The Recombination Genes *addAB* Are Not Restricted to Gram-Positive Bacteria: Genetic Analysis of the Recombination Initiation Enzymes RecF and AddAB in *Rhizobium etli*

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Received 8 June 2004/Accepted 1 September 2004

Single-strand gaps (SSGs) and double-strand breaks (DSBs) are the major initiation sites for recombination. In bacteria, the SSGs are repaired by RecFOR, while the DSBs are processed by RecBCD in gram-negative bacteria and AddAB in gram-positive bacteria. Unexpectedly, instead of *recBCD* genes, the *addAB* genes were found in members of the α -proteobacteria group (gram negative). Taking *Rhizobium etli* as a model, the role of *recF* and *addAB* genes in homologous recombination and repair of damaged DNA was evaluated. Inactivation of either *recF* or *addA* provoked strong sensitivity to UV radiation and mitomycin C, while an additive effect was observed in the *recF-addA* mutant. The DSBs generated by nalidixic acid caused low viability only in the *addA* mutant. The recombination frequency of large and small plasmids was reduced in the *recF* mutant (24- and 36-fold, respectively), whereas a slight decrease (threefold) in the *addA* mutant was observed. Moreover, an additive effect (47- and 90-fold, respectively) was observed in the double mutant, but it was not as dramatic as that in a *recA* mutant. Interestingly, the frequency of deletion and Campbell-type recombination was slightly affected in either single or double mutants. These results suggest that another pathway exists that allows plasmid and Campbell-type recombination in the absence of *recF* and *addA* genes.

Homologous recombination is a key process involved in the repair of damaged DNA, the appropriate segregation of chromosomes, and the generation of genetic diversity (11, 29). The genetic and biochemical studies in prokaryotes and eukaryotes have allowed the division of the process of DNA recombination into four successive steps, initiation, homologous pairing, branch migration, and resolution (29).

In the gram-negative bacterium *Escherichia coli*, about 20 gene products are implicated. In the initiation step, several protein complexes (RecBCD, RecFOR, RecJ, RecQ, RecN, etc.) act alternatively to generate the crucial 3' single-stranded DNA (ssDNA) end. This end is required for the RecA-dependent pairing between homologous sequences, producing a four-strand intermediate known as a Holliday junction. The Holliday junction is processed by either the RuvAB complex (causing branch migration) or the RecG protein (favoring resection of the Holliday intermediate). Finally, RuvC resolves the RuvAB-cruciform complex specifically (29). Alternatively, replication enzymes (DNA polymerase III and primosome proteins) are recruited to resolve recombinants (30, 44). Alternative use of different protein complexes in the initiation step has revealed the existence of the RecBCD, RecFOR, and RecE recombination pathways (29). The first two pathways are widely distributed in *E. coli*, while the last pathway (encoded on a cryptic prophage) is restricted to some K-12 strains. Additionally, the RecBCD pathway is involved in the majority of conjugational recombination and in repairing double-stranded DNA breaks (DSBs) in wild-type bacteria. In contrast, the

RecFOR pathway is implicated in plasmid recombination as well as in repairing single-stranded DNA gaps (SSGs) (30, 31).

A similar number of recombination genes, classified into five epistatic groups (α , β , γ , ϵ , and ζ), have been described in mutational studies of the gram-positive bacterium *Bacillus subtilis*. The α and β groups have functional counterparts in *E. coli* corresponding to the RecFOR and RecBCD pathways, respectively (1, 2, 17).

In bacteria, the 3' ssDNA ends required for recombination are principally generated by the processing of SSGs and DSBs. These lesions are generated as a by-product of normal DNA metabolism (replication, transcription, etc.); their occurrence can be increased by DNA-damaging agents and other environmental conditions (11, 29, 31). Upon encountering an SSG, the RecFOR complex binds the 5' terminus at the ssDNA-double-stranded DNA junction of the SSG, allowing the loading of the RecA protein onto ssDNA; alternatively, the helicase-exonuclease proteins (RecQ and RecJ) can process this structure to generate the 3' ssDNA end required to recombinationally repair the SSGs (5, 43, 61, 63, 64). These genes are widely distributed in eubacteria (13).

In *E. coli*, the helicase-exonuclease activity of the RecBCD complex is needed to generate 3' ssDNA ends at the DSBs in a process modulated by the RecD subunit in a sequence-dependent manner (45). The heterodimeric *B. subtilis* AddAB enzyme is the functional equivalent of RecBCD (26, 42). The distribution of *recBCD/addAB* genes is believed to be group specific; previous evaluations identified *recBCD* homologs in gram-negative bacteria, while the *addAB* genes are thought to be restricted to gram-positive bacteria (9).

Our interest in the study of recombination in the gram-negative α -proteobacterium *Rhizobium etli* is motivated by the fluidity displayed by the rhizobial genomes, where many rear-

