Roles of ruvA, ruvC and recG Gene Functions in Normal and DNA Damage-Inducible Replication of the Escherichia coli Chromosome

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ABSTRACT

Induction of the SOS response in Escherichia coli activates normally repressed DNA replication which is termed inducible stable DNA replication (iSDR). We previously demonstrated that initiation of iSDR requires the products of genes, such as recA, recB and recC, that are involved in the early stages of homologous recombination. By measuring the copy number increase of the origin (oriM1) region on the chromosome, we show, in this study, that initiation of iSDR is stimulated by mutations in the ruvA, ruvC and recG genes which are involved in the late stages of homologous recombination. Continuation of iSDR, on the other hand, is inhibited by these mutations. The results suggest that Holliday recombination intermediates, left on the chromosome due to abortive recombination, arrest replication fork movement. Low levels of iSDR and sfiA (sulA) gene expression were also observed in exponentially growing ruvA, ruvC and recG mutants, suggesting that the SOS response is chronically induced in these mutants. We propose that replication forks are arrested in these mutants, albeit at a low frequency, even under the normal (uninduced) conditions.

DNA replication and recombination occur concurrently in Escherichia coli cells and are tightly interrelated. The relationship becomes evident in SOS-induced cells. Blockade of an active replication fork by DNA-damaging treatments or thymine deprivation induces the SOS response [for a review, see Walker (1984)]. The response profoundly alters several aspects of DNA replication [see Witten (1991) for a review]. Initiation of chromosome replication usually occurs at oriC and requires DnaA protein, transcription and translation [for a review see McMacken et al. (1987)]. After induction of the SOS response, however, the chromosome can be replicated in the absence of these factors (Kogoma and Lark 1970, 1975; Ciesla and Jonczyk 1980; Magee et al. 1992) [for a review, see Asai and Kogoma (1994)]. This type of DNA replication, which is termed inducible stable DNA replication (iSDR), thus occurs in the presence of rifampicin (RIF) and chloramphenicol (CAM), inhibitors of transcription and translation, respectively. Two origins (oriM1A and oriM1B) for iSDR are mapped in two separable regions within the minimal oriC (Asai et al. 1994). The terC region also contains another origin (oriM2) for iSDR (Magee et al. 1992). The replication originating from these origins proceeds bidirectionally (Magee et al. 1992).

Initiation of iSDR requires gene functions that are involved in the early stages of homologous recombination. iSDR is not induced in recA428 mutant cells which are defective in homologous recombination but proficient in SOS induction (Asai et al. 1993), indicating that the recombinase activity of RecA is necessary for iSDR initiation. The helicase activity of RecBC or RecBCD enzyme, which unwinds linear double-stranded (ds) DNA from the ends, is also essential for iSDR induction (Magee and Kogoma 1990; Asai et al. 1993). This was concluded from the observation that recD(Null) mutants are proficient in iSDR induction whereas recB and recC mutations block the induction completely. The only enzymatic activity thought to remain in the RecBC enzyme prepared from recD(Null) mutant cells is the helicase activity (Palas and Kusher 1990) [for a review, see Rosenberg and Hastings (1991)]. Recently, in vivo experiments have also demonstrated that RecBC enzyme in recD mutant cells is capable of DNA unwinding, but not DNA degradation (Rinken et al. 1992).

Defects in the RecBCD pathway of homologous recombination in recB and recC mutants are suppressed by a mutation in the sbcA gene or by mutations in the sbcB and sbcC genes, which activate the RecE or RecF alternative pathways, respectively [for reviews, see Smith (1988) and Clark (1991)]. Inactivation of the sbcA gene leads to the expression of exonuclease VIII (Exo VIII), the recE gene product, and also the recT gene product, both of which are essential for the RecE pathway (Clark et al. 1993). Exo VIII digests one strand of dsDNA processively from the 5' end (Joseph and Kohondner 1993). We have previously demonstrated that defects of recBC mutants in iSDR induction are also suppressed by an sbcA mutation and that the suppression is abolished by a mutation in the recE gene (Asai et al. 1993). These
results strongly support the conclusion that homologous recombination is involved in iSDR initiation and indicate that the RecE pathway can mediate the initiation reaction. Similarly, iSDR is induced in recB/C sbeB sbeC mutant cells and the inducibility is eliminated by a mutation in the recE gene, which is involved in the RecE pathway of homologous recombination (T. Asai and T. Kogoma, manuscript in preparation). This indicates that the RecE pathway can also catalyze the initiation reaction of iSDR.

