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# Molecular cloning and nucleotide sequence of the *Rhizobium phaseoli recA* gene

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**Summary.** A recombinant λ phage carrying the *recA* gene of *Rhizobium phaseoli* was isolated from a *R. phaseoli* genomic library by complementation of the Fec$^-$ phenotype of the recombinant phage in *Escherichia coli*. When expressed in *E. coli*, the cloned *recA* gene was shown to restore resistance to both UV irradiation and the DNA alkylating agent methyl methanesulphonate (MMS). The *R. phaseoli recA* gene also promoted homologous recombination in *E. coli*. The cloned *recA* gene was only weakly inducible in *E. coli recA* strains by DNA damaging agents. The DNA sequence of the *R. phaseoli recA* gene was determined and compared with published *recA* sequences. No LexA-binding site was detected in the *R. phaseoli recA* upstream region.

**Key words:** *Rhizobium phaseoli* – *RecA* gene – Recombination – DNA sequence – Fec

*Rhizobium phaseoli* is the nodulating microsymbiont of common beans. The variability of the symbiotic properties of *R. phaseoli* (Soberón-Chávez et al. 1986; Flores et al. 1988) is a major problem when dealing with this species, especially when used as an inoculum. *R. phaseoli* possesses a high level of reiterated sequences (Flores et al. 1987). Some of these reiterated sequences, like *nifH* (Quinto et al. 1982), are involved in nitrogen fixation, while others have not yet been characterized. Homologous recombination between reiterated elements may contribute to the genomic instability of this biovar and therefore enhance its symbiotic variability. As a first step in the study of the molecular mechanisms of instability we isolated and characterized the *R. phaseoli recA* gene.

A gene bank of *R. phaseoli* strain CNPAF512 (EMBRAPA collection) was constructed in λ phage EMBL3 (Frischauf et al. 1983). *R. phaseoli* chromosomal DNA was prepared as reported by Morett et al. (1981) and partially digested with *Sau*3A. λ EMBL3 DNA was digested with *Bam*HI and *Eco*RI. Vector and insert were ligated and packaged in bacteriophage lambda heads yielding $1.5 \times 10^4$ independent phage clones as determined on *Escherichia coli* NM539 (Frischauf et al. 1983). Recombinant phages were amplified on NM539. This strain allows selection for the Spi$^-$ phenotype of the recombinant phages.

During the replication of λ *gam* phages (in *recBCD*$^+$ hosts) only monomeric λ are produced. These monomeric λ must recombine to produce packageable dimeric λ. This can be achieved through either the *E. coli* RecBCD pathway, involving the RecA protein, or the λ Red pathway. Recombinant phages are *gam red* and therefore need the recombination system of the cell to support their growth. 12000 independent recombinant λ EMBL3 clones were plated on the *E. coli recA* host HB101 (Maniatis et al. 1982). 0.25% of the phages were able to produce plaques and were therefore assumed to contain the *R. phaseoli recA* gene. λ DNA was purified from eight plaque-forming phages and restriction fragment analysis revealed that all clones contained overlapping DNA restriction fragments.

DNA of one such clone, λRprecAl, was partially digested with *Eco*RI and ligated to *Eco*RI-linearized pSUP102 (Simon et al. 1983). HB101 cells carrying recombinant plasmids were screened for colonies resistant to the mutagen methyl methanesulphonate (MMS). Plasmid DNA was purified from five MMS-resistant colonies and further analysed by means of restriction analysis. The different clones showed very similar *Eco*RI restriction patterns. Two of these plasmids, pJM112 and pJM113, were selected for further analysis. PJM112 contained a 6 kb insert of *R. phaseoli* DNA and pJM113 one of approximately 4.5 kb.

