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This paper is dedicated to the memory of Professor Karol Taylor

A method for isolation of plasmid DNA replication intermediates from unsynchronized bacterial cultures for electron microscopy analysis°

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Electron microscopy is a powerful technique for analysis of DNA replication intermediates. However, isolation of replicating DNA molecules from living cells is tricky and difficult, especially in the case of small DNA molecules (such as bacterial plasmids) whose initiation of replication is not easily synchronized. Here a relatively simple and rapid method for efficient isolation of replicating plasmid molecules from unsynchronized *Escherichia coli* cultures is described. The efficiency of this procedure is high enough for electron microscopy analysis of plasmid replication intermediates appearing in living cells in normal growth conditions. Under optimal conditions, using standard procedures of isolation of plasmid DNA, it is possible to achieve a content of only as few as 0.02 percent of replication intermediates in a plasmid DNA sample. The described method allowed us to enrich up to 100-fold the fraction of replication intermediates suitable for microscopic analysis among all plasmid molecules.

Electron microscopy is a powerful technique for studies on the mechanisms of DNA replication. Whereas it is relatively easy to find replication intermediates in a reaction mixture in *in vitro* replication systems (see ref. [1] and references therein), isolation of replicating DNA molecules from living cells appears to be tricky and inefficient. It is all the more difficult in the case of relatively small DNA molecules such as bacterial plasmids. There are several reasons for such a difficulty. First, small DNA molecules replicate very quickly,

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i.e. the replication round is completed within a few seconds. Even if a plasmid exists in several copies per cell, a single DNA molecule replicates usually once and only once per cell cycle, and thus it is only a little chance to find a replicating plasmid DNA molecule in the cell at a given moment. Second, one should remember that molecules just after the start of replication and shortly before the end of a replication round are usually not appropriate for the analysis of replication intermediates by electron microscopy. Therefore, the number of molecules useful in electron microscopy studies is lower than expected. Third, although it is possible to synchronize the initiation of replication of some plasmids [2, 3], which usually may help to obtain an increased number of replications [4], the efficient synchronization methods are currently available only for a small fraction of plasmids. Moreover, most, if not all, of the synchronization methods (e.g. thymine starvation, temperature shock and others) provoke conditions which are far from normal bacterial growth. In consequence, one might expect some artifacts when studying plasmid replication upon such a synchronization. Finally, replicating DNA molecules contain short single-stranded DNA fragments in the region of replication forks and are very fragile. Therefore, isolation of such delicate molecules in the intact form is very difficult and inefficient.

We have met all the above described problems in the course of our studies on plasmid DNA replication in *E. coli* cells and have found that the currently available methods are not efficient enough for isolation of plasmid DNA replication intermediates from unsynchronized cultures in such an amount that could be suitable for their analysis. The most important problem was too small fraction of plasmid replication intermediates among all DNA molecules (at best we were able to find one replication intermediate per a few thousand of plasmid molecules). One possibility to resolve this problem is to prepare a plasmid DNA sample by one of previously described methods, perform two identical two-dimensional (2D) gel electrophoreses, develop one of the gels using a labeled probe and try to cut out replication intermediates from the second gel, assuming that both gels run perfectly identically. This method is, however, tricky (two perfectly identical 2D gel electrophoreses must be performed) and time consuming (it takes more than a week). Therefore, the aim of this work was to develop a relatively simple and rapid procedure for efficient isolation of plasmid DNA replication intermediates from unsynchronized bacterial cultures. The method is based on gentle cell lysis, DNA purification on a Qiagen column, agarose gel electrophoresis, and isolation of a piece of agarose containing a fraction of DNA molecules rich in replication intermediates. A further optimized purification of DNA makes the material efficient enough for electron microscopy studies. Using this method, it is possible to obtain a sample of DNA suitable for electron microscopy within three days.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *E. coli* wild-type MG1655 strain [5] was used. The *recA13* allele was transferred by P1 transduction from strain BM223 [6] harboring a kanamycin-resistance marker close to *recA* locus (*zfi3134*::Tn*10kan*). Plasmids derived from bacteriophage λ: pAW6, pCB104 and pAS1 have been already described (refs. [7, 8] and [9], respectively).

