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Cloning and expression of recombinase A from *Meiothermus ruber*

Milda Pleckaityte[1],

Lina Baranauskiene[1],

Gintautas Zvirblis[1],

Violeta Popendikyte[2],

Aurelija Zvirbliene[1]*

[1] *Institute of Biotechnology, V. A. Graičiūno 8, LT-02241 Vilnius, Lithuania*

[2] *UAB Fermentas, V. A. Graičiūno 8, LT-02241 Vilnius, Lithuania*

The recombinase A (RecA) plays a critical role in the repair and maintenance of DNA. Interaction of the RecA protein with DNA and a nucleotide triphosphate cofactor, such as ATP, is essential for its ability to catalyze homologous pairing and subsequent transfer of strands among a variety of DNA substrates *in vitro*. Both mesophilic and thermophilic microorganisms were found to express the RecA protein. Proteins from thermophilic bacteria are of particular interest in the recent years because of their heat-stable properties. In the current study, the RecA protein from moderately thermophilic bacteria *Meiothermus ruber* has been cloned, expressed in *E. coli* and purified. The recombinant RecA from *M. ruber* has been found in the soluble fraction of transformed *E. coli* cells. The RecA from *M. ruber* is the first protein of this family, identified in moderately thermophilic bacteria so far. It shows a high sequence homology with the RecA protein from *T. thermophilus*. The RecA from *M. ruber* purified to homogeneity might be useful for the optimization of PCR-based assays.

Key words: *recA* gene, *Meiothermus ruber*, recombinase, thermophilic bacteria

INTRODUCTION

Recombinases represent a large family of proteins that participate in DNA replication, recombination and reparation [1]. RecA protein, a representative of the family of recombinases, catalyses homologous DNA pairing and strand exchange reactions that are crucial for recombination and recombinational repair in the cell [2, 3]. Homologous pairing requires formation of a complex of RecA and single-stranded DNA. This nucleoprotein filament can pair the bound single strand with the complementary strand of an incoming duplex, resulting in homologous recombination [2]. RecA is a DNA-dependent ATPase and contains a site for binding and hydrolyzing ATP [2].

The *E. coli recA* gene and protein are well characterized [2–4]. A number of other bacterial genes with sequence homology to the *E. coli recA* gene have been described, both in closely related enteric bacteria, e. g., *Proteus mirabilis* [5], and in more distant genera such as *Bacillus subtilis* [6], thermophilic bacteria [7, 8] and cyanobacteria.

Proteins from thermophilic bacteria are of particular interest in the recent years because of their heat-stable properties. A heat-resistant RecA protein from the thermophilic bacterium *Thermus thermophilus* has been isolated and fully characterized [7, 8]. *T. thermophilus* RecA was shown to eliminate a non-specific PCR product that creates the potential to perform multiplex PCR which is highly applicable in modern PCR-based diagnostic tests [9].

In the current study, we describe the cloning, expression in *E. coli* and purification of RecA protein from the moderately thermophilic bacterium *Meiothermus ruber* isolated from hot springs of Kamchatka. *M. ruber* is a gram-negative bacterium that does not form spores and has an optimal growth temperature in the range of 60–65 °C, versus 70–75 °C for *T. thermophilus* [10]. RecA proteins from moderately thermophilic bacteria have not been reported yet. We have also characterized the *M. ruber recA* gene and

* Corresponding author. E-mail: azvirb@ibt.lt

identified the putative *recA* promoter sequence. Recombinant RecA protein from *M. ruber* might be useful for the optimization of PCR-based assays.

MATERIALS AND METHODS

Cloning of *recA* gene from *M. ruber*

Genomic DNA was isolated from the RFL-1 strain of *M. ruber* (a kind gift of Fermentas, Vilnius, Lithuania) by standard procedures [11]. Several sets of degenerated primers were designed to be complementary to the conserved regions of RecA from *E. coli* and several thermophilic bacteria:
LEN-direct (dir) 5'-CTGGARAACGCCCTSAAG,
FGK-dir 5'-TTCGGCAARGGGGCGGTKATG,
VVD-dir 5'-GTRGTGGAYTCGGTGGCCGCYTTGGTSCC,
VVD-reverse (rev) 5'-GGSACCAARGCGGCCACCGARTCCACYAC,
QAR-dir 5'-CAGGCCCGGCTCATGAGCCAG,
QAR-rev 5'-CTGGCTCATGAGCCGGGCCTG,
VVK-dir 5'-TGGTGAAGAACAAGCTSGCCCCSCCCTTC,
VVK-rev 5'-GAAGGGSGGGGCSAGCTTGTTCTTCACCAC,
GSW-rev 5'-CCGTAGGAGAACCAGGAGCC, PEL-rev 5'-GATCTCCTCCARAAGCTCSGG; LGQ-rev 5'-TTCTCCTTCCCCTGGCCCAG

(R designates A/G; S – C/G; Y − G/A; K – G/T).

