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# Roles of ExoI and SbcCD Nucleases in "Reckless" DNA Degradation in *recA* Mutants of *Escherichia coli*[▽]

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**Exponentially growing *recA* mutant cells of *Escherichia coli* display pronounced DNA degradation that starts at the sites of DNA damage and depends on RecBCD nuclease (ExoV) activity. As a consequence of this "reckless" DNA degradation, populations of *recA* mutants contain a large proportion of anucleate cells. We have found that both DNA degradation and anucleate-cell production are efficiently suppressed by mutations in the *xonA* (*sbcB*) and *sbcD* genes. The suppressive effects of these mutations were observed in normally grown, as well as in UV-irradiated, *recA* cells. The products of the *xonA* and *sbcD* genes are known to code for the ExoI and SbcCD nucleases, respectively. Since both *xonA* and *sbcD* mutations are required for strong suppression of DNA degradation while individual mutations have only a weak suppressive effect, we infer that ExoI and SbcCD play partially redundant roles in regulating DNA degradation in *recA* cells. We suggest that their roles might be in processing (blunting) DNA ends, thereby producing suitable substrates for RecBCD binding.**

The RecA protein plays a central role in homologous recombination and recombinational DNA repair in *Escherichia coli*, as well as in other bacterial species. It catalyzes the key stages of the recombination process—homologous pairing and DNA strand exchange. Cells carrying null mutations in the *recA* gene are completely deficient for homologous recombination and are extremely sensitive to DNA-damaging agents (for a review, see references 21, 24, and 25). Populations of *recA* null mutants contain a large proportion (50 to 60%) of nonviable cells, reflecting the inability of these mutants to repair spontaneously occurring DNA damage (31). Also, exponentially growing *recA* cells display pronounced spontaneous DNA degradation that presumably starts at the sites of DNA damage and that depends on RecBCD nuclease (ExoV) activity (5, 48). This phenotype of *recA* cells is aggravated after DNA-damaging treatment, such as UV irradiation (48).

According to the present data, the majority of RecA-catalyzed DNA transactions in *E. coli* start with binding of the RecA protein onto single-stranded DNA (ssDNA) substrates. This binding is mediated by the RecBCD and/or RecFOR protein, which helps RecA to overcome hindrance imposed by the SSB protein during competition for the DNA substrate. The RecBCD and RecFOR proteins begin RecA polymerization on ssDNA, giving rise to a nucleoprotein filament that is indispensable for further recombination reactions (3, 33; reviewed in reference 44).

The RecBCD enzyme is crucial for initiation of recombinational processes at double-stranded DNA (dsDNA) ends (or breaks [DSBs]) in wild-type *E. coli* (a set of reactions known as the RecBCD pathway) (9, 43, 44). Upon recognizing a blunt or nearly blunt dsDNA end and binding to it, RecBCD acts as a combination of powerful helicase and nuclease, thus unwinding and simultaneously degrading both strands of the DNA duplex. After encountering a specific octanucleotide sequence designated Chi, the strong 3′-5′ nuclease activity of the enzyme is attenuated and a weaker 5′-3′ nuclease activity is upregulated (1). This Chi-dependent modification allows RecBCD to create a long 3′ ssDNA tail and to direct the loading of RecA protein onto it (2, 3). In vivo data suggest that this transition of RecBCD from a nuclease to a recombinase mode of action requires the presence of the RecA protein, suggesting that the two proteins might interact (27).

In wild-type *E. coli* cells, the RecFOR protein complex works predominantly on DNA gaps, which may arise in chromosomes due to replication forks passing over the noncoding lesions (e.g., UV-induced pyrimidine dimers) or may be present in replication forks stalled at different obstacles in DNA (44). On the other hand, the RecFOR complex has an important role in *recBC sbcBC*(*D*) mutant cells, replacing the RecA-loading activity of RecBCD during recombination reactions starting from dsDNA ends. Recombination reactions mediated by RecFOR proteins are termed the RecF (or RecFOR) pathway (44).

Cells mutated in the *recB* and/or *recC* gene exhibit strong deficiency in conjugational and transductional recombination, as well as in the repair of DSBs (8, 21). These defects can be rectified by extragenic *sbcB* and *sbcC*(*D*) suppressor mutations that inactivate two nucleases, thus enabling full efficiency of the RecF pathway on dsDNA ends (21, 44). The *sbcB* gene (also designated *xonA*) encodes exonuclease I (ExoI), the enzyme that digests ssDNA in the 3′-5′ direction (23). The *sbcC* and *sbcD* genes encode subunits of the SbcCD nuclease, which acts both as an endonuclease that cleaves hairpin structures and as an exonuclease that degrades linear dsDNA molecules (10, 11). Inactivation of either of the two subunits leads to the loss of SbcCD enzyme activity (18).

The exact mechanism of activation of the RecF pathway by *sbc* mutations is not completely understood. A plausible expla-

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† Mirjana Petranović passed away on 14 October 2008, after a long and courageous struggle with severe illness. She was a great teacher and a true friend to all of us.

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TABLE 1. *E. coli* strains

| Strain[a] | Relevant genotype | Source or reference |
|---|---|---|
| AB1157 | Wild type[b] | 4 |
| BW13635 | *proC677*(Tet$^r$)::Tn*5-132* | M. Berlyn |
| JC5519 | *recB21 recC22* | 4 |
| JJC260 | *sbcD300*::*kan* | B. Michel |
| JJC979 | *recF332*::Tn*3* | B. Michel |
| MG1655 | *proAB*$^+$ | 4 |
| N2691 | *recA269*::Tn*10* | R.G. Lloyd |
| STL113 | *recJ2052*::Tn*10kan* | 39 |
| STL2694 | Δ*xonA300*::*cat* | 39 |
| LMM1112 | *recF332*::Tn*3* | P1.JJC979 × AB1157 to Ap$^r$ UV$^s$ |
| LMM1123 | *recB21 recC22 recF332*::Tn*3* | P1.JJC979 × JC5519 to Ap$^r$ UV$^s$ |
| LMM1245 | *recA269*::Tn*10* | P1.N2691 × AB1157 to Tc$^r$ UV$^s$ |
| LMM1246 | Δ*xonA300*::*cat* | P1.STL2694 × AB1157 to Cm$^r$ |
| LMM1247 | *sbcD300*::*kan* | P1.JJC260 × AB1157 to Km$^r$ |
| LMM1248 | Δ*xonA300*::*cat sbcD300*::*kan* | P1.JJC260 × LMM1246 to Km$^r$ |
| LMM1249 | Δ*xonA300*::*cat sbcD300*::*kan recA269*::Tn*10* | P1.N2691 × LMM1248 to Tc$^r$ UV$^s$ |
| LMM1250 | Δ*xonA300*::*cat recA269*::Tn*10* | P1.N2691 × LMM1246 to Tc$^r$ UV$^s$ |
| LMM1251 | *sbcD300*::*kan recA269*::Tn*10* | P1.N2691 × LMM1247 to Tc$^r$ UV$^s$ |
| LMM1254 | *recB21 recC22 recA269*::Tn*10* | P1.N2691 × JC5519 to Tc$^r$ UV$^s$ |
| LMM1292 | *recB21 recC22 sbcD300*::*kan* | P1.JJC260 × JC5519 to Km$^r$ |
| LMM1315 | Δ*xonA300*::*cat sbcD300*::*kan recF332*::Tn*3* | P1.JJC979 × LMM1248 to Ap$^r$ UV$^s$ |
| LMM1317 | *recB21 recC22 sbcD300*::*kan* Δ*xonA300*::*cat* | P1.STL2694 × LMM1292 to Cm$^r$ UV$^r$ |
| LMM1318 | *recB21 recC22 sbcD300*::*kan* Δ*xonA300*::*cat recA269*::Tn*10* | P1.N2691 × LMM1317 to Tc$^r$ UV$^s$ |
| LMM1326 | *recB21 recC22 sbcD300*::*kan* Δ*xonA300*::*cat recF332*::Tn*3* | P1.JJC979 × LMM1317 to Ap$^r$ UV$^s$ |
| LMM1651 | *recJ2052*::Tn*10kan* | P1.STL113 × AB1157 to Km$^r$ |
| LMM1652 | *recJ2052*::Tn*10kan recA269*::Tn*10* | P1.N2691 × LMM1651 to Tc$^r$ Uv$^s$ |
| LMM1638 | *proAB*$^+$ | P1.MG1655 × AB1157 to Pro$^+$ |
| LMM1639 | *proAB*$^+$ *proC677*(Tet$^r$)::Tn*5-132* | P1.BW13635 × LMM1638 to Tc$^r$ Pro$^-$ |
| LMM1646 | *proAB*$^+$ *proC677*(Tet$^r$)::Tn*5-132 recJ2052*::Tn*10kan* | P1.STL113 × LMM1639 to Km$^r$ |
| LMM2040 | *proAB*$^+$ *recJ2052*::Tn*10kan proC*$^+$ *sbcD300*::*kan* | P1.LMM1247 × LMM1646 to Pro$^+$ λ*pal*$^s$ |
| LMM2041 | *proAB*$^+$ *recJ2052*::Tn*10kan proC*$^+$ *sbcD300*::*kan recA269*::Tn*10* | P1.N2691 × LMM2040 to Tc$^r$ UV$^s$ |

