Mutation of R Factors Capable of Specifying Hypersynthesis of Penicillinase

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When strains harboring R factor and showing ampicillin (APC) resistance were inoculated on plates containing various concentrations of APC, mutants carrying various levels of APC resistance occurred at high frequencies. Increases in the level of APC resistance were due to a quantitative increase in the formation of penicillinase. By conjugation experiments and transduction analysis, the mutation was found to affect the gene (*amp*) governing APC resistance on the R factor. The R-factor mutants carrying high and stable APC resistance were conjugally transferred at the same frequency as their parent R factors, and the level of their resistance to drugs other than APC was not distinct from that conferred by their parents. Such R-factor mutants could easily be obtained from wild-type R factors carrying low APC resistance. The hypersynthesis of penicillinase by such R-factor mutants was considered to be due to the replication of the *amp* gene on the R factor at hyper-rates and the integration of multiple copies of the *amp* gene (*amp*-hyper) in the R-factor genome.

R factors capable of conferring resistance to ampicillin (APC), in addition to other drugs such as tetracycline (TC), chloramphenicol (CM), streptomycin (SM), kanamycin (KM), and sulfanilamide (SA), were reported first by English workers (1) and later from many other laboratories (2, 8, 14, 31). Enzymological studies have disclosed that APC resistance conferred by an R factor is mainly due to the induction of penicillinase synthesis (6, 10). The penicillinases produced by R factors were mostly similar to each other in their enzymological and physicochemical properties, and were designated type I penicillinase (28, 29). Most of the penicillinases mediated by R factors belong to this group and are quite similar to that reported by Datta and Richmond (7). We reported another penicillinase mediated by R factors, designated type II penicillinase, which differed from type I penicillinases in its physicochemical, enzymological, and immunological properties (32).

Recently, a strain of *Klebsiella pneumoniae* carrying R_{GN823} was isolated which was capable of mediating APC resistance. The penicillinase mediated by R_{GN823} was found to be a known type I penicillinase, but the penicillinase activity per bacterial weight in an *Escherichia coli* strain harboring this R factor was about 20 times higher than those of *E. coli* strains carrying the known R factors which mediate the synthesis of type I penicillinase (25, 29). It was also reported that

strains carrying R-TEM (6, 7) were capable of producing penicillinase at a rate about 20-fold higher than those harboring the known R factors. This paper deals with our recent findings and demonstration of R-factor mutants capable of hypersynthesis of penicillinase as well as genetic studies of the mutants.

MATERIALS AND METHODS

Bacterial strains. The substrains of *E. coli* K-12 W3630, AB1157 *rec⁺ str^r* (resistant to streptomycin), AB2463 *str^r recA*, and ML 1410 *met⁻ nal^r* (resistant to nalidixic acid), and *Salmonella typhimurium* LT₂ were used.

R factors and T-tet factor. The six R factors used were R_{M201} (TC.CM.SM.SA.APC), R_{M89} (KM.APC), R_{M00} (TC.CM.SM.SA.KM.APC), R_{M01} (SM.SA.APC), R_{GN14} (TC.CM.SM.SA.APC), and R_{GN238} (TC.CM.SM.SA.APC). Strains carrying these R factors were isolated from clinical specimens. An R-factor mutant, R_{M201-2}, isolated from R_{M201} was used in most of our experiments. T-tet factor is the recombinant between T factor and the TC resistance (*tet*) determinant derived from R₁₀ (TC.CM.SM.SA) factor (19, 20).

Media. Brain heart infusion (BHI) broth was used for both the propagation of bacterial strains and the conjugation experiments. For the determination of drug resistance, heart infusion (HI) agar (Eiken, Tokyo, Japan) and peptone-water were used. Peptone-water consisted of 1,000 ml of distilled water, 5 g of NaCl, and 10 g of peptone. Semisynthetic medium (13) was used for the

assay of resistance to SA. Bromothymol blue (BTB) agar containing APC was used as the selective medium for the isolation of mutants with high APC resistance. Eosin methylene blue (EMB)-maltose agar (16) containing the selective drugs TC (25 $\mu\text{g/ml}$), CM (25 $\mu\text{g/ml}$), SM (12.5 $\mu\text{g/ml}$), APC (25 $\mu\text{g/ml}$), and NA (50 $\mu\text{g/ml}$), in the concentrations indicated, was used as the selective medium for R-factor transfer from *E. coli* W3630 R⁺ to ML1410. EMB agar containing both SM (1 mg/ml) and each selective drug was used as the selective medium for R-factor transfer from W3630 R⁺ to either AB1157 or AB2463. AL agar medium consisted of 1,000 ml of medium A (9), 10 g of lactose, 40 ml of 0.2% bromothymol blue, and 14 g of agar. AL agar containing each of the selective drugs was used for R-factor transfer from ML1410 R⁺ to W3630. L broth and L broth agar (17) were used for transduction experiments.

