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Composite Circular Forms of R Factor Deoxyribonucleic Acid Molecules

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Two R factors, one (*R15*) conferring resistance to streptomycin and sulfonamide (SM^rSU^r) and the other (*222/R3*) to streptomycin, sulfonamide, and chloramphenicol ($SM^rSU^rCM^r$), were transferred to a *Proteus mirabilis* strain, and deoxyribonucleic acid (DNA) extracted from these strains was subjected to density-gradient centrifugation. *R15*-DNA formed a single satellite band at a density of 1.709 g cm^{-3}. Electron microscopy of samples from this band showed circular molecules of one type, with a contour length of 18 μm (35 $\times$ 10^6 daltons). *222/R3*-DNA formed a satellite band with three peaks at densities 1.708, 1.711 and 1.717 g cm^{-3}. Electron micrographs revealed circular structures from each band with contour lengths, respectively, of 29 (54 $\times$ 10^6 daltons), 36 (68 $\times$ 10^6 daltons), and 6 μm (12 $\times$ 10^6 daltons). "Supertwisted" forms of several molecular species were found. It is suggested that *222/R3* DNA comprises either a single 36-μm molecule or two individual molecules, 29 and 6 μm in length, and that this may reflect the evolutionary development of R factors.

Multiple drug-resistance transfer factors or R factors are extrachromosomal genetic elements freely transmissible between many strains of *Enterobacteriaceae* (13). They have the properties of sex factors, enabling cells which harbor them to conjugate with other cells, as a result of which the R factor may be transferred with high efficiency, setting up a similar donor state in the newly infected cell. Since the R factor element, in addition to determinants of fertility, also carries determinants controlling resistance to a number of antibiotics, the infected cells acquire similar drug resistances. These properties, which may be freely transferred from nonpathogens, such as *Escherichia coli*, to pathogens, such as *Salmonella typhi*, account for the potential clinical importance of these entities. So far, the origin and evolution of these factors are poorly understood, although it seems clear that their incidence among strains involved in pathogenic outbreaks and the number of drugs to which one factor may carry resistance are increasing rapidly (2).

By density-gradient analysis of deoxyribonucleic acid (DNA) isolated from *Proteus mirabilis* strains [guanine plus cytosine (GC) base ratio, 39%] infected with one of a series of R factors, the molecular nature of several R factors has been identified as DNA with a GC base ratio of 50% and in some instances, DNA with a GC base ratio of 56% (6, 15). Two R factors, one (*R15*) carrying SM^rSU^r and the other (*222/R3*) $SM^rSU^rCM^r$, have been transferred to a *P. mirabilis* strain. The DNA satellite bands have been isolated by density-gradient centrifugation and examined by electron microscopy. "Circular" DNA molecules were found in each case. A minority of molecules with tertiary turns (supertwisted; 16) was found in several of the molecular species.

MATERIALS AND METHODS

R-factors and bacterial strains. The R factors utilized were obtained from *P. mirabilis* strain PM1, harboring either *R15* or *222/R3*. The latter factor is a derivative of R factor *222*, having lost the character of tetracycline resistance (6, 18). Each R factor was first transferred to a strain of *E. coli* and then from this strain to a non-R factor-harboring, nicotinic acid-requiring (*nia*$^-$) strain of *P. mirabilis* PM1. The *Proteus* strains were kindly supplied by S. Falkow. The *E. coli* strains comprised the standard Hfr "Cavalli" strain, which is methionine-requiring (*met*$^-$) and streptomycin-sensitive (*str-s*; 4), and RC 578, a 58-161 strain also *met*$^-$*str-s*, which was made F^- by acridine orange treatment (10).

Media. Culture media were as described previously (14). Antibiotic levels used were as follows: streptomycin (SM; Sigma Chemical Co., St. Louis, Mo.) at 25 μg/ml for *E. coli* and 100 μg/ml for *Proteus*; chloromycetin (CM; Parke, Davis & Co., Detroit, Mich., chloramphenicol) at 100 μg/ml; and sulfonamide (SU; Sigma sulfanilamide) at 500 μg/ml for both *E. coli* and *Proteus*. All media used for colonial growth of *Proteus* were supplemented with 0.1%

phenol to prevent swarming. Other media included TES buffer [0.01 M tris(hydroxymethyl)aminomethane salts, 0.01 M ethylenediaminetetraacetate and 0.2 M NaCl at *p*H 8.0 (9)] and urea-agar (Difco).

Transfer and growth conditions. The strain carrying the R factor (R^+) and the strain to be infected (R^-) were grown independently overnight in nutrient broth. Each culture was diluted 1:50 in fresh broth and incubated with aeration until a concentration of about 5×10^8 cells/ml was reached. Equal volumes of the R^+ and R^- cultures were then mixed and incubated together without aeration for 2 hr. When the R^- strain was *E. coli*, an appropriate dilution was plated for growth of isolated colonies on minimal agar (MA) supplemented with methionine (20 μg/ml) and streptomycin (25 μg/ml), with lactose (0.2%) substituted for glucose. After incubation for 48 hr, 50 or more of the emerging colonies were tested for growth on SU in the case of *R15*, and SU or CM in the case of *222/R3*. When the R^- strain was *P. mirabilis*, the mixture was spread on MA supplemented with phenol (0.1%), nicotinic acid (20 μg/ml), and streptomycin (100 μg/ml). After incubation for 48 hr, 50 or more of the emergent colonies were tested for growth on SU (*R15*) or SU + CM (*222/R3*).

