African Journal of Microbiology Research Vol. 4 (9), pp. 896-902, 4 May, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 © 2010 Academic Journals

Full Length Research Paper

Function analysis of the predicted protein-tyrosine phosphatases gene (DR2161) in *Deinococcus radiodurans*

Shu Haiyan* and Tian Baoming

Biology Department, Zhengzhou University, Zhengzhou, China.

Accepted 13 April, 2010

Protein phosphorylation on tyrosine has been demonstrated to occur in a wide array of bacterial species and appears to be ubiquitous among prokaryotes. In *Deinococcus radiodurans*, after the predicted protein-tyrosine phosphatase (PTP) gene DR2161 was deleted, the radiation resistance of the bacterium changed a little. The natural resistance-associated macrophage protein gene DR1709 was the possible target of PTP. But the radio resistance of the double mutant (DR1709 and DR2161 that were deleted) was almost the same as that of M1709. When the strain protease secretion was measured, the clearing area of M2161 was smaller than that of the wild type. But the protease secretion of the double mutant was similar to that of M1709. The influence on the bacterium DR2161, that was deleted seemed to have been covered by DR1709 being disrupted. The four strains (the wild type, M1709, M2161 and the double mutant) sensitivity to high concentrate of Mn^{2+} and Fe^{2+} were very similar, showing that they had similar resistance to high concentrate of Mn^{2+} or Fe^{2+}. In liquid defined minimal medium (DMM) with 200 nM Mn, M1709 and the double mutant almost can not grow. But in DMM with 200 nM Fe, the two strains grew as quickly as the wild type. M1709 reaction to low concentrate of Mn^{2+} and Fe^{2+} was not affected by DR2161. The expression of DR1709 was not regulated by DR2161.

Key words: *Deinococcus radiodurans*, protein-tyrosine phosphatase, natural resistance-associated macrophage protein, DR1709, DR2161.

INTRODUCTION

Deinococcus radiodurans was one of the most radiation resistant bacteria (Daly et al., 1994). As this bacterium can be engineered to clear the radioactive waste and offer clue to tumor controlling, it is important to study the bacterium's radio resistance mechanism (Longtin, 2003; Makarova et al., 2001; Brim et al., 2006; Deepti et al., 2006). There were at least three prevailing hypose to explain the extraordinary radiation resistance of *D. radiodurans*: (i) chromosome alignment, morphology and repeated sequences facilitate genome reassembly (Daly et al., 1995) (ii) a subset of uncharacterized genes encode functions that enhance the efficiency of DNA repair (Zahradka et al., 2006) and (iii) non-enzymic Mn (II) complexes present in resistant bacteria protect proteins from oxidation during irradiation (Daly et al., 2007, 2009); although the third hypose was most convictive and accepted by most people. There were many questions to be answered, such as how was Mn (II) transported in *D. radiodurans?* Protein-tyrosine phosphorylation of proteins was an important signal transport in prokaryote (Cozzone et al., 2004; Klumpp et al., 2002). It occurred in a wide array of bacterial species and appeared to be ubiquitous among prokaryotes (Cozzone et al., 2004; Klumpp et al., 2002). Now, more and more evidences show that phosphatase may participate in Mn (II) transportation and radiation resistance in *D. radiodurans.* This hypose mainly come from the following research: (I) ionizing radiation frequently leaves DNA strand-breaks that are not feasible for ligation and thus require end-healing by a 5'-polynucleotide kinase or a 3'-phosphatase (Blasius, 2007) (II) Many tyrosine phosphatases were Mn (II)-dependent (Shi, 2004). At the same time, Mn (II) plays important roles in *D. radiodurans* radiation resistance (III) *D. radiodurans* has an unusual envelop, whose main composition was peptidoglycan (Makarova, 2001). Some reports found that certain protein-tyrosine kinases

*Corresponding author. E-mail: shuhy@zzu.edu.cn.

Table 1. Strains and plasmids used in this paper.

