosphate effect on the response to AF is due to impurities in the reagents.

We next attempted to examine the relationship between the phosphate concentration in the medium and the AF sensitivity of the cells. Viability was highest at 8.4 mM-phosphate and lower at increased concentrations (Fig. 3). Without AF, viability was not affected by the phosphate concentrations used.

The phosphate effect is unlikely to be caused by an osmotic imbalance between the inside and outside of the plasma membrane, since 0.1 M-NaCl (osmotically higher than 0.1 M-phosphate) had no significant effect on the sensitivity of cells to AF.

The AF binding capacity of cells, which correlates with their sensitivity to AF in terms of survival and plasmid curing, is dependent upon the pH of the medium (Hirota, 1960; Nakamura, 1966, 1967). To investigate the effect of pH upon the phosphate effect, N544 was inoculated into two series of AF containing PGY media, the one buffered by 0.1 M phosphate and the other by 0.1 M-tris-HCl control cultures containing no AF. After 60 min

Fig. 2

Fig. 3

Fig. 2. Mutation from sensitivity to resistance to AF+phosphate in AB311. $\circ$, $\bullet$, Strain AB311; $\square$, $\blacksquare$, its resistant mutant. DK medium contained AF+phosphate (closed symbols) or AF alone (open symbols). Survivors were determined as in Fig. 1.

Fig. 3. Effect of phosphate concentration in the medium on AF sensitivity of N709. Medium S₁-glucose was modified so as to have a series of graded phosphate concentrations (67 mM at maximum) and 2 μg AF/ml. Fresh cells of the strain were inoculated into these media and, after 60 min shaking, viable cells were counted by plating on PGY agar medium.

shaking, samples were plated on PGY medium (pH 7.4) to count viable cells. As shown in Fig. 4, the pH-dependence of sensitivity to AF was greater when phosphate was present than when it was replaced by tris. The control experiments indicated that at high pH values, tris, like phosphate, acts synergistically with AF, but to a much lesser extent.

Genetic analysis of sensitivity to AF plus phosphate

In mating experiments, w1895 was used as the donor of Acp^+ , and N701, N705, N707 and N709 as Acp^- recipients. The donor strain is able to form colonies on DK agar medium (pH 7.4) containing AF (15 $\mu\text{g/ml}$), both with and without the addition of phosphate. The recipients, however, can only form colonies on DK agar medium (pH 7.4) containing AF (15 $\mu\text{g/ml}$) if no phosphate is added.

After 2 h mating of the donor and recipients, $\text{lac}^+ \text{met}^+$ and $\text{ara}^+ \text{met}^+$ recombinants were selected. The recombinants, after purification, were tested for sensitivity to AF+phosphate. As a control experiment, a cross was performed between strains w1895 ($\text{AcrA}^+ \text{Acp}^+$) and N43 ($\text{AcrA}^- \text{Acp}^-$). Table 2 shows that the Acp^+ phenotype is closely linked to lac^+ (about 80 %) and less closely to ara^+ (about 45 to 50 %). The linkage of Acp^+ to both lac^+ and ara^+ is about the same as the linkage of acrA^+ to these same markers (cf. w1895 $\times$ N43). Furthermore, no recombinant sensitive to AF alone appeared on selection for either lac^+ or ara^+ .

Fig. 4

Fig. 4. Effect of phosphate on the AF sensitivity at various pH values. PGY media were buffered by 0.1 M-phosphate (circles) and 0.1 M-tris-HCl (squares). ○, □: No AF; ●, ■, 1 µg AF/ml; ●, ■, 2 µg AF/ml.

Fig. 5

Fig. 5. Effect of phosphate on plasmid curing mediated by acridine in strain N2025. Cells were inoculated into PGY (pH 7.8), containing acridine orange (1 µg/ml) and various concentrations of phosphate, and shaken. Samples were plated on BTB-galactose to determine the frequency of Gal⁺ cells. ○, □, No addition of phosphate; ●, ■, △, phosphate at concentrations of 10⁻⁴ M, 10⁻³ M, 5 × 10⁻³ M, and 10⁻² M, respectively.

Table 2. Genetic constitution of the recombinants from crosses between an *Acp*⁺ donor and *Acp*⁻ recipients

Exponentially growing cultures of the donor and recipient strains were mixed in the proportion 1 Hfr to 20 F⁻. After incubation for 2 h without shaking, the mixture was diluted and plated on the selective media, followed by purification of the recombinants. Sensitivity to AF and to AF+phosphate was determined by point-inoculation on DK agar plates containing 15 µg AF/ml, with and without the addition of 0.1 M-inorganic phosphate.

