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Extrachromosomal Deoxyribonucleic Acid in R Factor-Harboring *Enterobacteriaceae*

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Extrachromosomal deoxyribonucleic acid (DNA) from 24 different R factor-harboring *Enterobacteriaceae* was isolated and characterized by analytical ultracentrifugation and electron microscopy. The R factors represented 15 different patterns of transferable drug resistance found in enterobacteria from an enclosed geographic area. All of the strains contained extrachromosomal, circular DNA molecules within the range of 0.4 to 52 μm. More than one size class of circular DNA molecules was observed in the majority of the extrachromosomal DNA preparations. The buoyant density of the extrachromosomal DNA ranged from 1.700 to 1.720 g/cm^3. The majority of the bacteria contained extrachromosomal DNAs of various densities. Three-fourths of the R factors were classified as *fi*$^+$. The investigation illustrates the extensive variability in the physical characteristics of plasmid DNA from R factor-harboring strains.

Most of the work on the physical characterization of plasmid deoxyribonucleic acid (DNA) in R factor-harboring enterobacteria has been done on genetically interesting R factors in *Escherichia coli* and *Proteus mirabilis* (15). These studies have shown the existence of monomolecular (21, 27) and multimolecular (18, 20) R factors. No definite correlation has been demonstrated between the physical size of the R factors and the number of drug resistance traits transferred (2, 6-10, 12-16, 18, 20-25, 27). An unequivocal correlation between the buoyant density of the plasmid DNA and the type of drug resistance traits transferred is also lacking, although Falkow et al. (10) have shown that the loss of different resistance traits due to deletions of DNA from the R factor R5 was accompanied by specific loss of different buoyant density profiles. Likewise, the *fi*-type of the R factor-harboring bacteria has not been associated with any specific physical appearance of the R factor DNA. On the other hand, there seems to be a close resemblance in the contour length of R factor molecules within the same compatibility group (13).

The differences in the physical appearances of R factors investigated thus far might imply that the origin of R factors has been multifocal. An opposite conclusion, however, has been suggested by Barth and Grinter (2), who found a close similarity in the physical characteristics of plasmids, from organisms from different parts of the world, with linked resistance to sulfonamide and streptomycin.

This article reports a screening of the physical characteristics of plasmid DNA from a clinical material of R factor-harboring strains. The work was done to establish whether the differences observed in the physical appearances of R factors investigated thus far might exist in plasmid DNA from R factor-harboring enterobacterial strains from an enclosed geographic area.

MATERIALS AND METHODS

Bacteria. The R factor-harboring strains investigated were *Enterobacteriaceae* obtained from hospitalized patients and outpatients in the county of Aarhus, Denmark (19).

R factor transfer and fi character. Conjugation experiments and the assay of R factor transfer were as described by Moller et al. (19).

For *fi* typing, R factors were transferred from the original strains to one of two different derivatives (CSH 62-N or CSH 62-K) of the HfrH K-12 strain CSH 62 (*thi*$^-$).

Mutants CSH 62-N (Nal^R) and CSH 62-K (Kan^R) were obtained by incubating strain CSH 62 for 1 h at 37 C in 5 ml of a citrate buffer (0.05 M, pH 5.5) containing 400 μg of *N*-methyl-*N*-nitroso-*N*-nitroguanidine. The R factor-harboring HfrH strains were then tested for the constitutive production of F pili by using male-specific phage MS2 (Miles Laboratories) in a cross-streaking method (17).

Isolation of extrachromosomal DNA. For isolation of extrachromosomal DNA, the bacteria were grown in 100-ml portions of brain heart infusion broth (Difco). After being harvested in the exponential phase, the organisms were washed, converted to spheroplasts, and lysed by the method of Bazaral and

Helinski (3, 4). Ethidium bromide-cesium chloride gradient centrifugation of the spheroplast lysates was performed as described by Bak et al. (1). The band containing the closed circular DNA was isolated through a needle, dialyzed against TES buffer [0.05 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane, 0.005 M ethylenediaminetetraacetic acid (EDTA), pH 8], and characterized by analytical ultracentrifugation and electron microscopy.

