

# Analysis of Chromosomal Replication Progression by Gel Electrophoresis

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## 1. Introduction

An absolute requirement for life is the preservation of genome integrity and the faithful duplication of chromosomes before segregation. A proliferating cell must duplicate its entire complement of DNA with exquisite precision facing a barrage of impediments of different nature. Single-strand breaks (SSBs) in the template DNA, either pre-existing or arising from abnormal DNA structures (folded DNA, cruciform structures or cross-links, etc.), collisions with DNA-bound proteins such as transcription complexes or DNA structural barriers restrain replication progression. For instance, during *Escherichia coli* DNA replication, the two forks initiated at the single origin of replication, *oriC*, move along the chromosome with high probability of pausing, stalling or even collapse (Maisnier-Patin et al., 2001). Replication arrest is a source of genetic instability in all types of living cells (Michel, 2000; Carr, 2002; Kolodner et al., 2002). As a consequence, cells have developed several effective strategies to tackle with replication fork arrest and/or repairing the double strand breaks (DSBs) generated at the stalled replication forks (Bierne et al., 1994; Kuzminov, 1995). Considerable evidence has been accumulated in the past decade demonstrating the involvement of recombination proteins in either direct or bypass repair of the lesions or structures blocking replication fork progression (reviewed in Courcelle et al., 2004; Kreuzer, 2005; Hanawalt, 2007; Michel et al., 2007 and references herein).

Conventional agarose DNA electrophoresis is one of the most frequently used techniques in molecular biology for the isolation or identification of DNA fragments. However, Pulse Field Gel Electrophoresis (PFGE) and Two-Dimensional (2D) Agarose Gel Electrophoresis techniques have been used to study biological processes such as the progression of the replication fork along a DNA fragment. In this work we introduce how these techniques has been used in bacteria to (i) verify and quantify the presence of stalled replication forks (ii) recognize DNA structure at the stalled replication fork, and (iii) understand how the replication fork could be restarted.

## 2. Pulse Field Gel Electrophoresis (PFGE)

Separation of DNA fragments by standard agarose gel electrophoresis is based on the capacity of the molecules to pass through the pore generated inside the matrix gel. Using

this feature as the only separation mechanism, the large DNA molecules cannot be discriminated from each other. The practical range of resolution is up to approximately 50kb; making impossible the direct genomic analysis of large DNA molecules as those generated by the presence of complex DNA structures, or the DSBs involved in stalled replication forks in the *E. coli* chromosome.

In 1983, PFGE was developed as a method to circumvent this limitation, allowing fractionation of very large DNA molecules up to a million base pairs in size (Schwartz & Cantor, 1984; Herschleb et al., 2007). PFGE allows the separation of these large DNA molecules through abrupt electrical perturbations to the paths crawling molecules take through the gel. In PFGE, the *direction of the electrical field* is periodically changed (usually 120°), requiring electrophoresing molecules to reorientate (Fig. 1). The *time* required to complete the orientation process scales with the size of the DNA, so that increasing the size of the DNA molecules, it takes more time between changes in the direction of the electric field (Fig. 1). These intervals vary depending on the size of the fragments that have to be resolved, a few seconds for small fragments to hours for fragments larger than 5Mb. The principle of PFGE is that large DNA fragments require more time to reverse the direction in an electric field than small DNA fragments. Alternating current direction during gel electrophoresis can resolve DNA fragments of 100 to 1,000 kb.

The *equipment* required to perform PFGE is also different from that used in traditional electrophoresis. The tank contains a set of *electrodes* (6-8), instead of a couple of them, being thicker and disposed to allow the different orientations of the electric field. Maintaining a constant the *temperature* (usually 14°C) during the process is important to avoid temperature variations through the gel, which could affect the resolution of DNA fragments. Accordingly, the system should include a cooling device. Agarose gel preparation does not differ from that reported for conventional electrophoresis.

Due to the fragility of the very large DNA fragments to be separated, preparing the *sample* is the most critical step for PFGE. To avoid breakage of genomic DNA during manipulation, the DNA is not extracted, but the cells are embedded in agarose plugs and then fixed into the wells.

