JOURNAL OF BACTERIOLOGY, Feb. 1973, p. 1026-1033
Vol. 113, No. 2

Printed in U.S.A.

Molecular Structure of an R Factor, Its Component Drug-Resistance Determinants and Transfer Factor

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Received for publication 27 June 1972

Plasmid DNA from *Escherichia coli* strains harboring drug resistance either of the infectious or noninfectious kind has been separated by CsCl centrifugation of crude cell lysates in the presence of ethidium bromide and examined by electron microscopy. Plasmid deoxyribonucleic acid (DNA) from an S^+ strain (which has the property of noninfectious streptomycin-sulfonamide resistance) consists of a monomolecular covalently closed circular species of 2.7 μm in contour length (5.6×10^6 atomic mass units; amu). DNA from a strain carrying a transfer factor, termed Δ, but no determinant for drug resistance, is a monomolecular covalently closed circular species of 29.3 μm in contour length (61×10^6 amu). DNA from either Δ^+A^+ or Δ^+S^+ strains, (which are respectively ampicillin or streptomycin-sulfonamide resistant, and can transfer this drug resistance), shows a bimodal distribution of molecules of contour lengths 2.7 μm and 29.3 μm, whereas DNA from a $(\Delta\text{-T})^+$ strain (showing infectious tetracycline resistance) contains only one species of molecule measuring 32.3 μm (67×10^6 amu). We conclude that ampicillin resistance is carried by a DNA molecule (the A determinant) of 2.7 μm, and streptomycin-sulfonamide resistance is carried by an independent molecule (the S determinant) of similar size. These molecules are not able to effect their own transfer, but can be transmitted to other cells due to the simultaneous presence of the transfer factor, Δ, which also constitutes an independent molecule, of size 29.3 μm. In general, there appears to be little recombination or integration of the A or S molecules into that of Δ, although a small proportion (5–10%) of recombinant molecules cannot be excluded. In contrast, the third drug-resistance determinant, that for tetracycline resistance (denoted as T), is integrated in the Δ molecule to form the composite structure Δ-T of size 32.3 μm, which determines infectious tetracycline resistance. The Δ^+A^+ and Δ^+S^+ strains are defined as harboring *plasmid aggregates*, and the $(\Delta\text{-T})^+$ strain is defined as carrying a *plasmid cointegrate*; the properties of all three strains are characteristic of strains harboring R factors. These results are compatible with the previously published genetic data. The number of Δ molecules per cell appears to be equal to the chromosomal number irrespective of growth phase, and this plasmid can thus be defined as stringently regulated in DNA replication. In contrast, S and A exist as multiple copies, probably in at least a 10-fold excess of chromosomal copy number. S and A can thus be defined as relaxed in the regulation of their DNA replication.

Infectious drug-resistance (R) factors isolated in different parts of the world differ considerably in their genetic and physiological properties. Two major series of investigations have stemmed from work by Watanabe and his colleagues (42) on R factors originally isolated in Japan, and by Anderson and co-workers on R factors isolated in Great Britain (3, 4).

Watanabe's studies are concentrated on an R factor transmitting resistance to streptomycin, sulfonamide, chloromycetin, and tetracycline ($Sm^rSu^rCm^rTc^r$), first isolated in a strain of *Shigella flexner* 2b (no. 222) by Nakaya et al. (32) and termed 222 by Watanabe and Fukasawa (43). Host cells containing the 222 factor transmit by conjugation all four drug resist-

ances concomitantly to most recipient cells, which then become active donors for the four drug resistances (43, 44). The three drug resistances Sm^r, Su^r, and Cm^r can be co-transduced from 222^+ host strains in *Salmonella typhimurium* by phage P22, whereas Tc^r is transduced independently. In *Escherichia coli*, phage P1 can co-transduce all four drug-resistance markers and, although the drug-resistant P22 transductants are no longer infectious, the P1 transductants retain their self-infectious property (45).

The 222 factor has therefore been proposed to comprise a single genetic linkage group incorporating all four drug-resistance (r) determinants together with the genes defined as responsible for transfer-factor properties and termed resistance transfer factor (RTF) (42). In *E. coli*, this conclusion has recently been verified by molecular studies which show the 222 factor to be in the form of a monomolecular, covalently closed circular deoxyribonucleic acid (DNA) species of molecular weight 70×10^6 atomic mass units (amu) (34).

