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Drug sensitivity and mutability to drug resistance associated with the presence of an R factor

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SUMMARY

Studies on the growth kinetics of R^+ and R^- cultures of *Escherichia coli* in the presence of nalidixic acid (NA), acriflavine (AF) and kanamycin (Kan) showed that each drug caused a decline in viability of both R^+ and R^- cells for several hours. During further incubation the viability rose rapidly for the R^+ cultures, but either rose less rapidly (AF and Kan) or continued to decline (NA) for R^- cultures. Distribution curves of the resistances of individual clones of R^+ and R^- bacteria to atabrine, NA, AF and Kan suggested that the presence of an R factor in the host bacterium increased its mutation rate to resistance to these drugs: this would account for the more rapid growth rate of R^+ cells during the latter stages of incubation in their presence. The mutations causing increased resistance to NA and to Kan were located in the bacterial chromosome and not in the R factor.

1. INTRODUCTION

The R factors responsible for transmissible resistance to many different antibiotics confer sensitivity to atabrine upon the host cells (Yoshikawa & Sevag, 1967). Thus it seemed that atabrine might be useful in infections caused by R^+ bacteria. However, it was subsequently found that when R^+ and R^- cells were grown in the presence of 60 μg/ml atabrine, the initial decline in the numbers of R^+ cells was always followed by an increased growth rate so that ultimately the R^+ cells overgrew the R^-. The R factor was thus assumed to confer not only sensitivity to atabrine but also higher mutability to resistance to the drug.

The present report shows that the R factor also confers initial sensitivity to nalidixic acid and acriflavine with higher mutability to resistance. It also gives higher mutability to kanamycin resistance but without the detectable initial sensitivity.

2. MATERIALS AND METHODS

Bacterial strains used. Three substrains of *Escherichia coli* K-12, W-3630 (F^-, Hfr_3^-, *met*), W-3104 (F^-, *gal*$^-$) and CSH-2 (F^-, *met*, *pro*), were used with their R^+ derivatives obtained by conjugal transfer. The R factors, R_{100} and R_{203}, determined resistance to sulphonamides (Sul), streptomycin (Str), chloramphenicol (Cml) and tetracycline (Tet).

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Drugs. Kanamycin (Kan) from Takeda Pharm. Co., Japan, acriflavine (AF) from the Tokyo Kasei Co., Japan, nalidixic acid (NA) kindly given by the Winthrop Laboratories of Japan, and atabrine (AT) kindly given by the Sterling-Winthrop Research Institute, Renselaer, N.Y., U.S.A., were used.

Media. Nutrient broth (pH 7·2) was used as liquid medium and EMB-lactose agar as solid, except when nutrient agar (pH 7·2) was used for the distribution curves of resistance to AT and AF. Dilutions were made in unbuffered 0·85% saline.

Growth kinetic study. Cells grown overnight in 5 ml of broth were used as inoculum after centrifugation and resuspension in 5 ml of saline to give a final reading of 100 in a Klett–Summerson photometer with a filter of 540 mμ.

Penicillin screening method to select R^- segregants. One tenth ml of an overnight culture of R^+ cells was inoculated in 5 ml of broth containing 500 units/ml of penicillin G and 25 μg/ml of chloramphenicol. It was incubated at 37 °C for 3 h with gentle shaking. Cells were centrifuged, washed, resuspended in saline, appropriately diluted and plated on EMB-lactose agar. After 24 h of incubation at 37 °C colonies were examined for drug resistance by replica-plating to EMB-agar containing chloramphenicol.

