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Differential roles of proteins involved in migration of Holliday junctions on recombination and tolerance to DNA damaging agents in *Rhizobium etli*

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ABSTRACT

The recombination genes involved in Holliday junction migration (*ruvB*, *recG*, *radA*) and heteroduplex editing (*mutS*) were studied in the α-proteobacterium *Rhizobium etli*. The genes were interrupted with a *loxP*Sp interposon and *R. etli* mutants, either single or in combination, were constructed by marker exchange. Our results show that these systems play a differential role in sensitivity to DNA damaging agents and recombination in *R. etli.* RuvB appears to be the main system for tolerance toward agents instigating single- or double-strand breaks (such as UV light, methyl methanesulphonate and nalidixic acid) while the RecG and RadA systems play minor roles in tolerance to these agents. Using five different recombination assays, we have found that a *ruvB* null mutant showed a notable reduction in recombination proficiency, while a *radA* mutant was only weakly affected. A null mutation in *recG* had the opposite effect, enhancing recombination in most of our assays. This effect was more clearly seen in an assay that measured recombination between divergent sequences (i.e. homeologous), but is unaffected by inactivation of *mutS*. These data indicate that RecG in *R. etli* limits intra- and intergenomic plasticity.

1. Introduction

Homologous recombination is an important process for all organisms, promoting genetic diversity and allowing the repair of damaged DNA. For the most studied models, (*Escherichia coli* and *Bacillus subtilis*), about 20 genes participate in this process (Michel et al., 2007; Sánchez et al., 2007). For the presynaptic step, different enzymatic complexes (AddAB/RecBCD; RecFOR-RecQJN; RecE) act alternatively to generate the crucial 3′ single-stranded DNA end. The 3′-end invades and anneals another homologous DNA sequence, with the aid of RecA, resulting in the formation of a Holliday junction; movement of this structure by unwinding and rewinding allows the travel of the junction, leading to heteroduplex extension.

Genetic redundancy has also been found for systems in charge of moving the Holliday junction (RecG, RuvAB and RadA). *In vitro* studies of heteroduplex processing by RecG and RuvAB systems revealed that RecG promotes heteroduplex extension, but also its regression (Whitby and Lloyd, 1998). This last activity was proposed to participate in restarting damaged replication forks (McGlynn and Lloyd, 2001). The RuvAB complex promotes mainly heteroduplex extension (Sharples et al., 1999). The *radA/sms* gene also participates in the processing of the heteroduplex structure (Beam et al., 2002; Carrasco et al., 2004), although its mechanism of action remains unknown.

The *in vivo* role assigned to these proteins, however, depends on the assay system used. For instance, for homologous conjugational recombination in *E. coli*, similar reductions in recombination were observed for single mutants in *ruvA*, *recG* or *radA* (Beam et al., 2002; Lloyd and Buckman, 1991), indicating that the products of these genes play an equivalent role in homologous recombination. Notably, for recombination-dependent adaptive mutation, RecG inhibits, but RuvAB facilitates adaptive mutation (Harris et al., 1996). Based on the opposing strand polarities of these helicases, it was proposed that a 3′-end invasion intermediate may be extended by RuvAB, but this intermediate may be unwound by RecG; and that the converse happens to a 5′-end invasion intermediate (Harris et al., 1996). A similar situation may apply to recombination between divergent sequences (i.e. homeologous recombination) as well (Stambuk and Radman, 1998).

Genomic studies have revealed a high level of conservation of these proteins in bacteria (Rocha et al., 2005). For the presynaptic steps, the RecFOR–RecQJN system is present in most bacterial genomes; the RecBCD complex is present in many bacteria, but is substituted by the analogous AddAB system in Firmicutes and α-proteobacteria (Rocha et al., 2005; Zuñiga-Castillo et al., 2004). The

Abbreviations: aa, amino acid(s); kb, kilobase(s); Cb, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; MMS, methyl methanesulphonate; Nal, nalidixic acid; Sp, spectinomycin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; LB, Luria–Bertani (medium); PY, Peptone-Yeast (medium); ORF, open reading frame; PCR, polymerase chain reaction; pSym, symbiotic plasmid; SD, standard deviation; UV, ultraviolet light.