Strains and plasmids	Description	Reference
D. radiodurans R1	ATCC13939	Anderson et al., 1956
M1709	As R1 but ↵*dr1709*	Chang et al., 2009
M2161	As R1 but ↵*dr2161*	This study
M1709-2161	As M1709R1 but ↵*dr2161*	This study
E. coli JM109	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1, ↵(*lac-proAB*)/F' [*traD*36, *proAB*+,*lac I* q, *lacZ* ↵M15]	TaKaRa, Dalian, China
pMD19-T Vector	pUC19 deriative with an EcoR V site insert, Ap^R	TaKaRa, Dalian, China
pT2161UP		This study
pT2161DW		This study
pT2161DIS		This study
pKatCAT	Catr Ampr	*Funayama et al.,* 1999

phosphatases have close relationship with the production and transport of surface polysaccharides (Cozzone, 2005; 2004; Obadia, 2007; Grangeasse, 2003) (IV) Gomez et al. (2007) found that murine Nramp-1 (natural resistance-associated macrophage protein family) expression has an effect on macrophage PTP activity, in turn modulating the phosphorylation/activation of signaling proteins associated with nitrogen oxide production. All these suggested that tyrosine phosphates had close relationship with *D. radiodurans* radiation resistance.

DR2161 was the only predicted PTP gene in *D. radiodurans*. In this study, DR2161 was deleted. A close-related gene, DR1709, which belong to NRAMP family, was also deleted. It was found that after the predicted protein-tyrosine phosphatase (PTP) gene DR2161 was deleted, the radiation resistance of the bacterium changed little. The four strains' (the wild type, M1709, M2161 and the double mutant) sensitivity to high concentrate of Mn^{2+} and Fe^{2+} were very similar, showing that they had similar resistance to high concentrate of Mn^{2+} and Fe^{2+}. M1709 reaction to low concentrate of Mn^{2+} and Fe^{2+} was not affected by DR2161. The expression of DR1709 was not regulated by DR2161.

MATERIALS AND METHODS

Strains and growing condition

The bacterial strains and plasmids used in this article were listed in Table 1. All genes were identified as described in the published genome sequence (http://www.tigr.org/tigr-scripts/CMR2/genome Page3.spl?database=gDR).

Unless grown in liquid defined minimal medium (DMM) (Venkateswaran et al., 2000), all *D.radiodurans* strains were grown at 30℃ in undefined rich media TGY broth (1% bactotryptone, 0.5% yeast extract and 0.1% glucose) or TGY agar (1.5% agar). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB plates at 37 ℃.

UV irradiation and cell survival

Measurement of UV irradiation and cell survival rates were performed as described earlier (Matthew et al., 2007). Cells were grown in TGY broth at 30℃ until OD_{600} = 0.5. They were serially diluted with PBS (137 mM NaCl, 2.7 mmol/LM KCl, 5.3 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4 at pH 7.4) and spread onto TGY plates. After the medium was absorbed, the plates were opened and exposed to UV light (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 30℃ for 3 days and then colonies were counted.

Growth in liquid defined minimal medium (DMM)

Cells were typically inoculated into liquid medium at ~1 × 10^6 CFUs/ml followed by incubation at 30℃. For each trial in liquid media, or CFU assays on solid medium, three replicates were carried out and the average was used. Liquid defined minimal medium (DMM) was prepared as described in Table 1 (Venkateswaran et al., 2000). DMM-Mn and DMM-Fe were also prepared according to this table (Venkateswaran et al., 2000), except Mn^{2+} and Fe^{2+}. In DMM-Mn, the concentration of Mn^{2+} was 200 nM and DMM-Fe contained 200 nM Fe^{2+}.

Construction of the mutants

The upstream of DR2161 was amplified by PCR (94℃, 1 min; 60℃, 50 s; 72℃, 1 min; 40 cycles) using the primers PF1-1 (5′-

