PREFERENTIAL INHIBITION OF THE GROWTH OF *ESCHERICHIA COLI* STRAINS CARRYING EPISOMES

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During the screening studies of antibacterial agents against *Escherichia coli* strains harboring episomes, namely F-*lac*-*tet*, R100, and T-*kan*, it was found that the strains carrying these episomes were more sensitive to macarbomycin than their original strains without episomes.

As described by MITSUHASHI[1], R factors have become wide-spread among most genera of *Enterobacteriaceae*, which are isolated from humans and livestock all over the world. R factors carrying drug-resistance against tetracycline (TC), chloramphenicol (CM), streptomycin (SM), sulfanilamide (SA), and their combinations[2,6] have been frequently observed. Furthermore, those carrying resistance to ampicillin (AB-PC)[7] or to kanamycin (KM)[8], or to both in addition to the aforementioned 4 drugs were recently demonstrated. Thus, studies of R factors which are transferred by conjugation have great importance in clinical chemotherapy.

Moreover, it was found that R factors were lost spontaneously from their host strains during storage of these strains in cooked meat media[9] and were eliminated artificially by treating such strains with acridine dyes[10~12]. It was also reported that sensitivity of *E. coli* strains to atabrine was enhanced by the presence of an R factor[13]. On the basis of these facts, we started screening studies of antibacterial agents, which would act selectively on bacterial strains carrying episomes including R factor. This paper deals with the effect of macarbomycin[14] exhibiting preferential inhibition against *E. coli* strains carrying episomes such as F, R, and T[15] factors.

Materials and Methods

Antibiotics: Chloramphenicol, tetracycline and kanamycin were used at the concentrations of 25, 50 and 50 mcg/ml. The properties of macarbomycin have been described by UMEZAWA in another paper[14]. A sample of macarbomycin (4,000 units/mg) was supplied by Dr. K. MAEDA, National Institute of Health, Tokyo.

Host strains and episomes: *Escherichia coli* K12 W3630 was used as host cells for various episomes. Episomes used were R100, T-*kan*, and F-*lac*-*tet* factors. R100 factor was originally isolated by NAKAYA and carries resistance to four drugs; tetracycline, chloramphenicol, streptomycin and sulphonamides[16]. F_{13} factor was isolated by HIROTA[17] and it transfers with high frequency *lac*$^+$, *pur*$^+$ and *pho*$^+$ (ability to synthesize

alkaline phosphatase). F-*lac-tet* factor is a recombinant between F_{13} factor and tet_{23} determinant[18]. The non-transferable tet_{23} determinant is responsible for resistance to tetracycline (TC) and was derived from R_{10} (TC·CM·SM·SA) factor by transduction with bacteriophage epsilon[19]. It is known that non-infectious drug-resistance determinants acquire conjugal transmissibility by the formation of recombinants with T (transfer) factors. T-*kan* factor is a recombinant between T_{95} factor and non-infectious kanamycin (KM)-resistance determinant[15,20].

Media: Brain heart infusion broth (BHI, Difco) and EMB-lactose agar[21] were used. Soft EMB-lactose agar was the same as EMB-lactose agar except that the concentration of agar was a half.

Replica plating method: LEDERBERG's method was employed[22].

Identification of the bacteria carrying episomes: For the identification of R100, T-*kan*, and F-*lac-tet* factors, the conjugal transmissibilities of the drug resistance in each episome, namely the transmissibilities of CM-, KM-, and TC-resistance, were examined respectively.

Viable count of bacteria: Bacterial culture was diluted with saline, and 0.1 ml was mixed with 2 ml of soft EMB-lactose agar and overlayed on EMB-lactose agar plate. After overnight cultivation, the colonies which appeared were counted.

Bacterial growth was determined photometrically every 5 minutes by using Biophotometer (Bonet-Maury and Jouan Co., Ltd., France).

