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Isolation of Circular DNA Molecules from Whole Cellular DNA by Use of ATP-Dependent Deoxyribonuclease

(plasmid/open and closed circular DNA/sedimentation)

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ABSTRACT **A technique is described for isolation of plasmid DNA in closed and open circular double-stranded forms from bacterial cells, by use of ATP-dependent deoxyribonuclease purified from *Micrococcus luteus*. This DNase, acting only upon linear DNA molecules, degrades all bacterial chromosomal DNA extracted in the linear form. Circular plasmid DNAs are left intact, and are then separated by sedimentation through a sucrose gradient. Unlike previous techniques for analysis of plasmid DNA, this technique can be used to isolate not only closed circular DNA but also open circular DNA. Several plasmids, such as those from phage (λdv1 and λdv21), a colicinogenic factor (Col E2), a sex factor (F_8' gal), and "minicircles" in *Escherichia coli* 15, in both the open and closed circular forms, were well separated from chromosomal DNA by this technique.**

Various double-stranded DNAs are circular. These include bacterial chromosomal DNA (1), extrachromosomal DNAs (colicinogenic factors, drug-resistance factors, sex factors, phage λdv, and several other nontransmissible plasmids), animal-virus DNA, as well as mitochondrial and kinetoplast DNA from higher organisms (2, 22). It is thought that the circular form of the DNA must be important in replication and regulation of replicons.

Two types of circular DNA molecule are known: the closed circular (cc) form, in which both strands of the DNA duplex are covalently closed, and the open circular (oc) form, which is also circular but in which one or both strands have breaks. Nicking one strand of a cc molecule converts it to the oc form (3). cc DNAs have constraints in the molecule, so that they differ from those of the corresponding linear molecules in hydrodynamic behavior. They also differ from the latter in ability to bind intercalating dye, and it is by these properties that circular forms have been detected. oc DNA molecules are free from constraints and so do not differ from linear molecules in these characters.

Recently, it was shown that ATP-dependent deoxyribonuclease acts upon linear DNA molecules and single-stranded circular molecules, hydrolyzing them into small, acid-soluble fragments. However, the enzyme does not attack double-stranded circular DNA molecules either of the cc or oc form (4–6). Due to this unique property, the enzyme can be used to isolate both cc and oc molecules from a mixture containing a large amount of linear DNA molecules, such as that originating from chromosomes as a result of shear fragmentation.

Abbreviations: cc DNA, closed circular DNA; oc DNA, open circular DNA; TEAE-cellulose, triethylaminoethyl cellulose; standard saline citrate, 0.15 M NaCl–0.015 M sodium citrate.

This report describes the use of ATP-dependent DNase of *Micrococcus luteus* to detect and estimate the contents of cc and oc molecules in several *Escherichia coli* K12 derivatives that carry plasmids. The plasmids tested included λdv, F'gal, colicin E2, and *E. coli* 15 "minicircles".

MATERIALS AND METHODS

Bacterial Strains. All strains except *E. coli* 15 were derivatives of *E. coli* K12, and are listed in Table 1.

Medium. PB medium contained per liter: 10 g of polypeptone, 1 g of extract of bonito, and 2.5 g of NaCl. The pH was adjusted to 7.0 with NaOH. Thymine was added at a concentration of 1 μg/ml to the medium for thymine-requiring bacteria.

Enzymes. ATP-dependent deoxyribonuclease was purified from *M. luteus* as reported (7). The concentrated hydroxylapatite fraction was used in all experiments. A trace of endonuclease activity was detected by the denaturation–filtration technique (8) in eluate from the second TEAE-cellulose column with 0.25 M NaCl, but the concentrated fraction from hydroxylapatite was free from any contaminating endonuclease. Exonuclease I from *E. coli* B was purified as described (9).

