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Genetics

Identification of the lactococcal exonuclease/recombinase and its modulation by the putative Chi sequence

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ABSTRACT **Studies of RecBCD–Chi interactions in *Escherichia coli* have served as a model to understand recombination events in bacteria. However, the existence of similar interactions has not been demonstrated in bacteria unrelated to *E. coli*. We developed an *in vivo* model to examine components of dsDNA break repair in various microorganisms. Here, we identify the major exonuclease in *Lactococcus lactis*, a Gram-positive organism evolutionarily distant from *E. coli*, and provide evidence for exonuclease–Chi interactions. Insertional mutants of *L. lactis*, screened as exonuclease-deficient, affected a single locus and resulted in UV sensitivity and recombination deficiency. The cloned lactococcal genes (called *rexAB*) restored UV resistance, recombination proficiency, and the capacity to degrade linear DNA, to an *E. coli recBCD* mutant. In this context, DNA degradation is specifically blocked by the putative lactococcal Chi site (5′-GCGCGTG-3′), but not by the *E. coli* Chi (5′-GCTGGTGG-3′) site. RexAB-mediated recombination was shown to be stimulated ≈27-fold by lactococcal Chi. Our results reveal that RexAB fulfills the biological roles of RecBCD and indicate that its activity is modulated by a short DNA sequence. We speculate that exonuclease/recombinase enzymes whose activities are modulated by short DNA sequences are widespread among bacteria.**

The DNA repair machinery of *Escherichia coli* includes RecBCD, a powerful, double-stranded (ds) DNA exonuclease. It degrades dsDNA ends that may be produced, for example, by breaks at the replication fork (1, 2). Exonucleolytic activity of RecBCD is modulated by the sequence 5′-GCTGGTGG-3′ (3), called Chi (referred to as Chi_{Ec}), when properly oriented on a dsDNA linear substrate. The exonuclease appears to stall at Chi_{Ec}, and the enzyme continues on the DNA as a helicase, with modified exonuclease activity (4). Single-stranded DNA is generated, and recombination is stimulated downstream (with respect to the direction of DNA degradation) of Chi_{Ec} (see ref. 5 for review).

Despite its important role in *E. coli*, the extent to which an exonuclease–Chi interaction is involved in DNA repair in other organisms is unknown. *In vitro* studies suggest that RecBCD–Chi interactions are conserved in enterobacteria closely related to *E. coli* (6). Exonuclease analogs of RecBCD have been identified by activity and/or sequence homology in less closely related organisms, including *Haemophilus influenzae*, *Bacillus subtilis*, and *Mycobacterium tuberculosis* (refs. 7–10, EMBL/GenBank/DDBJ database). The *B. subtilis* exonuclease, AddAB, which has been studied extensively (11–13), shares little sequence similarity with RecBCD (mainly in ATP-binding motifs and helicase motifs present in RecB and AddA). However, it is functionally similar to RecBCD, because it has exonuclease and helicase activity, and is necessary for transduction and transformation (11). To date, exonuclease–Chi interactions have not been shown in these or any other organisms distant from *E. coli*.

We previously reported the sequence of the putative Chi site (5′-GCGCGTG-3′, referred to as Chi_{Ll}, and/or its complement) of *Lactococcus lactis*, a Gram-positive organism unrelated to *E. coli* (14). The sequence assignment was based on the capacity of Chi_{Ll} to modulate exonuclease activity on a linear dsDNA substrate provided by rolling circle (RC) plasmids (15). This finding strongly suggested that a RecBCD analog exists. Here, we confirm the existence of a RecBCD analog in *L. lactis* and show that it interacts specifically with the lactococcal Chi_{Ll} site to attenuate exonuclease activity and stimulate recombination. Our results argue for the existence of couples like RecBCD and Chi_{Ec} in a broad spectrum of microorganisms.

MATERIALS AND METHODS

Bacterial Strains and Plasmid Constructions. Plasmid pRC1-*nuc* was constructed as follows: plasmid pBS::*nuc* (16), digested with *Pst*I and filled in, was fused to *Pvu*II-linearized pVS41 (17). The resulting plasmid was cut by *Sma*I-*Eco*RV and religated to eliminate the pBS (pBluescript) replicon, resulting in a pVS41 replicon fused to *nuc*. To construct plasmid pRexAB, the small *Hin*cII-*Bam*HI fragment of pBR322 was first cloned into *Hin*cII-*Bam*HI-treated pGB2 (low-copy-number plasmid; ref. 18) to generate the vector. An *Fsp*I-*Cla*I *L. lactis* chromosomal DNA fragment containing *rexB* (recovered from the cloned junction of transposon insertion 1; see Fig. 2) was inserted in the *Sma*I-*Cla*I cut vector. The resulting plasmid was cut by *Hin*cII and *Cla*I, and a *Bsr*GI (fill-in)-*Cla*I *L. lactis* chromosomal DNA fragment (recovered from the cloned junction of transposon insertion 2; Fig. 2) containing *rexA* was inserted. This construct contains the whole *rexAB* operon. To construct pGB2:P_{Ara}*gam*, which expresses the bacteriophage λ *gam* gene (19) under control of an arabinose-inducible promoter, a blunted *Bam*HI-*Sal*I fragment containing *gam* was cloned into *Sma*I and *Sal*I cut vector pBAD18 (20). The expression cassette, present on a *Cla*I-*Hin*dIII fragment, was recloned in *Acc*I-*Hin*dIII sites of pGB2. Strains and plasmids used directly in experiments are presented in Tables 1 and 2, respectively.

