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Chromosomal Lesion Suppression and Removal in *Escherichia coli* via Linear DNA Degradation

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ABSTRACT

RecBCD is a DNA helicase/exonuclease implicated in degradation of foreign linear DNA and in RecA-dependent recombinational repair of chromosomal lesions in *E. coli*. The low viability of *recA recBC* mutants *vs. recA* mutants indicates the existence of RecA-independent roles for RecBCD. To distinguish among possible RecA-independent roles of the RecBCD enzyme in replication, repair, and DNA degradation, we introduced wild-type and mutant combinations of the *recBCD* chromosomal region on a low-copy-number plasmid into a Δ*recA* Δ*recBCD* mutant and determined the viability of resulting strains. Our results argue against ideas that RecBCD is a structural element in the replication factory or is involved in RecA-independent repair of chromosomal lesions. We found that RecBCD-catalyzed DNA degradation is the only activity important for the *recA*-independent viability, suggesting that degradation of linear tails of σ-replicating chromosomes could be one of the RecBCD's roles. However, since the weaker DNA degradation capacity due a combination of the RecBC helicase and ssDNA-specific exonucleases restores viability of the Δ*recA* Δ*recBCD* mutant to a significant extent, we favor *suppression* of chromosomal lesions via linear DNA degradation at reversed replication forks as the major RecA-independent role of the RecBCD enzyme.

TWO-STRAND DNA lesions affect both strands of a DNA duplex opposite each other. In contrast to more common one-strand DNA lesions, two-strand DNA lesions threaten chromosomal integrity, block chromosomal replication, and interfere with chromosomal segregation; in this sense, they can be viewed as chromosomal lesions (Kuzminov 2001). Because of their two-strand nature, chromosomal lesions are recalcitrant to excision repair, which relies on the intact opposite strand to remove a one-strand DNA lesion. The only way to faithfully repair chromosomal lesions, and the major way employed by bacteria, is via homologous strand exchange with an intact sister duplex, promoted in *Escherichia coli* by the RecA protein (reviewed in Kuzminov 1999; Cox 2001). *recA* mutant cells are completely deficient in recombinational repair and therefore cannot mend chromosomal lesions. The two major configurations of chromosomal lesions are: (1) unfillable single-strand gaps (intermediates of a crosslink excision, daughter-strand gaps) and (2) double-strand ends (double-strand breaks, collapsed or broken replication forks; reviewed in Kuzminov 1999). Repair of both unfillable single-strand gaps and double-strand ends depends on RecA; however, it also depends on two independent sets of activities. RecF, RecO, and RecR proteins assist RecA in repairing single-strand gaps, whereas the RecBCD enzyme assists RecA in repairing double-strand ends (reviewed in Kuzminov 1999).

Consistent with the important role of homologous strand exchange in DNA damage repair, *rec* mutants are sensitive to DNA-damaging treatments. For example, *recBC* and *recF* mutants are sensitive to UV and show synergistic effect if combined in a double mutant, defining the two major independent pathways of recombinational repair (Horii and Clark 1973). In contrast, the *recA* mutation, which by itself makes cells extremely sensitive to UV (Clark and Margulies 1965), is epistatic to *recBC* or *recF* mutations for UV survival (Willetts and Clark 1969; Horii and Clark 1973), demonstrating the central role of RecA protein in recombinational repair. In addition to being sensitive to DNA-damaging treatments, *recA* mutant cultures exhibit lower viability, indicating the occurrence of endogenous chromosomal damage even during growth in laboratory conditions. The same epistatic interactions are observed between *recA* and *recF* in the case of endogenous chromosomal damage, assessed by the viability of cultures grown in the absence of exogenous DNA damage. However, the effects of *recA* and *recBC* mutations on viability are additive, rather than epistatic (Capaldo *et al.* 1974), suggesting that *recBC* genes define additional mechanisms relevant for viability.

The RecBCD enzyme is a multifunctional helicase-nuclease, also known as ExoV (reviewed in Taylor 1988; Kowalczykowski *et al.* 1994; Kuzminov 1999). *In vitro*, RecBCD rapidly degrades linear duplex and

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single-strand DNA (ssDNA) in reactions dependent on ATP. The unusual ATP dependence of the DNA degradation by RecBCD is explained by the powerful DNA helicase activity of the enzyme (DNA unwinding requires ATP): apparently, RecBCD can degrade only those DNA strands that are passed through its helicase domain in an ATP-dependent manner (reviewed in Telander-Muskavitch and Linn 1981; Taylor 1988). Although the only nuclease domain of the holoenzyme is found in the C-terminal part of the RecB polypeptide (Yu *et al.* 1998a,b), mutational inactivation of the RecD subunit results in the RecBC enzyme that is completely deficient as a nuclease yet continues to be a potent helicase (Palas and Kushner 1990; Anderson *et al.* 1997) with *in vitro* rates of DNA unwinding ~25% of RecBCD rates (Korangy and Julin 1994). Recombinational repair of double-strand breaks in recD mutants becomes dependent on ssDNA-specific exonuclease RecJ (Lloyd *et al.* 1988; Lovett *et al.* 1988). The RecD subunit is reported to have no activity of its own *in vitro* (Masterson *et al.* 1992) although it plays a regulatory role *in vivo* (Yu *et al.* 1998a; Amundsen *et al.* 2000).

Biochemical characterization of linear DNA processing by the RecBCD enzyme in the presence of RecA (Anderson and Kowalczykowski 1997) corroborated earlier proposals that RecBCD acts on double-strand DNA (dsDNA) ends to prepare them for RecA polymerization (Kuzminov 1996). According to the accepted models, the only role of RecBCD in *E. coli* is to help RecA in recombinational repair (Kowalczykowski 2000; Smith 2001). However, as mentioned above, the RecBCD enzyme must have an additional role in the chromosomal metabolism of *E. coli*, because additional inactivation of *recBC* genes reduces the viability of *recA* mutants from 50% to a mere 20% (Capaldo *et al.* 1974). In fact, the drop in viability due to the *recBCD* inactivation in wild-type cells is ~3.5-fold, an effect much stronger than that of *recA* inactivation (Capaldo *et al.* 1974).

There are two competing models for the RecA-independent role of RecBCD in *E. coli* chromosomal metabolism. The first model suggests that the major nonrepair role of RecBCD is in removal of linear tails of the σ-replicating chromosomes, thus returning the chromosomes to θ-, replication (Figure 1, D to A; Horiuchi and Fujimura 1995; Uzest *et al.* 1995; Kuzminov and Stahl 1997). This is important, because a σ-replicating chromosome cannot produce a circular daughter chromosome (Figure 1, E to F)—a situation described as a "σ-replication trap" (Kuzminov 1999). According to this idea, the RecBCD-catalyzed linear DNA degradation in *recA* mutant cells is the only way out of the σ-replication trap. According to the other model, RecBCD acts to suppress chromosomal lesions at reversed replication forks by attacking the short linear duplex formed by extruded newly replicated DNA strands (Figure 1C) and thus eliminating the Holliday junction (Figure 1, C to B), which would otherwise be resolved by RuvABC, leading to replication fork breakage (Figure 1, C to D; Seigneur *et al.* 1998). We tested these and other conceivable ideas regarding RecA-independent functions of RecBCD by employing various alleles and combinations of genes in the *recBCD* chromosomal region.

MATERIALS AND METHODS

Media, growth conditions, and general methods: Cells were grown in Luria broth (LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl, 0.25 ml 4 M NaOH/1 liter) or on LB plates (15 g agar/1 liter of LB). When cells were carrying plasmids, the media were supplemented with 100 μg/ml ampicillin. Other antibiotics, when required for strain construction, were used in the following concentrations: 100 μg/ml spectinomycin, 50 μg/ml kanamycin, 12.5 μg/ml chloramphenicol, or 10 μg/ml tetracycline. TM buffer is 10 mM Tris HCl pH 8.0, 10 mM $MgSO_4$. Hershey (H) agar (13 g tryptone, 8 g NaCl, 2 g sodium citrate, 1.3 g glucose, 15 g agar/1 liter) was used for T4 plating (Carlson and Miller 1994). Top agar is prepared immediately before plating by mixing equal amounts of the bottom agar and the dilution buffer used in this experiment.

Bacterial strains: Bacterial strains are *E. coli* K-12 (Table 1). Individual *recA*, *recBCD*, and *recF* mutants were confirmed by their characteristic UV sensitivities. In addition, *recBCD* mutants were confirmed by their ability to plate T4 *2* mutant phage (Silverstein and Goldberg 1976). Replacement of genes in the chromosome with either chloramphenicol or kanamycin resistance markers, as well as subsequent generation of in-frame deletions, was according to Datsenko and Wanner (2000). Deletion-replacement alleles of *recBCD* and *recF* were also confirmed by PCR.

Oligonucleotides used to replace *recF* with a *cat* insertion are:
AATTCGACATCAACGTTTCTCGCTCATTTATACTTGG
GTTTGTGTAGGCTGGAGCTGC and
CAGAGCGCGGCTTATGTTGTCATGCCAATGAGACTGT
AATCATATGAATATCCTCCTTAG.

Oligonucleotides used to replace the *recC-ptr-recB-recD* region with a *kan* insertion are:
TGCGTAACACTCGTACGTCGCATCCGGCAATTACGTT
TATTCCGTGTAGGCTGGAGCTGCTTC and
GACCCGCCTGCATTGCCCGAATCGTCAGTAGTCAGGA
GCCGCCATATGAATATCCTCCTTA.

Oligonucleotides used for insertion of a chloramphenicol-resistant gene between *yajD* and *tsx* at the position 430,320 bp on the *E. coli* chromosome are:
CGCATCCGGCATGAACAAAGCACACGTTGTTAACAAT
CAGAATGTGTAGGCTGGAGCTGC and
CAACTTCTGATTATGAAAATGCCGGGATTTATTCCCG
GCATATGAATATCCTCCTTAG.

In all cases, parts homologous to the chromosome are underlined.

Plasmids: A general description of the plasmids is in Table 1. Derivation of the plasmids built for this study is given below:

pAMP1: the 18.3-kbp *recBCD* region from pDWS2 cloned as the *Bam*HI fragment into the *Bam*HI site of pWSK29 in such orientation that the *recC-ptr-recB-recD* region is transcribed in the direction opposite to the *lac* promoter of the vector.

pAMP1B: as pAMP1, but the orientation of the *Bam*HI insert is reversed.

pAMP2: 921-bp *Eco*RI deletion removes the promoter and the 5′ portion of the *addB* gene from pWSK2988 (Kooistra and Venema 1991; Kooistra *et al.* 1993).

pAMP3: the 11.7-kbp *recC-ptr-recB* region from pSA122 cloned into the *Bam*HI site of pWSK29 in the same orientation as in pAMP1.

