The RuvAB Branch Migration Translocase and RecU Holliday Junction Resolvase Are Required for Double-Stranded DNA Break Repair in *Bacillus subtilis*

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ABSTRACT

In models of *Escherichia coli* recombination and DNA repair, the RuvABC complex directs the branch migration and resolution of Holliday junction DNA. To probe the validity of the *E. coli* paradigm, we examined the impact of mutations in Δ*ruvAB* and Δ*recU* (a *ruvC* functional analog) on DNA repair. Under standard transformation conditions we failed to construct Δ*ruvAB* Δ*recG*, Δ*recU* Δ*ruvAB*, Δ*recU* Δ*recG*, or Δ*recU* Δ*recJ* strains. However, Δ*ruvAB* could be combined with *addAB* (*recBCD*), *recF*, *recH*, Δ*recS*, Δ*recQ*, and Δ*recJ* mutations. The Δ*ruvAB* and Δ*recU* mutations rendered cells extremely sensitive to DNA-damaging agents, although less sensitive than a Δ*recA* strain. When damaged cells were analyzed, we found that RecU was recruited to defined double-stranded DNA breaks (DSBs) and colocalized with RecN. RecU localized to these centers at a later time point during DSB repair, and formation was dependent on RuvAB. In addition, expression of RecU in an *E. coli ruvC* mutant restored full resistance to UV light only when the *ruvAB* genes were present. The results demonstrate that, as with *E. coli* RuvABC, RuvAB targets RecU to recombination intermediates and that all three proteins are required for repair of DSBs arising from lesions in chromosomal DNA.

I N all organisms, structural aberrations in the DNA template or strand breaks induce arrest or collapse of replication forks and their restoration relies on recombination functions (HABER 1999; KUZMINOV 1999; COX *et al.* 2000; MICHEL *et al.* 2004). In *Escherichia coli*, stalled forks can reverse to form a four-stranded Holliday junction (HJ) intermediate (SEIGNEUR *et al.* 1998). Fork regression, which might also occur spontaneously, involves RecG or potentially RecA, the latter loaded onto single-stranded DNA (ssDNA) by the RecFOR complex (ROBU *et al.* 2001, 2004; SINGLETON *et al.* 2001; MCGLYNN and LLOYD 2002a,b). Once formed, the HJ can be processed in a number of ways: (i) The extruded duplex end can be removed by either RecBCD or RecQ and RecJ to reset the fork; (ii) DNA synthesis on the extruded partial duplex end followed by restoration of the fork by RecG or RuvAB branch migration provides a means of translesion bypass; and (iii) branch migration away from a block or lesion and HJ resolution by RuvC generates a broken fork (as is the case when the replisome encounters a strand break). RecA then mediates invasion of this broken end into the

intact chromosome arm to rebuild the replication fork (HABER 1999; KUZMINOV 1999; COX *et al.* 2000; MICHEL *et al.* 2004). Mechanisms for direct fork rescue, which do not invoke the formation of a HJ intermediate, have also been proposed and rely on the action of RecFOR, RecJ, and RecQ recombinases (COURCELLE and HANAWALT 1999; COURCELLE *et al.* 2001; DONALDSON *et al.* 2004).

The models for recombination-dependent replication highlight the important role played by RecBCD, RecQ, RecJ, and RecFOR in processing the ends of collapsed forks and loading of RecA (CLARK and SANDLER 1994; KOWALCZYKOWSKI and EGGLESTON 1994; KUZMINOV 1999; AMUNDSEN and SMITH 2003; MICHEL *et al.* 2004). RecBCD preferentially degrades double-stranded DNA (dsDNA) ends to expose a 3′ single-stranded tail. Similar reactions can be catalyzed by unwinding the end using RecQ helicase coupled with strand removal by the RecJ 5′-3′ exonuclease (COURCELLE *et al.* 2001; AMUNDSEN and SMITH 2003). RecA can be loaded directly onto this resected ssDNA by RecBCD (ANDERSON and KOWALCZYKOWSKI 1997, 2000; CHEDIN and KOWALCZYKOWSKI 2002; AMUNDSEN and SMITH 2003; XU and MARIANS 2003) or by the RecFOR complex when the strand is coated with Single-stranded DNA binding (SSB) protein (UMEZU and KOLODNER 1994; SHAN *et al.* 1997; KANTAKE *et al.* 2002; IVANCIC-BACE *et al.* 2003). Formation of a RecA nucleoprotein filament allows homologous pairing and strand

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exchange between the broken end and its undamaged partner. Invasion of the homologous duplex by the processed 3′-tail creates a D-loop upon which the replication apparatus can be reloaded by PriA (Kowalczykowski 2000; Marians 2000; McGlynn and Lloyd 2002a,b). At this stage the chromosomes are still interconnected so further extension of the DNA joint to form a HJ is needed so that RuvABC resolution can complete the fork restoration process.

In *Bacillus subtilis* the recombination genes, other than *recA*, which is central to all pathways of recombinational repair, have been placed into six different epistatic groups: α [comprising *recF*, *recL*, *recO*, and *recR* (*recFLOR*) and *recN*], β (*addA* and *addB*), γ (*recP* and *recH*), ε (*ruvA*, *ruvB*, *recD*, and *recU*), ζ (*recS*), and η (*recG*) (Alonso *et al.* 1993; Fernandez *et al.* 1998, 1999, 2000; Chedin *et al.* 2000; Ayora *et al.* 2004; Carrasco *et al.* 2004). Throughout this article, unless stated otherwise, the indicated genes and products refer to those of *B. subtilis* origin. The *recA*, *recF*, *recG*, *recJ*, *recN*, *recO*, *recQ*, *recR*, *ruvA*, and *ruvB* genes each have a homologous counterpart in *E. coli* with the same gene designation. In addition, the *addAB* and *recU* genes encode functional equivalents of *E. coli recBCD* (*recBCD$_{Eco}$*) and *ruvC$_{Eco}$* genes, respectively (Fernandez *et al.* 2000; Ayora *et al.* 2004). However, several recombination genes (*recL*, *recD*, *recH*, and *recP*) have no known equivalent in *E. coli* and, along with *recS* (a RecQ-like helicase), remain uncharacterized (Fernandez *et al.* 1998). Hence products classified within the α, β, ε, and η groups have their *E. coli* counterparts in RecN-FOR, RecBCD, RuvABC, and RecG, respectively (Ayora *et al.* 2004; Carrasco *et al.* 2004; Kidane *et al.* 2004), while those within the γ and ζ epistatic groups have yet to be assigned a function in DNA repair and recombination (Fernandez *et al.* 2000). Additionally, genetic analysis has not been undertaken with RecJ and RecQ and so neither has been assigned to any of these groupings.

