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UVSC of *Aspergillus nidulans* is a Functional Homolog of RAD51 in Yeast

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A defect in *uvsC* of *Aspergillus nidulans* caused high methyl methansulfonate (MMS)-sensitivity, hypo-recombination, and a lack of UV-induced mutation. The *uvsC* gene of *Aspergillus nidulans* shares a sequence similarity with the *RAD51* gene of *Saccharomyces cerevisiae*. In this study, *in vitro* and *in vivo* tests were conducted in order to determine whether or not the UVSC protein had functional similarities to RAD51, the recombination enzyme in yeast. The purified recombinant UVSC protein, following expression in *Escherichia coli*, showed binding activity to single-stranded DNA (ssDNA), when both ATP and magnesium are present. In addition, ATPase activity was also demonstrated and its activity was stimulated in the presence of ssDNA. The UVSC protein that was expressed under the ADH promoter in *S. cerevisiae* suppressed in part the sensitivity to MMS of the *rad51* null mutant. Similarly, when the *uvsC* cDNA was expressed from the *nmt* promoter, the MMS sensitivity of the *rhp51* null mutant of *Schizosaccharomyces pombe* was partially complemented. These results indicate that the *A. nidulans* UVSC protein is a functional homologue of the RAD51 protein.

Keywords: *Aspergillus nidulans*, ATPase, MMS sensitivity, RAD51, ssDNA binding, UVSC

Introduction

Since the *recA* mutant of *Escherichia coli* causes severe defects in genetic recombination, and is sensitive to DNA-damaging agents (Clark and Margulies, 1965), the RecA protein has been extensively studied. It is involved in homologous recombination, cellular SOS response to DNA damage, mutagenesis, proper partitioning of newly replicated chromosomes, and so on (reviewed in Miller and Kokjohn, 1990; Roca *et al.*, 1990; West, 1992; Kowalczykowski and Eggleston, 1994). The RecA protein plays a central role in genetic recombination. In the presence of ATP, RecA is able to polymerize DNA to form a right-handed helical nucleoprotein filament and promote homologous pairing and strand exchange. Also, it plays a regulatory role in the SOS response to DNA damage by catalyzing autoproteolysis of the LexA repressor, leading to the induction of about 20 genes that are involved in DNA repair.

In the yeast *S. cerevisiae*, genes of the RAD52 epistatic group are required for both homologous recombination and the repair of DNA double strand breaks (reviewed in Game, 1983; Paques and Haber, 1999). Mutations in these genes cause severe sensitivity to ionizing radiation and alkylating agents, reduced spontaneous and DNA damage-induced mitotic recombination, and a poor yield of viable spores (Petes *et al.*, 1991). *Rad51*, belonging to the RAD52 epistatic group, is a key component of these cellular processes. As expected from the sequence similarity between *RecA* and *RAD51*, the Rad51 protein has some of the RecA functional activities that include binding of single- and double-stranded DNA, ATP hydrolysis, formation of nucleoprotein filaments, and formation of heteroduplex DNA (Sung, 1994; Sung and Robberson, 1995; Sugiyama *et al.*, 1997). *RAD51* homologues have been subsequently identified in a wide range of higher eukaryotes as diverse as the lily, *Xenopus laevis*, mice, and humans. The sequences of the *RAD51* of all of the eukaryotic species are conserved within the core domain. Their role for recombination and repair is also conserved (reviewed in Baumann and West, 1998). In contrast to yeast *RAD51*, the *rad51* null mutant of mice shows early embryonic lethality, implying its essential role in cell viability (Lim and Hasty, 1996). The interaction of human RAD51 with several tumor suppressor genes, including p53, BRCA1, and BRCA2, implies its possible role in monitoring genome integrity and tumorigenesis (Sturzbecher *et al.*, 1996; Marmorstein *et al.*, 1998).

In *Aspergillus nidulans*, DNA repair defective mutants defined four *uvs* epistatic groups, based on the sensitivities of the single and double mutants to UV or MMS (Kafer *et al.*, 1986; Chae and Kafer, 1993). However, considering the

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properties of the mutants of each epistatic group of *A. nidulans*, none of the groups corresponds well to the RAD52 epistatic group of yeast. The *uvsC* and *uvsE* mutants of the UvsC epistatic group of *A. nidulans* are defective in meiosis and abolish spontaneous mitotic recombination, but they increase spontaneous mutation and usually lack UV-induced mutation (Jansen *et al.*, 1970; Jansen *et al.*, 1972; Kafer and Mayor, 1986; Chae and Kafer, 1997).

