Plasmid-instability in *acrA* Mutants of *Escherichia coli* K12

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SUMMARY

The effect of a mutation at *acrA*, which alters the bacterial membrane, on plasmid stability was studied. F-*gal*⁺ residing in the mutant was remarkably unstable in the presence of acridine dyes or at high temperature (42 °C) as compared with wild-type (*acrA*⁺) strains. Following suppression or back-mutation to acridine resistance, cells became capable of stably hosting the plasmid in the presence of acridine. Lipophilic substances, such as phenethyl alcohol and sodium dodecylsulphate, enhanced the curing-action of acridine in the F'/*acrA* strain. Certain fatty acids promoted curing of the mutant bacteria in the absence of acridines. It is suggested that the stability of F-*gal*⁺ may depend on some feature of the plasma membrane.

INTRODUCTION

Studies in this laboratory have demonstrated that a mutation at *acrA*, which maps near *lac* on the *Escherichia coli* chromosome, leads to sensitivity to acriflavine and lipophilic substances (Nakamura, 1965, 1967, 1968*a*). Physiological and, in the presence of acriflavine, morphological alterations occur in the plasma membrane as a result of a mutation at *acrA*. These changes, however, are restored when the mutant gene is reverted to *acrA*⁺ (Nakamura & Suganuma, 1972).

Jacob, Brenner & Cuzin (1963) have proposed that the autonomously replicable DNA unit, the replicon, is attached at a particular site on the plasma membrane. It is well known that acridines cure *E. coli* of autonomous sex factors (Hirota, 1960). This had been explained as the selective inhibition of the replication of the sex factor by acridines and the dilution of the plasmid during subsequent cell division (Yamagata & Uchida, 1969).

The purpose of the work reported in this paper was to study the effect of an *acrA* mutation on sex factor stability. The results suggested that the stability of F-*gal*⁺ in *E. coli* is related to the organization of the plasma membrane determined by the *acrA* gene.

Notation for dyes is as follows: AF, acriflavine; AO, acridine orange; PF, proflavine; QC, quinacrine; AY, acridine yellow; 9AA, 9-amino acridine; AC, acridine; and BTB, bromothymol blue.

METHODS

Bacterial strains. All the strains used were derivatives of *E. coli* K12. Strains N43 (F⁻, *acrA lac gal str*) and N90 (F⁻, *acrA⁺ lac gal str*) have been described in a previous paper (Nakamura, 1965). F-*gal*⁺ donors used were strains F8/1170 (F8/*gal str*) and N2196 (F8/*gal thr leu ilv*). Strains N2307, N2308, N2309 and N2310 were AF-resistant revertants of N43 induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Adelberg, Mandel & Chein (1965). As the *acrA*⁺ donor for them w1895 (HfrC, *met*) was used. Strains N2025 and N2035 were F-*gal*⁺ merozygotes of N43 and N90, respectively, derived by mating with F8/1170.

Media. Broth media PGY and L, synthetic medium D, and BTB-galactose agar for plasmid diagnosis were prepared as described by Nakamura (1965), Lennox (1955), Davis (1950) and Nakamura (1971), respectively.

Diagnosis of F-gal⁺ strains and cured clones. As EMB medium is lethal to *acrA* mutants because they are sensitive to methylene blue (Nakamura, 1965), BTB-galactose agar (Nakamura, 1971) was used. This medium is not lethal to either *acrA* or *acrA*⁺ strains. Gal⁺ colonies appeared yellow and Gal⁻ colonies blue.

Bacterial matings. A freshly-grown F-prime strain, F8/1170 or N2196, was mated with recipients in a 1:20 ratio and, after 60 min standing at 37 °C, the mixtures were plated on D-galactose minimal agar medium selective for Gal⁺ conjugants. These were purified by streaking on the selective medium. The presence of the plasmid was determined by growing the merozygotes in PGY-AO medium (pH 7.8; 3.8 and 110 µM for *acrA* and *acrA*⁺ strains respectively) and then streaking on BTB-galactose agar and incubating at 37 °C. The ability to donate F-gal⁺ was examined by cross-streaking the F-prime cells (F-gal⁺/gal) and the recipient KY130 (F⁻ gal ton) on requirement-supplemented D-galactose agar on which a T1 phage lysate (about 2 × 10⁸ particles/dish) had been spread, and incubating for 48 h at 37 °C.