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
<i>R. etli</i>		
CFN42	Wild type, Nal ^r	50
CE3	CFN42 derivative, Str ^r	50
CFNX101	CE3 derivative, <i>recA::Sp</i>	39
CFNX693	CE3 derivative, <i>recF::loxP</i> Sp	This work
CFNX694	CE3 derivative, <i>addA::loxP</i> Sp	This work
CFNX695	CE3 derivative with a deletion-substitution of <i>addAaddB</i> by $\Delta addAB::loxP$ Sp	This work
CFNX696	CFNX693 derivative, but <i>recF::loxP</i>	This work
CFNX697	CFNX696 derivative, but <i>addA::loxP</i> Sp	This work
<i>E. coli</i>		
DH5 α	<i>recA1</i>	Stratagene
S17.1	<i>recA13</i> , mobilization host	56
Plasmid		
pBluescript II SK(+)	Cb ^r , cloning vector	Stratagene
pSUP202	Cm ^r Tc ^r Cb ^r , suicide vector	56
pKOK4	Cm ^r Tc ^r Cb ^r , suicide vector	23
pJQ200SK+	Gm ^r <i>sacB</i> , suicide vector	47
pBX404-7	Tc ^r 5' del <i>neo/3'</i> del <i>neo</i> Km ^s plasmid recombination system	65
pBBMCS5	Gm ^r , broad-host-range vector	28
pJMS2	Sp ^r , source of <i>loxP</i> Sp cassette	40
pJMS08	vector containing the <i>loxP</i> site-specific recombinase Cre	Martínez-Salazar et al., unpublished
pLGsym56	pSUP202 derivative harboring a 6.0-kb BamHI fragment from <i>R. etli</i> pSym	19
pLG506	pSUP202 derivative containing 2.0-kb HindIII Ω Km and MCS from pBluescript SK(+) into BglIII	Girard et al., unpublished
pRK2013	Km ^r , helper vector	56
c1343	pLAFR1 cosmid harboring <i>R. etli</i> <i>recF</i> region	This work
c2486	pLAFR1 cosmid harboring <i>R. etli</i> <i>addAB</i> region	This work
pJZC1	pKOK4 derivative harboring the 3.0-kb EcoRI fragment of <i>R. etli</i> <i>recF</i> gene	This work
pJZC2	pJZC1 derivative containing the <i>R. etli</i> <i>recF::loxP</i> Sp allele	This work
pJZC3	pSK derivative harboring a 6-kb EcoRI fragment of <i>R. etli</i> <i>addA</i> gene	This work
pJZC4	pSK derivative harboring a 1.5-kb EcoRI PCR product with 225 bp upstream and 1,188 bp of <i>R. etli</i> <i>addA</i> gene	This work
pJZC5	pJZC4 derivative harboring the <i>R. etli</i> <i>addA::loxP</i> Sp allele	This work
pJZC6	pJQ200SK derivative harboring the 3.5-kb EcoRI <i>addA::loxP</i> Sp from pJZC5 into MCS	This work
pJZC7	pJQ200SK derivative containing the 3.3-kb EcoRI-SalI 5' del <i>neo/3'</i> del <i>neo</i> Km ^s from pBX404-7 into MCS	This work
pJZC8	pJZC7 derivative containing the 6.0-kb BamHI fragment from pLGsym56 into MCS	This work
pJZC9	pBBMCS5 derivative harboring the <i>addA</i> gene in the same orientation to <i>lacZ</i> promoter	This work
pJZC10	pBBMCS5 derivative harboring the <i>addA</i> gene in the opposite orientation to <i>lacZ</i> promoter	This work
pJZC11	pBBMCS5 derivative harboring the <i>addAB</i> genes in the same orientation to <i>lacZ</i> promoter	This work
pJZC12	pSK derivative containing the 1.2-kb SalI from pJZC3, corresponding to 585 bp of 3' end of <i>addA</i> and 672 bp downstream (<i>trxA</i> and <i>folC</i>)	This work
pJZC13	pJZC12 derivative harboring the 2.0-kb SalI <i>loxP</i> Sp into the SalI site located at aa 988 of <i>addA</i>	This work
pJZC14	pSK derivative harboring the 1.2-kb EcoRI-XhoI, corresponding to aa 63 to 469 of <i>addB</i>	This work
pJZC15	pJZC13 derivative harboring 1.2-kb EcoRI-XhoI from pJZC14 into EcoRI-SalI, $\Delta addAB::loxP$ Sp	This work
pJZC16	pLG506 derivative harboring the 4.4-kb EcoRI-XhoI from pJZC15 ($\Delta addAB::loxP$ Sp)	This work

rangements can occur by homologous recombination between repeated sequences present in the entire genome; these rearrangements may affect the symbiotic interaction of *Rhizobium* with leguminous plants (39–41, 51–54, 62). Therefore, the identification of genes that participate in the initial steps of recombination is particularly important. Although clear *recF* homologs were identified during this study, no convincing *recBCD* homologs were found. Surprisingly, BLAST searches in which the *addAB* sequences from *B. subtilis* or the *rexAB* sequences of *Lactococcus lactis* were used as queries revealed the presence of *addAB* homologs in several species of the α -proteobacteria group, including *R. etli*. In this work, we characterize the role of both *recF* and *addA* in the repair of damaged DNA and in the recombination of small and large plasmids in *R. etli*. Surprisingly, the inactivation of these two *R. etli*

recombination pathways did not lead to a complete loss of recombination capacity, although repair of damaged DNA was seriously affected. This finding may indicate that *R. etli* possess additional recombination activities such as helicases or prophage recombinases.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. *R. etli* strains were grown in PY medium supplemented with CaCl₂ (6) at 30°C. *E. coli* strains were grown in Luria-Bertani medium (55) at 37°C. Antibiotics were used at the following concentrations: nalidixic acid, 20 to 150 μ g ml⁻¹; spectinomycin, 100 μ g ml⁻¹; kanamycin, 30 μ g ml⁻¹; gentamicin, 5 μ g ml⁻¹; and tetracycline, 5 μ g ml⁻¹. Mitomycin C (Sigma-Aldrich) was used at concentrations ranging from 0.5 to 1 μ g ml⁻¹. UV treatment was carried out by using the UVC500 cross-linker (Hoefer Scientific Instruments). After DNA damage treatment (mitomycin C, UV radiation, or nalidixic acid), the cells were incubated at 30°C in the dark. β -Galactosidase-

positive strains were identified by growth on plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). For positive selections involving the *sacB* gene, sucrose-resistant strains were selected in presence of 12.5% (vol/vol) sucrose.

