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Research article

Accumulation of large non-circular forms of the chromosome in recombination-defective mutants of *Escherichia coli*

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Abstract

Background: Double-strand breakage of chromosomal DNA is obviously a serious threat to cells because various activities of the chromosome depend on its integrity. However, recent experiments suggest that such breakage may occur frequently during "normal" growth in various organisms – from bacteria through vertebrates, possibly through arrest of a replication fork at some endogenous DNA damage.

Results: In order to learn how the recombination processes contribute to generation and processing of the breakage, large (> 2000 kb) linear forms of *Escherichia coli* chromosome were detected by pulsed-field gel electrophoresis in various recombination-defective mutants. The mutants were analyzed in a rich medium, in which the wild-type strain showed fewer of these huge broken chromosomes than in a synthetic medium, and the following results were obtained: (i) Several *recB* and *recC* null mutants (in an otherwise *rec*$^+$ background) accumulated these huge linear forms, but several non-null *recBCD* mutants (*recD, recC1001, recC1002, recC1003, recC1004, recC2145, recB2154*, and *recB2155*) did not. (ii) In a *recBC sbcA* background, in which RecE-mediated recombination is active, *recA, recJ, recQ, recE, recT, recF, recO*, and *recR* mutations led to their accumulation. The *recJ* mutant accumulated many linear forms, but this effect was suppressed by a *recQ* mutation. (iii) The *recA, recJ, recQ, recF* and *recR* mutations led to their accumulation in a *recBC sbcBC* background. The *recJ* mutation showed the largest amount of these forms. (iv) No accumulation was detected in mutants affecting resolution of Holliday intermediates, *recG, ruvAB* and *ruvC*, in any of these backgrounds.

Conclusion: These results are discussed in terms of stepwise processing of chromosomal double-strand breaks.

Background

Double-strand (ds) breakage of chromosomal DNA is obviously a serious threat to cells because various activities of the chromosome – gene expression, replication and partition – depend on its integrity. However, recent experiments suggest that such chromosomal ds breakage may occur relatively frequently during "normal" growth in several organisms – in bacteria [1,2], yeast [3] and chicken cells [4].

In *Escherichia coli*, spontaneous breakage and degradation of the chromosome associated with a replication fork were predicted from early genetic analysis and were detected under various conditions of altered replication (for review, see [5]). DNA ds breaks play a key role in homologous recombination. From a DNA ds break, RecBCD enzyme starts degrading DNA (for review, see [6]). When it encounters a specific sequence called Chi, it promotes its pairing with a homologous DNA. Even in the absence of RecBCD enzyme, *sbcA* mutation confers other recombination pathway, called RecET pathway. The *recE* gene product of the Rac prophage converts dsDNA ends into 3' protruding single-stranded form and the *recT* gene product promotes recombination by annealing them with a homologous DNA in its vicinity (for review, see [7,8]). This recombination may result in one progeny DNA (non-conservative recombination) or two progeny DNAs (conservative double-strand break repair) [9]. In a *recBC sbcBC* background, a ds end stimulates homologous recombination that results in only one progeny DNA (non-conservative recombination) [10]. Analysis of the stimulation of recombination by replication (for review, see [11]) and analysis of altered chromosomal replication (for review, see [12]) led to the proposal that a chromosomal ds break formed during replication fork arrest triggers homologous recombination, which would reconstitute a replication fork (for review, see [5]).

Game and his colleagues have developed a sensitive means of detecting chromosomal ds breakage using a circular chromosome [3]. Under most conditions of pulsed-field gel electrophoresis, a circular yeast chromosome and circular bacterial chromosomes will not enter the gel, very likely because they are trapped by the branches of the network of agarose [3,13,14]. One double-strand break transforms this circular form into a linear form, which can now move slowly in the gel [3]. We used this procedure to detect double-strand breakage of a circular bacterial chromosome occurring spontaneously or after loss of a restriction-modification gene complex [15,16]. We found increased chromosome breakage in *recBC*-null and *recC1002* mutants of *E. coli* under both conditions [1]. Michel and her colleagues used pulsed-field gel electrophoresis to detect degraded chromosomal DNAs arising spontaneously in *recBC* mutants and arising during replication fork arrest [2]. RuvABC proteins, which catalyze migration and cleavage of Holliday junctions, are responsible for the occurrence of the degraded DNAs following replication fork arrests [17].

In this work, we employed the pulsed-field gel electrophoresis procedure to measure large non-circular forms of the chromosome obtained from various recombination-defective mutants in *rec*+, *recBC sbcA*, and *recBC sbcBC* genetic backgrounds.

