A Mur Regulator Protein in the Extremophilic Bacterium Deinococcus radiodurans

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Abstract

Ferric uptake regulator (Fur) is a transcriptional regulator that controls the expression of genes involved in the uptake of iron and manganese, as well as vital nutrients, and is essential for intracellular redox cycling. We identified a unique Fur homolog (DR0865) from Deinococcus radiodurans, which is known for its extreme resistance to radiation and oxidants. A dr0865 mutant (Mt-0865) showed a higher sensitivity to manganese stress, hydrogen peroxide, gamma irradiation and ultraviolet (UV) irradiation than the wild-type R1 strain. Cellular manganese (Mn) ion (Mn2+) analysis showed that Mn2+, copper (Cu2+), and ferric (Fe3+) ions accumulated significantly in the mutant, which suggests that the dr0865 gene is not only involved in the regulation of Mn2+ homeostasis, but also affects the uptake of other ions. In addition, transcriptome profiles under MnCl2 stress showed that the expression of many genes involved in Mn metabolism was significantly different in the wild-type R1 and DR0865 mutant (Mt-0865). Furthermore, we found that the dr0865 gene serves as a positive regulator of the manganese efflux pump gene mntE (dr1236), and as a negative regulator of Mn ABC transporter genes, such as dr2283, dr2284 and dr2523. Therefore, it plays an important role in maintaining the homoeostasis of intracellular Mn (II), and also other Mn2+ zinc (Zn2+) and Cu2+ ions. Based on its role in manganese homeostasis, DR0865 likely belongs to the Mur sub-family of Fur homolog.

Introduction

Metal ions, such as manganese (Mn2+) and iron (Fe2+), are essential micronutrients for many microorganisms and act as enzyme cofactors for a wide range of proteins in processes such as DNA synthesis, DNA repair, reactive oxygen species (ROS) scavenging and electron transport [1]. However, when in excess, they are toxic to cells. Excess iron induces the over-production of harmful ROS, such as super-oxide anion radicals (O2−) and hydrogen peroxide (H2O2) [1]. High levels of ROS may target DNA, RNA, proteins and lipids through the hydroxyl radicals (HO•) that are generated from H2O2 in the Fenton reaction, which uses divalent ions [2]. Inhibition of RNA and protein synthesis occur when high intracellular levels of manganese are reached [3]. Therefore, microorganisms have evolved efficient mechanisms to maintain metal ion homeostasis [4].

The uptake of metal ions is controlled by the ferric uptake regulator (Fur) or the Diphtheria toxin repressor (DtxR) family of proteins [5]. The Fur superfamily comprises different proteins with distinct regulatory roles [6]. Fur and Zur (zinc uptake regulator) [7], which respond to iron (Fe2+) or zinc (Zn2+), respectively, repress the expression of genes involved in Fe2+ or Zn2+ uptake. The PerR protein, which has been found in Gram-positive bacteria such as Bacillus and Staphylococcus, regulates several genes that are involved in the oxidative stress response [8,9]. Another Fur homolog, named Irr, which can repress the heme biosynthesis pathway, was first found in Bradyrhizobium japonicum [10,11]. In 2004, Johnston’s group identified a new fur-like protein named Mur (manganese uptake regulator) in Rhizobium leguminosarum. This protein represses the transcription of the sitABCD genes in response to Mn2+ [12].

Deinococcus radiodurans is a well-known bacterium that has extraordinary resistance to ionizing radiation (IR), ultraviolet radiation (UV), various DNA-damaging agents, oxidative stress and desiccation [13]. Ionizing radiation can directly damage biomacromolecules and also produces ROS, which can attack both proteins and DNA [14]. Recently, it has been shown that D. radiodurans has a special Mn/Fe regulatory system, which
accumulates exceptionally high levels of intracellular Mn$^{2+}$ and low levels of Fe$^{2+}$. Mn$^{2+}$ may act as an antioxidant to strengthen or support the antioxidant enzyme system, which protects the bacteria from oxidative stress [15,16]. It was shown that there are three types of Mn$^{2+}$-dependent transport genes in D. radiodurans: dr1236 (Mn$^{2+}$ efflux genes) [17], dr1709 (Nramp family transporters) and three ATP-dependent transporters (dr2283, dr2284 and dr2525). The genes that are involved in Fe$^{2+}$-dependent transport encode an ABC-type hemin transporter (drb0016), an ABC-type Fe(III)-siderophore transporter (drb0017), two Fe(II) transporters (dr1219, dr1220) and two DNA protection proteins [Dps] (dr2263, drb0092) [1]. Furthermore, D. radiodurans also has three oxidation-related regulators: OxyR (DR0615), DtxR (DR2539), and a Fur homolog (DR0865) [1]. The DR0615 protein is both a transcriptional activator of the katE and drb0125 genes and a transcriptional repressor of the dps and mntH genes [10]. The DR2539 protein acts as a negative regulator of a Mn$^{2+}$ transporter gene (dr2283) and as a positive regulator of Fe$^{2+}$-dependent transporter genes (dr1219 and drb0125) [19]. However, the function of the Fur homolog (DR0865) is still unknown.

In this study, we aimed to elucidate the function of the Fur homolog (DR0865) and demonstrate its role in maintaining the homoeostasis of intracellular Mn. The results showed that DR0865 is not only a Mur protein, but is also vital for the homoeostasis of intracellular Mn$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$ ions.

Results

D. radiodurans gene encodes a putative Fur family protein

There is a potential fur homolog (dr0865), which encodes a protein that contains 132 amino acids, in D. radiodurans genome. A BLASTP analysis showed that DR0865 exhibits 24% identity to Helicobacter pylori Fur (Hpy-Fur) and 26% identity to the E. coli Fur protein. Further comparison with the Hpy-Fur sequence showed that DR0865 has three similar metal-binding domains. Domain I consists of amino acid residues C82, C85, C121 and C124, domain II comprises the residues E70, H77 and H79 and domain III is formed from residues H76, H92, T97, H113 and H78 (Figure S1 in File S1). The predicted structure of DR0865 is based on the crystal structure of Hpy-Fur (Figure 1). Previous data showed that ZnS$_4$ binding by domain I stabilizes β3-β4-β5 structures. Domain II is a metal sensing site, which can regulate DNA binding ability in response to changes in metal concentrations. Domain III is not necessary for DNA binding, however, mutation of this domain reduces the DNA binding ability [20].

