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DNA double-strand breaks caused by replication arrest

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We report here that DNA double-strand breaks (DSBs) form in *Escherichia coli* upon arrest of replication forks due to a defect in, or the inhibition of, replicative DNA helicases. The formation of DSBs was assessed by the appearance of linear DNA detected by pulse-field gel electrophoresis. Processing of DSBs by recombination repair or linear DNA degradation was abolished by mutations in *recBCD* genes. Two *E.coli* replicative helicases were tested, Rep, which is essential in *recBC* mutants, and DnaB. The proportion of linear DNA increased up to 50% upon shift of *rep recBTS recCTS* cells to restrictive temperature. No increase in linear DNA was observed in the absence of replicating chromosomes, indicating that the formation of DSBs in *rep* strains requires replication. Inhibition of the DnaB helicase either by a strong replication terminator or by a *dnaBTS* mutation led to the formation of linear DNA, showing that blocked replication forks are prone to DSB formation. In wild-type *E.coli*, linear DNA was detected in the absence of RecBC or of both RecA and RecD. This reveals the existence of a significant amount of spontaneous DSBs. We propose that some of them may also result from the impairment of replication fork progression.

Keywords: DnaB/helicase/homologous recombination/ RecBCD/Rep

Introduction

DNA double-strand breaks (DSBs) can have multiple origins including exogenous factors, such as radiation or chemical DNA-damaging agents (Krasin and Hutchinson, 1977; d'Andrea and Haseltine, 1978), or site-specific endonucleases (Murialdo, 1988; Haber, 1995). Since they represent a lethal lesion, all organisms have developed repair pathways. The most economical is a simple ligation with another available DNA end. This pathway is preponderant in higher eukaryotes but has serious disadvantages, since eventually it is accompanied by the loss of genetic material and may even lead to gross chromosomal rearrangements (Roth and Wilson, 1989; Lobrich *et al.*, 1995). The most elegant repair pathway is conservative and uses recombination with an intact homologous copy. These recombination events require RecA and RecBCD in *Escherichia coli* and proteins of the Rad 52 pathway in yeast (reviewed in Clark and Sandler, 1994; Kowalczykowski *et al.*, 1994; Myers and Stahl, 1994; Haber, 1995). The RecBCD protein presumably acts at the initiation stage of homologous recombination, combining degradation (exonuclease V) and helicase activities to render the DNA single-stranded, and therefore accessible to the RecA protein (reviewed in Kowalczykowski *et al.*, 1994). It is essential for the repair of DNA DSBs induced by DNA-damaging agents (Wang and Smith, 1983, 1986; Sargentini and Smith, 1986) and binds to double-stranded DNA ends *in vitro* (Ganesan and Smith, 1993).

It has frequently been observed that only a minority of *recB* or *recC* mutant cells in culture form colonies when plated (Capaldo-Kimball and Barbour, 1971). This was thought to result from a defect in the repair of spontaneously occurring DSBs, the origin of which remained mysterious. Recently, several observations suggested that DSBs may occur during replication. Two classes of models were proposed, depending on whether the break precedes or follows the passage of the replication fork. DSBs may result from the encounter of a replication fork with a pre-existing nick or gap present in the template strand of the DNA (Kuzminov, 1995a). Such events are probably rare in wild-type cells, since *E.coli* synthesizes enough ligase to repair a large number of nicks (Heitman *et al.*, 1989). However, they could take place following the action of specific *E.coli* endonucleases, such as the SbcCD endonuclease, which cuts hairpin structures formed at palindromic DNA sequences (Connelly and Leach, 1996), or in ligase- or *polA*-deficient strains (Cao and Kogoma, 1995; Kuzminov, 1995a). In addition, Howard *et al.* (1994) observed *in vitro* that a T4 topoisomerase II cleavage complex formed in the presence of a topoisomerase inhibitor is converted to a DSB following the action of a helicase.

DSBs could also result from the breakage of arrested replication forks. Progression of the replication fork may be impeded by DNA secondary structures, DNA damage or DNA-bound proteins, which inhibit the polymerases or the helicases. These replication arrests could lead to DNA breakage (Bierne *et al.*, 1991; reviewed in Bierne and Michel, 1994; Kuzminov, 1995b). Experiments supporting the existence of a link between a defect in a replicative helicase and the occurrence of DSBs were reported for two *E.coli* helicases, DnaB and Rep. DnaB is the only helicase essential for chromosome replication, and two independent observations suggest that DnaB inactivation or blockage creates entry sites for the RecBCD complex: (i) in several *dnaBTS* mutants, *recB*-dependent DNA breakdown has been observed upon shift to restrictive temperature (Buttin and Wright, 1968; Fangman and Russel, 1971; Weschler and Gross, 1971); (ii) the replication terminator sites of *E.coli*, *Ter* sites, which arrest replication by inhibiting DnaB action (Khatri *et al.*, 1989; Lee *et al.* 1989), are hotspots for RecBCD-dependent

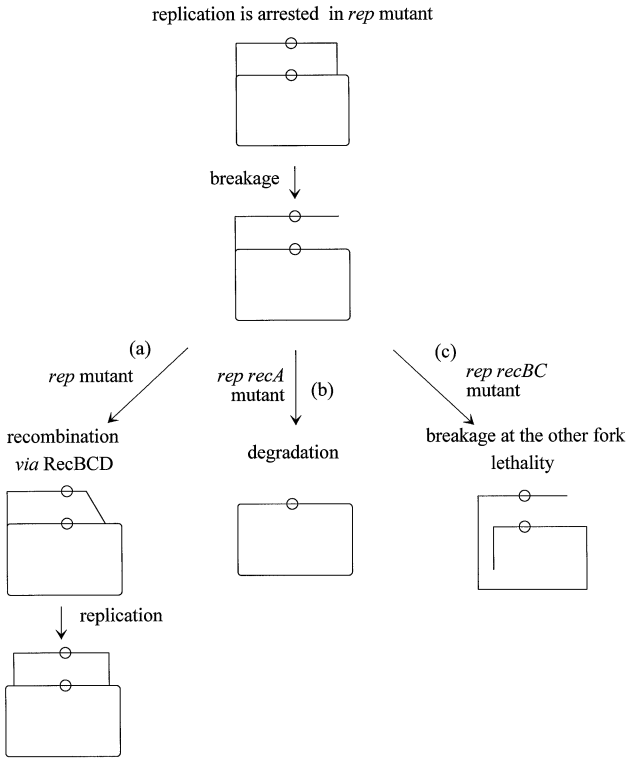

Fig. 1. Model for lethality of *rep recBC* and *rep recA recD* strains. Arrest of a replication fork due to the lack of the Rep helicase leads to breakage of the fork. In the presence of RecBCD and RecA (a) the DSB is repaired and replication can restart. In *rep recA* strains (b), the broken arm of the chromosome is degraded by RecBCD, and replication can restart from the chromosomal origin. When RecBC or both RecA and RecD are defective (c), no degradation or repair can take place, and a second breakage will lead to the formation of a linear chromosome (adapted from Uzest *et al.*, 1995).