Determination of acridine uptake by bacteria. Acridine uptake was determined as described previously (Nakamura, 1966) except that the dye-containing culture was passed through membrane filters (0.45 µm pore size) and the filtrates were used for the determination. The absorption maxima used for the spectrophotometry were 452, 454, 445, 426, 454, and 260 nm for AF, AO, PF, QC, AY and 9AA respectively.

RESULTS

Acridine-sensitivity of acrA and acrA⁺ strains carrying the F-gal⁺ plasmid

Three of each of the F-gal⁺/*acrA* and F-gal⁺/*acrA*⁺ clones derived from the matings F8/1170 × N43 and F8/1170 × N90 were compared with their parents for sensitivity to acridines such as AF, AO, QC, AY, 9AA and AC. The F' strains were more resistant to the acridines (except AF) than the parent strains. As no difference was found between the three clones derived from each mating, one of each was named N2025 (F-gal⁺/*acrA*) and N2035 (F-gal⁺/*acrA*⁺).

Twelve F' clones, including N2025 and N2035, carrying either the *acrA* or *acrA*⁺ allele, were cured by culturing in PGY (pH 7.8) containing 3.8 and 110 µM-AO respectively. Cured derivatives were tested for sensitivity to AF, PF and AO using the gradient plate method (Nakamura, 1965). Practically no difference was found between the cured clones and the original F⁻ parents, N43 and N90. Therefore, in the following experiments, N43 and N90 were used instead of cured clones.

Permeability to acridines. PGY medium (pH 7.8) containing AF, PF, QC, AO, AY or 9AA and with or without 0.33 % phenethyl alcohol (PEA), was inoculated with freshly grown cells of strains N2025, N2035, N43 and N90. After incubation for various periods at 37 °C, samples were withdrawn and the dye uptake by cells was determined. Figure 1 shows the results with strains N2025 and N2035. Uptake of AF by the *acrA* and *acrA*⁺ cells was enhanced by PEA, the effect being greater in the *acrA*⁺ cells. However, cellular uptake of the dyes was not significantly affected by PEA. The same tendency was observed with strains N43 and N90. The *acrA* cells took up more AF than the *acrA*⁺ cells (Nakamura, 1966), but practically no difference was observed for the other acridines tested.

Plasmid stability in acrA and acrA⁺ strains. Freshly grown N2025 and N2035 were inocu-

Fig. 1. Uptake of acridines by strains N2025 and N2035. Freshly grown bacteria were suspended in PGY (pH 7.8), containing (○) or lacking (●) 0.33% PEA, in the presence of an acridine at the concentration indicated. After incubation at 37 °C, the dye concentration of the culture filtrate was determined spectrophotometrically.

lated into PGY medium (pH 7.8) containing various concentrations of acridines and incubated for 30 h at 37 °C. Samples were spread on BTB–galactose agar to determine the frequency of bacteria cured of the plasmid. The control culture without dyes lost the plasmid with a frequency of 1% or less after 30 h incubation. Figure 2 shows that the F' *acrA* strain lost its plasmid at a lower concentration of acridines and with higher frequencies than the F' *acrA*⁺ strain. However, AC induced no curing of the merozygotes. It may be noted that a number of acridines (AO, AY, AF, QC) cured the F' *acrA* strain with remarkably similar efficiencies in spite of differences in their toxicity (unpublished observations).

