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Selective enrichment of R⁻ segregants as the main mechanism of 'curing' of the R factor by acridine dyes

By MASANOSUKE YOSHIKAWA

Department of Bacterial Infection, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan

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SUMMARY

By the use of appropriate strains of *Escherichia coli*, *Shigella flexneri* and *Salmonella typhimurium* with and without an R factor, R_{100}, the mechanism of 'curing' of R factor by acridine dyes was examined. This R factor was shown to confer increased sensitivity to acriflavine upon the host cells. *E. coli* strain W-3630, once infected with R_{100}, has never been observed to segregate R⁻ cells. When mixtures of R⁺ and R⁻ cells of this strain were grown in acriflavine broth, the proportion of R⁻ cells increased and was also correlated with the proportion in the initial inoculum. Other bacterial strains carrying R_{100} segregate R⁻ cells spontaneously. Growth tests starting with varying proportion of R⁺ and R⁻ cells of these strains in acriflavine broth also gave a marked correlation between the initial and final proportions of R⁻ cells, and indicated that the main cause of 'curing' the R factor was the selective enrichment of R⁻ segregants present in the initial inocula or arising spontaneously during growth of the R⁺ culture. These results suggest that the mechanisms underlying the 'curing' of F and R factors are different. Tests with several acridine dyes gave results similar to those with acriflavine.

1. INTRODUCTION

'Curing' of F⁺ cultures by growth in acridine dyes results from inhibition of F factor replication (Hirota, 1960; H. Yamagata & H. Uchida, personal communication). An R factor, R_{100}, has also been observed to be eliminated during growth of R⁺ cultures in acriflavine or acridine orange (Mitsuhashi, Harada & Kameda, 1961; Watanabe & Fukasawa, 1961). We have found that R_{100} confers sensitivity to atabrine as well as other drugs, including nalidixic acid and several acridine dyes (Yoshikawa & Sevag, 1967; Yoshikawa, 1971), which raises the question of whether the apparent curing of this R factor may in reality result not from elimination of the R factor but from selective enrichment of the more acriflavine-resistant R⁻ segregants which have arisen spontaneously.

2. MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1 with their derivation and relevant genetic markers.

Drugs and media. Acridine dyes were purchased from Tokyo Kasei, Tokyo, and

chloramphenicol from Sankyo, Tokyo. Difco Penassay broth (PAB), pH 7·6, and EMB-lactose agar (Eiken, Tokyo) were used as the liquid and solid media, respectively. Dilutions of cultures were made in unbuffered 0·85% saline.

General experimental procedure. Overnight cultures of R^+ and R^- strains were appropriately diluted in PAB with or without acridine dyes in test-tubes, which were covered with aluminium foil to keep the cultures dark and incubated in a water-bath at 37 °C without aeration. Viable counts were made on EMB-lactose agar, and the numbers of R^- segregants were measured by replica-plating to EMB-agar with or without 25 μg/ml of chloramphenicol.

Table 1. *Bacterial strains used in experiments*

Abbreviations: *cys*$^-$, cysteine-requiring; *met*$^-$, methionine-requiring; *pro*$^-$, proline requiring; *str*^{r}, chromosomally determined streptomycin resistance; *mal*$^-$, maltose non-fermenting; *sul*, sulphonamide resistance (on R); *str*, streptomycin (on R); *cml*, chloramphenicol resistance (on R); *tet*, tetracycline resistance (on R).

