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R-Factor Mutant Capable of Specifying Hypersynthesis of Penicillinase

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The physical characteristics of a mutant, R_{M201-2}, capable of conferring high and stable ampicillion resistance was analyzed. The R_{M201-2} and its parent R-factor deoxyribonucleic acid (DNA) could be isolated as an extrachromosomal and covalently closed circular form. Their buoyant densities were both 1.712 g/cm^3, and their molecular weights were about 82 $\times$ 10^6 and 64 $\times$ 10^6, respectively, when measured by CsCl and sucrose density gradient analyses. The contour lengths by electron microscopy were 35.9 $\pm$ 0.6 and 31.0 $\pm$ 0.6 μm, respectively. By using the extracted R-factor DNA, the mutant and parent characters were transformable to another *Escherichia coli* strain. The mutant R factor showed an increased amount of DNA even after conjugal transfer to *Proteus*. An increase in the size of R-factor DNA was thus considered to be the cause of the high level of ampicillin resistance.

In a previous paper (19), we analyzed an R-factor mutant that showed a high level of ampicillin (AMP) resistance. The phenotype did not change after conjugal transfer or transduction of the R factor. The high level of AMP resistance was caused by a high specific activity of β-lactamase whose quality was the same as that coded by the parent R factor, indicating that the structure gene did not change. The possibility of an increased number of R copies as was reported by Nordström et al. (17) or Morris et al. (16) was excluded, since only the ampicillin gene (*amp*) was affected. Two alternative possibilities remained. One was an effective transcription or translation of the *amp* gene as was reported for β-galactosidase (1), and the other was that repeats of the *amp* gene caused a gene dosage effect as in a case of transition reported by Rownd et al. (7, 22). The latter possibility was strongly suggested by two genetic results: (i) the mutation did not occur in a *rec*$^-$ host; and (ii) when a hypermutant was formed in a cell carrying two R factors, highly effective transposition of an *amp* gene from one R factor to the other was observed. These data proved a necessity of recombination and the existence of *amp* gene amplification in the course of hypermutant formation. This paper presents molecular analyses to demonstrate a larger size of the mutant R factor as a possible result of recombinational dissociation and reassociation.

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MATERIALS AND METHODS

Bacterial strains and R factors. The substrains of *Escherichia coli* K-12 W3630 *mal*, ML1410 *met nal*^{r} (resistance to nalidixic acid), and *Proteus mirabilis* ϕS38 *thy gal tet*^{s} were used. The R factors R_{M201} and its mutant R_{M201-2} were described previously (19). *E. coli* K-12 Km605 λdv-1 was kindly given by K. Matsubara, School of Medicine, Kyushu University, and used for the preparation of a marker deoxyribonucleic acid (DNA) of known size.

Media. Brain heart infusion broth was used for the propagation of bacteria and conjugation experiment. For the determination of drug resistance, heart infusion agar (Eiken, Tokyo) and peptone water were used. Peptone water consisted of 10 g of peptone, 5 g of NaCl, and 1,000 ml of distilled water (pH 7.0). Semisynthetic medium (13) was used for the assay of resistance to sulfanilamide (SA). Eosin methylene blue-maltose agar (15) containing tetracycline (TC) (25 μg/ml), chloramphenicol (CM) (25 μg/ml), or AMP (25 μg/ml) was used as a selective medium for the transformation with R-factor DNA and for the R-factor transfer from *E. coli* W3630 R$^+$ to ML1410. CY medium consisted of a minimal salts medium containing 0.2% Casamino Acids (Difco), 0.1% yeast extract, and 0.5% glucose (15). Minimal medium consisted of K_2HPO_4, (7 g), KH_2PO_4 (3 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), $(NH_4)_2SO_4$ (1 g), trisodium citrate (0.5 g), glucose (5 g), and distilled water (1,000 ml) (pH 7.0). AG agar medium consisted of medium A (1,000 ml) (8), glucose (10 g), nicotinic acid (1 g), 0.2% bromothymol blue (40 ml), and agar (14 g). AG agar containing TC (25 μg/ml) and thymine (20 μg/ml) was used for the R-factor transfer from ML1410 R$^+$ to *P. mirabilis* ϕS38.

Drugs. CM, TC, dihydrostreptomycin (SM), (SA), AMP, and nalidixic acid were all working standards

for the assay of drug resistance.

Determination of drug resistance and conjugal transfer of R factor. The methods were the same as described previously (20).

