Down-regulation of radioresistance by LexA2 in *Deinococcus radiodurans*

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The extremely radioresistant bacterium *Deinococcus radiodurans* contains two LexA homologues (LexA1 and LexA2) that are possible transcriptional regulators associated with the DNA damage response. In this study, resequencing revealed that there was an additional cytosine nucleotide (nucleotide position 612) in the *D. radiodurans* *lexA2* gene. Purified LexA2 possessed proteolytic activity that could be stimulated by RecA. In an effort to gain an insight into the role of LexA2 in the radiation response mechanism, *recA*, *lexA1* and *lexA2* disruptant strains were generated and investigated. The intracellular level of RecA increased in *lexA1* and *lexA2* disruptant strains following γ -irradiation as in the wild-type strain. These results indicated that the two LexA homologues did not possess functional overlap regarding the induction of RecA. The *lexA2* disruptant strains exhibited a much higher resistance to γ -rays than the wild-type strain. Furthermore, a luciferase assay showed that *pprA* promoter activation was enhanced in the *lexA2* disruptant strain following γ -irradiation. The *pprA* gene encoding the novel radiation-inducible protein PprA plays a critical role in the radioresistance of *D. radiodurans*. The increase in radioresistance of the *lexA2* disruptant strain is explained in part by the enhancement of *pprA* promoter activation.

Received 16 May 2006
Revised 3 August 2006
Accepted 17 August 2006

INTRODUCTION

Deinococcus radiodurans exhibits extraordinary resistance to the lethal and mutagenic effects of ionizing and UV radiation, as well as many other DNA-damaging agents. This resistance has been attributed to its highly proficient DNA repair capacity (Battista, 1997; Narumi, 2003; Cox & Battista, 2005). The most noteworthy characteristic of *D. radiodurans* is its capacity for repairing DNA double-strand breaks induced by ionizing radiation (Dean *et al.*, 1966; Kitayama & Matsuyama, 1968; Grimsley *et al.*, 1991). The highly efficient double-strand break repair process in *D. radiodurans* is radiation-inducible and is dependent on *de novo* protein synthesis following irradiation (Kitayama & Matsuyama, 1971). Several DNA-damage-inducible proteins that may be necessary for DNA repair have been detected in cell extracts of *D. radiodurans* using 2D-PAGE analysis (Hansen, 1980; Tanaka *et al.*, 1996). Recent transcriptome and proteome analyses have revealed the comprehensive expression profiles of radiation-inducible

genes and proteins (Lipton *et al.*, 2002; Liu *et al.*, 2003; Tanaka *et al.*, 2004; Zhang *et al.*, 2005). It has therefore been established that *D. radiodurans* possesses a DNA damage response mechanism. Additionally, analysis of DNA-damage-sensitive strains IRS24 (Mattimore *et al.*, 1995) and KH840 (Kitayama & Matsuyama, 1975) identified a novel regulatory protein (DR0167; PprI protein, also called IrrE protein) that plays a role in regulating *recA* and *pprA* gene expression following γ -irradiation (Earl *et al.*, 2002; Hua *et al.*, 2003; Ohba *et al.*, 2005). Furthermore, in a previous study we identified the radiation-responsive minimal promoter of the *pprA* gene encoding the novel radiation-inducible protein PprA, which plays a critical role in radioresistance of *D. radiodurans* (Narumi *et al.*, 2004; Ohba *et al.*, 2005).

RecA and LexA proteins in *Escherichia coli* play important roles in the DNA damage response repair mechanism (the SOS system) (Walker, 1984; Friedberg *et al.*, 1995). The SOS response in *Bacillus subtilis* progresses in a similar manner, with *B. subtilis* RecA having an identical role in controlling the SOS regulon together with a cellular repressor protein that is functionally homologous to the *E. coli* LexA repressor (Wojciechowski *et al.*, 1991). SOS-like processes have been conserved in a wide variety of eubacterial species (Miller &

Abbreviations: FL, firefly luciferase gene; RL, *Renilla reniformis* luciferase gene.

The GenBank/EMBL/DDBJ accession number for the *lexA2* sequence reported in this paper is AB256032.

Kokjohn, 1990; Roca & Cox, 1990). On the one hand, RecA is the sole protein required for LexA1 (DRA0344) cleavage, although LexA1 is not involved in RecA induction in *D. radiodurans* (Narumi *et al.*, 2001; Bonacossa de Almeida *et al.*, 2002). The *D. radiodurans* DNA-repair-deficient mutant KI696 carrying the *recA424* mutation was only slightly sensitive to γ -rays in comparison with the wild-type. RecA424 completely lacked the recombination activity but retained the co-protease activity. On the basis of this finding we suggested that the co-protease activity rather than the recombination activity of RecA contributed to the extraordinary radiation resistance of *D. radiodurans* (Satoh *et al.*, 2002). Bioinformatic analysis of the *D. radiodurans* genome sequence showed that the genome encodes a second, diverged copy of LexA1, LexA2 (DRA0074), which retains the same arrangement of the helix–turn–helix DNA-binding domain and the autocleavage domain (Makarova *et al.*, 2001). Sheng *et al.* (2004) suggested that the product of gene *dra0074* is involved in metabolic pathways other than the DNA repair response elicited by irradiation. However, the exact role of LexA2 and the relationship between LexA1 and LexA2 in the radiation response are poorly understood. The biochemical function of LexA2 therefore requires further clarification.

