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Cloning, sequencing and complementation analysis of the *recA* gene from *Prevotella ruminicola*

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Abstract

Degenerate PCR primers based on conserved RecA protein regions were used to amplify a portion of recE from *Prevotella ruminicola* strain 23, which was used as a probe to isolate the full-length *recA* gene from the *P. ruminicola* genomic library. The *P. ruminicola recA* gene encoded a protein of 340 amino acids with a molecular mass of 36.81 kDa. *P. ruminicola* RecA was highly similar to other RecA proteins and most closely resembled that of *Bacteroides fragilis* (75% identity). It alleviated the methyl methanesulfonate and mitomycin C sensitivities of *Escherichia coli recA* mutants, but did not restore the resistance to UV-light irradiation. Mitomycin C treatment of otherwise isogenic *E. coli* strains showed a higher level of prophage induction in a *recA* harboring lysogen.

Keywords: RecA; *Prevotella ruminicola*; *Escherichia coli*; Complementation

1. Introduction

The RecA protein plays an essential role in cellular metabolism, being directly involved in homologous recombination, DNA strand exchange, repair of damaged DNA, and coprotease activity resulting in the SOS response, prophage induction, and mutagenesis subsequent to LexA cleavage [1–5]. Cloning and molecular analysis of *recA* is one of the main steps in genetic characterization of bacterial strains. It creates the possibility for construction of mutants by gene disruption or gene replacement techniques.

To date, there have been no reports of the isolation of *recA* from ruminal bacteria. We present here our results on cloning and characterization of the *recA* gene from *Prevotella ruminicola*, which is a major bacterial species in the rumen [6–8].

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

Bacterial strains are listed in Table 1. The type strain of *P. ruminicola* subsp. *ruminicola* JCM8958[T]

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Table 1
Bacterial strains, plasmids, and phages used

Designation	Characteristics/genotype	Reference or source
Bacterial strains		
Escherichia coli		
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac*[F′*proA+B+, lacI*^{q}*ZΔM15,* Tn*10(tet*^{r}*)*]	Stratagene
XL1-Blue MR	*recA1, endA1, gyrA96, thi-1, supE44, relA1, lac, Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173*	Stratagene
XL1-Blue MR (λ)	XL1-Blue MR (λ (*cI857Sam7*) lysogen)	This study
XL1-Blue MRF′	*recA1, endA1, gyrA96, thi-1, supE44, relA1, Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, lac* [F′*proA+B+, lacI*^{q}*ZΔM15,* Tn*10(tet*^{r}*)*]	Stratagene
HB101	*recA13, Δ(mcrC-mrr), HsdS-(r-m-), supE44, ara14, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1*	Bachmann [9]
JM107	*endA1, gyrA96, thi-1, hsdR17, supE44, relA1, e14-(mcrA), Δ(lac-proAB),* [F′*traD36, proA+B+, lacI*^{q}*ZΔM15*]	Yanish-Perron et al. [10]
JM109	JM107 (*recA1*)	Yanish-Perron et al. [10]
*Prevotella ruminicola*JCM8958^T	ATCC 19189^T, the type strain of *Prevotella ruminicola*	JCM[a] [6]
Phages		
Lambda ZAPII (4/1)	Contains 8.2 kb insert with *recA* of *P. ruminicola*	This study
Lambda	*cI857Sam7*	Toyobo
Plasmids		
pSK4/1	In vivo excision product of Lambda ZAPII(4/1)	This study
pNHE1	4.1 kb fragment from pSK4/1 subcloned into pBluescript KS+, P_{lac}-*recA* transcriptional fusion	This study
pNHEΔE1	pNHE1, contains the mutant RecA with 71 amino acids deleted from the C-terminus	This study
pACYCREC7	8.2 kb fragment with *recA* of *P. ruminicola* cloned into low copy number plasmid pACYC184	This study
Charomid 9-20	Contains a spacer of seven 2074 bp direct repeats	Toyobo

[a]JCM, Japan Collection of Microorganisms, Wako, Japan.

