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Full Length Research Paper

Functional analysis of a putative transcriptional regulator gene *dr2539* in *Deinococcus radiodurans*

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The radiation resistant bacterium *Deinococcus radiodurans* R1 possesses a high intracellular Mn/Fe concentration ratio. Non-enzymic Mn(II) in *D. radiodurans* acts as an antioxidant to scavenge reactive oxygen species which contributes to its extreme radioresistance. The gene *dr2539* encodes a transcriptional regulator that is predicted to be involved in regulating the transportation of Mn(II) or Fe(II) in *D. radiodurans* R1. In this study, we constructed a *dr2539*disruption mutant with *D. radiodurans* R1 and compared them in growth rates and in intracellular Fe and Mn ions concentrations. We also investigated the phenotypes of the two strains including protease secretion and resistance to heavy metal ions, H_2O_2 and MV. The results showed that *D. radiodurans* R1 did not exhibit strong resistance to Hg(II), Ag(I), Cr(VI) and Pb(IV). Disruption of the gene *dr*2539 in *D. radiodurans* R1 resulted in an obvious growth defect in Mn-depleted medium and a remarkably-increased sensitivity to Mn(II). The disruption mutant obviously accumulated the intracellular Mn ion and raised the intracellular Mn/Fe ratio in Mn(II)-replete medium, but it had the similar intracellular Mn/Fe ratio with the wild strain in Fe(II)-replete medium. Furthermore, although the mutant accumulated high levels of intracellular Mn/Fe ratio in Mn(II)-replete medium, disruption of the gene *dr*2539 had no apparent effect on its resistance to H_2O_2 and MV. These results suggest that the gene *dr*2539 in *D. radiodurans* R1 plays an important role in regulating the transportation of Mn(II) and the correlation between the intracellular Mn/Fe ratio and the antioxidant capacity in *D. radiodurans* need further study.

Key words *Deinococcus radiodurans*, intracellular Mn/Fe concentration ratio, reactive oxygen species, minimal inhibitory concentration.

INTRODUCTION

Deinococcus radiodurans is an extreme bacterium, well known for its resistance to UV light, -ray radiation, oxidants and desiccation (Makarova et al., 2001). In addition to its highly efficient DNA damage repair systems, these resistant phenotypes are attributed to its powerful antioxidant system which consists of catalases (CAT), superoxide dismutases (SOD), peroxidases (POD), carotenoids (Cox and Battista, 2005; Battista et al., 1999; Chen et al., 2008) . Recently, Daly has proposed that non-enzymic Mn(II) also plays a crucial role in its antioxidant system (Daly, 2009). Compared with radiation sensitive bacteria, *D. radiodurans* R1 possesses a high total intracellular Mn/Fe concentration ratio (Ghosal et al., 2005; Daly, 2004) . Mn(II) in *D. radiodurans* R1 is predicted to act as an antioxidant to scavenge reactive oxygen species (ROS) generated during irradiation, as a result, proteins are protected from oxidative damage and the DNA repair systems function with great efficiency (Daly et al., 2007).

D. radiodurans R1 exploits at least two types of transporters to import Mn(II): the MntABC-type transpoter (DR2523, DR2283, DR2284) and the MntH-type transporter (DR1709) (Ghosal et al., 2005; Chang et al., 2009). However, the genes involved in regulating transportation of Mn(II) to maintain its intracellular homeostasis in *D. radiodurans* remain unclear. The remarkable radiation resistance and easily genetic manipulation of *D. radiodurans* R1 make it an attractive candidate for bioremediation of nuclear waste sites that comprise large of heavy metals such as Cr, Pb and Hg (Daly, 2000; Brim et al., 2000). Heavy metals, especially