## 2.1 Verifying replication fork reversal by detection of DNA breakage at the stalled replication forks

The progression of the replication fork can be halted by several causes, including deficiencies in replication enzymes and obstacles such as DNA-bound proteins, transcription complexes, nicks, gaps, DNA damage or topological constrictions. Replication arrest is a source of DNA breakage and rearrangement in all organisms (reviewed in Aguilera & Gómez-González, 2008); consequently stalled replication forks create the need for replication reactivation, and different ways of restarting replication have been proposed (Michel et al., 2004; Michel et al., 2007). In bacteria, the consequences of replication blockage have been studied mainly in *E. coli*. In several *E. coli* replication mutants, the stalled forks generated upon inactivation of the mutant enzyme are reversed and result in the formation of a Holliday junction (HJ) adjacent to a DNA double strand end, a reaction called 'replication fork reversal' (RFR) (Fig. 2A) (Michel et al., 2004; Seigneur et al., 1998; Seigneur et al., 2000). In a *rec* proficient background this intermediary could be processed without

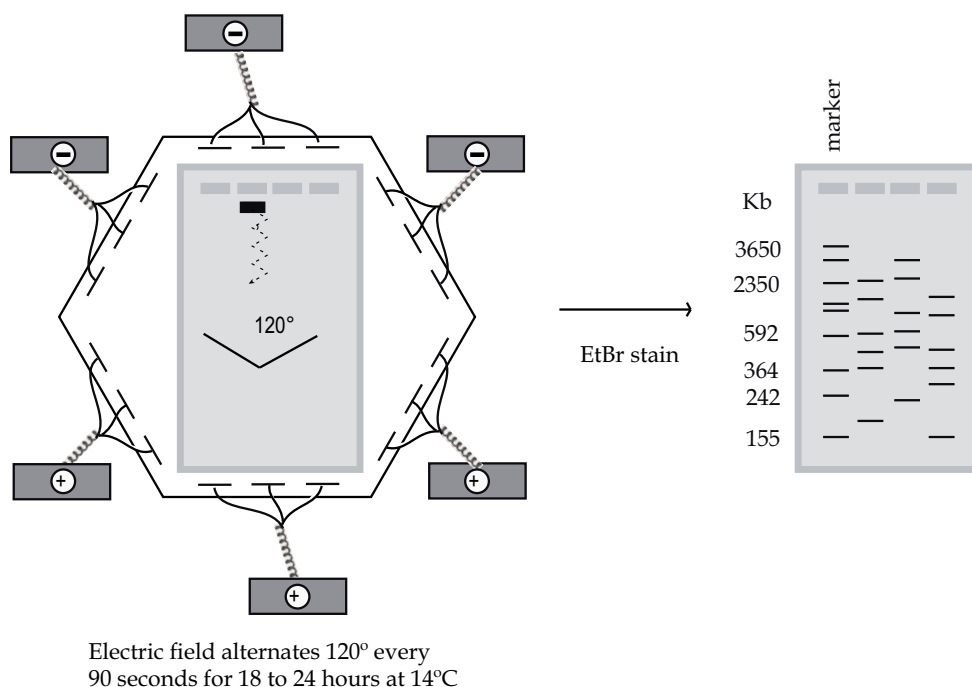


Fig. 1. Schematic diagram of PFGE instrumentation. Contoured clamped homogeneous electric field (CHEF) systems use a hexagonal gel box that alters the angle of the fields relative to the agarose gel. After running the gel by PFGE, DNA fragments are visualized by staining with ethidium bromide.

generating DSBs by using the recombination proteins RecBCD, RecA, and by the HJ-specific resolvase RuvABC (Fig. 2B) (Seigneur et al., 1998). This is a key aspect of the RFR model as it allows restarting of the blocked forks without generating chromosome instability. Nevertheless, in the absence of RecBCD activity (Fig. 2C), resolution of the RFR-produced HJ is done by RuvABC resolvase and leads to fork breakage. These particular DSBs are dependent on RuvABC activity in a *recB* deficient background.

If RFR does not take place at the stalled fork, at least two situations may arise. On the one hand, there would be an increase of DSBs independent of RuvABC activity and generated by another unknown endonuclease (Fig. 2E) as in the case of the thymine starvation (Guarino et al., 2007b). On the other hand, there would be no increase in the amount of DSBs probably because the stalled forks are not susceptible to the endonuclease action, and the restarting of the forks would take place without the generation of fork breakage. This situation has been described in *gyrB* mutants (Grompone et al., 2003), and when replication termination sequences *ter* were placed at ectopic positions on the bacterial chromosome (Bidnenko et al., 2002). Using the system described above, the fate of the stalled replication forks caused by any condition can be studied.

