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A copper-responsive gene cluster is required for copper homeostasis and contributes to oxidative resistance in *Deinococcus radiodurans* R1

Zhongchao Zhao,a,b Zhengfu Zhou,a,b Liang Li,b Xianyi Xian,b Xiubin Ke,b Ming Chen*b and Yuxiu Zhang*a

**Abstract**

Excess copper is toxic to organisms, and therefore, copper homeostasis is important for the limitation of its cellular levels. However, copper homeostasis has not been studied to date in the bacteria *Deinococcus radiodurans* R1, which exhibits extreme resistance to various environmental stresses. We have identified a copper-responsive gene cluster that encodes CopA, which is a copper-transporting P1-type ATPase, CopZ, which is a copper metallochaperone, and CsoR, which is a copper-sensing repressor. Copper induces the transcription of genes in this cluster. Mutants lacking *copA* exhibited reduced copper resistance and the overaccumulation of copper compared with the wild-type strain. Additionally, in both the absence and presence of copper, the *copZ* mutation increased the expression of *copA* and led to the accumulation of lower levels of copper compared with the wild type. The bioinformatic analysis showed that CsoR in *D. radiodurans* R1 shares high sequence similarity and identity with the CsoR of *Mycobacterium tuberculosis* and *Bacillus subtilis*. We also demonstrated through DNase I footprinting and electrophoretic mobility shift assays that CsoR binds to the promoter of the cluster and that copper ions eliminate this interaction. This implies that CsoR is the repressor of this cluster and that CopA, CopZ and CsoR participate in the regulation of copper homeostasis. Our data also indicate that after treatment with *H*$_2$*O$_2$* and cumene hydroperoxide, the viability of the *copA* mutants was significantly reduced. This suggests that copper homeostasis plays an important role in oxidative resistance in *D. radiodurans* R1.

Key words: Copper homeostasis, *Deinococcus radiodurans* R1, oxidation resistance, toxicity, gene regulation.

**Introduction**

As an essential micronutrient, copper is required by all organisms and is used as a co-factor by many proteins and enzymes. It also participates in redox reactions in many biological processes. However, excess copper may be extremely toxic to organisms because of its avid binding to sulfur and nitrogen donors, resulting in the disruption of vital biological processes. Therefore, copper homeostasis is extremely important to ensure the maintenance of the proper concentrations of copper ions in organisms and the avoidance of copper toxicity under conditions of excess. To achieve this, organisms must possess copper-sensing transcriptional regulators that have high binding affinities for copper ions. When these ions are present in excess, these regulators are able to sense the overabundance and induce the expression of detoxification systems, including copper-exporting P1-type ATPases and copper metallochaperones.

Several copper homeostasis systems have been identified in bacteria. A widespread system for maintaining the copper ion balance in cells consists of a P1-type ATPase protein, a copper chaperone protein and a copper-sensing regulator. In many bacterial species, such as *Escherichia coli* and *Enterococcus hirae*, P1-type ATPase proteins participate in efflux by exporting copper ions from the cytoplasm and play an essential role in copper resistance. Copper chaperone proteins are mainly involved in sequestration mechanisms by binding copper ions and intracellularly chaperoning them for incorporation/use/efflux by other copper-binding proteins. To date, two types of copper-sensing regulators have been identified in Gram-positive bacteria. One includes the CsoR copper-sensing repressors that have been found in *Mycobacterium tuberculosis* and *Bacillus subtilis* and the other is the CopY copper-sensing regulator family that has been observed in *E. hirae* and *Streptococcus* spp. Among all known species, *Deinococcus radiodurans* R1 is unparalleled in its capacity to resist extreme ionizing radiation, UV radiation, oxidative stress, and a variety of DNA damaging agents. For *D. radiodurans* R1 to survive in various environments, it must be able to sense and adapt to considerable variations in environmental conditions, including the altered concentrations of metal ions that are found in extreme environments. To date, numerous copper-related proteins have been reported in many Gram-positive bacteria; however, these proteins have not been identified in the *D. radiodurans* R1 genome. Although there is a lack of evidence that the *D. radiodurans* R1 requires copper, this organism must possess mechanisms for maintaining copper homeostasis in cells because it regularly encounters fluctuating levels of copper in its environment to which it must adapt.

