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SHORT PAPER

The plasmid curing action of imipramine in *Escherichia coli* K12

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SUMMARY

Curing of an F-prime plasmid by imipramine was most efficient on bacteria growing semianaerobically at 37 °C. The plasmid curing effect of imipramine was increased in the presence of methylene blue, whilst fluorescein, chlorpromazine-sulphoxide and tetraoxyanthrachinon antagonized the plasmid curing action of the drug. In addition to its plasmid curing effect, imipramine treatment selected for Lon mutants at high frequency. Lon mutants show increased resistance to the drug but cured strains, if Lon$^+$, are not resistant.

1. INTRODUCTION

Acridine dyes have been used extensively to eliminate various extrachromosomal genetic elements such as sex factors (Hirota, 1960), and R factors (Mitsuhashi, Harada & Kameda, 1961) from bacterial cells. The efficiency of curing generally varies from less than 0·1 % to more than 99 % depending upon the element involved, the bacterial strain, and the conditions used and it is generally assumed that curing activity is related to the ability of these compounds to intercalate into the DNA molecule (Lerman, 1961). Nevertheless the actual mechanism is still completely obscure. In our previous studies it was observed that the major tranquillizer chlorpromazine caused curing of an R-factor and of F′*lac*$^+$ from strains of *E. coli* (Molnár, Király & Mándi, 1975; Mándi, Molnár, Holland & Béládi, 1976). Phenothiazines are tricyclic, almost planar molecules similar in structure to acridines and are capable of intercalation into DNA (Yamabe, 1973). Unexpectedly, we have now found that some antidepressant tricyclic drugs, e.g. imipramine, which although not planar molecules are also capable of eliminating an F-prime plasmid of *E. coli*. In the present work some experiments were done to study the optimal conditions for plasmid curing by imipramine.

2. MATERIALS AND METHODS

E. coli K12, LE140 (*lac*Δ, *mal*, *str*, λ^R) carrying F′*lac*$^+$ was grown in MTY medium (Alföldi, Raskó & Kerekes, 1968) containing 0·1 % glucose. For the differentiation of *lac*$^+$ colonies from colonies formed by *lac*$^-$, plasmidless bacteria, dilutions of cultures were plated on EMB lactosè agar containing 2 % lactose (Clowes & Hayes, 1968). Strain *E. coli* C600 carrying the R-factor R144 or its derepressed derivative R144*drd*-3

were provided by Dr B. M. Wilkins. Imipramine was obtained from EGYT Pharmaceutical Works, Budapest, Hungary; methylene blue produced by Reanal, Budapest; tetraoxyanthrachinon obtained from Merck; chlorpromazine sulphoxide was prepared by the method of Fishman & Goldenberg (1963). For curing of plasmids, bacteria from overnight cultures were added (final concentration of 10^3 cells/ml) to 5 or 10 ml MTY media containing 50–160 μg/ml imipramine. The samples were incubated for 24 h at 37 °C, diluted and plated on EMB agar. The plates were incubated for 24 h at 37 °C and scored for *lac*$^-$ and *lac*$^+$ colonies. To determine the minimal inhibitory concentration of drugs, serial dilutions of imipramine were prepared in 5 ml aliquots of MTY medium; 5×10^3 bacteria from an overnight culture were added and the cultures incubated without shaking at 37 °C for 24 h. The minimum concentration of drug which completely inhibited growth was then scored.

3. RESULTS AND DISCUSSION

(*a*) *Curing action of imipramine.* The minimal inhibitory concentration of the drug (see Methods) was found to be 190 μg/ml. The bacteriocidal action of imipramine was demonstrated with an exponential culture of strain LE140 in MTY medium; at concentrations of 200 μg/ml and over, more than 90 % of the bacteria are killed after a 60 min treatment. When such cultures or cultures treated with 150 μg/ml are plated out on EMB lactose plates, all the surviving bacteria are *lac*$^+$, indicating that extensive growth of the bacteria in the presence of the drug is important for curing. Strain LE140 (Δ*lac*, F′*lac*) was therefore grown without aeration in MTY medium at 37 °C in the presence of a subinhibitory concentration (150 μg/ml) of imipramine. Bacteria were plated on EMB agar at intervals and after 8 h incubation a low frequency of *lac*$^-$ colonies is detected. The number of *lac*$^-$ bacteria then increases, particularly towards stationary phase when the proportion reaches 10–20 % of the population. Control experiments demonstrate that these *lac*$^-$ clones fail to grow on lactose minimal agar and are resistant to F-factor specific bacteriophages and we conclude that they have lost the F′-prime plasmid. As shown in Fig. 1, exponential cultures of both F′*lac* bearing and cured derivatives of strain LE140 are equally sensitive to 250 μg/ml imipramine. In addition, the frequency of curing of the R-factor R144 and a derivative (R144*drd*) derepressed for pilus synthesis, although low, was essentially the same for the two plasmids (data not shown). These results therefore indicate that imipramine curing does not result from selection of drug resistant, plasmid free clones.

