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Screening Method of Agents Against the R Factor by the Use of an Hfr Made by Integrative Suppression with an R Factor

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By the use of an Hfr strain made by integrative suppression of a derepressed R factor, R100-1 in a *dnaA*-ts mutant of *Escherichia coli* and several other appropriate control strains with and without the R factor, a screening system was established which can be used to detect agents which are either inhibitory to the replication of the R factor and lead to its "curing" or are preferentially inhibitory to the growth of the R-carrying bacteria. The validity of this system was shown with several agents already known to be experimentally effective.

Drug resistance in *Enterobacteriaceae* and in some other families is determined by extrachromosomal deoxyribonucleic acid (DNA) elements termed R factors. These may be contagiously transferred to drug-sensitive bacteria and thus increase the incidence of drug-resistant bacteria. The increase of drug-resistant bacteria due to this mechanism has been a grave obstacle in chemotherapy. Extensive efforts have been made to obtain agents active against the R factor. Agents have been reported which eliminate R factors (3, 4, 7), preferentially kill those bacterial cells carrying them (5, 8, 10-12), and inhibit their transfer (1, 2, 9). However, none of these has yet been recognized as valuable for clinical application. An easy screening system for these anti-R factor agents is urgently required.

Nishimura et al. (6) reported that an F^+ strain, with a temperature-sensitive mutation in the chromosomal gene (*dnaA*) responsible for the initiation of chromosomal replication, produced temperature-resistant revertants through integration of the F factor into the chromosome. Many of these revertants were shown to be Hfr. They interpreted this phenomenon as showing that the defective chromosome has become part of the F replicon and replicates under F control at the nonpermissive temperature. They termed this phenomenon as integrative suppression. It was also shown that agents such as acridine orange inhibitory to F factor replication were also inhibitory to the growth of this type of Hfr strain at nonpermissive temperature, confirming the idea that the replication of the host chromosome is under F control at nonpermissive temperature.

In order to extend this phenomenon to R factors, an Hfr strain was made by integration of the R factor, R100-1, and characterized (M. Yoshikawa, submitted for publication). When this Hfr and other appropriate control strains are grown at permissive and nonpermissive temperatures in the presence of an agent to be tested, it is to be expected that the growth of the Hfr strain will be inhibited at nonpermissive, but not at permissive, temperatures, if this agent inhibits the replication of the R factor. Growth of all strains carrying the R factor would be inhibited by the agents irrespective of the growth temperature if this agent preferentially kills bacteria carrying the R factor.

In this communication we examine the effect of agents known to be inhibitory to the replication of the R factor or to the growth of the R-carrying bacteria and confirm the mode of action of these agents. By making these examinations, the validity of this system as a screening method will be shown.

MATERIALS AND METHODS

Bacterial strains used. Substrains of *Escherichia coli* K-12, CR34 (F^-, *thi, thr, leu, thy, lac*, λ^-), its derivative to which has been transferred a derepressed mutant of the R factor, R100 (*fi*$^+$, *sul, str, cml, tet*) (gene markers used for drug resistance were: *sul* for sulfonamide, *str* for streptomycin, *cml* for chloramphenicol, and *tet* for tetracycline), designated as R100-1, CRT46 (F^-, *thi, thr, leu, ilv, thy, lac, mal*, λ^-) carrying a thermosensitive mutation of a chromosome gene, *dnaA* and its derivative carrying R100-1 were kindly given by Y. Nishimura. From the latter strain, an Hfr strain designated Hfr (R100-1)#2 was obtained and characterized (M. Yoshikawa, submitted for publication).

Media and chemicals. Penassay broth (Difco) supplemented with 10 μg of thymine per ml and adjusted pH to 7.6 with 1 N NaOH was used as the growth medium (PAB). For agar plates, PAB was solidified with 1.5% powdered agar. The acridine dyes were purchased from Tokyo Kasei, Tokyo. Ethidium bromide, macarbomycin, and flavomycin were kind gifts from the manufacturers.

Tests on PAB agar plates. Each strain was inoculated into PAB and grown overnight at 30 C with shaking, and 0.02-ml samples were spotted on two PAB agar plates containing various concentrations of agents to be tested. One of them was incubated at 30 C and the other at 42 C. After overnight incubation, the growth was scored.

Tests in liquid PAB. Overnight cultures in PAB grown at 30 C were centrifuged, and cells were resuspended in fresh PAB to give an OD of 0.6 with a Bausch & Lomb, Inc. spectrophotometer at 600 nm. A 0.1-ml portion was inoculated into 5 ml of PAB with and without added agents to be tested. These tubes were incubated without shaking at 30 and 42 C for 18 h, and the growth was scored by the OD reading with a Bausch & Lomb, Inc. spectrophotometer at 600 nm.

