

Multiple-Size Plasmids in *Agrobacterium radiobacter* and *A. tumefaciens*

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This article is the eighth in a series of studies on *Agrobacterium tumefaciens* (17).

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ABSTRACT

SHEIKHOESLAM, S., B.-C. LIN, and C. I. KADO. 1979. Multiple-size plasmids in *Agrobacterium radiobacter* and *A. tumefaciens*. *Phytopathology* 69: 54-58.

Tumorigenic *Agrobacterium tumefaciens* and naturally avirulent *A. radiobacter* strains harbor large plasmids with molecular weights ranging from 27 to 154 megadaltons, and mini plasmids with molecular weights of 1.0 to 12.0 megadaltons. Replicating plasmid molecules were observed by electron microscopy, indicating that these molecules are autonomous. The guanine-cytosine composition of these large plasmids (58.8 mole %) is

equivalent to that of chromosomal DNA. The number of total plasmid copies per cell is about two; they may be either two large plasmids or one of each size class. The small plasmid may have arisen from the large plasmid and represent a mechanism for amplification of virulence genes that could account for the various degrees of virulence that have been observed commonly.

Agrobacterium tumefaciens strains, which are capable of inducing crown gall tumors in higher dicotyledonous plants, harbor large plasmids averaging 100–120 megadaltons in molecular weight (3,12,24,25,27,28). The functional roles of the plasmid remain unknown, but the expression of tumorigenic phenotype of this organism depends on the existence of a plasmid for the following reasons: (i) virulent strains can be converted to avirulent forms by growth at supraoptimal temperatures (7,8,17,25,27), with either the concomitant loss of the plasmid (25,27) or partial loss owing to a deletion in the plasmid (16,17,24), and (ii) tumorigenicity (virulence) can be restored in avirulent plasmidless *Agrobacterium* strains by conjugation with virulent strains, along with the reappearance of the plasmid in the recipient strains (4,9,12,17,26,27).

It is uncertain whether the plasmid itself is the tumorigenic substance (ie, the plasmid DNA causes tumors directly) or whether it simply confers virulence to *Agrobacterium* (ie, invasive properties whereby the bacterial cell releases the tumorigenic substance). Matthyse and Stump (18) and Chilton et al (5), however, have reported plasmid sequences in bacteria-free crown gall tumors, in which plasmid sequences are transcribed (6,15). This would indicate that the plasmid plays a definitive role in tumor maintenance.

In this article, we report the existence of large plasmids in naturally avirulent *A. radiobacter*, their base composition relative to that of plasmids in virulent strains, and the occurrence of autonomous miniplasmids in both virulent and avirulent *Agrobacterium*. The presence of plasmids in *A. radiobacter* was reported (16) concomitantly with others (19,20); portions of the electron microscopy presented herein appeared in a preliminary report (24) and have been extended in this communication.

MATERIALS AND METHODS

Strains.—*Agrobacterium tumefaciens* ID135 (ATCC 27912) has been described previously (13). R. S. Dickey, of Cornell University, provided strain C-58. Both of these strains use nopaline, induce its synthesis in crown gall tumors, and are highly virulent on tomato, tobacco, and sunflower. Avirulent derivatives of these strains were obtained by subculture at 36.5 C or by

ethidium bromide treatment as described previously (17). Plasmids from both strains are similar (24). *Agrobacterium radiobacter* strains TR-5 and TR-6 were obtained from M. P. Starr, K22 and K84 from A. Kerr, S1005 from J. Schell, and AR-2, AR-3, AR-5, and AR-11 from M. N. Schroth.

Plasmid isolation.—Cultures were grown overnight at 30 C, with shaking, in 10 ml of medium 523 (13) in a 50-ml nepheloflask to a cell concentration of 100 Klett units (green filter), which represents approximately 7×10^8 colony-forming units (CFU)/ml. The cells were washed twice with distilled water and used immediately. Plasmids were isolated as described by Lin and Kado (17), with minor modifications. The cell lysates were treated with DNase-free pancreatic RNase (50 μ g/ml) for 15 min at 25 C and with proteinase K (Merck) (100 μ g/ml) for 20 min at 37 C. This treatment releases plasmids from the folded chromosome matrix and cellular material that usually floats to the meniscus during centrifugation in CsCl (11). Plasmid DNA was banded by isopycnic density centrifugation in a CsCl gradient in a 12-ml polyallomer tube (precleansed with 95% ethanol) that contained 3.62 g of CsCl, 3.5 ml of lysate, and 0.3 ml of ethidium bromide (5 mg/ml), and centrifuged at 77,200 g for 48 hr at 20 C in a Beckman/Spinco Ti 60 rotor. Plasmid DNA was removed by introducing a No. 18 gauge syringe needle through the side of the centrifuge tube just below the plasmid band, which can be visualized by long-wave ultraviolet light. Ethidium bromide was removed by extraction with water-saturated *n*-butanol. Plasmid preparations were dialyzed exhaustively against 10 mM Tris, pH 8.0, 1 mM EDTA.