Results

The strains carrying episomes were tested for sensitivity to macarbomycin compared with the original strains without the episomes. *E. coli* K12 W3630 and a strain carrying an episome were mixed in the ratio of 1 : 10. If the strains carrying the episomes were more sensitive to macarbomycin than the original strains, it would be expected that the percentage of the drug-sensitive cells in the mixed culture would increase during growth in the presence of macarbomycin compared with growth in the absence of the antibiotic. As shown in Table 1, the percentage of drug-sensitive cells in the mixed culture after overnight cultivation was 2~16 % in the absence of macarbomycin but increased to about 50~99 % in the presence of 10 or 20 mcg/ml of macarbomycin.

From these results it was assumed that macarbomycin inhibited the growth of cells harboring episomes more markedly than cells without an episome. The growth

Table 1. Effect of macarbomycin on the growth of bacteria carrying episomes

Bacterial strains contained in the mixed culture	Macarbomycin added					
	0 μg/ml		10 μg/ml		20 μg/ml	
	Growth of the mixed culture	Sensitive cells (%)*	Growth of the mixed culture	Sensitive cells (%)*	Growth of the mixed culture	Sensitive cells (%)*
W3630+W3630 R100+	+++	16	++	55	+	99
W3630+W3630 T-*kan*+	+++	7	++	86	+	98
W3630+W3630 F-*lac-tet*+	+++	2	++	77	+	99

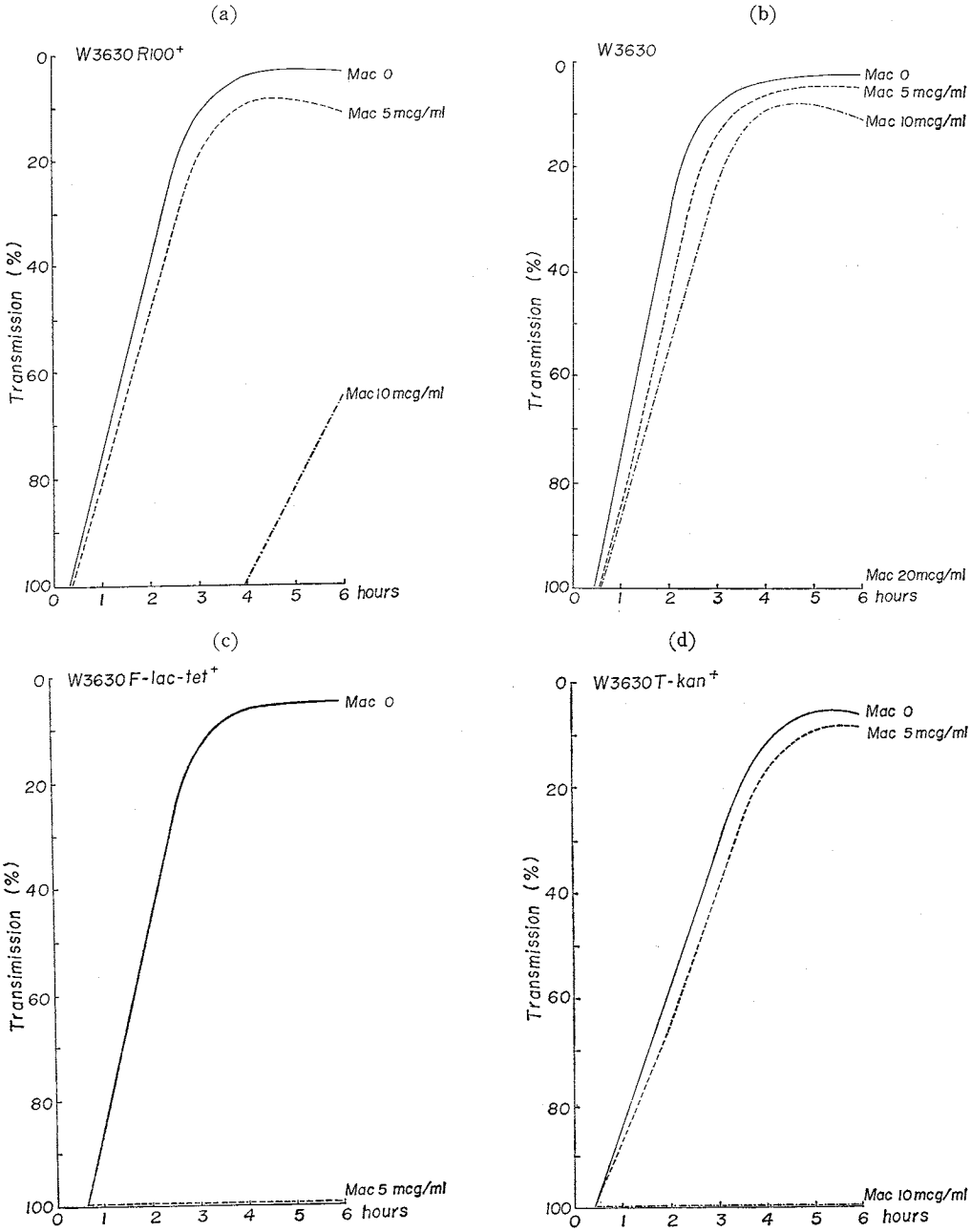
Drug-sensitive bacteria and those carrying an episome were mixed in the ratio 1 : 10, and were inoculated in BHI broth with or without macarbomycin. After an overnight incubation at 37℃, the growth level of the mixed culture was examined, and the ratio of drug-sensitive to total cells was assayed by the replica plating method as described.

