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Genome-Wide Response of *Deinococcus radiodurans* on Cadmium Toxicity

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***Deinococcus radiodurans* is extremely resistant to various genotoxic conditions and chemicals. In this study, we characterized the effect of a sublethal concentration (100 μM) of cadmium (Cd) on *D. radiodurans* using a whole-genome DNA microarray. Time-course global gene expression profiling showed that 1,505 genes out of 3,116 total ORFs were differentially expressed more than 2-fold in response to Cd treatment for at least one timepoint. The majority of the upregulated genes are related to iron uptake, cysteine biosynthesis, protein disulfide stress, and various types of DNA repair systems. The enhanced upregulation of genes involved in cysteine biosynthesis and disulfide stress indicate that Cd has a high affinity for sulfur compounds. Provocation of iron deficiency and growth resumption of Cd-treated cells by iron supplementation also indicates that CdS forms in iron-sulfur-containing proteins such as the [Fe-S] cluster. Induction of base excision, mismatch, and recombinational repair systems indicates that various types of DNA damage, especially base excision, were enhanced by Cd. Exposure to sublethal Cd stress reduces the growth rate, and many of the downregulated genes are related to cell growth, including biosynthesis of cell membrane, translation, and transcription. The differential expression of 52 regulatory genes suggests a dynamic operation of complex regulatory networks by Cd-induced stress. These results demonstrate the effect of Cd exposure on *D. radiodurans* and how the related genes are expressed by this stress.**

Keywords: *Deinococcus radiodurans*, cadmium, transcriptome, stress response, iron

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Cadmium (Cd) is widely used in various industrial processes, as a pigment for certain paints and plastics as well as in the production of nickel-Cd batteries [18]. However, Cd is a biologically nonessential divalent hazardous metal that causes serious cellular damages. The molecular mechanisms underlying Cd toxicity are diverse and complex. Unlike many other trace metals (*e.g.*, Cr, Fe, and Cu), Cd is not a redox active metal, since it does not directly catalyze electron transfer reactions to produce reactive oxygen species (ROS). However, it has been speculated that Cd causes damage to cells indirectly by the generation of ROS, which leads to unspecific oxidation of proteins and membrane lipids or causes DNA damage by depleting free-radical scavengers such as glutathione, metallothioneins, and protein-bound sulfydryl groups [37]. Intracellular Cd is also known to inhibit the DNA mismatch repair system [19] and the synthesis of nucleic acids and proteins [25, 26] as well as to damage single-stranded DNA and membrane integrity [25, 34]. Considering the increasing release of Cd into the environment and its toxic effects on living organisms, it is necessary to understand how organisms respond to Cd exposure.

Living organisms have developed various defense mechanisms to cope with environmental stresses by regulating the expression of genes involved in the defense response [29]. Alterations of global gene expression patterns and proteome composition during Cd stress have been described for a number of organisms. In *Escherichia coli*, Cd exposure induces several stress response systems, including those for heat shock, oxidative stress, stringent response, cold shock, and SOS [4, 10, 41]. Global response to Cd exposure involves profound changes in gene expression, such as an arrest in the synthesis of ribosomal proteins and zinc-binding proteins, a shift to anaerobic metabolism, and

upregulation of genes related to the damage repair system, cysteine and iron-sulfur cluster biosynthesis, and storage of iron and detoxification of Cd by efflux [16, 42]. A recent transcriptomic analysis in *E. coli* suggested that Cd toxicity results from its higher affinity to sulfur compounds, such as glutathione that is necessary for full Cd resistance, than to nitrogen- or oxygen-rich biological compounds [16]. *Saccharomyces cerevisiae* exposed to Cd substantially reprogram their transcriptome and proteome to redirect various biological pathways [39]. The major Cd responsive functional categories were those related to sulfur and glutathione biosynthesis, transcription, intracellular transport and sorting, regulation of carbon metabolism, and metal-ion homeostasis and transport [39]. Cd also induces protein glutathionylation that might lead to apoptosis of mutated cells [13].

D. radiodurans is a well-known extremophile characterized by resistance to a number of agents and conditions that damage DNA, including high-level ionizing radiation, desiccation, and DNA-damaging chemicals [8, 36]. Robust DNA repair processes reconstituting a functional genome from heavily damaged DNA fragments are the key elements for this resistance. Cd is one of the most toxic heavy metals able to produce genotoxic and mutagenic events by inhibiting various DNA repair processes [12]. However, the response of *D. radiodurans* to this potent mutagen has not yet been reported. In this study, we exposed *D. radiodurans* to a sublethal concentration of Cd and analyzed a time-course transcriptome to assess the genome-wide response of Cd toxicity.

Materials and Methods

Strain, Growth Conditions, and Medium

D. radiodurans R1 ATCC13939 was used throughout this work. Cultures were grown in TGY (1.0% tryptone, 0.5% yeast extract, and 0.1% glucose) liquid medium or on TGY plates supplemented with agar (1.5%). A single colony of *D. radiodurans* was suspended in 10 ml of TGY medium and grown as a preculture in a shaker (200 rpm) at 30°C for 24 h.

