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Double-Strand Break Generation under Deoxyribonucleotide Starvation in *Escherichia coli*[▽]

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Stalled replication forks produced by three different ways of depleting deoxynucleoside triphosphate showed different capacities to undergo "replication fork reversal." This reaction occurred at the stalled forks generated by hydroxyurea treatment, was impaired under thermal inactivation of ribonucleoside reductase, and did not take place under thymine starvation.

Stalled replication forks create the need for replication reactivation, and different ways of restarting replication have been proposed (19). In several replication mutants, the stalled forks generated upon the inactivation of the mutant enzyme are reversed and result in the formation of a Holliday junction (HJ) adjacent to a DNA double-strand end, a reaction called "replication fork reversal" (RFR) (Fig. 1A) (19, 29, 30). In a *rec*-proficient background, this intermediary could be processed without generating DNA double-strand breaks (DSBs) by using the recombination proteins RecBCD and RecA and by the HJ-specific resolvase RuvABC (Fig. 1B) (16). In contrast, in the absence of RecBCD activity (Fig. 1C), resolution of the RFR-produced HJ is done by RuvABC resolvase and leads to fork breakage. These particular DSBs are dependent on RuvABC activity in a *recB*-deficient background.

To verify the RFR process, a *recB*-deficient background should be used (i) to inhibit the degradation or the recombinational repair of the DNA tail created by the regression of the fork (20) (Fig. 1B), allowing RuvABC resolvase to transform this tail in a DSB, and (ii) to inhibit the repair of the DSBs generated by RuvABC resolvase (Fig. 1C). According to the RFR model, the occurrence of this process at the stalled forks can be verified by testing whether there is an increase of DSBs in a *recB*-deficient background and determining whether these DSBs are dependent on RuvABC resolvase activity by measuring the levels of DSBs in *recB*- and *recB ruvABC*-deficient backgrounds (Fig. 1C) (29). The occurrence of RFR at the stalled forks has been verified by this system in several replication mutants, such as in the helicase mutants *rep* and *dnaBts* (18, 29), in the *holD*^{G10} mutant (6), in the *dnaEts* mutant at 42°C, and in the *dnaNts* mutant at 37°C (11).

If RFR does not take place at the stalled fork, at least two situations may arise. On the one hand, there would be an increased level of DSBs independent of RuvABC activity and generated by another, unknown endonuclease (Fig. 1E), as in the case of *dnaBts recB ruvABC* at 42°C in the absence of RecA protein (30). On the other hand, there would be no increase in the level of DSBs, probably because the stalled forks are not susceptible to the endonuclease action, and the restarting of the forks would take place without the generation of fork breakage. This situation has been described in *gyrB* mutants (10) and when *ter* replication termination sequences were placed at ectopic positions on the bacterial chromosome (3).

Using the system described above, in the present work we studied the fate of the stalled replication forks caused by deoxynucleoside triphosphate (dNTP) depletion generated by chemical or structural inactivation of the ribonucleoside diphosphate reductase (NDP reductase) and by thymine starvation.

NDP reductase is the only specific enzyme required for the enzymatic formation of dNTP, the precursors of DNA synthesis in *Escherichia coli*, and has been proposed to be a structural component of the replication hyperstructure (12). It is a 1:1 complex of two subunits called proteins R1 and R2, coded by genes *nrdA* and *nrdB*, respectively (for a review, see reference 5). Inactivation of NDP reductase was attained in two ways: by the addition of hydroxyurea (Hu), which is a specific inhibitor of NDP reductase activity (26, 27, 31), or by the incubation of an *nrdA101* thermosensitive mutant strain at 42°C, which destroys the active structure of the enzyme (9). Thymine starvation was achieved by removing the exogenous thymidine from the growth medium, as the strains used were *thyA* mutants requiring thymine or thymidine for growth.