Results

Effect of growth medium on the accumulation of large chromosomal fragments

Large linear chromosomal fragments were measured by pulsed-field gel electrophoresis. In our analysis, growing *E. coli* cells are harvested, embedded in an agarose plug, and lysed *in situ*. The chromosomes in a plug are electrophoresed in varying electric fields. An example of such a gel is shown in Figure 1. The DNA is partitioned in three places in the gel – the well, the area just below the well (marked by a bar to the right of the gel), and the lower area. Intact circular chromosomes stay in the well [3,14] likely because they are trapped in the branches of agarose resin [13]. Large linear forms generated by a ds break in this circle would escape from agarose trap and form broad bands beneath the well (marked by the bar). This area corresponds to unbranched linear forms DNA of more than 2000 kb when compared with yeast chromosome markers. When the DNAs become smaller by degradation, they will migrate further. These interpretations are based on a previous work with a yeast circular chromosome and on our analysis of *E. coli* chromosomal breakage after loss of restriction-modification genes [3,15,16]. In this work, we focus on the second DNA species – the huge linear forms in the area just below the well (Figure 1, marked by the bar).

In the experiment shown in Figure 1, a *rec*+ strain (in AB1157 background) grown in minimal medium (M9) (Figure 1, lane 2) gave rise to some of these huge linear DNAs in this area. There was less of this DNA species when the cells were grown in a rich medium (LB) (Figure 1, lanes 3). In an isogenic *recB21 recC22* strain, the amount was larger than in *rec*+.

We do not know why the medium makes such a difference. It could reflect properties of the spontaneous DNA damages, the replication fork, the number of replication forks, the number of chromosomes, the organization and structure of the chromosomes, the repair machinery, or the availability of homologous chromosomes for repair. All of these features will influence the chromosome stability not only in *rec*+, but also in mutants. This medium-dependence is in the opposite direction to what is simply expected from generation of a double-stranded chromosomal end by collapse of a replication fork with another, replication fork moving in the same direction [18], because replication initiation should be more frequent in a rich medium than in a poor medium. Whatever the reason, we chose to use the rich medium in which the *rec*+ strain produce less linear forms, because the background is clear and may allow sensitive detection of their increase in a survey of various recombination-defective mutants.

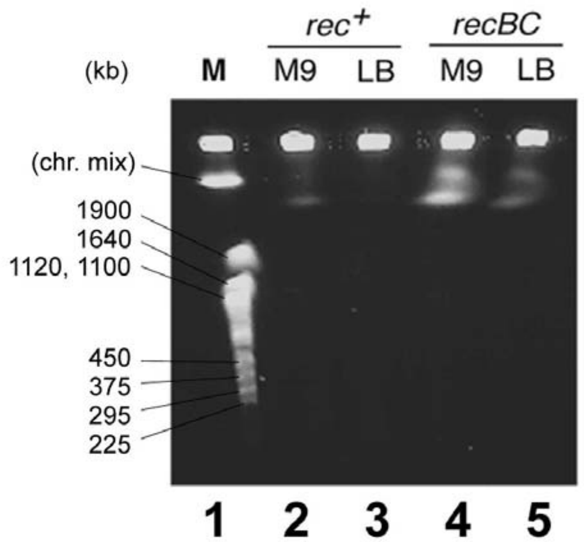

Figure 1
Detection of large non-circular forms of the chromosome by pulsed-field gel electrophoresis. AB1157 (= *rec*+) and JC5519 (= *recBC*) cells were grown either in M9, a minimal medium, or in LB, a rich broth (Materials and Methods). The cells were harvested, embedded in agarose, lysed *in situ*, and analyzed by pulsed-field gel electrophoresis. Giant circular chromosomes stay in the well. Huge non-circular forms generated by ds breakage will band just below the well (bar in the right). Lane "M" contains *Saccharomyces cerevisiae* chromosomes as linear size markers.

rec *and* ruv *mutations*

The accumulation of huge linear DNAs was also seen in other *recBC* null mutants in this AB1157 genetic background (*recB21*, *recC22* and *recC73* (Figure 2A)) and in another, V66, genetic background (*recC73* (Figure 2B)), as observed earlier [1,2]. A *recD* mutant showed no accumulation. The other non-null *recBCD* alleles examined (*recC1001*, *recC1002*, *recC1003*, *recC1004*, *recC2145*, *recB2154*, and *recB2155*) did not accumulate the huge linears (Figure 2B). We do not know why the same mutant allele, *recC73*, shows more accumulation in V66 background than in AB1157 background in a reproducible manner (Figures 2A and 2B).

