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The LexA Protein from *Deinococcus radiodurans* Is Not Involved in RecA Induction following γ Irradiation

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The involvement of LexA in induction of RecA was investigated in *Deinococcus radiodurans*. As in the wild-type strain, an increase in RecA protein synthesis following γ irradiation was detected in a *lexA* disruptant, indicating that LexA is not involved in the induction of RecA in *D. radiodurans*.

Deinococcus radiodurans is characterized by its extraordinary radiation resistance phenotype, which is considered to be due to a highly proficient DNA repair capacity (3, 25, 34). The most striking feature of *D. radiodurans* is that it can mend over 100 double-strand breaks (DSBs) of genomic DNA during postirradiation incubation (2, 18). As the rejoining of DSBs can be prevented by adding chloramphenicol to the incubation mixture, proteins induced by irradiation are necessary for the rejoining of DNA breakages (18). Several DNA damage-inducible proteins that may be required for DNA repair have been detected in the cell extract of *D. radiodurans* by two-dimensional polyacrylamide gel electrophoresis (PAGE) (12, 35). These observations suggest that *D. radiodurans* possesses a DNA damage response mechanism. However, little is known about the molecular basis for the control of the inducible proteins.

In *Escherichia coli*, the inducible DNA repair system (the SOS system) is regulated by two key proteins; RecA and LexA (8, 38). *E. coli* RecA is activated by DNA damage to mediate the proteolytic cleavage of the *E. coli* LexA repressor, resulting in derepression of the SOS regulon. 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 (42). The *B. subtilis* repressor (termed DinR) binds the promoter regions of several *din* genes and *B. subtilis recA* (20, 23, 40, 41) and undergoes autodigestion under alkaline conditions and RecA-mediated cleavage under more physiological conditions (23, 41). It has also been shown that the intracellular level of intact DinR is significantly reduced following DNA damage (23). Thus, the basic mechanism of the SOS response seems to be conserved between *E. coli* and *B. subtilis*. *Deinococcus* species form a coherent phylogenetic cluster related to the *Thermus-Meiothermus* lineage (30), indicating that the *Deinococcus* lineage is distinct from the lineages of both proteobacteria and gram-positive bacteria. Although SOS-like processes have been documented in a wide variety of eubacterial species (24, 32), the involvement of RecA and LexA in the SOS response is poorly understood in *Deinococcus* and closely related bacterial species.

As expression of the deinococcal *recA* gene is enhanced after γ irradiation (4), the *recA* gene seems to be a member of a DNA damage response regulon in *D. radiodurans*. In the present study, *D. radiodurans* LexA was purified from *E. coli* cells and its ability to cleave itself was examined. The changes in intracellular levels of the LexA and RecA proteins following γ irradiation were also investigated by using *lexA* and *recA* disruptant strains to gain insight into the DNA damage response mechanism in *D. radiodurans*. Our results indicated that deinococcal LexA undergoes RecA-mediated cleavage but is not involved in the regulation of deinococcal RecA.

Expression plasmid construction. pDC144 is a cosmid clone in a genomic library of *D. radiodurans* strain KD8301 (Table 1). pZA8 (16) contains *D. radiodurans lexA* in a 6,005-bp *Sal*I-*Sac*I fragment from pDC144. The nucleotide sequence of this region was confirmed to be perfectly consistent with the corresponding region of *D. radiodurans* (39). To isolate the *lexA* coding region, PCR was carried out by using pZA8 DNA with the specific oligonucleotides 5′-GGCAAACTGCGCGCATATGCCGCCTGAACTG-3′ and 5′-GTCGGGATCCTACTCGGTCACGCGGTGGCTCACG-3′, containing restriction sites (*Nde*I and *Bam*HI, which are underlined). PCR products were then digested with *Nde*I and *Bam*HI to adapt the termini for the in-frame insertion of *lexA* into the *Nde*I-*Bam*HI sites in the pET3a vector. The resultant expression plasmid was designated pET3lexAwt (Table 1). The DNA sequence of the expression plasmid was checked to confirm the lack of introduction of errors by PCR.

