Supplementary Information

DrwH, a novel WHy domain-containing hydrophobic LEA5C protein from *Deinococcus radiodurans*, protects enzymatic activity under oxidative stress

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Supplementary Materials and Methods

**Bioinformatics analysis.** General protein characteristics, such as amino acid content, molecular weight and protein GRAVY, were predicted using the ProtParam tool (http://web.expasy.org/protparam/). A hydrophathy plot was established with the ProtScale program (http://web.expasy.org/protscale/) using the Kyte and Doolittle algorithm\(^1\). SOPMA was used to predict the secondary structure of the protein\(^2\) (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and regions of protein disorder were predicted using the Cspritz web server\(^3\) (http://protein.bio.unipd.it/cspritz/) and the Gensilico Metadisorder service\(^4\) (http://iimeb.genesilico.pl/metadisorder). Amino acid sequences harboring the Water stress and Hypersensitivity response domain were predicted from the SMART non-redundant database\(^5\) (http://smart.embl-heidelberg.de/). Signal peptide prediction was performed using SignalP4.1\(^6\) (http://www.cbs.dtu.dk/services/SignalP/) and PrediSi\(^7\) (http://www.predisi.de/).

**qRT-PCR for gene expression.** Total cellular RNA was extracted from cultures at the beginning of the exponential phase using TRIzol Reagent (Invitrogen, Carlsbad, CA), and its quality and quantity were evaluated by UV absorbance at 260 and 280 nm. The RNA samples were reverse-transcribed using a Protoscript First Strand cDNA Synthesis Kit (New England Bio-Labs) as described in the manufacturer’s protocol. The qRT-PCR assays were performed using total RNA samples obtained from three independent cultures. Optimized primers were designed using primer software and are listed in Table S3. The PCR reactions were carried out with an AB 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s recommendations. The 16S rRNA gene was used as the endogenous reference control for normalization of differences in total RNA quantity, and relative gene expression was determined using the comparative threshold cycle \(2^{-ΔΔCT}\) method. All cDNA samples were assayed in triplicate\(^8\).
Construction of the plasmid-mediated complementation strain of the *drwH* mutant.

The plasmid carrying the wild-type *drwH* gene with its endogenous promoter was constructed using the *E. coli-D. radiodurans* shuttle plasmid pRADZ3 as a vector⁹ (see Supplementary Fig. S8 online) to complement the constructed R1-01 deletion strain. Briefly, an 835-bp DNA fragment of the wild-type *drwH* gene with its endogenous promoter and terminator regions was amplified from genomic DNA of strain R1 with the primer P11 with a *Hind*III site and P12 with a *Bam*HI site (see Supplementary Table S3 online). The amplicon was double-digested with *Hind*III and *Bam*HI, and ligated into the corresponding site of pRADZ3 to yield the complementation plasmid pRA-*drwH*. Correct recombination was checked by PCR, followed by nucleotide sequencing of the amplicon obtained. pRA-*drwH* was transformed into R1-01 deletion strain to generate the plasmid-mediated complementation strain, designated R1-11 (Δ*drwHcomp*) (see Supplementary Table S2 online).

**Abiotic stress-resistance assays.** The *D. radiodurans* cells were grown in TGY medium with the appropriate antibiotics to the beginning of the exponential phase (OD₆₀₀≈0.6) at 30°C. Cultures were then pelleted from 1 mL cultures by centrifugation to remove the growth medium. For H₂O₂ treatment, the pelleted cells were treated with fresh TGY medium containing various concentrations (20, 40, 60, 80, 100 mM) of H₂O₂ in the dark for 30 min. For NaCl treatment, the prepared cells were resuspended in fresh TGY medium containing different concentrations (ranged from 0 to 5 M) of NaCl with shaking for 5 h. Desiccation stress assays were carried out as previously described¹⁰ with some modifications. Briefly, 100 µL of cell suspension was placed inside a sealed desiccator at 25°C. Relative humidity within the desiccator was measured as less than 5% with a hygrometer. The desiccators were sealed, and the dried cultures were stored undisturbed at 25°C for 60 days. The samples were rehydrated by the addition of 1 mL TGY at regular intervals (ranged from 0 to 60 days) under sterilized conditions for 30 min. At the times indicated, 10 times serial dilutions were made, and 100 or 10 µL of each serial dilution of cell suspensions was spread/dripped onto TGY
agar plates. These plates were incubated at 30°C for 3 days before colony growth was observed and enumerated. The survival rate was expressed as the percentage of the number of colonies in the treated samples compared with those in the untreated controls. All the experiments are performed three times, and the values are shown as the mean ± standard deviation.