Analytic ultracentrifugation.—The method of Schildkraut et al (22) was used to estimate the buoyant density of plasmid DNA in CsCl. A Beckman Model E analytic ultracentrifuge equipped with scanning optics and a Xe-Hg high-intensity lamp was used. Charcoal-Epon centrifuge cells were filled with a solution containing 2.13 ml of plasmid DNA (3 μ g/ml), 0.04 ml of *Micrococcus lysodeikticus* DNA (15 μ g/ml), and 2.85 g of CsCl (Merck). The solution had a refractive index of 1.4012, which corresponds to a density of 1.720 g/cm³. The samples were centrifuged in an An-F rotor at 44,000 rpm at 25 C for 60 hr; scans were made at 266 nm.

Electron microscopy.—Plasmid DNA was dialyzed overnight against 10 mM Tris-HCl, pH 8.2, 1 mM EDTA buffer (filtered through 0.2 μ pore size Millipore® filters) at 4 C. After dialysis, the DNA was adjusted to 1 μ g/ml in the same buffer. The hyperphase solution containing 50 μ l of formamide; 20 μ l of 1 M Tris-HCl, pH 8.0, 0.1 M EDTA; 40 μ l of DNA (1 μ g/ml); and 3 μ l of 1%

cytochrome C (the cytochrome solution was filtered as above) was applied to the edge of an exposed part of an acid-washed glass microscope slide that had been placed sloping in a new acid-washed 50-ml Griffin beaker (Bellco) filled to the brim with double glass-distilled water. The rim of the beaker was previously coated with paraffin. Water was used as a hypophase in these studies to maximize linear expansion (14). The DNA solution was allowed to run down the slide onto the water surface; after 1 min, which allowed formation of the basic protein (cytochrome C) film on the hypophase, a 300- or 400-mesh copper electron microscope grid coated with 3% parlodion was touched to the surface of the film about 3 mm from the glass slide. The grid then was immersed into a fresh solution of 5 mm acidic uranyl acetate (in 0.05 M HCl-90% ethanol) for exactly 30 sec and then immersed into 95% ethanol for 5 sec and air dried. The DNA specimens were shadowed by rotating the specimen at 20 rpm under a uranium metal (45-mg) source placed at a distance of 8 cm and a height of 1 cm. Contour lengths were measured with the use of Col E1 plasmid DNA as an internal standard, as described previously (24).

RESULTS

Multiple size plasmids in *A. radiobacter* and *A. tumefaciens*.—Covalently closed circular DNA (CCC-DNA) was separated from linear DNA in the presence of ethidium bromide in isopycnic CsCl density gradients (1). When cleared-cell lysates of *A. radiobacter* strains AR-2, AR-3, AR-5, AR-11, TR-5, K22, K84, S1005, and TR-6 were analyzed by this procedure, a satellite band, which contained CCC-DNA that banded below linear DNA, was observed in each strain examined except for strains AR-3 and AR-11. Analysis of the DNA in this band by electron microscopy confirmed the presence of large and small CCC-DNA (Fig. 1 and

2). The largest CCC-DNA of *A. radiobacter* was found in strain TR-6 and had a molecular weight of 154×10^6 daltons based on its contour measurement from electron micrographs of fully relaxed circles. The molecular weights of the large class plasmids in *A. radiobacter* ranged from 12 to 154×10^6 daltons (Fig. 3). The molecular weights of the small class plasmids (less than 10×10^6 daltons) in these strains ranged from 2.2×10^6 to 6.2×10^6 daltons (Fig. 2). The above results on the large plasmid class are an extension of previous work (16,17,24) and represent measurements of greater numbers of CCC-DNA with Col E1 DNA as an internal standard.

