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Genetic Recombination in *Bacillus subtilis* 168: Contribution of Holliday Junction Processing Functions in Chromosome Segregation

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Bacillus subtilis **mutants classified within the ε (*ruvA*, Δ*ruvB*, Δ*recU*, and *recD*) and η (Δ*recG*) epistatic groups, in an otherwise *rec*$^+$ background, render cells impaired in chromosomal segregation. A less-pronounced segregation defect in Δ*recA* and Δ*sms* (Δ*radA*) cells was observed. The repair deficiency of *addAB*, Δ*recO*, Δ*recR*, *recH*, Δ*recS*, and Δ*subA* cells did not correlate with a chromosomal segregation defect. The sensitivity of ε epistatic group mutants to DNA-damaging agents correlates with ongoing DNA replication at the time of exposure to the agents. The Δ*sms* (Δ*radA*) and Δ*subA* mutations partially suppress the DNA repair defect in *ruvA* and *recD* cells and the segregation defect in *ruvA* and Δ*recG* cells. The Δ*sms* (Δ*radA*) and Δ*subA* mutations partially suppress the DNA repair defect of Δ*recU* cells but do not suppress the segregation defect in these cells. The Δ*recA* mutation suppresses the segregation defect but does not suppress the DNA repair defect in Δ*recU* cells. These results result suggest that (i) the RuvAB and RecG branch migrating DNA helicases, the RecU Holliday junction (HJ) resolvase, and RecD bias HJ resolution towards noncrossovers and that (ii) Sms (RadA) and SubA proteins might play a role in the stabilization and or processing of HJ intermediates.**

Cells have evolved several mechanisms to maintain the structural and informational fidelity of their DNA and to participate in sister chromatid segregation. UV and certain chemical compounds (e.g., 4-nitroquinoline-1-oxide [4NQO] and methyl methanesulfonate [MMS]), generate deleterious obstacles to DNA replication. Stalling of the replication fork due to such obstacles or the collapse of the replication machinery with resulting unrepaired single-strand nicks or double-strand breaks (DSBs) blocks replication fork progression in all organisms (13, 21, 54). The block must be repaired or removed, and replication must be restarted. Current models for DSB repair involve the formation of Holliday junctions (HJs) that need to be resolved to allow the repaired chromosomes to separate. The *Escherichia coli* RuvAB ($RuvAB_{Eco}$) helicase, together with the $RuvC_{Eco}$ HJ-specific endonuclease, target the HJ at the stalled fork and cleave on opposite strands. If the symmetric HJs are resolved at random, crossovers and noncrossover products are generated. In circular chromosomes, the outcome will be a dimeric chromosome or two monomeric chromosomes, respectively. Dimeric chromosomes are lethal and need to be resolved before cell division. This is accomplished by bacterial Xer-like site-specific recombination systems that catalyze the resolution of the dimers (55). It has been shown in vitro that the orientation of the $RuvABC_{Eco}$ complex determines the direction of cleavage (60), and it is proposed that the repair of broken replication forks is biased to the generation of noncrossover products (14, 41). However, in *E. coli*, chromosome dimers are formed by homologous recombination (HR) between sister chromosomes in about 14% of cells growing under standard laboratory conditions (46, 58).

In *Bacillus subtilis*, the recombination genes other than *recA* have been classified into six different epistatic groups (α, β, ε, γ, ζ, and η). Mutations in genes classified within the α (*recF*, *recL*, *recO*, and *recR* [known collectively as *rec*FLOR] and *recN*), ε (*recU*, *recD*, and *ruvA* [formerly termed *recB*] and *ruvB*), and η (*recG*) epistatic groups markedly affect the viability of cells exposed to DNA-damaging agents, whereas mutations in genes classified within the β (*addA* and *addB* [collectively known as *addAB*]), γ (*recH* and *recP*), and ζ (*recS*) epistatic groups slightly reduce the viability of cells exposed to DNA-damaging agents (reference 16 and this study). The *recA*, *recF*, *recO*, *recR*, *recN*, *ruvA*, *ruvB*, and *recG* genes have their counterparts in *E. coli* in genes with identical names, whereas the *addAB*, *recU*, and *sms* genes have their counterpart in the $recBCD_{Eco}$, $ruvC_{Eco}$, and $radA_{Eco}$ genes, respectively (3, 16). The *B. subtilis recL*, *recD*, *recH*, *recP*, *recS*, and *subA* genes have no obvious counterpart in genes in *E. coli*. The products classified within the α, β, ε, and η groups have their functional counterparts in the $RecN\text{-}FOR_{Eco}$, $RecBCD_{Eco}$, $RuvABC_{Eco}$, and $RecG_{Eco}$ products, respectively (3, 8, 10, 16, 25). The role of the functions classified within the γ and ζ epistatic groups in DNA repair and HR remains unknown (16). Unless otherwise stated, the indicated genes and products are of *B. subtilis* origin.

In *E. coli* cells, 18 to 50% of cells require replication fork reloading during a single round of chromosomal replication in the absence of any exogenous DNA-damaging agent (13, 34). Using an indirect measurement (measurement of repair centers as a measurement of blocked replication forks), we assumed that replication fork reloading might occur with a similar frequency in *B. subtilis* cells (25). The rate of formation of RecN-RecOF repair centers in the absence of any exogenous DNA-damaging agent was found to be about 35 and 5% in exponentially growing Δ*recA* and Δ*recU* cells, respectively (25).

A defect in the HJ resolvase RecU (3) (also termed penicillin-binding protein [PBP]-related factor A [designated PrfA]) or in the DNA organizer SMC complex (formed by the Smc,

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ScpA, and ScpB proteins) in an otherwise wild-type (wt) background, leads to the accumulation of anucleate cells (~3 and 10%, respectively) (7, 20, 35, 42, 45, 56). The Δ*recU* Δ*smc* double mutant does not seem to be viable. Genetic analysis of a synthetic conditional *recU* mutant combined with the Δ*smc* mutant at a permissive temperature indicated the accumulation of ~24% anucleate cells (45). These data suggest a role for the SMC complex and RecU in chromosomal segregation. Finally, it has been shown that the *recU* segregation phenotype is greatly exacerbated by the additional loss of PBP1 but not by the loss of other PBPs (e.g., PBP2c or PBP4), suggesting a possible role for *recU* in septum formation or as a chaperone in DNA-cell wall interaction (24, 45). Furthermore, genetic evidence suggests that the Δ*sms* (also termed Δ*radA*) and Δ*subA* mutations partially suppress the DNA repair and recombination defect of ε epistatic group mutants (8).