Recombinant DNA procedures. Restriction enzymes, alkaline phosphatase, and T4 DNA ligase were purchased from Amersham Biosciences or Invitrogen Life Technologies and were used as recommended by the manufacturer. Plasmid DNA was isolated by an alkaline-sodium dodecyl sulfate lysis method and transformed into *CaCl*₂-treated *E. coli* cells; other general DNA methods were done according to standard protocols (55). The transfer of plasmids from *E. coli* to *R. etli* was done by biparental or triparental matings with S17.1 or pRK2013 plasmid as a helper. Chromosomal DNA from *R. etli* and Southern blot hybridization were carried out as previously described (40). Probes were labeled with [α -³²P] dCTP by using a Rediprime II kit (Amersham Biosciences).

The PCRs were carried out with *Taq* DNA polymerase (Invitrogen Life Technologies) as follows: 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles, with an extension step at 72°C for 10 min. For long PCR products (3 to 7 kb), the reactions were carried out with a PCR-XL kit (rTth polymerase; Perkin-Elmer) with the following cycling regime: 15 cycles of 92°C for 30 s, 60°C for 1 min, and 68°C for 10 min and 16 cycles of 92°C for 30 s, 60°C for 1 min, and 72°C for 10 min.

The oligonucleotides used to obtain the PCR products were the sense FrF1 (5'-GGC GAG AAC GGT GCG GGC AA-3') and antisense RrF3 (5'-GTC GAG AAA GCG CCT GCG GT-3') (corresponding to amino acids 33 to 40 and 137 to 144, respectively) of *Sinorhizobium meliloti* *recF* and the sense ReBUSLA (5'-GCG AGG ACG ACG ATG AGG-3') and antisense ReBLSLA (5'-TCG AGC GGG AAC TGG TGC-3') (corresponding to 12 bp upstream to aa 2 and 143 to 149, respectively) of the *S. meliloti* *addA* (SMc02760) gene. The primers used to obtain PCR products of the *R. etli* *addAB* region were the sense JadKp1 (5'-GCG GGT ACC GAA GCG GAG GAA ACA ATC-3') (corresponding to 30 to 56 bp upstream of *R. etli* *addB*), the antisense JaddAE (5'-TCA GCG AAT TCG CGG TGC GGG TGA TCA GG-3') (corresponding to aa 389 to 396 of the *addA* gene), and the antisense JadSp1a (5'-TGA GAC TAG TTT TCA CGG TAG CCA TAG G-3') (corresponding to 76 to 96 bp downstream of the *R. etli* *addA* gene). The KpnI, EcoRI, and SpeI sites introduced are underlined. The sequencing primers were synthesized by Unidad de Síntesis, Instituto de Biotecnología, Universidad Nacional Autónoma de México.

Nucleotide sequence. The *R. etli* cosmids c1343 and c2486, containing the *recF* and *addAaddB* genes respectively, were subcloned into pBluescript II SK(+) and sequenced by the Sanger dideoxy chain termination method using a Thermosequenase cycle sequencing kit (Amersham Biosciences). Primers for sequencing were 5' end labeled with [γ -³²P]dATP and T4 DNA kinase. For automated sequencing, a DYEnamic ET dye terminator cycle sequencing kit (Amersham Biosciences) and the ABI-prism 310 (Applied Biosystems) were used.

Sequence analysis. BLAST searches were performed with BLASTX, BLASTP, and PSI-BLAST programs at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). Multiple protein alignment analyses were carried out with the ClustalW program (60), and the phylogenetic relationships were obtained by using the PHYLIP program (16) and visualized with TreeView software (46).

Plasmid construction. pJZC1 is a pKOK4 derivative (23) containing the 3.0-kb EcoRI fragment from c1343. This vector is mobilizable by conjugation but unable to replicate in *R. etli*. pJZC2 is a pJZC1 derivative where the *recF* gene was interrupted by insertion of the 2.0-kb SalI *loxP*Sp interposon from pJMS2 into the SalI site located at codon 240 (40). pJZC3 is a pBluescript II SK(+) derivative harboring the 6.0-kb *R. etli* *addA* gene-containing EcoRI fragment from c2486. pJZC4 is a pBluescript II SK(+) derivative containing a 1.5-kb EcoRI PCR product using the JaddAE and -20 primers from pJZC3 (having the 225 bp upstream and 1,188 bp of the coding *addA* gene). pJZC5 is a pJZC4 derivative with the 2.0-kb BamHI *loxP*Sp interposon inserted into the BglII site located at codon 95 of *addA*. pJZC6 is a pJQ200SK+ (47) derivative carrying the 3.5-kb EcoRI fragment from pJZC5 (*addA::loxP*Sp) in the multiple cloning site (MCS). pJZC7 is a pJQ200SK+ derivative containing the 3.3-kb EcoRI-SalI fragment from pBX404-7 (65) (contains the truncated but overlapping version of the *kan/neo* gene) in the MCS. pJZC8 is a pJZC7 derivative containing the 6.0-kb BamHI fragment from pLGsym56 (fragment of *R. etli* pSym) (19) in the MCS. This suicide and mobilizable plasmid allows measurement of Campbell-type recombination with pSym. After its integration, large-plasmid recombination with pSym (385 kb) was evaluated by restoration of the kanamycin gene or deletions that confer sucrose resistance and gentamicin sensitivity.

The 6-kb EcoRI fragment from pJZC3 was cloned into pBBRMCS5 in both orientations to place the *addA* gene in the same direction (pJZC9) or in the opposite direction (pJZC10) with respect to the *lacZ* promoter.