CsCl density gradient centrifugation of R-factor DNA. R factors were transferred to *P. mirabilis* φS38, and DNA from the R^+ bacteria was analyzed by CsCl density gradient equilibrium centrifugation. The cells were grown in Penassay broth and harvested after 8 h in shaking culture in the stationary phase. Six billion cells (about 3 ml) were centrifuged and washed in 3 ml of cold saline ethylenediaminetetraacetate (EDTA) (0.1 M disodium EDTA, 0.1 M NaCl, pH 8.0). After centrifugation, the cells resuspended in 1.0 ml of cold saline-EDTA were lysed by adding 0.08 ml of 25% sodium dodecyl sulfate and incubated for 10 min at 37 C. A 0.12-ml amount of 3 M sodium acetate (pH 8.0) was added to each lysate. After gentle mixing, 0.64 ml of isopropanol was overlaid, and the DNA was collected on a small glass rod and dissolved in 1.0 ml of 0.1 SSC, 0.015 M NaCl and 0.0015 M trisodium citrate, pH 7.0. The samples were heated at 65 C for 30 min in order to inactivate any nucleases that might be present in the DNA preparations, and were stored at 4 C. A sample of 8 to 16 μg of DNA was added to 0.8 ml of saturated CsCl (Nakarai, optical grade) solution in 0.02 M tris(hydroxymethyl)aminomethane (pH 8.0), and the final volume was adjusted to 1.00 ml by addition of 0.1 SSC (0.15 M NaCl + 0.015 M sodium citrate). The amount of DNA used for centrifugation was selected so that the maximal intensities of the chromosomal DNA bands were within the linear range of film response. The DNA solutions were centrifuged in a Spinco model E analytical ultracentrifuge at 44,000 rpm at 25 C for 20 h. For the ultraviolet absorption photograph, Kodak commercial film was used. The absorbance photographs were traced with a Canalco model E microdensitometer. Buoyant densities were calculated as described by Schildkraut et al. (25), using light *P. mirabilis* chromosome DNA (1.700 g/cm^3) as a reference density.

Lysis of radiooactive culture and ethidium bromide-CsCl gradient centrifugation. Cell lysis of the *E. coli* R^+ strain was performed by the lysozyme-Brij 58-Sarkosyl technique as described by Cohen and Miller (6), with some minor modifications. *E. coli* K-12 strains were grown overnight at 37 C in CY medium in an L tube and incubated with aeration to obtain about 2×10^8 cells/ml. Deoxyadenosine (250 μg/ml) and [^{3}H]thymidine (10 μCi/ml) or [^{14}C]thymidine (2 μCi/ml) were added to the culture and incubation with aeration continued until a cell concentration of 8×10^8 cells/ml was achieved. The cells were then sedimented, washed twice in TES [0.05 M tris-(hydroxymethyl)aminoethane, 0.05 M NaCl, and 0.005 M EDTA, pH 8.0] buffer, and resuspended in 0.1 ml of a solution containing 25% sucrose in TES buffer. One-tenth milliliter of lysozyme (10 mg/ml) and 0.5 M EDTA (pH 8.0) were added to the cell suspension, and the cells were incubated for 10 min at 25 C. The resulting spheroplasts were treated with Brij-58 (final concentration, 0.5%) and incubated for an additional 10 min at 25 C. Lysates were then incubated with ribonuclease (final concentration, 50 μg/ml) and with Sarkosyl (final concentration, 0.25%) at 37 C for 60 min to achieve complete lysis. The resulting lysates were sheared by drawing up and down slowly with a 1-ml pipette 15 times. One milliliter of the sheared lysate thus produced and 2.0 ml of ethidium bromide solution (700 μg of ethidium bromide/ml in water) were added to 4.0 ml of sterile water in a centrifuge tube, and then crystalline CsCl (6.55 g) was added and gently mixed. The mixture was ultracentrifuged at 44,000 rpm for 40 h at 20 C with a Hitachi RT65T angle roter in a Hitachi 65P preparative ultracentrifuge. Each 0.1-ml fraction was collected from the bottom of the tube into small test tubes. The fractions whose radioactivity showed a denser satellite band were pooled, cleared of ethidium bromide by extraction with *n*-octanol, and dialyzed against SSC. Solutions containing covalently closed circular (CCC) DNA were thus prepared and stored at 2 C.