(*b*) *The effect of temperature and aeration on curing by imipramine.* The curing action of the drug was the most effective at 37 °C whilst the drug was practically ineffective at 20 °C (see Table 1). It should be noted that the bacteria were not able to grow at 41 °C in the presence of the concentration of imipramine used (160 μg/ml). In shaken cultures in MTY medium at 37 °C for 24 h, the frequency of *lac*$^-$ bacteria varied from 0·0 to 0·2 %. In contrast, in cultures incubated without aeration, the frequency of curing varied from 50–70 %. The frequency was increased to 90 % in several experiments when the medium was supplemented with sodium thioglycolate (0·05 %, final concentration). Thus efficient plasmid curing by imipramine requires semianaerobic growth conditions.

(*c*) *Effect of other tricyclic compounds on curing by imipramine.* Fluorescein, tetraoxyanthrachinon chlorpromazine-sulphoxide and methylene blue when present alone were found to have no plasmid curing effect on *E. coli* LE140. All these compounds, although similar in structure to imipramine, are relatively polar molecules and may fail to penetrate the bacterial surface effectively. Treatment of bacteria with these compounds nevertheless had significant effects on the curing ability of imipramine. Thus as summarized in Table 1, fluorescein antagonized the plasmid curing action of imipramine.

Chlorpromazine sulphoxide and tetraoxyanthrachinon had the same effect over a similar range of concentrations, although none of these compounds antagonize the bacteriocidal effects of the drug (data not shown). In contrast, methylene blue had an additive effect on curing by imipramine.

Table 1. *Effect of temperature and structural analogues on imipramine curing*

(Overnight cultures of LE140(F'*lac*) diluted to 10^8 bacteria/ml in MTY medium were treated with 150 μg/ml imipramine and incubated without shaking at different temperatures for 24 h before plating on EMB-agar and screening for *lac*$^-$ clones. Similar cultures treated with imipramine at 37 °C were also grown for 24 h with varying amounts of methylene blue or fluorescein before plating on EMB-agar.)

Temperature (°C)	Viable cells after 24 h	No. of colonies tested	Lac$^-$ colonies	Cured bacteria (%)
20	$7{\cdot}5 \times 10^6$	2109	1	0·05
26	$6{\cdot}0 \times 10^7$	1641	21	0·13
30	$2{\cdot}0 \times 10^8$	1057	11	1·0
37	$5{\cdot}0 \times 10^8$	1101	209	19·0
Treatment				
Imipramine	$8{\cdot}7 \times 10^6$	544	364	67
Methylene blue (40 μg/ml)	$1{\cdot}8 \times 10^7$	838	0	0
Imipramine + methylene blue	9×10^6	1010	1008	100
Imipramine + fluorescein (100 μg/ml)	$1{\cdot}6 \times 10^7$	228	26	11
Imipramine + fluorescein (500 μg/ml)	$1{\cdot}9 \times 10^7$	298	6	2
Fluorescein (100 μg/ml)	$1{\cdot}2 \times 10^7$	952	0	0

(*d*) *Isolation of mucoid mutants by imipramine treatment.* Previous studies with chlorpromazine revealed that mucoid mutants appeared at high frequency in treated cultures (Molnár, Holland & Mándi, 1977). Similar results were obtained using imipramine (150 μg/ml) and thus after 24 h at 37 °C in the presence of the drug up to 50% of plated colonies were found to be mucoid. Only rarely had such strains also lost the plasmid. As Fig. 1 shows, mucoid strains are clearly more resistant to imipramine than are wild-type bacteria and are presumably greatly favoured by the selective conditions operating in cultures grown in the presence of the drug. Mucoidy was maintained in the strains after purification and such strains were shown to be lysed by the virulent *E. coli* phage, m-59, (Stirm *et al.* 1974) which is specific for binding to colanic acid. The mutants were also sensitive to UV-irradiation and therefore are probably Lon$^-$ as are the mucoid strains obtained by chlorpromazine treatment. It is of interest that acridine orange did not produce such mutants suggesting that the mechanism of acridine uptake and or binding to the bacterial envelope is distinct from that of imipramine and chlorpromazine.

