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Full Length Research Paper

Radioresistance analysis of *Deinococcus radiodurans* gene *DR1709* in *Escherichia coli*

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***Deinococcus radiodurans*' extraordinarily strong radiation resistance was attributed to its high Mn^{2+} content. DR1709 was one predicted Mn^{2+} transporter, but after it was disrupted, there were at least ten proteins whose expressions changed markedly, suggesting that the proteins which were expressed differently between the wild type and the mutant may play key roles in this bacterium's radiation resistance, while DR1709 was only a switch to activate these proteins. To identify if this deduction was true or not, *DR1709* was isolated from *D. radiodurans* and transformed into *Escherichia coli* BL21, whose genomic background is hugely different from that of *D. radiodurans*. Results showed that the transformed *E. coli* had higher resistance to and UV radiation than the original strain. After being treated with 150 Gy radiation, *E. coli* containing *DR1709* had 70% survival fraction, while only 17% of the control cells can be found on LB plate. DR1709 had the ability to protect cells directly from being damaged by and UV radiations. *E. coli* containing *DR1709* had higher Mn content than the initial strain. Although the transformed strain had higher survival than the original *E. coli*, its survival rate decreased with UV dose increasing. After being transformed with *DR1709*, *E. coli* BL21's Fe content had not changed. DR1709 may be specific for Mn^{2+} and was not responsible for transporting Fe^{2+}. Radiation resistance was controlled by multistep in *D. radiodurans*. Those genes whose expressions were different between the wild type and the *DR1709*-disrupted mutant were downstream of DR1709. These genes might also play some roles in radiation resistance, but such roles were much less than that played directly by DR1709.**

Key words: *Deinococcus radiodurans*, DR1709, *E. coli*, Mn, radiation resistance.

INTRODUCTION

Deinococcus radiodurans is famous for its extraordinary resistance to radiation (Patel et al., 2009; Sun et al., 2009; Makarova et al., 2001; Bouthier et al., 2009 and Khairnar and Misra , 2009). It can survive acute exposures to gamma radiation that exceed 1,500 krads without dying or undergoing induced mutation (Daly et al., 1994). Even in the presence of high-level chronic irradiation (6 kilorads/h), it still displays luxuriant growth (Lange et al., 1998; Venkateswaran et al., 2000) without there being any effect on its growth rate (Brim et al., 2000). Understandingly, its radioresistance mechanism had potential use in the treatment of radioactive waste and tumor control (Longtin, 2003; Makarova et al., 2001; Hassan et al., 2006; Deepti et al., 2006). The high DNA repairment capability was thought to play key roles in keeping high survival after the bacterium was radiated (Funayama et al., 1999). However, although many genes have been predicated to participate in DNA repair, the number of such function-known genes in *D. radiodurans* is still much less than that in *E. coli* (White et al., 1999; Daly, 2006), while *E. coli*'s radiation resistance was much lower than that of *D. radiodurans* (Lange et al., 1998; Makarova et al., 2001). Moreover, there were many *D. radiodurans* genes whose homologues can not be found in other species (Makarova et al., 2001; White et al., 1999).

All of these suggested that the radiation resistance may be attributed to the function-unknown genes (Liu et al., 2003; Hua et al., 2003 and Chang et al., 2008). But after 12 genes of *D. radiodurans*, which were implicated in resistance by transcriptional profiling following irradiation, they were knocked out respectively. The radiation

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resistance of the novel mutants still remained high (Cox et al., 2005; Liu et al., 2003 and Harris et al., 2004), demonstrating that possibility of this inference was also very little. Recently, Daly et al. found that the degree of irradiation resistance has been determined by the level of oxidative protein-damage caused during irradiation (Daly et al., 2007; Daly, 2009). Mn^{2+} can protect proteins from being oxidized in *D. radiodurans* (Daly et al., 2007; Daly, 2009). But how Mn^{2+} was assimilated from the medium, who participated in such a process, where they played such roles and how they protect the proteins from being oxidized are all still unknown.

