The helicases DinG, Rep and UvrD cooperate to promote replication across transcription units in vivo

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How living cells deal with head-on collisions of the replication and transcription complexes has been debated for a long time. Even in the widely studied model bacteria *Escherichia coli*, the enzymes that take care of such collisions are still unknown. We report here that in *vivo*, the DinG, Rep and UvrD helicases are essential for efficient replication across highly transcribed regions. We show that when rRNA operons (*rrn*) are inverted to face replication, the viability of the *dinG* mutant is affected and over-expression of RNase H rescues the growth defect, showing that DinG acts *in vivo* to remove R-loops. In addition, DinG, Rep and UvrD exert a common function, which requires the presence of two of these three helicases. After replication blockage by an inverted *rrn*, Rep in conjunction with DinG or UvrD removes RNA polymerase, a task that is fulfilled in its absence by the SOS-induced DinG and UvrD helicases. Finally, Rep and UvrD also act at inverted sequences other than *rrn*, and promote replication through highly transcribed regions in wild-type *E. coli*.

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Introduction

Replication fork arrest is a recognized source of genetic instability in all types of living cells. To limit the danger of replication arrest, eukaryotes induce checkpoint proteins that stabilize and protect blocked replication forks (reviewed in Branzel and Foiani, 2007; Tourriere and Pasero, 2007). Prokaryotes behave differently, they constitutively express replication restart proteins that are associated with the replication machinery (Sandler, 2000; Lecointe et al, 2007). Replication arrest can occur for many reasons, including collisions with DNA-bound proteins and particularly transcription complexes. As bacterial chromosomes are simultaneously transcribed and replicated, and because the velocity of the replication machinery (800 NT/s) is more than 10 times higher than that of the transcription machinery (50 NT/s), the problem raised by collisions between replication and transcription has been studied for decades (French, 1992; reviewed in Mirkin and O’Donnell, 2008 and references therein). In contrast, it is well established that head-on collisions between replication and transcription, that is the presence of an active RNA polymerase on the lagging strand template, arrest replication forks *in vitro* and *in vivo* (Deshpande and Newlon, 1996; Takeuchi et al, 2003; Mirkin and Mirkin, 2005). Genetic instability following head-on collisions of replication and transcription has been documented in bacteria and yeast (Vilette et al, 1995; Torres et al, 2004; Prado and Aguilera, 2005).

To limit head-on collisions between replication forks and the highly expressed rRNA genes, yeasts and eukaryotic cells use replication fork barriers, which are DNA sites where binding of a specific protein prevents replication from entering the rDNA region in the direction opposed to transcription (Brewer et al, 1992). In bacteria, to avoid head-on collisions ribosomal operons (*rrn*) are transcribed in the direction of replication (Brewer, 1988; Rocha and Danchin, 2003). *rrn* operons are highly expressed and their promoter regions carry regulatory elements that adapt their level of expression to the growth rate, so that transcription is more efficient in rich than in minimal medium (MM) (Condon et al, 1992; reviewed in Paul et al, 2004). Ribosomal-RNA transcripts are not translated and premature transcription arrest is prevented by the association of the RNA polymerase with ‘an anti-termination’ machinery, which increases the transcription speed to 90 NT/s (reviewed in Condon et al, 1995). The universality of the presence of *rrn* operons on the leading strand template in bacteria suggests that *rrn* inversion impairs bacterial growth. Surprisingly, *Escherichia coli* viability was not affected by inverting large chromosomal regions that carry several *rrn* operons, even when the main homologous recombination DNA repair protein, RecA, was inactivated (Esnault et al, 2007). This observation suggested that bacteria...
encode proteins other than RecA that facilitates the progression of replication forks through oppositely oriented highly transcribed genes. We describe here the identification of helicases that have such a role.

Helicases are enzymes that associate with DNA unwinding and capacity to translocate on DNA. Most helicases translocate on single-stranded DNA (ssDNA) to unwind double-stranded DNA, several also unwind DNA–RNA hybrids, or can remove proteins from DNA during translocation. The first helicase described to remove an RNA polymerase from the path of replication forks was the T4 dda helicase (Bedinger et al., 1983). In yeast, this function is fulfilled by the superfamily 1 (SF1) helicase Rrm3, a 5′-3′ helicase required for efficient replication at numerous protein-bound sequences such as in RNA and tRNA genes, centromeric and telomeric regions (Azvolinsky et al., 2006; reviewed in Boule and Zakian, 2006). In this study, we show that in bacteria the three helicases DinG, Rep and UvrD facilitate replication of the chromosome through oppositely oriented highly transcribed ribosomal operons.

DinG belongs to the SF2 family of helicases and translocates in the 5′-3′ direction on ssDNA (Voloshin et al., 2003). In vitro, it unwinds a wide variety of substrates with a preference for D-loops and R-loops (Voloshin and Camerini-Otero, 2007). DinG is present in most prokaryotes and is related to the DNA helicases Chl1 and Rad3 from Saccharomyces cerevisiae, Rad15 from Schizosaccharomyces pombe and the human helicases XPD and BACH1 (Koonin, 1993; Rudolf et al., 2006; Voloshin and Camerini-Otero, 2007; Liu et al., 2008). Rad3 and XPD are components of the transcription factor IHF, they function in transcription initiation and nucleotide excision repair, and XPD defects are responsible for several human diseases (Liu et al., 2008 and references therein). Although DinG is an SOS-inducible protein (Lewis et al., 1992; Courcelle et al., 2001), its absence does not render E. coli sensitive to DNA damaging agents and to date the function of DinG in vivo is totally unknown.

Rep and UvrD are also the founders of a large family of helicases, homologous to Srs2 in yeast. They belong to the SF1 superfamily, share 40% identity and translocate in the 3′-5′ direction on ssDNA. The uvrD gene was originally identified for its crucial role in nucleotide excision repair and mismatch repair. In addition, UvrD (but not Rep) can remove the replication terminator Tus protein from its cognate site, Ter, and the recombination protein RecA from ssDNA (Flores et al., 2005; Veau et al., 2005; Bidnenko et al., 2006). Rep assists replication because in its absence chromosome replication takes twice as long when compared with wild-type cells, and arrested replication forks undergo a remodelling reaction called replication fork reversal (Lane and Denhardt, 1975; Seigneur et al., 1998). Rep was hypothesized to facilitate replication across DNA-bound proteins because it can dislodge a DNA-bound repressor during translocation in vitro (Yancey-Wrona and Matson, 1992). The rep uvrD double mutant is lethal and rescued by the inactivation of the pre-synaptic recombination proteins RecQ, RecJ and RecFOR (Petit and Ehrlich, 2002; Lestini and Michel, 2008); one of the physiological roles of UvrD is thus to remove RecQJFOR-dependent RecA filaments from stalled replication forks, or to prevent their formation. Finally, replication forks that have been inactivated restart with the use of the major restart protein PriA; in a priA mutant, replication restart is catalysed by an Rep-PriC-dependent pathway (Sandler, 2000; Heller and Marians, 2005).

