微生物学报 *Acta Microbiologica Sinica*
49(4):438 – 444;4 April 2009
ISSN 0001 – 6209;CN 11 – 1995/Q
http://journals.im.ac.cn/actamicrocn

Disruption of manganese ions [Mn(Ⅱ)] transporter genes *DR1709* or *DR2523* in extremely radio-resistant bacterium *Deinococcus radiodurans*

Shenghe Chang[1], Haiyan Shu[2], Zongwei Li[1], Yanping Wang[1], Linhai Chen[1], Yuejin Hua[3], Guangyong Qin[1]*
([1] Ion Beam Bio-engineering Lab, School of Physics, Zhengzhou University, Zhengzhou 450052, China)
([2] Department of Biology, Zhengzhou University, Zhengzhou 450001, China)
([3] Institute of Nuclear-Agricultural Science, Zhejiang University, Hangzhou 310029, China)

Abstract: [Objective] The primary objective of this study was to identify whether the manganese ions [Mn(Ⅱ)] transporter genes *DR1709* and *DR2523* played roles in *Deinococcus radiodurans's* radiation resistance. The second objective was to study the relationships among manganese ions, manganese ions transporter genes and the bacterial radioresistance. [Methods] We constructed mutants of *DR1709* and *DR2523*. The wild type and the mutants were treated with UV and hydrogen peroxide (H_2O_2). The survival fractions of the three strains were analyzed. [Results] Disruption of *DR2523* hardly affected the growth of *D. radiodurans* in tryptone-glucose-yeast extract (TGY) broth. But at each site of the logarithmic stages, the OD_{600} values of *DR1709* mutant (M1709) were much lower than those of the wild type. After being treated with H_2O_2 and UV, the survival rates of M1709 cells at each dose were much lower than those of the wild type. However, the *DR2523* mutant (M2523) and wild type had the similar appearance after being treated with H_2O_2 and UV, though the wild type had the higher survival than M2523. [Conclusion] *DR1709* and *DR2523* could protect *D. radiodurans* from irradiation and superoxide radicals. In *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 the similar function as *DR1709*.

Keywords: *Deinococcus radiodurans*; Mn(Ⅱ) transporter; gene disruption; radioresistance
CLC number: Q933 **Document code**: A **Article ID**: 0001-6209(2009)04-0438-07

Deinococcus radiodurans is a non-spore-forming bacterium notable for its capacity to tolerate exposure to ionizing radiation[1,2]. Exploring the mechanisms of *D. radiodurans's* radiation resistance had potentially practical purposes in cleaning up and stabilization of radioactive waste sites[3-5].

D. radiodurans's radiation resistance had been attributed to a highly proficient DNA repair capacity[6]. However, the data from cryoelectron microscopy showed that the DNA fragments in *D. radiodurans* were mobile and that the arrangement of its nucleoids did not play the key role in radioresistance[7-8]. The degree of radiation resistance was determined by the level of oxidative protein damage caused during irradiation and Mn(Ⅱ) ions can protect proteins from less oxidative damage in *D. radiodurans*[9]. But mechanism of Mn(Ⅱ) ions on irradiation-tolerance remained still unclear.

DR1709 (Nramp family) and *DR2523* (ATP-dependent ABC-type transporter) were two predicted Mn(Ⅱ) transporter genes in *D. radiodurans*. Study the functions of DR1709 and DR2523 was helpful to reveal the mechanisms about Mn(Ⅱ) ions' protection the proteins. The aim of this paper was to test whether *DR1709* and *DR2523* could provide a protection for the bacterium against irradiation and superoxide radicals. We found that the bacterium was affected little after DR2523

Supported by the Key Project of China National Programs for Fundamental Research and Development (2004CB719604)
* Corresponding author. Tel/Fax: +86-371-67761947; E-mail: shchang@zzu.edu.cn
Received: 9 October 2008/ Revised: 13 December 2008

was disrupted. But the bacterium's tolerance to UV and H_2O_2 was reduced heavily when *DR1709* was mutated. The OD_{600} values of M1709 were also much lower than those of the wild type at each site of the logathmic stages. *DR1709* and *DR2523* could protect *D. radiodurans* from irradiation and superoxide radicals. The functions of *DR2523* might be replaced partially by *DR2283* and/or *DR2284*. But the possibility that other genes played the similar roles as *DR1709* was very little.