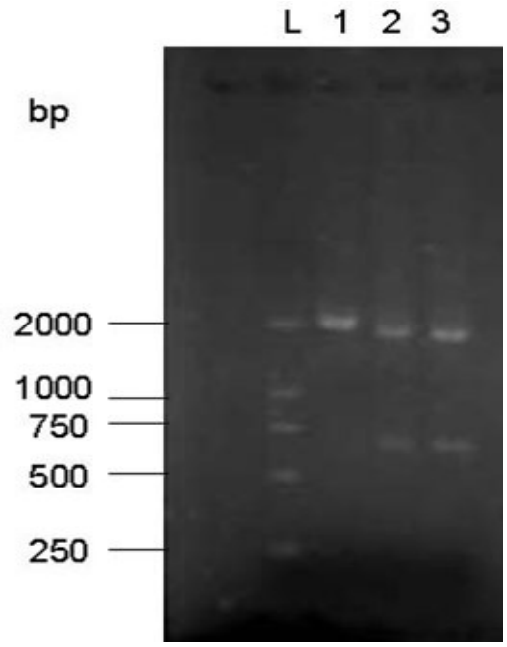

Figure 1. Identification of M2161 construction. L, DNA ladder 1; the fragments corresponding to DR2161 was digested with Bam HI. 1 was the wild type. 2 and 3 came from the possible mutants.

GACACAGGGGCTCGACATCC-3′) and PR1-2-2(5′--TATATAGGATCCCAGGGCGGTAGGAATCCAGT-3', the digestion site Bam HI was shown by underline). DR2161 downstream was gotten with the primers PF2-1-1(5′---TAGATACTCGAGGCGGCAGGAGCAGTTTATTCG—3′, the digestion site Xho I was shown by underline) and PR2-1 (5′---GCGAGATAAATCCGCTCAATCA---3′). The amplification products were ligated into pMD19-T (TaKaRa) and transformed into *E. coli* JM109 (TaKaRa). The upstream plasmid was named as pT2161UP and the downstream plasmid was named as pT2161DW. After being extracted from the transformed *E. coli*, pT2161UP was digested with *Bam* HI and pT2161DW was treated with Xho I. The catalase resistant gene *Cat* with the promoter *Kat* was amplified from pCATKAT (Funayama et al., 1999). PCATKAT was treated with Bam HI and Xho I. The digested products of pT2161UP, pT2161DW and pCATKAT were ligated and transformed into *E. coli* JM109. The plasmid was named as pT2161DIS. After being extracted from the transformed *E. coli* cultures, pT2161DIS was transformed into *D. radiodurans* R1 as described earlier (Chang et al., 2008). Chromosomal DNA was isolated from the wild type and possible mutants. PCR was performed using the primers PF1-2 and PR2-2 (94℃, 1 min; 61℃, 50 sec; 72℃, 4 min, 40 cycles). The PCR products were digested with Bam HI and electrophoresed.

DR1709-disrupted mutant M1709 was constructed as described earlier (Chang et al., 2009). The double mutant (M1709-2161) was constructed as M2161, except the original strain which was M1709.

Protease secretion assays

Tests for secretory proteases were carried out on indicator plates containing skimmed milk according to the method described earlier (Ghosal et al., 2005). After the bottom layer has solidified, top layer liquid were poured on it. When the top layer congealed, autoclaved steel tubes (diameter 5 mm) were placed on the plate. 10 µl bacterium culture was pipetted into the tubes. The plates were incubated at 30℃ for 48 h.

Cation sensitivity

Cation sensitivity was measured according to Rosch et al. (2009). Manganese chloride (1M) and iron chloride (1 M) (Sigma-Aldrich, St Louis, MO) were prepared in Milli-Q water and sterile filtered. Bottom TGY solid plates were prepared and cooled. The top layer of TGY media was prepared and cooled to 50℃ before it solidified, and the top layer media and the bacteria culture (OD_{600}=1.0) were mixed. The mixture was poured on the bottom plate. After the top layer has congealed, sterilely steel tubes (5 mm) were placed on the plate. The manganese chloride solution (200 µl) and iron chloride solution (200 µl) were pipetted into the tubes, respectively. The plates were incubated in 30℃ for 24 h and inhibition zones were measured.

RESULTS

DR2161 and DR1709 was disrupted in *D. radiodurans*

If M2161 was constructed successfully, the DNA corresponding to DR2161 in the mutant genome had one Bam HI site. After the fragment was digested with Bam HI and electrophoresed, there were two bands (1680 and 660 bp) in 0.8% agarose gel. For no Bam HI site in the fragment corresponding to DR2161 in the wild type, only one 2340 bp band was seen in the gel after the PCR product was treated with Bam HI. The result shows that DR2161 had been deleted successfully in the mutant (Figure 1).