Strain code	Relevant chromosome markers	Resistance pattern of the R factor	Derivation
Shigella flexneri 2b (R_{100})	*met*,$^-$ *trp*$^-$	(*sul*, *str*, *cml*, *tet*)	Naturally isolated R^+ strain, obtained from NIH of Japan
S. flexneri 2b	*met*$^-$, *trp*$^-$		Spontaneous R^- segregant
Salmonella typhimurium LT-2, Cys-36	*cys*$^-$, *str*^{r}		Obtained from NIH of Japan
S. typhimurium LT-2, Cys-36 (R_{100})	*cys*$^-$, *str*^{r}	(*sul*, *str*, *cml*, *tet*)	LT-2, Cys-36 strain carrying R_{100}
Escherichia coli CSH-2	F$^-$, *met*$^-$, *pro*$^-$		Obtained from Dr T. Watanabe
E. coli CSH-2 (R_{100})	F$^-$, *met*$^-$, *pro*$^-$	(*sul*, *str*, *cml*, *tet*)	CSH-2 strain carrying R_{100}
E. coli W-3630	F$^-$, Hfr_3^-, *mal*$^-$		Obtained from Dr Y. Hirota
E. coli W-3630 (R_{100})	F$^-$, Hfr_3^-, *mal*$^-$	(*sul*, *str*, *cml*, *tet*)	W-3630 strain carrying R_{100}

3. RESULTS

(i) *Growth rates of R^+ and R^- bacteria in acriflavine (AF) and population changes in a mixture of R^+ and R^- cells*

Strain W-3630(R_{100}) was chosen for this experiment since, over a period of 9 years study, no R^- segregants have ever been observed in this strain even after treatment with AF and penicillin screening. Overnight cultures of W-3630 and W-3630(R_{100}) in PAB were diluted 10^{-5}, and either 0·1 ml of the R^+ or the R^- strain or 0·05 ml of both strains were added to 5 ml PAB with or without 2·5 μg/ml of AF. Viable counts were made and the proportion of R^- bacteria in the mixture of R^+ and R^- cells measured at intervals during incubation.

R^+ and R^- bacteria grew at the same rate in the absence of AF, but AF reduced the growth rate of R^+ more than that of R^- bacteria (Fig. 1): the mean generation time of R^+ bacteria (36 min) in the presence of the drug was about 20% longer during the exponential growth phase than for R^- bacteria (30 min). Similar difference in generation time were consistently obtained with any inoculum size

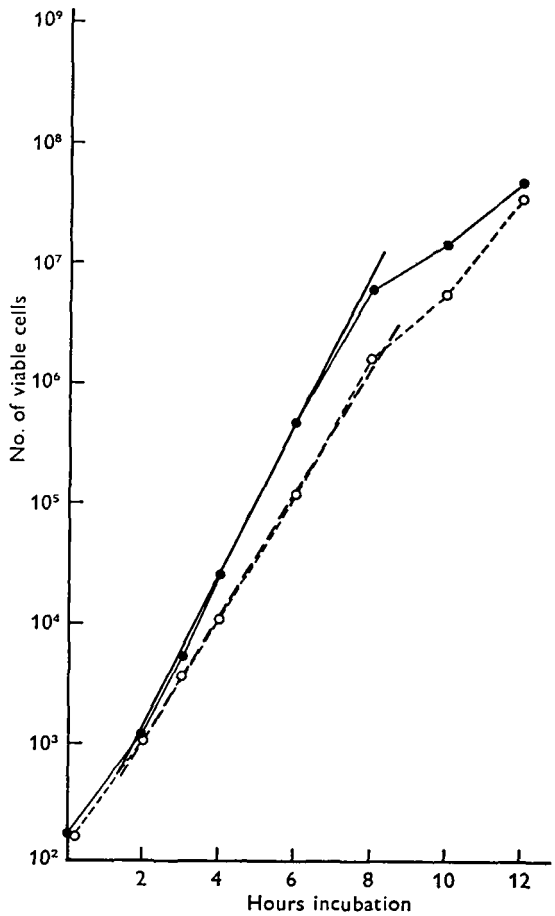

Fig. 1. Growth kinetics of W-3630 and W-3630 (R_{100}) in the presence of acriflavine. ——, W-3630 in PAB with 2·5 μ/ml AF; – – –, W-3630 (R_{100}) in the same medium; —— and – – – –, mean growth curves which correspond to the generation times of 30 and 36 min for R^- and R^+ cells, respectively.

and at any concentration of AF so far tested. Table 2 shows that the proportion of R^- bacteria in the mixed culture increased during the exponential growth phase in the presence of AF. The extent of this population change corresponds to that expected on the generation time of 30 min for R^- and 36 min for R^+ bacteria. During further incubation in AF the proportion of R^+ bacteria tended to rise again, probably due to an increased mutability of the host cells to AF resistance, conferred by the R factor (Yoshikawa, 1971).