[a] All strains except BW13635, MG1655, STL113, and STL2694 are derivatives of AB1157.
[b] Markers are F$^-$ *thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2* λ$^-$ *rac hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1 qsr'*.

nation is that inactivation of ExoI and SbcCD nucleases is necessary to prevent the degradation of recombinogenic 3′ DNA ends created in a RecBCD-independent manner (8, 23, 38, 45, 46). It was recently shown that the *sbcB15* mutant allele (encoding a protein without nucleolytic activity) (37) is a better suppressor of the RecBCD$^-$ phenotype than an *sbcB* deletion (50), suggesting that some nonnucleolytic activity of ExoI may also contribute to the efficiency of the RecF pathway (46, 50).

ExoI and SbcCD are usually viewed as enzymes with inhibitory roles in recombination due to their deleterious actions on the RecF pathway. However, some results suggest that these enzymes could also have stimulatory roles in recombination reactions proceeding on the RecBCD pathway. Genetic experiments with UV-irradiated *E. coli* cells indicated that ExoI and SbcCD might be involved in blunting radiation-induced DNA ends prior to RecBC(D) action (38, 45, 46). Such a role of ExoI and SbcCD seems to be particularly critical in *recD recF* mutants, in which the majority of DSB repair depends on the RecBC enzyme (38). It was also suggested that the blunting roles of the two nucleases may be required during conjugational recombination (16, 46).

In this work, we studied the effects of *sbcB* (*xonA*) and *sbcD* mutations on DNA degradation occurring spontaneously in exponentially growing *recA* mutant cells, as well as on DNA degradation induced in *recA* mutants by UV irradiation. We have demonstrated that in both cases DNA degradation is strongly reduced in *recA* mutants that carry in addition a combination of *xonA* and *sbcD* null mutations. The results described in this paper suggest that ExoI and SbcCD play partially redundant roles in regulating DNA degradation in *recA* cells.

## MATERIALS AND METHODS

**Strains and media.** The *E. coli* strains used in this study are mainly derivatives of AB1157 (4) and are listed in Table 1. The bacteria were grown in LB liquid medium or on LB plates (30). New strains were constructed by P1 transduction, as described by Miller (30). For selection of transductants, LB plates were supplemented with appropriate antibiotics: tetracycline (Tc), 10 μg/ml; chloramphenicol (Cm), 15 μg/ml; kanamycin (Km), 50 μg/ml; and ampicillin (Ap), 50 μg/ml. When necessary, transductants were checked for their UV sensitivity phenotypes. The phenotype of *sbcD300*::*kan* transductants was confirmed by the increased efficiency of plating (EOP) of λ phage carrying a 571-bp palindrome (18).

**Microscopy.** Cells were grown overnight in LB medium at 37°C with shaking. Overnight cultures were diluted 1,000-fold in LB medium and grown until they reached an optical density at 600 nm ($OD_{600}$) of 0.2. At that point, 1 ml of each culture was taken and pelleted by a brief centrifugation. The cells were fixed for 20 min in 0.5 ml of 0.1% $OsO_4$ solution prepared in 0.2 M cacodylate buffer (pH 7.0). After fixation, the cells were centrifuged again and resuspended in 0.5 ml of cacodylate buffer. Their nucleoids were stained by adding the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) to a final concentration of 1 μg/ml. After 20 min of staining (under conditions of subdued light), the cells were centrifuged and concentrated in 30 μl of cacodylate buffer. Three-microliter portions of DAPI-stained cells were spotted on microscope slides that had been previously covered with a thin layer of 2% low-melting-point agarose (a procedure described by Woldringh et al. [49]). Cover glasses were carefully put onto the cell samples, and their edges were sealed with transparent nail polish. The cells were observed with a Zeiss Axiovert 35 microscope adjusted for combined phase-

contrast and fluorescence microscopy. Cell images were captured with a digital camera.

**Determination of the EOP of the T4 *2* phage.** Fresh overnight cultures were diluted 100-fold in LB medium and grown with shaking at 37°C until they reached an $OD_{600}$ of 0.3. Four separate 1-ml portions were taken from each culture and spun down in a microcentrifuge. Each pellet was resuspended in 1 ml of TM buffer (10 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.2). A 100-μl volume of T4 *2* phage stock (appropriately diluted in TM buffer) was added to each cell sample. Infectious mixtures were incubated at room temperature for 15 min. A 6-ml volume of LB soft agar prewarmed at 46°C was added to each mixture, and the mixtures were poured onto LB plates. (The composition of LB soft agar is equal to that of standard LB plates except for the halved agar content). The plates were kept for 5 min at room temperature and then transferred to 37°C. Plaques were counted after 24 h of incubation.

**Measurement of DNA degradation.** Overnight bacterial cultures were diluted 500-fold in LB medium supplemented with 3 μCi/ml of [$^3$H]thymidine (specific activity, 90 Ci/mmol; Amersham Biosciences, United Kingdom) and 200 μg/ml deoxyadenosine and grown at 37°C to an $OD_{600}$ of 0.5. The cells were pelleted by centrifugation, washed three times with cold 67 mM phosphate buffer, and finally diluted 50-fold in nonradioactive LB medium. The cells were grown with shaking at 37°C, and at 30-min intervals, 0.5-ml aliquots were withdrawn into 1.5 ml of cold 10% trichloroacetic acid. Samples were kept on ice for 1 h and then collected by suction onto Whatman GF/C filters, followed by washing with 5% trichloroacetic acid and 96% ethanol. The filters were dried overnight at room temperature and placed in 5 ml of scintillation liquid. The precipitated counts were measured in a liquid scintillation counter (1209 Rackbeta; Wallac, Pharmacia). The specific radioactivity of labeled cells at the beginning of measurement was at least $10^3$ cpm/$10^6$ cells.

