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In Vivo Evidence for Two Active Nuclease Motifs in the Double-Strand Break Repair Enzyme RexAB of *Lactococcus lactis*

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In bacteria, double-strand DNA break (DSB) repair involves an exonuclease/helicase (exo/hel) and a short regulatory DNA sequence (Chi) that attenuates exonuclease activity and stimulates DNA repair. Despite their key role in cell survival, these DSB repair components show surprisingly little conservation. The best-studied exo/hel, RecBCD of *Escherichia coli*, is composed of three subunits. In contrast, RexAB of *Lactococcus lactis* and exo/hel enzymes of other low-guanine-plus-cytosine branch gram-positive bacteria contain two subunits. We report that RexAB functions via a novel mechanism compared to that of the RecBCD model. Two potential nuclease motifs are present in RexAB compared with a single nuclease in RecBCD. Site-specific mutagenesis of the RexA nuclease motif abolished all nuclease activity. In contrast, the RexB nuclease motif mutants displayed strongly reduced nuclease activity but maintained Chi recognition and had a Chi-stimulated hyper-recombination phenotype. The distinct phenotypes resulting from RexA or RexB nuclease inactivation lead us to suggest that each of the identified active nuclease sites in RexAB is involved in the degradation of one DNA strand. In RecBCD, the single RecB nuclease degrades both DNA strands and is presumably positioned by RecD. The presence of two nucleases would suggest that this RecD function is dispensable in RexAB.

In bacteria, double-stranded DNA breaks (DSB) are frequent events that may be provoked, for example, by pauses in the replication fork (36, 43). Such genomic disruptions are lethal in the absence of DNA repair. In *Escherichia coli* DSB repair requires the activity of a large enzyme complex, known as RecBCD, that has ATP-dependent helicase and exonuclease activities (see reference 32 for a review). The enzyme degrades both strands, starting from the DNA break until it reaches an octanucleotide sequence, known as Chi, that attenuates degradation and stimulates recombination (44, 46). The enzyme exhibits helicase activity and residual exonuclease activity with an altered polarity after Chi (4, 16); the remaining activity provides a single-stranded DNA substrate for recombination enzymes to mediate repair.

Organization of the three-subunit exonuclease/helicase (exo/hel) RecBCD. Structure-functional studies of RecBCD have revealed some of the roles of each subunit. RecB seems to possess two key activities of the enzyme. The N-terminal 929 amino acids (out of 1,180 total) have confirmed ATPase and helicase activities (13, 54); this region is similar to that of UvrD helicase. RecBCD helicase activity was recently proposed to function via a mechanism similar to that determined for UvrD (6). Nuclease activity was recently localized to the C-terminal 251 amino acids of RecB and is associated with the presence of a conserved motif, G-i-i-D-x(12)-D-Y-K-t-d (amino acids in small letters show less conservation) (51, 53, 54). This motif is present in numerous bacterial and eukaryotic enzymes (5). RecBCD was shown to have a single nuclease catalytic center in RecB that works on both DNA strands (51). Little is known about the roles of RecC, except that it appears to greatly enhance activities and processivity of RecB (11, 38); mutations in the RecC gene can also result in loss or modification of Chi recognition, as do mutations in genes of all subunits (1). RecD is an ATPase with similarity to a helicase involved in conjugational transfer of an enteric bacterial plasmid; its homologues seem to be broadly distributed in bacteria (determined by BLAST comparisons; http://www.ncbi.nlm.nih.gov/BLAST/). As part of RecBCD, RecD appears to regulate exonuclease activity. Recent data suggest that RecD maintains RecBCD incompetent for homologous recombination prior to Chi; at Chi, RecD is suggested to undergo a conformational change that attenuates exonuclease activity and stimulates recombination (2, 3, 12, 33, 48). A swing model was proposed in which RecD assures proximity of the RecB nuclease with both DNA strands prior to Chi and a repositioning of the nuclease after Chi (51, 54).

Organization of the two-subunit exo/hel enzymes. To date, models of exo/hel activities are based on those of RecBCD. Numerous RecBCD homologues have been identified in gram-negative enterobacteria and in the high-guanine-plus-cytosine-content mycobacteria. However, the functional RecBCD analogue in the low-guanine-plus-cytosine-content branch of gram-positive bacteria is structurally distinct from

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RecBCD. Using *Lactococcus lactis* as a model, a two-subunit enzyme called RexAB (comprising 1,073- and 1,099-amino-acid subunits, respectively) is necessary and sufficient to confer exo/hel activity and interacts with the *L. lactis* Chi site (22). RexAB bears homologues in at least six other gram-positive low-guanine-plus-cytosine-content bacteria as well as in the gram-negative bacterium *Porphyromonas gingivalis* (determined by BLAST comparison; http://www.ncbi.nlm.nih.gov/BLAST/). As studied in *L. lactis* or in *Bacillus subtilis* (AddAB), the two-subunit exo/hel enzymes display biological and/or biochemical activities equivalent to those of RecBCD (i.e., ATP-dependent exonuclease, helicase, exonuclease blocking at Chi, and Chi-stimulated recombination; 8, 9, 10, 22, 23, 28, 30, 31). RexA and its analogues in other gram-positive bacteria have homology with PcrA helicase (determined by BLAST comparison; http://www.ncbi.nlm.nih.gov/BLAST/), whose mechanism has been deduced from structural determinations (49). In addition, the nuclease motif described above for RecB is conserved in *L. lactis* RexA and in all known two-subunit exo/hel enzymes (5, 28, 53). This similarity has lead to the hypothesis that all exo/hel enzymes function via similar mechanisms.