This study began with the purpose of elucidating the role of LexA2 in relation to the radiation response mechanism of *D. radiodurans*. To accomplish this purpose, we generated *lexA1* and *lexA2* disruptant strains in wild-type and *recA* backgrounds. Our results suggest that LexA2 contributes to the regulation of the radiation response promoter of the *pprA* gene in *D. radiodurans* and that it is not involved in RecA induction.

METHODS

Bacterial strains, cosmids, plasmids and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *D. radiodurans* cells were grown with agitation at 30 °C in TGY broth, containing 0.5% (w/v) tryptone-peptone, 0.1% (w/v) glucose and 0.3% (w/v) yeast extract, or on TGY agar solidified with 1.5% (w/v) agar. When necessary, 3 μ g chloramphenicol ml⁻¹, 16 μ g kanamycin ml⁻¹ and 350 μ g spectinomycin ml⁻¹ were added. *E. coli* strains were grown in Luria–Bertani (LB) broth–Miller or on LB agar–Lennox at 37 °C. Selective drug concentrations for *E. coli* were 50 μ g ampicillin ml⁻¹, 34 μ g chloramphenicol ml⁻¹ and 25 μ g kanamycin ml⁻¹.

Construction of gene disruptant strains. In an effort to gain an insight into the role of LexA2 in the radiation response mechanism, we generated *recA*, *lexA1* and *lexA2* disruptant strains within a wild-type background. The disruptants were generated using a previously described directed insertional mutagenesis technique (Funayama *et al.*, 1999; Narumi *et al.*, 2001; Nishida & Narumi, 2002). The previously constructed disruption plasmid pKSCR3 (carrying the *recA229::cat* mutation) (Narumi *et al.*, 2001) was used to disrupt the *recA* gene. To construct plasmids carrying the *lexA1* null mutation, a 975 bp *HincII* fragment (KatAPH cassette) from pKatAPH3 (Ohba *et al.*, 2005) or a 948 bp *HincII* fragment (KatAAD cassette) from pKatAAD2, which is a spectinomycin-resistant version of pKatAPH3, was ligated to *EagI*-digested pZA8 (Kikuchi *et al.*, 1999).

The resulting plasmids were designated pLKN2 (carrying the *lexA1-166::aph* mutation) and pLAN2 (carrying the *lexA1-166::aad* mutation), respectively.

pDC201 is a cosmid clone of the genome library (Narumi *et al.*, 1997), and it was used to clone the *D. radiodurans* *lexA2* gene. The 5.6 kb *EcoRI* fragment carrying the *D. radiodurans* *lexA2* locus was isolated from pDC201 and subcloned in the *EcoRI* site of pUC19. The resulting plasmid was designated pLE44. Plasmid pLE44 was digested with *BsrGI* and *HindIII*, blunted with T4 DNA polymerase, and then self-ligated to yield plasmid pLEB7. Plasmid pLEB7 was subjected to site-directed mutagenesis to create a unique *SmaI* site in *lexA2* (T at nucleotide position 365 was changed to G). Site-directed mutagenesis was carried out using the QuikChange Site-directed Mutagenesis kit (Stratagene) with primers T365G-F (5'-GTGCAGCAGGTCCCGGGCTACATCGCCGGTCTAG-3') and T365G-R (5'-CTGACCGCGATGTAGCCCGGGACCTGCTGCAC-3'), and the resulting plasmid was designated pLSM2. The KatAPH cassette was ligated to *SmaI*-digested pLSM2 to construct plasmids carrying the *lexA2* null mutation, and the resulting plasmid was designated pLKN2 (carrying the *lexA2-362::aph* mutation).

All disruptant strains were generated from *D. radiodurans* R₁ (ATCC 13939). The *recA229::cat*, *lexA1-166::aph* and *lexA2-362::aph* mutations were introduced into the genome of *D. radiodurans* and the resulting disruptant strains were designated XRA1 (*recA* disruptant), XLK1 (*lexA1* disruptant) and XL2K1 (*lexA2* disruptant), respectively. We introduced the *lexA1* null mutation into the *recA* disruptant strain and the resulting *recA lexA1* double-disruptant strain was designated XRL11. Additionally, the *recA* null mutation was introduced into the *lexA2* disruptant strain to yield the *recA lexA2* double-disruptant strain XL2R8, and the *lexA1* null mutation was introduced into the *lexA2* disruptant strain to yield the *lexA2 lexA1* double-disruptant strain WXL1.

Construction of expression plasmid and DNA sequencing of the *D. radiodurans* *lexA2* gene.

To isolate the *lexA2* coding region, PCR was carried out using *PfuTurbo* HotStart DNA polymerase (Stratagene) with pLEB7 and specific oligonucleotides A74EX-F3 (5'-CTCAGTCTAATTCGCATATGCGAACATTTC-3') and A74EX-R2 (5'-GCAGGGATCCGGGCAGAGGCTCAGGC-3'), possessing *NdeI* and *BamHI* restriction sites (underlined in the sequences), respectively. PCR products were then digested with *NdeI* and *BamHI* to adapt the termini for in-frame insertion of *lexA2* into the *NdeI*–*BamHI* sites in the pET3a vector (Novagen). The resulting expression plasmid was designated pET3lexA2. The DNA sequence of the expression plasmids was checked to confirm the absence of errors by PCR using T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3').