**Estimation of cell viability.** Bacterial cultures were grown at 37°C in LB medium to an $OD_{600}$ of 0.2. The cells were appropriately diluted in 67 mM phosphate buffer and plated on LB plates. Colonies were counted after 24 to 48 h of growth at 37°C. The number of CFU obtained for each strain was expressed as a percentage of the CFU value of the wild-type strain and served as a measure of cell viability.

**DNA degradation and chromosome morphology after UV irradiation.** For measurement of UV-induced DNA degradation, cells were radioactively labeled, pelleted, and washed as described above. Five milliliters of bacterial suspension in phosphate buffer was exposed to 50 J/m$^2$ of UV light (wavelength, 254 nm) at a dose rate of 2 J/m$^2$/s. Irradiation was performed in a petri dish with shaking. The irradiated cells were diluted 50-fold in nonradioactive LB medium and further grown and treated as described above. To analyze UV-irradiated cells by fluorescence microscopy, exponentially growing cells (at an $OD_{600}$ of 0.1) were centrifuged, resuspended in phosphate buffer, and irradiated with UV light as described above. The irradiated cells were pelleted, resuspended in the same volume of LB medium, and grown for 3 h at 37°C. Cell samples were prepared for microscopy as described above.

## RESULTS

**Δ*xonA* and *sbcD* mutations reduce anucleate-cell production in *recA* mutants.** Exponentially growing populations of *recA* null mutants contain a high proportion (about 17%) of nondividing cells that are partially or completely devoid of DNA (5). The loss of DNA in such cells was attributed to DNA degradation initiated by the RecBCD (ExoV) nuclease at sites of unrepaired DNA breaks (5, 42, 48). As estimated by microscopic analysis of bacteria with DAPI-stained chromosomes, exponential cultures of *recA* mutants produce 5 to 13% anucleate cells (20, 51) (Table 2). Such a frequency is enormously high, given the fact that anucleate cells represent less than 0.03% of the cell populations of wild-type strains (19).

During analysis of chromosome morphology in various *E. coli* mutants affected in homologous recombination, we noticed that Δ*xonA300*::*cat* and *sbcD300*::*kan* mutations reduced the production of anucleate cells in exponential-phase *recA269*::Tn*10* cultures (Fig. 1 and Table 2). While the effects of the individual Δ*xonA* and *sbcD* mutations were moderate, the combination of these mutations almost completely abolished the appearance of anucleate cells in the *recA* culture. Hence, we concluded that Δ*xonA* and *sbcD* mutations display strong synergism in suppressing the production of DNA-less cells. The combined effects of Δ*xonA sbcD* mutations were strikingly similar to those of *recB21 recC22* mutations (Fig. 1 and Table 2). Consistent with previous observation by Capaldo and Barbour (5), inactivation of the RecBCD enzyme by *recBC* mutations led to a strong reduction in the number of DNA-less cells in the *recA* culture.

TABLE 2. Production of anucleate cells in *E. coli* strains

| Strain | Relevant genotype | Total no. of cells counted | Anucleate cells (%)[a] |
|---|---|---|---|
| AB1157 | Wild type | 2,405 | 0 |
| LMM1245 | *recA* | 2,188 | 5.2 |
| LMM1246 | *xonA* | 1,866 | 0 |
| LMM1250 | *xonA recA* | 2,293 | 1.7 |
| LMM1247 | *sbcD* | 1,975 | 0 |
| LMM1251 | *sbcD recA* | 2,272 | 1.4 |
| LMM1248 | *xonA sbcD* | 2,020 | 0 |
| LMM1249 | *xonA sbcD recA* | 2,921 | 0.1 |
| JC5519 | *recBC* | 1,542 | 0 |
| LMM1254 | *recBC recA* | 2,890 | 0.07 |
| LMM1651 | *recJ* | 1,752 | 0 |
| LMM1652 | *recJ recA* | 2,560 | 4.3 |
| LMM2040 | *recJ sbcD* | 1,830 | 0 |
| LMM2041 | *recJ sbcD recA* | 2,551 | 1.4 |

[a] Only cells showing no trace of DAPI fluorescence were considered to be anucleate.

In addition to *xonA* and *sbcD* mutations, we examined the effect of the *recJ2052*::Tn*10kan* mutation, which abolishes the activity of the RecJ protein, the main 5′-3′ exonuclease in *E. coli* (47). The *recJ* mutation showed a negligible effect on anucleate-cell production in *recA* cultures (Table 2). Also, it had no further effect on the frequency of anucleate cells in *sbcD recA* mutants (Table 2).

**DNA degradation is strongly reduced in Δ*xonA sbcD recA* triple mutants.** Since *xonA* and *sbcD* mutations inactivate two nucleases, and since DNA degradation was suggested to be implicated in the formation of anucleate cells, we measured the effects of these mutations on the kinetics of spontaneous DNA degradation in *recA* cells. The DNA of *E. coli* cells was labeled with [$^3$H]thymidine, and DNA degradation was estimated from the loss of radioactivity during growth in nonradioactive medium. The results of these measurements are presented in Fig. 2. In accord with previous data (42, 48), the *recA* mutant cells exhibited rather pronounced DNA degradation, which led to a loss of about 40% of the radioactive label within 150 min of measurement (Fig. 2A). Also in agreement with an earlier report (48), this DNA degradation was almost completely suppressed by *recBC* mutations (Fig. 2B), supporting the notion that ExoV activity is responsible for extensive chromosome degradation in the *recA* background. Individual *xonA* and *sbcD* mutations did not significantly affect DNA degradation in *recA* cells (Fig. 2A). However, a combination of these mutations strongly repressed DNA degradation, an effect that was quite similar to that observed in a *recBC recA* triple mutant (Fig. 2B). Finally, a *recBC xonA sbcD recA* multiple mutant showed a phenotype similar to those of *recBC recA* and *xonA sbcD recA* strains (Fig. 2B).