There were two types of predicted Mn^{2+} transporters in *D. radiodurans* (Nramp family and ATP-dependent ABC-type transporter) (Daly et al., 2004). DR1709 belonged to the Nramp family. Many Nramp family members, homologues to DR1709 in other species (such as yeast, *Xenopus oocytes* and mammals), can efficiently transport Mn^{2+} (Nevo and Nelson, 2006; Supek et al., 1996, 1997; Liu et al., 1997; Chen et al., 1999; Forbes and Gros, 2003 and Goswami et al., 2001). After *DR1709* is dis-rupted, the mutant cells have a much less survival than wild type when treated with irradiation (Chang et al., 2009). Using *DR1709*-disrupted mutant and the defined minimal medium, it was found that when Mn^{2+} was defi-cient, DR1709 became the only candidate responsible for Mn^{2+} assimilation (to be published). All of these indicated that DR1709 can play radiation resistance roles efficiently in *D. radiodurans*.

However, Tian et al recently reported that after two-dimensional electrophoresis was performed, there were at least ten protein spots whose intensities were remarkably different between wild type and the *DR1709*-disrupted mutant (Tian et al., 2009). This suggests that the increased radiation sensitivity of the mutant cells might not only be due to *DR1709*, but those genes which had different expressions between the wild type and the mutant may also play important roles in protecting *D. radiodurans* from being damaged by irradiation (Tian et al., 2009).

Is it *DR1709* that protect the proteins from being injured, or the genes whose expressions are different between the mutant and the wild type that play such roles, while DR1709 is only a switch to activate the expressions of these genes? To answer this question, in this paper, *DR1709* is isolated from *D. radiodurans* and transformed into *E. coli* BL21, whose genomic back-ground is enormously different from that of *D. radiodurans* (Makarova et al., 2001; White et al., 1999). It was found that when the transformed *E. coli* BL21 was treated with UV and radiation, its survival was higher than the wild type.

Although the transformed strain had higher survival than the original *E. coli*, its survival rate decreased with UV dose increasing. The intracellular Mn content of the strain containing *DR1709* was higher than that of the initial *E. coli*. DR1709 does have the ability to protect cells from being damaged by and UV radiation. After being transformed with *DR1709*, *E. coli* BL21's Fe content had not changed. DR1709 may be specific for Mn^{2+}, but was not responsible for transporting Fe^{2+}.

MATERIALS AND METHODS

Bacterial strains and their growth conditions

The *D. radiodurans* R1 and the mutant cells were grown at 31 in TGY broth (0.5% bacto tryptone, 0.1% glucose and 0.3% bacto yeast extract). *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB plates at 37°C. Plasmids were routinely propagated in *E. coli* strain JM109. The gene sequences used in this paper can be found in http://www.tigr.org/tigr-scripts/CMR2/genomePage3.spl?database=gdr. The construction process of the *DR1709*-disrupted mutant can be found in the study's previous paper (Chang et al., 2009).

Chromosomal DNA isolation

TGY broth (5 ml) was inoculated with a single colony of *D. radiodurans* from TGY agar plate. After 48 h, 1 ml of the cells were harvested by centrifugation at 4°C and 6,000 × for 15 min. Pellets were resuspended in 600 µl lysis buffer (10 mmol/L Tris-Hcl, 0.1 mmol/L EDTA [pH 8.0], 0.5% sodium dodecyl sulfate [SDS], 0.1 mg/ml proteinase K). After being incubated in 37°C water bath for 1 h, 100 µl of 5 mol/L NaCl and 80 µl of CTAB/NaCl were added to the suspension and incubated for 10 min at 65°C. Lysed cells were extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and twice with equal volumes of chloroform-isoamyl alcohol (24:1). Then, DNA was precipitated from the extracted materials with 50 µl of 3 mol/L sodium acetate (pH 7.0) and 1 ml of ice-cold 100% ethanol. Pellet was collected by centrifugation at 4°C at 6,000 × *g* for 15 min and washed twice with 70% ethanol. The DNA was air dried and dissolved in 100 µl of TE buffer (10 mmol/L Tris-Hcl, 0.1 mmol/L EDTA [pH 8.0]) and stored at 4°C.