Sucrose gradient centrifugation. Sucrose gradient centrifugation was performed in an RPS65T swinging-bucket rotor at 15 C for 60 min with a Hitachi 65P centrifuge. A mixture of [^{3}H]R_{M201}, [^{14}C]R_{M201-2}, and [^{3}H]λdv-1 DNA (0.1 ml) was layered on 4.8 ml of 5 to 20% (wt/vol) linear sucrose gradient in TES buffer. Two-drop fractions were collected from the tube directly on filter papers, washed, and dried. The radioactivity was counted as reported by Goebel and Schrempf (9).

Sedimentation constants were calculated by assuming a linear relationship between the distance sedimented and the sedimentation coefficient. Molecular weights were calculated from the $s_{20,w}$ value according to the data of Clayton and Vinograd (2). The marker λdv DNA is 31*S* in the CCC form and the molecular weight is 8.6×10^6 (K. Matsubara, personal communication).

Electron microscopy. The R-factor DNA in TES buffer was mixed with an equal volume of a 0.04% solution of cytochrome *c* and twice this volume of 4 M ammonium acetate. About 0.01 ml of this solution was spread on the surface of distilled water. The monolayer of cytochrome *c*, including the concerned DNA, was transferred to a specimen grid coated with carbon and shadowed with platinum-palladium at a distance of 4 cm by an angle of 6°. The preparations were examined with a Nihondenshi electron microscope, JEOL type 7. Their contour lengths were measured by enlarging the photographs of the circular DNA molecules. The molecular weight was calculated as 2.07×10^6 per μm of contour length (3, 14).

Transformation experiments. The methods described by Cohen et al. (5) were followed. CCC R-factor DNA prepared by ethidium bromide-CsCl gradient centrifugation was used in this experiment. *E. coli* W3630 was grown at 37 C in brain heart infusion broth to an optical density of 0.85 at 590 nm. The cells were then chilled quickly, sedimented, and washed once with 0.5 volume of 10 mM NaCl. The pellet was resuspended to the original volume of cold 0.03 M $CaCl_2$, kept at 0 C for 20 min, harvested, and then resuspended in 1 ml of 0.03 M $CaCl_2$ solution. A 0.2-ml amount of the competent cells was mixed with 0.2 ml of chilled DNA sample in SSC supplemented

with 0.1 volume of 0.3 M $CaCl_2$. After 60 min of incubation at 0 C, the mixture was subjected to a heat pulse at 42 C for 2 min and then plated directly onto eosin methylene blue-maltose agar containing TC (25 μg/ml), CM (25 μg/ml), or AMP (25 μg/ml).

RESULTS

As reported in a previous paper, R_{M201-2} was originally isolated from colonies developed on a nutrient agar plate containing 3,200 μg of AMP per ml on which a log-phase culture of *E. coli* W3630 $R_{M201}{}^+$ had been plated. The experiment was repeated and another mutant, R_{M201-3}, was isolated at a similar frequency of 10^{-8}. These mutants conferred similarly high levels of AMP resistance on a different *E. coli* host, ML1410, after a conjugal transfer. Only AMP resistance was affected in these mutants. Biochemical properties of β-lactamases produced by these R factors are shown in Table 1. Qualitatively, β-lactamases coded by R_{M201-2} and R_{M201-3} were not different from that coded by the parent, R_{M201}. The 13- to 14-fold increase in the specific enzyme activity of the mutants paralleled the increase in the level of AMP resistance. W3630 $R_{M201-2}{}^+$ was used for further molecular analyses.

TABLE 1. *Comparison of β-lactamase produced by R_{M201} and its hypermutants*[a]

Biochemical properties of β-lactamase	R_{M201}	R_{M201-2}	R_{M201-3}
Level of AMP resistance in *E. coli* W3630 (μg/ml)	200	3,200	3,200
Specific enzyme activity (units/mg/protein)	67	888	963
Substrate specificity[b]			
PC-G	100	100	100
AMP	132	125	127
PE-PC	27	29	26
6-APA	97	101	103
MCI-PC	1.6	1.5	1.8
CER	137	137	156
Optimal pH[c]	6.5-7.0	6.5-7.0	6.5-7.0
Optimal temperature[d] (C)	45	45	45
Rate of heat inactivation[e]	Similar	Similar	Similar
Isoelectric point[f]	5.2	5.3	5.2

[a] Method of preparation and assay of the crude enzyme were as described previously (19).

[b] Rate of hydrolysis of five penicillins and cephaloridine were expressed as percentage of hydrolysis of penicillin G. Abbreviations: PC, penicillin; PC-G, penicillin G.