R factors showing genetic properties similar to 222 are now known to be widely distributed. The DNAs of a number have been isolated from *E. coli*, each taking the form of circular molecules of uniform size (19, 20, 28, 30, 33, 34, 37, 40). However, it should be noted that in *Proteus mirabilis*, the 222 factor segregates into two independent replicons of about 58×10^6 amu and 12×10^6 amu (33). Similar data have also been presented for the factor R1 (20, 37) and R6 (20). From strains carrying either the R1 or R6 factor, drug-sensitive segregants have been isolated (20, 37) which carry a plasmid, inferred to be the transfer factor, of similar size to the larger of the two segregant plasmids from *Proteus*. However, since in *Salmonella*, 222 segregates a plasmid which carries tetracycline resistance and is self-transmissible but of 46×10^6 amu (34), the relationship between the larger 58×10^6 amu segregant from *Proteus* and RTF is not clear.

A second type of R factor was observed in Great Britain, characteristic of a type predominant in bovine infection by *S. typhimurium* (5, 6). Its behavior was such that it was difficult to envisage stable linkage between the constituents, since not only were drug resistances transferred together with the transfer factor, but transfer of independent resistance (r) determinants and of the transfer factor alone was common. In this second type of R factor (termed class II), the r determinants and the transfer factor are postulated to be independent of each other in the host cell and to be separate plasmids (3, 4). R factors of a similar type may also be common in porcine enteropathogenic strains of *E. coli* (38, 39).

This class of R factors was originally identified in strain RT1, a line of *S. typhimurium* phage-type 29 resistant to ampicillin (A), streptomycin (S), sulfonamides (Su), and tetracyclines (T) (5). Streptomycin and sulfonamide resistances are always transferred together and are proposed to lie on a single r determinant, SSu (5, 6), whereas ampicillin resistance is transferred independently and is proposed to be located on a separate plasmid (the A determinant). The A and SSu determinants (the latter symbol is shortened to S) are transferred to *E. coli* K-12 (= K-12) independently at a frequency of about 10^{-2} in overnight crosses. The transfer factor, designated Δ, is fi^- and I-like (25) and is transmitted to K-12 at a higher frequency, often in excess of 5×10^{-1} (2, 3, 6). In addition to its ability to mobilize plasmids that cannot transfer of their own accord, Δ can be detected in recipients by a characteristic phage-restriction pattern (1, 2, 6). In interrupted crosses, transfer of the A or S determinant in the absence of Δ transfer can be demonstrated. T was initially transferred from RT1 to K-12 on one occasion, which has not been reproducible. Once mobilized, T became highly transferable at the same frequency as Δ (2, 5, 6). It was assumed that the initial mobilization of T had occurred as the result of recombination between T and Δ, to generate the structure which is termed Δ-T.

The present study investigates the molecular nature of DNA molecules isolated from bacterial strains carrying Δ-mobilized R factors and resistance determinants. A preliminary report of this work has already appeared (Smith, Anderson, and Clowes, Bacteriol. Proc., p. 60, 1970).

MATERIALS AND METHODS

Bacterial strains and R factors. Two host strains were used. One (RC703) is the prototrophic wild K-12 strain, cured of the F factor by acridine orange, and the other is the methionineless non-λ-lysogenic strain, RC85, derived from strain W1655 and used in previous studies (30, 34). The R factors and derivatives were initially transferred to strain RC703 from strain RT1, the wild-type 29 of *S. typhimurium* carrying the determinants A, S, and T and the transfer factor Δ (5) by E. S. Anderson. Thus, strains carrying the A or S determinants are designated A^+ or S^+ in the text, whereas those carrying Δ in addition are termed Δ^+A^+ and Δ^+S^+, respectively. The symbols Δ^+ and $(\Delta\text{-T})^+$ are self-explanatory. A number of the R factors and their derivatives were then transferred from strain RC703 to strain RC85. A further description of the genetic properties of these strains and their correlation with the molecular nature of the DNA of their plasmids will be found in

the Discussion section.

Characterization of R-factor DNA. Media and reagents were used as detailed in previous publications (33, 34). Culture and labeling conditions were as described previously (34). R-factor DNA was isolated from *E. coli* host strains by the direct dye-buoyant density centrifugation of crude sarkosyl lysates in the presence of ethidium bromide as described previously (34). After removal of the ethidium bromide by dialysis (34), electron microscopy of the separated R-factor DNA from fractions of the heavy satellite band was carried out by the DNA spreading technique (29) by using 0.15 M ammonium acetate in the hypophase (bulk solution) and 0.5 M ammonium acetate in the spreading solution.