Primers JadKp1 and JadSp1a were used to obtain a 6-kb PCR product from the *R. etli* genome (containing the 76 bp upstream of *addB* and the entire *addB*

and *addA* genes) that was cloned into pCR2.1-TOPO (Invitrogen Life Technologies) to create pTOPO::*addAB*. Plasmid pJZC11 is a pBBRMCS5 derivative containing the 6-kb SpeI fragment from pTOPO::*addAB* with both genes in the same direction as the *lacZ* promoter. Plasmid pJZC12 is a pBluescript II SK(+) derivative containing a 1.2-kb SalI fragment from pJZC3 comprising 585 bp of the 3' end of the *addA* gene (aa 988 to 1183) and 672 bp downstream (corresponding *trxA* and *folC* genes). pJZC13 is a pJZC12 derivative with the 2.0-kb SalI *loxP*Sp interposon from pJMS2 cloned into the SalI site located at codon 988 of the *addA* gene. Plasmid pJZC14 is a pBluescript II SK(+) derivative containing the *addB* 5' end (corresponding to aa 63 to 469) in a 1.2-kb EcoRI-XhoI fragment. The pJZC14 1.2-kb EcoRI-XhoI fragment was inserted between the EcoRI and SalI sites of pJZC13 to generate pJZC15. The resulting plasmid has the deletion-substitution of the *addAB* genes by the *loxP*Sp interposon (*ΔaddAB::loxP*Sp) with a 2.5-kb deleted region comprising codon 406 of AddB (C terminus) to codon 195 of AddA (N terminus). Plasmid pJZC16 is a suicide plasmid derivative of pLG506 (M. L. Girard et al., unpublished data) harboring the 4.4-kb EcoRI-XhoI fragment from pJZC15 (*ΔaddAB::loxP*Sp).

Construction of *recF*, *addA*, and *recF-addA* mutants. The *R. etli* *recF* (CFNX693), *addA* (CFNX694), and deletion-substitution *ΔaddAB* (CFNX695) mutants were obtained by homologous gene replacement of the wild-type allele with the *recF::loxP*Sp (pJZC2), *addA::loxP*Sp (pJZC6), and *ΔaddAB::loxP*Sp (pJZC16) alleles, respectively. To that end, the corresponding plasmid was transferred by triparental mating from *E. coli* to *R. etli* CE3, and the double recombinants were selected on the basis of their respective phenotypes. The double mutant *recF::loxP-addA::loxP*Sp (CFNX697) was generated in two steps. First, the *loxP*Sp interposon in the CFNX693 strain was deleted by using the *loxP*-specific Cre recombinase expressed from pJMS08 (J. M. Martínez-Salazar et al., unpublished data). The *loxP*Sp deletion and pJMS8 curing plasmid strain was selected by screening for the Sp^r Tc^r phenotype. In a second step, the *addA::loxP*Sp allele was introduced by marker exchange in the CFNX696 (*recF::loxP*) strain, and the double mutant was selected by the Sp^r Gm^r Sac^r phenotype. All mutants were verified by Southern blot hybridization using the corresponding intermediate plasmids as probes.

Integrative recombination assay. Campbell-type recombination was evaluated in proficient and mutant strains of *R. etli* by using the conjugative, nonreplicative pJZC8 plasmid (14.3 kb). This plasmid has a 6-kb region homologous to the symbiotic plasmid (371 kb), which generates pSym::pJZC8 after integration.

Small-plasmid recombination determination. The small, broad-host-range vector pBX404-7 (13 kb) (65) was introduced into wild-type and mutant strains by triparental matings using pRK2013 as a helper. pBX404-7 contains two truncated but overlapping copies of the *kan/neo* resistance gene. Plasmid recombination (intermolecular and unequal sister exchange) frequency was measured by the restoration of the kanamycin resistance gene as described previously (40, 65).

Large-plasmid recombination assays. To evaluate recombination on a large plasmid, pSym::pJZC8 was obtained by integration of pJZC8 into pSym in wild-type and mutant strains (see above). The strains were verified for sensitivity to DNA-damaging agents and normal plasmid profile by the Eckhardt method (6).

Nucleotide sequence accession numbers. GenBank accession numbers for the nucleotide sequences of *recF* and *addAB* regions are AY552333 and AY552334, respectively.

RESULTS

Isolation of *R. etli* *recF* and *addAB* genes. A bioinformatic approach was used to identify crucial genes involved in the initiation of recombination in the gram-negative α -proteobacteria group (*Agrobacterium tumefaciens*, *Bradyrhizobium japonicum*, *Brucella melitensis*, *Brucella suis*, *Mesorhizobium loti*, *Rickettsia prowazekii*, and *S. meliloti*). To that end, BLAST searches were performed by using the *E. coli* *recF*, *recO*, *recR*, *recB*, and *recC* genes; the *B. subtilis* *addA* and *addB* genes; and the *L. lactis* *rexA* and *rexB* genes as queries.

The analysis revealed that the *recF*, *recO*, and *recR* genes were present in this group of bacteria as well as in other reported bacterial genomes (13). Unexpectedly, *recB* and *recC* were absent in members of the α -proteobacterial group sequenced to date; instead, homologs of the *addA* and *addB* genes were found (see below). In addition, the *addA* and *addB* genes were found in another gram-negative bacterium (*Ralstonia solanacearum*, a β -proteobacterium). The AddAB and RecBCD enzymes