The objective of this study was to investigate the mechanisms of copper homeostasis in *D. radiodurans* R1. A transcriptional profiling analysis of the genomic sequences of this organism revealed a chromosomal copper-responsive gene cluster encoding a homolog of the CsoR regulator family (*DR_2449*), a copper metallochaperone...
CopZ (DR_2452), a copper-transporting P1-type ATPase (DR_2453) and two proteins with unknown functions (DR_2450 and DR_2451). A real-time quantitative reverse transcription (RT)-PCR analysis demonstrated that the expression of genes in the copper cluster is induced specifically by copper and is regulated by CsoR. Furthermore, a copA mutant strain was constructed that showed an extreme sensitivity to copper and decreased viability following exposure to H$_2$O$_2$ oxidative stress, signifying that copper homeostasis plays a vital role in the physiology and oxidative stress resistance of D. radiodurans R1.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids that were used in this study are described in Table 1. The D. radiodurans strains were grown at 30 °C in TGY broth (0.5% tryptone, 0.1% glucose and 0.3% yeast extract) or on TGY plates supplemented with agar (1.5%). The Deinococcus mutants were grown in the presence of kanamycin (8 µg/mL) as required. The E. coli strains were grown at 37 °C in LB broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) or on LB plates supplemented with kanamycin (50 µg/mL) or ampicillin (100 µg/mL) as required. All molecular biology-grade chemicals were purchased from New England Biolabs, TaKaRa Bio Inc, Promega, and Sigma Chemicals Co.

Table 1 Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
<th>Use</th>
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<tr>
<td>FU1pCopZ</td>
<td>CCGATTCACCCGAGCCGTC</td>
<td>PCR</td>
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<tr>
<td>RU1pCopZ</td>
<td>GTTTTTCTATCGATGTACTCTATAGTCACGCCGCAATCGAC</td>
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<tr>
<td>RDnCopZ</td>
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<tr>
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<td>GGGATGTTCAAGGAGGCAAGAGCAGA</td>
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<td>ATATCGAGTTCTAGTTAACTACGCCGCGACGCAGGACTAGGAGTACCTGATTAAAGACCAC</td>
<td>PCR</td>
</tr>
</tbody>
</table>