The tumorigenic *A. tumefaciens* strains 1D135 and C-58 both possess a large 117×10^6 dalton plasmid. These strains also harbor a smaller plasmid that is about 1×10^6 daltons, a representative of which is shown in Fig. 2 for strain 1D135. Note that the small plasmids of avirulent strains TR-6 and virulent strain 1D135 were in the stage of replication. When the virulent strains C-58 and 1D135 were made avirulent by heat treatment or by exposure to ethidium bromide, plasmids were still retained but were of sizes smaller than in the wild-type strains (Fig. 3). This confirms our earlier observations that curing of virulence does not necessarily free strains of their plasmid in toto. The size of these plasmids was comparable to those found naturally in avirulent *A. radiobacter* strains.

Guanine-cytosine content of plasmid.—The guanine-cytosine (GC) content of the large plasmids of *A. tumefaciens* and *A. radiobacter* was determined by analytic ultracentrifugation. They were first isolated from the smaller plasmids by velocity sedimentation in neutral isokinetic sucrose density gradients and then analyzed for their buoyant densities in CsCl by analytic ultracentrifugation. Plasmids of *A. radiobacter* TR-6 and *A. tumefaciens* 1D135 have densities of 1.718 g/cm^3 , which is identical to the

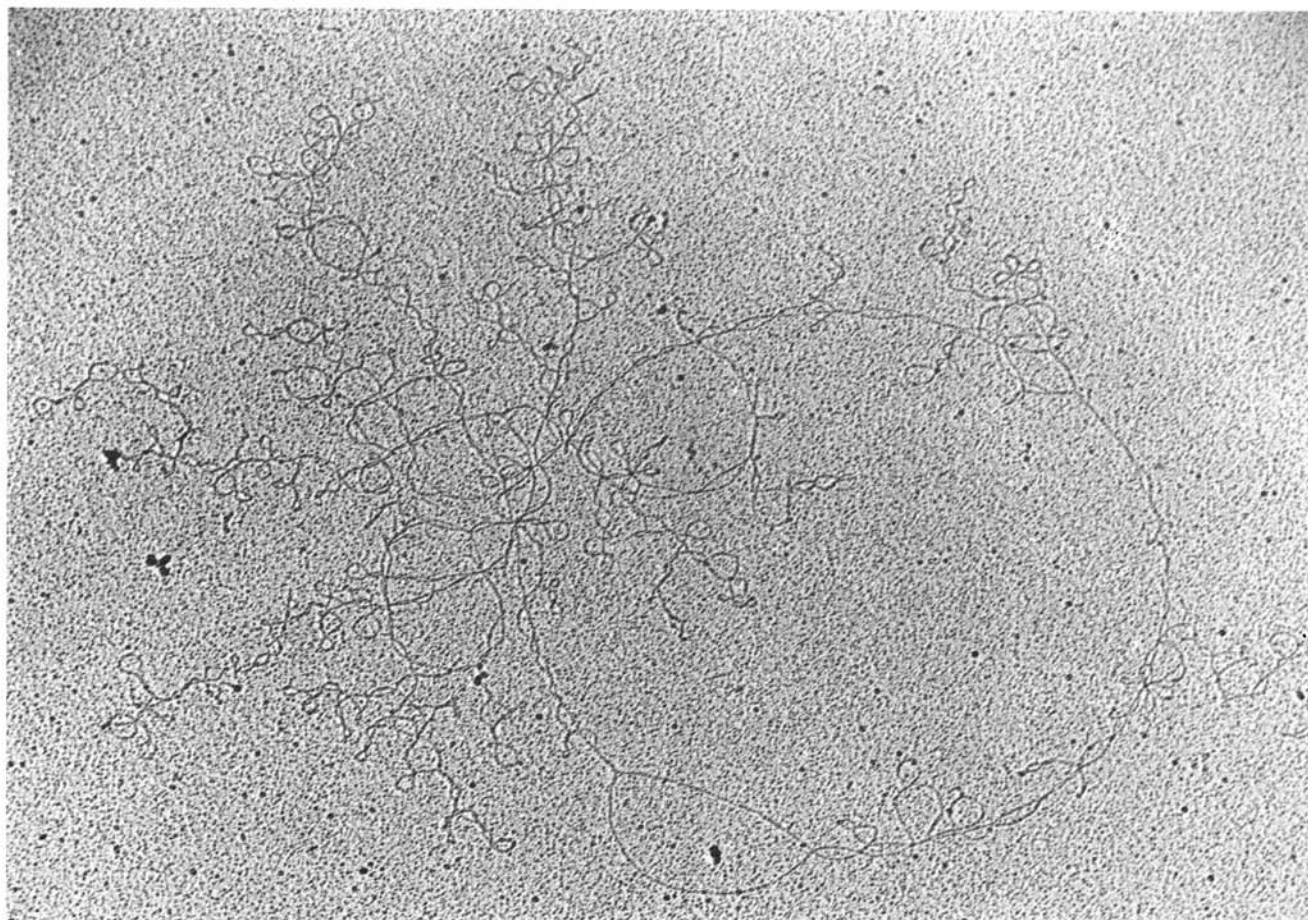