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Table 2. *Changes in the percentage of R⁻ cells during growth of a mixture of W-3630 and W-3630(R_{100}) in the presence of AF*

These results were obtained by examining the chloramphenicol sensitivity of the colonies on the plates for the mixture of R⁻ and R⁺ cells and are comparable to Fig. 1, whose results were obtained by making viable counts of each of R⁻ and R⁺ cultures with AF starting simultaneously with the experiments on this Table using the same cultures.

Incubation (h)	No. of total viable cells/ml	R⁻ (%)	Calculated viable counts of R⁻	Calculated viable counts of R⁺
0	$2{\cdot}2 \times 10^2$	51	$1{\cdot}1 \times 10^2$	$1{\cdot}1 \times 10^2$
2	$1{\cdot}1 \times 10^3$	48	$5{\cdot}0 \times 10^2$	$5{\cdot}5 \times 10^2$
3	$4{\cdot}1 \times 10^3$	65	$2{\cdot}8 \times 10^3$	$1{\cdot}5 \times 10^3$
4	$1{\cdot}9 \times 10^4$	65	$1{\cdot}3 \times 10^4$	$6{\cdot}5 \times 10^3$
6	$2{\cdot}8 \times 10^5$	79	$2{\cdot}2 \times 10^5$	$6{\cdot}0 \times 10^4$
8	$4{\cdot}3 \times 10^6$	85	$3{\cdot}7 \times 10^6$	$6{\cdot}5 \times 10^5$
10	$1{\cdot}2 \times 10^6$	75	$9{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^6$
12	$4{\cdot}1 \times 10^7$	71	$2{\cdot}9 \times 10^7$	$1{\cdot}2 \times 10^7$

(ii) *Effect of growing the inoculum in chloramphenicol and correlation of 'curing' with the presence of R⁻ segregants in the inoculum*

R⁺ cells of most bacterial strains segregate R⁻ cells spontaneously. In experiments to isolate R⁻ cells by treatment with acridine dyes, cultures grown without any antibiotic have generally been used. In view of the finding given in the previous section the existence of spontaneous R⁻ segregants in the inoculum might be an important factor in obtaining R⁻ cells after acridine treatment. In order to test this possibility two experiments were performed, both of which were designed to compare the effect of acridine treatment when inocula were previously grown with or without chloramphenicol.

Single colony isolates of *Salmonella typhimurium* LT-2 Cys 36(R_{100}), *Shigella flexneri* 2b(R_{100}) and *Escherichia coli* CSH-2(R_{100}) from EMB plates containing 25 μg/ml chloramphenicol were serially subcultured 5 times in PAB with and without chloramphenicol. About 500 colonies of each strain from each medium were then inoculated into 5 ml of PAB containing 0, 1·25, 2·5 or 5 μg/ml AF. Viable counts were made after 12 and 24 h incubation and the colonies were examined for chloramphenicol sensitivity. Table 3 shows that the percentage of R⁻ cells was always higher when the inoculum had been grown without chloramphenicol. The frequency of 'curing' of the R factor was higher in *Salmonella typhimurium* than in *Escherichia coli* and *Shigella flexneri*, an effect which probably related to differences in the frequency of spontaneous loss of the R factor in the different hosts.

Salmonella typhimurium LT-2 Cys 36(R_{100}), which gave the highest spontaneous or acridine-induced 'curing' of R factors, was grown for 24 h with and without chloramphenicol and 0·05 ml of a 10^{-5} dilution of the culture was inoculated into 5 ml of PAB containing 0, 1·25, 2·5 or 5 μg/ml AF and incubated for 16 h. At the same time the proportion of R⁻ segregants in each inoculum was determined. The

results given in Table 4 show that the extent to which the R factor was eliminated in cultures with AF was correlated with the proportion of R^- bacteria in the inoculum.