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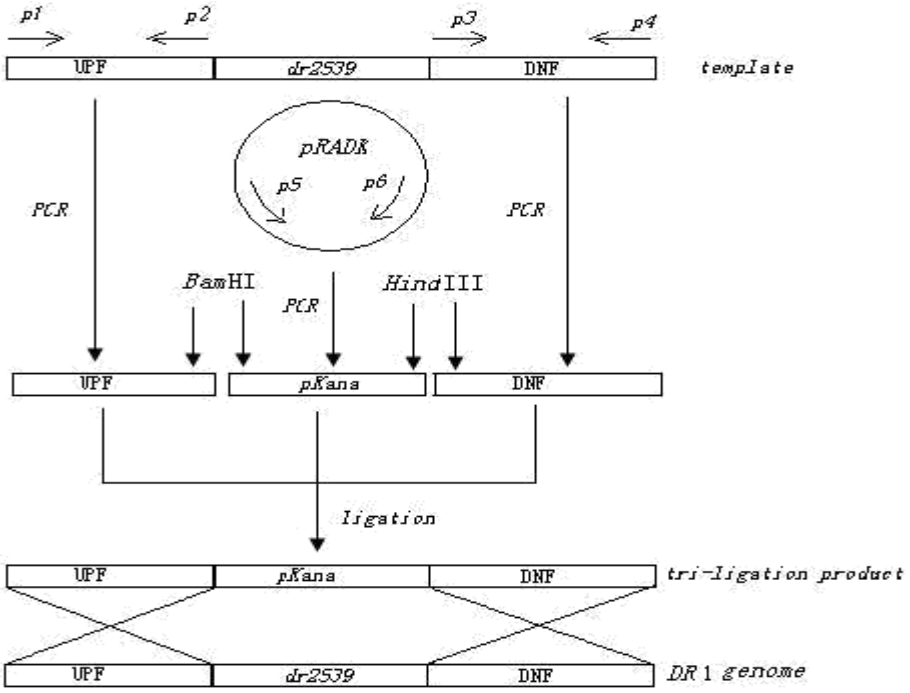

Figure 1. The scheme of M2539 construction.

at high concentrations, are toxic to organisms (Jakubovics and Jenkinson, 2001). But the resistance of *D. radiodurans* to heavy metals is still undefined.

The gene *dr2539* in *D. radiodurans* R1 is a putative Mn-dependent transcriptional regulator that may partici-pate in modulating the transportation of Mn(II) or Fe(II) (Ghosal et al., 2005; White et al., 1999). In this study, we constructed a *dr2539* disruption mutant with *D. radiodurans* R1 and compared them in growth rates and in intracellular Fe and Mn ions concentrations. We also investigated the phenotypes of the two strains including protease secretion and resistance to heavy metal ions, H_2O_2 and MV.

MATERIALS AND METHODS

Strains, plasmid and growth conditions

D. radiodurans R1 strain (ATCC 13939) and the plasmid of pRADK were stored in our lab (Chen et al., 2008). Unless otherwise indicated, strains were grown at 30°C aerobically in undefined replete medium TGY broth (1% tryptone, 1% glucose, 0.5% yeast extract) or on TGY plates solidified with 1.5% agar. Kanamycin (20 g/ml) was added to the medium to screen transformant. Chemical reagents were, if not otherwise specified, purchased from Takara (Dalian China). To make the competent cells of *D. radiodurans* R1, a modified $CaCl_2$ technique described previously was utilized (Nishida and Narumi, 2002).

Disruption of *dr2539* in *D. radiodurans* R1

Disruption of the gene *dr2539* in *D. radiodrans* R1 (Figure 1) was performed using a modified three-step gene splicing technique by overlap extension (Huang et al., 2007). Briefly, the upstream fragment (UPF) of *dr2539* with a *Bam*HI site was amplified with primers p1 (TAAAGCGGATACGAGACGACCTCCAACC) and p2 (ACGACCGGATCCATCTTCCCGTCCACTG); the downstream fragment (DNF) of *dr2539* with a *Hind*III site was obtained with primers p3 (ACTGGTAAGCTTCCACAGCACGTCGTCA) and p4 (CTACTACGATGCTCGCCCTGCAAACCCT) . The PCR was performed with 1 cycle at 94°C for 5min for initial denaturation; 42 cycles of denaturation at 94°C for 1min, annealing at 62°C for 50s, and extension at 72°C for 1min; and a final elongation step at 72°C for 8min.

The kanamycin resistant cassette with *Bam*HI and *Hind*III sites was seperated from a shuttle vector plasmid named pRADK using the primers p5 (CACACAGGAAACAGCTATGACCA) and p6 (ACAGACGGATCCTAGAAAAACTCATCGAGCATCAAATG) and the PCR was conducted as the following conditions: 42 cycles of 1 min at 94°C, 50 s at 62°C and 90 s at 72°C. The above three fragments were purified by 0.8% agarose gel electrophoresis and then digested with restriction enzyme seperately. The three digested DNA fragments were ligated at 4°C overnight. The ligation product was used as a template for PCR with the primers p1and p4, and then the resulting product was used to transform the competent cells of *D. radiodurans* R1.