A physical map of pJM112 was constructed using several restriction endonucleases (Fig. 1). Subcloning of specific restriction fragments of pJM112 and pJM113 was used to localize the *R. phaseoli recA* gene. While both plasmids contained two *Eco*RI fragments in common, one of 4 kb and one of 0.8 kb, pJM112 possessed an additional *Eco*RI fragment of 1.5 kb. PJM112 or

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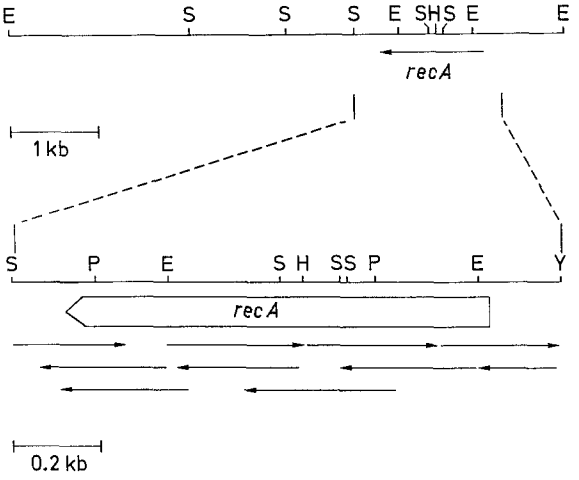


**Fig. 1.** Sequencing strategy and restriction map of the *Rhizobium phaseoli recA* containing DNA fragment. The 6 kb *Eco*RI fragment insert of pJM112 is shown at the top. The extent and direction of the open reading frame (ORF) are presented. The sequencing strategy is indicated by *solid arrows*. Symbols: E, *Eco*RI; S, *Sal*I; H, *Hin*dIII; P, *Pst*I; Y, *Sty*I

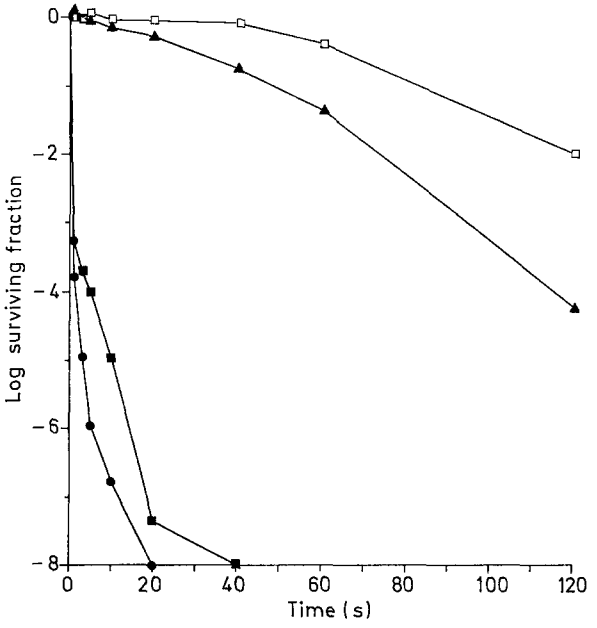


**Fig. 2.** UV survival of *recA*$^+$ and *recA*$^-$ *Escherichia coli* strains. Cells were grown to approximately $5 \times 10^8$/ml, spread onto solid medium and irradiated for the indicated times. Survivors were determined after overnight incubation at 37° C. Symbols: □, RR1(RecA$^+$); ■, HB101(RecA$^-$); HB101 harbouring the recombinant plasmid pJM131 (▲) and pJM132 (●)

pJM113 were digested to completion with *Eco*RI, religated and transformed into HB101. Subclones carrying only one of the three *Eco*RI fragments were MMS-sensitive (MMS$^s$), suggesting that the *recA* gene was located on the central 0.8 kb *Eco*RI fragment, and extended into one or both of the other two fragments.