However, several lines of evidence argue against a common mechanism of DSB repair. The exo/hel-Chi couples show remarkably little conservation from one bacterium to another. Furthermore, Chi sites are not the same in different species, and their genome distribution properties differ for each organism (7, 8, 10, 21, 45). This suggests that the Chi features of high frequency and overrepresentation on the genome had to arise independently in each case (7, 21). In addition, although the enzymes have equivalent biological functions, their structures are strikingly different. Similarities between two- and three-subunit enzymes are restricted to ATPase, helicase, and nuclease motifs present, for example, in RecB (of RecBCD) and RexA (of RexAB); no similarity is detected in the other exo/hel subunits. For comparison, RecA proteins of *Escherichia coli* and *L. lactis* are 56% identical (19). Recent in vitro studies with the two-subunit AddAB exo/hel may suggest that its activities differ from those of RecBCD (9). Unlike RecBCD, where a Chi encounter affects the degradation pattern of both strands, attenuation at Chi of AddAB-mediated degradation seems to affect only the Chi-containing strand (9, 16); however, exo/hel activities in vitro are very sensitive to assay conditions, which could explain these observations. The above considerations raise the possibility that the two- and three-subunit exo/hel enzymes are programmed differently to carry out their functions.

We examined the divergence between exo/hel enzymes of different microorganisms. Analyses of the *L. lactis* RexAB enzyme reveals the presence of two potential nuclease activities on the enzyme, one on each subunit. Each nuclease motif was modified by site-specific mutagenesis. The mutants show clear phenotypic differences in vivo, revealing that each nuclease motif has a key functional role in DNA degradation. Our results lead us to suggest that RexAB exo/hel has two distinct nuclease activities that may each degrade one double-stranded DNA (dsDNA) strand. Differences between the two- and three-subunit enzymes further suggest that the ubiquitous DSB repair strategy may undergo selective pressures that increase divergence.

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* strains used were TG1 [F′ *traD36 lacI*^{q} Δ*(lacZ)M15 proA*$^+$*B*$^+$*/supE* Δ*(hsdM-mcrB)5*(r_K^- m_K^- *mcrB) thi* Δ*(lac-proAB)*], AB1157 (*argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-5 thi-1 rpsL31 tsx-33 supE44*), and KM21 (AB1157 isogenic strain containing [*recC ptr recB recD*]::kan, referred to here as Δ*recBCD*) (37). The *rexAB* genes were derived from *L. lactis* strain MG1363 (25) and were cloned on low-copy-number plasmid pGB2 (confers spectinomycin resistance) to generate pRexAB (22).

Rolling circle (RC) plasmid pRC2 (confers chloramphenicol resistance) corresponds to a pVS41 derivative (50) equipped with a polylinker (5′-CTGGAATTCGTCGACGGATCC-3′) (*Eco*RI and *Bam*HI sites are underlined) (22). Complementary primers 5′-AATT*CACGCGC*TGCAG*GCGCGTG*G-3′ and 3′-*GTGCGCG*ACGTC*CGCGCAC*CCTAG-5′ containing two *L. lactis* Chi (Chi_{LI}) sites (one in each orientation on the primer, in italics) were cloned between the *Eco*RI and *Bam*HI sites to give rise to pRC2-Chi_{LI} (22).

Media. *E. coli* strains were grown in Luria broth at 30 or 34°C, as specified below. Antibiotics were used in *E. coli* as follows: ampicillin at 100 μg/ml, spectinomycin at 50 μg/ml, tetracycline at 15 μg/ml, kanamycin at 40 μg/ml, and chloramphenicol at 15 μg/ml.

***rexAB* mutant constructions.** To generate mutants affected in the nuclease motifs of RexAB, fragment mutagenesis was performed using modified primers and pRexAB as template DNA. RexAB^{D910A} was constructed by replacing the *Sfc*I-*Cla*I fragment that contains the 3′ end of *rexB* with a corresponding PCR fragment that was modified by a point mutation. The *Sfc*I-end primer was 5′-CTTTCTACAGATTACTTAGGGGCGATTG**CG**TATA-3′ (the *Sfc*I site is underlined; the GAC aspartate codon, RexB position 910, is replaced by the G**CG** alanine codon; changes are in bold). The *Cla*I-end primer was 5′-TCGACAAATCGATTTGAGAGGACAATATCGACA-3′ (the *Cla*I site is underlined). The resulting fragment was first subcloned onto an intermediate vector and then was cloned to replace the wild-type segment in pRexAB. The RexAB$^{\Delta DYK}$ mutant was constructed essentially in the same way except that the *Sfc*I-end primer was 5′-CCAACTTTCTACAGATTACTTAGGGGCGATT//TCAAGTGCTCATTCATT-3′ (the 9-codon deletion, RexB amino acid positions 910 to 912, is indicated by double slashes).