[c] Acetate buffers (0.1 M) were used for pH range of 5.0 to 5.9, and 0.1 M phosphate buffers were used for pH range of 6.0 to 8.9.

[d] Enzyme solution in 0.1 M phosphate buffer, pH 7.0, containing 8 mM AMP as substrate was incubated for 10 min at each temperature.

[e] Enzyme solution in 0.1 M phosphate buffer (pH 7.0) was incubated at 60 C. After various time intervals a portion of the sample was withdrawn and immediately frozen, and the residual enzyme activity was estimated iodometrically.

[f] Determination was carried out by agar gel electrophoresis as reported by Sawai et al. (23).

E. coli W3630 carrying either RM201 or RM201-2 was grown in a [^{3}H]- or [^{14}C]thymidine medium, lysed, and ultracentrifuged in a dye-buoyant density gradient. The DNA profile from an *E. coli* strain carrying either R_{M201} or R_{M201-2} had a distinct satellite CCC DNA band whose amount was 4% of the total DNA with R_{M201} and 6% with R_{M201-2} (Fig. 1). No such satellite band was present with DNA from an R^- strain. The mutant R factor comprised much more of the CCC DNA than did the parent R factor.

To examine the size difference between the parent and the mutant R factor, the CCC DNA fractions of [^{3}H]R_{M201} and [^{14}C]R_{M201-2} were mixed and analyzed on a 5 to 20% sucrose gradient (Fig. 2). Each R-factor DNA sedimented as two fractions that were assumed to correspond to CCC and open circular fractions. The calculated mean $s_{20,w}$ values for CCC and open circular peaks of [^{14}C]R_{M201-2} DNA were 84.1*S* and 55.3*S*, respectively, and the molecular weight was calculated as 82×10^6. As for R_{M201} DNA, the rapidly sedimenting band was 75.7*S* and assumed to be the CCC form. The molecular weight was calculated as 64×10^6. According to these findings, the significant difference in *S* value between R_{M201} and its mutant R_{M201-2} factor accounts for an increase in the size of the mutant R-factor DNA derived from the R_{M201} factor.

The DNA samples from the dye-buoyant density gradient were then examined by electron microscopy (Fig. 3A and B). The average contour lengths of open circular molecules of R_{M201-2} and R_{M201} were 35.9 ± 0.6 and 31.0 ± 0.6 μm, respectively. The molecular weights of R_{M201-2} and R_{M201} were calculated as about 74×10^6 and 64×10^6, respectively. Thus, the mutant R factor R_{M201-2} was much larger in size than the parent R_{M201} factor.

The DNA samples prepared by dye-buoyant density gradient centrifugation and used for the analyses of the size determination were then used for transformation of *E. coli* W3630. The transformation frequency of both R-factor DNAs was almost the same when selected with TC, CM, or AMP (Table 2). Most of the transformants had five-drug resistance even when selected with either TC or AMP, and 0.5 to 1.7% of the transformants were found to be segregants resistant to only TC and AMP or to AMP alone. All of the transformants with R_{M201-2} DNA showed a level of AMP resistance

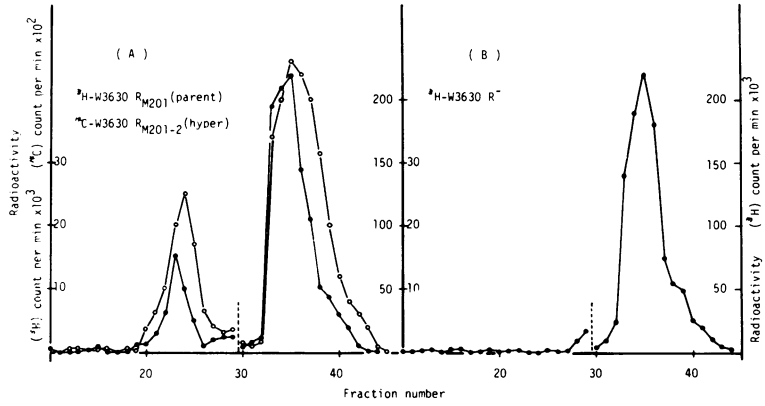

FIG 1. *Ethidium bromide-CsCl gradient analysis of E. coli carrying either* R_{m201} *or its hypermutant. Radioactive cultures were lysed and analyzed separately on an ethidium bromide-CsCl gradient. After centrifugation, fractions were collected from the bottom of the tube in small vials. Portions of each fraction (0.01 ml) were spotted on filter disks and assayed for radioactivity. (A) Strains of W3630 labeled with [*^3H*]thymidine. Symbols: O,* R_{M201-2}^+ *W3630 labeled with [*^{14}C*]thymidine; ●,* R^- *and* R_{M201}^+ *W3630 were labeled with [*^3H*]thymidine.*