Microarray Procedures

Time-course microarray experiments were repeated with two different biological samples and dye swapping using total RNAs obtained from *D. radiodurans* cultivated in the presence of 100 μM $CdCl_2$ (Sigma, St. Louis, MO, USA) for 15, 30, 60, and 120 min. The seed culture was diluted 100-fold and grown at 30°C with shaking (200 rpm) until an optical density ($OD_{600\ nm}$) of 0.6 was reached. The culture was equally divided into two Erlenmeyer flasks and $CdCl_2$ was added to one sample to a final concentration of 100 μM. After the addition of Cd, both the Cd-treated and untreated cultures were harvested at the indicated times by centrifugation. An aliquot of bacterial culture was immediately added to an ice-cold 5% phenol–95% ethanol mixture at a 1:10 (v/v) ratio and gently inverted to arrest bacterial growth and preserve RNA integrity. Samples were allowed to remain on ice for 5 min and then spun at 4,000 rpm for 10 min. The pellets were immediately frozen in liquid nitrogen and stored at −80°C until use.

Microarray experiments, including RNA extraction and purification, were carried out as described previously [20]. Briefly, total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further purified with an RNeasy mini purification kit and an RNase-Free DNase I set (QIAGEN, Valencia, CA, USA). The quality and amount of the total RNAs were measured using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA (50 μg) was converted to Cy3- or Cy5-labeled cDNA using SuperScript II reverse transcriptase (Invitrogen) and an aminoallyl post RNA labeling kit (GenChem, Korea). The labeled cDNA pools were mixed, dried, and dissolved with hybridization buffer. The probes were denatured by heating and hybridized simultaneously to a prehybridized DNA microarray slide for 16 h at 42°C in a MAUI Hybridization System (BioMicro Systems, Salt Lake City, UT, USA) with gentle mixing.

The cDNA microarray of *D. radiodurans* contained about 97.8% of the total open reading frames that were spotted as duplicated sets on a slide. Detailed information on the design of *D. radiodurans* cDNA microarrays is described in Joe *et al*. [20].

Data Analysis

The hybridized slides were scanned on a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA) at both 532 nm and 635 nm visible light. Using GenePix Pro 6.0 software (Molecular Devices), the spot intensities were quantified for each channel. Actual signal intensity was calculated by subtracting the local mean background intensity of each spot from the mean signal intensity of each spot. Any spot with an adjusted signal intensity lower than its median background intensity in both channels was excluded from further analysis. All of the calculations and normalizations were performed using the GeneSpring GX 11.0 software package (Agilent Technologies). The intensity-dependent locally weighted linear regression (Lowess) normalization method was used to adjust the control value for each measurement [33]. The t-test (p-value cutoff < 0.05) was then applied to the data to find genes with highly reproducible gene expression. Genes with a p-value of > 0.05 were excluded from consideration. Genes that had more than a 2-fold increase or decrease in expression as compared with the reference were classified as up- or down-regulated. The Institute for Genomic Research Comprehensive Microbial Resource was used for the functional categorization of the data (TIGR, http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi). The data obtained from this experiment are available using the accession number GSE20383 in the Gene Expression Omnibus Database (GEO, http://www.ncbi.nlm.nih.gov/geo/).

Confirmation of Microarray Data

Validation of microarray data was performed by real-time quantitative PCR (qRT-PCR) on 30 differentially expressed genes, including 17 upregulated and 13 downregulated genes. Real-time PCR was performed on a SmartCycler system (Cepheid, Sunnyvale, CA, USA). Primers for the qRT-PCR analysis (Table S1) were designed using the Web-based Primer3 software (http://frodo.wi.mit.edu/primer3/). The cDNA was produced with the same total RNA used for the microarray experiments. SYBR green qRT-PCR assay was performed with a 25-μl PCR mixture volume containing 12.5 μl of SYBR

Premix Ex Taq (Takara Bio Inc., Japan), 0.2 pmol specific primer sets, and 2 μl of cDNA sample. PCR reactions were performed as follows: one cycle of 94°C for 5 min, and then 45 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 20 s. Fluorescence was measured at the end of the 72°C incubations and analyzed using the SmartCycler software (Cepheid). Following the amplifications, melting-curve analysis (with the temperatures ranging from 60 to 95°C in 0.5°C increments) of PCR products was performed to ensure the specificity of the PCR. The relative expression level of each gene was normalized to *gap*, the gene encoding a glyceraldehyde 3-phosphate dehydrogenase, of *D. radiodurans* whose expression level remained stable irrespective of Cd addition in microarray data. Relative gene expression levels were obtained by using the comparative threshold cycle ($\Delta\Delta C_T$) method [22]. Each gene was assayed in duplicate from three independent biological samples and the average C_T value was used for further comparison. Gene expression was calculated as $2^{-\Delta\Delta CT}$, where values for $\Delta\Delta C_T$ ($\Delta C_{T,Cd-} - \Delta C_{T,Cd+}$) were obtained by subtracting the mean C_T value of the specific gene tested from the mean C_T value of the *gap* gene. Controls to ensure that there was no contaminating genomic DNA in the cDNA (cDNA reaction mixtures generated using RNA with no reverse transcriptase) as well as controls to identify any possible primer dimer artifacts (qRT-PCR mixtures containing primers alone) were also run with each set of qRT-PCR.

