ISSN 1330-9862
(FTB-3189)

original scientific paper

Comparison of Intraplasmid Rearrangements in *Agrobacterium tumefaciens* and *Escherichia coli*

Luka Bočkor[1], *Srećko Jelenić*[†], *Nenad Malenica, Jelena Mlinarec, Višnja Besendorfer and Ivana Ivančić-Baće**

University of Zagreb, Faculty of Science, Department of Molecular Biology, Horvatovac 102a, HR-10000 Zagreb, Croatia
[1]Current address: ICGEB, AREA Science Park, Padriciano 99, IT-34149 Trieste, Italy

Received: September 4, 2012
Accepted: July 15, 2013

Summary

In this work we have constructed a plasmid to compare intraplasmid recombination efficiency in *Agrobacterium tumefaciens* and *Escherichia coli*. The plasmid contains two directly repeated copies of spectinomycin resistance gene, one lacking 5′ and the other lacking 3′ end. These two copies share a 570-bp region of homology and are separated by the ampicillin resistance gene. Homologous recombination between repeated copies of incomplete spectinomycin resistance genes results in the restoration of spectinomycin resistance. During this process, ampicillin resistance gene is either deleted or incomplete spectinomycin genes are amplified along with the ampicillin resistance gene. This experimental system enabled us to follow for the first time the generation of deletions and amplifications during intraplasmid recombination in *A. tumefaciens*. We show here that predominantly RecA-independent mechanism contributes to the formation of deletion and amplification products in both, *A. tumefaciens* and *E. coli*. Additionally, deletion and amplification products were detected at similar frequencies, suggesting that amplifications and deletions probably occur by a similar mechanism.

Key words: RecA, intramolecular recombination, *Agrobacterium tumefaciens*, *Escherichia coli*

Introduction

Direct short repeat sequences are common and unstable genetic elements in chromosomes of prokaryotes and eukaryotes because they cause genome instability by promoting DNA rearrangements such as deletions or duplication of the repeat itself and any intervening sequence. Indeed, several human disorders have been caused by rearrangements between repetitive sequences, both in nuclear and mitochondrial genes (*1*). Molecular genetic analysis of repeated sequence rearrangements in *Escherichia coli* has revealed that multiple mechanisms participate in the process. They can be roughly divided into RecA-dependent and RecA-independent mechanisms. The RecA-dependent mechanism is based on homologous recombination, which can lead to unequal crossing over on two DNA molecules yielding different products (*2*,*3*). The RecA-independent mechanisms are believed to involve replication misalignment. These RecA-independent mechanisms can be further divided based on their genetic properties into three types: simple slipped misalignment, sister chromosome exchange-associated replication misalignment and single-strand annealing (*4*). The properties of RecA-independent recombination are: it occurs intramolecularly and it is dependent on the proximity of the repeat sequences, which have to be of perfect homology because small sequence differences between repeats reduce deletion efficiency by several orders of magnitude (*3*), as well as on their length. Most importantly, it is independent of almost all known recombination functions (*2*,*5*). However, *recB* and *recC* mutations have been shown to stimulate deletion formation in a *recA*$^-$ background (*6*).

*Corresponding author; Phone: ++385 1 4606 273; Fax: ++385 1 4606 286; E-mail: ivana.ivancic.bace@biol.pmf.hr

Intramolecular recombination has been typically studied by using plasmid-based assays where duplicated sequences are on the same DNA molecule. One of the expected products of recombination between direct repeats involves the deletion of one of the repeats and any intervening sequence between them (deletion or monomeric product, known as M). Alternatively, heterodimeric forms of the plasmid can be generated having duplicated or triplicated repeats in addition to the deleted repeat, named 1+2 and 1+3, respectively (Fig. 1) (*2*,*5*–*7*). In *E. coli* it has been shown that both deletion and amplification (duplication or expansion) occur at roughly similar rates (approx. 10^{-4}), and by similar molecular mechanisms (*8*). The rates of monomeric/heterodimeric forms can be further modified by the environmental stresses, such as concentrations of antibiotic, incubation temperature, oxygen fluctuations or growth in the minimal medium (*9*).