To verify the RFR process by PFGE, a *recB* deficient background should be used (i) to inhibit the degradation or the recombinational repair of the DNA tail created by the regression of the fork (Miranda & Kuzminov, 2003), allowing RuvABC resolvase to transform this tail in a DSB; (ii) to inhibit the repair of the DSBs generated by RuvABC resolvase (Fig. 2C). According to the RFR model, the occurrence of this process at the stalled forks generated under restrictive conditions can be verified by testing whether there is an increase of DSBs in a *recB* deficient background, and determining whether these DSBs are dependent on RuvABC resolvase activity by measuring the amount of DSBs in a *recB* and *recB ruvABC* deficient background (Fig. 2C) (Seigneur et al., 1998). The occurrence of RFR at the stalled forks has been detected by this system in several replication mutants, such as in the helicase mutants *rep* and *dnaBts* (Michel et al., 1997; Seigneur et al., 2000), in *holD<sup>G10</sup>* (Flores et al., 2001, 2002), in *dnaEts* at 42°C and in the *dnaNts* mutant at 37°C (Grompone et al., 2002) and finally in *nrdA101ts* (Guarino et al., 2007a, 2007b).

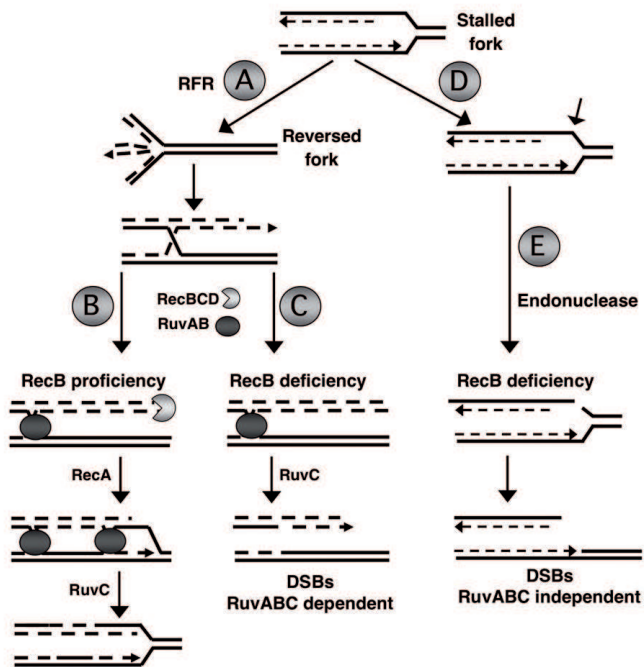


Fig. 2. The fate of the stalled forks. In the first step (A), the replication fork is arrested, causing fork reversal. The reversed fork forms a HJ (two alternative representations of this structure are shown - open X and parallel stacked X). In  $Rec^+$  cells (B), RecBCD initiates RecA-dependent homologous recombination, and the resulting double HJ is resolved by RuvABC. In the absence of RecBCD (C), resolution of the HJ by RuvABC leads to DSBs at the stalled replication fork. Alternatively, the replication fork is arrested without being regressed (D) and it is susceptible to be cut by an endonuclease, generating DSBs at the stalled replication fork (E). Continuous line (parental chromosome); dashed lines (newly synthesized strands); disk (RuvAB); incised disk (RecBCD). Adapted from Guarino et al., 2007b.

The amount of linear DNA resulting from DSBs can be estimated by using PFGE combined with cell lysis in agarose plugs (Michel et al., 1997). Briefly, cultures of *recB* and *recB ruvABC* strains growing in M9 minimal medium are labelled by addition of 5  $\mu\text{Ci/ml}$  [*methyl*- $^3\text{H}$ ] thymidine (100 Ci/mmol). When cultures reached 0.2  $\text{OD}_{450\text{nm}}$ , 1ml of cells were collected, washed in cold minimal medium and resuspended in 100 $\mu\text{l}$  of TEE buffer. Cells were incubated at 37°C for ten minutes, mixed with 100  $\mu\text{l}$  of low melting agarose 2% in TEE at 55°C and poured into the mould. Once agarose had solidified, cell lysis was performed in the plugs. This ensures only linear chromosomes to enter the gels, while circular molecules remain in the wells (Michel et al., 1997; Seigneur et al., 1998). Plugs were incubated with lysozyme at 5 mg/ml and sarcosyl 0.05% in TEE for 2 h with gently shaking. Then, plugs were retrieved and incubated with lysis solution (1 mg/ml Proteinase K, 1% SDS in TEE) at 56°C overnight. PFGE were run for 48 h at 4°C as described (Seigneur et al., 1998); initial run 500 sec, final run 500 sec, 3 volts/cm and 106° reorientation angle. DNA was visualized by ethidium bromide staining. Lanes were cut into slices and the proportion of migrating DNA was determined by calculating the amount of tritium present in each slice with respect to the total amount of tritium present in the corresponding lane plus the well (Fig. 3A). All the PFGE linear DNA data were analyzed by the least-squares statistical approach, considering measures as highly significantly different if  $p < 0.01$ .

