Full Length Research Paper

Function analysis of two Mn(II) ion transporter genes (DR1709 and DR2523) in *Deinococcus radiodurans*

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Deinococcus radiodurans* is best known for its extraordinary radiation resistance. Manganese(II) ions can protect proteins from being damaged by super radicals, which is supposed to be the main mechanism through which *D. radiodurans* has the strong radiation resistance. But the detailed molecular and physiological processes about how Mn(II) ions are transported in this bacterium is still not clear. In this paper, two Mn(II) ions transporter gene (DR1709 and DR2523) mutants were used to study the Mn(II) ions transporting mechanism. Results showed that M1709 was much more sensitive to low concentration of Mn(II) ions than M2523. But it was less sensitive to low concentration of Fe(II) ions than M2523. When there were enough Mn(II) ions in the medium, other genes were also activated to uptake them. But when Mn(II) ions was deficient, DR1709 became the only candidate responsible for Mn(II) assimilation. DR1709 was in charge of Mn(II) ions absorbing, no matter wheather Mn(II) ions supply was enough or not. DR1709 can also control the protease production or secretion to prevent over production. The functions of protease secreted by the strains was limited in radiation resistance. DR2523 was responsible for Mn(II) ions efflux, while DR1709 was a Mn(II) influx transporter, which cannot transport Fe(II) ions.

Key words: *Deinococcus radiodurans*, radiation resistance, Mn(II) ion transporter, DR1709, DR2523.

INTRODUCTION

Deinococcus radiodurans is well known for its strong radiation resistance (Daly, 1994). Understanding its radio-resistance mechanism had made it a potential agent used in the treatment of radioactive waste and tumor control (Longtin, 2003; Makarova et al., 2001; Hassan et al., 2006; Deepti et al., 2006). The multiple genome of *D. radiodurans* R1 was thought to facilitate its homologous recombination after irradiation (Battista et al., 1999; Daly et al., 1995). The ring-like structure of *D. radiodurans*'s chromosomes may play a key role in its radiation resistance too (Ghosal et al., 2005; Levin-Zaidman et al., 2003). It was also proposed that *D. radiodurans* use

conventional DNA repair system as prokaryotes but with high efficiency (Ghosal et al., 2005). Recent research showed that Mn(II) ions protect proteins from being damaged by superradicals (Daly et al., 2007, 2009), which was proposed to be the main mechanism that *D. radiodurans* had the strong radiation resistance (Daly et al., 2007, 2009). But what genes and how these genes participate to regulate the Mn(II) in *D. radiodurans* is unknown. There were two types of predicted Mn(II) trans-porters in *D. radiodurans* (Nramp family and ATP-depen-dent ABC-type transporter). DR1709 belonged to the Nramp family. DR2523 and DR2283-DR2284 were pre-dicted ATP-dependent and ABC-type transporters. After *DR1709* was disrupted, the growth of *D. radiodurans* was inhibited heavily, while M2523 had the similar survival fraction with the wild type (Chang et al., 2009). But the detailed process about Mn(II) ions transportation in this bacterium is still not clear.

In this paper, the detailed process of Mn(II) trans-portation in *D. radiodurans* was analyzed. It was suggested that there were other genes responsible for Mn(II) ions uptake when there were enough Mn(II) in the

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Abbreviations: DMM, Defined minimal medium; **TGY,** tryptone-glucose-yeast; ICP-MS, inductive coupled plasma mass spectrometry; **PBS,** phosphate buffered saline; **EDTA,** ethylenediaminetetraacetic acid; **TEM,** transmission electron microscopy; **ROS,** reactive oxygen species.

Table 1. Intracelluar Mn and Fe levels in *D. radiodurans* strains.

