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Original Article

The key residue for SSB–RecO interaction is dispensable for *Deinococcus radiodurans* DNA repair *in vivo*

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The RecFOR DNA repair pathway is one of the major RecA-dependent recombinatorial repair pathways in bacteria and plays an important role in double-strand breaks repair. RecO, one of the major recombination mediator proteins in the RecFOR pathway, has been shown to assist RecA loading onto single-stranded binding protein (SSB) coated single-stranded DNA (ssDNA). However, it has not been characterized whether the protein–protein interaction between RecO and SSB contributes to that process *in vivo*. Here, we identified the residue arginine-121 of *Deinococcus radiodurans* RecO (drRecO-R121) as the key residue for RecO–SSB interaction. The substitution of drRecO-R121 with alanine greatly abolished the binding of RecO to SSB but not the binding to RecR. Meanwhile, SSB-coated ssDNA annealing activity was also compromised by the mutation of the residue of drRecO. However, the *drRecO-R121A* strain showed only modest sensitivity to DNA damaging agents. Taking these data together, arginine-121 of drRecO is the key residue for SSB–RecO interaction, which may not play a vital role in the SSB displacement and RecA loading process of RecFOR DNA repair pathway *in vivo*.

Keywords DNA repair; *Deinococcus radiodurans*; RecFOR; RecO; single-stranded binding protein

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Introduction

Homologous recombination (HR) is a fundamental biological process that participates in DNA repair, restarting the stalled replication forks, proper segregation of chromosomes, maintenance of genomic integrity, and generation of genomic diversity [1–3]. The RecBCD and RecFOR pathways are two dominant RecA-dependent recombinatorial DNA repair pathways in bacteria. They respond mainly to double-strand breaks (DSB) and single-strand gaps, respectively [3–5]. Comparative and evolutionary analyses of bacterial HR systems have shown that the RecFOR pathway is a common HR pathway in bacteria [6], which is a back-up pathway for DSB repair in *Escherichia coli* with deficiency of *recBC* and *sbcB* [7]. Some bacteria lacking native RecBCD pathway, such as *Deinococcus radiodurans* and *Thermus thermophilus,* have an intact RecFOR pathway [5]. These findings suggest that the RecFOR pathway plays a fundamental role in DNA repair in bacteria.

To initiate HR repair, the loading of RecA proteins onto single-stranded DNA (ssDNA) is critical [5]. In the RecFOR pathway, after end processing by RecJ and RecQ [8–10], ssDNA is protected by single-stranded binding proteins (SSBs). SSBs bind to ssDNA with high affinity and little sequence specificity [10], greatly inhibiting RecA nucleation by means of steric obstruction [11,12]. Therefore, the displacement of SSB from ssDNA in preparation for RecA nucleation appears to be an essential step in the HR initiation process [13].

Deinococcus radiodurans, one of the most radiation-resistant organisms on earth [14], has a set of highly conserved homologs associated with the RecFOR pathway, including *recF* (*DR1089*), *recR* (*DR0198*), *recO* (*DR0819*), *recJ* (*DR1126*), *recQ* (*DR1289*), *ssb* (*DR0100*), and *recA* (*DR2340*) [15]. *Deinococcus radiodurans* cells devoid of any of the *recO*, *recF*, or *recR* genes are extremely radiosensitive, indicating that these genes are all vital in HR [16,17]. However, *D. radiodurans* lacks RecB and RecC, the key enzymes in the RecBCD pathway [18], making it an excellent model organism for RecFOR pathway study.

RecR and RecF are assumed to play adjunctive roles with RecO during SSB displacement in HR because only RecO, unlike RecR and RecF, can interact directly with SSB [19].

Previous studies have shown that the highly acidic region of the *E. coli* SSB C-terminal tail (SSB-Ct) is involved in the interaction between SSB and RecO [20,21]. Recent research on *T. thermophilus* RecO (ttRecO) revealed that not only the acidic C-terminal but also the OB fold of *T. thermophilus* SSB (ttSSB) could interact with ttRecO [22]. In *D. radiodurans*, no obvious interaction between SSB-Ct and RecO was identified [20], indicating the existence of diverse interaction sites. The crystal structure suggests that drRecO interacts with SSB through its highly positively charged C-terminus [20,23,24]. However, few specific interaction sites on drRecO have been characterized. The study of ttRecO also revealed that the residue arginine-127 (R127) is the key binding site for ttSSB [22]. However, its biological role has not been investigated *in vivo*.