FIG. 1. Phylogenetic relationships of the AddA and RecB subunits of the helicase-exonuclease type V protein family. A. The neighbor-joining cladogram was constructed with PHYLYP by using a CLUSTALW multiple-protein alignment of gram-negative bacteria [Gram (-)] RecB and gram-positive bacteria [Gram (+)] AddA sequences. The corresponding bootstrap values (1,000 replications) are shown at the edge of each clade. The GenBank accession numbers corresponding to each species are included in parentheses. Abbreviations for gram-negative bacteria: *Mtub*, *Mycobacterium tuberculosis* (accession number CAB07119); *Xfast*, *Xylella fastidiosa* (accession number AAF83233); *Nmeni*, *Neisseria meningitidis* (accession number AAF41198); *Paeru*, *Pseudomonas aeruginosa* (accession number AAG076672); *Vchlo*, *Vibrio cholerae* (accession number AAF95464); *Ypest*, *Yersinia pestis* (accession number CAC89863); *Ecoli*, *E. coli* (accession number AAC75859); *Stypb*, *Salmonella enterica* serovar Typhi (accession number CAD02818); *Stypm*, *Salmonella enterica* serovar Typhimurium (accession number AAL21870); *Hinfli*, *H. influenzae* (accession number ACC22966); *Pmult*, *Pasteurella multocida* (accession number AAK02600); *Buchn*, *Buchnera* sp. (accession number AP001119); *Bburg*, *Borrelia burgdorferi* (accession number AAC66981); *Ccres*, *Caulobacter crescentus* (accession number AAK25500); *Mloti*, *Mezorhizobium loti* (accession number BAB51593); *Bmeli*, *Brucella melitensis* (accession number AAL53204); *Bsuis*, *Brucella suis* (accession number ANN30993); *Smeli*, *Sinorhizobium meliloti* (accession number CAC41421); *Atume*, *Agrobacterium tumefaciens* (accession number AAK85848); *Retli*, *R. etli* (accession number AY552334); *Rsola*, *Ralstonia solanacearum* (accession number CAD14892). Abbreviations for gram-positive bacteria: *Saure*, *Staphylococcus aureus* (accession number BAB42067); *Bsubt*, *B. subtilis* (accession number AAA22200); *Linno*, *Listeria innocua* (accession number CAC97575); *Lmono*, *Listeria monocytogenes* (accession number EAL05437); *Caceti*, *Clostridium acetobutylicum* (accession number AAK80219); *Cperfi*, *Clostridium perfringens* (accession number BAB79727); *Llaet*, *L. lactis* (accession number AAK04102); *Spneu*, *Streptococcus pneumoniae* (accession number AAK99844); *Spyog*, *Streptococcus pyogenes* (accession number AAK33717). B. Conserved domains among AddA homologs of α -proteobacteria, AddA of gram-positive bacteria and RecB of *E. coli* (25, 27, 48). The asterisks indicate conserved amino acids, and colons indicate conservative replacements. C. Alignment of the nuclease motif present at the C termini of AddB homologs of α -proteobacteria and gram-positive bacteria proteins (48). The asterisks indicate conserved amino acids, and colons indicate conservative replacements.

(composed of two and three subunits, respectively) are both members of the helicase-exonuclease type V protein family and fulfill similar roles in the initiation of homologous recombination. Previous studies suggested that the *recBCD* genes were present only in gram-negative species, while *addAB* genes appear to be restricted to gram-positive bacteria (9).

To study the initiation of recombination in our *R. etli* CE3 strain model system, the *recF* and *addA* genes were isolated. An *R. etli* cosmid library (constructed with EcoRI fragments in pLAFR1) (40) was screened by Southern blot hybridization using PCR products corresponding to the *recF* and *addA* (SMc02760) genes from the related bacterium *S. meliloti* as

FIG. 2. Sensitivity of *R. etli* mutants to mitomycin C (A), UV radiation (B), and nalidixic acid (C). Data are the averages of three independent experiments. The surviving fraction is the number of viable cells after treatment with DNA-damaging agents divided by the number of viable cells without treatment. Symbols: CE3 (wild type), $\circ$; CFNX693 (*recF::loxP*Sp), $\square$; CFNX694 (*addA::loxP*Sp), $\bullet$; CFNX697 (*recF::loxP-addA::loxP*Sp), ∇ ; CFNX101 (*recA::Sp*), $\blacktriangledown$.

probes. The cosmids c1343 (*recF*) and c2486 (*addAB*) were subcloned, and regions containing the genes were sequenced.

Conserved functional motifs in α -proteobacterial AddA proteins. To gain insight into the identity of *R. etli* AddA and other homologs in α -proteobacteria, the corresponding amino acid sequences were compared with the reported AddA and RecB proteins. Figure 1A shows the phylogenetic relationships for RecB and AddA proteins in bacteria. Interestingly, the AddA homologs in α -proteobacterial species are more similar to those found in gram-positive species than to RecB genes of gram-negative bacteria. Moreover, the seven conserved helicase domains (25, 27) and the nuclease motif (48) of the protein family described previously are present (Fig. 1B). Convincing homologs of the *addB* genes are present upstream of all the α -proteobacterial *addA* genes. This organization mirrors the one found for *addAB* genes in the gram-positive group. The proteins encoded by these putative *addB* genes also contain a nuclease motif (Fig. 1C) characteristic of members of this family. Furthermore, the α -proteobacterial *addB* genes display a phylogenetic distribution fully congruent with that of the *addA* genes (data not shown). Despite the existence of a similar organization and high similarity of the α -proteobacterial *addAB* genes with members of the gram-positive clade, sequence features of the α -proteobacterial genes, such as codon usage and GC content, are consistent with the consensus found for each species (data not shown). These data do not support a recent horizontal transfer event from gram-positive bacteria as the origin of the α -proteobacterial homologs.

Conversely and in concordance with the distribution previously reported (13), the phylogenetic distribution for the α -proteobacterial *recF* homologs is similar to that reported for *recF* genes of gram-negative bacteria (data not shown).

Inactivation of *addA* and *recF* genes. To determine the biological role of AddA and RecF proteins in α -proteobacteria, the corresponding *R. etli* CE3 genes were inactivated. Mutants harboring the *recF::loxP*Sp (CFNX693), the *addA::loxP*Sp (CFNX694), or a deletion-substitution Δ *addAB::loxP*Sp (CFNX695) allele were generated by homologous gene re-

placement (see Materials and Methods). Additionally, inactivation of both recombination pathways was achieved by sequential introduction of mutations into both genes. To that end, the *loxP*Sp interposon was excised from the strain CFNX693 (*recF::loxP*Sp) by using the specific Cre recombinase. These excisants are still inactivated in the *recF* gene due to the retention of a 181-bp insertion. The introduction of the *addA::loxP*Sp allele in these excisants by marker exchange gave rise to the *recF::loxP-addA::loxP*Sp (CFNX697) mutant (see Materials and Methods). The inactivation of the *addA* gene showed a dramatic reduction in cell viability (20 to 30% of the wild type), while the effect was slight (80 to 90% of the wild type) in *recF* mutants.