Strain	Total Mn (nmol Mn/mg cell) in TGY	Total Mn (nmol Mn/mg cell) in DMM	Total Fe (nmol Fe/mg cell) in TGY	Total Fe (nmol Fe/mg cell) in DMM	Mn/Fe in TGY	Mn/Fe in DMM
W	1.14 ± 0.21	0.42 ± 0.17	3.22 ± 0.57	1.63 ± 0.35	0.316	0.257
M2523	1.02 ± 0.19	0.40 ± 0.11	5.54 ± 0.62	3.01 ± 0.55	0.184	0.133
M1709	0.57 ± 0.17	0.20 ± 0.15	3.21 ± 0.55	1.62 ± 0.34	0.178	0.123

medium. DR1709 was in charge of Mn(II) ions absorption, no matter if Mn(II) ions content was deficient or ample. But when Mn(II) ions concentration was low, DR1709 became the only gene responsible for Mn(II) assimilation. DR1709 can also control the protease production or secretion to prevent over production. The role of protease secreted by the strains was limited in radiation resistance. DR2523 was responsible for Mn(II) ions efflux, while DR1709 was a Mn influxer. DR1709 cannot transport Fe(II). It may be specific to Mn(II).

MATERIALS AND METHODS

Strains and growing conditions

The information about the mutants' construction and the wild-type *D. radiodurans* can be found in our previous paper (Chang et al., 2009). Undefined rich media (TGY) was made as the standard protocol (1% bactotryptone, 0.5% yeast extract, and 0.1% glucose). Liquid defined minimal medium (DMM) was prepared as described in Table 1 by Venkateswaran et al. (2000). DMM-Mn and DMM-Fe were also prepared according to this table, except Mn(II) and Fe(II). In DMM-Mn, the concentration of Mn(II) was 200 nM. DMM-Fe contained 200 nM Fe(II). Cells were typically inoculated in liquid medium at ~1×10^6 CFUs/ml followed by incubation at 32°C. For each trial in liquid media, or CFU assays on solid medium, three replicates were carried out and the averages were used. Chemical reagents were, if not otherwise specified, purchased from Takara (Dalian, China).

Protease secretion assays

Tests for secretory proteases were carried out on indicator plates containing skimmed milk (Ghosal et al., 2005). For individual plates, 30 ml bottom layer (0.3% Beef Extract (Sigma), 0.5% Tryptone Peptone (Difco), 1.5% Bacto-Agar (Difco)) was prepared and poured into the plate. After the bottom layer solidified, 5 ml top layer (3.3% Skimmed Milk Powder (Difco), 0.3% Beef Extract (Sigma), 0.5% Tryptone Peptone (Difco), 1.2% Bacto-Agar (Difco)) was poured on it. The wild type and the mutants were all cultivated to OD_{600} = 1.0 in tryptone-glucose-yeast (TGY) broth. Then, they were centrifuged for 20 min (12000 rpm, 4°C). The supernatants were filtered through millipore filteration membrane (0.22 μm). Oxford cups (diameter was 0.5 cm) were placed on the solidified top layer. Each of them was loaded with 200 μl of the above supernatant. The plates were incubated for 36 h at 32°C.

Cation sensitivity

Cation sensitivity was measured mainly according to the method described (Rosch et al., 2009). 1 M solutions of $MnCl_2$ and $FeSO_4$ (Sigma-Aldrich, St Louis, MO) were prepared in Milli-Q water and sterile filtered. *D. radiodurans* R1 and mutants were grown in TGY broth till OD_{600} = 0.5. Then an aliquot of each culture were diluted to 1% with fresh sterilized TGY broth. 100 μl of the cell suspensions were spread onto the TGY agar plates. After the suspension was absorbed, two oxford cups (diameter was 0.5 cm) were placed on the TGY plate. 200 μl of $MnCl_2$ (1M) was loaded into one cup and $FeSO_4$ (1 M) was loaded into the other cup. The plates were incubated at 30°C for 24 h. The zone of inhibition was measured for each disc. Statistical significance was determined by Friedman test.