On the basis of the above and other observations, we previously proposed a model (the D-loop model) (Asai et al. 1993) that, upon induction of the SOS response, a double-strand break is introduced into the chromosome within or near the origin of iSDR. Single-stranded (ss) DNA generated from the break by RecBC(D) helicase or Exo VIII would then be assimilated into an uncut homolog by the action of RecA recombinase. This reaction, i.e., D-loop formation [for reviews see Rosenberg and Hastings (1991), Clark (1991), Kowalczykowski (1991) and West (1992)], accomplishes the initial duplex opening essential for initiation of chromosome replication. Actual synthesis of daughter DNA molecules would then be primed by a PriA-mediated mechanism [for a review, see Asai and Kogoma (1994)]. The D-loop model is supported by our recent observation that an artificial double-strand break introduced into plasmid DNA induces, in the presence of an intact homolog, extensive DNA replication that is dependent on homologous recombination functions. The replication is completely dependent on homologous recombination functions.

The model implies that both homologous recombination and iSDR initiation share D-loop structures as a common intermediate, and suggests that the number of D-loops available for initiation determines the level of iSDR. In support of this contention, introduction of additional copies of oriM1 into a cell, which would result in an increase of D-loops in the cell, stimulates iSDR (Asai et al. 1994). Mutations in the ruvA, ruvB, ruvC and recG genes also enhance iSDR activity. The products of these genes are involved in the stages in homologous recombination that follow D-loop formation. RuvA and RuvB constitute the RuvAB helicase which catalyzes bidirectional branch migration (Tsaneva et al. 1992). RuvC, a resolvase, catalyzes resolution of Holliday junction intermediates (Connolly et al. 1991; Dundurda et al. 1991; Iwasaki et al. 1991). RecG is another helicase which promotes reversible branch migration of Holliday junctions (Whitby et al. 1993). Recently, a mutation, designated rus, has been identified which activates an additional factor, most likely a resolvase, that works with RecG to resolve Holliday junctions independently of RuvAB and RuvC proteins (Mandal et al. 1993). The observation that inactivation of these genes stimulates iSDR (Asai et al. 1993) strongly suggests that the failure to process Holliday junctions also results in an increase in the number of D-loops available for iSDR initiation.

To obtain further insight into the roles of homologous recombination in DNA replication in SOS-induced cells, we have analyzed effects of various rec mutations on the elongation stage of DNA replication. The results suggest that activities of RuvA, RuvC and RecG are necessary for replication fork movement in SOS-induced cells. Moreover, we have found that the SOS response is chronically induced in exponentially growing ruvA, ruvC and recG mutants, suggesting that the activities of these genes are important for uninterrupted replication fork movement even in uninduced cells.

**MATERIALS AND METHODS**

**Media and growth conditions:** Unless otherwise stated, cells were grown at 37°C in M9 salts-glucose medium (Miller 1992) supplemented with casamino acids (0.2%; Difco Laboratories, Detroit, Michigan), required amino acids (50 μg/ml), thymine (8 μg/ml) and thiamine hydrochloride (2 μg/ml).

**Chemicals and radioisotopes:** Chemicals were purchased from Sigma Chemical (St. Louis, Missouri). [methyl-3H]Thymine and [α-32P]deoxyctydine-5'-triphosphate were from New England Nuclear Corp. (Boston, Massachusetts).

**E. coli strains:** E. coli strains used in this study are listed in Table 1. Strains were constructed by phage P1-mediated transduction. The presence of the recD1903::mini-tet mutation was confirmed by the sensitivity to phage T4 gene 2 mutants (Chaudhury and Smirnoff 1984). The presence of the recJ28kTn10, ruuc1, recG258::Tn10kan and ruvA606::Tn10 mutations was confirmed by sensitivity to UV irradiation. The ruuC mutation was transduced by virtue of its linkage to the ada51::Tn10 mutation. Tetracycline-sensitive derivatives from Tn10-carrying strains were isolated by the method of Bogin et al. (1980).