homologous recombination (Horiuchi *et al.*, 1994; Horiuchi and Fujimura, 1995). A second replicative helicase in *E.coli* is the Rep helicase. Although not essential for *E.coli* viability, it is required for the 'normal' replication of the chromosome (Lane and Denhart, 1974). About 80 min are required for the replication of the entire chromosome in *rep* mutants, versus 40 min in wild-type strains (Lane and Denhart, 1975; Cosalanti and Denhart, 1987). Based on *in vitro* data, it was proposed that Rep might facilitate the progression of replication forks by removing DNA-bound proteins (Yancey-Wrona and Matson, 1992; Matson *et al.*, 1994). We recently reported that *rep recB* and *rep recC* double mutants are inviable and proposed that, in *rep* strains, DSBs occur at a higher rate than in wild-type strains, thus rendering the RecBCD-dependent recombination repair essential (Uzest *et al.*, 1995). Since chromosome replication is slowed down in *rep* cells, breakage could result from frequent replication pausing. The breaks could either be repaired or lead to cell lethality, as described in the model shown in Figure 1.

In the present work, the occurrence of chromosome breakage upon inactivation of replicative helicases was investigated by pulse-field gel electrophoresis (PFGE) of bacterial chromosomes. In contrast to linear DNA which migrates in pulse-field gels, *E.coli* chromosomes do not enter the gels, probably due to their large size and circular structure (Birren and Lai, 1993). Forked DNA, which is Y-shaped or σ-shaped, also remains in the wells (Petit *et al.*, 1992; Nakayama *et al.*, 1994). Consequently, PFGE allows the detection and quantification of intracellular linear DNA. Our data confirm the occurrence of spontaneous DSBs in strains defective for the Rep helicase. DSBs are detectable only in the absence of the repair enzymes RecA and/or RecBCD. They are associated with ongoing replication since a chromosomal initiation block, such as a *dnaATS* mutation, abolishes breakage. Blockage of the replication forks by a *dnaBTS* mutation or insertion of an improperly oriented *Ter* site in the chromosome also results in increased breakage.

Results

Linear DNA can be detected in recBC and recA recD E.coli mutants

In order to study the effect of helicase mutations on the breakage of *E.coli* chromosomal DNA, a method allowing the direct detection of DSBs has been developed. The presence of linear DNA *in vivo* was investigated by using PFGE combined with cell lysis in agarose plugs. Cells of *E.coli* grown in the presence of [^{3}H]thymidine were lysed in agarose plugs. The plugs were subjected to PFGE, and the gels were stained with ethidium bromide to visualize the DNA and then sliced. The tritium label present in each slice was counted, and the proportion of migrating DNA was determined. Under the conditions used, the majority of chromosomal DNA from wild-type cells remained in the well (Figure 2; Table I). When the same plugs were treated with a restriction enzyme, nearly all the DNA entered the gel (not shown). This indicates that chromosomes remain intact upon gentle lysis in plugs and that only linear DNA migrates in these experiments, as reported previously (Birren and Lai, 1993).

recBC mutants are defective for both the RecBCD-mediated recombination repair of DSBs and the degradation of linear DNA by exonuclease V (exoV) activity. In *recBC* mutants, linear DNA was detected, mostly migrating as 2–3 Mb controls (Figure 2). The total amount of linear DNA reached 15–20% (Table I). *recD* strains are devoid of exoV activity but proficient for homologous recombination (Amundsen *et al.*, 1986). They showed low amounts of migrating DNA (Table I). However, in the *recA recD* mutant, where both recombination repair and degradation of linear DNA are inhibited, a significant proportion (25%) of DNA entered the gel (Figure 2; Table I). These experiments indicate that upon inactivation of RecBC or RecA and RecD, broken chromosomes accumulate *in vivo*.

rep recBTS recCTS mutants accumulate linear DNA upon incubation at the restrictive temperature

It was proposed previously, on the basis of genetic evidence, that DSBs form frequently in cells lacking the Rep helicase (Uzest *et al.*, 1995). To follow directly the amount of broken DNA produced in *rep* mutants, it appeared necessary to prevent the degradation and repair of these DSBs by controlling the level of RecBCD activity. *recBTS recCTS* mutations, which inactivate the RecBCD protein at 42°C, were used for this purpose. Δ*rep*::*kan recBTS recCTS* cells were grown at 30°C in the presence of [^{3}H]thymidine. After two generations, part of the culture was shifted to 42°C, where inactivation of the RecBC