Isolation of plasmid DNA replication intermediates for electron microscopy analysis

Bacteria harboring plasmids were grown in LB medium to A_{575} of about 0.5–0.6; 100 ml of the culture was centrifuged (10 min, 2500 × *g*, 4°C), the bacterial pellet was suspended in 3 ml of a buffer consisting of 50 mM Tris/HCl (pH 8.0) and 25% sucrose, and transferred to a

polypropylene tube. Following addition of lysozyme (0.6 ml of a 5 mg/ml lysozyme solution in 0.25 M Tris/HCl, pH 8.0) the sample was chilled in an ice-bath for 5 min. Then, 1.2 ml of EDTA solution (0.25 M EDTA, pH 8.0) was added slowly (the suspension was stirred continuously during addition of EDTA solution) and the sample was kept again in an ice-bath for 5 min. SDS solution was added slowly to a final concentration of 1% (we used 0.54 ml of 10% SDS solution; the suspension was stirred continuously during addition of the SDS solution). The sample was transferred to room temperature and was kept until the opaque suspension became clear (it takes usually about 5 min). The sample was transferred again into an ice-bath and NaCl solution was added slowly to a final concentration of 1 M (we used 1.5 ml of 5 M NaCl and 0.6 ml of H_2O; the suspension was stirred continuously during addition of the NaCl solution). Following overnight incubation in an ice-bath, the sample was centrifuged (30 min, 17000 × *g*, 4°C) and the supernatant was transferred to a new polypropylene tube. An equal volume of 96% ethanol was then added and the sample was centrifuged (10 min, 11000 × *g*, 20°C). After washing with 70% ethanol and centrifugation as described above, the pellet was dried at room temperature (overdrying must be avoided) and resuspended in 2 ml of Qiagen buffer G2 (0.8 M guanidine·HCl, 30 mM EDTA, 30 mM Tris/HCl, 5% Tween-20, 0.5% Triton X-100, pH 8.0). 0.2 mg of RNase A (DNase-free) was added and the sample was incubated at 37°C for 30 min. Following addition of 1 mg of Proteinase K, the suspension was incubated at 50°C for 60 min (the sample was stirred gently from time to time). Then, the sample was centrifuged (10 min, 6500 × *g*, 4°C) and an equal volume of Qiagen buffer QBT (0.75 M NaCl, 50 mM Mops, 15% ethanol, 0.15% Triton X-100, pH 7.0) was added to the supernatant. The solution was loaded on a previously equilibrated (with 4 ml of Qiagen buffer QBT) Qiagen column (Qiagen Plasmid Midi Kit) and allowed to pass through by gravity flow. The column was washed three times with 10 ml of Qiagen buffer QC (1 M NaCl, 50 mM Mops, 15% ethanol, pH 7.0). The DNA was eluted with 2 × 5 ml of prewarmed (50°C) Qiagen buffer QF (1.25 M NaCl, 50 mM Tris/HCl, 15% ethanol, pH 5). Following addition of 6 ml of isopropanol to 10 ml of DNA solution, the sample was centrifuged (30 min, 16000 × *g*, 20°C), the pellet was washed with 70% ethanol and centrifuged again (10 min, 16000 × *g*, 20°C). Ethanol was removed carefully and as accurately as possible (the pellet has not been dried). Then, 0.1 ml of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) was added and the sample was incubated for 90 min at room temperature. Plasmid DNA was digested with an appropriate restriction enzyme [10] and in the case of resultant cohesive ends, they were filled-in with T4 DNA polymerase [10]. The whole sample was loaded into a well of 1% agarose gel (Sea Plaque GTG Agarose, FMC) 0.5 cm thick, size: 15 cm × 15 cm in TAE buffer 40 mM Tris/acetate, 2 mM EDTA, pH 8.0 [10] and electrophoresis was carried out very slowly (2 V per 1 cm of gel) overnight in this buffer. The gel was stained with 0.5 μg/ml ethidium bromide and appropriate agarose fragments (about 2–3 mm wide), expected to contain the replication intermediates, were cut out from the gel. Agarose slices were transferred into Eppendorf tubes and an equal volume of β-agarase buffer (10 mM Tris/HCl, 10 mM EDTA, pH 6.5) was added. Following 2 ×10 min incubation in an ice-bath (after the first 10 min the buffer was discarded and replaced with a new portion of the buffer), the sample was transferred to 65°C for 10 min and then to 40°C for 10 min. β-Agarase (Biolabs) was added (1 unit per 0.1 ml) and the sample was incubated at 40°C for 2 h (the sample was stirred from time to time). Following addition of 0.1 volume of 3 M sodium acetate and 10 min incubation at room temperature, the sample was centrifuged (15 min, 10000 × *g*, 20°C) and 2.5 volume of 96% ethanol was added to the supernatant. The sample was incubated for 30 min at −20°C and