Sequence alignment and structure analysis indicated that the residue R121 was the conserved residue of ttRecO R127 in *D. radiodurans* RecO (drRecO) [22] (**Supplementary Fig. S1**). In this study, to check the role of R121 in RecO–SSB interaction in *D. radiodurans*, drRecO R121A mutant was constructed and its SSB binding and DNA annealing activities were evaluated. Moreover, a drRecO-R121A mutant *D. radiodurans* strain was constructed successfully and the role of drRecO-R121 in DNA damage repair was further investigated.

Materials and Methods

Plasmids and bacterial strains

The bacterial strains and plasmids used in this study are listed in **Table 1**. To prepare drRecO mutant R121A, *drrecO* gene was amplified from *D. radiodurans* R1 strain genome and ligated into pMD18-T cloning vector (TaKaRa, Dalian, China) and point mutation was incorporated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, USA). The point mutation primers, R121A-F and R121A-R, are showed in **Table 2**. Mutation was confirmed by DNA sequencing. Both the wild-type (wt) *recO* gene and mutated gene *recO* (*R121A*) were cut from T vector by restriction endonucleases (*Nde*I and *Bam*HI) and ligated into pET28-HMT [26]. The result expression plasmid contains a 6× His tag, maltose binding protein (MBP), and tobacco etch virus protease (TEV) cleavage site at N-terminal of RecO (R121A). *drssb* and *drrecR* were amplified and the expression plasmids pET28-HMT-drSSB, pET28-drSSB, and pET28-drRecR were constructed. These expression plasmids were transformed into *E. coli* BL21(DE3). All *E. coli* strains were grown at 37°C in Luria–Bertani (LB) media (1% bacto-tryptone, 1% sodium chloride, 0.5% bacto-yeast extract) with the appropriate antibiotics (ampicillin, 100 μg/ml; kanamycin, 30 μg/ml) or on LB plates supplemented with 1.3% agar.

Table 1. Strains and plasmids used in this experiment

Strain and plasmid	Relevant feature	Reference or source
Strains		
E. coli		
DH(5α)	*supE44*, *ΔlacU169 (φ80lacZΔM15)*, *hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Invitrogen
BL21(DE3)	*F⁻, ompT, hsdS$_B$ ($r_B^-m_B^-$), dcm (DE3), gal (λcI857, ind1, Sam7, nin5, lacUV5-T7gene1)*	Invitrogen
D. radiodurans		
R1	Wild-type strain	ATCC13939
RecO⁻	R1 but *recO::aadA*	[25]
R1-O (wt) aadA	R1 but *recO(580bp+)::aadA*	This study
R1-O (R121A) aadA	R1-O (wt) aadA but 121-R was replaced by A	This study
Plasmids		
pMD18-T vector	For TA cloning, *lacZ, Amp^r*	TaKaRa
pET28-HMT	T7 promoter, T7 transcription start, His•Tag coding sequence, T7•Tag coding sequence, multiple cloning sites (*Bam*HI—*Xho*I), T7 terminator, *lacI* coding sequence, pBR322 origin, *Kan* coding sequence, f1 origin, 6His-tag coding sequence, maltose binding protein, TEV protease site	[26]
pET28-HMTO	As pET28-HMT but ligated with *drrecO*	This study
pET28-HMTdrSSB	As pET28-HMT but ligated with *drssb*	This study
pET28-HMTO (R121A)	As pET28-HMT but ligated with *recO(R121A)*	This study
pET28-drSSB	pET28a ligated with *drssb*	This study
pET28-drRecR	pET28a ligated with *drrecR*	This study

Table 2. Primers or oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]
R121A-F	CCGCCTCGCTGGCCGGCGTGGCGC
R121A-R	GCGCCACGCCGGCCAGCGAGGCGG
P1	CATATGCGCTCACGCACCGC
P2	AAGCTTTGACAAAAGAATGGC
P3	CTCGAGATGACCTCGCCCCGC
O1 (FAM)	GTCCAGGCTCTCGTTCAGGGTCTTTTTGGTGGATTGGCTTTCATCA
O2 (Dabsyl)	TGATGAAAGCCAATCCACCAAAAAGACCCTGAACGAGAGCCTGGAC

[a]Underlines indicate restriction sites.

To construct *D. radiodurans* drRecO-R121A *in situ* point mutated strains, a 580 bp downstream fragment of *recO* gene was amplified by polymerase chain reaction (PCR) with primer P2 and primer P3 (**Table 2**) and digested with *Hin*dIII and *Xho*I. *recO* or *recO* (*R121A*) and downstream fragments were ligated with streptomycin resistance gene-*aadA* to make a 2270 bp product. The *aadA* gene containing its own promoter for transcription was inserted inversely, which will not affect vicinal gene expression. The ligated product was taken as template for PCR with primer P1 and primer P3 (**Supplementary Fig. S2A**). The sequence of P1, P2, and P3 are listed in **Table 2**. The amplified products were transformed into *D. radiodurans* R1 strain. Through HR, the point mutated *recO* gene ligated with streptomycin resistance gene could insert into the genome of R1 and the mutant could be selected in 0.5% tryptone–0.1% glucose–0.3% yeast extract (TGY) plate (streptomycin, 8 μg/ml) at 30°C. The *in situ* point mutated strains were finally confirmed by PCR and DNA sequencing (**Supplementary Fig. S2B,C**).