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

ICP-MS was performed as described (Horsburgh et al., 2002). *D. radiodurans* R1 and the mutants were activated in TGY broth, and then reinoculated in TGY and DMM, respectively, and cultured to the stationary phase. After centrifugation at 10000 g, 4°C for 10 min, the pellets were washed three times with phosphate buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and twice with PBS. The cell dry weight was measured and changed 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 deioned water as 1:10. These samples were analyzed for Mn Fe concentration by inductive coupled plasma mass spectrometry (ICP-MS). A blank control was prepared in the same manner but without cells. All data were replicated three times and the means were used as representative value.

RESULTS

### M1709 was more sensitive to low content Mn(II) ions than M2523 and the wild type

In DMM with 200 nM Mn, M2523 grew slower than the wild type (Figures 1 and 2). But M1709 almost can not grow. In the corresponding solid agar plate, no M1709 clone could be observed even after the plates had been placed in 32°C for 13 days (Figure 1). These showed that M1709 was much more sensitive to low concentration of Mn(II) ions than M2523 and the wild type.

M2523 was more sensitive to low concentration of Fe(II) ions than M1709

In DMM with 200 nM Fe(II) ions, M1709 grew slower than the wild type between 10 and 25 h (Figure 3). They both entered the stationary phase after being cultured for 25 h
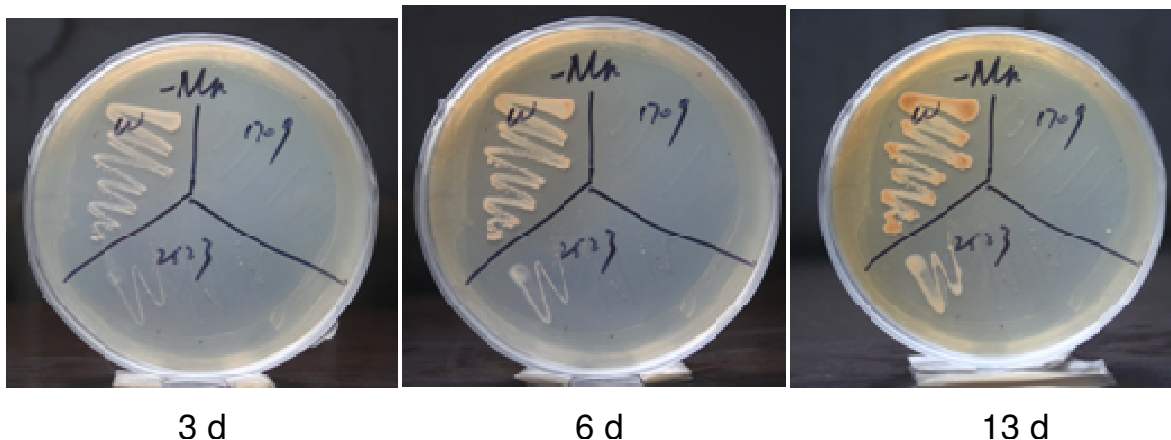
Figure 1. The growth of the strains in solid DMM (with 200 nM Mn). W = wild type *D. radiodurans*; 1709 = M1709; 2523 = M2523.
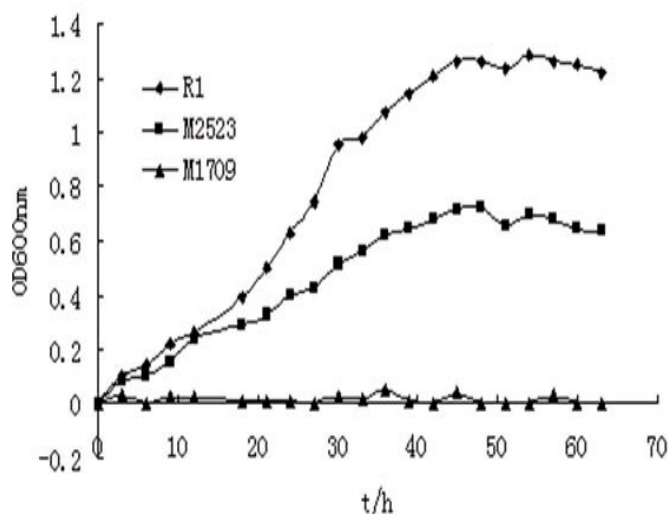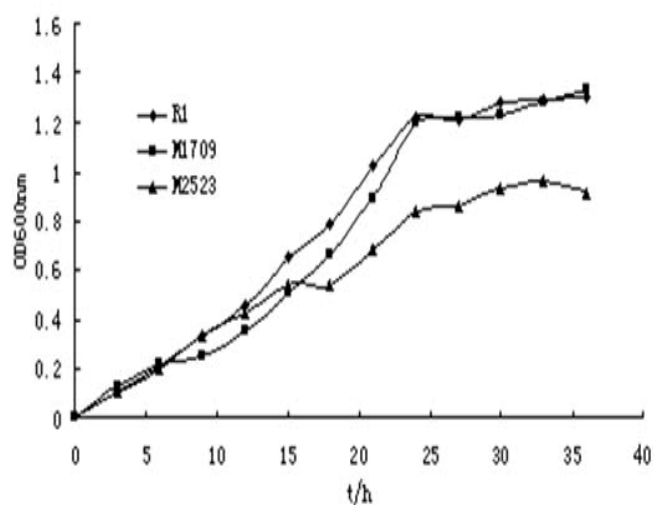