Because multiple attempts to mutagenize *recA* by insertion of Tn*5* were unsuccessful, a mutagenesis strategy was designed to inactivate the *recA* gene by inserting

**Table 1.** Recombination in JC14604 harbouring *recA*-containing plasmids

| Plasmid | Average number of Lac$^+$ papillae per cfu[a] | | |
|---|---|---|---|
| | Without DNA damage | After DNA damage[b] | |
| | | MMS | UV light |
| pSUP202 | 0.0 | NG[c] | 0.0 |
| pJM131 | 10.9 | 14.8 | 15.6 |
| pJM132 | 0.0 | NG | 0.0 |

[a] The Lac$^+$ papillae from 30 equally-sized colonies were scored after 48 h incubation at 37° C. All colonies containing pJM131 displayed recombinant papillae. None of 3000 cfu of JC14604 harbouring pSUP202 or pJM132 displayed Lac$^+$ papillae
[b] Induced recombination was assayed on medium containing 0.01% MMS or after irradiation with UV light for 1 s
[c] NG, no growth

**Table 2.** Growth of Fec$^-$ λ phage λF−1[a]

| Strain | pfu/ml |
|---|---|
| NM539 | $4.1 \times 10^5$ |
| HB101 | <10 |
| HB101(pJM131) | $7.0 \times 10^4$ |
| HB101(pJM132) | <10 |

[a] *E. coli* were grown to an $OD_{600}$ of 0.5 in L-broth containing 0.2% maltose. Cells were harvested by centrifugation and resuspended in half volume of 10 mM $MgSO_4$. The cells (0.2 ml) were mixed with various dilutions of a phage stock of $4.1 \times 10^5$ pfu/ml and incubated for 20 min at 37° C. 3 ml of top agarose was added to the cells and spread on λ plates. The number of pfu was determined after overnight incubation at 37° C

a kanamycin resistance gene cassette into the unique *Hin*dIII site.

PJM112 contains two *Dra*I sites, both in the vector, thus allowing removal of the insert as a 6 kb fragment. This fragment with vector sequences at both ends was subcloned in pUC18-2 (pUC18 containing the *Hin*dIII fragment of Tn*5*) vector DNA, linearized with *Hin*cII, yielding pJM121. The entire *recA* region was removed from pJM121 as a 6 kb *Bam*HI fragment, with approximately 0.1 kb Tn*5* flanking sequence at one of the ends, and subcloned into pSUP202-1 (*Hin*dIII site of pSUP202 filled in with Klenow polymerase). This plasmid, called pJM131, conferred MMS and UV resistance on HB101, indistinguishable from those conferred by pJM121 (results not shown).

The *recA* gene was inactivated by inserting the *Hin*dIII fragment of Tn*5* into the unique *Hin*dIII site of the gene, conferring kanamycin resistance on the construct. This plasmid, called pJM132, no longer complemented the RecA$^-$ phenotype of HB101 as shown by testing the UV resistance (Fig. 2), homologous recombination (Table 1) and the plating efficiency of Fec$^-$ λ phage (Table 2).

Several reports have indicated that the functions of RecA in both gram-negative and gram-positive organ-

isms are probably very well conserved (Sherry et al. 1984; Ball et al. 1990; Owttrim and Coleman 1989). We determined whether the cloned gene was able to promote homologous recombination and to complement a defective SOS response.

Complementation of a defective UV response was determined in HB101 harbouring the recombinant plasmids pJM131 and pJM132 (Fig. 2). PJM131 was found to restore the UV resistance of HB101 to a level approaching that of the RecA$^+$ *E. coli* strain RR1 (Maniatis et al. 1982). RR1 is isogenic to HB101 except for *recA*. This effect was abolished when the *Hin*dIII fragment of Tn*5* was inserted into the gene (pJM132).

The ability of the *R. phaseoli recA* gene to promote homologous genetic recombination in *E. coli* was evaluated in two ways. First recombination was evaluated in the *E. coli recA* strain JC14604 (Clark and Margulies 1965). This strain contains two defective *lac* operons. Lac$^+$ recombinants can arise as a result of recombination between these operons. Recombinants were scored on lactose MacConkey medium. Transformant colonies carrying pJM131 displayed Lac$^+$ papillae after 48 h, whereas transformants harbouring pJM132 or pSUP202 (Simon et al. 1983) did not show the Lac$^+$ phenotype (Table 1).