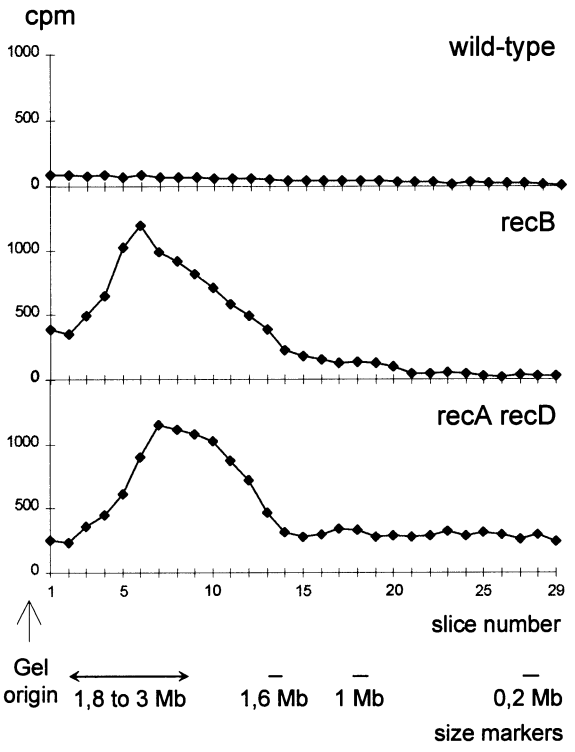

Fig. 2. The proportion of linear DNA is increased in *recB* and in *recA recD* mutants. Overnight *E.coli* cultures were diluted in minimal medium supplemented with 0.4% glucose, 0.2% casamino acids and 5 μg/ml of deoxyadenosine at 37°C at OD_{650} 0.01 or 0.02 and grown until OD_{650} 0.2–0.5. Agarose plugs were prepared and used for PFGE as described in Materials and methods. Gels were cut in 3 mm slices and the amount of [^{3}H]thymidine present in each slice was measured. The total amount of tritium per lane varied from ~45 000 to 55 000 c.p.m., hence, in order to render the different lanes directly comparable, the values shown in the figure were corrected for a total amount of 50 000 c.p.m. in each lane. The gel origin is not shown. The position of the size markers is indicated. Under the conditions used, *S.cerevisiae* and *H.wingei* 0.2–1 Mb chromosomes migrated as individual bands, while 1.8–3 Mb chromosomes co-migrated as a diffuse band. Results from a representative experiment are shown.

protein leads to the loss of cell viability (Uzest *et al.*, 1995; Figure 3A and B). Aliquots were removed periodically for PFGE analyses (Figure 4). An increasing amount of linear DNA, migrating as 2–3 Mb linear controls, appeared upon incubation at 42°C. The proportion of linear DNA increased to as much as 50% compared with 15% in the *recBTS recCTS* mutant (Figure 5). Similar results were obtained in *rep71 recBTS recCTS* cells (not shown). These results show that the chromosomes of *rep*-defective cells suffer more breaks than those of wild-type cells, and the resulting linear DNA can be detected in the absence of RecBCD.

Little or no DSBs were detected in *rep* single mutants (Table II), indicative of efficient DSB repair and/or linear DNA degradation by the RecBCD enzyme. As expected, in *rep recA* strains, which are proficient for exoV activity, or in a *rep recD* mutant, which is proficient for homologous recombination, the amount of migrating DNA was unchanged compared with the isogenic *rep* strains (Table II). The *rep recA recD* triple mutants could not be tested, as they are non-viable (Uzest *et al.*, 1995) and thus DSBs in *rep* mutants can only be detected in *recBTS recCTS* mutants, where both exoV and recombination can be inactivated.

Table I. Linear DNA in *recBCD* cells

Strain	Genotype	Linear DNA (%)
JJC40	wild type	2.7 ± 0.2
JJC315	*recB*::Tn*10*	17.5 ± 2.7
JJC316	*recC*::Tn*10*	14.9
JJC273	recD::Tn10	3 ± 0.8
JJC432	*recD*::Tn*10 recA*::*cam*	25.5 ± 6.6

Cells were grown at 37°C. Results shown with standard deviation are the mean of two independent determinations.

Generation of linear DNA in rep strains in the presence of the Gam protein

Induction of the λ Gam protein, which inactivates RecBCD (Unger and Clark, 1972), is also lethal for *rep* mutants (Uzest *et al.*, 1995), although residual RecBCD-dependent recombination activity is detectable in Gam-containing cells (Friedman and Hays, 1986; Murphy, 1991). We therefore used Gam to investigate further the formation of Rep-dependent DSBs. Cultures of cells containing a temperature-inducible *gam* gene (plasmid pSF117, Friedman and Hays, 1986) were grown at 30°C (Gam^-). An aliquot of the culture was shifted to 42°C (Gam^+) for 3 h and the chromosomes were analysed by PFGE (Table III). The proportion of migrating DNA was at most 2-fold higher upon Gam induction in *rep* than in wild-type strains, in spite of a clear loss of viability (Figure 3C).

To check that residual recombination is responsible for the relatively low amount of linear DNA in *rep* Gam^+ cells, *rep recA* gam^+ cells, where homologous recombination is totally inactivated by the *recA* mutation, were used. The lethality upon Gam induction is increased compared with that of *rep* single mutants (compare Figure 3C and D). The proportion of linear DNA in the *rep recA* gam^+ strain reached 50–60% of total DNA (Table III). The migration properties of linear DNA in the *rep recA* gam^+ strain were similar to those observed in the *rep recBTS recCTS* strain (see Figure 4).

In *recA* mutants, both SOS induction and homologous recombination are abolished. To determine which of the two effects caused the accumulation of linear DNA, we used a *lexA ind*$^-$ strain, which is proficient for recombination but defective in the SOS response. Inactivation of only the SOS response did not lead to a significant increase in the amount of linear DNA (compare *rep lexA ind*$^-$ gam^+ and *rep* gam^+ strains, Table III). We conclude that the accumulation of linear DNA in a *rep recA* gam^+ strain results from the inactivation of the homologous recombination function of RecA.

In *rep* Gam^+ cells, recombination could proceed by the RecF pathway or result from the residual RecBCD activity. The amount of linear DNA was similar in *rep* gam^+ and *rep recF* gam^+ cells (Table III). This suggests that, in the *rep* gam^+ cells, the conversion of linear DNA to a non-migrating form is mediated by RecA and residual RecBCD activity.