Many of these functions and pathways of recombination resemble those encountered in the *E. coli* system. In wild-type cells, the loading of RecA protein onto SSB-coated ssDNA (presynaptic step) relies on AddAB or RecN-RecFLOR proteins (Chedin *et al.* 2000; Kidane *et al.* 2004). Recently it has been shown that: (i) ~35% of the cells in a Δ*recA* and ~5% in a Δ*recU* mutant contain unrepaired DSBs under normal growth conditions (Kidane *et al.* 2004); (ii) the *ruvA* gene complements the defect of the *recB*2 mutation classified within the ε epistatic group [hence *recB*2 was renamed *ruvA*2 (Ayora *et al.* 2004)]; (iii) purified RecU protein binds preferentially to three- and four-strand junctions and cleaves Holliday junction substrates to produce nicked duplexes (Ayora *et al.* 2004); and (iv) in the absence of the RuvAB or RecG branch-migration activities, RecU and the poorly characterized RecD bias exchange toward crossovers (CO) (Carrasco *et al.* 2004).

To shed light on the importance of HJ branch migration and resolution in *B. subtilis*, we constructed a *ruvAB* null mutant (Δ*ruvAB*) and analyzed its sensitivity to different DNA-damaging agents. The Δ*ruvAB*, Δ*recG*, and *recF* strains showed similar sensitivities to DNA damage, which were significantly increased when Δ*ruvAB* was combined with *add*AB, *recF*, *recH*, or Δ*recJ*. Previously it was shown that in the absence of RuvAB, RecU, or RecG a clear chromosomal segregation defect was observed (Carrasco *et al.* 2004). Under standard transformation conditions we failed to construct Δ*ruvAB* Δ*recG*, Δ*ruvAB* Δ*recU*, and Δ*recU* Δ*recG* double-mutant strains. Expression of RecU could replace the repair function of RuvC$_{Eco}$ in the heterologous *E. coli* system if the RuvAB$_{Eco}$ complex were present. Formation of RecU foci on nucleoids also required the presence of RuvAB. Our data support the notion that RuvAB works in concert with the junction-resolving enzyme RecU, in a similar manner to the RuvABC$_{Eco}$ resolvasome complex, and that RuvAB, RecD, and RecU play a vital role in DNA DSB repair.

MATERIALS AND METHODS

Bacterial strains and plasmids: 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 acetyl transferase gene (*cat*) (Carrasco *et al.* 2004) was introduced within the coding sequence of *recJ*, *recQ*, *recG*, and *ruvA ruvB* to generate the *recJ:six-cat-six*, *recQ:six-cat-six*, *recG:six-cat-six*, and *ruvAB:six-cat-six* disruptions. These disruptions were transferred into the chromosome of wild-type cells to generate Δ*recJ*, Δ*recQ*, Δ*recG*, and Δ*ruvAB* strains, and expression of the β-gene provoked deletion of the *cat* gene. The null Δ*recU* or Δ*ruvAB* mutation was transferred into the isogenic *rec*-deficient derivatives and the double mutants generated by a double CO event as previously described (Alonso *et al.* 1993).

Chromosomal DNA from Δ*ruvAB* (*ruvAB:six-cat-six*), Δ*recU* (*recU:six-spc-six*), or Δ*recS* (*recS:cat*) strains were used to transform the wild type and the mutants Δ*ruvAB*, Δ*recU*, Δ*recG*, or Δ*recJ* strains with selection for chloramphenicol (conferred by the *cat* gene) or spectinomicyn (conferred by the *spc* gene) resistance. The Δ*recS* mutation could be transferred into all transformed strains, showing that the mutant strains are competent for transformation. An equivalent amount of chromosomal DNA from Δ*ruvAB* or Δ*recU* mutants transforms wild-type cells with similar efficiency, but no *bona fide* transformants were obtained for the Δ*ruvAB*, Δ*recU*, Δ*recG*, or Δ*recJ* mutant strain. Few tiny colonies after 72 hr of incubation times were obtained, detailed analysis of several of these transformants suggested that some of them contained a single CO with one copy of the wild type and one of the mutant gene, and a few with a double CO contained suppressor mutations (see below).

E. coli K12 *ruv* mutants strains, SR2210 (*ruvA200*), N1057 (*ruvB4*), N2057 (*ruvAB60*::Tn*10*), GS1481 (Δ*ruvC*::*kan*), CS85 (*ruvC53 eda*::Tn*10*), AM888 (Δ*ruvAC65* Δ*rusA*::*kan*), and N4454 (Δ*ruvABC*::*cat*) are derivatives of the *ruv*$^+$ wild-type strain, AB1157 (Sargentini and Smith 1989; Sharples *et al.* 1990; Mandal *et al.* 1993; Mahdi *et al.* 1996; Seigneur *et al.* 1998). Plasmid pCB564 was constructed by transferring the 1.2-kb *Bsp*EI-*Bam*HI DNA segment containing the *recU* gene into pHP13 (pRecU). pCB593 contains the 4.9-kb *Stu*I (*ruvA*) fragment inserted into pUC18 (pRuvA), pCB594 the 4.6-kb

HindIII (*ruvB*) fragment inserted into pUC18 (pRuvB), and pCB559 the 5.5-kb *Bam*HI-*Eco*RI (*ruvAB*) fragment inserted into pUC18 (pRuvAB). A pUC18 clone carrying RecU (pFC204) was obtained by polymerase chain reaction (PCR) from pCB564 using 5′-AGAATTCTAAGGAGGATGAGATAATGATTC-3′ and 5′-TCTGACATAGGATCCCAACCTTTCG and *Eco*RI and *Bam*HI restriction endonucleases (underlined). To create a C-terminal fusion of RecU with YFP for single crossover integration into the chromosome, the 3′ region (500 bp) of *recU* was amplified by PCR using primers 5′-ATCGGGCCCTCGCGGAATGACCC TCG-3′ and 5′-CTAGAATTCACCTTTCGCACGCAGATGATG-3′ and was cloned into *Apa*I and *Eco*RI sites of plasmid pYSG that carries *yfp* and *cat* genes and a xylose promoter for transcription of downstream genes (D. KIDANE and P. L. GRAUMANN, unpublished data), resulting in pYDK6. By transforming pyDK6 into PY79, we created the strain DK53. To move the *recU-yfp* fusion in different mutant backgrounds, DK53 was transformed with chromosomal DNA from the Δ*recN* strain, giving strain DK55, and the Δ*ruvAB* strain was transformed with chromosomal DNA of strain DK53, resulting in strain DK56. For the colocalization experiments, strain DK53 was transformed with chromosomal DNA from *recN-cfp*, generating strain DK54. Plasmid pGS739 was constructed by transferring the 1.7-kb *Bgl*II (*Sst*II)-*Eco*RV fragment containing the *ruvC* gene (obtained from a derivative of pFB512; BENSON *et al.* 1988) into pACYC184 cleaved with *Bam*HI and *Hinc*II. This clone does not fully restore UV resistance to *ruv* mutants, possibly due to a slight negative effect on cell survival after UV exposure. Other plasmids used were pGS762, pGS711, and pPVA101 (SHARPLES *et al.* 1990; SHARPLES and LLOYD 1991).