Media, Bacterial Transformation, and DNA Preparation. *E. coli* strains were grown in Luria–Bertani (LB) broth; 0.01% thymidine was added for strain V186 and derivatives. *L. lactis* strains were grown at 30°C in M17 medium containing 0.5% glucose (M17-glu). Antibiotics were used as follows: 50 μg/ml streptomycin (Str), 125 μg/ml rifampicin (Rif), 5 μg/ml erythromycin (Ery) for *L. lactis* and 150 μg/ml for *E. coli*, 100 μg/ml ampicillin (Amp), 15 μg/ml kanamycin (Kn); 50 μg/ml spectinomycin (Spc), 10 μg/ml chloramphenicol (Cm) for *E. coli* and 3 μg/ml for *L. lactis*, and 10 μg/ml tetracycline (Tet). *L.*

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Abbreviations: HMW, high-molecular-weight linear plasmid multimers; RC, rolling circle.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database [accession no. U76424 (*rexAB*)].
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Table 1. Strains used in this study

Strain	Description	Source
L. lactis		
MG1363	Derivative of natural isolate ML3 cured of plasmids and prophages	21
VEL 1122	*rec*A derivative of MG1363, Tet^r	22
CL56-5	Ery^r marker chromosomally integrated in MG1363	P. Le Bourgeois
REX2	Stable exonuclease-defective IS*S1*-insertional MG1363 mutant (insertion "2" in Fig. 2)	This work
E. coli		
AB1157	*thr-1 leu-6 proA2 his-4 thi-1 argE3 lacYI galK2 ara-14 xyl-5 mtl-1 tsx-33 supE44 rspL31 kdgK51*	A. J. Clark
JC5519	As AB1157 but *recB21recC22*	A. J. Clark
BW6175	HfrPK3, PO137, *argE86*::Tn10	23
V186	Δ*(thyA-argA)232* IN(*rrnD-rrnE*)1	24
V182	As V186 plus pDWS2	24
TG1	F′ *traD36* $lacI^q$Δ(lacZ)*M15* $proA^+B^+/supE$ Δ(*hsdM-mcrB*) ($r_K^- m_K^-$ $McrB^-$) *thi* Δ(*lac-proAB*)	

lactis strains were electrotransformed as described (26). *E. coli* strains were electrotransformed (27) or transformed by the Hanahan method (28). Whole-cell DNA minilysates were prepared as described (29).

Detection of High-Molecular-Weight Linear Plasmid Multimers (HMW). HMW accumulation was detected on whole-cell lysates after agarose gel electrophoresis (15). Plasmid DNA, labeled by chemiluminescence by using the ECL system (Amersham), was used as probe. Southern blot hybridization was performed as recommended by kit suppliers.

DNA Sequencing. An 11-kb fragment containing the putative lactococcal exonuclease genes was amplified from genomic DNA by long accurate PCR (30). A random library of *Alu*I partial subfragments (500–1,500-bp size range) of the 11-kb segment was inserted into *Sma*I-linearized and dephosphorylated M13mp18. Single-stranded DNA was prepared (Beckman Biomek 1000 laboratory workstation; ref. 31). DNA sequencing was performed by using the Applied Biosystems PRISM sequencing kit on the Catalyst station. To complete the sequence, dye terminator method (protocol supplied by Applied Biosystems) was used. We used XBAP and XNIP programs (32) for fragment assembly and consensus sequence analyses, and BLAST algorithm (on the National Center for Biotechnology Information e-mail server) for homology searches.

Table 2. Plasmids used in this study*

Plasmids	Description	Reference
pGh9:IS*S1*	Transposon delivery vector	25
pRC1-*nuc*	RC plasmid pVS41, containing *nuc* reporter gene	This work
pDWS2	pBR322 plasmid containing cloned *recBCD* genes of *E. coli*	24
pRC2	pVS41 *Pvu*II joined to large *Eco*RV-*Pvu*II fragment of pACYC184 and containing MCS linker cloned into *Pvu*II site	This work
pRC2-Chi-†	Chi- linker cloned into *Bam*HI-*Eco*RI sites of pRC2	This work
pRexAB	pGB2 joined to *Fsp*I-*Bsr*GI fragment, which carries the *rexAB* genes of *L. lactis*	This work
pΔB1a	pBR322 with a 111-bp (*Sca*I-*Pvu*I) deletion in the *bla* gene	This work
pGB2:P_{Ara}*gam*	*gam* expression controlled by arabinose-inducible promoter	This work

*Details on vectors and oligonucleotide sequences in *Materials and Methods*.

†Chi- corresponds to either Chi_{Ll}^{m}, Chi_{Ll}^{1}, Chi_{Ll}^{2}, $\mathrm{Chi}_{Ll}^{1\text{-}2}$ or Chi_{Ec}, as noted in text.