1 MATERIALS AND METHODS

1.1 Strains and growing condition

The bacterial strains and plasmids used were listed in table 1. All genes were identified as described in the published genome sequence (http://www.tigr.org/tigr-scripts/CMR2/ genomePage3.spl?database=gDR). 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℃. Plasmids were routinely propagated in *E. coli* strain JM109.

Table 1 The genotypes of the strains and plasmids used in this paper

Strains or plasmids	Description	Source or reference
D. radiodurans R1	ATCC13939	[10]
M1709	As R1 but △*dr1709*	This study
M2523	As R1 but △*dr2523*	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]	Dalian, China
pMD19-T Vector	pUC19 derivative with an *Eco*R Ⅴ site, Amp^r	TaKaRa Dalian, China
pTK1709	pT1709 derivative with a Kan insert	This study
pTK2523	pT2523 derivative with a Kan insert	This study
pT1709	pMD19-T Vector derivative carrying *DR1709* gene (chromosome I positions 1733183-1734493), treated with *Aor*51 HI and dephosphorylated	This study
pRADK	pRADZ3 derivative with a Kan insert, Cat^r Kan^r Amp^r	[11]

1.2 Growing curves

Growing curves were measured according to the published methods[12]. The cell density was determined at 600 nm using a Beckman spectrophotometer, with approximately 2×10^8 CFU/mL present at 1 OD. *D. radiodurans* and the mutants were grown overnight and frozen at −80℃ in TGY supplemented with glycerol to a concentration of 15% (V/V). Before growth in liquid TGY, the frozen strains were immediately transferred to TGY plates. All cultures on TGY plates were checked for contamination by streaking for single colonies. For liquid growth, cultures were shaken at 180 r/min at 30℃, with the culture volume kept at 10% ~ 15% of the flask volume. Cultures were first grown in a culture tube until $OD_{600} = 0.5$, and then 50 μL cultures were transferred to a flask containing 200 mL fresh TGY broth, and reinoculated. The OD_{600}s of the cultures were measured each 1 h interval. All data were measured for triplicates and the average values were used. The curves were drawn using Microsoft Excel.

1.3 UV irradiation and cell survival

Measurement of UV irradiation and cell survival rates was performed as described before[13]. Cells were grown in TGY broth at 30℃ until $OD_{600} = 0.5$, and then 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 74) in triplicate and spread onto TGY plates. After the medium was absorbed, the plates were opened and exposed to UV light from a LP UV lamp (15 W; Philip) at a rate of 0.295 $J \cdot s^{-1} \cdot m^{-2}$ (Laser power meter, Gentec, Canada, Model-PSV-3303). The plates were incubated at 30 ℃, and colonies were counted after 2 to 3 days.

1.4 H_2O_2 treatment and cell survival rates

H_2O_2 treatment of strains was prepared according to Huang et al[14]. The cells were grown in TGY broth at 30℃ until $OD_{600} = 0.5$, and then various volumes of H_2O_2 were added to the growing cultures. After the mixtures were vortexed briefly and put in darkness for 1 h at 30℃, katalase (Sigma) was added to stop the

reaction. Samples were withdrawn for immediate dilution and plated on TGY agar plates. Survival rates were calculated as a percentage of the number of colonies obtained to untreated cells.