Treatment of *E. coli* with DNA-damaging agents induces *recA* transcription. This induction depends upon the cleavage of the LexA repressor, a process that is stimulated by activated RecA protein. JC14604 transformants carrying pJM131 were irradiated with UV light or grown in the presence of 0.01% MMS. In both cases the recombinational proficiency was increased 1.4-fold as compared with the non-treated control plates (Table 1).

Homologous recombination was also determined by measuring the plating efficiency of a *red gam* $\lambda$ phage ($\lambda$F-1) displaying a Fec$^-$ phenotype (Table 2). The plating efficiency of $\lambda$F-1 on HB101 is less than $10^{-4}$ relative to that of the RecA$^+$ strain NM539. Introduction of pJM131 into HB101 restored the plating efficiency of $\lambda$F-1 to 17% as compared with that of NM539. Inactivation of the *R. phaseoli recA* gene (pJM132) reduced the plating efficiency of $\lambda$F-1 to the level of HB101.

In order to determine the nucleotide sequence of the *R. phaseoli recA* gene, the 6 kb insert fragment of pJM112 was digested with various restriction endonucleases and ligated into the appropriate restriction sites of pUC19 (Vieira and Messing 1982). Recombinant pUC19 clones were sequenced essentially as described by Chen and Seeburg (1985) using [$^{35}$S]dATP. Sequencing reactions of both strands of the cloned fragments were primed with the pUC universal and reverse sequencing primers. The *Sal*I, *Hin*dIII and *Eco*RI restriction sites were confirmed by overlapping sequencing of pJM121 probed with synthetic oligonucleotide primers. Sequence data were analysed using the software packages of PCGene.

The sequencing strategy and nucleotide sequence of the coding strand are shown in Figs. 1 and 3. Alignment of the sequence with the *E. coli recA* sequence (Sancar et al. 1980) revealed the presence of one start and one stop codon consistent with an open reading frame (ORF) of 1080 nucleotides. The putative ATG initiation codon is preceded by a potential ribosome binding site, AAGG, at position −12. Potential transcription regula-

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    CAAGAATCTTCTTCTTCATTCAACCGCCTTCCGATGCCGAAGCGCGAATCGAGACGGGC  -183
CTTCTGCGGCCAAGGCCGGCGCATCTGGCCTAAGCCGTTGCTTGCATCGATAAAACAAATATA  -120
GAACACGGGCTGTTTTACCTTTTTCTTTCAATCGTTTGATCGCCTATTGCGAGAGTGGAACAA   -57
ATAGGGTACAAACTCGACATTGCTTGGGCGGCTTCAATAACCTAAAGGTGGATCAGATG TCT     6
                                                        Met Ser

CAG AAT TCA TTG CGG CTG GTA GAG GAC AAA TCG GTG GAC AAA AGC AAG    54
Gln Asn Ser Leu Arg Leu Val Glu Asp Lys Ser Val Asp Lys Ser Lys

GCG CTT GAA GCG GCA CTC TCA CAG ATT GAG CGG TCG TTC GGC AAG GGC   102
Ala Leu Glu Ala Ala Leu Ser Gln Ile Glu Arg Ser Phe Gly Lys Gly

TCG ATC ATG AAA CTC GGT TCC AAC GAG AAC GTC ATC GAA ATC GAG ACG   150
Ser Ile Met Lys Leu Gly Ser Asn Glu Asn Val Ile Glu Ile Glu Thr

ATT TCG ACG GGT TCG CTG GGG CTC GAT ATC GCG CTT GGC GTC GCG CCT   198
Ile Ser Thr Gly Ser Leu Gly Leu Asp Ile Ala Leu Gly Val Ala Pro

GCG AAG GGC CGC ATC ATC GAA ATC TAC GGG CCG GAA AGC TCA GGC AAG   246
Ala Lys Gly Arg Ile Ile Glu Ile Tyr Gly Pro Glu Ser Ser Gly Lys