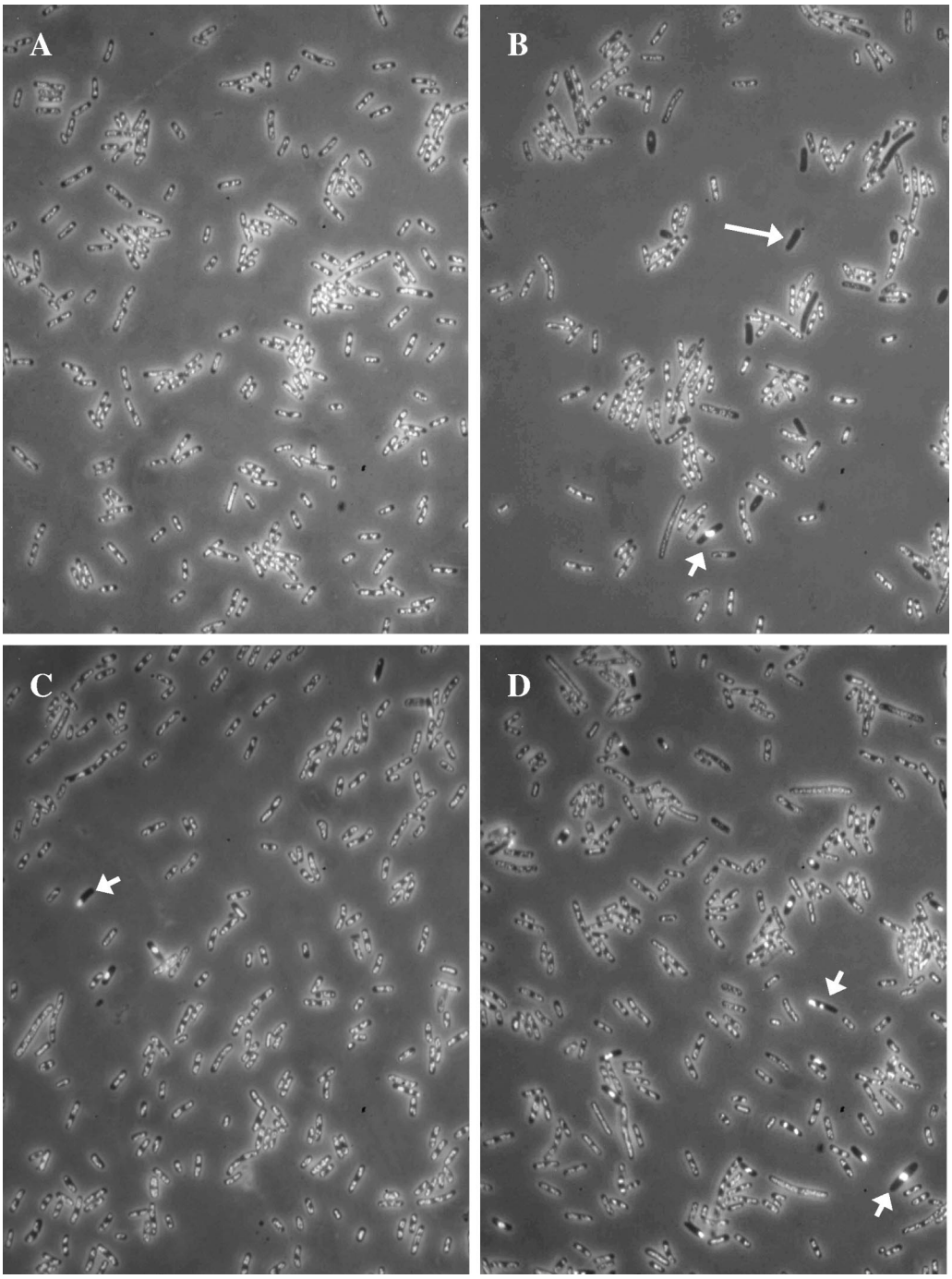


FIG. 1. Nucleoids of *E. coli recA* derivatives visualized by fluorescence microscopy. Exponentially growing cells were fixed with osmium tetroxide, and their DNA was stained with DAPI. (A) AB1157 (wild type). (B) LMM1245 (*recA*). (C) LMM1254 (*recBC recA*). (D) LMM1249 (*xonA sbcD recA*). The long arrow indicates one of the anucleate cells that are typical of *recA* cultures. The short arrows indicate cells with aberrant chromosome structure and positioning.

The *recJ* mutation, either alone or in combination with *sbcD*, did not affect strong DNA degradation in *recA* cells (Fig. 3). This result is consistent with high frequencies of anucleate cells that have been observed with *recJ recA* and *sbcD recJ recA* strains (Table 2).

**A combination of *xonA* and *sbcD* mutations does not affect the EOP of the T4 *2* phage.** The above-mentioned results showed that inactivation of the ExoI and SbcCD enzymes suppressed spontaneous DNA degradation in *recA* mutant cells. Since it was shown that this degradation depends on ExoV activity (42, 48) (Fig. 2), the suppressive effects of *xonA* and *sbcD* mutations could indicate a general involvement of ExoI and SbcCD in DNA degradation processes mediated by ExoV. In order to test this possibility, we measured the joint

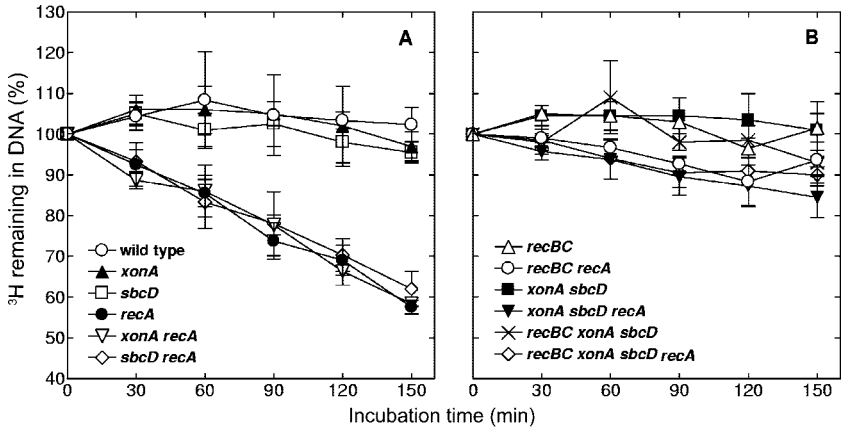


FIG. 2. Kinetics of spontaneous DNA degradation in various *E. coli* strains. A full description of the strains is given in Table 1. Cells were pretreated with [$^3$H]thymidine and then grown in nonradioactive medium. Samples were withdrawn at the indicated times, and the amounts of acid-precipitable $^3$H were determined. The values are averages of two to four independent measurements, with error bars representing standard deviations.

effects of *xonA* and *sbcD* mutations on the EOP of the T4 *2* mutant phage. It is known that the T4 *2* phage plates with reduced efficiency on wild-type cells but plates well on cells deficient for ExoV activity (i.e., on *recB*, *recC*, and *recD* mutants) (41). This phenomenon is due to the lack of the pilot protein 2, which protects the ends of T4 DNA from nucleolytic attack by ExoV (34, 41).

Consistent with previous studies (27, 31, 41), we found that the EOP of the T4 *2* phage was approximately 800-fold higher on the *recBC* mutant than on the wild-type strain (Table 3). In contrast, the EOP of T4 *2* on the *xonA sbcD* double mutant was only slightly higher (less than twofold) than on wild-type cells. Furthermore, the EOP was somewhat increased (about fourfold) on *recA* cells, which is basically in accord with a previous observation by Kuzminov and Stahl (27). The modest increase in the T4 *2* EOP in *recA* cells was suggested to be a consequence of ExoV engagement in the "reckless" chromosome degradation (i.e., partial titration of the enzyme on the chromosome) (27). In an *xonA sbcD recA* triple mutant, the EOP was reduced to a level similar to that observed with an *xonA sbcD* mutant (Table 3), suggesting that inactivation of the ExoI and SbcCD enzymes prevented modest increase of EOP in *recA* cells. Taken together, the above-mentioned results show that ExoI and SbcCD are not required for ExoV-mediated inactivation of T4 *2* DNA either in wild-type or in *recA* cells. Hence, these results imply that in the absence of ExoI and SbcCD, ExoV retains the capacity to bind phage DNA, as well as to perform its degradation, at least to a level sufficient to prevent phage propagation. These findings suggest that the necessity for ExoI and SbcCD during chromosome degradation in *recA* cells cannot be attributed to a direct involvement of these enzymes in extensive DNA degradation. It is more likely that ExoI and SbcCD play auxiliary roles in ExoV-mediated DNA degradation. Such a role could involve limited DNA degradation at sites of DNA breakage, thus producing the blunt DNA ends required for ExoV action.