1.5 Disruption of *DR1709* in *D. radiodurans*

The frame diagram of *DR1709* mutant construction was shown in Figure 1. The gene *DR1709* of *D. radiodurans* was amplified by PCR (94℃, 1 min; 52℃, 50 s; 72℃, 3 min. 40 cycles) using the primers P1 (5′-GTGGCGGACTGGTAGATG-3′) and P2 (5′-CGGCGAGGACTTCAGC-3′). The amplification product was ligated into the pMD19-T cloning vector (TaKaRa) and transformed into *E. coli* JM109 (TaKaRa) following the standard procedure[15]. After being extracted from the transformed *E. coli*, the plasmid was digested with *Aor*51 HI and dephosphorylated. The dephosphorylated plasmid was named pT1709. The kanamycin resistant gene *Kan* with the promoter *GroEL* was amplified from pRADK[11] by PCR (94℃, 1 min; 55℃, 50 s; 72℃, 1.5 min. 40 cycles) using the primers P3 (5′-ACAGACAGCGCTTAGAAAAACTCATCGAGCATCAAATG-3′) and P4 (5′-TTCTAGAGCGCTCGCCAAGCTCGCGAGGCC-3′) (*Aor*51 HI digestion sites were underlined). The PCR product was digested with Aor51 HI and ligated into pT1709. The new plasmid was named pTK1709-1 and also transformed into *E. coli* JM109. After being extracted from the transformed *E. coli* cultures and digested with *Aor*51 HI, those plasmids containing the 1.2-kb band were selected and sequenced. The plasmid in which the transcriptional direction of the kan gene was opposite to that of *DR1709* was selected and named pTK1709.

After *D. radiodurans* R1 was transformed with pTK1709 as described above[16], chromosomal DNA was isolated from the wild type and the possible mutants. PCR was performed using the primers P1 and P2 (94℃, 1 min; 52℃, 50 s; 72℃, 3 min. 40 cycles). The PCR products were digested with Aor51 HI and electrophoresed. If the mutant had been constructed successfully, three bands (800 bp, 1205 bp and 1250 bp) would be observed. Since it was difficult to distinguish 1205 bp and 1250 bp bands in 1% agarose gel, two bands would be seen in the gel. However, the predicted digestion result of the wild type contained three bands (800 bp, 1205 bp and 300 bp).

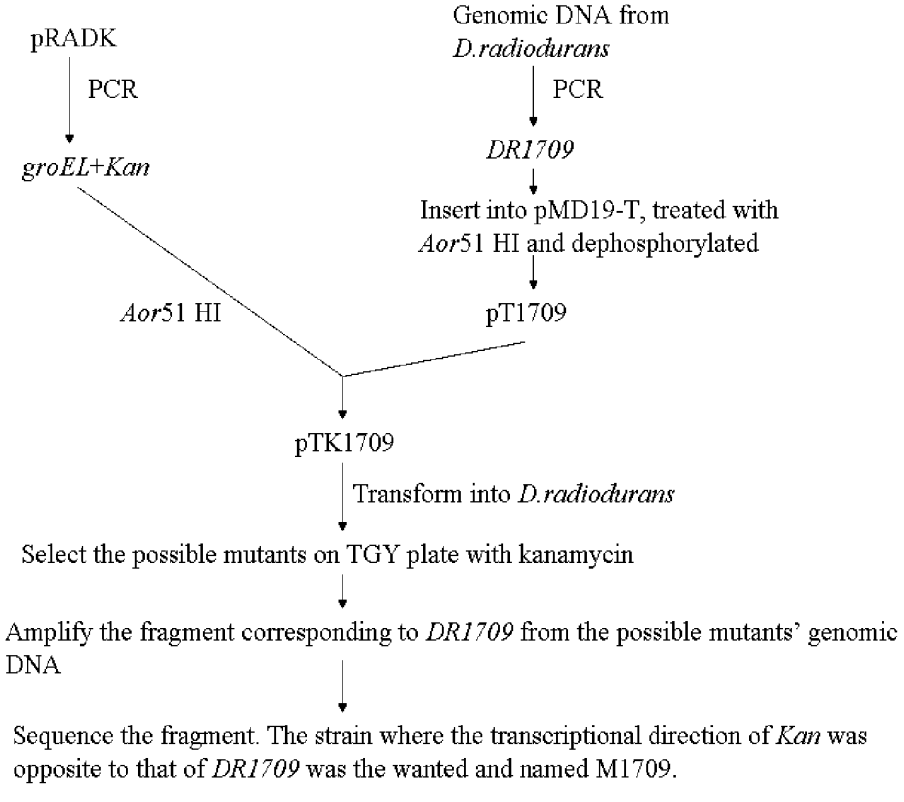

Fig. 1 The frame diagram of M1709 construction.