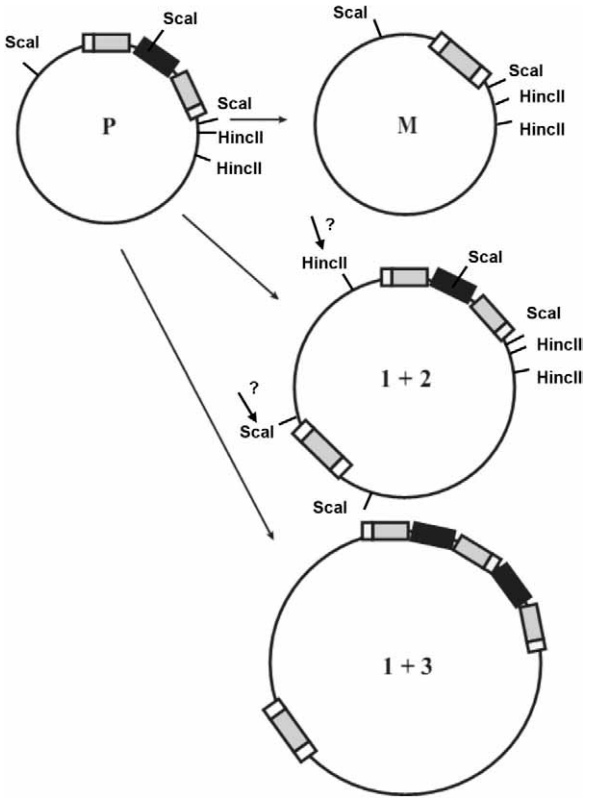

Fig. 1. A scheme depicting the monomeric (M) and heterodimeric (1+2) and (1+3) products formed by recombination of a parental plasmid that harbours direct repeats with long homologies (grey boxes) and intervening sequence (black box). Restriction sites for restriction enzymes HincII and ScaI are depicted. Arrows indicate predicted restriction sites in the heterodimeric 1+2 product based on the restriction analysis

To shed more light onto the phenomenon of intramolecular recombination in *Agrobacterium tumefaciens*, we compared it to that in *E. coli*. For that purpose, we constructed a pC2300 plasmid derivative consisting of two directly repeated *aadA* (spectinomycin resistance) genes separated by the ampicillin resistance gene. Deletion products were detected by the restoration of spectinomycin resistance and the loss of ampicillin resistance at relatively high rates (approx. 10^{-3}) in *E. coli* and a hundredfold reduced frequency in *A. tumefaciens* (approx. 10^{-5}). Amplification products occurred at reduced frequency compared to deletion recombinants in wild type (wt) and the *recA* mutant of *A. tumefaciens* or at similar frequency in wt, *recA* and *recB* mutants of *E. coli*. This suggests that deletion with amplification (duplication) occurred at similar rates probably by a similar intramolecular event.

Materials and Methods

Media and enzymes

Agrobacterium strains were grown at 28 °C and *Escherichia coli* strains were grown at 37 °C in Luria Bertani (LB) medium composed of (in g per 1000 mL): bactotryptone 10, yeast extract 5, and NaCl 10. Solid media were supplemented with 15 g/L of agar. Appropriate antibiotics kanamycin (Kan; 50 μg/mL), ampicillin (Amp; 100 μg/mL) and spectinomycin (Spc; 100 μg/mL) were added when required. Restriction enzymes, Klenow fragment, RNase A, Proteinase K, 1 kb ladder GeneRuler and T4 DNA ligase were purchased from Fermentas Inc., Burlington, Ontario, Canada, and New England Biolabs, Ipswich, MA, USA. Chloroform was purchased from Lach-Ner, s.r.o., Neratovice, Czech Republic, phenol was purchased from Carl Roth GmbH, Karlsruhe, Germany, isoamylalcohol and agarose were from Sigma-Aldrich Chemie GmbH, Munich, Germany, isopropanol was purchased from T.T.T., Sveta Nedjelja, Croatia, and ethanol was purchased from Kefo d.o.o., Sisak, Croatia. PCR purification kit and DNA extraction kits were purchased from Qiagen GmbH, Hilden, Germany.