Neither Bam HI nor Xho I site was found in the fragment sequence corresponding to DR2161 and DR1709 in the genome of wild *D. radiodurans*. But the double mutant contained one Bam HI site in the fragment corresponding to DR2161 and one Xho I site in the fragment corresponding to DR1709. The fragments corresponding to DR2161 from the double mutant and the wild type were treated with Bam HI and the fragments corresponding to DR1709 were digested with Xho I. The results showed that the double mutant had been constructed successfully (Figure 2).

After DR2161 was deleted, *D. radiodurans*'s performance changed little

Under the treatment of UV, M2161 had a similar appearance with the wild type (Figure 3). Although M1709's radiation resistance was much lower than that of the wild type, the double mutant appearance was much like that of M1709. In liquid TGY, when the growth curves of the double mutant and M1709 were compared, no obvious change was observed (data not shown). It seemed that DR2161's is being disrupted and had little effect on the expression of DR1709.

The growth of the strains in DMM mediums

In DMM-Fe, the differences among the four strains'

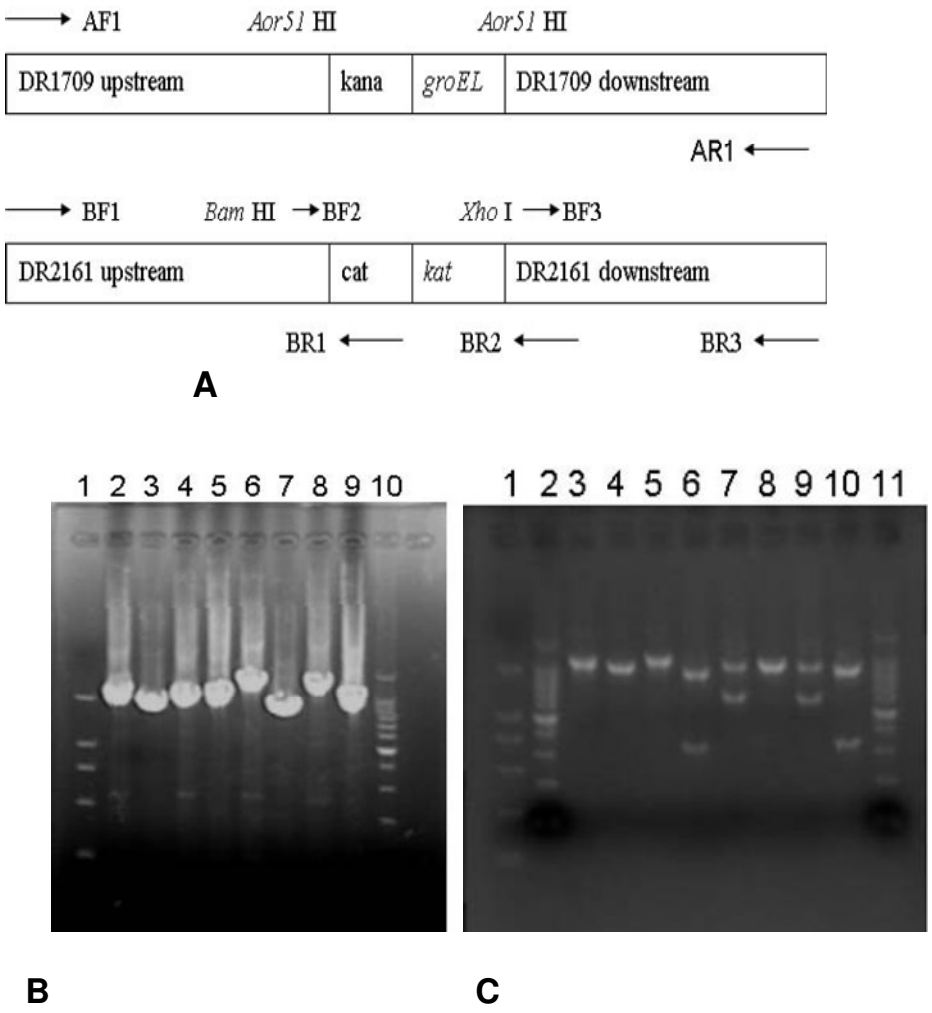

Figure 2. Construction and identification of the double mutant. A is the double mutant construction frame. B is the PCR products corresponding to DR2161 or DR1709 from the genomic DNA of *D. radiodurans* strains. 1 is the DNA ladder 1. The molecular weight corresponding to the bands was 2000, 1000, 750, 500, 250 and 100 bp, respectively. 10 was the DNA ladder 2. The molecular weight corresponding to the bands was 4000, 2200, 2000, 1800, 1600, 1400, 1200, 1000, 800, 600, 400 and 200 bp, respectively. 