The wild-type and recombinant *E. coli* strains were grown in LB broth supplemented with kanamycin (50 μg/mL) and 0.1 mM isopropyl-thiogalactopyranoside (IPTG) at 37°C to an OD₆₀₀ of 0.5. Cells were then pelleted from 1 mL cultures by centrifugation to remove the growth medium and resuspended in 1 mL fresh LB medium. For oxidative stress, 1.5 μL 30% H₂O₂ was added to a final concentration of 15 mM in the cell suspensions for 10 min. For freezing-thawing stress, 1 mL cell culture was frozen two times at -80°C for 20 min and thawed at room temperature for 20 min. After incubation, serial dilutions of 10 times were made. Ten microliters of each dilution was dripped onto LB agar plates at 37°C overnight. All assays were performed in triplicate.

**Preparation of the Dr-WHy protein.** The recombinant strain BL21-1 expressing Dr-WHy protein was grown in LB broth supplemented with kanamycin (50 μg/mL) at 37°C to an OD₆₀₀ of 0.5 and then induced with 0.5 mM IPTG at 16°C overnight. Then cells were harvested and resuspended in 20 mM Tris-HCl, pH 8.0, and 300 mM NaCl. Cells were lysed by sonication on ice, and the lysates were cleared by centrifugation at 13,000 g for 20 min at 4°C. The supernatant was then subjected to affinity chromatography on Ni²⁺-NTA agarose (Qiagen). After washing, the proteins were eluted with buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl) containing 300 mM imidazole and dialyzed overnight. The purity of purified Dr-WHy proteins was greater than 95%, as judged by electrophoresis analysis on a 15% polyacrylamide gel containing SDS that was then stained with Coomassie Blue (see Supplementary Fig. S6 online).
MDH and LDH enzymatic activities measurement under oxidative stress in vitro.

MDH from porcine heart (Sigma) and LDH from rabbit muscle (Sigma) were used to test the protective effects of the Dr-WHy (truncated-DrwH) protein against oxidative stress according to a previously published method\textsuperscript{1,12}. Experiments were performed in Eppendorf tubes to avoid protein adsorption to glass. The enzymes and corresponding protein were diluted in 50 mM potassium phosphate buffer (pH 7.2) (MDH) or 25 mM Tris-HCl stabilizer solution (pH 7.5) (LDH) to a final concentration of 1.0 mg/mL following the manufacturer’s recommendations. To measure the protective effect of the Dr-WHy protein on MDH or LDH activity after oxidative treatment, Dr-WHy or BSA was added to equal volumes of MDH or LDH (10 μg/mL final concentration) at a molar ratio of 1:1 (test protein: enzyme). The stock solution of Tris-HCl buffer was used as a control. Two concentrations of H\textsubscript{2}O\textsubscript{2} (1.0 and 2.0 mM) were added to the enzyme mixture and incubated at room temperature for 1 hour\textsuperscript{12}. At the end of the treatment, the samples were transferred to wet ice until enzyme activity was determined. Assays were performed in triplicate, and the appropriate buffer solution without Dr-WHy protein was used as the blank. To determine MDH enzyme activity, 10 μL of LDH/stabilizer solution was added to 1 mL of 150 mM potassium phosphate buffer (pH 7.5) containing 200 μM oxaloacetate (Sigma) and 200 μM NADH (Sigma). For LDH, enzymatic activity was assayed in 100 mM KCl (pH 7.5) with 150 μM NADH (Sigma) and 2 mM pyruvate (Sigma). MDH and LDH activities were monitored as the rate of decrease in absorbance $A_{340}$ nm for 1 min due to the conversion of NADH into NAD$^+$ at 25°C. Residual activity after treatment was expressed as percent of the initial activity. Enzyme activity for each sample shown was determined in at least three independent tests, as indicated in the figure legend for each experiment.
Supplementary Figures

Figure S1. Phylogenetic tree of DrwH and related protein sequences.