Bacterial strains and plasmids

The following *E. coli* strains were used in this study: AB1157 (*10*), N3071 (*recB268*::Tn*10* derivative of AB1157) and N3072 (*recA269*::Tn*10* derivative of AB1157), which are from our lab collection. XL1-Blue strain was used for cloning. *A. tumefaciens* strain was NT1 (*autC58* (pAtC58)) and its derivative *recA*$^-$ strain UIA143 (*autC58 recA*::*Ery 140* (pAtC58)) (*11*).

The assay plasmid pLB10 is a derivative of pCAMBIA2300 (pC2300; BiOS, Canberra, Australia), a large (8742 bp) high-copy plasmid which is stably replicated in *A. tumefaciens* and in *E. coli*. pBluescript SK was used as a source for ampicillin resistance gene, while plasmid pPZP200 (*12*) was used as a source for the *aadA* gene. The plasmid pLB10 was constructed by the following steps: primers Spec1 and Spec2 (see the list of primers) were used to generate a 942-bp fragment of *aadA* gene without its 5′ end. Primers Spec3 and Spec4 were used to generate a 972-bp fragment of the *aadA* gene without its 3′ end (Fig. 2a). Both PCR fragments were cloned into SmaI restriction site in pCP2300, giving plasmids pCSpecES-Sma and pCSpecHS-Sma, respectively. In a similar way, the ampicillin gene was amplified by primers AmpI and AmpII and cloned into EcoRV restriction site of the pBluescript SK plasmid, resulting in the construct pSK(EV)Amp containing two copies of the ampicillin gene. Next, the ampicillin resistance gene (1138 bp) was removed from pSK(EV)Amp by cleaving with PstI and

SalI and ligated into the same restriction sites of the plasmid pCSpecES-Sma, thus creating the plasmid pCSpecESAmp. In this construct, the spectinomycin fragment and the ampicillin resistance gene were next to each other. Then the fragment containing ampicillin and *aadA* genes was excised from the plasmid pCSpecES-Sma by EcoRI and inserted into the plasmid pCSpecHS-Sma in which 30 bp between BamHI and HindIII were removed and sticky ends were filled with the Klenow fragment. The newly generated plasmid, pCSpecHS-SmaΔHB, had ampicillin resistance gene in opposite orientation from the plasmid promoter, so the final plasmid was obtained by the excision of the ampicillin resistance gene by SmaI and religation. Then screening of plasmids was done for the plasmid with the insert in the correct orientation by the restriction digestion with XbaI and HindIII. The final plasmid was designated as $pC_{\Delta s}$SpecAmpi-ΔHB or for simplicity pLB10, and was about 12 kb long (Fig. 2b). Primers used in this study were:

Spec1 5′ GAATTCCGGGGTCTGACGCTCAGTGG 3′
Spec2 5′ GAGCTCCGGCTCCGCAGTGGATGGCG 3′
Spec3 5′ GTCGACATGACGGGCTGATACTGGGC 3′
Spec4 5′ AAGCTTATTCGGGCACGAACCCAGTG 3′
AmpI 5′ CCCGGGGATCTTTTCTACGGGGTCTG 3′
AmpII 5′ CCACCATCGTGGCACTTTTCGGGGAAATG 3′

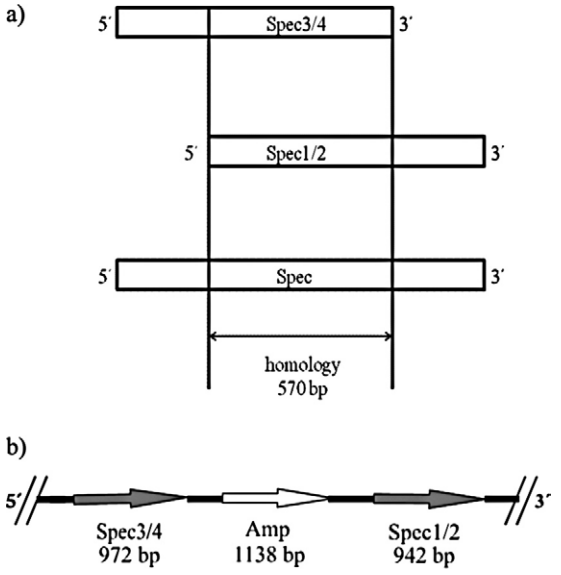

Fig. 2. Diagrams representing: a) incomplete spectinomycin fragments from the plasmid pLB10 showing 570-bp internal region of homology, and b) the same region from the plasmid pLB10 showing the positions of spectinomycin fragments and ampicillin resistance gene. pLB10 ($pC_{\Delta s}$SpecAmpi-ΔHB) is approx. 11790 bp long, see details in the text. Fragment Spec3/4 lacks its 3′ end and fragment Spec1/2 is deleted at its 5′ end