Preparation of DNA. A 50-ml amount of nutrient broth containing either SM or SM + CM was inoculated with a single colony of PM1(*R15*) or PM1(*222/R3*), respectively, and incubated overnight at 37 C. The culture was then added to 1 liter of similar drug-containing broth in an Erlenmeyer flask, which was shaken vigorously at 37 C until a titer of about 5×10^8 cells/ml was attained. The cells were then harvested and lysed. The DNA was extracted by the method of Hickson et al. (9) and assayed by absorption at 260 nm, using calf-thymus DNA as a standard. Yields were usually about 60 ml of DNA (60 μg/ml) per liter of culture. At the time the cells were harvested, they were checked for drug resistance by plating for single colonies on nutrient-agar. After overnight incubation, two or more plates, each containing several hundred colonies, were replicated to MA plus nicotinic acid containing either *SM + SU* (for *R15*) or *CM + SM + SU* (for *222/R3*). All colonies were able to replicate.

Analytical CsCl density-gradient centrifugation. An analytical ultracentrifuge (model E, Beckman Instruments, Inc., Fullerton, Calif.) equipped with an ultraviolet (UV) light source and a monochromator was used. Samples were centrifuged at 44,000 rev/min at 25 C for 24 to 48 hr. A mark IIIc (Joyce-Loebl) (Gateshead, England) microdensitometer was used for the evaluation of the photographs. As a reference, DNA from *Bacillus subtilis* phage SP01 was used. The density of this DNA is 1.7355 g cm^{-3} (W. Szybalski, *personal communication*). However, since previous workers (6, 15) have used densities calculated according to Marmur et al. (12), where the density of SP01 DNA corresponds to 1.742 g cm^{-3}, all densities quoted subsequently will use the latter value.

Preparative CsCl density-gradient centrifugation. Separation of DNA was achieved by centrifuging DNA-cesium chloride solutions in a Ti-50 angle rotor (Beckman preparative centrifuge) at $95,000 \times g$ at 25 C for 40 to 60 hr. Tubes contained 4.5 ml of CsCl (950 mg/ml) and DNA (40 μg/ml) in TES buffer at *p*H 8.0. After centrifugation, 50 to 60 samples of about 0.08 ml each were collected through a hole in the bottom of the tube and each sample was diluted to 1 ml with TES. Absorption at 260 nm was then measured in each sample.

Electron microscopy. DNA samples in TES buffer were dialyzed twice against 0.15 M ammonium acetate for 30 min at 5 C. Grids for electron microscopy were prepared from solutions of 0.15 M ammonium acetate by the diffusion method to a cytochrome *c* film at a final DNA concentration of 0.2 μg/ml, as described by Lang et al. (11). Circular molecules whose contours could be unambiguously seen were centered on the fluorescent screen and photographed with a Siemens Elmiskop 1A. Usually, about 20 to 30 separated molecules were photographed. The microphotographs were optically projected on a 4 by 3-ft screen and the molecular contours were traced on paper. Lengths of all molecules with clearly defined contours were measured with a map measurer (curvimeter). The measured lengths were finally corrected for "pincushion" distortion of the electron microscope projective lens (11).

RESULTS

Characteristics of drug-resistance determinants. Under the conditions described, the two R factors were transferred from the *Proteus* to the *E. coli* strains 58-161 or Hfr Cavalli at frequencies of 10^{-4} for *R15* and 10^{-7} for *222/R3*. All drug resistance characters shown by the original PM1 strain were found in both types of *E. coli* recipients. The *fi* character of the R factor could be determined in the Hfr strain by its sensitivity to a spot test by use of the male-specific phage μ2 (5). Confirming previous reports, the presence of *R15* in the *E. coli* Hfr strain did not repress its sensitivity to this phage, whereas the effect of *222/R3* was to abolish phage sensitivity (17, 19). This was taken to indicate that *R15* did not repress the fertility characteristics of the Hfr strain and is fi^-, and that *222/R3* represses Hfr fertility and is an fi^+ type-factor.

To avoid transfer of *E. coli* chromosomal DNA to *Proteus*, which might have occurred had Hfr donors been used (7), the R factors were transferred from *E. coli* 58-161 strains to a nonfactor-carrying strain of *Proteus* PM1. Transfer occurred at frequencies of 10^{-3} for *R15* and 10^{-6} for *222/R3*. The colonies were confirmed as being *Proteus* by their ability to produce a red zone characteristic of urease-positive strains in Difco urea agar. Again, all selected *Proteus* colonies showed all of the drug resistance characteristics of the parental *E. coli* strain. The *fi* character of the transferred R factors was again checked and shown to be unchanged after further transfer to Hfr Cavalli. After contact of PM1 cells with *E. coli*

RC578(*R15*), clones of PM1 were found which were SM^rSU^r. One of these clones was picked and termed PM1(*R15*)*; this clone was used for all DNA preparations involving *R15*. A similar, newly isolated strain, *PM1(222/R3)**, was used as the source of all *222/R3* DNA.

When cultures of *P. mirabilis* strains carrying R factors were grown in broth containing either SM (in the case of *R15*) or SM + CM (in the case of *222/R3*) and then plated for single colonies on nutrient agar, all colonies emerging were able to grow when replicated on MA plus nicotinic acid containing either SM + SU or SM + SU + CM, respectively; each of the 500 or more colonies examined from each culture maintained the original drug resistance spectrum. Thus, there was no evidence for segregation of SU^s cells. DNA for our studies was always extracted from cultures in such conditions.