RESULTS

Contour length measurements and estimates of molecular weights. After centrifugation in ethidium bromide-cesium chloride of a crude lysate of the host strain RC703 carrying no known plasmid, fractionation showed a DNA profile with a single peak, as previously found with strain RC85 (34), inferred to be due to the chromosome and the absence of any satellite peak. Similar centrifugation profiles of strains RC703 or RC85 infected with one or other of the determinants Δ, S, A, or Δ-T, showed, in addition to the chromosomal peak, a single satellite peak which, on the basis of our previous studies with other R factors (30, 34), was inferred to be due to R-factor DNA. A sample of this DNA band was examined by electron microscopy after dialysis. DNA molecules were observed predominantly in the form of "circles," some of which were supercoiled. The contour length of a number of molecules which were not supercoiled (open circles, see Fig. 1) was measured. Table 1 shows details of contour length measurements of "circular" DNA molecules isolated from a series of R-factor satellite bands. No differences were noted using DNA isolated from either RC703 or RC85 host strains harboring the same factor(s), and the data are pooled. Each result represents pooled data from three to six DNA preparations, each derived from independent cultures, centrifuged, and fractionated at different times. The molecular weights are calculated from the mean contour lengths (MCL) on the assumption that 1 μm of double-stranded DNA is equivalent to 207×10^4 amu (31). The size distributions of the two classes of molecules, the large Δ and Δ-T molecules and the small A and S molecules, are shown as histograms in Fig. 2 and 3, respectively. An electron micrograph taken of a preparation of ΔS DNA showing both large and small molecules of Δ and S, respectively, is shown in Fig. 1.

Estimation of the "copy number" of plasmids. From the molecular weight of a plasmid, calculated as above, and assuming the molecular weight of the *E. coli* chromosome to be 2.5×10^9 (21), the relative numbers of copies of plasmid per copy of chromosome can be estimated from the relative amounts of plasmid and chromosomal DNA as measured by radioactive thymidine uptake over several generations of growth (Table 2). However, since Helinski and collaborators (12) have shown that not all plasmid DNA is found in the form of covalently closed circular DNA molecules, the satellite DNA isolated in the presence of ethidium bromide may represent only a fraction of the total plasmid DNA of the cell, and these estimations therefore would give only a lower limit to the copy number.

DISCUSSION

In cells harboring only the transfer factor (Δ), the satellite DNA shows a monomolecular species of MCL of 29.1 μm equivalent to 60×10^6 amu (Table 1). The size of this molecule is thus of the same order as that of other transfer factors isolated and measured in a similar way, such as F (13, 16, 40) and colIb (12, 16), and the R-factors 222 (34), R1 and R6 (19, 37), R64 and R538 (40).

In cells harboring only the noninfectious streptomycin-sulfonamide resistance determinant (S), a monomolecular DNA species is again seen about one-tenth the size of Δ, with a MCL of 2.9 μm (6.0×10^6 amu). The size of this element is similar to that of other noninfectious extrachromosomal elements such as ColEl (4.5×10^6 amu [11]).

The Δ^+S^+ strains transfer both Δ and S plasmids. Such strains yield molecules of two sizes. One is inferred to be Δ, its MCL being 29.4 μm, similar to the 29.1 μm value previously determined for Δ. The other is deduced to be that of S, since its size (2.7 μm) is similar to that of the single molecular species (2.9 μm) in the S^+ strain. In general terms, therefore, when Δ and S coexist in the same cell, they exist as independent molecules. This conclusion is in accordance with the genetic behavior of Δ^+S^+ cells which, although they can transfer the S determinant only because of the simultaneous presence of the transfer factor, can transfer S or Δ independently or together (2, 5). A similar situation has been known for some years in the case of transfer of ColEl (which is itself noninfections [15]) by mobilization by the classical sex-factor F. F^+ColEl^+ cells usually transmit both F and ColEl together, but occasionally they transmit them independently. ColEl