In this study, we show that chromosomal inversion, including E. coli rRNA operon(s) renders DinG essential for growth in rich medium. Moreover, the inactivation of the helicases DinG, Rep and UvrD has synergistic effects on replication blockage at an inverted rRNA locus. In the natural chromosome configuration, E. coli cells lacking these three helicases are viable only if the stability of the RNA polymerase is compromised and RecA binding is prevented by an RecF mutation. These results suggest that these helicases exert a fork-clearing function at inverted rrr loci and also at other transcription units.

Results

dinG inactivation confers rich medium sensitivity to strains that carry inverted rrr operons

The lambda attR and attL attachment sites were used to construct strains carrying a chromosome inversion (Valens et al., 2004; Esnault et al., 2007). The InvA mutant carries a 18 kb inversion encompassing the rrrA operon (Figure 1). It carries only 11 genes in addition to rrrA, among which 4 are naturally oriented in opposition to replication. rrrA is the only transcription unit that is highly expressed and whose expression is increased in rich medium in InvA strains (Corbin et al., 2003; Lopez-Campistrous et al., 2005). The InvBE mutant carries a 138.3 kb inversion containing rrrB and rrrE; about 100 genes are present in the inverted region, among which 67% are originally co-directional with replication and may be sites of transcription–replication collisions after inversion. As rrr expression is growth-rate regulated, these two Inv mutants allowed the analysis of three kinds of head-on replication–transcription collisions: (i) in highly expressed rrr, (ii) in moderately expressed rrr, (iii) in genes other than rrr. As previously observed for similar inversions (Esnault et al., 2007), InvA and InvBE were

![Figure 1 Schematic representation of the inverted region in the mutants InvA (top) and InvBE (bottom). Numbers indicate the sequence coordinates in the wild-type E. coli MG1655 chromosome. The large black arrows indicate the inversion end points (lambda att sites). The grey arrows indicate the position of rrr operons (the coordinates of rrrA, and of rrrE and rrrB 3′ ends are indicated). The vertical arrows show the position of NotI sites (used for PFGE).](image-url)
fully viable on MM and on rich medium (Luria broth, LB) (Figure 2A and B; Supplementary Table S2). DinG, Rep and UvrD were inactivated in InvA and InvBE mutants to test whether these helicases are required for replication across oppositely oriented genes.

dinG inactivation did not affect the formation of Inv mutant colonies on MM; however, colony formation on LB was strongly impaired for InvA dinG and delayed for InvBE dinG (Figure 2A and B). To test whether transcription is responsible for the LB sensitivity of InvA dinG and InvBE dinG mutants, we used the rpoC^{215-220} mutation (called rpoC\* thereafter). By mimicking the presence of ppGpp, this mutation reduces the stability of transcription elongation complexes (Bartlett et al, 1998, 2000; Trautinger and Lloyd, 2002; Trautinger et al, 2005). rpoC\* restored 100% overnight colony formation on LB in both Inv dinG mutants (Figure 2C and D). In InvBE, the oriC-distal att site removes rrnE P1 Fis-binding sites (Supplementary Figure S1A), so that the promoter is weakened about seven-fold during steady-state growth in rich medium, but remains growth-rate regulated (Appleman et al, 1998; Hirvonen et al, 2001). Specifically, deleting the highly expressed rrnE operon in the InvBE dinG mutant fully restored the plating efficiency on LB (Supplementary Table S2). We conclude that DinG is required for efficient colony formation on rich medium when a highly expressed rrn operon is inverted on the chromosome, and that the growth defect observed in Inv dinG mutants is completely overcome by reducing the transcription level (growth on MM or inversion of only rrnB, which is deprived of Fis sites in this construction).

**rep inactivation causes cell elongation in rich medium**

Most of the rep mutants were constructed in the presence of a conditional Rep\* plasmid (IPTG dependent) that was cured before each experiment, (Supplementary Table S1; Lestini and Michel, 2008). Inactivation of rep in InvA or InvBE mutants did not cause any loss of plating efficiency (Figure 2A and B), although InvBE rep overnight colonies on LB were quite small. The introduction of rpoC\* or the deletion of rrnE in InvBE rep suppressed this slow-growth phenotype, again suggesting a deleterious effect of the inverted highly expressed rrn operons (not shown). This idea was confirmed by the use of a strain with a large inverted region carrying the three operons rrnA, rrnB and rrnE (InvABE, 277.3 kb inverted): InvABE rep was sensitive to LB and this defect was fully suppressed by the rpoC\* mutation (Figure 2C). In contrast, all Inv uvrD mutants were fully viable on LB as on MM (Figure 2). We conclude that the Rep helicase (and not UvrD) is required for colony formation on LB when at least three highly transcribed rrn operons are oriented opposite to replication. In contrast, a 277 kb inversion does not impair growth of the rep mutant providing that the rrn operons are only moderately expressed (InvABE cells grown in MM) or that the stability of the RNA polymerase is compromised (rpoC\* mutant on LB, Figure 2).

Formation of a visible colony requires about 24 generations and to determine whether the rep mutation affects Inv cell growth at early times, Inv rep cells were analysed by differential interference contrast (DIC) microscopy. Both InvA rep and InvBE rep cultures, shifted for 1 h from MM to LB, contained a high percentage of elongated cells, higher than rep or Inv single mutants (Table I). Cell elongation was