ACG ACG CTG GCG CTG CAG ACC ATC GCG GAA TCG CAG AAG AAG GGC GGC   294
Thr Thr Leu Ala Leu Gln Thr Ile Ala Glu Ser Gln Lys Lys Gly Gly

ATC TGC GCC TTT GTC GAC GCC GAA CAT GCG CTC GAT CCG GTC TAT GCC   342
Ile Cys Ala Phe Val Asp Ala Glu His Ala Leu Asp Pro Val Tyr Ala

CGC AAG CTT GGC GTC GAT CTC CAG AAC CTT CTG ATC TCG CAG CCC GAT   390
Arg Lys Leu Gly Val Asp Leu Gln Asn Leu Leu Ile Ser Gln Pro Asp

ACC GGC GAG GAG GCG CTT GAG ATT ACC GAT ACG CTG GTG CGC TCC GGC   438
Thr Gly Glu Gln Ala Leu Glu Ile Thr Asp Thr Leu Val Arg Ser Gly

GCC GTC GAC GTT CTG GTC GTC GAC TCG GTC GCC GCA CTG ACG CCG CGT   486
Ala Val Asp Val Leu Val Val Asp Ser Val Ala Ala Leu Thr Pro Arg

GCC GAA ATC GAA GGT GAA ATG GGC GAC AGC CTT CCC GGC TTG CAG GCA   534
Ala Glu Ile Glu Gly Glu Met Gly Asp Ser Leu Pro Gly Leu Gln Ala

CGT CTG ATG AGC CAG GCG CTG CGC AAG CTG ACC GCC TCG ATC TCC AAG   582
Arg Leu Met Ser Gln Ala Leu Arg Lys Leu Thr Ala Ser Ile Ser Lys

TCG AAT ACG ATG GTG ATC TTC ATC AAC CAG ATC CGC ATG AAG ATC GGC   630
Ser Asn Thr Met Val Ile Phe Ile Asn Gln Ile Arg Met Lys Ile Gly

GTC ATG TTC GGT TCG CCT GAG ACA ACG ACG GGC GGC AAT GCG CTG AAA   678
Val Met Phe Gly Ser Pro Glu Thr Thr Thr Gly Gly Asn Ala Leu Lys

TTC TAT GCC TCC GTG CGC CTG CAC ATC CGT CGC ATC GGC TCG GTC AAG   726
Phe Tyr Ala Ser Val Arg Leu His Ile Arg Arg Ile Gly Ser Val Lys

GAG CGC GAA GAG GTG ATC GGC AAC CAA ACC CGC GTC AAG GTC GTC AAG   774
Glu Arg Glu Glu Val Ile Gly Asn Gln Thr Arg Val Lys Val Val Lys

AAC AAG ATG GCG CCT CCC TTC AAG CAG GTC GAA TTC GAC ATC ATG TAT   822
Asn Lys Met Ala Pro Pro Phe Lys Gln Val Glu Phe Asp Ile Met Tyr

GGC GAA GGC GTA TCG AAG ACC GGC GAA CTC GTC GAT CTC GGC GTC AAG   870
Gly Glu Gly Val Ser Lys Thr Gly Glu Leu Val Asp Leu Gly Val Lys

GCC GGC ATC GTC GAA AAG TCG GGC GCC TGG TTT TCC TAT AAC AGC CAG   918
Ala Gly Ile Val Glu Lys Ser Gly Ala Trp Phe Ser Tyr Asn Ser Gln

CGT CTC GGC CAG GGC GGC GAA AAC GCC AAG ACC TTC CTG CGC GAC AAT   966
Arg Leu Gly Gln Gly Gly Glu Asn Ala Lys Thr Phe Leu Arg Asp Asn

CCC GAT CTC GCC CGT GAG ATC GAG CTG GCG TTG CGC GAA AAT GCC GGT  1014
Pro Asp Leu Ala Arg Glu Ile Glu Leu Ala Leu Arg Glu Asn Ala Gly