Cloning of Oligonucleotide Linkers. Six oligonucleotide pairs were synthesized (DNA synthesizer Oligo 1000, Beckman): MCS, 5′-CTGGAATTCGTCGACGGATCC-3′ and 3′-GACCTTAAGCAGCTGCCTAGG-5′; Chi_{Ll}^{m}, 5′-AATTCCTGCAG**C**CGCGTGG-3′ and 3′-GGACGTC**G**GCGCACCCTAG-5′; Chi_{Ll}^{1}, 5′-AATTCCTGCAGCACGCGCG-3′ and 3′-GGACGTCGTGCGCGCCTAG-5′; Chi_{Ll}^{2}, 5′-AATTCCTGCAGGCGCGTGG-3′ and 3′-GGACGTCCGCGCACCCTAG-5′; Chi_{Ll}^{1-2}, 5′-AATTCACGCGCTGCAGGCGCGTGG-3′ and 3′-GTGCGCGACGTCCGCGCACCCTAG5′; Chi_{Ec}^{2}, 5′-AATTCCTGCAGGCTGGTGGG-3′ and 3′-GGACGTCCGACCACCCCTAG-5′. Chi sites are underlined; the base in bold creates a mutation in Chi_{Ll}, resulting in Chi_{Ll}^{m}. Chi_{Ll}^{2}, Chi_{Ll}^{m}, and Chi_{Ec}^{2} are oriented for recognition by a RecBCD molecule entering through the tail of a σ-form RC plasmid replication intermediate formed during plus-strand replication. Chi_{Ll}^{1} is in the opposite orientation. Chi_{Ll}^{1-2} contains sites in both orientations. Linker sequences were verified by DNA sequencing after cloning into plasmid pRC2.

Transposition Mutagenesis and Mutant Screening Test. Plasmid pGh9:IS*S1* was used to perform transposition mutagenesis (25) on strain MG1363 containing the HMW-reporter plasmid pRC1-*nuc*; potential exonuclease-defective mutants were screened (Fig. 1). pRC1-*nuc* encodes the staphylococcal nuclease (Nuc), a secreted enzyme (33). Because Nuc has no intracellular activity in *L. lactis* (Y. Le Loir and I. Poquet, this laboratory, unpublished results), its use does not affect intracellular DNA metabolism. Exonuclease-deficient mutants would accumulate HMW and therefore be detected as Nuc overproducers by plate tests by using an overlay containing Toluidine blue and denatured DNA (16). Accumulation of HMW in mutant candidates was verified by Southern hybridization by using pRC1-*nuc* DNA as probe.

A stable, exonuclease-defective IS*S1*-generated mutant, called REX2, was constructed by excision of the transposed vector (of transposition "2" in Fig. 2) as described (25); its structure was confirmed by Southern hybridization.

T4 *gene 2* Test for Exonuclease Activity. Bacteriophage T4 *gene 2am mutant* (T4*g2*; provided by W. Wackernagel, Univ. of

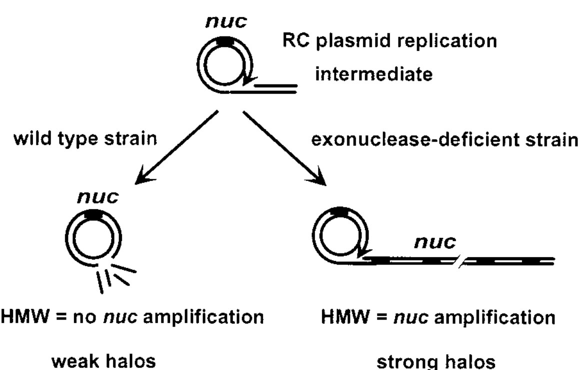

FIG. 1. Screening system to isolate exonuclease-defective mutants of *L. lactis*. RC plasmid replication may generate a σ-shaped molecule with dsDNA tail (*Upper*; arrow indicates direction of replication). This tail may be degraded by exonuclease, thereby restoring a monomeric circle (*Left Lower*). Expression of the *nuc* reporter gene (present on pRC1-*nuc*) is low and results in weak halos around colonies. If the exonuclease is inactivated, the σ form is extended and HMW accumulates, amplifying the *nuc* gene. Amplification leads to overexpression of Nuc, which gives rise to strong halos of DNA digestion around colonies in plate tests (*Right Lower*).

Oldenburg, Germany) was used to examine exonuclease activity (34, 35). Plaque-forming units were measured in *E. coli recB21recC22* strain JC5519 containing pRexAB, JC5519, or wild-type AB1157, as described (15) except that cultures and plates were incubated at 30°C. This temperature was used because RexAB exonuclease activity appears to be thermosensitive (note that optimal growth temperature of *L. lactis* is 30°C).