centrifuged (15 min, 10000 × *g*, 20°C). The pellet was washed with 70% ethanol, centrifuged as described above, dried under vacuum and resuspended in 10–20 μl of TE buffer.

DNA electron microscopy

DNA samples, obtained as presented in the preceding paragraph, were prepared for electron microscopy as described by Burkardt & Lurz [11] and analyzed in the CM100 Philips electron microscope.

RESULTS AND DISCUSSION

General features of the method of the isolation of plasmid DNA from unsynchronized bacterial culture, suitable for electron microscopy analysis

The method of isolation of plasmid DNA replication intermediates suitable for electron microscopy analysis is described in detail in Materials and Methods. In order to develop the efficient method we: (i) adopted and modified the gentle lysis procedure described by Guerry *et al.* [12], (ii) used a technique of plasmid DNA purification on a Qiagen column, as it has been earlier reported [13] that this method is very gentle (DNA sample is loaded on the column and allowed to pass through by gravity flow) and suitable for purification of DNA replication intermediates from eukaryotic cells, and (iii) enriched a DNA sample in replication intermediates by agarose gel electrophoresis and cutting out agarose slice(s) just above the plasmid monomer band (replication intermediates are expected to migrate more slowly during agarose gel electrophoresis than monomer plasmid DNA molecules). The general scheme of this procedure is presented in Fig. 1. The most important steps of the procedure are discussed below.

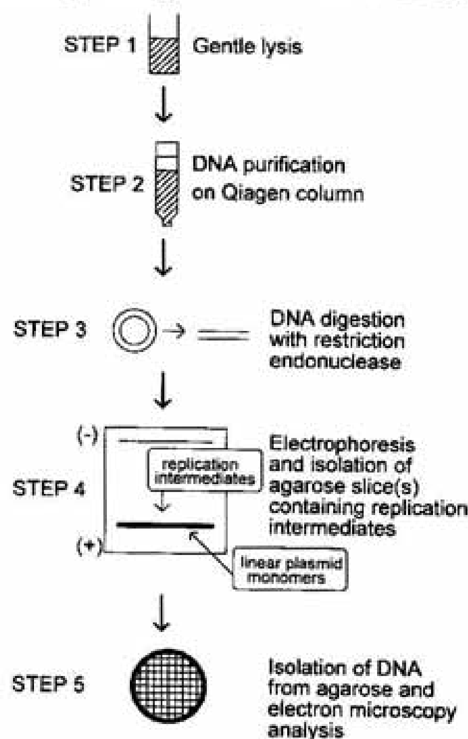

Figure 1. A scheme of the method of isolation of plasmid DNA replication intermediates for electron microscopy analysis.

Step 1: Cultivation of bacteria and bacterial cell lysis

We used *E. coli* cells bearing different λ plasmids (plasmids derived from bacteriophage λ). These plasmids are of medium copy number (20–40) in *E. coli* [14] and their replication is not easy synchronizable. In most of the experiments we used *E. coli recA* mutant. In such a mutant the plasmids are known to exist as monomers if monomeric forms are introduced by transformation (generally, however, the monomeric forms are predominant only relatively shortly, i.e. during a few passages, after transformation) [15]. Since we expected that plasmid monomers should be the most appropriate forms for electron microscopy analysis (especially for the analysis of Y-shaped molecules appearing after digestion of replication intermediates with restriction endonucleases), we introduced appropriate plasmid monomers into the *recA* strain. However, the method of isolation of replication intermediates works well also in the case of *recA*⁺ strains (not shown). In all experiments, unsynchronized bacterial cultures were used.