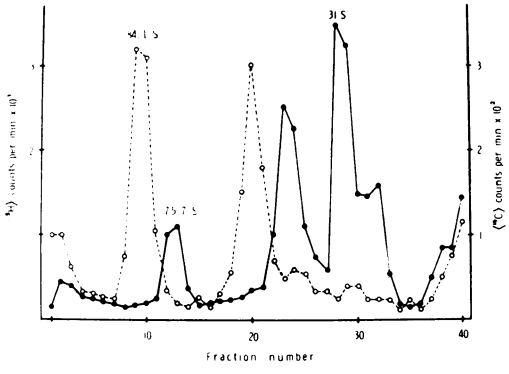

FIG. 2. *Neutral sucrose gradient centrifugation of R-factor DNA from E. coli. Radioactive DNA of* ^3H*-labeled* R_{M201} *and* ^{14}C*-labeled* R_{M201-2} *was collected by pooling satellite fractions shown in Fig. 1 (no. 21 to 25 for* R_{M201}*, no. 20 to 26 for* R_{M201-2}*). Each 0.05-ml sample of both R-factor DNAs was mixed, layered onto 4.8 ml of a neutral 5 to 20% sucrose gradient, and centrifuged for 60 min at 45,000 rpm in a RPS65T rotor at 15 C. The λdv-1 DNA (*$s_{20,w}$ *31S) was used as a marker. Symbols: ●,* ^3H*-labeled* R_{M201} *DNA and λdv-1 DNA; O,* ^{14}C*-labeled* R_{M201-2} *DNA.*

as high as in strain from which the R-factor DNA had been prepared, indicating that the increased number of *amp* genes was stably integrated in the R-factor genome. The R-factor DNAs were then analyzed in *Proteus*. R_{M201} and R_{M201-2} were transferred first to ML1410 and then to *P. mirabilis* φS38. TC was used as a selective drug. The transfer frequency was 10^{-7} in each case. The level of AMP resistance was 6.3 and 200 μg/ml in R_{M201}^+ and R_{M201-2}^+ *Proteus*, respectively. The levels were similarly lower than those in *E. coli*. The levels of TC, CM, or SM resistance were also lower in *Proteus* than in *E. coli*. The R_{M201-2}^+ cells were incubated in a broth containing 400 μg of AMP per ml, and the R-factor DNAs were analyzed by equilibrium centrifugation in a CsCl gradient (Fig. 4). A *P. mirabilis* φS38 R_{M201-2}^+ strain possessed two detectable DNA species (Fig. 4A). The major band of DNA corresponded to *P. mirabilis* chromosomal DNA, whose buoyant density was 1,700 g/cm³ (guanine plus cytosine, 40%). The satellite band of R_{M201-2} DNA, which was about 16% as large as the chromosomal DNA band exhibited a buoyant density of 1.712 g/cm³ (guanine plus cytosine, 52%). The R_{M201} satellite DNA exhibited the same density of 1.712 g/cm³, but the amount of the satellite band was about 7% that of the chromosome (Fig. 4B).

The same culture from which DNAs were isolated was examined for the level of drug resistances. CM and SM resistance did not differ, but the level of AMP resistance was 400 μg/ml by R_{M201-2} and 6.3 μg/ml by R_{M201}. There was a 64-fold difference between the two. The level of CM and SM resistance did not differ between the two cultures.