Drugs. CM, TC, SM, SA, APC, and nalidixic acid were all working standards for the assay of drug resistance. Benzylpenicillin, APC, phenethicillin, 6-amino-penicillanic acid, cloxacillin, and cephaloridine were used as substrates of β -lactamase.

Determination of drug resistance and conjugal transfer of R factor. The methods used to determine drug resistance and conjugal transfer of R factor were described previously (24). Transfer frequency of R factor was expressed by the number of R⁺ conjugants per number of input cells after 40 min of mixed culture. Transferability of drug resistance was also examined by the cross-brushing method with the use of a mixed culture of the donor and W3630.

Transduction of R factor. Transduction was carried out by the method of Lennox (17) in the system of P1vir (12) and *E. coli*. W3630 *mal*⁻ R⁺ was used as the donor of R factor and ML 1410 *met*⁻ *nal*^b was used as the recipient of R factor. EMB-maltose agar containing both nalidixic acid (50 $\mu\text{g/ml}$) and CM (25 $\mu\text{g/ml}$), TC (25 $\mu\text{g/ml}$), or APC (25 $\mu\text{g/ml}$) was used as the selective medium to detect resistant transductants. The titer of input phage and the number of recipient cells were adjusted to about 10⁹ plaque-forming units per ml and about 10¹⁰ microorganisms per ml, respectively. The multiplicity of infection was approximately 0.1. Transduction frequency was expressed as the number of resistant transductants divided by the number of input phages. As controls, phage lysates without recipient were plated to detect the contamination of the donor cells.

Preparation of β -lactamase. W3630 R⁺ was inoculated in BHI broth at 37 C. After 18 h of incubation, 10 ml of the culture was added to 200 ml of fresh BHI broth and incubated with shaking. After incubation for 3 h, the cells were harvested by centrifugation and washed once with physiological saline. The cells were resuspended in 0.1 M phosphate buffer (pH 7.0) to a density of about 2 to 3 mg (dry weight) per ml, and the cell suspension was treated for 3 min at 20 kc with an ultrasonic disintegrator. The suspension of sonically

disrupted cells was centrifuged at 105,000 $\times g$ for 120 min at 5 C, and the supernatant fluid was used for β -lactamase studies.

Determination of β -lactamase activity. β -Lactamase activity was determined iodometrically at 30 C according to the method of Perret (27). Specific activity of the enzyme was expressed in units per milligram of protein, 1 unit being equivalent to the micromoles of penicillin G hydrolyzed by β -lactamase in 1 h at 30 C. Hydrolysis of cephaloridine was measured similarly, except that 1 mole of hydrolyzed cephaloridine was calculated to be equivalent to 4 mol of iodine.

Assay of CM acetyltransferase. Cultures at the exponential phase of growth in BHI broth were harvested by centrifugation. After the cells had been washed once with physiological saline, they were resuspended in tris(hydroxymethyl) aminomethane-hydrochloric acid buffer (pH 7.8) and subjected to ultrasonic disintegration at 20 kc for 3 min. The disrupted cells were removed by centrifugation at 105,000 $\times g$ for 2 h, and the supernatant fraction obtained was used for the assay of CM acetyltransferase. The assay of CM acetyltransferase was conducted by the method of Shaw (30). Specific enzyme activity was expressed by micromoles of CM inactivated by CM acetyltransferase per minute per milligram of protein at 37 C.

Protein determination. The protein concentration in the enzyme solution was determined by the method described by Lowry et al (18).

Isolation of R factor mutants with high APC resistance. *E. coli* W3630 R⁺, AB1157 R_{M201}⁺, or AB2463 R_{M201}⁺ was inoculated in BHI broth and incubated overnight at 37 C. A 1-ml amount of the cultures was added to 10 ml of BHI broth and incubated with shaking. After 3 h of incubation, samples of appropriate dilutions of the cultures were spread on BTB agar plates containing various concentrations of APC. After 18 h of incubation at 37 C, the colonies were counted. Each of the colonies was picked and the levels of APC resistance were determined after purification by three successive single-colony subcultures on a plate containing the same concentration of APC as that used for the selective plate. The frequency of mutation was expressed as the number of mutants with high APC resistance divided by the number of colonies on an APC-free plate.