Protein purification. *D. radiodurans* LexA was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) in *E. coli* strain BL21(DE3) carrying pLysS and pET3lexAwt. Cells were harvested, washed, and resuspended in a buffer containing 20 mM

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TABLE 1. Strains, cosmids, and plasmids used in this study

Designation	Relevant description	Source or reference
D. radiodurans		
KR_1	Wild type (γ ray^r)	17
KD8301	KR_1 Ade$^-$ DNase$^-$ Sm^r (γ ray^r)	28
XE1	KD8301 *lexA166*::*cat*; generated by insertional mutagenesis with pXKE6	This work
RN201	KR_1 *recA229*::*cat*; generated by insertional mutagenesis with pKSCR3	This work
E. coli		
JM109	Host of pUC- and pET3a-based plasmids	Takara
BL21 (DE3)	Host for gene expression	Novagen
Cosmids		
SuperCos 1	Cosmid vector; 7.6 kb; Ap^r Km^r	Stratagene
pDC144	SuperCos 1 with 34.4-kb *D. radiodurans* DNA; *lexA*$^+$	28
Plasmids		
pUC18	*E. coli* cloning vector; 2.7 kb; Ap^r	Takara
pUC19	*E. coli* cloning vector; 2.7 kb; Ap^r	Takara
pET3a	*E. coli* expression vector; 4.6 kb; Ap^r	Novagen
pLysS	*E. coli* plasmid containing T7 lysozyme gene; 4.9 kb; Cm^r	Novagen
pKatCAT	pUC19 containing *cat* and *D. radiodurans katAp*; 3.6 kb; Ap^r Cm^r	9
pZA8	pUC19 with 6,005-bp *Sal*I-*Sac*I fragment from pDC144	16
pET3lexAwt	pET3a *Nde*I-*Bam*HI::635-bp PCR product from pZA8	This work
pXKE6	pZA8 *Eag*I::915-bp *Hin*cII fragment from pKatCAT; *lexA166*::*cat*	This work
pKS1	pUC18 with 4,402-bp *Apa*LI-*Sph*I fragment containing *recA*	29
pKSCR3	pKS1 *Bgl*II::915-bp *Hin*cII fragment from pKatCAT; *recA229*::*cat*	This work

sodium phosphate (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, and 0.1% (wt/vol) protease inhibitor cocktail. The suspension was sonicated for 10 min, and debris was removed by centrifugation. Ammonium sulfate was added to the supernatant to give 30% saturation. The suspension was stirred for 1 h and then centrifuged for 30 min. The pellets were resuspended in a buffer containing 20 mM sodium phosphate (pH 7.4) and 0.1 mM EDTA, and the suspension was dialyzed for 18 h. The protein was further purified to apparent homogeneity by column chromatography on HiTrap Heparin HP and Resource S (Fig. 1). The N-terminal amino acid sequence of the purified protein was found to be Pro-Pro-Glu-Leu-Thr-Pro-Thr-Arg-Arg-Ser-Ile-Leu-Gln-Ala-. This was completely consistent with the sequence from residue 2 to residue 15 of the primary structure predicted from the DNA sequence data. Thus, the purified protein was confirmed to be *D. radiodurans* LexA.

Autodigestion and RecA-mediated cleavage. First, whether the purified protein has autodigestion and RecA-mediated cleavage activity was investigated. For this purpose, antiserum raised against the purified *D. radiodurans* LexA protein was generated. The autodigestion reaction was assayed by incubating 0.4 μM LexA in 50 mM Tris-HCl (pH 10) at 37°C and monitored by Western analysis. As shown in Fig. 2, LexA was autodigested to yield two breakdown products. For RecA-mediated cleavage, reactions were carried out in a buffer consisting of 20 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ with 6 μM oligonucleotide (35-mer) and 1 mM adenosine-5′-*O*-[γ-thio]triphosphate (ATPγS). LexA (0.4 μM) was incubated with *D. radiodurans* RecA (4.2 μM) at 37°C for 1 h and sampled for Western analysis. The purification of the RecA protein will be reported elsewhere. LexA was cleaved by incubation with RecA to yield two breakdown products the sizes of which were identical to those observed in autodigestion. When the RecA, oligonucleotide, or ATPγS was omitted from the reaction mixture, no breakdown product was observed (Fig. 2). From these results, we concluded that *D. radiodurans* LexA maintains proteolytic activity and that *D. radiodurans* RecA can promote the proteolytic activity of LexA.