Analytical density-gradient centrifugation. *Proteus DNA.* Equilibrium centrifugation of DNA from the parental PM1 strain uninfected with an R factor or from either PM1(*R15*)* or PM1(*222/R3*)* after acriflavine-curing of the R factor gave rise to a single band with a mean density calculated as 1.698 g cm^{-3} (from reference peak of SPO1 DNA taken as 1.742 g cm^{-3}).

R15-DNA. After centrifugation to equilibrium, DNA from PM1(*R15*)* preparations showed a main band with its mean density calculated as 1.698 g cm^{-3} and a denser satellite peak at 1.709 g cm^{-3} (Fig. 1A).

222/R3-DNA. Four separate preparations of DNA, each from a separate culture of PM1(*222/R3*)*, were analyzed. Each tracing showed a main band with a mean density calculated as either 1.698 or 1.699 g cm^{-3} and a denser shoulder. In the shoulder, three peaks were seen with mean densities calculated as 1.708, 1.711 and 1.717 g cm^{-3} (Fig. 1B).

Preparative, density-gradient centrifugation. *R15-DNA.* A typical UV absorption pattern of 56 samples from one of five runs is shown in Fig. 2A. It shows a major band with its peak in fraction 38 and a minor denser band with its peak at fraction 34. Fractions 33, 34, and 35 were pooled and examined by electron microscopy. Corresponding fractions were examined from the satellite peak of a separately centrifuged preparation of DNA from a different subculture of PM1(*R15*)*.

222/R3-DNA. After centrifugation for 40 hr, the DNA from cultures of PM1(*222/R3*)* gave rise to the UV absorption curve showing a major peak, taken as corresponding to *Proteus* chromosomal DNA, together with a denser shoulder which did not show clearly defined peaks. From this shoulder, three fractions were taken, pooled, and examined by electron microscopy. Two DNA preparations from separate subcultures of PM1(*222/R3*)* were analyzed in this way. The

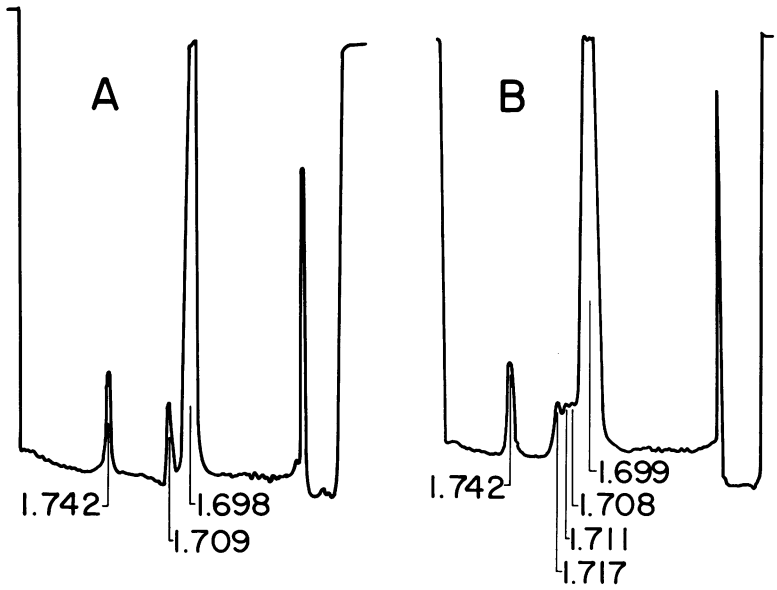

FIG. 1. *Microdensitometer tracings after analytical, cesium chloride, density-gradient centrifugation at 44,000 rev/min for 27 hr. The reference DNA peak at density of 1.742 is of Bacillus subtilis phage SPO1. (A), R15-DNA; (B), 222/R3-DNA.*

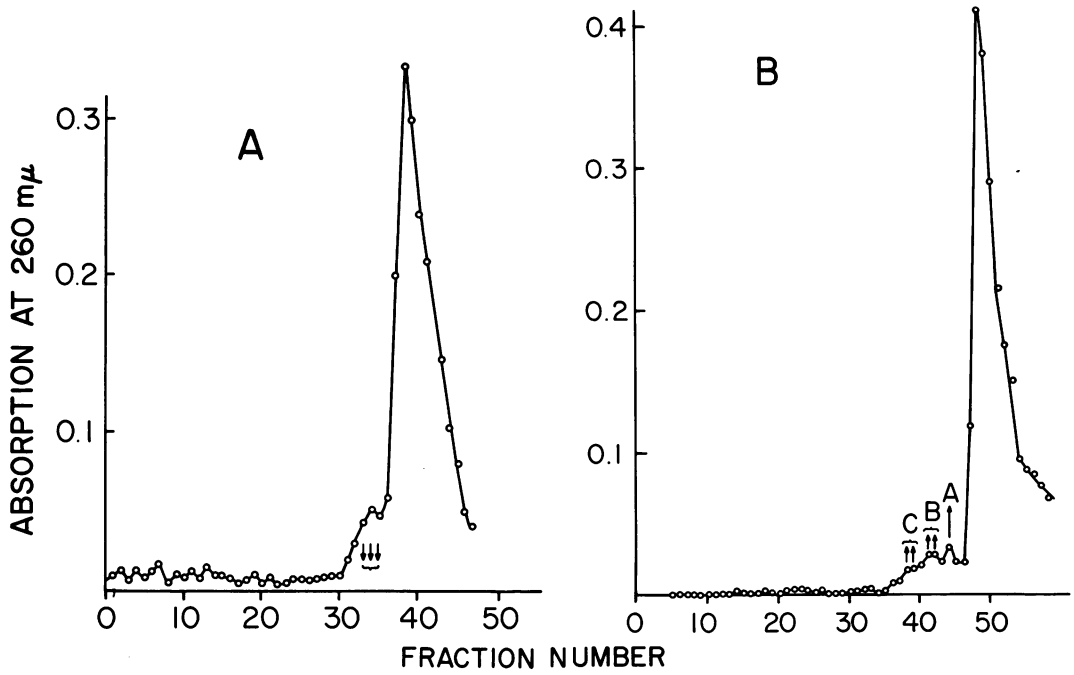

FIG. 2. *UV absorption of fractions after preparative cesium chloride density-gradient centrifugation for 60 hr. (A), R15DNA; (B), 222/R3 DNA. Fractions A, B, and C were examined by electron microscopy.*