Isolation of the recombinant T-tet-amp factor with high APC resistance. W3630 T-tet⁺ was mixed with ML1410 *nal*^b carrying R_{M91} (SM. SA.APC) in BHI broth. After 18 h of incubation at 37 C, the mixed culture was spread on AL agar containing TC (25 $\mu\text{g/ml}$) and APC (25 $\mu\text{g/ml}$). W3630 R_{M91}⁺ T-tet⁺ thus obtained was purified by three successive single-colony subcultures on AL agar plates containing both APC (25 $\mu\text{g/ml}$) and TC (25 $\mu\text{g/ml}$) and was inoculated in BHI broth. After 18 h of incubation at 37 C, samples of appropriate dilutions of the culture were spread on either a BTB agar plate containing APC (1,600 $\mu\text{g/ml}$) or an APC-free BTB agar plate. The fre-

quency of mutation with high APC resistance was calculated by the method described above. Five colonies developed on each plate with or without APC were randomly picked and mixed in 0.1 ml of BHI broth. For the propagation of phage P1, equal volumes (0.1 ml) of both bacterial suspension and phage lysate were mixed with 2 ml of soft agar and inoculated on an L broth agar plate by the method of Lennox (17). ML 1410 *nal^r* was used as the recipient of transduction. EMB-maltose agar containing TC (25 $\mu\text{g}/\text{ml}$) was used as the selective medium to detect resistance transductants.

RESULTS

The log-phase culture of W3630 R_{M201}^+ was plated on BTB agar containing various concentrations of APC. After 18 h of incubation at 37 C, the colonies developing on each plate were counted. As shown in Fig. 1, mutants carrying various levels of APC resistance were obtained. The levels of their resistance generally corresponded to the concentrations of drug used for selection, but a few mutants showed resistance to a drug concentration much higher than that used for selection. The isolation frequency of these mutants was found to decrease in parallel with the increase in the concentrations of drug used for selection. As can be seen in Fig. 2, the levels of APC resistance in such mutants were accounted for by their penicillinase activity.

We next investigated whether this mutation affected a gene on the host chromosome or on the R factor itself. Ten colonies from the plate containing 3,200 μg of APC per ml (Fig. 1) were randomly picked and purified, and their R factors were conjugally transferred to various host strains. High levels of APC resistance in such mutants were found to be conjugally transferred to new host strains, and the increase in the APC resistance in both W3630 and the new hosts could be explained by the increase in the penicillinase activity governed by R-factor mutants derived from R_{M201} . Representative results are shown in Table 1.

Thus, the mutation was concluded to affect the gene on R_{M201} , and one of the R-factor mutants was denoted R_{M201-2} . By contrast, there were no differences in the level of resistance to TC, CM, and SM in W3630 and in various host strains carrying either R_{M201} or its mutants. Similarly, we could not detect any difference in the activity of CM acetyltransferase in various host strains carrying either R_{M201} or its mutants. These findings show that the increase in APC resistance conferred by R_{M201} mutants was not caused by an increase in the copies of the R-factor genome in its host.

Further, the two penicillinases produced by

FIG. 1. *R*-factor mutants of various levels of ampicillin (APC) resistance. W3630 R_{M201}^+ was inoculated in BHI broth at 37 C. After 18 h of incubation, 10 ml of fresh BHI broth was added to 1 ml of the culture, and it was further incubated at 37 C with shaking. After 3 h of incubation, the samples of appropriate dilutions of the culture with broth were spread on BTB agar plates containing various concentrations of APC as shown in the figure. After 18 h of incubation, the colonies on the plates with or without APC were counted. The survival rate indicates the number of colonies on the APC plate divided by the number of colonies on the drug-free plate.

the R_{M201-2} and R_{M201} factors were very similar in substrate specificity for five penicillins and cephaloridine, as well as in optimal pH, optimal temperature, rate of heat inactivation, kinetic aspects (K_m and V_{max}), and isoelectric point. These facts strongly suggested that there was no change in the structural gene responsible for penicillinase production by these two R factors.