Conjugation Tests in *L. lactis* and *E. coli*. Str^r-Rif^r derivatives of REX2 and of VEL1122 were isolated by first plating cells on Rif. Rif^r clones were then plated on Str to select double Str^r-Rif^r mutants. Str^r-Rif^r strains were used as recipients in surface mating experiments (36, 53). Strain CL56–5 was the Hfr donor and IL1776 was the plasmid donor. CL56–5, a derivative of MG1363, contains a chromosomally integrated Ery^r marker near the initiation site of chromosomal transfer (P. Le Bourgeois, Univ. Paul Sabatier, France). IL1776, an IL1403 derivative, contains plasmid pIL205, which confers Cm resistance; pIL205 is conjugative in the absence of homologous recombination (37). Mid-log phase donor and recipient cells were mixed at a ratio of 1:2, then 0.1 ml was spread on an M17-glu agar plate. After overnight incubation, cells were resuspended and dilutions were plated on M17-glu medium containing Ery, Cm, Ery-Str-Rif, or Cm-Str-Rif, as appropriate, or nonselective medium, for donor, recipient, and transconjugant cells. Control plates containing donor or recipient cells only were also plated. Conjugation frequency was determined as the number of transconjugants divided by the total number of recipients.

E. coli matings (38) were performed by using Hfr strain BW6175 as donor. Because the *L. lactis* exonuclease is thermosensitive, recipient strains were grown at 30°C. Matings were performed at 35°C for 30 min (pili are poorly formed at 30°C) and plated at either 35 or 30°C (conjugation efficiencies were similar at both temperatures).

Ultraviolet Light-Sensitivity Tests in *L. lactis* and in *E. coli*. Tests (22) were performed on cells maintained at 30°C, except that irradiation was carried out with a Stratalinker 2400 UV source (Stratagene).

Chi$_{Ll}$-Stimulated Recombination Tests. The strategy described by Dabert and Smith (39) to examine Chi$_{Ec}$-stimulated homologous recombination by gene conversion was modified to examine the effect of Chi$_{Ll}$. We constructed a deletion derivative of the β-lactamase gene (*bla*) of pBR322 so that a double crossover event would convert cells to Amp^r. For this purpose, the *Sca*I-*Pvu*I internal fragment of plasmid pBR322 (positions 3735 through 3846 of the pBR322 sequence) was deleted to give rise to pΔBla. Primers were designed to PCR-amplify a *bla* gene fragment (positions 3374 through 4214 on the pBR322 sequence) covering the DNA deleted from pΔBla, plus ≈360 bp additional *bla* gene DNA (see Fig. 4); primer tails immediately flanking *bla* gene DNA contained double Chi$_{Ll}$ or Chi$_{Ec}$ sites. Primer couples generating double Chi$_{Ll}$ sites were: 5′-CAAATAGGTCTTATTGAAAATGATGGAGTA**CACGCGCTCACGCGC**AA*TGTAGATAACTACGATACGGGAGGGC*-3′ and 5′-CATTCAGCACAATGGTTGAATAATTACAAA**CACGCGCTCACGCGC***TATCCGCTCATGAGACAATAACCCTG*-3′. Primer couples generating double Chi$_{Ec}$ sites were: 5′-CAAATAGGTCTTATTGAAAATGATGGAGTA**CCACCAGCTCCACCAGC**A*TGTAGATAACTACGATACGGGAGGGC*-3′ and 5′-CATTCAGCACAATGGTTGAATAATTACAAA**CCACCAGCTCCACCAGC***TATCCGCTCATGAGACAATAACCCTG*-3′. Chi site complements are in bold, and *bla* sequences are in italics. The resulting PCR fragments were cloned into the *Sma*I polylinker site of a pBS derivative deleted for the entire *bla* gene and replaced by a Kn^r marker (provided by P. Renault, Institut National de la Recherche Agronomique, France). The final linear fragment containing DNA internal to the *bla* gene flanked by Chi sites and surrounded by heterologous DNA was excised on a *Pvu*II fragment, and its structure was confirmed by sequencing. Linear DNA samples were quantitated on agarose gels by using known quantities of marker DNA.

To determine the efficiency of gene conversion, electrocompetent cells were prepared. Known amounts of pACYC184 DNA were used to test competence. For electrotransformation of linear fragments, ≈200 ng DNA was used. Incubation after electrotransformation was performed at 35°C for 90 min. Colonies were counted after 48 hr at 35°C. Numbers of Amp^r transformants obtained with Chi$_{Ll}$- or Chi$_{Ec}$-containing linear DNAs were compared for each strain. *E. coli* strain JC5519 *recB21C22* containing pRexAB and pΔBla was used to test whether Chi$_{Ll}$ stimulates RexAB-mediated homologous recombination. Electrocompetent *recB21C22* containing pΔBla was used as negative recipient control, and TG1 containing pGB2:P$_{Ara}$*gam* and pΔBla was used as positive control. Electrocompetent cells of this strain were prepared in LB containing 0.02% L-arabinose.