1.6 Disruption of *DR2523* in *D. radiodurans*

The total procedure of DR2523 mutant construction was similar with M1709 (Fig. 1). A PCR fragment the DR2523 gene of *D. radiodurans* R1 was amplified from the purified chromosomal DNA (94℃, 1 min; 58℃, 50 sec; 72℃, 2 min; 40 cycles). Primers P5 (5′-GCAACAGGCACTGGAACCG-3′) and P6 (5′-CCACC CTTGAAGACCTTGAGC-3′) were designed for amplification. The PCR product was ligated into pMD19-T vector and the new plasmid was named with pT2523. After being transformed into *E. coli* JM109, the pT2523 plasmids were extracted, digested with *Apa* Ⅰ and electrophoresed. If a 1.5-kb band was observed in the agarose gel, the plasmid was afterward digested with *Aor*51 HI and dephosphorylated. The *Kan* gene with the promoter *GroEL* was amplified from pRADK[11] by PCR (94℃, 1 min; 55℃, 50 s; 72℃, 1.5 min. 40 cycles). The primers P7 (5′-ACAGACAGCGCTTAGA AAAACTCATCGAGCATCAAATG-3′) and P8 (5′-TTCTAGAGCGCTCTGC-AGACGCGTCATCTG-3′) were designed and the *Aor*51 HI digest sites were also underlined. The amplified product was digested with *Aor*51 HI, ligated into the dephosphorylated pT2523 transformed into *E. coli* JM109, and spread on the LB plates containing 30 μg kanamycin per ml. The plasmids were extracted from the *E. coli* colonies growing on the LB plates, digested with *Aor*51 HI and electrophoresed. If a 1.2-kb band was seen in the 1% agarose gel, the corresponding colonies were cultured and sequenced. The plasmid where the transcriptional direction of *DR2523* was opposite to that of kan gene was named with pTK2523.

D. radiodurans R1 was transformed into pTK2523. After the chromosomal DNA was isolated from the wild type and the possible mutants, the PCR fragments were amplified using the primers P5 and P6 (94℃, 1 min; 58℃, 50 s; 72℃, 2 min; 40 cycles). The PCR products were digested with Aor51 HI and electrophoresed. If DR2523 had been disrupted successfully, three bands (1442 bp, 1104 bp, 872 bp) would be seen in 1% agarose gel, but two bands (1448 bp and 872 bp) would be observed in the wild type.

2 RESULTS

2.1 Construction of M1709 and M2523

There were two *Aor*51 HI sites in the genome sequence of *DR1709*. The fragment corresponding to DR1709 was amplified from the possible mutants and digested with *Aor*51 HI. After being electrophoresed in 1% agarose gel, two bands were seen, suggesting that the mutant had been constructed successfully (Fig. 2B). The length of PCR fragment from the mutant was larger than that in the wild type, which also illustrated the inference (Fig. 2B). The fragment corresponding to *DR1709* in the possible mutant was further amplified and sequenced. We found that the transcriptional direction of the *Kan* gene was opposite to that of *DR1709* (data not shown). These results showed that the gene *DR1709* had been disrupted successfully. The *DR1709*-disrupted strain was named M1709 (Table 1).