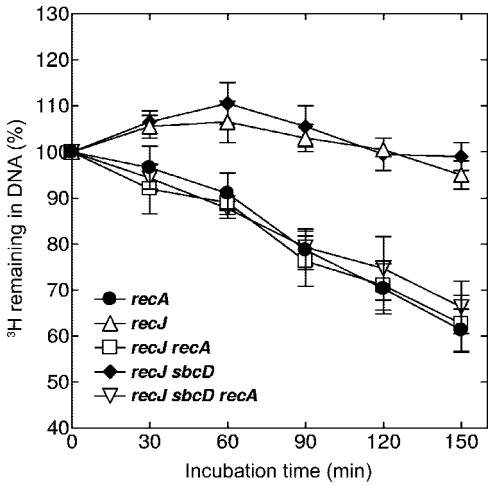


FIG. 3. Kinetics of spontaneous DNA degradation in *recJ* derivatives of *E. coli*. Cells were pretreated with [$^3$H]thymidine and then grown in nonradioactive medium. Samples were withdrawn at the indicated times, and the amounts of acid-precipitable $^3$H were determined. The values are averages of two or three independent measurements, with error bars representing standard deviations.

**Effects of *xonA* and *sbcD* mutations on cell viability.** If ExoI and SbcCD are necessary to prepare a DNA substrate for RecBCD (ExoV) during "reckless" DNA degradation in *recA* mutants, they could also be important for RecBCD-mediated DNA repair in wild-type cells. To check this assumption, we

TABLE 3. EOP of T4 phage on different *E. coli* mutants

| Strain | Relevant genotype | Relative EOP of T4[a] | Relative EOP of T4 *2*[b] |
|---|---|---|---|
| JC5519 | *recB recC* | 1 | 1 |
| AB1157 | Wild type | 0.91 ± 0.18 | 0.0012 ± 0.0002 |
| LMM1248 | *xonA sbcD* | 0.94 ± 0.15 | 0.0019 ± 0.0003 |
| LMM1245 | *recA* | 0.93 ± 0.28 | 0.0055 ± 0.0024 |
| LMM1249 | *xonA sbcD recA* | 0.90 ± 0.15 | 0.0025 ± 0.0007 |

[a] The EOP of T4 on each strain is expressed relative to the EOP on control *recB recC* strain JC5519, which averaged $3.2 \times 10^{10}$. The values are averages of four independent determinations ± standard deviations.

[b] The EOP of T4 *2* on each strain is expressed relative to the EOP on control *recB recC* strain JC5519, which averaged $1.9 \times 10^{10}$. The values are averages of six independent determinations ± standard deviations.

TABLE 4. Cell viabilities of *E. coli* strains

| Strain | Relevant genotype | Viability (%)[a] |
|---|---|---|
| AB1157 | Wild type | 100 |
| LMM1245 | *recA* | 43.29 ± 8.62 |
| LMM1246 | *xonA* | 97.39 ± 4.43 |
| LMM1250 | *xonA recA* | 38.34 ± 5.74 |
| LMM1247 | *sbcD* | 98.36 ± 5.42 |
| LMM1251 | *sbcD recA* | 41.55 ± 6.82 |
| LMM1248 | *xonA sbcD* | 95.83 ± 4.57 |
| LMM1249 | *xonA sbcD recA* | 19.15 ± 7.21 |
| JC5519 | *recBC* | 29.95 ± 3.40 |
| LMM1254 | *recBC recA* | 18.97 ± 3.09 |
| LMM1317 | *recBC xonA sbcD* | 45.25 ± 4.89 |
| LMM1318 | *recBC xonA sbcD recA* | 11.70 ± 2.62 |
| LMM1112 | *recF* | 78.89 ± 8.03 |
| LMM1123 | *recBC recF* | 23.37 ± 1.71 |
| LMM1315 | *xonA sbcD recF* | 67.86 ± 7.03 |
| LMM1326 | *recBC xonA sbcD recF* | 13.20 ± 1.67 |
| LMM1651 | *recJ* | 89.11 ± 4.11 |
| LMM1652 | *recJ recA* | 44.14 ± 9.42 |
| LMM2040 | *recJ sbcD* | 92.60 ± 7.76 |
| LMM2041 | *recJ sbcD recA* | 38.81 ± 6.90 |

[a] Viability was determined for cultures grown to an $OD_{600}$ of 0.2 and is expressed relative to the number of CFU per milliliter in cultures of the wild-type strain AB1157, which averaged $9.2 \times 10^7$ CFU/ml. The values are averages ± standard deviations of at least eight measurements.

measured the effects of *xonA* and *sbcD* mutations on the viability of exponentially growing cells. A decrease in cell viability would serve as an indication of unsuccessfully repaired chromosomal lesions. As shown previously (6, 31), inactivation of the RecBCD enzyme in an otherwise wild-type background reduced cell viability to less than one-third of that observed in a *recBCD*$^+$ strain (Table 4). In contrast, *xonA sbcD* mutants exhibited the same high viability as the wild-type strain, clearly showing that DNA repair of spontaneously occurring DNA damage is not compromised in the absence of ExoI and SbcCD activities. Furthermore, a significant reduction in cell viability (to approximately 45% of the wild-type value) was observed with the *recBC xonA sbcD* mutant (Table 4) (50), suggesting that even in the absence of ExoI and SbcCD, the RecBCD enzyme plays a crucial role in maintaining the viability of exponentially growing cells. This conclusion was further strengthened by an experiment with *recF* and *xonA sbcD recF* mutants, in which DNA repair was expected to rely mainly on the RecBCD pathway. Both *recF* derivatives showed similarly high viabilities (70 to 80% of the wild-type value) (Table 4), indicating that the RecBCD pathway remains highly efficient in the absence of ExoI and SbcCD. Consistent with this, the viability of *xonA sbcD recF* mutants was strongly reduced in the presence of additional *recBC* mutations (Table 4). Therefore, the results described above argue against the assumption that the ExoI and SbcCD nucleases are indispensable for RecBCD-mediated repair of spontaneous DNA damage in *recA*$^+$ cells.

It was previously shown that *recA recBC* mutants display lower cell viability than *recA* single mutants, indicating the existence of a RecA-independent role(s) for the RecBCD enzyme (6, 31). An extensive genetic analysis revealed that DNA degradation is the only activity of RecBCD that is important for the maintenance of *recA*-independent viability (31). It was suggested that RecBCD (ExoV) activity removes dsDNA tails, which pose a threat to cell survival in the absence of RecA-mediated DNA repair (31). Our results confirmed that *recA recBC* cells have significantly lower viability than *recA* cells (Table 4).

Individual *xonA* and *sbcD* mutations only mildly reduced the viability of *recA* cells (Table 4). However, a combination of *xonA* and *sbcD* mutations decreased the viability of *recA* cells to an extent similar to that with *recBC* mutations (Table 4). This finding, together with our observation that *xonA sbcD* mutations reduce "reckless" DNA degradation (Fig. 2), is in accord with the assumption that DNA degradation contributes to the viability of *recA* cells. Consistent with this, the *recJ* and *recJ sbcD* mutations, which showed no significant effect on DNA degradation (Fig. 3), also did not markedly reduce the viability of *recA* cells (Table 4).

Interestingly, the *recBC xonA sbcD recA* strain showed somewhat lower cell viability than the *recBC recA* mutant (Table 4). In addition, the *recBC xonA sbcD recA* strain produced colonies with ragged edges that differed significantly from the normal round colonies of the *recBC recA* strain (data not shown). Similar differences in viability and colony shape were also observed between *recBC xonA sbcD recF* and *recBC recF* strains (Table 4). These results suggest that ExoI and SbcCD are not only involved in RecBCD-mediated DNA degradation, but also play RecBCD-independent roles in maintaining the viability of cells deficient in either RecA function or RecA loading activity.