The *uvsC* gene is known to share a sequence homology to RAD51 (Seong *et al.*, 1997; van Heemst *et al.*, 1997). Targeted gene disruption of *uvsC* caused slow-growth phenotype and mutator effects (Seong *et al.*, 1997). Since *uvsC* affects both recombination and mutation processes, it was therefore of interest to analyze the function of the UVSC protein. In this study, the possible function of the UVSC protein on the recombination process was assayed *in vitro* by testing its binding activity to single-stranded DNA and ATPase activities. Moreover, an *in vivo* cross-species complementation test was also carried out by introducing the *uvsC-coding* region under appropriate promoters into *rad51* and *rph51* null mutants of yeast.

Materials and Methods

Strains and media *E. coli* DH5α (*supE44ΔlacU169* (φ*80lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was generally used for propagation and preparation of the recombinant plasmid. *E. coli.* HMS174(DE3) (F^-*recA1* *hsdR*($r_{k12}^-m_{k12}^+$)Rif^R (DE3)) was used for the over-expression of the UVSC protein in *E. coli.* All *E. coli* strains were grown in a standard LB medium (0.5% yeast extract, 1% bacto-tryptone, 1% NaCl) that was supplemented with ampicillin (50 μg/ml), if necessary. *Saccharomyces cerevisiae* XS803-3A/51Δ (*MATa leu2-3 ura3-52 his1-1 trp2 rad51Δ::LEU2*), SL560-3A (*MATa leu2-1 ura3-52 his3 trp1 met8-1*), and *Schizosaccharomyces pombe* JAC1/51Δ (*h⁺ ade6-704 leu1-32 ura4-D18 rhp51::ura4⁺*), ED665 (*h⁻ ade6-704 leu1-32 ura4-D18*) strains were kindly provided by Dr. S. D. Park at the School of Biological Sciences, Seoul National University. *S. pombe* strains were grown under standard growth conditions (Alfa *et al.*, 1993; Cho *et al.*, 2000; Hwang *et al.*, 2000).

Transformation The transformation of *E. coli* was performed by the $CaCl_2$ method with a slight modification (Sambrook *et al.*, 1989). The transformation of *S. cerevisiae* was performed by the alkaline cation method (Ito *et al.*, 1983) with a slight modification. An overnight culture of 0.5 ml was transferred to 50 ml of YPD broth and further incubated till OD_{600} = 1.5-2.0 at 30°C with vigorous shaking. The cells were harvested, washed once with 10 ml of DW, and resuspended in 5 ml of a LA solution (0.1 M lithium acetate in TE buffer). After incubation for 2 h at room temperature with gentle agitation, the cells were harvested and resuspended in 1.5 ml of a LA solution. Then 0.2 ml of the cell suspension was mixed with 1-2 μg of the transforming DNA and 50 μg of the carrier DNA (sonicated denatured salmon sperm DNA). After 5 min, 0.7 ml of the PEG solution (40% PEG 3350 in LA solution) was added to the mixture. The mixture was incubated for 1 h at room temperature. The mixture was heat-shocked at 42°C for 15 min. The cells were plated onto selective media (SD plus appropriate amino acids) and incubated at 30°C. Transformation of *S. pombe* was carried out according to the procedure by Okazaky *et al.* (1990).

Purification of the UVSC protein The IPTG induced 200 ml cell culture was harvested by centrifugation at 4°C, 6,000 rpm for 10 min. The cell mass was resuspended in 5 ml of a native binding buffer (20 mM phosphate, 500 mM NaCl, pH 7.8) and disrupted by repeating freeze-thawing three times. The lysate was sonicated with two or three 10-sec bursts at medium intensity. After centrifugation at 4°C, 12,000 rpm for 20 min, the supernatant was loaded onto a His-affinity column. The column was washed with 5 volume of a column washing buffer (2 0mM phosphate, 50 0mM NaCl, pH 6.0), and 5 volume of 350 mM imidazole in a column washing buffer. The UVSC protein was finally eluted with 4 ml of 500 mM imidazole in a column washing buffer, and dialyzed against Buffer A (20 mM Tris-HCL, 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol).