Table 3. *The effect of the inoculum cells grown with or without chloramphenicol on curing of the R factor*

Three different R^+ strains were serially grown 5 times in the presence and absence of chloramphenicol and treated with AF. The numbers of viable cells were counted and the colonies examined for their chloramphenicol-sensitivity.

		Length of incubation (h)			
		12		24	
AF (μg/ml)	Pretreat. in CM*	No. of viable cells/ml	% CM^s cells†	No. of viable cells/ml	% CM^s cells†
		Salmonella typhimurium (R_{100})			
0	+	$2{\cdot}9 \times 10^8$	12	$9{\cdot}3 \times 10^8$	9
	−	$2{\cdot}8 \times 10^8$	44	$7{\cdot}9 \times 10^8$	34
1·25	+	$2{\cdot}7 \times 10^8$	13	$5{\cdot}9 \times 10^8$	6
	−	$2{\cdot}9 \times 10^8$	53	$7{\cdot}0 \times 10^8$	33
2·5	+	$2{\cdot}2 \times 10^8$	10	$5{\cdot}9 \times 10^8$	5
	−	$2{\cdot}4 \times 10^8$	51	$4{\cdot}9 \times 10^8$	36
5	+	$1{\cdot}3 \times 10^8$	14	$7{\cdot}5 \times 10^7$	20
	−	$1{\cdot}4 \times 10^8$	52	$3{\cdot}1 \times 10^8$	66
		Shigella flexneri (R_{100})			
0	+	$3{\cdot}5 \times 10^8$	0	$5{\cdot}1 \times 10^8$	0
	−	$3{\cdot}5 \times 10^8$	0	$4{\cdot}6 \times 10^8$	0
1·25	+	$2{\cdot}0 \times 10^7$	0	$3{\cdot}0 \times 10^8$	0
	−	$1{\cdot}9 \times 10^7$	3	$3{\cdot}1 \times 10^8$	0
2·5	+	$1{\cdot}1 \times 10^4$	0	$6{\cdot}5 \times 10^7$	2
	−	$6{\cdot}8 \times 10^4$	12	$1{\cdot}2 \times 10^8$	34
5	+	$< 5{\cdot}0 \times 10^2$	−	$< 5{\cdot}0 \times 10^2$	−
	−	$< 5{\cdot}0 \times 10^2$	−	$< 5{\cdot}0 \times 10^2$	−
		Escherichia coli (R_{100})			
0	+	$7{\cdot}2 \times 10^7$	0	$4{\cdot}5 \times 10^8$	0
	−	$8{\cdot}0 \times 10^7$	0	$4{\cdot}2 \times 10^8$	0
1·25	+	$3{\cdot}8 \times 10^7$	0	$1{\cdot}3 \times 10^8$	0
	−	$3{\cdot}6 \times 10^7$	0	$1{\cdot}3 \times 10^8$	0
2·5	+	$8{\cdot}0 \times 10^6$	3	$1{\cdot}0 \times 10^7$	8
	−	$8{\cdot}6 \times 10^6$	6	$1{\cdot}1 \times 10^7$	9
5	+	$1{\cdot}1 \times 10^5$	0	$1{\cdot}0 \times 10^6$	4
	−	$1{\cdot}2 \times 10^6$	1	$1{\cdot}2 \times 10^6$	6

One hundred colonies were scored to estimate the percentage of CM^s cells.

* +, With chloramphenicol; −, without chloramphenicol.

† CM^s indicates sensitivity to chloramphenicol.

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Table 4. *Evidence indicating correlation between 'curing' and the numbers of the R⁻cells pre-existing in the inoculum,* Salmonella typhimurium (R_{100})

Growth in CM for inocula	R⁻ in inocula (%)	AF treatment		
		AF (μg/ml)	No. of viable cells/ml	R⁻ (%)
+	0 (< 0·05)	0	$4·6 \times 10^8$	7·5
		1·25	$2·6 \times 10^8$	9
		2·5	$2·3 \times 10^8$	8
		5	$1·0 \times 10^8$	23
−	14	0	$4·1 \times 10^8$	45
		1·25	$3·7 \times 10^8$	52
		2·5	$3·4 \times 10^8$	55
		5	$2·4 \times 10^8$	66

