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Method for Obtaining More-Accurate Covalently Closed Circular Plasmid-to-Chromosome Ratios from Bacterial Lysates by Dye-Buoyant Density Centrifugation

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A method that gives high recovery of deoxyribonucleic acid (DNA) from crude bacterial lysates using ethidium bromide-cesium chloride density gradient centrifugation is presented. After Pronase digestion and shearing of the lysate, essentially 100% recovery of chromosomal DNA and a reproducible recovery of covalently closed circular (CCC) plasmid DNA is obtained for a specific plasmid in a given strain. This method should be useful for comparing the CCC plasmid/chromosome ratio of various plasmid-host combinations.

Dye-buoyant density centrifugation (18) of bacterial lysates has been used as a convenient and simple method for detecting and isolating covalently closed circular (CCC) plasmid deoxyribonucleic acid (DNA) (2, 4, 5, 8, 9). CCC plasmid DNA does not bind as much of an intercalating dye as nicked circular plasmid DNA or linear chromosomal DNA (1). Its buoyant density in a cesium chloride gradient is consequently not lowered to the same extent as that of linear DNA (1). This allows the CCC form of the plasmid DNA to be separated from the linear chromosomal DNA.

Ethidium bromide-cesium chloride (EB-CsCl) density gradient and alkaline sucrose gradient centrifugation are often used to estimate the ratio of CCC plasmid DNA to the host chromosomal DNA in bacterial cells (5, 8, 9). However, these methods have been rather imprecise, and large variations have been reported in the CCC plasmid/chromosome ratio, even for a specific plasmid in the same host strain in experiments reported by the same authors (3, 5–8, 16). These variations might be due to the nicking of CCC plasmid DNA under certain experimental conditions (6, 12, 14). Alternatively, plasmid and chromosomal DNA might be recovered to different extents in different experiments, which could lead to the variation in the CCC plasmid/chromosome ratios observed by many laboratories. Unfortunately, most workers who have used this method for the analysis of the ratio of plasmid to chromosomal DNA have not reported the recovery of total DNA on the gradients. It is therefore difficult to assess the validity of their measurements. We report here a method that reproducibly gives essentially 100% recovery of the total DNA applied to EB-CsCl gradients and significantly reduces the variation in measured CCC plasmid/chromosome ratios.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* strains CR34A$_1$ (Thy$^-$, nalidixic acid resistant) (13; D. Taylor, unpublished data), CT28 (temperature-sensitive *dnaC* mutant) (19), and ML1410 (20) and *Proteus mirabilis* strain Pm15 (10) were used in these studies. These strains harbored one of the following R plasmids: NR1 (60×10^6 daltons; confers resistance to chloramphenicol, streptomycin/spectinomycin, sulfonamides, and tetracycline) (15); NR1-C (a chloramphenicol-sensitive mutant of NR1) (D. Taylor, unpublished data); and ROR12, ROR21, ROR22, and ROR51 (all round-of-replication [ROR] mutants of NR1 with an increased number of plasmid copies per chromosome) (13; D. Taylor, L. Ponton, and R. Rownd, unpublished data).

Growth and labeling conditions. Cells were cultured in M9 medium containing 0.2% glucose, 0.5% vitamin-free Casamino Acids (Difco), and appropriate growth supplements (11). DNA was labeled by including [^{3}H]thymine or [^{14}C]thymine (New England Nuclear Corp.) in the medium (final concentration, 2 μg/ml).

EB-CsCl centrifugation of bacterial lysates. Cells were harvested by centrifugation, washed twice with SV buffer (0.15 M NaCl, 0.10 M disodium ethylenediaminetetraacetic acid, pH 10.2), and resuspended in 1.0 ml of SV buffer. A 0.1-ml amount of lysozyme (Calbiochem, 3 × crystallized) [5 mg/ml in TES; TES = 50 mM each tris(hydroxymethyl)aminomethane, disodium ethylenediaminetetraacetic acid, and NaCl, pH 8.0] was added, and the mixture was incubated at 37°C for 10 min. A 10-