ATPase Assay ATPase activity was measured according to the procedure of Kmiec *et al.* (1994) with a slight modification. The reaction mixture contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM denatured herring sperm DNA (as nucleotide), and 20 μCi of [α-^{32}P]ATP. The reaction was initiated by the addition of the UVSC protein at 37°C. Ten μl of aliquots were taken from the mixture every 10 min, and quenched by the addition of 20 μl of 10 mM potassium phosphate, 20 μl of acetone, 10 μl of 50 mM ammonium molybdate in 4 N H_2SO_4, and 140 μl of isobutanol-benzene (1 : 1). The mixtures were then vortexed and the phase separated by centrifugation at 12,000 rpm, 4°C for 10 min. One hundred μl of the organic phase (top) was removed and the radioactivity was determined by liquid scintillation counting.

Single-stranded DNA binding assay Ten μM of the UVSC protein and 120 ng of single-stranded M13 DNA were mixed in a reaction buffer that contained 6.6 mM of ATP, 40 mM of KCl, and 20 mM of $MgCl_2$. The reaction mixture was incubated at 37°C for 30 min, then analyzed by agarose gel (0.7%) electrophoresis. As a negative control, ATP or $MgCl_2$ was omitted from the reaction buffer.

Complementation of the MMS sensitivity of the *rad51* null mutant of *S. cerevisiae* The *A. nidulans uvsC* cDNA was inserted into the YEpACU vector under the ADH promoter, resulting in pATH1 (Fig. 1A). This plasmid was transformed into *S. cerevisiae*. The growth of transformants carrying pATH1 in a YPD medium that contained various concentrations of MMS was assayed. After cultivating the transformants for 2 d in YPD, the absorbance at 600 nm of each culture was measured.

Complementation of the MMS sensitivity of the *S. pombe rhp51* null mutant with *uvsC* of *A. nidulans* The *A. nidulans uvsC* cDNA was inserted into the pREP1 shuttle vector, which resulted in pLOS5 (Fig. 1B) under the *nmt* (no message in thiamine) promoter that was constructed by Maundrell (1993). The transformants were selected with a *leu-2* marker and the expression of the *uvsC* cDNA

