5−15. −5) 佐々学(1955)：日本の蚊．DDT協会，東京．−6）山口左仲他（1956）：岡山市に於ける1955年日本脳炎流行と蚊の燈火採集成績について．衛生動物，7(2)：94−95．−7 七村 清（1968）：日本における衛生上重要な蚊の分布と生態．衛生動物，19(1)：15−34

Summary

The seasonal occurrence of mosquitoes collected by a light-trap was studied in Yamanashi Prefecture, during the period from July to December 1967. The total number of mosquitoes were 2,266 belonging to 6 species of 3 genera. The majorities of the specimens collected were 1,072 *Culex pipiens* complex (47.3%), 777 *C. tritaeniorhynchus* (34.3%) and 398 *Anopheles hyrcanus sinensis* (17.6%).

Fifteen cases of Japanese encephalitis were recorded in Yamanashi Prefecture in 1967. The peak of the outbreak was observed in the late of August which was 3 weeks later than the highest prevalence of *C. tritaeniorhynchus*.

（本論文は衛生動物19巻2号に掲載）

3. 細 菌 血 清 科

1) Recombination between a Thermosensitive kanamycin Resistance Factor and Nonthermosensitive Multiple Drug Resistance Factor

TAKESHI YOKOTA, YOSHIRO KANAMARU, REIKO MORI AND TOMOICHIRO AKIBA

Yamanashi Prefectural Institute of Public Health, Kofu and Research Loboratories of Chugai Pharmaceutical Co. Ltd., Tokyo, Japan

Abstract

When the thermosensitive kanamycin resistance factor, designated as R(KM)^t, discovered by Terawaki et al. (18), and a nonthermosensitive multiple drug resistance factor, named by R_{100}, were simultaneously introduced into a host cell of *E. coli* or *S. typhimurium*, the temperature sensitivities of the both R factors remained unchanged as long as they replicated independently. Under certain conditions, however, a new thermosensitive R factor harbouring resistance markers for kanamycin (KM), streptomycin (SM) and sulfanilamide (SA) was obtained by recombination between the R(KM)^t and R_{100} factors. Furthermore, R factors carrying resistance markers for KM and SA only, or SM and SA were obtained from the recombinant, R(KM. SA. SM)^t, by spontaneous segregation.

Though the R_{100} factor has been known as an fi^+ (positive for F- mediated fertility inhibition of its host) type and this R factor does not restrict any coexisting phages as far as examined, the thermosensitive recombinants of it with the R(KM)^t and their segregants were found to be fi^- and restrict the replication of all T-even phages, as well as the R(KM)^t factor.

No double infection immunity was observed between the R(KM)^t and R_{100} factors.

Introduction

Since Akiba et al. (2) first reported an extrachromosomal, transferable drug resistance factor (R factor)

in *Enterobacteriaceae*, many studies on the R factor have been performed. It is now known that bacterial R factors carry resistance to many drugs including the following : kanamycin(KM), aminobenzyl penicillin(3), spectinomycin and gentamycin(20) as well as streptomycin(SM), chloramphenicol(CM), tetracycline(TC) and sulfonamide(SA) resistnce. (10, 12)

It is also established that R factors can be transmitted from one strain to another by conjugation among all species of *Enterobacteriaceae*(7), *Pseudomonas aeruginosa*(12), some species of *Pasteurella*(6), *Vibrio comma*, *Vibrio eltor*(11), nonagglutinable vibrio and *Aeromonas hydrophila*(1).

From the clinical view point, bacterial drug resistance due to R factors has been considered to play an important role in infections of not only the gastro-intestinal tract but also the urinary tract(17).

Terawaki, Takayasu and Akiba(15) recently discovered, in a clinically isolated strain of *Proteus vulgaris*, a kanamycin resistance factor, R(KM)^t, whose replication is thermosensitive. That was the first report concerning the temperature sensitivity of an R factor and also the first report of an R factor carrying only KM resistance.

Another type of temperature sensitive drug resistance due to an R factor has been reported in 1967 by Mise and Suzuki(14). They treated a multiple drug resistance factor with nitrosoguanidine and induced a mutant producing a temperature sensitive CM- acetylase. It has been concluded that the R(KM)^t factor is completely different in genetic nature from that of Mise and Suzuki, since the former R factor can be never recovered from the host bacteria sensitized at higher temperatures, while the cells carrying the latter R factor change to sensitive to CM at higher temperatures but return to resistant if cultured again at lower tempratures.

This paper deals with the behaviors of thermosensitive and nontermosensitive R factors which have been simultaneously introduced into bacterial cells.

Material and Method

Media. Penassay broth (Difco) was used as the liquid medium throughout the experiments. Mac Conkey agar (Eiken, Japan) and minimal eosin methyleneblue agar (the EMS medium of Lederberg(13)) containing either 1% lactose or 1% maltose as the sole carbon source were employed as plating media. In cases of selecting certain auxotrophic bacteria, the EMS agar was supplemented with appropriate vitamins or amino acids.

Microorganisms. *E. coli* JE948 carrying the R_{100} factor and *E. coli* YA2 having the R(KM)^t factor were employed as initial donors of the respective R factors. The former strain was bestowed from Dr. Yoshikawa(24) and the latter was given by Dr. Terawaki(18). *E. coli* W3630, *E. coli* YA1, *E. coli* CSH2, *E. coli* YA10, *E. coli* YA11 and *Salmonella typhimurium* LT2 were used as original recipient strains of the R factors. *E. coli* YA5 and YA6 were derived from *E. coli* W3630 and *E. coli* JE948 by receiving the R(KM)^t factor from *E. coli* YA2 by conjugation, respectively. *E. coli* YA3 and YA4 were substrains of *E. coli* YA1, which had been infected by the R_{100} factor from *E. coli* JE948. *Salmonella typhimurium* YA7 and YA9 were induced from *S. typhimurium* LT2 by inheriting R(KM)^t from *E. coli* YA2 and R_{100} from *E. coli* JE948, respectively. The phage T4b was presented from Dr. Terawaki, and phages T2h, T4d and T6 were given by Dr. Uchida