CTC ATC GCA GAC CGC TTC CTG CAG AAC GGC GGG CCG GAT GCA GAC GAC  1062
Leu Ile Ala Asp Arg Phe Leu Gln Asn Gly Gly Pro Asp Ala Asp Asp

GGC GAT GGC GCG GAC ATG TAA ATTCCGCGTTTGAATGCATCGACCATTCCAGAGCAT 1119
Gly Asp Gly Ala Asp Met  *

TCCTGATGAGAATGCGGCCGGTTTCGTTTGTCGGCTGGACAGTGGCGGAGGCGAACGTTAAAA  1182
GCCGATGGATTTGTATTATCTGTCCGGCAGCATTGAAGGGCATAGAATGAGTGGTGTGAACGA  1245
TATCCGGTCGAC                                                      1257
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**Fig. 3.** Nucleotide sequence and deduced amino acid sequence of the *Rhizobium phaseoli recA* gene. The nucleotides are numbered starting from the first nucleotide of the ATG initiation codon. The termination codon is at nucleotide 1081. The putative ribosome binding site and the putative transcriptional regulatory sequences are *underlined*. The corresponding amino acid sequence is presented

tory sequences are located at positions −118 and −141. No *rho*-independent terminator structure was found. One additional putative ATG start codon is located at position 109. It would determine an ORF of 324 codons. However the resulting polypeptide would lack the amino-terminal region found in other RecA proteins (Sancar et al. 1980; Owtthrim and Coleman 1989; Ball et al. 1990).

The *R. phaseoli recA* ORF of 1080 nucleotides codes for a protein of 360 amino acids with a predicted molecular mass of 38.61 kDa. Interestingly this protein has an extension of 9 amino acids at its amino-terminus when compared with the *E. coli* RecA protein. A computer search in GeneBank of this extended sequence did not reveal any significant homology with other amino-terminal sequences. No *E. coli*-like LexA repressor binding site was detected in the upstream region of the *R. phaseoli recA* gene.

The cloned *R. phaseoli recA* gene restored the ability of an *E. coli recA* mutant to repair DNA damaged by UV irradiation or by the DNA alkylating agent MMS. Both treatments are known to induce the SOS response in wild-type *E. coli*. The UV sensitivity of the *E. coli recA* mutant HB101 was only partially suppressed when compared with the wild-type level. A similar situation has been reported for the *recA* genes of *Rhizobium meliloti* (Better and Helinski 1983) and *Agrobacterium tumefaciens* (Miles et al. 1986). These differences may be attributed to differences in expression or activity of the cloned genes in *E. coli*. Secondly, homologous recombination was restored in the *E. coli* strain JC14604 as concluded from the formation of $Lac^+$ papillae in transformant colonies. The 6 kb *Eco*RI fragment carrying the *recA* gene was able to suppress the $Fec^-$ phenotype of *red gam* phages, showing that the *R. phaseoli recA* gene was responsible for the growth of the originally isolated λRprecA clones on HB101.

Comparison of the deduced amino acid sequence with the *RecA* proteins of *R. meliloti* and *E. coli* confirmed the identity of the *R. phaseoli* RecA protein (Fig. 4). The *R. phaseoli* RecA protein is highly homologous to both RecA proteins. When aligned with the *R. phaseoli* RecA protein, the *E. coli* and *R. meliloti* RecA proteins contained 65% and 90% identical amino acid residues respectively. Comparison of the *R. phaseoli* RecA protein with the RecA protein sequences of *E. coli* (Sancar et al. 1980), *R. meliloti* (Buikema 1985), *Serratia marcescens* (Ball et al. 1990), *Anabaena variabilis* (Owtthrim et al. 1989) and *Pseudomonas aeruginosa* (Sano and Kageyama 1987) revealed the conservation of stretches of identical amino acid residues (Fig. 4). Most conserved are two blocks of 12 and 18 amino acids at positions 73 to 84 and 216 to 233. The frist of these blocks contains the consensus pattern [A/G]-X(4)-G-K-[S/T]. This sequence, known as the 'A' consensus sequence, is thought to interact with one of the phosphate groups of ATP in a great number of ATP or GTP binding proteins (Walker et al. 1982). Divergence between the RecA proteins is found primarily in the C-terminal region.