The absence of dr0865 inhibits cell growth

To confirm the specific roles of DR0865 in D. radiodurans, the null mutant of dr0865 (Mt-0865) and the complemented strain (C-0865) were constructed. The coding region of the dr0865 gene was replaced with a kanamycin resistance cassette under a constitutively expressed D. radiodurans groEL promoter (Figure S2 in File S1). As shown in Figure 2, the cell growth of Mt-0865 was approximately two-fold lower than that of the wild type strain at 30°C, whereas the growth rate of the complemented strain C-0865 was similar to that of the wild-type strain. This result indicates that the dr0865 gene is necessary for cell growth and other metabolic activities.

Loss of dr0865 causes Mn (II) ion sensitivity in D. radiodurans

To test whether the growth inhibition was caused by a disruption of ion homeostasis, a metal ion sensitivity assay was carried out as described previously [17]. As shown in Figure 3 and Figure S3 in File S1, the growth of Mt-0865 was strongly inhibited by Mn$^{2+}$ but not by the presence of other metal ions. The C-0865 showed the same growth phenotype as the wild-type R1 strain, which indicates that mutation of the dr0865 gene disrupts Mn$^{2+}$ homeostasis.

To further confirm the Mn sensitivity of Mt-0865, we measured the effect of various concentrations of Mn$^{2+}$ on the growth of Mt-0865 (Figure 4). In comparison with the wild-type R1 strain, the
growth of Mt-0865 was inhibited in the presence of low concentrations of Mn$^{2+}$ in TGY medium. When the Mn$^{2+}$ concentration was increased, the growth defect phenotype became more pronounced. An analogous was observed in previous studies, in which the growth of a *Streptococcus pneumoniae* mutant with a disrupted calcium efflux system was more severely inhibited at higher calcium concentrations [21]. Therefore, we inferred that the Mt-0865 strain may either have a higher rate of Mn$^{2+}$ uptake or is unable to efficiently remove excess Mn$^{2+}$.

The effect of H$_2$O$_2$, UV and gamma irradiation on the survival of Mt-0865

The response of the Fur regulator to oxidative stress is very complicated in some microorganisms [5]. Because the Mt-0865 strain exhibits a growth defect and is sensitive to Mn stress, we further investigated the sensitivity of Mt-0865 to H$_2$O$_2$, UV and gamma irradiation. First, the survival of these strains was measured under oxidative stress. The results showed an increased sensitivity of Mt-0865 to H$_2$O$_2$, whereas the C-0865 strain exhibited a similar survival rate as the wild-type R1 strain (Figure 5 and Figure S4 in File S1). Furthermore, the survival rate was measured under UV and gamma irradiation. The D$_{10}$ value, which represents the irradiating dose required to reduce the population by 90%, was used to assess the resistance of the wild-type R1 and Mt-0865 strains to gamma and UV irradiation. As shown in Figure 6A and B, the wild-type R1 strain showed higher resistance to gamma irradiation and UV radiation than the Mt-0865 strain. Similarly, the mutant strains show higher sensitivity to hydrogen peroxide, as shown in the Figure 6C.

Loss of *dr0865* results in an accumulation of intracellular Mn

It has been previously reported that a high intracellular Mn$^{2+}$/Fe$^{2+}$ ratio in *D. radiodurans* helps to protect proteins from oxidative damage, and contributes to its extreme resistance [15,22]. Because the Mt-0865 is sensitive to Mn stress, H$_2$O$_2$ stress, as well as UV and gamma irradiation, inductively coupled plasma mass spectrometry (ICP-MS) analyses were performed to show whether the Mt-0865 strain had lost its ability to maintain homeostasis of manganese and other ions.

As expected, even on TGY medium, the Mn (II) level in the Mt-0865 strain was almost three-fold higher than in the wild-type R1 strain (Figure 7A). Similar results were obtained when the two strains were grown on TGY medium supplemented with Mn$^{2+}$. Furthermore, we also found that the Mn$^{2+}$ level was increased when the wild-type R1 strain was grown on manganese-rich TGY medium, compared to normal medium (Figure 7A). In contrast, there was no significant difference in Fe$^{2+}$ concentrations between the Mt-0865 and wild-type R1 strains. However, the Fe$^{2+}$
concentrations in both wild type and mutant strains increased under Mn stress, indicating that *D. radiodurans* has a system to regulate Mn/Fe homeostasis (Figure 7B). Collectively, these results verified that the Mt-0865 strain is sensitive to Mn2+ stress and that the sensitivity of the mutant to damaging agents may be caused by excess manganese.

Transcriptome changes in the *dr0865* mutant under manganese stress

Because DR0865 is homologous to transcriptional regulators and its disruption resulted in sensitivity to excess manganese, RNA sequencing (RNA-seq) was used to assess changes in transcripts of the wild-type R1 and Mt-0865 mutant strains when cultured in the presence of high (20 mM) levels of MnCl2. In total, 12.7 million (M) and 13.3 M pair-end reads were obtained for the wild-type R1 and Mt-0865 strains, respectively. After cleaning the reads, 9.3 M and 9.8 M reads mapped to the genome, and, after removing rRNA sequences, the unique sequence reads were 4.3 M and 4.4 M (Table 1).

A total of 3,090 of the 3,167 open reading frames (ORFs) of the wild-type R1 strain, and 3,060 of the 3,167 ORFs of Mt-0865 mutant strain were detected by RNA sequence data. In the Mt-0865 strain, 246 genes were up-regulated more than two-fold (Table S1 in File S1) and 317 genes were down-regulated more than two-fold in comparison to the wild-type R1 strain (Table S2 in File S1). The significantly expressed genes were classified in accordance with the Cluster of Orthologous Groups (COG) of proteins database (Table 2). The top three categories were transcription (22%), inorganic ion transport and metabolism (19.2%), and nucleotide transport and metabolism (18.5%).