2 and 3 were from the wild type, 4 and 5 from M2161, 6 and 7 were from M1709 and 8 and 9 was from the double mutant (M1709-2161). 2, 4, 6 and 8 were the PCR results using the primers corresponding to DR1709. 3, 5, 7 and 9 were the PCR products with the primers corresponding to DR2161. C is the identification of the double mutant. 1 is the DNA ladder 1, 2 and 11 are the DNA ladder 2, 3 and 4 are from the wild type, 5 and 6 are from M2161, 7 and 8 are from M1709, 9 and 10 are from the double mutant M1709 (2161). 3, 5, 7 and 9 were the digested results using Xho I. The original genomic DNA corresponded with DR1709. 4, 6, 8 and 10 were the digested results using Bam HI. The original genomic DNA corresponded with DR2161.

growth curves were not obvious (Figure 4). In DMM-Mn, although M2161 grew slower than the wild type, the difference was not much. But M1709 and the double mutant almost could not grow (Figure 5). These suggested that M1709 was very sensitive to low content of Mn. But it was not sensitive to Fe starvation. M2161 was not sensitive to low content of Mn or Fe. These results also show that DR2161 was deleted or had no obvious effect on the expression of DR1709.

Protease secretion assays of the strains

When the protease secretions of the strains were measured, the clearing area of M2161 was smaller than that of the wild type. But M1709 had bigger clearing area

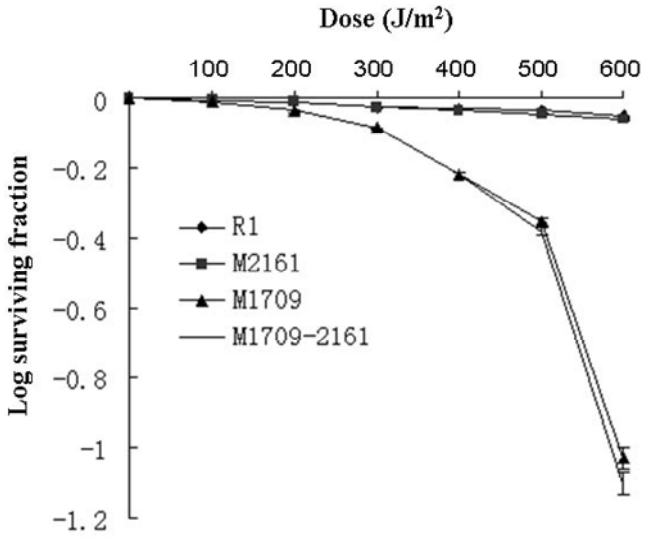

Figure 3. The strains performances under UV treatment.

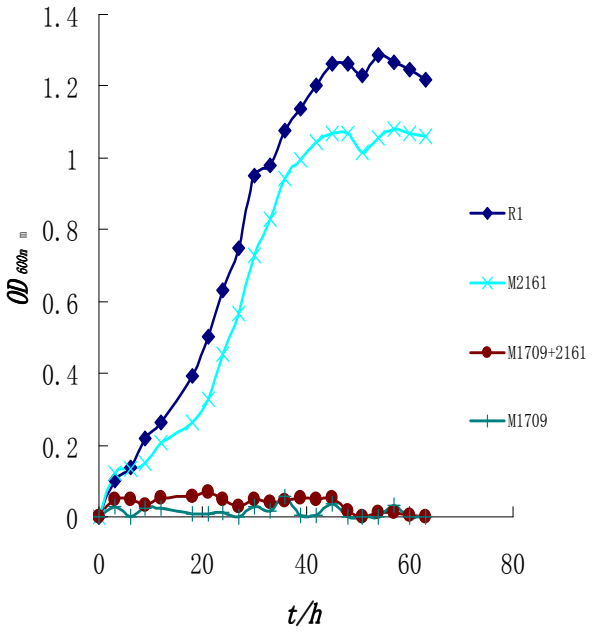

Figure 5. The growth of the strains in DMM-Mn.