The Gam protein also inactivates the SbcCD protein (Kulkarni and Stahl, 1989), which exhibits endo- and exonucleolytic activities *in vitro* (Connelly and Leach, 1996). Both the presence of a high level of linear DNA in *rep recA* gam^+ cells and the lack of effect of *sbcCD* mutations in the *rep71 recBTS recCTS* strain (strains

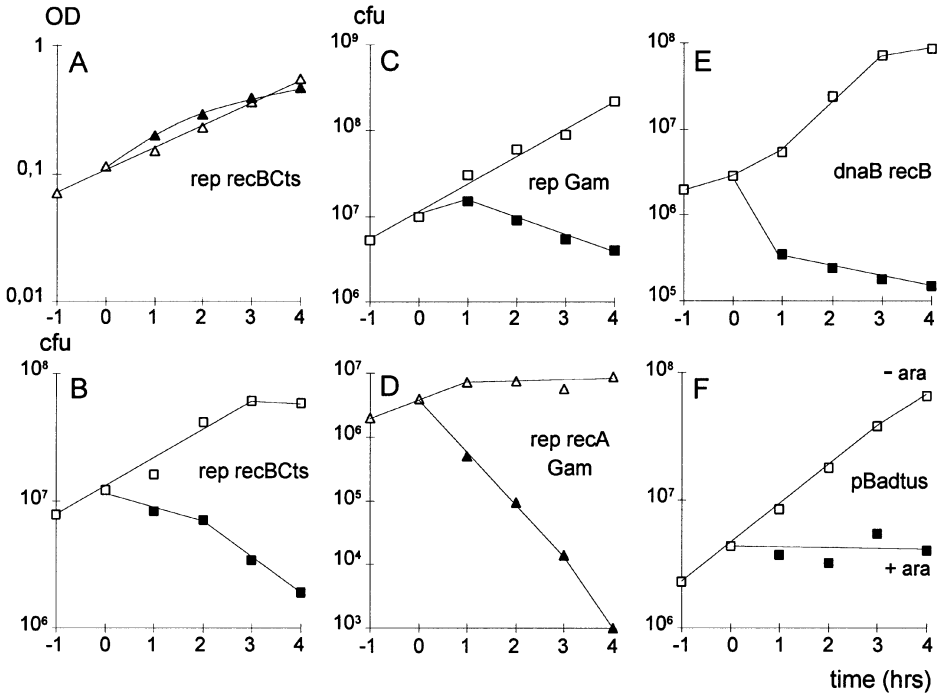

Fig. 3. Loss of viability in various strains upon shift to restrictive conditions. (**A**) JJC505 (*rep recBTS recCTS*), OD_{650}; other panels, colony-forming units (c.f.u.). (**B**) JJC505. (**C**) JJC213 [pSF117] (*rep*). (**D**) JJC356 [pSF117] (*rep recA*). (**E**) JJC507 (*dnaB8 recB*). (**F**) JJC438 [pBadTus] (*InvTer recB*). Overnight cultures were diluted in minimal medium supplemented with 0.4% glucose, 0.2% casamino acids and 5 μg/ml of deoxyadenosine at 30°C at OD_{650} 0.01 or 0.02, with the exception of JJC507, grown in LB, and JJC438 grown at 37°C. For plasmid-containing cultures, 100–200 μg of ampicillin were added. When the cultures reached OD_{650} ~0.1, they were divided in two, and one part was incubated at 42°C (time 0); for JJC438 (F), 0.2% of arabinose was added at time 0. Aliquots were taken every hour to measure the OD_{650} and c.f.u. Plates were incubated for 24–48 h at 30°C; for JJC438 at 37°C. Results from representative experiments are shown. □, 30°C; ■, 42°C. In (F), JJC438, open symbols show no arabinose and closed symbols 0.2% arabinose. For the *rep recA* strain (D), although the OD_{650} increased 24-fold in 5 h at 30°C (not shown), the c.f.u. only increased 4-fold during that time, reflecting the low plating efficiency of the strain (Uzest *et al.*, 1995). Growth of all control strains was as expected (see Uzest *et al.*, 1995).

JJC484 and JJC498, Table IV; data not shown) indicate that the SbcCD nuclease does not have a major role in the formation of DSBs in *rep* cells.

The accumulation of linear DNA in rep recA gam$^+$ cells is DnaA dependent

In a previous report, we proposed that these breaks could occur upon blockage of the replication forks (Uzest *et al.*, 1995; Figure 1). To test directly whether *rep*-dependent formation of linear DNA requires the presence of replication forks, a *dnaATS* mutation was used. The DnaA protein is essential for the initiation of chromosomal replication but not for its propagation. Therefore, after shifting a *dnaATS* mutant to the restrictive temperature, ongoing replication is not affected while no new replication rounds are initiated at the origin (Kornberg and Baker, 1992). As we could not construct a *rep recBTS recCTS dnaATS* mutant (not shown), we took advantage of the Gam-inducible system to test the involvement of replication in DSB formation. The *dnaATS rep recA* strain, (strain JJC481, Table IV) carrying the plasmid encoding Gam (pSF117) was used. In this strain, incorporation of tritiated thymidine was arrested after 1–2 h at the restrictive temperature, in contrast to the *dnaA*$^+$ control strain (not shown). During the first 1.5 h at 42°C, while replication still took place, the amount of linear DNA increased. When replication stopped, the amount of linear DNA ceased to increase (Figure 6). As expected, in the control *dnaA*$^+$ strain, the amount of linear DNA continuously increased during 3 h (Figure 6). This shows that formation of linear DNA requires the presence of replication forks.

Inactivation or inhibition of the DnaB helicase leads to the formation of DSBs

DnaB is the only essential helicase in *E.coli*, as it catalyses the unwinding of replicating chromosomes. Several lines of evidence, including RecB-dependent DNA degradation in *dnaBTS* mutants (Buttin and Wright, 1968) and elevated rates of RecBCD-dependent recombination in the region next to replication arrest sites (Horiuchi *et al.*, 1994), suggested that inhibition of the DnaB helicase might also induce DSB formation. A *recB*::Tn*10* mutation was introduced in a *dnaB*$^+$ and a *dnaBTS* isogenic strains (JJC506 and JJC507, Table IV). The amount of linear DNA was measured at different times after shift to 42°C by PFGE (Figure 7). The percentage of linear DNA increased rapidly in the *dnaBTS* mutant at the restrictive temperature, confirming the hypothesis that replication block effectively leads to DSB.