In this study, our analysis focused on the genes involved in (i) Mn/Fe metabolism, (ii) ROS production, (iii) DNA damage response genes, and (vi) cell cleaning genes (Table 3).

(i) Proteins involved in Mn2+ and Fe2+ metabolism

The expression of five genes involved in Mn/Fe metabolism was significantly changed under MnCl2 adaptation conditions (Table 2), *dr1236* (*mntE*), which encodes a putative manganese efflux family protein that controls the removal of excess Mn2+, was repressed (Table 2). However, all ATP-dependent Mn2+ transporter genes, including *dr2283*, *dr2284*, and *dr2523*, were induced. The Mn2+ transporter gene expression pattern suggested that Mn2+ concentration was increased in the mutant, which is in agreement with our ICP-MS data. Furthermore, a previous DNA binding assay also supported that ATP-dependent Mn2+ transporter genes and *mntE* are regulated by DR0865 [23].

(ii) Proteins associated with the production of ROS

Manganese is among the essential enzyme cofactors because it protects cells from oxidative damage. However, it can be toxic at high concentrations and, therefore, its level should be strictly regulated [17,23]. Previous research showed that cytochromes, flavoproteins, iron-sulfur proteins, NADPH and NADH-dependent enzymes are regarded as the major generators of ROS [1,2]. Under Mn2+ stress, nine cytochrome-related genes and 12 NADH-dependent enzymes were repressed in the Mt-0865 mutant compared to the wild-type R1 strain. This indicates that the mutant strain compensates for its Mn sensitivity by dampening the production of ROS.
Figure 7. Analysis of the intracellular ion content of the wild-type R1 and Mt-0865 strains, cultured in a medium supplemented with or without 50 μM manganese; (A) manganese, (B) iron, (C) zinc, (D) copper. The data represent the mean ± standard deviation of three independent experiments.

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Table 1. Summary of sequence reads statistics resulting from Illumina deep sequencing of mutant and wild type strain.

|                        | Mutant Strain | Wild type strain |
|------------------------|---------------|------------------|
| Raw data (read number) | 13289865      | 12798354         |
| Raw data (length, bp)  | 200           | 200              |
| Clean data (read number) | 10260088     | 9998560          |
| Clean data (length, bp) | 176           | 178              |
| Mapping to genome (read number) | 9831262  | 9299761          |
| Mapping to rRNA        | 5289176 (53.8%) | 4728594 (50.85%) |
| Unique mapping         | 4441705 (45.18%) | 4387406 (47.18%) |

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Proteins associated with the DNA damage response:

There were 15 induced genes associated with stress responses, but these did not include katE or recA. However, three antioxidant proteins, ahpC (dr1209), grxA (dr1209, dra0072), were up-regulated in the Mt-0865 strain (Table 3), which indicates that the mutant strain may be under oxidative stress. In addition to the well-characterized components of stress response systems, D. radiodurans encodes several proteins whose specific roles are unknown but are likely to be important for the multiple stress resistance phenotypes of the bacterium. An example of a poorly studied, but potentially important, system is the “addiction module” response, which is encoded by two genes, mazE (dr0417) and mazF (dr0416). MazF is a stable protein that is toxic to bacteria, whereas MazE protects cells from the toxic effect of MazF, and is degraded by the ClpX serine protease (dr0202) (Table 3). When the Mt-0865 mutant was under Mn stress, all of these genes were induced, which suggests that the mutant strain activates the antidote-toxin system to reduce cell growth to avoid the production of ROS. This result is also consistent with the expression patterns of ROS generating genes.

Cell cleaning proteins:

When the Mt-0865 mutant strain was under Mn stress, we found that the cellular cleansing system, including the export of damaged DNA components and sanitization of intracellular mutagenic precursors, was also induced. First, it was observed that six ABC transporter permease genes, which may control oligonucleotide export, were activated (Table 3) [24]. The export of damaged nucleotides outside the cell might protect the organism from elevated levels of mutagenesis by preventing the reincorporation of damaged bases during DNA synthesis [25]. Second, 15 of 20 mutT/nudix family genes were induced, five (dr0092, dr0192, dr0261, dr0274, dr0784) of which were up-regulated significantly (Table 3). The MutT protein has an 8-oxo-dGTPase activity, which can limit mutation of DNA by hydrolyzing the oxidized products of nucleotide metabolism. The remaining intracellular mutagenic precursors could be sanitized via this superfamily [26]. Finally, it was also found that Lon protease (DR1974) and ClpX protease (DR0202) were induced approximately twofold (Table 3). These ATP-dependent proteases help with cellular sanitization by degrading damaged proteins [27].

Table 2. Classification of the genes with different levels of expression according to the Cluster of Orthologous Groups of proteins (COG) database.

| COG_type           | Total genes | Induced genes | Repressed genes | Total rate |
|--------------------|-------------|---------------|-----------------|------------|
| Information storage and processing: |             |               |                 |            |
| J                  | 165         | 10            | 6               | 9.6%       |
| K                  | 149         | 13            | 21              | 22.8%      |
| L                  | 138         | 5             | 12              | 12.3%      |
| B                  | 1           | 0             | 0               | 0          |
| Cellular processes and signaling: |             |               |                 |            |
| D                  | 26          | 0             | 4               | 15.4%      |
| V                  | 50          | 1             | 7               | 16%        |
| T                  | 119         | 5             | 9               | 11.2%      |
| M                  | 110         | 6             | 9               | 13.6%      |
| N                  | 21          | 0             | 2               | 9.5%       |
| U                  | 40          | 0             | 3               | 7.5%       |
| O                  | 108         | 6             | 12              | 16.7%      |
| Metabolism:        |             |               |                 |            |
| C                  | 140         | 7             | 28              | 25%        |
| G                  | 114         | 6             | 10              | 14%        |
| E                  | 251         | 13            | 15              | 11.2%      |
| F                  | 92          | 10            | 7               | 18.5%      |
| H                  | 110         | 8             | 9               | 15.5%      |
| I                  | 99          | 5             | 3               | 8%         |
| P                  | 130         | 14            | 11              | 19.2%      |
| Q                  | 53          | 4             | 2               | 11.3%      |
| Poorly characterized: |             |               |                 |            |
| S                  | 251         | 19            | 23              | 16.7%      |
| R                  | 333         | 19            | 23              | 12.6%      |

1. J: Translation, ribosomal structure and biogenesis; K: Transcription; L: Replication, recombination and repair; B: Chromatin structure and dynamics; D: Cell cycle control, cell division, chromosome partitioning; V: Defense mechanisms; T: Signal transduction mechanisms; M: Cell wall/membrane/envelope biogenesis; N: Cell motility; U: Intracellular trafficking, secretion, and vesicular transport; O: Posttranslational modification, protein turnover, chaperones; C: Energy production and conversion; G: Carbohydrate transport and metabolism; E: Amino acid transport and metabolism; F: Nucleotide transport and metabolism; H: Coenzyme transport and metabolism; P: Inorganic ion transport and metabolism; Q: Secondary metabolites biosynthesis, transport and catabolism; S: Function unknown; R: General function prediction only.