The antibacterial effect of imipramine was not apparently antagonized by several structural analogues (unpublished experiments) although these compounds antagonized the plasmid curing action of the drug. This suggested that the bacteriocidal action and plasmid curing effects are independent properties of imipramine. The results obtained with methylene blue were surprising since the dye enhanced the plasmid curing activity of imipramine, whilst this compound, which is structurally analogous

to acridine orange, antagonizes the elimination of the F-plasmid by acridine (Hirota, 1960). The F^+ and cured F^- cells appear equally sensitive to the drug and therefore curing is unlikely to involve any selective mechanism, as is found when sodium dodecyl-sulphate is used as a curing agent (Salisbury, Hedges & Datta, 1972). Nevertheless, curing by imipramine does appear to require multiplication of the bacteria and this may reflect the gradual emergence of plasmid-free bacteria as plasmid replication or segregation is inhibited by the drug. A differential effect of imipramine upon plasmid replication could reflect the interaction of the drug with plasmid specific membrane binding sites in the bacterial envelope. Alternatively, direct interaction with plasmid

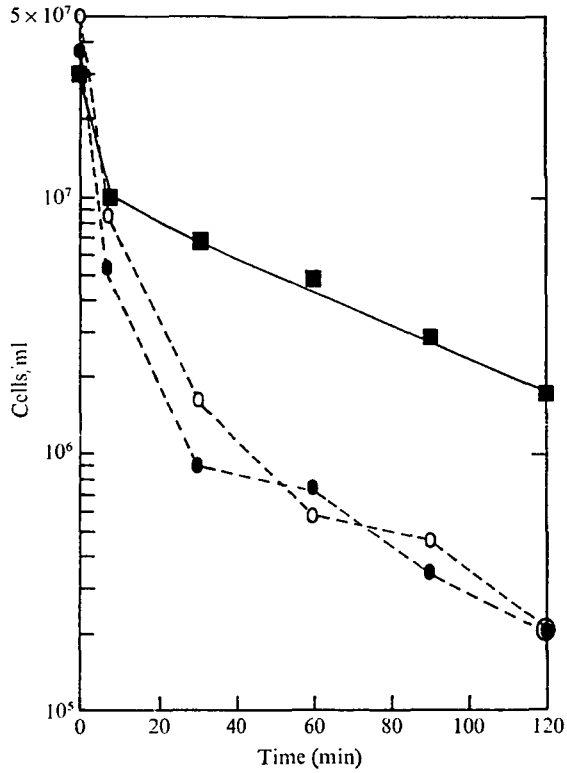

Fig. 1. Survival of different *E. coli* strains treated with imipramine. Bacteria were grown to 5×10^8 bacteria/ml at 37 °C in MTY medium, cultures were then transferred to 30 °C and each treated with 250 μg/ml imipramine. Samples were removed at intervals, diluted in saline and plated on MTY agar for viable counts. ■——■, a mucoid strain isolated after imipramine treatment of *E. coli* LE140; ○---○, LE140, F'*lac*; ●---●, a non-mucoid derivative of LE140 cured of the F'*lac* by growth in imipramine.

DNA is also possible. Intercalation into DNA appears unlikely, however, in view of the non-planar arrangement of the three ring structures in imipramine. In any case the degree to which a particular compound is able to penetrate the bacterial surface is also likely to be of primary importance in determining any potential curing activity. Thus methylene blue and fluorescein intercalate into DNA *in vitro* (Yamaba, 1973) but fail to show curing activity in our system (see also Hirota, 1960). The optimal activity of imipramine observed in semianaerobic cultures may also reflect some change in the cell surface favourable to uptake or binding of the drug.

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