UV absorption curve from one of these DNA preparations, after centrifugation for 60 hr, is shown in Fig. 2B. A major band is seen at fraction 48 with three minor peaks in fractions 39, 41, and 44. Three pools containing fractions 44 (A), 41 and 42 (B), and 38 and 39 (C), respectively, were taken for electron microscopy.

Electron microscopy. *R15-DNA.* From the pooled fractions (33, 34, and 35) from the preparative run shown in Fig. 2A above, several grids were prepared and examined, and 10 closed structures corresponding roughly to about 10% of the molecules were seen. The lengths of the preponderant linear molecules varied extensively from small fragments about 1 μm to those apparently larger than the circular molecules. The lengths of five of the "circular" molecules with clearly defined contours were measured. From fractions obtained from the independently prepared and centrifuged DNA preparation, 20 additional circular molecules were photographed, and the contour lengths of 11 of these were measured. The contour lengths of the 16 molecules had a mean of 18.3 ± 0.12 μm (SD). A histogram of the lengths reduced to the nearest 0.5 μm is shown in Fig. 3A. Figure 4A is an electron micrograph of one of these circular molecules.

222/R3 DNA. The pools of the fractions taken from the denser shoulder to the major peak appearing after centrifugation of the two DNA preparations for 40 hr were examined by electron microscopy. In both preparations, circular molecules were seen. Twenty molecules from each pool were photographed, and 11 of each were found suitable for contour length measurement. The circular molecules from both preparations were found to be of two distinct contour lengths (Table 1), one molecular species of about 36 and another of about 29 μm. When centrifugation was continued for 60 hr to form three satellite peaks (Fig. 2B), circular molecules were seen in samples from all three peaks. In pool A, 10 molecules were photographed and the contour lengths of five were measured. From the B pool, 10 photographs were taken and five contour lengths were measured. In the C pool, a third major class of about 6 μm, in addition to other sizes of a smaller number of circular DNA molecules, were seen. A total of 44 molecules were photographed and, due to their small size, contour lengths of all could be measured (Table 1). In all of these fractions, the circular molecules formed about 10% of the total number of molecules. A histogram of the origins and sizes of all circular molecules from *222/R3* DNA preparations is shown in Fig. 3B. The mean values for the lengths of each of three major classes of molecules are (i) 35.8 ± 0.3 μm, found in pools A and B, (ii) 28.5 ± 0.3 μm found only in

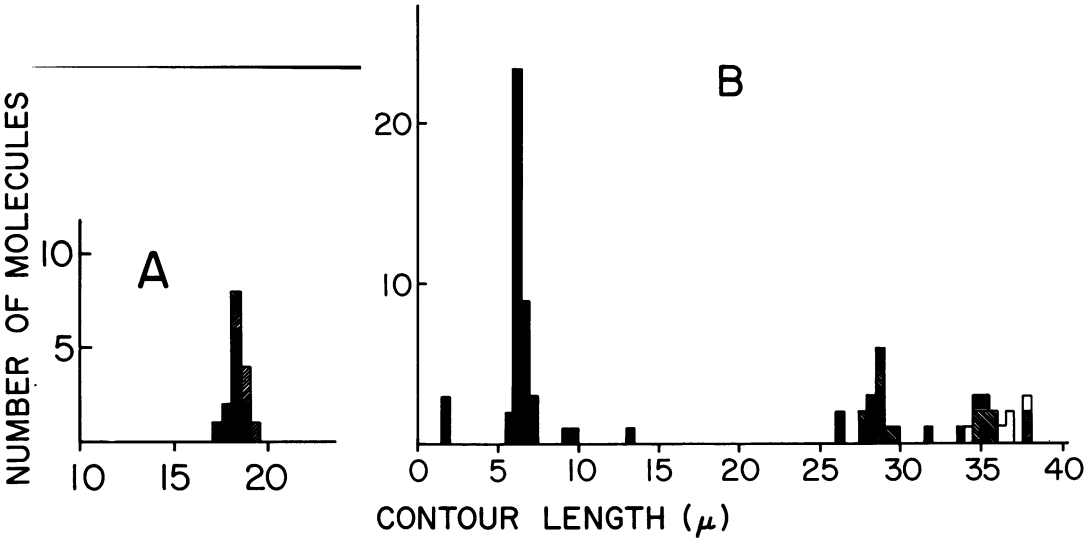

FIG. 3. *Frequency distributions of contour lengths of circular DNA molecules plotted to nearest 0.5 μm. (A), 16 R15 DNA molecules. Hatched bars are from a gradient shown in Fig. 2A. Solid bars are from a separate DNA preparation and centrifugation. (B), 75 222/R3 DNA molecules. Upper left to lower right crosshatching represents 11 molecules from the first 40-hr run; lower left to upper right crosshatching, 11 molecules from the second 40-hr run; horizontal hatching, 4 molecules in 60-hr "A" fraction; open bars, 5 molecules in 60-hr "B" fraction; and solid bars, 44 molecules in 60-hr "C" fraction.*

pool A, and (iii) 6.4 ± 0.1 μm, found only in pool C (Table 1). Electron micrographs of these molecules are shown in Fig. 5A, 5B, and 5C, respectively.

