ISOLATION OF *E. COLI* R FACTOR DNA BY MOLECULAR SIEVE CHROMATOGRAPHY IN 4 M GUANIDINE HYDROCHLORIDE

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1. Introduction

As reported previously [1–4] the R factor DNA from *E. coli* K-12 may be isolated in a single circular form having molecular weight of approx. 55–65 × 10^6 daltons. It is however difficult to separate because of its presence in low proportion (about 1%) [5] and its large molecular size which make it sensitive to nicking [6].

Previous work from our laboratory has indicated that bacterial particles can be solubilized in 4 M guanidine hydrochloride [7]. In this study 4 M GHCl has been used for lysing the *E. coli* K-12 mutant HK-26 Rl and parent R^- strains followed by the fractionation of lysates by molecular sieve chromatography into total DNA (chromosomal + R factor DNA), RNA, proteins and other cellular components. The R factor DNA was then separated and characterized.

2. Materials and methods

The R^- *E. coli* was the strain HK-26 developed by Hubáček et al. [8] The R^+ strain was obtained by infecting the strain HK-26 by the plasmid Rl drd-19 as described by Braná et al. [9]. The strains were grown in the medium (bacto beef extract 1.5 g, yeast extract 1.5 g, bactopeptone 5.0 g, dextrose 1.0 g, NaCl 3.5 g, $K_2HPO_4 \cdot 3\,H_2O$ 3.68 g, KH_2PO_4 1.32 g, in 1 l) containing 20 μg/ml of chloramphenicol (in the case of Rl strain) for 21 hr. The cells were harvested by centrifugation and washed once with cold buffer (0.05 M NaCl, 0.05 M Tris, 0.005 M EDTA pH 8).

Labelling of the cells was carried out in the same medium using [^{3}H]methyl thymidine at a concentration of 20 μg/ml (the specific activity was 48 μCi/mmole) in the presence of 4 μg/ml of 5-fluorodeoxyuridin. The cells were grown for 24 hr to a cell density of 2 × 10^8 cells/ml.

0.5 g of wet bacterial pellet was suspended in 20 ml of glycerol and lysed at 4°. Five minutes later an equal volume of ether ethanol mixture was added and the suspension was centrifuged. The sediment was suspended in a medium containing 4 ml of glycerol + 1.5 ml Tris buffer pH 8 (0.05 M Tris, 0.01 M EDTA in SSC). 3.84 g guanidine-HCl in 2 ml of Tris buffer pH 8 was added slowly to the cell suspension to bring the concentration of the latter up to 4 M (the absorbance of 4 M guanidine-HCl against dest. H_2O was $A^{1\,cm}_{260} = 0.05$). The lysate was gently rocked at 4° for 30 min to homogenize it and then centrifuged at 1700 *g* for 10 min to remove the few remaining clumps. The lysate was fractionated at room temp. on Sephadex G-150 (Pharmica, Uppsala, Sweden).

The total DNA from Sephadex column (fig. 1 peak 1) was dialysed against SSC and treated with pronase (Calbiochem) at a conc. 200 μg/ml for 1 hr at 37°. The DNA solution was then purified in a methylated albumin kieselguhr column [10, 11]. (Prior to chromatography the kieselguhr was cleared of particles finer than 60 μ.)

The circular DNA of R factor was identified by its resistance to alkaline pH [12, 13] and separation was

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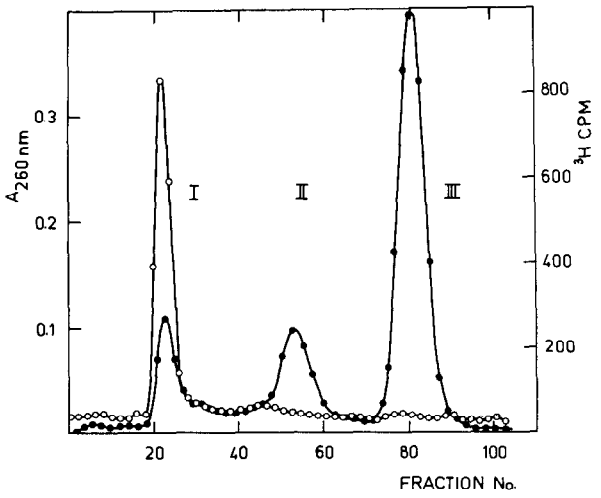

Fig. 1. Molecular sieve chromatography of 10 ml lysate of *E. coli* K-12-26 Rl strain labelled with [^{3}H]methyl thymidine at an A_{260} = 1.8 on a column of Sephadex G-150 (2 × 100 cm). Flow rate 1 ml per cm^2 of column cross section. The absorbancy of the fractions (3 ml) was measured at 260 nm (•—•—•). Five drop fractions were collected from each tube and assayed for trichloroacetic acid-precipitable radioactivity (○—○—○). I – total DNA, II – RNA, III – proteins and other cellular components. Every second fraction is plotted.