**Induction of the SOS response and determination of the iSDR activity:** These were described previously (Asai et al. 1993). Cells were grown to exponential phase in the presence of [3H]thymine (10 μCi/8 μg/ml) for three generations. Radioactive thymine was then removed by filtration and the cells were resuspended in medium lacking required thymine. Immediately, a sample (90 μl) was immediately added to one half of the suspension (uninduced control). The other half of the suspension was starved of thymine for a duration approximately equivalent to two doublings (shown in the legend to Figure 1) and then the mixture was added. Samples (90 μl) were taken at the indicated times and the radioactivity of the TCA-insoluble fraction was determined by liquid scintillation counting as described previously (Kogoma et al. 1979). The cell suspension was split into two halves and a mixture of [3H]thymine (10 μCi/8 μg/ml), Rif (200 μg/ml) and CAM (150 μg/ml) was immediately added to one half of the suspension (uninduced control). The other half of the suspension was starved of thymine for a duration approximately equivalent to two doublings (shown in the legend to Figure 1) and then the mixture was added. Samples (90 μl) were taken at the indicated times and the radioactivity of the TCA-insoluble fraction of each sample was determined as described above. The relative increase of DNA during iSDR was determined by dividing the radioactivity of each sample taken after the addition of Rif and CAM by the initial radioactivity. The average of standard error of means of relative DNA increases obtained by this method was calculated from a number of independent experiments. It was 4.3%.

**Extraction of chromosomal DNA and Southern blot hybridization:** These were described previously (Mages et al. 1992). Cells were grown to exponential phase in the presence of [3H]thymine (10 μCi/8 μg/ml). Radioactive thymine was then removed by filtration and the cells were resuspended in me-
dium lacking required thymine. The suspension was starved of thymine as described above. A mixture of RIF (200 pg/ml) and CAM (150 pg/ml) was then added and samples (500 µl) were taken at the indicated times. Chromosomal DNA was extracted from each sample according to AUSUBEL et al. (1987) except that the cells were treated with lysozyme at a final concentration of 1 mg/ml for 60 min at 37° before the addition of SDS and proteinase K. Amounts of DNA samples normalized to give equal ³H counts were digested with restriction enzymes and analyzed on agarose gel. Southern blot hybridization was carried out as described by MILLER et al. (1987) with nylon membranes (Hybond-N; Amersham, Arlington Heights, Illinois). The PstI (co-localize -1500 (MEIJER et al. 1979; SUGIMOTO et al. 1979) BamHI (~100) fragment carrying the gidA sequence (WALKER et al. 1984) and the EcoRI-HindIII fragment (1.3 kb of pMTR1560 (Amersham) containing the lacP gene) were used for the preparation of probes to determine the copy number of the oriM1 and lac regions, respectively. Probes were prepared by using Random Primers DNA Labeling System (BRL, Gaithersburg, Maryland) and [α³²P]deoxyctydine-5'-triphosphate. The copy number of each region was determined by counting the corresponding bands from Southern blots and counting radioactivity by liquid scintillation. The average of standard error of mean number determinations was calculated from a number of independent experiments. It was 9.5%.

**Determination of sfiA::lacZ expression:** β-Galactosidase activity was measured as described by MILLER (1992).

**RESULTS**

**Effects of rec mutations on the initiation of iSDR:** By monitoring the incorporation of [³H]thymine into the acid-insoluble fraction, we previously demonstrated that the initial rate of iSDR was stimulated by mutations in the recD, recJ, ruwA, ruwB, ruwC and recG genes (ASI et al. 1993). Similar results were obtained in the present study (Figure 1). To test whether or not the stimulation by these mutations is due to an increase in the initiation frequency of iSDR, we measured the copy number increase of the oriM1 region on the chromosome in the presence of RIF and CAM after SOS induction (Figure 2A). The result shows that the copy number of the oriM1 region in ruwC and recG mutants is about twice as high as that in wild type (wt). The ruwA::Tn10 mutation had a larger effect on the stimulation of iSDR initiation than the ruwC::Tn10 mutation. The effects of the ruwC and recG mutations were additive, though introduction of the recG mutation into the ruwA mutant did not show appreciable effects. recD mutations also stimulate the copy number increase of the oriM1 region (ASI et al. 1993). These results support our postulate that the enhanced iSDR in these rec mutants is a result of the elevated frequency of initiation.