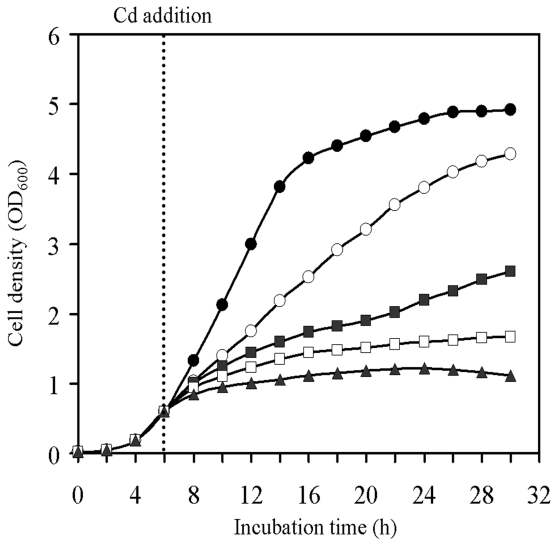

Fig. 1. Effect of Cd on the growth of *D. radiodurans*.
Cells were grown in TGY medium to an optical density ($OD_{600\ nm}$) of 0.6 at 30°C and various concentrations of Cd were added to the cultures: the concentrations were 0 μM (solid circle), 10 μM (open circle), 25 μM (solid square), 100 μM (open square), and 1 mM (solid triangle). The mean values of the results from three experiments are shown. Growth was monitored as the absorbance at 600 nm.

RESULTS AND DISCUSSION

Effect of Cd on the Growth of *D. radiodurans*

To analyze the changes in gene expression in response to Cd stress, an optimal sublethal Cd concentration was experimentally determined in order to induce extensive differential gene expression without compromising the viability of the cells. Initially, the effects of Cd treatment on the growth and viability of exponentially growing *D. radiodurans* were investigated to find the proper condition. Exponentially growing *D. radiodurans* cultures were treated with $CdCl_2$ at concentrations ranging from 10 μM to 1.0 mM. As shown in Fig. 1, the growth of *D. radiodurans* was gradually retarded with increasing concentrations of Cd and almost completely inhibited at concentrations above 100 μM. However, *D. radiodurans* maintained cell viability when the Cd concentration was increased up to 1.0 mM, as indicated by the CFUs at 2, 4, 6, and 24 h (data not shown). Cd (273 μM) exposure caused transient growth inhibition of *E. coli*, and it resumed growth at a slower rate than before the addition of Cd [10]. *S. cerevisiae* also showed growth retardation after Cd (300 μM) addition, but cell death was observed with the addition of 1 mM of Cd [28]. It appears that *D. radiodurans* has no adaptive response to Cd. Therefore, for time-course transcriptome analyses, a sublethal Cd concentration was chosen (100 μM) that impacted *D. radiodurans* growth rate but was not lethal, as determined by viable cell counts.

Overview of Transcriptomic Response in Response to Cd

To counteract Cd toxicity, living organisms have evolved several defense mechanisms that are mainly mediated by massive changes in gene expression [16, 22, 29, 39]. To gain insights into the cytotoxic effects in *D. radiodurans* in response to Cd exposure, cDNA microarray analysis was utilized to monitor temporal changes in transcriptional profiles. We used *D. radiodurans* cDNA microarrays, which contain 3,116 probes corresponding to 97.8% of all *D. radiodurans* ORFs in duplicate [20]. Total RNA samples were collected from *D. radiodurans* cells after 15, 30, 60, and 120 min treatments with 100 μM Cd. The RNA samples from the untreated cells cultivated at the same time were used as a reference. For all analyses, dye-swapping experiments were performed to avoid dye bias, and thus eight intensity values were obtained for each ORF. After the filtering process, a total of 2,061 transcripts were subjected to further analyses.

The microarray data revealed that Cd caused a significant impact on gene expression, with 1,505 genes from Cd-treated cells being up- or down-regulated more than 2-fold ($p<0.05$) during at least one timepoint compared with reference cells (Table S2). In total, 893 genes (401 upregulated and 492 downregulated) at 15 min, 1,068 genes (486 upregulated and 582 downregulated) at 30 min, 1,105 genes (536 upregulated and 569 downregulated) at 60 min, and 1,036 genes (523 up regulated and 513 downregulated) at 120 min displayed differential expression (≥2-fold) in

response to Cd treatment. This corresponds to 47% of all *D. radiodurans* genes. To obtain an overview of the impact of Cd exposure on bacterial physiology, differentially expressed genes of known function were classified according to their biological role (Fig. 3 and Table S3). Our data suggest that the expression of genes involved in essential components of protein and membrane synthesis, posttranslational modification, iron uptake, and DNA repair were significantly affected. Clear biases towards Cd-dependent expression of genes involved in few specific cellular processes, such as DNA metabolism and protein synthesis, were observed and the functional categories exhibiting significant changes in gene expression will be discussed individually in the following sections.

Validation of Microarray Data

We used qRT-PCR to verify the microarray data by analyzing transcript levels for 30 genes (Fig. 2). These included genes whose expression appeared to be increased (DR_0268, DR_0332, DR_0577, DR_0821, DR_1885, DR_2165, DR_2276, DR_2535, DR_2623, DR_A0162, *ddrB*, *ftsH-1*, *mrp*, *mazG*, *ribD*, *ruvB*, and *uvrB*) and decreased (DR_0115, DR_0116, DR_0201, DR_0337, DR_0399, DR_1545, DR_1935, DR_2362, DR_2559, DR_A0141, *lspA*, *hbs*, and *hpcD*) over time after Cd exposure. The expression ratio of the selected genes at each timepoint correlated well (R^2>0.9) with those obtained from the microarray results, suggesting that the time-course microarray data were an accurate reflection of the temporal gene expression profile in *D. radiodurans*.