3. RESULTS

(i) *Distribution curve of resistance to atabrine*

Dilutions of overnight broth cultures of W-3630 and W-3630(R_{100}) were plated on nutrient agar containing various concentrations of AT and incubated at 37 °C for 48 h. A distribution curve of the resistance of each strain was made from the

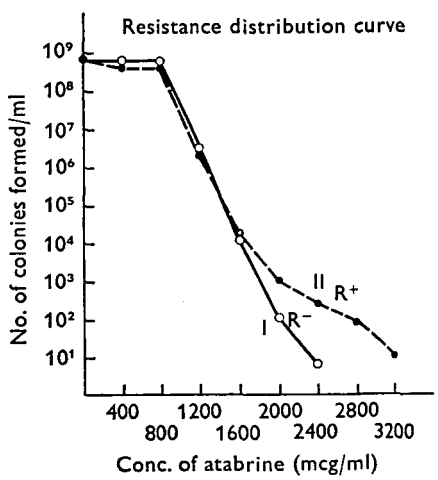

Fig. 1

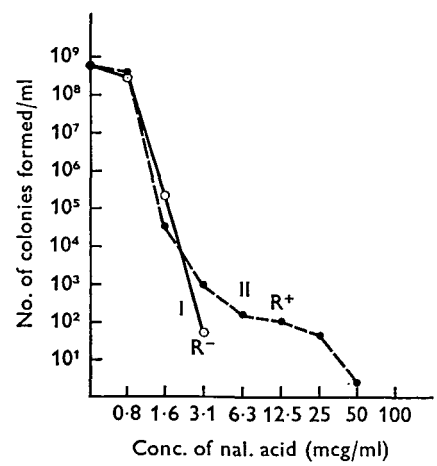

Fig. 2

Fig. 1. Resistance distribution curve to atabrine. Curve I indicates W-3630 and curve II, W-3630 (R_{100}).

Fig. 2. Resistance distribution curve to nalidixic acid. Curve I indicates W-3630 and curve II, W-3630 (R_{100}).

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numbers of colonies formed on each concentration (Fig. 1). The concentration of AT at which the number of colonies began to decrease was almost the same with both R^+ and R^- cells. However, R^+ cells gave more survivors than R^- cells on atabrine concentrations above 1600 μg/ml. Two other comparisons of R^- and R^+ cells (W-3630 and W-3630(R_{203}) and W-3104 and W-3104(R_{100})) gave a similar picture. Essentially the same results were obtained with NA (Fig. 2), with AF (Fig. 3) and with Kan (Fig. 4). R^+ cells are more sensitive than R^- cells to AF, and these differences must be taken into account in comparing the curves in Fig. 3.

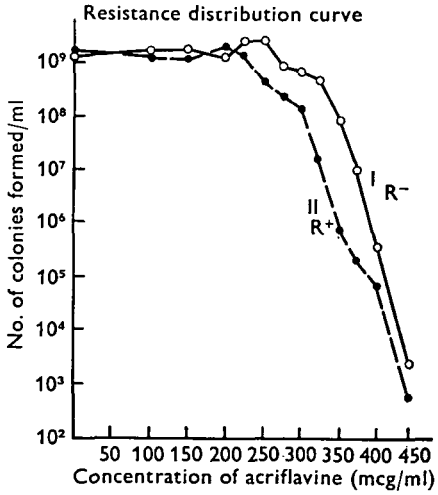

Fig. 3

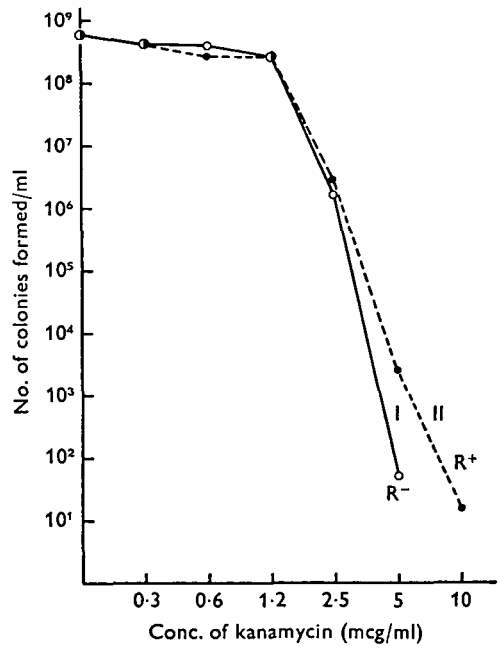

Fig. 4

Fig. 3. Resistance distribution curve to acriflavine. Curve I indicates W-3630 and curve II, W-3630(R_{100}).