In this paper, we analyze the effect on segregation of the different repair-deficient *B. subtilis* epistatic groups, as well as the putative suppression of the segregation phenotypes by the Δ*sms* (Δ*radA*) and Δ*subA* mutations. Our results indicate that the functions of genes classified within the ε and η epistatic groups, which are involved in the processing of an HJ, are required for proper chromosomal segregation in wt cells under normal growth conditions. It is likely that the replication and subsequent segregation of chromosomes bearing unrepaired DNA lesions can seriously compromise genome stability. This is consistent with the hypothesis that *B. subtilis* RuvAB-RecU-RecD and RecG proteins in an otherwise wt background under normal growth conditions (this work) and *E. coli* RuvABC proteins in UV-irradiated, *rep* or *recBC sbcBC* backgrounds (22, 36, 41) prevent dimer formation in vivo. Finally, the suppression of the segregation defect of HJ processing functions by Δ*sms* (Δ*radA*) and Δ*sub* mutations point to the role for both proteins in the stabilization or processing of branched DNA molecules.

MATERIALS AND METHODS

Bacterial strains. All *B. subtilis* strains used in this study are listed in Table 1 and are isogenic to strain YB886 (*rec*$^+$ control). A 2-kb *six-cat-six* cassette containing two directly repeated copies of the β site-specific recombinase target site (*six*) surrounding the chloramphenicol acetyltransferase gene (*cat*) was introduced within the coding sequences of *recG* and *ruvAB*. The disruptions were then transferred into the chromosomes of wt cells to generate Δ*recG* and Δ*ruvAB* strains. Their isogenic *rec*-deficient derivatives, as well as the Δ*recA* Δ*recU* and Δ*recU* Δ*recO* double mutants, were generated by a double-crossover event as previously described (1). Expression of the β gene mediated deletion of the *cat* gene. The *att*SKIN and *att*PBSX (62) regions were moved into wt and Δ*recU* backgrounds by chromosomal transformation as previously described (2).

Survival studies. Cells were grown overnight at 37°C to obtain stationary-phase cultures as previously described (2). Exponentially growing cells were obtained by inoculating overnight cultures in fresh Luria-Bertani (LB) medium and growing them to an optical density at 560 nm of 0.4 at 37°C. When indicated, chloramphenicol (CM) (20 μg/ml) was added to the exponentially growing cells and further incubated for 2 h to stop protein synthesis (11), thereby preventing new rounds of DNA replication. 4NQO was from Sigma, and MMS was from Merck. The chemical treatment (100 μM 4NQO or 10 mM MMS) of exponential- and stationary-phase *recU*, *ruvA*, *ruvB*, *recD*, *recR*, and *uvr*A mutant and wt cells was performed as previously described (11), except that LB medium was used for growing cells, and plating was done on LB agar.

Fluorescence and electron microscopy of *B. subtilis* cells. Exponentially growing cells were obtained by inoculation of overnight cultures in fresh LB medium and growing them to an optical density at 560 nm of 0.4 at 37°C. The mid-log-phase cells were then fixed with 2% formaldehyde, 4′,6′-diamino-2-phenylindole (DAPI) (1 μg/ml) was added for nucleoid visualization, and cells were analyzed by fluorescence microscopy as previously described (7). For electron microscopy sectioning, cells were fixed with glutaraldehyde, treated with osmium tetroxide, and embedded in Spurr's low-viscosity medium (57).

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype[b]	Reference or source
YB886	*trpC2 metB5 amyE sigB37 xin-1 att*SPβ	63
YB1005	+ *uvrA42*	19
YB1290	+ *ruvA2* (formerly *recB2*)	19
BG119	+ *recH342*	2
BG121	+ *recD41*	2
BG123	+ *recU40*	2
BG127	+ *recR13*	2
BG189	+ *addA5 addB72*	1
BG190	+ Δ*recA*	9
BG427	+ Δ*recU*	18
BG651	+ Δ*recU* Δ*recA*	This work
BG425	+ Δ*recS*	18
BG439	+ Δ*recO*	17
BG501	+ Δ*recU* Δ*sms* (Δ*radA*)	8
BG545	+ Δ*recU* Δ*subA*	8
BG621	+ Δ*recU* Δ*recO*	This work
BG503	+ *ruvA2* Δ*sms* (Δ*radA*)	8
BG543	+ *ruvA2* Δ*subA*	8
BG547	+ *recD41* Δ*sms* (Δ*radA*)	8
BG547	+ *recD41* Δ*subA*	8
BG699	+ Δ*ruvAB*	This work
BG691	+ Δ*recG*	This work
BG745	+ Δ*recG* Δ*sms* (Δ*radA*)	This work
BG747	+ Δ*recG* Δ*subA*	This work
BG500[a]	+ *att*SKIN *att*PBSX	This work
BG575[a]	+ *att*SKIN *att*PBSX Δ*recU*	This work

[a] The original background has a mutation in the PBSX-encoded *xin* gene (*xin-1*), whereas in the BG500 and BG575 strains the entire PBSX prophage was deleted.

[b] +, relevant genotype is that of strain YB886 plus the indicated gene(s).

RESULTS

Nucleoid phenotype of *B. subtilis* recombination mutants during exponential-phase growth. To assess the effect on nucleoid morphologies of any recombination-deficient strain in an otherwise wt background, mutant strains representative of each of the epistatic groups (α [Δ*recO* and Δ*recR*], β [*addA5 and addB72*], γ [*recH342*], ε [Δ*recU*, Δ*ruvAB*, and *recD41*], ζ [Δ*recS*], and η [Δ*recG*]) as well as the Δ*rec*A strain (Table 2) were collected during exponential phase, and as a measure of a segregation defect, the frequency of anucleate cells was quantified after the cells were stained with DAPI. Anucleate cells in the *addA5 addB72*, *recH342*, and Δ*recS* strains were rare (Table 2). However, diffuse and "linked" nucleoids that occupied almost the whole cell were visible in 6% of *addA5 addB72* cells, 10% of Δ*recO* cells, and 26% of *recH342* cells (Fig. 1). Very little is known about the biochemical role of RecH on DNA repair and recombination.