Growth: +++, 10^8-10^9; ++, 10^7-10^8; +, 10^6-10^7 cells per ml.

* The ratio of drug-sensitive cells to total cells in %.

Fig. 1. Effect of macarbomycin on the growth of *E. coli* strains carrying episomes.

One ml of overnight culture of bacteria in BHI broth was added to 9 ml of BHI broth shaken at 37℃ until the optical density at 660 mμ of the culture became 0.7, 0.62, 0.7, and 0.65, in the case of W3630 R100$^+$ (1a), W3630 T-*kan*$^+$ (1b), W3630 F-*lac-tet*$^+$ (1c), and W3630 (1d), respectively. These values corresponded to about 5×10^8/ml viable cells. A 0.1 ml sample of each culture was withdrawn and added to 10 ml of BHI broth with or without macarbomycin, and the growth curve was examined with Biophotometer (Bonet-Maury and Jouan, France).

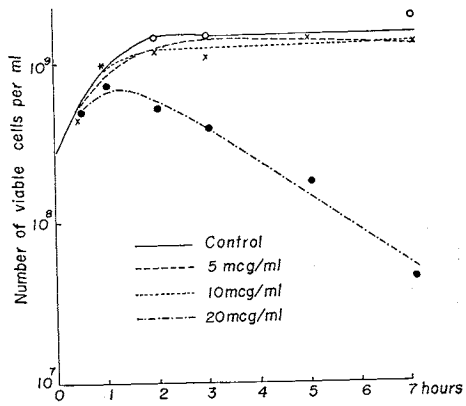


curves of each strain in the presence of various concentrations of macarbomycin are shown in Fig. 1. The growth of W3630 was completely inhibited by 20 mcg/ml of macarbomycin but only slightly inhibited by 5 or 10 mcg/ml under the conditions

employed. In contrast, the growth of W3630 F-*lac-tet*$^+$ was completely inhibited even by the addition of 5 mcg/ml of macarbomycin and with strains W3630 R100$^+$ and W3630 T-*kan*$^+$, 20 and 10 mcg/ml caused complete inhibition of growth, and 5 mcg per ml of macarbomycin resulted in slight inhibition. Thus, it was found that growth of the strains harboring episomes was more sensitive to macarbomycin than was growth of the original strain without an episome, and the inhibitory effect of macarbomycin on growth of W3630 harboring episomes decreased in the following order: F-*lac-tet*, T-*kan*, and R100.