Construction of gene disruptant strains. To test our assumption mentioned above, we investigated the in vivo interaction of LexA and RecA by detecting changes in intracellular protein levels following γ irradiation. For this purpose, we generated a *lexA* disruptant strain and a *recA* disruptant strain

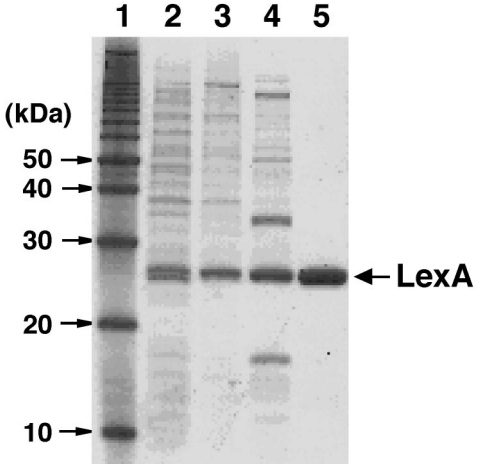

FIG. 1. Purification of *D. radiodurans* LexA protein. Samples were subjected to sodium dodecyl sulfate–15% PAGE and stained with Coomassie brilliant blue. Lanes: 1, 10-kDa protein ladder (Invitrogen); 2, total cellular proteins from *E. coli* BL21(DE3)/pLysS/pET3lexAwt induced by IPTG; 3, resuspension from 30% ammonium sulfate precipitation; 4, pooled LexA fractions from a HiTrap Heparin HP column; 5, pooled LexA fractions from a Resource S column. The position of the 25-kDa band of LexA is indicated on the right.

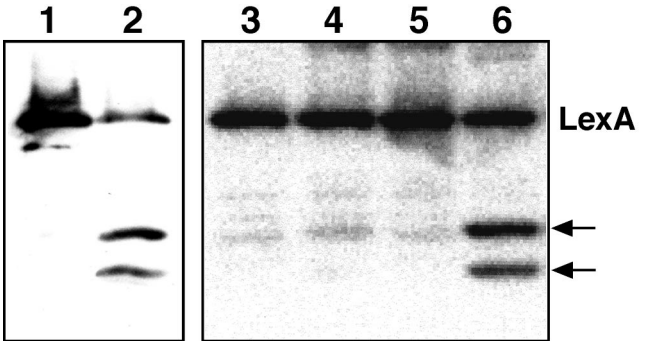

FIG. 2. Autodigestion and RecA-mediated cleavage of purified LexA protein visualized by Western analysis with *D. radiodurans* LexA antiserum (diluted 1:10,000). LexA (0.4 μM) was incubated in 50 mM Tris-HCl (pH 10) for 0 h (lane 1) and 8 h (lane 2). For RecA-mediated cleavage, LexA was incubated with *D. radiodurans* RecA under the conditions described in the text. Lanes: 3, RecA omitted; 4, oligonucleotide omitted; 5, ATPγS omitted; 6, complete reaction mixture. The arrows on the right indicate the positions of breakdown products of LexA.

by the direct insertional mutagenesis technique (9). The disruptant strains were isolated on TGY plates (28) containing chloramphenicol at 3 μg/ml and designated XE1 (carrying *lexA166*::*cat*) and RN201 (carrying *recA229*::*cat*), respectively. Disruption of the genes was confirmed by amplifying the targeted allele by PCR. The DNA damage sensitivity phenotype of strain RN201 was confirmed by measuring cell survival following γ irradiation (Fig. 3). RN201 exhibited extreme γ ray sensitivity, as observed in the *recA* disruptant strain 1R1A constructed previously (10) and mutant strain rec30 (10, 26, 35) carrying a *recA670* mutation (29). In addition, strain RN201 had a slow growth rate compared with that of its parental strain. On the other hand, the growth rate of strain XE1