The activity of Δ*ruvA*, Δ*recU*, and Δ*recG* cells and the uncharacterized activity of *recD41* cells, all impaired in the processing of HJs, showed a clear defect in chromosomal segregation (Table 2). The most severe segregation defect was found in Δ*recG* cells: >10% of Δ*recG* cells had abnormally condensed nucleoids, and ~7% of the cells were anucleated (see below) under normal growth conditions.

In all experiments, the lysogenic prophage SKIN encoding a RusA-like HJ resolvase protein (52) was present in the genetic

TABLE 2. Anucleate cell production of recombination mutants

Genotype of mutant strain (epistatic group)	% of anucleate cells (no. of cells counted)[a]
rec$^+$	<0.1 (1,400)
Δ*recA*	1 (2,240)
Δ*recO* (α)	<0.2 (652)
Δ*recR* (α)	<0.1 (328)
addA5 addB72 (β)	<0.1 (3,079)
recH342 (γ)	<0.1 (1,614)
Δ*recS* (ζ)	<0.3 (338)
Δ*recG* (η)	7.4 (1,630)
Δ*recU* (ε)	4.4 (510)
recD41 (ε)	4.3 (387)
ruvA2 (ε)	4.9 (485)
Δ*ruvAB* (ε)	5.2 (218)
Δ*recU* Δ*recA*	<0.2 (511)
Δ*recU* Δ*recO*	4.1 (200)
rec$^+$ *att*SKIN *att*PBSX	<0.1 (400)
Δ*recU att*SKIN *att*PBSX	4.5 (410)

[a] Cells were grown and the percentage of anucleate cells were determined as described in Materials and Methods.

background used. To learn whether the RusA-like protein could play any role in chromosomal segregation, SKIN-free wt and Δ*recU* strains were constructed. Similar segregation patterns were observed with SKIN-free and SKIN-containing cells (Table 2). The percentage of anucleate cells in the Δ*recU* mutant that lacks bacterially encoded HJ resolvase was unaffected by the absence of the SKIN prophage (Table 2). Therefore, it is likely that the SKIN-encoded RusA-like protein either is not expressed or has no effect on chromosomal segregation under normal growth conditions.

Unlike a $recA_{Eco}$ mutant that shows ~10% anucleate cells (64), a Δ*recA* mutant shows a moderate segregation defect (~1% of cells) (Table 2) (28). The presence of the Δ*recA* null allele in the Δ*recU* background suppressed the segregation phenotype (Table 2). This is consistent with the observation that in both *E. coli* and *B. subtilis* cells, chromosome dimer formation is not observed and the Xer-like site specific recombinase is not needed in the absence of the RecA protein (6, 27, 28).

The $RecO_{Eco}$, RecO, and RecU proteins can catalyze D-loop formation (3, 32). A Δ*recO* Δ*recU* double mutant strain was constructed to assess whether the absence of DNA strand invasion could suppress the chromosomal segregation phenotype. The Δ*recO* Δ*recU* double mutant strain showed a segregation defect similar to that of the Δ*recU* single mutant (~4% of anucleate cells) (Table 2). These results suggest a strand-invading accessory role for both RecO and RecU proteins and confirm that RecA is primarily responsible for the formation of HJ in vivo.

The DNA damage sensitivity of *recU*, *ruvAB*, and *recD* cells correlates with DNA replication. Previously, it has been shown that UV-generated DNA damages are removed by the nucleotide excision repair (NER) machinery in *E. coli* wt cells (12). The NER proteins are involved in the repair of UV-generated DNA damage independently of the replication state of the cell.

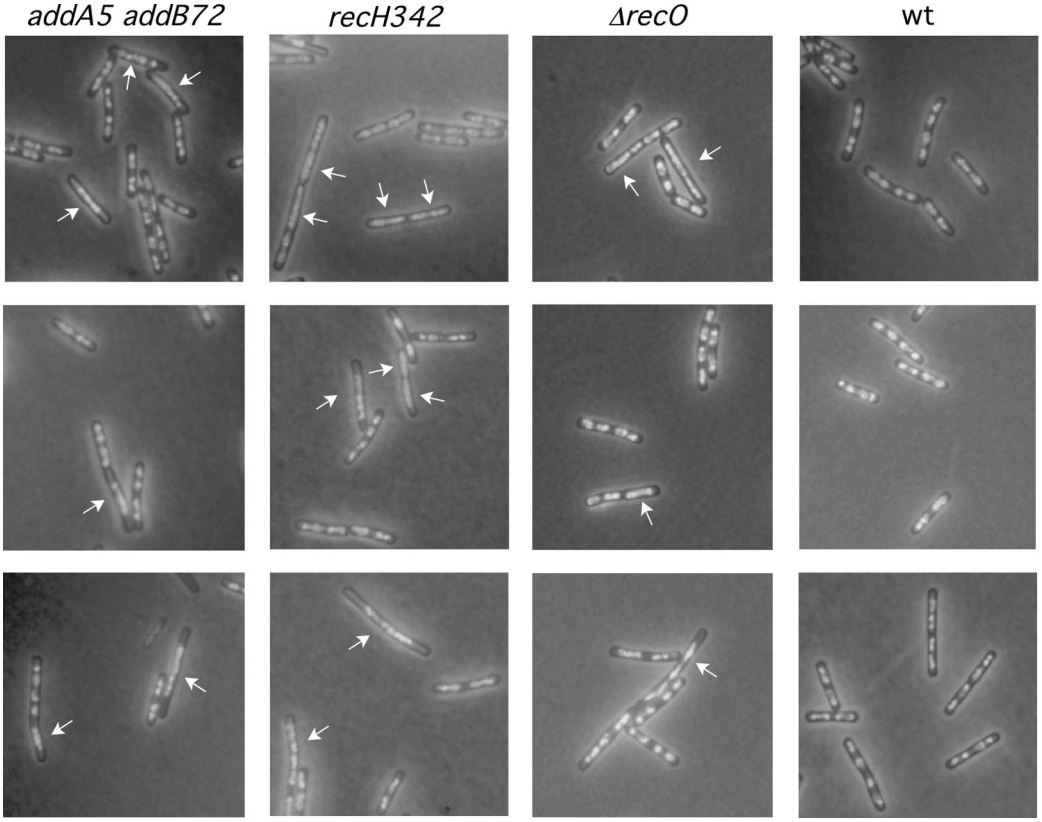

FIG. 1. Nucleoid morphologies of *addA5 addB72*, *recH342*, and Δ*recO* cells. Exponentially growing cells were fixed, stained with DAPI, and analyzed by fluorescence microscopy to visualize the nucleoids. White arrows point to diffuse and linked nucleoids.