Two lines of evidence suggest that, when expressed in *E. coli*, the *R. phaseoli* RecA protein is not transcriptionally regulated by the *E. coli* LexA repressor. First, no *E. coli* SOS box was found in the 5′ upstream region of the *R. phaseoli recA* gene. The absence of a LexA repressor binding site may result in constitutive expression of the *R. phaseoli recA* gene in *E. coli*. Second, UV or MMS mediated activation of the RecA protein resulted in a 1.4-fold increase in the recombination frequency of *E. coli* JC14604 harbouring a *recA* containing plasmid. This increase is small when compared with the 2-fold to 15.3-fold enhancement of recombination frequency found with the *recA* genes of *Aeromonas caviae* (Resnick and Nelson 1988) and *Vibrio anguillarum* (Singer 1989) in *E. coli* in the presence of DNA damaging agents. The 1.4-fold increase in recombination frequency can possibly be attributed to the generation of single stranded DNA regions as a result of DNA damage.

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Rp   MSQNSLRLVEDKSVDKSKALEAALSQIERSFGKGSIMKLGSNENVIEIET      50
Rm    A                                      AKDS VK       50
Ec   ---------MAIDEN Q   A   G   KQ       R  -EDRSMDV      40

Rp   ISTGSLGLDIALGVAP-AKGRIIEIYGPESSGKTTLALQTIAESQKKGGI    99
Rm   V          IGGLP                                A     100
Ec         S     AGGLPM   V              T  V  AA RE KT    90

Rp   CAFVDAEHALDPVYARKLGVDLQNLLISQPDTGEQALEITDTLVRSGAVD    149
Rm    G                  E                            I    150
Ec      I        I        ID   C            C A A         140

Rp   VLVVDSVAALTPRAEIEGEMGDSLPGLQARLMSQALRKLTASISKSNTMV    199
Rm   I  I      V                M                      C   200
Ec    I         K      I   HM  A  M    M   AGNLKQ   LL    190

Rp   IFINQIRMKIGVMFGSPETTTGGNALKFYASVRLHIRRIGSVKEREEVIG    249
Rm                                        D              V   250
Ec                  N                     D     A   G N V   240

Rp   NQTRVKVVKNKMAPPFKQVEFDIMYGEGVSKTGELVDLGVKAGIVEKSGA    299
Rm                                        I                  300
Ec   SE          I A    A  Q L     INFY          EKLI  A     290

Rp   WFSYNSQRLGQGGENAKTFLRDNPDLAREIELALRENAGLIADRFLQNGG    349
Rm             R    L   E  E L    T   Q          E          350
Ec    Y  KGEKI    KA  TAW K   ET K    KKV  LLLSNPNSTPDFSV  340

Rp   PDADDGDGADM
Rm    ES GDEA                                              360
Ec   D SEGVAETNEDF                                         361
                                                            353
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**Fig. 4.** Comparison of the *Rhizobium phaseoli* (Rp) RecA protein with the RecA proteins of *Escherichia coli* (Ec) and *Rhizobium meliloti* (Rm) and mapping of the most conserved regions of the RecA protein. Insertions made to align both sequences are indicated by *dashes*. The deduced amino acid sequences of the *recA* genes of *R. phaseoli, E. coli, R. meliloti, Serratia marcescens, Anabaena variabilis* and *Pseudomonas aeruginosa* were aligned, and identical amino acids are *underlined*

Homologous genetic recombination between repeated elements may be a major source of symbiotic variability of commercial inocula of *R. phaseoli*. Therefore, the isolation of the *R. phaseoli recA* gene may constitute an important step in the engineering of stable *R. phaseoli* inocula.

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