Next, R_{M201} and R_{M201-2} in W3630 were transferred to ML1410 by conjugation, and the genetic properties of the conjugants were examined. As shown in Table 2, the transfer frequency of both factors was almost the same when selected for by TC, CM, or APC. All conjugants showed the same resistance patterns as those in W3630, and ML1410 R_{M201-2}^+ showed a high level of APC resistance as in W3630, indicating that high levels of APC resistance were conferred by the R_{M201-2} factor itself.

Transduction of R_{M201-2} to ML1410 with phage P1 showed that most of the transductants had

FIG. 2. Relation between the level of APC resistance and the β -lactamase activity in W3630 carrying various R-factor mutants. Six strains of W3630 R^+ showing various levels of APC resistance were randomly selected from those obtained in the experiment shown in Fig. 1. After three successive single-colony isolations on the plates containing the same concentration of APC as that used for selection, drug resistance and β -lactamase activity were determined.

five-drug resistance, as in W3630, even when selected by TC, CM, or APC (Table 3). When selected by either TC or APC, 4 to 8% of the transductants were found resistant to both TC and APC. All of the transductants from W3630 R_{M201-2}^+ showed as high a level of APC resistance as in the donor strain, indicating that the high APC resistance conferred by R_{M201-2} could be explained by mutation on the site responsible for APC resistance on R_{M201} factor.

Various strains carrying R factors conferring APC resistance were randomly picked from our stock cultures of clinical origin, and the occurrence of R-factor mutants carrying high APC resistance was examined after growth on BTB agar containing 1,600 μ g of APC/ml. As shown in Table 4, we could easily demonstrate mutants showing a high level of APC resistance at a frequency of 10^{-7} to 10^{-5} , and this resistance was conjugally transferred by mixed cultivation with another resistance marker(s) as well as with the original R factors. These results indicated that the R-factor mutants carrying high APC resistance could be readily demonstrated along with wild-type R factors carrying a low level of APC resistance.

According to the results shown above, the mutation of R factors from a low to a high level of APC resistance can be explained by either hyperreplication of the gene (*amp*) responsible for APC resistance or hyperproduction of the messenger ribonucleic acid (RNA) coding the formation of penicillinase. If the former assumption is true, the recombination frequency is increased between the hyperreplicated *amp* genes and an episome that exists in a cell. To investigate the possibilities, W3630 $T-tet^+ R_{M91}^+$ was obtained by superinfection of both factors and was plated on BTB agar containing only TC (25 μ g/ml) or both TC (25 μ g/ml) and APC (1,600 μ g/ml). After 18 h of incubation at 37 C, five colonies were randomly picked from each plate, mixed together in L broth, and used as the donor of transduction. As shown in Table 5, all transductants selected by TC from W3630 $T-tet^+ R_{M91}^+$ after treatment with only TC were found to be resistant only to TC. By contrast, 96% of the transductants from the donor after treatment with both TC and APC were resistant to both TC and APC, even when selected only by TC, indicating the extremely high rate of recombinant formation between *T-tet* and the *amp* gene(s) in the W3630 $T-tet^+ R_{M91}^+$ donor. A possible interpretation of these findings is (i) that the *amp* gene of R_{M91} factor is hyperreplicated, resulting in the appearance of the mutants showing various levels of APC resistance, and (ii) that the cells carrying both *T-tet* factor and the multiple copies of the *amp* gene are selected for by inoculating W3630 $T-tet^+ R_{M91}^+$ in the medium containing both TC and a high concentration (1,600 μ g/ml) of APC. Consequently, the recombinants between *T-tet* factor and the *amp* gene(s) are formed at extremely high frequencies.

It should be noted further that the mutants showing various levels of APC resistance can be demonstrated at a high frequency by inoculating the strains harboring an R factor conferring APC resistance in the media containing various concentrations of APC. The demonstration of R-factor mutants showing high and stable APC resistance can be interpreted by the prediction that the *amp* gene of the R factor is replicated at hyper-rates, resulting in the appearance of mutants showing various levels of APC resistance, and the multiple copies of the *amp* gene become stable after integration into the R-factor genome. This possibility was investigated by infecting the *rec^-* and *rec^+* strains with R_{M201} carrying APC resistance and comparing the frequency of occurrence of R-factor mutants carrying high and stable APC resistance on plates containing various concentrations of APC. The number of colonies that appeared on the plates

TABLE 1. Levels of drug resistance and drug-inactivating activities in various hosts carrying a mutant R_{M201-2} ^a