Construction of D. radiodurans copA and copZ mutant strains

The copA and copZ mutants were generated by replacing the target gene with an antibiotic determinant via a fusion PCR protocol. The primers that were used in this study are listed in Table 2. In brief, the regions that were located 628 bp upstream and 524 bp downstream of DR_2452 were amplified with the primers FU1pAcoP and RU1pAcoP and with FDnAcoP and RDnAcoP, and the regions 437 bp upstream and 300 bp downstream of DR_2453 were amplified with the primers FU1pAcoP and RU1pAcoP and with FDnAcoP and RDnAcoP from the D. radiodurans R1 genome. The kan cassettes for the copA and copZ mutants were amplified with the primers FKatA coP and RKanZ and with FKanA and RKanA from the pKatAPH3 plasmid. The primers RU1pAcoP, FDnAcoP, RU1pAcoP and FDnAcoP were designed such that the amplification reaction produced fragments possessing tails with identities to the amplification kan cassettes. The two DR_2452 fragments and two DR_2453 fragments were mixed with their respective kan cassettes and fused together by PCR using nested primers (FU1pAcoP, RDnAcoP and FU1pAcoP, RDnAcoP), creating two linear fragments that were suitable for transformation. The mutant strains containing the kan cassettes in the target copZ and copA genes were constructed by transforming the wild-type D. radiodurans R1 with the linear fragments. The disruption of the genes was confirmed by diagnostic PCR using the appropriate primers (FDiaZ, RDiaZ and FDiaA, RDiaA) and subsequent sequencing assays.
promoters in both the presence and absence of 10 mM glycerol). Increasing concentrations of CsoR were incubated for 10 min at room temperature with the labeled Tris/HCl pH 8.0, 50 mM using T4 polynucleotide kinase (T4 PNK; NEB). EMSA reactions were carried out in 10 µl EMSA buffer (20 mM γ-32P]ATP using T4 polynucleotide kinase (T4 PNK; NEB). EMSA reactions were carried out in 10 µl EMSA buffer (20 mM Tris/HCl pH 8.0, 50 µg BSA ml⁻¹, 50 mM NaCl, 1 mM DTT, 5 µg salmon sperm DNA ml⁻¹, and 5% (v/v) glycerol). Increasing concentrations of CsoR were incubated for 10 min at room temperature with the labeled promoters in both the presence and absence of 10 µM CuSO₄. DTT was added to the reactions. Metal sensitivity tests
The TGY liquid cultures were inoculated at an OD₆₀₀ of 0.1 from overnight cultures and supplemented with the desired CuSO₄ concentrations as indicated in the figures. The cells were grown for 16 h, and the OD₆₀₀ measurements were recorded.
Copper accumulation in D. radiodurans cells
Thirty milliliters of liquid TGY cultures of both the wild-type and mutant strains in late exponential phase were supplemented with 0.2 mM CuSO₄ and incubated for 2 h. The OD₆₀₀ values were then determined, and the cells were harvested, and washed with 0.9% NaCl. The pellets were acid digested with 10 ml HNO₃ (trace metal grade) overnight at 80 °C. The copper concentrations of the digested samples were measured by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo X II).
Gene expression determinations
The D. radiodurans R1 wild-type cells from the 2 ml TGY liquid cultures (supplemented with 1 mM CuSO₄) were harvested. RNA was isolated with the FastRNA® Pro Blue Kit (MP Biomedicals). The gene expression was studied using quantitative real-time RT-PCR (7500 Real-Time, Bio-Rad). The primers that were used are indicated in Table 1. cDNAs were obtained with the PrimeScript™ RT Reagent Kit (TaKaRa) following the manufacturer’s instructions. qPCR reactions were carried out with SYBR® Premix Ex Taq™ (TaKaRa) in final volume of 20 µl. The qPCR program consisted of a 10-min incubation at 95 °C followed by 40 cycles of 15 s at 95 °C, 34 s at 60 °C and 30 s at 72 °C. The specificity of the PCR amplification was verified using a heat dissociation protocol (from 70 °C to 100 °C) after the final PCR cycle. The efficiencies of the primer sets were evaluated using performed real-time PCR on dilutions of cDNA. The results were normalized to the 16s rRNA levels. RT-PCR determinations were carried out with RNA extracts from three independent biological samples and the threshold cycle (Ct) was determined in triplicate. The relative transcriptional levels were calculated using the 2-∆∆Ct method. In all experiments, a control sample that was not treated with reverse transcriptase was included to detect any possible DNA contamination.

Plasmid Construction, Protein Expression, and Purification
The csoR (DR_2449) coding regions were amplified with the Fcsor and Rsor primers (Table 2) from the D. radiodurans R1 genome and ligated to pJET1.2/blunt, prior to subcloning into the NdeI/BamHI site of pET-28a. The CsoR protein were expressed in E. coli BL21 (DE3) for 4 h at 30 °C by adding 0.3 mM 1-thio-β-d-galactopyranoside (IPTG) into the LB culture when the cells reached OD₆₀₀ of 0.6. The cells were recovered by centrifugation, resuspended in NTA-0 buffer (20 mM Tris-HCl, 0.5 M NaCl, and 10% Glycerol, pH 7.9) and sonicated. The extracts were clarified by centrifugation, and the hexahistidine-tagged recombinant protein that was bound to the HisPur Cobalt Resin was eluted with NTA-400 buffer (20 mM Tris-HCl pH 6.0, 50 mM NaCl, 1 mM DTT, 5 µg salmon sperm DNA ml⁻¹, and 5% (v/v) glycerol). The fractions were analyzed by tricine-SDS-PAGE, the pure fractions were harvested, and washed with 0.9% NaCl. The pellets were acid digested with 10 ml HNO₃ (trace metal grade) overnight at 80 °C. The copper concentrations of the digested samples were measured by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo X II).

Electrophoretic mobility shift assays (EMSA)
PCR fragments containing the copper gene cluster promoter (145 bp) were amplified and labeled with [γ-32P]ATP using T4 polynucleotide kinase (T4 PNK; NEB). EMSA reactions were carried out in 10 µl EMSA buffer (20 mM Tris/HCl pH 8.0, 50 µg BSA ml⁻¹, 50 mM NaCl, 1 mM DTT, 5 µg salmon sperm DNA ml⁻¹, and 5% (v/v) glycerol). Increasing concentrations of CsoR were incubated for 10 min at room temperature with the labeled promoters in both the presence and absence of 10 µM CuSO₄. DTT was added to the reactions. All of the
samples were loaded onto an 8% polyacrylamide gel and electrophoresed for 1 h at 100 V in 45 mM Tris/borate buffer (without EDTA) at pH 8.0. The gel was scanned with a luminescent image analyzer (ImageQuant LAS400 mini).