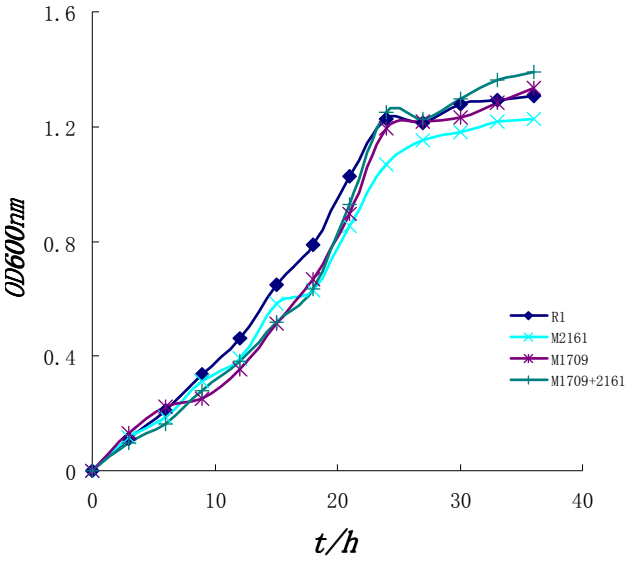

Figure 4. The strains' growth in DMM-Fe.

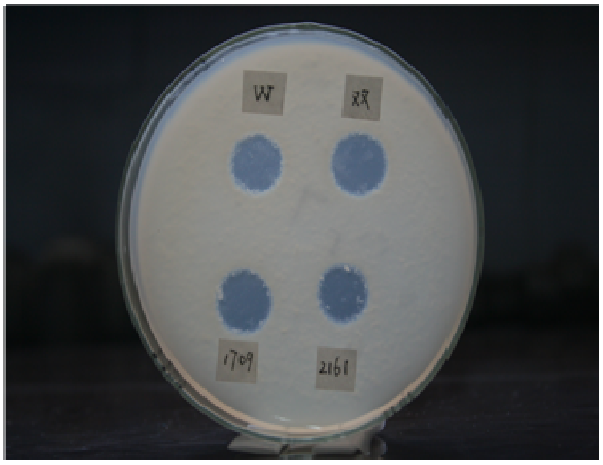

Figure 6. Protease secretion measure of wild type, M1709, M2161 and the double mutant.

than wild strain. The clearing area of the double mutant was similar to that of M1709 (Figure 6). In the double mutant, the effect of DR2161's deletion seemed to be covered by DR1709's disruption.

The strain sensitivity to Mn and Fe

The inhibition bacteria circles of M2161 to Mn^{2+} and Fe^{2+} were very similar to those of the wild type, showing that M2161 had similar resistance to high concentrate of Mn^{2+} and Fe^{2+} with the wild type (Figure 7A). The same instances happened when DR1709 was disrupted (data not shown) or DR2161 and DR1709 were both disrupted (Figure 7B). These results suggested that DR1709 and DR2161 had no relationship with the bacterium's sensitivities to high content of Mn^{2+} and Fe^{2+}.

DISCUSSION

D. radiodurans is one of the most radiation resistant microbes. But the mechanism was still unclear. Protein phosphorylation on tyrosine is an important signal transport and regulates a large array of cellular functions