(= ATCC 19189^T), strain 23 [6], was obtained from the Japan Collection of Microorganisms (JCM) and maintained as described earlier [11]. *Escherichia coli* strains were grown in LB medium. Solid media contained 1.8% agar. Ampicillin (50 μg/ml) or chloramphenicol (30 μg/ml) was added when required.

2.2. DNA techniques

Techniques for manipulating DNA, including plasmid preparations, chromosomal DNA isolation, restriction endonuclease digests, ligations, transformation into *E. coli*, and gel electrophoresis, were performed according to standard protocols [12]. The Gene Amp kit (Perkin-Elmer Cetus) was used for the PCR. PCR primers and PCR conditions for the amplification of the 360 bp internal fragment of *recA* were as described [13]. DNA fragments were purified from agarose gels using the Geneclean II kit (Bio 101 Inc., USA).

2.3. DNA library construction and screening

An *Eco*RI partially digested genomic library of *P. ruminicola* JCM 8958 was constructed in Lambda Zap II (Stratagene) and screened using the randomly labeled DNA fragment of *recA*, obtained by PCR amplification, as a probe.

2.4. DNA sequence analysis

The dideoxy chain termination method of DNA sequencing was carried out on double-stranded DNA templates with the *Taq* Dye Primer and Dye

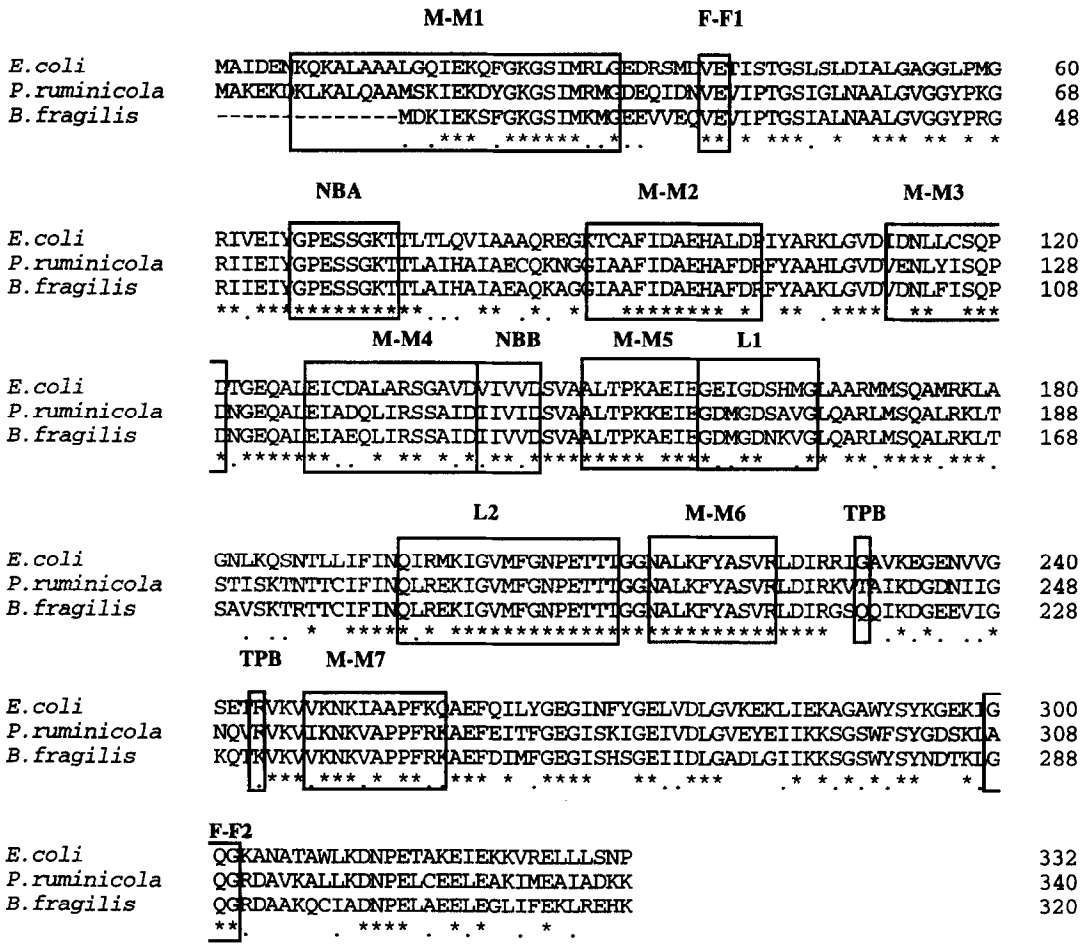

Fig. 1. Comparison of the amino acid sequences of RecA from *B. fragilis* [14], *P. ruminicola* and *E. coli*, numbered according to [15]. Amino acid sequence alignment was achieved using the program CLUSTAL W [16]. Residues from 7 to 14 and from 333 to 353 in the *P. ruminicola* and *E. coli* amino acid sequences were omitted to improve the alignment quality. The numbers denote the positions of the residues within their respective full-length sequences. Identical (*) and similar (·) residues are indicated. Functional regions, as have been compiled recently [17], are boxed and indicated: NBA, nucleotide binding domain; NBB, nucleotide hydrolysis region; M-M1 to M-M,-monomer-monomer interaction regions; F-F1 and F-F2, filament-filament contact positions; TPB, target protein binding site; L1 and L2, DNA binding loops.

Terminator Cycle sequencing kits (Applied Biosystems) on a Perkin-Elmer PCR apparatus. Sequencing reactions were analyzed on an automatic sequencer (373A DNA sequencer; Applied Biosystems). Sequencing primers were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer. The sequence was determined completely in both strands.