Analytical ultracentrifugation. Isolated extrachromosomal DNA was analyzed in a Beckman model E analytical ultracentrifuge equipped with monochromator, electronic speed regulation, and photoelectric scanner.

The CsCl gradients were prepared by dissolving CsCl (Merck Suprapur) in a solution of plasmid DNA in TES (1.5 μg of DNA/ml) to a density of 1.700 g/cm^3. To 1 ml of this solution, 1 μg of the marker DNA (DNA from *Micrococcus lysodeikticus*; density, 1.731 g/cm^3) was added and the density was readjusted.

Conditions for centrifugation and calculation of buoyant densities were as described previously (1).

Electron microscopy. The extrachromosomal DNA in TES buffer was mixed with 1 volume of a 0.04% solution of cytochrome *c* (Fulka AG, Buchs, Germany) and with 2 volumes of a 1 M ammonium acetate solution. This solution was spread onto the surface of a cooled (+4 C) solution of 0.25 M ammonium acetate. The monolayer was transferred to 3-mm copper grids (LKB, Sweden) covered with Parlodion (Struers, Denmark), dipped into 5×10^{-5} M uranyl acetate (RP Carlo EBBA, Italy) in 90% ethanol for 30 s, and fixed in 90% ethanol for a few seconds. Rotary shadowing and examination in a Jeol JEM-100 B electron microscope were done as described previously (1).

The contour lengths of the photographed circular DNA molecules were measured on paper copies (magnification, ×5) with a Hewlett-Packard digitizer, model 9107A.

RESULTS

Of 632 strains isolated from hospitalized patients and outpatients (19), 77 transferred drug resistance, comprising 18 different patterns of resistance. To determine whether there were any differences in the physical characteristics of these R factors, we chose for study at least one R factor-harboring strain representative of 15 of the 18 different resistance patterns. A total of 24 strains was examined. In all instances the extrachromosomal DNA was isolated from the original bacterial hosts.

The R factors are listed in Table 1 according to patterns of transferable drug resistance; the results of the *fi* typing, analytical ultracentrifugation, and electron microscopy of the extrachromosomal DNA from the 24 R factor-harboring strains are also shown.

The majority of the R factors were *fi*$^+$ (17 of 24). Most of the extrachromosomal DNA preparations (17 of 24) contained more than one buoyant density profile (Table 1, Fig. 1). The buoyant densities ranged from 1.700 to 1.720 g/cm^3. All plasmid preparations contained at least one buoyant density profile in the interval of 1.708 to 1.712 g/cm^3. No correlation between the patterns of resistance transferred and the number of buoyant density profiles or values could be found.

The extrachromosomal DNA contained almost exclusively supercoiled molecules when examined immediately after isolation. The preparations contained no linear DNA molecules. After a few weeks of storage at 4 C, an increasing number of molecules were present as open circles. Only molecules that could be seen as open circular forms were traced and measured (Fig. 2). As indicated in Table 1, more than one (maximum of five) distinct molecular size class could be found in the majority (17 of 24) of the DNA preparations. The average contour length of molecules within the same size class was calculated and the standard deviation was found to be generally less than 5% of the average contour length. In some strains a broader distribution of molecular contour lengths was found. In those cases the minimum and the maximum length is indicated (Table 1). The length of the molecules obtained varied between 0.4 and 52 μm. It is characteristic that all plasmid preparations contained at least some molecules with a contour length of 13 to 16 μm or greater. This length is the minimum length thus far associated with the ability of conjugational transfer.

The molecules could be divided into two groups of size classes according to their contour lengths. One group comprised size classes within the range of 0.4 to 4.5 μm; the second comprised size classes with contour lengths of more than 10 μm. These molecules are probably transfer factors or composite R factors able to promote their own transfer and the transfer of the smaller molecules.

No unambiguous correlation was found between the contour length and the number of resistance traits transferred. Molecules from strains harboring R factors conferring resistance to the same number of antibiotics may thus have the same, or widely different, contour length.