The other mutants tested – *recA*, *recF*, *recG*, *recJ*, *recN*, *recO*, *recQ*, *recR*, *ruvAB*, and *ruvC* – did not accumulate huge linear DNAs. The *recF* mutation partially suppressed the effect of the *recC73* mutation in accumulating the huge linear chromosomes (Figure 2B).

recBC sbcA *background*

In the *recBC sbcA* strain, an *sbcA* mutation on the Rac prophage expresses *recET* genes, which promotes homologous recombination at a ds end [7]. The accumulation of the huge linears was seen with *recA*, *recE*, *recT*, *recJ*, *recQ*, *recF*, *recO* and *recR* strains (Figure 2C). Mutations in genes involved in processing Holliday structures – *recG*, *ruvAB* and *ruvC* – did not lead to their accumulation. The accumulation by *recJ* mutation was suppressed by a *recQ* mutation (Figure 2C, lanes 7 and 15).

recBC sbcBC *background*

In the *recBC sbcBC* strain, RecBCD enzyme is inactive and RecFOR and RecQJ proteins promote recombination together with RecA [19]. In the *recBC sbcBC* background, *recA*, *recF*, *recJ*, *recQ* and *recR* mutants accumulated these huge linears to varying extents (Figure 2D). However, again the *ruvC* mutation did not lead to accumulation.

Control experiments

These assays were carried out more than twice for each strain, and the extent of accumulation of the linear forms was reproducible. The DNA in the area just below the origin was also measured by densitometry to confirm the above results (data not shown).

When the chromosomal DNA in the agar plug was digested with a restriction enzyme (*Xba*I) before the pulsed-field gel electrophoresis, all the strains examined produced comparable amounts of DNA (Figure 3). This amount is much larger than the large linear forms. This indicates that the total amount of undegraded DNA associated with the cells is comparable for all the strains.

Discussion

We found that large, non-circular forms of the chromosome accumulate in varying amounts in various recombination-defective mutants of *Escherichia coli*.

Our operational definition of the non-circular forms is their presence in an area just below the well in our pulsed-field gel, as marked by a bar in Figure 1. The molecular species in this area may not be limited to a simple linear form of varying lengths. If a chromosome carries multiple replication forks as usual at 37°C in rich media, more than one double-strand break may be necessary to form a non-circular, branched species, which should be able to move through the gel. Finding out macroscopic forms of these giant molecules would be a technical challenge (see [20], for example). We do not know why DNAs make two broad bands in this area (Figure 1, 4th lane, for example), either. Depending on the electrophoresis condition, one narrow band, a pair of two bands or one very broad band was observed (data not shown).