To check the sequence of the *lexA2* locus in plasmid pLEB7 and the *D. radiodurans* genome, direct sequencing was carried out using the oligonucleotide primer sets A74DI-F (5'-ATGGCCTGATCGCAGAACG-TATG-3') and A74DI-R (5'-TGTCACCTCCGTGAAAGGTGCTAGA-3').

RNA isolation and primer extension assay. *D. radiodurans* cells were grown in TGY broth at 30 °C with agitation to early stationary phase. Total RNA was isolated from the harvested cells using a FastRNA Pro Blue Kit (Qbiogene) with a DNA-free kit (Ambion). The specific oligonucleotide IRD800lexA2 (5'-CCTTCGCGCGCTGGTAGCAAG-3') was used to map the transcriptional start site of the *lexA2* mRNA. RNA (10 μ g) and the IRD800 infrared dye-labelled primer (1 pmol) were incubated in a buffer containing 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and 250 mM KCl at 64 °C for 1 h. After annealing, reverse transcription was carried out using Superscript III RNase H⁻ reverse transcriptase (Invitrogen) as recommended by the manufacturer. Analysis of the primer extension product was performed using a DNA Analyser model 4200-1G

Table 1. Strains, cosmids and plasmids

Designation	Relevant description	Source or reference
<i>D. radiodurans</i>		
R ₁	Wild-type (ATCC 13939)	ATCC
MR ₁	Wild-type	Moseley (1967)
KR ₁	Wild-type	Anderson <i>et al.</i> (1956)
KD8301	Ade ⁻ DNase ⁻ Sm ^r	Narumi <i>et al.</i> (1997)
XLK1	R ₁ <i>lexA1-166::aph</i> ; generated by insertional mutagenesis with pLKN2	This study
XL2K1	R ₁ <i>lexA2-362::aph</i> ; generated by insertional mutagenesis with pL2KN2	This study
XRA1	R ₁ <i>recA229::cat</i> ; generated by insertional mutagenesis with pKSCR3	This study
XRL11	XRA1 <i>lexA1-166::aph</i> ; generated by insertional mutagenesis with pLKN2	This study
XL2R8	XL2K1 <i>recA229::cat</i> ; generated by insertional mutagenesis with pKSCR3	This study
WXL1	XL2K1 <i>lexA1-166::aad</i> ; generated by insertional mutagenesis with pLAN2	This study
<i>E. coli</i>		
JM109	Host for plasmid subclones	Takara Bio
BLR(DE3)	Host for expression plasmid	Novagen
Cosmids		
SuperCos I	Cosmid vector; 7.6 kb; Ap ^r Km ^r	Stratagene
pDC201	SuperCos I with 33.4 kb <i>D. radiodurans</i> KD8301 DNA; <i>lexA2</i> ⁺	Narumi <i>et al.</i> (1999)
Plasmids		
pUC19	<i>E. coli</i> vector; 2.7 kb; Ap ^r	Takara Bio
pET3a	<i>E. coli</i> vector; 4.6 kb; Ap ^r	Novagen
pLE44	pUC19 with 5589 bp <i>EcoRI</i> fragment from pDC201	This study
pLEB7	pUC19 with 3385 bp <i>EcoRI</i> – <i>BsrGI</i> fragment from pLE44	This study
pLSM2	pLEB7 containing a unique <i>SmaI</i> site	This study
pET3lexA2	pET3a with <i>NdeI</i> – <i>BamHI</i> ::747 bp PCR product from pLEB7	This study
pKatAPH3	pUC19 containing <i>aph</i> and <i>D. radiodurans katA</i> promoter; 2.3 kb; Km ^r	Ohba <i>et al.</i> (2005)
pL2KN2	pLEB7 <i>SmaI</i> ::975 bp <i>HincII</i> fragment from pKatAPH3; <i>lexA2-362::aph</i>	This study
pZA8	pUC19 with 6005 bp <i>D. radiodurans</i> DNA; <i>lexA1</i> ⁺	Kikuchi <i>et al.</i> (1999)
pLKN2	pZA8 <i>EagI</i> ::975 bp <i>HincII</i> fragment from pKatAPH3; <i>lexA1-166::aph</i>	This study
pKSCR3	pUC19 containing <i>recA229::cat</i>	Narumi <i>et al.</i> (2001)
pKatAAD2	pUC19 containing <i>aad</i> and <i>D. radiodurans katA</i> promoter; 2.2 kb; Spc ^r	This study
pLAN2	pZA8 <i>EagI</i> ::948 bp <i>HincII</i> fragment from pKatAAD2; <i>lexA1-166::aad</i>	This study
pKatHPH4	pUC19 containing <i>hph</i> and <i>D. radiodurans katA</i> promoter; 2.5 kb; Hyg ^r	This study
pHOB12R	<i>E. coli</i> – <i>D. radiodurans</i> shuttle vector containing <i>D. radiodurans pprA</i> promoter-regulated FL and <i>D. radiodurans groE</i> -promoter regulated RL; 9 kb; Cm ^r , Ap ^r	Ohba <i>et al.</i> (2005)
p12R-Hyg	pHOB12R but Hyg ^r	This study

(LI-COR). The size of the primer extension product was determined by comparison with the DNA sequence generated using the same primer with plasmid pLEB7 as a template.