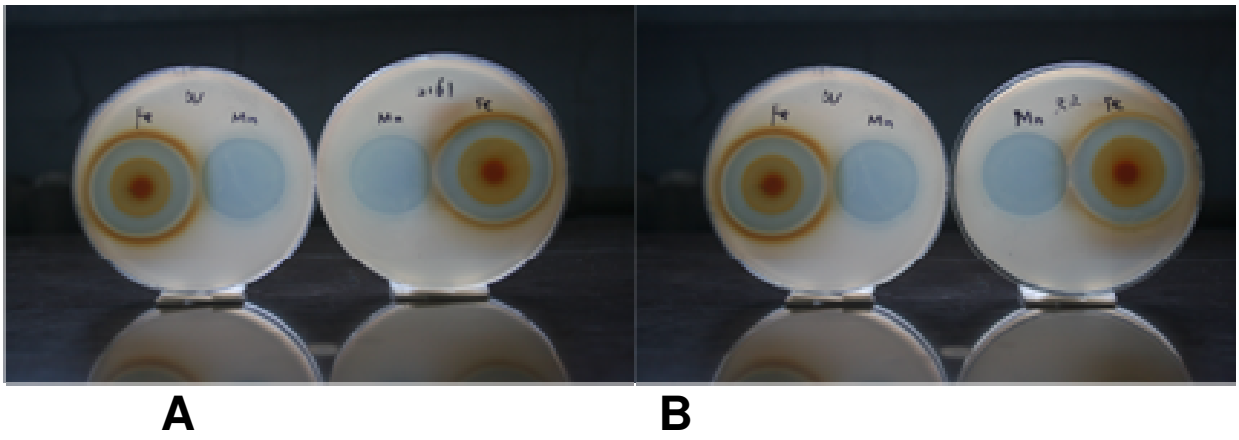

Figure 7. Cation sensitivity of the wild type, M2161 and the double mutant (M2161-1709).

(Cozzone et al., 2004; Klumpp et al., 2002). Many research suggested that it may play a role in *D. radiodurans* radiation resistance. However, after DR2161, the only predicted protein-tyrosine phosphatase gene in *D. radiodurans*, was deleted, M2161 had the similar appearance with the wild type after being treated with UV. DR1709, whose homologous can be regulated by protein-tyrosine phosphates in murine, was also deleted in *D. radiodurans*. Oddly enough, although M1709's radiation resistance was much lower than that of the wild type, the double mutant appearance was much like that of M1709. The four strains' (the wild type, M1709, M2161 and the double mutant) inhibition circle to Mn^{2+} and Fe^{2+} were very similar, showing that they had the similar resistance to high content Mn^{2+} and Fe^{2+}. In DMM with 200 nM Mn, M1709 and the double mutant almost could not grow.

But in DMM with 200 nM Fe, the two strains grew as quickly as the wild type. These showed that M1709's reaction to low concentrate of Mn^{2+} and Fe^{2+} was not affected by DR2161. They also suggested that when there was little Fe^{2+}, it can be used, the function of Fe was substituted by Mn (Anjem et al., 2009) but the function of Mn can not be replaced by Fe.

D. radiodurans encodes 18 peptide transporters and has numerous expanded protein families for branched-chain amino acid transport, many of which are unregulated during recovery from radiation (Liu et al., 2003). The halo clearing area of M1709 was larger than that of the wild type, indicating that more protease was secreted after DR1709 was disrupted. The role of DR1709 was able to control the protease production or secretion to prevent too much protease produced. The clearing area of M2161 was smaller than that of the wild type. But the clearing area of the double mutant was similar with that of M1709. The influence on the bacterium that DR2161 was deleted seemed to have been covered by DR1709 that is being disrupted. It seemed that the expression of DR1709 was not regulated by DR2161.

REFERENCES

Anderson AW, Nordon HC, Cain RF (1956). Studies on a radio-resistant micrococcus. I, Isolation, morphology, cultural characteristics, and resistance to gamma radiation. Food Technol., 10: 575-578.

Anjem A, Varghese S, Imlay JA (2009). Manganese import is a key element of the OxyR response to hydrogen peroxide in Escherichia coli. Mole. Microbiol., 72(4): 844-858.

Blasius M, Buob R, Shevelev I, Hubscher U (2007). Enzymes involved in DNA ligation and end-healing in the radioresistant bacterium Deinococcus radiodurans. BMC Mole. Biol., 8: 69-81:

Brim H, Osborne JP, Kostandarithes HM, Fredrickson JK, Wackett LP, Daly MJ (2006). *Deinococcus radiodurans* engineered for complete toluene degradation facilitates Cr(VI) reduction. Microbiol., 152: 2469-2477

Chang S, Shu H, Lu H, Hua YJ, Li ZW, Wang YP, Qin GY (2008). Construction of the gene drb0099 deleted mutant and adversity analysis in the extremely radioresistant bacterium *Deinococcus radiodurans*. Acta Microbiol. Sin., 48(1): 57-62