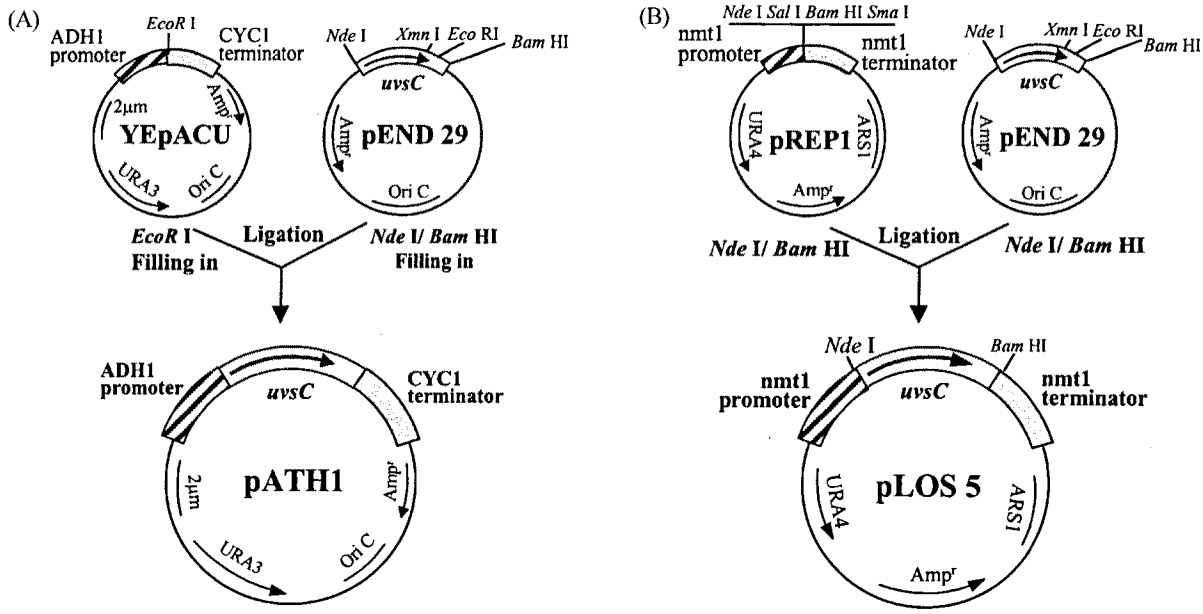

Fig. 1. Diagrammatic representation of the construction of plasmids. (A) pATH1 plasmid for expression of the *uvsC* gene in *S. cerevisiae* was constructed by filling in the ends of the *Nde*I*Bam*HI fragment from pEND29 carrying the *uvsC* cDNA. It was ligated into YEpACU that was cut with the *Eco*RI and filled in with DNA polymerase Klenow fragment. (B) pLOS5 plasmid for expression of the *uvsC* gene in *S. pombe* was constructed by ligating the *Nde*I*Bam*HI fragment that carries the *uvsC* cDNA from pEND29 into *Nde*I, *Bam*HI sites of the pREP1 vector.

was controlled by the addition of thiamine (final conc. of 5 μM). The growth of the transformants, which carried pLOS5 in an EMM medium that contained various concentrations of MMS, was assayed. After cultivating the transformants at 30°C for 2 days, the absorbance at 600 nm of each culture was measured.

Results and Discussion

Purification of the UVSC protein We previously cloned the *E. coli* RecA and yeast RAD51 homolog from *A. nidulans*, *radA*, by screening genomic and cDNA libraries with a PCR-amplified probe (Seong *et al.*, 1997). *radA* is an allele of *uvsC* and has three introns in its N-terminus. To explore the biochemical functions of the UVSC protein, we purified it following the expression in *E. coli*. For expression of the UVSC protein in *E. coli*, *uvsC* cDNA was cloned into the pET-15b expression vector that included codons for an N-terminal hexahistidine tag (6X His-tag) and a thrombin cleavage site in its leader sequence. These modifications added 20 amino acids to the N-terminus of the UVSC protein. The resulting plasmid, pEND29, was transformed into *E. coli* HMS174(DE3) and the expression of the UVSC protein was induced with 0.5 mM of IPTG at 27°C for 2.5 hrs. The majority of the overexpressed recombinant protein formed insoluble inclusion bodies (Fig. 2, lane 3). Nevertheless, a significant fraction of the recombinant protein was soluble (Fig. 2, lane 2). This fraction was used for purification by nickel chelate chromatography (Fig. 2, lane 5). The purified protein had a molecular mass of about 37 kDa, as determined by SDS-PAGE.

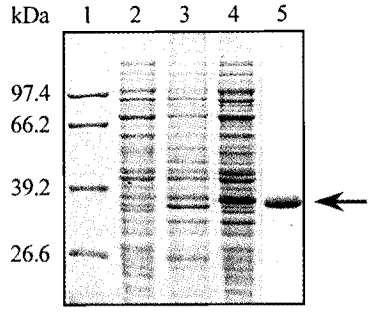

Fig. 2. Purification of UVSC protein. Coomassie Blue-stained SDS-polyacrylamide gel extracts of HMS174(DE3) cells transformed with pEND29 after IPTG induction (lane 4). The majority of the induced protein formed insoluble inclusion bodies (lane 3). The soluble lysate (lane 2) was purified by nickel chelate chromatography (lane 5). The position of the recombinant protein is indicated by an arrow on the right. Molecular mass standards are shown in lane 1. Their sizes in kDa are shown on the left.

UVSC protein binds to single-stranded DNA *S. cerevisiae* RAD51, and human RAD51 proteins synthesized in *E. coli*, have single-stranded DNA (ssDNA) and double-stranded DNA binding activities (Benson *et al.*, 1994; Sung 1994; Baumann *et al.*, 1996). The binding activity of the UVSC protein to ssDNA was assayed by DNA mobility shift experiments. The UVSC protein was incubated with M13 ssDNA in the presence or absence of ATP and magnesium. Nucleoprotein complexes were separated from free DNA in