*Plasmid stability in revertants of the *acrA* strain.* Strains N2307, N2308, N2309 and N2310, which were AF-resistant revertants of N43, were crossed with strain W1895 and plated on medium selective for *lac*⁺ *met*⁺ bacteria. The recombinants from the crosses with N2307, N2308 and N2309 showed AF sensitivity with frequencies of 46% (130 clones tested), 41% (130 clones tested) and 28% (670 clones tested), respectively, when streaked on PGY (pH 7.8) agar containing 104 μM-AF. Hence, they must contain a suppressor mutation. On the

Fig. 2. Curing of the F-gal⁺ factor from *acrA* and *acrA*⁺ strains by acridines. Freshly grown (a) N2035 and (b) N2025 bacteria were inoculated into PGY (pH 7.8) containing the indicated concentration of acridine. After 30 h incubation at 37 °C, samples were plated on BTB-galactose agar to determine the frequency of Gal⁻ bacteria. ●, AO; ○, 9AA; ■, PF; ▼, AF; ▲, AY; △, AC; ◇, QC.

other hand, as none of 278 recombinants from the cross involving N2310 showed AF sensitivity, N2310 seems to be either a back-mutant or to carry a second suppressing mutation very close to the lesion at *acrA*.

F8-merozygotes of strains N2307, N2308, N2309 and N2310 were prepared by mating with strain N2196 and plating on medium selective for *Thr*⁺ *Leu*⁺ *Ily*⁺ *Gal*⁺ bacteria. The plasmids residing in the AF-resistant revertants were found to be within a few per cent as stable to AF as those in strain N2035, when tested by the method described in the previous section.

Effect of high temperature

Strains N2025 and N2035 were inoculated in PGY medium and shaken at 42 °C. The cell density during the culture was low, about 10⁸ to 10⁹ bacteria/ml, to minimize the introduction of the F' factor into the cured cells. Figure 3 indicates that as the cells divide at 42 °C the *acrA* strain loses the plasmid at a higher rate than the wild-type strain. The growth rates at 42 °C of the F' strains, N43 and N90, were found to be indistinguishable.

Fig. 3. Effect of incubation at high temperature on plasmid stability in strains N2025 (▲) and N2035 (●). Freshly grown cells were inoculated at a low cell density into PGY (pH 7.4) prewarmed at 42 °C and shaken. Samples were withdrawn at intervals and plated on BTB-galactose agar.

Effect of lipophilic substances on the action of acridines

It has been suggested that PEA may disorganize the plasma membrane by affecting its lipid components (cf. Masker & Eberle, 1972; Silver & Wendt, 1967). It was thus expected that, if the plasmid, like the bacterial chromosome (Masker & Eberle, 1972), is stably connected with the plasma membrane of the host cell, PEA might disturb the attachment and thus the replication of the plasmid. However, experiments using N2025 and N2035 showed that PEA alone was not effective in eliminating the plasmid.

An attempt was made to see whether PEA increased the effect of acridines in curing. PGY medium (pH 7.8) containing 3.5 μ M-AF and various concentrations of PEA was lightly inoculated with N2025 and N2035 and shaken for a few generations at 37 °C. Figure 4 shows that strain N2025 is cured by AF as a function of number of generations, and the presence of 0.04% or more of PEA increased the curing effect. Similar experiments on the PEA effect were performed using PF (4 μ M) and QC (16 μ M). PEA raised the curing effect of these dyes also (Fig. 4).

To determine whether the acridine effect in eliminating the plasmid was increased by the presence of a detergent, sodium dodecylsulphate (SDS), strains N2025 and N2035 were inoculated into PGY (pH 7.8) containing 3.5 μ M-AF and 0.001% or more of SDS. As shown in Fig. 5, N2025 cells lost their plasmids with increasing frequency as the SDS concentration was raised. SDS did not promote curing of N2035 by AF. SDS alone did not influence the

Fig. 4. Effect of PEA on plasmid-curing mediated by (a) AF, (b) PF, and (c) QC, in strain N2025. The cells were inoculated in PGY (pH 7.8) containing AF ($3.5 \mu\text{M}$), PF ($4 \mu\text{M}$) or QC ($16 \mu\text{M}$) and various concentrations of PEA and shaken at 37°C . Samples were plated on BTB-galactose agar to determine the frequency of Gal^+ cells. $\times$, No additions. PEA added: $\circ$, 0.06% (no dye); $\blacktriangle$, 0%; $\bullet$, 0.01%; $\blacktriangledown$, 0.02%; $\blacksquare$, 0.04%; $\blacklozenge$, 0.06%. (No curing occurred under the same conditions in N2035.)