In most of the extrachromosomal DNA preparations, the number of different size classes of contour length and buoyant density profiles was found to be the same. In the DNA preparations in which this was not the case, fewer density profiles than size classes were obtained. However, it may be noted from Table 1 that some

TABLE 1. *Physical nature of extrachromosomal circular DNA molecules from different R factor-harboring Enterobacteriaceae*

Pattern of resistance transferred[a]	Plasmid	Host	*fi* type	Buoyant density (g/cm^3)	Contour length[b] (μm)
Tc	pJM 6	*Escherichia coli*	+	1.700 1.710	0.7 (11) 16.6 (11) 20.8 (15)
Tc	pJM 11	*E. coli*	+	1.710 1.715	2.7 (3) 39.1 (7)
Sm	pJM 7	*E. coli*	+	1.703 1.707 1.710	1.7 (18) 12.3 (14) 24–29 (12)
Ap	pJM 9	*E. coli*	–	1.701 1.703 1.709 1.712	1.6 (3) 3.4 (16) 10.9 (6) 14.1 (5) 20.5 (4)
Su,Sm	pJM 3	*E. coli*	+	1.711	21.6 (29)
Su, Sm	pJM 25	*E. coli*	+	1.708	1.0 (3) 29.9 (3) 34.5 (9)
Su,Tc	pJM 21	*E. coli*	–	1.711	38.1 (3)
Su,Ap	pJM 23	*E. coli*	–	1.706 1.708 1.711	1.5 (53) 3.5 (30) 18–27 (10)
Sm,Tc	pJM 22	*E. coli*	+	1.710 1.720	2.5 (57) 21.0 (6) 23.1 (11)
Sm,Tc	pJM 26	*E. coli*	+	1.708	31.5 (19)
Tc,Ap	pJM 17	*E. coli*	+	1.708	21.2 (24)
Su,Sm,Tc	pJM 5	*E. coli*	+	1.707 1.709	0.9 (5) 1.6 (5) 12.5 (21) 26–34 (7)
Su,Sm,Tc	pJM 15	*Proteus mirabilis*	+	1.705 1.711	10.0 (48) 15.8 (16)
Su,Sm,Cm	pJM 12	*E. coli*	+	1.711 1.713	1.3 (12) 2.1 (20) 25–29 (12)
Su,Sm,Cm	pJM 19	*E. coli*	+	1.710 1.712	1.0 (4) 24–32 (11)
Su,Sm,Ap	pJM 4	*Klebsiella pneumoniae*	–	1.709 1.715	3.9 (17) 17.5 (19)
Su,Sm,Ap	pJM 14	*K. pneumoniae*	–	1.710 1.715	4.0 (20) 18.0 (6)

TABLE 1.—*Continued*

Pattern of resistance transferred[a]	Plasmid	Host	*fi* type	Buoyant density (g/cm³)	Contour length[b] (μm)
Su,Sm,Ap	pJM 16	*E. coli*	–	1.704	1.2 (18)
				1.710	3.9 (59)
				1.715	10.8 (7)
					17.0 (12)
Sm,Tc,Ap	pJM 18	*Enterobacter cloacae*	–	1.705	1.6 (6)
				1.712	17–21 (10)
				1.715	
Su,Sm,Cm,Ap	pJM 20	*E. coli*	+	1.711	27.0 (18)
Su,Sm,Cm,Ap	pJM 24	*E. coli*	+	1.712	24.7 (15)
Su,Sm,Tc,Cm,Ap	pJM 10	*E. coli*	+	1.708	2.1 (16)
				1.711	19.1 (9)
Su,Tc,Cm,Ap,Km	pJM 1	*E. coli*	+	1.710	50.6 (3)
Su,Tc,Cm,Ap,Km	pJM 8	*E. coli*	+	1.703	0.4 (6)
				1.711	11.6 (5)
					18.0 (5)
					22.3 (10)
					40–52 (5)

[a] Su, Sulfonamide; Sm, streptomycin; Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; and Ap, ampicillin.