Drugs. Kanamycin sulfate (Takeda pharm. Co. Ltd., Osaka, Japan), dihydrostreptomycin sulfate(Takeda), tetracycline hydrochloride (Japan Lederly, Tokyo, Japan), chloramphenicol (Yamanouchi Pharm. Co. Ltd., Tokyo, Japan) and sulfisoxazole (Yamanouchi) were employed and abbreviated as KM, SM, TC, CM and SA, respectively

Method for testing the temperature sensitive replication of R factors. Bacterial strains bearing R factors were purified by repeating 3 times single colony isolations on MacConkey plates containing 50 μg/ml of KM only or KM and 25 μg/ml each of CM, TC, or SM, or 1,000 μg/ml of SA. Then the

TABLE 1

List of strains and their properties

Strain names		Taxon	Phenotypic properties*									Episomes & Plasmids		Other Properties
Current	Former		Lac	Mal	Met	CM	TC	SM	SA	KM	Az	F	R	
YA1	20S0	*E. coli*	−	+	+	S	S	R	R	S	R	F^-	R^-	Resistance to SM, SA and Az are not conferred by an R factor
YA2	〃	*E. coli*	−	+	+	S	S	R	R	R	R	F^-	R(KM)^t**	Derived from YA1
YA3	〃	*E. coli*	−	+	+	R	R	R	R	S	R	F^-	R_{100}***	〃 〃 〃
YA4	〃	*E. coli*	−	+	+	R	R	R	R	R	R	F^-	R(KM)^t R_{100}	〃 〃 〃
W3630		*E. coli*	+	−	+	S	S	S	S	S	S	F^-	R^-	Hfr_3 ($♀_3$) see Richter, A. 1961. Genet. Research, 2 : 333-345.
YA5		*E. coli*	+	−	+	S	S	S	S	R	S	F^-	R(KM)^t	Derived from W3630
JE948		*E. coli*	+	−	+	R	R	R	R	S	S	F^-	R_{100}	Derivative of W3630. see Yoshikawa and Sevag, 1967. J. Bacteriol. 93 : 245-253
YA6		*E. coli*	+	−	+	R	R	R	R	R	S	F^-	R(KM)^t R_{100}	Derived from W3630
LT2		*S. typhimurium*	−	+	?	S	S	S	S	S	S	F^-	R^-	
YA7		*S. typhimurium*	−	+	?	S	S	S	S	R	S	F^-	R(KM)^t	Derived from LT2
YA8		*S. typhimurium*	−	+	?	R	R	R	R	S	S	F^-	R_{100}	〃 〃 〃
YA9		*S. typhimurium*	−	+	?	R	R	R	R	R	S	F^-	R(KM)^t R_{100}	〃 〃 〃
YA10	58-161	*E. coli*	+	+	−	S	S	S	S	S	S	F^+	R^-	Bio^-
YA11	Hfr-C	*E. coli*	+	+	−	S	S	S	S	S	S	Hfr	R^-	
W677		*E. coli*	−	−	+	S	S	S	S	S	S	F^-	R^-	Thi^-, Thr^-, Leu^-, Xyl^-, Tre^-
CSH2		*E. coli*	+	+	−	S	S	S	S	S	S	F^-	R^-	

* The following abbreviations are used : Lac, lactose ; Mal, maltose ; Met, methionine ; Bio, biotin ; Tre, trehalose ; Xyl, xylose ; Thr, threonine ; Thi, thiamin ; CM, chloramphenicol ; TC, tetracycline ; SM, streptomycin ; SA, sulfonamide ; KM, kanamycin ; Az, azide ; 〃−〃, nonfermenting with abbreviatiations for sugar, 〃requiring〃 with abbreviations for growth factors ; 〃+〃, opposite of 〃−〃 ; R, resistance ; S, sensitivity.

** R(KM)^t confers KM-resistance on its host and undergoes thermosensitive replication.

*** R_{100} confers the resitance to CM, TC, SM and SA on its host and is nonthermosensitive.

thermosensitivity of an R factor was tested by the method for elimination of the R(KM)^t factor(18).

Method for simultaneous transfer of the R(KM)^t and R_{100} factors. Purified *E. coli* YA6 and YA1 were cultured in Penassay broth for 18 hours without shaking. Separate cultures were incubated at 25°C, 37°C and 43°C. Equal vulumes of the donor (YA6) and recipient (YA1) cultures and fresh Penassay broth were mixed in all combinations, and aliquots were incubated at 25°C, 37°C and 43°C for 6 hours without shaking. One tenth ml of each mating mixture was spread on MacConkey plates containing 50 μg/ml of KM and 100 μg/ml of sodium azide for selecting KM^r(Az^r) conjugal progeny inheriting R(KM)^t, and on those containing sodium azide and 25 μg/ml of CM to select CM^r(Az^r) conjugational progeny inheriting R_{100}. On these plates, the donor srain, YA6, could not grow because of the inhibitory effect of sodium azide, and recipient strain, YA1, was also suppressed by the bacteriostatic action of KM or CM. The colonies which appeared on the selective plates were further identified as the recipient cells inheriting the R factor and not spontaneous azide resistant mutants of the donor, by their inability to ferment lactose.

Method for mutual transfer between a strain carrying the R(KM)^t and one carrying the R_{100} factor. In this experiment, *E. coli* JE948 was employed as the donor of R_{100} and the recipient of R(KM)^t, and *S. typhimurium* YA7 was used as the opposite donor and recipient. The strains were cultured separately in Penassay broth at 25°C for 18 hours, and an equal volume of each culture and fresh Penassay broth were mixed, and incubated at either 25°C, 37°C or 43°C for 6 hours without shaking. The conjugation progeny inheriting the R_{100} or R(KM)^t factor were selected with MacConkey plates containing both 50 μg/ml of KM and 25 μg/ml of CM. Differentiation of progeny derived from *E. coli* W3630 from those derived from *S. typhimurium* was easily performed by observing lactose fermentation.