The AddA and RecF proteins are required for repair of damaged DNA. It is well known that recombination proteins play an active role in the recombinational repair of damaged DNA (11, 31). To study the roles of *addA* and *recF* in DNA repair, the corresponding *R. etli* mutants were evaluated for sensitivity towards several mutagenic agents. Mutants in either the *recF* or *addA* gene displayed a marked hypersensitivity to both mitomycin C and UV radiation (Fig. 2A and B), agents that may generate SSGs and DSBs (31). Interestingly, the sensitivity of the double mutant *recF::loxP-addA::loxP*Sp was increased in an additive way (Fig. 2A and B), suggesting a joint participation of both systems in DNA repair.

On the other hand, while inactivation of the *addA* gene led to increased sensitivity to nalidixic acid, the *recF* mutant remained as resistant as the wild-type strain towards this antibiotic (Fig. 2C). Strains harboring the simultaneous inactivation of both *recF* and *addA* (*recF::loxP-addA::loxP*Sp) or with a loss of both *addA* and *addB* (Δ *addAB::loxP*Sp) showed sensitivity to nalidixic acid similar to that of the *addA::loxP*Sp mutant (Fig. 2C and data not shown, respectively).

Complementation tests of the Δ *addAB::loxP*Sp mutant strain were performed by using vectors containing either the *addA* or *addAB* genes (see Materials and Methods). Ectopic expression of *addA* was sufficient to complement the *addA* mutant to wild-type levels with respect to nalidixic acid sensitivity and viability

FIG. 3. *R. etli* DSB repair is dependent on both *addA* and *addB* genes. The CFNX695 ($\Delta addAB::loxP$ Sp) mutant was complemented by the *addAB* genes located in plasmid. The surviving fraction is the number of viable cells divided by the number of total cells. Each point is the average of three independent experiments. The arrows indicate that the *addA* gene is in the same ($\rightarrow$) or in the opposite ($\leftarrow$) orientation to the *lacZ* promoter of the vector.

(Fig. 3 and data not shown). Full complementation of the $\Delta addAB$ strain requires simultaneous expression of both *addA* and *addB* (Fig. 3). It is known that nalidixic acid causes an increase in DSB formation (58). The strong sensitivity towards nalidixic acid observed by inactivation of *addAB* genes, coupled with the results of complementation experiments, suggests that this enzymatic complex has a prime role in DSB repair.

Recombination of both small and large plasmids is dependent on RecF and AddA. To gain further insight into the physiological relevance of RecF and AddA, we examined the effect of *R. etli* mutants on homologous recombination. To this end, reporter systems on plasmids were used to evaluate different recombination events. The recombination of small plasmids was evaluated with pBX404-7 (65), which harbors two unequally truncated and overlapping fragments of the *kan/neo* resistance gene, arranged in a direct orientation. A restoration of kanamycin resistance could be achieved by either intermolecular recombination of two plasmids or intramolecular unequal sister exchange recombination.

To evaluate if large plasmids have differential gene depen-

dence for recombination, pBX404-7 (see above) was modified (pJZC8) to allow integration into one of the large plasmids (pSym, 371 kb) of *R. etli*, as described in Materials and Methods.

A mutation in *recF* led to a marked reduction in recombination frequency for both small and large plasmids (36- and 24-fold, respectively [Table 2]); in contrast, inactivation of *addA* showed only a slight decrease (threefold) in recombination in both systems. Moreover, the *recF-addA* strain showed an additive effect on recombination frequency, with 90- and 47-fold decreases on small and large plasmids, respectively. Both events were more negatively affected by a *recA* mutation (100-fold for small plasmids and 117-fold for large plasmids [Table 2]).

These results led us to conclude the following: (i) RecF constitutes the major pathway for recombination of small plasmids in *R. etli*, as shown in other systems (10, 22, 32, 36, 37); (ii) although the AddA route plays a minor role in small-plasmid recombination, the near-additive effect observed in the double mutant indicates that the two pathways are required in this system; (iii) large- and small-plasmid recombinations are achieved in similar ways, processed principally by a RecF pathway with a minor contribution of the AddA pathway; and (iv) other activities may participate in large-plasmid recombination (as seen for a *recA* mutant) since it is not abolished in the double mutant.

Participation of RecF and AddA in deletions. To evaluate the deletion frequency in the *recF* (CFNX693), *addA* (CFNX694), and *recF-addA* (CFNX697) mutants, a cointegrate pSym::pJZC8 was selected for each mutant (data not shown). In these large plasmids (385 kb), the whole pJZC8 is flanked by two 6-kb direct repeats generated during cointegration. Unequal sister exchange recombination or intramolecular recombination between the 6-kb direct repeats led to the deletion of pJZC8, which was evaluated by scoring for sucrose-resistant and gentamicin-sensitive derivatives.

Inactivation of *recF*, *addA*, or both had a weaker effect on deletion recombination in large plasmids (two-, three-, and eightfold, respectively [Table 2]) compared to plasmidic recombination. Interestingly, the reduction in recombination frequency observed in a *recA* mutant was also more modest in this system (18-fold) than in the large plasmidic assay (117-fold).