DISCUSSION

A physical characterization of a mutant, R_{M201-2}, capable of conferring high and stable AMP resistance was carried out. The R_{M201-2}-factor DNA could be isolated as an extrachromosomal, CCC form. Its molecular weight was about 8×10^7 by sucrose gradient analyses. The

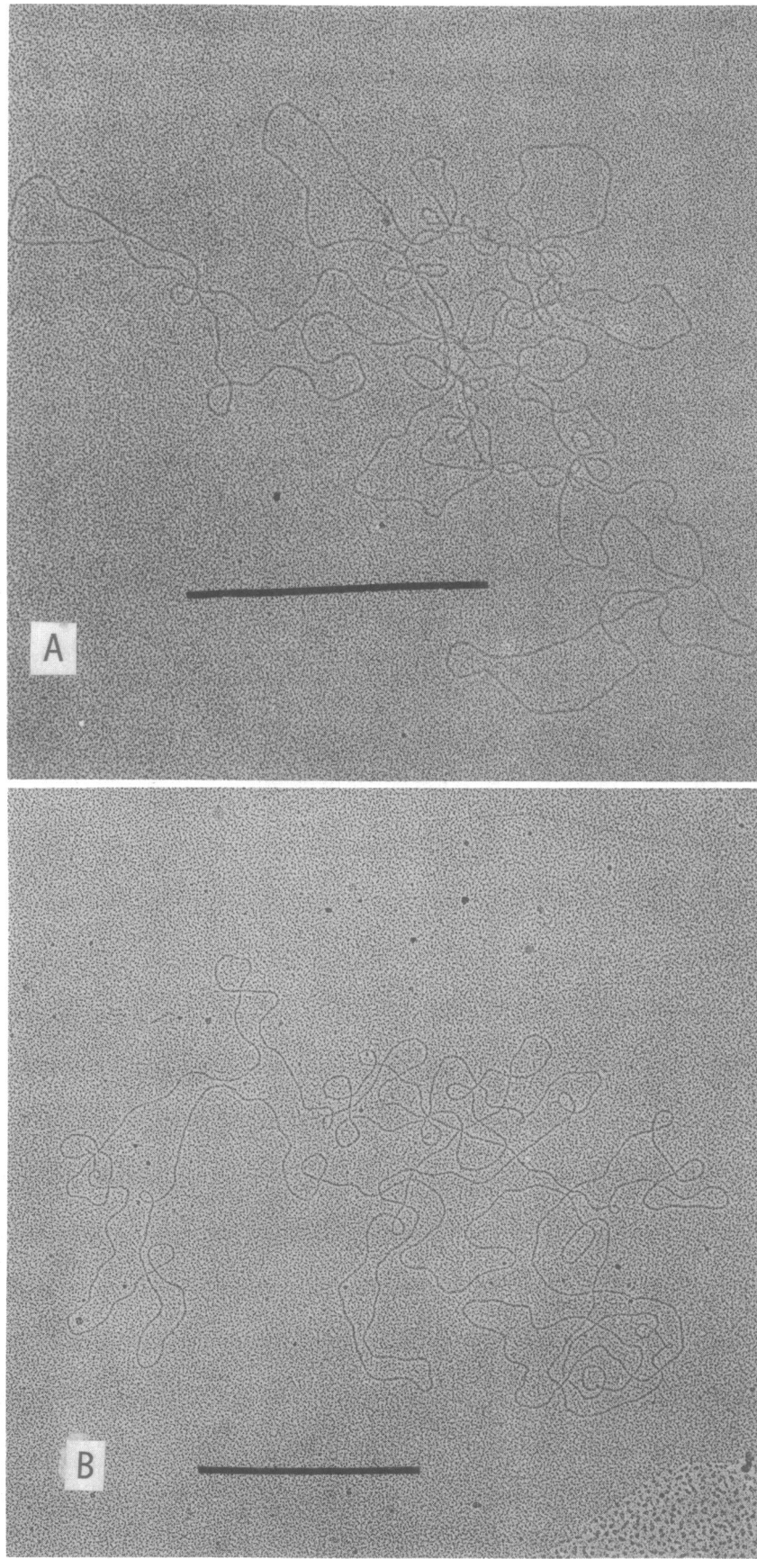

FIG. 3. *Electron micrographs of* R_{M201} *(A) and* $R_{M201\text{-}2}$ *(B) DNAs. Bar indicates 1 μm.*