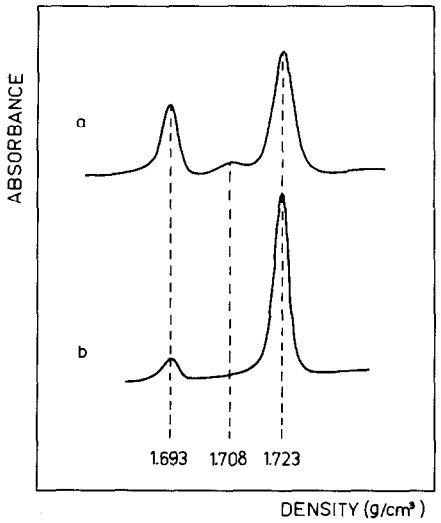

Fig. 2. The sedimentation of alkali denatured and neutralized DNA in CsCl density gradient. 4 μg of DNA was centrifuged at 44 770 rpm at 20° for 24 hr. *Staphylococcus aureus* DNA at density 1.693 g/cm^3 was used as a standard. (a) DNA *E. coli* HK-26 Rl strain, (b) DNA of parent R$^-$ strain.

achieved by bulk nitrocellulose technique [3].

Sedimentation analysis of DNA (10–12 μg/ml in SSC) was performed in a Spinco Model E analytical ultracentrifuge at 35 600 rpm. The average sedimentation coefficients $S_{20,w}$ of total DNA were evaluated. The sedimentation of alkali denatured and renatured DNA in CsCl density gradient was carried out according to Meselson et al. [14].

DNA samples were obtained from the column of methylated albumin (in 1 M NaCl + phosphate buffer pH 6.8) and prepared for electron microscopy by a modified method [7] of Kleinschmidt and Zahn [15] which uses 50% formamide.

3. Results and discussion

The partial removal of lipopolysacharides and lipoproteins from cell wall by washing it with ether and ethanol makes it susceptible for the action of 4 M guanidine-HCl without using any enzyme or detergent. The presence of glycerol in the lysing medium preserves the DNA during the rapid lysis of the cells. All operations should be carried out at low temperature.

As shown in fig. 1 the bulk cellular DNA labelled by [^{3}H]thymidine is eluted in the first peak (I). The absorbance of total DNA in 4 M guanidine-HCl indicates the presence of some protein impurities (A_{260}/A_{280} = 1.6, A_{260}/A_{230} = 1.5). After pronase treatment and purification of DNA on the column of methylated albumin the absorbancy ratios increased to 2. No RNA was detected on the column. The sedimentation coefficients of total purified DNA show a broad distribution with average $S_{20,w}$ value of 32 and hyperchromicity of 32% was obtained [16]. The fractions of peaks II and III were not analysed in detail. However, we assume from their ultraviolet spectra that peak II contains RNA (A_{260}/A_{280} = 1.8, A_{260}/A_{230} = 2) while the third peak III contains the rest of low molecular weight RNA, proteins and other cellular substances.

The sedimentation in the neutral CsCl gradient (fig. 2) of alkali denatured (pH 12.3) and neutralyzed (pH 7.2) DNA purified by nitrocellulose shows a satellite