Transforming DR1709 into *E. coli* BL21

Using the genomic DNA of *D. radiodurans* as template, PCR was performed as the following procedure (94°C, 1 min, 60°C, 1 min, 72°C, 1.5 min, 40 cycles). The primers were C1709F:5 -GTCGTCCATATGATGGATTCCCGTTCTC-3 (the restriction enzyme *Nde*I site was underlined) and K1709R:5'---TATATTCTCGAGGCCCCCCAGCAGCTCC--- 3' (the restriction enzyme *Xho*I site was also underlined). The plasmid vector (pET-29a, Novagen, catlogue number: 69871- 3) was digested with *Nde*I and *Xho*I. *DR1709* was ligated into the digested vector using T4 DNA ligase. The ligation was transformed into *E. coli* BL21 as the standard method (Sambrook et al., 1989). The transformed cells were screened on LB plate containing 50 mg/L Kanamycin. The plasmid was extracted from the cells and digested with *Nde*I and *Xho*I. Those samples containing 1.3 kb fragments were the ones wanted.

Cell survival measurement after being treated with UV and irradiation

UV irradiation and cell survival measurement were performed as described before (Servinsky and Julin, 2007). Cells were grown in TGY PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 5.3 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4 at pH 7.4) in triplicate and spread on

LB plates. After the medium was absorbed, the plates were opened and exposed to UV light from an LP UV lamp (15 W; Philip) at a rate of 0.295 J sec^{-1} m^{-2} (Laser power meter, Gentec, Canada, Model-PSV-3303). The plates were incubated at 37°C and colonies were counted after 16 h. For irradiation, cell suspensions (200 L) of various strains were irradiated at room temperature for 1 h with ^{60}Co -rays at various distances from the source. And then, the cell suspensions were spread on TGY plates (for *D. radiodurans* strains) or LB plates (for *E. coli* strains). TGY plates were incubated at 31°C for 2 to 3 days. LB plates were incubated at 37°C for 16 h. Colonies were counted and the survival fractions were calculated.

Analysis of intracellular Mn and Fe concentrations by inductive coupled plasma mass spectrometry (ICP-MS)

ICP-MS was performed as described before (Horsburgh et al., 2002). *E. coli* strains were grown in TGY medium to OD_{600} = 0.9. After centrifugation at 10000 g, 4°C for 10 min, the pellets were washed three times with PBS containing 1mmol/L EDTA and twice with PBS. The cell dry weight was measured and accurated to milligram after the pellets were incubated at 80°C overnight. 2 ml of 30% nitric acid was added and the cells were digested in an 80°C water bath for 4 h. The digestion was filtered and diluted with deionized water as 1:10. These samples were analyzed for Mn and Fe concentrations by ICP-MS. A blank control was prepared in the same manner but without cells. All data were triplicated and the means were used as the representative values.

RESULTS

DR1709 was transformed into *E. coli* BL21

A: *DR1709* was isolated from *D. radiodurans*. B: After *DR1709* was transformed into *E. coli*, the plasmids were extracted and digested with *Nde*I and *Xho*I. L was the DNA marker (Tiangen, catalogue number: MD115-01). The molecular weight of the bands was 4000, 2200, 2000, 1800, 1600, 1400, 1200, 1000, 800, 600, 400 and 200 bp, respectively.

DR1709 was isolated from the genomic DNA of *D. radiodurans* (Figure 1a). The PCR product was digested with *Nde*I and *Xho*I and ligated into plasmid vector pET-29a. The ligation was transformed into *E. coli* BL21. After being extracted from the transformed cells, the plasmids were digested with *Xho* I and *Nde* I. There were two bands (5.37 and 1.32 kb) that appeared in sample 5. The 5.37-kb band was corresponding to the plasmid vector and the 1.32-kb band was corresponding to the gene *DR1709*, indicating that sample 5 may be the one wanted (Figure 1b). So, sample 5 was sequenced and identified. Result showed that it was the strain that was transformed successfully.

E. coli BL21 containing *DR1709* was more resistant to UV and radiation than the control

A: after being irradiated with -ray, and the survival fractions of *D. radiodurans* R1 wild type and the mutant M1709. B: the survival lines of *E. coli* BL21 strains after being treated with UV. C: the survivals of the *E. coli* BL21 strains after being irradiated with -ray. BL21 referred to the original *E. coli*. BL21-DR1709 containing the *D. radiodurans*'s gene *DR1709*. R1 referred to *D. radiodurans* R1 wild type.