Effect of Cd on Genes Involved in Cell Growth

Cellular growth was arrested in cells treated with Cd (Fig 1). When cell growth is impaired, restriction of protein synthesis is often observed. The majority of the genes for protein translation machinery underwent the greatest changes in expression following exposure to Cd (Fig. 3 and Table S3). Specifically, 50 of the 55 genes encoding ribosomal proteins showed continuous downregulation by Cd, suggesting a shutdown of protein synthesis. The

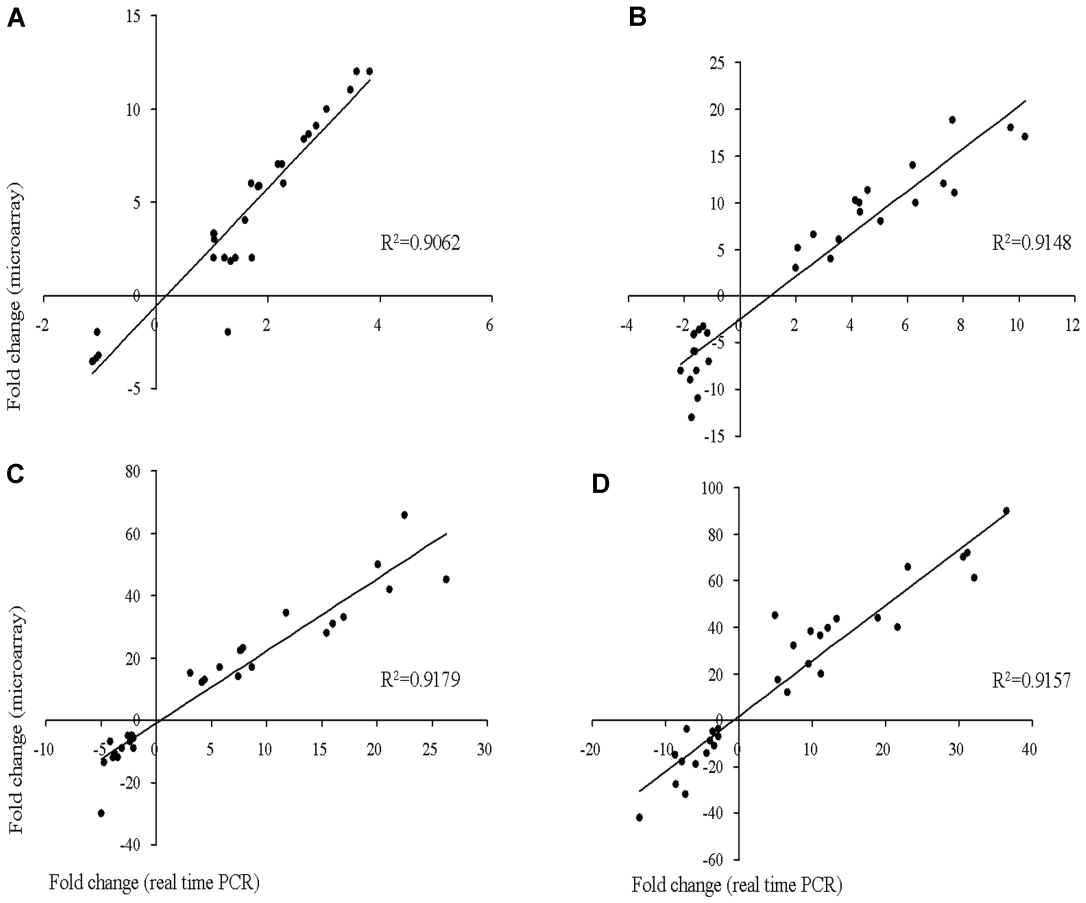

Fig. 2. Validation of microarray results by qRT-PCR.

Shown are 17 genes upregulated and 13 genes downregulated by Cd exposure. Fold changes are shown [(expression level without Cd)/(expression level with Cd)] at each time profile: A, 15 min; B, 30 min; C, 60 min; and D, 120 min after Cd treatment. The level of each transcript was normalized to that of *gap* (DR1343), a relatively stable transcript. The induction of the transcripts in each sample is expressed relative to the amount of the transcripts from the corresponding non-treated sample. This result is a representative of three independent experiments.