Fig. 2. Effect of macarbomycin on the viability of W3630 R100$^+$

E. coli W3630 R100$^+$ was grown in BHI broth with shaking at 37°C. When the number of bacteria reached 2.8×10^8 per ml, macarbomycin was added to the culture and the number of viable cells was then counted.



The effect of marcarbomycin on the viability of W3630 R100$^+$ was also examined. As shown in Fig. 2, the number of viable cells of W3630 R100$^+$ was decreased by the addition of 20 mcg/ml of macarbomycin after about 90 minutes lag, but the addition of either 5 or 10 mcg/ml did not show any effect on the viability of W3630 R100$^+$. These results are accounted for by the facts that macarbomycin has two effects on W3630 carrying episomes, namely a killing effect and an inhibitory effect on growth.

Discussion

Macarbomycin has been reported to inhibit the cell wall synthesis of *Staphylococcus aureus*[23]. It is known that *E. coli* carrying either F or R factor produces f$^+$ [24] or r$^+$ [25,26] antigen respectively, which is not detected in the strain without such episomes. Furthermore it has been found that the f$^+$ antigen of *E. coli* F$^+$ is due to F pili which were produced on the surface of F$^+$ bacteria[27]. The strains of *E. coli* carrying R factor have also been found to produce R pili similar to F pili, and the pili formation has been considered to participate in the conjugal transfer of either episome or the host chromosome[28,29]. It was reported from this laboratory that T factors have many genetic properties similar to R factors, although the detailed studies still await clarification[20]. Considering these facts, the preferential effect of macarbomycin on the bacterial strains carrying episomes may be explained by its effect on a specific material on the cell surface of bacteria carrying episomes. The detailed studies will be described elsewhere.

References

1) Mitsuhashi, S.: Epidemiology of R factors. *In* Transferable drug-resistance factor R. Tokyo University Press, Tokyo, 1970
2) Ochiai, K.; K. Yamanaka, K. Kimura & O. Sawada: Studies on inheritance of drug-resistance between *Shigella* and *Escherichia coli* strains. (in Japanese) Jap. Med. J. No. 1861 : 34~46, 1959
3) Akiba, T.; T. Koyama, Y. Isshiki, S. Kimura & T. Fukushima: Studies on the mechanism of development of multiple drug-resistant *Shigella* strains. (in Japanese) Jap. Med. J. No. 1886 : 45~50, 1960