2.5. *Mitomycin C and methyl methanesulfonate survival measurements*

E. coli cells were grown to $OD_{600} = 0.6$ in LB medium, serially diluted, and spread directly onto LB agar containing mitomycin C (MC) or methyl methanesulfonate (MMS) at different concentrations. Viability was determined after 20–24 h growth at 37°C.

Table 2
Survival of *E. coli* strains on plates containing methyl methanesulfonate (0.01%, final concentration)

Strains and plasmids	Fraction surviving (%)
E. coli JM109 (pBluescript KS)	0.15
E. coli JM107 (pBluescript KS)	2.0
E. coli JM109 (pNHE1)	1.88
E. coli JM109 (pNHE1)+IPTG[a]	1.13
E. coli JM109 (pNHEΔE1)	0.08
E. coli XL1-Blue (pBluescript KS)	0.39
E. coli XL1-Blue (pNHE1)	26.0
E. coli XL1-Blue (pNHE1)+IPTG	18.0
E. coli XL1-Blue (pNHEΔE1)	1.13
E. coli XL1-Blue (pNHEΔE1)+IPTG	0.06
E. coli HB101 (pBluescript SK)	< 0.03
E. coli HB101 (pSK4/1)	18.33

[a]Transcription from the external *lac* promoter was induced by adding IPTG to a final concentration of 1 mM and incubating for 30 min before plating. Triplicate counts were made for each sample.

2.6. UV-light sensitivity test

Exponentially growing *E. coli* cells ($OD_{600} = 0.6$) were plated at several dilutions on LB plates and UV-light irradiated at different doses using the Stratalinker® UV Crosslinker (Stratagene) operated in energy mode. Plates were incubated at 37°C in the dark for 20–24 h, and UV sensitivity was evaluated by colony counting.

2.7. Lysogenic induction

The lysogenic *E. coli* strain, XL-1 Blue MR (λ) (Table 1), transformed either by a control vector or by the *recA* harboring recombinant plasmid, was grown at 26°C to $OD_{600} = 0.6$ and the SOS response was induced by adding MC (0.5 μg/ml) or MMS (0.01%, v/v). Control thermal induction was done by increasing the cultivation temperature to 37°C. 100 μl aliquots were taken at 10 min intervals and the phage titer was determined by standard methods [12].

2.8. SDS-PAGE and protein sequencing

E. coli cultures were grown to $OD_{600} = 0.6$ in LB medium, chilled on ice and washed once in cold TE buffer containing phenylmethylsulfonyl fluoride (40 μg/ml), resuspended in the same buffer and sonicated on ice using a Branson Sonifier (Branson, USA). Cell debris was removed by centrifugation and samples were frozen at −80°C. SDS-PAGE was performed in 12% resolving gels. For protein sequencing RecA was fractionated by SDS-PAGE and electroblotted onto a PVDF membrane (BioRad).