Neighbor-joining (NJ) tree method was used to compute the distances of LEA5C proteins using the MEGA 7.0 program. The different LEA groups are shown by different colors. The *D. radiodurans* DrwH protein and the dWHy1 protein from uncultured bacteria are indicated with red arrows.
**Figure S2. Multiple alignments of WHy domain from LEA5C proteins.**

Alignment was performed using ClustalX2 and then manually refined. The bacterial sequences are labeled in red, archaea in blue, and plants in green. The consensus sequences with >60% identity were reported below the alignment. The lengths and identities of different WHy domains were calculated and listed.
Figure S3. Construction and verification of the ΔdrwH mutant.

(A) Schematic representation of the ΔdrwH mutant generated by replacing the drwH region with the kanamycin resistance gene nptII (Kan’). The open boxes represent the putative promoter regions of the flanking genes and nptII genes. (B) PCR verification of the ΔdrwH mutant. Lane M: Trans2K PlusII DNA marker; lanes 1 & 4: PCR products amplified from the ΔdrwH mutant using primers P7/P8 and P9/P10, respectively; lanes 2 & 5: PCR products amplified from *D. radiodurans* WT using primers P7/P8 and P9/P10, respectively; and lanes 3 & 6: PCR products amplified from sterile water using primers P7/P8 and P9/P10, respectively. (C) Effect of the drwH deletion on the expression of its flanking genes (*dr1371* and *dr1373*) under normal growth and oxidative stress conditions. Relative levels of transcripts are presented as the mean values ± standard deviation, calculated from three sets of independent experiments and normalized to levels in the WT strain. Different letters indicate significant differences (*P* < 0.05).
Figure S4. Survival phenotype plate assay upon oxidative or salinity stresses.

(A) Growth curves for *D. radiodurans* WT and ΔdrwH mutant in TGY rich medium under normal conditions. All experiments are performed three times and values are mean ± standard deviation. (B) Serial 10-fold dilutions of OD-standardized *D. radiodurans* WT and ΔdrwH mutant were spotted onto TGY plates after exposure to 80 mM H$_2$O$_2$ or 4 M NaCl. CK, untreated culture control. All experiments were performed three times.
Figure S5. Survival phenotype plate assay upon oxidative stress.

Serial 10-fold dilutions of the OD-standardized wild-type *E. coli* BL21 and recombinant strains (the intact DrwH-expressing strain BL21-3, the truncated DrwH-expressing strain BL21-1, and the control strain BL21-0 harboring the empty vector pET28a) were spotted on LB plates after exposure to 15 mM H$_2$O$_2$. All experiments were performed three times.
Figure S6. SDS-PAGE analysis of purified Dr-WHy.

Cultures were incubated with 0.5 mM IPTG at 16°C overnight. Lane M, molecular weight standards (kDa); lane 1, supernatant after lysis; lane 2, flow through; lane 3, elution with 10 mM imidazole; lane 4, elution with 50 mM imidazole; and lane 5, elution with 300 mM imidazole.
Figure S7. A proposed working model for DrwH in *D. radiodurans*.

The figure shows the proposed model in which DrwH effectively protect activities of various intracellular enzymes from damage caused by oxidative stress. Solid arrow and blocked arrow represent protection and damage effects, respectively. For more details, see the results or discussion of text.
Figure S8. Complementation assays under oxidative stress.