A typical profile of gel migration for the different strains analyzed is shown in figure 3B. In this case, results indicate that the amount of DSBs in the strain *nrdA101 recB* was greater than in the strain *nrdA+ recB*, suggesting an increase in the number of the stalled forks induced by the presence of a defective ribonucleotide reductase (RNR) at the permissive temperature. To establish the possible origin of the DSBs induced by the *nrdA101 recB* background we investigated whether the formation of DSBs resulted from the action of the RuvABC resolvase (Fig. 2). The DSBs levels estimated in *nrdA101 recB ruvABC* and *nrdA+ recB ruvABC* strains were markedly lower than in the respective Ruv+ counterpart strains. As RuvABC is a specific resolvase for HJ, according the RFR model (Fig. 2), it generates DSBs at arrested replication forks in *recB* deficient background (Seigneur et al., 1998); these results indicated the occurrence of replication fork reversal in *nrdA101* mutant. As RFR is one of the mechanisms to restart the stalled replication forks, we could infer that the *nrdA101* strain growing at 30°C increases the number of stalled replication forks that would proceed with the help of RFR process in a Rec+ proficient context (Guarino et al., 2007a).

## 2.2 Replication fork collapse at natural arrest sites

In *E. coli*, replication termination occurs by the encounter of two opposite migrating forks at the terminus region or at specific arrest sites named *Ter*, when one of the forks reaches the terminus before the other. Tus protein binds the *Ter* sites forming a complex that acts as a polar replication fork barrier by preventing the action of the DnaB helicase (Neylon et al., 2005).

PFGE was used to determine the analysis of replication forks blocked at terminator sequences *Ter* inserted at ectopic positions on the bacterial chromosome (Bidnenko et al., 2002). This strain requires the RecBCD pathway of homologous recombination for viability, although replication forks blocked at *Ter* are not broken nor reversed (Fig. 2A, D). The analysis of the structure of the chromosomes by PFGE showed linear fragments of about 2 Mb, corresponding to the distance between the origin and the ectopic *Ter* sites. A model of a



### 2.3.1 Reinitiation events under thymine starvation

Thymine starvation results in cellular death in thymine requiring strains. This is a phenomena know as *thymineless death* (TLD), first described in the 50's (Cohen & Barner 1954). Some proposals postulated the formation of branched DNA as the source of the toxic effect of thymine starvation (Nakayama, 2005). Nakayama and co-workers demonstrated the presence of complex DNA structures by digesting DNA from cultures under thymine starvation with the restriction enzyme *XbaI* and separating it by PFGE (Nakayama et al., 1994). They called these structures "non-migrating DNA" (nmDNA), defined as the DNA that is unable to enter the gel and gets stuck in the well. The nmDNA was characterized as having single-stranded tails or gaps and branching with single-stranded arms. TLD has been related to DNA replication (Maaloe & Hanawalt, 1961; Hanawalt & Maaloe, 1961); nevertheless, ongoing replication does not appear to be required for TLD as same lethality is observed under thymine starvation in the presence of hidroxyurea (Morganroth & Hanawalt, 2006), an inhibitor of the DNA synthesis. By contrast, TLD is suppressed by the addition of rifampicin or cloramphenicol, both inhibitors of the new initiation events of the *E. coli* chromosomal DNA (Hanawalt, 1963).