Viability test: *B. subtilis* recombination-deficient strains were plated and incubated in Luria broth (LB) medium overnight. At least six independent colonies from each strain were resuspended in fresh LB medium and shaken for 30 min to minimize aggregation. Appropriate dilutions were plated on LB and colony-forming units (CFUs) were counted or stained with membrane-permeable SYTO 9 and membrane-impermeable propidium iodide and subjected to conventional direct count of total cells. SYTO 9, which labels bacteria with green fluorescence, and propidium iodide, which stains membrane-compromised bacteria with red fluorescence, were purchased from Molecular Probes (Leiden, The Netherlands).

DNA repair survival studies: Exponentially growing *B. subtilis* cells were obtained by inoculating overnight cultures in fresh LB media and grown to an A_{560nm} of 0.4 at 37°. These were exposed to 10 mM methyl methanesulfonate (MMS) and the fraction surviving was determined with reference to an unexposed control plate. Alternatively, the sensitivity to MMS, 4-nitroquinoline-1-oxide (4NQO), or mitomycin C (MMC) was determined by growing cultures to an A_{560nm} of 0.4 and spotting 10 μl of serial 10-fold dilutions (1×10^{-2} to 1×10^{-5}) on LB agar supplemented with the indicated concentrations of the DNA-damaging agent and incubating overnight at 37°.

E. coli strains carrying appropriate clones were measured for UV resistance by growing cells in LB media to an A_{650nm} of 0.4 and spotting appropriate dilutions onto agar plates. These were exposed to UV light at a dose rate of 1 J/m²/sec and the fraction surviving was determined with reference to an un-irradiated control plate.

DNA lesions generated by MMS, which reacts with single reactive groups in adenine (N3-alkyladenine), guanine (N7-alkylguanine) and 4NQO, which is a potent mutagen that induces two main guanine adducts at positions C_8 and N_2 in damaged dsDNA or ssDNA. MMC results in the formation of interstrand crosslinks and UV light primarily induces pyrimidine dimers. All of these lesions act as DNA replication roadblocks, inducing replication fork arrest and DSBs.

Image acquisition: Fluorescence microscopy was performed on an Olympus AX70 microscope. Cells were grown in minimal medium and were mounted on agarose pads containing S750 medium on object slides as described in KIDANE *et al.* (2004). Images were acquired with a digital MicroMax CCD camera; signal intensities were measured using the META-MORPH 4.6 program. DNA was stained with 4′,6 diamidino-2-phenylindole (DAPI; final concentration 0.2 ng/ml) and membrane with FM4-64 (final concentration 1 nM).

RESULTS

Defects in the α, ε, and η epistatic groups render cells extremely sensitive to DNA-damaging agents: To gain insight into the involvement of HJ processing in the repair of DNA damage, we constructed a null Δ*ruvAB* mutant strain and analyzed its phenotype in parallel with mutations in the α (*recF*15, *recL*16, Δ*recO*, Δ*recR*), β (*addA*5 *addB*72, termed here *addAB*), γ (*recH*342), ε (*recD*41, Δ*recU*, Δ*ruvAB*), ζ (Δ*recS*, Δ*recQ*, Δ*recJ*), and η (Δ*recG*) epistatic groups as well as in the Δ*recA* strain (Table 1).

The recombination-deficient cells, when present in an otherwise Rec⁺ strain, were exposed to the killing action of alkyl groups generated by MMS and their phenotypes were recorded. The Δ*recS*, Δ*recQ*, and Δ*recJ* cells (epistatic group ζ) showed a similar degree of sensitivity to MMS; hence, only the former is shown. Figure 1 shows that Δ*recS*, *addAB*, and *recH*342 cells displayed a moderate and/or sensitive phenotype to the killing action of 10 μM MMS when compared to the wild-type control.

The *recF*15, *recL*16, Δ*recO*, and Δ*recR* cells (group α) showed a similar degree of sensitivity to MMS or 4NQO (ALONSO *et al.* 1993); hence, only the sensitivity of the former mutant strain is shown. The *recF*15, Δ*recG*, *recD*41, Δ*recU*, Δ*ruvAB*, and Δ*recA* cells were extremely sensitive to the killing action of 10 mM MMS when compared to the wild-type control. The *recF*15 (group α), *recD*41, Δ*recU*, Δ*ruvAB* (group ε), and Δ*recG* (group η) strains were less sensitive to 10 mM MMS than the Δ*recA* strain (Figure 1).

Branch migration and resolution of Holliday junctions is essential for DNA repair: Previously, it was shown that *addAB*, *recH*342, and Δ*recS* mutations increased the sensitivity of Δ*recU* cells to DNA damage (FERNANDEZ *et al.* 1998). Furthermore, strains lacking RecF, RecU, or both are extremely sensitive to MMS and 4NQO (ALONSO *et al.* 1993). These results indicate that RecA assembly factors (*e.g.*, RecFLOR) and the RecU HJ resolvase facilitate repair of DSBs (FERNANDEZ *et al.* 1998; AYORA *et al.* 2004). We therefore investigated whether RecU was required for repair of DSBs generated by different DNA-damaging agents. The Δ*recU* null mutation was transferred into representatives from each of the epistatic groups (α, *recF*15 and Δ*recO*; β, *addA*5 *addB*72; γ, *recH*342; and ζ, Δ*recS* and Δ*recQ* strains), but we were unable to recover the Δ*recU* allele in Δ*ruvAB* (epistatic group ε), Δ*recG* (η), and Δ*recJ* (ζ) backgrounds without the appearance of undesired mutations. In our

TABLE 1

B. subtilis strains used in this study

Bacterial strain	Epistatic group	Relevant genotype	Source or reference
YB886	NA	*trpC2 metB5 amyE sigB37 xin*-1 *att*SPβ	YASBIN *et al.* (1980)
BG190	NA	Δ*recA*	CEGLOWSKI *et al.* (1990)
BG129	α	*recF15*	ALONSO *et al.* (1988)
BG189	β	*addA5 addB72*	ALONSO *et al.* (1993)
BG119	γ	*recH342*	ALONSO *et al.* (1988)
BG633	ε	Δ*recU*	FERNANDEZ *et al.* (1998)
BG121	ε	*recD41*	ALONSO *et al.* (1988)
BG703	ε	Δ*ruvAB*	This work
BG707	η	Δ*recG*	This work
BG425	ζ	Δ*recS*	FERNANDEZ *et al.* (1998)
BG705	ζ	Δ*recQ*	This work
BG675	ζ	Δ*recJ*	This work
BG501	NA + ε	Δ*sms* Δ*recU*	CARRASCO *et al.* (2002)
BG651	NA + ε	Δ*recA* Δ*recU*	CARRASCO *et al.* (2004)
BG703	NA + ε	Δ*recA* Δ*ruvAB*	This work
BG817	NA + η	Δ*recA* Δ*recG*	This work
BG717	α + ε	*recF*15 Δ*ruvAB*	This work
BG735	β + ε	*addA5 addB72* Δ*ruvAB*	This work
BG783	γ + ε	*recH342* Δ*ruvAB*	This work
BG711	ε + ζ	Δ*ruvAB* Δ*recJ*	This work
BG709	ε + ζ	Δ*ruvAB* Δ*recQ*	This work
DK1	NA	*recN-cfp*	KIDANE *et al.* (2004)
DK35	α	Δrec*N*	KIDANE *et al.* (2004)
DK53	NA	*recU-gfp*	This work
DK54	NA	*recU-yfp recN-cfp*	This work
DK55	α	*recU-gfp* ΔrecN	This work
DK56	ε	*recU-gfp* Δ*ruvAB*	This work