TABLE 2. *Transformation of drug resistance by R-factor DNAs*

DNA[a]	Selected by	No. of transformants/10 μg of DNA	Genetic properties of the transformant			
			Resistance pattern	No. of transformants (%)[b]	Level of AMP resistance (μg/ml)[c]	Conjugal transferability[d]
R_{M201}	TC	66	TC, CM, SM, SA, AMP	195/196 (99.5)	200 (30/30)	+
			TC, AMP	1/196 (0.5)	200 (1/1)	+
	CM	11	TC, CM, SM, SA, AMP	72/72 (100)	200 (30/30)	+
	AMP	48	TC, CM, SM, SA, AMP	165/168 (98.2)	200 (30/30)	+
			TC, AMP	1/168 (0.6)	200 (1/1)	+
			AMP	2/168 (1.2)	200 (2/2)	+
R_{M201-2}	TC	96	TC, CM, SM, SA, AMP	199/200 (99.5)	≧3,200 (30/30)	+
			TC, CM, AMP	1/200 (0.5)	≧3,200 (1/1)	+
	CM	20	TC, CM, SM, SA, AMP	95/95 (100)	≧3,200 (30/30)	+
	AMP	59	TC, CM, SM, SA, AMP	182/186 (97.8)	≧3,200 (30/30)	+
			SM, SA, AMP	1/186 (0.5)	≧3,200 (1/1)	+
			AMP	3/186 (1.7)	≧3,200 (3/3)	+

[a] R-factor DNA used in this experiment was isolated from *E. coli* W3630 R^+ by ethidium bromide-CsCl gradient centrifugation, and the DNA concentration was 20 μg/0.1 ml.

[b] Denominator indicates the number of transformants examined and numerator indicates the number of transformants carrying the indicated resistance pattern.

[c] Denominator indicates the total number of colonies tested and numerator indicates the number of colonies carrying the AMP resistance shown in the table.

[d] All colonies whose AMP resistance was examined were picked, and their conjugal transferability was tested by the cross-brushing method with ML1410.

size of the DNA molecule of this mutant R factor was almost 1.3 times that of the parent R factor. By using the extracted R-factor DNA, the high level of AMP resistance was transformable to another *E. coli* strain. The mutant R factor showed an increased level of AMP resistance after conjugal transfer to *Proteus*. Increase in the size of the R-factor DNA is thus considered to be the cause of the high level of AMP resistance, and a gene dosage increase due to repeated *amp* genes on the R factor is assumed to be responsible for the hypersynthesis of penicillinase. A linear relationship between the amount of DNA and the expressed enzyme activity has been reported by Rownd et al. (21, 22), Nordstrom et al. (17), and Jacob et al. (12). If the 18×10^6-dalton difference in the DNA size between R_{M201} and R_{M201-2} corresponds to the 13-fold difference in their enzyme activities (Table 1), then the size of the *amp* segment is calculated as about 1.5×10^6 ($18 \times 10^6/12$) daltons. This size is large enough to code one R_{M201} β-lactamase whose molecular weight is 2.5×10^4 (S. Yamagishi, personal communication). The characterization of this segment in its amplifying state is necessary in order to know the mechanism of the *amp* hypermutant formation, and this is still unknown. The amount of the mutant R-factor DNA was about 1.3 times as much as that of the parent DNA in *E. coli* and 2.4 times that in *Proteus*. The discrepancy may be accountable for by the difference in selective force of ampicillin in the different host. In *Proteus*, the mutant DNA was isolated after the R^+ cells were incubated in a broth containing 400 μg of AMP per ml. The concentration might have been high enough to select cells that had undergone further gene amplification. The increase in the level of AMP resistance after the treatment of $R_{M201-2}{}^+$ cells suggests such a possibility.

For the mechanism of hyperformation of penicillinase, evidence for specific amplification of an *amp* gene and necessity of recombination was presented in a previous report (19). Dissociation of a part of the R genome including the *amp* gene might be necessary before the gene amplification. This paper presents evidence for the association of the hypersynthesizing *amp* genes in the parental R genome, resulting in the formation of a larger R factor. Thus, recombination may be necessary for dissociation and reassociation. The same density of the hyper and parental R-factor DNAs indicates a similar DNA density between the parental R_{M201} and the segment containing the *amp* gene.

In *Proteus* strains carrying the NR1 factor, Rownd et al. (7, 22) reported the phenomenon of a density shift of the R-factor DNA, termed transition, when an R^+ strain was grown for a