Figure 2. The growth of the strains in liquid DMM with 200 nM Mn.

Figure 3. The growth of strains's curve in DMM with 200 nM Fe.

(Figure 3). Although M2523's OD_{600} values were bigger than those of M1709 between 6 and 14 h, M2523 grew worse than M1709 and the wild type in general. Especially after being cultured for 24 h, the concentration of M2523 at every site was much lower than those of M1709 and wild type. These indicated that M2523 was more sensitive to low concentration of Fe(II) ions than M1709.

Protease secretion assays of the strains

As shown in Figure 4, the halo-shaped clearing area of M2523 was smaller than that of the wild type, after DR2523 was disrupted, *D. radiodurans* secreted less protease to the media. The expression of the protease

might be up-regulated by DR2523. On the contrary, the halo clearing area of M1709 was larger than that of the wild type, demonstrating that more protease was secreted after DR1709 was disrupted. The role of DR1709 was possible to control the protease production or secretion to prevent the production of too much protease.

The strains' sensitivity to high concentration of Mn(II) and Fe(II) ions

The inhibition bacteria circle of M2523's resistance to Mn(II) was larger than that of the wild type, indicating that M2523 was more sensitive to high content Mn(II) ions (Figure 5A). But the circle of M2523's resistance to Fe(II) was similar to that of the wild type (Figure 5A). The inhibition bacteria circle of M1709 to Mn^{2+} and Fe^{2+} was
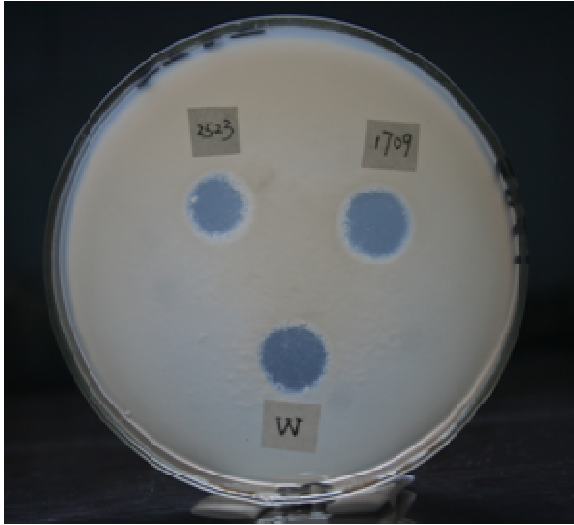
Figure 4. Protease secretion measured of the strains. W = wild type *D. radiodurans*. 1709 referred to M1709. 2523 referred to M2523.