Protein purification. *E. coli* BLR(DE3) carrying pET3lexA2 was cultivated in LB broth-Miller containing ampicillin. At an OD₆₀₀ of approximately 0.5, IPTG was added to a final concentration of 0.4 mM and growth was continued for an additional 2 h. The cells were then harvested by centrifugation. The cell pellet was resuspended in cold buffer P1 (20 mM sodium phosphate pH 6.0 and 0.1 mM EDTA) containing 1 mM PMSF and 0.1% (w/v) protease inhibitor cocktail (Calbiochem) in 1/100 of the original culture volume, and stored at –80 °C. All subsequent steps were carried out at 4 °C unless otherwise indicated.

Cells were allowed to thaw with occasional stirring and the suspension was sonicated for 10 min on ice; debris was removed by centrifugation at 10 000 g, 4 °C for 30 min. The supernatant was then applied to a HiPrep 16/10 Heparin FF column (GE Healthcare) equilibrated with

buffer P1. The column was washed with four column volumes of buffer P1. Following elution with 10 volumes of a linear NaCl gradient (0 to 1 M), the fractions containing LexA2 were pooled. The majority of LexA2 was eluted at approximately 0.6 M NaCl. The pooled fractions were concentrated and desalted using an Amicon Ultra-15 filter unit (Millipore) with buffer P1, and the fraction was then loaded onto a Mono S HR 5/5 column (GE Healthcare) equilibrated with buffer P1. The column was washed with 10 column volumes of buffer P1. Following elution with 20 volumes of a linear NaCl gradient (0.3 to 1 M), the fractions containing LexA2 were pooled. The majority of LexA2 was eluted at approximately 0.5 M NaCl. The pooled fractions were concentrated using an Amicon Ultra-15 filter unit with buffer P1.

Autodigestion and RecA-mediated cleavage reaction. The autodigestion reaction was assayed by incubating 8 µM LexA2 in 50 mM Tris/HCl (pH 10.0) at 37 °C for 16 h. For the cleavage of LexA2 mediated by *D. radiodurans* RecA, the reaction mixture contained 20 mM Tris/HCl (pH 6.0), 10 mM MgCl₂, 6 µM oligonucleotide (5'-GAGAACCATGGATGGACCAATAATAATGACTAGAG-3';

35-mer), 1 mM ATP γ S, 4.2 μ M RecA (Satoh *et al.*, 2002) and 8 μ M LexA2. To activate RecA by binding with ATP γ S and oligonucleotide, the mixture without LexA2 was first combined in a microtube and preincubated at 37 °C for 10 min. The reaction was then initiated by addition of LexA2, and the reaction mixture was incubated at 37 °C for 1 h. After quenching by addition to SDS sample buffer and heating at 95 °C for 10 min, the reaction products were subjected to 15% (w/v) SDS-PAGE. LexA2 and breakdown products were visualized by staining with GelCode Blue Stain Reagent (Pierce).

Detection of intracellular levels of *D. radiodurans* RecA, LexA1 and LexA2. *D. radiodurans* cells were grown in TGY broth at 30 °C with agitation to early stationary phase. Cells were harvested by centrifugation at 5000 g, 4 °C for 5 min, washed twice with 10 mM sodium phosphate buffer (pH 7.0) and resuspended in the same buffer. Aliquots (20 ml) of the cell suspension were irradiated at room temperature for 1 h with ⁶⁰Co γ -rays at a dose rate of 2 kGy h⁻¹. The cells were then centrifuged at 5000 g, 4 °C for 5 min and resuspended in 200 ml TGY broth. The cell suspension was incubated at 30 °C for different post-irradiation times (0, 0.5, 1 and 2 h). Aliquots (80 ml) of the culture were then harvested as needed. To obtain crude protein extract, the cells were resuspended in lysis buffer (0.5 ml) containing 50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1 mM PMSF, 0.1% (w/v) protease inhibitor cocktail (Calbiochem) and 1% (w/v) SDS, and disrupted using a FastPrep Cell Disruptor FP120 (Savant Instruments) with a FastProtein Blue Kit (Qiogene). Following removal of cell debris by centrifugation, the supernatant was subjected to 12.5 or 15% (w/v) SDS-PAGE. The resolved proteins were transferred onto a PVDF membrane (Millipore). *D. radiodurans* RecA, LexA1 or LexA2 were detected using *D. radiodurans* RecA antibody (diluted 1:10 000), *D. radiodurans* LexA1 antibody (diluted 1:10 000) (Narumi *et al.*, 2001) or *D. radiodurans* LexA2 antibody (diluted 1:4000) together with alkaline-phosphatase-conjugated anti-mouse or anti-rabbit IgG antisera (Applied Biosystems). The *D. radiodurans* RecA and LexA2 antibodies (mouse, polyclonal) were raised against purified *D. radiodurans* RecA (Satoh *et al.*, 2002) and purified *D. radiodurans* LexA2, respectively, at Immuno-Biological Laboratories (Gunma, Japan). Chemiluminescent signals on the PVDF membrane were visualized using a Lumi-Imager F1 Workstation (Roche Diagnostics). *D. radiodurans* LexA1 and LexA2 antibodies did not cross-react with each antigen.

Measurement of cell survival rate. *D. radiodurans* cells were grown, harvested, washed and resuspended as described above. Aliquots (0.1 ml) of the cell suspension were dispensed into test tubes and irradiated at room temperature for 1 to 3 h with ⁶⁰Co γ -rays at dose rates from 0.1 to 5 kGy h⁻¹ that were regulated by changing the distance of the samples from the γ -ray source. Irradiated samples were appropriately diluted with 10 mM phosphate buffer, spread onto TGY agar, and incubated at 30 °C for 3 days prior to the enumeration of colonies.