Protein expression and purification

For RecO purification, the constructed expression strains were grown in LB media at 37°C to OD_{600} of 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.5 mM. The cells were harvested after 6 h incubation at 30°C and then re-suspended in buffer containing 20 mM Tris-HCl (pH 8.0), 1 M NaCl, 100 mg/ml lysozyme, and 2 mM amidinophenylmethylsulfonyl fluoride A (PMSF). The cells were lysed using ultrasonic disruption followed by centrifugation at 15,000 *g* for 40 min to remove the insoluble debris. For His-tag RecO purification, the supernatant of cell lysate was loaded onto Ni-NTA column (GE Healthcare, Wisconsin, USA). After being washed with washing buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM imidazole), His-RecO was eluted with elution buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 200 mM imidazole). The protein was further purified using HiTrap Heparin column and Superdex75 column. His-RecO protein was dialyzed against 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol and stored at −20°C. For MBP-RecO purification, the supernatant of cell lysate was loaded onto Amylose column (GE Healthcare) and MBP-RecO was eluted with 20 mM Tris-HCl (pH 8.0), 1 M NaCl, 10 mM amylose. After TEV cleavage with 0.1 mM TEV, 1 mM dithiothreitol (DTT), and 1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C overnight, protein was dialyzed against dialysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT], and further purified by Ni-NTA column (GE Healthcare). The flow through fraction was loaded onto Amylose column and the subsequent flow through fraction was purified by HiTrap Heparin column. The eluted protein was then purified by Superdex75 (GE Healthcare). RecO protein was dialyzed against 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10% glycerol and stored at −20°C. Protein SSB without any tag used in this experiment also was purified with similar protocol as RecO.

For His-RecR purification, the constructed expression strain was grown in LB media at 37°C to OD_{600} of 0.6. IPTG was added to final concentration of 0.5 mM. The cells were harvested after 5 h incubation at 30°C and then re-suspended in buffer containing 20 mM Tris-HCl (pH 8.0), 1 M NaCl, 10 mM imidazole, 100 mg/ml lysozyme, and 2 mM PMSF. The cells were lysed using ultrasonic disruption followed by centrifugation at 15,000 *g* for 40 min to remove the insoluble debris. The supernatant was loaded onto Ni-NTA column and the target protein was eluted with buffer containing 20 mM Tris-HCl (pH 8.0), 1 M NaCl, and 300 mM imidazole. Then the protein was further purified by Hitrap Q (GE Healthcare) and Superdex200 (GE Healthcare). Protein His-SSB used in this experiment was purified with similar protocol as His-RecR.

Concentrations of the proteins were assessed by Bradford assay and the purities of the proteins were assessed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Circular dichroism spectra

Circular dichroism (CD) spectra of the wt and mutant RecOs were recorded at 20°C on MOS-450 Spectrometer (Bio-Logic, Claix, France). Protein concentrations were 0.05 mM.

The spectra were recorded over the range of 190–250 nm using a cuvette with an optical path length of 0.1 cm. Origin 8.0 and Dicroprot software were used to create the map and predict the folding.

Native PAGE assay

Native PAGE assay was performed as previously described [13,22] but with some modifications. drSSB (5 μM) and drRecO (5, 10, 20, 40, and 60 μM) were incubated in 20 μl reaction system in reaction buffer [50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT] at 30°C for 10 min. The interaction of drRecR (5 μM) and drRecO (5, 10, 25, and 50 μM) was analyzed in the same manner. Samples were then analyzed by 10% PAGE at non-denaturing conditions. The gels were stained with Coomassie brilliant blue R250 dye.

Immunodot blotting assay

Immunodot blotting assay was performed as previously described [27] with some modifications. drRecO, drRecO (R121A), and bovine serum albumin (BSA) were spotted in increasing amounts from 0.1 to 100 nmol of monomer on a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5% defatted milk powder at 4°C for 2 h, and then incubated with 1 μM purified SSB at 4°C overnight. Membrane was washed by TBST (TBS containing 0.05% Tween 20) for four times, followed by incubation with the primary antibody anti-SSB at a 1 : 1000 dilution at 4°C for 4 h. Again, the membrane was washed by TBST for four times. The membrane was subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at a 1 : 10,000 dilution at 4°C for 4 h. Finally, the membrane was washed four times with TBST. SSB signal was detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific, USA).