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**Figure 2** The helicases Rep, UvrD and DinG are required for colony formation in Inv mutants. Appropriate dilutions of overnight cultures at 37\(^\circ\)C in MM (OD 1.0–1.5) were plated on MM and LB plates, which were incubated at 37\(^\circ\)C. Unmarked positions on the left of (A) (InvA), (B) (InvBE) and (C) (InvABE) are data points for Inv mutants that express all helicases. White boxes: colony forming units (cfu)/ml on MM plates after 48 h incubation; dark grey boxes: cfu/ml on LB plates after 16–24 h incubation; light grey boxes: cfu/ml on LB plates after 48 h of incubation. The hatched box indicates cfu/ml on MM after 3 days incubation. The results are also presented in Supplementary Table S2.
weaker in MM and was strongly decreased by the \textit{rpoC}^* mutation or the deletion of \textit{rrnE} and \textit{rrnB} (from 29 to 3% in InvA \textit{rep} and from 48 to 5–11% in InvBE \textit{rep}, Table I), indicating that it is caused by the strong expression of inverted \textit{rrn}. It was also specific for the \textit{rep} mutation, as Inv \textit{uvrD} cells were no more elongated than single \textit{uvrD} mutants (6–11% elongated cells, Table I), and Inv \textit{dinG} cells were not (InvBE) or only slightly (InvA) elongated (4 and 16% of elongated cells, respectively, Table I). The contrast between the elongated phenotype of Inv \textit{rep} cells after a shift to LB and a wild-type efficiency of colony formation overnight on LB plates suggest an early defect followed by a recovery. Conversely, the absence of cell elongation of the Inv \textit{dinG} mutants after a shift to LB contrasts with their plating defect suggests late, possible cumulative defects. These ideas were tested by analysing micro-colony formation by time-lapse microscopy (Supplementary Figure S2). InvA \textit{rep} microcolonies grown for a few hours on LB contained normal-sized cells, owing to the splitting of some elongated cells. Conversely, InvA \textit{dinG} normal-sized cells growing on an LB agar pad produced micro-colonies composed of non-dividing, mostly elongated cells (Supplementary Figure S2).

The effects of the \textit{dinG}, \textit{rep} and \textit{uvrD} mutations are additive

To analyse whether DinG, Rep and UvrD have independent or overlapping roles, we tested whether the inactivation of two of these helicases is synergistic. Cells that do not carry a chromosome inversion were tested first, showing that \textit{dinG} \textit{uvrD} and \textit{dinG} \textit{rep} double mutants are fully viable (Figure 2E). As described earlier (Petit and Ehrlich, 2002), non-inverted \textit{rep uvrD} cells were (i) nearly lethal on MM (small colonies appeared in 3 days), (ii) lethal on LB and (iii) mainly rescued by \textit{recF} inactivation (Figure 2E). Therefore, Inv \textit{rep uvrD} mutants were tested in a \textit{recF} mutant background. As \textit{recF} inactivation \textit{per se} does not affect the growth of Inv strains (Supplementary Table S3, see below) and is beneficial to \textit{rep uvrD} cells, we consider thereafter that the growth defects of \textit{rep uvrD recF} mutants carrying an inversion result from the inactivation of the \textit{rep} and \textit{uvrD} genes and not from the \textit{recF} mutation.

All Inv mutants lacking two helicases were sensitive to rich medium as they formed colonies on LB plates with a very low efficiency (Figure 2A and B). As the InvA \textit{dinG} mutant was already quite sensitive to rich medium, the deleterious effect

### Table I: Cell elongation after a shift to LB

| Strain | Relevant genotype | MM^a | LB 1 h^b |
|--------|------------------|------|---------|
|        | \textit{dinG}   | \textit{rep} | \textit{uvrD} | \textit{1 N}^* | \textit{2 N} | \textit{> 3 N} | \textit{1 N} | \textit{2 N} | \textit{> 3 N} |
| JJC3524 | +               | +    | + | 73 | 27 (20) | 0 | 62 (1) | 36 (20) | 2 |
| InvA strains |                |      |    |  |  |  |  |  |  |
| JJC4010/4802 |                |      |    | 58 | 41 (19) | 1 (1) | 61 | 36 (19) | 3 (2) |
| JJC4678/4881 S |                |      |    | 60 | 36 (12) | 4 (1) | 40 | 44 (12) | 16 (8) |
| JJC4408 | +               | +    | + | 51 | 39 (8) | 10 (9) | 23 | 44 (5) | 29 (12) |
| JJC4873 | +               | +    | \textit{uvrD} | 68 (1) | 29 (1) | 3 (3) | 42 | 47 (16) | 11 (7) |
| JJC4880 | \textit{dinG} | +    | \textit{uvrD} | 48 | 39 (1) | 12 (2) | 17 | 26 (2) | 56 (7) |
| JJC4828 S | \textit{dinG} | rep | + | 60 | 29 (3) | 10 (6) | 24 | 30 (1) | 46 (3) |
| JJC4879 S | +               | rep | \textit{uvrD} | 11 | 25 | 64 (5) | 4 | 24 | 72 (1) |
| InvA \textit{rpoC}^* |                |      |    |  |  |  |  |  |  |
| JJC4962 | \textit{dinG} | +    | + | 75 (5) | 23 (13) | 0 | 43 (3) | 33 (23) | 12 (8) |
| JJC4995 | +               | rep | + | 64 (3) | 33 (23) | 3 (2) | 45 (3) | 33 (15) | 8 (3) |
| JJC4963 | \textit{dinG} | +    | \textit{uvrD} | 54 (5) | 30 (12) | 17 (10) | 17 (10) | 33 (9) | 13 (1) |
| JJC4914 S/4919 | \textit{dinG} | rep | + | 54 (5) | 33 (9) | 13 (1) | 43 | 39 (5) | 18 (0) |
| JJC5140 S/5143 | +               | rep | \textit{uvrD} | 71 (0) | 24 (5) | 5 (4) | 15 | 36 (2) | 49 (5) |
| InvA \textit{lexA} |                |      |    |  |  |  |  |  |  |
| JJC5096 | +               | rep | + | 56 | 38 (12) | 6 (4) | 17 | 55 (4) | 28 (9) |
| InvBE strains |                |      |    |  |  |  |  |  |  |
| JJC4349 | +               | +    | + | 83 (4) | 17 (11) | 1 (1) | 52 (1) | 40 (11) | 7 (5) |
| JJC4920 | \textit{dinG} | +    | + | 77 (1) | 23 (14) | 0 | 50 (1) | 45 (13) | 4 (2) |
| JJC4700 S/4978 S | +               | rep | + | 33 | 50 (9) | 17 (13) | 17 | 35 (3) | 48 (10) |
| JJC4870 S/4997 | +               | +    | \textit{uvrD} | 78 (4) | 22 (14) | 0 | 45 | 48 (11) | 7 (3) |
| JJC4981 | \textit{dinG} | +    | \textit{uvrD} | 63 (1) | 34 (14) | 3 (2) | 25 | 43 (7) | 27 (10) |
| JJC4746 S/5009 S | \textit{dinG} | rep | + | 52 | 34 (3) | 14 (6) | 7 | 37 (1) | 56 (7) |
| InvBE \textit{rpoC}^* |                |      |    |  |  |  |  |  |  |
| JJC4997 | +               | rep | + | 46 (1) | 49 (23) | 5 (3) | 55 (2) | 26 (10) | 18 (9) |
| JJC4966/4979 | \textit{dinG} | +    | \textit{uvrD} | 55 (2) | 26 (10) | 18 (9) | 58 (6) | 36 (19) | 5 (3) |
of inactivating urd in this mutant can be deduced from the increased level of elongated cells after only 1 h of propagation in LB (Table I). A high percentage of elongated cells is observed in all Inv mutants lacking two helicases. It is accompanied by a decrease in the number of cells with a visible septum (number in parenthesis in Table I), in agreement with a cell division defect. Therefore, dinG, rep and urd mutations are synergistic, indicating overlapping functions.