Recombination test and product analysis

The plasmid pLB10 was transformed into *A. tumefaciens* NT1 (wt) or its *recA* derivative strain UIA143 by electroporation, and the transformants were selected on LB plates containing kanamycin at 28 °C. Five individual colonies of each strain were resuspended in sterile distilled water and serially diluted. Appropriate amounts of cells were plated on LB plates containing kanamycin (Kan plate) to obtain viable cells, spectinomycin (Spc plate) to select cells with recombinant plasmid, and ampicillin-spectinomycin (AmpSpc plate) to select cells with heterodimeric plasmids. The cells were counted after 3 days of incubation at 28 °C. *E. coli* cells were transformed in the same way but incubated overnight at 37 °C. The frequency of recombination was measured as the ratio of the number of colonies on the Spc or AmpSpc plates to the number of colonies on the corresponding Kan plate. For each strain, two to five independent experiments were performed, and the frequency of recombination of a deletion or amplification product was calculated as the mean of the results of all experiments with standard deviations (*13*).

Plasmid DNA isolation and analysis

Individual colonies that formed on Kan or Spc plates were grown to saturation in 4 mL of LB broth supplemented with kanamycin at appropriate temperatures for *E. coli* and *A. tumefaciens*. Plasmids from *E. coli* were extracted from 2 mL of culture by alkaline lysis followed by precipitation with 1.4 mL of isopropanol and washing with 70 % ethanol. The pellet was resuspended in 30 μL of sterile distilled water and samples of 5 μL were used for restriction digests. In the case of *A. tumefaciens*, the whole 4 mL of culture were centrifuged and resuspended in Tris-EDTA (TE) buffer with Proteinase K (10 mg/mL). Afterwards, the cells were lysed by standard alkaline lysis, followed by the extraction of DNA from the supernatant by the addition of the same volume of phenol/chloroform/isoamyl solution (25:24:1). After centrifugation, the upper aqueous phase was collected in a new tube and vortexed with the same volume of chloroform/isoamyl (24:1). Separation of phases followed as in the previous step. The plasmid DNA recovered from the upper aqueous phase was precipitated with 1.4 mL of isopropanol and the pellet was washed with 70 % ethanol. The pellet was resuspended in 30 μL of sterile distilled water and samples of 15 μL were used for restriction digests. Plasmids were digested with either HincII or ScaI and analyzed by agarose gel electrophoresis. Ten individual colonies were analyzed from each *E. coli* strain from Spc plates and 5 from Kan plates. In the case of *A. tumefaciens*, due to problems with plasmid purification, 5 individual colonies of each mutant from Spc plates and 3 from Kan plates were analyzed.

Results and Discussion

To study intramolecular recombination between direct repeats separated by an intervening sequence in *A. tumefaciens* and *E. coli*, the derivative of plasmid pC2300 was used. This plasmid replicates in both species of bacteria, which enabled plasmid construction and recombination analysis in both species. The recombination tests were performed as described in Materials and Methods. The diagram of pLB10, which shows direct repeats of *aadA* genes separated by ampicillin resistance gene, is shown in Fig. 2b. The two genes share 570 bp of homology (Fig. 2a), and recombination between these repeats is expected to restore a functional *aadA* gene (1400 bp long), which confers resistance to spectinomycin and eliminates the ampicillin resistance gene. This plasmid enabled simple detection of deletion and/or duplication

products of recombination by plating bacteria on appropriate selective plates. The deletion product was expected to exhibit $Amp^{S}Spc^{R}$ resistance, while the heterodimeric products were expected to have $Amp^{R}Spc^{R}$ resistance. This plasmid was introduced into wt and *recA* and *recB* mutant derivatives of *E. coli*, and wt and *recA* mutant derivative of *A. tumefaciens*.