RESULTS AND DISCUSSION

Tests on PAB agar plates. Five strains, CR34, its R100-1$^+$ derivative, a *dnaA*-ts mutant, its R100-1$^+$ derivative, and Hfr (R100-1)#2 (with R100-1 inserted into the host chromosome between 81 and 90 min on the standard map [M. Yoshikawa, submitted for publication]) were examined by tests on PAB agar plates with and without agents to be tested at 30 and 42 C. The results are shown in Table 1. Acriflavine, atabrine, and ethidium bromide were all more inhibitory to Hfr (R100-1)#2 than to CR34 at 42 C, but not at 30 C. This might be due to their effect on the integrated R factor which controls the replication of the host chromosome at 42 C. Because the replication of the host chromosome is not under R control at 30 C, these agents inhibited growth of the Hfr and CR34 to almost the same extent. As we have already reported (11, 12), acridine dyes, including atabrine, were more inhibitory to the R$^+$ bacteria than to the R$^-$ corresponding strains, irrespective of temperature. However, this effect could not markedly be observed by the present method.

Tests in liquid PAB. The agar plate method is not suitable for screening of the agents inhibitory to the replication of the R factor, because these strains gave different extent of growth even at permissive temperature and without any of these inhibitory agents. For more objective interpretations of the results it is necessary to normalize the results for this effect. To judge the effect of the agent for the replication of the R factor, growth was expressed by the OD reading in liquid PAB and normalized by

TABLE 1. *Penassay broth agar plate tests*[a]

Agents added	Concn (μg/ml)	Strains				
		CR-34	CR-34 R100-1	CRT-46	CRT-46 R100-1	Hfr (R100-1) #2
Acriflavine	0	+/+	+/+	+/−	+/−	+/+
	6.25	+/+	+/+	+/−	+/−	+/+
	12.5	+/+	+/+	+/−	+/−	+/+
	25	+/+	+/+	+/−	+/−	+/−
	50	+/+	+/+	+/−	+/−	+/−
	100	+/+	+/+	+/−	+/−	+/−
Atabrine	0	+/+	+/+	+/−	+/−	+/+
	50	+/+	+/+	+/−	+/−	+/−
	100	+/+	+/+	+/−	+/−	+/−
	200	+/+	+/+	+/−	+/−	+/−
Ethidium bromide	0	+/+	+/+	+/−	+/−	+/+
	4	+/+	+/+	+/−	+/−	+/−
	8	+/+	+/+	+/−	+/−	+/−
	16	+/+	+/+	+/−	+/−	+/−
	32	+/+	+/+	+/−	+/−	+/−
	64	+/+	+/+	+/−	+/−	+/−
	128	+/+	+/+	+/−	+/−	+/−

[a] Overnight cultures of the five strains indicated were spotted in duplicate on agar plates containing various concentrations of agents, and one was incubated at 30 C and the other at 42 C. A + indicates positive growth and a − indicates negative growth. Nominator and denominator were for the results at 30 and 42 C, respectively.

calculating the ratio: OD at 600 nm with agent at 42 C × OD at 600 nm without agent at 30 C divided by OD at 600 nm without agent at 42 C × OD at 600 nm with agent at 30 C.

Figure 1A shows the OD values of cultures grown overnight at 30 and 42 C in the presence and absence of various concentration of acriflavine. The strain, Hfr (R100-1) #2, was markedly inhibited at 42 C in the presence of acriflavine in contrast to its growth at 30 C and to that of CR34 both at 30 and 42 C. This effect can be observed in Fig. 1B, which shows the ratios calculated as described above with the use of the results obtained in Fig. 1A. The ratios were constantly 1.0 for CR34, but markedly lower than 1.0 for the Hfr. Figure 2 shows the results of similar experiments using acridine yellow, acridine orange, acridine red, acrinol, atabrine, and ethidium bromide. All of these agents were shown to be inhibitory for replication of the R