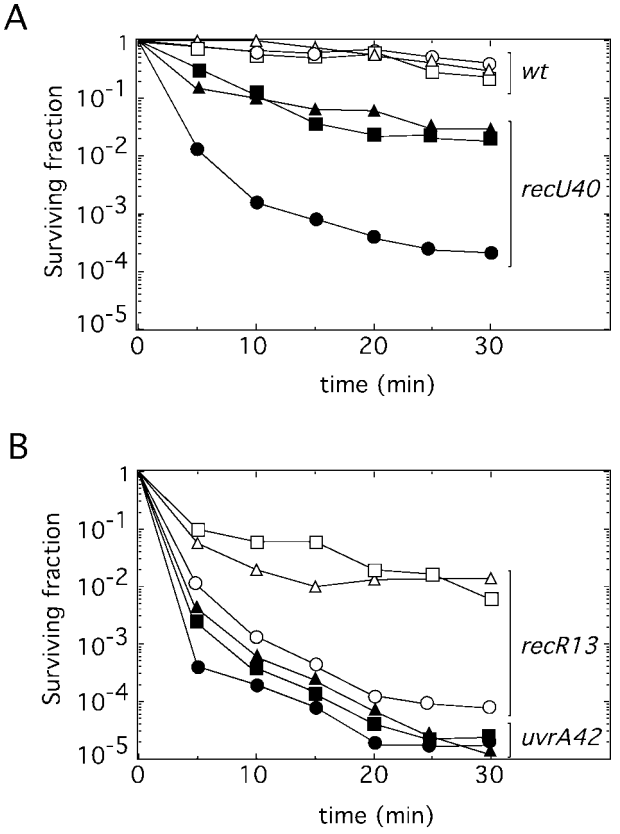

FIG. 2. DNA damage sensitivity of *recU* replicating cells. The survival of wt cells (open symbols) and *recU40* cells (filled symbols) (A), and of *recR13* cells (open symbols) and *uvrA42* control cells (filled symbols) (B) after exposure to the killing action of 100 μM 4NQO under different growth conditions is shown. Triangles, stationary-phase cells; circles, exponential-phase growing cells; squares, cells pretreated with CM (20 μg/ml) 120 min before 4NQO treatment. Survival curves represent the averages of results of three or more independent experiments.

UV-irradiated cells resume DNA synthesis after a transient inhibition by a process called replication restart that has been shown to involve $recF_{Eco}$, $recO_{Eco}$, and $recR_{Eco}$ gene products (11). These results suggest a close interplay between recombination repair and DNA replication and suggest that the failure of $recFOR_{Eco}$ and perhaps *recFLOR* cells arises from a defect in rescuing a stalled replication fork (12, 13, 26). To learn whether the high sensitivity of *recU*, *ruvA*, and *recD* cells to DNA-damaging agents also correlates with ongoing DNA replication, different assays were undertaken. First, wt, *recR13*, *uvrA42* (*uvrA42* is the counterpart to $uvrB_{Eco}$ mutants), and *recU40* cells were grown in LB medium until mid-exponential or stationary phase and exposed to 100 μM 4NQO for various times, and then the numbers of viable cells were measured. Independently of the growth phase, wt cells were resistant to the killing action of 100 μM 4NQO, whereas *uvrA42* cells, deficient in NER, were sensitive (1). As previously reported, exponentially growing *recU40* and *recR13* cells were sensitive to DNA-damaging agents (1), but stationary-phase *recU40* and *recR13* cells were ~100-fold more resistant to 4NQO (Fig. 2) than were the exponentially growing cells (1, 18). Stationary-phase *ruvA2* and *recD41* cells were also ~100-fold more resistant to 100 μM 4NQO than were exponentially growing cells (8) (data not shown). Furthermore, stationary-phase *recU40*, *ruvA2*, *recD41*, and *recR13* cells were also 80- to 100-fold more resistant to other DNA-damaging agents, such as 10 mM MMS, than were exponentially growing cells (data not shown).

To further confirm that the defect observed with the *recU40*, *ruvA2*, and *recD41* mutants was due to a defect in replication restart recovery, wt, *recU40*, *recR13*, and *uvrA42* cells were grown in LB medium until mid-exponential phase. DNA replication was reversibly halted by blocking protein synthesis with CM (20 μg/ml), the cells were then exposed to the killing action of 100 μM 4NQO for various times, and the numbers of viable cells were measured. The *recU40* and *recR13* cells, pretreated with CM for 120 min before exposure to 4NQO, were ~100-fold more resistant to the DNA-damaging agent than were cells untreated with CM (Fig. 2). Similar results were observed when the *ruvA2*, Δ*ruvAB*, *recD41*, or Δ*recO* cells were pretreated with CM for 120 min before exposure to 4NQO (data not shown). Pretreated *uvrA42* cells were as sensitive as untreated cells (Fig. 2B), whereas wt cells were resistant to 100 μM 4NQO exposure. Therefore, it is likely that (i) the failure of *recU40*, *ruvA2*, and *recD41* cells arises from a defect in rescuing a stalled replication fork until the lesion can be removed by NER, (ii) the defect of both $recR_{Eco}$ (11) and *recR13* cells correlates with ongoing DNA replication at the time of exposure to the agent, and (iii) DNA-damaged *uvrA42* cells do not recover normally independently of the growth phase.

Previously, a direct correlation between increased damage sensitivity of $recFOR_{Eco}$ cells and DNA replication has been established (11). Therefore, it is likely that the rescue of arrested replication forks in exponentially growing cells occurs via HR in both *E. coli* cells (11, 12) and *B. subtilis* cells (Fig. 2).

Nucleoid and cell morphology phenotypes of *recU*, *recD*, and *ruvA* cells during exponential-phase growth. To investigate whether the chromosome segregation defect may be due to a defect in replication fork progression, the wt strain and its isogenic derivatives (Δ*recU*, *recU40*, *ruvA2*, and *recD41* cells) were grown to mid-exponential phase in rich medium and either stained with DAPI, fixed, and visualized by fluorescence microscopy or fixed, processed, and visualized by electron microscopy.