The RexA$^{\Delta DYK}$B mutant was constructed by two-step mutagenesis using four primers. A *rexAB Pst*I-*Eco*RI fragment can be generated using two outside primers: A, 5′-GAAGTTCAACCAGTCAGTGAGTTTGTTCG-3′ (the *Pst*I site present in *rexA* is 46 nucleotides downstream of this primer), and B, 5′-GGGAATTCGGTACCATTGTTCTTCCTCCCTAACAGC-3′ (the PCR-amplified fragment contains an added *Eco*RI site [underlined] at the end of the *rexA* gene). To generate an internal DYK codon deletion, overlapping oligonucleotides that prime in opposite directions were designed: C, 5′-CACATTTGTAAATCTGTCCGT//AAATAATATAATCTTGTCAGC-3′ (the 9-codon RexA deletion, positions 1114 to 1116, is indicated by double slashes; this oligonucleotide generates a PCR fragment when coupled with primer A), and D, 5′-GACAAGATTATATTATTT//ACGGACAGATTTACAAATGTG-3′ (the 9-codon RexA deletion, positions 1114 to 1116, is indicated by double slashes; this oligonucleotide generates a PCR fragment when coupled with primer B). To generate a *Pst*I-*Eco*RI fragment in which the DYK tricodon is missing, separate PCRs were first performed using primers A plus C and B plus D. The template was pRexAB. These fragments were purified, combined, and used as templates in a PCR containing oligonucleotides A plus B. The resulting band of the expected size was purified and cloned, and the *Pst*I-*Eco*RI fragment was finally recloned into *Pst*I-*Eco*RI-cut pRexAB. The resulting pRexA$^{\Delta DYK}$B clone was confirmed by sequencing.

The plasmid pRexAB$^{\Delta 771-1063}$ was constructed by digesting pRexAB with *Cla*I-*Sca*I filling in and religation. This resulted in an in-frame deletion in *rexB*.

UV sensitivity tests for *E. coli*. The *E. coli* Δ*recBCD* strains containing mutated or wild-type *rexAB* alleles were maintained at 30°C. Note that exonuclease activity of RexAB is thermosensitive in *E. coli*, possibly reflecting an optimal growth temperature of *L. lactis* of 30°C. Tests to determine UV resistance were performed as described previously (18).

T4*g2* test for exonuclease activity in *E. coli*. The bacteriophage T4*g2* amber mutant (kindly provided by W. Wackernagel, University of Oldenburg, Oldenburg, Germany) was used to evaluate exonuclease activity. The gene 2 product encodes a protein which protects phage DNA extremities from RecBCD degradation (34). The phage stock was prepared on a *recBC* strain not containing a *supE* mutation, so DNA ends of this phage mutant are exonuclease sensitive. Therefore, the number of PFU is low when this phage is titrated on strains expressing RecBCD. Lactococcal exonuclease activity expressed from pRexAB wild-type and mutated alleles was evaluated in the *E. coli* Δ*recBCD* strain and compared to that of plasmid-free AB1157 (wild type) and *E. coli* Δ*recBCD* strains essentially as described (52), except that cultures and plates were incubated at 30°C.

FIG. 1. Alignment of nuclease motifs present in each subunit of the two-subunit exo/hel enzymes. (A) RexA subunit homologue alignments are presented for the region surrounding the nuclease motif (corresponding to positions 1085 to 1164 of the 1,173-amino acid *L. lactis* RexA subunit). (B) RexB subunit homologies in the region surrounding the nuclease motif (corresponding to positions 871 to 949 of the 1,099-amino acid *L. lactis* RexB subunit). Highly conserved motifs are enclosed in a rectangle, and amino acids that are totally conserved are in bold. The black bar over amino acids DYK corresponds to the region deleted in lactococcal RexA or RexB mutants; the arrow over position 910 in RexB indicates a point substitution from aspartic acid to alanine generated in RexB (see the text). Llac, *L. lactis*; Spyo, *Streptococcus pyogenes*; Spnu, *Streptococcus pneumoniae*; Efae, *Enterococcus faecalis*; Cdif, *Clostridium difficile*; Bsub, *B. subtilis*; Saur, *Staphylococcus aureus*.

Detection of HMW. High-molecular-weight linear plasmid multimer (HMW) accumulation was detected on whole-cell lysates after agarose gel electrophoresis (14). Plasmid DNA, labeled by chemiluminescence using the ECL system (Amersham), was used as a probe. Southern blot hybridization was performed as recommended by the kit supplier.

Recombination test. We used a previously described strategy to measure Chi-stimulated homologous recombination using short dsDNA substrates (15). The plasmid target (named pΔBla) is a pBR322 derivative with a 111-bp deletion in the β-lactamase gene (*bla*). The intact *bla* gene is restored via a double-exchange event with a linear DNA fragment (see reference 22 for details of its construction). In brief, primers were designed to PCR amplify a *bla* gene internal fragment covering the DNA deleted from pΔBla plus an additional 360-bp flanking homology with *bla*. Primer couples generating double Chi_{LL} sites or no Chi sites (Chi_{LL}^{0}) were as described previously (22). Chi_{LL} sites are located about 300 bp from heterologous dsDNA ends. Linear DNA used for experiments was recovered by PCR using the primers 5′-GTTGGGAAGGGCGATCGGTG-3′ and 5′-CACTCATTAGGCACCCCAGGC-3′. The final fragment sizes were ~1.3 kb.

Electrocompetent cells of the Δ*recBCD* strain with or without plasmids encoding the different RexAB alleles plus pΔBla were prepared at 34°C, and control strain TG1 carrying pΔBla was prepared at 37°C. Cells were incubated at 34°C for 90 min after electrotransformation, and colony counts were determined after a 2-day incubation. Competence was determined by transforming cells with known amounts of pACYC184 DNA, selecting for chloramphenicol. Strains were transformed with about 200 ng of linear DNA as described previously (17). Linear DNA samples were quantitated on agarose gels using marker DNAs of known quantities. Electrotransformation into TG1 carrying pΔBla was used to evaluate DNA quality. It was previously shown that electroporation totally inactivates wild-type *E. coli* RecBCD exonuclease but allows *E. coli* Chi-independent recombination (20); the recombination capacity of the two fragments (regardless of Chi_{LL}) was compared in this way. Although the reason why electroporation inactivates RecBCD exonuclease is unknown, it is possible that the electric shock induces the SOS response, which is known to diminish exonuclease activity and retain recombination proficiency (41). Both fragments were found to transform this strain with about equal efficiencies (see Table 2).