Labeling and Preparation of Lysates. Cells were grown in 7 ml of PB medium at 37° to a concentration of 1×10^9 per ml. After addition of 60 μCi of [^{3}H]thymidine, incubation was continued for another 90 min. When strains Km 723, Km 724, and Km 1000 were being labeled, 300 μg/ml of uridine was

TABLE 1. *Bacterial strains and plasmids*

Strain	Properties	Plasmids	Molecular wt. of plasmids	Ref.
Km 723	str, his, recA1, gal^{del}			12
Km 724	str, his, recA1, gal^{del}	λdv21	$\approx 4 \times 10^6$	12 and this work
Km 605	str, his, recA1, thy, gal, try	λdv1	9×10^6	10
Km 1000	gal	Col E2	5×10^6	13
Km 55	thr, leu, thi, thy	F_8' gal	7.8×10^7	14
E. coli 15T⁻D3	thy	"Minicircles"	1.5×10^6	15

added to the medium at the same time as radioactive thymidine. After labeling, cells were harvested by centrifugation, washed twice with cold 20 mM Tris·HCl buffer (pH 8.0) containing 0.8% NaCl, and suspended in 0.75 ml of 40 mM Tris·HCl buffer (pH 8.5) containing 30 mM EDTA. Then 0.3 mg of lysozyme was added. After the mixture remained for 20 min at 0°, 0.8 ml of Sarkosyl solution [0.75% in 10 mM Tris·HCl buffer (pH 7.4) containing 1 mM EDTA] was added. The suspension was incubated for 8 min at 65°. Then 0.3 mg of Pronase (2 mg/ml, autodigested) was added and incubation was continued for 60 min at 37°. The lysate was sheared by drawing it in and out of a syringe with a micropipette tip 10 times. The sheared lysate (about 1.7 ml) was extracted twice with an equal volume of freshly distilled phenol, saturated with 50 mM Tris·HCl buffer (pH 7.5). Then the aqueous layer was dialyzed against 10 mM Tris·HCl buffer (pH 7.4) containing 1 mM EDTA, and was used as the radioactive DNA preparation.

Enzyme Treatment. The radioactive DNA preparation was incubated at 35° for 30 min with 5–10 units of ATP-dependent DNase in reaction medium (0.15 ml) containing 10 μmol of glycine–NaOH buffer (pH 9.4), 3 μmol of $MgCl_2$, 1.3 μmol of 2-mercaptoethanol, 10 μg of bovine-plasma albumin, and 50 nmol of ATP. After 15 min, 0.4 unit of exonuclease I was added and incubation was continued for another 15 min. The reaction mixture was then chilled and immediately layered on top of a sucrose gradient.

Sucrose Gradient Centrifugation and Measurement of Radioactivity. A linear 4.3-ml sucrose gradient [5–20% (w/v)] over 0.3 ml of 30% sucrose solution saturated with CsCl was used. For sedimentation at low salt concentration, the buffer contained 10 mM Tris·HCl buffer (pH 7.5) and 2.5 mM EDTA, while for sedimentation at high salt concentration, 1 M NaCl was also added. Centrifugation was performed in a Hitachi model 55P ultracentrifuge with an RPS 40 swinging bucket rotor at 37,000 rpm, at 20°. After centrifugation the bottom of each tube was pierced with a needle, and about 25 fractions were collected directly into vials. Toluene–DPO–Triton X100 was added to each vial, and radioactivity was measured in a Hitachi–Horiba, model LS-500, liquid scintillation counter.