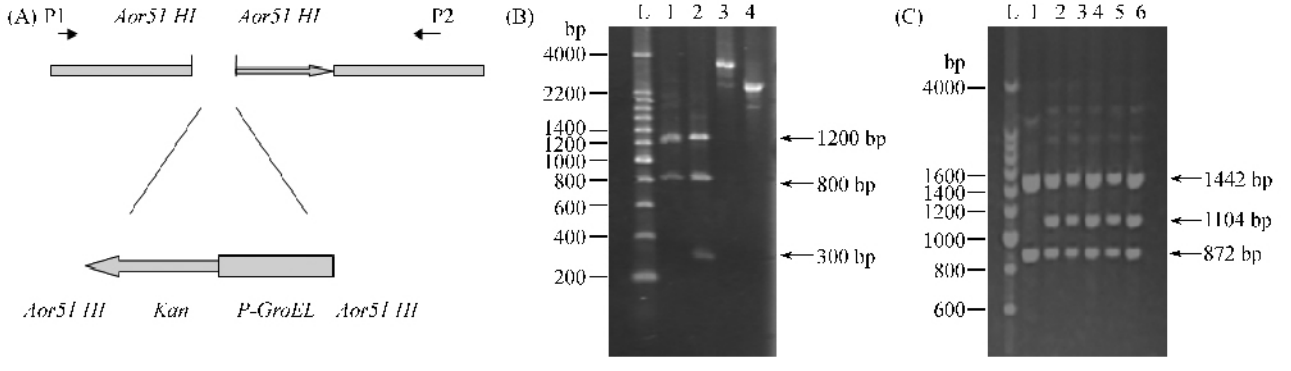

Fig. 2 The construction of M1709 and M2523. A was the blueprint of mutants construction. B was the confirmation of *DR1709* disruption by PCR and digestion. The enzyme was *Aor*51 HI. Lanes L, DNA ladder; 1, the digestion result of *D. radiodurans* R1; 2, the digestion results from the mutant M1709; 3, PCR product from M1709; 4, PCR product from the wild type. C referred to the confirmation of DR2523 disruption by PCR and enzyme digestion. The enzyme was *Aor*51 HI. Lanes L, DNA ladder; 1, the digestion result of the wild type; 2-6, the digestion results from the mutant M2523.

After being transformed with the disrupted plasmid pTK2523, the fragment corresponding to DR2523 was amplified from the DNA of the possible mutants and the wild type, and then digested with *Aor*51 HI. If the *DR2523* gene was inserted as expected, three bands (1442 bp, 1104 bp and 872 bp) would be found in the agarose gel, otherwise two bands (1448 bp and 872 bp) would be observed (Fig. 2C). The fragment corresponding to *DR2523* from the possible mutant was sequenced and found that the transcriptional direction of the kan gene was opposite to that of *DR2523* (data not shown), which showed that the gene *DR2523* had been disrupted successfully. After *DR2523* was disrupted, the strain was named M2523 (Table 1).

2.2 The growth in TGY broth

As shown in Fig 3, the growth curves of the wild type and the two mutants were all S types, which all had the logarithmic stages. The curve of M2523 was consistent with that of the wild type. But the OD_{600} value of the wild type was higher than that of M2523 at each site of logarithmic stage. These indicated that after *DR2523* was disrupted, the growth of *D. radiodurans* in TGY broth was affected little. However, the OD_{600} values of M1709 at each site in logathmic stage were much lower than those of the wild type. M1709 entered logarithmic stage 4 hours later than the wild type, showing that the growth of *D. radiodurans* was badly inhibited in logathmic stage after *DR1709* was mutated.

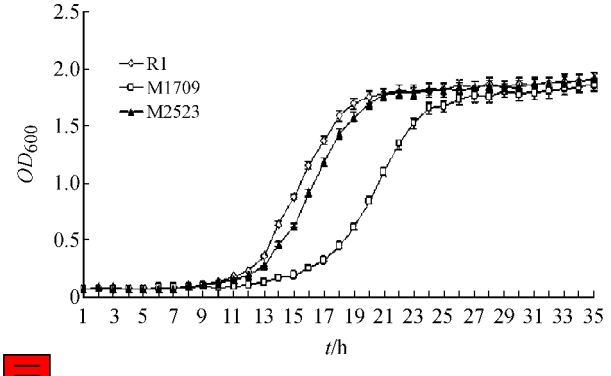

Fig. 3 Growth curves of the three strains in liquid TGY.