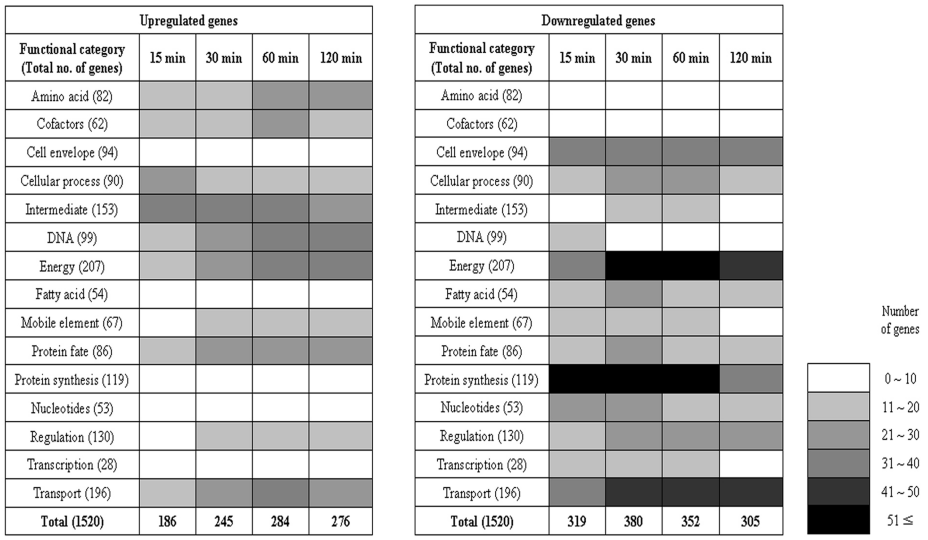

Fig. 3. Distribution of differentially expressed genes.
The up- or down-regulated genes at each timepoint were counted after classification into the following functional categories. Amino acid: amino acid biosynthesis and metabolism; Cofactors: biosynthesis of cofactors, prosthetic groups, and carriers; Cell envelope: biosynthesis and degradation of cell envelope components; Cellular process: cell division, adaptation, and detoxification; Intermediate: central intermediary metabolism; DNA: DNA replication, recombination, repair, restriction, and modification; Energy: energy metabolism; Fatty acid: fatty acid and phospholipid metabolism; Mobile element: mobile and extrachromosomal element functions; Protein fate: protein modification, repair, folding, stabilization, and degradation; Protein synthesis: tRNA aminoacylation, synthesis, and modification of ribosomal proteins; Nucleotides: nucleotide biosynthesis and metabolism; Regulation: regulatory function, putative regulatory proteins; Transcription: transcription, RNA processing, and degradation; Transport: transport and binding proteins, putative transporter. The number of total genes is indicated at the bottom of each heat map.

degree of downregulation of ribosome-related genes gradually increased over time but decreased slightly during the longest treatment period (120 min). Previously, a reduction in the growth rate of the *ΔrecG* strain of *D. radiodurans* as well as the repression of 12 r-protein related genes in the *ΔrecG* mutant has been reported [45]. In addition, treating the *ΔrecG* mutant with hydrogen peroxide caused significant downregulation of 33 r-protein related genes. Similarly, downregulation of ribosome-related genes in Cd-treated *E. coli* and *S. cerevisae* was also observed [16, 28, 42]. This finding indicates that the inhibition of protein synthesis is one of the main reasons for the Cd-induced growth impairment and is consistent with the reduction in the overall rate of protein synthesis by Cd [3]. Likewise, a large number of genes involved in biosynthesis of the cell envelope and energy production were also significantly downregulated (Fig. 3 and Table S3). The expression of genes involved in biosynthesis of murein sacculus and peptidoglycan (*murBCDGI*, *mraY*, and *ddlA*), biosynthesis of surface polysaccharides and lipopolysaccharides (*asmG*, *bcsp31*, *glmU*, *kdtB*, *manC*, *mrp*, *mtfB*, *rfbABD*, *tmbC*, and *yvyH*), and surface structure (*hpi* and *pilT*) showed constitutive downregulation by Cd. The expression patterns of genes involved in energy metabolism underwent dynamic changes in response to Cd. Twelve genes of the *nuo*-operon encoding the NADH dehydrogenase complex I, which is responsible for coupling redox chemistry to generate the proton-motive force necessary to provide energy for ATP synthesis, were downregulated by Cd. In addition, a putative operon (DR_0695~DR_0701) encoding V-type ATP synthase units was also significantly downregulated. The repression of genes responsible for energy production also explains the reduction in growth rate caused by Cd exposure. Taken together, the expression profile of genes involved in cell growth could represent a survival strategy of stressed cells to repress their most energy-consuming cellular processes for the benefit of other protective functions.

Expression of Genes Encoding Protein Modifications

A variety of genes encoding proteins required for the refolding and stabilization of denatured proteins underwent significant changes (Fig. 3 and Table S3). The expression of *msrA* encoding methionine sulfoxide reductase involved in the repair of methionine sulfoxide containing proteins was rapidly increased by Cd. Methionine residues in proteins are one of the most sensitive amino acid targets of oxidation by reactive oxygen species (ROS) [5]. Although Cd is unable to generate ROS directly *via* redox reactions with molecular oxygen, the upregulation of *msrA* suggests that Cd can increase the cellular ROS content, which results in oxidative damage of proteins. The expression of genes encoding one of the major molecular chaperone systems, GroEL and GroES, gradually increased over time. The