DNA molecules containing tertiary turns ("supertwists"). In all preparations examined by electron microscopy where "circular" DNA molecules were seen, a small proportion (about 5%) possessed a twisted tertiary structure (16). A twisted *R15* DNA molecule is shown in Fig. 4B. Two twisted forms of *222/R3* DNA are shown in Fig. 5; one, shown in Fig. 5E from pool C, probably belongs to the 6-μm class; the other (Fig. 5D) from pool B is assumed to be a 36-μm class molecule.

DISCUSSION

Stability of R factors. Factors *R15* and *222/R3*, originally present in the *Proteus* strain PM1, maintain their phenotypic characteristics when transferred to *E. coli* strains. Each is infective and can again be freely transmitted to a non-R-carrying strain of *Proteus* PM1. When further transferred from the newly infected PM1 strains, *R15* shows the *fi*⁻ phenotype, in that it does not suppress the male phage sensitivity of *E. coli* Hfr, whereas *222/R3* acts as an *fi*⁺ factor in suppressing male phage sensitivity of this strain. The original drug resistances carried by the factors SM^rSU^r on *R15* and $SM^rSU^rCM^r$ on *222/R3* are also transmitted to the *E. coli* strains and from these strains to uninfected (R^-) *Proteus* PM1. When these newly infected *Proteus* strains are lysed and their DNA is extracted, at least 99.8% of the cells maintain all of the original drug resistance markers, and no more than about 0.2% of drug-sensitive segregant cells could have been present in any culture which was lysed to prepare DNA.

Density-gradient profiles. As previously shown (6, 15), samples of DNA extracted from R factor-carrying strains of *Proteus* show a satellite band at a higher density than the band corresponding to the chromosomal DNA. In the case of *R15*, the satellite band formed a single peak at a density of 1.709 g cm^{-3}, compared to a single peak at 1.710 g cm^{-3} found by Falkow et al. (6) for the same R factor. In the case of *222/R3*, where these authors found two peaks at densities of 1.711 and 1.716 g cm^{-3}, our studies show three peaks at 1.708, 1.711, and 1.717 g cm^{-3}.

Electron-microscopy studies. Previously reported studies (6, 15) use a method of DNA isolation which is said to result in fragmentation of DNA to sizes estimated as approximately 10×10^6 daltons (6). Since the sizes of R factors have been estimated as about 25×10^6 daltons (15), it seems likely that most R molecules would have been fragmented during isolation. In the case of *R15*, the previous report suggests that the frag-