Inv dinG urd mutants were fully viable on MM whereas a significant plating defect of both Inv dinG rep mutants on MM indicates replication impairment by moderately expressed rna in this mutant and suggests overlapping functions of Rep and DinG (MM, Figure 2A and B). Inv rep urd recF cells were also impaired on MM; the plating defect was stronger for InvBE than for InvA, suggesting a possible replication impairment also at non-rna sequences (MM, Figure 2A and B; Inv rep urd RecF 

rrn expression is responsible for the growth defects of helicase mutants on LB

rrn* and rrn deletion alleles (ArrnE and ArrnB, Supplementary Figure S1) were used to ascertain the role of rna in the observed growth defects. rpoC* was first tested in a non-inverted rep urd mutant. Importantly, rpoC* rescued colony formation of rep urd cells on MM and on LB, regardless of the recF status (Figure 2E). This result indicates that (i) in E. coli the presence of both Rep and UvrD is required because of a high level of transcription and (ii) decreasing transcription by affecting the stability of RNA polymerase bypasses the need for RecFOR inactivation.

In InvA mutants, rpoC* restored the viability of both dinG urd and dinG rep cells (although InvA dinG urd rpoC* remained slightly impaired on LB) and the InvA rep urd rpoC* mutant formed colonies on LB in 2 days (Figure 2C–E; Table I). Therefore, the growth defects of all the three InvA mutants lacking two helicases result from the high level of rna expression.

In InvBE dinG rep, introduction of the rpoC* allele improved viability and decreased cell elongation in LB, as observed for the InvA strain (Figure 2D; Table I). Accordingly, deletion of both rrnE and rrnB also fully rescued the InvBE dinG rep mutant, confirming that these highly expressed operons are the only deleterious sequences in this mutant (Figure 2D, Table I). In contrast, rpoC* did not

Table 1

| Strain                  | Relevant genotype | MM≤ | LB 1 h≥ |
|------------------------|-------------------|-----|---------|
|                        | dinG             | rep | urd     | 1 N 2 N > 3 N | 1 N 2 N > 3 N |
| InvBE ArrnE            |                   |     |         |               |               |
| JJC4951                | dinG             | +   | +       | 80 (4)        | 19 (11)       | 0             |
| JJC4973                | dinG             | +   | urd     | 39 (0)        | 48 (11)       | 13 (5)        |
| InvBE ArrnE ArrnB      |                   |     |         |               |               |
| JJC5125                | dinG             | +   | +       | 82 (5)        | 19 (11)       | 0             |
| JJC5154                | +                | rep | +       | 44 (1)        | 45 (18)       | 11 (10)       |
| JJC5158                | dinG             | +   | urd     | 39 (5)        | 50 (11)       | 11 (10)       |
| JJC5156S               | dinG             | rep | +       | 56 (5)        | 41 (14)       | 3 (3)         |
| JJC5175S              | +                | rep | urd     | 80 (7)        | 19 (17)       | 1             |
| InvBE recA             |                   |     |         |               |               |
| JJC4631                | +                | +   | +       | 69 (7)        | 26 (15)       | 4 (1)         |
| JJC5036                | dinG             | +   | +       | 59 (1)        | 36 (13)       | 4 (2)         |
| JJC5058S               | +                | rep | +       | 35 (3)        | 43 (10)       | 22 (8)        |
| JJC5034               | +                | +   | urd     | 54 (2)        | 30 (5)        | 15 (7)        |
| Non-inverted rpoC*     |                   |     |         |               |               |
| JJC4645/S165S          | +                | rep | urd     | 63 (1)        | 26 (15)       | 11 (3)        |
| JJC4629               | recA             |     |         | 79 (6)        | 16 (5)        | 2 (1)         |

'S': the pAM-rep plasmid was cured before the experiment, the strain number is followed by an 'S' to indicate that experiment was performed after the plasmid has been segregated. In each medium, the smallest wild-type cells produced by division (baby cells, 1.5 μM in MM and 2.1 μM in LB) were used as cell unit and their size was, as expected, half that of the smallest cells with a detectable septum. Numbers indicate the percentage of cells in each of the following categories: 1 N: cells whose length was from baby wild-type cells to twice as long; 2 N: cells whose length was between twice and three times that of baby wild-type cells; > 3 N: cells longer than three times the size of baby wild-type cells. With few exceptions, 150–300 cells were counted. Numbers in parentheses indicate the percentage of cells with a visible septum in formation. Data in bold differ at least three-fold from their parental values (InvA and InvBE single mutants, and non-inverted cells carrying the same helicase or recA mutations); for these mutants, results are the average of two independent experiments.

*Cells in exponential phase in MM.

≥Cells in exponential phase shifted for 1 h in LB.

JJC4879, JJC5157 and JJC4878 are rep urd recF mutants.
Helicases that dislodge RNA polymerase

H Boubakri et al

rescue InvBE dinG wvrD, but deletion of both rrnE and rrnB allowed a full recovery of colony formation on LB (Figure 2D; Table I, Supplementary Table S2). These observations allow us to conclude that rrn are also the only deleterious sequences in InvBE dinG wvrD, but that even in the presence of the rpoC* mutation, inverted rrn impair growth of this mutant on rich medium.

In InvBE rep wvrD cells, introduction of rpoC*, deletion of rrnE or of both rrn allowed colony formation on MM but cells remained sensitive to LB, even in a recF context (Figure 2D; Supplementary Table S2). Therefore, the inversion of genes other than rrn is deleterious in rich medium in rep wvrD and rep wvrD recF mutants.