μl amount of 25% sodium dodecyl sulfate (SDS) was added, followed by 100 μl of Pronase (Calbiochem, B grade) (20 mg/ml in TES; autodigested for 10 min at 80°C), and the mixture was incubated for 2 h at 37°C. The lysate was incubated for 30 min at 65°C and then sheared by being gently drawn 20 times in and out of a 1-ml serological pipette (Scientific Products, no. P4305-1X). A 0.5-ml amount of ethidium bromide (Calbiochem) (2.5 mg/ml in TES) was added. TES was added to a final volume of 3.7 ml, and 3.45 g of solid CsCl (Harshaw, optical grade) was added. The refractive index was adjusted (if necessary) to 1.3890 to 1.3900, and the mixture was poured into a polyallomer tube, topped up with mineral oil, and centrifuged to equilibrium at 23°C in a Beckman 50Ti rotor at 45,000 rpm for 36 h. The gradient was fractionated through a hole punctured in the bottom of the tube by collecting drops on strips of Whatman 3MM filter paper (1 inch [about 2.5 cm] wide with 1-inch squares marked off). The filter strips were washed three times in ice-cold 7.5% trichloroacetic acid and 95% ethanol and dried. The filter squares were cut off the strips into scintillation vials containing 3 ml of 2.5-diphenyloxazole (PPO)-toluene (19 g of PPO per gallon [about 3.8 liters] of toluene) and counted.

RESULTS AND DISCUSSION

In our initial experiments to estimate the CCC plasmid/chromosome ratios in *E. coli* harboring R plasmids, spheroplasts of the cells were lysed with SDS and prepared for EB-CsCl gradient centrifugation as described in Materials and Methods except that the lysates were not sheared and, in many cases, also not treated with Pronase. The shearing step was not included because we felt that this might increase the breakage of the CCC DNA of high-molecular-weight R plasmids. In these experiments, however, a large amount of variation was observed in the recovery of the total DNA in an EB-CsCl gradient and in the CCC plasmid/chromosome ratio. In the experiments summarized in Table 1, samples of spheroplasts prepared from the same culture of CT28/NR1-C were lysed, using four different detergents. Depending on the detergent used for lysis, the fraction of total recovered DNA that was in the CCC form varied from 0.013 to 0.23 (column 4, Table 1). The density profile of the DNA from the sample lysed with Triton X-100 is shown in Fig. 1A. The CCC plasmid/chromosome ratio was 0.23 in this experiment. However, the recovery of total DNA (plasmid + chromosome) from the EB-CsCl gradients was less than 20% after lysis with these four detergents (column 3, Table 1). If there were one copy of the R plasmid DNA (60×10^6 daltons) per chromosome (2.5×10^9 daltons), the plasmid/chromosome ratio should have been approximately 0.025 if there had been complete recovery of R plasmid and

TABLE 1. *Comparison of recovery of DNA from lysates of samples of the same culture of CT28/NR1-C made with various detergents*[a]

Sample	Detergent	% Recovery of total DNA[b]	CCC plasmid/chromosome ratio[b] of:	
			Recovered DNA	Total applied DNA
A	2% SDS	18.0	0.095	0.017
B	0.5% Brij, 0.25% DOC	11.6	0.19	0.022
C	0.05% Triton X-100	8.0	0.23	0.018
D	1% Sarkosyl	3.4	0.013	0.0004

[a] A culture of CT28/NR1-C was labeled with [^{3}H]thymine at 30°C. Four 2-ml samples (approximately 5×10^8 cells/ml) were harvested, lysed, and centrifuged as described in Materials and Methods, except as follows: Pronase digestion and shearing steps were omitted, the 37°C incubation with detergent was for 10 min, and the detergents added were (A) 100 μl of 25% SDS in water; (B) 1.0 ml of 1% Brij 58 and 0.5% deoxycholate (DOC) in TES; (C) 1.0 ml of 0.1% Triton X-100 in TES; (D) 100 μl of 10% Sarkosyl in TES.

[b] Calculated as described in Table 2.

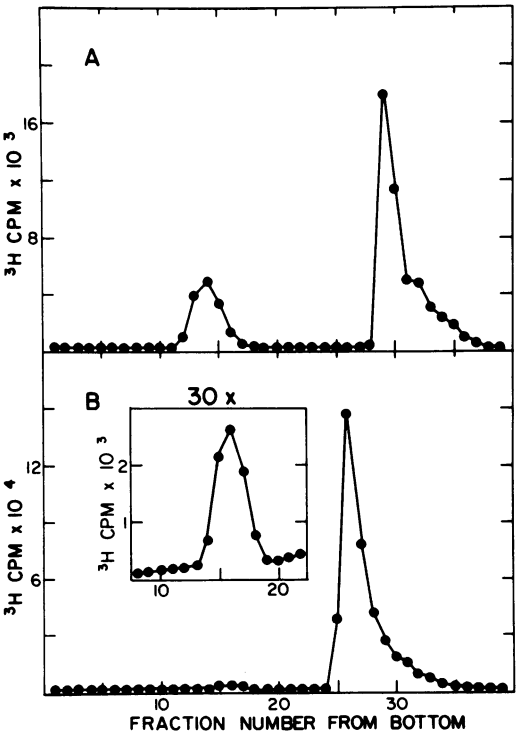

FIG. 1. *EB-CsCl gradient density profiles of DNA from lysates of E. coli harboring R plasmids. (A) Sample lysed with Triton X-100 and not sheared or treated with Pronase (Table 1, sample C). (B) Sample lysed with SDS, sheared, and treated with Pronase (Table 3, sample 2). Inset in (B) is a 30-fold magnification of the CCC region.*