**Effects of *xonA* and *sbcD* mutations on DNA degradation in UV-irradiated *recA* cells.** It was previously shown that UV irradiation stimulates "reckless" DNA degradation in *recA* mutants (48). We examined the effects of a UV dose of 50 J/m$^2$ on DNA content in different *recA* derivatives. Our results showed that during 3 h of postirradiation incubation, the cells of the single *recA* mutant lose about 90% of radioactively labeled DNA (Fig. 4A). This severe DNA degradation is only slightly reduced in *xonA recA* and *sbcD recA* mutants (Fig. 4A). In contrast, the *xonA sbcD recA* triple mutant displayed pronounced inhibition of DNA degradation, limiting DNA loss to about 40% (Fig. 4B). These results indicate that the *xonA* and *sbcD* mutations have synergistic inhibitory effects on DNA degradation after UV irradiation, similar to their effects on spontaneous DNA degradation in unirradiated cells. Even stronger suppression of the degradation process was observed with *recBC recA* derivatives (Fig. 4B), in which about 25% of the DNA was degraded. This level of DNA degradation is only slightly higher than that observed with wild-type cells (Fig. 4A).

A great difference in the extents of DNA degradation between the *recA* single mutant on one side and *recBC recA* and *xonA sbcD recA* cells on the other was also revealed by fluorescence microscopy analysis of cell samples taken 3 h after exposure to UV (Fig. 5). In the sample from the *recA* mutant, 91% of 2,475 cells counted contained no DAPI-stained DNA (Fig. 5B). Among *recA* cells that showed DAPI fluorescence, DNA was often present in the form of small fluorescent grains that probably represented the remains of degraded nucleoids. In contrast to *recA* single mutants, in the *recBC recA* culture, only 0.9% of 2,255 cells were devoid of DNA (Fig. 5C). In DNA-containing *recBC recA* cells, nucleoids were clearly visible, although they adopted a dispersed form that differed significantly from normal, undamaged nucleoids. The number of

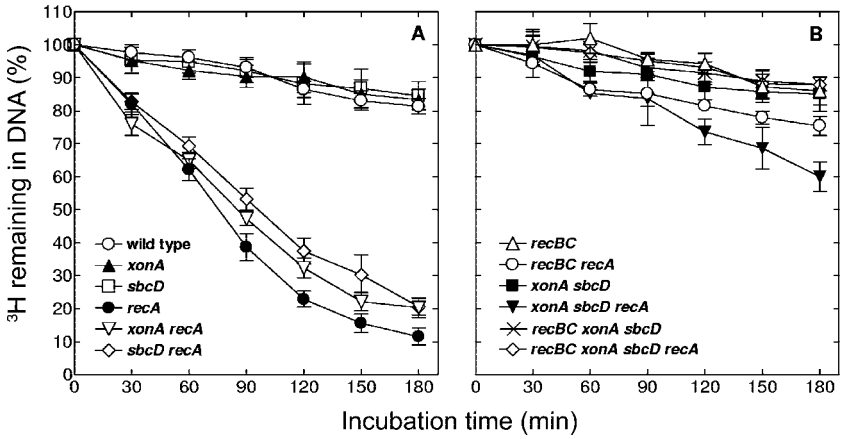


FIG. 4. Kinetics of UV-induced DNA degradation in various *E. coli* strains. Cells were pretreated with [$^3$H]thymidine, irradiated with 50 J/m$^2$ of UV light, and then grown in nonradioactive medium. Samples were withdrawn at the indicated times, and the amounts of acid-precipitable $^3$H were determined. The values are averages of two or three independent measurements, with error bars representing standard deviations.

DNA-less cells was also significantly reduced in the *xonA sbcD recA* culture (Fig. 5D), but not to the level observed in the *recBC recA* culture. Of a total of 2,144 *xonA sbcD recA* cells analyzed, 13.5% were completely devoid of DNA. In addition, a significant proportion of *xonA sbcD recA* cells (14.7%) contained tiny amounts of DAPI-stained material, indicating an extensive yet unfinished DNA degradation process. Anucleate cells were also observed in UV-irradiated wild-type cultures (Fig. 5A); of 2,050 cells, 8.5% had no DNA. However, in this case, the appearance of anucleate cells was obviously associated with a chromosome segregation defect caused by UV. The culture of wild-type cells contained numerous filaments with

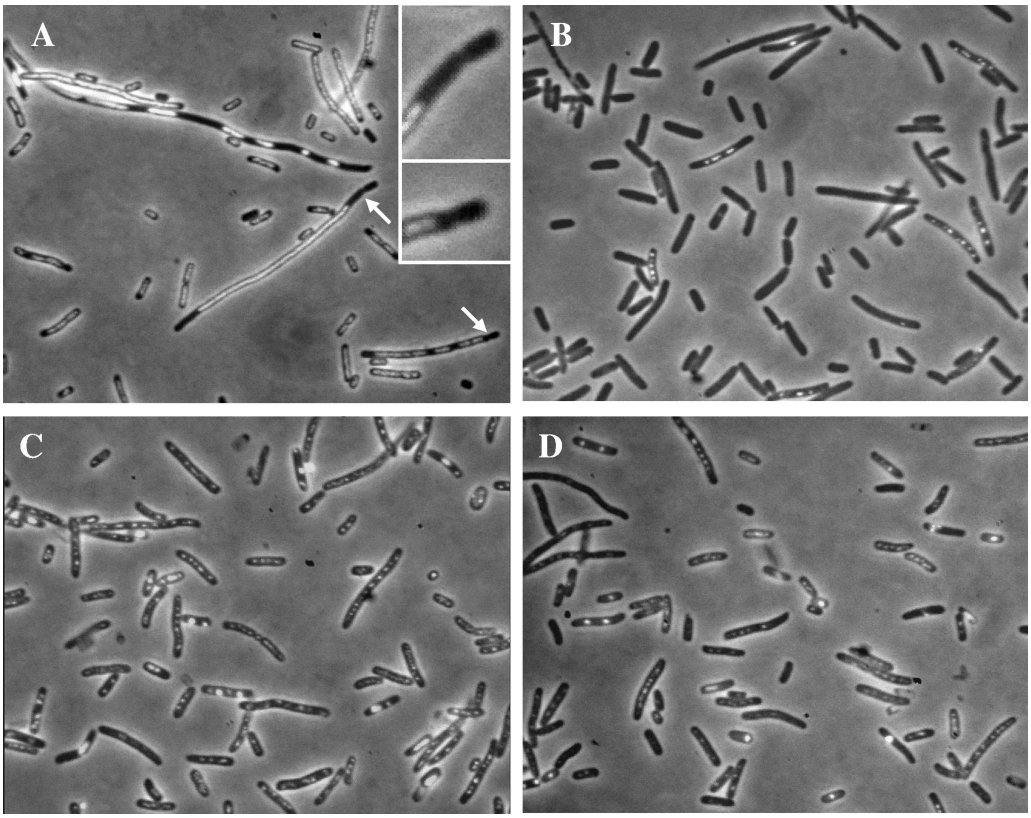


FIG. 5. Nucleoids of *E. coli* cells grown for 3 hours after exposure to 50 J/m$^2$ of UV radiation. The cells were fixed with osmium tetroxide, stained with DAPI, and observed under a fluorescence microscope. (A) AB1158 (wild type). (B) LMM1245 (*recA*). (C) LMM1254 (*recBC recA*). (D) LMM1249 (*xonA sbcD recA*). The arrows in panel A indicate septation events that produced anucleate cells in UV-irradiated wild-type *E. coli* (enlarged in the insets).

large DNA aggregates. These filaments often pinched off small DNA-less cells from their ends (Fig. 5A).