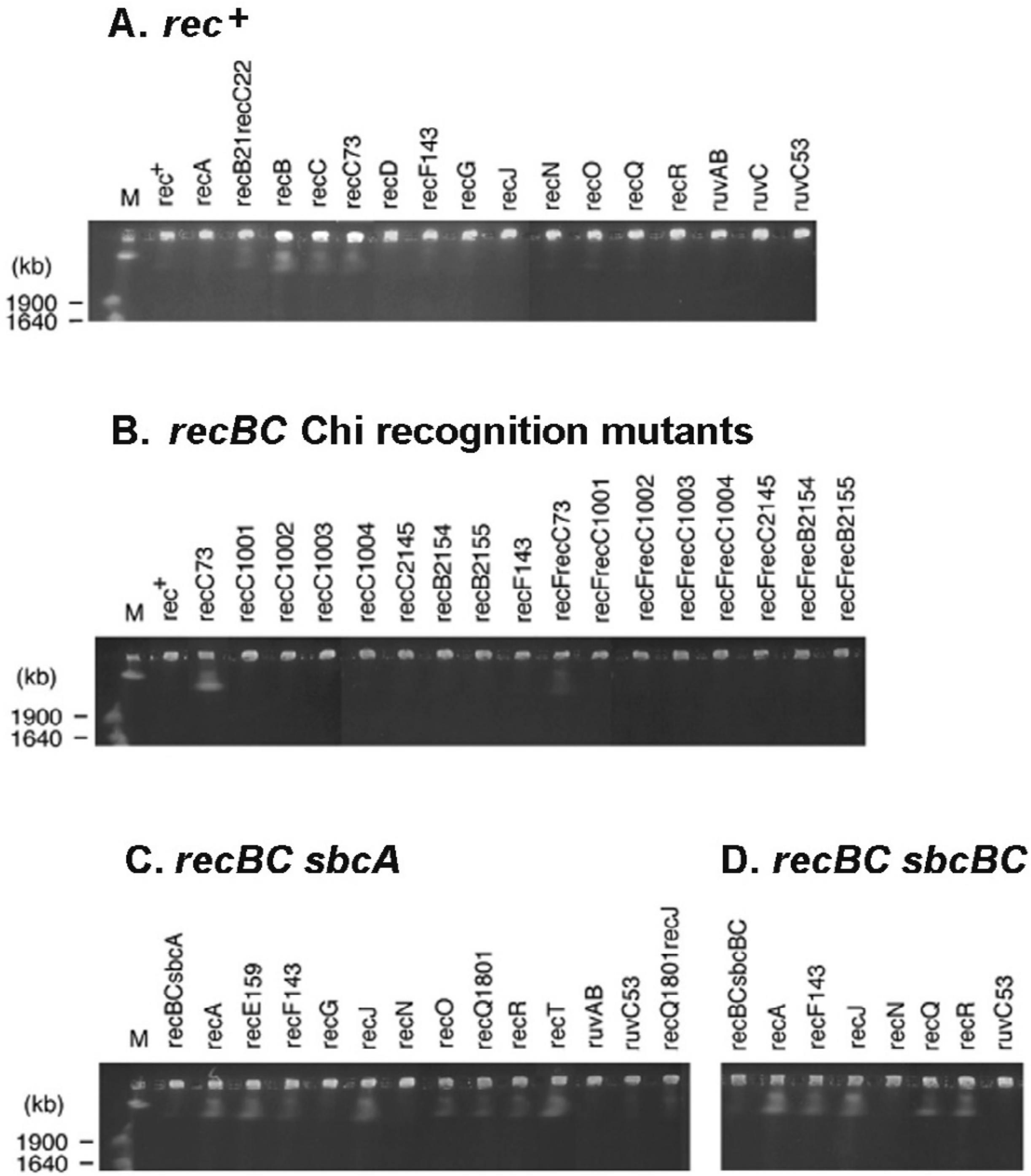

Figure 2

Accumulation of large non-circular forms of the chromosome in recombination-defective mutants. A: In an otherwise *rec*$^{+}$ background. The mutation alleles are as follows: Δ*recA306*::Tn*10*, *recB21 recC22, recB268*::Tn*10*, *recC266*::Tn*10*, recC73, *recD1901*::Tn*10*, *recF143, recG258*::mini-Tn*10* Kan, *recJ284*::Tn*10*, *recN1502*::Tn*5*, *rec01504*::Tn*5*, *recQ1803*::Tn*3*, *recR252*::mini-Tn*10*Kan, Δ*ruvAB100*::Cm, Δ*ruvC100*::Cm, *ruvC53 eda*::Tn*10*. B: Various *recBCD* alleles in V66 background. C: In a *recBC sbcA* background. The mutation alleles are the same as in A except for *recE159, recQ1801, recT101*::Tn*10*, and Δ*ruvAB*::Tc. D: In a *recBC sbcBC* background. The mutation alleles are the same as in A except for *recN262 tyrA16*::Tn*10*.

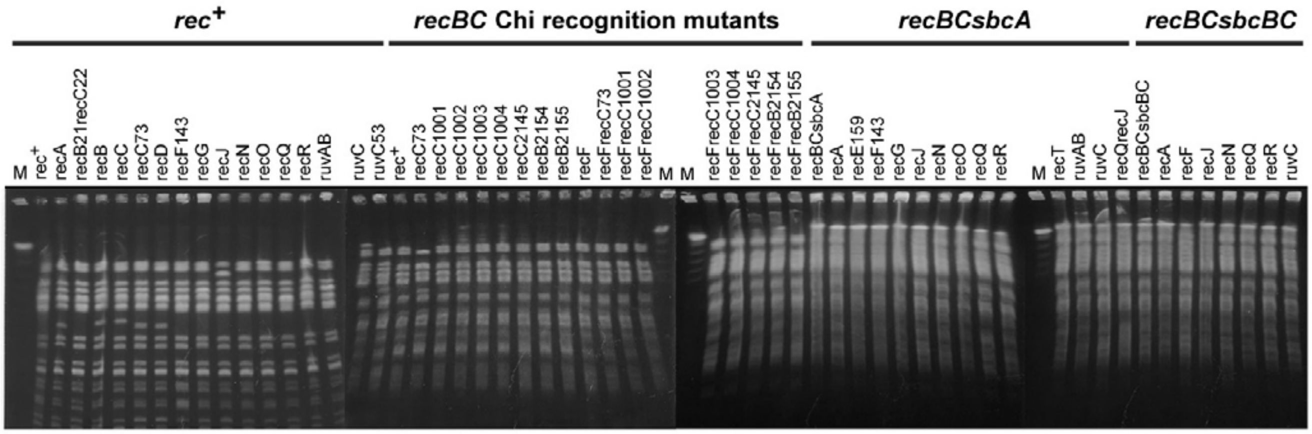

Figure 3
Pulsed-field gel electrophoresis of the chromosomes after restriction enzyme digestion. The cells were lysed in an agarose plug and were treated with *Xba*I before pulsed-field gel electrophoresis. M indicates yeast chromosome marker.