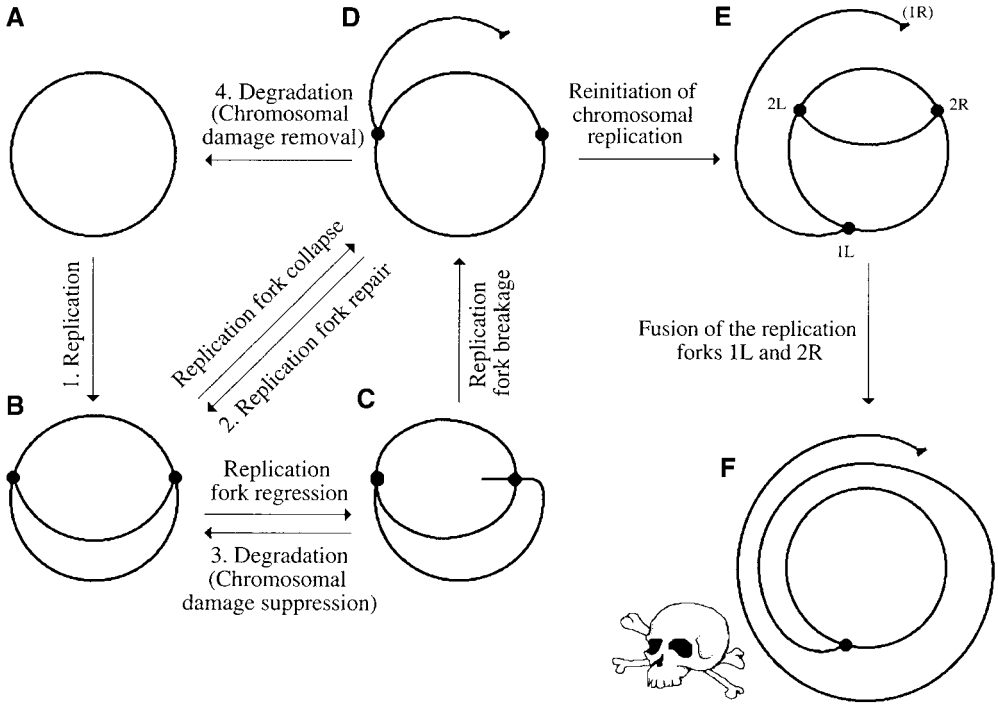

FIGURE 1.—The hypothetical RecA-independent roles of RecBCD in repair/removal of chromosomal lesions. The roles of the RecBCD enzyme are indicated by the numbered text in the grid of normal and abnormal chromosomal processes. DNA duplex is shown as a single line, the chromosome is shown as a circle, replication forks are marked by the solid circles at the branching points. (A–D) *recA*− RecBCD+; (E and F) *recA*− *reBCD*−. (A) A circular chromosome. (B) A θ-replicating chromosome. (C) The right replication fork of the θ-replicating chromosome has reversed, forming a Holliday junction from which the short linear duplex with an open end has extruded. (D) Resolution of the Holliday junction leads to breakage of the reversed replication fork. The chromosome is replicating in the σ-mode. (E) The σ-replicating chromosome initiates a new round of θ-replication from the origin. The old and new replication forks (including the broken one) are numbered. (F) Replication fork fusion lengthens the tail of the σ-structure (the σ-replication trap), dooming the cell.

pAMP5: the 18.3-kbp *recC-ptr-recB-recD* region from pB1082CD cloned into the *Bam*HI site of pWSK29 in the same orientation as in pAMP1. The presumed $recB^{K1082Q}$ mutation could not be confirmed by sequencing; since the non-wild-type behavior of the construct suggested an uncharacterized mutation, we designated the allele *recB*CD*.

pAMP7: the 18.3-kbp *recC-ptr-recB-recD* region from pMY330 cloned into the *Bam*HI site of pWSK29 in the same orientation as in pAMP1.

pAMP8: the [*Xho*I-*Bam*HI 4854 bp] fragment from pAMP1, containing *recD* and the 3′ part of *recB*, cloned at the *Xho*I and *Bam*HI sites of pWSK29. The orientation of the *recD* gene in pAMP8 is the same as the orientation of the whole *recBCD* region in pAMP1.

pEAK1: the big *Eco*RI-*Hin*dIII fragment of pHGS415, carrying the *bla* gene and the replication origin, combined with the small *Eco*RI-*Hin*dIII fragment of pMTL21 (CHAMBERS *et al.* 1988), carrying a multiple cloning site.

pK134: *Bam*HI-cleaved pEAK1 first combined with the [*Bam*HI-*Bam*HI 11.7-kbp] fragment from pSA122 carrying the *recC-ptr-recB* region; the resulting plasmid was cleaved with *Sma*I, and the 14.8-kbp fragment was circularized, thus removing one of the *Bam*HI sites and putting the *recC* gene under the *cat* promoter, left from pHSG415.

pK135: pK134, into the unique *Bam*HI site of which the [*Bam*HI-*Bam*HI 3274 bp] fragment from pBEU14 containing the wild-type *recA* gene has been inserted such that *recA* is co-oriented with the *recC-ptr-recB* genes.

T4 2 mutant plating: A fresh overnight culture is diluted 1000-fold into 2 ml of LB (supplemented with 250 μg of ampicillin if the strain harbors a plasmid) and grown with shaking at 28° to 2×10^8 cells/ml. A total of 100 μl of the growing culture is mixed with 10 μl of either T4 wild-type or T4 *2* mutant phages, diluted in TM buffer to produce approximately the same number of plaques on wild-type cells. After a 3-min incubation at room temperature, 1 ml of top H agar is added and the mixture is plated on a 60 × 15 petri plate with H agar and incubated at 37° for 16 hr. The number of plaques of T4 *2* mutant phage is divided by the number of plaques of the T4 wild-type phages on the same strain the same day to determine the normalized T4 *2* mutant plating.

UV survival: A fresh overnight culture is diluted 100-fold into 2 ml of LB and grown with shaking at 28° to 2×10^8 cells/ml. Tenfold serial dilutions are made in 1% NaCl and spotted by 10 μl in rows of six onto a square petri dish with LB agar. The plate is dried, partially covered with a screen, and exposed to a gradient of doses of UV light in the direction perpendicular to the dilution gradient, so that every dilution column (from 10^{-1} to 10^{-6} dilutions) receives its own dose. UV crosslinker (Amersham-Pharmacia), in which all the lamps except the central one are removed and 90% of the remaining lamp is shielded, is used to deliver precise doses of UV (measured by the internal UV sensor). Immediately after the exposure, the plate is covered with aluminum foil and incubated at 37° for 16–24 hr. The titer of the culture at the zero dose is used to determine the survival at various UV doses.

Quantitative P1 transduction: In a 1.5-ml microcentrifuge tube, 400 μl of a fresh overnight culture in LB is mixed with 500 μl of 30 mM $MgCl_2$, 15 mM $CaCl_2$, 200 μl of LB, and 30 μl of a P1 lysate of AM6 and incubated for 20 min at 28° with shaking. The cells are pelleted by 1 min centrifugation in a microcentrifuge, resuspended in 1.2 ml of LB supplemented with 20 mM sodium citrate, and incubated with shaking for 1 hr at 28°. A total of 100 μl of the culture is plated on LB + 20 mM sodium citrate plates with single selections, whereas cells from the remaining 1 ml are collected by centrifugation, resuspended in 100 μl of LB, and plated on an LB + 20 mM sodium citrate plate with the double (chloramphenicol + tetracycline) selection. The plates are incubated at 37° for 48 hr before transductants are counted.

Viability: A fresh overnight culture is diluted 100-fold into 2 ml of LB and grown with shaking at 28° to 1–5 × 10^8 cells/ml. Two 100-μl aliquots of the culture are taken at the same time: one aliquot is diluted 100-fold into 1% NaCl to stop

TABLE 1

Bacterial strains and plasmids

Strain no.	Relevant genotype[a]	Reference/derivation
	Previous studies	
AB1157	rec$^+$	Bachmann (1987)
JC2926	*recA13*	Clark and Margulies (1965)
JC5519	*recB21 recC22*	Willetts and Clark (1969)
JC5547	*recA13 recB21 recC22*	Willetts and Clark (1969)
JC10287	Δ(*recA-srlR*)*304*	Czonka and Clark (1979)
JC12123	*recJ284*::Tn*10*	Lovett and Clark (1984)
STL2694	Δ*xonA300*::*cat*	Viswanathan and Lovett (1998)
N3033[b]	*lacZ98*::Tn*10*	CGSC no. 6660
	This study	
JB1	Δ(*recC-ptr-recB-recD*)*3*::*kan*	Deletion replacement in AB1157
AM1	Δ(*recA-srlR*)*304* Δ(*recC-ptr-recB-recD*)*3*::*kan*	JC10287[c] × P1 JB1
AM2	Δ(*recA-srlR*)*304* Δ(*recC-ptr-recB-recD*)*4*	In-frame deletion (AM1 Δ*kan*)
AM3	Δ*recF20*::*cat*	Deletion replacement in AB1157
AM4	Δ(*recA-srlR*)*304* Δ*recF20*::*cat*	JC10287[c] × P1 AM3
AM5	Δ*recF20*::*cat* Δ(*recC-ptr-recB-recD*)*3*::*kan*	AM3 × P1 JB1
AM6[b]	*lacZ98*::Tn*10 tsx*::*cat*	Deletion replacement in N3033
AK132	Δ(*recA-srlR*)*304* Δ(*recC-ptr-recB-recD*)*3*::*kan recJ284*::Tn*10*	AM1[d] × P1 JC12123
AK133	Δ(*recA-srlR*)*304* Δ(*recC-ptr-recB-recD*)*3*::*kan* Δ*xonA300*::*cat*	AM1[d] × P1 STL2694