The PCR fragments of appropriate length were generated using three sets of the following primers: FGK-dir and VVD-rev; VVR-dir and VVK-rev; FGK-dir and QAR-rev, corresponding to amino acids 20–150, 141–255 and 20–166 in *T. thermophilus* RecA. The obtained PCR fragments were cloned into the plasmid pUC57/T (Fermentas) and sequenced. The sense primer 5'-CGGATCATCGAGATCTACGGCCCCG (BglII site underlined) resulting from the sequenced fragment FGK-QAR and antisense primer LGQ generated a 720 base pairs (bp) PCR fragment. The ewly generated 720 bp fragment was ligated with the FGK-QAR fragment using the BglII site.

The southern hybridization of restriction endonucleases cleaved genomic DNA was carried out using an [α-^{33}P] dCTP-labeled 850 bp fragment (PCR product obtained using FGK-dir and LGQ-rev primers). The putative promoter sequence and 120 nucleotides downstream of the stop codon of *recA* gene were determined by the inverted PCR method [12]. The PCR product of 1.3 kilobase pairs (kbp) was obtained using primers 5'-CGATGCCCAGGGCCATATCCA and 5'-GACCTGGTGACGGTAGCCATC. The nucleotide sequence of the PCR product was determined at the Sequencing Center of the Institute of Biotechnology, Vilnius.

To verify the cloned *recA* sequence, genomic DNA of *M. ruber* was digested with PstI, and the fragment of 2.0 kbp was cloned into the PstI-digested pUC18 plasmid. The insert was sequenced, and the sequence was aligned with the *recA* sequence obtained by PCR.

Nucleotide sequence accession numbers

The complete nucleotide sequence of the *M. ruber recA* gene has been deposited in the GenBank database under the Accession No. GU226763.1.

Expression of RecA from *M. ruber*

The NdeI and BamHI sites were introduced by PCR at the 5'- and 3'-ends of the *recA* gene, respectively. The amplified *recA* gene was ligated into the NdeI and BamHI sites of pET21b(+) plasmid (Merck, Darmstadt, Germany).

M. ruber RecA was expressed in the *E. coli* strain BL21(DE3). Protein expression was induced with 1 mM IPTG (isopropyl-b-D-thiogalactopyranoside). After induction, the cell pellet was disrupted by sonication and centrifuged. The supernatant (soluble fraction) and the cell pellet (insoluble fraction) were then analyzed by 12.5% polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of recombinant RecA from *M. ruber*

Purification of the RecA from *M. ruber* was performed as described in Shigemori et al. [9], with minor modifications. A biomass obtained from 1 L of induced culture was resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 25% (w/v) sucrose and 1 M KCl, and disrupted by sonication. The debris was removed by centrifugation, and the supernatant was heated 60 min at 55 °C and centrifuged to remove the precipitate. The supernatant was dialyzed against 20 mM of potassium phosphate (pH 6.5) buffer containing 10% of glycerol and 1 mM EDTA, and applied to phosphocellulose (P11 Cellulose; Whatman, Japan). The RecA protein was eluted with a linear gradient of KCl (0–1.5 M). Fractions containing RecA were combined. The sample was dialyzed against a buffer containing Tris-HCl (pH 7.8), 0.1 mM EDTA, 1.5 M KCl.

RESULTS AND DISCUSSION

Characterization of the *recA* gene of *M. ruber*

The *M. ruber recA* gene was cloned using the degenerated oligonucleotide primers designed to be complementary to the conserved regions of RecA from several thermophilic bacteria and *E. coli*.

Southern blot hybridization revealed two restriction endonuclease PstI sites flanking the *recA* gene. The *M. ruber recA* sequence obtained by PCR was verified by sequencing the 2.0 kbp DNA fragment of *M. ruber* genomic DNA obtained by PstI digestion. Comparison of these two *recA* sequences did not indicate any sites of discrepancy. We have deposited *M. ruber recA* gene with flanking sequences in the GenBank database under the Accession No. GU226763.1.