Abundance of these huge non-circular forms is expected to be affected by several factors, which might work potentially in opposite directions, such as: (i) breakage in the cell; (ii) degradation in the cell; (iii) repair in the cell; (iv) breakage and degradation out of the cell. Each term is, in turn, affected by other factors such as chromosome organization, number of the replication forks, speed of the replication forks, abundance of specific proteins, and so forth. Therefore, our finding of accumulation of more of the non-linear forms in a rich medium than in a poor medium (Figure 1) does not immediately allow us to conclude that starving conditions induce a chromosomal double-strand breakage.

Spontaneous DNA damages, repair and degradation are expected to be the key processes in interpreting our data. Spontaneous DNA damages may interfere with replication fork progression and produce chromosomal double-stranded breaks. This would lead to extensive exonucleolytic degradation. Complete repair at some of these steps would reconstitute a circular chromosome, which will stay in the well. On the other hand, further degradation of the huge, non-circular forms would result in shorter or no fragments, which will run faster in the gel. The presence of huge linear forms, therefore, probably indicates both the absence of complete repair and the absence of further degradation. The absence of the large linears could either mean the presence of complete repair or the presence of extensive degradation activity. Our control experiments demonstrated that restriction digestion of chromosome DNAs before the electrophoresis results in release of comparable amounts of DNA from the wells in all the strains examined (Figure 3). This result, at least, excludes the possibility that the absence of the large, non-circular chromosomes in some strains (Figure 2) reflects the absence of DNAs in the wells during the process or by extensive and general nuclease action. Of course, we cannot exclude the possibility that the broken chromosomes specifically have suffered extensive degradation.

In spite of these potential complexity and essential ambiguity, our measurements provided a unique clue to the action of recombination-associated enzymes in the chromosome metabolism. Indeed, some of our observations in the mutants can be readily related to the established properties of the affected enzyme.

Accumulation of the huge linear DNAs in the *recBC* null mutants can be interpreted from the known properties of RecBCD enzyme in a straightforward way. These null mutant enzymes cannot degrade DNA from a ds break nor can they repair DNA by recombination [6]. We assume that they cannot repair the broken chromosomes to form intact circular chromosomes and that they cannot degrade them into smaller pieces. The *recD* mutant does not show nuclease activity but is recombination-proficient and able to repair the broken DNA molecules [6]. This explains why it does not accumulate the huge linear forms. The other non-null *recBCD* mutants (*recC1001*, *recC1002*, *recC1003*, *recC1004*, *recC2145*, *recB2154*, and *recB2155*) are all nuclease positive [21,22]. They would be expected to degrade the huge linears. They retain some to nearly complete recombination proficiency [21,22], which may contribute to repair of the large linears into circles. The other recombination-defective mutants, in otherwise *rec*+ background, did not accumulate the huge linears probably because the DNA was degraded by active RecBCD enzyme or was not produced.

Partial suppression of the accumulation of the huge linears in a *recBC* null allele by a *recF* mutation (Figure 2B) leads to several possible explanations. For example, RecF-mediated homologous recombination may transform a circular chromosome, possibly with a spontaneous damage, into some type of non-circular forms. This is expected because RecF-mediated recombination is non-conservative in the sense that it generates only one progeny DNA molecule from two parental DNA molecules [10]. Alternatively, RecF function may somehow help generation of broken chromosomes or maintenance of break to load RecA [23].

In the *recBC sbcA* and the *recBC sbcBC* backgrounds, the absence of RecBCD nuclease may prevent faster degradation of the large non-circular DNAs. However, we see only little accumulation of the broken forms. One might expect that the accumulation of the huge linears may correlate with the capacity for recombination repair that reconstitutes a circular form. Indeed, the effects of *recA*, *recJ* and several other *rec* mutations on accumulation of the huge linear chromosomes in these two *recBC* backgrounds (Figures 2C and 2D) were similar to their negative effects on conjugational recombination [19] with interesting exceptions (see next paragraph). This accords with the concept that a huge linear fragments of the chromosome is involved in recombination following conjugation. However, any of the recombination mutants that lead to accumulation of linear DNA could affect the probability of breaks occurring in the first place.