Pull down assay

For MBP-RecO-SSB pull down assay, 200 μl MBP-RecO (0.5 mM) was incubated with 20 μl amylose beads and washed with washing buffer A [100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.05% Tween 20] three times and then incubated with 400 μl SSB (0.5 mM, with 0.5 mM lysozyme as control) at 4°C for 3 h. The beads were washed with washing buffer four times to completely remove the lysozyme. Proteins were eluted with 50 μl elution buffer [10 mM amylomaltose, 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM DTT] and analyzed by 12% SDS–PAGE.

For His-SSB-RecO pull down assay, 200 μl His-SSB (0.5 mM) was incubated with 20 μl Ni-NTA beads and washed with washing buffer A three times and then incubated with 400 μl wt or mutant RecO (0.5 mM, with 0.5 mM BSA as control) at 4°C for 3 h. The beads were washed with washing buffer four times when BSA was completely washed off. Proteins were eluted with 50 μl elution buffer [300 mM imidazole, 200 mM NaCl, 20 mM Tris-HCl (pH 7.5)] and analyzed by 12% SDS–PAGE.

DNA annealing assay

ssDNA was prepared by heat denaturation from 3000 bp double-stranded DNA (dsDNA). The ssDNA (10 μM) was incubated with SSB (0.5 μM) at 4°C for 30 min. Then, the SSB-coated ssDNA were added into 10 μl reaction systems with reaction buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT). Reactions were started by adding various concentrations of RecO (0.5, 1, 2, and 4 μM). After incubation at 30°C for 20 min, the reaction was stopped by adding 0.5 μl of 5% SDS and 0.5 μl of 20 mg/ml proteinase K, incubated at 37°C for another 5 min. The result of annealing was assessed by electrophoresis on 1.0% agarose gels.

Fluorescence intensity values assay was performed as previously described [20]. Oligonucleotides O1 with fluorescein (FAM) labeling at the 5′-end (20 nM, nucleotide concentration) and an equal amount of Dabsyl-labeled complementary sequence O2 at the 3′-end (**Table 2**) were incubated with SSB (200 nM) separately at 4°C for 30 min. FAM-labeled ssDNA was added into the reaction buffer [50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT], which contains various concentrations of RecO (200, 400, 800, and 1600 nM). Immediately, reactions were started by adding the same amount of Dabsyl-labeled SSB-coated complementary DNA. The annealing was monitored by the quenching of FAM signal using BioTek (Beijing, China) plate reader at 30°C for 30 min. Fluorescence intensity values were inverted and normalized using the equation: $I = -1 \times (I_i - I_0)/I_0$, I_0 and I_i are fluorescence intensity values at initial timepoint and the specific timepoint, respectively. The assay was repeated three times. Annealing figures were created by using Origin 8.0 software.

Electrophoresis mobility shift assay

The binding ability of drRecO protein with ssDNA and dsDNA were tested by electrophoresis mobility shift assay (EMSA). Ten microliters reaction mixture containing 20 μM M13mp18 ssDNA (New England Biolabs, Beijing, China) or 20 μM 2350 bp dsDNA (PCR products) was incubated with various concentrations of RecO (wt or R121A mutant) (0, 2, 4, and 6 μM) in a reaction buffer [50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT] at 30°C for 10 min. Samples were separated by electrophoresis on 1.0% agarose gels and visualized by using UV transilluminator.

Cell survival study

Cells were grown in TGY media with the appropriate antibiotics to exponential phase ($OD_{600} = 0.6–0.8$). For gamma irradiation, cells were irradiated on ice at different doses from 0 to 4 kGy and then diluted to appropriate concentrations and plated on TGY plates. For UV treatment, cells were first diluted to appropriate concentrations and plated on the TGY plates. After absorbed completely, the plates were exposed to UV light at different dose from 0 to 1000 J/M^2 followed by plating on the TGY plates. For mitomycin-C (MMC) treatment, MMC was added to final concentration of 20 mg/l and the cells were cultured at 30°C for 10–40 min. Cells were then diluted to appropriate concentrations and plated on the TGY plates. Colonies were counted after incubation at 30°C after 2–3 days.

Results

RecO (R121A) mutation weakened the RecO–SSB interaction but not RecO–RecR interaction

A previous study has indicated that residue R127 of ttRecO is the major binding site for ttSSB [22]. Sequence alignment suggests that drRecO-R121 is the conserved residue in *D. radiodurans* [22] (**Supplementary Fig. S1**). Here, it was found that the expression of ttRecO can restore the phenotype of RecO-deficient *D. radiodurans* in response to gamma rays and UV light (**Supplementary Fig. S3**), indicating that these two proteins are highly homologous. To determine whether drRecO-R121 is the major SSB binding site, arginine was substituted with alanine and the effect of R121A mutation on RecO–SSB or RecO–RecR interaction was further investigated. The purity of the proteins was assessed by SDS–PAGE (**Supplementary Fig. S4**). To assess the influence of mutation on protein structure, gel filtration and CD spectra assays were also conducted and no difference was identified in these analysis between RecO (wt) and RecO (R121A), indicating that the R121A mutation does not influence the protein structure of RecO (**Supplementary Fig. S5**).