The N-terminal sequence was determined by automated Edman sequencing using a 470 gas-phase sequencer (Applied Biosystems).

3. Results

3.1. Cloning of recA

RecA fragment of *P. ruminicola* was PCR amplified from the total DNA preparation using a pair of primers complementary to highly conserved regions of the protein [13]. This fragment was cloned, sequenced and found to be similar to other *recA* genes, with highest identity to the *B. fragilis recA* gene [14]. The fragment was randomly labeled and used to screen a *P. ruminicola* Lambda ZAPII (Stratagene) library. Seven clones were selected this way and two of them were purified further using the same probe. After in vivo excision, the plasmids were subjected to restriction mapping, subcloning and sequencing steps.

3.2. Sequence features

The *P. ruminicola recA* sequence has been submitted to GenBank, accession number U61227. An open reading frame (ORF) having high similarity to published *recA* sequences can be identified within the sequenced region. This ORF encodes a 36810 Da protein containing 340 amino acid residues. However, there was also the possibility to translate this frame from the second methionine residue, generating thereby a 34403 Da protein containing 318 amino acid residues which would have the same size and higher similarity (81% identity) to *B. fragilis* RecA [14]. RecA from the recombinant *E. coli* HB101 strain was therefore fractionated on SDS-PAGE, and the sequence of the first 10 N-terminal amino

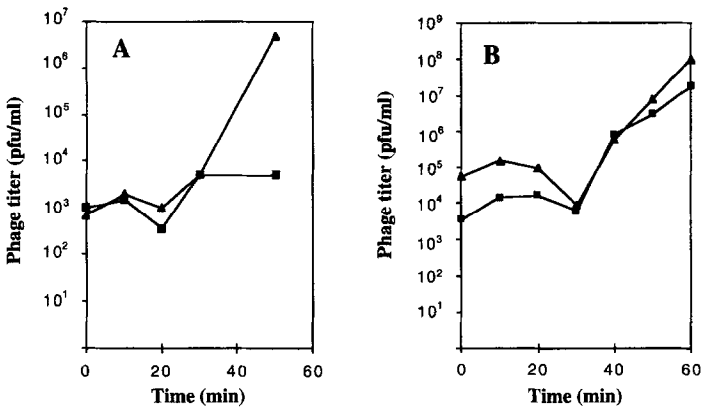

Fig. 2. (A) Lysogenic induction of *E. coli* strains by MC (0.5 μg/ml) treatment: squares, XL1-Blue MR (λ); triangles, the isogenic strain carrying *recA* of *P. ruminicola*. MC was added at 0 min. (B) Thermoinduction of the lysogenic *E. coli* strains by increasing the cultivation temperature from 26°C to 37°C: squares, XL1-Blue MR (λ); triangles, the isogenic strain carrying *recA* of *P. ruminicola*. Cultures were transferred to 37°C at 0 min.

acid residues was determined. The amino acid sequence obtained, AKEKDEALSP, corresponds to the larger ORF, the initial methionine residue presumably having being removed posttranslationally from the protein. A number of conserved amino acid residues of functional importance can be identified in the amino acid sequence when compared with other published data (Fig. 1).

The ORF is preceded by a 50 bp AT-rich (82%) region with no apparent ribosome binding site. Many of the *recA* genes isolated from Gram-negative bacteria present an SOS box ($CTG(N_{10})CAG$) like that of *E. coli* [18] which is the consensus binding site for the LexA repressor. This nucleotide sequence is not represented in the 500 bp upstream from the translational start of *P. ruminicola recA*. Downstream of the ORF, a putative rho-independent terminator with a free energy of $\Delta G = -27.8$ kcal/mol can be detected.