Colony counts were performed after 48 h of incubation. The numbers of Amp-resistant transformants obtained with Chi_{LL}- or Chi_{LL}^{0}-containing linear DNAs were compared for each strain. Linear DNA samples were quantitated on agarose gels using marker DNAs of known quantities. Electrocompetent *E. coli* Δ*recBCD* containing pΔBla was used as a negative recipient control.

RESULTS

Conserved regions of two- versus three-subunit exo/hel enzymes. Alignments of several two-subunit exo/hel enzymes reveal that these enzymes are poorly homologous, even in closely related species. They bear very little homology with the three-subunit exo/hel. Nevertheless, a short nuclease motif was previously revealed in the AddA subunit of the *B. subtilis* AddAB enzyme and in the RecB subunit of RecBCD (28, 53). In RecBCD, this motif corresponds to the sole nuclease activity of the exo/hel enzymes (51). We found that this motif is actually present in both subunits of the two-subunit enzymes (Fig. 1). In the RexA subunit, a consensus is G-i-i-D-x(12)-D-Y-K-t-d (amino acids in small letters show some variation); in RexB it is G-r-i-D-R-i-D-x(9–12)-v-D-Y-K-S-s. The striking similarities between these motifs lead us to ask whether RexAB may bear two active nuclease sites, one in each subunit.

Site-specific mutagenesis of putative nuclease motifs in RexA and RexB. The *rexAB* operon (*rexB* is followed by *rexA*) was previously cloned on a low-copy-number plasmid and shown to fulfill the biological roles of RecBCD in an *E. coli* Δ*recBCD* strain (22). The putative RexA nuclease motif ($RexA^{Nuc}$) was modified by a 3-amino-acid deletion removing the tripeptide DYK in positions 1114 to 1116 (called $RexA^{\Delta DYK}B$) (Fig. 1). The putative RexB nuclease motif ($RexB^{Nuc}$) was modified by alteration of D^{910} to A (called $RexAB^{D910A}$) or by a 3-amino-acid deletion, removing the tripeptide DYK in positions 910 to 912 (called $RexAB^{\Delta DYK}$) (Fig. 1). In addition, a 293-amino-acid in-frame deletion of RexB that removed C-terminal amino acids 771 to 1063 (called $RexAB^{\Delta 771–1063}$) was constructed. The cloned *rexAB* mutated genes gave rise to pRex plasmids bearing the name of the mutation and were established in an *E. coli* Δ*recBCD* strain (37).

UV resistance conferred by the different pRexAB mutants was examined (Table 1). The Δ*recBCD* strain shows greater UV resistance in the presence of pRexAB (22) or $pRexAB^{D910A}$. However, UV resistance of the Δ*recBCD* strain was not at all or was only very slightly improved in the presence of $pRexA^{\Delta DYK}B$, $pRexAB^{\Delta 771–1063}$, or $pRexAB^{\Delta DYK}$ compared to that of the control strains. These results indicate that the introduced mutations affect the DNA repair capacity of RexAB exo/hel.

TABLE 1. Capacity of RexAB nuclease mutants to complement a Δ*recBCD* strain

E. coli strain (plasmid)	Relative survival after UV irradiation[a]	Relative PFU of T4*g2*[b]
recBCD (pRexAB)	1	0.000062
recBCD (pRexAB^{D910A})	0.4	0.0014
recBCD (pRexAB$^{\Delta DYK}$)	0.09	0.015
recBCD (pRexAB$^{\Delta 771-1063}$)	0.05	0.17
recBCD (pRexA$^{\Delta DYK}$B)	0.01	0.2
recBCD (pGB2)[c]	0.01	1

[a] Cells were irradiated at 100 J/m^2. Note that survival of the wild-type strain (AB1157) was 4×10^{-1}, while that of *recBCD* (pRexAB) was 3×10^{-2}. Results are means of two experiments.

[b] The values given are relative to a plaque titer of $\sim 4 \times 10^{10}$ PFU/ml on the Δ*recBCD* strain; AB1157 gave a relative plaque titer of 0.0001. Results are means of at least three experiments.

[c] pGB2 is the vector used to clone *rexAB* genes. For both experiments, determination values were within twofold of presented values.

Changes in RexA or RexB nuclease motifs affect RexAB exonuclease activities. Phage T4*g2* is susceptible to exo/hel degradation. As nuclease activity inhibits plaque-forming ability, plaque formation would reflect diminished nuclease activity in the *rexAB* mutants (Table 1). The *recBCD* mutant containing pRexAB efficiently inhibits phage multiplication, whereas this strain lacking *rexAB* genes is totally permissive (22). The strain containing pRexA$^{\Delta DYK}$B allowed efficient phage multiplication. Thus, inactivation of the nuclease motif of RexA essentially abolishes all DNA degradation activity by RexAB.

For strains containing pRexAB^{D910A} and pRexAB$^{\Delta DYK}$, T4*g2* infectivity was increased 10- and 100-fold, respectively (Table 2), indicating that nuclease activity is significantly reduced in these strains. Note that nuclease inactivation may even be underestimated in this assay, as unwinding activity alone may have a modest inhibitory effect on T4*g2* multiplication (40). This result shows that the RexB nuclease motif DYK is a functionally active component of the exo/hel enzyme. The strain containing pRexAB$^{\Delta 771-1063}$ is totally permissive for phage multiplication, indicating that a large deletion in RexB abolishes in vivo nuclease activity.