Plasmid	Replicon/drug resistance/other genes	Reference/derivation
	Previous studies	
pBEU14	R1(Ts)/*bla*/*recA*	Uhlin and Clark (1981)
pDWS2	pBR322/*bla*/*recC-ptr-recB-recD*	Ponticelli *et al.* (1985)
pB1082CD	pBR322/*bla*/*recC-ptr-recB*^{K1082Q}-*recD*	Wang *et al.* (2000)
pMY330	p15A/*cat*/*recC-ptr-recB*^{D1080A}-*recD*	Yu *et al.* (1998b)
pSA122	p15A/*cat*/*recC-ptr-recB*	Amundsen *et al.* (2000)
pWSK29	pSC101/*bla*/−	Wang and Kushner (1991)
pWSK2988	pSC101/*bla*/*addAB*	Kooistra *et al.* (1993)
pHSG415	pSC101(Ts)/*bla cat kan*	Hashimoto-Gotoh *et al.* (1981)
	This study	
pAMP1	pSC101/*bla*/*recC-ptr-recB-recD*	pWSK29::*recBCD*(WT)
pAMP2	pSC101/*bla*/Δ*addAB*	pWSK2988 Δ*Eco*RI
pAMP3	pSC101/*bla*/*recC-ptr-recB*	pWSK29::*recBC*
pAMP5	pSC101/*bla*/*recC-ptr-recB**-*recD*	pWSK29::*recB***CD*
pAMP7	pSC101/*bla*/*recC-ptr-recB*^{1080}-*recD*	pWSK29::*recB*^{1080}*CD*
pAMP8	pSC101/*bla*/*recD*	pWSK29::*recD*
pEAK1	pSC101(Ts)/*bla*	pHSG415 Δ*Eco*RI-*Hin*dIII
pK134	pSC101(Ts)/*bla*/*recC-ptr-recB*	pEAK1::*recBC*
pK135	pSC101(Ts)/*bla*/*recA, recC-ptr-recB*	pEAK134::*recA*

[a] Full genotype: F-λ-rac- *thi-1 hisG4* Δ(*gpt-proA*)*62 argE3 thr-1 leuB6 kdgK51 rfbD1 araC14 lacY1 galK2 xylA5 mtl-1 tsx-33 glnV44 rpsL31.*

[b] Other mutations are λ-, *IN(rrnD-rrnE)1.*

[c] The JC10287 *recA* defect was complemented for the P1 transduction with the pBEU14 plasmid, which was cured once the construct was confirmed.

[d] AM1 *recA* and *recBCD* defects were complemented for the P1 transduction with the pK135 plasmid, which was cured once the construct was confirmed.

growth, and the other one is mixed with 0.8 μl of 4 M NaOH to stop cell movement. A total of 5 μl of the NaOH-treated aliquot is used to count cells under the microscope in a Petroff-Houser counting chamber. The 1% NaCl-diluted aliquot is diluted further to achieve the final dilution of 10^{-5}, 100 μl of the final dilution is combined with 3.5 ml of top LB agar, and the mixture is poured over an LB plate. The plate is incubated at 37° for 16–36 hr, and the titer of the colony-forming units is divided by the titer of the microscopic count to determine the viability.

Field-inversion gel electrophoresis: Growing cultures of strains to be tested were normalized to OD_{600} = 0.5–0.6. Cells from 1.5 ml of the normalized cultures were pelleted by centrifugation and resuspended in 60 μl of TE buffer. After incubation at 37° for 2–10 min, 5 μl of 10 mg/ml Proteinase K was added, followed by 65 μl of 1.2% molten agarose in 0.2% sarcosyl,

TABLE 2

The viability of *E. coli* strains carrying point mutations or deletions of *recA*, *recBCD*, and/or *recF* genes

Strain	Relevant genotype	Viability[a]
	Point mutants	
AB1157	Rec+[b]	100.0 ± 2.8
JC2926	*recA13*	47.6 ± 3.8
JC5519	*recB21 recC22*	27.7 ± 1.9
JC5547	*recA13 recB21 recC22*	18.0 ± 1.7
	Deletions	
AB1157	Rec+[b]	93.8 ± 4.7
JC10287	Δ*recA304*	46.0 ± 2.4
JB1	Δ*recBCD*	27.8 ± 2.3
AM1	Δ*recA304* Δ*recBCD*	18.8 ± 1.9
AM3	Δ*recF*	70.8 ± 5.2
AM4	Δ*recA304* Δ*recF*	45.6 ± 4.0
AM5	Δ*recBCD* Δ*recF*	29.6 ± 7.9

[a] Viability is expressed as mean ± standard error. The number of independent measurements, done on different days, is between 8 and 20.

[b] Both strains are AB1157, but the data were collected at different times and treated separately.

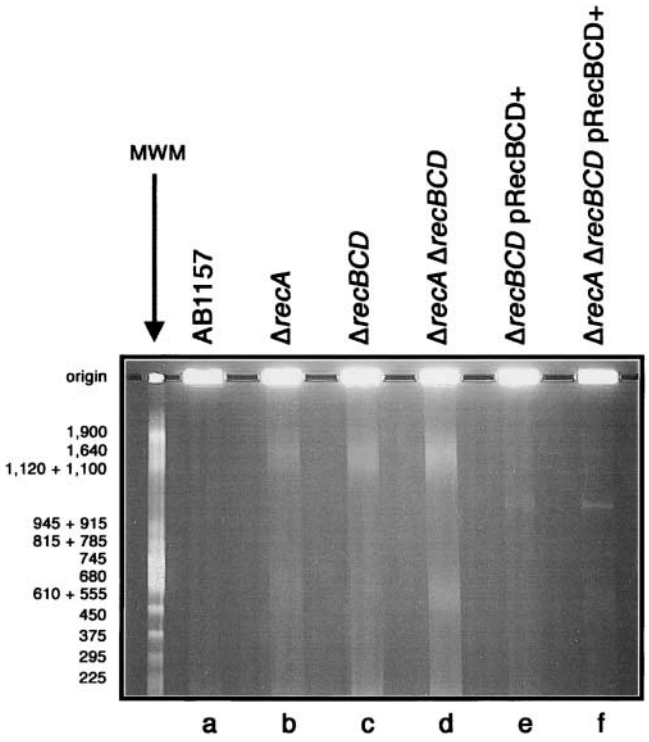

FIGURE 2.—Physical evidence for the chromosomal fragmentation in *rec* mutants. Field-inversion gel electrophoresis of chromosomal DNA isolated from exponential cells of the indicated genotype is shown. MWM, molecular weight markers (yeast chromosomes). The size of the MWM bands in kilobase pairs is shown on the left. The extent of chromosome fragmentation in various strains is reproducible; however, due to the inherent lack of linearity over a broad range of fluorescence intensities, comparison of the data from different gels is not appropriate. Δ*recA*, JC10287; Δ*recBCD*, JB1; Δ*recA* Δ*recBCD*, AM1; pRecBCD+, pAMP1.

10 mM Tris HCl pH 8.0, 5 mM EDTA, kept at 70°. After vortexing and mixing by pipetting, 110 μl of the cell suspensions were pipetted into plug molds and let solidify for 2–10 min. Each plug was then placed in a small glass tube containing 1 ml of 1% sarcosyl, 50 mM Tris HCl pH 8.0, and 25 mM EDTA and incubated for 1 hr at 60°. Plugs were inserted into a 1% agarose gel on 0.5× TBE buffer and run at room temperature in a regular agarose gel box with field inversion factor 3:1 at 4 V/cm for 5 hr with ramping 1–20 sec, then for 5 hr with ramping 20–60 sec, and then for 5 hr with ramping 60–100 sec. Gels were stained with ethidium bromide before being photographed in UV light.

RESULTS

The phenomenon and possible explanations: When grown under the standard laboratory conditions, *recA* mutants are only 46–48% viable (Table 2), indicating the frequency of chromosomal damage and demonstrating the importance of repairing it. The two pathways of *recA*-dependent repair in *E. coli* are controlled by the *recBC* and *recFOR* genes. A Δ*recF* mutant has ~71% viability (Table 2), which is consistent with inactivation of one of the two recombinational repair pathways. A Δ*recA* mutation is epistatic to the Δ*recF* mutation for viability (the Δ*recA* Δ*recF* double mutant has the viability of a single Δ*recA* mutant), indicating that RecF has no effect on viability outside the RecA-dependent processes. In contrast, *recBC* mutants are only ~28% viable, suggesting an additional role for RecBCD beyond the RecA-controlled recombinational repair. Indeed, compared with 46–48% viability of *recA* mutants, *recA recBC* combinations are only 18–19% viable (Table 2), supporting a RecA-independent role for RecBCD. *recBCD* inactivation is epistatic to *recF* inactivation (Table 2).

Pulsed-field gel electrophoresis of undigested chromosomal DNA is employed to detect chromosomal fragmentation: under pulsed-field gel conditions, intact chromosomes stay at the origin, whereas linear subchromosomal fragments migrate into the gel, forming a smear (MICHEL *et al.* 1997; THOMS and WACKERNAGEL 1998). Pulsed-field gel electrophoresis reveals a background level of chromosomal fragmentation in wild-type cells (Figure 2, lane a). Chromosomal fragmentation is visibly increased by inactivation of either *recA* or *recBCD* and is most evident in a *recA recBCD* mutant (Figure 2, lanes b–d). Thus, the chromosomal fragmentation results coincide with the viability results and suggest that the low viability of *recA recBCD* mutants is due to the failure to prevent or repair chromosomal fragmentation.

We conceived four possibilities for the RecA-independent role of RecBCD, numbered from 1 to 4 irrespective of their likelihood (Figure 1). Possibility 1 is that

RecBCD is an integral part of a supramolecular complex at the replication factory (Figure 1, A to B), and that its absence destabilizes other components of the factory, negatively affecting chromosomal replication. Indeed, RecBCD is occasionally reported to purify from cells in complexes with replication enzymes (Hendler *et al.* 1975; Syvaoja 1987). Possibility 2 is that the RecBCD enzyme promotes RecA-independent recombinational repair of disintegrated replication forks (Figure 1, D to B). There are types of homologous recombination in the chromosome that are independent of RecA, although these either require inactivation of RecBCD as well (Wackernagel and Radding 1974; Weisberg and Sternberg 1974; Ellis *et al.* 2001) or are independent of RecBCD (Lovett *et al.* 1993; Bierne *et al.* 1997). Possibility 3 is that the RecBCD-promoted linear DNA degradation is important in preventing replication fork breakage (Figure 1, C to B) as a chromosomal damage *suppression* mechanism (see Introduction). And last, possibility 4 is that RecBCD degrades linear tails of σ-replicating chromosomes (Figure 1, D to A) as part of a chromosomal damage *removal* mechanism (see Introduction).