During preparation of DNA samples for electron microscopy the lysis procedure should be extremely gentle. It is all the more important during isolation of replication intermediates as these fragile molecules have to be preserved for further analysis. The bacteria were collected by centrifugation from late logarithmic culture, since it was earlier reported that some plasmids replicate more frequently at this stage of cell growth [16]. The lysis should be performed in polypropylene tubes in order to protect DNA molecules from sticking to the walls of a tube. We adopted and modified a lysis procedure described by Guerry *et al.* [12] as follows: since it is important to avoid long incubation of bacterial cells in 25% sucrose solution, lysozyme was added immediately after the bacterial pellet was suspended of in the Tris-sucrose buffer. One should remember that all necessary stirring must be very gentle (by inverting the tubes only) to avoid disruption of replication intermediates. The addition of RNase (DNase free) and Proteinase K is crucial. Otherwise, the quality of preparations for electron microscopy analysis is poor and the number of replication intermediates drops dramatically.

Step 2: DNA purification on Qiagen columns

As during the lysis procedure, DNA purification must be gentle enough to preserve replication intermediates. Most of commonly used DNA purification methods (including ultracentrifugation in CsCl gradient) appeared to be inappropriate (not gentle enough). Therefore, we prefer a method based on using a Qiagen column as it has been reported [13] that such a procedure is very gentle and suitable for purification of fragile DNA molecules. In most of our experiments the Qiagen Plasmid Midi Kit was used, but we found that the Qiagen Plasmid Mini Kit works as well when a lower amount of bacterial culture is used.

Step 3: DNA digestion with restriction endonuclease

For most of DNA electron microscopy analyses (such as mapping of the origin of replication and investigation of directionality of replication), linear plasmid DNA forms are more suitable than the circular ones, especially if digestion with a restriction endonuclease produces a linear molecule with asymmetrically placed *origin* of replication. However, this step does not seem to be crucial and if one needs to analyze circular plasmid forms the digestion can be omitted. Nevertheless, when plasmid DNA is digested and cohesive ends are produced, they should be filled in (we used T4 DNA polymerase) to avoid formation of circular forms.

Step 4: Electrophoresis and isolation of agarose slice(s) containing replication intermediates

This is a key step allowing significant enrichment of an investigated DNA sample in replication intermediates. It is based on the expected slower migrations of replication intermediates during agarose gel electrophoresis than linear plasmid DNA molecules. It is important to run the electrophoresis slowly (2 V per 1 cm of gel). Following staining with ethidium bromide, a few slices of agarose are cut out just above the band of plasmid monomers (Figs. 1 and 2). Generally, replication intermediates placed closer to monomers contain shorter already replicated fragments.

Step 5: Isolation of DNA from agarose and electron microscopy analysis

We found that the Sea Plaque GTG and Nu Sieve GTG agaroses give the best results in this preparation procedure. DNA was isolated from agarose using β-agarase. Other methods of isolation of DNA from agarose appeared not to be gentle enough for the preservation of

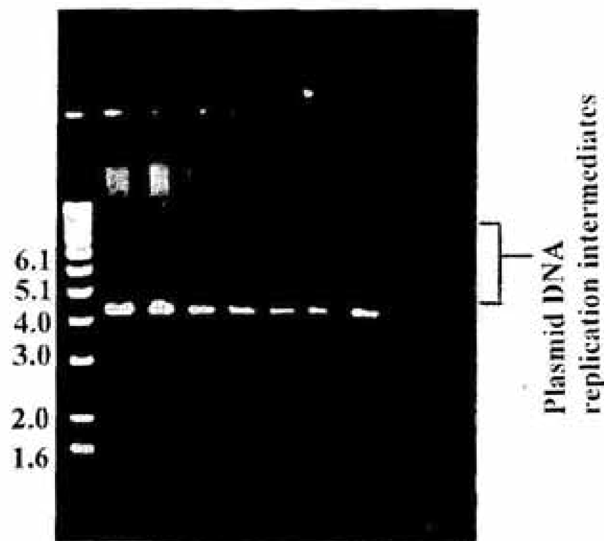

Figure 2. An example of the agarose gel electrophoresis (Step 4) of plasmid DNA isolated as described in the text.