Fig. 4. Resistance distribution curve to kanamycin. Curve I indicates W-3630 and curve II, W-3630(R_{100}).

(ii) *Growth of R^+ and R^- bacteria in nalidixic acid and in acriflavine*

Overnight broth cultures of W-3630 and W-3630(R_{100}) were inoculated at a concentration of about 2×10^7 cells/ml into separate lots of 5 ml broth, either containing 12·5 μg/ml of NA or with no antibiotic, and viable counts were made at intervals during incubation at 37 °C. Fig. 5 shows that, in the presence of NA, the numbers of R^+ colonies at first decreased slowly at about the same rate as the R^-. They then fell off more steeply from about the 12th to 18th h, but finally rose to reach about 5 times the initial cell concentration after 35 h, by which time the overall number of R^- colonies had reached 10-fold. Very similar kinetic curves were obtained with AF (Fig. 6). Atabrine had already been shown to produce

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a similar effect on growth (Yoshikawa & Sevag, 1967), the only difference being that the R^+ cells were relatively more sensitive to AT than the R^- from the beginning. Essentially the same results were also obtained with NA and AF, using two other comparisons of R^- and R^+ strains: W-3630 compared with W-3630(R_{203}) and W-3104 compared with W-3104(R_{100}).

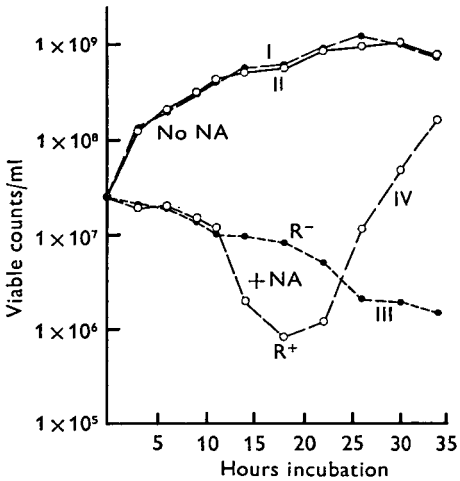

Fig. 5

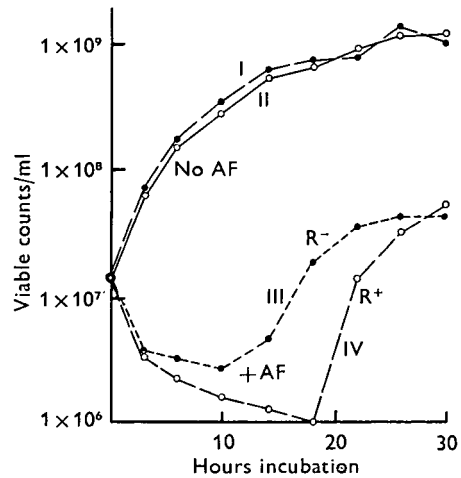

Fig. 6

Fig. 5. Growth (viable counts) of R^- and R^+ cells in the presence of nalidixic acid. Curve I indicates W-3630 in the absence of the drug; curve II, W-3630(R_{100}) in the absence of the drug; curve III, W-3630 in the presence of 12·5 μg/ml of the drug; and curve IV, W-3630(R_{100}) in the presence of 12·5 μg/ml of the drug.

Fig. 6. Growth (viable counts) of R^- and R^+ cells in the presence of acriflavine. Curve I indicates W-3630 in the absence of the drug; curve II, W-3630(R_{100}) in the absence of the drug; curve III, W-3630 in the presence of 80 μg/ml of acriflavine; and curve IV, W-3630(R_{100}) in the presence of 80 μg/ml of acriflavine.