Fig. 5. Effect of SDS on plasmid-curing mediated by AF ($3.5 \mu\text{M}$) in strain N2025. The conditions were the same as for Fig. 4 except that SDS was used in place of PEA. $\times$, No additions. SDS added: $\circ$, 0.001% (no dye); $\bullet$, 0.0015% (no dye); $\triangle$, 0%; ∇ , 0.001%; $\square$, 0.0015%.

stability of the plasmid in either strain up to 0.0015%, but at higher concentrations it effectively cured the merozygotes.

Effect of fatty acids on curing

The following saturated and unsaturated fatty acids (Na-salts) were examined: butyrate, caproate, caprylate, caprate, laurate and myristate (saturated); and oleate and linoleate (unsaturated). PGY media (pH 7.8), each containing one of the fatty acids at 1 mg/ml, were inoculated with N2025 and N2035. Overnight incubation of the cultures at 37 °C was followed by determination of the extent of episome-curing. Both saturated and unsaturated fatty acids cured strain N2025 with frequencies of up to 10% but they were non-effective for strain N2035.

DISCUSSION

An *acrA* mutation in *E. coli* leads to pleiotropic alterations in cellular functions, including a change in the membrane (Nakamura, 1968*a, b*; Nakamura & Suganuma, 1972). The present data indicate that the F-*gal*⁺ plasmid residing in *acrA* cells is less stable under the action of acridines and other agents than it is in *acrA*⁺ cells. Revertants of the *acrA* strain carrying a suppressor or back mutation stably hosted the plasmid like the wild-type strain. Thus, it can be concluded that instability of the plasmid in the *acrA* cells is related to membrane defects.

A relationship between plasmid stability and membrane organization is suggested by the following observations. Plasmid-curing by acridines in the *acrA* cells was stimulated by the presence of PEA and SDS. It has been demonstrated that PEA is more toxic to *acrA* cells than to *acrA*⁺ cells (Nakamura, 1967, 1968*a*), disturbs biosynthesis of membrane lipid (Nunn & Tropp, 1972), and destroys the permeability barrier reversibly (Silver & Wendt, 1967). PEA is also known to release the chromosomal DNA from the plasma membrane (Masker & Eberle, 1972) and inhibit the initiation of DNA replication (Lark & Lark, 1966). Furthermore, PEA significantly eliminates the plasmid from *E. coli* C600 (Wada & Yura, 1971), but it did not do so in the K12 strains used in the present experiments. On the other hand, SDS has been shown to be more toxic to *acrA* cells than to *acrA*⁺ cells (Nakamura, 1968*a*), to denature membrane proteins (Putnam, 1948), and to dissociate the bacterial chromosome and phage DNA from the plasma membrane (Fielding & Fox, 1970; Knippers & Sinsheimer, 1968; Rosenberg and Cavalieri, 1968). SDS can also eliminate some kinds of plasmids from bacterial cells (Sonstein & Baldwin, 1972*b*; Tomoeda, Inuzuka, Kubo & Nakamura, 1968).

The present results show that, with the exception of AF, the dye-binding capacity of the *acrA* mutant is no greater than that of the wild-type cells and that incubation either at high temperature (42 °C) or in the presence of fatty acids promotes the elimination of the plasmid from the *acrA* merozygotes. These observations demonstrate that plasmid instability in the *acrA* cells is not due to the enhanced uptake of acridines but to plasmid-hosting defects.

It is not clear why the acridines selectively inhibit the replication or segregation of the plasmid. *In vitro* experiments have shown that acridines are intercalated between specific adjacent base pairs in DNA (Lerman, 1963) and inhibit synthesis of DNA (Hurwitz, Furth, Malamy & Alexander, 1962). The base specificity of the affinity has been demonstrated to differ according to the kinds of acridines (Tubbs, Eitmars & van Winkle, 1964; Weisblum & de Haseth, 1972). However, the present data show that some acridines are equally effective in plasmid-curing in *acrA* merozygotes within a range of dye concentrations. It has been suggested that acridines do not readily penetrate through the plasma membrane but adsorb

on the cell surface (Nakamura, 1966; Silver, Levine & Spielman, 1968). These features support the hypothesis that acridines primarily act on a specific site of the plasma membrane rather than on the DNA molecule in order to inhibit plasmid replication.

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