Native PAGE assay was performed to analyze the interaction between RecO and SSB or RecR. Since both SSB and RecR are negatively charged proteins at neutral pH, they could enter the native gel. RecO is positively charged and cannot enter the gel. If RecO interacts with RecR or SSB, the complex could form a new band in the gel or the intensity of SSB (or RecR) band could be decreased. As shown in **Fig. 1A**, drSSB could enter the gel and formed a clear band, while drRecO or drRecO R121A remained in the gel well. When drSSB was mixed with different concentration of drRecO, the intensity of the drSSB band decreased in a drRecO concentration-dependent manner. In contrast, when drSSB was mixed with drRecO R121A mutant protein, the intensity of the drSSB did not change even when the RecO : SSB molar ratio reached 12 : 1. Meanwhile, both wt and mutant protein can bind to RecR (**Supplementary Fig. S6**).

Furthermore, immunodot blotting assay was carried out. RecO, RecO (R121A), and BSA were spotted in increasing amounts from 0.1 to 100 nmol of monomer on a nitrocellulose membrane. The membrane was incubated with 1 μM purified SSB followed by incubation with the primary antibody, anti-SSB, and was subsequently incubated with the secondary antibody for signal detection. The signal increased as the RecO concentration increased, while in R121 mutant, no signal was detected (**Fig. 1B**).

Moreover, pull down assays were performed to further verify the protein–protein interaction. To prevent any non-specific interactions of RecO and SSB, 0.05% Tween 20 and 0.5 mM lysozyme (or BSA) were added. In MBP-RecO-SSB pull down assay, although the binding affinity of SSB was not very well in these conditions, a small amount of SSB could interact with MBP-RecO but not R121A mutant (**Fig. 1C**). At the same time, a small amount of RecO but not R121A mutant was co-purified with His-SSB by Ni-NTA beads (**Fig. 1D**).

These data indicated that drRecO R121 is the key residue for drSSB binding, in concordance with ttRecO R127.

R121A reduced SSB-coated ssDNA annealing activity of RecO

It has been suggested that drRecO shows SSB-coated ssDNA annealing activity via RecO–SSB interaction [28]. Given that the SSB binding activity of R121A was suppressed, R121A should show much lower SSB-coated ssDNA annealing ability than the wt. To test this hypothesis, the SSB-coated ssDNA annealing activity was compared between wt RecO and the R121A mutant.

The annealing assay results showed that drRecO exhibited very strong SSB-coated ssDNA annealing activity (**Fig. 2A**, Lanes 4–7). More than 90% of the substrate was rapidly annealed within 20 min at 30°C by mixing wt RecO and SSB at different molar ratios: 1 : 1, 2 : 1, 4 : 1, or 8 : 1. In contrast, the R121A mutant showed much weaker annealing activity (**Fig. 2A**, Lanes 8–11). Less than 50% of the ssDNA was annealed when the ratio was below 2 : 1 (**Fig. 2A**, Lanes 8 and 9). The activity was increased to the level of the wt protein only when the ratio was increased to 8 : 1 (**Fig. 2A**, Lanes 7 and 11).

To investigate the SSB-coated ssDNA annealing activity dynamically, a fluorescence quenching assay was carried out. The results were consistent with the electrophoresis assay. At low concentrations, R121A displayed reduced SSB-coated DNA annealing activity. This could be recovered when the molar ratio of R121A and SSB was increased to 8 : 1 (**Fig. 2B**).

To eliminate the possibility that the R121A-ssDNA or R121A-dsDNA interaction itself affects SSB-coated