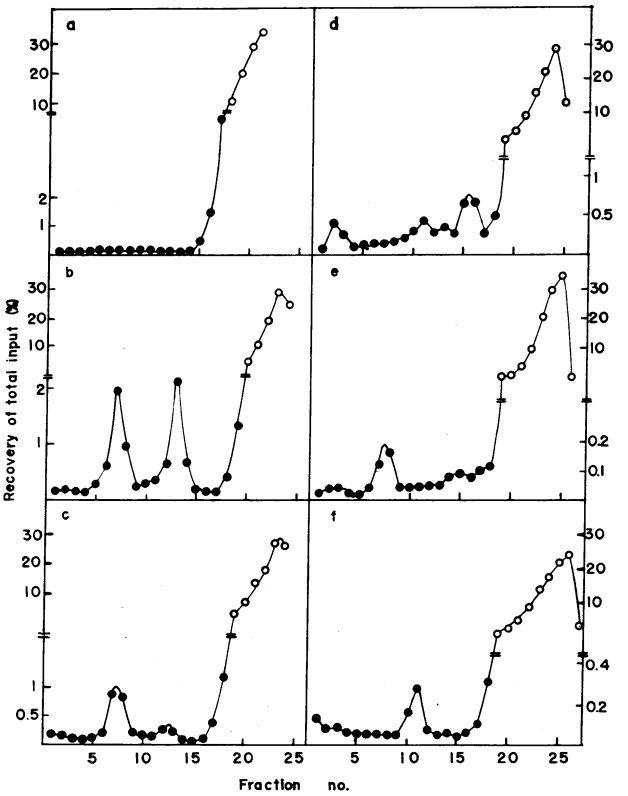

FIG. 1. Sedimentation profiles in neutral sucrose gradient of ^{3}H-labeled plasmid-carrier bacterial DNA after treatment with ATP-dependent DNase. Bulk bacterial DNA was extracted with Sarkosyl from the following bacteria: (*a*) Km 723, which carries no plasmids; (*b*) Km 605 (λdv1); (*c*) Km 724 (λdv21); (*d*) Km 1000 (Col E2); (*e*) Km 55 (F_8' gal); (*f*) *E. coli* 15T⁻D3 ("minicircles"). After phenol treatment and dialysis, radioactive DNA preparations containing 1–4 μg of DNA were incubated with *M. luteus* ATP-dependent DNase and exonuclease I. After incubation, reaction mixtures were immediately layered on top of sucrose gradients and centrifuged for: (*a*) 210 min; (*b*) 150 min; (*c*) 210 min; (*d*) 180 min; (*e*) 150 min; and (*f*) 360 min. Samples in *a*, *b*, *c*, and *d* were centrifuged at low salt concentration, and those in *e* and *f* at high salt concentration.

RESULTS

After phenol treatment the DNA preparation contained over 90% of the DNA found in the Sarkosyl lysate. Phenol-treated radioactive DNA samples from various bacterial strains containing plasmids were incubated with *M. luteus* ATP-dependent DNase and exonuclease I, and the products were analyzed by sucrose gradient centrifugation.

A sample of *E. coli* Km 723, which carries no plasmids, gave the sedimentation profile shown in Fig. 1*a*. All the radioactivity was seen near the meniscus, indicating that it was in low-molecular-weight degradation products. Thus *M. luteus* ATP-dependent DNase eliminated bacterial chromosomal DNA isolated in linear form due to shearing forces during DNA preparation. Omission of exonuclease I from the incubation mixture resulted in the appearance of a broad peak around fraction 10 of the figure, which contained about 5% of the total radioactivity of DNA. The nature of this material is not known.

A sample from *E. coli* Km 605, which carries plasmid λdv1 of phage origin with a molecular weight of 9×10^6 (10), gave two clear peaks in the sedimentation profile (Fig. 1*b*). Unlike linear DNA, the faster sedimenting component was not denatured upon heating in 0.2 × standard saline citrate at 85° for 4 min (8), so that it was double-stranded cc DNA. In neutral buffer containing a high salt concentration (1 M NaCl) or no salt, the sedimentation rate of the slower component was essentially constant, but the faster component sedimented 1.3-times and 1.5-times faster than the slower component in the presence of a high salt concentration and no salt, respectively. This relationship is identical to that of the cc and oc forms of λDNA reported by Bode and Kaiser (11). Moreover, the rates of sedimentation of the faster and slower components were the same as those of the cc and oc forms, respectively of purified λdv1 DNA.

The DNA preparation from strain Km 724 carries another plasmid, λdv21 (12), which is half the size of λdv1. This preparation was treated with ATP-dependent DNase and analyzed in the same way. The result (Fig. 1*c*) shows the presence of two components, which represent cc and oc DNA molceules with the expected molecular weight.

Colicinogenic factor E2 (Col E2; molecular weight 5×10^6) is another extrachromosomal genetic element, determining

TABLE 2. *Percentage of plasmid DNA in total cellular DNA*

Plasmid	cc DNA*				oc DNA
	ATP-dependent DNase sucrose gradient	Dye–buoyant centrifugation	Previously reported†		
λdv1	3.5	9.0	6	(10)	3.4
λdv21	1.9	4.6	—		0.53
Col E2	0.54	0.74	0.85	(13)	0.96
F_8' gal	0.26	0.57	—		0.04
E. coli 15 "Minicircles"	0.39	0.55	0.64–0.80	(15)	0.01

* Figures represent contents of cc DNA as percentages of total bacterial DNA.