In the wt and ruwC and recG single mutant cells, the relative increase of chromosomal DNA measured by [³H]thymine incorporation (Figure 1B) is about three-fold higher than the relative copy number increase at oriM1 (Figure 2A). This is because the DNA synthesis measured by [³H]thymine incorporation reflects DNA replication that is originated not only from oriM but also from oriM2 and other minor origins (MACEE et al. 1992). The difference in relative increase of DNA between the two assays for other mutants could be explained by the inhibition of isDR elongation (see below).

**Effects of rec mutations on the elongation of iSDR:** In recD1903::mini-tet mutant cells, the rate of DNA synthesis decreased gradually, but iSDR continued at a relatively high level until the end of an 8-hour incubation (Figure 1A). A very high level of incorporation of [³H]thymine during the first few hours is due mainly to the increased number of D-loops accumulated during thymine

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**Table 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source, reference or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQ634</td>
<td>F&quot; ilo metB his-29 trpA9605 pro thyA deoB (or C)</td>
<td>OGAWA et al. (1984)</td>
</tr>
<tr>
<td>AQ1802</td>
<td>As AQ634 but rec1284::Tn10</td>
<td>AQ634 × Pl.AQ9029 select Tc' UV⁺</td>
</tr>
<tr>
<td>AQ8202</td>
<td>As AQ634 but rec1284::Tn10 (Tc')</td>
<td>AQ634 × Pl.TNM554 select Tc' UV⁺</td>
</tr>
<tr>
<td>AQ8070</td>
<td>As AQ634 but rec1284::Tn10 recD1903:mini-tet</td>
<td>AQ3862 select Tc' on Bochner plates.</td>
</tr>
<tr>
<td>AQ1107</td>
<td>F&quot; (pro lac) pro tpsl. Ip[sfiA::lacZ, c(lnd-)]</td>
<td>AQ8022 × Pl.DPB271 select Tc', T4.2⁺</td>
</tr>
<tr>
<td>AQ8194</td>
<td>As AQ634 but recG258::Tn10</td>
<td>HUSSMAN and D'ARI (1981)</td>
</tr>
<tr>
<td>AQ8195</td>
<td>As AQ634 but recG258::Tn10 recG258::Tn10</td>
<td>AQ9317 × Pl.N2731 select Km', UV⁺</td>
</tr>
<tr>
<td>AQ8134</td>
<td>As AQ634 but rec12903::mini-tet</td>
<td>AQ834 × Pl.AQ8020 select Tc', T4.2⁺</td>
</tr>
<tr>
<td>AQ8144</td>
<td>As AQ634 but ruwC51 eda-51::Tn10 recG258::Tn10kan</td>
<td>AQ7960 × Pl.N2731 select Km'</td>
</tr>
<tr>
<td>AQ8194</td>
<td>As AQ7960 × Pl.N2731 select Km'</td>
<td>AQ8022 × Pl.N2731 select Km'</td>
</tr>
<tr>
<td>AQ8198</td>
<td>As AQ8107 but ruwC51 eda-51::Tn10 recG258::Tn10kan</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>AQ8360</td>
<td>As AQ8107 but ruwC51 eda-51::Tn10 recG258::Tn10kan</td>
<td>AQ8119 × Pl.N2731 select Km'</td>
</tr>
<tr>
<td>AQ8917</td>
<td>As AQ634 but ruwA60::Tn10</td>
<td>AQ8198 × Pl.N2731 select Km'</td>
</tr>
<tr>
<td>AQ9335</td>
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<td>AQ854 × Pl.N2057 select Tc', UV⁺</td>
</tr>
<tr>
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<td>AQ8130 × Pl.N2057 select Tc'</td>
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<tr>
<td>AQ9439</td>
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<tr>
<td>DP8071</td>
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<td>BUCK and COHEN (1986)</td>
</tr>
<tr>
<td>N2057</td>
<td>ruwA60::Tn10</td>
<td>SHARPLES et al. (1990)</td>
</tr>
<tr>
<td>N2731</td>
<td>recG258::Tn10kan</td>
<td>SHARPLES and BUCKMAN (1991)</td>
</tr>
<tr>
<td>TNM554</td>
<td>ruwC51 eda-51::Tn10</td>
<td>G. J. SHARPLES</td>
</tr>
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UV⁺, sensitivity to UV; T4.2⁺, sensitivity to phage T4 gene 2 mutants.
starvation (see Introduction). The recJ304::Tnl010 mutant also showed a similar incorporation profile (Figure 1A).