The mutations in Holliday-structure-processing enzymes – RecG, RuvAB, and RuvC – did not result in accumulation of the huge linears even in the *recBC*-minus background. The complex intermediate forms accumulating in these mutants may be trapped in the agarose gel (see [24,25]). An alternative interpretation could be that these enzymes may be involved in generation of double-strand breaks as hypothesized by Seigneur *et al.* [17].

The accumulation by the *recJ* mutation in the *recBC sbcA* background is suppressed by a *recQ* mutation (Figure 2C). Kusano et al. [26] found that both sensitivity to DNA damaging agents and decreased association of crossing-over with double-strand break repair in a *recBC sbcA recJ* strain are suppressed by mutant *recQ* alleles. Such suppressing relationship was interpreted to suggest that RecQ acts prior to or concurrently with RecJ. Pulsed-field gel electrophoresis analysis of chromosomes after ultraviolet irradiation has revealed extensive chromosome degradation dependent on *uvrA* incision enzyme [27]. A report [28] showed that RecQ and RecJ proteins process nascent DNA at replication forks blocked by ultraviolet irradiation prior to the resumption of DNA synthesis (see also [29]).

The accumulation of the non-circular, broken chromosomes correlated with the growth rate or DNA damage response in most of the *recBC*-minus background [30]. The *recB* or *recC* null mutation showed low viability even in the absence of exogenous DNA damage [31,32]. A simple interpretation of these data is that RecA, RecFOR, and RecQJ functions (and RecET functions for the *sbcA* background) repair chromosome breakage and/or prevent generation of the breakage. The major contradiction observed here is the phenotype in *ruv* mutants. The *ruv* mutants in all the background did not show any accumulation of the broken chromosome. This may suggest that the possible role of Ruv protein is making a break into dsDNA [33].

Conclusions

Our sensitive measurements of the large non-circular forms of the chromosome – which should be able to detect one ds break out of 4 million bp – provided unique sets of data that would help in further elucidating the mechanisms of chromosome double-strand break repair. A simplest interpretation of our data is that RecBCD enzyme is involved in repair and degradation of broken chromosomes, and that RecA, RecFOR, RecQJ and RecET functions are involved in prevention and/or repair of the breakage. Interaction was observed between a *recC* mutation and a *recF* mutation and between a *recQ* mutation and a *recJ* mutation. *ruvABC* mutants and a *recG* mutant did not accumulate broken chromosomes. Further molecular analysis would bring about interpretation of the present data in detailed molecular terms.

Methods

Bacteria

Escherichia coli K-12 strains used are listed in Table 1.

Media

E. coli cells were grown in M9 medium (1 × M9 salts [34], 0.2% glucose, 0.05 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.2% casamino acids and 1 microgram/ml vitamin B1) and LB broth (1.0% Bacto-tryptone, 0.5% Yeast extract and 1.0% NaCl) with antibiotics at the following concentrations when necessary: ampicillin (Amp) at 50 microgram/ml together with methicillin at 200 microgram/ml, chloramphenicol (Cml) at 25 microgram/ml, kanamycin (Kan) at 10 microgram/ml and tetracycline (Tet) at 10 microgram/ml.