Cultures of strains JK626 (*thyA arg his recB258*::Tn*10*), JK707 (*thyA arg his recB258*::Tn*10* Δ*ruvABC*::*Cm*) and their isogenic *nrdA101* counterparts JS628 and JS705, respectively, were grown at 30°C in M9 minimal medium (MM9) containing 5 μg/ml thymidine, 5 μCi/ml [*methyl*-^{3}H]thymidine (100 Ci/mmol), 20 μg/ml of required amino acids, and 0.2% of Casamino Acids. By the time the cultures reached an optical density at 450 nm (OD_{450}) of 0.2, one portion each of the JK626 (*recB*) and JK707 (*recB ruvABC*) cultures was treated with Hu 50 mM (freshly prepared at 1 M in MM9), and a second portion was thymidine starved at 30°C by collecting the cells on a Millipore filter and washing and resuspending them in MM9 free of thymidine. To achieve the thermal inactivation of NDP reductase, cultures of JS628 and JS705 *nrdA101* strains growing at 30°C were transferred to 42°C at an OD_{450} of 0.2. To determine the extent of DSBs, the amount of linear DNA

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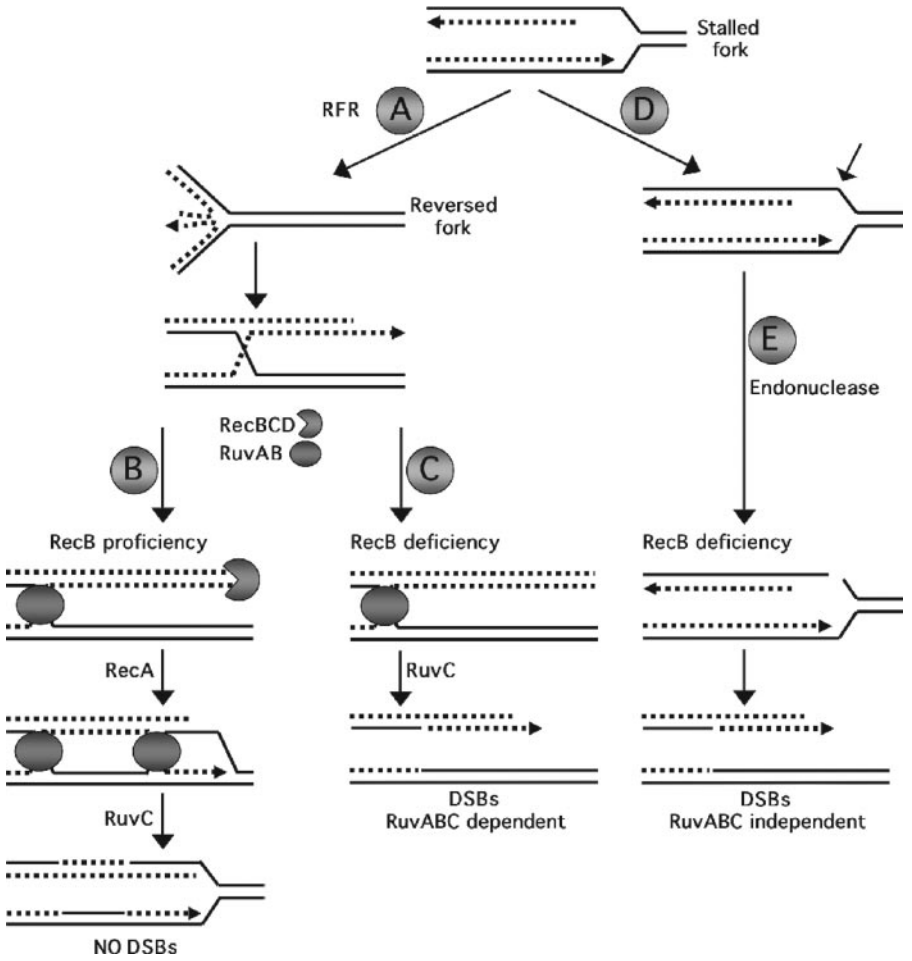

FIG. 1. The fate of the stalled forks. In the first step (A), the replication fork is arrested, causing fork reversal. The reversed fork forms an HJ (two alternative representations of this structure are shown—an open X and a parallel stacked X). In Rec^+ cells (B), RecBCD initiates RecA-dependent homologous recombination, and the resulting double HJ is resolved by RuvABC. In the absence of RecBCD (C), resolution of the HJ by RuvABC leads to DSBs at the stalled replication fork. Alternatively, the replication fork is arrested without being regressed (D), and it is susceptible to be cut by an endonuclease, generating DSBs at the stalled replication fork (E). Continuous lines show parental chromosomes and dashed lines show newly synthesized strands.

was quantified before beginning the treatment, after two hours of treatment for Hu addition and for 42°C incubation, and after 30 min for the thymidine starvation treatment.