TABLE 2. Recombination frequencies of *R. etli* strains^a

Strain	Recombination frequency			
	Campbell-type recombination ^b (10^{-4})	Small plasmidic recombination ^c (10^{-4})	Large plasmidic recombination ^d (10^{-5})	Deletion recombination ^e (10^{-4})
CE3 (wild type)	6.17 $\pm$ 0.91 (1.0)	4.71 $\pm$ 0.77 (1.0)	5.76 $\pm$ 1.05 (1.0)	5.57 $\pm$ 0.99 (1.0)
CFNX693 (<i>recF</i>)	2.05 $\pm$ 0.87 (0.33)	0.13 $\pm$ 0.007 (0.027)	0.24 $\pm$ 0.02 (0.042)	2.92 $\pm$ 1.06 (0.52)
CFNX694 (<i>addA</i>)	5.73 $\pm$ 1.00 (0.92)	1.64 $\pm$ 0.65 (0.34)	1.60 $\pm$ 0.82 (0.27)	1.86 $\pm$ 0.89 (0.33)
CFNX697 (<i>recFaddA</i>)	4.13 $\pm$ 0.48 (0.66)	0.05 $\pm$ 0.005 (0.011)	0.12 $\pm$ 0.00 (0.021)	0.69 $\pm$ 0.55 (0.12)
CFNX101 (<i>recA</i>)	0.0009 $\pm$ 0.0007 (0.0015)	0.02 $\pm$ 0.005 ^f (0.01)	0.049 $\pm$ 0.033 (0.0085)	0.30 $\pm$ 0.07 (0.053)

^a Data reported are the averages of four independent experiments plus the standard deviations. The numbers in parentheses correspond to frequencies relative to the wild type.

^b The Campbell-type recombination frequency is the number of gentamicin-resistant colonies divided by the total number of viable cells.

^c The small plasmidic recombination frequency is the number of kanamycin-resistant colonies divided by the number of tetracycline-resistant cells.

^d The large plasmidic recombination frequency is defined as the number of kanamycin-resistant colonies divided by the number of viable cells.

^e The deletion recombination frequency is the number of sucrose-resistant colonies divided by the total number of viable cells. The deletion was verified by gentamicin sensitivity.

^f Data previously reported (39).

These results indicate that for the substrate analyzed, *recF* and *addA* play a minor role in the generation of deletions. This finding raises the possibility of alternative recombination pathways for the processing of this substrate (see Discussion).

Effect of *recF* and *addAB* genes on Campbell-type recombination. The *recF* and *addA* weak effect observed on deletion recombination prompted us to evaluate other types of recombination. One of these is Campbell-type recombination leading to cointegration. The frequency of this event was scored by cointegration of pJZC8 into pSym using a 6-kb homologous region. The recombination frequency of this event was slightly decreased in the *recF* and *recF-addA* mutants (30 and 60%, respectively, with respect to wild type) but was not significantly affected in the *addA* mutant (Table 2). However, the Campbell-type integration was decreased by 666-fold by inactivation of the *recA* gene. These results suggest the existence of additional recombination pathways responsible for Campbell-type integration (see Discussion).

DISCUSSION

We employed a bioinformatic approach to identify crucial recombination genes in members of the gram-negative α -proteobacteria group. The study focused on genes implicated in the presynaptic formation of the 3' ssDNA ends at SSGs and DSBs, the major sites of initiation for recombination in bacteria. We found *recF*, *recO*, and *recR* genes in this group, as expected due to their ubiquitous distribution in other bacterial genomes (13). In a previous report, the distribution of *recBCD* genes was clustered in gram-negative bacteria, while *addAB* was found exclusively in gram-positive bacteria (9). However, *recB* and *recC* were absent in members of the α -proteobacteria, and *addA* and *addB* homologs were found (see Results). The α -proteobacterial homologs are part of a single operon, with a genetic organization similar to that observed in the gram-positive bacteria (14, 24). Moreover, both homologs are expressed in *R. etli* from an upstream promoter of the *galF*-like gene, which encodes a nucleotidyl transferase not involved in the repair-recombination process (unpublished data). In addition, the GC content and codon usage analysis do not indicate a recent lateral transfer event from gram-positive bacteria to α -proteobacteria (data not shown).

Although RecBCD and AddAB/RexAB perform a similar role, several differences are observed: (i) RecBCD is constituted by three subunits, while AddAB/RexAB have two; (ii) although the identity between AddA and RecB is low, both share conserved regions corresponding to helicase and nuclease motifs (25, 27, 48) (Fig. 1B); (iii) only one nuclease motif is present in the RecB subunit of RecBCD, while AddAB have two active nuclease motifs, one in each subunit (48) (Fig. 1C); and (iv) RecBCD has two helicase activities performed by the RecB and RecD subunits, while in AddAB, only AddA has helicase activity (12, 20, 27, 49, 59). Based on these features, the homologs found in the α -proteobacteria group (including *R. etli*) and in *Ralstonia solanacearum* (β -proteobacterium) correspond to AddAB. These data indicate that the AddAB pathway is distributed in both gram-positive and gram-negative bacteria.

To evaluate the role of the RecF and AddA proteins in α -proteobacteria, the corresponding genes were interrupted in

R. etli CE3. The single *recF* and *addA* mutants showed a hypersensitivity to both mitomycin C and UV radiation, both inducers of SSGs and DSBs (31), while the *recF-addA* double mutant showed an additive effect to these agents (Fig. 2A and B, respectively). Furthermore, only *addA* and *addAB* mutants were markedly sensitive to nalidixic acid (DSB inducer) (Fig. 2C and 3). Moreover, ectopic expression of both *addA* and *addB* in the $\Delta addAB::loxP$ Sp mutant background was needed to repair DSBs. The results observed for *R. etli* are in agreement with those reported for other bacteria; in both *E. coli* (gram negative) and *B. subtilis* (gram positive), the inactivation of *recB* or *addA* genes produces a marked decrease in the viability and hypersensitivity to DSB inducers (i.e., nalidixic acid and γ radiation), while the viability of the *recF* mutant is slightly affected but a strong sensitivity to SSGs inducers is observed (UV radiation and mitomycin C). Finally, a double knockout in either *recF-recB* (*E. coli*) or *recF-addA* (*B. subtilis*) shows a synergistic effect to DNA-damaging agents (1, 17, 29, 31, 42, 43).

