Novel DNA-protection Protein (Dps) Originated From *Deinococcus Geothermalis*

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Abstract

We characterized two interrelated proteins from the *Deinococcus geothermalis* strain with regard to the role of DNA-binding and protection, such as a novel Dps, Dgeo_0257, and a Dps orthologous protein, Dgeo_0281. Despite the lack of conserved amino acid sequence for ferroxidase activity, Dgeo_0257 exhibited high DNA-binding affinity and formed a dodecameric conformation. In contrast, Dgeo_0281 protein showed less effective DNA-binding and was mainly observed monomeric or dimeric forms. Electrophoretic mobility shift assay showed that both purified proteins are non-specifically bound to DNA to protect against DNA degradation and the properties of Dps responding to specific metal ions. In the presence of ferrous and ferric ions, Dgeo_0257 or Dgeo_0281 protein does not readily bind to DNA. However, in Zn and Mn ions are present, both proteins had more stable DNA-binding. From the Dps gene disrupted mutant test, each showed a higher sensitivity to oxidative stress than the wild-type strain. In addition, the expression level of each gene was correlated with the presence of each other. In this study, we characterized and confirmed the functional roles of Dgeo_0257 for sensing metal ions and binding to DNAs along with the Dps orthologous Dgeo_0281. Based on various observational evidence, we propose that Dgeo_0257 is a novel Dps protein with no ferroxidase activity.

Introduction

Deinococcus species are extremophiles that can survive under various harsh conditions, such as oxidative stress, desiccation, and extreme radioactive environments beyond normal bacterial environmental adaptation zone (Ferreira et al., 1997; Makarova et al., 2007). *Deinococcus geothermalis* is one of more than 90 species of the intense radiation-resistant Genus *Deinococcus* including mesophile *Deinococcus radiodurans*, which can optimally grow at 48°C and survive under acute and chronic exposures of ionizing radiation of in total 10 kGy of 60 Gy/hour, 1 kJ/m² of ultraviolet light, and years in desiccation (Daly, 2009; <https://lpsn.dsmz.de/>). *D. geothermalis* can reduce Fe (III)-nitrilotriacetic acid, U (VI), Cr (VI), and curtail Hg (II) in the presence of radiation and high temperature and utilized in the bioremediation (Brim et al. 2003; Daly et al., 2004; Rainey et al., 2005; Gerber et al., 2015; Ranawat & Rawat, 2017). The DNA-binding protein from starved cell (Dps) is one of the significant factors for DNA damage protection, toxic metal ions storage and homeostasis, and reactive oxygen species (ROS) scavenger (Chiancone & Ceci, 2010).

Dps has been described as a DNA-binding protein that regulate and protects the DNA of starved *Escherichia coli* (Almirón et al., 1992). It plays an essential role in multifaceted protection by nonspecific DNA binding, iron sequestration, and ferroxidase activity (Calhoun & Kwon, 2010). These properties appear to correlate with the structure and function of iron storage proteins of ferritin (FtnA) and bacterioferritin (Bfr). *E. coli* Dps has a shell-like structure and a spherical hollow cavity in the center. This hollow cavity of Dps acts as an iron storage compartment, and iron sequestration is essential in iron detoxification and homeostasis. As in Ftns, the internal cavity of the Dps protein is also negatively charged amino acid residues (Asp and Glu), which have a foundation for storing metal ions by electromagnetic force. Expression of Dps is dominantly induced both during starvation and in response

to oxidative stress during the exponential phase (Almirón et al., 1992; Altuvia et al., 1994; Lomovskaya et al., 1994). The *dps* gene can be induced during OxyR-dependent growth by processing exponentially growing cells from *E. coli* with low concentrations of H₂O₂ (Altuvia et al, 1994).

D. radiodurans has two Dps proteins: DrDps1 (DR2263) with 207 amino acid (aa)-long and DrDps2 (DRB0092) with 241 aa-long. DrDps1 and DrDps2 share just 16% amino acid (aa) sequence identity. Both proteins have a common dodecameric structure forming a hollow spherical cavity (Santos et al., 2015). The N-terminal region of DrDps1 has a metal-binding site suggested to be important for DNA-binding (Nguyen & Grove, 2012; Reon et al., 2012; Santos et al., 2017). The first 30 amino acid residues of DrDps2 are expected to be signal peptides and were located in the cell periphery region based on intracellular iron localization and protected against exogenously derived ROS in *D. radiodurans* (Reon et al., 2012; Santos et al., 2015). Application of H₂O₂ during the exponential cell growth phase results in changes in the oligomeric formation of DrDps1, which appears to be related to manganese homeostasis and detoxification of ROS. On the other hand, H₂O₂ causes the accumulation of DrDps2 as a dodecameric cage form in stationary phases, which could be considered a 'typical Dps' response (Santos et al., 2015, 2019).