Table 5. *Effect of the R⁻ cells mixedly inoculated with the R⁺ cells on the final increase of the fraction of the R⁻ cells*

Inoculum mixture			Grown without AF		Grown with 2·5 μg/ml AF		
R⁺	R⁻	R⁻ (%)	No. of viable cells/ml	R⁻ (%)	No. of viable cells/ml	R⁻ obtained (%)	R⁻ if no selection (%)
600	560	48	$7·6 \times 10^8$	56	$2·8 \times 10^7$	100	53
600	56	9	$3·7 \times 10^8$	5	$2·7 \times 10^7$	70	18
600	6	1	$4·0 \times 10^8$	2	$4·6 \times 10^6$	14	11
600	0	0	$4·3 \times 10^8$	0	$1·9 \times 10^6$	10	—

(iii) *Effect of mixing R⁻ with R⁺ cells*

Shigella flexneri 2b (R_{100}) and a spontaneous R⁻ segregant were grown with and without chloramphenicol, respectively. The *Shigella* strains were the most suitable for this experiment among three genera adopted in the previous section because they gave rise to a relatively high proportion of R⁻ segregants without accompanying cells carrying partially segregated R factors. About 500 R⁺ cells mixed with 500, 50, 5 or 0 R⁻ cells were inoculated into 5 ml of PAB with and without 2·5 μg/ml AF and incubated for 16 h, when viable counts were made and the colonies examined for chloramphenicol-sensitivity by replica-plating. The results are presented in Table 5, where the figures in the last column were calculated as follows. The expected percentage of R⁻ cells arising by elimination of the R factor will be 10%, as shown by the percentage of R⁻ cells present when no R⁻ cells were introduced in the inoculum. Thus, if the inoculum consisted of A % R⁻ cells and (100 − A) % R⁺ cells, the expected percentage of R⁻ cells after treatment with AF should be $A + 0·1\ (100 - A)$, if R⁻ cells had no selective advantage. However, a remarkable difference can be seen between the percentage calculated on this assumption and the percentage actually observed, indicating that selective enrichment of R⁻ cells contributed more markedly than any other possible curing mechanism to increase the proportion of R⁻ cells in the culture.

(iv) *Effect of other acridine dyes*

Escherichia coli CSH-2(R_{100}) and a mixture of *Escherichia coli* W-3630 and W-3630(R_{100}) in the proportion of 9 to 75 were tested in the usual way in 5 ml of PAB containing various concentrations of the dyes. Table 6 shows that all the dyes led to an increase in the percentage of R^- cells in both cultures, the former culture giving an index for 'curing' and the latter mixture for selective enrichment in the presence of acridine dyes.

Table 6. *Effect of various acridine dyes on curing (CSH-2(R_{100})) and on selective enrichment of the R^- cells (W-3630 + W-3630(R_{100}))*