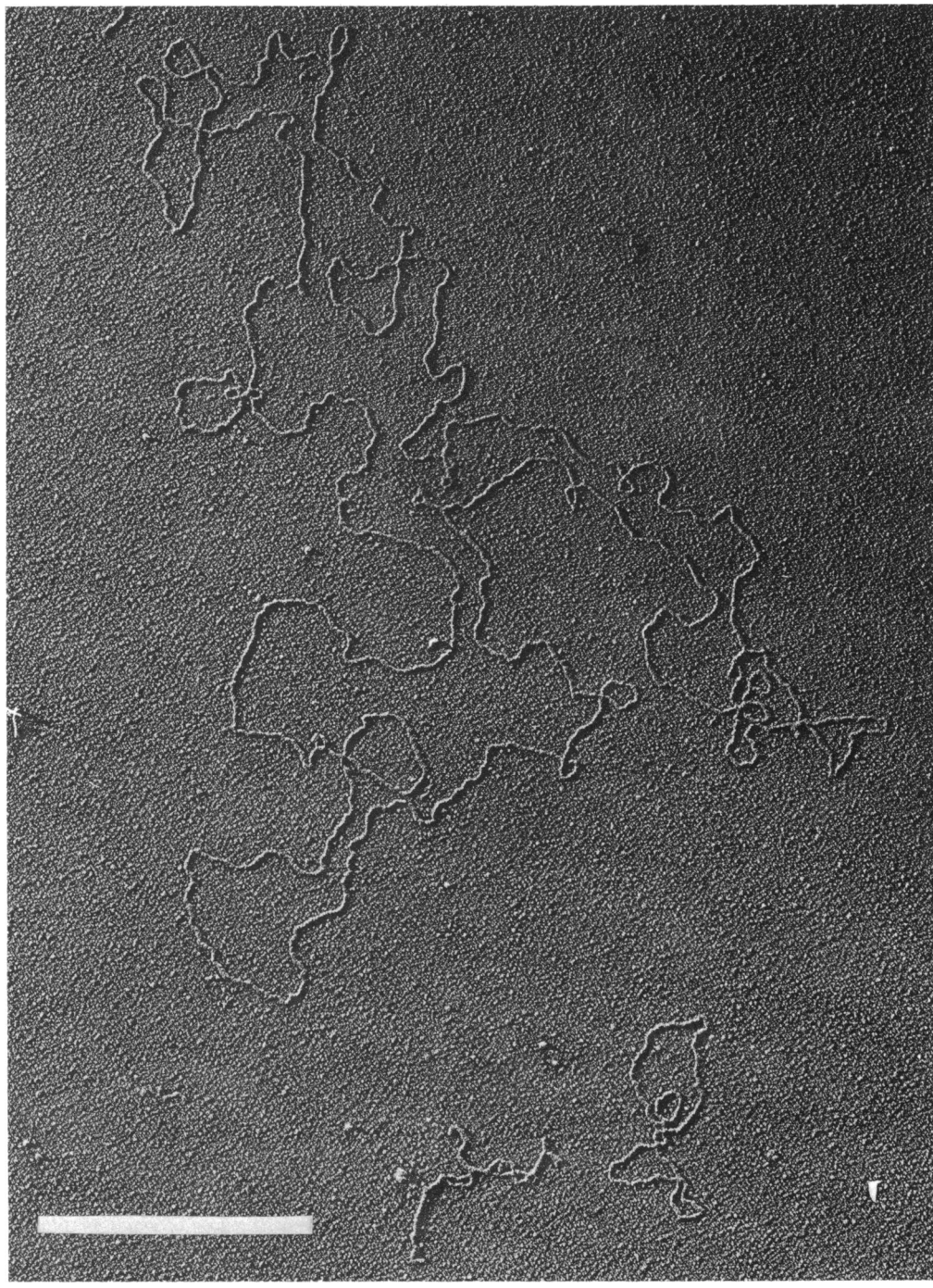

FIG. 1. *Electron micrograph of DNA molecules isolated from a Δ^+S^+ strain of E. coli K-12. At the bottom right is a small "open circular" molecule inferred to be S, and to its left a supercoiled molecule also inferred to be S. Above is a large, "open circular" molecule inferred to be that of Δ. Bar represents 1 μm.*

transfer from Hfr strains is found at a similar level (14). Since the F factor is not usually transferred in this latter cross, it may be concluded that there is no physical association between F and ColEl in either the F^+(ColEl)$^+$ or Hfr(ColEl)$^+$ cell (15). This is consistent with