**DNase I footprinting assay with FAM-labeled primers**

DNase I footprinting assays were performed according to Wang et al. After agarose gel electrophoresis, the FAM-labeled probes were purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and were quantified with the NanoDrop 2000C (Thermo, USA). For each assay, 1 pmol (250 ng) of probes was incubated with differing amounts of CsoR protein in a total volume of 40 µl in the same buffer that was used for the previously described EMSAs. After incubation, 10 µl of a solution containing 0.015 U of DNase I (Promega) and 100 nmol of freshly prepared CaCl₂ were added, and a further incubation was performed for 1 min at 25°C. The reaction was stopped by adding 140 µl of DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% sodium dodecyl sulfate). The samples were extracted with phenol-chloroform and precipitated with ethanol, and the pellets were dissolved in 10 µl of Milli-Q water. Preparation of the DNA ladder, electrophoresis and data analyses were performed as described above, with the exception that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

**H₂O₂ and cumene hydroperoxide (CHP) sensitivity test**

The TGY liquid cultures were inoculated at an OD₆₀₀ value of 0.1 using overnight cultures. When an OD₆₀₀ value of 0.6 was reached, aliquots (1 ml) of the cultures were supplemented with 0 mM or 40 mM H₂O₂ or 0 mM or 1 mM CHP. Seven microliters of each dilution was spotted onto TGY solid plates after the addition of H₂O₂ or CHP for 30 min.

**Results**

**D. radiodurans** R1 encodes a number of putative copper homeostasis genes

The bioinformatic analysis showed that the majority of sequenced *Deinococcus* genomes contain a unique copper gene cluster. The *D. radiodurans* R1 copper gene cluster encodes a putative copper-sensing repressor protein (designated CsoR), a putative copper chaperone (designated CopZ) and an ATPase (designated CopA), which are conserved in all other *Deinococcus* species, in addition to two genes of unknown functions that are unique to *D. radiodurans* R1. Analyses of the flanking regions of the copper gene cluster identified promoter sequences and a GC-rich pseudo-inverted repeat sequence between the *copA* and *copZ* genes (Fig. 1(a)). RT-PCR that was performed using intergenic primer sets confirmed that the four genes (*copZ*, DR_2451, DR_2452 and *csoR*) are transcribed as a polycistronic operon (Fig. 1(b)).

The *copA* gene (DR_2453) encodes a probable heavy metal transporting ATPase with 40% identity to *E. coli* K-12 CopA²⁰ and 47% identity to *B. subtilis* CopA²¹. The predicted CopA polypeptide contains four conserved domains, including two amino terminal heavy metal-associated domains, an E1-E2 ATPase domain and a carboxyl-terminal haloacid dehalogenase-like hydrolase domain. *D. radiodurans* R1 CopZ (DR_2452) shares significant sequence similarity with CopZ of *B. subtilis* (46%, identity), which is a metallochaperone that plays a demonstrated role in copper homeostasis and has been shown to interact and exchange copper with the *B. subtilis* copper exporter, CopA.¹¹,²² The probable CopZ has a heavy metal-associated domain that may bind copper. The *copR* gene (DR_2449) encodes a 102-amino acid protein that belongs to the DUF156 superfamily similar to the *M. tuberculosis* and *B. subtilis* CsoR proteins,⁴,⁷ which show high homologies to the CsoR of *D. radiodurans* R1 with identities of 36% and 47%, respectively (Fig. 2). Of the three residues (C36, H61 and C65) that are involved in copper ion binding by *M. tuberculosis* CsoR, two are conserved in the DR_2449 protein (C49 and H74) (Fig. 2). Unlike *copA*, *copZ* and *csoR*, the two other genes (DR_2451 and DR_2450) in the *D. radiodurans* R1 copper gene cluster are not conserved between species. DR_2451 and DR_2450 are predicted to encode two proteins of unknown function and thus have been designated as ORF1 and ORF2, respectively (Fig. 1).