genes encoding DnaJ-1, DnaJ-2, DnaK, and the GrpE co-chaperone system were also induced but at a lower level than the GroEL and GroES co-chaperone system. The upregulation of *groEL* and *groES* was also detected in heavily irradiated *D. radiodurans* [38]. Among the genes required for protein refolding and stabilization, DR_1114, a putative heat shock protein, is the most highly upregulated gene by Cd, which was also observed in gamma ray exposed cells [38]. Therefore, these results indicate that the GroEL/GroES co-chaperone system and DR_1114 could be the main chaperone system for proteins damaged by Cd in *D. radiodurans*. Four genes encoding subunits of the Clp ATP-dependent protease complex, including ClpB that works with DnaK to prevent protein aggregation, were highly upregulated. In addition, the genes encoding another ATP-dependent protease system, *lon1* and *lon2*, that play a major role in protein quality control by degrading misfolded and oxidatively modified proteins, were highly increased [6]. Lon proteases are also known to serve as a chaperone and to be involved in repairing quinolone-mediated chromosomal DNA lesions [23, 40]. Among the genes involved in proteolysis of abnormal proteins, *aat* encoding a Leu/Phe-tRNA-protein transferase (L/F transferase) is the most highly induced gene. The L/F transferase conjugates a recognition determinant for protein degradation on the N-terminal residue of proteins and produces N-end rule substrates. Finally, modified proteins with the N-terminal signal are degraded by the Clp protease [27]. Additionally, *trxB* and *trxC* encoding thioredoxin reductase and thioredoxin, respectively, which is an important antioxidant system, were highly upregulated. The DR_2085, a putative glutaredoxin catalyzing reduction of protein disulfide bonds, was also highly upregulated. However, genes encoding putative serine proteases (DR_0812, DR_2322, DRA_0064, DRA_0283, DRA_0341, and DRB_0069) and oligopeptidases (*opdA*, *pepQ*, DR_1627, DR_2055, and DRA_0206) were downregulated. The downregulation of genes encoding serine proteases and oligopeptidases may indicate that these proteases are not required for Cd-induced proteolysis but required for normal growth conditions. Regulation of intracellular proteolysis is crucial in many physiological processes, including elimination of abnormal proteins, the maintenance of amino acid pools in cells during stresses, the initiation of appropriate transcriptional responses to cellular stress, and the control of protein life span [14, 15, 27]. The expression profiles of genes involved in various posttranslational mechanisms indicates that *D. radiodurans* rapidly modulates a number of genes required for protein repair and degradation to protect cells from Cd toxicity.

Expression Profile of Genes Involved in Cysteine and Sulfur Metabolism

Sulfur-containing amino acids, such as methionine and cysteine, function as antioxidants and are key components in the regulation of cell metabolism. Cysteine chemistry is important to the enzymatic mechanism of the thiol-disulfide oxidoreductases of the thioredoxin superfamily, such as thioredoxins, glutaredoxin, and protein disulfide isomerase [11]. The expression patterns of all of the genes involved in cysteine biosynthesis, including *cysS* encoding a cysteinyl-tRNA synthetase and *cysA-1* encoding thiosulfate sulfurtransferase, in *D. radiodurans* were highly upregulated by Cd exposure (Fig. 3 and Table S3). However, upregulation of another sulfur amino acid, methionine, was not detected. Upregulation of genes involved in cysteine and cellular thiol biosynthesis by Cd has been frequently observed in other organisms [16, 42, 45], and one of the most obvious responses of *Bacillus subtilis* to disulfide stress is the strong induction of cysteine biosynthesis genes [17]. The most highly upregulated gene involved in sulfur metabolism is the *frnE* encoding a putative dithiol-disulfide isomerase, a member of the thioredoxin superfamily, which catalyzes formation of protein disulfide bonds. The expression of *frnE* increased to a peak induction of 253-fold at 90 min after Cd treatment and was also induced in response to ionizing radiation [38]. Additionally, as mentioned above, the expression of the thioredoxin system and glutaredoxin was also increased by Cd. In *E. coli*, glutaredoxins are ubiquitous thiol-disulfide oxidoreductases, which catalyze the reduction of glutathione–protein mixed disulfides and therefore protect proteins from irreversible oxidative damage. In most eukaryota and many Gram-negative bacteria, the major low molecular weight thiol is glutathione [9]. However, many Gram-positive bacteria, including *D. radiodurans*, lack glutathione but produce other low molecular weight thiols, such as mycothiol, bacillithiol, and cysteine [17, 30, 31]. Although the presence of bacillithiol was confirmed previously in *D. radiodurans*, the biosynthetic genes were not found [31]. In the representative Gram-positive bacterium *B. subtilis*, cysteine and bacillithiol are the major low molecular weight thiols [17, 30, 31]. Moreover, *S*-cysteinylation is known as a general response for thiol protection of *B. subtilis* proteins after oxidative stress [21]. The activation of cysteine biosynthesis after Cd stress is thought to be caused by depletion of the intracellular cysteine pool resulting from increased consumption of free cysteine by oxidation to cystine and the formation of mixed disulfides with proteins to protect redox-sensitive thiols from irreversible oxidation. Therefore, these results suggest a kind of protective mechanism of *D. radiodurans* proteins from Cd-induced disulfide stress. Furthermore, the results are in good agreement with the previous report that Cd has higher affinity to sulfur compounds than nitrogen- or oxygen-rich biological compounds [16].

Upregulation of Genes Responsible for Iron Uptake

Interestingly, treatment with Cd resulted in iron starvation of *D. radiodurans*. The majority of genes required for iron

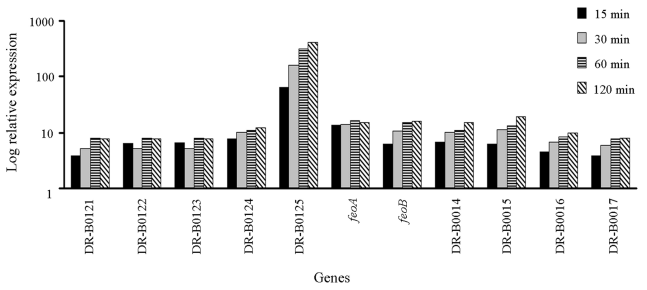

Fig. 4. Expression level of genes encoding iron uptake systems in response to Cd exposure at different timepoints.
The expression levels of DR_B0014 to DR_B0017, DR_B0121 to DR_B0125, and *feoAB* of *D. radiodurans* at each timepoint (15, 30, 60, and 120 min) after Cd treatment were revealed by qRT-PCR analyses. The level of each transcript was normalized to that of *gap* (DR1343), a relatively stable transcript. The induction of the transcripts in each sample is expressed relative to the amount of the transcripts from corresponding non-treated samples. This result is a representative of three independent experiments.