Method for selection of bacterial clones bearing recombinant R factors derived from crossing over between R(KM)^t and R_{100}. *E. coli* YA6 which carries both R(KM)^t and R_{100}, was cultured in a series of test tubes containing 8 ml of Penassay broth at 25°C for 18 hours. Thereafter, KM (50 μg/ml final concentration) and different combinations of CM, TC, SM (all at 25 μg/ml final concentration) and SA (1,000 μg/ml final concentration) were added, and then incubated at 43°C for 18 to 72 hours. Samples were streaked from each tube on drug-free MacConkey plates at one day interval. The drug resitance patterns of colonies which appeared on the plates were tested by the replica method, and any survivors showing resistance to KM and CM, TC, SM or SA were carefully purified. Bacterial cells carrying both the separately replicating R(KM)^t and R_{100} factors might grow at least during the initial period of the 43°C incubation with the drugs. The R(KM)^t factor, however, would be lost from host cells at their division because of the inhibitory effect of 43°C on R(KM)^t replication. Any host cell losing R(KM)^t would be killed by the bactericidal action of KM. On the other hand, if bacterial cells bearing an integrated R(KM)^t and R_{100} factor appeared, their number might increase when the recombinant R factor multiplied nonthermosensitively, or cells sensitive not only for KM but also to CM, TC, SM and SA might appeared, even in small number, when the integrated R factor was thermosensitive. Furthermore, if cells carrying a recombinant R factor with only some of the resistance characters of R_{100} were produced, their number might increase or be preserved constant according to the combination of resistance markers integrated into the recombinant R factor and the combination of drugs added to the culture at 43°C.

Method for testing R factor inhibition of F-mediated fertility. R(KM)^t, R_{100} and their recombinants were transferred by conjugation from W3630 substrains to F$^+$ strain YA10(formerly 58-161) and to Hfr strain YA11(formerly Hfr-C). To select YA10 or YA11 derivatives inheriting one of the R factors, EMS-maltose agar plates supplemented with 50 μg/ml of methionine and 0.1 μg/ml of biotin and containing KM, TC, CM, SM or 100 μg/ml of SA were employed. On these plates, the donor cells could not grow because of their inability to ferment maltose, and the recipient cells not inheriting R factors were not incapable of colony formation because of the bacteriostatic effect of added antibiotics. The obtained R$^+$ subclones of the F$^+$ and Hfr strains were purified by successive streaking on MacConkey plates

containing or not containing drugs, and their biological characteristics were rechecked to differentiate them from conjugational progeny produced by chance donation from the F^+ and Hfr cells to the R^+ cells. Chromosomal recombination between R^-F^+, R^+F^+, R^-Hfr or R^+Hfr strains and W677 was performed by mixing culture method(22), and their prototrophic recombinants were selected with minimum EMS-lactose plates(13). The viable cells of each strain were counted with drug-free MacConkey plates and EMS-glucose plates supplemented with methionine, biotin, thiamine, threonine and leucine.

Method for testing the plating efficiency of phage T4b against R^+ derivatives of W3630. R^+ derivatives of 3630 were cultured at 25°C for 18 hours, diluted 20 fold with fresh Penassay broth and incubated without shaking at 25°C and 43°C for 5 hours. The plating efficiency of phage T4b on these R^+ strains was determined at 37°C by the ordinary method(23). T4b lysates were prepared using W3630, which is R^-.

Results

Thermosensitivity of the $R(KM)^t$ and R_{100} factors in E. coli and S. typhimurium strains which carry both. If a small number of cells of *E. coli* strains YA4 or YA6, or the *S. typhimurium* strain YA9, each of which carries both $R(KM)^t$ and R_{100} are inoculated into a liquid medium and incubated at 43°C for 24 hours, only the $R(KM)^t$ factor was completely eliminated from the host, while the R_{100} factor was stably maintained in the host bacteria at either 43°C or 25°C without any relation to coexisting $R(KM)^t$. (Table 2)

It was concluded that the temperature sensitivity of $R(KM)^t$ was expressed even in the presence of the temperature insensitive R_{100} factor. This reinforces the impression that the two R factors replicate independently of each other.

Simultaneous transfer of the $R(KM)^t$ and R_{100} factors from YA6 to YA1. When YA6 was subcultured at 25°C, 37°C or 43°C and then mixed and cultured with YA1 at 25°C, 37°C or 43°C in accordance with the temperatures of the subcultre, $R(KM)^t$ was transferred with the highest frequency at

TABLE 2

Loss of the thermosensitive $R(KM)^t$ factor from E. coli and S. typhimurium strains carrying $R(KM)^t$ alone or $R(KM)^t$ and nonthermosensitive R_{100} factors, and grown in a liquid medium at different temperatures.

Strain names	Culture temperature	Number of colonies sensitized/Total		Elimination frequency in %	
		$R(KM)^t$	R_{100}	$R(KM)^t$	R_{100}
JE948	25°C	—	0/33	—	0.
	43°C	—	0/30	—	0.
YA5	25°C	0/27	—	0.	—
	43°C	26/26	—	100.	—
YA6	25°C	0/29	0/29	0.	0.
	43°C	28/28	0/28	100.	0.
YA4	25°C	0/35	0/35	0.	0.
	43°C	40/40	0/40	100.	0.
YA9	25°C	0/24	0/24	0.	0.
	43°C	22/22	0/22	100.	0.

TABLE 3

Simultaneous transfer of the thermosensitive R(KM)^t and nonthermosensitive R_{100} factors from YA6 carrying both R factors to YA1 at various temperatures.