After *D. radiodurans* R1 was transformed, colonies that were able to grow on TGY plate containing 20 g/ml of kanamycin were collected as the candidate mutants. Firstly, used the genomic DNA isolated from R1 strain and candidates as the templates to PCR with the primers p1 and p4 to select the mutant for the resulting product of strain R1 was estimated to be 2405 bp, while the product of the mutant was 2342 bp. Secondly, identified the above products by double enzyme digestion with *Bam*HI and *Hind*III. If the mutant had been constructed successfully, three bands would be seen. However, the product generated from R1 strain can not be digested for it does not contain the above two restriction enzyme sites. The disruption mutant of *dr2539* was designated as M2539.

Table 1. Concentration gradients of each metal ion for MIC tests.

Metal type	Concentration gradients (M)
$MnCl_2$	500-1000-2000-4000-6000-8000
$FeSO_4$	500-1000-2000-4000-6000-8000
$HgCl_2$	150-300-600-1200
$K_2Cr_2O_7$	200-400-800-1600-3200-6400
$Pb(CH_3 COO)_2$	200-400-800-1600-3200- 6400
$AgNO_3$	150-300-600-1200

Growth rate assay

Growth rates were measured according to the published method (Leibowitz et al., 1976). *D. radiodurans* R1 and M2539 were grown in TGY broth till OD_{600}s were 0.8. After centrifugation at 3000 rpm, at 4°C for 10 min, the cell pellets were washed twice and resuspended with phosphate-buffered saline (PBS) . Then the cell suspensions were reinoculated in defined replete media (DRM) modified with different concentrations of Mn(II) and Fe(II) (Venkateswaran et al., 2000). DRM medium was used without $FeSO_4$ (DRM-Fe) and $Mn(CH_3 COO)_2$ (DRM-Mn), with $Mn(CH_3COO)_2$ at a final concentration of 5 M (DRM- 5.0) or 10 M (DRM-10) or 50 M (DRM-50). The growth rates were monitored by measuring the OD_{600}s each, 12 h intervals. All the data were measured for replications and the average values were used. The curves were drawn using Microsoft Excel.

Minimal inhibitory concentration assay

Assays of minimal inhibitory concentrations (MICs) of metals were performed as described before (Filali et al., 2000). *D. radiodurans* R1 and M2539 were grown in TGY broth till early stationary phase. Then aliquots of each culture were diluted to 1% with fresh sterilized TGY broth. 100 l of the cell suspensions were spread onto the TGY plates, added or non-added (control) with the res-pective metal element at different concentrations. The concentration gradients of each metal ion were listed in Table 1.

All the plates were incubated at 30°C for 48 h. The minimal inhibitory concentration (MIC) is defined as the lowest concentration that causes no visible growth.

Assay of intracellular Mn and Fe ions concentration

The protocol for determining intracellular concentrations of Mn and Fe ions was identical to that previously reported (Horsburgh et al., 2002). *D. radiodurans* R1 and M2539 were activated in TGY broth then reinoculated in TGY, TGY- Mn (TGY broth amended with $MnCl_2$ at a final concentration of 500 M), TGY-Fe (TGY broth amended with $FeSO_4$ at a final concentration of 500 M) respectively and cultured to the stationary phase. After centrifugation at 10000 g, 4°C for 10min, the pellets were washed three times with PBS containing 1 mM EDTA and twice with PBS. The cell dry weights were mea-sured and accurated to milligram after the pellets were incubated at 80°C overnight. 2 ml of 30% nitric acid were added and the cells were digested in a water bath at 80°C for 4 h. The digestions were filtered and diluted with deioned water as 1:10. These samples were analyzed for the total concentrations of Mn and Fe ions by inductived coupled plasma mass spectrometry (ICP-MS). A blank control was prepared in the same manner but without cells. All the data were replicated for twice, and used the means as the representative values.