Host	R factor	Level of resistance				β -Lactamase activity (APC)	CATase activity
		TC	CM	SM	APC		
<i>E. coli</i> W3630	R_{M201}	100	200	12.5	100	89.3	0.803
	R_{M201-2}	100	200	12.5	$\geq 3,200$	1,035.4	0.750
<i>E. coli</i> ML1410	R_{M201}	50	200	25	200	103.3	0.626
	R_{M201-2}	50	200	25	$\geq 3,200$	1,665.1	0.720
<i>E. coli</i> AB2463	R_{M201}	100	100	— ^b	100	102.2	0.752
	R_{M201-2}	100	100	—	$\geq 3,200$	1,055.2	0.739
<i>S. typhimurium</i> LT ₂	R_{M201}	50	200	—	200	98.2	0.640
	R_{M201-2}	50	200	—	$\geq 3,200$	1,328.1	0.670

^a R_{M201-2} is one of the mutants obtained in the experiment shown in Fig. 1 and purified by single-colony isolation on a plate containing 3,200 μ g of APC/ml. The R factors were transferred by conjugation from their original host W3630 to various strains. TC = tetracycline; CM = chloramphenicol; SM = streptomycin; APC = ampicillin; CATase = chloramphenicol acetyltransferase.

^b SM resistance was not determined because of SM resistance of both host strains.

TABLE 2. Conjugal transfer of a mutant R_{M201-2} ^a

Donor ^b	Recipient	Selected on the plate containing	Transfer frequency	Genetic properties of conjugants	
				Resistance patterns ^c	Levels of APC resistance (μ g/ml)
W3630 R_{M201-2}^+	ML1410	TC	6.3×10^{-5}	TC.CM.SM.SA.APC (100)	$\geq 3,200$ (50/50) ^d
		CM	4.1×10^{-5}	TC.CM.SM.SA.APC (100)	$\geq 3,200$ (50/50)
		APC	6.2×10^{-5}	TC.CM.SM.SA.APC (100)	$\geq 3,200$ (50/50)
W3630 R_{M201}^+	ML1410	TC	2.4×10^{-5}	TC.CM.SM.SA.APC (100)	200 (50/50)
		CM	2.1×10^{-5}	TC.CM.SM.SA.APC (100)	200 (50/50)
		APC	2.4×10^{-5}	TC.CM.SM.SA.APC (100)	200 (50/50)

^a After 40 min of incubation at 37 C, the mixed culture was streaked on BTB agar containing both nalidixic acid (50 μ g/ml) and each of the selective drugs.

^b See footnote a of Table 1.

^c One hundred colonies developing on the selective plate were picked and their resistance patterns were examined after single-colony isolation on plates containing the same selective drugs. The number in parentheses indicates the percentage of conjugants carrying the resistance patterns shown. For drug abbreviations, see footnote a of Table 1.

^d Denominator indicates the total number of colonies tested and numerator indicates the number of colonies carrying the indicated APC resistance.

containing various concentrations of APC varied, and the number of colonies developed from AB2463 $rec^- R_{M201}^+$ was found to be about 100-fold less than that from AB1157 $rec^+ R_{M201}^+$. But the high APC resistance of mutants developed from AB2463 $rec^- R_{M201}^+$ was easily lost after growth, and we did not find any mutants showing high and stable APC resistance among those examined. By contrast, mutants showing high and stable APC resistance developed from

AB1157 $rec^+ R_{M201}^+$ at a frequency of 10^{-4} to 10^{-7} , and the stable and high APC resistance of such mutants was transmissible by conjugation (Table 6).

DISCUSSION

Epidemiological studies from this laboratory have shown that R factors isolated from clinical specimens had high and stable APC resistance, and their resistance was due to the increase in the

TABLE 3. Transduction of a mutant *R*_{M201-2} with phage *P*1

Donor ^a	Recipient	Selected on the plate containing	Transduction frequency	Genetic properties of transductants	
				Resistance patterns ^b	Levels of APC resistance (μg/ml) ^c
W3630 <i>R</i> _{M201-2} ⁺	ML1410	TC	2.3×10^{-6}	TC.CM.SM.SA.APC (92)	≥ 3,200 (50/50)
				TC.APC (8)	≥ 3,200 (8/8)
		CM	2.8×10^{-6}	TC.CM.SM.SA.APC (100)	≥ 3,200 (50/50)
		APC	4.9×10^{-8}	TC.CM.SM.SA.APC (93)	≥ 3,200 (50/50)
W3630 <i>R</i> _{M201} ⁺	ML1410			TC.APC (7)	≥ 3,200 (7/7)
		TC	3.3×10^{-6}	TC.CM.SM.SA.APC (96)	200 (50/50)
				TC.APC (4)	200 (4/4)
		CM	3.7×10^{-6}	TC.CM.SM.SA.APC (100)	200 (50/50)
		APC	4.1×10^{-8}	TC.CM.SM.SA.APC (95)	200 (50/50)
				TC.APC (5)	200 (5/5)