TABLE 1. *Contour length measurements of circular DNA molecules isolated from E. coli host strains harboring various plasmids*

Plasmid(s)	No. molecules measured	Range of lengths (μm)	MCL ± SSD[a] (μm)	SSD as % MCL	Molecular weight (amu × 10^6)[b]
Δ	33	27.7-31.2[c]	29.1 ± 0.8	2.8	60
S	63	2.6-3.4[d]	2.9 ± 0.13	4.5	6.0
ΔS	52	26.6-34.4[c, e]	29.4 ± 1.3	4.4	61
	296	2.3-3.2[d, f]	2.7 ± 0.13	4.8	5.6
ΔA	32	27.0-32.7[c]	29.5 ± 1.4	4.8	61
	210	2.3-3.3[d, g]	2.7 ± 0.15	5.3	5.6
Δ-T	80	29.5-35.1[c]	32.3 ± 1.1	3.3	67

[a] MCL, mean contour length; SSD, sample standard deviation.

[b] Molecular weight calculated on the assumption that 1 μm DNA = 207 × 10^4 amu (31).

[c] Distribution of contour lengths shown in Fig. 2.

[d] Distribution of contour lengths shown in Fig. 3.

[e] In addition, another molecule was measured, of size 56.9 μm, which is not included in the calculation of MCL.

[f] In addition, other moelcules were measured, of sizes 5.2 μm, 5.4 μm, 7.9 μm, and 10.3 μm, which were not included in the calculation of MCL.

[g] In addition, other molecules were measured, of sizes 5.4 μm, 5.5 μm, 6.0 μm, and 9.1 μm, which were not included in the calculation of MCL.

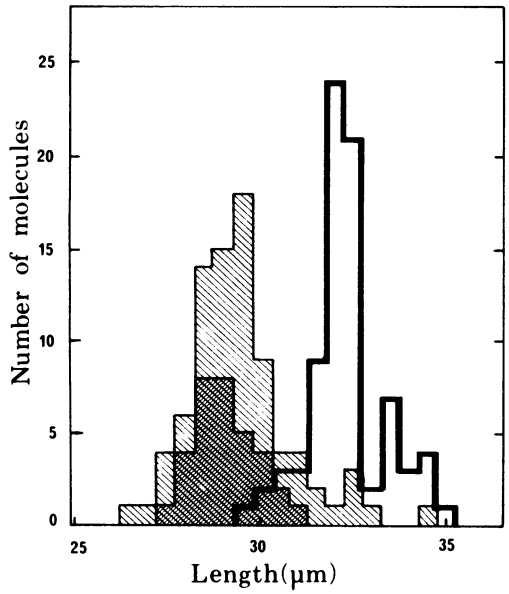

FIG. 2. *Distribution of contour lengths (to nearest ½ μm) of circular molecules measured in DNA isolated from Δ⁺ strains (double hatched), (Δ-T)⁺ strains (thick outline), and of all large circular molecules from the DNA of Δ⁺A⁺ and Δ⁺S⁺ strains (single crosshatched).*

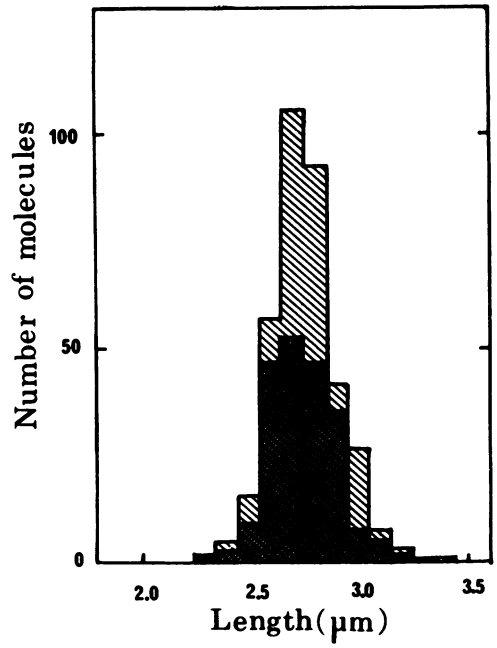

FIG. 3. *Distribution of contour lengths (to 0.1 μm) of circular molecules measured in DNA from S⁺ strains, together with those of small circular molecules in DNA from Δ⁺S⁺ strains (single crosshatched), and small circular molecules in DNA from Δ⁺A⁺ strains (double hatched).*

the curing of F from 100% of F⁺ColEl⁺ cells by acridine orange, which does not cure any of the cells of ColEl (17).