Luciferase reporter assay. The luciferase reporter plasmid pHOB12R contained engineered firefly luciferase gene (FL), *Renilla reniformis* luciferase gene (RL) and the chloramphenicol resistance gene as a genetic marker. The expression of FL and RL was regulated by the *D. radiodurans* *pprA* and *groE* minimal promoters, respectively (Ohba *et al.*, 2005). The luciferase reporter plasmid p12R-Hyg, which carries a hygromycin-resistance gene instead of a chloramphenicol-resistance gene, was constructed as a pHOB12R-based plasmid. A 1199 bp *HincII* fragment (KatHPH cassette) from pKatHPH4, which is a hygromycin-resistant version of pKatAPH3, was ligated to *MscI*- and *FspI*-digested pHOB12R. *D. radiodurans* cells carrying the resulting plasmid p12R-Hyg were grown, harvested, washed and resuspended as described above. Aliquots (1 ml) of the cell suspension were irradiated at room temperature for 2 h with ⁶⁰Co γ -rays at a dose rate of 4 kGy h⁻¹. The cells were then centrifuged at 5000 g, 4 °C for 5 min, and resuspended in 4 ml TGY broth. The

cell suspension was incubated at 30 °C for different post-irradiation incubation times, with agitation. FL and RL activities in cells (20 μ l) were measured using the Dual-Glo Luciferase Assay System (Promega). Chemiluminescent signals were visualized using a Lumi-Imager F1 Workstation. The relative reporter activity was calculated as the ratio of the luminescence from FL (experimental reporter) to the luminescence from RL (control reporter).

RESULTS

Sequence and structure of *D. radiodurans* *lexA2*

To construct the disruptant plasmid carrying the *lexA2* null mutation, we tried digesting plasmid pLEB7 with *Aor51HI*, based on the DRA0074 sequence deposited in the public database (GenBank accession no. AE001862). However, pLEB7 surprisingly could not be digested with *Aor51HI*. We therefore decided to check the nucleotide sequence of the *lexA2* locus in pLEB7. We found that there is an additional cytosine nucleotide (nucleotide position 612 in Fig. 1) that was not present in the DRA0074 sequence. Furthermore, the *lexA2* loci in *D. radiodurans* strains MR₁, KR₁ and R₁, which are different isolates of the wild-type strain (Islam *et al.*, 2003), were resequenced using primers A74DI-F and A74DI-R. In each case, the sequence was identical to our *lexA2* sequence, suggesting an error in the previous sequence. We deposited our *lexA2* sequence in GenBank under accession no. AB256032. The addition of one nucleotide affected the open reading frame; 44 amino acid residues were altered, and the predicted amino acid sequence of *lexA2* was 8 amino acid residues longer than the previous sequence (Fig. 1). Fig. 2 shows the alignment of the amino acid sequence of *E. coli* LexA and *D. radiodurans* LexA1 and LexA2. Although *D. radiodurans* LexA2 exhibited low similarity to LexA of *E. coli* and *D. radiodurans* (23.4% and 24.4% identity, respectively), LexA2 retained the amino acid residues involved in self-cleavage of the protein (Ala-130, Gly-131, Ser-163 and Lys-203) and a helix–turn–helix DNA-binding motif (Ile-38 to Leu-100) (Fig. 2). The C-terminal region of *E. coli* LexA (Phe-186 to Arg-197) participates in dimeric interactions (Fig. 2) (Luo *et al.*, 2001). The C-terminal region of LexA2 revealed in this study may also be involved in dimeric interactions in a manner similar to *E. coli* LexA. The amino acid sequence alignment shows that *D. radiodurans* LexA2 has a longer N-terminal sequence than *E. coli* and *D. radiodurans* LexA1 proteins (Fig. 2). The primer extension assay revealed that the transcription start site of *lexA2* was located at –97 and –98 upstream of the translation start site (Fig. 3). No promoter-like sequence was found between the translation start and transcription start sites for *lexA2*. These results support the existence of an extended N-terminal region in *D. radiodurans* LexA2.

Autodigestion and RecA-mediated cleavage

The purified *D. radiodurans* LexA2 migrated on SDS-polyacrylamide gel with an apparent molecular mass of

Fig. 1. Nucleotide sequence of the *D. radiodurans* *lexA2* gene. The predicted amino acid sequence is shown above the nucleotide sequence of DRA0074 described in a previous study (White *et al.*, 1999) in single-letter code. The predicted amino acid sequence of *D. radiodurans* *lexA2* determined in this study is shown below the nucleotide sequence. Numbers on both sides represent the coordinates of each sequence. The filled triangle indicates the nucleotide that differs between the two sequences. The reading frame affected by the previous error is shown in boxes.

approximately 26 kDa (Fig. 4a). This was very close to the molecular mass (26 750 Da) calculated from the amino acid sequence of LexA2.

As described above, LexA2 retained the amino acid residues involved in self-cleavage by the amino acid alignment (Fig. 2). Similar to *E. coli* LexA, *D. radiodurans* LexA1 undergoes autodigestion when subjected to alkaline conditions and RecA-mediated cleavage (Narumi *et al.*, 2001). We investigated whether these proteolytic cleavage properties are conserved in *D. radiodurans* LexA2. Purified LexA2 was autodigested at pH 10 to yield two breakdown products (Fig. 4b, lane 2). LexA2 was cleaved by incubation with *D. radiodurans* RecA to produce two breakdown products whose sizes are the same as those observed in

autodigestion (Fig. 4b, lane 6). No breakdown product was observed when RecA, oligonucleotide or ATP γ S was omitted from the reaction (Fig. 4b, lanes 3 to 5). These results support the conclusion that *D. radiodurans* LexA2 maintains proteolytic activity that can be stimulated by *D. radiodurans* RecA.