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RecE pathway is restricted to *E. coli* and closely related bacteria (Rocha et al. 2005). In contrast, proteins that participate in migration of the Holliday junction are nearly ubiquitous. Both the RuvAB and RecG systems are prevalent, being absent only in a few species (Rocha et al., 2005). The RadA system is also present in most bacteria (Beam et al., 2002; Lovett, 2006).

Widespread occurrence of these proteins is taken to imply that they should have roles in all bacteria similar to the ones described in *E. coli* and *B. subtilis*. However, the corresponding genes have a phylogenetic history consistent with the genome in which they reside, with scant evidence of horizontal transfer (Rocha et al., 2005). Thus, it is conceivable that, during their long evolutionary history, their role in each species has been differentially adapted to its needs. Evidence supporting this hypothesis was found in bacteria with high rates of intragenomic recombination.

In the ε-proteobacterium *Helicobacter pylori*, a null mutation in *recG* did not affect survival to DNA damaging agents and showed an enhanced recombination (Kang and Blaser, 2008; Kang et al., 2004), while the *E. coli recG* mutant showed reduced recombination (Lloyd and Buckman, 1991). A similar effect was also seen in the γ-proteobacterium *Acinetobacter baylyi* (Gore et al., 2006). In the β-proteobacterium *Neisseria gonorrhoeae*, *recGruvB* mutants are lethal, due to their inability to process an intermediate in pilin antigenic variation (Sechman et al., 2006). These data support the view that bacteria with significant intragenomic recombination may be ideal systems to seek for alternative roles in proteins for migration of the Holliday intermediate.

The nitrogen-fixing α-proteobacterium *Rhizobium etli* is a model for symbiotic plant–microbe interactions. Besides this interest, its high genomic plasticity makes it an attractive model for mechanisms of homologous recombination. A significant fraction of its genome (*ca.* 35%) is represented by large plasmids (180 to 640 kb) (Brom et al., 2000; González et al., 2006). Both plasmids and the chromosome harbor a wealth of repeated sequences (Romero et al., 1999; González et al., 2006), comprised by 133 families of repeated elements larger than 100 bp (González et al., 2006). Homologous recombination between repeated sequences provokes high-frequency genomic rearrangements, including cointegrations (Brom et al., 1991, 2004), deletions, duplications, and gene conversion events (Rodríguez and Romero, 1998; Romero and Palacios, 1997; Santoyo et al. 2005; Valencia-Morales and Romero, 2000).

Previous studies about genes for homologous recombination in this organism revealed the existence of a conventional RecFOR system, but also the presence of an AddAB pathway (Zuñiga-Castillo et al., 2004). Here, we aimed to evaluate the effect of mutations in genes involved in migration of the Holliday intermediate. Our results show that these systems play a differential role in sensitivity to DNA damaging agents and recombination. RuvB appears to be the main system for tolerance toward agents instigating single- or double-strand breaks (UV light, methyl methanesulphonate and nalidixic acid) while the RecG and RadA systems play minor roles. Using different recombination assays, we found that a *ruvB* null mutant showed a notable reduction in recombination proficiency, while a *radA* mutant was only weakly affected. A null mutation in *recG* had the opposite effect, enhancing recombination in most of our assays. This effect was more clearly seen in an assay that measured recombination between divergent sequences (i. e. homeologous). These data indicate that RecG in *R. etli* limits intra- and intergenomic plasticity.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The bacterial strains and plasmids used are listed in Supplementary Table 1. *E. coli* strains were grown in Luria–Bertani (LB) medium (Sambrook et al., 1989) at 37 °C, and *Rhizobium* strains in PY medium (Martínez-Salazar and Romero, 2000) at 30 °C. Antibiotics were used at the following concentrations (in μg ml^{-1}): carbenicillin (Cb), 150; chloramphenicol (Cm), 25; tetracycline (Tc), 5; kanamycin (Km), 30; gentamicin (Gm), 15; spectinomycin (Sp), 100; nalidixic acid (Nal), 25 to 200. Methyl methanesulphonate (MMS) was used at concentrations ranging from 0.005 to 0.03% (v/v). β-galactosidase-positive strains were identified by growth on LB plates with 30 μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). For selections involving the *sacB* gene, sucrose-resistant (Sac^r) strains were selected in presence of 12.5% (w/v) sucrose. UV irradiation was performed with a UVC500 cross-linker (Hoefer Scientific Instruments).