Chang SH, Shu HY, Li ZW, Wang YP, Chen LH, Hua YJ, Qin GY (2008). Disruption os manganese ions [Mn(II)] transporter genes DR1709 or DR2523 in extremely radio-resistant bacterium Deinococcus radiodurans. Acta Microbiol. Sin. 49(4): 438-444

Cozzone AJ (2005). Role of protein phosphorylation on serine/threonine and tyrosine in the virulence of bacterial pathogens. J. Mol. Microbiol. Biotechnol., 9(3-4): 198-213.

Cozzone AJ, Grangeasse C, Doublet P, Duclos B (2004). Protein phosphorylation on tyrosine in bacteria. Arch Microbiol., 181(3): 171-181.

Daly MJ (2009). A new perspective on radiation resistance based on *Deinococcus radiodurans*. Nature Rev. Microbiol., **7:** 237-245

Daly MJ, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, Leapman RD, Lai B, Ravel B, Li SM, Kemner KM, Fredrickson JK (2007). Protein oxidation implicated as the primary determinant of bacterial radioresistance. PLoS Biol., 5(4): 0769-0779.

Daly MJ, Minton KW (1995). Resistance to radiation. Science, 270: 1318.

Daly MJ, Ouyang L, Fuchs P, Minton KW (1994). In vivo damage and recA-dependent repair of plasmid and chromosomal DNA in the radiation-resistant bacterium Deinococcus radiodurans. J. Bacteriol., 176: 3508-3517.

Deepti A, Amara SR, Shree KA (2006). Engineering of *Deinococcus radiodurans* R1 for bioprecipitation of Uranium from dilute nuclear waste. Appl. Environ. Microbiol., 72(12): 7873-7878.

Funayama T, Narumi I, Kikuchi M, Kitayama S, Watanabe H, Yamamoto K (1999). Identification and disruption analysis of the recN gene in the extremely radioresistant bacterium Deinococcus radiodurans. Mutation Res., 435(2): 151-161.

Ghosal D, Omelchenko MV, Gaidamakova EK, Matrosova VY, Vasilenko A, Venkateswaran A, Zhai M, Kostandarithes HM, Brim H, Makarova KS, Wackett LP, Fredrickson JK, Daly MJ (2001). How radiation kills cells: survival of Deinococcus radiodurans and Shewanella oneidensis under oxidative stress. FEMS Microbiol. Rev.. 29(2): 361-375.

Gomez MA, Li S, Tremblay ML, Olivier M (2007). NRAMP-1 expression modulates protein-tyrosine phosphatase activity in macrophages: impact on host cell signaling and functions. J. Biol. Chem., 282(50): 36190-36198.

Grangeasse C, Obadia B, Mijakovic I, Deutscher J, Cozzone AJ, Doublet P (2003). Autophosphorylation of the Escherichia coli protein kinase Wzc regulates tyrosine phosphorylation of Ugd, a UDP-glucose dehydrogenase. J. Biol. Chem., 278(41): 39323-39329.

Klumpp S, Krieglstein J (2002). Phosphorylation and dephosphorylation of histidine residues inproteins. Eur. J. Biochem., 269: 1067-1071.

Liu Y, Zhou J, Omelchenko MV, Beliaev AS, Venkateswaran A, Stair J, Wu L, Thompson DK, Xu D, Rogozin IB, Gaidamakova EK, Zhai M, Makarova KS, Koonin EV, Daly MJ (2003). Transcriptome dynamics of Deinococcus radioduransrecovering from ionizing radiation. Proc. Natl. Acad. Sci. USA, 100: 4191-4196.

Longtin R (2003). *Deinoccocus radiodurans*: Getting a Better Fix on DNA Repair. J. Natl. Cancer Inst., 95(17): 1270-1271.

Makarova KS, Aravind L, Wolf YI, Tatusov RL, Minton K, Koonin EV, Daly MJ (2001). Genome of the extremely radiation resistant bacterium Deinococcus radiodurans viewed from the perspective of comparative genomics. Microbiol. Mol. Biol. Rev., 65: 44-79.