Plasmid pAW6 was digested with *Eco*RI restriction endonuclease, the cohesive ends were filled-in with T4 DNA polymerase and 1% agarose (Sea Plaque GTG agarose) gel electrophoresis was performed (at 2 V per 1 cm of a gel). Following staining with ethidium bromide, slices of agarose containing replication intermediates (as indicated) were cut out.

replication intermediates. Samples for electron microscopy analysis were prepared by a standard method [11]. Examples of the replication intermediates isolated by the above described method are presented in Fig. 3.

In our electron microscopy studies, standard procedures of isolation of plasmid DNA allowed us to observe only one replication intermediate per about 5000 plasmid molecules, i.e. the fraction of replication intermediates

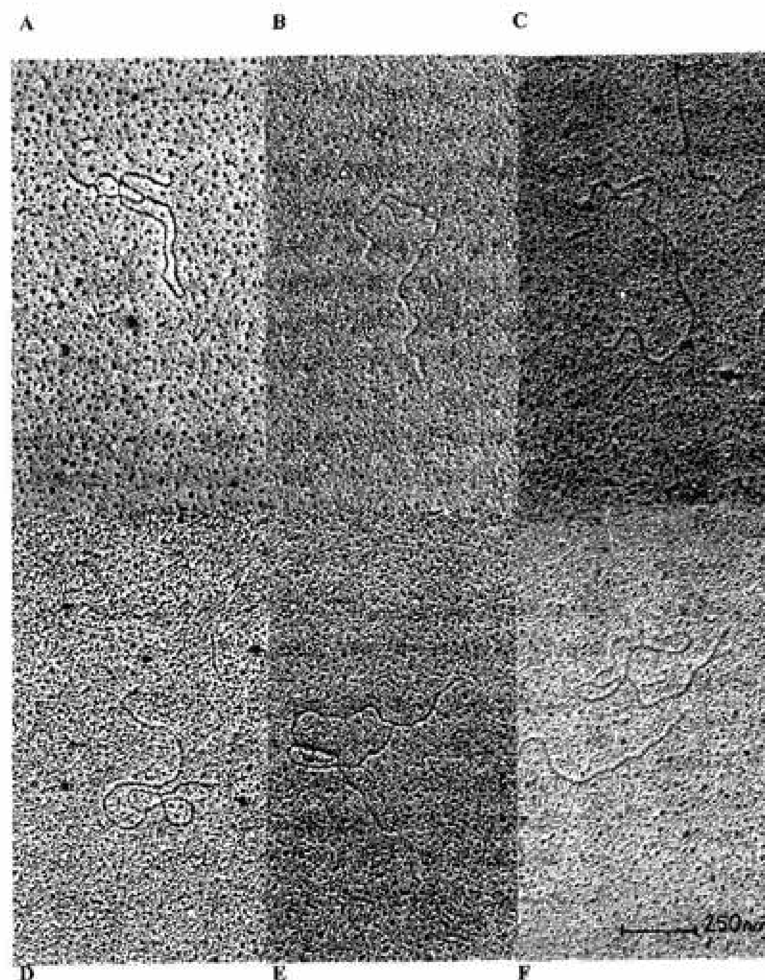

Figure 3. Electron microscopy of λ plasmid replication intermediates isolated by the method described in this paper.

Plasmids pAS1 (panel A) and pAW6 (panels B–F) were digested with *Bam*HI and *Eco*RI restriction endonucleases, respectively.

among total molecules in a sample was as small as 0.02%. Using the method described in this paper, we were able to enrich the fraction of replication intermediates suitable for analysis up to 2–3% (thus we obtained an at least 100-fold enrichment). An alternative method based on two identical two dimensional gel electrophoreses is tricky and time consuming (it takes more than a week). In comparison to that method, the procedure described in this paper is relatively simple and rapid (one can obtain a sample suitable for electron microscopy analysis within three days). Therefore intention was to optimize a method for isolation of plasmid DNA replication intermediates from unsynchronized *E. coli* cultures, and we have found that the procedure proposed here is effective, at least for λ plasmids (Fig. 3) and ColE1-type replicon, pBR322 (not shown). However, the method may be, of course, used also for isolation of replicating plasmid molecules from synchronized cultures. Moreover, we believe that, with some modifications, the method should be suitable for isolation of replication intermediates from other organisms as well.

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