NA, not applied.

attempt to construct Δ*recU* Δ*ruvB,* Δ*recU* Δ*recG,* and Δ*recU* Δ*recJ* double mutants, we obtained a few colonies after prolonged incubation. Analysis of several of these transformants suggested that they contained either single CO or suppressor mutations (*e.g.,* Δ*recU* Δ*recG sms*; results not shown). To confirm that no other un-selected mutations accumulate in these strains, DNA from a plasmid-borne *recG:six-cat-six* was used to trans-form *B. subtilis* BG501 (Δ*recU* Δ*sms*) competent cells selecting for chloramphenicol resistance. Using this approach, we succeeded in making a Δ*sms* Δ*recU* Δ*recG* strain. This fits with the earlier observations that Δ*sms* (also termed Δ*radA*) partially suppresses the Δ*recU* de-fect (CARRASCO *et al.* 2002).

Unlike *Streptococcus pneumoniae* in which the *recU* gene (TIGR SP0370) is apparently essential (THANASSI *et al.* 2002), a *B. subtilis* Δ*recU* mutant is viable, although it grows poorly (Table 2) and accumulates suppressor mutations at a high frequency (PEDERSEN and SETLOW 2000; CARRASCO *et al.* 2002, 2004). Therefore, the Δ*ruvAB* null mutation was transferred into representatives from the different epistatic groups (α, *recF*15 and Δ*recO*; β, *addA5 addB72*; γ, *recH342*; ε, *recD41*; and ζ, Δ*recS,* Δ*recQ,* and Δ*recJ* strains), the double and triple mutants were exposed to MMS, 4NQO, or MMC, and their phenotypes were recorded.

In the absence of any DNA-damaging agent, the num-ber of viable cells per colony of strains grouped in the α, β, γ, or ζ epistatic group was affected <1.5-fold when compared to wild-type cells (data not shown), whereas the Δ*ruvAB* and Δ*recA* strains showed a similar reduced number of viable cells per colony (4- to 5-fold) when compared to the wild-type strain (Table 2). Exponentially growing cells were stained with SYTO 9, and only ~4% of these wild-type cells were also stained with propidium iodide (an indicator of membrane-compromised "dead" bacteria). The proportion of Δ*ruvAB* and Δ*recA* cells stained with propidium iodide increased 3- to 4-fold when compared with wild-type cells (Table 2). A similar reduced number of viable cells per colony was observed when the Δ*recU* or Δ*recG* cells were analyzed (Table 2).

The Δ*ruvAB* cells were extremely sensitive to 10 μg/ml of MMS, 0.75 μg/ml of 4NQO, or 12 ng/ml of MMC (Figure 2), whereas the wild-type strain showed a minimal defect in the presence of 250 μg/ml MMS, 24 μg/ml of 4NQO, or 150 ng/ml of MMC relative to an unexposed control (data not shown). The DNA damage sensitivity of Δ*ruvAB recD41* cells (epistatic group ε) was similar to that obtained with the Δ*ruvAB* mutant strain (Figure 2). The recombination mutants classified within the β (*addAB*) and ζ (Δ*recS*) groups marginally increased
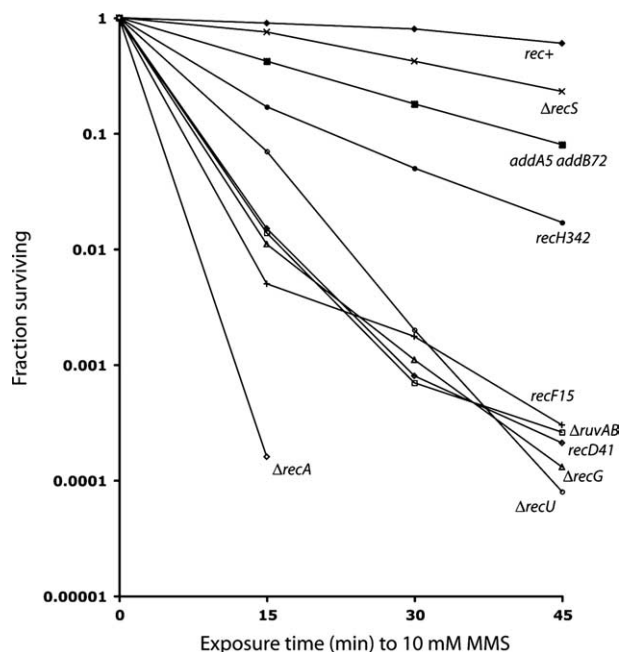
FIGURE 1.—Survival of strains after exposure to MMS. The strains used, which are identified by the indicated relevant genotype, were exposed to 10 mM MMS for a variable time.

the sensitivity of $\Delta ruvAB$ cells following exposure to 10 μg/ml MMS, 0.75 μg/ml of 4NQO, or 12 ng/ml of MMC (Figure 2), but they were slightly less sensitive than $\Delta recA$ cells. It is likely that acting, in concert, RecQ and RecJ initiate DNA recombination in $\Delta ruvAB$ cells.

The $recF15$ and the poorly characterized $recH342$ mutation reduced the survival of $\Delta ruvAB$ cells to a level comparable to the $\Delta recA$ strain following exposure to 5 μg/ml MMS, 0.35 μg/ml of 4NQO, or 6 ng/ml of MMC (Figure 2). The $\Delta ruvAB$ mutation did not increase the sensitivity of the $\Delta ruvAB\ \Delta recA$ strain (Figure 2).

RecU resolves a HJ by endonucleolytic cleavage (AYORA *et al.* 2004), while it is believed that RuvAB, perhaps in concert with the unknown activity associated with RecD, recognizes and branch migrates HJs. The $recU$, $recD$, $ruvA$, and $ruvB$ mutants all belong to the ε epistatic group. Since we failed to construct a $\Delta recU$ $\Delta ruvAB$ strain, yet successfully made $\Delta recA\ \Delta recU$ (CARRASCO *et al.* 2004), $\Delta recA\ \Delta ruvAB$ (this work), and $\Delta recA\ \Delta recG$ strains (H. SANCHEZ and J. C. ALONSO, unpublished results), we propose that the $\Delta recU\Delta ruvAB$ combination leads to accumulation of "toxic" recombination intermediates during strain construction.