As shown in Table 1, the frequency of recombination for the deletion of monomeric product ($Amp^{S}Spc^{R}$) was approx. $4{\cdot}10^{-5}$ for wt cells of *A. tumefaciens* (strain NT1), and a hundredfold higher, approx. $4{\cdot}10^{-3}$, for wt cells of *E. coli*. The obtained frequencies for *E. coli* were about fourfold higher than the rate of $8.2{\cdot}10^{-4}$ previously reported in the literature for 559-bp repeats and 2669-bp intervening sequence (*5,14*). Given that the length of the repeat and the distance separating the repeats affect the type of products and frequency of recombination, shorter intervening sequence (in our case 1138 bp) was expected to increase the frequency of recombination. On the other hand, another report showed that the frequency of recombination in wt *E. coli* was $4{\cdot}10^{-5}$ for 787-bp repeats and 659-bp intervening sequence (*8*). The plasmids used in these two studies were different, so direct comparisons of recombination frequencies are not possible.

Table 1. Relative deletion and amplification frequencies of pLB10 in isogenic mutants of *A. tumefaciens* and *E. coli*

Strain (genotype)	Relative deletion frequency ($Amp^{S}Spc^{R}$)	Relative amplification frequency ($Amp^{R}Spc^{R}$)
NT1 (wt, *A. tumefaciens*)	$(4\pm1){\cdot}10^{-5}$	$(1.2\pm0.6){\cdot}10^{-5}$
UIA143 (*recA*, *A. tumefaciens*)	$(4\pm1){\cdot}10^{-5}$	$(1.9\pm0.1){\cdot}10^{-5}$
AB1157 (wt, *E. coli*)	$(4\pm1){\cdot}10^{-3}$	$(4\pm1){\cdot}10^{-3}$
N3071 (*recB*, *E. coli*)	$(2\pm1){\cdot}10^{-3}$	$(18.0\pm0.9){\cdot}10^{-4}$
N3072 (*recA*, *E. coli*)	$(2.0\pm1){\cdot}10^{-3}$	$(20.0\pm0.3){\cdot}10^{-4}$

Deletion frequencies were determined by the number of $Amp^{S}Spc^{R}$ cells in Kan^{R} populations as described in Materials and Methods and the mean deletion frequency with standard deviation was determined for all independent experiments.
Amplification frequencies were determined by the number of $Amp^{R}Spc^{R}$ cells in Kan^{R} populations and calculated as for deletion frequencies

However, the reason for the discrepancy in the deletion (and amplification) and recombination frequencies of the same plasmid between *A. tumefaciens* and *E. coli* at present is unknown, but it could be due to the differences in the genome replication (such as the speed of replication), genome organisation, or differences in recombination proteins. However, other factors such as the plasmid copy number and environmental stress could also modulate recombination frequencies (*9*). The copy number of many shuttle plasmids is known to be lower in *A. tumefaciens* in comparison with *E. coli* (*15*). We could not directly compare the plasmid yields between *A. tumefaciens* and *E. coli* since we used different protocols for plasmid extraction due to problems with lysis of *A. tumefaciens* cells. However, we did observe that plasmid yields from *A. tumefaciens* were much lower and plasmids were very unstable and prone to degradation (data not shown). In addition, the difference in the growth temperatures between *A. tumefaciens* (28 °C) and *E. coli* (37 °C) cultures may also affect either the plasmid copy number and/or recombination frequencies (*9*).

Interestingly, mutations in *recA* or *recB* reduced the frequency of recombination for the same deletion product about twofold ($2{\cdot}10^{-3}$) in *E. coli*, and deletion frequency in the *recA* mutant of *A. tumefaciens* (strain UIA143) was almost the same as in wt cells (Table 1). This indicates that the formation of deletion products between large repeats occurred predominantly independently of RecA and RecB homologous recombination functions (*2, 3,5,7*). Overall, these data show that common molecular mechanism contributed to intramolecular plasmid recombination in both *A. tumefaciens* and *E. coli*.

In addition to deletion/monomer product, we also measured the recombination frequency of repeat amplifications or heterodimeric products that were expected to have both resistances ($Amp^{R}Spc^{R}$). The recombination frequencies of heterodimeric $Amp^{R}Spc^{R}$ product were reduced about threefold in comparison with the corresponding deletion product in both wt and *recA* mutant of *A. tumefaciens* (Table 1). On the other hand, almost the same recombination frequencies for heterodimeric and deletion products were obtained for wt, *recA* and *recB* mutants of *E. coli*, suggesting that amplification formation was accompanied by deletion probably by a similar intramolecular event, which is in agreement with previous reports (*2,13*).