To elucidate the role of RecF and AddA, several homologous recombination events were evaluated in *recF*, *addA*, and *recF-addA* genetic backgrounds. The 36-fold reduction of small-plasmid recombination observed in *R. etli* clearly established its dependence on *recF*, while the inactivation of *addA* caused only a slight effect (threefold). In the double mutant, an additive effect was observed (90-fold), but it was not as dramatic as that in the *recA* mutant. The results are in agreement with the RecBCD independence of small plasmids lacking chi sequences in *E. coli* (10, 22, 32, 37). However, plasmid recombination shows RecBCD dependence in an *sbcB* background if a chi sequence is present (66). Furthermore, by using plasmids lacking a chi sequence, the recombination rate in the *recB-recF* mutant is similar to that observed in the *recF* mutant (10, 22, 32, 37), or an enhanced activity is observed in the wild-type or *recF* backgrounds after *recB* inactivation (36).

Recombination pathways for large plasmids (>100 kb) have been infrequently explored in bacteria, even though a high frequency of rearrangements of R-type plasmids, mainly through an active RecF pathway, was observed in *E. coli* (38). In *R. etli*, large-plasmid recombination was mainly *recF* dependent (24-fold), with a minor participation of *addA* (threefold). Moreover, an additive effect was observed in the double mutant *recF-addA* (47-fold). These results indicate that small- and large-plasmid recombination in *R. etli* uses primarily the RecF pathway, with a minor role for the AddAB pathway, and suggest that pBX404-7 and pSym (371 kb) may have a low density of the *R. etli* chi sequence. However, the remaining recombination levels still observed in the *recF-addA* mutant (compare with the *recA* mutant [Table 2]) suggest the participation of an additional large-plasmid recombination route.

Our evaluation of deletion events in *R. etli* indicates that they are slightly affected in the *recF*, *addA*, and *recF-addA* mutants. These results indicate that *recF* and *addA* have a minor but additive effect in deletions. Interestingly, this event was affected only 18-fold in the *recA* mutant. It was previously reported that a 120-kb deletion generated by recombination between 5-kb direct repeats in the same plasmid was affected about 100-fold in a *recA* mutant (39, 53). Together, these results suggest that in *R. etli*, (i) closely spaced repeated sequences recombine in a *recA*-independent way and (ii) the

degree of *recA* dependence for deletion is affected by the distance between the repeated sequences. The results are in agreement with those reported for *E. coli*, where the distance between and the length of homologous repeats determine the RecA dependence of recombination (3, 34–36).

It has been reported that inactivation of either *addA* (*B. subtilis*) or *recB* (*E. coli*) produces a strong decrease in post-conjugational recombination events (Hfr and plasmid integration). Moreover, the effect is synergistic in the corresponding double knockout *recF-addA* (*B. subtilis*) or *recF-recB* (*E. coli*) (1, 29). In *R. etli*, the postconjugational integration of pJZC8 was unaffected by *addA* inactivation and only weakly dependent on the RecF pathway (Table 2) although it was *recA* dependent (666-fold [Table 2]). Similar recombinational frequencies were obtained by using other sites for integration in pSym or in the chromosome (data not shown). These results indicate the existence of another presynaptic route for Campbell-type recombination.

The unaltered rate of Campbell-type recombination in the *recF-addA* mutant raises the possibility that other as-yet-undescribed activities participate in the initiation of homologous recombination. For example, some helicase activities like RecQ, UvrD, HelD, etc., could be used with this type of substrate, or the absence of some exonucleases, like RecJ, ExoI, ExoX, etc., could allow the persistence of this type of substrate. In fact, the inactivation of the ssDNA exonucleases *recJ*, *exoI*, and *exoX* improves the recombination frequency of both homologous and homeologous substrates in *E. coli* (18, 21).

BLAST searches of the *Rhizobiaceae* genome database have identified *recN*, *recQ*, and *recJ* genes whose products suppress the absence of RecBCD and RecFOR systems at the initiation steps in *E. coli* (30). However, their role in repair and recombination is observed only when the two principal systems (RecBCD/AddAB and RecFOR) are mutated (17, 29).

The RecBCD and AddAB systems are modulated by a chi sequence. This species-specific sequence has been reported for *E. coli*, *Haemophilus influenzae*, *B. subtilis*, and *L. lactis* (4, 7, 8, 14, 15, 33, 49, 57). Furthermore, the basal level of plasmid recombination in *E. coli* and *L. lactis* is increased by incorporation of a chi sequence (14, 66). Therefore, it is possible that the integrative plasmids used in our work lack the *R. etli* chi sequence.

It is clear from the results presented that other systems for initiation of homologous recombination, beside those described in this paper, seem to be present in *R. etli*. Thus, our system provides the tool for the search of genes that, when mutated, provoke dramatic decrease in the recombinational capacity of *R. etli*.

ACKNOWLEDGMENTS

Partial financial support was provided by grants 31753-N from CONACyT and IN203897 from PAPIIT-DGAPA and 202302 from PAEP-DGEP (both from de Universidad Nacional Autónoma de México). J.Z.-C. received scholarships from Consejo Nacional de Ciencia y Tecnología (México) and Dirección General de Estudios de Posgrado (Universidad Nacional Autónoma de México).

We are indebted to Sonia Davila for phylogenetic analysis, Rosa Elena Gómez for sequencing assistance, Paul Gaytán and Eugenio López for oligonucleotide synthesis, and Laura Cervantes and Javier Rivera for skillful technical assistance. We thank R. Myers and the two anonymous reviewers for helpful comments on the manuscript.

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