Previously, it was shown that 3 to 5% of Δ*recU* cells have a chromosomal segregation phenotype (45). A similar chromosomal segregation defect was observed with the *ruvA*, Δ*ruvAB*, and *recD* mutants (Fig. 3; Table 2). This observation is consistent with the classification of *ruvA*, *ruvB*, and *recD* in the same epistatic group as *recU* (3). An absence of DAPI-stained material was observed for 3 to 5% of the *ruvA2* and *recD41* cells, whereas <0.05% of wt cells were anucleate under identical growth conditions (Fig. 3A). In addition to a higher abundance of cells showing no nucleoids, a high proportion of Δ*recU*, *recU40*, *ruvA2*, and *recD41* cells had defects in nucleoid structure. The one or two normally compact, condensed, and regular nucleoid bodies seen in fixed wt cells often appeared as highly condensed nucleoids asymmetrically located in *recU40*, *ruvA2*, and *recD41* cells (Fig. 3A). Δ*recU*, *ruvA2*, and *recD41* cells had many more nucleoids of much higher DNA content and with large cytoplasmic spaces free of nucleoid bodies than did wt cells. Similar results were obtained when the Δ*ruvAB* strain was analyzed.

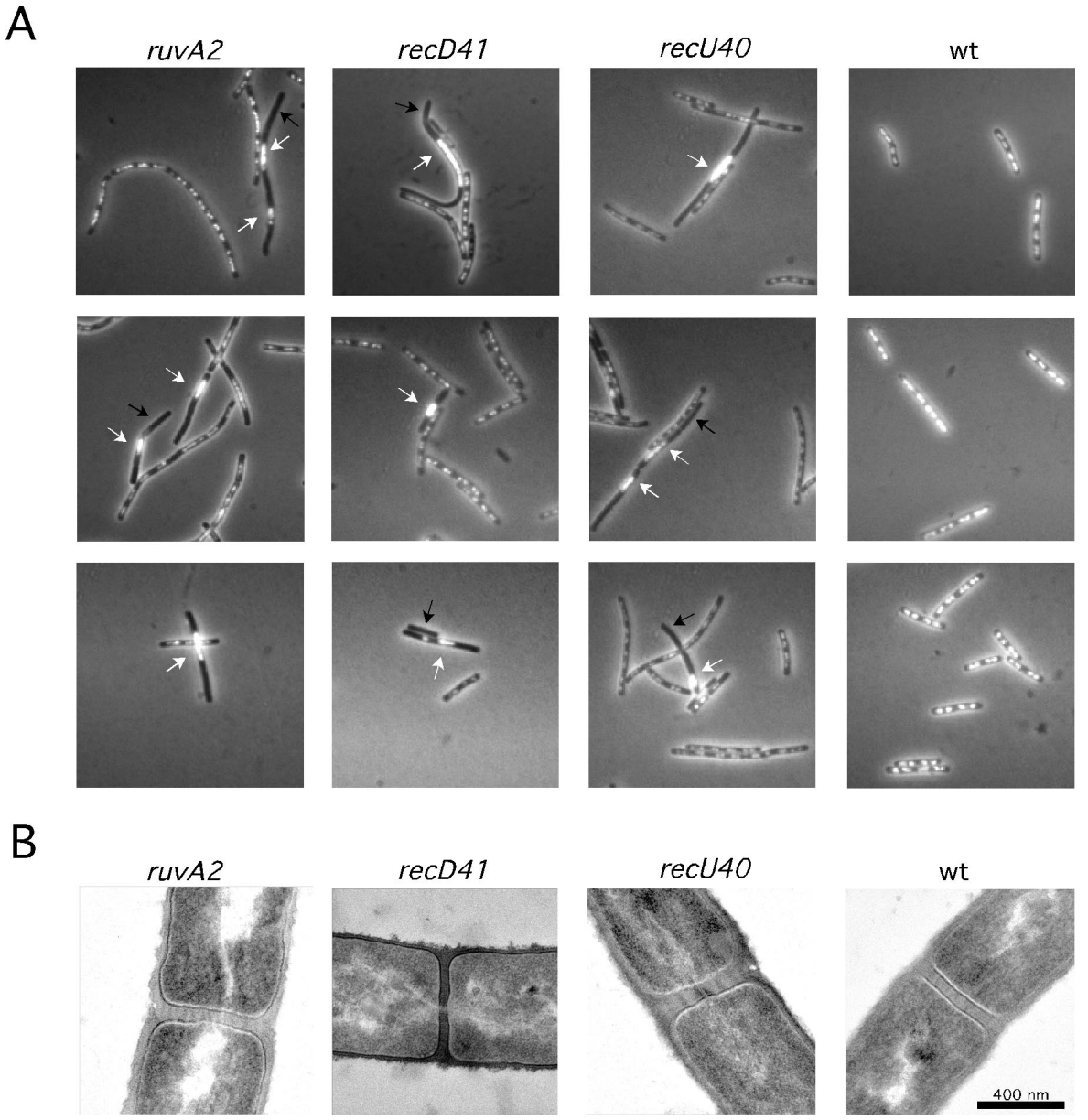

FIG. 3. *recU*, *recD*, and *ruvA* mutations produce anucleate cells and aberrant nucleoids. (A) Exponentially growing cells were fixed, stained with DAPI, and analyzed by fluorescence microscopy to visualize the nucleoids. Black arrows indicate anucleate cells, whereas white arrows show aberrant and misplaced nucleoids. (B) Electron micrographs of cross-sectioned processed cells. The nucleoids appear as light material in the cytoplasm.

The chromosomal segregation defect of *recU40*, *recD41*, and *ruvA2* cells was more apparent when cells were visualized by electron microscopy, and nucleoids that appear bisected by the septum were observed (Fig. 3B).

The *recU* gene maps upstream and forms an operon with *ponA*, which encodes PBP1. As shown in Fig. 3, the absence of the genetically unlinked *recU*, *ruvAB*, and *recD* genes has the same profound effect on both chromosomal structure and segregation. It is likely, therefore, that the segregation defects observed with *recU*, *recD*, and *ruvAB* cells are unlinked to PBP1 and therefore not due to the PBP1 defect in septation and its localization at sites of cell division (45).