RESULTS

Isolation of Exonuclease-Defective Mutants of *L. lactis*. A screening test was devised to isolate exonuclease-minus insertional mutants of *L. lactis* strain MG1363. RC plasmid DNA replication generates a linear dsDNA substrate that can be attacked by exonucleases (Fig. 1) (14, 15). If exonuclease activity is absent, a σ-form replication intermediate accumulates, giving rise to HMW. DNA amplification by HMW accumulation can be scored if a reporter gene is on the plasmid. An *L. lactis* MG1363 strain was constructed containing plasmid pRC1-*nuc*, which has a marker gene whose activity is detected by a plate overlay (*Materials and Methods*). pRC1-*nuc*, which lacks both Chi$_{Ec}$ and Chi$_{Ll}$, is present only in monomeric form, and gives rise to weak halos. If HMW is generated through exonuclease inactivation, strong halos are

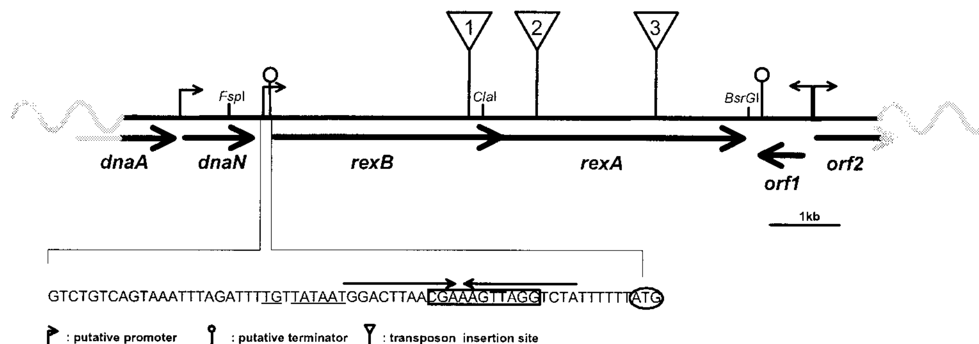

FIG. 2. Organization of the exonuclease genes. *rexA* and *rexB* are organized in tandem, and their ORFs overlap by 5 bp. Putative promoters, terminators, and transposon insertion sites are shown. Bold arrows indicate ORFs. Sequence of the *rexB* upstream region is shown. Underlined nucleotides, putative extended −10 promoter; boxed letters, putative Shine–Dalgarno sequence; arrows above nucleotides indicate a potential hairpin; start codon is circled. Restriction sites used in cloning are shown.

produced because of marker gene amplification (Fig. 1). Insertion sequence delivery plasmid pGh9:IS*S1* (25) was established in the strain to perform insertional mutagenesis.

From approximately 20,000 mutants screened for marker gene activity, ≈30 colonies produced large halos. Hybridization tests confirmed that three mutants accumulated HMW. Because our previous studies in *E. coli* indicated that HMW accumulates if the host strain is exonuclease-defective (15), we considered it likely that the three mutants interrupted a *L. lactis* gene encoding a major exonuclease.

Sequence Determination of Putative Exonuclease Genes. DNA regions flanking the transposon insertion sites of the three mutants were recovered by cloning. Hybridization experiments revealed that the insertion sites were closely spaced (within ≈3 kb; Fig. 2), indicating that all three may be localized within a single genetic locus. Sequencing of the junctions distal to the transposon insertion sites delimited an 11-kb fragment that presumably encompasses the exonuclease gene(s). Long-range PCR was performed on genomic DNA extracted from the parental MG1363 strain to generated this 11-kb segment that was sequenced. Two ORFs of 1,100 and 1,174 aa were identified and found to share 23 and 34% overall identity with AddB and AddA of *B. subtilis*, respectively. AddAB is known to have several functions equivalent to those of RecBCD, including exonuclease, helicase, and recombinase activities (11, 12). These sequence similarities provided further evidence that the three mutants identified by the RC plasmid assay inactivate the major exonuclease of *L. lactis*. We tentatively assigned the name RexA and RexB (for **R**ecombination **ex**onuclease) to the identified ORFs encoded by *rexA* and *rexB*, respectively. The identification of a single genetic locus by our screening procedure confirms the use of RC plasmids as a biological probe of RecBCD-like exonuclease activity.

Sequence Analyses of the *rexAB* Operon. The operon is localized near the *dnaA* homolog of *L. lactis* (Fig. 2) and is immediately adjacent to *dnaN* (encoding a DNA polymerase III subunit), thus placing *rexAB* close to the replication origin (ref. 40; A. Wind, Chr. Hansen, Denmark, personal communication). In other genomes, this position is occupied by *recF* (40). Like *addAB* of *B. subtilis*, *rexAB* genes are organized in tandem, with translational overlap, and are likely to be cotranscribed. The putative transcriptional initiation site of *rexAB* is characterized by an extended −10 promoter (41). In addition, a putative rho-independent transcriptional terminator of the upstream *dnaN* gene exists just downstream of the −10 promoter and overlaps the *rexAB* ribosome binding site (Fig. 2). This organization suggests that transcription of the exonuclease genes is affected by *dnaN* expression.