The requirement for UvrD in Inv dinG mutants is not because of its anti-RecF-RecA action

An recF null mutation was used to test whether UvrD is required in Inv dinG mutants to counteract a deleterious DNA binding of RecFOR, and in turn RecA. recF inactivation did not improve the growth of dinG, wvrD or dinG wvrD Inv mutants (Supplementary Table S3). We conclude that in Inv dinG wvrD mutants, the deleterious effect of the absence of UvrD is not because of the lethal binding of RecFOR-RecA to DNA. We propose that the synergistic effects of dinG and wvrD inactivation in cells carrying a highly expressed inverted rrn operon reflect a redundant function of these two helicases.

In agreement with a previous report, we observed that recA inactivation did not affect the viability of InvA and InvBE single mutants (Esnault et al., 2007; Supplementary Table S3). However, recA deletion prevented growth of InvBE dinG and Inv rep mutants on LB (Supplementary Table S3). Furthermore, no plasmid-less colony could be obtained from Inv dinG rep recA; [pAM-rep] cells even on MM, indicating that in both Inv backgrounds the dinG rep recA combination of mutations is lethal (Supplementary Table S3; wvrD recA colonies were slow growing on LB and were not affected by inversion, Supplementary Table S3). This suggests that the lack of Rep and/or DinG in Inv mutants generates ssDNA that renders homologous recombination and/or SOS induction crucial for viability. Notably, in Inv rep mutants the inactivation of the SOS response by a lexAind mutation also delayed (InvA) or prevented (InvBE) colony formation on LB (Supplementary Table S3; Supplementary Figure S2), indicating that the plating defect of Inv rep recA mutants may mainly result from the absence of SOS induction.

The combination of rep wvrD dinG recF mutations is lethal in non-inverted strains and rescued by rpoC*

We attempted to construct a rep wvrD dinG recF mutant by eliminating the pAM-Rep plasmid from rep wvrD dinG recF [pAM-Rep+] cells. Small plasmid-less colonies were obtained in 3 days on MM but some failed to grow in overnight cultures and others exhibited variable plating efficiencies, indicating that the simultaneous inactivation of the three helicases Rep, UvrD and DinG is nearly lethal in a recF E. coli mutant (Supplementary Table S2). Therefore, the viability of each helicase double mutant relies on the presence of the third helicase when all genes are in their original orientation. The rpoC* mutation also failed to restore rep wvrD dinG colony formation. Therefore, in cells lacking all three helicases, neither decreasing RNA Pol stability nor preventing RecA binding to blocked forks is sufficient to allow colony formation, even in slow-growth conditions (MM). However, when the stability of the RNA polymerase was compromised by the rpoC* mutation and recF was inactivated, the resulting rep wvrD dinG rpoC* recF mutant formed colonies on MM and on LB in 2 days (Figure 2E; Supplementary Table S2). This result indicates that an E. coli mutant lacking all three helicases is killed by collisions between replication and transcription complexes; in the presence of the rpoC* mutation, the triple helicase mutant is killed by RecFOR-RecA binding to arrested forks.

Pulse field gel electrophoresis analysis of Inv helicase mutants

To investigate the effects of helicase inactivation on the progression of replication forks across inverted sequences, chromosomes of Inv mutants were analysed using two approaches, pulse-field gel electrophoresis (PFGE) and 2D gels.

Y or X structures, that is replication or recombination intermediates, prevent migration of linear DNA fragments in PFGE (Ayzvolinsky et al., 2006 and references therein). Therefore, by measuring the proportion of DNA fragments that remain trapped in the wells after PFGE, we could quantify the formation of abnormal DNA structures in the inverted region. Chromosomes were digested by a rare cutting enzyme (NotI), and a probe specific for the NotI fragment carrying the inverted region (named Inv-fragment below) was used (Figure 3A).

In the InvA background, after a 1 h shift to LB the percentage of Inv-fragment trapped in the wells increased from 3% (InvA) to 41% in a dinG mutant and 52% in a rep mutant, whereas it remained weak in the InvA wvrD mutant (Figure 3B). The level of trapped DNA was still high after 2 h in LB for the dinG mutant, whereas it decreased slightly in the rep mutant, suggesting an adaptation to LB in this mutant. All double helicase mutants exhibited a high level of trapped Inv-fragment after 1 h of propagation in LB (56–86%; Figure 3B). DNA trapping was abolished (in the rep mutant), or decreased (in wvrD dinG and rep dinG mutants) by the rpoC* mutation (compare Figure 3B and D), indicating that trapping of the Inv-fragment results mainly from the high level of rrrA transcription in LB.

In the InvBE rep mutant the percentage of trapped Inv-fragments increased from 15 to 79% after 1 h in LB and, as in the InvA rep mutant, this increase was transient (Figure 3C). Trapping was only increased after 2 h of propagation in LB in the InvBE dinG mutant (35%), and remained weak in InvBE wvrD (Figure 3C). In contrast, InvBE mutants lacking two helicases exhibited a high level of DNA trapping (64–90%; Figure 3C; InvBE rep wvrD recF grew too poorly to be tested). The rpoC* mutation had partial effects (Figure 3D). Deleting rrnE and rrnB suppressed Inv-fragment trapping in dinG (Supplementary Table S4) and dinG wvrD cells, confirming that rrn are the only inverted genes that perturb replication in these mutants (Figure 3D; Supplementary Table S4). Although InvBE rep and InvBE dinG rep lacking both rrnE and rrnB were also fully viable (Figure 2D; Supplementary Table S2), they retained a weak but significant level of DNA trapping when propagated in LB for 1 or 2 h (Figure 3D; Supplementary Table S4). Finally, the LB sensitivity of the InvBE rep wvrD recF ΔrrnE ΔrrnB mutant correlates

150 The EMBO Journal VOL 29 NO 1 | 2010 ©2010 European Molecular Biology Organization
with a high level of Inv-fragment trapping (Supplementary Table S4), confirming a role for Rep and UvrD at other inverted genes, as well as at rrn.

Interestingly, the recovery of normal DNA migration in Inv rep mutants propagated for 2 h in LB was not observed in a recA or lexAind context, indicating it requires SOS induction (Supplementary Table S4). In other mutants, the proportion of Inv-fragments trapped in wells was only marginally affected by the recA mutation (Supplementary Table S4). The observation that the proportion of non-migrating Inv-fragments is similar in the absence of RecA indicates that these non-linear structures are not recombination intermediates but rather replication intermediates.