The experimental system: To find the nature of the RecBCD effect on the cell's viability outside the RecA-dependent recombinational repair, we transformed a Δ*recA* Δ*recBCD* strain with plasmids carrying various alleles of the *E. coli recBCD* genes or genes with similar functions from *Bacillus subtilis* (Table 1, plasmids; this study) and determined viability of the resulting strains, looking for the constructs that would restore viability to the ~50% level of a single *recA* mutant. As controls, we used the same plasmids to transform a $RecA^+$ $RecBCD^+$ strain, as well as single Δ*recA* or Δ*recBCD* deletion mutants. As a vector for all our constructs, we used pSC101-derived pWSK29, which has a high stability and a low copy number (Wang and Kushner 1991).

To assess the functionality of the constructs, we determined to what extent defects of a Δ*recBCD* mutant are complemented by the introduced constructs. As mentioned in the Introduction, the two major functions of the RecBCD enzyme are: (1) participation in the RecA-promoted recombinational repair and (2) degradation of linear duplex DNA (ExoV). To determine the recombinational repair capacity of Δ*recBCD* mutants transformed with the constructs, we measured their resistance to UV irradiation. Relative to the wild-type cells, Δ*recBCD* mutants are quite sensitive to UV due to their defect in repair of double-strand breaks (Wang and Smith 1983, 1986). Thus, restoration of UV resistance signals that the plasmid construct carries an enzyme proficient in recombinational repair. To evaluate the ExoV activity of the constructs, we measured the ability of *recBCD* mutant cells, transformed with the constructs, to plate a gene *2* mutant of bacteriophage T4. The gene *2* product binds to the ends of the linear T4 chromosome and protects them from exonuclease degradation inside the cell. T4 *2* mutant phages plate with reduced efficiency on $ExoV^+$ cells, but plate well on null *recBCD* mutants, which are deficient in ExoV (Silverstein and Goldberg 1976). Thus, poor plating of T4 *2* mutant phages signals that the plasmid construct carries an enzyme proficient in linear DNA degradation.

To simultaneously evaluate both the recombination capacity and the ExoV activity of the constructs in functional interaction with each other, we measured the frequency of generalized P1 transduction in these strains. During P1 transduction, an ~100-kbp piece of a donor chromosome, carrying a selectable marker, is introduced into a recipient cell, and recombinants, with the donor marker inserted in the recipient chromosome, are selected for. The formation of these recombinants depends on both RecA and RecBCD functions in the recipient cells (Willetts and Mount 1969; Zieg and Kushner 1977); the transduction is promoted by the recombination activity of RecBCD, while being somewhat inhibited by the DNA degradation activity (Chaudhury and Smith 1984). When incorporation of a single marker is selected for, the incorporated piece of the donor chromosome can be almost as short as the marker itself. However, when two separate donor markers are simultaneously selected for, the incorporated piece needs to be at least as long as the distance between the two markers (unless the recombinant is formed by two independent integration events, which is rare). Under these circumstances, the wild-type levels of linear DNA degradation should decrease the frequency of double transductants, while having little effect on the levels of single transductants. The two markers that we used were *lacZ*::Tn*10*(*tet*) and a *cat* insertion between *yajD* and *tsx* in the *E. coli* chromosome, placing the distance between the two markers at ~64 kbp. By selecting for growth on tetracycline plus chloramphenicol after transduction, we were selecting for incorporation of the central 64 kbp of the injected 100 kbp (as well as for rare independent double transductants).

We verified how the system works with Δ*recBCD* cells transformed with an empty vector or the vector carrying a complete wild-type *recBCD* region of the chromosome. In the DNA degradation test (T4 *2* mutant phage plating), the vector alone behaved essentially as the Δ*recBCD* control strain, whereas the *recBCD*+ plasmid conferred nearly wild-type levels of DNA degradation capacity (Figure 3A). In the recombinational repair test (UV resistance) the vector alone did not provide any resistance over the Δ*recBCD* levels, whereas the *recBCD*+ plasmid conferred close to wild-type levels of UV resistance at doses 27 J/m^2 and lower (Figure 3B). With the combined recombinational repair/DNA degradation test, vector alone did not contribute to transduction over the Δ*recBCD* levels, whereas the *recBCD*+ plasmid significantly increased transduction over the background levels, although it failed to bring it to the wild-type levels (Figure 3C). Finally, we measured the viability of wild-

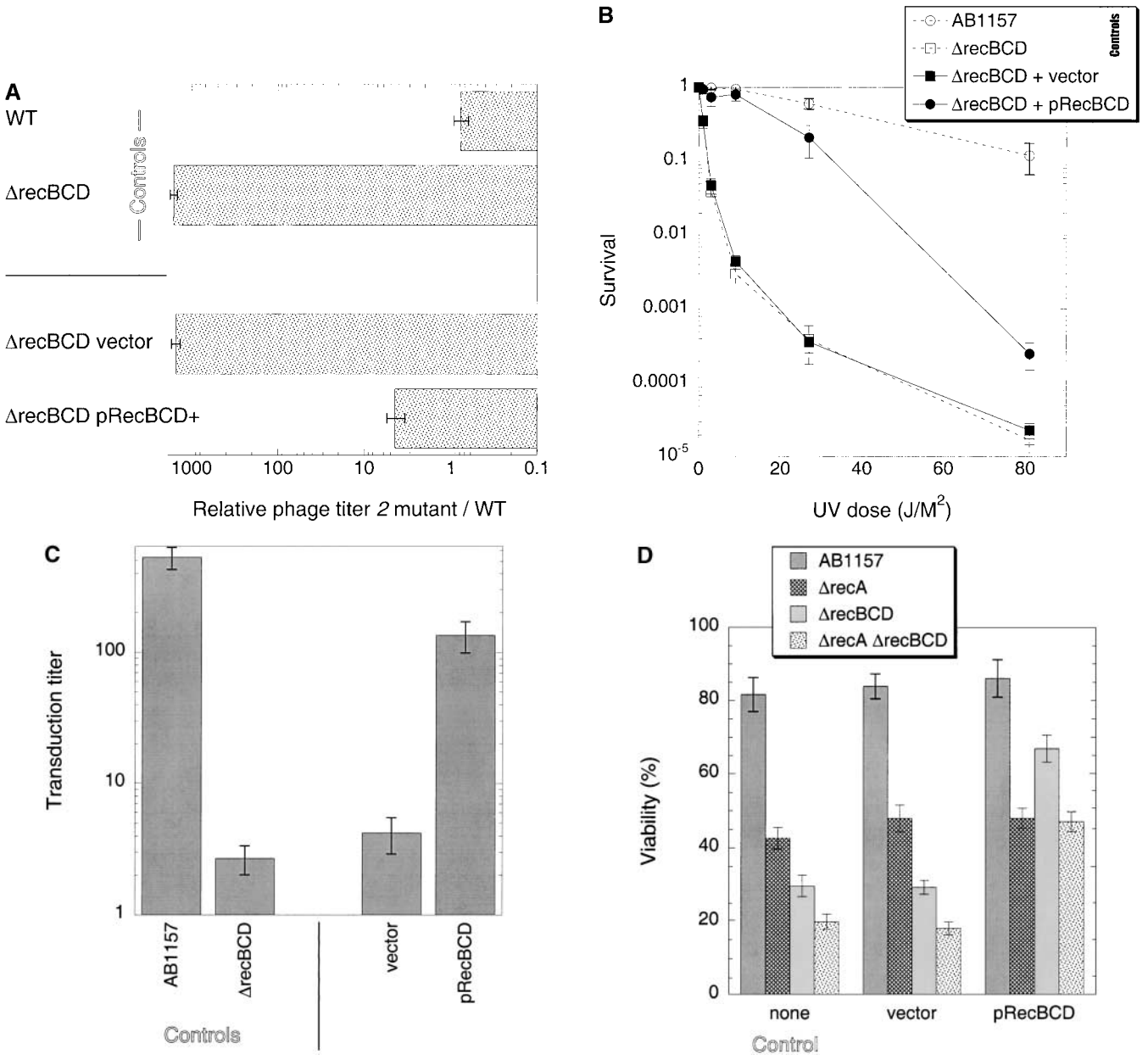

FIGURE 3.—pRecBCD+ plasmid fully complements the linear DNA degradation defect and partially complements the recombinational repair defect of *recBCD* mutant cells. The assays in A–C employ wild type, Δ*recBCD* mutant cells, and Δ*recBCD* mutant complemented with the vector alone or with the pRecBCD+ plasmid. The viability assay in D employs wild-type, Δ*recA*, Δ*recBCD*, and Δ*recA* Δ*recBCD* strains. (A) Linear DNA degradation capacity, as gauged by plating efficiency of T4 *2* mutant phage. (B) Recombinational repair capacity, as gauged by survival of UV irradiation. (C) Recombination repair capacity *vs.* linear DNA degradation activity in functional interaction with each other, as gauged by a two-marker P1 transduction. (D) Viability expressed as colony-forming efficiency. The strains are: AB1157, the wild-type control; Δ*recA*, JC10287; Δ*recBCD*, JB1; Δ*recA* Δ*recBCD*, AM1. The plasmids are: vector, pWSK29; pRecBCD+, pAMP1.

type cells, as well as single Δ*recA* or Δ*recBCD* deletions and of the double Δ*recA* Δ*recBCD* deletion strains, transformed with vector alone or with the *recBCD*+ plasmid. Vector alone did not significantly change the viability of the four strains (Figure 3D). In contrast, the *recBCD*+ plasmid, although having no effect on the viability of wild-type and Δ*recA* cells, increased the viability of the Δ*recBCD* mutant to the level intermediate between the mutant and the wild type and increased the viability of the Δ*recA* Δ*recBCD* mutant to the level of the Δ*recA* mutant (Figure 3D). Subjecting chromosomal DNA to pulsed-field gels revealed that the *recBCD*+ plasmid decreases chromosomal fragmentation in both *recBCD* and *recA recBCD* mutant cells (Figure 2, lanes e and f). We conclude that (1) the tests for the RecBCD functions are highly sensitive, allowing a distinguishing power of

two to three orders of magnitude; (2) the vector itself does not contribute to the RecBCD-attributed characteristics and to the viability; and (3) the *recBCD*-carrying plasmid does restore some characteristics to the levels of the wild-type cells and some other characteristics to intermediate levels (addressed in the DISCUSSION).