Biological Activities of an Exonuclease-Deficient *L. lactis* Strain. REX2, a stable derivative of one of the insertional exonuclease mutants, showed UV light sensitivity intermediate between that of the parental MG1363 strain and the UV-sensitive *recA* mutant of MG1363 (ref. 22; Table 3). *E. coli recBC* (42) and *B. subtilis addAB* (12) mutants have similar phenotypes. Recombination capacity of REX2 was examined (Table 3) by using a lactococcal Hfr conjugation test. A Str^r-Rif^r derivative of REX2 was isolated and used as recipient. Compared with the parental strain recipient, conjugational transfer of a chromosomal marker was reduced ≈300-fold in REX2. Two other independently obtained Str^r-Rif^r REX2 derivatives gave comparable conjugation frequencies. As expected, transfer requires a *recA*-proficient recipient. Frequencies of plasmid conjugation, which does not require homologous recombination, were about the same in all recipients. These results indicate that the major exonuclease has a role in *L. lactis* repair and recombination that is equivalent to that reported in *E. coli* and *B. subtilis*.

Complementation of *E. coli recBCD*-Deficient Strains by the Cloned *L. lactis* Exonuclease Genes. Plasmid pRexAB, carrying the two putative exonuclease subunit genes, was established in *E. coli* V186 Δ*recBCD* or JC5519 *recB21recC22* strains. Bacteriophage T4*g2* DNA is sensitive to exonuclease degradation (34), and its plaque-forming ability provides a simple test to evaluate host exonuclease activity (35). A wild-type (wt) *E. coli* strain expressing RecBCD is resistant to T4*g2* phage infection, whereas the *recB21recC22* strain is sensitive (Table 4). When *rexAB* genes are present in the *recB21recC22* strain, T4*g2* multiplication is inhibited, thus showing that the lactococcal exonuclease is active in *E. coli*.

Table 3. Biological characteristics of the *L. lactis* REX2 mutant

L. lactis strain	Relative survival and frequencies: UV irradiation*	Conjugational recombination†	Plasmid conjugation‡
Wt (MG1363)	1	1	1
rexB (REX2)	0.27	0.0034	0.97
recA (VEL1122)	0.02	0.00047	1.06

For each study, results are means of at least three experiments, and standard deviation <50% of mean values.
*Cells were irradiated at 20 J/m^2. Survival of wt strain was 0.11.
†Recombination frequency of wt strain was 1.25×10^{-4} per viable recipient.
‡Plasmid pIL205 conjugational transfer in wt strain was 1.0×10^{-3}.

UV sensitivity of the RecBCD-deficient strain was partially alleviated by the lactococcal exonuclease (Table 4), showing that the DNA repair defect can be complemented. Hfr crosses were performed to examine conjugational recombination efficiency in the presence of *rexAB* (Table 4). In the presence of pRexAB, recombination frequencies were within 2.5-fold of those measured in the wt recipient, and 100-fold higher than those in the *recBCD*-defective host (43). These results show that *rexAB* provides the functions necessary for efficient repair and recombination in *E. coli*.

Introduction of *L. lactis* Exonuclease Genes into a Δ*recBCD* *E. coli* Strain Directs Chi Specificity to the Putative Lactococcal Chi_{Ll} Site. The accumulation of HMW is an accurate reflection of the presence of Chi on an RC plasmid (15). Plasmid pRC2 containing either Chi_{Ll} or Chi_{Ec} was established in an *E. coli* strain containing *rexAB* genes. pRC2 accumulated HMW when it contained Chi_{Ll} in either orientation but not when it contained Chi_{Ec} (Fig. 3). At present, we cannot explain the effect of Chi_{Ll} in both orientations (which was also observed in *L. lactis*; ref. 14). When the same plasmids were established in the wild-type *E. coli* strain, only plasmids containing Chi_{Ec} accumulated HMW (Fig. 3). These results show that RexAB exonuclease activity is modulated specifically by the putative Chi_{Ll} site. They also show that modulation of RexAB does not require other host-specific factors. These results lead us to propose that regulation of a major exonuclease by interaction with a specific DNA sequence is a feature conserved among diverse microorganisms.

Table 4. pRexAB complements a *recBCD* defect in *E. coli*

E. coli strain [plasmid]	Relative values for: pfu of T4*g2**	Survival after UV irradiation†	Frequency of conjugational recombination‡
Wt	0.0005	1	1
recBCD [pRexAB]	0.0002	0.2	0.4
recBCD	1	0.0073	0.0038

For each study, results are means of at least three experiments, and standard deviation <50% of mean values.
*Values given are relative to a plaque titer of 10^{10} pfu/ml on JC5519 (*recBCD*). Wt strain is AB1157.
†Cells were irradiated at 25 J/m^2. Survival of wt strain (V182) was 2.4 $\times 10^{-1}$. *recBCD* strain is V186.
‡Recombination frequency of wt strain (AB1157) was 1.6×10^{-3} per viable recipient. *recBCD* strain is JC5519.