2. the total number of significant genes/Number of total genes in this COG.
Table 3. The significant genes were classified into three classes, Mn/Fe metabolism, ROS production genes, and Damage response genes.

| ORF | Name          | Description                                      | M^1  |
|-----|---------------|--------------------------------------------------|------|
|     | Mn/Fe metabolism: |                                                  |      |
| DR2283 | dr2283       | Mn ABC transporter permease                      | 1.99 |
| DR2284 | dr2284       | Mn ABC transporter permease                      | 2.39 |
| DR2523 | fimA          | Mn/Fe transport system substrate-binding protein | 3.79 |
| DR1236 | mntE          | manganese efflux protein                        | −1.69|
| DR1220 | feoA          | ferrous iron transport protein A                 | 1.35 |
|     | ROS production genes |                                          |      |
| DR0342 | dr0342       | cytochrome complex iron-sulfur subunit           | −1.45|
| DR0344 | ccmH          | cytochrome c-type biogenesis protein             | −1.09|
| DR0346 | ccmF          | cytochrome c-type biogenesis protein             | −1.09|
| DR0347 | ccmE          | cytochrome c-type biogenesis protein             | −1.30|
| DR0348 | dr0348        | cytochrome c-type biogenesis heme exporter protein C | −1.46|
| DR2095 | dr2095        | c-type cytochrome                               | −1.39|
| DR2617 | ctaA          | cytochrome AA3-controlling protein               | −1.11|
| DRC0001 | drc0001      | cytochrome P450-related protein                  | −∞²  |
| DRC0041 | drc0041      | Cytochrome P450                                  | −∞   |
| DR1492 | dr1492        | NADH dehydrogenase I subunit N                  | −1.03 |
| DR1493 | dr1493        | NADH dehydrogenase I subunit M                  | −1.68 |
| DR1494 | dr1494        | NADH dehydrogenase I subunit L                  | −1.55 |
| DR1497 | dr1497        | NADH dehydrogenase I subunit I                  | −2.54 |
| DR1498 | dr1498        | NADH dehydrogenase I subunit H                  | −2.28 |
| DR1499 | dr1499        | NADH dehydrogenase I subunit G                  | −1.57 |
| DR1500 | dr1500        | NADH dehydrogenase I subunit F                  | −1.99 |
| DR1501 | dr1501        | NADH dehydrogenase I subunit E                  | −1.76 |
| DR1503 | dr1503        | NADH dehydrogenase I subunit D                  | −2.59 |
| DR1504 | dr1504        | NADH dehydrogenase I subunit C                  | −2.32 |
| DR1505 | dr1505        | NADH dehydrogenase subunit B                    | −1.79 |
| DR1506 | dr1506        | NADH dehydrogenase I subunit A                  | −1.44 |
| DRA0243 | Hmp          | Haemoglobin-like flavoprotein                   | −1.33 |
|     | Damage response genes: |                                                |      |
| DR1208 | Bcp           | Antioxidant type thioredoxin fold protein        | −∞   |
| DR1209 | ahpC          | Thiol-alkyl hydroperoxide reductases             | 1.87 |
| DRA0072 | graA         | Glutaredoxin                                     | 3.38 |
| DR2056 | hsU           | Related to heat shock protein                    | 1.18 |
| DR0194 | htpX          | Predicted Zn-dependent proteases                 | 1.69 |
| DR0416 | mazE          | Regulatory protein, MazF antagonist              | 1.66 |
| DR0417 | mazF          | ppGpp-regulated growth inhibitor                 | 1.32 |
|     | Cell cleaning genes: |                                                  |      |
| DRA0123 | arcC         | Arsenate oxidoreductase                          | −1.31|
| DR0455 | strA          | Streptomycin resistance protein                  | −1.62|
| DR2234 | dr2234        | involved in multidrug resistance                | −2.41|
| DR1695 | gloA          | Lactoylglutathion lyase, fosphomic resistance protein | 1.49|
| DRA0599 | BS._yokD     | amino glycoside N3-acetyltransferase             | 1.52 |

Note: M^1 represents the fold change in expression; −∞² indicates expression below the detection limit.
Table 3. Cont.

| ORF   | Name     | Description                        | M1     |
|-------|----------|------------------------------------|--------|
| DR0261| dr0261   | MutT/nudix family protein          | 3.33   |
| DR0274| dr0274   | MutT/nudix family protein          | 3.02   |
| DR0784| dr0784   | MutT/nudix family protein          | 1.18   |
| DR0202| clpX     | ATPase subunit of Clp protease     | 1.20   |
| DR1974| Lon      | ATP-dependent Lon serine protease  | 1.40   |
| DR0958| dr0958   | peptide ABC transporter permease   | 1.65   |
| DR0959| dr0989   | peptide ABC transporter permease   | 1.44   |
| DR1358| dr1358   | outer membrane protein             | 1.18   |
| DRA0168| dra0168| ABC transporter permease           | 1.54   |
| DRA0268| dra0268| adenine deaminase-like protein     | 1.21   |
| DRA0323| dra0323| urea/short-chain amide ABC transporter ATP-binding protein | 1.07   |

1. M value means log2 Ratio, Ratio = FPKM(Mutant)/FPKM(WT)
2. ≠ means gene's expression level is not detected in one sample, but detected in another sample.