In contrast, ruvC and recG mutants lost iSDR activity rapidly after two hours of steady increase at high rates and ceased completely by the end of the incubation (Figure 1B). Similar results were obtained with recD recG and recf recG double mutants (Figure 1C). The inhibition of iSDR was particularly severe in ruvA mutant cells (Figure 1D). Introduction of the recG258::Tnl10kan mutation into the ruvC and ruvA mutants aggravated the effect (Figures 2C and D). Since the frequency of initiation of iSDR is elevated in these mutants (Figure 2A), these results strongly suggested that the activities of the ruvA, ruvC and recG genes are necessary for elongation of iSDR. This idea was examined by measuring the copy number increase of the lac region (located at 8 min on the chromosome) (Figure 2B). In contrast to the oriM1 region (84 min), the copy number increase of the lac region in these mutants was lower than that of wt. The levels of the copy number increase were in good agreement with the rate of [3H]thymine incorporation seen in Figure 1. This indicates that the progress of replication forks started from oriM1 was arrested in these mutants before they reached the lac region. Similar results were obtained with a galK probe (data not shown; for a result obtained with ruvC recG double mutant cells, see Asai et al. 1994), suggesting that replication forks started from oriM2 (~31 min) as well as oriM1 were also arrested before they reached the galK region (17 min).

The relative copy number increase of the lac region in wt cells is about four times as high as that of the oriM1 region. The second reason is that the lac region is replicated by replication forks originated not only from oriM1 but also from oriM2.

Chronic induction of the SOS response in ruvA, ruvC and recG mutants: In ruvA, ruvC and recG mutants,
DNA synthesis continued, albeit at a slow rate, after the addition of RIF and CAM without SOS-inducing treatment (open symbols in Figure 1). This can be clearly seen in Figure 3A which depicts the incorporation of \(^{3}H\)thymine in uninduced wt, \(recG\) and \(ruvC\) \(recG\) cells in an expanded ordinate. The level of drug-resistant DNA replication observed in several \(rec\) mutants without SOS-inducing treatments is summarized in Figure 3B. The result suggests that signals for iSDR induction are constitutively generated in these mutants when they grow exponentially. We further found that the level of \(sfiA\) expression is significantly high in \(ruvA\), \(ruvC\) and \(recG\) mutants compared with wt (Figure 3B). Elevated expression of the \(sfiA\) gene in exponentially growing \(recG\) mutants was previously reported (Lloyd and Buckman 1991). These observations indicate that the SOS response is chronically expressed in these mutants.

We considered the possibility that the condition that leads to the chronic SOS expression and constitutive iSDR activity in uninduced cells (Figure 3) might also give rise to propensity of the mutant cells to express higher levels of the SOS response than the wt level of induction. This could simply be a reason for the elevated iSDR activity observed in induced cells of these mutants (see Figure 1). The possibility was tested by analyzing the expression of the \(sfiA\) gene in \(ruvC\) and \(recG\) mutants after treating the cells with mitomycin C (MMC). The result (Figure 4) shows that the SOS inducibility in these mutants was in fact lower than that in wt. The reduced induction of the SOS response in MMC-treated \(recG258::Tn10kan\) mutant cells was previously observed with a different genetic background (Lloyd and Buckman 1991). These results make the above possibility very unlikely.