As expected, the percentage of DNA trapping was low in non-inverted strains, or when a probe hybridizing with a Nott fragment other than the Inv-fragment was used as a control (Supplementary Table S4). DNA trapping was also low when Inv cells were grown in MM; the presence of abnormal DNA structures only when cells are grown in LB indicates that this non-migrating DNA only forms when cells are propagated at a high growth rate (Supplementary Table S4).

**In helicase mutants, replication intermediates accumulate in the inverted rrn operon**

As increased DNA trapping correlates with a high level of rrn transcription, we examined replication progression in the rrn operon by 2D gel analysis (Brewer and Fangman, 1987). rrnA and rrnB operons were each analysed after DNA cleavage with two different restriction enzymes and a specific probe just downstream of the rrn operon (Figure 4A). As replication forks move at a speed of about 800 bp/s in *E. coli*, replication intermediates are not detectable in the chromosome of wild-type cells. Actually, we never detected replication intermediates in control 2D gels performed with an Inv mutant that expresses all helicases (InvA and InvBE single mutants) and with non-inverted strains (wt chromosome) lacking one or two helicases (see for instance non-Inv rep Figure 4C; and data not shown).

A ‘simple Y’ arc corresponding to the accumulation of Y-shaped replication intermediates was clearly detected in all mutants exhibiting more than 40% Inv-fragment trapped in wells in PFGE, indicating replication fork arrest within the transcribed region of the restriction fragment. Interestingly, an intense enlarged spot was observed on the simple Y arc.
indicating a specific accumulation of replication intermediates. The spot position moves along the simple Y arc depending on the restriction fragment analysed, allowing us to map this replication arrest zone to the 3' end of the rrn operon, including the transcription terminator (Figure 4). The spot was of weaker and variable intensity in InvA dinG and InvBE rep mutants. The replication arrest zone was often prolonged 100–300 base pairs downstream of the operon calculated according to computer simulations (Viguera et al., 1998), particularly for rrnE, suggesting a possible impairment of replication by transcription-induced supercoiling or a defect in transcription termination. Replication intermediates were not detected with cells growth in MM and were of weak intensity in the rpoC* context, confirming that replication is strongly impaire only when rrn are highly expressed (Figure 4; and data not shown).

**Figure 4** 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. (A) Schematic representation of the restriction fragments used for 2D gels, left InvA, right InvBE. Top line, the position of rrn and of restriction sites are shown; bottom lines, schematic representation of the forked fragments when replication is arrested at the 5' end of rrn, distances from the restriction sites to the 5' end of rrn and the relative size of the forked fragments compared with linear fragments are indicated. (B, C) DNA from various InvA (B) and InvBE (C) mutants were digested with the indicated restriction enzyme, analysed by 2D gels and probed for the sequence just downstream of the analysed rrn. The left panel shows a simulation of replication arrest in the entire restriction fragment with an increased arrest in about 500 pb around the rrn transcription terminator sequence. The mutants used are indicated above each panel. In the InvBE rep and rep dinG rpoC* mutants, the signal of increased replication arrest in the transcription termination region was not always observed, independently of the restriction enzyme used, and one example of each situation is shown.

(Dinh is required to remove R-loops and RNA Pol)

In addition to their very high level of expression, a characteristic of rrn operons is the production of non-translated RNA, which favours the formation of R-loops by the annealing of RNA with its template DNA. Therefore, replication blocks within actively transcribed rrn can result from collisions of replication forks with RNA Pol and/or R-loops. To determine whether R-loop formation has a role in the defects of Inv helicase mutants, we used a multicopy plasmid that carries the rnhA gene encoding RNase H, which degrades R-loops (pEM001, Masse et al., 1997, or an ApR derivative pEM-Ap). Vectors pACYC184 and pBR322 were used as controls. In the InvA dinG mutant, over-expression of RNase H clearly suppressed the plating defect on LB and the trapping of Inv-fragments (Figure 5; RNase H over-expression also suppressed the growth delay of InvBE dinG cells, Supplementary.
In these mutants, replication impairment is observed on the occurrence of collisions of replication forks with RNA Pol. Tants that overproduce RNase H can logically be interpreted as Inv-fragment in InvA then the growth defect and the high level of non-migrating progression in oppositely oriented highly transcribed ribosomal operons. Furthermore, these helicases are also crucial in wild-type E. coli, where replication and rrn transcription are co-directional. Although DinG, Rep and UvrD helicases have overlapping functions, our results show that they do not act on exactly the same molecular substrate, and do not act at exactly the same time.

**Discussion**

When replication and transcription proceed in opposite directions, the DnaB helicase collides on the lagging strand with RNA Pol and, as shown here and elsewhere, replication progresson is hampered. In this work, we show that the three E. coli helicases DinG, Rep and UvrD are recruited to the replication fork to allow replication across oppositely oriented highly transcribed ribosomal operons. Furthermore, these helicases are also crucial in wild-type E. coli, where replication and rrn transcription are co-directional. Although DinG, Rep and UvrD helicases have overlapping functions, our results show that they do not act on exactly the same molecular substrate, and do not act at exactly the same time.

**The helicases do not prevent replisome disassembly**

In E. coli, restart of inactivated replication forks involves the reloading of the replication machinery by ‘replication restart’ proteins. The main restart pathway is catalysed by PriA and its partners (Sandler, 2000). We constructed InvA priA, InvBE priA and InvABE priA mutants. priA mutants devoid of chromosome inversion were used as a control (Supplementary Table S1). All mutants were constructed in the presence of a PriA + IPTG-dependent plasmid, pAM-priA, which can be cured by growing cells in the absence of IPTG (Grompone et al., 2004). No plasmid-less colony could be recovered by growing Inv priA mutants in MM devoid of IPTG, whereas plasmid-less priA colonies were obtained in the non-inverted strains as expected (Supplementary Table S2). Therefore, the PriA pathway is essential for viability in Inv mutants. Importantly, this result indicates that DinG, Rep and UvrD do not prevent replication arrest. We propose that these helicases act after replisome disassembly and allow PriA-dependent restarted forks to replicate across the obstacle created by the inversion.

Defects conferred by the single dinG mutation in cells that carry an oppositely oriented rrn are suppressed by RNase H over-expression and by the rpoC* mutation. These findings indicate that R-loops form within highly expressed rrn operons and block replication, and that DinG is the only helicase that removes them in vivo. The identification of R-loops as an in vivo target for DinG is in full agreement with the in vitro properties of the purified protein (Voloshin and Camerini-Otero, 2007), and with the deleterious phenotype of a dinG mutation in an rrn background, attributed to an excess of R-loops (Yasuda et al., 1996). The stronger defects of the InvA mutant compared with InvBE suggest that R-loops are more prone to form in rrnA than in rrnE. Either R-loops may form more often when rrn are facing the direction of replication, or they may form at a similar efficiency in Inv and wild-type cells but they may be deleterious only when replication and transcription move through the operon in the opposite orientation. However, because our data indicate that neither Rep nor UvrD act on R-loops in Inv mutants, the synergistic effects of inactivating dinG in rep or uvrD mutants indicate that R-loops are not the only target of DinG.