RecBCD enzyme does not play a structural role in supramolecular assemblies: As elaborated above, RecBCD can boost the viability of *E. coli* independently of RecA in at least four possible ways (Figure 1): (1) by serving as a structural element at the replication factory, (2) by promoting RecA-independent recombinational repair of damaged chromosomes, (3) by suppressing chromosomal damage via degradation of abnormal replication forks, and (4) by returning σ-replicating chromosomes to θ replication via degradation of linear tails. First, we tested the idea that RecBCD could be an integral part of a supramolecular assembly involved in chromosomal replication/repair (replication factory) and that its absence destabilizes the assembly, negatively affecting the replication process.

To do that, we provided the four test strains with the AddAB enzyme from *B. subtilis*, the powerful exonuclease/helicase that plays the same role in Bacillus as RecBCD does in *E. coli*, but has little sequence homology with the *E. coli* RecBCD and therefore is unlikely to become a part of any supramolecular structure in *E. coli*. Somewhat surprisingly, the AddAB enzyme complemented not only the DNA degradation deficiency of the Δ*recBCD* mutant (Figure 4A), but also restored its recombinational repair proficiency (KOOISTRA *et al.* 1993; Figure 4B). The combined test for DNA degradation/recombination revealed lowered proficiency of the AddAB enzyme, likely due to the high degradation power of this enzyme (Figure 4C; KOOISTRA *et al.* 1993). This hyperactive DNA degradase and a potent recombinase from a very different organism restore the viability of both the Δ*recBCD* mutant (to the wild-type levels) and the Δ*recA* Δ*recBCD* mutant (to the *recA* mutant levels; Figure 4D), while slightly decreasing the viability of wild-type cells. Inactivation of *addAB* genes by deletion of the promoter region, as well as the very 5′-terminal part of the *addB* gene, eliminates all the complementation (Figure 4, A–C), elevating the viability of all three mutant strains somewhat (Figure 4D). This shows that the effect of the addAB clone is due to the functional AddAB enzyme, rather than to some sequences in the cloned region. The restoration of the Δ*recA* Δ*recBCD* mutant viability by the completely nonhomologous AddAB enzyme therefore contradicts the idea that the RecA-independent role of RecBCD is one of a structural element in a replication factory or another supramolecular complex involved in DNA replication.

As another test of this idea, we employed the RecB^{1080}CD mutant enzyme of *E. coli*, which has a single-amino-acid change completely inactivating the only nuclease active center of the enzyme, situated on the RecB subunit. Although presumably structurally very similar to the wild-type enzyme, the mutant enzyme does not show any DNA degradation activity *in vitro* (YU *et al.* 1998b). In our *in vivo* DNA degradation, recombinational repair, and combined DNA degradation/recombination tests, the *recB^{1080}CD* allele behaves essentially as a null allele (Figure 5, A–C), confirming observations of others (AMUNDSEN *et al.* 2000). If such a (presumably) structurally preserved but functionally inactive enzyme would restore the viability of Δ*recBCD* mutant cells, this could be a strong indication for the structural role of RecBCD. However, the RecB^{1080}CD mutant enzyme moderately increases the viability of *recBCD* mutant cells (and, surprisingly, of wild-type cells), but does not improve the viability of a *recA* single mutant and a *recA recBCD* double mutant (Figure 5D). These results with the RecB^{1080}CD mutant enzyme argue against the structural role for the RecBCD enzyme in *E. coli*'s DNA replication process.

Wild-type levels of DNA degradation or DNA unwinding are important for viability of *recA* mutants: The RecBCD enzyme exhibits two groups of enzymatic activities: those important for recombination and those important for DNA degradation, both of which could contribute to the viability of *recA* mutants, as demonstrated by the effect of the analogous AddAB enzyme from *B. subtilis*. To see which activity of RecBCD is important for *recA*-independent survival, we employed the RecB*CD enzyme, which does not restore UV resistance and P1 transduction proficiency to the Δ*recBCD* mutant cells, but does restore wild-type levels of DNA degradation, as measured by the T4 *2* mutant survival (Figure 5, A–C). Thus, the recombination activities of RecBCD are selectively inactivated by the *recB** mutation. If the RecB*CD mutant enzyme fails to restore the viability of the Δ*recA* Δ*recBCD* mutant cells, this would argue against the role of linear DNA degradation in *recA*-independent survival. However, we found that the RecB*CD mutant enzyme restores the viability of the Δ*recBCD* mutant to wild-type levels and upgrades the viability of the *recA recBCD* mutant to the *recA* mutant level (Figure 5D), arguing that it *is* the DNA degradation activity of RecBCD that is important for its RecA-independent contribution to viability.

To verify this conclusion, we employed a *recBC+* allele; deletion of *recD* should selectively inactivate the DNA degradation activities of the RecBCD enzyme, leaving the DNA helicase activity (important for recombinational repair) intact (AMUNDSEN *et al.* 1986; KORANGY and JULIN 1994; CHURCHILL *et al.* 1999). Indeed, our *in vivo* tests show selective removal of the bulk of DNA degradation activity of the enzyme (Figure 6, A–C, pRecBC variant): the RecBC-supplemented Δ*recBCD* mutant cells behave as wild-type cells in the recombinational repair (UV survival) test, are grossly defective in linear DNA degradation (50% permissivity for T4 *2* mutant), and hyper-rec in the combined DNA degrada-

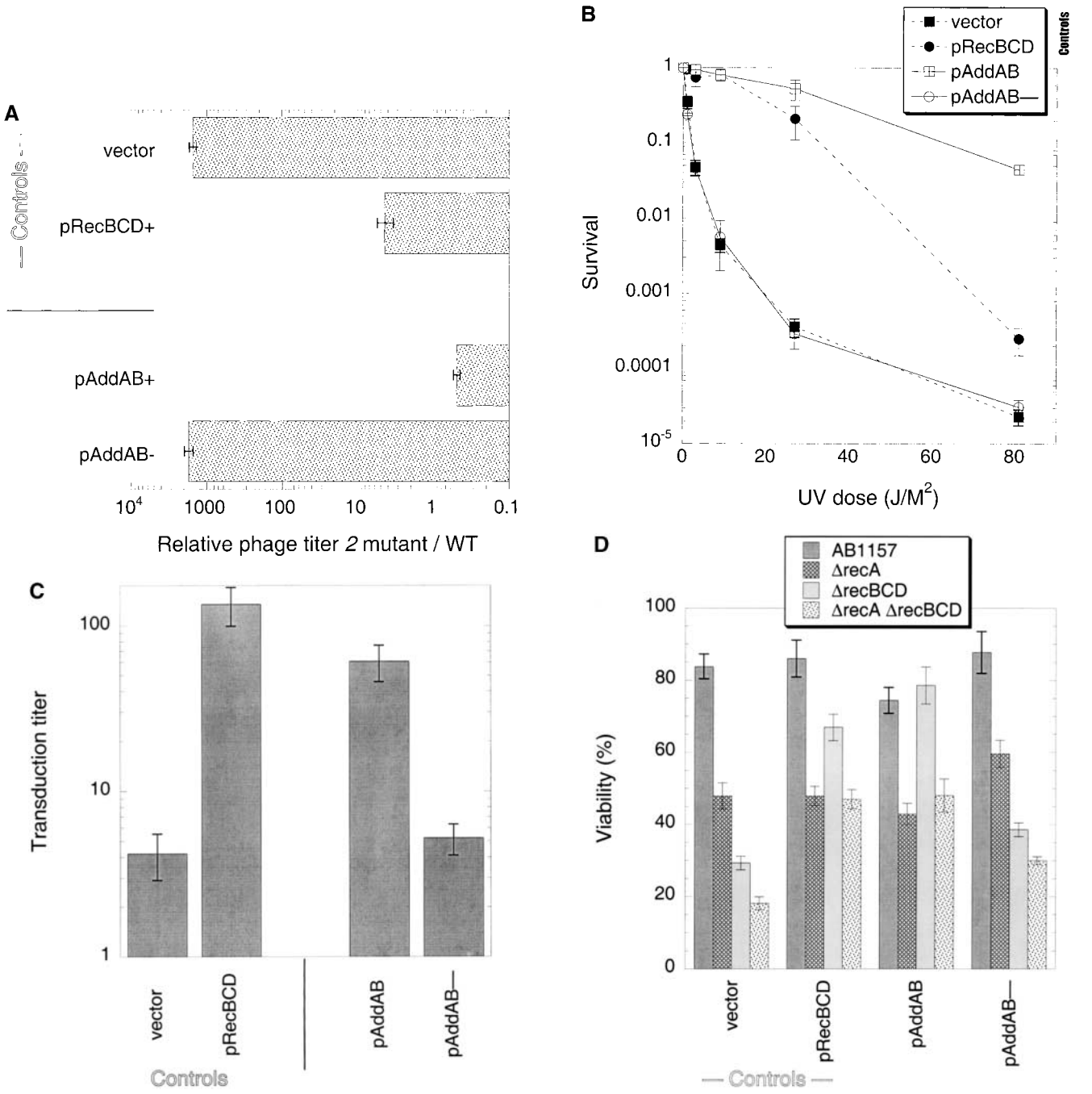

FIGURE 4.—The effect of the *B. subtilis* AddAB nuclease. The four assays are the same as in Figure 3. The assays in A–C employ Δ*recBCD* mutant cells complemented with various plasmids. The viability assay in D employs wild-type, Δ*recA*, Δ*recBCD*, and Δ*recA* Δ*recBCD* strains (identified in the legend to Figure 3). The plasmids are: vector, pWSK29; pRecBCD+, pAMP1; pAddAB+, pWSK2988; pAddAB−, pAMP2.

tion/recombination test (two-marker P1 transduction), reflecting their defect in linear DNA degradation. If the wild-type levels of DNA degradation were important for the *recA*-independent contribution to viability by RecBCD, the RecBC enzyme would not contribute significantly to the viability of *recA recBCD* mutants. The plasmid expressing only *recBC* genes does not influence the viability of the wild-type or *recA* mutants cells, but, remarkably, it almost doubles the viability of *recBCD* mutant cells and restores the viability of *recA recBCD* mutant cells to the levels of *recA* mutants (Figure 6D). As a control we used a plasmid that expresses only the RecD polypeptide. As expected, this plasmid did not restore any RecBCD-specific activity of the Δ*recBCD* mutant cells, nor did it change the viability of the three mutants (Figure 6). Characteristically, the RecD-producing plas-