2.2. Microbiological and recombinant DNA methods

Plasmid DNA was isolated by an alkaline-SDS lysis method and transformed into $CaCl_2$-treated *E. coli* cells using standard protocols (Sambrook et al., 1989). Plasmid mobilization from *E. coli* to *Rhizobium* was done by mating using S17.1 as donor strain (Simon et al., 1983). All enzymes, [α^{32}-P]-CTP, [γ^{33}P]-ATP, hybridization buffer and labeling systems were purchased from Amersham Biosciences. Southern blot hybridizations were carried out as previously described (Martínez-Salazar and Romero, 2000). Probes were labeled with [α^{32}P]-CTP by using a Rediprime II kit (Amersham Biosciences). Oligonucleotides used in this work are shown in Supplementary Table 2.

2.3. Plasmid construction

A 1.3 kb XhoI–SalI fragment from pBS39 (Sauer, 1994), containing the phage P1 *cre* gene, was cloned into SalI site of pBluescript SK+, to give pSKcre. Plasmid pJMS8 is a pRK7813 derivative (Jones and Gutterson, 1987) harboring a 1.3 kb EcoRI–SalI fragment (*cre* gene) from pSKcre. pJMS10 is a pSUPKm (Supplementary Table 1) derivative with the 3.5 kb *R. etli recG* gene-containing EcoRI fragment. All mutants were generated by interruption with the 2.0 kb *loxP*Sp interposon from pJMS2 (Martínez-Salazar and Romero, 2000), cut with the appropriate enzymes. pJMS11 is a pJMS10 derivative harboring the *recG* gene interrupted (SalI site) by the *loxP*Sp interposon. The 1.2 kb *R. etli mutS* gene-containing PstI fragment was cloned into pSUPKm, to give pJMS12. In pJMS12 the *mutS* gene was interrupted (SalI site) with the *loxP*Sp interposon, resulting in pJMS13. The 2.0 kb *R. etli radA* region was amplified by PCR from strain CE3 and cloned into EcoRI and HindIII pK18mobsacB plasmid (Schäfer et al., 1994) to give pJMS14. pJMS15 is a pJMS14 derivative harboring the *R. etli radA* gene interrupted (ClaI site) by the *loxP*Sp interposon.

2.4. Construction of *R. etli* mutants

The *R. etli radA*, *recG* and *mutS* mutants were obtained by allelic exchange with the *radA::loxP*Sp (pJMS15), *recG::loxP*Sp (pJMS11) or *mutS::loxP*Sp (pJMS13) alleles, respectively. To that end, the corresponding plasmid was mobilized from *E. coli* to *R. etli* by biparental mating; double recombinants were screened by Sp^rKm^s phenotype. The *ruvB-radA*, *ruvB-recG*, *ruvB-mutS*, *recG-radA* and *recG-mutS* double mutants were generated by a two-step procedure. In the first step, Sp^r marker was excised from the *loxP*Sp interposon present in strains CFNX635 (*ruvB::loxP*Sp) and CFNX705 (*recG::loxP*Sp) by using the Cre recombinase located in pJMS8. Losses of Sp^r marker as well as pJMS8 were selected by screening single-colony isolates for a Sp^sTc^s phenotype. In a second step, *radA::loxP*Sp (pJMS15), *recG::loxP*Sp (pJMS11) or *mutS::loxP*Sp (pJMS13) alleles were introduced in strain CFNX707 (*ruvB::loxP*) by marker exchange. Double recombinants were selected by their respective phenotype. Using the same strategy, the *radA::loxP*Sp (pJMS15) or *mutS::loxP*Sp allele (pJMS13) were introduced into strain CFNX708 (*recG::loxP*). To generate the *ruvB-recG-radA* mutant, the Sp^r marker was excised from strain CFNX709 (*ruvB::loxP-recG::loxPSp*), introducing afterwards the *radA::loxP*Sp allele by