Cultured microbes	Culture temperature		Transfer frequency : R^+ recipient/Donor	
	Donor subculture	Mixed culture	R(KM)^t	R_{100}
YA6	25°C	25°C	5.2×10^{-1}	$<10^{-7}$
+	37°C	37°C	2.6×10^{-4}	4.1×10^{-3}
YA1	43°C	43°C	7.7×10^{-8}	1.3×10^{-2}
YA6	25°C	25°C	5.2×10^{-1}	$<10^{-7}$
+	25°C	37°C	4.6×10^{-2}	6.3×10^{-4}
YA1	25°C	43°C	1.5×10^{-6}	7.5×10^{-5}

TABLE 4

Mutual transfer of the thermosensitive R(KM)^t and nonthermosensitive R_{100} factors between E. coli and S. typhimurium.

Cultured microbes	Culture temperature		Transfer frequency : R^+ recipient/Donor	
	Donor subculture	Mixed culture	R(KM)^t to *E. coli*	R_{100} to *Salm.*
JE948	25°C	25°C	4.3×10^{-1}	$<1.0\times10^{-9}$
+	25°C	37°C	2.2×10^{-2}	4.1×10^{-7}
YA7	25°C	43°C	3.7×10^{-5}	8.0×10^{-5}
JE948	25°C	25°C	—	$<1.2\times10^{-9}$
+	25°C	37°C	—	8.1×10^{-3}
LT2	25°C	43°C	—	2.7×10^{-4}

TABLE 5

Transfer of the thermosensitive R(KM)^t factor from YA5 to R$^-$ strain YA1 or to R_{100} carrying strain YA3.

Cultured microbes	Culture temperature		Transfer frequency : R(KM)$^{t+}$ recipient/Donor
	Donor subculture	Mixed culture	R(KM)^t
YA6	25°C	25°C	1.2×10^{-1}
+	25°C	37°C	4.8×10^{-2}
YA1	25°C	43°C	3.1×10^{-6}
YA5	25°C	25°C	9.8×10^{-2}
+	25°C	37°C	5.5×10^{-2}
YA3	25°C	43°C	2.7×10^{-6}

TABLE 6

Survivors of YA6. which is carrying both R(KM)^t and R_{100}. incubated at 43°C with KM and various combinations of CM, TC, SM and SA.

Tube code	Drugs added	Number or survivors	Resistance patterns	Number	Temporary code
A.	KM, SM	124	KM^r, CM^r, TC^r, SM^r, SA^r.	107	No. 1
			KM^r, CM^r, TC^r, , SA^r.	17	No. 14-1 ---No. 14-4
B.	KM, CM	68	KM^r, CM^r, TC^r, SM^r, SA^r.	53	No. 2
			KM^r, , TC^r, SM^r, SA^r,	3	No. 16-5
			KM^r, , , SM^r. SA^r.	12	No. 17-1
C.	KM, TC	0			
D.	KM, SA	34	KM^r, CM^r, TC^r, SM^r, SA^r.	33	No. 3
			KM^r, CM^r, TC^r, SM^r, .	1	No. 15-1
E.	KM, SM, CM	52	KM^r, CM^r, TC^r, SM^r, SA^r.	41	No. 4
			KM^r, , TC^r, SM^r, SA^r.	3	No. 16-4
			KM^r, , , SM$_s$, SA^r.	3	No. 17-2
			KM^r, , TC^r, , .	5	No. 18-1
F.	KM, SM, TC	0			
G.	KM, SM, SA	31	KM^r, CM^r, TC^r, SM^r, SA^r.	24	No. 5
			KM^r, CM^r, TC^r, , SA^r.	6	No. 14-5
			KM^r, CM^r, TC^r, SM^r, .	1	No. 15-2
H.	KM, CM, TC	0			
I.	KM, CM, SA	29	KM^r, CM^r, TC^r, SM^r, SA^r.	16	No. 6
			KM^r, , TC^r, SM^r, SA^r.	5	No. 16-3
			KM^r, , , SM^r, SA^r.	1	No. 17-3
			KM^r, , TC^r, , .	7	No. 18-2
J.	KM, TC, SA	79	KM^r, CM^r, TC^r, SM^r, SA^r.	78	No. 7
			KM^r, CM^r, , SM^r, SA^r.	1	No. 13
K.	KM, SM, CM, TC	0			
L.	KM, SM, TC, SA	0			
M.	KM, SM, CM, SA	80	KM^r, CM^r, TC^r, SM^r, SA^r.	33	No. 8
			KM^r, , TC^r, SM^r, SA^r.	11	No. 16-1, 2
			KM^r, , TC^r, , .	36	No. 18-2--5
N.	KM, CM, TC, SA	0			
O.	KM, CM, TC, SM, SA	19	KM^r, CM^r, TC^r, SM^r, SA^r.	19	No. 9, 10 No. 11, 12
Total survivors		516	KM^r, CM^r, TC^r, SM^r, SA^r.	404	No. 1----12
			KM^r, CM^r, , SM^r, SA^r.	1	No. 13
			KM^r, CM^r, TC^r, , SA^r.	23	No. 14-1---5
			KM^r, CM^r, TC^r, SM^r, .	2	No. 15-1---2
			KM^r, , TC^r, SM^r, SA^r.	22	No. 16-1---5
			KM^r, , , SM^r, SA^r.	16	No. 17-1---5
			KM^r, , TC^r, , .	48	No. 18-1---5
				516	

25°C, whereas, the highest transfer frequency of R_{100} was found to be at 43°C. However, if the donor was subcultured only at 25°C, the transfer frequency of R(KM)^t at 25°C was not much different from that at 37°C. (Table 3)

This fact may suggest that the donor cells subcultured at 25°C (in comparison to those subcultured at 37°C) may carry more number of R(KM)^t, so that the R(KM)^t factor can be transmitted with a high frequency even when the mixed culture is incubated at 37°C.

The similar result was obtained when *S. typhimurium* strain LT2 was used as the recipient.