Hydrogen peroxide and methyl viologen sensitivity assays

The sensitivity assays were conducted as the publication described (Horsburgh et al., 2002; Que and Helmann, 2000). *D. radiodurans* R1 and M2539 were cultured in TGY, TGY-Mn (TGY broth amended with $MnCl_2$ at a final concentration of 500 M) and TGY-Fe (TGY broth amended with $FeSO_4$ at a final concentration of 500 M) respectively. When the cultures reached to the early stationary phase, aliquots of each culture were diluted to 4% with the corresponding agar plates prewarmed to 45°C. 5 ml of the cell suspensions were spread onto the corresponding agar plates. 50 l of 3% H_2O_2 or 25 l of 0.5 mg/ml methyl viologen (MV) were placed in the oxford cups laid on the plates. The diameter of the inhibition zones were measured after the plates were incubated at 30°C for 24 h. All the data were measured for triplicates and the average values were used.

Protease secretion assay

Test for protease secretion was carried out on indicator plate which contained 0.3% Beef Extract, 0.5% Trptone, 1.5% Agar powder and 2% Skimmed milk powder (Ghosal et al., 2005). The wild strain and M2539 were grown to the stationary phase in TGY broth and then centrifuged at 12000 rpm at 4°C for 20 min. The supernatants were spinned again then filtered by 0.22 m millipore filter. Each oxford cup on the indicator plates was loaded with 200 l of the above supernatants then incubated at 30°C for 36 h.

RESULTS

Disruption mutant of *dr* 2539 in *D. radiodurans* R1 constructed successfully

The potential mutant of *dr2539* was selected on the TGY plates amended with 20 g/ml of kanamycin. As the amplified products from wild strain and potential mutant with primers p1 and p4 were 2405 bp and 2342 bp res-pectively, they could not be separated clearly by agarose gel electrophoresis (Figure 2). However, the product from potential mutant were double digested with *Bam*HI and *Hind*III which indicated that the above two restriction-enzyme sites resulted from modifying the primers p2 and p3 were introduced into *D. radiodurans* R1 successfully (Figure 2). This result validated that the disruption mutant of the gene *dr2539* had been constructed successfully for the wild strain does not contain these two restrition-enzyme sites. The disruption mutant of *dr2539* was denominated as M2539.

Disruption of *dr2539* results in *D. radiodurans* cell growth defect in Mn (II)- depleted medium

Growth rates of *D. radiodurans* R1 and M2539 in DRM modified with different concentrations of Mn(II) and Fe(II) were shown in Figure 3. The results showed that R1 strain and M2539 could grow with trace levels of Mn(II) and Fe(II). However, they grew more slowly in the Mn(II)-depleted (DRM-Mn) or Fe(II)-depleted (DRM-Fe) DRM as compared to be grown in DRM (DRM 5.0). This result

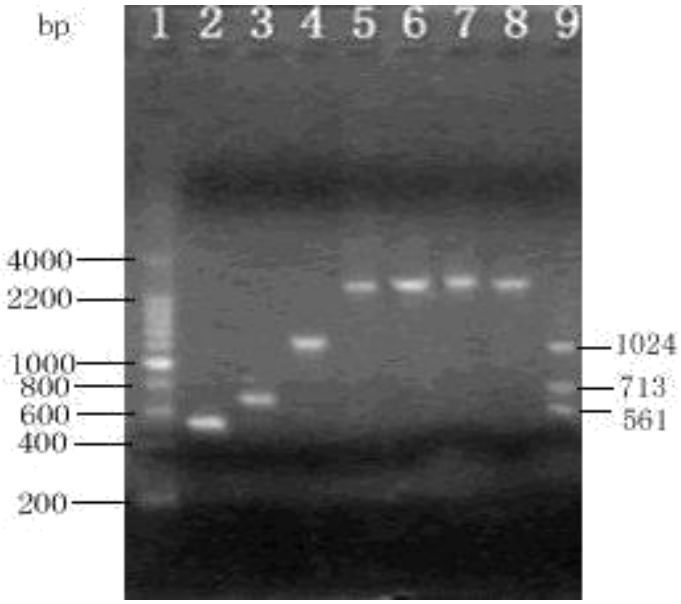

Figure 2. Construction and identification of M2539. Lanes:1,DNA ladders;2,upstream fragment of the target gene(561bp);3,downstream fragment of the target gene(713bp); 4,kanamycin resistant cassette(1024bp);5,ligation product of the above three fragment (2342bp);6,product from wild type with p1and p4(2405bp);7,double digestion of the above fragment;8,product from candidate mutant with p1and p4;9,double digestion of the above fragment.