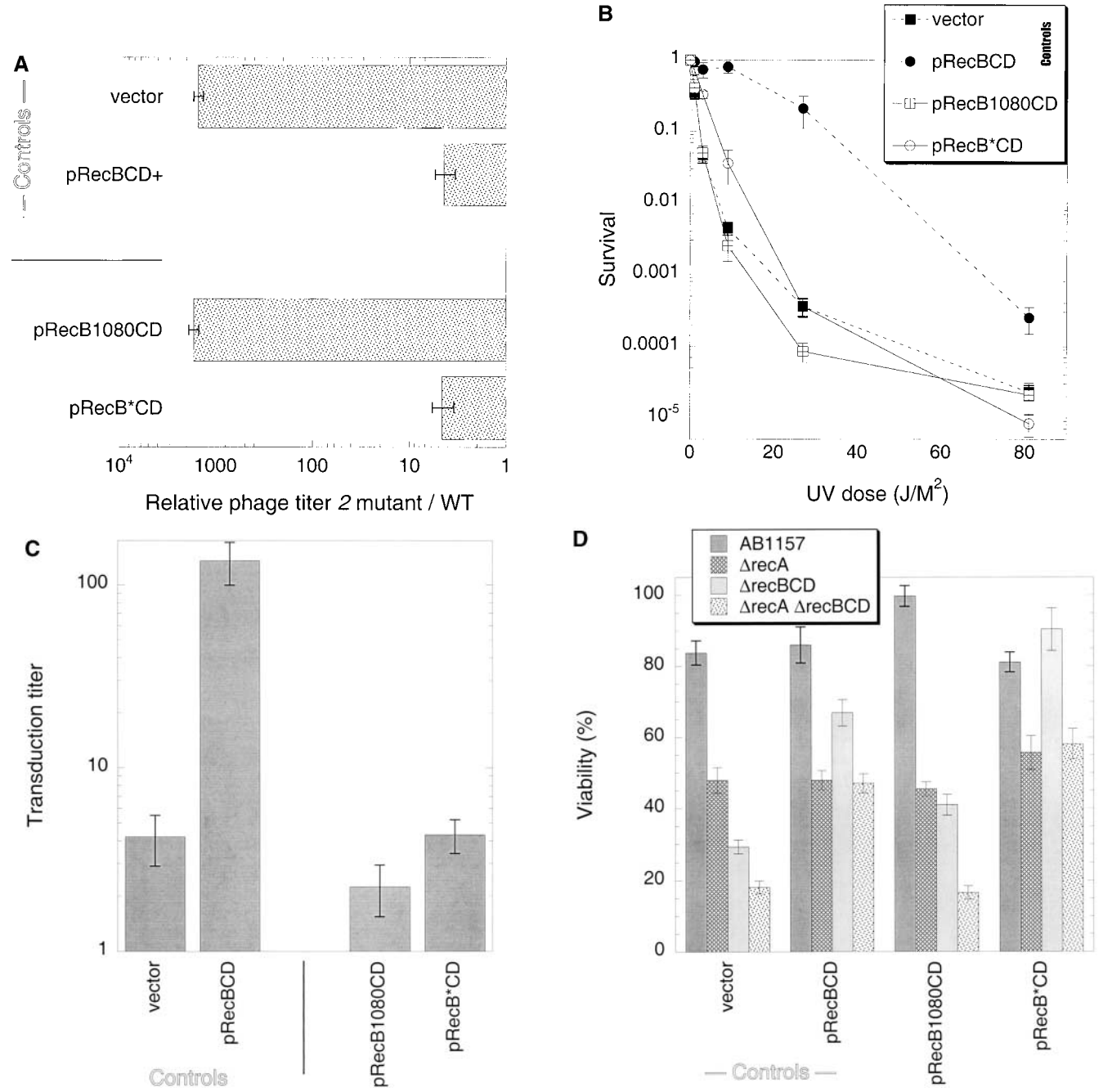

FIGURE 5.—The effect of two RecBCD enzymes with point mutations. The four assays are the same as in Figure 3. The assays in A–C employ Δ*recBCD* mutant cells complemented with various plasmids. The viability assay in D employs wild-type, Δ*recA*, Δ*recBCD*, and Δ*recA* Δ*recBCD* strains (identified in the legend to Figure 3). The plasmids are: vector, pWSK29; pRecBCD+, pAMP1; pRecB1080CD, pAMP7; pRecB*CD, pAMP5.

mid lowered the viability of the wild-type cells, probably due to the lack of regulation of the DNA degradation, observed in strains overproducing the RecD subunit (BRCIC-KOSTIC *et al.* 1992).

DNA unwinding restores viability only if ssDNA-specific degradation is available: At face value, the result with the RecBC enzyme contradicts the previous result, arguing that the wild-type level of DNA degradation is not as important for viability in the absence of RecA as the recombination-relevant DNA helicase activity of the RecBC(D−) mutant enzyme is. To see if the RecBC enzyme or the complete RecBCD enzyme could promote recombinational repair in the absence of RecA, we verified whether the corresponding plasmids confer any degree of UV resistance or P1 transduction proficiency to the *recA recBCD* mutant. We found no changes

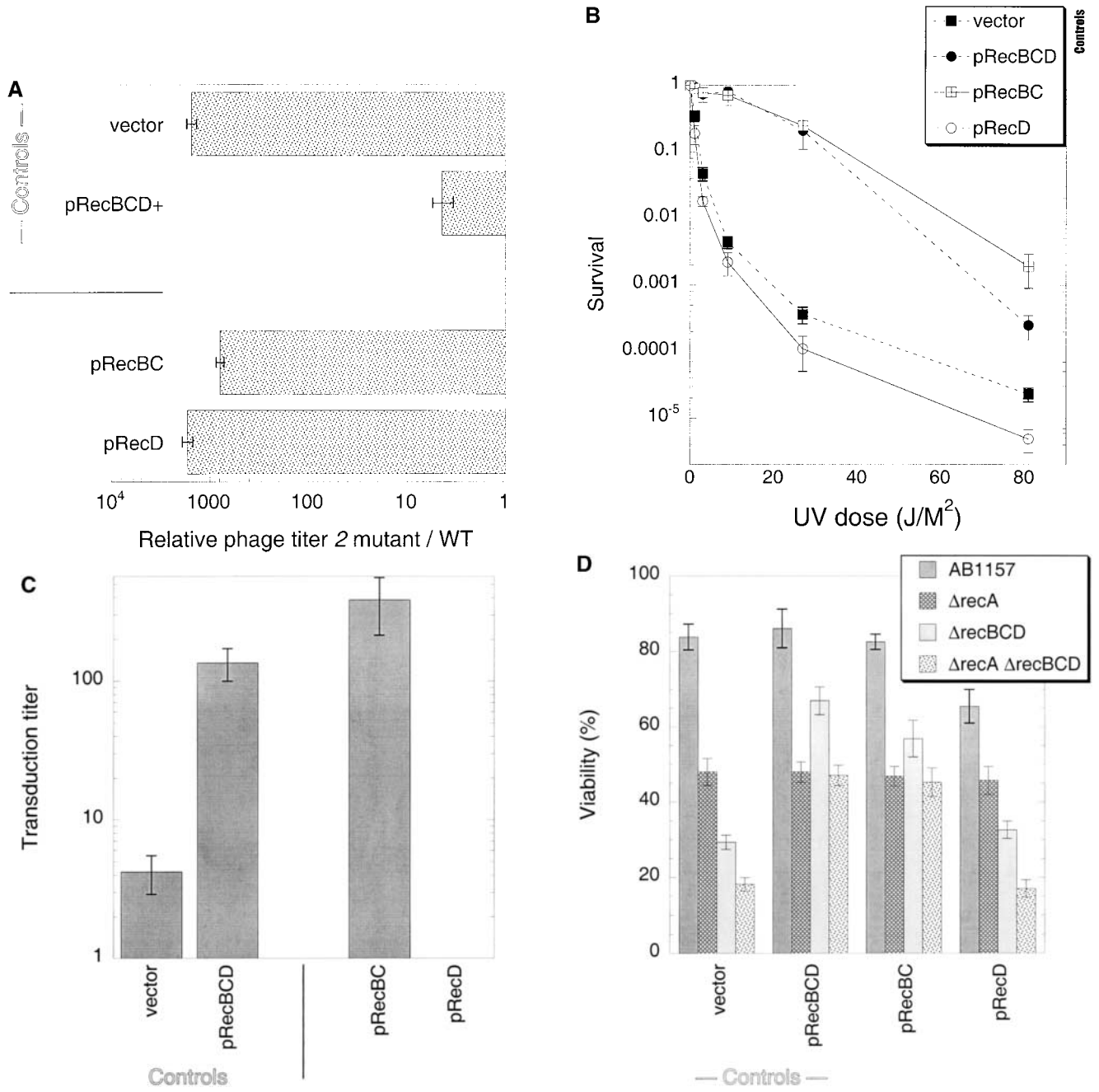

FIGURE 6.—The effect of expressing the RecBC enzyme *vs.* the RecD subunit. The four assays are the same as in Figure 3. The assays in A–C employ Δ*recBCD* mutant cells complemented with various plasmids. The viability assay in D employs wild-type, Δ*recA*, Δ*recBCD*, and Δ*recA* Δ*recBCD* strains (identified in the legend to Figure 3). The plasmids are: vector, pWSK29; pRecBCD+, pAMP1; pRecBC, pAMP3; pRecD, pAMP8.

in UV resistance and a very low level of P1 transduction promoted by the RecBCD or RecBC enzymes in the absence of RecA (Figure 7). Thus, the only possible explanation for the surprising restoration of the viability of the Δ*recA* Δ*recBCD* mutant by the RecBC-producing plasmid is that the attenuated DNA degradation, catalyzed by the combination of RecBC helicase and ssDNA-specific nucleases (Figure 8A; RINKEN *et al.* 1992; KORANGY and JULIN 1994), is enough to restore the viability of *recA recBCD* mutants to the level of *recA* mutants. The prediction is that inactivation of the ssDNA-specific exonucleases in the RecBC-complemented Δ*recA* Δ*recBCD* mutant would diminish the degree to which the viability is restored.