These data show unambiguously that both the DYK motif in RexB (positions 910 to 912) as well as that in RexA (positions 1114 to 1116) are involved in nuclease activities of RexAB. Thus, the RexAB enzyme appears to differ from RecBCD, in which a single nuclease locus is involved.

RexB^{Nuc} mutants recognize Chi. We previously developed an in vivo test to detect Chi attenuation of exo/hel exonuclease activity by using an RC plasmid as substrate. A σ-formed replication intermediate of RC plasmids provides a dsDNA end as an entry point for exo/hel enzyme. If the RC plasmid contains a Chi site in the orientation recognized by exo/hel, degradation is attenuated and σ-form replication results in accumulation of HMW (14, 26). In the absence of Chi on the plasmid, HMW do not accumulate as long as exo/hel is active. However, if exo/hel is nuclease defective, any RC plasmid will accumulate HMW (e.g., RC plasmids accumulate large amounts of HMW in an *E. coli recD* mutant; our unpublished data).

We examined the activity of *rexAB* mutants in the *E. coli* Δ*recBCD* background on RC plasmids with or without an *L. lactis* Chi site (called Chi$_{Ll}$) (Fig. 2). In the presence of pRexAB, HMW accumulation was observed only if Chi$_{Ll}$ was present on the RC plasmid. In the absence of any exo/hel enzyme, HMW accumulated regardless of the presence of Chi$_{Ll}$ on the plasmid (Fig. 2) (22). The strain containing pRexA$^{\Delta DYK}$B behaved like the exo/hel-deficient strain. These results are consistent with the nuclease-negative phenotype conferred by RexA$^{\Delta DYK}$B in the phage infection test. Other mutated *rexA* alleles in which the nuclease motif was deleted gave rise to similar phenotypes (L. Rezaïki and A. Gruss, unpublished observations). We also observed Chi$_{Ll}$-independent HMW accumulation in the strain expressing RexAB$^{\Delta 771-1063}$, confirming that nuclease activity is deficient in this enzyme.

The two *rexB*^{Nuc} mutants exhibit a markedly different phenotype (Fig. 2). As expected for a nuclease-defective enzyme, the presence of pRexAB^{D910A} resulted in more HMW than did pRexAB. However, HMW accumulation remained Chi$_{Ll}$ dependent. Greater amounts of HMW were also observed when pRexAB$^{\Delta DYK}$ was present; a modest effect of Chi$_{Ll}$ in increasing accumulation was still observed. These results are consistent with our hypothesis that the RexB^{Nuc} motif is necessary

TABLE 2. Recombination efficiency in the presence of RexAB nuclease mutants

pRexAB derivatives in *E. coli* Δ*recBCD* (pΔBla)	Transformation efficiency with pACYC184 (1 μg)	No. of ampicillin-resistant transformants with:[a]		Chi$_{Ll}$ stimulation[b]
		Chi$_{Ll}^{0}$[c]	Chi$_{Ll}$[c]	
pRexAB	2×10^6	10	199	30
pRexAB^{D910A}	2×10^6	482	3,012	10
pRexAB$^{\Delta DYK}$	1×10^6	130	309	4
pRexAB$^{\Delta 771-1063}$	8×10^5	63	112	3
pRexA$^{\Delta DYK}$B	5×10^6	8	6	1
pGB2[d]	2×10^6	30	19	1
TG1 control	1.5×10^7	416	272	1

[a] Values represent the total number of transformants obtained in three transformation experiments. For each experiment 200 ng of the appropriate linear DNA was used.

[b] Chi$_L$ stimulation was determined as the ratio of ampicillin-resistant transformants obtained with linear DNA fragments containing Chi$_{Ll}$ compared to those with no Chi$_{Ll}$ (designated Chi$_{Ll}^{0}$). To determine the capacity of each fragment to effect homologous recombination, we used conditions of TG1 electroporation that abolish nuclease activity; the ratio obtained in TG1 was used to correct for Chi activity ratios in the test strains (20) (see Materials and Methods).

[c] Chi$_{Ll}$ and Chi$_{Ll}^{0}$ correspond to the linear fragments used for gene conversion, which do and do not, respectively, contain the double Chi$_{Ll}$ sites flanking the region of *bla* homology.

[d] pGB2 was the vector used to clone *rexAB* genes.