A   B
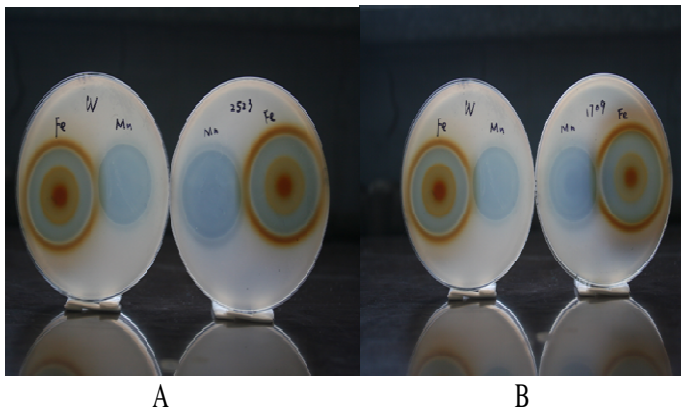
Figure 5. Cation sensitivity of strains. W = wild type *D. radiodurans*; 1709 = M1709; 2523 = M2523.

very similar to those of the wild type respectively, showing that M1709 and the wild type had the similar resistance to high concentration of Mn(II) and Fe(II) (Figure 5B). DR1709 had no relationship with the bacterium's resistance to high concentration of Mn(II) and Fe(II).

Analysis of intracellular Mn and Fe contents by ICP-MS

From Table 1, it was found that strains grown in TGY contained more Mn or Fe than those grown in DMM. The former had higher Mn/Fe ratio than the latter. M2523 contained less Mn and more Fe than the wild type. The Mn in M1709 was about half of the wild type. But for Fe, they were similar. These results suggested that DR1709

was a Mn influx transporter and it cannot transport Fe. It may be specific to Mn.

DISCUSSION

There were three prevailing hyposes now to explain the extraordinary radiationresistance of *D. radiodurans* (i) Chromosome alignment, morphology and/or 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 inresistant bacteria protect proteins from oxidation during irradiation (Daly et al., 2007; 2009). According to the first propose, transmission electron microscopy (TEM) of chromosomal DNA from *D. radiodurans* should reveal evidence of structures linking chromosomes. However, no linking structures have been observed by TEM-based optical mapping (Lin et al., 1999).   12 genes of *D. radiodurans*, which were implicated in resistance by transcriptional profiling following irradiation, were knocked out. The radiation resistance of the novel mutants still remained high (Cox and Battista, 2005; Liu et al., 2003; Harris et al., 2004), demonstrating that the possibility of the second hypose was very little. Daly et al. (2004) found that *D. radiodurans* accumulates higher concentration of Mn(II) ions than radiation-sensitive bacteria. The role of Mn ions in *D. radiodurans* R1 was performed as an antioxidant to scavenge reactive oxygen species (ROS) generated during irradiation. Mn(II) ions were proposed to protect proteins from being oxidized. Therefore, repair systems of *D. radiodurans* survived and functioned with far efficiency than in sensitive bacteria (Daly et al., 2007; 2009). DR1709 and DR2523, two predicted Mn(II) ions transporter genes in *D. radiodurans* was deleted respectively in our previous experiments (Chang et al., 2009). It was found that DR1709 and DR2523 indeed can protect *D. radiodurans* from irradiation and superoxide radicals. *D. radiodurans* transporting Mn(II) from the medium was possibly controlled by several different steps. The roles of DR2523 might be partially substituted by DR2283 and/or DR2284, while no other genes could exercise similar function as DR1709 (Chang et al., 2009). However, the detailed molecular and phosylogical process about Mn(II) movement in this bacterium is still unknown.