To test this idea, we constructed a Δ*recA* Δ*recBCD* variant, with the two major single-strand DNA-specific exonucleases, ExoI (gp*xonA*) and RecJ, inactivated. To accomplish this, we complemented the strain with a temperature-sensitive plasmid, carrying both *recA*+ and

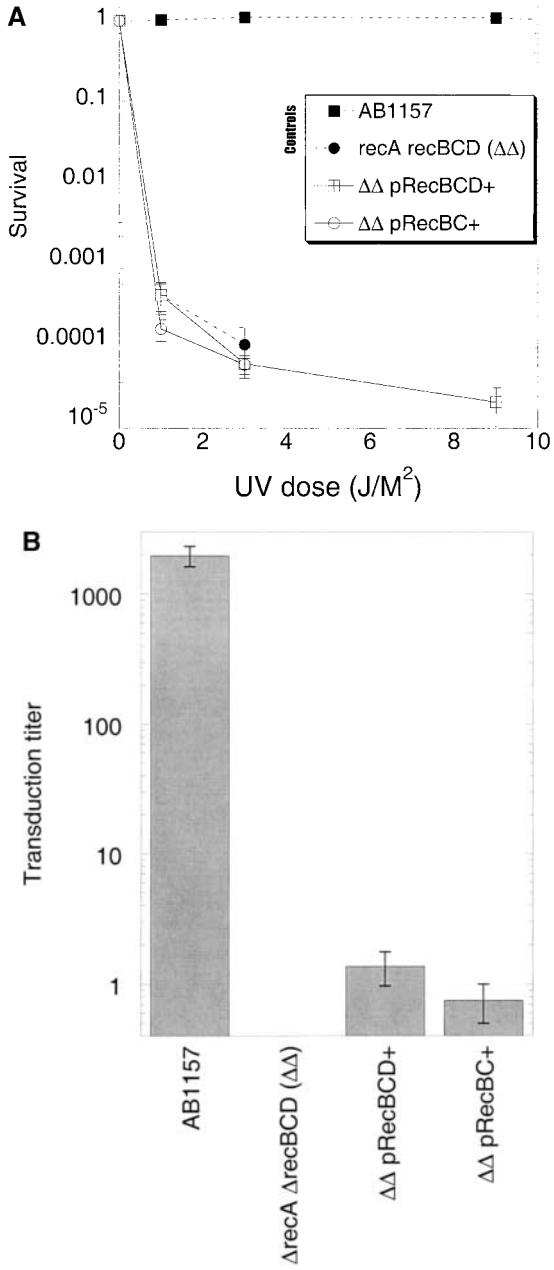

FIGURE 7.—RecBCD or RecBC enzymes are unable to promote recombinational repair in the absence of RecA protein. The assays employ wild-type, Δ*recA* Δ*recBCD* mutant cells, and the Δ*recA* Δ*recBCD* mutant complemented with the pRecBCD+ plasmid or with the pRecBC+ plasmid. (A) Survival of UV irradiation. (B) One-marker P1 transduction. The strains are: AB1157, the wild-type control; Δ*recA* Δ*recBCD*, AM1. The plasmids are: pRecBCD+, pAMP1; pRecBC+, pAMP3.

recBCD+ genes, introduced either a Δ*xonA* or a *recJ* mutation by P1 transduction, and, finally, lost the complementing plasmid. The plasmid was easily lost from the Δ*recA* Δ*recBCD* Δ*xonA* mutant, was lost with some difficulty from the Δ*recA* Δ*recBCD recJ* mutant, but could not be lost, even after repeated attempts, from the Δ*recA* Δ*recBCD* Δ*xonA recJ* mutant, suggesting that this combination is inviable (in fact, even the Δ*recA* Δ*recBCD recJ* mutant has an extremely low viability; Figure 8C). Since the double Δ*xonA recJ* mutant was inviable in the Δ*recA* Δ*recBCD* background, we had to do the experiment in Δ*recA* Δ*recBCD* Δ*xonA* and Δ*recA* Δ*recBCD recJ* mutant strains.

To confirm the ssDNA-exonuclease defects, we introduced the RecBCD+ or the RecBC+ plasmids into the ssDNA-exonuclease-deficient Δ*recA* Δ*recBCD* cells and measured the level of linear DNA degradation in these cells by T4 *2* mutant plating. As expected, T4 *2* mutant plating was high on the Δ*recA* Δ*recBCD* mutant and its Δ*xonA* or *recJ* derivatives (Figure 8B). T4 *2* mutant plating decreased to the same low level in all three mutants supplemented by the RecBCD+ (Hel+/Exo+) plasmid (RecBCD degrades dsDNA without the help of ssDNA-specific exonucleases) and was intermediate in the presence of the RecBC+ (Hel+/Exo−) plasmid in the Δ*recA* Δ*recBCD* cells (Figure 8B). However, compared with the Δ*recA* Δ*recBCD* strain, T4 *2* mutant plating in the presence of pRecBC+ plasmid was four times higher on Δ*recA* Δ*recBCD* Δ*xonA* and seven times higher on Δ*recA* Δ*recBCD recJ* mutant cells, corroborating their defect in ssDNA-specific exonucleases (Figure 8B).

As expected, the RecBCD+ plasmid completely restored the viability of the ssDNA-exonuclease-deficient Δ*recA* Δ*recBCD* cells to the viability levels of *recA* mutants (Figure 8C). RecBC+ plasmid also restored the viability of the Δ*recA* Δ*recBCD* control strain almost to the *recA* mutant level. However, the viability of the Δ*recA* Δ*recBCD* Δ*xonA* mutant, and especially of the Δ*recA* Δ*recBCD recJ* mutant, although improved, was still two- to threefold below the viability of the same mutants, complemented with the complete RecBCD+ region (Figure 8C). In other words, the effects on the two graphs (Figure 8, B *vs.* C) were exactly reversed: the higher T4 *2* mutant survival (indicating lower DNA degradation levels) translated into the lower viability of the strain. Thus, ssDNA-specific exonucleases become essential for viability if the complete RecBCD enzyme is replaced with the exonuclease-deficient but helicase-proficient RecBC enzyme. Therefore, the relatively high viability of *recA* mutants relies on the high affinity of the RecBCD or RecBC enzymes toward double-strand ends and depends on some DNA degradation from these ends.

DISCUSSION

RecBCD of *E. coli* is a large protein complex with DNA helicase and exonuclease activities, which are implicated in degradation of linear DNA and in recombinational repair of double-strand breaks in the chromosome. *recA* null mutants are 50% viable, whereas *recA recBCD* mutants are only 20% viable, indicating a role for the RecBCD enzyme in a *recA*-independent survival. We conceived four possibilities for such a role (Figure 1): (1) RecBCD is a structural element in the replication

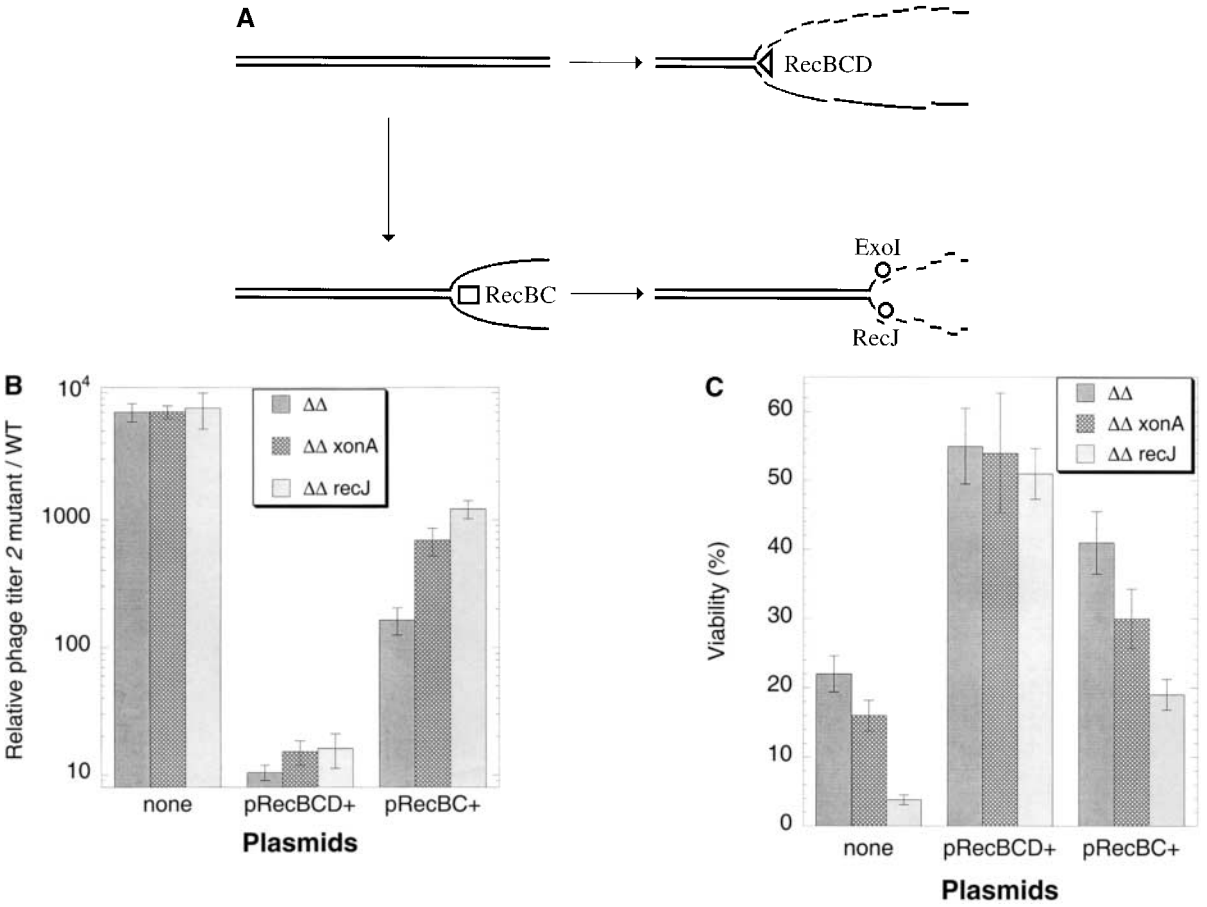

FIGURE 8.—The viability of *recA recBCD* mutant cells complemented with the RecBC+ enzyme depends on the ssDNA-specific exonucleases. The assays employ *recA recBCD* (ΔΔ), *recA recBCD xonA* (ΔΔ xonA), and *recA recBCD recJ* mutants (ΔΔ recJ), either uncomplemented or complemented with pRecBCD+ or pRecBC+ plasmids. (A) The scheme of linear DNA degradation by the RecBCD nuclease or by the combination of the RecBC helicase and ssDNA-specific exonucleases ExoI and RecJ. (B) Linear DNA degradation capacity, as gauged by plating efficiency of T4 *2* mutant phage. (C) Viability expressed as colony-forming efficiency. The strains are: Δ*recA* Δ*recBCD*, AM1; Δ*recA* Δ*recBCD recJ*, AK132; Δ*recA* Δ*recBCD xonA*, AK133. The plasmids are: pRecBCD+, pAMP1; pRecBC+, pAMP3.

factory assembly; (2) RecBCD has RecA-independent recombinational repair activity (uses its DNA helicase activity to reassemble disintegrated replication forks); (3) RecBCD is a chromosomal damage suppressor (uses its high affinity to double-strand ends to attack abnormal replication structures whose alternative processing would lead to chromosomal lesions); and (4) RecBCD has chromosomal damage-removal activity (uses its powerful exonuclease activity to degrade long linear tails of σ-replicating chromosomes, thus returning chromosomes to θ-replication).