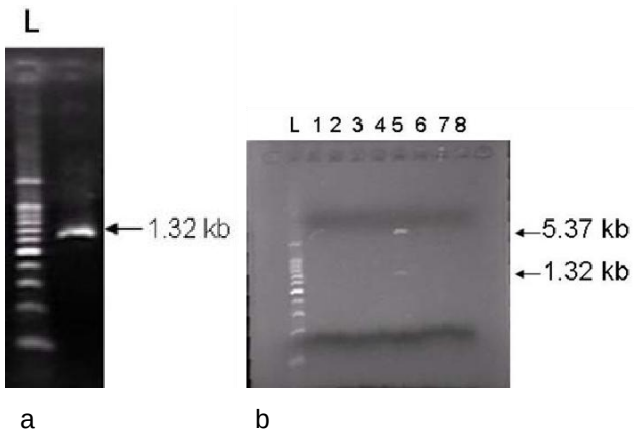

Figure 1. Identification of the transformed *E. coli* strains.

After *DR1709* was deleted, the *D. radiodurans* cells had lesser survival than the wild type when treated with -ray irradiation (Figure 2a). This was in agreement with the previous studies (Chang et al., 2009). With the UV dose increasing, the survivals of the original *E. coli* decreased sharply (Figure 2b). When treated with 10 J/m^2, the survival fraction of the transformed cells was 90%, while the corresponding survival value of the initial *E. coli* strain was only 50% (Figure 2b). At 20 J/m^2, the corresponding data was 80 and 3%, respectively (Figure 2b). The bacterium transformed with *DR1709* had higher survivals than the control (Figure 2b) . After being treated with 150 Gy of the ray, the *E. coli* cells that contained *DR1709* had 70% survival fraction, while only 17% of the control cells could be found on LB plates (Figure 2c). The strain transformed with the empty vector had almost the same survival rate with the original strain. To prevent being confused with the original strain, the survival fractions of the strain containing the empty vector was not shown. These indicated that after being transformed with *DR1709*, the *E. coli* cells had higher resistance to UV and radiation. *DR1709* in *E. coli* can protect the cell from being damaged by UV and radiation.

E. coli containing *DR1709* had higher content Mn and Mn/Fe ratio than the original strain

A: the intracellular Mn content of the *E. coli* strains. B: the intracellular Fe content of the *E. coli* strains. C: the intracellular Mn/Fe concentration ratios of the *E. coli* strains. BL21 referred to the original *E. coli*, BL21-V referred to the *E. coli* containing the empty vector and BL21-DR1709 referred to the *E. coli* containing the *D. radiodurans*'s gene *DR1709*.