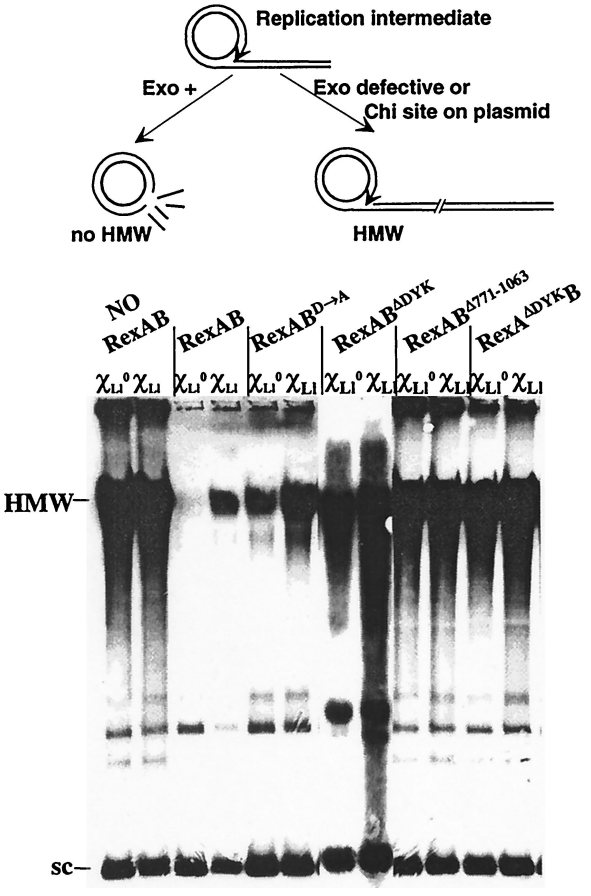

FIG. 2. HMW accumulation in the presence of wild-type or mutant RexA or RexB subunits. The upper portion shows the schema of HMW accumulation. RC plasmid replication may result in formation of a σ-shaped intermediate with a dsDNA tail. This tail is susceptible to exonuclease (Exo) degradation, and a monomeric circle is restored (left). If the strain is exonuclease defective, or if Chi is present on the RC plasmid, the σ form is extended and HMW accumulates (right). The bottom portion shows *E. coli* Δ*recBCD* derivatives containing plasmid pRC2 with Chi_{L1} (marked χ_{L1}) or without (marked $\chi_{L1}{}^0$) as well as a plasmid that carries a *rexAB* allele or no *rexAB*, as indicated above the wells. Cells were grown to mid-logarithmic phase at 34°C, and whole-cell lysates were prepared. HMW were detected by Southern hybridization using a pRC2 DNA fragment as the probe. Positions of HMW and supercoiled monomer plasmid (sc) migration are indicated. Note that results shown for RexAB$^{\Delta DYK}$ are from a separate gel.

for nuclease activity of the enzyme. In addition, the Chi_{L1}-dependent increase in HMW accumulation shows that the remaining nuclease activity is still attenuated at Chi_{L1}. Possibly, the RexB subunit degrades just one of the two dsDNA strands from the 5′ end (i.e., the strand containing the Chi complement) (23).

These results confirm the nuclease-defective phenotype of RexB^{Nuc} as seen in the T4*g2* test. They further show that although RexB^{Nuc} nuclease activity is reduced, its Chi_{L1} recognition is maintained. In contrast, the RexA^{Nuc} nuclease change abolishes all nuclease activity, regardless of Chi_{L1}. Thus, the phenotypes of the RexA and RexB nuclease changes are clearly distinguishable in vivo.

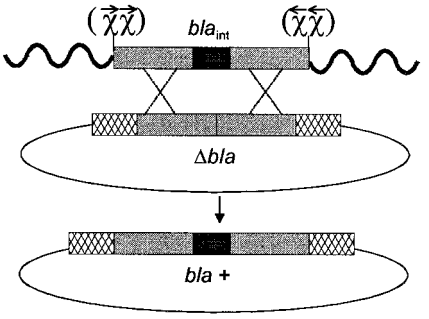

FIG. 3. Strategy to test Chi_{L1} effect on RexAB mutant-mediated homologous recombination. The recombination target plasmid pΔBla bears an internal deletion of *bla* (Δ*bla*). Linear transforming DNA contains an internal fragment of *bla* which spans the *bla* deletion (black rectangle) plus an additional 360 bp of flanking homology (dark grey rectangle) with pΔBla (called bla_{int}). Where present, double Chi_{L1} sites on the linear fragments are oriented for recognition to enhance recombination and are represented by χχ (RexAB recognizes the arrow tail). Wavy lines represent heterologous dsDNA tails. Double-crossover homologous recombination is required to convert cells to being ampicillin resistant (*bla*$^+$). Hatched rectangles represent *bla* DNA outside the regions present on linear DNA. The figure is essentially the same as one published in reference 22, with permission from the National Academy of Sciences.

RexB^{Nuc} but not RexA^{Nuc} mutants display a Chi-stimulated hyperrecombination phenotype. Gene replacement with linear DNA fragments is stimulated if correctly oriented Chi sites are present in the linear DNA flanking regions of homologies (15, 22, 23). Using this criterion, it was previously demonstrated that the presence of Chi_{L1} stimulates homologous recombination (22, 23). We examined the ability of mutant RexAB exo/hel to mediate Chi_{L1}-stimulated homologous recombination by using linear fragments with or without double Chi_{L1} sites at the ends (Fig. 3 and Table 2). The *E. coli* Δ*recBCD* recipient contained the recombination target plasmid (pΔBla) together with a plasmid encoding a mutated *rexAB* allele. As expected, very few recombinants were obtained in the Δ*recBCD* host, regardless of whether Chi_{L1} were present on the linear dsDNA ends. Recombination via wild-type RexAB was stimulated 30-fold by the presence of Chi on incoming linear fragments, in keeping with previous results (22, 23). In the presence of pRexA$^{\Delta DYK}$B, recombination was at background levels, further confirming that a change in the RexA nuclease motif results in total inactivation of RexAB biological activities in vivo.

RexB^{Nuc} nuclease mutants displayed a totally distinct hyperrecombination phenotype. Significant stimulation of homologous recombination as well as a Chi_{L1} effect were observed. The greatest stimulation was seen in the presence of pRexAB^{D910A}; a hyperrecombination phenotype was observed for the $Chi_{L1}{}^0$ fragment as well as a further 10-fold stimulation by Chi_{L1}. Chi-stimulated, elevated homologous recombination frequencies were also observed in the strain containing pRexAB$^{\Delta DYK}$. Thus, RexB^{Nuc} mutants have a stimulatory effect on recombination using short DNA fragments as substrates, and they also retain Chi recognition.