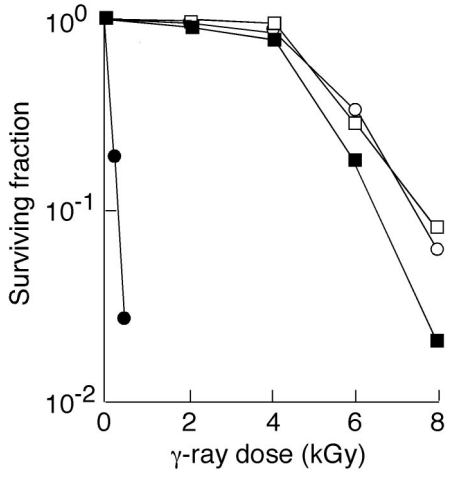

FIG. 3. Sensitivity of *lexA* and *recA* disruptant strains to γ rays. Cells grown to early stationary phase were resuspended in 10 mM sodium phosphate buffer (pH 7), challenged with ^{60}Co γ irradiation, spread on TGY plates, and incubated at 30°C. After 3 days, surviving colonies were counted. Each point represents the average result of three independent experiments. Symbols: open squares, strain KD8301; filled squares, strain XE1 (*lexA166*::*cat*); open circles, strain KR_1; filled circles, strain RN201 (*recA229*::*cat*).

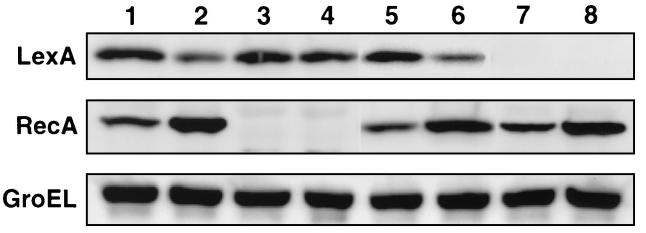

FIG. 4. Changes in intracellular LexA, RecA, and GroEL levels following irradiation. Each sample contained 10 μg of protein. Lanes: 1 and 2, strain KR_1; 3 and 4, strain RN201 (*recA229*::*cat*); 5 and 6, strain KD8301; 7 and 8, strain XE1 (*lexA166*::*cat*). Odd- and even-numbered lanes contained nonirradiated samples and those irradiated with 2 kGy, respectively.

carrying the *lexA166*::*cat* mutation was almost the same as that of the parental strain. Although XE1 cells showed a slightly higher rate of cell death than the parental strain at high doses (6 and 8 kGy) of γ rays, the disruption of *lexA* did not severely affect γ ray resistance (Fig. 3).

Changes in intracellular LexA and RecA levels following irradiation. The changes in LexA and RecA levels following γ irradiation were compared among the disruptants and their parental strains. Early stationary phase cells were resuspended in 10 mM sodium phosphate buffer (pH 7) and divided into two fractions. One fraction was irradiated at a dose of 2 kGy, and the other fraction was not irradiated. The cells were then incubated in fresh TGY broth for 2 h at 30°C with agitation. The protein extracts were subjected to Western analysis with *D. radiodurans* LexA antiserum (diluted 1:10,000) and *E. coli* RecA antiserum (1:500). The *E. coli* RecA antiserum was raised against purified *E. coli* RecA protein (Promega). As a control, *D. radiodurans* GroEL (22) was detected by using *E. coli* GroEL antiserum (diluted 1:2,000) (StressGen Biotechnologies Corp.) (Fig. 4).