Fig. 1. Plasmid of *Agrobacterium radiobacter* TR6 (naturally nontumorigenic strain) with some supercoiled structure. Contour length of plasmid in TR6 corresponds to average molecular weight of $154 \pm 6 \times 10^6$ daltons. Magnification, $\times 12,900$.

density of the chromosomal DNA reported previously (13). This density corresponds to a 58.8 mole % GC with *M. lysodeikticus* as reference DNA ($\rho = 1.731 \text{ g/cm}^3$) (Fig. 4).

Number of plasmid copies per cell.—Plasmid bands resolved by dye-CsCl density gradient centrifugation were used to estimate

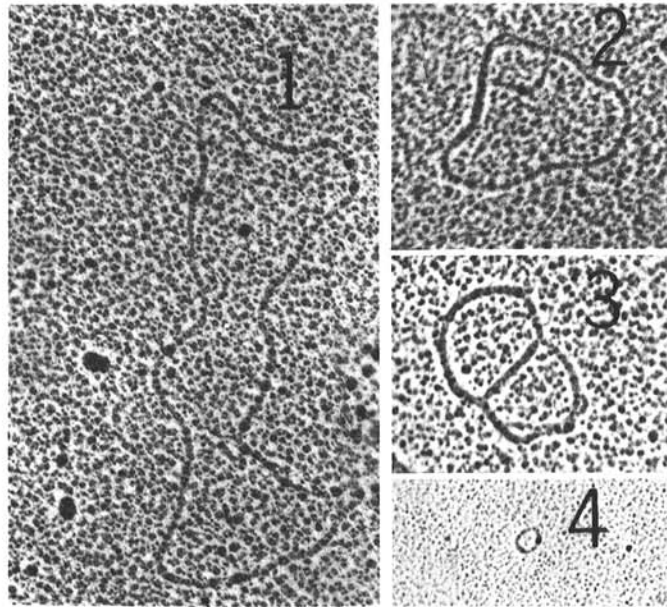


Fig. 2. Small replicating plasmids of *Agrobacterium radiobacter* TR6 and *A. tumefaciens* 1D135. On the basis of contour lengths, molecular weights are 6.2×10^6 (No. 1), 2.2×10^6 (No. 2) in TR6, 1.2×10^6 (No. 3), and 0.6×10^6 daltons (No. 4) in 1D135.

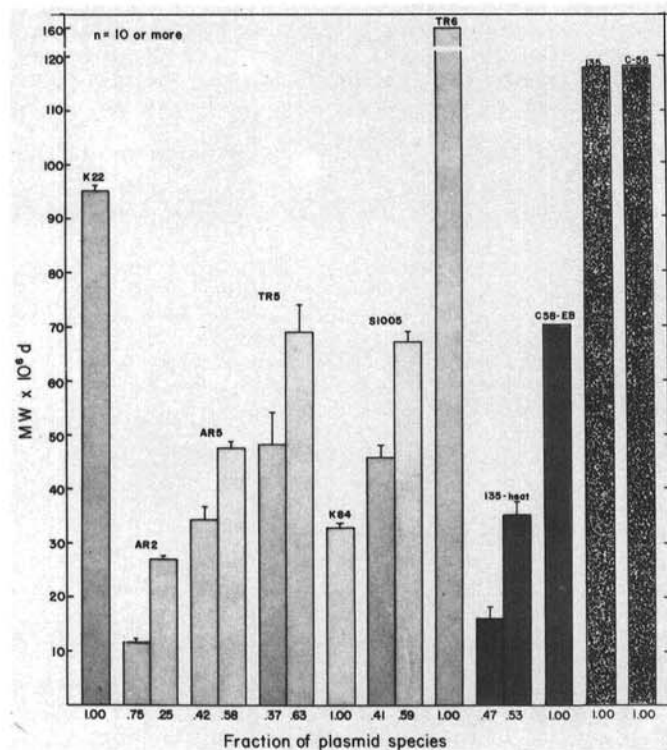


Fig. 3. Distribution of plasmids in *A. radiobacter* and *A. tumefaciens*. K22, AR-2, AR-5, TR-5, K84, S1005, and TR-6 are *A. radiobacter* strains. 135-Heat is strain 1D135 made avirulent by treatment at 36.5 C (16). C-58 EB is strain C-58 cured of virulence by ethidium bromide treatment (16). 135 and C-58 are virulent wild-type strains, from which only large plasmid was recovered. Lines in center of bars represent standard deviations of mean.