indicated that the ions of Mn(II) and Fe(II) have great influence on growth of *D. radiodurans*. The optimum growth concentration of Mn(II) for strain R1 was 10 M, but it was 5 M for M2539. The notable difference reflecting the influence of *dr2539* disruption on growth was that M2539 grew more slowly in Mn(II)-depleted DRM than R1 strain, while no significant difference was shown about the two strains grown in Fe(II)-depleted DRM. These results indicate that the role of *dr2539* in growth of *D. radiodurans* R1 is associated with Mn(II).

Disruption of the gene *dr*2539 renders *D. radiodurans* quite sensitive to Mn(II)

The above results showed that *dr2539* disruption resulted in a growth defect of the mutant in Mn(II)-depleted medium. Then we tested whether the *dr2539* disruption had an impact on its growth in media with high concentrations of Mn(II) or Fe(II). Minimal inhibitory concentrations (MICs) for heavy metal ions of the two strains were presented in Table 2. The results showed an obvious difference between the two strains that the MIC for Mn(II) of wild strain was 8000 M, threefold over than that of M2539 which indicated that *dr2539* disruption in *D. radioduran* R1 significantly increased its sensitivity to Mn(II). Moreover, compared with the bacteria isolated from metal-contaminated water, *D. radiodurans* R1 exhibited stronger resistance to Mn(II) and Fe(II) but higher sensitivity to others such as Hg(II), Ag(I), Cr(VI) and Pb(IV) (Filali et al., 2000). For example, the MIC for $HgCl_2$ of *Klebsiella pneymoniae* was 1200 M fourfold than that of *D.radiodurans* R1.

Disruption of *dr2539* results in a great increase of intracellular Mn and Mn/Fe ratio of *D. radiodurans* in Mn(II)-replete medium

Daly has proposed that high total intracellular Mn/Fe ion ratio in *D. radioduans* R1 is conducive to protecting proteins from oxidative damage and then contributes to its extreme resistance (Daly, 2009) . However, little is known about the influence of *dr2539* on the intracellular Mn/Fe ratio of *D. radioduans* R1. With this question, the total intracellular concentrations of Mn and Fe ions of the two strains at stationary phase were measured and listed in Figure 4 A, B, C. The results showed that when they grew in Mn(II)-replete medium (TGY-Mn) or Fe(II) -replete medium (TGY-Fe), the intracellular Mn-ion or Fe-ion concentration of the two strains would be increased respectively, but the increase of the total intracellular Mn-ion concentration of M2539 was obviously more than that of wild strain. In addition, when grown in the Fe(II)-replete medium, M2539 had similar intracellular Mn/Fe ratio with the wild strain, but when grown in Mn(II)-replete medium, it remarkably increased intracellular Mn/Fe ratio as compared with wild strain.

Disruption of the gene *dr*2539 has no apparent effect on oxidative resistance of *D. radiodurans*

Non-enzymic Mn(II) in *D.radiodurans* R1 was predicted as an antioxidant to clear ROS and contributed to its antioxidant ability (Chen et al., 2008). Since the total intracellular Mn-ion concentration of *D.radiodurans* was influenced by the gene *dr2539* and by the Mn(II) content of the medium where it grew, its antioxidant ability might also be affected by them.

To evaluate the effect of *dr2539* on oxidation resistance, the wild strain and M2539 were treated with H_2O_2 and MV in different media respectively. The results showed that the two strains had similar zone of inhibition, which indicated that disruption of *dr2539* in *D. radiodurans* R1 did not make notable difference to its antioxidant ability (Figure 5).