Despite differences in their DNA degradation patterns, the *recA*, *xonA sbcD recA*, and *recBC recA* mutants showed similar extremely low survival rates (approximately 0.0001%) after 50-J/m$^2$ UV irradiation. In addition, they showed similarly low residual growth rates after UV exposure, leading to only small increases in $OD_{600}$ values during 3 hours of postirradiation incubation (1.6-, 1.9-, and 2.1-fold increases for *recA*, *xonA sbcD recA*, and *recBC recA* strains, respectively). Under the same conditions, the wild-type strain displayed about 90% survival and approximately 12-fold increase in the $OD_{600}$ value.

## DISCUSSION

*E. coli* cells with the *recA* gene mutated are completely deficient in homologous recombination and recombinational DNA repair. One of the distinct phenotypes associated with the lack of functional RecA protein is the phenomenon of "reckless" DNA degradation, a tendency of *recA* cells to extensively degrade their own chromosomes starting from sites of DNA damage (48). According to the present data, the "reckless" DNA degradation in *recA* mutants is considered to be dependent primarily on RecBCD (ExoV) activity. Such a notion is based on the fact that inactivation of ExoV almost completely abolishes "reckless" DNA degradation (references 42 and 48 and this paper). In addition, this notion is supported by in vivo studies showing that in the absence of functional RecA protein, the activity of the RecBCD enzyme is not modulated at Chi sites, so that the enzyme remains permanently in its vigorous degradation mode (15, 26, 27). It was shown that such unconstrained degradation activity of RecBCD may lead to the loss of whole chromosomes (42).

**ExoI and SbcCD cooperate with RecBCD (ExoV) in "reckless" DNA degradation.** The major finding of this work is that concomitant inactivation of the *xonA* and *sbcD* genes results in suppression of "reckless" DNA degradation in *E. coli recA* mutants during normal exponential growth (Fig. 2). Under the experimental conditions that we used, the effect of *xonA sbcD* mutations on spontaneous DNA degradation strikingly resembled that of *recBC* mutations, suggesting that ExoI and SbcCD enzymes participate in ExoV-catalyzed degradation processes. However, the experiments measuring the EOP of the T4 *2* phage suggested that *xonA* and *sbcD* mutations do not affect ExoV-mediated degradation of phage DNA (Table 3), at least not to a level that could influence phage survival. These results indicate that "reckless" chromosome degradation and phage DNA degradation might have different requirements for ExoI and SbcCD activities. As a plausible explanation for this difference, we suggest that the necessity for ExoI and SbcCD in "reckless" chromosome degradation reflects their roles in blunting DNA ends and thus preparing a substrate for RecBCD (ExoV) nuclease.

The idea that ExoI and SbcCD are involved in DNA blunting originates from genetic experiments with UV-irradiated *E. coli* cells. Thoms and Wackernagel showed that exposure of wild-type cells to relatively high doses of UV light leads to a strong increase in the T4 *2* EOP (ITE) (45). They proposed that ITE arises from the combined effects of temporary RecBCD sequestration on damaged DNA and the permanent silencing of its ExoV activity upon interaction with Chi sites on DNA. In *xonA sbcCD*, *recJ*, and *recJ sbcCD* mutants, ITE was markedly suppressed, suggesting that the nuclease activities of the ExoI, RecJ, and SbcCD enzymes on DNA ends with single-stranded tails enable RecBCD to bind DNA and, subsequently, to interact with Chi sites. In accord with the blunting hypothesis, later work by Seigneur et al. showed that in UV-irradiated *recF* and *recD recF* mutants, RecBC(D)-dependent DNA repair required the activities of the ExoI and SbcCD nucleases (38). Our finding that a combination of *xonA* and *sbcD* mutations strongly suppressed "reckless" DNA degradation in UV-irradiated *recA* cells (Fig. 3) complements the studies mentioned above and is consistent with the original idea that ssDNases play an important role in preparing DNA ends for RecBCD enzyme. In line with this, a recent study of exponentially growing cells proposed a stimulative role of ExoI in RecBCD-mediated loading of RecA protein (7).

**Overlapping roles of ExoI and SbcCD in *recA* cells.** Since the strong effect of *xonA* and *sbcD* mutations on chromosomal-DNA degradation was observed only when both mutations were present, we suggest that ExoI and SbcCD play partially redundant roles in regulating DNA degradation in *recA* cells. Given that ExoI is known to work on 3′-ended ssDNA, the redundancy of ExoI and SbcCD functions could imply that the SbcCD nuclease might also degrade 3′ ssDNA tails. Such a possibility had already been proposed by Seigneur et al. (38) to explain the redundant activities of ExoI and SbcCD during RecBC-mediated DNA repair in UV-irradiated *recD recF* mutants of *E. coli*. However, the exonuclease activity of SbcCD on 3′ ssDNA tails has not been demonstrated in vitro so far, although weak exonucleolytic and strong endonucleolytic activities on 3′ ssDNA overhangs were reported for eukaryotic homologues of SbcCD, i.e., the Mre11/Rad50 complexes in yeast and humans (35, 36; reviewed in reference 22). On the other hand, it is well documented in vitro and in vivo that the SbcCD enzyme efficiently degrades hairpins in ssDNA (10, 11, 28). This activity could enable SbcCD to shorten 3′ overhangs in an indirect way, i.e., by attacking and cleaving hairpin-like secondary structures present in ssDNA.

An in vitro study also revealed that SbcCD has a single-strand endonuclease activity on short 5′overhangs (10). It was suggested that removal of 5′ overhangs in vivo by SbcCD may provide blunt-ended substrates for RecBCD (10). Interestingly, our results showed that inactivation of RecJ nuclease (which is supposed to abolish the major Exo activity on 5′ overhangs) had no significant effect on DNA degradation in either exponential *recA* or *sbcD recA* cultures (Fig. 3 and Table 2). This finding suggests that the crippling of 5′-3′ single-strand-specific exo/endo activity does not impede spontaneous DNA degradation in *recA* cells, thus supporting our notion that SbcCD contributes to this degradation primarily by acting on 3′ ssDNA. Within the frame of the DNA-blunting model, it could be further inferred that spontaneous DNA breakage in *recA* cells does not produce 5′ overhangs that could prevent binding of RecBCD. However, to address this issue in a more detailed way, additional experiments with *xse* mutants (deficient for ExoVII, a nuclease with 5′-3′ and 3′-5′ polarities) would be required. It was previously demonstrated that ExoVII may efficiently replace the RecJ function in recombination and DNA repair in *recD recJ* mutants of *E. coli* (16). Our prelim-

inary data show that *xseA recJ recA* and *xseA recJ sbcD recA* mutants give relatively high yields (2.8 and 1.3%, respectively) of anucleate cells during exponential growth. This suggests that further depletion of 5′-3′ exo activity due to inactivation of ExoVII does not significantly affect spontaneous DNA degradation.

**The frequency of anucleate cells reflects the extent of DNA degradation in Exo$^-$ RecA$^-$ cells.** The early work of Capaldo and Barbour revealed that exponentially growing *recA* mutants produce unusually high numbers of cells with little or no DNA (5). The lack of DNA in *recA* cells was attributed to excessive DNA degradation catalyzed by the RecBCD (ExoV) enzyme (5).