^a See footnote a of Table 1.^b One hundred colonies developing on each of the selective plates were picked, and their resistance patterns were examined after single-colony isolation on plates containing the same selective drug. The number in parentheses indicates the percentage of colonies carrying the resistance patterns shown. For drug abbreviations, see footnote a of Table 1.^c Denominator indicates the total number of colonies tested and numerator indicates the number of colonies carrying the APC resistance shown.TABLE 4. Demonstration of *R* factor mutants with a high level of ampicillin (APC) resistance^a

R factor	Source	Mutants showing high levels of APC resistance			Genetic properties of R factor mutants ^d	
		Isolation ^b frequency	Levels of APC resistance (μg/ml) ^c		Resistance patterns	Levels of APC resistance (μg/ml)
			Before mutation	After mutation		
<i>R</i> _{M20} (KM.APC)	<i>P. rettgeri</i>	1.2×10^{-6}	200	1,600	KM.APC	1,600
<i>R</i> _{M30} (TC.CM.SM.SA.KM.APC)	<i>S. flexneri</i>	1.4×10^{-6}	200	1,600	TC.CM.SM.SA.KM.APC	1,600
<i>R</i> _{M91} (SM.SA.APC)	<i>P. mirabilis</i>	3.0×10^{-6}	100	≥ 3,200	SM.SA.APC	≥ 3,200
<i>R</i> _{GN14} (TC.CM.SM.SA.APC)	<i>E. coli</i>	3.6×10^{-6}	100	1,600	TC.CM.SM.SA.APC	1,600
<i>R</i> _{GN235} (TC.CM.SM.SA.APC)	<i>E. coli</i>	3.5×10^{-7}	100	1,600	TC.CM.SM.SA.APC	1,600

^a W3630 carrying each of the R factors shown was inoculated on BHI agar plates containing 1,600 μg of APC per ml. After 18 h of incubation at 37 C, the colonies developed on plates without or with APC were counted. KM = kanamycin; TC = tetracycline; SM = streptomycin; SA = sulfanilamide.^b Isolation frequency indicates the number of colonies developed on APC plate divided by the number of colonies on a drug-free plate.^c APC resistance of each colony developed on an APC plate was determined as described in Materials and Methods.^d The R factors in W3630 R⁺ developed on APC plates were conjugally transferred to ML1410, and the resistance patterns and the levels of APC resistance in conjugants were determined.

specific activity of penicillinase (25). One of the present authors (Y.O.) also isolated an R factor carrying high APC resistance from *Proteus rettgeri*, and the level of APC resistance in an *E. coli* strain carrying this factor was 3,200 μg/ml, or more, which is about 16 times higher than that carried by other wild-type R factors showing APC resistance (unpublished data). However, the level of resistance to other drugs conferred

by these R factors was similar to that conferred by other known wild-type R factors with or without APC resistance.

During genetic studies of such R factors showing high and stable APC resistance, we demonstrated, by chance, such R-factor mutants at high frequencies by inoculating *E. coli* strains harboring a wild-type R factor of low APC resistance on plates containing high concentra-

TABLE 5. Formation of the recombinant *T-tet-amp* factor with high ampicillin (APC) resistance between the *amp* determinant and *T-tet* factor^a

Treatment of donor of transduction before selection by TC ^b	Transduction frequency	Genetic properties of transductants	
		Resistance pattern ^c	No. of transductants examined ^d
TC	4.5×10^{-6}	TC	650/650 (100)
TC+APC	5.3×10^{-6}	TC	61/1,560 (4)
		TC.APC	1,499/1,560 (96)

^a The original host, W3630 *T-tet*⁺ *R_{M91}*⁺, was obtained by mixed cultivation of W3630 *T-tet*⁺ with ML1410 *R_{M91}*⁺. For details, see Materials and Methods.