Disruption of the gene *dr*2539 makes little effect on protease secretion of *D. radiodurans*

Protease secretion of *D. radioduran* R1 may facilitate cell's recovery from ionizing radiation since it may provide the bacterium with lots of nutrition, which is conducive to its DNA repair (Ghosal et al., 2005). M2539 exhibited

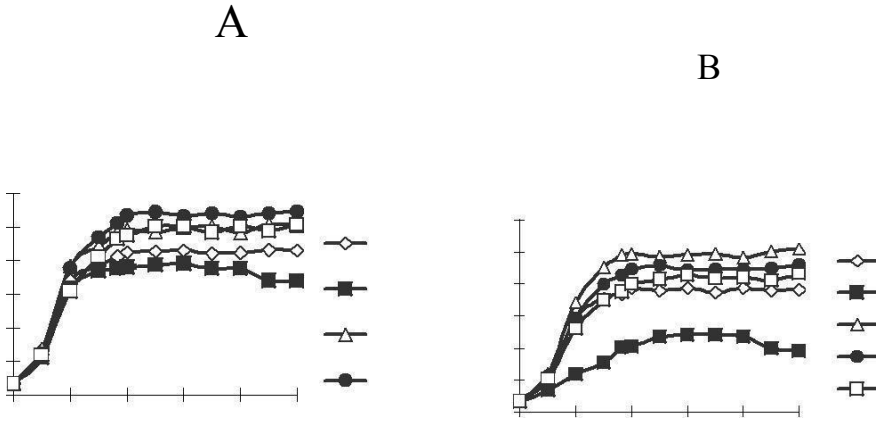

Figure 3. Growth rates of *DR*1 and M2539 in DRM with different concentrations of Mn and Fe.

Table 2. Minimal inhibitory concentrations (M) of heavy metal ions for wild strain and M2539.

Strains	$MnCl_2$	$FeSO_4$	$HgCl_2$	$K_2Cr_2O_7$	$AgNO_3$	$Pb(CH_3COO)_2$
D. radiodurans R1	8000	4000	300	1600	300	3200
M2539	2000	4000	ND[a]	ND	ND	ND

[a]ND,not determined

identical resistance to H_2O_2 and MV as compared with the wild strain. Can the disruption of the gene *dr2539* stimulate its protease secretion in *D. radiodurans*? However, the protease secretion assay (Figure 4D) showed that there was no visible difference between the two strains.

DISCUSSION

D. radiodurans R1 is well known for its extraordinary capacity to survive extreme doses of radiation without lethality (Makarova et al., 2001). Available evidences argue that: I) The multiple genomes of *D. radiodurans* R1 may facilitate its homologous recombination after irradiation, but the number of double-strand DNA breaks (DSBs) induced by irradiation is not associated with the genomic copy number since the numbers of DSBs per Gy per genome for a given dose of radiation in many bacteria are very similar (Battista et al., 1999; Daly et al., 2004). II) The ring-like structure of *D. radiodurans*'s chromosomes does not play a vital role in its radiation resistance (Ghosal et al., 2005; Levin-Zaidman et al., 2003). III) *D. radiodurans* uses a routine DNA repair system as prokaryotes but with high efficiency (Ghosal et al., 2005). IV) Early studies of radiation damage focused on DNA, but many prokaryotes were killed by irradiation which caused little DNA damage.

A new prospect is that the degree of bacterial radioresistance is determined by the level of oxidative protein damage caused during irradiation (Daly et al., 2007). The strong enzymic antioxidant system including CAT, SOD, POD in *D. radiodurans* plays an important role in clearing ROS caused by irradiation to protect proteins from oxidation which helps the bacterium in combating oxidative stress (Chen et al., 2008). V) Non-enzymic Mn(II) in *D. radiodurans* may scavenge ROS through its redox reaction (Ghosal et al., 2005). However, neither the state of the Mn(II) existed in *D. radiodurans* R1 nor the genes involved in regulating the Mn(II) transportation has been studied.

In *D. radiodurans*, two types of Mn(II) import systems have been determined, DR1709 belongs to the Nramp family transporter, another type of predicted Mn- ion trans-porter is a MntABC type transporter (DR2523, DR2283, DR2284) (Ghosal et al., 2005; Daly et al., 2004; Chang et al., 2009). The efflux system of Mn ion has not been reported in bacteria until recently when Rosch identified one in *Streptococcus pneumoniae* encoded by *mntE* (Rosch et al., 2009). But whether the homologous gene of *mntE* exists in *D. radiodurans* is still unknown.