To study whether the formation of nmDNA correlates with TLD under the above described replication conditions, we analyzed the generation of nmDNA after thymine starvation in the presence or absence of rifampicin, cloramphenicol and hidroxyurea (Mata & Guzmán, 2011). Mid-exponentially growing culture of *thyA* mutant MG1693 was starved for thymine in the presence or absence of rifampicin, cloramphenicol, or hidroxyurea. Two hours after the treatment, cells were collected, washed, embedded in agarose plugs, gently lysed and plugs treated with *XbaI* (50 U/100  $\mu$ l) for two hours before being used for PFGE (Matushek et al., 1996; Gautom, 1997). The visualization of DNA bands was achieved by ethidium bromide staining (Fig. 4A). The amount of nmDNA was quantified by densitometry of the PFGE by using the *Imagen J* program. The nmDNA values were expressed as the ratio (%) between the arbitrary densitometric units of the gel well and those of the gel line plus the well (Table 1, Figure 4B). By using this experimental approach, we showed that nmDNA was generated under thymine starvation, and it was absent in the presence of rifampicin or chloramphenicol, as previously reported (Nakayama et al., 1994). This might suggest that TLD correlates with the generation of nmDNA. However, we found no nmDNA under thymine starvation in the presence of hidroxyurea, indicating that the generation of nmDNA is not a requirement for TLD (Mata & Guzmán, 2011).

Treatment	% nmDNA <sup>1</sup>	Treatment effect <sup>2</sup>	Lethality
None -Exponential culture	8.6 $\pm$ 3.6	1	no
-Thymine	21.4 $\pm$ 4.7	2.48	yes
-Thy+ 75 mM hidroxyurea	11.2 $\pm$ 3.8	1.30	yes
-Thy+ 150 $\mu$ g/ml rifampicin	9.0 $\pm$ 3.9	1.04	no
-Thy+ 200 $\mu$ g/ml chloramphenicol	9.5 $\pm$ 4.4	1.10	no

Table 1. Percentage of nmDNA in MG1693 cells after 2 h of thymine starvation in the presence or absence of hidroxyurea, rifampicin or chloramphenicol. <sup>1</sup> The percentage of nmDNA is expressed as the mean  $\pm$  standard deviation. <sup>2</sup> The percentage of nmDNA relative to the exponential culture.