2.3 M1709 was more sensitive to UV radiation than wild type and M2523

After being spread on TGY plates, the strains were treated with UV radiation. Within 200 J/m^2, the three strains had the similar survival fraction. When treated with higher dose, however, the survival rate of M1709 was much lower than those of the wild type and M2523. After treated with 600 J/m^2, almost no survival M1709 cells can be found on TGY plates, while 89% of the wild type and 72% of M2523 cells were still alive (Fig. 4). M2523 had the corresponding survival fraction with the wild type, although the survival fraction of wild type was higher than that of M2523 (Fig. 4). M1709 was far more sensitive to UV radiation than the wild type and M2523.

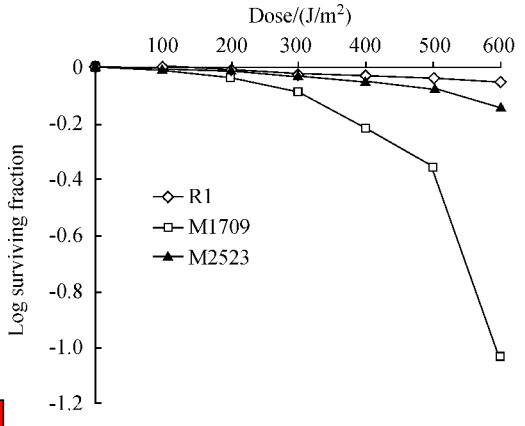

Fig. 4 The survival fraction of the three strains after being treated with UV.

2.4 The survival rates of the three strains after being treated with hydrogen peroxide (H_2O_2)

After treated with H_2O_2, the survival lines of the wild type and M2523 were almost the same (Fig. 5). But the survival rate of M1709 was lower than that of the wild type at each site, especially at high doses (25 mmol/L and 30 mmol/L) (Fig. 5). M2523 and the wild type had the similar resistance to H_2O_2. But M1709 was much more sensitive to H_2O_2 than the wild type and M2523.

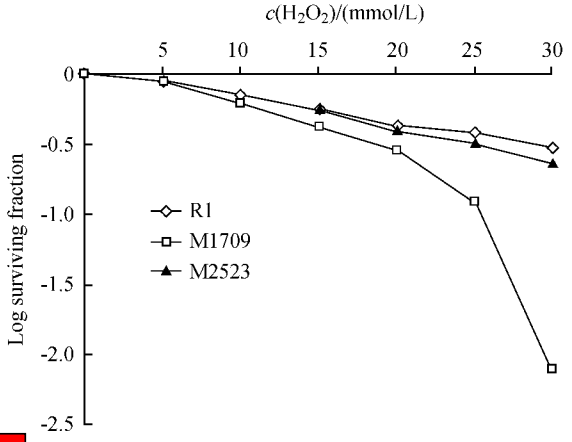

Fig. 5 The survival fractions of the three strains after being treated with H_2O_2.

3 DISCUSSION

D. radiodurans had an exceptional ability to withstand the lethal effects of ionizing radiation[17]. Although several explanations on the extreme resistance had been reported, the underlying molecular mechanism was poorly understood[3,18]. Rescently, it was found that Mn(Ⅱ) ions could alleviate the damage caused by ionizing radiation in *D. radiodurans*[9]. But how Mn(Ⅱ) ions protect proteins remained unclear.