Copper gene cluster is induced by copper

To examine the metal-responsive expression of this copper gene cluster, the wild-type *D. radiodurans* R1 strain was cultured in defined medium that was supplemented with 1 mM CuSO₄, and the expression levels of the three genes (*copA*, *copZ* and *csoR*) in the gene cluster were determined. CuSO₄ supplementation induced significant increases in the expressions of the three genes (Fig. 3(b)). Further analyses of the CuSO₄ responsiveness of the gene cluster revealed a gradual response with increasing concentrations of supplemented CuSO₄ (data not shown). Hence, this gene cluster is specifically induced in response to elevated copper levels.

**CopA is required for D. radiodurans** R1 copper resistance and oxidative resistance

To investigate the physiological functions of the *copA* gene, a *copA* mutant (*AcopA::kan*) was generated by the insertion of the *kan* cassette, resulting in the removal of 86% of the *copA*-coding region. The growth of this
strain in defined medium without CuSO₄ was nearly similar to that of the wild-type. However, copper is toxic to Deinococcus in high concentrations, as shown by the inhibition of growth at 2 mM CuSO₄ (Fig. 3(a)). In contrast, the copA mutant displayed significant growth inhibition at 0.1 mM CuSO₄ and therefore demonstrated substantially increased sensitivity to exogenous copper compared with the wild-type strain (Fig. 3(a)). The complementation of the copA::kan mutation with the pZ3CopA plasmid restored the ability of the mutant to grow in presence of CuSO₄ at comparable rates as the wild-type strain (data not shown), confirming that CopA confers copper resistance to D. radiodurans R1. Additionally, copA expression was significantly activated when the wild-type strain exposed to CuSO₄ (Fig. 3(b)). Taken together, these data confirm that the copper gene cluster responds specifically to copper, which is the only metal against which CopA affords protection.

The increased sensitivity of the copA-mutated strain to exogenous copper implies an increase in cytosolic copper levels that are consistent with the loss of CopA-mediated copper export. This finding was supported by the copper quotient of the wild-type and AcopA::kan mutant strains that were grown in defined medium with and without 0.2 mM CuSO₄ as shown by the ICP-MS analysis (Fig. 3(c)). The results revealed that the strain lacking copA accumulated more copper than did the wild-type strain following copper exposure, implying that the mutant was impaired in its ability to remove copper from the cell. These results therefore support the designation of CopA as a specific copper exporter in D. radiodurans R1.

Some types of copper transporting ATPases have been reported to be involved in oxidative resistance mechanisms, such as those that are associated with the assembly of cytochrome c oxidase, which is a copper-requiring redox enzyme. To test the oxidative role of CopA in D. radiodurans R1, the responses of the mutant and wild-type strains to oxidative stressors were measured. The copA mutant strain was observed to exhibit substantially increased sensitivity following the H₂O₂ treatment (Fig. 4). Similar results were observed when CHP was used for the oxidative treatment (Fig. 4).

CsoR binds to copper gene cluster promoter (Pcop) in vitro

To confirm that CsoR binds to the Pcop in vitro, EMSAs were performed with increasing concentrations of purified CsoR and a DNA fragment containing Pcop in a reducing electrophoresis buffer. The purification of CsoR has been described in the Materials and Methods section above. CsoR bound with high affinity to Pcop, and this binding was eliminated in the presence of 10 µM CuSO₄ and 1 mM DTT (Fig. 5). No shift in the mobility of the negative control fragment was observed at these concentrations of CsoR, indicating that binding was specific (Fig. 5).

The ESMA results showed that CsoR interacted with the Pcop in vitro. To determine the specific CsoR-binding sequences, we performed DNase I footprinting experiments using a DNA probe comprising the Pcop in the presence or absence of copper ions. As shown in Fig. 6, two CsoR binding sites (CCTCTCC-GG-GGGATGGTTGCA and CCTTGACATCCCCCCGGCTGGTCTACCC) were identified. In agreement with the EMSA experiments, the protection of the promoter regions was lifted once copper was supplied to the reaction (Fig. 6). These results are consistent with the role of CsoR as a repressor. Taken together, the EMSA and DNase I footprinting data suggest that CsoR specifically binds to the Pcop, acting as a repressor of the transcription of the copper gene cluster. However, in the presence of copper, CsoR is displaced from or unable to bind to the Pcop, resulting in the deregulation of the gene cluster.