Sequence alignment of deduced amino acid sequences of *M. ruber* RecA revealed a homology with proteins from several thermophilic bacteria, but the highest score was obtained for RecA from *T. thermophilus* (Table 1). The RecA protein from *M. ruber* revealed a 76% sequence homology with RecA protein from *T. thermophilus* and 56% with RecA from *E. coli*.

Alignment of deduced amino acid sequences of the RecA from *T. thermophilus*, *M. ruber* and *E. coli* revealed the presence of a highly-conserved ATP binding site (Fig. 1). The core of the ATP-binding site of the RecA protein is well-conserved, with four invariant amino acid residues (G / A) XXXXGK(T / S) [2]. The *E. coli* RecA sequence GPESSGKT matches the consensus sequence of amino acids for the Walker A box (also referred to as the P-loop) found in a number of nucleoside triphosphate (NTP)-binding proteins, such as representatives of the Hsp100 chaperone family [13] and kinases [14].

Table 1. **Comparison of deduced amino acid sequence of RecA from *M. ruber* with that of several thermophilic bacteria and *E. coli***

Strain	Degree of sequence identity, %
Bacillus stearothermophilus (Bacillales)	49.2
Streptococcus thermophilus (Lactobacillales)	52.7
Thermotoga maritima (Thermotogales)	54.3
Escherichia coli (Enterobacteriales)	56.5
Sphaerobacter thermophilus (Sphaerobacterales)	57
Natranaerobius thermophilus (Clostridia)	58
Aquifex pyrophilus (Aquificales)	58.6
Hydrogenobacter thermophilus (Aquificales)	60.0
Thermoanaerobacter sp. (Clostridia)	60.2
Rhodothermus marinus (Sphingobacteria)	60.5
Thermus thermophilus (Deinococci)	76.5