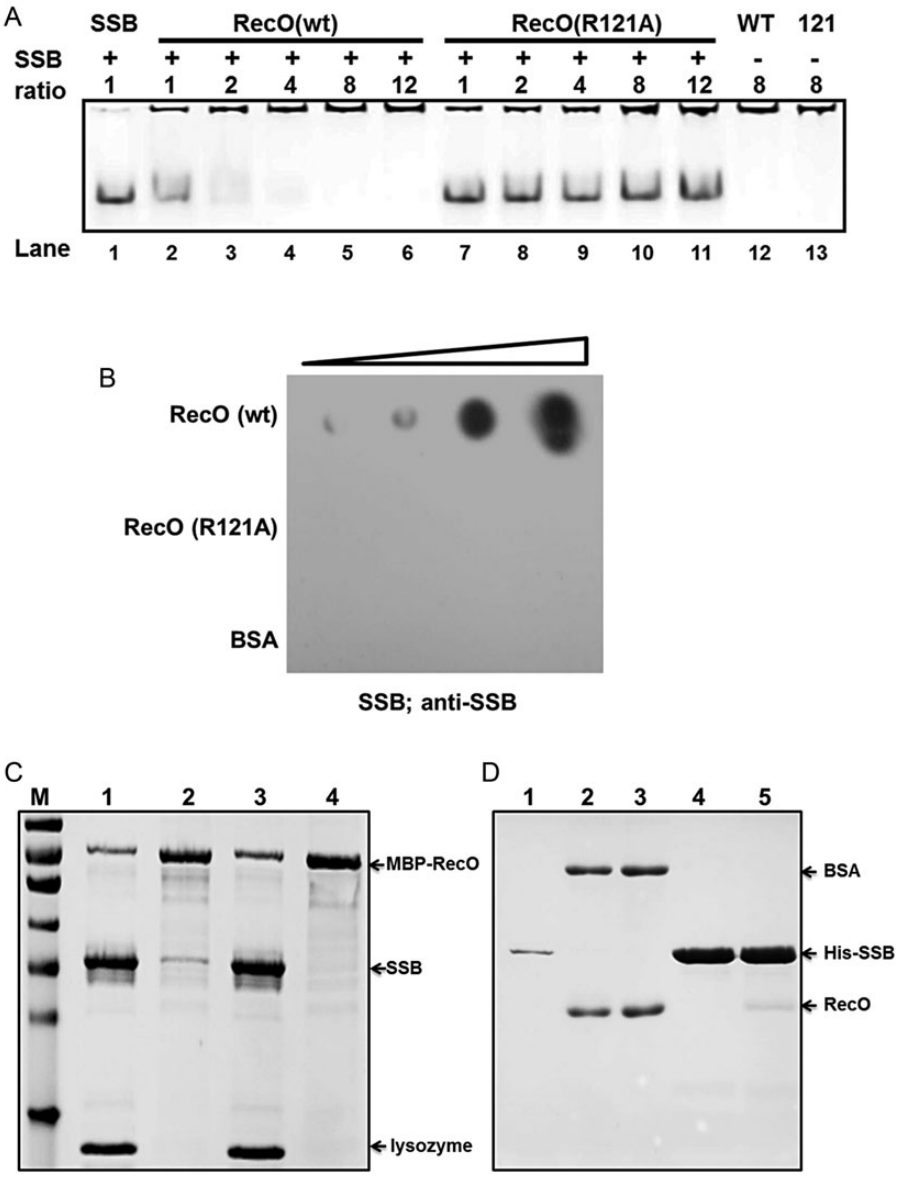

Figure 1. Interaction between RecO (R121A) and SSB (A) Native PAGE analysis of RecO–SSB interaction. Lane 1: SSB (5 μM) only; Lanes 2–11: SSB (5 μM) was incubated with different ratio (1 to 1, 2, 4, 8, 12) of wt or mutant RecO; Lanes 12 and 13: RecO (wt) and R121A only (25 μM). (B) Detection of direct RecO–SSB interaction using immunodot blotting. (C) SDS–PAGE result of MBP-RecO-SSB pull down assay. Lanes 1–2: the protein mix input [RecO (wt) and RecO (R121A)]; Lanes 3–4: The protein eluted from amylose beads [(RecO (wt) and RecO (R121A)]. (D) SDS–PAGE result of His-SSB-RecO pull down assay. Lane 1: His-SSB immobilized on Ni-NTA; Lane 2: RecO (R121A) input, with same amount of BSA; Lane 3: RecO (wt) input, with same amount of BSA; Lane 4: the eluted RecO (R121A) and SSB from Ni-NTA; Lane 5: the eluted RecO (wt) and SSB from Ni-NTA.

DNA annealing activity, we measured the ssDNA and dsDNA binding activities of either R121A or wt RecO. EMSA showed that there was no difference between wt RecO and the R121A mutant in ssDNA or dsDNA binding activity (**Fig. 2C**). This indicated that the reduced SSB-coated DNA annealing activity of the R121A mutant is totally attributable to the lack of RecO–SSB interaction.

The R121A mutant did not impair the DNA repair capability of *D. radiodurans*

Given that the R121A mutant showed highly decreased SSB binding activity and displayed reduced SSB-coated DNA annealing activity at low protein concentrations *in vitro*, one would expect its effect on DNA repair capability *in vivo*. Therefore, we investigated the cell survival of the mutant when treating with different DNA damaging agents.