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qRT-PCR analysis

To confirm the transcriptome assay results, gene expression in the Mt-0865 mutant and in the wild-type R1 strains was analyzed using quantitative real time PCR (qRT-PCR) analysis. Eight genes (dr2523, dr2283, dr1709, dr1236, dr1998, dr1506, dr0348, and dr0828) were quantified under normal growth conditions and after treatment with Mn(II). Four of these genes are Mn(II) transport genes (dr2523, dr2283, dr1709 and dr1236). The DR2523 and DR2283 proteins are ATP-dependent transporters, the DR1709 protein belongs to the Nramp family of transporters, and DR1236 is a Mn(II) efflux gene. The dr1998 gene encodes a major catalase (KatE), which plays an important role in the protection of D. radiodurans from oxidative stress and ionizing radiation [1]. The DR1506 protein is a NADH dehydrogenase and DR0348 is the cytochrome c-type biogenesis heme exporter protein C. It was previously shown that dr1506 and dr0348 are associated with the production of ROS [1]. The dr0828 gene encodes an isocitrate lyase, which is an enzyme in the glyoxylate cycle that catalyzes the cleavage of isocitrate to succinate and glyoxylate. Previous research has shown that when irradiated, D. radiodurans represses the tricarboxylic acid (TCA) cycle and activates the glyoxylate bypass [20].

It was observed that under normal growth conditions, the expression of dr2523, dr2283, and dr1709 increased 1.96-fold, 4.24-fold and 4.41-fold, respectively, in the Mt-0865 mutant strain compared to the wild-type R1 strain (Table S3 in File S1), while the dr1236 gene was significantly repressed 24.32-fold in the mutant strain. In addition, the transcript levels of dr1506 and dr0348 decreased, whereas the level of the dr0828 transcript increased, in the mutant strain (Figure 8A and Table S3 in File S1). Gene expression levels were also measured under Mn(II) stress. The expression of the Mn(II) transporter gene dr2539 increased 20.14-fold, while expression of dr1236 decreased 53.94-fold in the Mt-0865 mutant (Figure 8A). This suggests that under Mn(II) stress, the wild-type strain attempts to stop Mn(II) uptake and opens the Mn(II) efflux system, whereas this does not occur in the mutant strain. These results provide further evidence that Mn(II) transporter genes are not properly expressed in the mutant. In addition, the dr1506 and dr0348 genes were repressed and the dr0828 gene was induced. This suggests that, under Mn(II) stress, the wild-type strain lowers its metabolic rate to reduce ROS production and activates the glyoxylate bypass to provide energy, whereas these adaptations are defective in the mutant strain. Overall, the pattern of gene expression indicates that the mutant strain is likely subject to more damage than the wild-type strain under Mn(II) stress.

Discussion

Manganese is a trace element that is essential for many cellular functions in all organisms. For example, Mn(II) is required as a co-factor for super-oxide dismutase, which is critical for preventing cellular oxidative stress [29]. However, high manganese levels inhibit calcium influx and promote the exchange of accumulated Ca(II), and inhibit RNA and protein synthesis [3]. Thus, maintaining metal ion homeostasis is necessary for all organisms. D. radiodurans is well known for its extreme resistance to radiation and oxidants and its high intracellular Mn/Fc ratio is an important factor that contributes to this resistance. In this study, we identified a unique Mur homolog that is encoded by dr0865, and data showed that it is Mn(II)-specific regulator.

Sequence analyses showed that DR0865 contains three metal-binding domains that are present in the H. pylori Fur homolog. Previous data showed that the Cys2XXC150 motif is necessary for the construction of the Zn(II) [N/O]2 domain, while the Cys135XXXC138 motif is not important [20,30]. Further research is needed to discern the function of the Cys2XXC150 and Cys112XXXC115 motifs in D. radiodurans. Because the phenotype of the Mt-0865 mutant strain showed that DR0865 is a novel Mur protein, we compared the DR0865 amino acid sequence to the R. leguminosarum Mur protein [12]. The results showed that the R. leguminosarum Mur protein does not have domain I, while it contains domains II and III (data not shown). This indicates that domains II and III are important for Mn(II) ion regulation.

It has been suggested that the accumulation of Mn(II) or a higher Mn/Fc ratio benefit the radio-resistance of D. radiodurans. However, the excess of Mn(II) is toxic to the cell. Although the precise mechanism of Mn(II) toxicity is poorly understood, three mechanisms have been suggested previously. In the first mechanism, Mn(II) cell toxicity may be associated with its interaction with other essential trace elements, such as Fe(II), Zn(II) and Cu(II) [31]. Human studies have shown that chronic exposure to Mn(II) appears to be associated with similar increases in cellular Fe(II) uptake, which consequently produces cellular oxidative stress and...
also increases the concentration of Cu$^{2+}$ and Zn$^{2+}$ [31]. When the wild-type R1 strain was under Mn$^{2+}$ stress, the intracellular concentration of Mn$^{2+}$ and Fe$^{2+}$ increased significantly, whereas the concentrations of Zn$^{2+}$ and Cu$^{2+}$ increased slightly (Figure 7C and D). Under normal growth conditions, the Mt-0865 strain had higher Mn$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ contents than the wild-type R1 strain. These data further confirmed that mutation of the $dr0865$ gene causes a defect in the control of Mn$^{2+}$ metabolism, which also results in changes in the concentrations of Cu$^{2+}$ and Zn$^{2+}$. Interestingly, Fe$^{2+}$ concentration was not significantly different between the wild-type and mutant strains under Mn$^{2+}$ stress (Figure 7B), which may be due to the distinct D. radiodurans Fe$^{2+}$ regulation system, which utilizes OxyR and DtxR regulators.

The second mechanism is that high intracellular levels of Mn$^{2+}$ inhibit RNA and protein synthesis, and manganese may exert a toxic effect through such inhibition [3]. When Bacillus stea-totherophilus was grown in media containing excess Mn$^{2+}$, its doubling time increased more than two-fold. A similar effect on growth was also observed in the Mt-0865 mutant under normal growth conditions. The third mechanism suggests that Mn$^{2+}$ can participate in reactions that potentially increase ROS, which subsequently causes oxidative damage [32]. These three mechanisms may explain why the mutant was sensitive to different DNA damaging agents.