CopZ tempers transcription of copA

CopZ has been reported to be a metallochaperone that has the ability to exchange copper ions with CopA in B. subtilis. Given the characterized role of copper chaperones in copper channeling within the cell, a mutant lacking CopZ may exhibit reduced copper tolerance. To examine the role of CopZ in D. radiodurans R1, a copZ-mutated strain (AcopZ::kan) was generated, and the copper tolerance of the resulting strain was compared with that of the wild type when grown in defined media that were supplemented with various concentrations of CuSO₄. Compared with the copA mutant, no significant differences were detected in the abilities of the strains to tolerate copper exposure (Fig. 3(a)). An ICP-MS analysis of the copper quotients of these strains that were grown with and without copper supplementation revealed that the copZ mutant accumulated less copper than did the wild-type strain following copper exposure (Fig. 3(c)) and that the copper concentrations of both strains increased in response to the elevated copper levels in the medium (data not shown). However, in both the absence and presence of copper supplementation, the expression of copA in the copZ mutant strain was up-regulated significantly compared with that of the wild-type strain (data not shown).

Discussion
In this study, we have identified a copper gene cluster, which consists of several copper-responsive genes that play a vital role in copper resistance in \textit{D. radiodurans} R1 (Fig. 1(a)) and investigation of its regulatory mechanisms has revealed several novel features.

Our data indicate that CopA is a P1-type copper-transporting ATPase with high similarity to P1-type copper-transporting ATPases in other organisms. The designation of CopA as a specific copper-exporting protein is based on several pieces of evidence, including the following: (i) the hypersensitivity of the \textit{AcopA} mutant strain to exogenous copper but not other metals and the successful complementation of this phenotype by transformation using a plasmid containing \textit{copA}, (ii) the hyperaccumulation of copper in the \textit{AcopA} mutant strain; and (iii) the up-regulated transcription of \textit{copA} following copper exposure (Fig. 3(a)). All of these results are in agreement with previous studies involving \textit{copA} \textit{R1}, which was also found to be up-regulated in response to excess copper.

The transcription of the copper gene cluster in \textit{D. radiodurans} R1 is repressed by CsoR, which is the sole \textit{Deinococcus} ortholog of CsoR from \textit{M. tuberculosis} and \textit{B. subtilis}, under low copper conditions. We also revealed that \textit{D. radiodurans} R1 \textit{copR} transcription is induced by copper, which is similar to that which occurs with \textit{M. tuberculosis} \textit{csoR} but differs from that with \textit{B. subtilis} \textit{csoR}, which is not transcribed in response to copper. In support of previous findings by Liu \textit{et al.}, CsoR appears to be the principal copper regulator in \textit{D. radiodurans} R1 because this organism lacks other types of known bacterial copper regulators, including CopY and CueR, which are found in \textit{E. hirae} and \textit{E. coli}, respectively. CsoR \textit{R1} shares 36\% identity with CsoR \textit{R1}, although CsoR \textit{R1} lacks the C-terminal extension of CsoR \textit{R1}. The EMSA and DNase I footprinting assays demonstrated the specific binding of CsoR to the \textit{Pcop} \textit{in vitro} (Fig. 6). CsoR protects two regions of the \textit{Pcop}, and these results contrast with CsoR \textit{R1}, which protects only one 32-bp region of the \textit{Pcop}. One protected region includes an imperfect inverted repeat (CCTCTCC-GG-GGGGATGGTTGCA), and the other has no specific characteristics (CCTTGACATCCCCCCCCGCTGGGTCTACCC). One explanation for these results is that one of these two regions plays a role in the transcription of \textit{copA}, and the other involves the transcription of \textit{copZ} because \textit{copA} and \textit{copZ} have opposite transcriptional directions. An alternative explanation is that one of the two regions is a strong promoter, while the other is a weak promoter.