Mutual transfer of the R(KM)^t and R_{100} factors between E. coli strain JE948 and S. typhimurium strain YA7. Transfer frequencies of R(KM)^t from *S. typhimurium* to *E. coli* and of R_{100} from *E. coli* to *S. typhimurium* were highest at 25°C and 43°C, respectively, also in the mutual transfer experiment. (Table 4)

Similar results were obtained, even when the relationship between the donor and recipient was reversed.

No superinfection immunity between the R(KM)^t and R_{100} factors. Table 4 also shows that there is no difference in the transfer frequencies of the R_{100} factor to *S. typhimurium* carying or not carrying the R(KM)^t factor. Furthermore, it was demonstrated that R(KM)^t could be transmitted from YA5 to R^- strain YA1 or R_{100}^+ strain YA3 with the same frequency. (Table 5)

Selection of bacterial clones bearing a recombinant between the R(KM)^t and R_{100} factors. Most cells of YA6, which carries both R(KM)^t and R_{100}, were killed by incubation at 43°C with KM and various combinantions of the drugs, CM, TC, SM and SA. A small portion of the cells, however, survived, especially in the cultures containing KM and SM only. As total, 516 survivors were recovered. Among them, 48 colonies were resistant to KM and TC, 23 to KM, CM, TC and SA, 22 to KM, SM, SA and TC, 16 to KM, SM and SA, 2 to KM, CM, TC and SM, 1 to KM, CM, SM and SA, and remaining 404 survivors were resistant to all of KM, CM, TC, SM and SA. (Table 6)

Representative colonies of each resistance pattern were numbered as shown in Table 6, and their resistance patterns were rechecked after subculture in drug-free Penassay broth at 25°C and 43°C. (Table 7). A11 surviuors resistant to all of KM, CM, TC, SM and SA were judged to be ones bearing the original R factors, R(KM)^t and R_{100}, and hence to be unchanged from YA6, as far as examined. The No. 12 survior was presented in the table as a representative. A survivor resistant to KM, CM, SM and SA and sensitive to TC was considered to carry R(KM)^t and a segregant of R_{100} which harbours resistance markers for CM, SM and SA only (No. 13 suvivor).

Survivors resistant to KM, CM, TC and SA only and to KM, CM, TC and SM were also judged as cells bearing R(KM)^t and a segregant of R_{100} separately. (No. 14-1 and No. 15-1 survivors)

Survivors showing the resistance pattern to KM, TC, SM and SA were very complicated. No. 16-1, which had shown once such resistance pattern, changed its resistance during the subculture in drug-free Penassay broth at 25°C and 43°C, and segregated into 3 types of resistance patterns, KM^r, CM^r, TC^r, SM^r, SA^r; KM^r, TC^r, SM^r, SA^r; CM^r, TC^r, SM^r, SA^r. The reason why CM-resistance, which had once disappeared, revives in some subclones of this case is now unknown.

No. 16-2, which also had once shown the resistance pattern to KM, TC, SM and SA, changed its resistance during the subculture in drug-free Penassay broth, and it segregated into 2 types of resistance patterns, KM^r, SM^r, SA^r ; SM^r, SA^r. Anyhow, successive single colony isolation was required to obtain cells with a stable reistance pattern from the survivors showing initially the resitance pattern for KM, TC, SM and SA.

No. 17-1 survivor, which had been resistant to KM, SM and SA only, was stable after the subculture in drug-free Penassay broth at 25°C, but the resistance to KM, SM and SA was simultaneously lost at 43°C in drug-free Penassay broth.

Survivors resistant to KM and TC only were also stable even after the subculture in drug-free Penassay

TABLE 7

Confirmation of the resistance patterns of the survivors obtained from YA6 incubated at 43°C with KM, and various combinations of CM, SM, TC and SA, after subculture in drug-free Penassay broth for 18 hours at 25°C or 43°C.

Temporary code of survivors	Subculture temperature	Number of colonies tested						Elimination frequency in %	
		Total	KM-R	SM-R	CM-R	TC-R	SA-R	KM-R	Others*
No. 12**	25°C	14	14	14	14	14	14	0	0
	43°C	14	0	14	14	14	14	100	0
No. 13	25°C	38	38	38	38	0	38	0	0
	43°C	26	3	26	26	0	26	89	0
No. 14-1	25°C	47	47	0	47	47	47	0	0
	43°C	51	0	0	51	51	51	100	0
No. 15-1	25°C	33	33	33	33	33	0	0	0
	43°C	24	1	24	24	24	0	96	0
No. 16-1	25°C	23	23	23	18***	23	23	0	22
	43°C	21	4	21	21	21	21	81	0
No. 16-2	25°C	20	19****	20	0	0	20	5	0
	43°C	25	0	0	0	0	0	100	100
No. 17-1	25°C	69	69	69	0	0	69	0	0
	43°C	79	4	4	0	0	4	94	94
No. 18-1	25°C	41	41	0	0	41	0	0	0
	43°C	37	0	0	0	37	0	100	0

* Elimination frequency of the resistance to drugs other than KM means loss of the resistance only when difference between that at 25°C and that at 43°C is recognized.

** Temporary survivor numbers were shown in Table 6

*** Five subclones were resistant to KM, TC, SM and SA but not to CM (see Text pp 14-15)

**** Mainly Consists of cells resistant to KM, SM and SA, but one novel subclone was found to be resistant to SM and SA only.

broth at 25°C, but only the TC-resistance was retained after the subculture at 43°C.

No. 18-1 was presented in Table 7 as a representative of KM^r, TC^r survivors.

Each colony appeared on the drug-containing plates used for examinantion of resistance patterns of survivors, and whose resistance markers for KM and other drugs seemed to be simultaneously lost at 43°C (namely assumed to carry a new thermosensitive recombinant between $R(KM)^t$ and R_{100}), was repurified at 25°C on drug-free MacConkey plates, and assigned as shown in Table 8. Then, the effect of temperature on the inheritance of every resistance marker was examined again by the method for elimination of the $R(KM)^t$ factor (18).