R121A point mutants were constructed *in situ* to ensure that the expression level of the R121A mutant was equal to that of the wt strain (**Supplementary Fig. S2A**). As confirmed by PCR and sequencing (**Supplementary Fig. S2B,C**), the *recO* gene and its 580 bp downstream fragments (1320 bp) were replaced by *recO* (*R121A*) (or *recO*)-*aadA*-580 bp downstream fragments (2270 bp) on all copies of *D. radiodurans* chromosomes. Therefore, the *in situ* point mutant R1-O

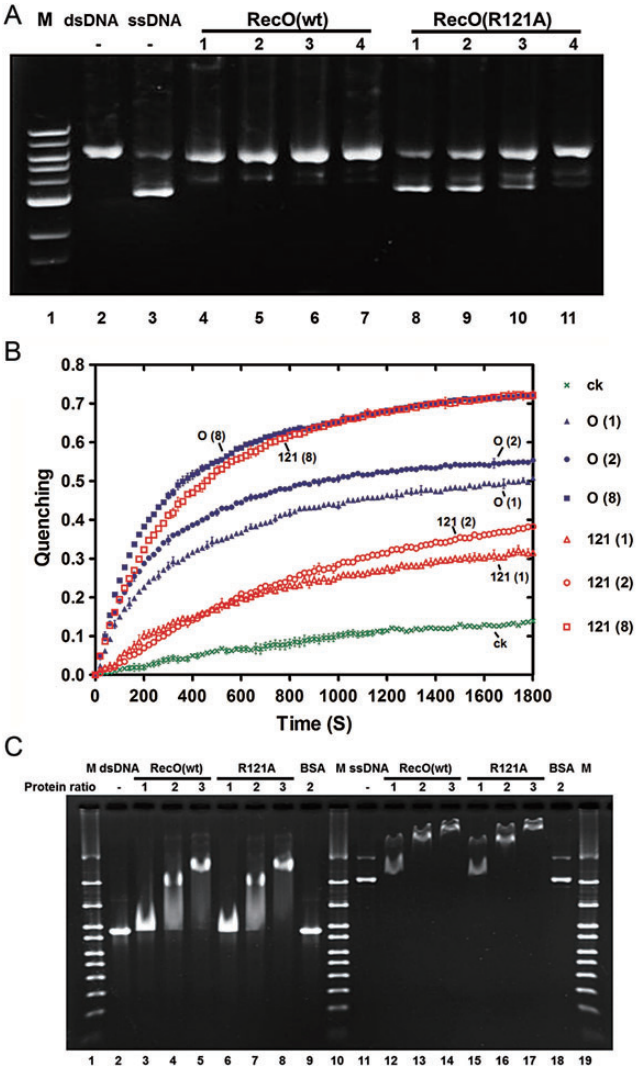

Figure 2. Effect of the R121A mutation on SSB-coated ssDNA-annealing (A) SSB-coated ssDNA annealing. Lane 1: DNA marker; Lane 2: dsDNA only (10 μM); Lane 3: ssDNA (10 nM) with SSB and stop treatment; Lanes 4–7: 0.5, 1, 2, and 4 μM RecO (wt) were added; Lanes 8–11: 0.5, 1, 2, and 4 μM R121A were added. (B) Fluorescence intensity values assay on ssDNA annealing. RecO (wt) is marked solid and blue, while R121A marked hollow and red. Controls are shown in green. Triangle stands for ratio of RecO to SSB is 1 : 1; Circle stands for the ratio of RecO to SSB is 2 : 1; Square stands for the ratio of RecO to SSB is 8 : 1; Cross stands for no RecO as control. (C) Effect of the R121A mutation on dsDNA and ssDNA binding activities. Lanes 1, 10, and 19: DNA marker; Lane 2: dsDNA only; Lanes 3–5: dsDNA was incubated with 2, 4, and 6 μM wt RecO; Lanes 6–8: dsDNA was incubated with 2, 4, and 6 μM R121A mutant RecO; Lane 9: dsDNA was incubated with 6 μM BSA. Lane 11: ssDNA only; Lanes 12–14: ssDNA was incubated with 2, 4, and 6 μM wt RecO; Lanes 15–17: ssDNA was incubated with 2, 4, and 6 μM R121A; Lane 18: ssDNA was incubated with 6 μM BSA.

(R121A) aadA as well as the control strain R1-O (wt) aadA, a wt strain containing an *aadA* cassette, were constructed successfully.