Table 1: Bacterial strains used here

Strain	Other name	Genotype	Source/Reference
AB1157	BIK788	*thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE44 rec*$^+$	[36]
TES1	BIK733	As AB1157, but *ΔrecA306*::Tn*10*	K. Yamamoto/[37]
JC5519	BIK751	As AB1157, but *recB21 recC22*	T. Kato/[38]
N2101	BIK2876	*recB268*::Tn*10*	R. Lloyd/[39]
N2103	BIK2877	*recC266*::Tn*10*	R. Lloyd/[39]
BIK3961		As AB1157, but *recB268*::Tn*10*	P1 (BIK2876) to AB1157
BIK3963		As AB1157, but *recC266*::Tn*10*	P1 (BIK2877) to AB1157
BIK806		As AB1157, but *recD1901*::Tn*10*	[40]
JC9239	BIK783	As AB1157, but *recF143*	A. J. Clark
BIK1538		As AB1157, but *recG258*::mini-Tn*10* Kan	P1 (BIK1400) to AB1157
JC12123	BIK 787	*recJ284*::Tn*10 his-4*	A. Clark/[41]
BIK2563		As AB1157, but *recJ284*::Tn*10*	P1 (BIK787) to AB1157
BIK2565		As AB1157, but *recN1502*::Tn*5*	P1 (BIK1044) to AB1157
KEN24	BIK1179	As AB1157, but *recO1504*::Tn*5*	K. Yamamoto/[40]
KD2216	BIK1048	*recQ1803*::Tn*3 ilv-145 metE46 his-4 trpC3 pro thi thyA*::Tn*5 thyR mtl-1 malA1 ara-9 galK2 lac-114 rpsL ton* F$^-$	H. Nakayama/[42]
BIK2680		As AB1157, but *recQ1803*::Tn*3*	P1 (BIK1048) to AB1157
BIK2577		As AB1157, but *recR252*::mini-Tn*10* Kan	P1 (BIK1399) to AB1157
HRS1004	BIK1331	*ΔruvAB*::Tc	T. Shiba & H. Shinagawa
HRS2302	BIK1620	As AB1157, but *ΔruvAB100*::Cm	H. Shinagawa/[24]
HRS1100	BIK1618	As AB1157, but *ΔruvC100*::Cm	H. Shinagawa/[43]
KEN72	BIK1051	As AB1157, but *ruvC53 eda*::Tn*10*	K. Yamamoto
JC8679	BIK813	As AB1157, but *recB21 recC22 sbcA23*	A. J. Clark/[44]
BIK1415		As JC8679, but *ΔrecA306*::Tn*10*	[26]
JC8691	BIK784	As JC8679, but *recE159*	A. J. Clark/[44]
JC9610	BIK786	As JC8679, but *recF143*	A. J. Clark/[44]
N2796	BIK1400	As JC8679, but *recG258*::mini-Tn*10* Kan	R. Lloyd/[45]
BIK814		As JC8679, but *recJ284*::Tn*10*	Kusano *et al.* (1994b)
BIK1044		As JC8679, but *recN1502*::Tn*5*	Takahashi *et al.* (1993)
BIK1192		As JC8679, but *recO*::Tn*5*	[26]
RDK1693	BIK1401	As JC8679, but *recQ1801*	S. Lovett/[46]
BIK1427		As JC8679, but *recQ1801 recJ284*::Tn*10*	[26]
BIK1224		As JC8679, but *recQ1803*::Tn*3*	[26]
AM265	BIK1399	As JC8679, but *recR252*::mini-Tn*10* Kan	R. Lloyd/[47]
BIK3884		As JC8679, but *recT101*::Tn*10*	N. Kobayashi-Takahashi
BIK1478		As JC8679, but *ΔruvAB*::Tc	P1 (BIK1331) to JC8679
BIK1050		As JC8679, but *ruvC53 eda*::Tn*10*	[26]
JC7623	BIK752	As AB1157, but *recB21 recC22 sbcB15 sbcC201*	T. Kato/[48,49]
BIK2176		As JC7623, but *ΔrecA306*::Tn*10*	P1 (BIK733) to JC7623
JC8111	BIK749	As JC7623, but *recF143*	A. J. Clark
BIK1772		As JC7623, but *recJ284*::Tn*10*	P1 (BIK814) to JC7623
BIK1212		As JC7623, but *recN262 tyrA16*::Tn*10*	[10]
BIK1774		As JC7623, but *recQ1803*::Tn*3*	P1 (BIK1224) to JC7623
BIK1776		As JC7623, but *recR252*::mini-Tn*10* Kan	P1 (BIK1399) to JC7623
KEN87	BIK1181	As JC7623, but *ruvC53 eda*::Tn*10*	K. Yamamoto
V66	BIK796	*recF143 his-4 met rpsL31 gal xyl(?) ara(?) argA21* F$^-$λ$^-$	A. Taylor/[21]
V68	BIK2411	As V66, but *recC73*	G. Smith/[50]
V73	BIK1275	As V66, but *recC73 recC1001*	G. Smith/[21,50]
V69	BIK1272	As V66, but *recC73 recC1002*	G. Smith/[21]
V71	BIK1273	As V66, but *recC73 recC1003*	G. Smith/[21,50]
V72	BIK1274	As V66, but *recC73 recC1004*	G. Smith/[21,50]
V1296	BIK1910	As V66, but *recC2145*	G. Smith/[22]
V1360	BIK1911	As V66, but *recB2154*	G. Smith/[22]
V1363	BIK1912	As V66, but *recB2155*	G. Smith/[22]
BIK1288		As V66, but *recF*$^+$*zic*::Tn*10*	[51]
BIK3713		As BIK1288 (tet^S)	tet^S selection from BIK1288
NK5992	BIK800	*IN (rrnD-rrnE)*1 λ$^-$ F$^-$ *argA81*::Tn*10*	N. Kleckner via A. Taylor
BIK3732		As BIK2411, but *argA81*::Tn10	P1 (BIK800) to BIK2411
BIK3738		As BIK3713, but *recC73 argA81*::Tn10	P1 (BIK3732) to BIK3713