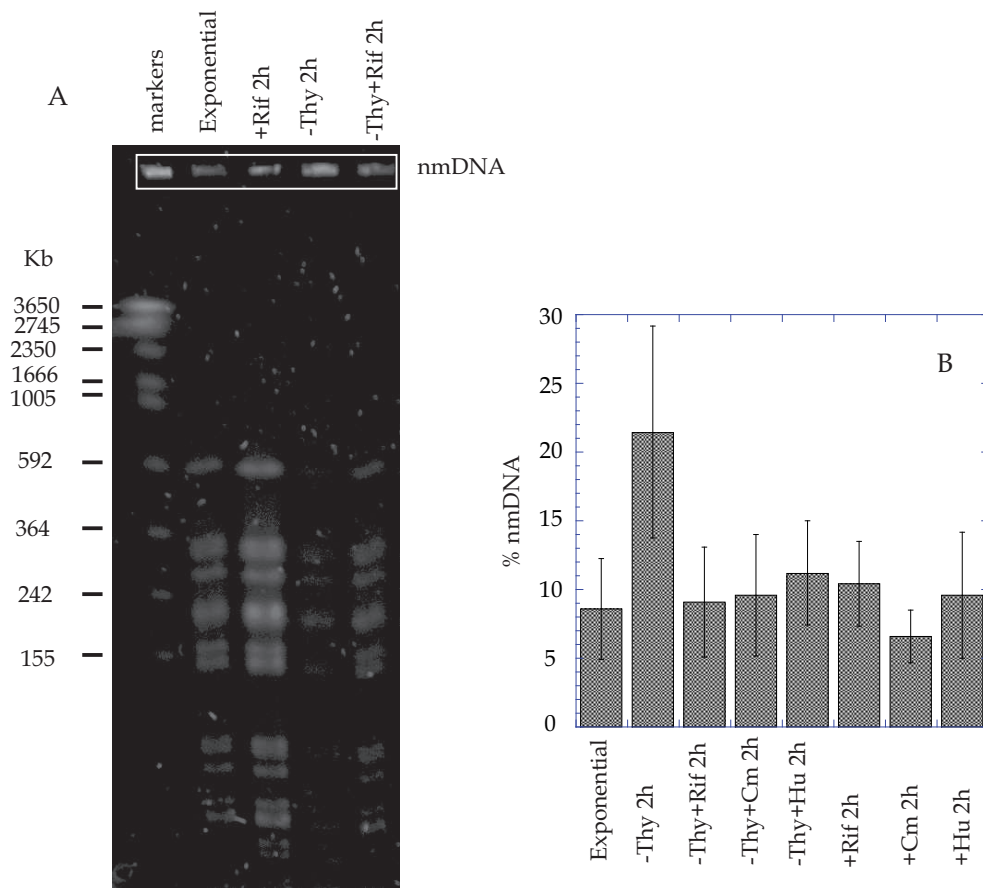


Fig. 4. Visualization and quantification of nmDNA by PFGE after treatment with *Xba*I. (A) Visualization of a representative agarose gel stained with ethidium bromide. (B) Quantification of the amount of nmDNA after different treatments of thymine starvation in the absence or the presence of the drugs, by using the data obtained by densitometry analysis. The standard deviation from 2-3 independent experiments is shown.

### 2.3.2 Collision between replication and transcription machines

PFGE has been also used for the characterization of collisions of the replication and transcription complexes. Genetic instability following head-on collisions of replication and transcription has been described in bacteria (Vilette et al., 1995) and yeast (Torres et al., 2004; Prado & Aguilera, 2005). Replication fork barriers are present on the rDNA of all eucaryotic cells described until now, for example in yeast (Brewer & Fangman, 1988; Kobayashi et al., 1992; López-Estraño et al., 1998; Sánchez-Gorostiaga et al., 2004), *Xenopus* (Wiesendanger et al., 1994) and plants (Hernández et al., 1993; López-Estraño et al., 1998) and they bound specific proteins. In yeast, these barriers are polar since they block replication forks facing transcription from the pre-rRNA 35S (Brewer & Fangman, 1988; Linkens & Huberman,



1988). It has been proposed that the function of those barriers could be to prevent head-on collisions between replication forks and the highly expressed rRNA genes (Takeuchi et al., 2003).

In order to avoid head-on collisions, ribosomal operons (*rrn*) are transcribed in the direction of replication in bacteria. By using genetic approaches together with PFGE analysis, the laboratory of B. Michel (Boubakri et al., 2010) identified that the three *E. coli* DNA helicases DinG, Rep and UvrD are recruited to the replication fork to allow replication across oppositely oriented highly transcribed ribosomal operons. Strains containing an inversion of an *rrn* operon were used in such a way that a region of increased head-on collisions between replication and transcription were created. Increased level of DNA trapping at wells in PFGE experiments was correlated with a high level of *rrn* transcription, suggesting the formation of abnormal structures in certain genetic backgrounds (Boubakri et al., 2010).

### 3. Two-dimensional agarose gel electrophoresis

The movement of a DNA molecule through an agarose gel is determined either by factors intrinsic to the electrophoretic conditions (agarose concentration, the strength of the electric field, the presence of intercalating agents, etc.) as well as the size and shape of the molecule.

The most evident example of the influence of the shape of a DNA molecule on the electrophoretic mobility in an agarose gel is observed when circular DNA molecules are analyzed: supercoiled DNA molecules and the corresponding relaxed-nicked DNA forms do not migrate necessarily at the same position than a linear DNA molecule of the same mass.

Taking into account this property, neutral/neutral 2D agarose gel electroforesis technique was developed to study the shape of recombination intermediates (Bell & Byers, 1983). Later on, it was adapted to study the DNA replication intermediates (RIs) (Brewer & Fangman, 1987). Since then, 2D agarose gel electrophoresis was used to map and characterize replication origins (Brewer & Fangman, 1988; Gahn & Schildkraut, 1989; Liu & Botchan, 1990; Schwartzman et al., 1990; Linskens & Huberman, 1990 b; Friedman & Brewer, 1995; Bach et al.; 2003), to analyze the progression of DNA replication along a DNA fragment (Azvolinsky et al., 2006), to characterize replication fork barriers (Brewer & Fangman, 1988; Linskens & Huberman, 1988; Hernandez et al., 1993; Wiesendanger et al., 1994; Samadashwily et al., 1997, López-Estraño et al., 1998, Possoz et al., 2006; Mirkin et al., 2006, Boubakri et al., 2010), replication termination (Zhu et al., 1992; Santamaría et al., 2000a,b), origin replication interference (Viguera et al., 1996), RIs knotting (Viguera et al., 1996; Sogo et al., 1999), fork reversal (Viguera et al.; 2000; Fierro-Fernandez et al., 2007a) or the topology of partially replicated plasmids (Martín-Parras et al., 1998; Lucas et al., 2001). See (Schvartzman et al., 2010) for an excellent review in plasmid DNA replication analyzed by 2D-gel.