To identify the products of recombination, plasmid DNA was extracted from several Kan- or Spc-resistant isolates of wt and *rec* mutants of both *E. coli* and *A. tumefaciens*. Uncut and plasmid DNA digested with HincII or ScaI restriction enzymes were examined by electrophoresis (Fig. 3). Interestingly, the restriction analysis of all recombinant plasmids from both species (more than 40 plasmids analyzed in total) showed only one amplification product (data not shown). HincII enzyme cleaves pCP2300 plasmid at two positions (571 and 1973) and the plasmid in lane 4 digested with HincII has an additional band. ScaI cleaves pCP2300 plasmid at two positions (35 and 6289) and also within the ampicillin resistance gene. The cleavage by ScaI in both plasmids results in an approx. 6.3-kb fragment from the plasmid and an approx. 2.5-kb fragment from the cleavage within the ampicillin resistance gene and the plasmid (other bands are probably partially digested products). On the basis of restriction patterns, we concluded that 1+2 heterodimeric recombinant products were generated. However, restriction analysis of recombinant plasmid by each enzyme showed fewer bands than expected if dimeric circles were formed. This suggested that additional rearrangements accompanied the formation of the heterodimeric product, which are the loss of one restriction site for HincII and ScaI and the inversion of newly generated restriction sites (Figs. 1 and 3). Since the plasmid we used was very large (about 12 kb), it may have been more prone to additional DNA rearrangements such as inversions and/or deletions than smaller plasmids. These ad-

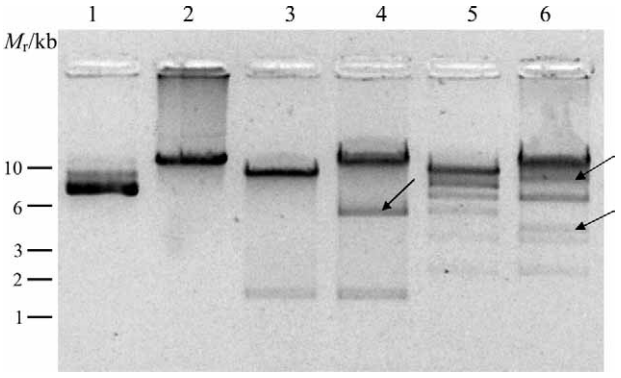

Fig. 3. Agarose gel showing restriction digest of two plasmid forms extracted from *E. coli*. The 1 kb DNA ladder (Fermentas) was resolved in parallel as size marker for linear DNA (not shown; only several bands are depicted). Lanes 1 and 2 contain undigested plasmids, lanes 3 and 4 contain HincII, and lanes 5 and 6 contain ScaI-digested plasmids of nonrecombinant and recombinant types, respectively. Arrows represent bands formed by cleavage within new restriction sites in 1+2 heterodimeric product which are predicted in Fig. 1

ditional rearrangements probably occurred by RecA-independent replication misalignment process (*8*). No monomers or 1+3 products were observed, which is in agreement with the previous study showing that if the length of the intervening sequence is greater than 100 bp, most of (>90 %) the products were 1+2, while the percentage of 1+3 or monomers was low (*13*). The formation of heterodimeric products (deletions associated with dimerization) has also been noted by others (*2*,*6*,*7*). Since we analyzed rather moderate number of plasmids, we were not able to detect other recombinant types.

Taken together, our results show that duplication accompanied by deletion probably resulted from a similar intramolecular event in both species through predominantly RecA-independent pathway. This study does not provide enough data to describe the mechanism of this event, but the obtained results are best explained by a model in which a blocked or stalled replication fork may promote in part RecA-dependent recombinational repair that can result in occasional unequal crossing-over, which on the other hand, provides opportunity for RecA-independent cross-fork misalignments that are associated with sister chromosome exchange events (*3*).