In wild-type strain KR_1, the level of LexA was decreased (2.7-fold) and the level of RecA was, in contrast, increased after γ irradiation (2.5-fold) (Fig. 4, lanes 1 and 2). These results suggested that RecA is activated during postirradiation incubation and promotes the proteolytic cleavage of LexA. In strain RN201, the RecA signal completely disappeared (Fig. 4, lanes 3 and 4), confirming the disruption of *recA*. Importantly, γ irradiation did not affect the level of LexA in RN201. These results supported our observation of in vitro RecA-mediated LexA cleavage and further suggested that RecA is the sole protein required for LexA cleavage. As in strain KR_1, a decrease in LexA (2.5-fold) and an increase in RecA (2.5-fold) were observed in strain KD8301 after irradiation (Fig. 4, lanes 5 and 6). In strain XE1, a derivative of strain KD8301, the LexA signal disappeared because of disruption of *lexA* (Fig. 4, lanes 7 and 8). If RecA represses the expression of *recA*, constitutive production of LexA at an elevated level can be seen in unirradiated *lexA* disruptant cells. However, this was not the case in strain XE1. The level of RecA in unirradiated XE1 cells was comparable to those in unirradiated KR_1 and KD8301 cells, and RecA induction following irradiation (2.0-fold) was observed in XE1 as in KR_1 and KD8301. The level of GroEL was constant irrespective of irradiation in all of the strains tested. Thus, the results of our experiments did not

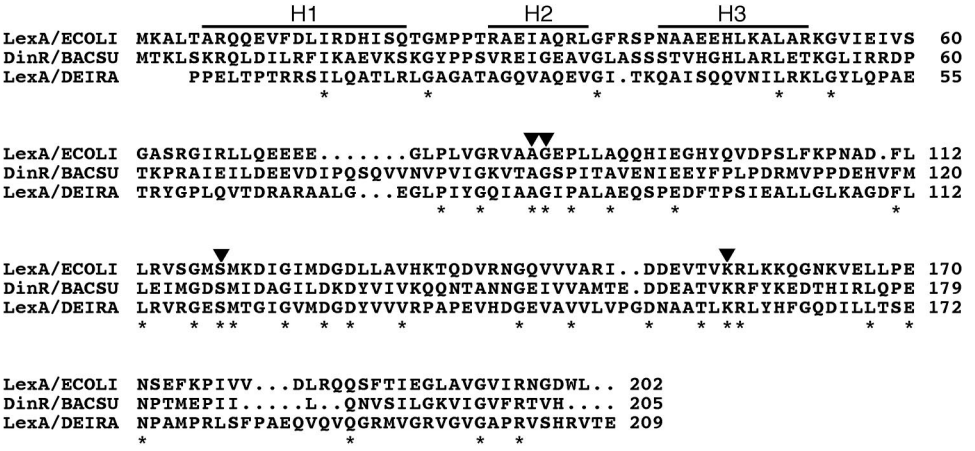

FIG. 5. Multiple amino acid sequence alignment of *E. coli* RecA (14), *B. subtilis* DinR (31), and *D. radiodurans* LexA. Multiple alignment was achieved with the CLUSTAL W program (37). Dashes indicate gaps in the alignment. Numbers on the right are the coordinates of the proteins. Asterisks indicate identical residues. The conserved Ala and Gly residues in the cleavage site and the Ser and Lys residues required for cleavage are indicated by filled triangles. The locations of three α helices (H1, H2, and H3) found in *E. coli* LexA are indicated according to the description of Fogh et al. (7).

support the involvement of LexA in the induction of RecA in *D. radiodurans*.

Discussion. The results obtained in this study indicate that *D. radiodurans* LexA undergoes RecA-mediated cleavage (Fig. 2) and RecA is the sole protein responsible for cleavage of LexA in vivo (Fig. 4). *E. coli* RecA mediates the proteolytic cleavage of the bond between Ala-84 and Gly-85 of LexA (13). Hydrolysis of the LexA Ala-Gly bond proceeds similarly to that of serine proteases, with Ser-119 acting as a nucleophile and Lys-156 acting as an activator (33). Alignment of the amino acid sequences of *E. coli* LexA, *B. subtilis* DinR, and *D. radiodurans* LexA (Fig. 5) revealed that the amino acid residues involved in the cleavage reaction are also conserved in *D. radiodurans* LexA (Ala-83, Gly-84, Ser-119, and Lys-158). Therefore, we assumed that the two breakdown products observed in the in vitro cleavage assays (Fig. 2) were the N-terminal and C-terminal fragments of LexA cleaved between Ala-83 and Gly-84.