The RNA-seq data identified 562 genes (approximately 17% of the genome) that showed at least a twofold change in expression between the Mt-0865 mutant and the wild-type R1 strain, which indicates that these genes were regulated by $dr0865$ either through direct or indirect mechanisms. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, we found that genes involved in metabolic pathways, the biosynthesis of secondary metabolites, oxidative phosphorylation and nitrogen metabolism were significantly repressed in the mutant strain. This indicates that the Mt-0865 mutant is likely to suffer more cellular damage under Mn$^{2+}$ stress than the wild-type strain. This phenomenon may be caused by higher Mn$^{2+}$ levels in the mutant, which would increase ROS levels and lead to DNA damage [33]. Five nudix family genes were also activated but no major DNA repair genes (such as $recA$ or $pprA$) were induced, which further confirms our hypothesis.

Interestingly, we found that the heme biosynthesis pathway (HemA, HemE and HemN) was slightly repressed in the Mt-0865 mutant (Table S2 in File S1). Hemes are biosynthesized from protoporphyrin and free ferrous iron [34] and are cofactors for cytochromes, catalases and peroxidases. This may explain why nine cytochrome genes were down-regulated under Mn$^{2+}$ stress in the mutant. In addition, two vitamin B12 biosynthesis proteins, $db0010$ (cobalamin biosynthesis protein) and $db0012$ (cobic acid synthase), were also repressed. Vitamin B12 is a water-soluble vitamin that is normally involved in DNA synthesis and regulation, as well as in fatty acid biosynthesis and energy production. The $dr0910$ and $dr1076$ genes, which encode cell wall protein and cell wall synthesis proteins, respectively, were also down-regulated. The reduction of vitamin B12 and cell wall proteins may be caused by high Mn (II) levels, which consequently results in the inhibition of cell growth.

Five ribonucleases ($dr0020$, $dr0859$, $dr1949$, $dr2374$ and $dr0107$) were induced at least two-fold in the Mt-0865 mutant (Table S1 in File S1). Ribonucleases in prokaryotic toxin-antitoxin systems are proposed to function as stress-response elements. The degradation of RNA within a cell leads to fragments of RNA that are no longer needed and can be cleaned up as part of the cellular protection system. Five cation transporter genes ($dr0748a$, $dr0816$, $dr0883$, $dra0168$, $dra0361$) were also activated, which explains why the mutant had higher ion concentrations.

Overall, our work presents a biochemical mechanism for Mn (II) sensing by the Mur homolog gene in D. radiodurans. Using qRT-PCR and global transcriptome analysis, we provided evidence that $DR0865$ functions as a positive ($dr1236$) and a negative regulator ($dr2283$, $dr2284$, $dr2523$) of different classes of Mn$^{2+}$ transporter genes. More research is needed to establish the detailed mechanism of Mur regulation of these important genes. The potential communication between OxyR and other regulators, such as DtxR ($dr2539$), should be explored to determine...
whether it is required for the intricate coordination of oxygen radical detoxification.

**Experimental Procedures**

**Strains, media and primers**

All the primers used in this study are listed in Table 4. The *E. coli* strains were grown in Luria-Bertani (LB) broth medium (1% tryptone, 0.5% yeast extract and 1% sodium chloride) with aeration or on LB agar plates (1.2% Bacto-agar) at 37°C supplemented with the appropriate antibiotics. All *D. radiodurans* R1 (ATCC 13939) strains used in this work were grown at 30°C in TGY medium (0.5% tryptone, 0.1% glucose and 0.3% yeast extract) with aeration or on TGY plates supplemented with 1.5% Bacto-agar.

**Sequence alignment**

The protein sequence of previously characterized Fur proteins found in *A. ferrooxidans*, *P. aeroginosa*, *E. coli*, *B. subtilis*, *M. marinum*, *D. radiodurans*, *H. pylori* were obtained from the NCBI database. The protein sequence alignment of selected Fur proteins was generated using ClustalW.

**Disruption of the DR0865 gene in *D. radiodurans***

The mutant strain was constructed as described previously [18]. Primer ME1 and ME2 were used to amplify a *Bam*HI fragment upstream of targeted genes, and primers ME3 and ME4 were used to obtain a *Hind*III fragment downstream of targeted genes respectively (Table 5). The kanamycin resistance cassette containing the groEL promoter was obtained from a shuttle plasmid, pRADK. After this three DNA fragments were digested and ligated. The ligation products were used as template for PCR to amplify the resulting PCR fragment (ME1 and ME5 used as primers), which was then transformed into exponential-phase cells by CaCl2 treatment [35]. The mutant strain was constructed as described previously [18]. Primer ME1 and ME2 were used to amplify a *Bam*HI fragment upstream of targeted genes, and primers ME3 and ME4 were used to obtain a *Hind*III fragment downstream of targeted genes respectively (Table 5). The kanamycin resistance cassette containing the groEL promoter was obtained from a shuttle plasmid, pRADK. After this three DNA fragments were digested and ligated. The ligation products were used as template for PCR to amplify the resulting PCR fragment (ME1 and ME5 used as primers), which was then transformed into exponential-phase cells by CaCl2 treatment [35]. The mutant strains were selected on TGY agar plates supplemented with 30 μg/ml kanamycin. Null mutants were confirmed by PCR product sizes, enzyme-digested electrophoresis, and DNA sequencing and the resulting mutant was designated Mt-0865.

**Complementation of DR0865 mutant**

Complementation strain was constructed as described previously [18,36]. Briefly, genome DNA was isolated from wild-type R1 strain. A 2500-bp region containing the *dr0865* gene was amplified by ME5 and ME6 (Table 5), and ligated to pMD-18 T-Easy vector (Takara, JP), designed as pMD-dr0865. After digested by *Nde*I and *Bam*HI, the target gene *dr0865* was ligated to *Nde*I and *Bam*HI-pre-digested pRADK, which named as pRKR. The complementation plasmid was confirmed by PCR and DNA sequence analysis, and transformed into Mt-0865, resulting in functional complementation strains. Selection for *D. radiodurans* complementation strain was achieved on TGY plates, supplemented with kanamycin (30 μg/ml) and chloramphenicol (5 μg/ml).