Replication in *E.coli* is naturally arrested at sites named *Ter*, located in the terminal region of the chromosome. *Ter* sites act by inhibiting DnaB action when bound by their cognate protein Tus. To investigate the effects of fork arrest at *Ter* sites on DSB formation, we made use of a strain carrying two inverted *Ter* sites in the terminus of *E.coli* (*Inv-Ter* structure) in which Y-structures typical of arrested replication forks accumulate in the presence of Tus (Sharma and Hill, 1995). As expected, overproduc-

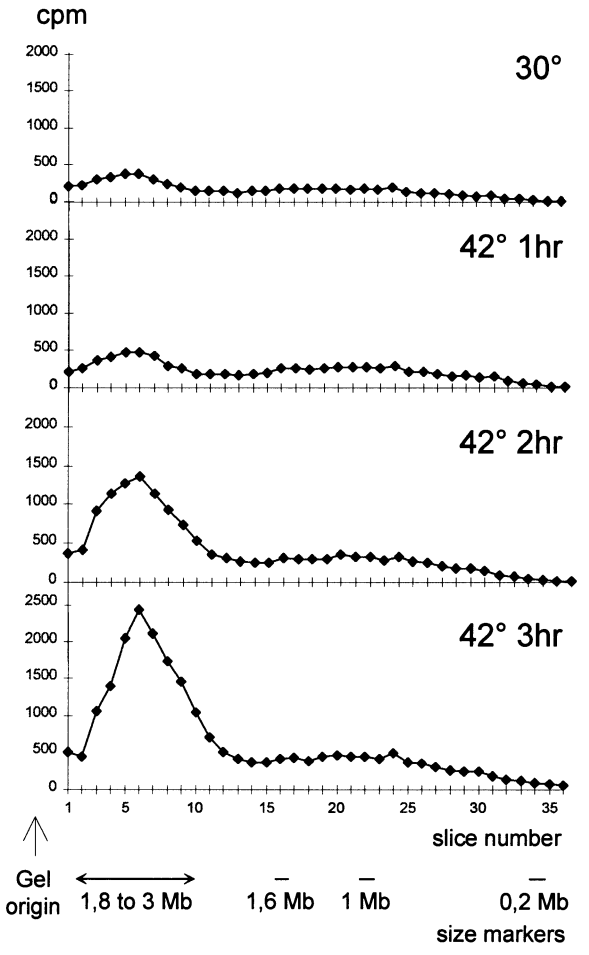

Fig. 4. DNA breakage in *rep recBTS recCTS* cells. JJC505 cells were grown as described in the legend to Figure 3 in the presence of 5 μCi of [^{3}H]thymidine. After 3, 4 or 5 h of growth at 30°C, at OD_{650} ranging from 0.05 to 0.2, cells were incubated for 3, 2 or 1 h at 42°C respectively. The control culture was grown at 30°C for 6 h. DNA was prepared and analysed as described in Figure 2. Results are the mean of two independent experiments.

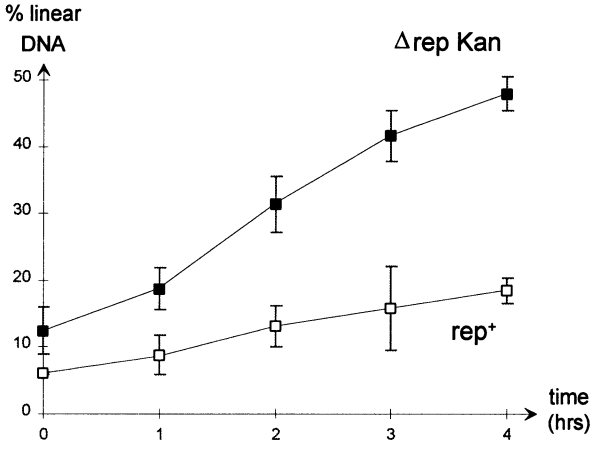

Fig. 5. The proportion of linear DNA in *recBTS recCTS* strains increases with incubation at 42°C. JJC330 (*rep*$^+$) and JJC505 (Δ*rep*::*kan*) cells were grown and treated as described in the legend to Figure 4. The proportion of linear DNA was calculated. Results are the mean of 2–5 independent determinations. □, JJC330; ■, JJC505.

Table II. Linear DNA in *rep* cells

Strain	Genotype	Linear DNA (%)		
		30°C	37°C	42°C
JJC489	*rep71*	7	7.4	5.3
JJC213	*rep*::*kan*	2.1	1.9	1.8
JJC502	*rep71 recA*::*cam*	5.4	ND	3.6
JJC356	*rep*::*kan recA*::*tet*	ND	5	ND
JJC269	*rep*::*kan recD*::Tn*10*	1.4	ND	1.7

ND, not determined.

Table III. The effects of Gam on linear DNA

Strain	Genotype	Linear DNA (%)	
		Gam– (30°C)	Gam+ (42°C)
JJC40	wild type	6.45 ± 0.4	8.1 ± 2.7
JJC16	*recA*::*tet*	17 ± 0.2	23.5 ± 0.5
JJC489	*rep71*	5.5 ± 0.5	16.7 ± 3
JJC502	*rep71 recA*::*cam*	15 ± 1	49.5 ± 0.8
JJC503	*rep71 recF*::*kan*	4.3 ± 1.3	11.8 ± 1.6
JJC213	*rep*::*kan*	4.35 ± 1.3	11.4 ± 1.4
JJC356	*rep*::*kan recA*::*tet*	11.4 ± 0.6	60 ± 1
JJC335	*rep*::*kan lexA ind3*	5.4 ± 0.8	16.6 ± 1.3

All cells contained the plasmid pSF117. Cells were grown in the presence of 100 or 200 μg of ampicillin. The average of two or three independent determinations are shown. The slightly high amount of linear DNA detected in *recA* cells at 30°C reflects the presence of some Gam protein which escapes repression.

tion of Tus, encoded from an arabinose-dependent promoter (plasmid pBadTus, Table IV), was lethal in the *InvTer* strain carrying a *recB* mutation (Figure 3F). In the absence of Tus, the proportion of linear DNA was 17%, as in other *recB* cells. This proportion increased up to 30% in 3 h of Tus induction. This confirms that DSBs are generated efficiently upon a replication fork block due to the inhibition of the main replicative helicase, DnaB.

Discussion

In this work, we examined the susceptibility of the chromosomes to DSB formation under conditions in which different replicative helicases were impaired. In the absence of exoV and recombination repair, a significant amount of linear DNA is detected, revealing a surprisingly high level of spontaneous DSBs. Inhibition of replicative DNA helicases further increased the amount of linear DNA, indicating that arrested replication forks are susceptible to breakage. This suggests a role for replication blockage in spontaneous DSBs and points to a role for helicases in the prevention of replication-associated DSBs, possibly by a limitation of replication pauses. The main phenotype of cells lacking such a helicase is a dramatic need for efficient DSB repair.