In every cases of 5 subclones (YA12-1 to YA12-5), which were repurified from the No. 16-1 survivor and resistant to KM, TC, SM and SA, the resistance to KM, SM and SA was concurrently eliminated by cultivation at 43°C in drug-free Penassay broth, although the resistance to TC remained persistently even at 43°C. The resistance to KM, SM and SA in subclones (YA13-1 to YA13-10) repurified from the No. 16-2 and No. 17, and the resistance to SM and SA in a subclones purified from No. 16-2 were also simaltaneously eliminated with a high frequency at 43°C (YA14). (Table 8)

TABLE 8

Designation of the pure subclones of E. coli YA6 assumed to carry a recombinant-R between R(KM)^t and R^{100}, and temperature sensitivities of their each resistance marker.

Strain names	Taxon	Phenotypic properties									Episomes & plasmids		Other properties
		Lac	Mal	Met	CM	TC	SM	SA	KM	Az	F	R	
YA12 (Subnumber 1---5)	*E. coli*	+	−	+	S	R	R	R	R	S	F$^-$	R^{ts}2* & R$_{100}$'**	Derived from No. 16-1 survivor of YA6
YA13 (1---10)	*E. coli*	+	−	+	S	S	R	R	R	S	F$^-$	R^{ts}2	Derived from No. 17-1 survivor of YA6
YA14	*E. coli*	+	−	+	S	S	R	R	S	S	F$^-$	R^{ts}3***	Derived from No. 16-2 survivor of YA6

* R^{ts}2 confers the resistance to KM, SM and SA on its host and undergoes thermosensive replication

** R$_{100}$' confers TC-resistance on its host and is nonthermosensitive

*** R^{ts}3 confers the resistance to SM and SA on its host and is thermosensitive

Strain names	Subculture temperature	Number of colonies tested						Elimination frequency (%)	
		Total	KM-R	SM-R	CM-R	TC-R	SA-R	R^{ts}	R
YA14	25°C	51	0	51	0	0	51	0	0
	43°C	49	0	1	0	0	1	98	—
YA13-1	25°C	50	50	50	0	0	50	0	0
	43°C	40	4	4	0	0	4	90	—
YA13-10	25°C	50	50	50	0	0	50	0	0
	43°C	50	0	0	0	0	0	100	—
YA12-1	25°C	50	50	50	0	50	50	0	0
	43°C	50	5	5	0	50	5	90	0
YA12-2	25°C	50	49	49	0	49	49	2	2
	43°C	50	1	1	0	50	1	98	0
YA12-3	25°C	51	51	51	3*	51	51	0	0
	43°C	44	12	12	0	44	12	73	0
YA12-4	25°C	50	50	50	0	50	50	0	0
	43°C	50	16	16	0	50	16	68	0
YA12-5	25°C	50	50	50	0	50	50	0	0
	43°C	50	0	0	0	50	0	100	0

* Despite performing successive single colony isolation, still CM- resistance revives in some cells of YA12-3 (see Text pp 14-15)

As the conclusion of these experiments, it seemed that two kinds of thermosensitive recombinants between R(KM)^t and R$_{100}$ could be obtained, one harbours resistance markers for KM, SM and SA, and the other contains resistance markers for SM and SA. Though many cells with resistance patterns for KM and various combinantions of CM, TC, SM and SA other than SM^r, SA^r; KM^r, SM^r, SA^r or KM^r, SM^r, SA^r, TC^r were obtained, almost all cells were judged to carry R(KM)^t and a segregant of R$_{100}$ separately.

*Thermosensitive cotransfer of the resistance to KM. SM and SA in YA*13. When *E. coli* YA13-1 was subcultured in drug-free Penassay broth at 25°C, and mixed with R$^-$ *E. coli* strain CSH2 at 25°C for 6 hours without shaking, resistances to KM, SM or SA were transferred with almost the same frequency, and the incubation temperature of the plated culture was not critical. Whereas, if subculture, mixed culture

TABLE 9

Thermosensitive co-transfer to F^-R^- E. coli strain CSH2 of the KM-, SM- and SA-resistances from YA13 suspected to carry a recombinant-R between $R(KM)^t$ and R_{100}.

Culture temperatures			Transfer frequencies determined with EMS-maltose plates containing		
Subculture	Mixed culture	Selective culture	KM 50 μg/ml	SM 25 μg/ml	SA 100 μg/ml
25°C	25°C	25°C	1.3×10^{-3}	1.1×10^{-3}	1.0×10^{-3}
25°C	25°C	37°C	1.9×10^{-3}	1.0×10^{-3}	1.2×10^{-3}
25°C	25°C	43°C	7.0×10^{-4}	10^{-5}	6.5×10^{-4}
25°C	25°C	37°C	1.9×10^{-3}	1.0×10^{-3}	1.2×10^{-3}
25°C	37°C	37°C	3.6×10^{-4}	3.0×10^{-4}	3.9×10^{-4}
25°C	43°C	37°C	$<10^{-7}$	4.8×10^{-7}	$<10^{-7}$
25°C	25°C	37°C	1.9×10^{-3}	1.0×10^{-3}	1.2×10^{-3}
37°C	37°C	37°C	7.7×10^{-6}	8.5×10^{-6}	5.4×10^{-6}
43°C	43°C	37°C	$<10^{-8}$	$<10^{-8}$	$<10^{-3}$

TABLE 10

Spontaneous segregation of the resistance markers in the thermosensitive $R^{ts}2$ factor at the time of transfer from YA13 to CSH2.

Temperatures concerning the R-transfer			Selective plates for R^+ recipient	Number of subcolonies retaining resistance to			
Subculture	Mixed culture	Selective culture		Total	KM-R	SM-R	SA-R
25°C	25°C	25°C	KM-plate	14	14	14	14
25°C	25°C	43°C	KM-plate	18	18	7	18
25°C	25°C	43°C	SA-plate	30	30	0	30
25°C	37°C	37°C	KM-plate	10	10	10	10
25°C	37°C	37°C	SM-plate	8	8	8	8
25°C	37°C	37°C	SA-plate	17	17	9	17

TABLE 11

Thermosensitive and nonthermosensitive transfers of the resistance markers from YA12 or YA14 to F^-R^- E. coli strain CSH2.