3.3. Complementation of MC and MMS sensitivities in E. coli recA mutants

Complementation of sensitivities to these DNA damaging agents by *P. ruminicola recA* was tested in various *E. coli* genetic backgrounds. Results on complementation of the MMS sensitivity are shown in Table 2: *E. coli recA* mutants, harboring the cloned *P. ruminicola recA* gene, exhibited a viability comparable with an *E. coli* $recA^+$ phenotype. The mutant with 71 amino acid residues deleted from the C-terminus was inactive in these complementation tests (Table 2). Strains which were induced by IPTG prior to the MC or MMS exposure did not show any difference in survival as compared with a non-induced state (Table 2). Heterologous expression of the gene in *E. coli*, therefore, is considered to be controlled by its own regulatory regions with a negligible effect of the vector sequences.

3.4. Resistance to UV-light irradiation

No difference in the survival rate after the UV-light exposure was found between the *E. coli recA* mutants carrying or not carrying the *recA* locus of *P. ruminicola*. At the same time, the recA-proficient *E. coli* strain JM107, which differs from the JM109 strain only by the presence of the functional gene, was almost unaffected by the doses used in these experiments (up to 65 J/m^2) (data not shown).

3.5. Lysogenic induction

Temperate bacteriophages are induced following treatment of lysogenic bacteria with DNA damaging agents. This induction is part of the SOS response and requires a functional RecA protein in *E. coli* [19]. This function of RecA from *P. ruminicola* was estimated in the lysogenic *E. coli* strain XL1-Blue MR (λ) upon MC (0.5 μg/ml) induction (Fig. 2A).

More than 1000-fold increase in the phage titer within 50 min was observed in the *recA* harboring strain, while in the isogenic *recA*-negative background it increased less than 10-fold. The control thermoinduction of lytic cycles in both strains produced kinetics similar to lysogenic induction, with a higher level of background induction in the case of the RecA producing strain (Fig. 2B). For unknown reasons, there was no apparent lysogenic induction when MMS was used as a DNA damaging agent (data not shown).

3.6. Recombination test

In these experiments two compatible plasmids, pACYC184 carrying *recA* of *P. ruminicola* and Charomid 9-20 having a spacer of seven 2047 bp direct repeats were cotransformed into the *recA* mutant *E. coli* strain JM109. It was presumed that RecA would promote strand exchange between the repeated regions of Charomid 9-20 and the recombination products would be seen as a ladder on gels after digestion at the single *Bam*HI site of the plasmid. These products, however, were not detected, either in untreated or in MC or MMS treated cultures (data not shown).

4. Discussion

The availability of increasing numbers of recA sequences opens new possibilities for cloning of the *recA* genes in addition to complementation techniques used in earlier studies. Here it was shown that *P. ruminicola recA* was unable to protect a *recA*-deficient *E. coli* strain against damage caused by UV-irradiation. The approach based on the amplification of the conserved regions of the gene with subsequent library screening may be helpful in such cases.

The DNA sequence 500 bp upstream of *P. ruminicola recA* is exceptional in that it appears to lack the SOS box ($CTG(N_{10})CAG$) characteristic of LexA-mediated promoters. This observation raises the possibility that a divergent promoter may be recognized by *P. ruminicola* LexA. There is also no apparent conventional ribosome binding site upstream from the translational start of *P. ruminicola recA*.

Functions encoded by *recA* appear to have been conserved during bacterial evolution. It was shown that dysfunctions in the *E. coli* gene can be complemented by *P. ruminicola recA*. At the same time, it failed to restore the resistance to UV irradiation and did not form recombination products from a template having extensive direct repeats. Since RecA of *P. ruminicola* alleviates other sensitivities (Table 2) and bears functionally important conserved aa residues (Fig. 1), this deficiency cannot be explained in terms of poor expression or of absence of functional domains. We found that the *P. ruminicola recA* gene is overexpressed in *E. coli*, producing RecA as a major protein product in recombinant strains (unpublished data). The constitutive increase of RecA protein in *E. coli* has been shown to inhibit pyrimidine dimer excision and reduce the survival rate of UV-irradiated cells [20]. These data support the view that overproduction of RecA may mask the dimers with subsequent reduction of their excision and switching off the SOS signal [20]. A similar scenario may take place in *E. coli* cells overproducing *P. ruminicola* RecA. Further investigations are necessary to identify the regulatory region(s) of the gene as well as the SOS response events in the original host.

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