CopZ has been identified as a copper chaperone that has the ability to shuttle intracellular copper ions to their intended targets, thus exporting excess copper and maintaining cellular homeostasis. For example, CopZ of \textit{B. subtilis} is able to interact with and donate copper ions to CopA, which is a copper exporter. Based on similarities with other bacterial CopZ sequences, the CopZ of \textit{D. radiodurans} R1 is expected to be involved in routing cellular copper to the CopA exporter and transferring copper to CsoR by ligand-exchange reactions in a manner that is analogous to the delivery of copper to the CopY of the \textit{E. hirae} \textit{cop operon}. However, our data suggest that this is not the case for the \textit{D. radiodurans} R1 CopZ. In fact, the \textit{copZ} mutation actually increased the transcription of \textit{copA} in the absence of copper and also substantially increased its transcription by several fold in its presence. These data imply that CopZ in \textit{D. radiodurans} R1 suppresses the expression of \textit{copA}. However, the deletion of \textit{copZ} results in the decreased accumulation of copper compared with the wild-type strain. The lack of altered copper tolerance and decreased copper accumulation in the \textit{copZ} mutant suggest that the elevated expression of \textit{copA} in this strain may compensate for the loss of CopZ function, resulting in lower cellular copper concentrations compared with the wild type, although also resulting in a similar copper tolerance. As shown in Fig. 3(a), \textit{copZ} was the mostly highly up-regulated gene compared with \textit{copA} and \textit{copA} following copper exposure. Hence, it is highly likely that CopZ plays a role in copper resistance by binding copper ions directly and transferring them to CopA, which could then export excess ions and suppress the transcription of \textit{copA} because CsoR is rendered unable to sense copper.

In this study, we have attained a preliminary understanding of the copper homeostasis system of \textit{D. radiodurans} R1. The core element in this system is a gene cluster that mainly consists of three genes, including \textit{copA}, \textit{copZ} and \textit{csoR} (Fig. 7). Unlike the genetic organization of the copper operon in \textit{E. hirae}, which is the most understood copper homeostasis system in Gram-positive bacteria, \textit{copA} and the other two genes, \textit{copZ} and \textit{csoR}, are transcribed in opposite directions. Whether these different gene organizations have effects on copper homeostasis in cells remain to be elucidated. To date, the manner by which copper ions enter the cell remains unknown, but in \textit{E. hirae}, they have been shown to enter cells via CopA. When excess cytoplasmic copper ions enter cells, the ions may bind to CopZ, which can then be provided to CopA for export and to the CsoR repressor to induce the transcription of the copper gene cluster. When copper ions are donated to CsoR, CsoR is released from the promoter and induces the transcription of the genes in the gene cluster. The \textit{copZ} gene is assumed to encode a copper chaperone that has a function that is similar to that of CopZ in \textit{E. hirae}. However, its function in \textit{D. radiodurans} R1 has not been precisely identified and thus requires further investigation. Although the exact pathway that is followed by the copper ions and the functioning of CopZ are unknown, we have demonstrated that the copper gene cluster enables \textit{D. radiodurans} R1 to grow in excess copper conditions and maintain homeostasis.
biosynthesis. Therefore, most organisms have developed mechanisms to maintain copper homeostasis within their cells and prevent the damage that occurs following the generation of reactive oxygen species.

The copper gene cluster in *D. radiodurans* R1 may counteract copper toxicity to cells. Our data showed that the copper gene cluster, including *copA*, *copZ* and *csoR*, was induced in response to copper stress and that is protected the cells from copper toxicity. Previously undefined mechanisms of copper toxicity may be demonstrated by this unique transcriptional response to copper. As an extremophile, *D. radiodurans* R1 is highly resistant to oxidative stress, such as that due to H$_2$O$_2$, but the mutant strain lacking *copA* was shown to be extremely sensitive to H$_2$O$_2$, which is similar to observations in other bacteria. Collectively, these results demonstrate that the copper gene cluster and the *copA* gene in particular are important for copper homeostasis and oxidative resistance in cells. However, the mechanisms underlying the regulation of the copper gene cluster and CopA in association with oxidative resistance require further investigation.

### Conclusion

The bacterium *D. radiodurans* R1 is known for its extreme tolerance to various environmental stresses. Copper homeostasis is very important to ensure for the maintenance of proper copper ions concentrations in cells, but it had not yet been studied in *D. radiodurans* R1. In this study, we identified a copper-responsive gene cluster that encodes CopA, which is a copper-transporting P1-type ATPase, CopZ, which is a copper metallochaperone, and CsoR, which is a copper-sensing repressor. Our data suggest that the copper-responsive gene cluster is required for copper homeostasis and that it contributes to oxidative resistance in *D. radiodurans* R1. This current analysis has provided molecular insight into the manner by which *D. radiodurans* R1 handles copper. This study sets the foundation for further research investigating the role of metal homeostasis in stress resistance in *D. radiodurans* R1.