2D agarose gel electrophoresis consists of two successive electrophoreses in which the second dimension occurs perpendicular to the first. Two different migration conditions are used so that the first dimension conditions (low voltage, low agarose concentration) minimize the effect of molecular shape on electrophoretic mobility, whereas this effect is maximized during the second dimension (high voltage and high agarose concentration, in the presence of an intercalating agent) (Friedman & Brewer, 1995). As a consequence, a

branched DNA molecule like a recombination or a replication intermediate is separated from a linear molecule of the same mass during the second dimension.

As DNA replication is a continuous process, a sample of DNA isolated from an exponentially growing culture should contain all the replication intermediates (RIs), ranging from the linear non-replicative forms (named 1.0X) to molecules almost completely replicated (2.0X) (Fig. 5A). See (Krasilnikova & Mirkin, 2004), for a detailed protocol of isolation of RIs in *E. coli* and *S. cerevisiae*.

The different migration patterns of a RI digested with a specific restriction enzyme are revealed after southern blotting hybridization with a specific probe and it indicates the mode it has been replicated (Fig. 5). Electrophoresis conditions must be adapted to the fragment size in order to obtain a good separation of the different patterns (Friedman & Brewer, 1995). Different situations can be discerned by using 2D gels. (i) A single fork that moves from one end to the other end of the fragment generates a simple-Y pattern indicating that the DNA fragment is replicated passively and does not contain neither a replication origin nor a replication terminus (Fig. 5A). (ii) Two forks that move convergently generate a double-Y pattern, indicating that replication termination occurs within the analyzed fragment (Fig. 5C); and (iii) two forks that have initiated at some specific point in the analyzed fragment and progress divergently, generate a bubble pattern, indicating that DNA replication has been initiated inside this fragment (Fig. 5B).

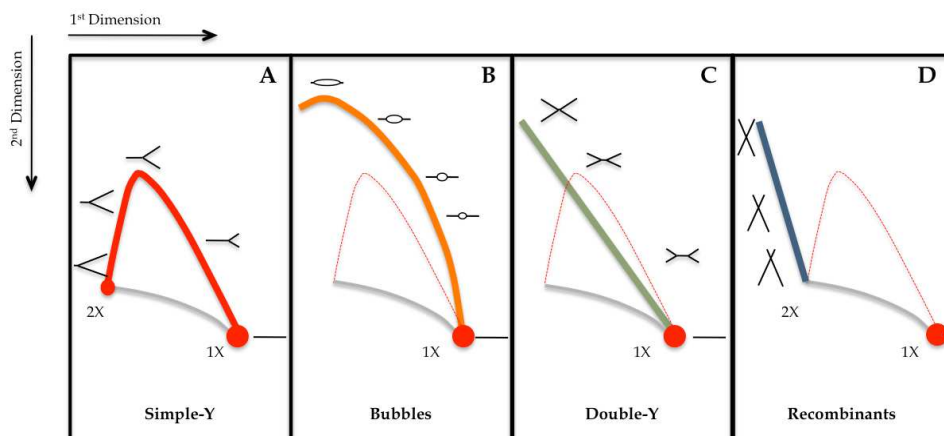


Fig. 5. 2-D gel hybridization patterns generated by replication and recombination intermediates after two-dimensional agarose gel electrophoresis. Replication and recombination intermediates of the restriction fragment are shown above the different 2D-gel pattern. In panels B, C and D, the simple-Y arc is presented as a reference. See text for details.

Moreover, the relative proportion of a particular RI in the population increases as a consequence of the stalling of the replication forks at a specific site. This accumulation is detected as a discrete signal on top of the corresponding arc produced by the RIs. In order to map the region where replication is paused, different restriction fragments must be

analyzed to confirm that the signal corresponding to the accumulated molecules move along the arc.

Recently, this technique was used to get insight into the nature of the elements that causes the trapping of the DNA in PFGE experiments in *E. coli* mutant strains containing an inverted *rrn* operon (Boubakri et al., 2010). No RIs were detected in the non-inverted strains or the inversion mutants that express all helicases. However, a simple-Y arc that corresponds to the accumulated Y-shape restriction intermediates was detected in all *dinG*, *rep* and *uvrD* helicase mutants in which the Inv-fragment was trapped in PFGE wells. Moreover, an intense elongated spot was observed over the simple-Y arc. These results indicate that a specific accumulation of RIs occur at the 3' end of the *rrn* operon (Figure 6).