[b] Numbers of molecules measured are given in parentheses.

size classes may contain dimers of smaller molecules.

It is obvious that no relationship between the contour lengths of the different size classes and the buoyant densities can be directly deduced from these results. However, a comparison of the physical characteristics of plasmid DNA from strains transferring the same pattern of resistance gives some information. For example plasmids pJM 4, 14, and 16, transferring resistance to sulfonamide, streptomycin, and ampicillin, respectively, all consist of molecules with contour lengths about 4 μm and 17 to 18 μm and buoyant densities of 1.710 and 1.715 g/cm³. All three R factors were further classified as fi^-, also indicating a close resemblance or identity. Furthermore, plasmid fraction pJM 16 contained molecules with contour lengths of 1.2 and 10.8 μm and a buoyant density profile of 1.704 g/cm³. It is characteristic that a buoyant density profile of less than 1.708 g/cm³ was found, with very few exceptions, in the extrachromosomal DNA preparations that contained molecules of less than 2 μm. Finally, all preparations containing composite R factors (one molecular size class only) seem to be associated with buoyant density values of 1.708 to 1.712 g/cm³.

DISCUSSION

The finding of a marked heterogeneity in base composition and contour length of the extrachromosomal DNA obtained from R factor-harboring strains suggests the existence of several different molecular types of R factors even in organisms from a small geographic area.

It is obvious that all extrachromosomal DNA preparations containing only one molecular size class must represent composite R factors. However, all of the molecules found in DNA preparations with more than one molecular size class are presumably not R factor molecules. Nontransferable drug resistance determinants isolated so far have been associated with molecular contour lengths of 2.4 to 4.5 μm (14, 25, 26). The smaller molecules with a contour length of less than 2 μm, obtained in this study, might be nontransferable resistance determinants but could also be plasmids not related to R factors. In this connection it is worth referring to the data of Christiansen et al. (5) for smaller molecules in all extrachromosomal DNA preparations from randomly picked enterobacteria with and without resistance to different antibiotics. Furthermore, several of the plasmid fractions (pJM 6, 7, 9, 23, 22, 5, 12, 16, 18, and 8)

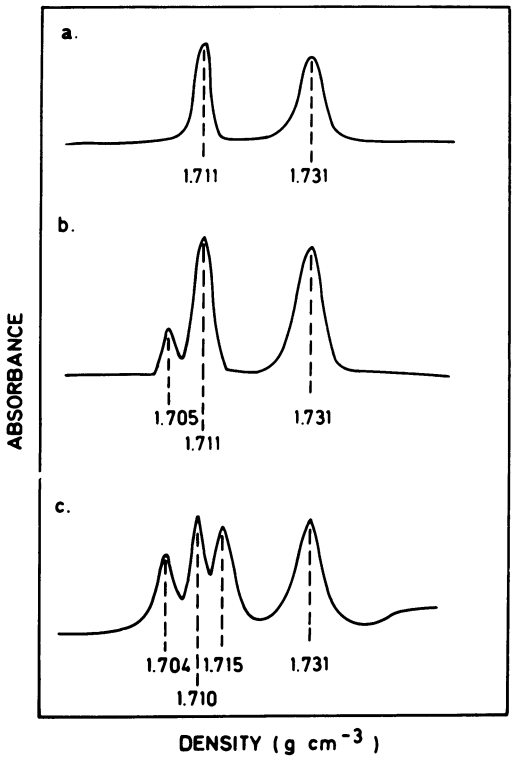

FIG. 1. *Tracings of analytical, neutral CsCl gradients of isolated, circular DNA from three different R factor-harboring strains. Reference marker is DNA from Micrococcus lysodeikticus (density 1.731 g/cm^3). (a) pJM 20 isolated from E. coli. (b) pJM 15 isolated from P. mirabilis. (c) pJM 16 isolated from E. coli.*