DSB repair

This study also allowed the investigation of DSB repair in the absence of any DNA-damaging agent. Linear DNA was only detected in significant amounts in *recBC* or *recA recD* mutants (see Table I). The elevated proportion of linear chromosomes found in these mutants correlates

Table IV. Strains and plasmids

Strains	Genotype/description	Source/reference
DG76	wild type, parent of PC8	B.Backman
FG252	*sbcD300*::*kan*	Gibson *et al.* (1992)
GC2687	*dnaATS Tna*::Tn*10*	R.D'Ari
GY9701	Δ*recA*::*cam*, [miniF *recA*]	R.Devoret
K1019	*rep 71 ilv*::Tn*10*	N.Zinder
N2101	*recB268*::Tn*10*	Lloyd *et al.* (1987)
N2103	*recC266*::Tn*10*	Lloyd *et al.* (1987)
N2364	*sbcC201, phoR*::Tn*10*	Lloyd and Buckman (1985)
PC8	*dnaB8*	B.Backman
SK129	*recB270 recC271*	Kushner (1974a,b)
TH347	Δ*tus*::*kan*,*Invter*::*spc*^{R}[pBadTus]	Sharma and Hill (1995)
WA576	*recF*::Tn*5*	W.Wackernagel
JJC16	Δ(*recA-srl*)::Tn*10*	laboratory stock
JJC40	wild type	laboratory stock
JJC41	as JJC40, but F′[*lacI*^{q}, *lacZ*Δ*M15, proAB*]	laboratory stock
JJC213	Δ*rep*::*kan*	Uzest *et al.* (1995)
JJC269	*recD1901*::Tn*10*, Δ*rep*::*kan*	Uzest *et al.* (1995)
JJC273	*recD1901*::Tn*10*	Uzest *et al.* (1995)
JJC315	*recB*::Tn*10*	P1 N2101 * JJC40 to Tet^R
JJC316	*recC*::Tn*10*	P1 N2103 * JJC40 to Tet^R
JJC335	*lexA1*(Ind$^-$), Δ*rep*::*kan*	Uzest *et al.* (1995)
JJC356	Δ(*recA-srl*)::Tn*10*, Δ*rep*::*kan*	Uzest *et al.* (1995)
JJC390	Δ*rep*::*kan*, *recA*::*Cam*	P1 GY9701 * JJC213 to Cam^R
JJC432	*recD*::Tn*10*, *recA*::*Cam*	P1 GY9701 * JJC273 to Cam^R
JJC438	Δ*tus*::*kan*, *Inv-Ter*::*spc recB*::*Tn10*	P1 N2101 * TH347, and additional screening for the loss of the plasmid pBadTus
JJC480	Δ*rep*::*kan*, *dnaATS*	P1 GC2687 * JJC213 to Tet^R screening for thermosensitivity
JJC481	Δ*rep*::*kan*, *dnaATS*, *recA*::*Cam*	P1 GY9701 * JJC480 to Cam^R
JJC482	*recB270*, *recC271*, *rep71*	P1 K1019 * SK129 to Tet^R screening for thermosensitivity
JJC484	*rep71*, *recB270*, *recC271*, *sbcD300*::*kan*, *ilv*::Tn*10*	P1 FG252 * JJC482
JJC489	*rep71*	P1 K1019 * JJC40 to Tet^R screening for defect M13 replication
JJC495	*rep71*, *recB270*, *recC271*, *sbcD300*::*kan*	*ilv*$^+$ revertant of JJC484
JJC498	*rep71*, *recB270*, *recC271 sbcC201*, *phoR*::Tn*10*	P1 N2304 * JJC495 to Tet^R screening for λDRL154 growth
JJC502	*rep 71*, *recA*::*cam*	P1 GY9701 * JJC489
JJC503	*rep 71*, *recF*::Tn*5*	P1 WA576 * JJC489
JJC505	Δ*rep*::*kan*, *recB270*, *recC271*	P1 JJC213 * SK129
JJC506	*recB*::Tn*10*	P1 N2101 * DG76
JJC507	*dnaB8 recB*::Tn*10*	P1 N2101 * PC8
Plasmid		
pBR322	cloning vector	
pSF117	pBR322 carrying pL-Gam	Friedman and Hays (1986)
pBadTus	pBR322 carrying *tus* under the control of the araC promotor	Sharma and Hill (1995)
Phage		
M13mp2		J.Messing
λDRL154	λ*pal*571,*delta-spi*6, *c*I857, *chi*-CII	D.Leach

Most of the JJC strains are JJC40 derivatives: *leu6, his4, argE3, lacY1, galK2, ara14, xyl5, mtl1, tsx33, rpsL31, supE44, hsdR; JJC438 is trpR, trpA905am, his29-am, ilv, pro-2, arg-427, thyA, deoB or deoC, tsx, rac-plus, IN(rrnD-rrnE) lac, del-tus 2474*::*kan*^{R}, *InvTer*::*spc*^{R}; JJC482 and derivatives and JJC505 are AB1157 derivatives (as JJC40 but *thrA1, proA2*, HsdR$^+$); JJC506 and JJC507 are *leuB6, thyA47, deoC3, rpsL153*. Abbreviations: Tet^R, tetracycline resistance; Kan^R, kanamycin resistance; Cam^R, chloramphenicol resistance; Spc^R, spectinomycin resistance.

with their low colony-forming ability. This confirms the central role of the RecBCD pathway in the repair of linear DNA in *E.coli*, via its dual action as a double-stranded DNA exonuclease and recombinase.

It is interesting to note that the amount of linear DNA increased with temperature in a *recB* mutant (Figure 7). This could reflect an increased number of replication forks at high temperature, and consequently more opportunities for fork breakage. DSB repair in the *rep* mutant proceeds via the same pathway, since (i) the proportion of linear DNA increases upon inactivation of RecBC in the *rep recBTS recCTS* strains and (ii) *rep recD* and *rep recA* strains which are viable do not accumulate linear DNA, while unrepaired DSBs may be responsible for the *rep recA recD* triple mutant inviability.