The *ruvA2* and *recD41* segregation defect is partially suppressed in Δ*sms* (Δ*radA*) cells. Previously it was shown that Sms (RadA), the counterpart of $RadA_{Eco}$, and SubA proteins play an active role in recombinational repair, most likely through the stabilization and/or processing of branched DNA molecules or blocked replication forks (5, 8). Mutations in both proteins partially suppress the recombination defect of mutations in proteins expressed by genes of the ε epistatic group (8). To learn whether the chromosomal segregation defect of Δ*recU*, *recD41*, and *ruvA2* cells may be also suppressed by mutations in the *sms* (*radA*) and *subA* genes, we constructed double mutant strains and investigated their segregation phenotypes. The Δ*sms* (Δ*radA*), Δ*subA*, Δ*recU* Δ*sms* (Δ*radA*), *recD41* Δ*sms* (Δ*radA*), *ruvA2* Δ*sms* (Δ*radA*), Δ*recU* Δ*subA*, *recD41* Δ*subA*, and *ruvA2* Δ*subA* cells were grown to mid-exponential phase in rich medium and stained with DAPI

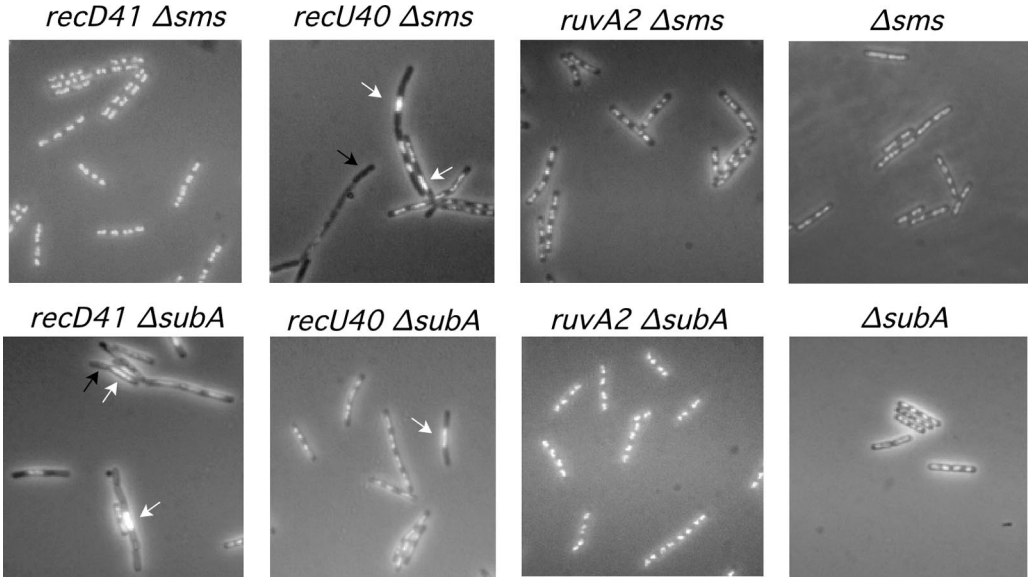

FIG. 4. Effect of the Δ*sms* (Δ*radA*) and Δ*subA* suppressors in the segregation defect of the ε epistatic group mutants. Exponentially growing cells were fixed, stained with DAPI, and analyzed by fluorescence microscopy to visualize the nucleoids. Black arrows indicate anucleate cells, whereas white arrows show aberrant and misplaced nucleoids.

and either fixed and visualized by fluorescence microscopy (Fig. 4) or fixed, processed, and visualized by electron microscopy (data not shown). The Δ*sms* (Δ*radA*) strain contains a low number of anucleate cells (~0.5% of total cells). The chromosomal segregation defect observed with *ruvA2* and *recD41* cells was partially suppressed if the Δ*sms* (Δ*radA*) mutation was present in the background (Fig. 4). In contrast, the Δ*recU* segregation defect was not suppressed by the presence of the Δ*sms* (Δ*radA*) mutation.

Anucleate cells in the Δ*subA* strain were rare. The presence of the Δ*subA* mutation suppressed the segregation phenotype of Δ*ruvA* cells, but Δ*subA* did not suppress the segregation defect of Δ*recU* and *recD* cells (Fig. 4). This finding is consistent with the observation that Δ*subA* partially overcomes the repair defect of *ruvA2* cells but fails to suppress the recombinational defect of *recD41* cells (8).

The Δ*subA* mutation partially suppressed DNA repair and segregation phenotypes of Δ*recG* cells. The $RuvAB_{Eco}$ and $RecG_{Eco}$ helicases, in concert with the HJ endonuclease $RuvC_{Eco}$, are involved in the formation and processing of branched recombination intermediate structures (38, 40). Above, we showed that *ruvA*, Δ*ruvAB*, Δ*recU*, and *recD* cells have a segregation defect that can be, in some cases, partially suppressed either in Δ*sms* (Δ*radA*), Δ*subA*, or both genetic backgrounds. To determine whether Δ*recG* cells show any segregation and DNA repair phenotype and if the Δ*sms* (Δ*radA*) or the Δ*subA* null mutation has any influence in the segregation pattern of Δ*recG* cells, Δ*recG* single and double mutant strains (Δ*recG* Δ*sms* [Δ*radA*] and Δ*recG* Δ*subA* mutants) were constructed and analyzed.

The Δ*recG* strain failed to form colonies in the presence of 20 μg of MMS/ml. Δ*subA* and Δ*sms* (Δ*radA*) strains formed colonies in the presence of 250 μg of MMS/ml, and the wt strain formed colonies in the presence of 300 μg of MMS/ml (8). The Δ*recG* Δ*sms* (Δ*radA*) strains failed to form colonies in the presence of 20 μg of MMS/ml, whereas the Δ*recG* Δ*subA* double mutant strain was able to form colonies in the presence of 250 μg of MMS/ml. Therefore, it is likely that the Δ*subA* mutation partially suppresses the recombinational defect of Δ*recG* cells.

The absence of DAPI-stained material was observed for ~7% of exponentially growing Δ*recG* cells, and >30% of these cells had abnormally condensed nucleoids (Fig. 5). Similar results were observed with Δ*recG* Δ*sms* (Δ*radA*) cells (Fig. 5). The chromosomal segregation defect observed with Δ*recG* cells was partially suppressed if the Δ*subA* mutation was present in the background. The presence of the Δ*subA* null allele in the Δ*recG* background reduced the number of anucleated cells to ~1% (Fig. 5). This finding is consistent with the observed partial Δ*subA* suppression of the DNA repair defect of Δ*recG* cells.