RecU restores UV resistance to *E. coli ruvC* mutants: Recently it was demonstrated that RecU protein binds three- and four-stranded DNA branches, resolves Holliday junctions, and promotes joint molecule and D-loop formation *in vitro* (AYORA *et al.* 2004). Furthermore, the structure of RecU, which shows a striking similarity to a class of resolvase enzymes found in archaea and members of the type II restriction endonuclease family, was determined (MCGREGOR *et al.* 2005). To confirm the involvement of RuvAB and RecU in HJ processing, we examined their ability to replace the activities of their counterparts in the well-characterized *E. coli* system. Plasmid-borne $recU$, $ruvA$, $ruvB$, or $ruvAB$ genes were introduced into various *E. coli ruv* mutant combinations and exposed to varying doses of UV light (Figure 3 and Table 3). Plasmids carrying RecU restored full UV resistance to strains ($\Delta ruvC_{Eco}$ and $ruvC53_{Eco}$) deficient in the RuvC$_{Eco}$ HJ resolvase (Figure 3; Table 3; data not shown). RecU also conferred resistance to MMC at 0.2 and 0.5 μg/ml in these strains (data not shown). The results reveal for the first time that RecU functions as a HJ resolvase *in vivo*. Significantly, RecU only partially improved the UV sensitive phenotype in *E. coli ruvA*, $ruvB$, $ruvAB$, $ruvAC$, and $ruvABC$ mutants (Figure 3;

TABLE 2

Viability of $\Delta recA$ recombination-deficient mutants

Strain	Relevant genotype	CFU per colony[a]	CFU per colony relative to wild type[b]	% propidium-iodide-stained cells[c]
YB886	Wild type	$2.2 \times 10^7 \pm 0.1$	1	4 (96)
BG190	$\Delta recA$	$3.6 \times 10^6 \pm 0.2$	0.20	21 (79)
BG633	$\Delta recU$	$3.7 \times 10^6 \pm 0.2$	0.19	21 (79)
BG703	$\Delta ruvAB$	$3.7 \times 10^6 \pm 0.2$	0.24	28 (72)
BG707	$\Delta recG$	$4.2 \times 10^6 \pm 0.1$	0.25	26 (73)
BG651	$\Delta recA\ \Delta recU$	$1.5 \times 10^6 \pm 0.1$	0.07	90 (9)
BG703	$\Delta recA\ \Delta ruvAB$	$1.3 \times 10^6 \pm 0.2$	0.06	93 (6)
BG817	$\Delta recA\ \Delta recG$	$1.0 \times 10^6 \pm 0.1$	0.04	94 (6)

Cells were grown to midexponential phase in LB medium and plated. Individual colonies after overnight incubation were resuspended in LB and appropriate dilutions were plated.

[a] The number of CFUs (viable cells) per colony, reported as the mean ± standard deviation, averaged from 5 to 10 colonies.

[b] Ratio of CFUs per total number of cells for mutant strains relative to wild type.

[c] Percentage of propidium-iodide-stained cells per colony, averaged from more than three colonies. The percentage of *only* SYTO 9-stained cells is indicated within parentheses.
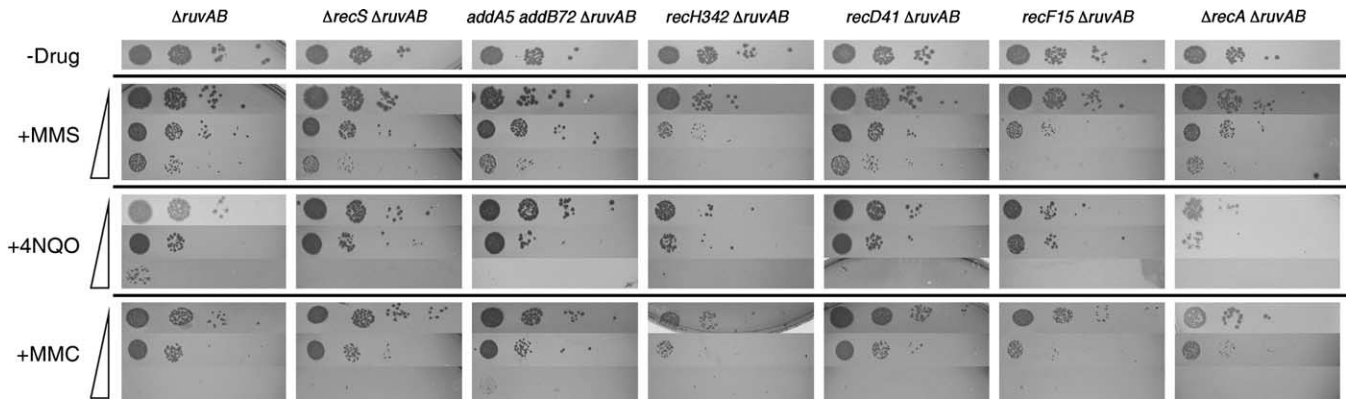
FIGURE 2.—Survival of $\Delta ruvAB$ mutants in combination with mutations from other epistatic groups after exposure to DNA-damaging agents. The strains used are identified by the indicated relevant genotype. A serial 10-fold dilution (10-μl sample) of a culture of each strain was spotted on LB agar plates containing the indicated concentration of the DNA-damaging agents. Dilution fractions were from 0.01 (left) to 0.00001 (right). Cells were exposed to different concentrations of MMS (2.5, 5, or 10 μg/ml), 4NQO (0.12, 0.35, or 0.75 μg/ml), or to MMC (6, 12, or 24 ng/ml) or plated in the absence of any drug (−drug).

Table 3; data not shown). Because RecU is as effective as RuvC$_{Eco}$ at promoting repair in a $ruvC_{Eco}$ mutant, the results establish that, as with RuvC$_{Eco}$, RecU depends on RuvAB branch migration for efficient HJ resolution.

The plasmid constructs carrying RuvA, RuvB, or RuvAB were unable to improve the UV sensitivity of the relevant ruv_{Eco} mutants (Table 3). Both RuvA and RuvAB clones produced an obvious negative effect on wild-type *E. coli* cells exposed to UV light. The results suggest that unlike RecU, RuvAB cannot replace the function of RuvAB$_{Eco}$ and that this may, in part, be due to a detrimental effect of RuvA expression. In fact, plasmids expressing high levels of RuvA$_{Eco}$ are known to confer an extreme negative effect on the UV sensitivity of wild-type cells (SHARPLES *et al.* 1990). This is probably a consequence of RuvA binding HJ DNA and preventing access of alternative junction processing enzymes such as RuvC$_{Eco}$ or RecG$_{Eco}$. These effects strengthen the argument that RuvAB cannot work properly with RuvC$_{Eco}$, rather than an expression and/or stability problem with the heterologous RuvAB complex.