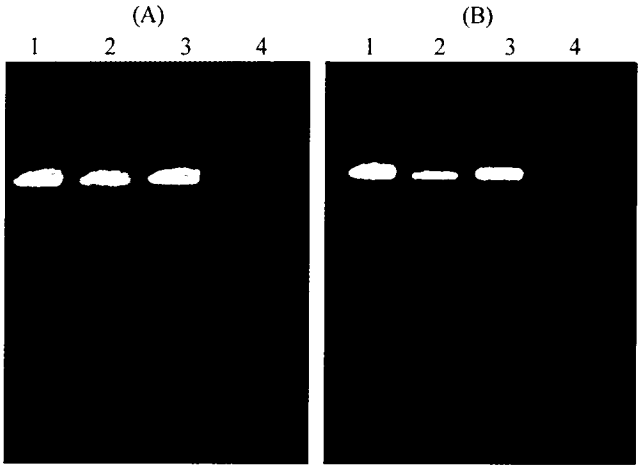

Fig. 3. Single-stranded DNA binding activity of UVSC protein. (A) ssDNA binding of UVSC protein is dependent on Mg^{2+}. One hundred twenty ng of M13 ssDNA were incubated with (lanes 3 and 4) or without (lanes 1 and 2) the UVSC protein (10 μM) in the presence (lanes 2 and 4) of Mg^{2+} (20 mM), or in the absence (lanes 1 and 3) of Mg^{2+}. All of the reaction mixtures were incubated at 37°C for 30 min in the presence of 6.6 mM ATP and analyzed by 0.7% agarose gel. (B) ssDNA binding of the UVSC protein is dependent on ATP. M13 ssDNA was incubated with (lanes 3 and 4) or without (lanes 1 and 2) the UVSC protein in the presence (lanes 2 and 4) of ATP or in the absence (lanes 1 and 3) of ATP.

an agarose gel and visualized by staining with ethidium bromide. The M13 ssDNA band was smeared in the presence of both ATP and magnesium (Fig. 3). However, we detected no ssDNA shift when magnesium (Fig. 3A) or ATP (Fig. 3B) was omitted from the reaction mixture. These results indicate that the UVSC protein is able to bind to ssDNA, and that both ATP and magnesium are required for the binding activity of the UVSC protein to ssDNA.

UVSC protein has ATPase activity The pairing and strand exchange reaction by RAD51 protein requires ATP, which is consistent with the presence of the DNA-dependent ATPase activity of *S. cerevisiae* and human RAD51 proteins (Benson *et al.*, 1994; Sung, 1994). The UVSC protein also contains the "Walker box" motif and is, therefore, predicted to be an adenine nucleotide binding protein (Thacker, 1999). The ATPase activity of the UVSC protein was measured in the presence or absence of ssDNA. For the assay of ATPase activity, 30 μl of the reaction mixture (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM denatured herring sperm DNA (as nucleotide), and 20 μCi of [α-^{32}P]ATP) and the UVSC protein was incubated at 37°C. Ten μl of the reaction mixtures were taken every 10 min, quenched and the increment of the hydrolyzed inorganic phosphate was measured by liquid scintillation counting. The UVSC protein was able to hydrolyze ATP (Fig. 4). Some ATPase activity was seen in the absence of DNA and the ATPase activity was

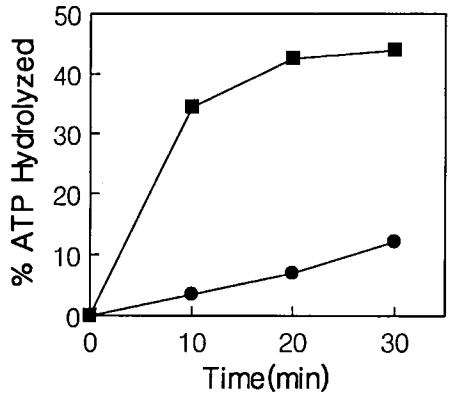

Fig. 4. UVSC is a ssDNA-stimulated ATPase. The UVSC protein was assayed for ATPase activity in the presence (■) or in the absence (●) of ssDNA, described under "Materials and Methods". Aliquots were taken from the reaction mixture at the indicated time points and the radioactivity was determined by liquid scintillation counting.

stimulated by ssDNA. This result indicates that UVSC is a DNA-stimulated ATPase.