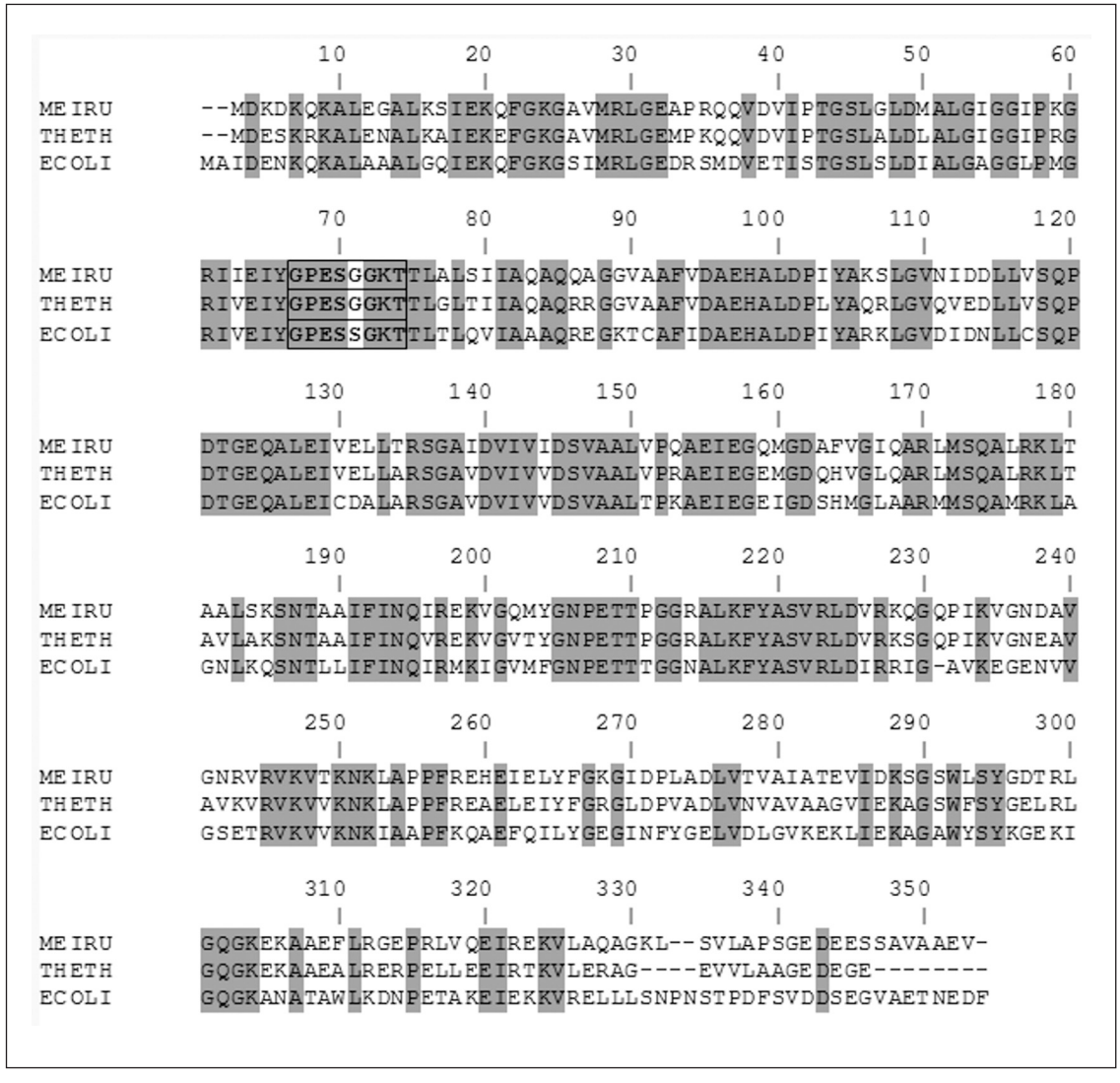

Fig. 1. Alignment of amino acid sequences of RecA from *M. ruber* (MEIRU), *T. thermophilus* (THETH) and *E. coli* (ECOLI). Identical amino acids are shown by letters on shaded background. Boxed letters indicate the ATP-binding site

Start	End	Score	Promoter Sequence
244	289	0.93	CAGTACCGTGTTTTGGAGCAGTTTCCACTACGTTGAATAG**C**CTCTGGTCG

Fig. 2. Promoter-like sequence upstream the *recA* gene determined by the Neural Network Promoter prediction program. Bold C indicates transcription initiation

Table 2. **Amino acid composition in RecA of thermophiles and mesophiles**

Strain	Content of amino acids			
	Proline, %	Cysteine, %	Arginine, %	Alanine, %
Escherichia coli (mesophile)	2.8	0.8	4	10.8
Proteus mirabilis (mesophile)	2.3	0.8	3.9	10.4
Bacillus subtilis (mesophile)	2.9	0	4.6	8.6
Meiothermus ruber (moderately thermophile)	4.3	0	4.9	11.7
Thermus thermophilus (extreme thermophile)	4.1	0	6.8	12.4
Aquifex pyrophilus (extreme thermophile)	3.4	0.6	5.7	8.3
Rhodothermus marinus (extreme thermophile)	3.5	0.6	6.6	12.1

The further analysis of a PCR fragment of *M. ruber* genomic DNA using the Neural Network Promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html) allowed us to identify a promoter-like sequence upstream the *recA* gene (Fig. 2).

Expression and purification of *M. ruber* RecA protein

Thermophilic proteins have shown an increase in the content of proline, charged amino acid residues (especially glutamic acid, arginine and lysine) and hydrophobic amino acids residues, namely alanine and leucine [15]. Differences in the amino acid composition of thermophilic and mesophilic RecA proteins are summarized in Table 2. As compared to mesophiles, RecA protein from *M. ruber* is characterized by an increased content of proline (4.3%), arginine (4.9%) and alanine (11.7%).

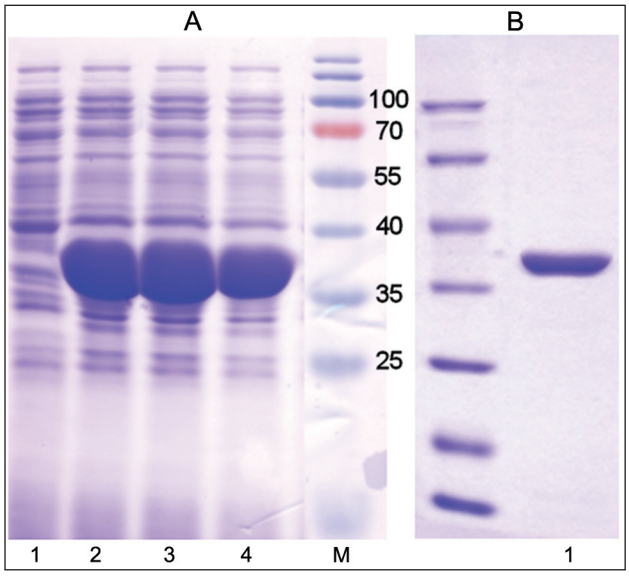

Fig. 3. SDS-PAGE of recombinant RecA protein expressed in *E. coli*.