Cross	Selected for	No. tested	Frequency of phenotype (%)			
			AcrA ⁺	Acp ⁻	Lac ⁺	Ara ⁺
W1895 × N701	<i>lac</i> ⁺ <i>met</i> ⁺	126	100	79	100	31
W1895 × N705	<i>lac</i> ⁺ <i>met</i> ⁺	134	100	81	100	28
W1895 × N707	<i>lac</i> ⁺ <i>met</i> ⁺	150	100	82	100	28
W1895 × N709	<i>lac</i> ⁺ <i>met</i> ⁺	150	100	79	100	24
W1895 × N43	<i>lac</i> ⁺ <i>met</i> ⁺	150	84	—	100	38
W1895 × N705	<i>ara</i> ⁺ <i>met</i> ⁺	150	100	49	61	100
W1895 × N707	<i>ara</i> ⁺ <i>met</i> ⁺	150	100	45	57	100
W1895 × N709	<i>ara</i> ⁺ <i>met</i> ⁺	150	100	49	63	100
W1895 × N43	<i>ara</i> ⁺ <i>met</i> ⁺	150	48	—	56	100

Fig. 6. Sections N544 and N545. (a) Strain N544 was incubated with PGY medium containing AF (50 $\mu\text{g/ml}$) and phosphate (0.1 M) for 30 min. The cytoplasm contains granular aggregates (G). (b) Strain N544 with AF (50 $\mu\text{g/ml}$) alone (without addition of phosphate). The cytoplasm (P) and the nucleus (N) show normal structure. (c), (d) Strain N544 treated as in (a) and (b), respectively. Both kinds of cells show small granular aggregates (G) in the cytoplasm, but the nucleus (N) shows normal configuration. (e), (f) Sections of normal cells of N544 and N545, respectively.

Electron microscopic studies

Strain N544 was incubated with PGY medium containing AF (50 µg/ml), with and without phosphate. After 30 min, samples were fixed and sectioned. Results are shown in Fig. 6(a) and (b). The control culture (Fig. 6e) was without the addition of AF or phosphate. We conclude tentatively that, in the presence of phosphate, the low electron density area spreads widely from the surface to the interior of cytoplasm. The cytoplasm contains granular aggregates, which are not seen in Fig. 6(b) and (e).

With strain N545, the small granular aggregates were observed in both AF+phosphate Fig. 6(c) and AF alone Fig. 6(d), while such aggregation was not observed in the control culture Fig. 6(f).

Plasmid instability to AF plus phosphate

The F' strain N2025 was inoculated into PGY media (pH 7.8) containing acridine orange (1 µg/ml) and various concentrations of phosphate. Samples were withdrawn at intervals and plated on to BTB-galactose agar medium to score the number of colonies with and without the plasmid. Fig. 5 indicates that elimination of the plasmid is stimulated by the presence of high concentration of phosphate.

DISCUSSION

The results indicate that some but not all strains of *E. coli* K12 are sensitive to AF+phosphate, even when they are resistant to AF alone. Genetic analysis demonstrated that the strain specificity of the sensitivity to AF+phosphate was determined by a gene closely linked to *lac*. The gene is thought to be *acrA*. Recently we found that the allele *acrA*⁺ directs biosynthesis of a protein constituent of the plasma membrane (unpublished data). It is therefore possible that the phenotype Acp⁻ is a manifestation of a difference in this protein from that of the wild-type Acp⁺.

The effect of phosphate in stimulating the action of AF may be due to the formation of a complex between cationic AF and anionic PO₄³⁻. The electron microscopic study suggests that, in the Acp cell, plasma membrane and the layer under it is disorganized by the presence of AF + phosphate but not by AF alone. These features resemble those of *acrA* mutant cells treated with AF (Nakamura & Suganuma, 1972), suggesting that the primary action point of the AF-phosphate complex is the same as that of AF alone.

Finally, the closeness of *acrA* to the genes *phoA* and *phoB* (Taylor & Trotter, 1972) is intriguing: *phoA* codes for enzyme alkaline phosphatase, and its expression is controlled by the regulator genes *phoB* and *phoS* together with inorganic phosphate (Echols *et al.* 1961; Aono & Otsuji, 1968).

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