**Rep, DinG and UvrD participate in RNA Pol removal**

Several data indicate that these three helicases share a common function. First, in Inv Rep + cells the combination of uvrD and dinG mutations leads to the accumulation of replication intermediates at early times after a shift to LB and prevents colony formation. This rich medium sensitivity persists when RNase H is overproduced and is only observed when the inversion carries a highly expressed rrn, indicating that together with Rep either UvrD or DinG is required for the...
dislodging of RNA Pol from inverted rrr operons but not from other genes. Second, in Inv rep mutants both DinG and UvrD must be present for colony formation, even on MM. We conclude that replication across inverted rrr requires the presence of two out of these three helicases. It is noteworthy that RecA and RecF, which are required for replication fork progression across DNA lesions (Courcelle and Hanawalt, 2003) are not required for replication across inverted highly transcribed sequences (Esnault et al., 2007, Supplementary Table S3).

**Rep acts early after a shift to rich medium**

In a strain that lacks Rep and carries an inverted rrr, a shift to LB induces cell elongation and the accumulation of replication intermediates. As the obstacles to replication are not R-loops (because they are not abolished by RNase H overproduction) they are most likely RNA Pols. Interestingly, Inv rep mutants spontaneously recover and eventually form 100% colonies on LB. The rescue of invA rep and InvBE rep mutants requires SOS induction and the presence of both DinG and UvrD (it is abolished in recA, lexAind, dinG and uvrD contexts). Actually, the SOS response is induced by replication impairment, caused by DNA lesions or various replication defects (Sassanfar and Roberts, 1990; Lestini and Michel, 2007). We propose that, after replication blockage, Inv rep mutants are rescued by the SOS-induced DinG and UvrD helicases. It is tempting to speculate that the Rep helicase acts early owing to an efficient targeting to blocked replication forks, whereas in its absence UvrD and/or DinG may be efficient at unblocking forks only when they are at a high concentration, that is SOS induced.

**Model for helicase action**

The lethality of Inv priA mutants implies that in Inv cells replication forks are arrested and disassembled. Therefore, the three helicases act after fork arrest, either on naked replication forks, or in conjunction with a reassembled, restarting replisome. As DinG migrates in the 5′–3′ direction on DNA it is conceivable that it acts on the lagging strand template whereas Rep and UvrD, which migrate in the 3′–5′ direction, progress on the leading strand template (Figure 6). In Inv cells inactivating only the uvrD has no deleterious effects and we propose that RNA Pols are dislodged by the concerted action of Rep and DinG (Figure 6A). If Rep is lacking, it is replaced by UvrD and the replication restart is then delayed because UvrD needs to be SOS induced to be efficient (Figure 6B). If DinG is lacking, the fork will recruit UvrD in addition to Rep and because these two helicasces progress on the same strand, they will efficiently remove a series of RNA Pol provided that no R-loop forms (R-loops form on the other strand) (Figure 6C). Rep alone (DinG and UvrD absent) and UvrD alone (DinG and Rep absent) can only progress through co-directional highly expressed rrr genes or through moderately expressed rrr, on which less RNA Pol travel and RNA Pol stability is compromised by ppGpp or by a mutation that mimics its presence (Figure 6D and E). Finally, DinG alone does not allow normal replication progression across an inverted rrr or across other inverted sequences (Inv rep uvrD recF mutants), whereas it is sufficient for the growth of non-inverted E. coli cells, provided that the stability of RNA polymerase is compromised (rep uvrD rpoC* cells, Figure 6F).

**Figure 6** Rescue of transcription-blocked replication forks by helicases. Schematic representation of a replication fork blocked by a transcription unit. Top, a replication fork encounters an oppositely oriented highly expressed rrr operon (Inv mutant in LB): (a) in cells proficient for all helicases and in a uvrD single mutant, Rep translocating towards the transcription unit on the leading strand template and DinG on the lagging strand template act in concert; (b) in a rep mutant both UvrD and DinG are required, UvrD translocating on the leading strand template and DinG on the lagging strand template act in concert; (c) in a dinG mutant, both Rep and UvrD are required, they both translocate towards the transcription unit on the leading strand template; because R-loops form on the lagging strand template (not shown) where no helicase is present, R-loops are deleterious. Middle, a replication fork encounters an oppositely oriented moderately expressed rrr operon (Inv mutant in MM): (d) Rep only (dinG uvrD mutant) or (e) UvrD only (dinG rep mutant) is sufficient for replication. Bottom, a replication fork encounters a normally oriented (co-directional) moderately expressed rrr operon (wild-type chromosome in MM): this is the only condition in which DinG alone (rep uvrD recF mutant) allows full viability. Full lines: template DNA; dashed lines: newly synthesized DNA; oval: replisome; yellow circles: DnaB helicase; green indented circles: RNA Pol; pink lines: rRNA. Helicases are shown as grey indented circles: hatched DinG, light grey Rep, dark grey UvrD.

**Replication arrest at inverted rrr operons**

The analysis of mutants in which both inverted rrr have been deleted shows that rrr are the main obstacle to replication in dinG uvrD and (to a lesser extent) in rep dinG mutants, and one of the obstacles to replication in the rep uvrD recF mutant. To get insight into the nature of the elements that slow down replication in the rrr operon we analysed replication intermediates by 2D gels. Replication forks are slowed down in the transcribed region in rrr. Replication intermediates are similar in the dinG and in the rep mutant, although they result from the encounter of R-loops in the former and RNA Pol in the latter. Interestingly, we observed a strong accumulation of replication intermediates at the very end of
the operon, including the transcription terminator, suggesting the encounter of replication forks with a highly stable nucleoprotein complex. To our knowledge such complexes have not been described so far in the rrn transcription termination region, even though RNA Pols may accumulate at the terminator and replication arrest was described at rrn terminators in *E. coli* plasmids during co-directional collisions (Mirkin *et al.*, 2006).