(A) Construction and verification of the complementation plasmid pRA-drwH. (B) Survival phenotype plate assay upon oxidative stress. Serial 10-fold dilutions of OD standardized *D. radiodurans* WT, ΔdrwH and ΔdrwHcomp were spotted onto TGY plates after exposure to 80 mM H₂O₂ for 30 min. CK, untreated culture control. (C) *drwH* transcription in *D. radiodurans* WT, ΔdrwH and ΔdrwHcomp under 80 mM H₂O₂. Relative levels of transcripts are presented as the mean values ± standard deviation, calculated from three sets of independent experiments and normalized to levels in the WT strain. Different letters indicate significant differences (*P* < 0.05). All experiments were performed three times.
Figure S9. Physical organization and comparative analysis of the six gene clusters containing \textit{drwH} of \textit{D. radiodurans} compared with \textit{D. geothermalis} DSM11300, \textit{D. actinosclerus} BM2, \textit{D. maricopensis} KR-23, \textit{D. deserti} VCD115, \textit{D. gobiensis} I-0, and \textit{D. peraridilitoris} KR-200.

Same colors indicate similar predicted functions of the depicted ORF. The numbers in parentheses indicate the distances between the two genes on the left and right sides. Orthologous genes of \textit{drwH} are shown in red. Orthologous genes of \textit{dr1370} and \textit{dr1371} are shown in yellow and green, respectively. Adjacent genes are labeled with other colors. The genome size is indicated at the end of physical organization.
## Supplementary Tables

### Table S1. Comparison of amino acid content (mol%), GRAVY and instability index of LEA

| LEA group | Residue type residues |
|-----------|-----------------------|
| 5C        | SR1172                |
|           | DGo-CA1605            |
|           | IBLEA14               |
|           | OxLEA5                |
|           | dWHy1                 |
|           | BN5_2959              |
| 5A        | M0PM25                |
|           | ECP31                 |
|           | ARuRab28              |
| 5B        | OBLEA5                |
|           | SAG21                 |
|           | ATTHF1                |

| LEA group | Residue type residues |
|-----------|-----------------------|
| 1         | D-19                  |
|           | AhLEA1-2              |
|           | EMB564                |
| 2         | DHN1                  |
|           | PDey1dinin K          |
|           | ERD10                 |
| 3         | OxLEA3                |
|           | LEA76                 |
|           | CRE-LEA-1             |
| 4         | P06700108             |
|           | GmPM29                |
|           | PAP260                |
| 6         | LEA-18                |
|           | AhLEA-1-1             |
| 7         | BPS1                  |
|           | GmASR                 |
|           | LLA23                 |