We attempted to construct a plasmid carrying all three *B. subtilis* HJ processing genes to test RuvAB and RecU functionality directly in an *E. coli ruvABC*-deficient strain. However, the clones obtained had suffered substantial deletions, indicating that this combination is highly deleterious. Similarly, we were unable to maintain pCB559 (RuvAB) and pCB564 (RecU) jointly in a strain lacking *ruvAC* (effectively a *ruvABC* mutant) and the cryptic HJ resolvase *rusA* (MAHDI *et al.* 1996; data not shown). It seems likely that too much RuvAB and RecU generates frequent lethal DSBs at regressed replication forks or additionally blocks their processing even in the absence of DNA-damaging agents.

RecU forms discrete foci on nucleoids after induction of DSBs and colocalizes with RecN: Previously, it was shown that RecN, RecO, and RecF proteins accumulate in discrete foci following induction of DSBs

(KIDANE *et al.* 2004). RecN foci were detected 15–20 min after treatment with MMC, RecO foci were first visible 30 min after induction, while RecF foci were not observed until after ∼60–90 min (KIDANE *et al.* 2004; our unpublished results).

A RecU-GFP fusion strain was constructed and localization of the RecU protein was investigated in the presence or absence of MMC. The fusion was the sole source of RecU in the cell and fully supported repair of DNA following addition of MMC, showing that the fusion retained activity. In exponentially growing cells, RecU-GFP was present throughout the cells (Figure 4A), with fluorescence levels barely above background. However, after addition of 100 ng/ml of MMC, RecU-GFP formed discrete foci in up to 45% of the >500 cells analyzed (Figure 4C). RecU-GFP foci were always present on the nucleoids (see arrowheads in Figure 4C) and cells generally contained a single focus; only 2.7% of the cells contained two foci. One hour after the addition of MMC, clear foci were observed in only 1.5% of the cells (Figure 4B), with the highest number of foci observed 120 min after induction of DSBs (Figure 4C), and became increasingly fewer and fainter thereafter. The foci therefore occurred at a later point during DSB repair than RecN or RecO foci.

To establish that RecU is recruited to the RecNOF DSB repair centers (RCs), we generated a RecU-YFP variant and combined it with a RecN-CFP fusion, such that both were simultaneously expressed within cells. Many dually labeled cells showed rather patchy areas on the nucleoids (shaded arrow, Figure 4F); only 10% of the cells showed clear RecU-YFP and RecN-CFP foci after MMC treatment (open arrowheads, Figure 4F), mostly because RecN-CFP fluorescence was extremely low. The formation of patches in many cells suggests that GFP labels on both proteins slightly interfere with the proper function of the proteins, although the single labels are fully functional. However, in all of the cells
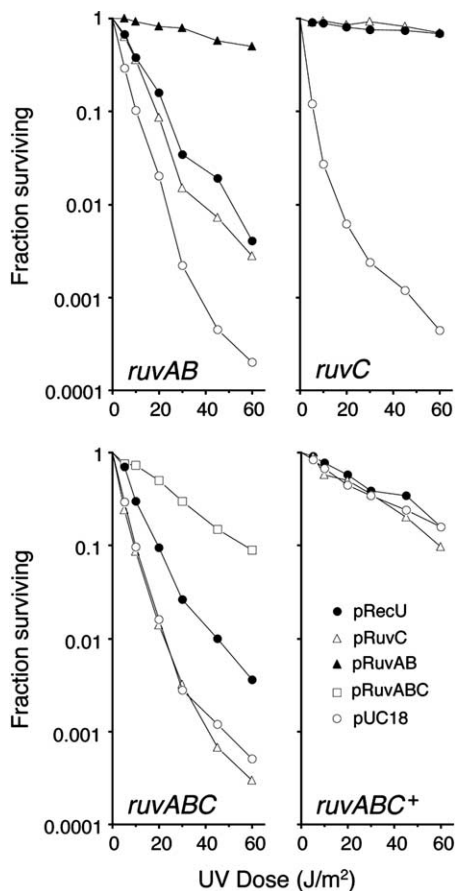
FIGURE 3.—Survival of UV-irradiated *E. coli ruv* mutants carrying the *recU* gene. The strains used were N2057 (*ruvAB*), GS1481 (*ruvC*), N4454 (*ruvABC*), and AB1157 (*ruv*⁺). Symbols for plasmids carrying RecU (pFC204), RuvC$_{Eco}$ (pGS762), RuvAB$_{Eco}$ (pGS711), RuvABC$_{Eco}$ (pPVA101), and the pUC18 vector are shown (bottom). The relevant vector control for pPVA101, pHSG415 (not shown), was UV sensitive.

with clear foci, both RecU-YFP and RecN-CFP signals were coincident, and in most cells containing fluorescent patches these signals were likewise at similar places within the cells, showing that RecN and RecU colocalize within the DSB RCs.

To test if accumulation of RecU requires other proteins, we moved the RecU-YFP fusion into a *ruvAB* or *recN* mutant background. Only 0.4% of the cells showed RecU-YFP foci in the absence of *ruvAB* (Figure 4D), while 37% of the *recN* mutant cells contained RecU-YFP foci after addition of MMC (Figure 4E). Interestingly, 25% of the *recN* mutant cells contained two foci, rather than one (Figure 4E). As with RecU-YFP, RecN-GFP forms two foci in only 4% of the cells and a single focus in the remaining cells (KIDANE *et al.* 2004). These experiments demonstrate that RecU is part of a dynamic response to DSBs in *B. subtilis* cells and is recruited into defined RCs at a late stage in a reaction dependent on RuvAB. RecU is recruited to RCs independently of RecN in agreement with data showing that different avenues

can lead to the formation of crossovers that are the substrate for RecU. However, on the basis of our previous findings suggesting that several DSBs are recruited to and repaired within a single RC (KIDANE *et al.* 2004), it is clear that RecN is a candidate for a factor combining different breaks into a single RC, because of the increase in the number of RecU-YFP foci in the absence of RecN.

DISCUSSION

This work provides evidence that inactivation of genes in epistatic group β (*addAB*) or in epistatic group ζ (*recQ, recS,* or *recJ*), when present in otherwise Rec⁺ cells, have rather modest effects on sensitivity to MMS. In contrast, elimination of those functions classified within the α (namely *recF*15, *recL*16, Δ*recO*, or Δ*recR*), ε (Δ*ruvAB*, *recD*41, and Δ*recU*) or η (Δ*recG*) epistatic groups shows a dramatic reduction in ability to repair DNA damage mediated by these agents, showing only slightly more resistance than the recombination-defective Δ*recA* strain. Previously it was shown that RecS shares 36 and 34% identity with RecQ and RecQ$_{Eco}$ proteins, respectively (FERNANDEZ *et al.* 1998). This homology is significantly greater (43 and 40% identity) if only the regions containing the seven conserved DExH-box DNA helicase motifs (the first 330 residues of RecQ and RecS) are compared (FERNANDEZ *et al.* 1998). It was shown that Δ*recS* does substitute for the Δ*recQ* defect as the double mutant is as sensitive as the single parent mutant (our unpublished results). RecQ$_{Eco}$ unwinds both partially dsDNA and fully duplex DNA with a 3′ to 5′ polarity, while RecJ$_{Eco}$ is a 5′ to 3′ ssDNA exonuclease, generating a 3′-terminated end subsequently coated with SSB$_{Eco}$ (KOWALCZYKOWSKI and EGGLESTON 1994; COURCELLE and HANAWALT 1999; AMUNDSEN and SMITH 2003). We have therefore tentatively placed *recJ* and *recQ* within the ζ epistatic group, together with *recS*, in a recombination pathway that can generate 3′-tailed ssDNA at broken forks akin to the activities of AddAB or RecBCD.