Changes in intracellular RecA, LexA1 and LexA2 levels in disruptants following irradiation

To determine if LexA2 is involved in RecA induction and whether cleavage of LexA2 is promoted by RecA *in vivo*, changes in RecA, LexA1 and LexA2 levels following γ -irradiation (2 kGy) were compared between parental and disruptant strains.

Fig. 2. Multiple amino acid sequence alignment of *E. coli* LexA (Hori *et al.*, 1981) and *D. radiodurans* LexA1 (Narumi *et al.*, 2001) and LexA2 (this study). Multiple alignment was determined using CLUSTAL W (Thompson *et al.*, 1994). Dashes indicate gaps in the alignment. Numbers on both sides represent the coordinates of each protein. Asterisks indicate identical residues. Filled triangles indicate conserved Ala and Gly residues in the cleavage site, as well as Ser and Lys residues required for cleavage. The locations of three α -helices (H1, H2 and H3) found in *E. coli* LexA are shown according to the description of Fogh *et al.* (1994). The underline indicates the helix-turn-helix DNA-binding motif of *D. radiodurans* LexA2 that was detected using the Search Motif program (Bateman *et al.*, 2004). The box indicates the region of dimeric interactions in *E. coli* LexA (Luo *et al.*, 2001).

Fig. 3. Transcription start site of *lexA2* determined by primer extension analysis. Sequence ladders of the 5'-flanking region of *lexA2* primed with the same primer as used in the primer extension reaction are shown in lanes A, C, G and T. The arrows indicate the primer extension products. Transcription start sites are marked with asterisks.

Fig. 5 shows the results of experiments in which cells treated with or without radiation were incubated in fresh TGY broth for different post-irradiation incubation times and then subjected to Western blot analysis using *D. radiodurans* RecA, LexA1 or LexA2 antibodies. The intracellular level of RecA increased in the *lexA1* disruptant strain following γ -irradiation as in the wild-type strain (Fig. 5a). The LexA1 level in the *recA* disruptant strain did not change for any of the post-irradiation incubation times examined (Fig. 5b). These results confirmed our previous conclusions that *D. radiodurans* LexA1 is not involved in RecA induction following γ -irradiation and that RecA is the sole protein required for LexA1 cleavage (Narumi *et al.*, 2001). RecA induction following γ -irradiation was also observed in the *lexA2* disruptant and *lexA1 lexA2* double-disruptant strains (Fig. 5a), clearly indicating that neither LexA1 nor LexA2 is involved in RecA induction. While a decreased level of LexA2 was observed in wild-type and *lexA1* disruptant strains following γ -irradiation for all post-irradiation incubation times (Fig. 5c), γ -irradiation did not affect the level of LexA2 in the *recA* disruptant strain. This result indicated that RecA is the sole protein required for LexA2 cleavage, and supported our observation of *in vitro* RecA-mediated LexA2 cleavage (Fig. 4b).

In the wild-type strain, the LexA1 level decreased to half the initial level at 1 h following irradiation, and increased to 62 % of the initial level 2 h following irradiation (Fig. 5b). Surprisingly, the LexA1 level in the *lexA2* disruptant strain almost recovered 1 h after irradiation, although the decrease

Fig. 4. *D. radiodurans* LexA2 protein. (a) Purification of LexA2. Samples were subjected to 15 % SDS-PAGE and stained with GelCode Blue Stain Reagent. Lane 1, 10 kDa protein ladder (Invitrogen); lane 2, total cellular proteins from *E. coli* BLR(DE3)/pET3lexA2wt induced by IPTG; lane 3, pooled LexA fractions from HiTrap Heparin HP column; lane 4, pooled LexA fractions from Mono S column. The arrow on the right indicates the position of the 26 kDa band of LexA2. (b) Autodigestion and RecA-mediated cleavage of purified LexA2. LexA2 was incubated in 50 mM Tris/HCl (pH 10.0) for 0 h (lane 1) and 16 h (lane 2). For RecA-mediated cleavage, LexA2 was incubated in the absence or presence of *D. radiodurans* RecA, oligonucleotide (5'-GAGAACCATGGATGGACCAATAATAATGACTAGAG-3'; 35-mer) and ATP γ S in a buffer containing 20 mM Tris/HCl (pH 6.0), 10 mM MgCl₂ at 37 °C for 1 h, and subjected to 15 % (w/v) SDS-PAGE. LexA2 and breakdown products were visualized by staining with GelCode Blue Stain Reagent. Lane 3, RecA was omitted; lane 4, oligonucleotide was omitted; lane 5, ATP γ S was omitted; lane 6, complete reaction. Arrows indicate the positions of breakdown products of LexA2.

in the LexA1 level was observed until 0.5 h in the case of the wild-type strain (Fig. 5b). The damaged DNA leads to the production of segments of ssDNA that act as a substrate for RecA activation. As in *E. coli*, the co-protease of *D. radiodurans* RecA could not activate in the absence of ssDNA (Fig. 4b, lane 4). The segments of ssDNA disappear as a consequence of various DNA repair processes. Since the decrease in the intracellular LexA1 level reflects the co-protease activity of RecA, the recovery to a basal level of LexA1 indicates the inactivation of RecA (Fig. 5b). This