The strain containing pRexAB$^{\Delta 771-1063}$ demonstrated recombination frequencies like those of pRexAB, except that $Chi_{L1}{}^0$ fragment frequencies were elevated; nevertheless, an

approximately threefold Chi stimulation effect was observed. These results suggest that the pRexAB$^{\Delta 771-1063}$ enzyme retains some Chi_{L1} recognition activity despite a large C-terminal RexB deletion.

Taken together, these results show that RexA and RexB subunits both contribute to the observed nuclease activity of RexAB. The RexA$^{\Delta DYK}$ mutant abolishes all detectable in vivo activity of the enzyme, including homologous recombination. The RexAB^{D910A} and RexB$^{\Delta DYK}$ mutants reduce exonuclease activity but retain Chi_{L1} recognition and display a Chi_{L1}-stimulated hyperrecombination phenotype when using short dsDNA fragments as substrates.

DISCUSSION

RexAB exo/hel enzyme function involves two active nuclease sites. The *E. coli* RecBCD-Chi couple has served as the prototype for bacterial DSB repair. Indeed, exo/hel-Chi couples identified in other bacteria were confirmed to fulfill the biological or biochemical functions established in *E. coli* (9, 22, 42, 52). However, RecBCD and the *L. lactis* exo/hel enzyme, RexAB, differ operationally: RecBCD relies on a single nuclease to effect DNA degradation (51, 53). In contrast, we have shown that RexAB contains two nuclease motifs, one in each subunit, both of which are required for full nuclease activity. Inactivation of the RexA nuclease motif results in total loss of exo/hel functions in vivo, whereas inactivation of the RexB nuclease motif reduces degradation while retaining Chi activity. As these two nuclease motifs are present in all identified (or predicted) two-subunit exo/hel enzymes, we predict that these enzymes will have properties similar to those described here.

We propose a model for RexAB activity based on our in vivo results (Fig. 4A). The two major features of the model are the following. (i) RexA, like RecB, has helicase and nuclease activities and drives the enzyme. Unwinding of the double helix is assured by RexA, which has significant homology with PcrA helicases. The RexB subunit could enhance activities of RexA helicase, just as RecC appears to increase RecB processivity and activities (11, 38). (ii) The RexA nuclease cleaves just one strand, from the 3′ end, while the RexB nuclease is positioned to degrade from a 5′ end. This model is consistent with our results showing that RexB^{Nuc} mutants degrade DNA but maintain Chi recognition and with recent in vitro studies reported for AddAB in which degradation is attenuated at Chi but only on the Chi-containing strand; degradation of the "bottom" strand continues after Chi (9). This model may be useful in understanding the absence of a third, RecD-like component in the two-subunit exo/hel enzymes. RecD is proposed to position the single nuclease of the RecBCD enzyme so that it degrades the two DNA strands (51); if, as proposed, the nucleases of RexAB each act on a DNA strand, this RecD function would not be needed. Alternatively, the two established nuclease motifs are both needed to degrade each strand. We consider this possibility unlikely, as our results show that the RexA nuclease is active even if RexB^{Nuc} is mutated. In vitro analyses will confirm whether this model is valid.

The above model can explain simply the behavior of the RexB^{Nuc} mutants (Fig. 4B). As mentioned above, we propose that RexA drives the enzyme. Our data suggest that inactiva-