**Note:** Grey shading represents the hydrophobic LEA5 protein family.
| Strains and plasmids | Genotype or description | Reference or source |
|----------------------|------------------------|---------------------|
| **Bacterial Strains** |                        |                     |
| *D. radiodurans* R1  |                        |                     |
| WT                   | Wild-type, Chinese Culture Collection: CGMCC 1.633 | Laboratory stock   |
| R1-01 (ΔdrwH)        | R1 *drwH*-deletion mutant, Kanr | This study          |
| R1-11 (ΔdrwH*comp*)  | R1-01 containing the complementation plasmid pRA-*drwH*, Kanr and Cmr | This study          |
| R1-21 (ΔirrE)        | R1 *irrE*-deletion mutant, Spe¢ | Zhang et al.¹³     |
| *E. coli*             |                        |                     |
| BL21                 | F- *ompT hsdS_B (rB mB) gal dcm (DE3) | TransGen Biotech |
| BL21-0               | The control strain harboring the empty vector pET28a, Kanr | This study         |
| BL21-1 (Dr-WHy)      | The recombination BL21 strain containing pET-Dr-WHy, Kanr | This study         |
| BL21-2 (dW-WHy)      | The recombination BL21 strain containing pET-dW-WHy, Kanr | This study         |
| BL21-3 (intact)      | The recombination BL21 strain containing pET-*drwH*, Kanr | Laboratory stock   |
| **Plasmids**         |                        |                     |
| pET28a (+)           | Kanr *ori*BR322 lacPT7p | Novagen            |
| pET-*drwH*           | pET28a carrying the wild-type *drwH* gene under the control of T7 promoter, Kanr | This study         |
| pET-Dr-WHy           | pET28a derivative plasmid carrying the truncated *Dr-WHy* gene with an intact WHy domain but without the predicted signal peptide, Kanr | This study         |
| pET-dW-WHy           | pET28a derivative plasmid carrying the truncated *dW-WHy* gene with an intact WHy domain but without the predicted signal peptide, Kanr | This study         |
| pRADZ3               | The *E. coli*-*D. radiodurans* shuttle vector, Ap¢, Cmr | Meima & Lidstrom⁹  |
| pRA-*drwH*           | pRADZ3 derivative carrying the wild-type *drwH* gene under the control of its endogenous promoter, Cmr | This study         |
| Gene/Fragment name | Primers | Sequence (5′–3′) | Amplicon size (bp) |
|--------------------|---------|----------------|-------------------|
| drwH-U             | P1      | GAGTTCGGCGAGCGGTGTT | 749               |
|                    | P2      | GTTTTCTAATCAGATCCTCTAGGCCCCGGTGC CGGCAACT |               |
| nptII              | P3      | AGTTTGCCGGCACCAGGCTTCAGAAGCATCCGATTAGAAAAAC | 1007             |
|                    | P4      | AAAGAGAGGGGAGACACTACACGGTACATGGTAAAGGTGAAT |               |
| drwH-D             | P5      | ATATCAGCCTACTGACCGGTGAGTCTCCCCTTCTTT | 594               |
|                    | P6      | GCGTGGTGGCTGAAGTCC |               |
| YZdrwH             | P7      | TGGCGGAAGTACGGCTAGCAACT | 346               |
|                    | P8      | TCAAAAACACCGATAAAAGGCG |               |
| YZdrwH-UKD         | P9      | GTGCCGGCAACTCCACCCGACTCCGATA | 2578             |
|                    | P10     | GCGTGGTGGCTGAAGTCC |               |
| drwHcomp           | P11     | CAAAAGCTTGCCTGAGTCCCGTCGC | 835               |
|                    | P12     | CCGGATCTCTCAAACACCGATAAA |               |
| dr06               | RT16s-F | ATTCCTGGTGAGCCGGTG | 146               |
|                    | RT16s-R | CATCGTTTAGGGTGTTGCA |               |
| dr1372 (drwH)      | RT1372-F | AACCAGTAACGCTTCCGGATAT | 164               |
|                    | RT1372-R | CGAGGTCAGCCCGGCTAGGATT |               |
| dr1998 (cat)       | RT1998-F | ACCAACATCCAGTCGCAG | 167               |
|                    | RT1998-R | CTTTACCTTGCTGTTG |               |
| drA0259 (cat)      | RTA0259-F | TGGGAAGATGGCTCTCG | 113               |
|                    | RTA0259-R | CAGCGGGTCTGGTGTTG |               |
| dr1279 (sod)       | RT1279-F | TGCCTCTGCTACGCTACGAG | 159               |
|                    | RT1279-R | CGAGCTGCTGAATGACTTGTG |               |
| dr1546 (sod)       | RT1546-F | GCACCGCCACCTTTAGC | 108               |
|                    | RT1546-R | TGACTGCTGGTCTTCTCG |               |
| drA0202 (sod)      | RTA0202-F | AACCAGAGCTGCTGTCTCATCC | 174               |
|                    | RTA0202-R | TTTCTTCCGCTCGTAGGCC |               |
| dr0644 (sod)       | RT0644-F | CTGGCTCTGCTGCTCTCG | 172               |
|                    | RT0644-R | GTAGCTGCTGGCAGGCTC |               |
| drA0145 (pod)      | RTA0145-F | TACCCAGTCGCTTCAAAAGTG | 133               |
|                    | RTA0145-R | TCGACGACTATGGTGCTACG |               |
| drA0301 (pod)      | RTA0301-F | CGCTTTCAGGAGGTGTTCG | 114               |
|                    | RTA0301-R | CGCCGAGTACCGGTCATA |               |
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