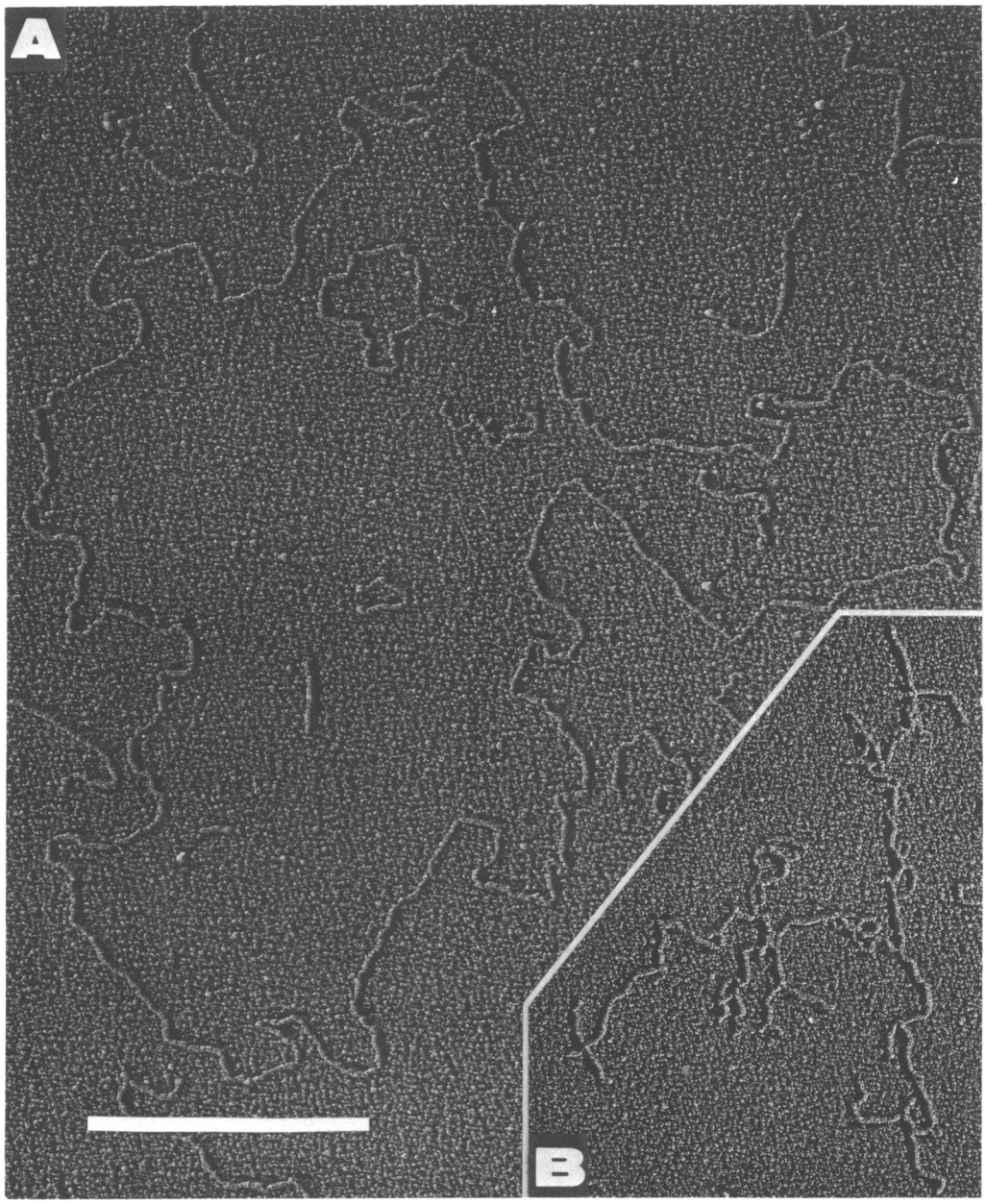

FIG. 4. *Electron micrographs from R15 DNA fractions. (A), Open circular molecule; (B), twisted molecule. Bar represents 1 μm.*

ments appear to be homogeneous in density, since only one satellite peak is seen, whereas *222/R3* DNA formed two density peaks. We used a less disruptive DNA isolation procedure and when the satellite fractions were studied in the electron microscope, "circular" DNA molecules corresponding to sizes greater than 10×10^6 daltons were seen. (In addition to these molecules, linear molecules were seen in preponderant numbers and varying sizes. No measurements of linear molecules were made.)

In the case of *R15*, 16 circular molecules showed

TABLE 1. *Contour lengths of 222/R3 DNA molecules*

DNA sample	Time centrifuged	Total no. of molecules	Class I[a]			Class II[b]			Class III[c]			Other classes		
			No. of molecules	Length (μm)		No. of molecules	Length (μm)		No. of molecules	Length (μm)		No. of molecules	Length (μm)	
				Min	Max		Min	Max		Min	Max		Min	Max
	hr													
1	40	11	6	34.8	37.6	5	27.9	30.0	0			0		
2	40	11	3	34.0	38.0	8	26.3	29.0	0			0		
	60(A)	5	2	34.7	35.2	2	26.3	29.0	0			1	31.7	
	60(B)	5	5	34.1	37.9	0			0			0		
	60(C)	44	0			0			38	5.9	7.2	3	2.3	2.5
												2	9.3	9.8
												1	13.0	

[a] Mean length of the 16 molecules was 35.8 ± 0.3 μm. (SD).
[b] Mean length of the 15 molecules was 28.5 ± 0.3 μm. (SD).
[c] Mean length of the 38 molecules was 6.4 ± 0.1 μm. (SD).