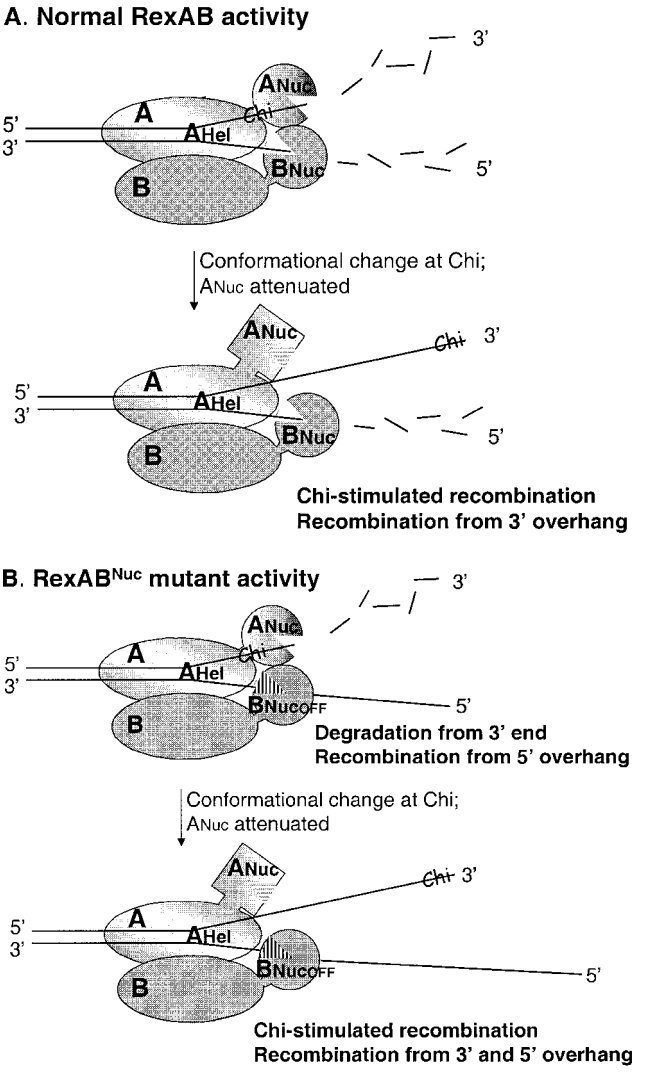

FIG. 4. Model for RexAB activity. (A) Normal RexAB. RexAB advances from an end on its dsDNA linear substrate via the RexA-driven helicase. RexB is proposed here to increase processivity, as does RecC of RecBCD (38). The RexA nuclease motif degrades from the 3′ end until the exo/hel enzyme reaches a Chi site. The conformational change at Chi attenuates RexA nuclease activity and thereby stimulates homologous recombination. The RexB nuclease degrades the dsDNA substrate from the 5′ end (bottom strand). At Chi_{L1}, degradation is essentially unchanged or possibly enhanced, as reported, in vitro for the bottom strand after RecBCD encounters an *E. coli* Chi site (3). (B) Mutant affected in the RexB nuclease motif. Activities are as in panel A, except that the RexB nuclease is inactive. As such, although the 3′ end is degraded by RexA nuclease, a 5′ end is generated that can act as a substrate in homologous recombination, as shown from previous in vitro and in vivo data (35, 39). This can explain elevated levels of recombination in the assay using linear DNA fragments lacking Chi_{L1} (Table 2). Upon a Chi_{L1} encounter, RexA nuclease is attenuated, thus making both DNA strands accessible for recombination. A and B refer to RexA and RexB subunits, respectively. ANuc and BNuc correspond to the nuclease domains surrounding the motifs presented in Fig. 1. An open mouth has an active nuclease, while a barred mouth represents an inactive nuclease. AHel corresponds to the RexA helicase domain (deduced from BLAST homologies with PcrA helicase).

tion of the RexB nuclease motif does not abolish other enzyme functions. As such, DNA strands would be unwound and the 3′ end degraded by RexA nuclease. The protruding 5′ end could act as a substrate for homologous recombination, as suggested by previous in vitro and in vivo data (35, 39), consistent with the hyperrecombination phenotype seen for the $RexB^{Nuc}$ mutants in the absence of Chi_{Ll} stimulation (Table 2). Upon Chi_{Ll} encounter, degradation from the 3′ end is inhibited and both strands are available for recombination.

In contrast to $RexB^{Nuc}$ mutants, the $RexA^{\Delta DYK}B$ mutant exhibited no in vivo biological activity and may thus correspond to a null mutant. Interestingly, in *B. subtilis* and in *E. coli*, mutations affecting nuclease motifs in RexA analogues AddA and RecB, respectively, retain helicase activities. For example, an AddAB mutant in which the C-terminal end of AddA is deleted lacks nuclease activity but retains some helicase proficiency in vivo and in vitro (28), and an *E. coli* $RecB^{D1080A}CD$ gene mutation inactivates the RecB nuclease (53); in vivo, this enzyme is nonfunctional (2). It was reasoned that $RecB^{D1080A}CD$ is unable to undergo a conformational change at Chi and thus remains locked in the "antirecombinase" position; this effect is alleviated by removing RecD (2, 12). Our preliminary results suggest that recombination is restored in a $RexA^{\Delta DYK}$ $RexB^{\Delta DYK}$ double mutant (data not shown), suggesting that the conformational change observed at Chi_{Ll} might involve interactions between RexB and RexA nuclease domains. Applying the above reasoning, we speculate that $RexA^{\Delta DYK}B$ could be locked in a nonrecombinogenic state that blocks the conformational change at Chi_{Ll} needed to render it recombinogenic. The alternative hypotheses concerning the $RexA^{Nuc}$ mutant will be examined by further genetic and biochemical tests.

Why is exo/hel so poorly conserved? The primordial need for an intact genome suggests that DNA genome repair mechanisms have been present early in evolution. Accordingly, generalized homologous recombination proteins such as RecA, SSB, and RecF are rather highly conserved (19) (comparisons analyzed using BLAST; http://www.ncbi.nlm.nih.gov/BLAST/).

It is surprising that components of an important survival function like DNA repair are so markedly divergent. The diversity in DSB repair enzymes may be related to genome plasticity: genome rearrangements are common events that may occur via intrachromosomal transposition, gene duplications and inversions, or entry of exogenous DNA. In *L. lactis*, there is a 4:1 orientation bias of Chi distribution with respect to the direction of DNA replication (exo/hel recognizes Chi in one orientation) (14,21,23,29,47). Inversion of a large DNA segment could considerably reduce the number of Chi sites available to stimulate repair if a replication fork break occurs in that region (36). Such rearrangements could impose selective pressure for exo/hel divergence. The constant need for the exo/hel enzyme to adapt to altered distributions of Chi on the genome (e.g., due to chromosomal inversions or mutations) could explain why these enzymes are so divergent, even in closely related species.

Is *E. coli* the right DSB repair model? The *E. coli* RecBCD exo/hel enzyme has been the paradigm for DSB repair over the last 30 years. But *E. coli* is a relatively young bacterium in terms of evolution; it seems to have evolved well after oxygen became abundant (27). In contrast, *L. lactis*, which thrives under low- or no-oxygen conditions, appears to have preceded *E. coli* evolutionarily (24, 27). To follow the evolution of the DSB repair system, we suggest that comparison with an older microorganism like *L. lactis* will be informative and may reveal minimum requirements for DSB repair.

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