(iii) *Growth of R^+ and R^- bacteria in kanamycin*

W-3630 and W-3630(R_{100}) were inoculated into broth containing 7·5 μg/ml Kan, starting from an inoculum of 10^7 cells/ml. Viable counts during the first 3 h showed no difference in the numbers of R^- and R^+ survivors. After this time the numbers of both kinds of cell began to increase, but the R^+ bacteria increased more rapidly than the R^- (Fig. 7A). Fig. 7B shows the results of an experiment independently performed under the same conditions as those of Fig. 7A, the difference being that more samples were taken during the early hours of incubation. The above statements, especially the final overgrowth of the R^+ cells and the lack of initial sensitivity phase of R^+ strains, were thus confirmed. Essentially the same result was obtained in 10 μg/ml Kan, but this concentration prevented any increase in R^- cells (Fig. 7C).

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(iv) *Stability of NA and Kan resistance acquired by R+ cells*

Five colonies resulting from plating W-3104(R_{100}) on 25 μg/ml NA were picked, purified and serially subcultured 5 times in the absence of NA. Subsequent testing on medium containing NA showed that the resistance was stable (Table 1). A similar experiment with Kan showed that Kan resistance acquired by the R+ bacteria was also stable.

To decide whether the determinants for acquired NA or Kan resistance were located on the bacterial chromosome or on the R factor, four R− segregants were obtained from NA-resistant W-3104(R_{100}) strains by the penicillin screening method and examined for their NA resistance. As shown in Table 2, they were still resistant

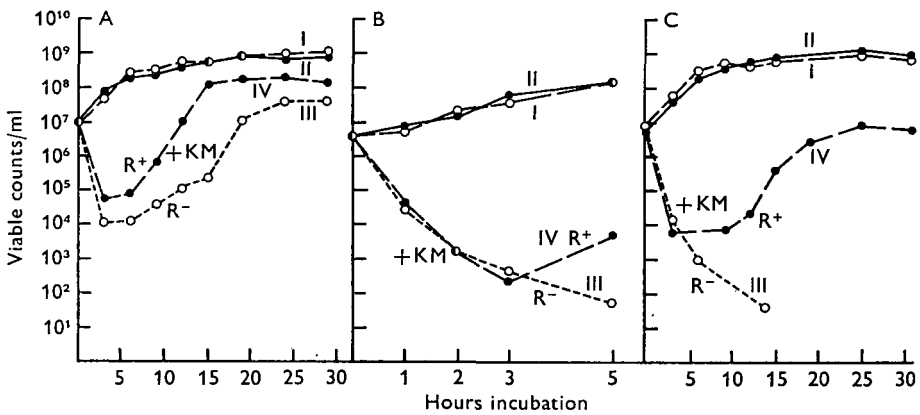

Fig. 7. Growth (viable counts) of R− and R+ cells in the presence of kanamycin. Concentrations of kanamycin (sulphate); 7·5 μg/ml in A and B and 10 μg/ml in C. In all of these three figures, curve I indicates W-3630 in the absence of the drug; curve II, W-3630(R_{100}) in the absence of the drug; curve III, W-3630 in the presence of the drug; and curve IV, W-3630(R_{100}) in the presence of the drug.

Table 1. *Stability of nalidixic acid resistance acquired by R+ cells*

Five nalidixic acid-resistant *Escherichia coli* W-3104(R_{100}) strains were obtained from EMB agar plates containing 25 μg/ml of nalidixic acid. These strains were grown 5 times successively in broth without nalidixic acid and then plated on EMB agar with or without 25 μg/ml of nalidixic acid.

Strains	No. of colonies formed per ml on EMB agar containing: No antibiotic	Nalidixic acid (25 μg/ml)
W-3104	$1{\cdot}2 \times 10^9$	$< 5{\cdot}0 \times 10^0$
W-3104(R_{100})	$1{\cdot}2 \times 10^9$	$1{\cdot}1 \times 10^2$
W-3104(R_{100}) N.A. resist.-1	$9{\cdot}6 \times 10^8$	$1{\cdot}1 \times 10^9$
W-3104(R_{100}) N.A. resist.-2	$9{\cdot}2 \times 10^8$	$9{\cdot}6 \times 10^8$
W-3104(R_{100}) N.A. resist.-3	$7{\cdot}4 \times 10^8$	$8{\cdot}3 \times 10^8$
W-3104(R_{100}) N.A. resist.-4	$8{\cdot}7 \times 10^8$	$5{\cdot}5 \times 10^8$
W-3104(R_{100}) N.A. resist.-5	$9{\cdot}1 \times 10^8$	$8{\cdot}4 \times 10^8$

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to 25 μg/ml of NA. The R factor from the NA-resistant W-3104(R_{100}) strain was then transferred to W-3104 R^- via another *Escherichia coli* strain, CSH-2, and its NA resistance examined. As shown in Table 3, the R^+ recipient bacteria did not show NA resistance. Similarly, acquired Kan resistance was retained by R^- segregants and could not be transferred with the R factor.