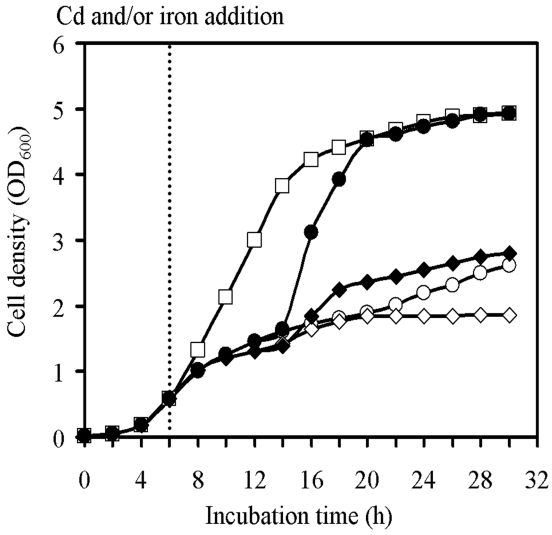

Fig. 5. Effect of iron supplementation on the growth of Cd-treated *D. radiodurans*.
Growth curves for cells treated with 25 μM of Cd (open circle), 25 μM of Cd and 100 μM of iron (solid circle), 50 μM of Cd (open diamond), 50 μM of Cd and 100 μM of iron (solid diamond), and without any treatment (open square) are shown. At an $OD_{600\ nm}$ of 0.6, Cd and/or Fe were added to the media. The mean values of the results from three experiments are shown. Growth was monitored as the absorbance at 600 nm.

uptake were highly upregulated by Cd (Fig. 3 and Table S3). Among them, the most upregulated gene is DR_B0125, the firstly transcribed gene in a putative operon (DR_B0121~DR_B0125). Expression of DR_B0125 was increased with a peak induction of 133-fold at 120 min after Cd exposure. However, DR_B0124 was only moderately induced (5-fold at 30 min) and the expression data of DR_B0122 and DR_B0123 were removed after a filtering process because of the low p-value associated with the t-test. To validate the expression levels of these genes including DR_B0122 and DR_B0123 in response to Cd, we examined their transcriptional levels by qRT-PCR. Consistent with the transcriptome data, the relative expression levels of DR_B0014~DR_B0017, DR_B0121, ~DR_B0125, and *feoAB* were increased by Cd and showed more sensitivity than the microarray results (Fig. 4). Therefore, this result indicates that *D. radiodurans* is inefficiently importing iron ions. In *S. cerevisae*, Cd treatment induced genes encoding functions in iron homeostasis, and iron addition rescued the Cd-sensitivity of certain yeast mutants [35]. Likewise, Cd exposure provoked iron deficiency in plants [46]. However, in the case of *E. coli*, all iron uptake systems were downregulated and all iron storage systems were upregulated by Cd [16]. In order to address the relationship between iron deficiency and Cd on growth, 100 μM of ferric chloride was added to exponentially growing cells treated with Cd at various final concentrations (Fig. 5). The cells grown with Cd (25 μM) and iron (100 μM) showed 8 h of stasis and resumed growth. The final OD_{600} was similar to that of the cell grown in plain TGY medium. The final OD_{600} was 2-fold higher than that of the cell grown with Cd (25 μM) alone. However, the positive effect of iron addition on growth was not observed in cells treated with Cd over 50 μM (data not shown). Therefore, this result indicates that iron deficiency could be an important determinant of Cd toxicity in *D. radiodurans*. Additionally, five genes (DR_0496, DR_1869, DR_1908, *fdx-2*, and *nirA*) encoding proteins involved in iron-sulfur [Fe-S] cluster assembly were upregulated by Cd, indicating damage or requirement of [Fe-S] cluster-containing proteins. The biogenesis of the [Fe-S] cluster requires the coordinated delivery of both iron and sulfide, which are involved in many cellular processes such as electron transfer, substrate binding/activation, and iron/sulfur storage [2]. As described above, Cd increased the biosynthesis of cyteine and induced disulfide stress. Therefore, the upregulation of genes required for iron uptake and [Fe-S] cluster assembly indicate that Cd-induced iron deficiency in *D. radiodurans* might be caused by iron substitution by Cd from [Fe-S] cluster-containing proteins.