R-donor	Culture temperature*	Transfer frequencies determined with complete EMS-maltose plates containing			
		KM 50 μg/ml	SM 25 μg/ml	SA 100 μg/ml	TC 25 μg/ml
YA12-1	25°C	4.1×10^{-4}	3.9×10^{-4}	3.9×10^{-4}	10^{-6}
	37°C	9.8×10^{-7}	1.8×10^{-6}	1.2×10^{-6}	9.4×10^{-5}
	43°C	10^{-8}	10^{-8}	10^{-8}	3.3×10^{-4}
YA14	25°C	—	8.7×10^{-4}	8.7×10^{-4}	—
	37°C	—	1.4×10^{-6}	8.0×10^{-7}	—
	43°C	—	10^{-8}	10^{-8}	—

* Culture temperatures indicated in this table were used for throughout the donor subculture and mixed culture, and the selective culture was performed uniformly at 37°C.

and selective culture were carried out uniformly at 25°C, 37°C or 43°C, the resistances to KM, SM, or SA were transferred with the highest frequency at 25°C and lowest at 43°C. When the donor strain subcultured at 25°C was incubated after mixing with the recipient, at 25°C, 37°C and 43°C, no significant difference in the transfer frequencies of the resistances to these three drugs at 25°C and 37°C was observed. (Table 9)

TABLE 12

Designation of E. coli male strains carrying various R factors, and influence of co-existing R factors in the male strains on the F-mediated chromosomal transmission.

Strain names		Taxon	Phenotypic properties										Episomes & piasmids	
Current	Origin		Lac	Mal	Met	CM	TC	SM	SA	KM	Az	Bio	F	R
YA10	58-161	*E. coli*	+	+	−	S	S	S	S	S	S	−	F^+	R^-
YA15	58-161	*E. coli*	+	+	−	S	S	S	S	R	S	−	F^+	$R(KM)^t$
YA16	58-161	*E. coli*	+	+	−	R	R	R	R	S	S	−	F^+	R_{100}
YA17	58-161	*E. coli*	+	+	−	R	R	R	R	R	S	−	F^+	$R(KM)^t R_{100}$
YA18	58-161	*E. coli*	+	+	−	S	S	R	R	R	S	−	F^+	$R^{ts}2$
YA19	58-161	*E. eoli*	+	+	−	S	S	S	R	R	S	−	F^+	$R^{ts}4$*
YA20	58-161	*E. coli*	+	+	−	S	S	R	R	S	S	−	F^+	$R^{ts}3$
YA11	Hfr-C	*E. coli*	+	+	−	S	S	S	S	S	S	?	Hfr	R^-
YA21	Hfr-C	*E. coli*	+	+	−	S	S	S	S	R	S	?	Hfr	$R(KM)^t$
YA22	Hfr-C	*E. coli*	+	+	−	R	R	R	R	S	S	?	Hfr	R_{100}
YA23	Hfr-C	*E. coli*	+	+	−	R	R	R	R	R	S	?	Hfr	$R(KM)^t R_{100}$
YA24	Hfr-C	*E. coli*	+	+	−	S	S	R	R	R	S	?	Hfr	$R^{ts}2$
YA25	Hfr-C	*E. coli*	+	+	−	S	S	S	R	R	S	?	Hfr	$R^{ts}4$*
YA26	Hfr-C	*E. coli*	+	+	−	S	S	R	R	S	S	?	Hfr	$R^{ts}3$

* $R^{ts}4$ was obtained in the cells of CSH2, which was mixingly cultured with YA13-1 at 25°C and selected with SA-plates at 43°C (Table 10). This R factor confers the resistance to KM and SA and undergoes thermosensitive replication.

TABLE 12 (continued)

Chromosomal donor	F^-R^- recipient	Recombination frequency : Prototrophs/Donor
YA10	W677	2.1×10^{-7}
YA15	〃	5.3×10^{-7}
YA16	〃	10^{-8}
YA17	〃	10^{-8}
YA18	〃	2.7×10^{-6}
YA19	〃	5.5×10^{-7}
YA20	〃	3.6×10^{-7}
YA11	W677	2.4×10^{-3}
YA21	〃	7.8×10^{-4}
YA22	〃	10^{-6}
YA23	〃	10^{-6}
YA24	〃	2.0×10^{-3}
YA25	〃	1.0×10^{-4}
YA26	〃	6.7×10^{-4}

Despite using KM, SM or SA alone as the selective agent, almost all recipient cells inherting one drug were found to inherit also the resistance to the other drugs, though a small portion of them inherited only resistance to KM and SA. (Table 10)

The same cotransfer experiments were also successfully carried out on the resisance to KM, SM and SA, and to SM and SA in YA12-1 and YA14. The resistance to TC in the former strain, however, was nonthermosensitively and independently transferred. (Table 11)

From the results obtained in these experiments and in the previous experiments, it was assumed that the resistances to KM, SM and SA, and that to SM and SA, in YA12 or YA13, and YA14 are integrated into a new thermosensitive R factor which is postulated as a recombinant-R between R(KM)^t and R_{100}.