Table 1: Bacterial strains used here *(Continued)*

BIK4034		As AB1157, but *recC73 argA81*::Tn*10*	P1 (BIK3732) to AB1157
A211	BIK1276	*IN (rrnD-rrnE)*1 λ^- F^- *lacZ*^{s20}Y^{const}*gyrB*$^+$*recF*$^+$*zic*::Tn*10*	A. Miura
BIK1286		As BIK1275, but *recF*$^+$*zic*::Tn*10*	P1 (BIK1276) to BIK1275
BIK1290		As BIK1272, but *recF*$^+$*zic*::Tn*10*	P1 (BIK1276) to BIK1272
BIK1282		As BIK1273, but *recF*$^+$*zic*::Tn*10*	[51]
BIK1284		As BIK1274, but *recF*$^+$*zic*::Tn*10*	[51]
BIK2445		As BIK1910, but *recF*$^+$*zic*::Tn*10*	[51]
BIK2446		As BIK1911, but *recF*$^+$*zic*::Tn*10*	[51]
BIK2447		As BIK1912, but *recF*$^+$*zic*::Tn*10*	[51]

Preparation of DNA samples in agarose gel

The cells were lysed in agarose gel by a modification of the method of Kusano *et al*. [35]. Cells were grown in 5 ml of L-broth with or without antibiotics to an OD_{660} of 0.4 and were harvested. This OD_{660} of 0.4 corresponds to 5 × 10E8 to 1 × 10E9 cells/ml depending on the strain. One milliliter of the culture was transferred to a micro-tube and mixed with 2,4-dinitrophenol (to the final concentration of 0.01%), which blocks energy metabolism. After centrifugation, the pellet was washed twice with a half volume of 10 mM Tris-HCl (pH 7.5), 1 M NaCl and 2,4-dinitrophenol. The cells were suspended in 0.5 ml of the same buffer, mixed with the same volume of 1.0% of InCert agarose (FMC), split into 10 molds, and allowed to solidify at 4°C. One agarose plug, thus obtained, corresponds to 0.04 OD_{660} of the culture. Six of these agarose plugs were treated at 37°C for 15 hrs with 2.5 ml of a solution containing 6 mM Tris-HCl (pH 7.5), 1 M NaCl, 0.1 M EDTA, Brij-58 (0.5%), sodium deoxycholate (0.2%), sodium lauryl sarcosinate (0.5%), lysozyme (1 mg/ml) and RNase A (20 mg/ml). The plugs were then washed with 0.5 M EDTA (pH 9.5), treated at 50°C for 48 hrs with 2.5 ml of a solution containing 0.5 M EDTA, 1% SDS and 2 mg/ml proteinase K (pH 9.5), and washed with 0.5 M EDTA (pH 9.5).

Pulsed-field gel electrophoresis

The sample plugs were placed in the wells of a running gel (1.0% (w/v) SeaKem GTG agarose (FMC)) and solidified with molten 1.0% agarose. Pulsed-field gel electrophoresis was carried out in a Pharmacia/LKB apparatus under the following conditions: electrophoresis buffer, 1 × TBE (45 mM Tris-borate/1.25 mM EDTA); 165V; pulse time, 120 sec; run time, 24 hrs; temperature, 10°C. As a size marker, a plug containing yeast (*Saccharomyces cerevisiae*) chromosomes (Pharmacia) was used. After the run, the gel was stained with ethidium bromide, and photographed under ultraviolet illumination. The DNA in the region of the huge linear chromosomes was quantified using a VILBER LOURMAT apparatus with BIO-PROFIL software.

The control experiment (*Xba*I digestion before the run) was done in a CHEF-DR III system (Bio-Rad) under the following conditions: electrophoresis buffer, 0.5 × TBE; 6 V/cm; angle, 120°; pulse time, 4 × 50 sec; run time, 20 hrs; temperature, 14°C. After the run, agarose gels were processed as described above.

Authors' contributions

NH carried out all of the experiment and drafted the manuscript. IK supervised the work and edited the manuscript. All authors read and approved the final manuscript.

List of abbreviations

ds, double-strand. *E. coli, Escherichia coli*. Amp, ampicillin. Cml, chloramphenicol. Kan, kanamycin. Tet, tetracycline.

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