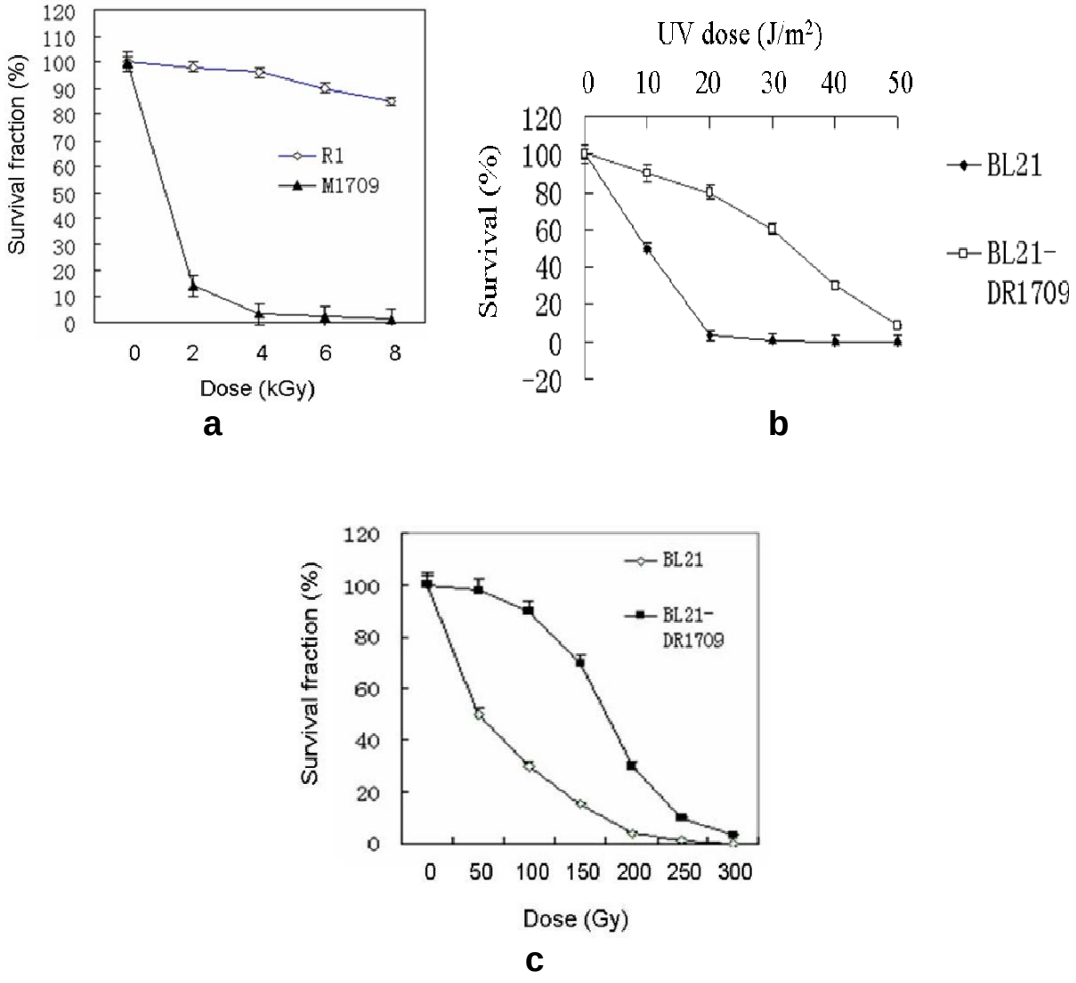

Figure 2. The survival lines of different strains.

When the *E. coli* strains were grown to OD_{600} 0.9 in TGY medium, the intracellular Mn content of BL21-DR1709 was about 0.025 nmol/mg protein, while the corresponding value of the control was 0.0194 nmol/mg protein (Figure 3a). The strain transformed with the empty vector had almost the same Mn concentration (Figure 3a), indicating that the vector had no effect on the bacterium's Mn uptake. The *E. coli* containing *DR1709* had marked higher Mn content than the initial strain, but the Fe content in these *E. coli* strains was very similar (Figure 3b), demonstrating that *DR1709* had no function in the uptake of Fe from the medium. The strain transformed with *DR1709* had higher Mn/Fe ratio than the original strain (Figure 3c). After being transformed with *DR1709*, the bacterium's Mn assimilating ability was improved.

DISCUSSION

Although there were several hypotheses to explain *D. radiodurans*'s extraordinary radiation resistance nowadays, more research results showed that this bacterium's strong radiation resistance should be attributed to its high content of Mn^{2+} (Daly and Minton 1995; Zahradka et al., 2006; Lin et al., 1999; Cox et al., 2005; Liu et al., 2003; Harris et al., 2004 and Daly et al., 2007; 2009) . But concrete details about how Mn^{2+} was transported, the physiological processes, the molecular mechanism, especially the genes participating in these procedures all still remained to be elucidated.

DR1709 was one of the predicted Mn^{2+} transporter genes in *D. radiodurans* (Daly et al., 2004). After this gene was disrupted, at each site of the logarithmic stage, the OD_{600} value of the mutant was much lower than that of the wild type (Chang et al., 2009). When they were treated with H_2O_2 or UV, the survival rates of the mutant cells at each dose were also much lower than those of the wild type (Chang et al., 2009). In DMM (defined minimum medium) with only 200 nmol/L Mn^{2+}, M1709 can not grow at all. In the corresponding solid agar plate, no