Induction of DNA Repair Mechanisms

Cd interferes with multiple DNA repair processes, as well as enhancing, at low biological relevant concentrations, the mutagenicity of other DNA damaging agents. Prevention of the mutagenic and lethal consequences of DNA damage requires the timely expression of DNA repair and protective genes, in order to maintain the integrity of the genome and viability of the cell. As expected, 57 genes involved in the group of DNA replication, repair, and modification showed significant changes in response to Cd exposure, 42 genes were upregulated and 16 genes were downregulated during at least one timepoint (Fig. 3 and Table S3). Reportedly, proteins participating in DNA repair systems, especially

base excision and mismatch repair, are sensitive targets of Cd toxicity [12]. The transcriptome experiments revealed that *mutLS* involved in mismatch excision repair and 10 genes encoding the Nudix family of DNA glycosylases that are required for base excision repair were upregulated in response to Cd. Some members of the Nudix family, such as MutT of *E. coli*, limit mutations by hydrolyzing oxidized products of nucleotide metabolism that are mutagenic when misincorporated into the genome [24]. Thus, induction of many Nudix family members may be one of the most important protective responses from Cd-induced mutagenic activity. Expression of eight genes (*recA*, *recD*, *recF*, *recQ*, *recR*, and *ruvABC*) involved in recombinational repair was also induced. *D. radiodurans* has 16 unique DNA damage response genes induced in response to ionizing radiation and desiccation, designated as *ddr* [38]. Among these, 12 genes (*ddrA*, *ddrB*, *ddrC*, *ddrD*, *ddrE*, *ddrF*, *ddrH*, *ddrI*, *ddrK*, *ddrN*, *ddrO*, and *ddrP*) were highly induced by Cd. Additionally, other upregulated genes required for DNA metabolism, *recN*, *recO*, *gyrAB*, DR_2410, *dnaC*, and *uvrBC*, were highly upregulated. Upregulation of genes encoding DNA polymerase subunits, *dnaC* and DR_2410, suggests a requirement for increased amounts of at least some DNA polymerase subunits for recovery from the DNA damage caused by Cd. Based on the transcriptome results, we can assume that *D. radiodurans* operates various types of DNA repair systems to prevent damage to DNA and subsequent mutagenesis.

Other Cellular Processes

Fifty-four genes categorized by regulatory function were differentially expressed upon Cd exposure (Fig. 3 and Table S3). Among them, the expression level of *smtB* encoding a metalloregulatory transcriptional repressor sensing Zn, Co, and Cd was the most highly upregulated gene (20-fold after 60 min of Cd exposure) [7]. Besides *smtB*, the expression of *lexA2*, *ddrI*, and *ddrO* involved in gene regulation was induced moderately. Interestingly, *rsbRST* that have been demonstrated to be involved in the regulation of an alternative sigma factor, σ^B, in Gram-positive bacteria were downregulated by Cd. In *B. subtilis*, σ^B is an alternate transcription factor implicated in controlling gene expression in response to various physical or nutritional stresses [44]. Although the mechanisms by which the presence of physical stress is conveyed to RsbR, RsbS, and RsbT are unknown, some reports indicated that ribosome-associated events may contribute to the process [44]. As mentioned above, transcriptome results revealed that ribosome synthesis was significantly decreased after Cd exposure in *D. radiodurans*. Thus, we can assume that if the reduction of ribosomes affected the function of RsbR, RsbS, and RsbT, then the transcription of *rsbRST* might be downregulated. In addition, *drRRA* encoding a response regulator required for radioresistance was downregulated. Deletion of *drRRA* caused significant reduction of antioxidant enzyme activities, and the transcription level of genes encoding catalases, superoxide dismutases, and peroxidase were also downregulated [43]. Despite the downregulation of *drRRA* by Cd, transcription of *katE*, *sodA*, and DR_A0145 encoding a peroxidase was upregulated, indicating the presence of other regulations. In contrast, *katA* was gradually downregulated. These results suggest a complex regulation of genes encoding these antioxidative enzymes. Expression of DR_1968 encoding a putative FMN-dependent nitroreductase was highly upregulated, with a peak induction of 150-fold at 120 min. Bacterial nitroreductases are associated with cellular defense against oxidative damage. The major FMN-dependent nitroreductase of *Salmonella* Typhimurium, SnrA, is induced by paraquat, a potent generator of superoxide [32]. NfsA, the major oxygen-insensitive nitroreductase of *E. coli*, reduces chromate [Cr(VI)] to the less-soluble and less-toxic Cr(III) and can also increase the chromate tolerance by reducing ROS generation [1]. Therefore, upregulation of DR_1968 indicates the induction of oxidative damage by Cd exposure and antioxidative role of DR_1968. Together with reduction of protein synthesis, 15 genes involved in transcription underwent downregulation by Cd. Among these, *nusG* encoding the transcription antitermination protein and two RNA helicase-encoding genes (*hepA* and DR_1624) were highly downregulated. In addition, *rpoABC* encoding RNA polymerase and *rpoD* encoding sigma factor A were also downregulated. These results also indicate minimization of cellular processes in order to prevent and repair Cd-induced damages.

The present study used a microarray approach to investigate global gene expression responses that are induced by Cd exposure in a radioresistant bacterium, *D. radiodurans*. In conclusion, the results revealed that exposure to Cd at a sublethal concentration caused an arrest in transcription and translation, growth retardation, iron deficiency, damages in protein and DNA, and induction of antioxidant enzymes, chaperone, and complex regulatory networks. Cd contamination to the environment should be carefully monitored and controlled to protect the ecosystem. Therefore, results from this study may be valuable not only for guiding future research on mechanisms of Cd toxicity and comparative analysis on gene expression in other organisms, but also for providing useful information to build up a stress induction system by cadmium.

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