Since the DNA-damaging agents used in this report act as replication roadblocks, inducing replication fork arrest and single-strand nicks or DSBs, we considered the possibility that replication restart in Δ*ruvAB* cells relies on the processing of DNA ends by the action of AddAB (CHEDIN *et al.* 2000) or by the combined action of the RecQ or RecS helicase and the RecJ ssDNA exonuclease. RecA protein could be loaded on the 3′-ssDNA by the AddAB enzyme (CHEDIN *et al.* 2000) or RecN-RecFLOR complex (KIDANE *et al.* 2004). This is consistent with the observation that the Δ*recU* Δ*recJ* strain did not seem to be viable and that RecN forms RCs at DSBs in concert with RecO and RecF (KIDANE *et al.* 2004). RecA bound to ssDNA promotes homologous pairing, D-loop formation, and strand exchange between one or both of the broken ends with an intact DNA molecule to generate HJs. RecG or RuvAB (alone

TABLE 3

Effect of plasmids carrying RecU on the survival of UV-irradiated ruv_{Eco} mutants

		Fraction surviving (60 J/m²)				
Plasmid	*E. coli* strain: Genotype:	SR2210 *ruvA*	N1057 *ruvB*	N2057 *ruvAB*	GS1481 *ruvC*	AB1157 *ruv*⁺
pRecU[a]		0.0014	0.000057	0.0013	0.19	0.26
Vector[a]		0.00077	0.000074	0.0014	0.00010	0.56
pRuvA[b]		0.000068	0.000022	0.000075	ND	0.0033
pRuvB[b]		0.00014	0.000098	0.00032	ND	0.138
pRuvAB[b]		Sensitive	0.000025	0.0010	ND	0.0055
Vector[b]		0.000047	0.000010	0.00064	ND	0.57
pRuvA, B, AB$_{Eco}$[c]		0.0080	0.079	0.091	ND	0.17
pRuvC$_{Eco}$[d]		0.013	0.00045	0.00048	0.036	0.075
Vector[d]		0.0011	0.00050	0.00058	0.00016	0.48

ND, not done.

[a] pRecU (pCB564: *recU* in pHP13).

[b] pRuvA (pCB593: *ruvA* in pUC18), pRuvB (pCB594: *ruvB* in pUC18), and pRuvAB, pCB559 (*ruvAB* in pUC18).

[c] pRuvA$_{Eco}$ (pGTI4: *ruvA$_{Eco}$* in pUC18, control for SR2210), pRuvB$_{Eco}$ (pGTI19: *ruvB$_{Eco}$* in pUC18, control for N1057), and pRuvAB$_{Eco}$ (pGS711: *ruvAB$_{Eco}$* in pUC18, control for N2057).

[d] pRuvC$_{Eco}$, (pGS739: *ruvC$_{Eco}$* in pACYC184). Relevant strain genotypes are indicated.

or in concert with RecD) branch migrates these junctions for RecU resolution. This model fits with the observations that: (i) *recF addAB* cells are impaired in DNA repair and genetic recombination to the level of *recA* cells and showed a similarly reduced viability in the absence of external damage, together with extreme sensitivity to the killing action of MMS, 4NQO, or MMC (Alonso *et al.* 1993), and (ii) *recF* or Δ*recO* mutations reduce the viability of Δ*ruvAB* cells to a greater extent than do *addAB* mutations (see Figure 2; our unpublished results).

To confirm the functionality of RuvAB and RecU in HJ processing, we studied their ability to complement the DNA repair defect *of E. coli ruv* mutants. We found that expression of the *recU* gene restores UV-light resistance to *ruvC$_{Eco}$* strains to a level similar to that of clones carrying RuvC$_{Eco}$. In contrast, RecU conferred only a slight improvement in UV survival of *ruvA$_{Eco}$*, *ruvB$_{Eco}$*, *ruvAC$_{Eco}$*, *ruvAB$_{Eco}$*, or *ruvABC$_{Eco}$* mutants. Since the *E. coli ruv* system is well defined, we can conclude that RecU does indeed function as a HJ resolvase as demonstrated by *in vitro* data (Ayora *et al.* 2004; McGregor *et al.* 2005). The improvement in resistance to UV when RecU is present in strains lacking *ruvAB$_{Eco}$* indicates that it can function to some extent in the absence of RuvAB. However, this may be artificially high due to overexpression of RecU, since clones carrying RuvC$_{Eco}$ also improve the survival of *ruvAB$_{Eco}$* strains following exposure to UV light. The dependence on RuvAB for full DNA repair activity does suggest that RecU normally functions together with the branch migration complex as is the case with *E. coli* RuvABC (Zerbib *et al.* 1998; van Gool *et al.* 1999). Any contacts that stabilize a RuvABC$_{Eco}$ or a RuvAB-RecU complex

must be conserved between these heterologous systems, if indeed they are important for stability of the tripartite complex. Consequently, the resolvasome model, where the resolution endonuclease (either RecU or RuvC$_{Eco}$) scans the junction for preferred target sequences, appears to be widely conserved in bacteria.