Results in the λ Gam-containing cells revealed more complex repair pathways. In *rep recA gam*$^+$ cells, there is a clear correlation between lethality and DSB formation. This is not the case for *rep Gam*$^+$ cells which contain only 11–17% linear DNA. The linear DNA could be converted to a non-migrating form by RecA-dependent homologous recombination. Alternatively, RecA could protect the chromosomes specifically from DSB formation in the *rep gam*$^+$ cells. Neither mechanism, however, restores cell viability, possibly because proteins remain bound to the DNA, leading to structures which do not

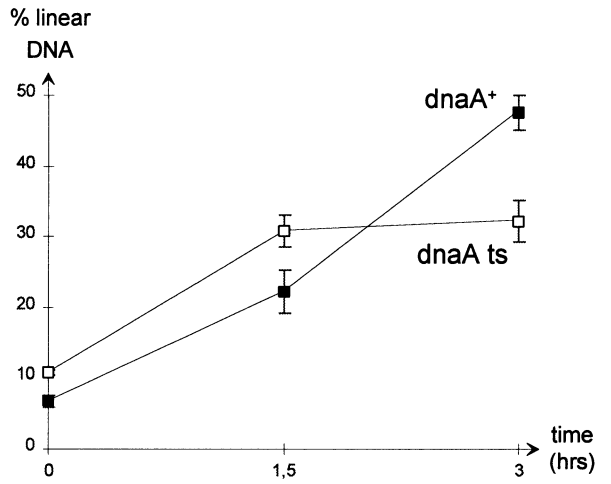

Fig. 6. Effect of a *dnaATS* mutation on the proportion of linear DNA in *rep recA* [pSF117]. Cells were grown as described in the legend to Figure 3. An aliquot was grown at 30°C (time 0) and two aliquots were shifted to 42°C for 1.5 and 3 h respectively. Results are the mean of two independent determinations for *rep recA* [pSF117] (■) and three independent determinations for *rep recA dnaATS* [pSF117] (□).

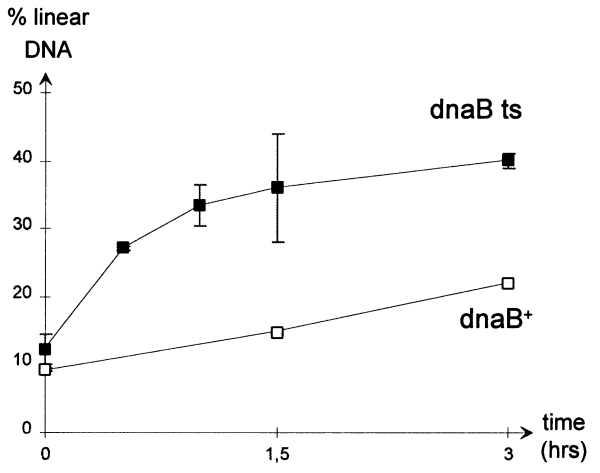

Fig. 7. The proportion of linear DNA increases upon incubation of *recB dnaBTS* cells at 42°C. Cells were grown in LB medium plus 5 μg of [^{3}H]thymidine. Part of the culture was grown at 30°C for 6 h. Aliquots were shifted to 42°C for 0.5, 1, 1.5 and 3 h for the *dnaBTS* mutant and for 1.5 and 3 h for the DnaB$^+$ control. Results are the mean of two independent determinations. □, JJC506; ■, JJC507.

allow replication restart. A similar 'lethal repair' may take place in *rep recBCD sbcBC* and *rep recBCD sbcA* strains, which would account for the intriguing observation that these mutants are inviable, although they are recombination proficient via the alternative RecF and RecE recombination pathways, respectively (Uzest *et al.*, 1995). Repair of DSBs by the wild-type RecBCD enzyme appears to be the only way to restore viable chromosomes in *rep* strains.

Among the proteins proposed to be involved in restarting stalled or repaired replication forks are the primosome assembly protein PriA and the RecF protein (Kogoma *et al.*, 1996; Sandler, 1996). PriA and RecF mutations, therefore, were tested for their effect in *rep* strains. However, as PriA is essential for RecBCD-dependent homologous recombination (Kogoma *et al.*, 1996; Sandler *et al.*, 1996), it is expected to be essential in *rep* strains. Effectively we could not construct a *rep3, priA*::*kan* double mutant (our unpublished result). RecF does not seem to play a major role in *rep* strains, as we detected no effect of the *recF*::Tn*5* mutation in either *rep*, *rep recD* or *rep gam*$^+$ strains. In addition, the high level of replication-dependent breakage reported here in cells which are proficient for both PriA and RecF indicates that neither of these two proteins allows replication restart in the absence of Rep.

DSB formation and helicase inhibition

The present work shows that impairment of the progression of replication forks leads to DSB formation. Inhibition of the *E.coli* helicase DnaB leads to chromosome breakage and replication-dependent DSBs occur in *rep* mutants. Studies on the progression of replication forks in *rep* mutants revealed a slow but constant rate of replication (Lane and Denhart, 1975; Cosalanti and Denhart, 1987). This suggests that replication pauses occur all over the chromosome. Replication pauses due to collisions with the transcription apparatus or DNA-bound proteins were observed *in vivo* and *in vitro* (French, 1992; Vilette *et al.*, 1992; Liu and Alberts, 1995), and the Rep protein removes DNA-bound proteins *in vitro* (Yancey-Wrona and Matson, 1992). Therefore, the Rep protein could prevent the formation of DSBs by limiting replication pauses.

The mechanism of chromosomal breakage is unknown. Are the breaks at arrested replication forks due to enzymatic cleavage of DNA, and in this case are there specific nucleases involved, or do DNA breaks occur spontaneously, as a result of tensions consequent to cell growth? The two best characterized single-stranded endonucleases in *E.coli* are RecBCD and SbcCD. However, if these endonucleases are responsible for the introduction of breaks at arrested replication forks, they are dispensable, since a significant amount of breakage occurred in the *rep recBTS recCTS sbcCD* mutants at restrictive temperature. Analysis of appropriate mutants should allow the identification of functions that promote breakage at arrested replication forks.