Influence of the R(KM)^t, R_{100} and their recombinant R factors on the F-mediated fertility. Nonthermosensitive R_{100} factor has been known as an fi$^+$ (capable of inhibiting F-mediated fertility of its host) type of R factor, while thermosensitive R(KM)^t has been reported as an fi$^-$ type of R factor(15, 16, 21). It is of interest to know the influence of R factors against F-mediated fertility, when both fi$^+$ and fi$^-$ R factors are simultaneously introduced into an F$^+$ or Hfr strain, or when a recombinant of both is carried by the male cells. It was demonstrated that the male strains, YA10 and YA11, carrying only R(KM)^t or its recombinant with R_{100} was capable of transferring chromosome with the same frequency as R$^-$ male strains, Strain harbouring the R_{100} factor alone or both R_{100} and R(KM)^t were much poorer chromosomal transmitters. (Table 12)

Efficiency of plating (EOP) of phage T4b against E. coli strains carrying or not carrying the R(KM)^t, R_{100} or their recombinant R factors. The plating efficiency of phage T4b propagated on W3630 was examined at 37°C against derivatives of W3630 bearing either or both R(KM)^t and R_{100} or

TABLE 13

Efficiency of plating of the T4b phage against E. coli W3630 derivatives carrying or not carrying R(KM)^t, R_{100} or both, or carrying their recombinant-R factors, and grown at 25°C or 43°C.

Indicator strains	Culture temperature		Efficiency of plating	
	Indicator subculture	Plating culture	Plaque count/ml	EOP
W3630	25°C	37°C	6.1×10^9	1.00
	43°C	〃	5.6×10^9	0.92
YA5	25°C	〃	3.1×10^7	0.005
	43°C	〃	1.1×10^9	0.18
JE948	25°C	〃	7.7×10^9	1.26
	43°C	〃	5.4×10^9	0.89
YA6	25°C	〃	2.7×10^9	0.44
	43°C	〃	1.8×10^9	0.30
YA13	25°C	〃	3.5×10^8	0.057
	43°C	〃	1.2×10^9	0.20
YA12	25°C	〃	2.3×10^9	0.38
	43°C	〃	1.7×10^9	0.28
YA14	25°C	〃	3.2×10^8	0.053
	43°C	〃	1.5×10^9	0.25

* T4d is a wild type of T4 phage employed by Dr. Doermann, A. H. (Cold Spring Harbour Symposia Quant. Biol. 18 : 3, 1953)

bearing the recombinant of these R factors. These derivatives were grown at 25°C or 43°C. As shown in Table 13, the R_{100} factor did not influence the EOP, although the EOP of phage T4b against the indicator carrying $R(KM)^t$ or its thermosensitve recombinant with R_{100} and grown at 25°C was found to be less than one-hundredth and one-tenth, respectively, of that obtained with an R^- strain. It was noteworthy that the restriction of phage T4b by $R(KM)^t$ and its recombinant was almost released by R_{100} when doubly R^+ strains were tested. The restriction was also diminished by growing the indicator cells at higher temperature, even when 5 hour incubation insufficient for elimiation of thermosensitive R factors was employed.

Discussion

Investigations were carried out on the genetic behavior of a thermosensitive kanamycin resistance factor, designated as $R(KM)^t$, when it was carried by various *E. coli* or *S. typhimurium* strains along with the nonthermosensitive multiple drug resistance factor, R_{100}. The $R(KM)^t$ factor was easily eliminated from the host by the high temperature culture (43°C) without any interaction with the coexisting R_{100} factor. For high percentage elimination of $R(KM)^t$, strict 43°C culture and a small bacterial inoculum were necessary. At 40°C, more than 3 successive subcultures with small inocula were required for a high percentage elimination, and no elimination occured at 37°C under any condition. Since the transfer frequency of $R(KM)^t$ at 25°C from the donor grown at 37°C was somewhat lower than that from the donor subcultured at 25°C, it was suggestive that the replication of $R(KM)^t$ was suppressed even at 37°C yet no elimination was observed. The temperature sensitive transfer of the $R(KM)^t$ factor was not influenced by nonthermosensitive R factors in either the simultaneous or the mutual transfer experiment. Jacob and Brenner proposed an hypothesis concerning the regulation of DNA replication in bacteria, *L'hypothese du replicon*, in 1963 (8). According to this hypothesis, the chromosome and extrachromosomal genetic elements, such as F and R factors, have been considered as independent replication untis, i. e. replicons. In other words, the replication of a certain genetic element (DNA chain) is regulated by initiator and replicator genes. An initiator was proposed to be active only against the homologous replicator gene where new duplication of DNA starts. If we suppose that the temperature sensitivity of $R(KM)^t$ depends upon its temperature sensitive initiator, then independent susceptibilities to temperature of $R(KM)^t$ and R_{100} may be understood as the result of a self-govering initiator-replicator system in each. In addition, we suppose that an initiator of one R factor is highly specific and will not interact with the replicator of a different R factor.

Contrary to these results, Terawaki et al. have recently found that the cell growth of certain *E. coli* strain was made temperature sensitive by infection with the $R(KM)^t$ factor(19). The mechanism of this phenomenon, however, has remained unknown.

It is of interest that only two kinds of new thermosensitive R factor harbouring resistance markers for KM, SM and SA and for SM and SA could be obtained as the recombinant between $R(KM)^t$ and R_{100}, from the 516 survivors of YA6 grown at 43°C with KM and various combinations of CM, TC, SM and SA. Though many survivors with various resistance patterns were recovered, i. e. TC^r, KM^r; SM^r, SA^r; KM^r, SM^r, SA^r; KM^r, CM^r, TC^r, SA^r; KM^r, TC^r, SM^r, SA^r; KM^r, CM^r, TC^r, SM^r; KM^r, CM^r, SM^r, SA^r or KM^r, CM^r, TC^r, SM^r, SA^r, almost all cells were judged to carry separately $R(KM)^t$ and R_{100} or its segregant (see Tables 6 and 7). Only the resistance markers for KM, SM and SA in the cells with resistance patterns of KM^r, SM^r, SA^r or of KM^r, SM^r, SA^r, TC^r were confirmed to be integrated into a thermosensitive R factor (see Tables 8, 9 and 10). And the other new thermosensitive R factor containing the resistance markers for SM and SA only was obtained as a spontaneous segregant of the former R factor. It is assumed that the thermosensitive R factor harbouring resistance markers for KM, SM and SA is most easily obtainable recombinant between the $R(KM)^t$ and R_{100} factors, eventhough it is not the only one.