4) MITSUHASHI, S.; H. HASHIMOTO, K. HARADA, R. EGAWA & T. MATSUYAMA: Studies on the drug-resistance of enteric bacteria. 7. Type of the transmissible drug-resistance factor. Gunma J. Med. Sci. 10 : 59~66, 1961
5) KIMURA, S.; T. MIZUNO, T. AKIBA & T. KOYAMA: New types of R factor. Med. Biol. 63 : 109~112, 1962
6) OMORI, G.; A. YOSHIMURA & S. IIDA: Transmissibility of naturally developed sulfonamide resistance in *Shigella*. Biken J. 5 : 87~98, 1962
7) ANDERSON, E. S. & N. DATTA: Resistance to penicillins and its transfer in *Enterobacteriaceae*. Lancet 1965-1 : 407~409, 1965
8) LEBEK, G: Ueber die Entstehung mehrfachresistenter Salmonellen. Ein experimenteller Beitrag. Zentralbl. Bakt. 188 : 494~505, 1963
9) MITSUHASHI, S.; H. HASHIMOTO, K. HARADA, M. SUZUKI, M. KAMEDA & T. MATSUYAMA: Multiple resistance of *S. flexneri* 3a and *E. coli* isolated from the epidemic in Gunma Prefecture. Japan. J. Bact. 15 : 844~848, 1960
10) MITSUHASHI, S.; K. HARADA & M. KAMEDA: Drug resistance of enteric bacteria. Elimination of transmissible drug-resistance by treatment with acriflavine. (in Japanese) Tokyo Med. J. 77 : 462, 1960
11) MITSUHASHI, S.; K. HARADA & M. KAMEDA: Elimination of transmissible drug-resistance by treatment with acriflavine. Nature 189 : 947, 1961
12) WATANABE, T. & T. FUKASAWA: Elimination of resistance factors with acridine dyes. J. Bact. 81 : 679~683, 1961
13) YOSHIKAWA, M. & M. G. SEVAG: Sensitivity of *Escherichia coli* to Atabrine conferred by R factor and its potential significance. J. Bact. 93 : 245~253, 1967
14) TAKAHASHI, S.; A. OKANISHI, R. UTAHARA, K. NITTA, K. MAEDA & H. UMEZAWA: Macarbomycin, a new antibiotic containing phosphorus. J. Antibiotics 23 : 48~50, 1970
15) MITSUHASHI, S.; M. KAMEDA, K. HARADA & M. SUZUKI: Formation of recombinants between nontransmissible drug-resistance determinants and transfer factors. J. Bact. 97 : 1520~1521, 1969
16) NAKAYA, R.; A. NAKAMURA & T. MURATA: Resistance transfer agents in *Shigella*. Biochem. Biophys. Res. Comm. 3 : 654~659, 1960
17) HIROTA, Y.: Mutants of the F-factor in *Escherichia coli* K12. Records Genetic Soc. Am. 28 : 75, 1959
18) HARADA, K.; M. KAMEDA, M. SUZUKI & S. MITSUHASHI: Drug-resistance of enteric bacteria. III. Acquisition of transferability of non-transmissible R(TC) factor in co-operation with F factor and formation of FR(TC). J. Bact. 88 : 1257~1265, 1964
19) HARADA, K.; M. KAMEDA, M. SUZUKI & S. MITSUHASHI: Drug resistance of enteric bacteria. II. Transduction of transmissible drug-resistance (R) factors with phage epsilon. J. Bact. 86 : 1332~1338, 1963
20) KAMEDA, M.; K. HARADA, M. SUZUKI & S. MITSUHASHI: Formation of transferable drug-resistance factor by recombination between resistance determinants and transfer factors. Japan. J. Microbiol. 13 : 255~262, 1969
21) LEDERBERG, J.: Isolation and characterization of biochemical mutants of bacteria. Methods Med. Res. 3 : 5~22, 1950
22) LEDERBERG, J. & E. M. LEDERBERG: Replica plating and indirect selection of bacterial mutants. J. Bact. 63 : 399~406, 1952.
23) SUZUKI, S.; M. HORI & H. UMEZAWA: Mode of action of macarbomycin. Abst. of the 42 nd General Assembly of Japan Biochem. Soc. p. 507, Oct. 6~9, 1969 (Hiroshima)
24) ØRSKOV, I. & F. ØRSKOV: An antigen termed f^+ occurring in F^+ coli strains. Acta Pathol. Microbiol. Scand. 48 : 37~46, 1960
25) HIROTA, Y.; Y. NISHIMURA, J. ØRSKOV & I. ØRSKOV: Effect of drug-resistance factor R on the F properties of *Escherichia coli*. J. Bact. 87 : 341~351, 1964
26) SEKIJIMA, Y. & S. ISEKI: On the r^+ antigens of bacteria having drug resistance transfer factor R. Proc. Japan Academy 42 : 984~988, 1966
27) ISHIBASHI, M.: F pilis as f^+ antigen. J. Bact. 93 : 379~389, 1967
28) NISHIMURA, Y.; M. ISHIBASHI, E. MEYNELL & Y. HIROTA: Specific piliation directed by a fertility factor and a resistance factor of *Escherichia coli*. J. Gen. Microbiol. 49 : 89~98, 1967
29) MEYNELL, E.; G. G. MEYNELL & N. DATTA: Phylogenetic relationships of drug-resistance factors and other transmissible bacterial plasmids. Bacteriol. Rev. 32 : 55~83, 1968