To identify the RecA-independent role for the RecBCD enzyme in the viability of *E. coli* cells, we employed several constructs, carrying the *recBCD* chromosomal region on a low-copy-number plasmid (Table 3). The constructs were characterized in Δ*recBCD* mutant cells for the DNA degradation capacity (T4 *2* mutant survival) and for recombinational repair proficiency (UV resistance) as well as in a combined test for both DNA degradation and recombination (two-marker P1 transduction). Some constructs restored both the DNA degradation and the recombinational repair capacity of the Δ*recBCD* mutant, some other constructs restored selectively either DNA degradation or recombinational repair, while yet other constructs restored neither property. In addition, the introduced enzymes were judged either structurally similar or dissimilar to the wild-type RecBCD enzyme on the basis of the sequence homology or the nature of the mutation (Table 3). We then introduced these different constructs into a Δ*recA* Δ*recBCD* mutant and determined viability of the resulting strains. We found that the RecBCD mutant enzyme lacking any activity due to a point mutation in the active site cannot restore the viability of the Δ*recA* Δ*recBCD* mutant, which argues against the idea that RecBCD plays the role of a structural element at the replication factory. The enzyme proficient only in DNA degradation restores the viability, which argues for the importance of DNA degra-

TABLE 3

Summary of the findings

Construct name	Structure	Nuclease	Recombinase[a]	Viability[b]
vector	NA	NA	NA	−
pRecBCD	+	+	+	+
pAddAB	−	+	+	+
pRecBC	±	±	+	+
pRecBC[c]	±	−[c]	?	−[c]
pRecB1080	+	−	−	−
pRecB*CD	+	+	−	+
pAddAB−	−	−	−	−
pRecD	−	−	−	−

The three properties of the RecBCD enzyme, relevant for the RecA-independent role in cell viability, are "structure," DNA degradation ("nuclease"), and recombinational repair ("recombinase"). "+," wild-type levels; "±," partial levels; "−," lack of property; "?," "unknown"; NA, not applicable.

[a] In Δ*recBCD* mutant cells.

[b] In Δ*recA* Δ*recBCD* mutant cells.

[c] In ssDNA-exonuclease-deficient Δ*recA* Δ*recBCD* mutant cells.

dation in prevention/removal of chromosomal lesions. Surprisingly, the enzyme proficient only in DNA unwinding can also restore the viability (Table 3), suggesting *recA*-independent recombinational repair. However, the constructs that restore the recombinational repair capacity to the Δ*recBCD* strain do not restore the recombinational repair capacity to the Δ*recA* Δ*recBCD* strain, suggesting that the only RecA-independent role of the RecBC enzyme is still in linear DNA degradation. The inability of the helicase-proficient but exonuclease-deficient RecBC enzyme to fully restore the viability of Δ*recA* Δ*recBCD* strains, deficient in either one of the two major ssDNA-specific exonucleases, confirms this idea. We conclude that the low viability of *recA recBCD* mutants is due to their loss of the capacity to target linear DNA for limited degradation.

We introduced our constructs on a pSC101-derivative plasmid, which is reported to have a copy number of five to seven per chromosome (Manen and Caro 1991). The constructs retain natural promoters, and since no regulation is known for any gene of the *recBCD* operon, we may assume that the actual level of the enzymes produced from these constructs is five to seven times higher than the wild-type level. Therefore, the incomplete complementation of the Δ*recBCD* mutant phenotype by the pRecBCD and pRecBC plasmids could be attributed to this severalfold overproduction of the enzyme from plasmid. On the other hand, the *recBCD* genes in all our plasmids are cloned in opposite orientation relative to the direction of transcription from the truncated *lacZ* gene, making it possible that the *recBCD* genes actually are underexpressed. However, when we inverted the wild-type *recBCD* fragment in the pAMP1 construct, the viability of the Δ*recBCD* deletion strain, complemented with the "inverted" construct (pAMP1B), was the same as the viability complemented by the "direct" construct (data not shown), suggesting that the possible *lacZ* expression does not interfere with the expression of *recBCD*. The incomplete complementation is unlikely to be due to the plasmid instability, because (1) plasmids are relatively stable in *recBC* mutants (Bassett and Kushner 1984) and (2) the pSC101 replicon has a dimer resolution site and therefore always exists in monomeric form, which increases plasmid stability (Cornet *et al.* 1994). Few examples of incomplete complementation notwithstanding, the combined results provide a coherent and compelling picture, allowing one to distinguish among the competing hypotheses with confidence.

What is the nature of chromosomal lesions suppressed or removed by the RecBCD-promoted DNA degradation? Pulsed-field gel electrophoresis reveals significant chromosome fragmentation in *recA* mutants, which is roughly doubled in *recBCD* and *recA recBCD* mutants (Figure 2; Michel *et al.* 1997; Grompone *et al.* 2002). Therefore, the chromosomal lesions must be double-strand ends: either double-strand breaks or disintegrated replication forks. Our original idea was that the main targets of the RecBCD-promoted degradation were long linear tails of σ-replicating chromosomes (Figure 1, D → A; Horiuchi and Fujimura 1995; Uzest *et al.* 1995; Kuzminov and Stahl 1997). The speed with which the RecBCD enzyme can unwind DNA *in vitro* [1000 bp/sec (Roman and Kowalczykowski 1989)—and presumably even faster *in vivo*] is higher than the speed of replication fork propagation in *E. coli* (600–900 bp/sec; Bremer and Dennis 1996), so, theoretically, RecBCD can overcome the remaining replication fork in the σ-replicating chromosome. However, our finding that a more modest DNA degradation activity, due to the combined action of the RecBC helicase plus ssDNA-specific exonucleases ExoI and RecJ (Rinken *et al.* 1992; Korangy and Julin 1994), restores the viability of the *recA recBCD* mutant almost to the same level that the wild-type RecBCD enzyme does, argues against the σ-replicating chromosome as the most frequent target of RecBCD. Two additional observations suggest that degradation of linear tails of σ-structures cannot be the sole RecA-independent pathway of RecBCD-promoted chromosomal repair: (1) in *polA recA*, *lig recA*, and *dam recA* mutants the chromosome is degraded by RecBCD, but the degradation does not make these mutants viable (Monk and Kinross 1972; Marinus and Morris 1975; Morse *et al.* 1976) and (2) there is no more chromosomal degradation in *rep recA* or *holD recA* mutants than in *recA* single mutants, but the single *rep* or *holD* mutants are dependent on RecBCD, while the double mutants (with *recA*) are even dependent on RecD (Seigneur *et al.* 1998; Flores *et al.* 2001). Apparently, there are situations when RecBCD does not have to degrade long

linear tails to contribute to viability. This raises a question about the chromosomal structure, in which a short linear piece of DNA makes the difference between a healthy chromosome and a chromosome with a lesion.

It was proposed that inhibited replication forks can reverse, extruding the newly synthesized DNA strands into a new duplex and forming a Holliday junction (Figure 1C; Scudiero and Strauss 1974; Higgins *et al.* 1976; Morgan and Severini 1990; Louarn *et al.* 1991). Formation of such reversed replication forks has been inferred from chromosomal fragmentation dependent on the Holliday-junction-resolving enzymes (Seigneur *et al.* 1998; Flores *et al.* 2001; Grompone *et al.* 2002), as well as observed by electron microscopy in preparation for replicating chromosomes from yeast checkpoint mutants, where DNA before isolation was crosslinked *in vivo* to preserve the original topology (Sogo *et al.* 2002). It was further proposed that the open double-strand end of the newly formed duplex is attacked by the RecBCD enzyme (Louarn *et al.* 1991) and that the duplex is either completely degraded, eliminating the Holliday junction (Figure 1, C → B; Kuzminov 1995), or recombined with the parental duplex ahead of the fork (Seigneur *et al.* 1998). Without such degradation, the Holliday junction is eventually cut by the RuvABC resolvasome, which breaks the replication fork (Figure 1, C → D; Seigneur *et al.* 1998). Interestingly, the chromosome fragmentation in *recB recA* mutants is suppressed to the *recA* levels by inactivation of the RuvABC resolvasome (Michel *et al.* 1997; Grompone *et al.* 2002), suggesting reversed replication forks as one possible chromosomal lesion suppressed by the RecBCD-promoted DNA degradation. However, in our hands, inactivation of RuvABC did not significantly improve the viability of *recA recBCD* mutants (data not shown), so there must be other chromosomal lesions, prevented or removed by the RecBCD-promoted degradation.

RecBCD is not the only enzyme hypothesized to be involved in both the formation and subsequent repair/removal of chromosomal lesions. As detailed above, RuvABC is another enzyme involved at both stages. The difference is that, while both RecBCD and RuvABC help to repair chromosomal lesions, RecBCD also suppresses their formation, whereas RuvABC promotes their formation. Still other recombinational repair enzymes, RecG and PriA helicases, are recognized by their *in vitro* affinity to branched DNA structures as potential early players around inhibited replication forks (McGlynn *et al.* 1997; Whitby and Lloyd 1998; McGlynn *et al.* 2001; Gregg *et al.* 2002). RecG and PriA recently have been proposed to repair stalled forks without breakage or recombination (Gregg *et al.* 2002), but we found no predicted decrease in viability caused by *recG* inactivation in the double *recA recBCD* mutants (data not shown).

In summary, we propose that *recBCD* mutant cells are unable (1) to suppress breakage of inhibited replication forks independently of RecA and (2) to degrade the linear tails of σ-replicating chromosomes in the absence of RecA. We propose that this double defect is the reason why the viability of *recA recBCD* mutants is significantly lower than that of *recA* mutants. If we add to these two defects the deficiency in repair of broken replication forks, in which RecBCD is involved together with RecA, it becomes clear why *recBCD* mutants have such a low viability.

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