The gene *dr2539* of *D. radiodurans* is presumed to encode a transcriptional regulator involved in regulating transportation of Mn(II) or Fe(II) (Ghosal et al., 2005; White et al., 1999). Our results reveal that it is more likely to be a DtxR-like protein as MntR in *Bacillus subtilis* which acts as a positive regulator in high Mn(II) condition and a negative regulator in low Mn(II) condition (Horsburgh et al., 2002). Mn(II) is required for the metabolisms of organisms, but it will inhibit its growth or even kill cells when it is at high concentration (Filali et al., 2000). Trace level of Mn(II) is essential to the growth of

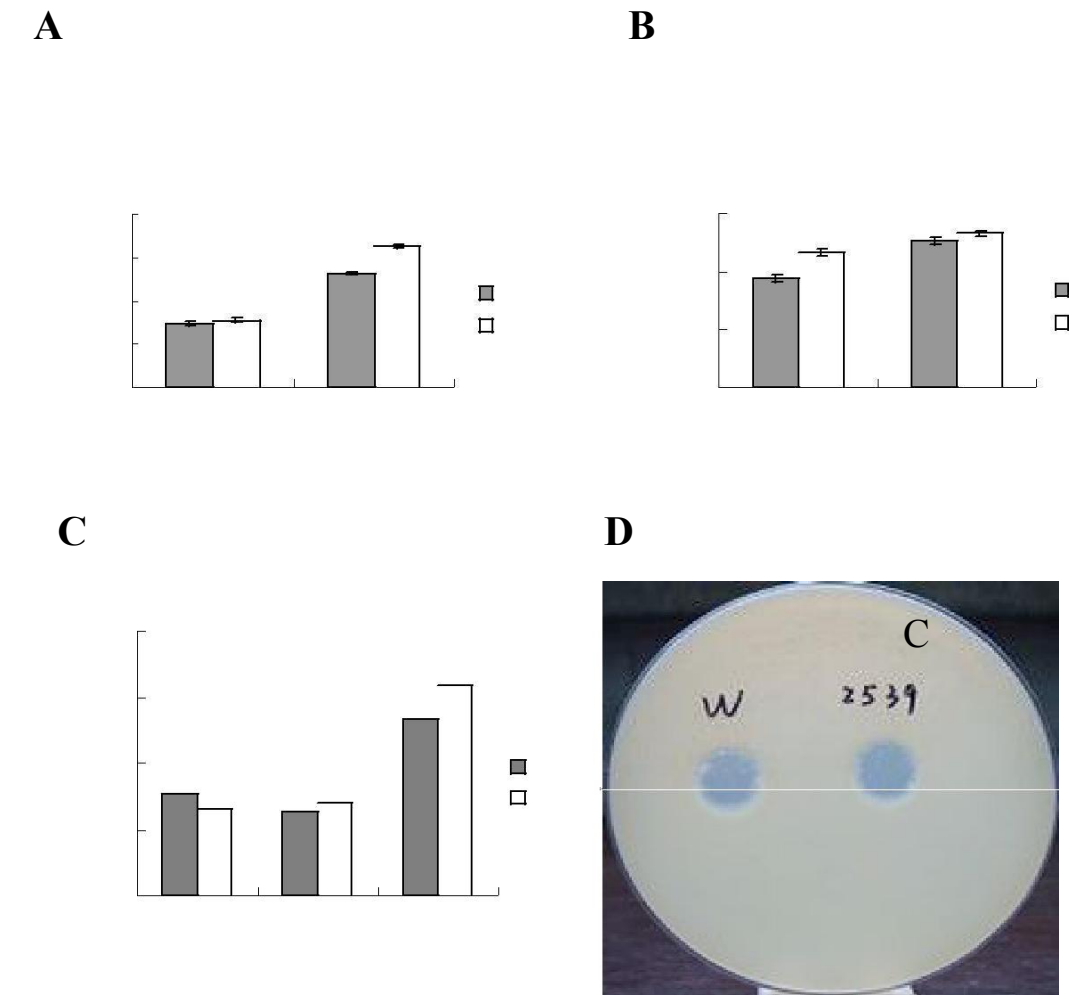

Figure 4. A: Intracellular concentrations of Mn of strains in different cultures; B: Intracellular concentrations of Fe of strains in different cultures; C: Intracellular Mn/Fe ratios of strains in different cultures; D: Protease secretion assay on an indicator plate containing skimmed milk. W represents for Wild strain; 2539 represents for M2539.