Cells labeled with [*methyl*-^{3}H]thymidine were gently lysed in agarose plugs, and their DNA was analyzed by pulse-field gel electrophoresis (PFGE), in which only linear chromosomes enter the gels and circular molecules remain in the wells (18, 29). All the PFGE linear DNA data were analyzed by the least-squares statistical approach, with measurements considered highly significantly different if P was <0.01.

The inactivation of NDP reductase by Hu addition increases by up to twofold the amount of linear DNA in the strain JK626 (*recB*) (JK626 with and without Hu, $P = 7.2\ 10^{-5}$), indicating the increase in DSBs (Fig. 2A). In order to test whether these DSBs resulted from the action of RuvABC, PFGE was performed with JK707 (*recB ruvABC*) in the presence of Hu. The level of linear DNA dramatically decreased (Fig. 3A), indicating that the DSBs induced by Hu addition resulted from the RuvABC resolvase activity in a *recB*-deficient background. Therefore, we conclude that RFR is induced at the stalled

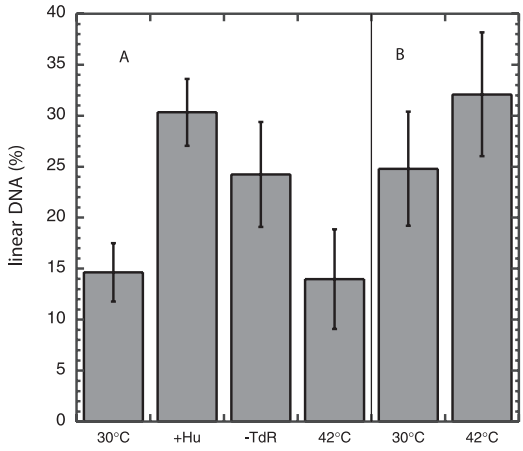

FIG. 2. Increases in levels of linear DNA in *recB*-deficient strains under depletion of dNTP supply by the addition of Hu (+Hu) or by thymidine starvation (−TdR) in the *nrdA*$^+$ strain (A) or by shifting the culture of the *nrdA101* strain to 42°C (B). Error bars indicate standard deviations of results from at least four independent experiments.

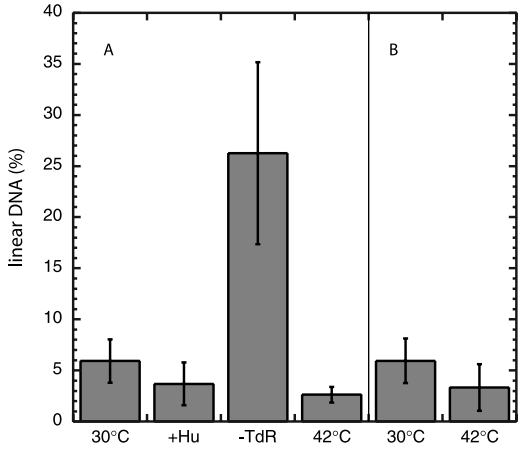

FIG. 3. Levels of linear DNA in *recB ruvABC*-deficient strains under depletion of dNTP supply. (A) *nrdA*$^+$ strain. (B) *nrdA101* strain. Treatments were the same as those described for Fig. 2. Error bars indicate standard deviations of results from at least four independent experiments.

forks generated by depletion of dNTP. However, when we tested whether incubation of the strain JS628 (*nrdA101 recB*) at 42°C led to DSB formation, we found only a slight increase in DSBs relative to the level at 30°C (Fig. 2B, compare JS628 at 30°C and at 42°C; $P = 0.006$). These DSBs generated at 42°C in the *nrdA101* mutant strain were still RuvABC-dependent (Fig. 3B), i.e., they resulted from the formation of an HJ at the stalled forks. That the *nrdA101* strain incubated at the restrictive temperature induced only a small increase in RuvABC-dependent DSBs would indicate that the stalled forks generated under thermal inactivation of the NDP reductase have a much lower propensity to be regressed and cut by RuvABC resolvase than the stalled forks generated under chemical inactivation of the enzyme.

To investigate the occurrence of RFR at stalled forks generated by TTP depletion without altering the NDP reductase, we measured DSBs in JK626 (*recB*) and JK707 (*recB ruvABC*) under thymine starvation using a similar approach to that performed with the other conditions. DNA breakage has been observed under thymine starvation, but whether DSBs occur has been controversial (22). Using neutral density gradients, Yoshinaga (32) was able to detect DSBs in thymine-starved *E. coli* cells, and, using neutral filter elution, DSBs were found in thymidilate synthase-negative mutants of mouse FM3A cells (2). In contrast, Nakayama et al. (23) using PFGE were unable to detect linear DNA from thymine-starved cells, but the technical and genetic system used in that work differed from our experimental conditions.