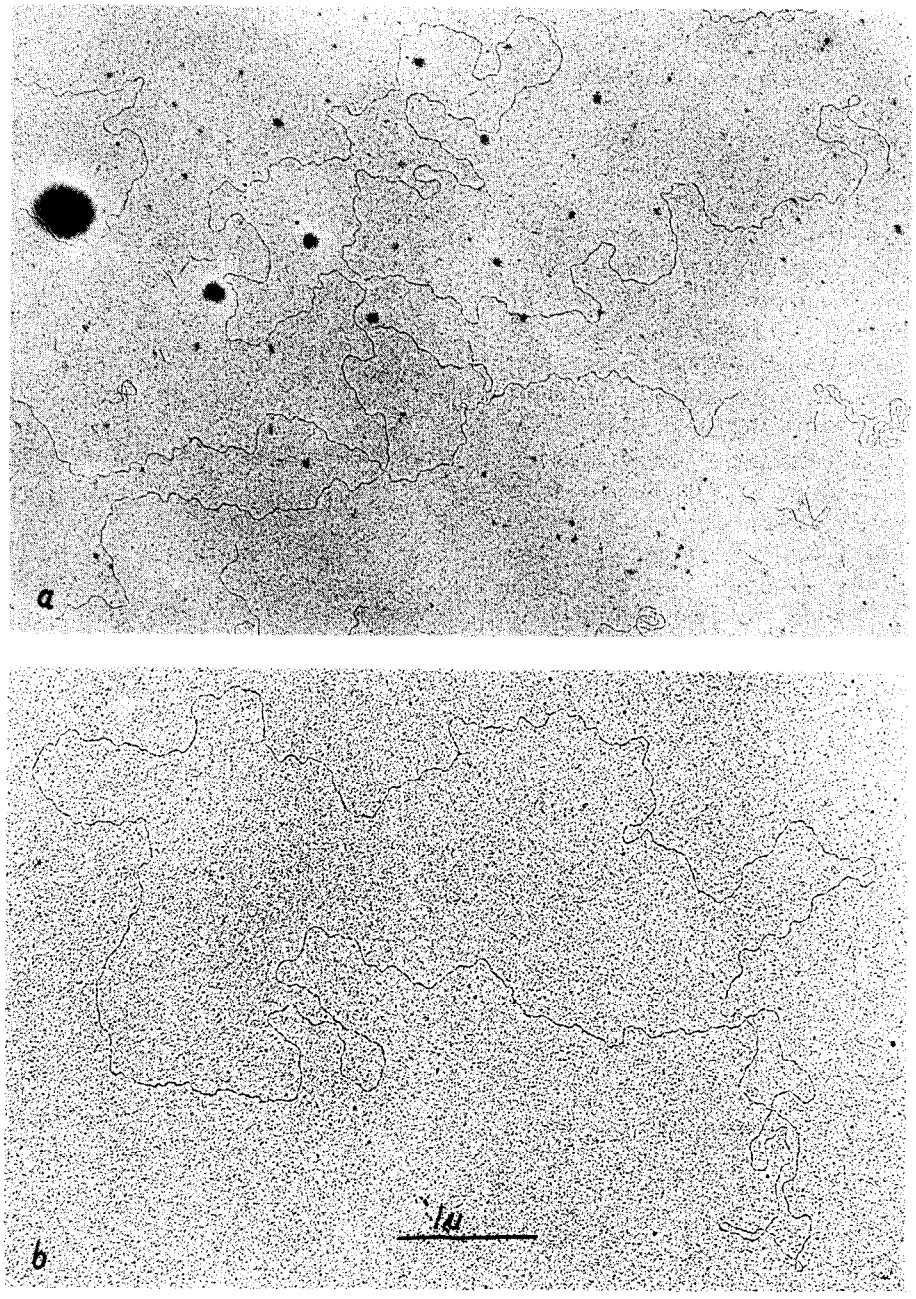

Fig. 3. Electron micrographs of DNA isolated from *E. coli* HK-26 Rl cells. The samples were stained with uranylacetate in ethanol and shadowed by Au/Pd and photographed in Tesla electron microscope. The contour lengths of the molecules were measured by a map measurer. (a) Linear form (27.4 μ); (b) circular form (27.6 μ) DNA after alkali denaturation and neutralization.

band of the buoyant density of native *E. coli* DNA (1.708 g/cm^3) corresponding to the covalently closed circular structure which is missing in the DNA isolated from *E. coli* strain R$^-$.

The electron micrographs of alkali denatured and neutralized DNA show open circular molecules with contour lengths in the range 26.8–28.6 μ which corresponds to molecular weights [17] of 52–56 $\times$ 10^6 daltons (fig. 3). The distribution of contour lengths of total native DNA after purification on the column of methylated albumin shows a significant fraction of linear molecules having similar contour lengths as the circular form. It seems that in the stationary phase of cell growth the total amount of R factor DNA including linear and circular forms is much higher than 1%. Similar observations were made for the Rl factor DNA of *Proteus mirabilis* [18]. This is supported by the fact that DNA isolated from parent R$^-$ strain by the same method shows only a broad statistical contour lengths distribution. The supercoiled molecules of R factor DNA [3] were not observed in our preparation.

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