Functional complementation of *uvsC* for MMS sensitivity of *rad51* and *rhp51* mutants We next tested whether the *A. nidulans uvsC* gene is a functional homolog of *S. cerevisiae Rad51* by examining its ability to complement for MMS sensitivity of *rad51*D mutation. The coding sequence of the *uvsC* gene was cloned downstream of the yeast ADH promoter. The resulting plasmid, pATH1, was transformed into *S. cerevisiae* XS803-3A/51Δ (*rad51* null mutant). Wild type as well as transformants that harbor pATH1 or empty vectors was cultivated in a YPD broth that contained various concentrations of MMS. The optical density at 600 nm was measured. The *rad51* null mutant showed high MMS sensitivity, and the *rad51* null mutant that harbored the *uvsC* gene showed an intermediate sensitivity compared to those for the wild type and *rad51* null mutant (Fig. 5A). This result indicates that the *uvsC* of *A. nidulans* could partially complement MMS sensitivity of the *rad51* null mutation of *S. cerevisiae.*

We also tested the ability of *uvsC* to complement MMS sensitivity of the *rph51* mutation in fission yeast *S. pombe.* The *S. pombe rph1* gene is a homologue of *E. coli recA* and *S. cerevisiae RAD51.* The coding sequence of the *uvsC* gene was cloned downstream of the thiamine-repressible *nmt* promoter. The resulting plasmid, pLOS5 that carried *A. nidulans uvsC* under the *nmt* promoter was transformed into the *S. pombe* JAC1/51Δ strain (*rhp51* null mutant). The MMS sensitivity was measured by cultivating transformants that harbor pLOS5 in the medium that contained 5 μM of thiamine or no thiamine. Various amounts of MMS were added to each batch. Transformants that expressed *A. nidulans uvsC* cDNA from the *nmt* promoter in the absence of thiamine showed MMS resistance, but they were sensitive to MMS in the presence of

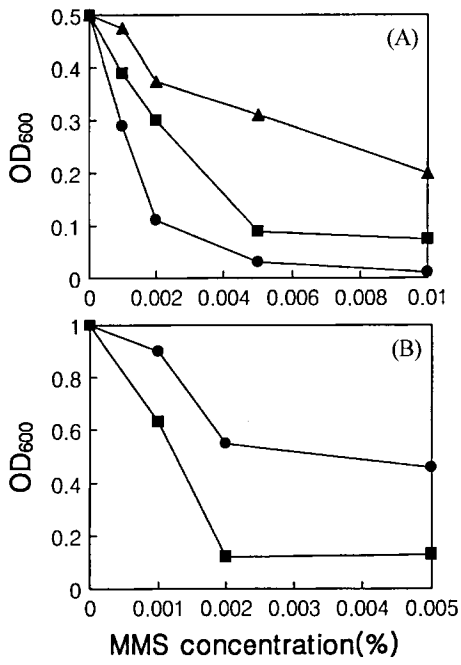

Fig. 5. Complementation for MMS sensitivity. (A) *S. cerevisiae* wild type (▲), *rad51* null mutant (●), and *rad51* null mutant that express *uvsC* of *A. nidulans* (■) from the ADH1 promoter were inoculated with a YPD medium that contained various concentrations of MMS, as indicated, and grown at 30°C for 2 days. The optical density was then measured. (B) *S. pombe rhp*51 null mutant, which carries a plasmid expressing *uvsC* of *A. nidulans* from a thiamine-repressible *nmt1* promoter, was inoculated with a EMM medium that contained various concentrations of MMS, as indicated, with thiamine (■) or without thiamine (●). After incubation at 30°C for 2 days, the optical density was measured.

5 μM of thiamine (expression of *uvsC* was repressed; Fig. 5B). These results indicate that *uvsC* of *A. nidulans* could complement the MMS sensitivity *rhp51* null mutation of *S. pombe* JAC1/51Δ.

In summary, the present results demonstrate that the UVSC protein has ssDNA binding activity and ssDNA-stimulated ATPase activity like RAD51 proteins, which suggests a similar role of UVSC in the recombination process. Moreover, the partial complementation of the *uvsC* gene for MMS sensitivity of *rad51* mutations suggests that UVSC could also be involved in the DNA repair of double strand break. However, UVSC has a role in the mutagenic process, which is different from RAD51 that plays only a small role in the mutagenic repair process. This suggests that there are some differences in the DNA repair systems between *Aspergillus* and yeast, although they have overlapping functions.

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