A *rep* homologue has been identified in *Staphylococcus aureus*, where this gene is essential (Iordanescu, 1993). Whether the absolute requirement for the *rep* gene in this species correlates with a limited capacity for DSB repair is unknown. The role proposed here for the Rep helicase is reminiscent of that proposed for the T4 Dda helicase which shares no homology with Rep (Bedinger *et al.*, 1983). Recently, a correlation between the defect in a putative helicase and increased DSBs was described in humans. Two genetic disorders, Bloom's syndrome and Werner's syndrome, were found to result from a defect in proteins sharing homologies with prokaryotic and yeast helicases (Ellis *et al.*, 1995; Yu *et al.*, 1996). Indicators of defective DNA metabolism in these syndromes include chromosomal instability and hyper-recombination phenotype, resulting from increased DSBs. In Bloom's syndrome cells, the rate of nascent DNA chain elongation is retarded (Hand and German, 1975; Gianelli *et al.*, 1977). This and several additional observations led to the proposal that the helicase defective in Bloom's syndrome is involved in DNA replication (Ellis *et al.*, 1995). The present work illustrates how, in *E.coli*, a defect in a replicative helicase can lead directly to increased DSB formation.

Materials and methods

Strains and plasmids

The *E.coli* K12 strains and plasmids used are listed in Table IV. All strains constructed during this work were made by P1 transduction. P1 stocks were prepared, and transductions performed as described (Miller, 1992). Transductions with stocks grown on strains carrying a *malF3089*::Tn*10*, *fuc3154*::Tn*10kan* or a *malF*::*cam* allele were used as controls for the Tet^R, Kan^R and Cam^R markers respectively. Unless otherwise indicated, all transductions described in this work gave the expected amount of clones with the expected phenotype. Compared with JJC482 (*rep71 recBTS recCTS*), strain JJC336 (Δ*rep*::*kan, recBTS recCTS*; Uzest *et al.*, 1995) displays better growth ability and less linear DNA at 30°C. P1 transduction of the *rep71* mutation in JJC336 revealed that this could not be attributed to the difference in the *rep* alleles, and was probably due to the presence of a compensatory mutation in JJC336, selected during growth at 30°C. For this reason, a new Δ*rep*::*kan, recBTS recCTS* strain, JJC505, was constructed and used in the present work. This strain grows as JJC482.

The Rep phenotype was verified by transforming $CaCl_2$-competent cells by M13mp2 RF DNA on a lawn of JJC41 (F′) indicator cells. The phenotype of *recA*, *recB* and *recC* mutants was verified by measuring UV sensitivity. Dilutions of cultures in late exponential phase (~10^8 cells per ml) were plated on LBAT (Luria broth agar thymine medium) and irradiated at 254 nm. Plates were irradiated with an energy of 20–120 J/m^2 at a rate of 2–4 J/m^2/s. Dose rate was measured with a Latarjet dosimeter. The exo^- phenotype of *recB*, *recC* and *recD* mutants was verified by measuring the plating efficiency of T4 gene 2^- phage, which is increased ~2000-fold in these mutants.

Rich medium was Luria broth (Miller, 1992) supplemented with 25 μg/ml thymine (LBT). Minimal medium was medium M63 (a medium devoid of NaCl, Miller, 1992), supplemented with 0.4% glucose and 0.2% casamino acids. Antibiotics were used at the following concentrations: ampicillin (Amp), 40 μg/ml; tetracycline (Tet), 15 μg/ml; kanamycin (Kan), 45 μg/ml; chloramphenicol (Cam), 25 μg/ml; and spectinomycin (Spc), 60 μg/ml. Overnight cultures were grown routinely in 5 ml of LBT broth. Cells were made competent by $CaCl_2$ treatment and transformed as described (Dagert and Ehrlich, 1979).

Preparation of plugs and PFGE migration

Overnight cultures were grown in LBT medium. The cells were diluted to OD_{650} 0.01 or 0.02 in M63 supplemented with 0.4% glucose, 0.2% casamino acids, 100 μg/ml deoxyadenosine and 5 μCi/ml of [^{3}H]thymidine (sp. act. 87 Ci/mmol, New England Nuclear). For JJC338 (*thyA deoC*), 5 μg/ml of cold thymidine were added, and, due to poor growth in minimal medium, JJC506 and JJC507 (*thyA deoC*) were grown in LB plus 5 μCi/ml of [^{3}H]thymidine. When 3 or 4 h of incubation at restrictive temperature were required, the culture was divided into two when the OD_{650} reached 0.07–0.08. One part was incubated further at 30°C, the other was shifted to 42°C. For shorter times of incubation at restrictive temperature, the shift was performed at a higher OD. Cells were collected (they reached OD_{650} 0.3–0.7), washed in cold minimal medium and resuspended in Tris 10 mM, EDTA 100 mM, EGTA 10 mM (TEE buffer) at a final OD of 0.7. After 10 min of incubation at 37°C, the suspension was mixed with an equal volume of agarose InCert 1.5% in TEE and allowed to solidify in plug molds. The plugs were then incubated for 2 h at 37°C in 20 ml of TEE buffer containing 0.05% sarcosyl, 5 mg/ml lysozyme solution, and overnight at 52°C in 10 ml TEE buffer containing 1 mg/ml proteinase K, 1% SDS. Plugs were kept in 10 ml of TEE buffer at 4°C and used within 2 weeks.

The 1% agarose gels were run for 40 h at 160 V (6–7 V/cm) in 0.3× TEB, using a PFGE ramp from 3 ms forward/1 ms backward to 180 ms forward/60 ms backward.

After migration, gels were stained with ethidium bromide and photographed. Plugs containing the chromosomes of the yeast *Saccharomyces cerevisiae* and plugs containing the chromosomes of *Hansenula wingei* were used routinely as molecular weight standards (Bio-Rad). The lanes were cut into 3 mm slices using a razor blades slicer. A total of 36–42 slices representing 12–14 cm of migration were usually made to allow the recovery of all fragments from the migration origin to 30–50 kb fragments. The amount of tritium in the slices corresponding to the smaller fragments was generally low or null. The agarose slices were melted at 90°C, and diluted 5-fold in 0.1 M HCl at the same temperature. Counting was performed in 10 ml of scintillation fluid (Beckman ready safe) in a Beckman scintillation counter. The fraction of migrating DNA was calculated by dividing the amount of tritium found in a lane by the total amount of tritium found in the lane plus the migration origin. This total amount generally did not differ significantly from the tritium present in an intact plug counted as a control.

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