chromosome DNA (11, 13). The ratio of counts per minute in the CCC plasmid band to the total counts per minute applied to the gradient is shown in column 5 of Table 1. Because this ratio approaches 0.025 in this and several of the following experiments, we assume that one copy of plasmid DNA per chromosome is a reasonable value for calculating the expected CCC plasmid/chromosome ratio. A majority of the R plasmid DNA was probably recovered in samples A through C. Thus, the elevated plasmid/chromosome ratio using these detergents is due primarily to the preferential loss of chromosomal DNA. There was a very low recovery of plasmid DNA using 1% Sarkosyl for lysis (sample D), even though the CCC/chromosome ratio of the recovered DNA of this sample (0.013) was about one-half of the expected value of 0.025. Thus, the loss of both plasmid and chromosome in sample D was about the same. This wide variation in the results obtained using a single culture shows that these techniques are unsuitable for measuring the ratio of CCC plasmid to chromosomal DNA.

Other experiments of this type have shown that the DNA that is lost from the gradients is present in a pad of cellular debris that forms at the interface between the CsCl solution and the mineral oil that was placed on top of the EB-CsCl solution to prevent evaporation or collapse of the tube during centrifugation. This pad may be formed by the adherence of DNA and cellular debris to the SDS that precipitates after the addition of CsCl to the cell lysate. This would explain other observations that the recovery of counts per minute from the gradient is improved by lowering the cell density and SDS concentration (data not shown). However, to get complete recovery of chromosomal DNA and a reproducible recovery of plasmid DNA, the lysates had to be treated with enzymes and sheared in a pipette. Table 2 shows the recovery of total (column 3) and CCC (columns 4 and 5) DNA from CR34A$_1$/NR1 lysed with 0.2% SDS. A combination of Pronase digestion and shearing resulted in the highest percentage of CCC plasmid DNA when there was essentially 100% recovery of total DNA (sample F). The plasmid/chromosome ratio of CR34A$_1$/NR1 that was cultured at 37°C was consistently higher (approximately 0.04 as compared with 0.025) than observed for the same strain cultured at 30°C or for other *E. coli* strains harboring NR1 when cultured at either temperature (D. Taylor, unpublished data). The reason for this is unknown.

Table 3 shows a comparison of the CCC plasmid/chromosome ratio of lysates prepared us-

TABLE 2. *Recovery of DNA from SDS lysates of samples of the same culture of CR34A$_1$/NR1 after various enzymatic and shearing treatments*[a]

Samples	Treatment[b]	% Recovery of total DNA[c]	CCC plasmid/chromosome ratio[d] of:	
			Recovered DNA	Total applied DNA
A	None	29.2	0.063	0.018
B	Pronase	36.0	0.031	0.011
C	RNase	56.2	0.026	0.015
D	Shear	98.1	0.024	0.024
E	RNase + shear	94.3	0.035	0.033
F	Pronase + shear	101	0.042	0.043

[a] A culture of CR34A$_1$/NR1 was labeled with [^{3}H]thymine at 37°C. At an optical density at 650 nm of 0.5 (approximately 5 × 10^8 cells/ml), six 2-ml samples were harvested, lysed, and centrifuged as described in Materials and Methods.

[b] (A) Sample not treated with Pronase or shear; (B) sample treated with Pronase but not shear; (C) sample treated with ribonuclease (RNase) (50 μg/ml) but not with Pronase or shear; (D) sample treated with shear but not Pronase; (E) sample treated with RNase and shear but not Pronase; (F) sample treated with Pronase and shear.

[c] Calculated by totaling the counts recovered on the gradient in the CCC and chromosomal bands, dividing by the total number of counts applied to the gradient, and multiplying by 100%. The counts applied to the gradient were calculated from the number of counts per minute in 10-μl samples taken after the cells were resuspended in SV buffer.

[d] Calculated by dividing the counts recovered in the CCC band by the total recovered counts or the total applied counts in the gradient.