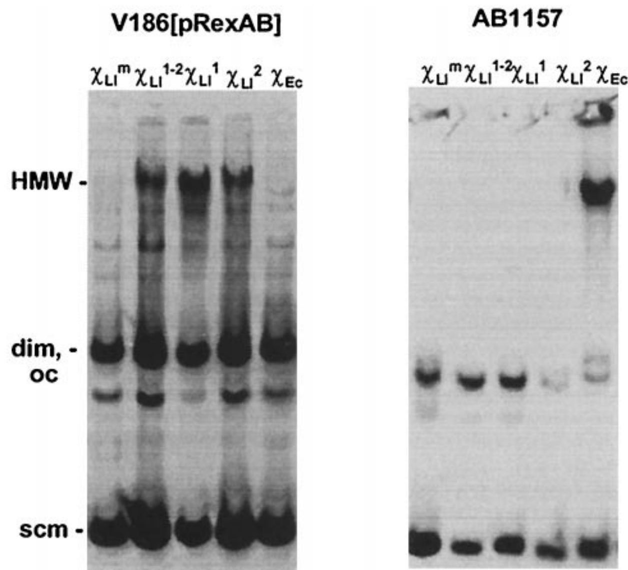

FIG. 3. Chi_{Ll} is specifically recognized by RexAB. *E. coli* strains containing plasmid pRC2 with Chi_{Ll} or Chi_{Ec} were grown to mid-logarithmic phase, and whole-cell lysates were prepared. HMW was detected by Southern hybridization by using pRC2 as probe. Plasmids are: pRC2-Chi_{Ll}^{m} (containing mutated Chi_{Ll}, χ_{Ll}^{m} lanes), pRC2-Chi_{Ll}^{1-2} (containing head-to-head copies of Chi_{Ll}, χ_{Ll}^{1-2} lanes), pRC2-Chi_{Ll}^{1} (containing Chi_{Ll} in orientation 1, χ_{Ll}^{1} lanes), pRC2-Chi_{Ll}^{2} (containing Chi_{Ll} in orientation 2, χ_{Ll}^{2} lanes), pRC2-Chi_{Ec}^{2} (containing Chi_{Ec} in orientation 2, χ_{Ec} lanes). Smear and bands below HMW in V186[pRexAB] strain appear to be secondary products of HMW accumulation.

RexAB-Mediated Recombination Is Stimulated by Chi_{Ll} in *E. coli*. It was reported in *E. coli* that gene replacement with linear DNA fragments is stimulated by correctly oriented Chi_{Ec} sites in the DNA-flanking regions of homologies (39). To determine whether Chi_{Ll} stimulates RexAB-mediated recombination in an *E. coli recB21recC22* strain, we modeled experiments on the published strategy. The target for gene replacement was on pΔBla. Restoration of *bla* on pΔBla by gene conversion with a linear molecule requires a double crossover event (Fig. 4). Constructions were made to produce linear fragments containing the internal region missing from pΔBla plus ≈360 bp of adjacent *bla* DNA. This DNA was flanked by sequences containing either double Chi_{Ll} or Chi_{Ec} sites on either side and heterologous DNA at the extremities (Fig. 4 and *Materials and Methods*). To first ensure that Chi_{Ll}- and Chi_{Ec}-containing fragments were potentially active for gene conversion, we used as recipient TG1 containing pGB2:P_{Ara}*gam* and pΔBla; in this strain, expression of λ *gam* inhibits Chi recognition but allows Hfr recombination to occur (19). The two fragments transformed this strain with about equal efficiencies (Table 5). Similar results were obtained in a *recBCsbcB* recipient, which is recombination-competent but Chi activity-defective; however, electrocompetence of the strain was poor (data not shown). In contrast, nearly no transformants were obtained in the *recB21recC22* recipient. We then tested for recombination in the presence of RexAB. *E. coli recB21recC22* cells containing pRexAB and pΔBla were transformed with equivalent amounts of linear DNA containing either the Chi_{Ll} or Chi_{Ec} sites (Table 5). In this context, DNA fragments containing Chi_{Ll} gave ≈27-fold more transformants than those containing Chi_{Ec}. These results demonstrate that Chi_{Ll} is a crossover instigator that requires RexAB [hot-spot activity in the vicinity of Chi (44), remains to be shown]. We hypothesize that a strategy of DNA degradation and repair by an exonuclease and a short sequence that modulates its activity is widespread among bacteria.

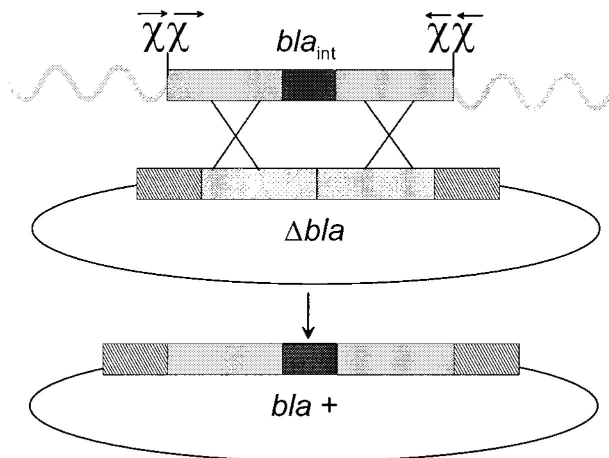

FIG. 4. Strategy to test Chi_{L1} effect on RexAB-mediated recombination. Linear transforming DNA contains an internal fragment of *bla* (*bla*$_{int}$, solid rectangle) and has an additional 360 bp flanking homology with *bla* (grey rectangle). Double Chi sites on the linear fragments, (either Chi_{L1} or Chi_{Ec}) oriented for recognition to enhance recombination, are represented by χχ. Wavy lines represent heterologous dsDNA tails. Plasmid pΔBla bears an internal deletion of *bla* (Δ*bla*), the target for gene conversion. Hatched rectangles represent ends of the *bla* gene not present on linear DNA. Double crossover homologous recombination would be required to convert cells to Amp^r (*bla*$^+$).