Table 2. *Persistence of nalidixic acid resistance in R^- segregants*

R^- segregants derived from nalidixic acid-resistant strains used in the experiment of Table 1 were obtained by screening in the presence of chloramphenicol and penicillin G. Acridine dyes were not used. The nalidixic acid sensitivity of these R^- segregants was examined.

Strains	No. of colonies formed per ml on EMB agar containing: No antibiotic	Nalidixic acid (25 mcg/ml)
W-3104	$1{\cdot}1 \times 10^9$	$< 5{\cdot}0 \times 10^0$
W-3104(R_{100})	$9{\cdot}3 \times 10^8$	$8{\cdot}0 \times 10^1$
W-3104-R^- from N.A. resist.-2	$9{\cdot}3 \times 10^8$	$8{\cdot}9 \times 10^8$
W-3104-R^- from N.A. resist.-3	$7{\cdot}1 \times 10^8$	$6{\cdot}3 \times 10^8$
W-3104-R^- from N.A. resist.-4	$7{\cdot}1 \times 10^8$	$7{\cdot}5 \times 10^8$
W-3104-R^- from N.A. resist.-5	$1{\cdot}0 \times 10^9$	$1{\cdot}0 \times 10^9$

Table 3. *Non-transmissibility of nalidixic acid resistance acquired by R^+ cells*

The R factors of the nalidixic acid-resistant W-3104(R_{100}) strains used in the experiment of Table 1 were transferred to W-3104(R^-) via another *Escherichia coli* strain, CSH-2, and their nalidixic acid sensitivity was examined.

Strains	No. of colonies formed per ml on EMB agar containing: No antibiotic	Nalidixic acid (25 μg/ml)
A. W-3104(R_{100})	$8{\cdot}4 \times 10^8$	$3{\cdot}0 \times 10^1$
B. W-3104(R_{100}) N.A. resist.-1	$9{\cdot}3 \times 10^8$	$1{\cdot}0 \times 10^9$
C. W-3104(R_{100}) N.A. resist.-2	$8{\cdot}1 \times 10^8$	$8{\cdot}3 \times 10^8$
D. W-3104 infected with R of (B)	$9{\cdot}2 \times 10^8$	$2{\cdot}5 \times 10^1$
E. W-3104 infected with R of (C)	$8{\cdot}8 \times 10^8$	$3{\cdot}0 \times 10^1$

4. DISCUSSION

In the present study, experiments with nalidixic acid and with acriflavine show that, although an R factor might confer increased initial sensitivity to nalidixic acid or acriflavine, this was followed by the emergence of resistant mutants at a higher rate than with R^- strains. Nevertheless, the mutation leading to resistance to nalidixic acid was located in the bacterial chromosome and not in the R factor itself. Experiments with kanamycin also revealed higher mutability to resistance in R^+ cells than in R^- but without any initial difference in sensitivity.

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The author cannot offer any explanation for these phenomena. The R factor might act as a mutator plasmid (Gundersen, Jyssum & Lie, 1962), increasing the mutation rate to drug resistance, but this cannot explain why R^+ cells are initially more sensitive than R^- to nalidixic acid and acriflavine as well as to atabrine. Furthermore, no difference in mutation rate for six other chromosomal markers was observed between R^- and R^+ strains. An alternative hypothesis would be that the R^+ cells are more permeable to the drugs and hence more vulnerable to killing as well as to any possible mutagenic action the drugs might have. However, the failure of these drugs to cause detectable increase in mutation rates in other genes is against this hypothesis also.

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