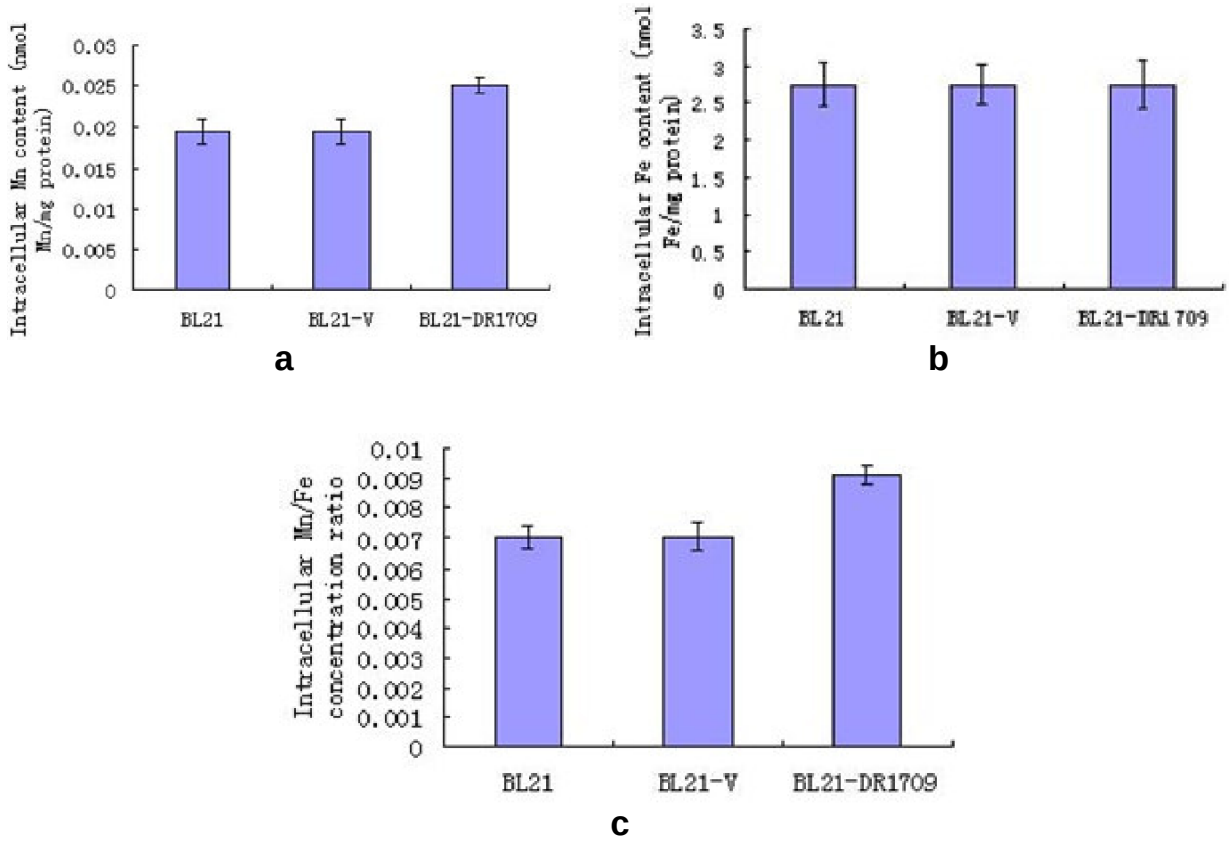

Figure 3. The intracellular Mn and Fe content of the *E. coli* strains.

M1709 clone can be observed even after the plates had been placed in 32°C for 13 days (to be published). These demonstrated that in *D. radiodurans*, when Mn^{2+} was limited, *DR1709* was the only gene responsible for Mn^{2+} assimilation. But in liquid TGY, M1709 can grow well, although its growth curve was lower than that of the wild type (Chang, 2009), suggesting that there were other genes responsible for Mn^{2+} uptake when Mn^{2+} was ample in the medium. All of these results showed that DR1709 played important roles in protecting *D. radiodurans* from being injured by radiation. However, Tian et al. recently reported that after *DR1709* was disrupted, the expressions of DR1120 (acetokinase), DR1691 (heat shock protein), DR1485 (putative lipase), DR2095 (putative c-type cytochrome) and other three hypothetical proteins (DR0124, DR0047 and DR2474) were repressed. The expression of DR1794 (putative nosX) was induced (Tian et al., 2009), suggesting that during *D. radiodurans* survived the radiation. There may be at least a second possibility: the proteins that were expressed differently between the wild type and the mutant played key roles, while DR1709 was only a switch to activate these proteins. To identify if this deduction was true or not, it is essential to isolate *DR1709* from *D. radiodurans* and transform it into other organisms. Many researches take *E. coli* as the representative of radiosensitive bacteria compared with *D. radiodurans* (Daly, 2006; Makarova et al., 2001; Lange et al., 1998; White et al., 1999; Sweet and Moseley, 1974; Daly et al., 2004) . In the exponential phase, *D. radiodurans* is 33-fold more resistant to UV than *E. coli* (Daly, 2006; Sweet and Moseley, 1974). Furthermore, there were many *D. radiodurans* genes whose homologues can not be found in *E. coli* (Makarova et al., 2001). *E. coli*'s genome was enormously different from that of *D. radiodurans* (Makarova et al., 2001; White et al., 1999). Therefore, *E. coli* was selected as the recipient to be transformed in the study's experiment.