plasmid copy number. The gradients were photographed and the negatives were scanned in a spectrophotometer and the percentage of total DNA present in the satellite band was determined for strain 1D135 (Fig. 5). The satellite band represented 7% of the total DNA, which corresponded to about two copies per cell based on their respective molecular weights as determined by electron microscopy and assuming a value of 3×10^9 for the chromosomal DNA (10).

DISCUSSION

An earlier observation (28) reported no plasmids in avirulent *A. tumefaciens* and *A. radiobacter* strains, which led to the conclusion that plasmids were the component required for virulence. Subsequently, large plasmids were discovered in naturally avirulent *A. radiobacter* (16,17,19,20,24). Beside large 100-megadalton plasmids, we found that *A. tumefaciens* and *A. radiobacter* strains also harbor small miniplasmids. Schilperoot (23) first observed small circular DNA in total DNA preparations from strains A6, B6, and EIII 9.6.1. Watson et al (27) also reported in one instance a few small circular DNA molecules in strain C-58, with a molecular weight of 17.7 megadaltons, in their plasmid preparation, but they conjectured that these smaller DNAs were possible cast-off components of the large plasmid. This mechanism of "casting off" by the organism quite likely may have a physiologic function. We postulate here that the organism simply amplifies

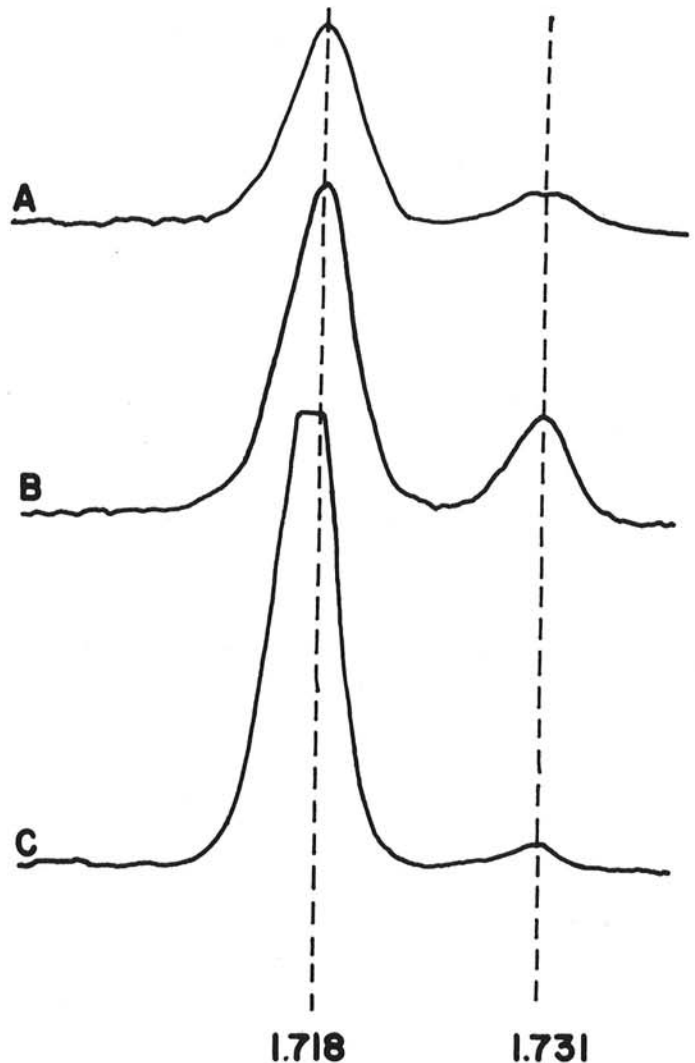


Fig. 4. Analytic CsCl density gradient profiles of: A) *Agrobacterium tumefaciens* 1D135 chromosomal DNA free from plasmid DNA, B) *A. tumefaciens* 1D135 plasmid and *A. radiobacter* TR-6 plasmid. *Micrococcus lysodeikticus* DNA ($\rho = 1.731 \text{ g/cm}^3$) served as the standard density marker.