TABLE 3. *Comparison of recovery of DNA from different detergent lysates of samples of the same culture of CT28/NR1-C with or without shear*[a]

Sample	Detergent[b]	Shear[c]	% Recovery of total DNA[d]	CCC plasmid/chromosome ratio[d] of:	
				Recovered DNA	Total applied DNA
1	0.2% SDS	−	28.3	0.025	0.007
2	0.2% SDS	+	79.0	0.021	0.016
3	0.05% Triton	−	30.2	0.040	0.012
4	0.05% Triton	+	54.2	0.029	0.016
5	0.5% Brij	−	61.5	0.031	0.019
6	0.5% Brij	+	98.5	0.025	0.024
7	1% Sarkosyl	−	45.2	0.018	0.008
8	1% Sarkosyl	+	90.8	0.023	0.021

[a] A culture of CT28/NR1-C was labeled with [^{3}H]thymine at 30°C. Eight 2-ml samples were harvested, lysed, and centrifuged as described in Materials and Methods, except as indicated for detergent and shear.

[b] Detergents other than SDS were as indicated in Table 1.

[c] Symbols: +, Sample was sheared; −, shearing was omitted.

[d] Calculated as described in Table 2.

ing various detergents and Pronase digestion with or without shear. In all cases, recovery of both chromosomal (column 4) and CCC (columns 5 and 6) plasmid DNA was improved by shearing the lysate. In the cases of the gradients that yielded high recovery of total DNA (samples 2, 6, and 8), the CCC plasmid/chromosome ratio of the recovered DNA was approximately 0.025, with a range of 0.021 to 0.025 (column 5). When the recovery of DNA was not as high, this ratio tended to be slightly larger, even when the DNA was sheared (i.e., sample 4). In subsequent experiments 0.2% SDS was chosen as the detergent for routine use because SDS treatment results in quick and complete lysis of *E. coli* as well as of other bacterial genera used in this laboratory, such as *Proteus mirabilis*. An example of a density profile of the DNA prepared using SDS and shear treatment (sample 2, Table 3) is shown in Fig. 1B.

Table 4 shows an analysis of *E. coli* containing various ROR mutant R plasmids that have an increased number of plasmid copies per chromosome compared with wild-type NR1 (13; Taylor, Ponton, and Rownd, unpublished data). These data show that the ROR phenotype was easily distinguished by this method, because the cells harboring the ROR mutants had a much higher CCC plasmid/chromosome ratio than the cells harboring NR1.

One possible explanation for the loss of DNA during EB-CsCl gradient centrifugation is that the DNA becomes entangled with the cellular debris in a lysate, which floats to the meniscus. To examine this possibility, [^{3}H]thymine-labeled spheroplasts were lysed with 0.2% SDS, and the lysate was treated with Pronase and sheared. The lysate was centrifuged in an EB-CsCl gradient, and the entire gradient was collected into a single tube with the exception of the pad of cellular debris at the meniscus, which was discarded. This gradient was then mixed with a lysate of [^{14}C]thymine-labeled cells, which has lysed with 0.2% SDS but not treated with Pronase or sheared. This mixture was then centrifuged, fractionated, and counted in the usual way. There was nearly complete recovery of both the total and the CCC ^{3}H-labeled DNA (Table 5). However, there was only a 14% recovery of the total ^{14}C-labeled DNA, and only about one-third of the applied ^{14}C-labeled CCC R plasmid DNA was recovered in the same gradient. Thus, once the ^{3}H-labeled chromosome and CCC R plasmid DNA was buoyant in an EB-CsCl gradient, it was not reentrapped when mixed with a nonsheared lysate and recentrifuged in an EB-CsCl gradient. This indicates that the DNA in a lysate must be attached to some cellular material, which results in its entanglement with the cellular debris that floats to the meniscus during centrifugation, unless this attachment is first broken by Pronase digestion and shear.

Another variable that could affect the CCC plasmid/chromosome ratio is nicking of the plasmid DNA in the gradient during centrifugation (6, 12, 14). To test the stability of CCC DNA with time in an EB-CsCl gradient, lysates of a culture of PM15/ROR12 were centrifuged in an EB-CsCl gradient for various lengths of time, and the CCC plasmid/chromosome ratio was measured. Although recovery of total DNA varied slightly from sample to sample (Table 6, column 3), the amount of CCC DNA actually recovered was relatively constant (Table 6, column 5). Thus errors due to breakage of CCC

TABLE 4. *Comparison of CCC plasmid/chromosome ratios from high-recovery lysates of ML1410 harboring ROR plasmids*[a]

Sample	Plasmid	% Recovery of total DNA[b]	CCC plasmid/chromosome ratio[b] of:		Relative CCC plasmid/chromosome ratio[c]
			Recovered DNA	Applied DNA	
A	NR1	126	0.024	0.030	1.00
B	ROR21	110	0.158	0.174	6.56
C	ROR22	139	0.163	0.227	6.76
D	ROR51	122	0.081	0.099	3.36

[a] Cultures of ML1410 harboring NR1 (A), ROR21 (B), ROR22 (C), or ROR51 (D) were labeled, harvested, lysed, and centrifuged as described in Materials and Methods.