DISCUSSION

Chromosomal segregation in presynaptic stage mutants is not affected. Several models to overcome the block of the replication fork progression have been proposed, depending on the nature of the lesion that encounters the replication fork (13, 14, 21, 26, 41, 54). $RecBCD_{Eco}$ (counterpart of AddAB) processes DSBs to produce the single-stranded DNA required for homologous pairing by the $RecA_{Eco}$ protein, and the $RecFOR_{Eco}$ proteins (counterparts of RecFLOR) load $RecA_{Eco}$ on single-stranded gaps and accelerate DNA strand exchange (13, 26, 40). Both processes lead to the formation of an HJ that will be resolved with a specific polarity (60). Some authors have proposed that RecBCD-dependent DSB repair leads to crossing over and subsequent dimerization and that $RecFOR_{Eco}$-dependent gap repair will not lead to crossover (14, 15). Based on the viability of *E. coli rep* mutants in the absence of XerC or *dif*, other authors have proposed that

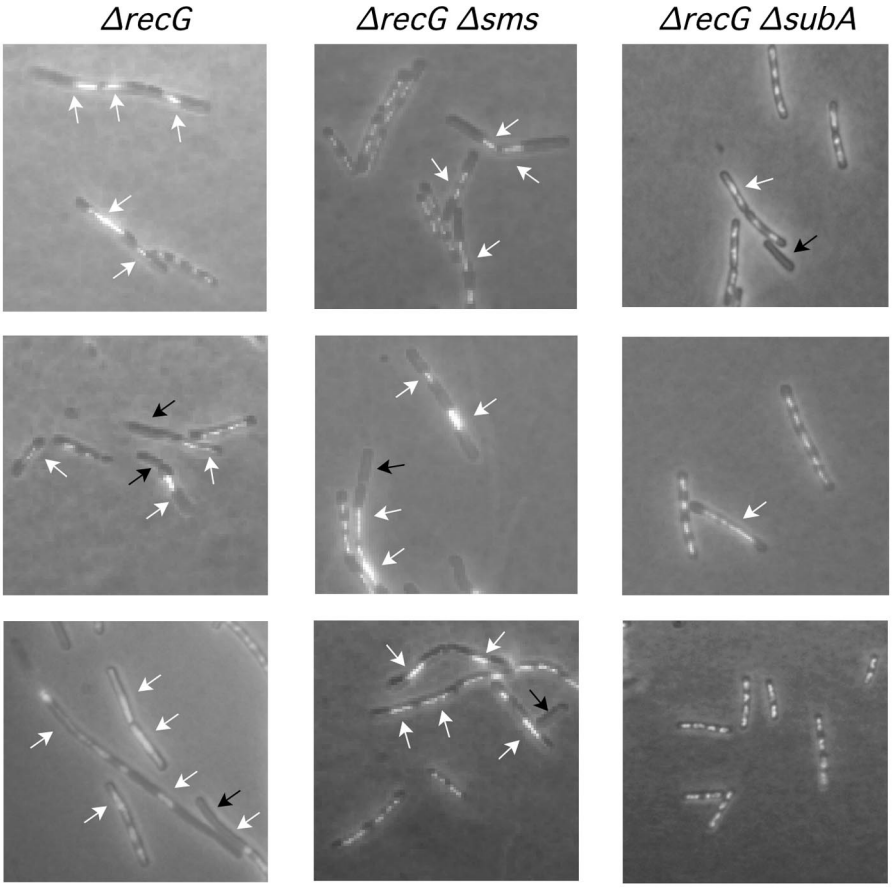

FIG. 5. The *recG* segregation defect is suppressed by the absence of the SubA product. Exponentially growing cells were fixed, stained with DAPI, and analyzed by fluorescence microscopy to visualize the nucleoids. Black arrows indicate anucleate cells, whereas white arrows show aberrant and misplaced nucleoids.

recombination events at arrested forks generally do not lead to the formation of dimers (41). Furthermore, it has been described that about half of the dimers appear to arise through RecBCD-dependent events in *E. coli* cells, while the other half arise from RecFOR-dependent recombination events (58). In order to clarify which of the recombination proteins could be involved in the formation of crossover or noncrossover events in *B. subtilis*, representatives of each of the described epistatic groups were examined by fluorescence microscopy after DAPI staining. The *recO* and *recR* (representatives of the α epistatic group), *addAB* (β), *recH* (γ), and *recS* (ζ) mutant cells did not show any chromosomal segregation phenotypes. We favor the hypothesis that recombination events catalyzed by RecFLOR, AddAB, RecH-RecP, and RecS might occur predominantly in the absence of crossing over. This hypothesis is consistent with the observation that $RecFOR_{Eco}$-dependent recombination events occur in the absence of crossing over (14) and with the viability of *E. coli rep* mutants in the absence of XerC (41). Alternatively, all presynaptic proteins can be considered RecA accessory proteins, and mutations in only one of the genes will never lead to a strong segregation phenotype.

Genes classified within the ε epistatic group are required for replication fork repair and chromosomal segregation. Genetic and biochemical evidence suggests that the genes classified within the ε epistatic group (*ruvAB*, *recU*, and *recD*) are involved in DNA repair and HR (3, 8, 18). We show here that the *recU*, *ruvA*, *ruvB*, and *recD* gene products are involved in recombinational repair of replicating cells and in proper chromosomal segregation. Furthermore, the results presented suggest a postsynaptic role for the unknown activity associated with the *recD41* mutation.

The recombinational repair of stalled or collapsed replication forks leads to the production and resolution of an HJ. In both *E. coli* and *B. subtilis*, the HJ resolvases RuvC and RecU, respectively, bind and resolve the HJ (3, 61). Depending on the particular binding orientation, $RuvC_{Eco}$ or RecU can resolve the symmetric HJs to crossover or noncrossover status. The defect of Δ*recU*, Δ*ruvAB*, and *recD41* mutations in chromosomal segregation might be a consequence of their inability to bias HJ resolution toward noncrossovers. In that case, the crossover product will produce a dimeric chromosome. Alternatively, the dimer is formed because the HJ remains unresolved in both $ruvABC_{Eco}$ cells (41) and *ruvAB recU recD* cells (this work). In both cases, dimers need to be resolved before cell division can occur. In *E. coli* and *B. subtilis* cells, specific site-specific recombinase systems, the XerCD/FtsK and CodVRipX/SpoIIIE complexes, respectively, act at *dif* to ensure the resolution of dimeric chromosomes (6, 27, 48, 49). This is

consistent with the observations that for both *E. coli* and *B. subtilis*, the segregation defect of $xerC_{Eco}$ Δ*dif* and Δ*ripX* mutants is suppressed by inactivation of the RecA protein (6, 27, 28) and that the absence of the RecA protein also suppresses the segregation defect of Δ*recU* cells. We propose that the HJs made in the absence of the RecA protein are resolved to noncrossovers. This proposal is consistent with the observation that chromosome dimer formation (crossovers) is prevented in Δ*recA* Δ*recU* mutants, in rep_{Eco} $ruvABC_{Eco}$ Δdif_{Eco} $recA_{Eco}$ or $priA_{Eco}$ $recA_{Eco}$ mutants, or in UV-irradiated $ruvC_{Eco}$ $recA_{Eco}$ cells (22, 36, 41).