After 30 min of thymidine starvation, we found induction of DSBs in the strain JK626 (*recB*) (Fig. 2A), and the level of DSBs was not reduced in JK707 (*recB ruvABC*) (Fig. 3A). Consequently, these DSBs were not dependent on RuvABC resolvase activity, as they occurred under RuvABC inactivation, indicating that replication forks stalled by thymine starvation do not undergo the RFR process. The DSBs found under this treatment could have been generated (i) by direct endonucleolytic cleavage of single-strand breaks at or near stalled forks (14, 15, 32) (Fig. 1D), (ii) by cleavage of single-strand breaks at places not related to the replication fork (22), or (iii) by both.

In addition to the formation of RuvABC-dependent DSBs in a *recB*-deficient context, the RFR model would imply differences in the viability of *rec*-deficient strains under Hu treatment and thymidine starvation. On one hand, if RFR occurred after Hu addition, (i) a lethality in the *recB* mutant strain due to the unrepaired DSBs and (ii) a suppression of this lethality by the inactivation of RuvABC resolvase in a *recB ruvABC*-deficient strain would be expected. On the other hand, if RFR was not taking place under thymidine starvation, thymineless death (TLD) in the *recB* mutant strain should not be alleviated by the deficiency of the RuvABC resolvase, as DSBs induced by thymidine starvation are not avoided in a *recB ruvABC*-deficient strain.

Viability experiments were performed by growing the strains JK607 (*rec*$^+$), JK626 (*recB*), and JK707 (*recB ruvABC*) up to an OD of 0.2 and treating them with Hu addition or thymidine starvation in the same experimental conditions as for the PFGE experiments. At various times, 0.05-ml aliquots were removed, appropriately diluted, and plated on rich medium plates (yeast extract, 3 g/liter; peptone, 5 g/liter; agar, 13 g/liter) for determination of viability (Fig. 4). We found that the viability of the *recB*-deficient strain was highly sensitive to the addition of Hu and thymidine starvation compared with the viability of the wild type (Fig. 4A and B), as expected from the increased level of unrepaired DSBs in the absence of RecBC activity. This detrimental effect was recovered by the inactivation of RuvABC resolvase only in the case of Hu addition, but not under thymidine starvation (Fig. 4C). These results support the proposal that RFR occurs when DNA replication is inhibited by Hu treatment but not when it is performed by thymidine starvation. Furthermore, these results would correlate the fate of the stalled replication forks with the viability of the cells under these treatments. According to the viability results presented in this work, the occurrence of RFR would provide a "scaffold" for recombination proteins to bind and proceed with some of the recombination pathways (19) or with the protection of the stalled forks from cleavage (4), alleviating the cells from the loss of viability under certain conditions, such as Hu treatment (Fig. 4). Recently, Morganroth and Hanawalt have observed that TLD occurs after Hu addition (21). According to our proposal, a recovery of TLD could be expected after Hu addition, but it has to be pointed out that our prediction would be only in the case that the stalled fork underwent RFR under these conditions. In the abovementioned work, Hu addition was performed at the same time as the culture was thymine starved (21), and under these conditions, the fate of the stalled forks is, at this moment, unknown. Nevertheless, our results agree with the more general observation of the Morganroth and Hanawalt work that active DNA synthesis is not required for TLD, as we suggest that TLD would be related to the fate of the stalled replication under thymine starvation rather than to the occurrence of the replication process itself.

The present data together show that stalled replication forks generated by the depletion of nucleotides display a differential capacity to undergo RFR after depletion of dNTPs brought about in three different ways. Replication fork reversal occurred at the stalled forks generated by Hu addition, was