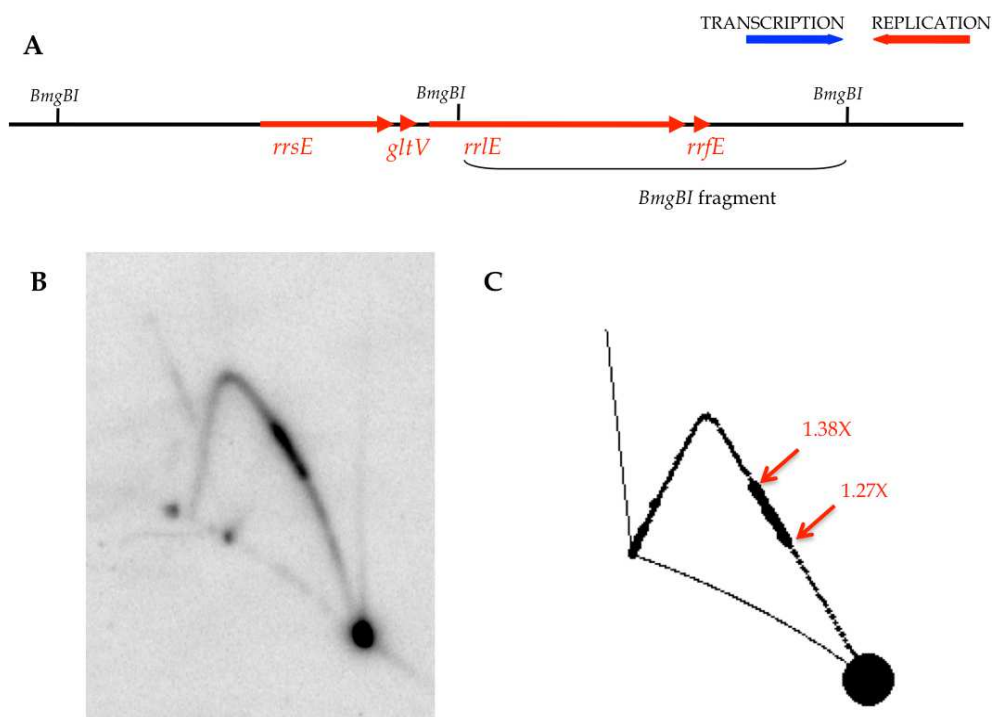


Fig. 6. Replication forks are arrested in inverted *rrn*. 2D-Gels were used to examine DNA replication in restriction fragments containing a large 3' region of *rrnA* in *InvA* mutants and of *rrnE* in *InvBE* mutants (Adapted from Boubakri et al., 2010). (A) Schematic representation of the restriction fragment used for 2D gels (only *InvBE* is presented in this figure). The position of *rrn* and restriction sites is shown. (B) DNA from *InvBE dinG rep* mutant was digested with *BmgBI*, analyzed by 2D gels and probed for the sequence just downstream of *rrnE*. A simple-Y arc is clearly detected. On top of this arc, an enlarged signal corresponding to arrested forks is detected. (C) A simulation of replication arrest in this fragment of about 500 pb around the *rrn* transcription terminator sequence was obtained by using the 2D-Gel computer program (Viguera et al., 1998).

#### 4. Concluding remarks

Replication arrest is a source of genetic instability in all types of living cells. As a consequence, cells have developed several effective strategies to tackle with replication fork arrest and/or repairing the double DSBs generated at the stalled replication forks. We have reviewed how PFGE and 2D gels can be used to elucidate some features related to the progression of the replication forks. By using these two non-conventional electrophoresis it can be verified the presence of stalled replication forks, understanding how they have been generated and how they could be restarted.

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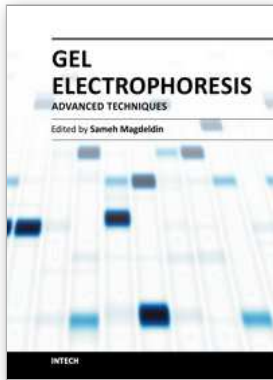
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As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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