[b] Calculated as described in Table 2.

[c] Calculated by dividing the CCC plasmid/chromosome ratio of recovered DNA (fourth column) of each sample by the value for sample A (NR1).

TABLE 5. *Recovery of DNA from a mixture of purified DNA and a lysate that was not sheared or treated with Pronase*

DNA source[a]	Isotope	% Recovery of total DNA[b]	CCC plasmid/chromosome ratio[b] of:	
			Recovered DNA	Total applied DNA
EB-CsCl	^{3}H	96.4	0.024	0.023
Cell lysate	^{14}C	13.9	0.054	0.008

[a] A [^{3}H]thymine-labeled sample of CT28/NR1-C cells was lysed, treated with Pronase, sheared, and centrifuged as described in Materials and Methods, except that the entire gradient was collected into a tube. This gradient was mixed with a lysate of [^{14}C]thymine-labeled cells that had not been sheared or treated with Pronase. The mixture was then centrifuged and fractionated as usual.

[b] Calculated as described in Table 2.

TABLE 6. *Stability of CCC ROR12 DNA with time in an EB-CsCl gradient*[a]

Sample	Hours at 55,000 rpm[b]	% Recovery of total DNA[c]	CCC plasmid/chromosome ratio[c] of:	
			Recovered DNA	Applied DNA
A	23	97	0.044	0.042
B	42.5	112	0.043	0.048
C	70.5	104	0.039	0.040
D	89.5	119	0.037	0.044

[a] A culture of Pm15/ROR12 was labeled, harvested, and divided into eight samples. Each sample was lysed and centrifuged as described in Materials and Methods except that the lysozyme step was omitted, and the samples were centrifuged in a Beckman 75Ti rotor at 55,000 rpm.

[b] After the times indicated, the centrifuge was stopped, two tubes were picked at random and fractionated, and the remaining tubes were recentrifuged until the next sample time.

[c] Calculated as described in Table 2. All values are averages of two samples.

DNA with time during centrifugation should be minimal.

This method involving Pronase treatment and shear has been used successfully to measure the quantity of various CCC R plasmids in different bacterial strains, and the results have been found to be quite reproducible. When the Pronase and shearing treatments are omitted, the percentage of recovery of total DNA is usually variable, with values ranging from a few percent to as high as 30%. In general, under these conditions the CCC plasmid/chromosome ratio varies inversely with the percentage of recovery of total DNA; that is, the ratio is higher for lower extents of recovery of total DNA. When the Pronase and shearing treatments are included as outlined in Materials and Methods, the method gives a high and a reproducible recovery of DNA from crude bacterial lysates in EB-CsCl gradients. Under these conditions, the variation in either the percentage of recovery of total DNA or the CCC plasmid/chromosome ratio is generally less than 10%. These conditions have now been used routinely in this laboratory in hundreds of experiments. The method has been used successfully for measuring the CCC plasmid/chromosome ratio for exponential- or stationary-phase cells that were cultured in M9 minimal medium or Penassay broth (Difco) and for temperature-sensitive replication mutants that were cultured at either the permissive or the nonpermissive temperature. The method has been used successfully with several *E. coli* strains, including a temperature-sensitive *dnaC* mutant, and also with *P. mirabilis*. It has also been possible to isolate replicating plasmid DNA molecules from *P. mirabilis* by this method (21).

In conclusion, this method should be useful for comparing the CCC plasmid/chromosome ratio of different plasmids in the same strain or of a specific plasmid in different strains. In cases where SDS cannot be used as the detergent for cell lysis because it will cause relaxation (nicking) of DNA-protein relaxation complexes (8, 9), other detergents (such as Brij) can be used to lyse the cells in order to obtain a high recovery of total DNA and a reproducible CCC plasmid/chromosome ratio. The observed ratio would, of course, be a minimum value if any nicking of the CCC plasmid DNA took place for other reasons during preparation of samples.

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