The N-terminal domain of *E. coli* LexA is involved in DNA binding (15), and the LexA DNA binding domain contains three α helices, of which helices 2 and 3 form a variant helix-turn-helix DNA binding motif (7). The N-terminal region of the *D. radiodurans* LexA sequence, however, showed very limited similarity to analogous regions in *E. coli* LexA and *B. subtilis* DinR (Fig. 5). If the N-terminal region of *D. radiodurans* LexA serves as a DNA binding domain, *D. radiodurans* possesses a distinct LexA binding motif that is different from the *E. coli* SOS box (38) and the *B. subtilis* DinR box (40). For the purification of *D. radiodurans* LexA, we found a heparin column to be very effective (Fig. 1), suggesting that the LexA protein retains DNA binding ability. In the preliminary gel mobility shift assay, we also found that the purified LexA protein could bind to the upstream region of its own gene (data not shown). A detailed analysis of the *D. radiodurans lexA* operator is ongoing in our laboratory.

Under our experimental conditions (irradiation with 2 kGy, followed by 2 h of incubation), the RecA induction ratio was 2.0- to 2.5-fold (Fig. 4). This induction ratio was much smaller than that observed in a previous study (50- to 100-fold; irradiation with 5 kGy, followed by 2 h of incubation) (4). It has been shown that, after irradiation at 3 kGy, a cell's recovery from DSBs is complete within 3 h (2). Therefore, one explanation for the induction ratio difference is the different γ ray doses used in the two experiments. It is conceivable that most DSB repair is completed under our experimental conditions; thereby, the induction ratio was smaller than that in the previous study. However, the 50- to 100-fold RecA induction ratio seems to be an overestimate because such radiation-induced protein was not detected by two-dimensional PAGE analysis in which cells were irradiated at 6 kGy and incubated for 2 h (35).

It has been shown that several eubacterial species lack a *lexA* gene (1, 6). A lack of inducibility of RecA has also been demonstrated in some genera (24). Thus, while the *recA* gene is clearly conserved in a wide variety of eubacterial species, the control mechanism of its expression is not. Our results indicated that the *recA* gene is part of a DNA damage response regulon in *D. radiodurans*. However, our findings did not support the suggestion that *D. radiodurans* LexA is involved in the induction of RecA. This, in turn, led us to speculate that *D. radiodurans* has an alternative DNA damage response mechanism with which to control *recA* expression. In *E. coli*, the *sulA* gene, whose products inhibit cell division, is under the direct control of *E. coli* LexA (38). Consequently, *E. coli lexA* (Def) mutants are viable only if they contain an additional mutation on the *sulA* gene. In addition, it has been shown that *B. subtilis lexA* (Def) mutants exhibit a strong filamentation phenotype, accompanied by significant loss of viability (11). On the other hand, the *D. radiodurans lexA* disruptant generated in this study was viable. This different behavior has been reported previously for *lexA* disruptants of *Xanthomonas campestris* (43) and *Rhodobacter sphaeroides* (36) in which LexA functions as a repressor of *recA* expression. The *D. radiodurans* genome does not encode a homolog of the *E. coli sulA* gene (39). However, it has been shown that the DNA damage-induced delay in *D. radiodurans* chromosomal DNA replication is dose dependent and that the length of the delay always exceeds the time re-

quired for repair of the DNA damage that caused the inhibition (5, 19, 27). Based on these observations, it has been proposed that there is a regulatory mechanism in *D. radiodurans* that controls chromosome replication and, as a consequence, controls cell division (2, 3). We suggest that *D. radiodurans* LexA is probably not involved in such a regulatory mechanism because of the behavior of LexA disruptant cells.

Interestingly, it has recently been shown that *D. radiodurans* encodes a second but diverged copy of LexA (DRA0074) that retains the potential DNA binding domain and the autocleavage domain (21). It would be interesting to determine whether DRA0074 is involved in the control of the DNA damage response in *D. radiodurans*. Recently, we succeeded in overproducing DRA0074 in *E. coli*. Characterization of this recombinant protein will provide useful information about control of the DNA damage response.

Nucleotide sequence accession number. The nucleotide sequence reported here (the *Dienococcus radiodurans* gene for aldehyde dehydrogenase, succinic semialdehyde dehydrogenase, partial and complete cds) has been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB003475.

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