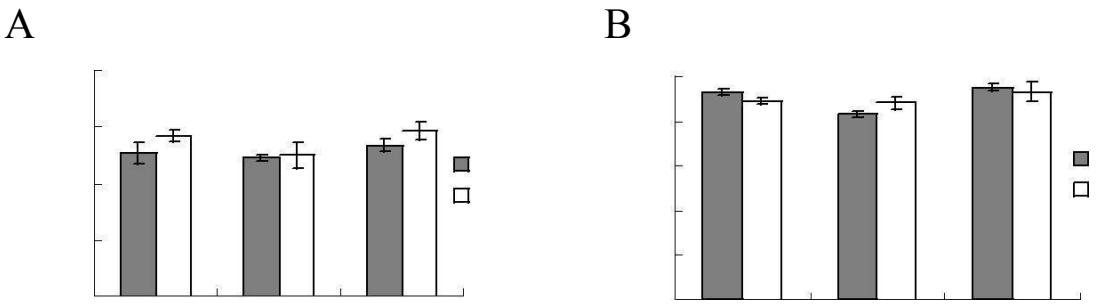

Figure 5. A: H_2O_2 sensitivtity assay of *DR*1 and M2539 in different cultures B: MV sensitivtity assay of *DR*1 and M2539 in different cultures.

D. radiodurans R1 for it grows slowly in Mn(II)-depleted medium. The *dr2539* disruption results in a growth defect indicates that the gene *dr2539* may play as a positive regulator to stimulate the Mn(II) transportation in *D.*

radiodurans R1 and then to contribute to its growth. However, in high Mn(II) condition, disruption of *dr2539* in *D. radiodurans* R1 raises its sensitivity to Mn(II). The mutant obviously accumulates high intracellular Mn ion and raises the intracellular Mn/Fe ratio in Mn(II)-replete medium, but it does not alter the intracellular Mn/Fe ratio significantly in Fe(II)-replete medium. These results indicate that *dr2539* disruption in *D. radiodurans* R1 may make it lose a negative regulation of Mn(II) uptake, for it maybe killed by excessive accumulation of intracellular Mn (II).

Bioremediation of the nuclear waste sites, which contain a variety of heavy metal ions such as Cr(VI), Pb(IV) and Hg(II), is an attractive proposition (Brim et al,. 2000) . The suitable candidate for bioremediation, such areas must be able to withstand cellular toxicity caused by irradiation and heavy metals (Daly, 2000). *D. radiodurans* R1 with notable radioresistance and easily genetic manipulation is the most prospective candidate for bioremediation. However, our results show that *D. radiodurans* R1 does not exhibit strong resistance to those heavy metal (Table 1). Therefore, in the process of remediation of those sites, the contents of heavy metals in the environment and the metal resistance of *D. radiodurans* R1 should be taken into consideration simultaneously.

Recently, Daly has proposed that the degree of radiation resistance is determined by the level of protein oxidation caused by irradiation (Daly et al., 2007). But Sukhi shows a contradiction that *D. radiodurans* R1 at different exponential phases exhibit identical radioresistance, but the intracellular Mn/Fe ratios alter remarkably and the degree of proteins oxidation are significant (Sukhi et al., 2009).

In this study, the intracellular Mn/Fe ratio of *D. radiodurans* changes with the media where it grows. Increasing the Mn(II)- concentration of the medium *D. radiodurans* grows in, can raise its intracellular Mn/Fe ratio.

Furthermore, the wild strain and M2539 cultured in Mn(II) -replete TGY broth possess high intracellular Mn/Fe ratios but exhibit similar oxidative resistance as compared to be cultured in TGY, which indicates that there is no direct correlation between the intracellular Mn/Fe ion ratio and the oxidation resistance of *D. radiodurans* R1. Recently, Imaly has proposed that Mn(II) may play an important role in replacing Fe(II) to combine with the metalloproteins which alleviates the cellular oxidative damage caused by irradiation (Anjem et al., 2009). Refreshing the culture of *D. radiodurans* R1 at late sta-tionary phase will increase its radiation resistance (Sukhi et al., 2009).

This result indicates that the metabolisms provided with energy for *D. radiodurans* R1 play an important role in its recovery from radiation damage. Disruption of the gene *dr2539* did not alter its ability of protease secretion. It maybe that the role of *dr2539* in *D. radiodurans*' metabolism are replaced by that of other unknown gene.

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