**DISCUSSION**

The major finding in this study is that the continuation of iSDR is severely inhibited in \(ruvA\), \(ruvC\), and \(recG\) mutants which have defects in late stages of homologous recombination (Figure 1). This effect is striking particularly in light of the fact that iSDR initiation is actually stimulated by these mutations and can continue for at least 4 hr in most mutants (Figure 2A). Therefore, it seems as if replication forks that are repeatedly formed at \(oriMs\) would continue to travel to a blockade at which they might pile up. Since DNA increase comes to a complete halt at later times, for example, after 6 hours in \(recG\) \(recD\) and \(recG\) \(recJ\) double mutants (Figure 2C), it is likely either that initiation eventually completely ceases or that replication forks travel so short a distance at later times that the replication does not significantly contribute to the overall DNA increase.

Several lines of evidence suggest that in *E. coli* the rate-limiting step in homologous recombination is often
formation of appropriately activated DNA substrate (recombinogenic DNA) (Konrad 1977; Zieg et al. 1978; Hays and Boehmer 1978; Korsa and Hays 1980; Zagursky and Hays 1983). In thymine-starved cells, ssDNA, which is a substrate of RecA-mediated strand exchange reaction (Kowalczykowski 1991; West 1992), could be generated at an elevated frequency due to the stalling of replication fork movement. The frequency of homologous recombination is thus thought to be enhanced in such cells. Whole recombination reactions, however, cannot be completed in ruvC and recG mutant cells, and the intermediates such as Holliday junctions would remain unresolved. The helicases associated with replication forks (e.g., DnaB helicase) could not overcome the obstacle. Consequently, the replication fork originated from oriMs upon restoration of thymine would be arrested at these sites. In vitro experiments (Pugh and Cox 1987) suggest that, even if Holliday junctions are resolved by RuwC, RecA filaments may still hold two DNA duplexes together in vivo. This could also result in the inhibition of fork movement. It has recently been suggested that the helicase activity of RuvAB could be employed to remove these "used" RecA filaments from DNA (Kuzminov 1993). This hypothesis accounts for our observation that elongation of ISDR is inhibited in ruvA mutant cells even in the presence of active RuwC. In fact, arrest of fork movement appears to be much more severe in a ruvA single mutant than in ruwC and recG single mutants. Since expression of the ruvA and ruwB genes is derepressed by SOS induction (Sharplles et al. 1990; Lloyd and Buckman 1991) can be accounted for to a large extent, if not solely, by the blockade of replication fork movement due to the unresolved recombination intermediates in these mutants.

Mutations in ruv genes have been shown to reduce the recovery of F-prime transconjugants (Benson et al. 1991). Since the reduction depends on recA+ and homologous sequences between conjugative plasmids and the chromosome, it has been proposed that abortive recombination in ruv mutants results in loss of viability. Our observations which suggest that replication forks are arrested in ruv mutants could account for the loss of viability.

The activities of the ruvA, ruwC and recG genes must be important not only for ISDR but also for normal DNA replication started from oriC in SOS-induced cells. We wish to propose, further, that the same activities are necessary for uninterrupted replication fork movement even under the normal (uninduced) conditions. The procedure employed in Figure 1 was unsuccessful in detecting replication fork arrest in uninduced cells. This is probably due to low frequency of recombination in uninduced cells and high frequency of replication initiation at oriC compared with the frequencies at oriMs. However, we observed chronic induction of the SOS response (induction of the sfiA gene and ISDR) in exponentially growing ruvA, ruwC and recG mutant cells. The chromosomes of aerobically growing cells suffer a constant insult inflicted by active oxygen species (Cox 1993). Homologous recombination initiated, albeit at a low frequency, from these damage sites in the mutants would leave some intermediates unresolved. These intermediates could arrest replication forks and induce the SOS response. In keeping with this proposal, exponentially growing cell populations of ruvA, ruwC and recG mutants contain a significant portion of filamented cells (Sharplles and Lloyd 1982, our unpublished observation).

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