Similar observations have been made on the transfer of the neomycin-kanamycin (K)-resistance determinant by K-12 strains carrying the F factor (7). Anderson et al. (7) therefore suggested a physical association between the K determinant and a specific region of the F factor during transfer which might be in the form of transient hydrogen bonding between a limited region of complementary base pairs of

F and K. This association would occur while each plasmid was in the single-stranded state needed for transfer (18, 40, 41), but it would require short regions of double strandedness for transfer of K to occur.

Another possibility is that there is no physical association between the transfer factor and mobilizable plasmids such as A, S, K, and ColEl during transfer, but that the transfer is potentiated by the conjugation process and by influences so far unidentified, one of which may be the proximities of the postulated cell attachment sites of these molecules. In any event, the results in Table 1 strengthen the view that the mobilizable determinant and the transfer factor in these systems are independent plasmids.

A similar bimodal distribution of molecules to that in Δ^+S^+ cells is found in Δ^+A^+ cells where two molecular species, a large 29.5 μm molecule (inferred to be Δ) and a small 2.7 μm molecule (inferred to be A) are found. The data from the measurements of the large molecules in Δ^+A^+ and Δ^+S^+ cells are compared to those from Δ^+ cells in Fig. 2. The size of a recombinant Δ+S or Δ+A molecule would be expected to be about 32 μm (29.1 + 2.9 μm). The data in Fig. 2 show that a small proportion (about 5%) of the molecules could be distributed about that mean, and one molecule of 34.4 μm was measured (Table 1; Fig. 2). Nevertheless, the differences in size between such a recombinant and Δ are too small, compared to the experimental variations, to exclude random variations in measurement. However, it seems clear that the majority of molecules in Δ^+S^+ or Δ^+A^+ cells represent discrete Δ or S and A molecules, as they are found in Δ^+, S^+, or A^+ cells.

In contrast to ΔA and ΔS, Δ-T DNA comprises only one size molecule of MCL 32.3 μm (67×10^6 amu) (Table 1; Fig. 2). No small molecules were seen in any DNA preparation from $(\Delta\text{-T})^+$ cells out of several thousand scanned on electron microscope grids. These observations are consistent with the genetic properties of $(\Delta\text{-T})^+$ cells which, in general, transfer infectious tetracycline resistance without transferring either the transfer factor or noninfectious tetracycline-resistance determinant (T) independently. (Although up to 1% of recipients in overnight crosses with $(\Delta\text{-T})^+$ donors receive the Δ factor properties only, these recipients also show the phage restriction pattern of Δ-T, which establishes that the transfer factor has come from the Δ-T complex [2]). The difference in size between Δ-T and Δ (MCL 32.3-29.1 = 3.2 μm) shown in Fig. 2 is perhaps indicative of the size of the T determinant alone.

TABLE 2. *Amounts of DNA found in satellite band relative to chromosomal band and the estimated numbers of plasmid copies per chromosome*

Plasmid	No. of experiments	Mean % label satellite/chromosomal band (range of values)	Mean ratio of copies[a] plasmid/chromosome (range of ratios)
Δ	6	1.8 (1.1-2.3)	0.8 (0.5-1.0)
S	8	3.1 (1.9-5.2)	14 (9-23)

[a] Isolated as covalently closed circular molecules (calculated from mean molecular weights; Δ 61 $\times 10^6$ amu, and S; 5.6 $\times 10^6$ amu).

Each of the strains Δ^+A^+, Δ^+S^+, and $(\Delta\text{-T})^+$ can transfer its drug resistance to a recipient strain, which thereby itself becomes drug resistant and can transfer this resistance. Thus, by definition, Δ^+A^+, Δ^+S^+, and $(\Delta\text{-T})^+$ strains each harbor an R factor. In Δ^+A^+ and Δ^+S^+ cells, the R factor is comprised of an aggregate of two independent plasmids, one carrying drug-resistance genes and the other carrying transfer factor genes. In $(\Delta\text{-T})^+$ cells, the R factor resembles other R factors previously examined, in which the drug-resistance genes and transfer genes are integrated into a single molecular structure. We will term these two R factor types as R-factor *plasmid aggregates* and R-factor *plasmid cointegrates*, respectively.