After *DR1709* was transformed into *E. coli*, the transformed strain's resistance to UV was improved markedly. At the dose of 20 J/m^2, there was almost no control of *E. coli* clones found on LB plates, but the strain transformed with *DR1709* still had an 80% survival rate. When cells were irradiated with -ray, similar results were gotten. As soon as the cells are irradiated, a lot of reactive oxygen species are generated (Von Sonntag, 1987).

The proteins in the cells will be damaged by these reactive oxygen species (Levine, 1983; Chou et al., 1995; Imlay, 2003). Mn^{2+} in cells can scavenge these reactive oxygen species (Daly et al., 2007; Daly, 2009). The transformed *E. coli* is more resistant to irradiation than

the control, suggesting that there are more Mn^{2+} uptaken into the cells. More reactive oxygen species are reacted with Mn^{2+} and then, they are scavenged. Fewer proteins were damaged and more cells survived (Von Sonntag, 1987; Imlay, 2003; Nauser et al., 2005), but the speed of reactive oxygen species generation might be higher than that of Mn^{2+} assimilated. As a result, although the transformed strain had higher survival than the original *E. coli*, its survival rate decreased with UV dose increasing.

To monitor what happened in the transformed cells, the intracellular Mn and Fe concentrations were measured. When the *E. coli* strains were grown to OD_{600} 0.9 in TGY medium, the intracellular Mn content of BL21-DR1709 was about 0.025 nmol/mg protein, while the corresponding value of the control was 0.0194 nmol/mg protein. The strain containing *DR1709* had marked higher Mn than the initial strain. This result demonstrated that the study's deduction above was right.

Iron is another essential trace-element for most organisms (Semsey et al., 2006). Many homologues of DR1709 in other bacteria species can uptake Mn^{2+} and Fe^{2+} at the same time (Patzer and Hantke, 2001; Kehres et al., 2000; Makui et al., 2000; Bearden and Perry, 1999; Boyer et al., 2002). However, after being transformed with *DR1709*, *E. coli* BL21's Fe content had not changed. This suggested that DR1709 may be specific for Mn^{2+}. It was not responsible for transporting Fe^{2+}.

And then, what is the relationship among *DR1709* and those genes whose expressions were different between the wild type and the *DR1709*-disrupted mutant? The study thought the possible mechanism might the following. Radiation resistance was controlled by multistep in *D. radiodurans* (Ghosal et al., 2005) . Those genes whose expressions were different between the wild type and the *DR1709*-disrupted mutant were downstream of DR1709.

These genes might also play some roles in radiation resistance (Tian et al., 2009). But such roles were much lesser than that of DR1709 played directly. This conclusion was based on the following four facts. (i), many DR1709's homologues in other species can efficiently transport Mn^{2+} (Nevo and Nelson, 2006; Supek et al., 1996, 1997; Liu et al., 1997; Chen et al., 1999; Forbes and Gros, 2003; Goswami et al., 2001). These species' genomic backgrounds were enormously different from that of *D. radiodurans*. (ii), after *DR1709* was isolated from *D. radiodurans* and transformed into *E. coli* in this paper, the transformed *E. coli* had higher radiation resistance than the initial strain. (iii), in *D. radiodurans*'s Mn^{2+} transporting cascade, no other gene can exercise similar functions as DR1709 (Chang et al., 2009). (iv), although, the genes whose expressions were different between the wild type and the *DR1709*-disrupted mutant, might play some roles in radiation resistance in *D. radiodurans* (Tian et al., 2009). This was only a possibility that the author supposed. Concrete evidences to support this deduction can not be found in this paper. Further experiments are needed to identify these roles.

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