Conclusions

Rearrangements between directly repeated sequences of various lengths are a major source of genome instability. These rearrangements can result in either genetic deletions or amplifications. To test the stability of plasmids in *A. tumefaciens*, we studied intramolecular recombination in *Agrobacterium tumefaciens* in comparison with that in *E. coli.* We showed that intramolecular recombination in *A. tumefaciens* is a hundredfold lower compared to *E. coli.* We have also shown for the first time that primarily RecA-independent mechanism contributed to the formation of the heterodimeric 1+2 products between direct repeats in *A. tumefaciens*. The obtained results confirm that the mechanisms of intramolecular recombination are common to both species, and that the plasmid stability when carrying direct repeats cannot be further increased by the introduction of *recA* mutation.

Acknowledgements

This work was supported by Grant 119-1191196-1201 of the Croatian Ministry of Science, Education and Sports. We are grateful to Dr. S. K. Farrand for providing bacterial strains of *Agrobacterium tumefaciens* and to Mia Arbanas for practical assistance.

References

1. M. Krawczak, D.N. Cooper, Gene deletions causing human genetic disease: Mechanisms of mutagenesis and the role of the local DNA sequence environments, *Hum. Genet. 86* (1991) 425–441.
2. S.T. Lovett, P.T. Drapkin, V.A. Sutera, T.J. Gluckman-Peskind, A sister-exchange mechanism for *recA*-independent deletion of repeated DNA sequences in *Escherichia coli*, *Genetics*, *135* (1993) 631–642.
3. S.T. Lovett, Encoded errors: Mutations and rearrangements mediated by misalignment at repetitive DNA sequences, *Mol. Microbiol. 52* (2004) 1243–1253.
4. M. Bzymek, S.T. Lovett, Instability of repetitive DNA sequences: The role of replication in multiple mechanisms, *PNAS*, *98* (2001) 8319–8325.
5. X. Bi, L.F. Liu, *recA*-independent and *recA*-dependent intramolecular plasmid recombination. Different homology requirement and distance effect, *J. Mol. Biol. 235* (1994) 414–423.
6. A.V. Mazin, A.V. Kuzminov, G.L. Dianov, R.I. Salganik, Mechanisms of deletion formation in *Escherichia coli* plasmids, *Mol. Gen. Genet. 228* (1991) 209–214.
7. G.L. Dianov, A.V. Kuzminov, V.A. Mazin, R.I. Salganik, Molecular mechanism of deletion formation in *Escherichia coli* plasmids. I. Deletion formation mediated by long direct repeats, *Mol. Gen. Genet. 228* (1991) 153–159.
8. A.S. Morag, C.J. Saveson, S.T. Lovett, Expansion of DNA repeats in *Escherichia coli*: Effects of recombination and replication functions, *J. Mol. Biol. 289* (1999) 21–27.
9. P.H. Oliveira, D.M.F. Prazeres, G.A. Monteiro, Deletion formation mutations in plasmid expression vectors are unfavored by runaway amplification conditions and differentially selected under kanamycin stress, *J. Biotechnol. 143* (2009) 213–238.
10. B.J. Bachman: Derivations and Genotypes of Some Mutant Derivatives of *Escherichia coli* K-12. In: *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, F.D. Neidhardt (Ed.), American Society for Microbiology, Washington, D.C., USA (1987) pp. 1190–1219.
11. S.K. Farrand, S.P. O'Morchoe, J. McMutchan, Construction of an *Agrobacterium tumefaciens* C58 *recA* mutant, *J. Bacteriol. 171* (1989) 5314–5321.
12. P. Hajdukiewicz, Z. Svab, P. Maliga, The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation, *Plant Mol. Biol. 25* (1994) 989–994.
13. X. Bi, L.F. Liu, A replicational model for DNA recombination between direct repeats, *J. Mol. Biol. 256* (1996) 849–858.
14. P.H. Oliveira, F. Lemos, G.A. Monteiro, D.M.F. Prazeres, Recombination frequency in plasmid DNA containing direct repeats-predictive correlation with repeat and intervening sequence length, *Plasmid, 60* (2008) 159–165.
15. A.A. Wise, Z. Liu, A. N. Binns: Nucleic Acid Extraction from *Agrobacterium* Strains. In: *Agrobacterium Protocols*, *Vol. 1; Methods in Molecular Biology*, K. Wang (Ed.), Humana Press Inc., New York, NY, USA (2006) pp. 67–76.