Previously, it has been shown that the Δ*sms* (Δ*radA*) and Δ*subA* mutations partially suppress the DNA repair defect of genes classified within the ε epistatic group (8). As shown in Fig. 4, the Δ*sms* (Δ*radA*) mutation suppressed the segregation phenotype of *ruvA2* and *recD41* cells but failed to suppress the segregation defect of Δ*recU* cells. We propose that, in the absence of the Sms (RadA) and RuvAB or RecD proteins, the branch migration RecG protein bound to an HJ intermediate will dictate the RecU resolution of the HJs in a way that should allow replication restart and noncrossover formation. This proposal is consistent with the observation that in *E. coli*, the *sms* (*radA*) and *ruv* mutations are synergistic with the *recG* mutation (5, 30). Alternatively, as previously proposed by McGlynn and Lloyd (38) for *E. coli* cells, the RecG protein in the *sms* (*radA*) *ruvAB* background would reestablish the fork ready for PriA-dependent reloading of the replisome. The Sms (RadA) protein shares a significant degree of identity with the RecA protein at its central region and with the Lon protease at its C-terminal region and plays a role in recombinational repair (5, 8). At present the biochemical activity(ies) associated with the Sms (RadA) protein remains to be elucidated.

The *recG* gene product is required for chromosomal segregation. The $RecG_{Eco}$ protein plays an essential role in the processing of recombination intermediates in *E. coli* cells (38, 40). Unlike the $recG_{Eco}$ mutation that confers moderate sensitivity to DNA-damaging agents (31), the Δ*recG* mutation markedly affects the viability of cells exposed to 20 μg of MMS/ml (M. C. Cozar and H. Sanchez, personal communication). Furthermore, Δ*recG* cells show a chromosomal segregation phenotype (Fig. 4), suggesting that the *recG* mutant failed to repair stalled or collapsed replication forks. Furthermore, as observed with *E. coli* cells, if positive supercoiling is allowed to accumulate ahead of the replication fork, the forks may be converted to HJs, which have to be converted back to forks if replication is to be completed (43, 47). Hence, in both *ruvAB recU (recD)* and *recG* cells, replication should be stalled and anucleate cells should accumulate.

It has been suggested that the Sms (RadA) and SubA proteins are involved in the formation, stabilization, or processing of branched DNA molecules or blocked replication forks (5, 8). Here, we show that the Δ*subA* mutation also partially suppresses the DNA repair and segregation phenotypes of Δ*recG* cells, but the Δ*sms* (Δ*radA*) mutation suppresses neither the DNA repair nor the segregation defect of Δ*recG* cells. Interestingly, the Δ*subA* mutation suppresses the DNA repair and segregation phenotypes of both previously described branch-migrating DNA helicases (RuvAB and RecG).

What is the role of the SubA protein? The *subA* and *mfd* genes form an operon (4, 8), and a *subA* counterpart in *E coli* is apparently absent. SubA shares a low degree of identity with the UvrA protein, and Mfd shares a significant degree of identity with the RecG and PriA proteins (4, 8, 33). Both Mfd and Mfd_{Eco} proteins recognize a stalled RNA polymerase (RNAP) at UV-induced lesions in the template DNA, dissociate RNAP from the DNA, and recruit UvrA to the site of damage, thereby facilitating excision repair of the transcribed strand (4, 44, 51). RNAP molecules stalled at lesions in the DNA are major obstacles to replication fork progression, and $RuvABC_{Eco}$ is required to promote the rescue of the stalled replication forks (39, 50, 59). With *E. coli*, it has been shown that elevation of ppGpp levels or certain RNAP mutations improves the survival of UV-irradiated RuvABC mutants, probably by minimizing stalling of RNAP at lesions (39).

$PriA_{Eco}$ loads the replisome at recombination intermediates to rescue arrested forks (29, 37). Although a mutation in the helicase motif of $PriA_{Eco}$ reduces the ability of *ruv* mutants to survive DNA damage, it suppresses the DNA repair defect in *recG* cells (23). Since (i) the Δ*subA* mutation suppresses the phenotype of mutations (*ruvA2*, Δ*ruvAB*, and Δ*recG*) in genes encoding the major branch migrating helicases and (ii) *ruvAB* and *recG* suppressors in *E. coli* are helicase-defective proteins, we hypothesize that Mfd alone or in concerted action with another factor(s) may recognize branched structures and translocate in such structures in the presence of SubA. This hypothesis is consistent with our previous failure to detect Mfd-specific binding to HJs and promotion of branch migration (4) and with the fact that the DNA translocation motifs of $RecG_{Eco}$ and Mfd_{Eco} are conserved (33). However, *E. coli mfd recG* and *mfd ruvAB* cells were two- to threefold-more UV sensitive than the *recG* or *ruvAB* cells (53). Furthermore, we predict that the low degree of identity of SubA with UvrA might correspond to the domain of interaction with Mfd. At present, the Mfd_{Eco}-interacting domain in $UvrA_{Eco}$ remains unknown.

A direct effect due to the absence of Mfd in the Δ*subA* strain can be ruled out because (i) the downstream *mfd* gene is under the control of an inducible promoter in the Δ*subA* cells (8) and (ii) the Δ*mfd* mutation increased the sensitivity to DNA-damaging agents of Δ*recU* cells (4), whereas the Δ*subA* mutation partially suppressed its defect (8). Alternatively, the *subA* gene might code for an Mfd repressor. However, a suppression of the Δ*recU* segregation defect was observed with Δ*subA* cells, even in the absence of induction, which will render low levels of Mfd. At present, the biochemical activity(ies) associated with the SubA protein remains to be elucidated.

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