† Values were obtained by the dye–buoyant technique.

production of the antiobiotic colicin (13). This plasmid DNA was also detected as circular molecules (Fig. 1*d*). The slower sedimenting component, which represents oc molecules, predominates over the faster sedimenting component. F_8' gal is a sex factor which carries the gal segment of the chromosome, and can be transferred upon conjugation. This element, with a molecular weight of 7.8×10^7 (14), is also detected as circular molecules in the Sarkosyl lysate after treatment with ATP-dependent DNase (Fig. 1*e*). A sucrose gradient containing a high salt concentration was used for analysis. The faster sedimenting peak was predominant and had the same sedimentation velocity as that of F_8' gal DNA in the cc form, purified with CsCl–ethidium bromide. Strains of *E. coli* 15 contain small closed circular plasmid DNA, "minicircles," with a molecular weight of 1.5×10^6, the function of which is unknown (15). Again, treatment of the whole DNA preparation of *E. coli* 15 with ATP-dependent DNase degraded all except the plasmid DNA (Fig. 1*f*). "Minicircles" purified with CsCl–ethidium bromide sedimented at the same rate as the rapidly sedimenting component. The amount of the slower sedimenting component (oc form) was not readily detectable.

The percentages of the cc and oc forms of plasmid DNA in the total cellular DNA are summarized in Table 2. The table also includes values for the contents of cc molecules in Sarkosyl lysates of the same bacterial strains, as assayed by dye–buoyant density equilibrium centrifugation (16). In these experiments, Pronase-treated Sarkosyl lysates were immediately mixed with CsCl. With all the plasmids tested, values for the content of cc molecules determined by the two techniques showed good agreement. With λdv21, F_8' gal, and *E. coli* 15 "minicircles," only one-fifth or less of the plasmid population was detected in oc form. On the other hand, with λdv1 and Col E2 a considerable amount of oc molecules was detected in the ATP-dependent DNase technique.

DISCUSSION

We have described conditions under which preparations of bulk DNA from bacterial cultures are treated with ATP-dependent DNase and all the chromosomal DNA is converted into oligonucleotides. The remaining material resistant to ATP-dependent DNase is plasmid DNA in the cc or oc form, which is detected by sedimentation through a sucrose gradient. We do not know why addition of exonuclease I is required for extensive degradation of linear DNA, because the mode of DNA hydrolysis by ATP-dependent DNase has not been clarified completely. However, the intermediates that accumulate during partial degradation of DNA with ATP-dependent DNase under various conditions have tails susceptible to exonuclease I (17; Takagi, Y., unpublished observation), and the transformability of *E. coli* strain K12 is strongly affected in combination with ATP-dependent DNase and exonuclease I (18).

The procedure for isolation of plasmid DNA using ATP-dependent DNase has the great advantage that it can be used to isolate DNA in the oc form, as well as in the cc form. Only cc DNA could be isolated by previously reported methods, which are based on the unique hydrodynamic properties of cc DNA. That is, unlike linear DNA molecules, DNA in the cc form is "collapsed" and sediments rapidly in alkali (19), renatures readily after denaturation (20), and binds little intercalating dye (16). When treated this way, oc molecules behave in the same way as linear DNA molecules. The ATP-dependent DNase method may be widely applicable to detection of episomal factors or other cytoplasmic circular DNAs in various organisms, including higher organisms, and will be particularly useful in systems in which many circular molecules are in the oc, not the cc, form.

The content of bacterial plasmid DNAs in the cc form measured by the ATP-dependent DNase technique agreed with that obtained by the dye–buoyant centrifugation technique. Plasmids λdv21, F_8' gal, and *E. coli* 15 "minicircles," which were labeled with [^{3}H]thymidine for several bacterial generations, are mostly in the cc form, and only a few molecules are in the oc form. It may be possible to analyze the *in vivo* event that influences interconversion of the cc and oc forms with this technique. A considerable fraction of plasmids λdv1 and Col E2 was detected as oc molecules, as measured by the ATP-dependent DNase technique. There is a possibility that some of the cc molecules detected by the dye–buoyant density gradient technique were measured as the oc form by the ATP-dependent DNase technique. This result is not due to the action of contaminating endonuclease during incubation with ATP-dependent DNase and exonuclease I, since no endonuclease activity was detected in either enzyme preparation in a parallel run using a cc DNA preparation, and since almost all of F_8' gal DNAs, the molecules of which are even larger than λdv1 or Col E2 DNA, are recovered as cc molecules by this technique. Though conversion of molecular forms in these plasmids is not the major concern of this report, it may be relevant to point out that some closed circular Col E2 molecules are converted to the oc form upon removal of protein (21). Such conversion in molecular form could have occurred in our procedure, as the ATP-dependent DNase treatment was preceded by treatment of Sarkosyl lysate with phenol.

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