Table 5. Chi_{Ll} stimulates RexAB-mediated recombination

E. coli strain (plasmids)	Transformation efficiency pACYC184 (1 μg)	No. Amp^r transformants* (No. tfo) Chi_{Ll}‡	Chi_{Ec}‡	Chi_{Ll} stimulation†
recB21C22 (pΔB1a, pRexAB)	3×10^9	650 (7)	22 (7)	27†
recB21C22 (pΔB1a)	8×10^9	8 (2)	7 (2)	1
Wt (pGB2:P_{Ara}*gam*, pΔB1a)	1.5×10^{10}	615 (4)	567 (4)	1†

*Values represent total number of transformants from multiple experiments (number in parentheses).
†Chi_{Ll} stimulation is the ratio of Amp^r transformants obtained with linear DNA fragments containing Chi_{Ll} compared with Chi_{Ec}. The presented ratio was corrected by taking into account transformation efficiencies in the strain depleted for Chi activity (TG1 containing pGB2:P_{Ara}*gam* and pΔB1a).
‡Chi_{Ll} and Chi_{Ec} correspond to double Chi sites present at ends of linear fragments used for gene conversion.

DISCUSSION

Conservation of Exonuclease–Chi Interactions in Bacteria. Demonstration in *E. coli* of exonuclease–Chi activities have led to the assumption that a strategy for recombination and DNA repair involving modulation of exonucleolytic activity by specific DNA sequences is ubiquitous among bacteria and possibly in higher organisms (45, 46). Our demonstration of exonuclease–Chi interactions in *L. lactis*, a Gram-positive organism distant from *E. coli*, provides support for these assumptions for bacteria, despite little conservation of enzyme sequences. Furthermore, demonstration of RexAB-Chi_{Ll}-mediated DNA protection and recombination in a heterologous host suggests that these processes do not require other host-specific factors.

The biological test based on RC plasmids we developed to analyze the exonuclease and Chi in *L. lactis* appears to be

specific for these two elements. It thus constitutes a simple test that could be used to study analogs in other organisms in which RC plasmids replicate (14).

Extent of Exonuclease Conservation. The functional conservation of exonuclease–Chi interactions is surprising in view of the structural divergence observed between the known enzymes. Three-subunit exonucleases like RecBCD are present in *E. coli* and other Gram-negative bacteria and, curiously, in Gram-positive bacterium *M. tuberculosis*, whereas two-subunit exonucleases are present in *L. lactis*, *B. subtilis*, and possibly in *Listeria monocytogenes*. Significant homologies are found in just one subunit, corresponding to RecB, AddA, or RexA, which contains helicase motifs, an ATP-binding site, and two C-terminal end motifs (10, 13). Experiments with AddAB suggest that these motifs are generally required for exonuclease activity (13). In contrast, the RexB subunit is only 23% identical to AddB and bears no apparent similarity with the three-subunit enzyme (compared with 61% identity between *L. lactis* and *B. subtilis* RecA proteins; ref. 47). Interestingly, BLAST analyses reveal 28 and 23% identity over 100- and 200-aa stretches, respectively, between RexA and human RAD50, a protein involved in repair of dsDNA breaks in eukaryotes (48).

RexAB, unlike the three other sequenced exonucleases, has one (rather than two) putative ATP-binding site, in RexA, which is presumably the equivalent of the ATP-binding site in RecB and AddA. Mutational analyses of both RecBCD and AddAB indicate that the common ATP-binding site is critical for activities (49, 50), whereas the second ATP-binding site, which is present in RecD and AddB (10), but not in RexB, had little and no effect, respectively, on enzyme activities (12, 51). The lack of the second ATP-binding site in RexAB argues that it is not essential for activity.

RexAB-Chi_{Ll} Interactions in DNA Repair. Our results show that RexAB and Chi_{Ll} conserve several biological properties originally found for RecBCD and Chi_{Ec}. Chi_{Ll} modulates RexAB-mediated DNA degradation. It is also a "crossover instigator" of RexAB-mediated homologous recombination. However, our test did not prove that Chi_{Ll} is a recombination "hot spot," which originally characterized Chi_{Ec} in bacteriophage λ crosses (44). Experiments by Stahl and coworkers with λ showed that $RecBC(D^-)$-mediated crossover events are localized at the phage extremity (52), suggesting that the hot spot of RecBCD-mediated recombination is the point at which linear dsDNA is no longer degraded. Although it remains to be proven, exonuclease attenuation and recombination–stimulation, demonstrated here for the RexAB-Chi_{Ll} couple, may be sufficient to produce the hot-spot effect of Chi.

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