		CSH-2 (R_{100})		W-3630 + W-3630 (R_{100})	
Addition	Concn. in μg/ml	No. of viable cells	R^- (%)	No. of viable cells	R^- (%)
None		$6{\cdot}2 \times 10^8$	0·37	$3{\cdot}3 \times 10^8$	13·0
Acriflavine	2·5	$3{\cdot}3 \times 10^7$	2·2	$2{\cdot}6 \times 10^8$	17·4
	5	$9{\cdot}0 \times 10^5$	13·5	$1{\cdot}0 \times 10^8$	25·4
	10	$< 5{\cdot}0 \times 10^0$	.	$2{\cdot}0 \times 10^6$	82·0
Acridine orange	2·5	$5{\cdot}8 \times 10^8$	1·2	$3{\cdot}2 \times 10^8$	17·0
	5	$3{\cdot}7 \times 10^8$	5·2	$1{\cdot}4 \times 10^8$	24·6
	10	$1{\cdot}6 \times 10^8$	6·6	$3{\cdot}5 \times 10^6$	42·6
Acrinol	1·25	$4{\cdot}3 \times 10^8$	3·0	$3{\cdot}4 \times 10^8$	15·1
	2·5	$3{\cdot}0 \times 10^8$	2·2	$3{\cdot}2 \times 10^8$	15·6
	5	$2{\cdot}7 \times 10^4$	1·9	$5{\cdot}7 \times 10^4$	0·3
Acridine red	2·5	$6{\cdot}0 \times 10^8$	1·6	$3{\cdot}0 \times 10^8$	19·6
	5	$6{\cdot}1 \times 10^8$	2·0	$2{\cdot}3 \times 10^8$	20·0
	10	$4{\cdot}0 \times 10^8$	0·51	$5{\cdot}2 \times 10^7$	30·6
Acridine yellow	2·5	$4{\cdot}2 \times 10^8$	1·9	$3{\cdot}3 \times 10^8$	12·3
	5	$2{\cdot}8 \times 10^8$	4·8	$2{\cdot}2 \times 10^8$	16·5
	10	$< 5{\cdot}0 \times 10^0$	.	$1{\cdot}4 \times 10^8$	23·9
Acridine	10	$5{\cdot}5 \times 10^8$	0·98	$3{\cdot}2 \times 10^8$	16·4
	20	$2{\cdot}7 \times 10^8$	1·6	$2{\cdot}9 \times 10^8$	16·0
	40	$2{\cdot}7 \times 10^8$	0·81	$2{\cdot}8 \times 10^8$	20·1

4. DISCUSSION

Some R factors have previously been shown to confer sensitivity to atabrine upon the host cells (Yoshikawa & Sevag, 1967). This observation raised the question of whether apparent curing of the R factor by acridine dyes may, contrary to the interpretation given by Mitsuhashi *et al.* (1961) and Watanabe & Fukasawa (1961), result from selective enrichment of the more acridine-resistant R^- segregants which have arisen spontaneously. The results of experiments performed to test this possibility may be summarized as follows.

The R factor, R_{100}, was found to confer increased sensitivity to acriflavine upon host cells. Thus, *E. coli* strain W-3630(R_{100}) has never segregated R^- cells even

after AF treatment; but, when a mixture of R^+ and R^- cells of this strain was grown in AF, the proportion of R^- cells increased.

Some other bacterial strains infected with R_{100} segregate R^- cells spontaneously. Growth of such R^+ strains in chloramphenicol (which inhibits growth of R^- cells) before testing with AF caused a marked reduction in the proportion of R^- cells found after AF treatment. The final proportion of R^- cells was then found to be correlated with the proportion immediately before AF treatment.

When a mixture of known proportions of R^+ and R^- cells was grown in AF, the proportion of R^- cells obtained was more than twice the proportion expected on the assumption that no selective enrichment of R^- cells had occurred. Other acridine dyes also led to an increase in the percentage of R^- cells, both in a mixture of R^+ and R^- cells and in an R^+ culture of a bacterial strain which spontaneously segregates R^- cells. It is concluded that the main, if not the only mechanism by which R_{100} is 'cured' by acridine dyes is through selective enrichment of R^- segregants which arise spontaneously.

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REFERENCES

HIROTA, Y. (1960). The effect of acridine dyes on mating type factors in *Escherichia coli*. *Proceedings of the National Academy of Sciences, U.S.A.* **46**, 57–64.

MITSUHASHI, S., HARADA, K. & KAMEDA, M. (1961). Elimination of transmissible drug-resistance by treatment with acriflavine. *Nature* **189**, 947.

WATANABE, T. & FUKASAWA, T. (1961). Episome-mediated transfer of drug resistance in Enterobacteriaceae. II. Elimination of resistance factors with acridine dyes. *Journal of Bacteriology* **81**, 679–683.

YOSHIKAWA, M. (1971). Drug sensitivity and mutability to drug resistance associated with the presence of the R factor. *Genetical Research* **17**, 1–7.

YOSHIKAWA, M. & SEVAG, M. G. (1967). Sensitivity of *Escherichia coli* to atabrine conferred by R factor and its potential clinical significance. *Journal of Bacteriology* **93**, 245–253.