In DMM with only 200 nM Mn, M1709 cannot grow at all. In the corresponding solid agar plate, no M1709 clone could be observed even after the plates had been placed in 32°C for 13 days. MntH, a homologue gene of DR1709 in *Bradyrhizobium japonicum* showed similar phenotype in Mn(II)-deficient medium (Hohle and O'Brian, 2009). However, in other bacteria species, mntH mutants show approximately wild type levels of growth in Mn(II)-limited media (Boyer et al., 2002; Domenech et al., 2002; Horsburgh et al., 2002; Kehres et al., 2000; Makui et al.,

2000; Que and Helmann, 2000). These demonstrated that in *D. radiodurans*, when Mn(II) ions was limited, DR1709 was the only gene responsible for Mn(II) 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(II) ions uptake when there were ample Mn(II) ions in the medium. Even if Mn(II) concentration was high or low, DR1709 was in charge of Mn(II) ions uptake. Consistent with this conclusion, we found that even if the strains were cultured in liquid TGY or DMM, the intracelluar Mn of M1709 was much lower than those in the wild type (Table 1). In liquid TGY, the growth curve of M2523 was similar to that of the wild type. But in DMM with 200 nM Mn(II) ions, its growth was much lower than the wild type. After the gene sitA, one homologous gene of DR2523 in *B. japonicum*, was disrupted, the mutant has a growth deficiency in Mn(II)-depleted media (Hohle and O'Brian, 2009). It is not yet clear why *D. radiodurans* contains at least two different Mn(II) uptake systems. In yeast, metal ion uptake is typically mediated by two or more specific transport systems often differing in affinity for the transported metalion (Eide., 1998). DR1709 and DR2523 may correspond to high- and low-affinity systems for uptake.

Iron is an essential trace-element for most organisms (Semsey et al., 2006). It is a highly versatile prosthetic component present in many key enzymes of major biological processes (Andrews et al., 2003). Many homologues genes of DR1709 and DR2523 in other bacteria species can take up Mn(II) and Fe(II) at the same time (Patzer and Hantke, 2001; Kehres et al., 2000; Makui et al., 2000; Bearden and Perry, 1999; Boyer et al., 2002). Radiation resistant bacteria species always had high Mn/Fe ratio (Daly et al., 2004). M2523 was more sensitive to low concentration of Fe(II) ions than M1709 and wild type, indicating that DR2523 can transport Fe(II). But M1709's sensitivity was very similar with that of the wild type. Fe(II) might not be a substrate for DR1709. Consistent with this thought, we found that the intracellular Fe content of M1709 was very near to the wild type, no matter the strains grown in liquid TGY or DMM. Mn(II) and Fe(II) can be toxic at high concentrations (Kehres and Maguire, 2003; Moore and Helmann, 2005; Papp-Wallace and Maguire, 2006; Que and Helmann, 2000). M2523 was more sensitive to high concentration of Mn(II) ions, showing that DR2523 was responsible for Mn(II) ions efflux when Mn(II) content was higher than the bacterium need. The inhibition bacteria circle of M1709 to Mn^{2+} and Fe^{2+} was very similar to those of the wild type, respectively. DR1709 had no relationship with the bacterium's resistance to high concentration of Mn(II) and Fe(II).

Although currently there is no evidence to support that proteases might facilitate recovery from ionizing radiation, at least 10 complete open reading frames with considerable sequence similarity to secreted subtilisin-like proteases of *B. subtilis* have been identified in *D.*

radiodurans (Makarova et al., 2001), which might relieve its amino acid auxotrophies (Ghosal et al., 2005). The halo-shaped clearing area of M2523 on indicator plates containing skimmed milk was smaller than that of the wild type, indicating that, after DR2523 was disrupted. *D. radiodurans* secreted less protease to the media than the wild type. The expression of the protease might be up-regulated by DR2523. On the contrary, the halo clearing area of M1709 was larger than that of the wild type, suggesting that more protease was secreted after DR1709 was disrupted. The role of DR1709 was possible to control the protease production or secretion. What interested us was that DR1708, just next to DR1709, was predicated to be a secreted protein. DR1709 can control the expression of DR1708. After DR1709 was disrupted, more DR1708 was produced and secreted than necessary. If proteases facilitate recovery from ionizing radiation, the recovery of M1709 should be faster than the wild type. But this experiment result was the contrary (Chang et al., 2009). Therefore, the role of protease secreted by the strains was limited in radiation resistance, if it really had any function.

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