We focused on a novel Dps protein, Dgeo_0257 (DgDps3) in *D. geothermalis*. Both Dps proteins Dgeo_0257 and Dgeo_0281(DgDps1) were characterized using conformation analysis, DNA-binding capacity, and metal ions effects. Besides, results of the qRT-PCR analysis showed that each Dps gene was expressed dependently on growth phases using oxidative stress and gene expression levels. Thus, the ability of Dps to perform in a regulatory capacity remains, to some extent, speculative. Dgeo_0257 is proposed as one of the Dps DNA-binding proteins in *D. geothermalis* and its roles probably differ somewhat from those of DgDps1.

Materials And Methods

Bacterial strains and culture conditions

The strain *D. geothermalis* KACC 12208 derived from DSM 11300^T was obtained from the Korean Agricultural Culture Collection (KACC, <http://www.genebank.go.kr>). Dps gene disrupted mutants $\Delta dgeo_0257$ and $\Delta dgeo_0281$ were constructed in the previous study using the homologous recombination procedure to determine cell viability, growth pattern, and RNA-Seq analysis (Table S1).

E. coli DH5 α was routinely used for cloning and protein purification procedures. *E. coli* strains containing cloned recombinant plasmids were cultivated in LB broth medium containing 100 ppm of ampicillin at 37°C. For protein expression at the late-log phase (O.D₆₀₀ 0.8) of *E. coli*, where the *lac* promoter is induced by 1 mM IPTG of end concentration and continuously cultured for 4 hours. After 4-hour induction, the culture was centrifuged at 5,000 rpm for 20 min and supernatants were discarded. Stored the pellets at -70°C for use in further experiments. *D. geothermalis* was cultured at 48°C in TGY medium contained 1%

tryptone, 0.5% yeast extract, and 0.1% glucose at 48°C. *E. coli* were cultured at 37°C in Luria-Bertani (LB) medium (MB Cell, Kisan Bio. Korea) contained 1% tryptone, 0.5% yeast extract, and 1% NaCl.

Construction of expression vector for Dgeo_Dps genes

The *dgeo_0257* and *dgeo_0281* genes were constructed and amplified using the PCR products encompassing both genes with different restriction enzyme sequences, double digested with *Nco*I and *Bgl*II for *dgeo_0257*, and *Nco*I and *Bam*HI for *dgeo_0281* and ligated into the pCS19 vector for the carboxyl-terminal His-tag version (Table S1). Then the recombinant plasmids were transformed into *E. coli* DH5α competent cells using the CaCl₂ chemical transformation method. The transformants were selected on a 100 µg/ml ampicillin-containing plate. Correct construction was detected using colony PCR with primer sets of target genes and finally decided by DNA sequence analysis (Macrogen, Korea).

Purification of a putative Dps protein (Dgeo_0257) and Dgeo_Dps1 (Dgeo_0281)

Cell pellets stored in a -70°C refrigerator were resuspended with 5 ml lysis buffer (300 mM NaCl, 50 mM NaH₂PO₄, pH8.0, and 10 mM Imidazole) per pellet obtained from 250 ml. Cell membranes were broken using a French Press (with 7,000 psi; Vision Biotech, Korea) or Sonicator (with Max 30% with 20 sec On and 40 sec Off time for three cycles, followed by Max 21%; Model CV334 of Sonics & Materials Inc., USA). Following the breakage of cells, cells were centrifuged at 5,000 rpm for 20 min, and supernatants were collected for further testing. Nickel-nitrilotriacetic acid (Ni-NTA) resin column chromatography was used to purify over-expressed C-terminal His-tagged target proteins. After a Ni-NTA resin column equilibrated with lysis buffer, the sample solution was loaded and pass-through was collected. Next, 10–20 ml washing buffer (300 mM NaCl, 50 mM NaH₂PO₄, pH 8.0, 20 mM Imidazole) was poured. Finally, 10 ml elution buffer (300 mM NaCl, 50 mM NaH₂PO₄, pH 8.0, 250 mM Imidazole) was poured into resin to recover the target proteins. The Bradford assay was used to confirm protein production. SDS-PAGE electrophoresis with 12% separation gel was applied to determine the target protein profiles. Electrophoresis was performed in 1x Tank buffer (25 mM Tris, pH 8.3, 192 mM glycine), followed by placement in staining buffer with 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% distilled water (DW), for at least 3 hours (usually left overnight). Destaining (10% acetic acid, 50% methanol, and 40% DW) was performed to confirm protein bands of interest visually. Protein dialysis was performed using a cellulose tubular membrane (CelluSep H1, Membrane Filtration Products Inc., USA) with 1x dialysis solution (25 mM Tris, pH 7.5, 150 mM NaCl). The membrane was placed in 1L dialysis solution and rotated to submerge it under water for 1 hour. The process was repeated three times, followed by protein concentration using the manufacturer's guidelines of VivaSpin (7,000 rpm for 15–20 mins, Vivaspin-6 of Sartorius, Germany).