**Growth curve assay**

To examine bacterial growth in vitro as described previously was little modified [37], the single clone of the wild-type R1, Mt-0865 and C-0865 strains were transferred into 5 ml liquid TGY medium. When the OD600 of the cultures reached 1.0, 1 mL of each culture was added to 100 mL fresh TGY medium. Three repeats were performed for each strain. The nine cultures were incubated with shaking at 30°C and samples were taken every two hours to measure the OD600 value. The cultures were incubated with 250 rpm at 30°C and samples were taken to measure the OD600 value at different time. All experiments were repeated in triplicate.

**Cation sensitivity assays**

Cation sensitivity assays were carried out as described previously [17]. Solutions (1 M) of manganese chloride, manganese sulfate, zinc chloride, copper chloride, copper sulfate, cobalt (II) chloride, nickel chloride, cadmium chloride, ferrous sulfate, ferric chloride, ferric chloride, magnesium chloride, calcium chloride (sigma) were prepared in milli-Q water and filter-sterilized by passing through 0.22-μm filters. Newly fresh clone was taken from the wild-type R1, Mt-0865 and C-0865 TGY plates, into 5 ml TGY fresh media, when the cells grown up to stationary phase. Then, the cells were plated on TGY plates and overlaid with 5-mm sterile discs containing 1 M various cation solutions. The plates were incubated for three days, and the inhibition zone of each disc was measured. All the data provided here represent the mean and standard deviation of at least three independent experiments (mean ± SD of three experiments).

Similarly, to ascertain the effect of Mn2+ on growth of Mt-0865 and wild-type R1, 1×106 CFU ml−1, were grown in TGY supplemented with increasing concentration of MnCl2. The...

| Table 4. Bacterial strains and plasmids used in this study. |
|---------------------------------|------------------|-----------------|-----------------|
| **Strain or plasmid** | **Relevant marker** | **Source** |
| **Strains** | | |
| *E. coli* DH5α | Propagation for plasmid | Invitrogen |
| *E. coli* BL21(pLysS) | DR0865 expression strain | Invitrogen |
| *D. radiodurans* R1 | ATCC13939 | This lab |
| Mt-0865 | As R1, but *dr0865*-kan | This study |
| C-0865 | Mt-0865 complemented with pRKR | This study |
| **Plasmids** | | |
| pMD18-T | TA cloning vector | Takara |
| pMR | pET-28a derivative recombinant expressing and *dr0865* | This study |
| pRADK | *E. coli*-D. radiodurans shuttle vector carrying *D. radiodurans* groEL promoter | [36] |
| pRKR | pRADK derivative expressing *D. radiodurans* *dr0865* | This study |

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The OD<sub>600</sub> value was measured after 12 h and 24 h post incubation. All the data provided here represent the mean and standard deviation of at least three independent experiments (mean ± SD of three experiments).

H<sub>2</sub>O<sub>2</sub> sensitivity assays (Oxidative stress assays)

The discs diffusion assay to test H<sub>2</sub>O<sub>2</sub> sensitivity, was performed as described previously with a little modification [38,39]. The strain was cultured up to log phase and 130 μl aliquots were spread on TGY plates. A sterile 5-mm-diameter filter discs, containing 4 μl and 6 μl of 1 M H<sub>2</sub>O<sub>2</sub> was placed on the surface of the TGY plate. After incubation at 30°C for three days, the size of the area cleared of bacteria (zone of inhibition) was measured. For the curve H<sub>2</sub>O<sub>2</sub> treatment, the cultures were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> for 30 min and then plated on TGY plates, as prescribed previously [40]. All the data provided here represent the mean and standard deviation of at least three independent experiments (mean ± SD of three experiments).

Gamma irradiation and UV sensitivity assays

Survival curves of the wild-type R1 and Mt-0865 cells were cultured in TGY broth to OD<sub>600</sub>, 1.0. For the Gamma radiation treatment, the 100 ml cultured was irradiated with different doses of 60Co gamma at room temperature, which correspond to doses from 0 to 16 kGy, as previously published [41,42]. After the irradiation treatment, the culture centrifuged and then re-suspended in phosphate buffer (1XPBS Buffer, pH 7.5). The cells were plated on TGY plates and incubated at 30°C for at least three days. The colonies were counted. All the data provided here represent the mean and standard deviation of at least three independent experiments (mean ± SD of three experiments).

For the UV treatment, the cells were cultured in TGY broth to OD<sub>600</sub>~1.0, as described previously [17,43]. The cells were re-suspended in 1XPBS buffer (pH 7.5), then plated on TGY plates and exposed to different doses of UV radiation at 254 nm. All the data provided here represent the mean and standard deviation of at least three independent experiments (mean ± SD of three experiments).

Assay of intracellular Mn, Fe, Zn and Cu ion concentration

The protocol for determining intracellular concentration of metals ions was identical to previously reports [17]. D. radiodurans R1 and Mt-0865 were cultured in 5 ml TGY broth and re-inoculated in 500 ml TGY broth which had been pretreated with Chelex to remove any cat-ion, and then supplemented with 50 μM manganese chloride. The cells were grown up to OD<sub>600</sub>~0.6–0.8 and harvested. After centrifugation at 10000 g, 4°C for 10 min, the pellets were washed three times with 1xPBS (pH 7.5), containing 1 mM EDTA and rinsed three times with 1xPBS, without EDTA. Cells (1/10 of the total volume) were withdrawn to measure the dry weight. For ion analysis, 1 ml of Ultrex II nitric acid (pH 2.0) was used to digest the pellet. The concentration of Mn, Fe, Zn and Cu ions were measured using a flame atomic absorption spectrophotometer.