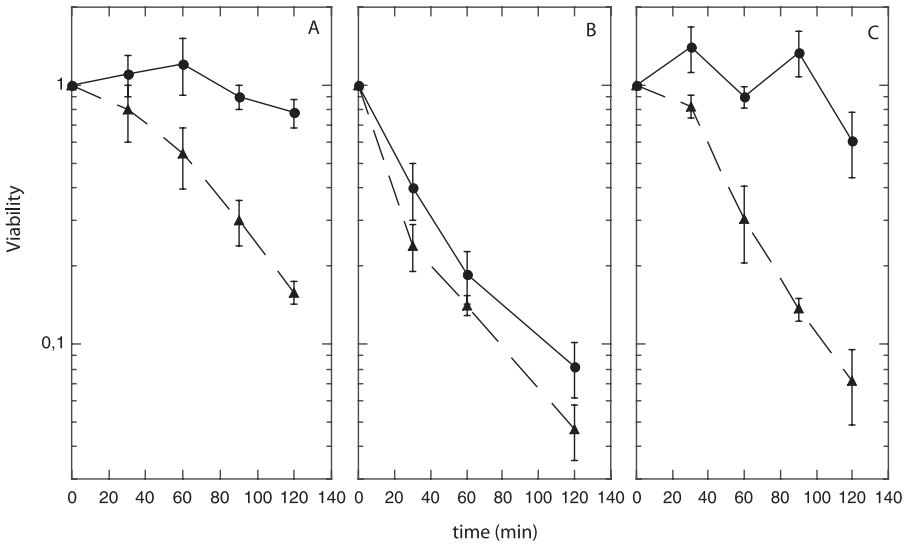

FIG. 4. Effects of Hu addition (●) and thymidine starvation (▲) on the viability of (A) the *rec*$^+$ strain, JK607; (B) the *recB*-deficient strain, JK626; and (C) the *recB ruvABC*-deficient strain, JK707. Error bars indicate standard deviations of results from at least four independent experiments.

impaired with thermally inactivated NDP reductase, and did not take place with thymine starvation.

These results are explained by positing a relationship between the presence of the replication proteins at the replication forks and the capacity of the replication forks to undergo RFR. Disassembly of the replication hyperstructure seems to be a requirement for the stalled replication forks to be regressed (7, 24). We propose that a "clean" disassembly of the replication hyperstructure would occur in the presence of Hu-inactivated NDP reductase and that this would allow the RFR reaction to take place. According to the proposal of NDP reductase being a component of the replication hyperstructure (12), structural changes in this enzyme induced by the incubation at 42°C (9) could account for a "distorted" disassembly of the hyperstructure. We propose that the presence of a structurally inactivated NDP reductase could cause some proteins to become "stuck" at the replication forks in such a way that the reversion of the replication fork would be impaired. In this sense, the incubation of the *nrdA101* mutant strain at 42°C generates a phenotype unique among replication mutants, since the induction of RFR by the thermal inactivation of proteins related to the replisome has been described (19). Only the *dnaN159* thermosensitive allele does not induce RFR after incubation at 42°C (although it does at 37°C), and in this case, β-clamp has been proposed to have a role in attracting the proteins that could catalyze fork reversal (11).

Under thymine starvation, no enzyme of the replication hyperstructure is likely to be affected, and we might think that when replication forks stop under this treatment, the disassembly of the replication hyperstructure would not take place. Replication proteins would stay at the replication fork, thereby hindering the reversion of the stalled forks. Nevertheless, thymine starvation is a pathological condition for the cell, which has a complicated web of responses (1), and it could not be ruled out that effects other than the proposed presence of the replication proteins at the replication fork could also account for the hindering of RFR at the stalled forks. We could tentatively propose the induction of some proteins capable of impairing RFR (1, 8, 28) or that the occurrence of changes in DNA supercoiling resulting from the observed DNA single-strand breaks (22) could interfere with the regression of the stalled replication forks (25).

Besides the absence of RFR at the replication forks under thymine starvation, the occurrence of DSBs independent of RuvABC endonuclease needs to be explained. At present, it is not known which protein(s) or cellular process might be involved in the production of these DSBs. It has been reported that a crude extract of thymine-starved *E.coli* cells exhibits an endonuclease activity not found in nonstarved cells (8). Furthermore, Nakayama and Hanawalt proposed that DNA breakage could be generated by the transcription process (22). We suggest that these DSBs (or at least a number of them) were originated by endonucleolytic cleavage at or near stalled replication forks (Fig. 1D and E). That these DSBs occurred at or near the replication forks would explain the connection between replication forks stalled by thymine starvation and TLD proposed by Maaloe and Hanawalt in the 1960s after showing that only those cells actively replicating DNA when thymine was removed underwent TLD (13, 17). Our proposal is that this connection is not by the replication process itself, as it has been shown not to be required (21), but through the fate of the stalled replication forks when thymine is removed.

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