^b W3630 *T-tet*⁺ *R_{M91}*⁺ in a hetero-R state was plated on BTB agar containing both tetracycline (TC, 25 µg/ml) and APC (1,600 µg/ml) or on BTB agar containing only TC (25 µg/ml). After 18 h of incubation at 37 C, five colonies were randomly picked from each plate and mixed in L broth for the propagation of phage P1. For details, see Materials and Methods.

^c The resistance pattern of the donor was TC.SM.SA.APC.

^d Denominator indicates the number of transductants examined and numerator indicates the number of transductants carrying the indicated resistance pattern. The number in parentheses indicates the percentage of colonies carrying the resistance patterns shown. The transferability of resistance of 160 randomly selected transductants was examined by the cross-brushing method with W3630, and in all resistance was transferable.

TABLE 6. Mutation frequency of *R_{M201}* to mutants with high and stable ampicillin (APC) resistance in either *rec*⁺ or *rec*⁻ strains

Host	APC concn in selective plate (µg/ml)	Survival rate ^a	Mutants showing high and stable APC resistance	
			Isolation frequency ^b	Transmissibility ^c
AB2463 <i>rec</i> ⁻	400	2.1×10^{-6}	$<4 \times 10^{-8}$	
	800	1.3×10^{-7}	$<3 \times 10^{-9}$	
	1,600	3.8×10^{-8}	$<2 \times 10^{-9}$	
AB1157 <i>rec</i> ⁺	400	1.1×10^{-4}	1×10^{-4}	+
	800	6.5×10^{-5}	4×10^{-5}	+
	1,600	8.9×10^{-7}	5×10^{-7}	+

^a The number of colonies developed on an APC-containing plate divided by the number of colonies developed on a drug-free plate.

^b All colonies developed on each selective plate were picked, and each of them was suspended in 0.2 ml of BHI broth. After 2 h of incubation at 37 C, each culture was streaked on BTB agar plate containing the same concentration of APC as that in the first selective plate to confirm whether or not the developed mutants carried stable and high APC resistance. Isolation frequency was expressed by the number of R-factor mutants showing high and stable APC resistance divided by the number of input cells carrying *R_{M201}* on drug-free plate.

^c Fifty colonies were randomly picked from each group of mutants and the transmissibility of their APC resistance was examined by the cross-brushing method with W3630.

tions of APC. The levels of APC resistance in such R-factor mutants generally corresponded to the concentration of APC used for selection, although the isolation frequency was decreased in parallel with the increase in the concentration of the drug.

Pearce and Meynell (26) reported that bacteria carrying an R factor conferring resistance to low concentrations of SM frequently gave highly

resistant variants, but they mentioned that the genes responsible for the high resistance were located either in the chromosome or in an R factor. The high and stable APC resistance conferred by those R-factor mutants obtained from wild-type R factors in the present investigation can be explained by the following possibilities: (i) an increased number of copies of the R-factor genome per bacterium, (ii) a new type of

R-factor mutant that harbors tandemly repeated sequences of the *amp* gene in its genome, (iii) an R-factor mutant capable of hyperproduction of the messenger RNA that codes penicillinase synthesis, and (iv) an increase in the specific activity of the enzyme as a result of mutation of the structural gene.

It was reported that mutation of *E. coli* K-12 R_{1a}^+ to high APC resistance occurred at a frequency of 10^{-8} to 10^{-4} after treatment with ethyl methane sulfonate and was accompanied by a simultaneous increase in resistance to CM, SM, and SA (22). Increased APC resistance was due to increased penicillinase activity of the bacteria. Further studies indicated that the increased resistance was due to a two- to fourfold increase in the number of R-factor copies per chromosome, and the production of drug-inactivating enzymes was linearly correlated to the gene dosage (23). Kontomichalou et al. (15) also reported that penicillinase assays of cultures of *P. mirabilis* strains carrying an R factor of high APC resistance at different phases of growth showed a correlation between the estimated number of R-factor copies present as covalently closed circular molecules and the enzyme activity per cell.

It was reported that the colicinogenic factor E_1 in *E. coli* JC411 continued to replicate by a semiconservative mechanism in the presence of CM in the medium for 10 to 15 h, long after chromosomal deoxyribonucleic acid (DNA) synthesis had terminated (3). On the basis of preparative buoyant density-equilibrium centrifugation, it was concluded that the number of copies of Col E_1 DNA molecules that accumulate under these conditions approached about 3,000 copies per cell, representing a 125-fold increase over the normal level of 24 copies per cell (3).