Mn homeostasis in bacteria depends upon regulation of Mn(Ⅱ) transport, which includes import and efflux. The coordinated expression and activities of the transporters are necessary for the cells to be able to respond to fluctuations in external Mn(Ⅱ)[19]. There were two types of Mn(Ⅱ) transporters in *D. radiodurans* (Nramp family and ATP-dependent ABC-type transporter). DR1709 belonged to the Nramp family and DR2523 was one of ATP-dependent ABC-type transporters[20]. Therefore, in D. radiodurans, utilizing Mn(Ⅱ) ions from the media was possibly controlled by several different steps. In the genome of *D. radiodurans*, only one predicted Nramp family gene (*DR1709*) was found[20]. In liquid TGY, M1709 grew much slower than the wild type in logarithmic stage. It showed that after *DR1709* was disrupted, the growth of *D. radiodurans* was inhibited heavily, suggesting that no other gene could exercise the similar function as *DR1709* in *D. radiodurans*. However, M2523 showed the alike appearance with the wild type, although it grew slower than the wild type. There were three possible ATP-dependent ABC-type transporter genes (*DR2283-DR2284*, *DR2523*) in *D. Radiodurans*[20]. When DR2523 was disrupted, its partial functions could be recovered by *DR2283* and/or *DR2284*.

After being treated with H_2O_2, the M1709 cells had a much less survival rate than wild type. In M1709 cells, the uptake of Mn(Ⅱ) ions from the medium was blocked. The concentration of Mn(Ⅱ) ions in them might be low. When treated with H_2O_2, the cells had less Mn(Ⅱ) ions to react with superoxide radicals[9]. Many cells were killed by the superoxide radicals, resulting that M1709 grew worse than wild type. After *DR2523* was disrupted, the partial functions of *DR2523* might be exercised by *DR2283* and/or *DR2284*. Therefore, M2523 had the similar survival fraction with the wild type. The survivals of the three strains under UV treatment were also measured and the similar results were found, which also approved the inference.

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耐辐射球菌基因 DR1709 与 DR2523 的突变分析

常胜合[1], 舒海燕[2], 李宗伟[1], 王雁萍[1], 陈林海[1], 华跃进[3], 秦广雍[1]*

(郑州大学, [1]物理工程学院离子束生物工程实验室, [2]生物工程系, 郑州 450052)

([3]浙江大学原子核农业科学研究所, 杭州 310029)

摘要: 【目的】检测在耐辐射球菌抵抗外来辐射和氧自由基的过程中, 锰离子转运蛋白基因 (*DR1709* 和 *DR2523*) 是否发挥了作用。探讨锰离子、锰离子转运蛋白基因与耐辐射球菌辐射抗性之间的关系。【方法】分别构建这两个基因的突变体。对突变体和野生型进行紫外线照射和过氧化氢处理。对处理后的菌株存活率进行分析。【结果】*DR2523* 被突变以后, 耐辐射球菌在 tryptone-glucose-yeast extract (TGY) 培养液中的生长受影响很小。而 *DR1709* 突变体 M1709 在对数生长阶段的生长速度远低于野生型。用紫外线和过氧化氢处理以后, 尽管野生型的存活率高于 *DR2523* 突变体 *M2523*, 不过二者的差别并不大。但是 M1709 在紫外线和过氧化氢处理后的存活率远低于野生型。【结论】在耐辐射球菌抵御辐射和氧自由基损伤的过程中, *DR2523* 和 *DR1709* 都在不同程度上发挥了作用。耐辐射球菌从培养基吸收锰离子分多步骤进行。在 *DR2523* 被突变以后, 其部分功能可能被 *DR2283* 和/或 *DR2284* 所取代。

关键词: 耐辐射球菌; 锰离子转运蛋白; 基因突变; 辐射抗性

中图分类号: Q933 **文献标识码**: A **文章编号**: 0001-6209 (2009) 04-0444-07

(本文责编: 王晋芳)

基金项目: 国家"973 项目"(2004CB719604)

* 通信作者。Tel/Fax: +86-371-67761947; E-mail: shchang@zzu.edu.cn

作者简介: 常胜合 (1974 –), 男, 河南唐河人, 副教授, 主要从事微生物遗传学研究。E-mail: shchang@zzu.edu.cn

收稿日期: 2008-10-09; 修回日期: 2008-12-13