Fig. 5. Changes in intracellular RecA, LexA1 and LexA2 levels following irradiation. Cells were irradiated with γ -rays at a dose of 2 kGy. Dots indicate the integrated density of chemiluminescent signals by Western blotting at the indicated times. Each point represents the mean $\pm$ SD of three independent experiments. (a) RecA increase. (b) LexA1 remaining. (c) LexA2 remaining. ●, Wild-type; ○, *recA* disruptant strain; □, *lexA1* disruptant strain; △, *lexA2* disruptant strain; ◆, *lexA1 lexA2* double-disruptant strain.

result suggested that the inactivation of RecA was faster in the *lexA2* disruptant strain than in the wild-type strain. Given that the inactivation of RecA is associated with the completion of DNA repair, the DNA repair capacity might

be enhanced in the *lexA2* disruptant strain relative to the wild-type strain.

Sensitivity of gene disruptant strains to γ -rays

To investigate DNA repair capacities, we examined the sensitivity of the disruptant strains to γ -rays. As shown in Fig. 6(a), the *lexA2* disruptant and *lexA1 lexA2* double-disruptant strains exhibited a much higher resistance than the wild-type strain at high doses (8 to 15 kGy) of γ -rays. The LD₉₀ values (12.7 kGy) of the *lexA2* disruptant and *lexA1 lexA2* double-disruptant strains were 1.6-fold higher than those for the wild-type strain (7.8 kGy). On the other hand, the radioresistance of the *lexA1* disruptant strain was equal to that of the wild-type strain.

We also examined the effect of *lexA1* and *lexA2* disruption on radioresistance in a *recA* background. As shown in Fig. 6(b), the *recA* disruptant strain exhibited extreme sensitivity (LD₉₀ value 0.09 kGy). In a *recA* background, neither *lexA1* nor *lexA2* disruption altered the radio-sensitivity of *D. radiodurans* (LD₉₀ values 0.09 and 0.12 kGy, respectively).

Change in *pprA* promoter activation in disruptants following γ -irradiation

The *pprA* gene encoding the novel radiation-inducible protein PprA plays a critical role in the radioresistance of *D. radiodurans* (Narumi *et al.*, 2004). To investigate whether LexA homologues are involved in PprA induction, changes in *pprA* promoter activation following γ -irradiation (8 kGy) were compared between parental and disruptant strains using the luciferase reporter assay. *pprA* promoter activation reached a maximum at 3 h post-irradiation incubation in all of the strains (Fig. 7). Remarkably, the maximum level of *pprA* promoter activation in the *lexA2* disruptant strain was approximately 1.4-fold higher than that recorded for the wild-type strain. The change in *pprA* promoter activation in the *lexA1* disruptant strain was very similar to that of the wild-type strain. On the other hand, the maximum level of *pprA* promoter activation in *recA* disruptant strains was lower than that of the wild-type strain. The maximum level of *pprA* promoter activation of the *recA lexA2* double-disruptant strain was slightly higher than that recorded for the *recA* disruptant strain. These results indicated that the *pprA* promoter activation was enhanced by *lexA2* disruption.

DISCUSSION

In this study, we resequenced the *lexA2* sequence of *D. radiodurans* R₁ (Fig. 1) and found a single base difference between our sequence and the published *D. radiodurans* R₁ genome sequence (White *et al.*, 1999). It was reported recently that the *D. radiodurans* strain used in the genome project was not the R₁ type strain that had been deposited in the American Type Culture Collection as ATCC no. 13939, but that it was the strain designated BAA-816 in which it was

Fig. 6. Sensitivity of *recA*, *lexA1* and *lexA2* disruptant strains to γ-rays. Each point represents the mean ± SD of three independent experiments. (a) ●, Wild-type; □, *lexA1* disruptant strain; △, *lexA2* disruptant strain; ◆, *lexA1 lexA2* double-disruptant strain. (b) ○, *recA* disruptant strain; ■, *recA lexA1* double-disruptant strain; ▲, *recA lexA2* double-disruptant strain.

suspected many mutations were acquired during years of separate culture (Mennecier *et al.*, 2004). Although resequencing of the *lexA2* locus in this study revealed the correct sequence, the fact remains that *D. radiodurans* contained a second, diverged copy of *lexA1*.

We purified *D. radiodurans* LexA2 to obtain a molecular mass of approximately 26 kDa (Fig. 4a), which was smaller than the molecular mass (approx. 33 kDa) of *D. radiodurans* DRA0074 purified by Sheng *et al.* (2004). For this reason it was thought that the *lexA2* stop codon of the expression plasmid pET3lexA2wt constructed in this study differed from the expression plasmid constructed by Sheng *et al.* (2004) because they had designed PCR primers for amplifying the *dra0074* gene on the basis of the published sequence (White *et al.*, 1999). Consequently, the DRA0074 protein purified by Sheng *et al.* (2004) may contain amino acid residues that originated from a vector at its C-terminal region.