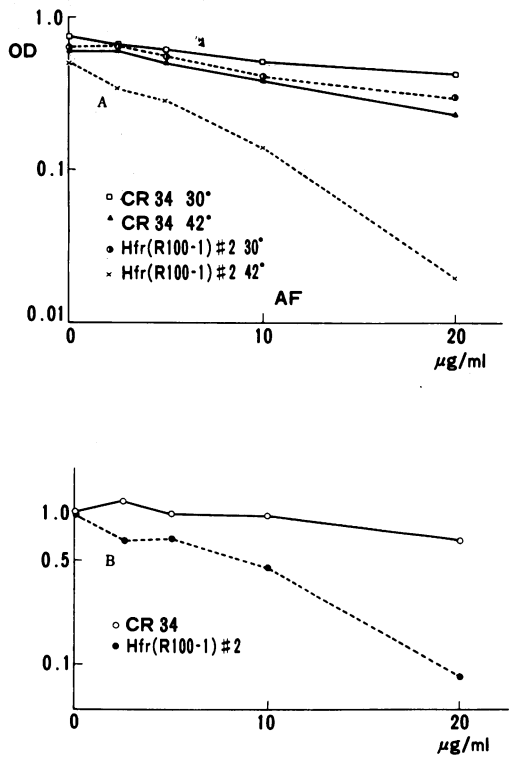

FIG. 1. *Growth of Hfr(R100-1)#2 and other control strains in the presence of acriflavine. (A) Growth was expressed after overnight incubation by an OD reading at 600 nm. (B) Growth was expressed after overnight incubation by the ratio: OD with acriflavine at 42 C × OD without acriflavine at 30 C divided by OD without acriflavine at 42 C × OD with acriflavine at 30 C.*

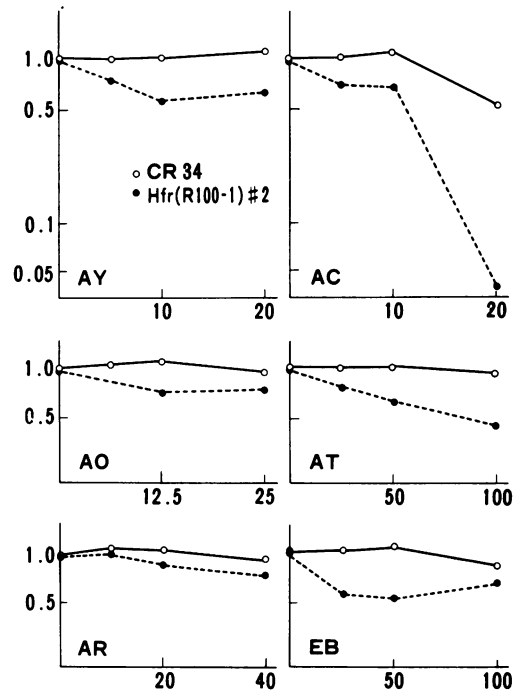

FIG. 2. *Growth of Hfr (R100-1)#2 and CR34 in the presence of various agents. Growth was expressed by the ratio described in the text and in the legend for Fig. 1. The abscissa and the ordinate were the concentration of the agents in micrograms per milliliter and the OD reading, respectively. Abbreviations for agents: AY for acridine yellow, AO for acridine orange, AR for acridine red, AC for acrinol, AT for atabrine and EB for ethidium bromide.*

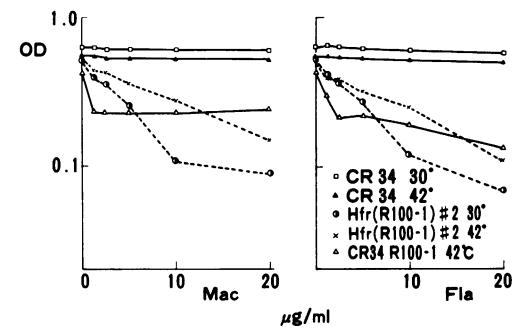

FIG. 3. *Growth of Hfr (R100-1)#2 and other control strains in the presence of macarbomycin (Mac) and flavomycin (Fla). Growth was expressed by OD reading at 600 nm.*

factor. This method should thus be applicable for screening of agents with similar modes of action.

Figure 3 is the results of similar experiments

using macarbomycin (5) and flavomycin (8), which are known to be preferentially inhibitory against bacteria carrying an R factor or an F factor. Both of these agents showed marked inhibition against the growth of bacteria carrying the R factor under the autonomous as well as the integrated state, whereas they were almost noninhibitory against bacteria without the R factor at the concentrations indicated. Thus, the effect of these agents against R-carrying bacteria was confirmed, and hence this method might be also useful for screening of this type of agent. Expression of the results by ratio was not suitable for this type of agent. The ratios were higher than 1.0 for the Hfr, suggesting that the effect of the presence of the R factor is more marked at 30 than at 42 C.

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