A – total lysates of *E. coli* cells transformed with the plasmid carrying *recA* gene, B – purified RecA protein. Lane M – protein molecular mass marker (Fermentas, #SM0671), lane *1* – the lysate of non-transformed *E. coli* cells, lane *2–3* – total lysate of transformed *E. coli* cells after induction with 1 mM of IPTG

The recombinant RecA protein from *M. ruber* was successfully expressed in *E. coli* under the control of inducible T7 promoter (Fig. 3, A). The RecA protein was recovered from the soluble fraction of the total *E. coli* cell lysates. The yield of the recombinant RecA protein was approximately 60% of the total cell protein. The RecA protein was purified from the soluble fraction of the cell lysate to 98% homogeneity using ion-exchange chromatography (Fig. 3, B). Current efforts are directed toward application of purified thermophilic RecA protein from *M. ruber* to eliminate nonspecific PCR products.

In summary, we have cloned, for the first time, the *recA* gene from the moderately thermophilic bacterium *M. ruber*, determined its complete nucleotide sequence and identified the putative *recA* promoter sequence. The alignment of the deduced amino acid sequence of RecA from *M. ruber* with that of thermophilic and mesophilic bacteria revealed a high sequence homology (76%) between *M. ruber* and *T. thermophilus* RecA proteins. We have successfully expressed in *E. coli* the recombinant RecA protein from *M. ruber* and purified it to homogeneity from the soluble fraction of transformed *E. coli* cells. The exploitation of RecA proteins from various sources is expected to have a potential for improving the efficiency of PCR-based assays. The cloning and expression of RecA from moderately thermophilic bacteria may expand the application profile of thermostable proteins.

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REKOMBINAZĖS A IŠ *MEIOTHERMUS RUBER* GENO KLONAVIMAS IR RAIŠKA

Santrauka

Rekombinazė A (RecA) yra vienas pagrindinių baltymų, dalyvaujančių DNR rekombinacijoje ir reparacijoje. Sąveikaudamas su DNR ir kofaktoriumi nukleotidtrifosfatu ATP, baltymas RecA katalizuoja homologinių DNR susiporavimą ir grandinines reakcijas, kurios yra ląstelės rekombinacijos ir reparacijos procesų pagrindas. RecA baltymas aptinkamas tiek mezofiliniuose, tiek termofiliniuose mikroorganizmuose. Domėjimąsi termofilinių mikroorganizmų baltymais skatina šių baltymų panaudojimo galimybės. Karščiui atsparus RecA baltymas anksčiau buvo klonuotas iš termofilinės bakterijos *T. thermophilus* ir išsamiai apibūdintas. Nustatyta, jog *T. thermophilus* RecA pašalina nespecifinius PGR produktus daugine PGR paremtuose metoduose, kurie pastaruoju metu plačiai naudojami moderniuose diagnostiniuose testuose. Pirmą kartą *recA* geną klonavome iš nuosaikiai termofilinės bakterijos *Meiothermus ruber*, išskirtos iš Kamčiatkos karštųjų versmių. *M. ruber* yra sporų neformuojanti gram-neigiama bakterija, kurios optimali augimo temperatūra yra 60–65 °C, tuo tarpu *T. thermophilus* optimali augimo temperatūra – 70–75 °C. *M. ruber* RecA baltymas yra pirmasis šios šeimos baltymas, nustatytas nuosaikiai termofilinėse bakterijose, o jo amino rūgščių seka labiausiai panaši (homologija siekia 76 %) į *T. thermophilus* RecA baltymo amino rūgščių seką. Rekombinantinis *M. ruber* RecA buvo aptiktas tirpioje *E. coli* ląstelių frakcijoje ir išgrynintas. Homogeniškas RecA baltymas bus naudojamas polimerazės grandinine reakcija (PGR) paremtiems metodams optimizuoti. RecA baltymų, gautų iš įvairių termofilinių mikroorganizmų, panaudojimas suteiktų naujų žinių apie karščiui atsparius baltymus ir jų taikymo galimybes.

Raktažodžiai: *recA* genas, *Meiothermus ruber*, rekombinazė, termofilinės bakterijos