The N-terminal region of *E. coli* LexA is involved in DNA-binding activity (Fogh *et al.*, 1994). The purification of *D. radiodurans* LexA2 showed a heparin column to be very effective (Fig. 4a), suggesting that LexA2 retains

Fig. 7. Changes in *pprA* promoter activation following irradiation. Cells were irradiated with γ-rays at a dose of 8 kGy. The relative reporter activity was calculated as the ratio of the luminescence from the experimental reporter (FL) to the luminescence from the control reporter (RL) at the indicated times. Each point represents the mean ± SD of three independent experiments. ●, Wild-type; □, *lexA1* disruptant strain; △, *lexA2* disruptant strain; ○, *recA* disruptant strain; ■, *recA lexA1* double-disruptant strain; ▲, *recA lexA2* double-disruptant strain. All strains carried plasmid p12R-hyg, in which the radiation-responsive minimal promoter region (−57 to −1 upstream of the initiation site) of the *pprA* gene was located upstream of FL.

DNA-binding ability. Amino acid sequence alignment revealed that the N-terminal region of *D. radiodurans* LexA2 did not possess similarities to the *E. coli* and *D. radiodurans* LexA1 proteins (Fig. 2). These results suggested that *D. radiodurans* possesses distinct LexA1- and LexA2-binding motifs that differ from the *E. coli* SOS box (Walker, 1984; Friedberg *et al.*, 1995). *D. radiodurans* LexA1 and LexA2 may be involved in different gene regulations.

A hypothesis concerning the radiation response mechanism in *D. radiodurans* is presented in Fig. 8 on the basis of the results obtained in the present and previous studies. Unlike *E. coli*, *D. radiodurans* LexA1 is not involved in RecA induction following γ-irradiation (Narumi *et al.*, 2001; Bonacossa de Almeida *et al.*, 2002). Furthermore, neither the *lexA1* gene deletion nor the mutation leading to a non-cleavable LexA1 affected survival or double-strand breaks repair, suggesting that LexA1 does not play a major role in the radiation response mechanism following γ-irradiation in *D. radiodurans* (Jolivet *et al.*, 2005). The results of radioresistance in the *lexA1* disruptant strains (Fig. 6) are consistent with these conclusions. A previous study assumed that LexA1 and DRA0074 have similar regulatory functions in the metabolic pathway because a *lexA1 dra0074* double-disruptant strain could not be constructed (Sheng *et al.*, 2004). However, we successfully constructed a *lexA1 lexA2* double-disruptant strain in the present study. The induction of RecA was observed in the *lexA1 lexA2* double-disruptant

strain (Fig. 5a), indicating that the two LexA homologues do not exhibit functional overlap in relation to RecA induction. These results support the notion that *recA* expression following γ -irradiation is regulated by DR0167 (also called PprI/IrrE) in *D. radiodurans* (Earl *et al.*, 2002; Hua *et al.*, 2003) (Fig. 8).

In a previous study we suggested that the co-protease activity rather than the recombination activity of RecA contributed to radiation resistance in *D. radiodurans*, given that a *D. radiodurans recA* mutant strain that completely lacked recombination activity but retained co-protease activity of RecA showed only slight sensitivity to γ -rays compared with the wild-type (Satoh *et al.*, 2002). RecA can accelerate autocleavage of *D. radiodurans* LexA1 (Narumi *et al.*, 2001), and LexA2 autocleavage is also accelerated by RecA (Fig. 4b, lane 6). This suggests that a certain gene down-regulated by LexA1 and/or LexA2 might be important for the radioresistance of *D. radiodurans*.

Sheng *et al.* (2004) examined the radiosensitivity of dra0074 disruptant strain M74 up to a dose of 8 kGy, and found no significant difference in survival curves between strain M74 and the wild-type. However, we found that *lexA2* disruptant strains exhibited a much higher resistance to γ -rays than the wild-type at high doses (8 to 15 kGy) (Fig. 6a). At least part of the increase in radioresistance of the *lexA2* disruptant strain may be explained by the enhancement of *pprA*

promoter activation (Fig. 8). *D. radiodurans* PprA preferentially binds to dsDNA carrying strand breaks (Narumi *et al.*, 2004). DNA strand breaks also result from the process of DNA replication. We suggest that the intracellular PprA level must be regulated. If PprA is induced constitutively, cell growth will be inhibited in *D. radiodurans* because PprA binds to DNA strand breaks that are generated by DNA replication. Therefore, the regulation by LexA2 contributes to the stable maintenance of cell growth in the undamaged state. While an enhancement of *pprA* promoter activation in the *recA lexA2* double-disruptant strain was not prominent (Fig. 7), *lexA2* disruption did not restore radiosensitivity in the *recA* background (Fig. 6b). This finding suggested that the radiation response mechanism proceeds in a RecA-dependent manner.

ACKNOWLEDGEMENTS

We thank Y. Kobayashi, M. Kikuchi, T. Funayama and S. Kitayama for valuable discussions. This work was funded in part by a grant to I. Narumi from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Fig. 8. Hypothesis concerning the radiation response mechanism in *D. radiodurans*. Following DNA damage, PprI up-regulates the induction of RecA and PprA (Earl *et al.*, 2002; Hua *et al.*, 2003; Ohba *et al.*, 2005). RecA is activated by binding with ssDNA segments generated from the damaged DNA. Activated RecA serves as a co-protease to stimulate LexA1 (Narumi *et al.*, 2001) and LexA2 cleavages. An unknown protein down-regulated by LexA2 enhances *pprA* promoter activation. RecA and PprA play crucial roles in recombination and end-joining repairs, respectively (Satoh *et al.*, 2002; Narumi *et al.*, 2004).

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