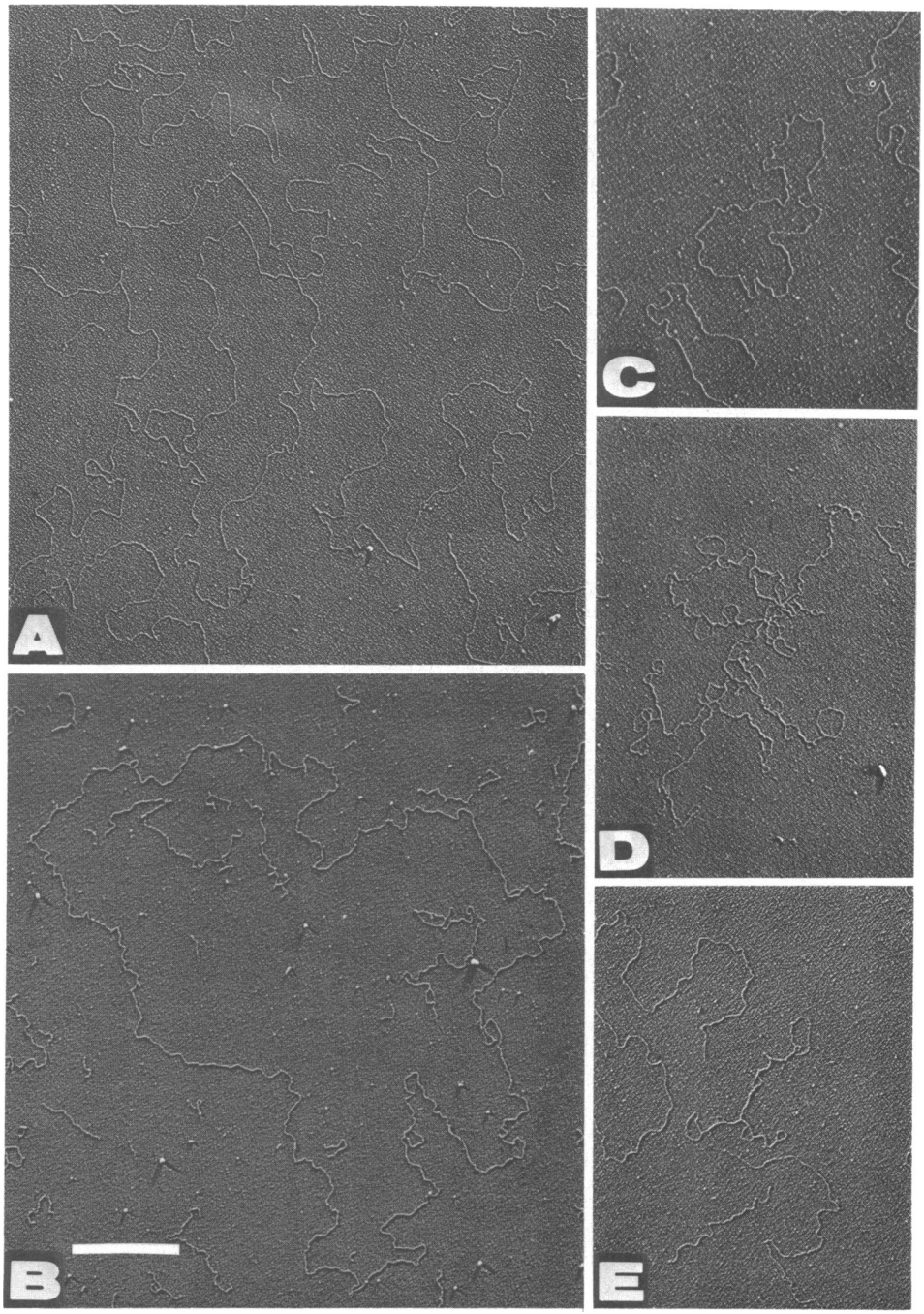

FIG. 5. *Electron micrographs from 222/R3-DNA fractions. (A), 35.8-μm molecule; (B), 28.5-μm molecule; (C), 6.4-μm molecule; (D), twisted molecule from B fraction (36-μm molecule?); (E), 6.4-μm molecule (twisted). Bar represents 1 μm.*

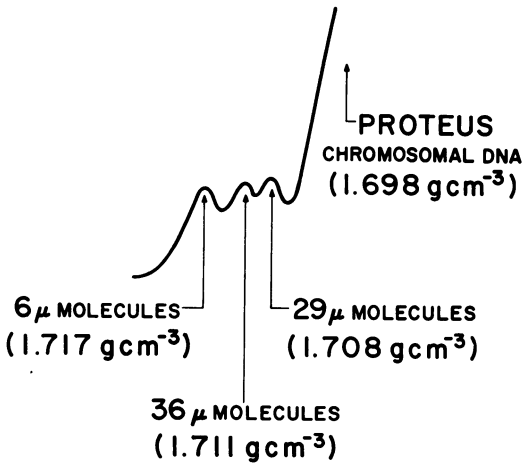

FIG. 6. *Schematic diagram of density-gradient separation and contour lengths of 222/R3 DNA molecular species.*

a contour length of 18.3 ± 0.1 μm (SD), corresponding to a molecular weight of 35 × 10^6 daltons (assuming a weight of 191 daltons per angstrom for the "B" configuration of the sodium salt of DNA).

From the satellite bands of *222/R3* DNA, circular molecules were also seen. However, here at least three distinct molecular species were recognized. One major species has a contour length of 35.8 ± 0.3 μm, corresponding to a molecular weight of about 68 × 10^6 daltons. A second has a length of 28.5 ± 0.3 μm, corresponding to a molecular weight of about 54 × 10^6 daltons, and a third has a length 6.4 ± 0.1 μm, corresponding to about 12 × 10^6 daltons. In general, molecules of 35.8 μm predominate at the intermediate density (1.711 g cm^{-3}) fraction, although they are also found together with those of 28.5 μm which occur exclusively in the lowest density (1.708 g cm^{-3}). The smallest molecules, predominantly of 6.4 μm, are found only in the densest fraction (1.717 g cm^{-3}; Fig. 6). It appears, therefore, that the smallest (6 μm) molecules are the most dense, those of intermediate (29 μm) size are the least dense, and the largest (36 μm) molecules have an intermediate density.

From the sizes of the three major molecular classes, it seems likely that there may be a physical interrelationship that takes into account the fact that the size of the largest molecule equals the sum of the sizes of the other two molecules within the experimental errors of measurement, if one bears in mind that, in addition to the standard deviations of the mean lengths of molecules (Table 1), further small variations in magnification (± 5%) occur when samples are prepared and examined for microscopy on different occasions. One possible explanation for this length interrelationship, which is also consistent with the fact that the largest molecule is intermediate in density to the two smaller ones, would be that the *222/R3* factor is composite and takes the form either of a single 36-μm structure or of two smaller (6 and 29 μm) structures in each R^+ cell. It would be expected that the density of this composite molecule would lie nearer to that of its larger component. This is, in fact, so. Moreover, within the variations in reproducibility of density peak measurements, which are estimated as ± 0.001 g cm^{-3}, the density data is consistent with this model. That both the smaller structures appear to persist and replicate appears likely from the finding that the mass of DNA in the three density peaks is of the same order (Fig. 1B), if these peaks can, in fact, be correlated with the three different molecules. This would suggest that the two smaller structures are themselves replicons, able to coexist in the same cell. Whether *222/R3* was initially (before its transmission to PM1 *222/R3**) a composite 36-μm structure, which later separated to the two smaller replicating molecules, or whether it was formed of two small replicons that later united cannot be stated from these results. However, if before its transfer to PM1 it was, in fact, formed of two molecules, then these molecules must have been very efficiently cotransferred, as has been shown in the cotransfer of certain pairs of sex factors [R. C. Clowes et al., Intern. Congr. Genet. (Tokyo), *in press*] or even under circumstances such as that found with F and Col E1, where only one element (F) is a sex-factor (1). It cannot yet be stated, therefore, whether both of these smaller molecules have sex factor activity, or only one. Neither can it be concluded whether both, or only one, carry drug resistance markers of any one kind, since the large majority of cells from which DNA was extracted retained all drug resistance markers. However, if drug resistance markers are in fact distributed between the two smaller molecules, it should be possible, by selecting drug-sensitive segregants, to isolate strains in which one replicon has been lost. Experiments along these lines are in progress.