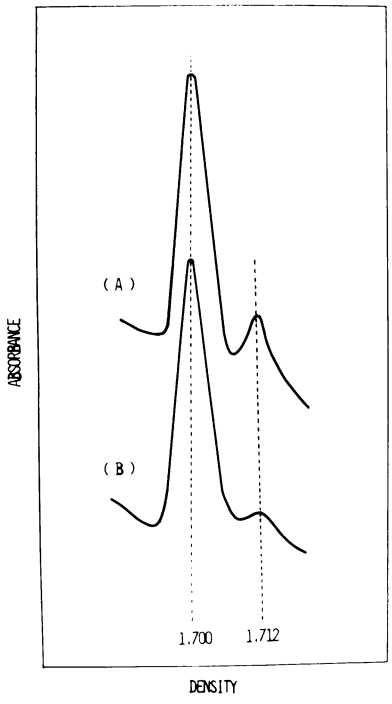

FIG. 4. *Microdensitometer tracings of an ultraviolet absorption photograph at equilibrium in a CsCl density gradient at 44,000 rpm. The ordinate represents DNA concentration as a function of distance from the axis of rotation. The cells were cultured in Penassay broth and harvested after 8 h in a shaking culture. (A) DNA from φS38 R* $_{M201-2}^{+}$*; (B) DNA from φS38* R_{M201}^{+}.

number of generations in either CM-, SM-, or SA-containing medium. Rownd assumed a dissociation of the specific genes coding CM, SM, and SA resistance, and their amplification and reassociation with each other, resulting in the density shift of R-factor DNA because of the different DNA densities between the parental R-factor DNA and the dissociated segment coding CM, SM, and SA resistance. However, the dissociated segment was easily lost after growth in drug-free medium, resulting in the loss of the shifted peak of R-factor DNA.

In our case, a segment possessing the *amp* gene is considered to have the same density as the total R-factor DNA and showed no density shift when this gene was specifically amplified. In contrast to the "transition" reported by Davies and Rownd (7), amplification of the *amp* gene reported in our studies can occur in an *E. coli* strain, and the reassociated *amp* genes in the R-factor genome are quite stable after transduction or transformation and even after a conjugal transfer from *E. coli* to *Proteus*. This difference may be accountable for by the difference between the *amp* gene and the CM, SM, and SA resistance genes, to the degree of linkage with the R-factor genes governing replication. The segment that undergoes transition is lost at a high frequency in *Salmonella* or after transduction (10), whereas AMP resistance is stable in *Salmonella*, and an AMP-sensitive transductant has never been isolated (9); close linkage of the *amp* gene with a replication locus was suggested (9). Rownd found two initiation sites of replication on an R factor, R100, one of which was located on the area undergoing transition. The other initiation point would be near the TC region, since Cohen et al. (4) isolated a miniplasmid carrying the TC gene from R6, whose TC region is almost identical to that of R100 in its base sequences (24). The reason for incapability of autonomous replication of the dissociated CM segment under physiological conditions would be too much or too little negative control, which does not allow the segment enough replication to adapt cell division. Only in *Proteus* and only under limited conditions might the segment replicate adequately. The *amp* gene of R_{M201} is closely linked to the TC gene, and the mode of replication of the *amp* segment may be similar to Cohen's TC plasmid, which has one initiation site for replication and adequate negative control. Then the occurrence and stability of a hypermutant in *E. coli* may be explained by a relatively stable replication of the *amp* segment in *E. coli* and an efficient selective force of ampicillin.

A quantitative increase in specific activity of an enzyme coded by a mutant R factor may be caused in different ways. Morris et al. (16) and Nordström et al. (17) reported mutants that had increased the number of R copies. In those cases, all of the phenotypes changed but the size of the R factor did not. Another type of quantitative increase in an enzyme activity may be caused by a mutation in a regulator gene. Bruenn and Hollingsworth (1) reported such a mutant for the β-galactosidase gene on the *E. coli* chromosome, and Sawai et al. suggested such a mechanism for an *amp* gene of wild-type R-factor R823 that expressed highly active β-lactamase (personal communication). In this case also the size of an R factor may not change.

Though the size increase in R_{M201-2} favors the recombinational dissociation-reassociation model assumed by Rownd et al. (21b), we have not yet proved that the dissociated *amp* segment replicates autonomously in a cell under physiological conditions. In a DNA sample from R_{M201}, we have with difficulty found minicircles whose size is under 5 μm by electron micros-

copy. Therefore, an R_{M201-2}^{+} *E. coli* may carry only reassociated *amp* genes. However, since the parent R_{M201} once dissociated an *amp* gene in order to form R_{M201-2}, an R_{M201-2} should dissociate *amp* genes more frequently than R_{M201} because of the increased site of homologous region in an R factor. The increase of the difference in the amount of R-factor DNA and the level of AMP resistance between the hyper and parent R factor in *Proteus* might indicate that the *amp* segment would easily dissociate and replicate autonomously in the host. Detailed analyses of this possibility are under way and the results would be published later.

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