certain plasmid genes that are necessary for its survival in nature. Two plasmids are liberated into the cytoplasm when *A. tumefaciens* undergoes various conditions of physiologic stress (11). Thus, additional copies of plasmid genes such as the hypothetical "tumor-inducing" genes might be advantageous during the infection process. These small, covalently closed circular DNAs in *A. tumefaciens* and *A. radiobacter* were in a state of replication (Fig. 2). This implies that these small molecules are independent replicons. It is uncertain whether these small plasmids were dissociated from the large 120 megadalton plasmid. If this is the case, then the large plasmid is a composite plasmid with multiple origins for DNA replication. Clearly, heat or ethidium bromide treatment causes loss of virulence and a decrease in the size of the existing plasmid (Fig. 3), which confirms our earlier observations (17). We can draw on the analogous existing situation of a composite R plasmid, which dissociates into smaller-sized plasmids when cells are grown in the presence of antibiotic, thus increasing the gene dosage for antibiotic resistance (21). The possibility exists that that smaller plasmid also may be necessary to maintain the highly virulent state of *A. tumefaciens*. The well-known variations in the degree of virulence (2) possibly are related directly to the degree of plasmid gene amplification, that is, the more copies of the tumor-conferring genes in the bacterium, the more virulent the bacterium.

Both virulent and naturally avirulent strains of *Agrobacterium* must have a specific need for these plasmids. Based on identical GC contents between the strains and their chromosomal DNA, the large plasmid seems to have had long association with these bacteria through the course of all the selection pressures that they encounter in nature. *Agrobacterium* cured of its large plasmid forms much larger colonies on agar medium (17). Thus, constraints

on growth placed by the harbored plasmid would seem to be a selective disadvantage on the basis of growth in the laboratory. Nonetheless, such constraints may not be operating in the natural habitat of *Agrobacterium* and perhaps certain plasmid genes are essential for survival maintenance.

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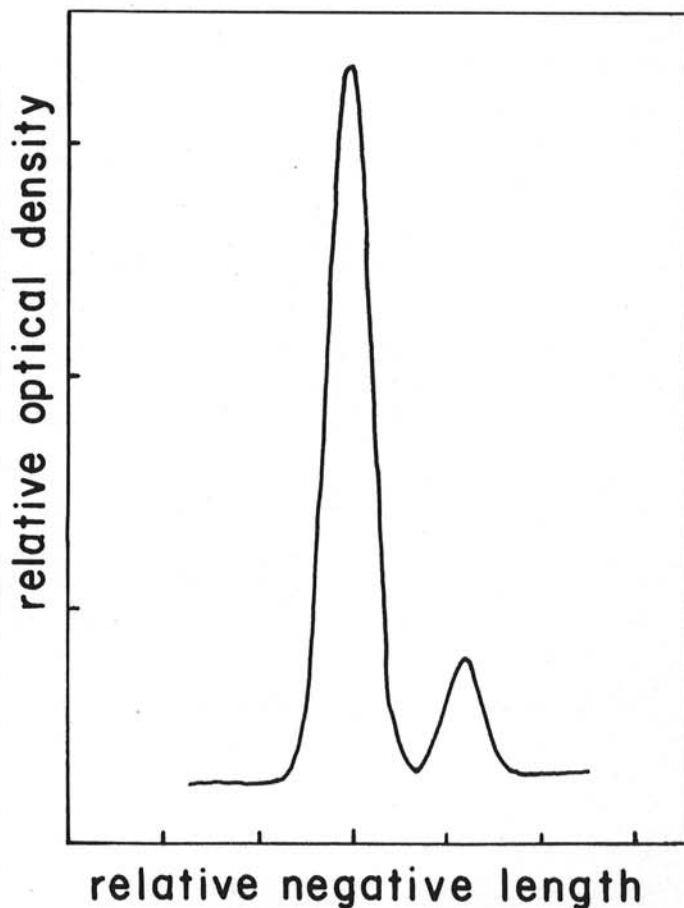


Fig. 5. Densitometric scan of a photographic negative (reversed) of plasmid (small peak) and chromosomal-linear DNA of strain 1D135 resolved in CsCl-ethidium bromide density gradient. Peak heights reflect relative optical densities at 550 nm as function of their position. Negative was assumed to be linear as judged from baseline absorbancy at 550 nm.

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