In ΔS DNA, two molecules larger than all others are seen (Table 1). One, of 34.4 μm, has already been considered as a possible Δ+S recombinant. The other, of 56.9 μm, may be a circular dimer of Δ. Several smaller molecules are seen in ΔS and ΔA DNA which fall between the sizes of Δ and A or S. In ΔS, the sizes are consistent with the idea that these molecules may represent three circular S dimers, (expected value 5.4 μm; measured 5.2 μm, 5.4 μm, 5.6 μm), one S trimer (expected value 8.1 μm; measured 7.9 μm) and a circular S tetramer (expected value 10.8 μm; measured 10.3 μm). In ΔA DNA, three molecules might similarly be circular A dimers (5.4, 5.5, and 6.0 μm) and another a trimer, (although this 9.1 μm molecule is larger than expected of a circular A trimer).

The size distributions of molecules found in S DNA together with the small molecules in ΔS DNA (concluded to be S) are shown in Fig. 3 together with the small molecules in ΔA DNA inferred to be A molecules. Both A and S appear to be similar in size.

Recent transduction experiments with P1 phage (8) support the postulates previously made of the genetic structure of the Δ-

mediated resistance transfer systems (3, 4). The Δ^+S^+ or Δ^+A^+ K-12 strains produce P1 lysates that yield noninfectious S^+ or A^+ transductants. No Δ^+S^+ or Δ^+S^+ transductants were found. The Δ can be introduced into these S^+ or A^+ transductants at its normal high frequency, and the determinant is then mobilized in the usual way, i.e., the Δ,S and Δ,A resistance transfer systems are reconstituted. These results support the idea that the S and A determinants are independent of Δ in the cell. In contrast, transduction of Δ-T by P1 follows the pattern expected of a plasmid co-integrate; it is transduced intact and can be transferred from transductants in the same way and at the same high frequency as from the donor strain from which the transducing phage was prepared. In contrast, previous transduction experiments using P22 had failed to transduce Δ-T intact, with only noninfectious tetracycline resistance being transduced (9). These results are presumably due to the size of the Δ-T molecule of 67×10^6 amu, which is larger than P22 DNA (27×10^6 amu [35]) but less than P1 DNA (78×10^6 amu [27]).

Table 2 shows measurements of relative radioactivity in the satellite and chromosomal bands in experiments involving host cells harboring a single plasmid type. As other workers have noted (24), the amounts of covalently closed circular DNA isolated by a number of different methods is subject to so far unexplained fluctuations and may also represent only a fraction of the plasmid molecules, since DNA-relaxation complexes (12) would not be isolated by this method. These measurements can therefore be taken only as a rough guide to copy number.

In experiments with Δ, the ratio of DNA under the two peaks is consistent with an average of one copy of Δ for every copy of chromosome, irrespective of the phase of growth at lysis. Thus, Δ is characteristic of a plasmid that is stringently regulated in the replication of its DNA so as to limit the numbers of plasmid copies to the equivalent of the numbers of chromosomes at all growth phases.

DNA measurements from S^+ cells were consistent with an average of 14 copies of S. It seems likely, therefore, that S is a plasmid that is relaxed in its DNA replication (30, 36), is present in multiple copies, probably in excess of ten, and thus differs in its replication control from Δ.

Unfortunately, the fluctuations in experimental DNA assays were even more marked when DNA was examined from host cells harboring Δ-T or two different sizes of plasmid such as Δ and S, and no conclusions can be drawn about the numbers of copies.

The physical studies of R-factor DNA described in this paper correlate well with many of the genetic experiments of the Δ-mediated R factors and the postulates on their genetic structure made from these studies. In particular, the hypothesis that resistance determinants such as A and S are essentially independent in the host cell of the factors that mediate their transfer (3, 4) receives strong support from the molecular observations. However, our conclusion that there are multiple copies of the independent drug-resistant determinant S per chromosome copy is contrary to that drawn from genetic experiments on incompatibility, when a single copy of both transfer factor and drug-resistance determinant was stated to be present in each host cell (4).

ACKNOWLEDGMENTS

We are indebted to E. S. Anderson for the provision of host strain RC703 infected with the plasmids used in this study, and to Dimitrij Lang for the use of electron microscopy facilities and for invaluable advice. This work was supported by Public Health Service research grants GM-14394 and GM13234 from the National Institutes of General Medical Sciences.

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