The patterns of spontaneous DNA degradation observed in this work are well correlated with the frequencies of anucleate cells in populations of particular *recA* derivatives. We found that *xonA sbcD* mutations suppressed anucleate-cell production in a *recA* mutant with the same efficiency as they suppressed DNA degradation. Furthermore, when DNA degradation in *recA* derivatives was stimulated by UV, the number of anucleate cells was correspondingly increased. These findings support the conclusion that anucleate cells of *recA* strains result mainly from extensive DNA degradation (5, 42), rather than being caused by uneven chromosome distribution during cell division, which was also proposed as a possibility (51). However, our results do not exclude certain involvement of RecA protein in the maintenance of chromosome structure, as well as in the regulation of chromosome positioning within a cell. It should be mentioned that cultures of all *recA* mutant derivatives used in this work contained a small proportion of cells with exceptionally condensed chromosomes, which were often placed asymmetrically, i.e., close to one of the cell poles (Fig. 1). Also, we occasionally observed dividing cells that formed septa across DNA (not shown), a phenomenon that is associated with a chromosome segregation defect (19). It remains to be elucidated whether these irregularities reflect the lack of some specific chromosome maintenance/segregation function of the RecA protein, as previously proposed (51), or represent an indirect consequence of unsuccessful DNA repair.

The microscopic analysis of *xonA sbcD recA* cells after UV irradiation revealed significant heterogeneity in DNA content among the cells. A majority of cells (~70%) contained dispersed but clearly visible chromosomes, similar to those observed in *recBC recA* cells (which suffered only moderate DNA degradation). The remaining 30% of the population consisted of cells that displayed strong reduction in visible DNA or even complete lack of DNA. These results suggest that in the latter fraction of the population, DNA degradation occurred rather efficiently even in the absence of the ExoI and SbcCD enzymes. If it is assumed that ExoI and SbcCD participate in blunting DNA ends for RecBCD, the above-mentioned observation suggests that some DSBs induced by UV are suitable for direct RecBCD binding or, additionally/alternatively, require processing by other exonucleases, including those with 5′-3′ polarity. Consistent with the latter possibility, the previous results of Thoms and Wackernagel suggested that RecJ nuclease has an important role in processing DSBs provoked by UV irradiation (45).

**Spontaneous DSBs in *recA* mutants and possible roles of ExoI and SbcCD.** The effects of *xonA* and *sbcD* mutations on DNA degradation in *recA* cells suggest that ExoI and SbcCD significantly influence the activity of the RecBCD enzyme in the absence of the RecA protein. The situation seems to be different in *recA*$^+$ cells, judging from the fact that *xonA* and *sbcD* mutations do not significantly affect the ability of the RecBCD enzyme to maintain cell viability (Table 4). These results indicate that DNA ends that serve as substrates for the RecBCD enzyme are different in wild-type and *recA* cells, further implying that the processes that lead to DNA breakage may be different in the two types of cells. It is possible that the RecA protein is involved not only in the repair processes once the damage to DNA is inflicted, but also in the mechanisms of avoidance of secondary lesions that may arise when the replication fork encounters damaged DNA template or other impediments to its progression (12, 13). Consistent with this, a novel *recA* mutation has recently been isolated that does not affect genetic recombination, recombinational repair, and mutagenesis but disables rescue of stalled or damaged replication forks (40).

To accommodate our data in current models proposed by other authors, we suggest the following hypothetical scenario. It is generally accepted that a fraction of spontaneous DSBs occur due to breakage of replication forks stalled at different obstacles on the DNA template (e.g., proteins bound to DNA, secondary structures, lesions associated with oxidative metabolism, and abasic sites) (14, 29, 32). The obstacles that simultaneously block DNA synthesis on both template strands are likely to give rise to a stalled fork with a gap on the nascent lagging strand (Fig. 6). If such a fork is broken within an ssDNA region (e.g., by endonucleolytic cleavage or mechanical forces), this would most probably produce a DSB with a 3′ protruding end. In cases where such an overhang is longer than 25 nucleotides, RecBCD would not be able to bind the DSB unless it was previously processed by ExoI and/or SbcCD. It is possible that the RecA protein temporarily stabilizes the stalled fork and diminishes its possibility of breakage. In that respect, RecA might act directly by coating ssDNA associated with the stalled replication fork, thus protecting it from endonucleolytic attack or other shearing forces. In addition, the binding of RecA onto ssDNA may have an indirect protective effect by helping template strands to reanneal, thus enhancing fork reversal (12, 13, 39) (Fig. 6). By doing so, RecA could preserve the replication fork until the obstacle to its progression is removed. In the absence of RecA, however, the stalled fork might be more susceptible to breakage and consequently subjected to the concerted nucleolytic actions of the ExoI, SbcCD, and RecBCD enzymes (Fig. 6).

The above-described model predicts that DNA blunting by ExoI and SbcCD is required primarily for the initial binding of the RecBCD enzyme to the DSB. However, it is likely that DNA degradation often occurs through a series of attacks and detachments of RecBCD complexes. The question arises whether there is any necessity for DNA blunting during later binding events, i.e., when the initial RecBCD complex has been dissociated from the DNA and DNA degradation continues through successive actions of other RecBCD molecules. Our results obtained by measuring the T4 *2* EOP suggest that once DNA degradation is successfully initiated, ExoI and

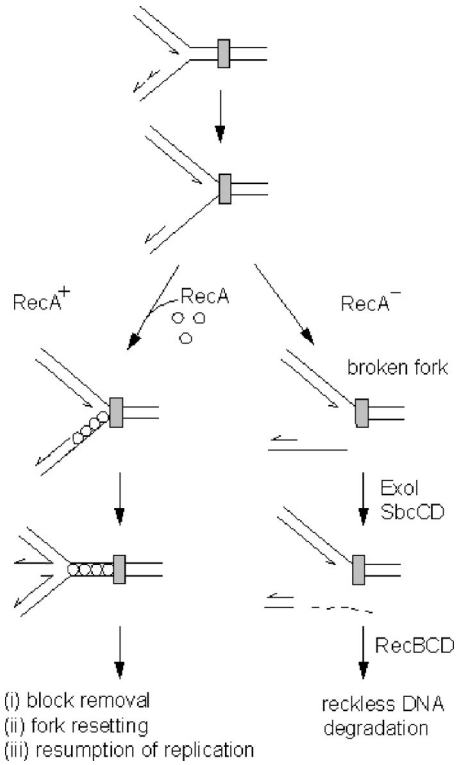


FIG. 6. Model depicting the roles of the ExoI and SbcCD nucleases in the regulation of "reckless" DNA degradation. At the top, an advancing replication fork approaches a block (gray rectangle) in its path. In wild-type ($RecA^+$) cells, a stalled replication fork is stabilized by RecA protein, thus avoiding DNA breakage. In the absence of the RecA protein ($RecA^-$), the unprotected ssDNA region of the stalled fork is prone to breakage. The detached arm of the broken fork may have a 3′ ssDNA tail that is removed by ExoI and/or SbcCD. A blunt DNA end produced in this way serves as an entry point for the RecBCD enzyme that initiates "reckless" DNA degradation. Further discussion of the model is given in the text.

SbcCD do not influence its progression. However, since the T4 *2* EOP is an indirect measure of DNA degradation, we cannot completely rule out the possibility that ExoI and SbcCD have some role in maintaining ongoing "reckless" DNA degradation. In vitro studies have shown that inhibition of RecBCD degradase activity by Chi sites may occur independently of RecA (17). If such RecA-independent modulation of RecBCD activity occurs occasionally in vivo (even with a low frequency), it could be a source of 3′ ssDNA tails that might prevent the next round of RecBCD binding. In that case, the blunting activities of ExoI and SbcCD would also be required to underpin ongoing RecBCD-mediated DNA degradation.

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