Suppression of the LB sensitivity of the *rep uvrD* mutant by *rpoC* indicates that in wild-type *E. coli* these two helicases are essential for replication across highly transcribed regions. On chromosome inversion, *Rep* and *UvrD* are also required to replicate across genes other than *rrn*, as the InvBE *rep uvrD recF* cells lacking *rrnE* and *rrnB* remain sensitive to LB. It is noteworthy that the InvBE inversion also carries genes encoding ribosomal proteins. Future work will tell whether replication is hampered by these or by other specific sequences, and/or by the high number of genes in an inverted orientation.

**RNA Pol removal in other contexts**

In *E. coli*, removal of RNA Pol from damaged DNA is performed during transcription coupled repair by the Mfd helicase, which also attracts the nucleotide excision repair machinery (reviewed in Selby and Sancar, 1994). Mfd localizes on DNA behind the RNA Pol in the direction of transcription, pushing a blocked RNA Pol forward, which causes its dissociation from DNA when its advance is prevented by a lesion or a DNA-bound protein (Park *et al.*, 2002). Mfd action on a series of RNA Pol is likely to be prevented by steric hindrance; however, it would be interesting to test whether Mfd participates in the dislodging of single RNA Pol from replication forks. It is noteworthy that Mfd would only dislodge RNA Pol as long as the replisome prevents its forward movement.

The presence in numerous organisms of DinG and UvrD homologues underlines the importance of these proteins. In several Gram-positive bacteria, *rep* and *uvrD* are a single, essential gene, *pcrA* (Petit and Ehrlich, 2002 and references therein). Interestingly, in a two-hybrid assay PcrA interacts with the *Bacillus subtilis* RNA polymerase (Noirot-Gros *et al.*, 2002). DinG in *B. subtilis* carries an N-terminal exonuclease domain, raising the possibility that DinG in *E. coli* functions in conjunction with an RNase (Moser *et al.*, 1997). DinG is homologous to Rad3 in *S. cerevisiae* and XPD (ERCC2) and is a single, essential essential gene, *pif1*. Helicases of the Pif1 family have a function similar to DinG and Rep (Lahaye *et al.*, 1993; Heller and Marians, 2007; Voloshin and Camerini-Otero, 2007).

The physiological role of DinG, Rep and UvrD helicases could only be deduced from detailed *in vivo* analyses. By revealing the relevant physiological substrates for these helicases, this study paves the way for future experiments aimed at understanding the molecular mechanism of action of enzymes that displace RNA Pol from the path of replication forks.

**Materials and methods**

**Strains and plasmids**

All *E. coli* strains are derivatives of MG1655. Plasmids and strains are described in Supplementary Table S1. MM is M9 (Miller, 1992) complemented with 0.04% glucose. Standard transformation and transduction procedures were as described earlier (Miller, 1992). Chromosome inversions were made as described earlier (Valens *et al.*, 2004). Briefly, strains were first P1 transduced for attR (linked to a kan^R^ marker) and attL (linked to a cm^R^ marker) to construct the non-inverted parental strain. For inversion, the attR attL carrying strain was transformed at 30°C with the plasmid pTSA29-CXI (ts replication, cph^C^–/Pc^C^–/int^C^–/Ap^R^); a transformant propagated at 30°C in exponential phase was shifted to 37°C for 10 min (a control culture was not shifted) and then incubated at 30°C for 1 h. Appropriate dilutions were plated on MM Ap X-gal plates and incubated for 3 days at 30°C, only the cultures that were shifted to 37°C gave rise to blue colonies (Lac^-^, about 50%). Blue colonies were streaked on MM and then cultured at 37°C with a 4-h shift to 42°C to cure pTSA29-CXI. The inversion was verified by PCR using the oligonucleotides shown in Supplementary Table S6. *uvrD* mutants were tested for UV sensitivity and mutator phenotype (100-fold excess of Rif^R^ colonies in overnight cultures). AttL insertions and gene insertions were verified using the oligonucleotides listed in Supplementary Table S6. Oligonucleotides used to synthesize PCR DNA fragments for strain construction, or probes, are listed in Supplementary Table S6. pAM-rep and pAM-priA plasmids were segregated before each experiment as published earlier (Grompone *et al.*, 2004). Briefly, overnight cultures propagated in the presence of 500 μg/ml IPTG and 100 μg/ml Ap were diluted 1000-fold and propagated for 7–8 h in MM at 37°C. Appropriate dilutions were then plated on MM and MM containing IPTG and Ap. Routinely, the number of colonies on MM was 10-fold higher than the number of clones on IPTG/Ap MM plates and more than 90% of the clones obtained on MM were sensitive to Ap.

**Measures of plating efficiencies**

Overnight cultures (OD₆₅₀ 1.0–1.5) were diluted and plated on MM or LB plates, incubated at 37°C. LB plates were counted after 24 and 48 h of incubation. MM plates were counted after 48 h incubation.

**Microscopy**

Cells were grown in MM to OD₆₅₀ 0.05–0.1, centrifuged, grown for 1 or 2 h in LB or MM to reach OD₆₅₀ 0.15–0.3 and then observed with a Zeiss microscope by DIC. Photographs were acquired with the Metamorph software; cell lengths were measured by hand under Image J software.

**PFGE and 2D gels**

Cells grown for microscopy analysis were lysed in plugs as described earlier (Seigneur *et al.*, 1998). Chromosomes embedded in plugs were treated with the appropriate restriction enzyme for 6 h at 37°C according to the instructions of the suppliers.

PFGE was performed in 1% agarose gels, TEB 0.5 ×, at 14°C, 5 V/cm, angle 120 deg, in a CHEF DR II apparatus (Bio-Rad). InvA gels: 11 h, switch time 1–6 s. InvB gels 19 h, switch time 5–30 s.

2D gel migration was in 1 × TEB and as follows: InvBE/BsmBI or BssHII and InvA/AflII: 1st dimension 0.4% agarose, 0.9 V/cm, 22 h at room temperature (RT); 2nd dimension 1% agarose, ethidium bromide (Et Br) 0.5 μg/ml, 5 V/cm, 9 h at 4°C. InvA/ BseEII: 1st dimension 0.35% agarose, 0.9 V/cm, 39 h; RT; 2nd dimension 0.8% agarose, Et Br 0.5 μg/ml, 1.7 V/cm, 27 h at 4°C.

DNA was transferred from PFG or 2D gels to a nylon membrane and hybridized by the classical Southern technique. Storage

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phosphor imaging was performed with a Typhoon; image analysis was performed with ImageQuant software.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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Conflict of interest
The authors declare that they have no conflict of interest.

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