RuvAB$_{Eco}$ or RecG$_{Eco}$ catalyze replication fork regression *in vivo* and play a critical role in promoting the recovery of replication when it is blocked by DNA damage (Bolt and Lloyd 2002; Gregg *et al.* 2002; Meddows *et al.* 2004). Other studies, however, indicate that RuvAB$_{Eco}$- or RecG$_{Eco}$-catalyzed fork regression is not essential for DNA synthesis to resume following arrest by UV-induced DNA damage *in vivo* (Donaldson *et al.* 2004). In this work, we also show that it is possible to visualize the place of action of RecU in live cells. We have found that RecU forms a single discrete center on the nucleoid upon induction of DSBs, as previously observed with RecNOF proteins (Kidane *et al.* 2004). RecN is the first to form the RCs, within 15–20 min with foci visible at defined DSBs in live cells (Kidane *et al.* 2004). RecU is recruited into RCs, since it colocalizes with RecN. Consistent with a role in resolution of HJs, RecU accumulated within the RCs after the formation of RecN, RecO, or RecF foci; RecU foci were clearly visible 120 min after induction of DSBs. These data indicate that repair of DSBs occurs over a long period of time during which several sequential processes take place. Recruitment of RecU was dependent on RuvAB proteins, strengthening the view that these proteins form a resolvasome complex. Interestingly, the number of RCs was increased in the absence of RecN protein, supporting our suggestion that RecN might organize different recombination events within a single center (Kidane *et al.* 2004).
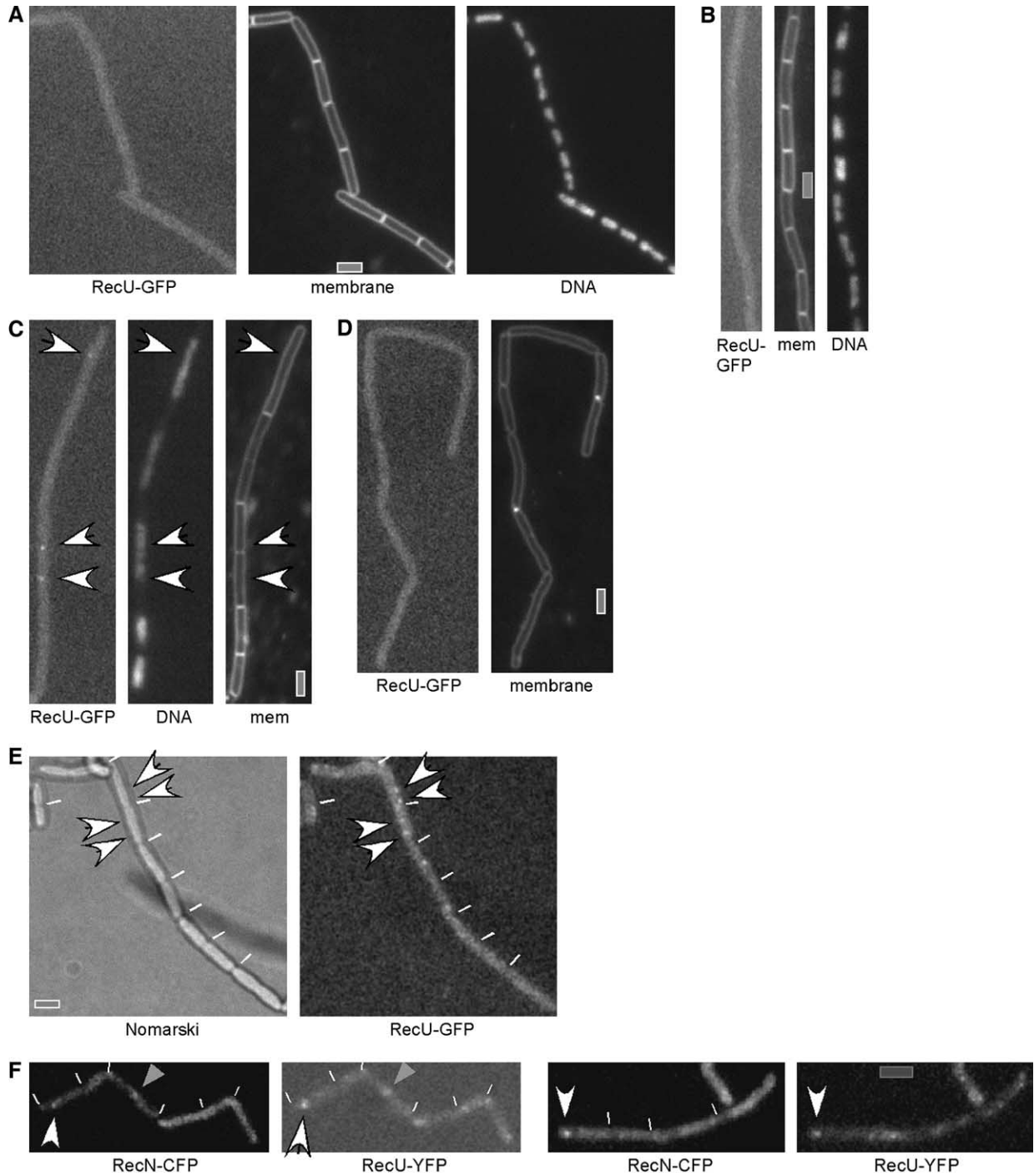
FIGURE 4.—Subcellular localization of RecU in live *B. subtilis* cells. (A) RecU-GFP in exponentially growing cells; (B) 60 min after addition of MMC; (C) 120 min after addition of MMC. Open arrowheads in C indicate RecU-GFP foci. (D) RecU-GFP in Δ*ruvAB* cells, 120 min after addition of MMC. (E) RecU-YFP in Δ*recN* cells, 120 min after addition of MMC; open arrowheads indicate two RecU-YFP foci per cell. (F) Colocalization of RecU-YFP and RecN-CFP 120 min after addition of MMC, indicated by open arrowheads; shaded arrowheads indicate the patch formed by RecN and RecU and open lines indicate the ends of cells. DNA is stained with DAPI and membranes (mem) by FM4-64. Bar, 2 μm.

Under standard transformation conditions we failed to construct Δ*ruvAB* Δ*recG*, Δ*ruvAB* Δ*recU,* and Δ*recU* Δ*recG* mutant strains. Previously we reported the construction of *ruvA*2 *recU*40 double-mutant strains (ALONSO *et al.* 1992) and here report the construction of the

*recD*41 Δ*ruvAB* strain. However, the *recU*40 strain is proficient in plasmid transformation and shows a doubling time similar to the wild-type strain, whereas a Δ*recU* is significantly impaired in plasmid transformation and shows a marked growth defect (FERNANDEZ

et al. 1998; PEDERSEN and SETLOW 2000; CARRASCO *et al.* 2002; Table 2). It is likely that the *recU*40 allele may encode only a partially defective HJ resolvase. Very little information is available concerning the *recD*41 strain. These two pieces of apparently conflicting data argue that RecU resolves HJ intermediates branch migrated by either RuvAB(RecD) or RecG DNA helicases. The improvements in UV resistance conferred upon *ruvAB*$_{Eco}$ mutants by clones carrying RuvC$_{Eco}$ and RecU support this idea, confirming that both these HJ resolvases can function *in vivo* without RuvAB branch migration. Alternatively, the apparent lethality of Δ*recG* Δ*ruvAB*, Δ*recG* Δ*recU*, and Δ*ruvAB* Δ*recU* double mutants arises from accumulation of "toxic" recombination intermediates (GANGLOFF *et al.* 2000). This is consistent with the observation that Δ*recA* Δ*recU*, Δ*recA* Δ*recG* (CARRASCO *et al.* 2004), and Δ*recA* Δ*ruvAB* were viable, albeit with a 14- to 25-fold reduced plating efficiency (see Table 2). In *E. coli*, *recG ruvAB*, *recG ruvC*, and *ruvABC* mutants are viable, the latter indistinguishable from single-mutant strains (LLOYD 1991; MANDAL *et al.* 1993). There are clearly important differences between Gram-negative and Gram-positive recombinational repair processes despite the apparent similarities in coordination of HJ resolution by RuvAB-RecU and RuvABC$_{Eco}$. This serves to highlight the importance of having more than one model system to evaluate the mechanics of complex repair, replication, and recombination processes.

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Communicating editor: S. T. Lovett