What are the physical events that lead to the association of two small replicons to form a larger one, or, by a reversal of the process, the separation of one large replicon to two smaller ones? Two major possibilities come to mind: (i) either a standard "recombination" event depending on two, intact (closed), circular, double-stranded DNA molecules which can pair in homologous regions leading, after breakage and reunion, to the formation of one, intact, double-stranded, circular structure, as in the case of the incorporation

of a λ molecule into the *E. coli* chromosome (3); or (ii) two linear molecules with single-strand terminal homologies (sticky ends), which can form "open" circular molecules with two single-strand breaks, could form "mixed dimers" if the homologies were in part identical, as has been shown to occur in the case of mixtures of λ and χ phage (M. Mitani and D. Lang, *personal communication*). However, from the fact that "supertwisted" and, by inference, covalently-closed circular molecules have been found, and in particular if it can be shown that some are of the 36-μm type, the latter mechanism involving a "mixed dimer" of 36-μm seems less likely. For the same reason, an in vitro origin of the 36-μm molecules during or after DNA extraction is unlikely. Moreover, preliminary experiments show that heat treatment of pool B (Table 1) for 5 min at 75 C in 0.15 M ammonium acetate, followed by quick cooling, fails significantly to reduce the proportion of 36-μm molecules, so that if cohesive ends are involved, they probably involve more extensive regions than those in λ or in χ phage (8). Furthermore, the areas under the curves in fractions A, B, and C in Fig. 2B are not consistent with the idea that the smaller structures are produced entirely by breakdown of preexisting 36-μm structures. For example, the amount of DNA forming the 6-μm molecules (A) is not equivalent to one-sixth or so of that forming the 36-μm molecules (B), but is in fact equal to or greater than this amount. These relative amounts of DNA suggest not only that the smaller molecules replicate independently, but also that the smallest (6 μm) replicon replicates more frequently than the other molecules.

More speculatively, if the 36-μm replicon can segregate by recombination into a 6- and a 29-μm replicon, this might suggest that the evolution of the more complex drug resistance factors may have resulted by recombination of smaller independent replicons.

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LITERATURE CITED

1. Alfoldi, L., F. Jacob, and E. L. Wollman. 1957. Zygose létale dans les croisements entre souches colicinogènes et non-colicinogènes d'*Escherichia coli*. Compt. Rend. 244:2974–2976.
2. Anderson, E. S. 1967. Facteurs de transfert et résistance aux antibiotiques chez les enterobactéries. Ann. Inst. Pasteur 112:547–563.
3. Campbell, A. M. 1962. Episomes. Advan. Genet. 11:101–145.
4. Cavalli, L. L. 1950. La sessulita nei batteri. Boll. Ist. Sieroterap. Milan. 29:281–289.
5. Dettori, R., and G. A. Maccacaro. 1961. Sex-specific bacteriophages of *Escherichia coli* K12. Gion. Microbiol. 9:141–150.
6. Falkow, S., R. V. Citarella, J. A. Wohlhieter, and T. Watanabe. 1966. The molecular nature of R-factors. J. Mol. Biol. 17:102–116.
7. Gemski, Jr., P., J. A. Wohlhieter, and L. S. Baron. 1967. Chromosome transfer between *Escherichia coli* Hfr strains and *Proteus mirabilis*. Proc. Natl. Acad. Sci. U.S. 58: 1461–1467.
8. Hershey, A. D., and E. Burgi. 1965. Complementary structure of interacting molecules at the ends of lambda DNA molecules. Proc. Natl. Acad. Sci. U.S. 53:325–328.
9. Hickson, F. T., T. R. Roth, and D. R. Helinski. 1967. Circular DNA forms of a bacterial sex factor. Proc. Natl. Acad. Sci. U.S. 58:1731–1738.
10. Hirota, Y. 1960. The effect of acridine dyes on mating type factors in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. 46:57–64.
11. Lang, D., H. Bujard, B. Wolff, and D. Russell. 1967. Electron microscopy of size and shape of viral DNA in solutions of different-ionic strengths. J. Mol. Biol. 23:163–181.
12. Marmur, J., R. Rownd, S. Falkow, L. S. Baron, C. Schildkraut, and P. Doty. 1961. The nature of intergeneric episomal infection. Proc. Natl. Acad. Sci. U.S. 47:972–979.
13. Meynell, E., G. G. Meynell, and N. Datta. 1968. Phylogenetic relationships of drug-resistance factors and other transmissible bacterial plasmids. Bacteriol. Rev. 32:55–83.
14. Monk, M., and R. C. Clowes. 1964. Transfer of the colicin I factor in *Escherichia coli* K12 and its interaction with the F fertility factor. J. Gen. Microbiol. 36:365–384.
15. Rownd, R., R. Nakaya, and A. Nakamura. 1966. Molecular nature of the drug-resistance factors of the enterobacteriaceae. J. Mol. Biol. 17:376–393.
16. Vinograd, J., and R. Lebowitz. 1967. *In* Macromolecular metabolism, The New York Heart Association, p. 103. Little Brown & Co., Boston.
17. Watanabe, T., and T. Fukasawa. 1961. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. I. Transfer of resistance factors by conjugation. J. Bacteriol. 81:669–678.
18. Watanabe, T., and T. Fukasawa. 1961. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. II. Elimination of resistance factors with acridine dyes. J. Bacteriol. 81:679–683.
19. Watanabe, T., H. Nishida, C. Ogata, T. Arai, and S. Sato. 1964. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. VII. Two types of naturally occurring R factors. J. Bacteriol. 88:716–726.