### Table 5. Primers used in this study.

| Primer          | Sequence (5’ → 3’ ) |
|-----------------|---------------------|
| **Mutation primers** |                     |
| 0865upF (ME1)   | CGAAGAAGTGGCCCAACAAC|
| 0865upR (ME2)   | GGATCCGAGGCAGGTAGCAAGCG |
| 0865downF (ME3) | AAGCTTGGGCGAACTTTACGCTTG |
| 0865downR (ME4) | ACATAACGGTTTTGCCATTTGCC |
| **Complement primers** |                   |
| 0865F (M56)     | CATATAGCCAGCCGCGCAAC |
| 0865R (M56)     | GGATCTTAGTGCCCGGCTT |
| **Real time PCR primers** |                 |
| RT-dr1506F      | GCGGGAAGGCTGAGCTAGAGG |
| RT-dr1506R      | CTGGTTGGGCGGCTTTTGGG |
| RT-dr0348F      | CCTCGGCTACCTATCATCCTGGC |
| RT-dr0348R      | TTGACCGGCTGCGCTGTTGAATG |
| RT-dr1236F      | CATCAATCTGTTGTCGGCAAC |
| RT-dr1236R      | CAAGCAGCGGTTCAAGGATGTG |
| RT-dr0828F      | GACACTTAGACACCCACCCCAC |
| RT-dr0828R      | GCGTACTAGATGGGGCGGTCG |
| RT-dr2523F      | CGACGCCATACCTTTCGAC |
| RT-dr2523R      | GTCACTGCTCTACCGGCCCAC |
| RT-dr2283F      | GAGCCTGCGGACATGA |
| RT-dr2283R      | GCGAGCAGCCAGCAGAAA |
| RT-dr1998F      | GGCGTGAGCAAGGATTTC |
| RT-dr1998R      | GATAGCAGGGGGCTCCTGCT |
| RT-dr1709F      | CGCATGGTGATTAGACAACT |
| RT-dr1709R      | GTTCGCGCTGAAATCCGTA |

Note: straight line represents restriction site.

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acid (Fluka AG., Buchs, Switzerland) was added to the rest cells and incubated at 100°C for 1 h. After centrifugation at 20,000 g for 20 minutes, the supernatant was filtered against 0.45 μM membrane. The concentration of samples was analyzed for ion content by inductive coupled plasma mass spectrometry (ICP-MS, Model Agilent 7500a, Hewlet-Packard, Yokogawa Analytical System, Tokyo, Japan). A control prepared in the same manner but without 50 μM manganese chloride. All the data provided here represent the mean and standard deviation of at least two independent experiments (mean ± SD of twice experiments).

Total RNA isolation

To see the effect of Mn2+ on the genome level, the total RNA was extracted from the three biological replicates of wild-type R1 and Mt-0865 strains under Mn2+ stress. Briefly, the wild-type R1 and Mt-0865 strains were cultured in a 5 ml TGY broth and re-inoculated in a 500 ml TGY broth. When the cells grow to OD600 0.865 strains were cultured in a 5 ml TGY broth and re-inoculated and Mt-0865 under Mn2+ was extracted from the three biological replicates of wild-type R1

Bacterial RNA sequence library construction

Total RNA from three wild-type R1 and mutant strain (Mt-0865) were pooled, respectively, and tRNA (include 16S and 23S) was removed from 4 μg total RNA by Microbexpress(TM) (Ambion AM1905), and the left RNA was chemically fragmented. The sequence library construction is according to ScriptSeq mRNA-Seq Library Preparation Kit (Illumina-compatible). Briefly, the fragmented RNA is reverse-transcribed into cDNA using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) with the addition of SuperScript III reverse transcriptase (Invitrogen), and random primers containing a tagging sequence at their 3'ends. This was followed by RNase A (Roche, Germany) treatment, phenol-chloroform extraction, and ethanol precipitation. The resulting cDNAs were ligated to 5’ DNA/DNA adaptor, and the di-tagged cDNAs was purified by PAGE gel, the insert fragment size is 150 bp~250 bp. The purification products were PCR amplified in 18 cycles using a high-fidelity DNA polymerase. PCR products were purified using the PAGE gel. Both direct cDNAs were sequenced simultaneously using a single flow cell of the Illumina HiSeq2000. All the sequence assays were performed in Zhejiang TianKe Company.

Transcriptome analysis

The images generated by the sequencers were converted into nucleotide sequences by a base-calling pipeline. The raw reads were saved in the fastq format. Three criteria were used to filter out the raw reads according to previously published [44], (i) Remove reads with sequence adaptors; (ii) remove reads with more than 20% ‘N’ bases; (iii) remove low-quality reads, which have more than 40% QA ≤20 bases. All subsequent analyses were based on clean reads. Only reads with high quality value were selected and used in the mapping using TopHat [45]. No more than 2-mismatches were allowed in the alignment for each read, and only the unique mapping reads used in the latter analysis. Cufflink and Cuff-diff were used to calculate Fragments Per Kilobase of transcript per Million mapped reads (FPKM), and find significantly expressed genes, respectively. The annotation of the D. radiodurans genome obtained from NCBI.

Reverse transcription-PCR (RT-PCR) analysis of expression of genes

QRT-PCR assay utilized RNA samples obtained from different condition and first-strain cDNA synthesis was carried out in 20 μl of reaction containing 1 μg of RNA sample combined with 3 μg of random hexamers using SuperScript III Reverse Transcriptase kit (Invitrogen). Each measurement was obtained for three replicate. Then Quant SYBER Premix TX TaqTM (TaKaRa Biotechnological (Dalian) Co. Ltd, China) was used to amplification following the manufactures instruction. As an internal control, dd0089 was used as a house-keeping, encoding the glycosyl transferase [18]. All primers used in QRT-PCR are shown in Table 5. All assays were performed using the STRAGENE Mx300PTM Real-time detection. Data analysis was carried out with iCycler software (Bio-rade Laboratories). The ratio of the copy number for the treatment to the control copy number was calculated. Differences in relative transcript abundance level were calculated using 2−ΔΔT [18].

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed on the raw data using paired student’s t-test; p values <0.05 were considered significant.

Supporting Information

File S1 Combined Supporting Information file.

Author Contributions

Conceived and designed the experiments: YH HC. Performed the experiments: AMUHS YZ YW GY QZ LW BT. Analyzed the data: AMUHS YZ HC. Contributed reagents/materials/analysis tools: YH HC. Wrote the paper: YH HC AMUHS.

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