In our studies of R-factor mutants, the level of resistance to other drugs, i.e., TC, CM, SM, and SA, which was conferred by these R-factor mutants capable of hypersynthesis of penicillinase was found not to be distinct from that conferred by their parent R factors. Similarly, we could not find any difference in CM acetyltransferase activity between mutant R factors and the parent R factor. Consequently, the first possibility, i.e., an increased number of copies of the R factor genome per bacterium, can be excluded.

We could demonstrate at high frequencies mutants showing various levels of APC resistance, but not resistance to other drugs, when *E. coli* strains carrying an R factor capable of a low level of penicillinase synthesis were inoculated in media containing various concentrations of APC. It should be noted that the APC resistance in such

mutant R factors was stable. Similarly, we could demonstrate at high frequencies recombinants between T-tet factor and the *amp* gene(s) derived from R factor which showed high and stable APC resistance. High frequencies of recombinant formation between T-tet (or R) factor and the *amp* gene(s) after growth in APC-containing medium could be accounted for by the presence of the extra copies of the *amp* gene in a cell. The inability to isolate recombinants between T-tet (or R) factor and the *amp* gene(s) in the *rec*⁻ host strongly suggests that the recombination mechanism is necessary to obtain such mutants possessing high APC resistance.

Enzymological studies did not show any differences except for their specific enzyme activities between the β -lactamases mediated by R_{M91} factor and T-tet (or R) factors carrying high and stable APC resistance. It was found further that the amount of mutant R_{M201} factor DNA carrying *amp*-hyper increased about two- to threefold over that of the parent R factor, and the closed circular DNA of the mutant R factor was sedimented faster than that of the parent R factor in alkaline sucrose gradient centrifugation, indicating that the size of the DNA of R_{M201} had been increased (unpublished data). These preliminary results also support the assumption of the selective application of the *amp* gene on R factor and of the subsequent integration of the *amp* gene(s) into the coexisting R (or T-tet) factor. The third and fourth possibilities, i.e., the effective production of messenger RNA caused by a change of the regulatory gene and the changes in the specific activity of the penicillinase caused by mutation of the structural gene, can be excluded.

When *P. mirabilis* NR1⁺ was cultured in drug-free medium for a long period, NR1 DNA formed a single satellite band with a density of 1.711 g/cm³. After prolonged growth in medium containing any of the drugs to which NR1 conferred resistance (except TC), a much larger satellite band with a density of 1.718 g/cm³ was observed. These two types of density profiles were found to be interconvertible, depending on the conditions of cell culture called "transition" (8). Studies of the density profiles of NR1 (renamed R_{100}), showed that it consisted of two DNA components with densities of 1.711 g/cm³ (51% guanine plus cytosine) and 1.718 g/cm³ (58% guanine plus cytosine; 5, 8, 21). Thus, the production of the extra copies of the *r* determinant conferring (CM.SM.SA) resistance caused an increase in resistance to SA, SM, and CM (accompanying an increase in the CM acetyltransferase activity) but did not influence TC resistance. The extra copies of the *r* determinant were, however,

unstable and thus were diluted passively by cell division in drug-free medium.

Thus, the demonstration of the R-factor mutants showing stable and high APC resistance may be explained by the following genetic events: (i) the extra copies of the *amp* gene are produced from an R factor carrying a low level of APC resistance and (ii) the repeating sequences of the *amp* gene are integrated in the R-factor (or T-tel factor) genome, resulting in the formation of a new episome showing stable and high APC resistance. These predictions can be supported also by the findings that the extra segments carrying the *lac* region in *E. coli* capable of hypersynthesis of β -galactosidase resulted in the hypersynthesis of the enzyme and were located in the chromosome near T6 and *pro* markers (11).

It was reported that the addition of CM or growth without glucose caused a random dissociation and association of ColE₁ and "relaxation" protein during or between rounds of ColE₁ replication, resulting in an increase of the copies of ColE₁ in a cell (4). However, it is not clear whether an increase in the copies of the *amp* gene from R factors showing APC resistance is caused after growth in APC-containing medium or whether APC merely plays a role in the selection of cells carrying extra copies of the *amp* gene. The hyperreplication of the *amp* gene could be seen in many R factors randomly selected from our stock culture, which produced either type I or type II penicillinase, but it is not known why the *amp* gene on R factor is selectively replicated at hyper-rates.

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