seem to contain more than one plasmid system because several distinct smaller and larger molecular size classes were present. Some of these plasmid fractions (pJM 6, 7, and 9) were isolated from strains with R factors mediating resistance to one antibiotic only, indicating that two different R factors in the same bacterium is not very likely. The other molecules might then represent either cryptic plasmids or known types such as, for example, the K 88 factor, the Hly factor, or the nontransferable colicin-producing plasmids. Plasmid DNA from three different strains (pJM 4, 14, and 16) harboring the R factors mediating resistance to sulfonamide, streptomycin, and ampicillin were found to contain a smaller molecule with a contour length of about 4 μm and a larger molecule with a contour length of about 17 to 18 μm. These three R factors were found in two *Klebsiella* strains and one *E. coli* strain.

Some of the other plasmid fractions (pJM 10, 11, 15, 18, and 23) may presumably also contain multimolecular R factors of the form: transfer factor molecules + nontransferable drug resistance determinant molecules.

Two of the plasmid preparations (pJM 9 and 22) might contain composite R factor molecules in addition to molecules representing the transfer factor and the nontransferable resistance determinant. Such a dissociation of plasmid molecules into a transfer factor and a phenotypic determinant has been observed for certain R factors (7, 20, 22), the K88 factor (1), and the Hly factor (11).

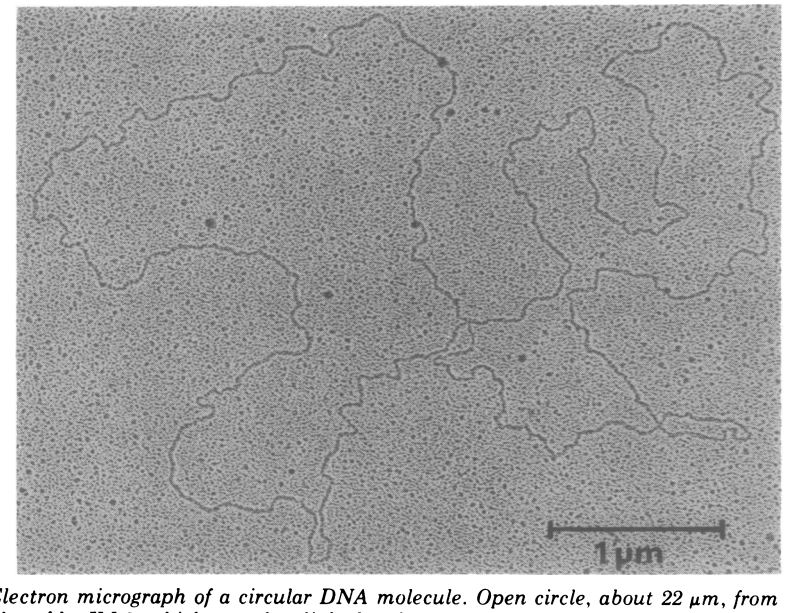

FIG. 2. *Electron micrograph of a circular DNA molecule. Open circle, about 22 μm, from an E. coli strain harboring plasmid pJM 3, which transfers linked resistance to sulfonamide and streptomycin.*

In this report two different strains with plasmids pJM 3 and 25 coding for transfer of linked resistance to sulfonamide and streptomycin, respectively, were found to harbor one molecular size class only, i.e., resistance determinants and transfer genes located on the same molecule (Fig. 2). This is in contrast to the findings of Barth and Grinter (2), who, in strains from widely different origins, found that linked resistance to sulfonamide and streptomycin was always located on nontransferable plasmids with contour lengths of about 5.7 μm.

In conclusion, the results of the present investigation reveal that plasmid molecules isolated from different, randomly chosen R factor-harboring strains vary greatly in number and physical nature of the different size classes. The physical nature of plasmids in strains isolated from the environment thus seem to be more complex than that of most laboratory strains (2, 6-10, 12-16, 18, 20-27). Probably the naturally occurring strains contain other plasmids in addition to those that determine the antibiotic resistance. Many of these other plasmids are presumably cryptic; i.e., their phenotypic expression is unknown at present.

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