To introduce different DNA damage forms, cells were treated with various doses of gamma radiation, UV light, and MMC. To sum up, R1-O (R121A) aadA was slightly more sensitive to DNA damaging agents than R1-O (wt) aadA or wt R1 (**Fig. 3**). However, when compared with the highly decreased cell survival rate the *recO* knockout strain showed, such a difference was negligible. And we cannot exclude the possibility that such minor difference was caused by the insertion of a streptomycin resistance gene-*aadA*. Such a phenotype strongly suggests that compared with the entire protein, site R121 contributes little to the *D. radiodurans* DNA repair capacity.

Discussion

The displacement of SSB from ssDNA in preparation for RecA nucleation appears to be an essential step in the HR repair initiation process. In RecFOR DNA repair pathway, it was found that RecO but not RecR or RecF interacts with SSB directly and could displace SSB from ssDNA *in vitro* [13,19,22]. Therefore, the interaction between RecO and SSB has been suggested to play a vital role in the SSB dissociation and RecA loading process in the RecFOR pathway. Due to that the key site for RecO–SSB interaction was not identified, the role of this interaction in this process was not verified *in vivo*.

Most recently, R127 of ttRecO was identified as the key residue for ttSSB binding [22]. Replacing this arginine with alanine greatly reduced SSB-coated ssDNA annealing activity, as well as RecA ATP hydrolysis [22]. However, the *in vivo* role of ttRecO-R127 has not been investigated. The drRecO R121 residue was the conserved site of ttRecO-R127 in *D. radiodurans*. Here, we found that the substitution of R121 with alanine could disrupt RecO–SSB interaction and weaken its SSB-coated ssDNA annealing activity in *D. radiodurans*. Nonetheless, this mutant still shows robust DNA repair activity as well as the wt strains. These results suggest that, although drRecO-R121 is the key residue for drRecO–SSB interaction *in vitro*, it might not be a vital drRecO functional site and contributes little to DNA repair activity *in vivo*.

SSB dissociation and RecA loading may be a very complicated process. Two distinct RecA loading pathways, RecOR and RecFOR, were proposed to co-exist in *E. coli*, in which RecOR pathway totally depends on the RecO–SSB interaction while RecFOR does not [21]. It was also found that the C-terminal of RecA would suppress the SSB dissociation since RecAΔC17 could greatly enhance the SSB displacing in the absence of RecOR in *E. coli* [29]. Recently, SSB was shown to be able to migrate on the ssDNA via a reptation mechanism [30] and could be possibly pushed off from the ssDNA by RecO or other proteins competing for the ssDNA binding sites [31]. Therefore, the interaction between RecO and SSB might only play a minor role to help localize RecO onto ssDNA. Meanwhile, RecO is a multifunctional protein. Besides its interaction with SSB, it also has RecR and DNA binding activities. R121A mutation only impaired the

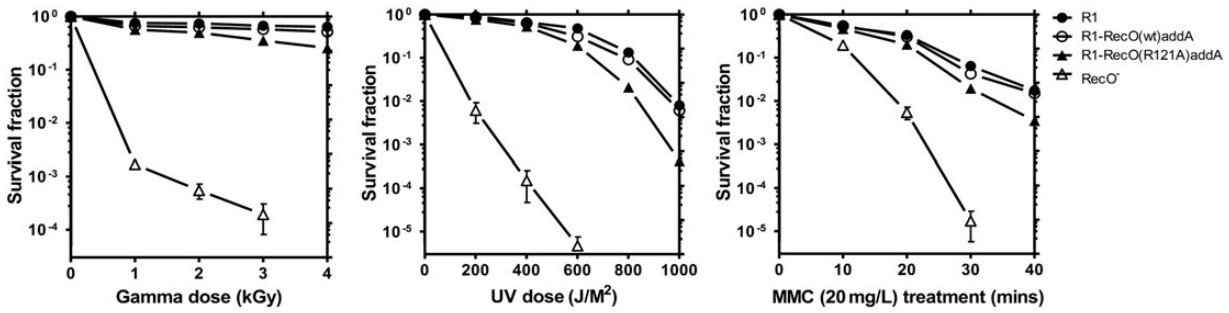

Figure 3. R121A compensated strain showed modest sensitivity to DNA damaging agents *in vivo* Survival fraction of gamma irradiation (0, 1, 2, 3, 4 kGy), UV treatment (0, 200, 400, 600, 800, 1000 J/M^2), and MMC (20 mg/l) treatment (10, 20, 30, and 40 min) compared among *in situ* complementary strains and RecO-deleted *D. radiodurans* cells.

RecO–SSB interaction but has no influence on RecR and DNA binding activities. Those may be the reasons for the *drRecO-R121A* strain showing only modestly decreased DNA damaging agents' resistance. Further experiments are needed to elucidate the molecular mechanism of RecO in RecFOR DNA repair pathway.

Supplementary Data

Supplementary data are available at *ABBS* online.

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