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### Notes and references

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### References

A copper-responsive gene cluster is required for copper homeostasis and contributes to oxidative resistance in *Deinococcus radiodurans* R1

Zhongchao Zhao,‡ a, b Zhengfu Zhou,‡b Liang Li,‡ Xianyi Xian,‡ Xibin Ke,‡ Ming Chen* b and Yuxiu Zhang* a

Fig. 1

![Diagram](image)

**Fig. 1.** (a) The structure of the *D. radiodurans* R1 copper gene cluster encoding *csoR*, which is a putative copper transcriptional regulator, *ORF1* and *ORF2*, which are two hypothetical proteins, *copZ*, which is a putative copper chaperone, and *copA*, which is a copper-translocating P-type ATPase. The intergenic region between *copZ* and *copA* is indicated between the two arrows. The nucleotides in bold indicate the core promoter sequences, and the boxed nucleotides denote the GC-rich pseudo-inverted repeat.

(b) RT-PCR analysis to confirm the polycistronic nature of the *D. radiodurans* R1 copper gene cluster. RT-PCR was performed on total RNA isolated from the *D. radiodurans* R1 wild-type strain that was grown in TGY + 1 mM CuSO₄ with (cDNA) and without (RNA) reverse transcriptase treatment using the 51-52, 50-51, and 49-50 intergenic region primer pairs (see Fig. 1(a)). gDNA was used as a positive control.
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Fig. 2

Fig. 2. Amino acid sequence alignments of D. radiodurans R1 (Dr), M. tuberculosis (Mt), B. subtilis (Bt) and T. thermophilus (Tt) CsoR proteins. The sequences were aligned using Clustal X2.23 The secondary structure was predicted using SWISS-MODEL,24 and the figure was generated with ESPript 3.25 The percent identities (id.) to Dr CsoR are indicated on the right. The positions of the conserved copper-coordinating residues of CsoR are indicated by asterisks.
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Fig. 3.

(a) Effects of increasing concentrations of Cu²⁺ on growth of *D. radiodurans* R1 (wild-type, □), ∆*copZ* (*copZ* mutant, ○) and ∆*copA* (*copA* mutant, ▲). Turbidity (OD₆₀₀) was determined after 16 h of growth in TGY liquid medium at 30 °C. (b) Cu-dependent regulation of *copA*, *copZ* and *csoR* expression. Gene expression levels were determined by qPCR that was performed using RNA extracted from cultures grown in the presence of Cu²⁺ and standardized to the expression levels of 16s rRNA. (c). copper concentrations of *D. radiodurans* strains exposed to 0.2 mM CuSO₄. Data represent the means ± SE of three independent experiments.
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Fig. 4

Fig. 4. Effects of oxidative stress on growth of *D. radiodurans* strains. Growth of serial dilutions (noted at the bottom) of *D. radiodurans* R1 (WT), *copZ* mutant (ΔcopZ) and *copA* mutant (ΔcopA) on solid medium after treatment with 40 mM H$_2$O$_2$ and 1 mM CHP, separately, for 30 min. These experiments were performed on at least three independent occasions, and representative results are shown.
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Fig. 5

![Image of CsoR EMSAs](image_url)

**Protein (ng):** 0 150 300 600 900 900 (Cu⁺) 0

**Shifted** →

**Free** →

**Fig. 5.** CsoR EMSAs were carried out with increasing amounts of protein and a constant amount of the specific copper gene cluster promoter Pcop; 10 μM CuSO₄ in the presence of 1 mM DTT was added to the last lane to test the effects of copper on DNA-binding activity.
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Fig. 6

Identification of the CsoR protected sequences in the D. radiodurans R1 copper gene cluster promoter region using a DNase I footprinting assay. The probes were labeled with the FAM dye and are described in Materials and Methods. The region protected by CsoR from DNase I cleavage is enlarged and shown in the panel. The four sequencing results (G, A, T and C) are indicated by four different colors separately and then merged together.
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Fig. 7

Copper homeostasis in *D. radiodurans* R1. The manner by which copper enters the cell and the exact function of CopZ are unknown. When excess copper ions enter the cell, they are provided to CopA for export or to the CsoR repressor to induce the transcription of the gene cluster. When CsoR senses the presence of copper ions, it is released from the promoter and induces the transcription of the genes in the copper gene cluster.