Determination of protein conformation by gel filtration

Size-exclusion chromatography (SEC) was performed using the BioLogic HR Chromatography system (Bio-RAD, USA) with a HiLoad 16/600 Superdex 75 prep grade column (GE Healthcare Life Sciences,

USA). The SEC column was filled with a running buffer solution (25 mM Tris-HCl, pH 7.5, 150 mM NaCl) to separate the molecules throughout the resin charged in the column, following the buffer to flow at a rate of 2.0 ml/min for 2 hours. The protein samples were loaded, first protein size markers such as ferritin (440 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), and anhydrase from erythrocytes, and purified proteins, with a flow rate of 0.5 ml/min for 1 min. The buffer was then drained at a flow rate of 2.0 ml/minute for 1 hour and dispersed in the fraction collector. Aliquots of critical parts that exceed the preset threshold values were collected and used in the following experiments.

Electrophoretic mobility shift assay (EMSA)

Three putative promoters and the same number of open reading frame (ORF) regions were amplified from *D. geothermalis* genomic DNA by PCR using a forward and reverse primer set (Table S2). For the EMSA, DNA probes with ca 150–350 nucleotides length were purified using the AccuPrep® PCR purification kit (Bioneer, Korea). Various amounts of DNA and Dgeo_0257 protein were mixed in 5 x EMSA binding buffer [100 mM Hepes (pH 7.2), 160 mM KCl, 0.5 mM EDTA (pH 8.0), 50% glycerol, 350 ng/μl BSA, 40 ng/μl poly(dI-dC), 12.5 mM DTT] in 10 μl volumes. Prepared metal ions included Fe (II) from ammonium iron (II) sulfate hexahydrate, $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (Sigma, USA), Fe (III) from ammonium iron (III) citrate, $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{Fe} \cdot \text{NH}_3$ (Sigma, USA), and other metal ions, including Zn (II), Mn (II), Cr (VI), Co (II), Cu (II), Cs, and Pb (II). The DNA-protein binding reactions were performed at 37°C for 30 min and loaded onto 6% native polyacrylamide gels buffered in 0.5 x Tris-borate-EDTA (TBE). After running the polyacrylamide gel electrophoresis (PAGE) at 60V for 120 min, stained DNA in the gels with SYBR™ Gold nucleic acid gel staining solution (Life Technologies Co., USA). Then the band positions and intensities were detected using Chemi-Doc (Bio-RAD, USA).

Analysis of qRT-PCR according to growth phases in wild-type, Δgeo_0257, and Δgeo_0281

After overnight incubation, pre-cultured both wild-type and *dps* gene disrupted mutants, then used new 5 ml TGY broth with differential inoculation sizes as learned from the delayed growth patterns of mutant strains (approximately 1 hour). After 3 hours, measurement was performed at O.D_{600} , adjusted to O.D_{600} 0.06, and started the main-culture in 50 ml TGY. When samples reached O.D_{600} at 2.0, 4.0, and 8.0, centrifuged as 5 ml, 2.5 ml, and 1.25 ml each to match cell number, then discarded supernatants and resuspended 5 ml 0.9% NaCl, then treated 50 mM H_2O_2 for 30 min. After the H_2O_2 challenge, samples were centrifuged at 10,000 rpm for 5 minutes and washed once more with 0.9% NaCl. Supernatants were discarded and stored pellets at -20°C. Prepared samples without H_2O_2 for the control group.

The cell wall was broken using phenol to determine the gene expression level. DNA digestion was performed using DNase I, and RNA was extracted using an RNA prep kit for RNA cleanup (RNeasy mini purification kit, Qiagen, Germany). After measuring the extracted RNA concentration, the concentration was made constant of 1,000 ng in 8 μl volume. Then cDNA synthesis was performed with dNTP mixture and 6-mer random primers using followed protocol: 60°C for 5 minutes, 4°C for 3 minutes, 30°C for 10

minutes, 42°C for 60 minutes, and finally 95°C for 5 minutes (PrimeScript™ 1st strand cDNA Synthesis Kit, TaKaRa, Japan). In the step of 3 minutes at 4°C, 4 µl 5x buffer, 4.5 µl RNase free water, 1 µl RTase, and 0.5 µl RNase inhibitor were added. Each primer prepared to determine the expression levels of *dgeo_0257* and *dgeo_0281* was added to the created cDNA. And then, qRT-PCR analysis was performed using TB Green® Premix Ex Taq™ (TaKaRa, Japan) on the Bio-Rad RT-PCR model CFX96™ Optics Module (Bio-Rad, USA). The expression level of GAPDH, a constitutively expressed gene on all growth phases, was normalized. The related expression levels of both *dps* genes were calculated from the previous study (Choo et al., 2020). The statistical analysis was done by probability *t*-test for differences between the samples in Prism™ program and it was considered significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Results

Biochemical properties of a putative DgDps

D. geothermalis has 222 aa-long DrDps1 orthologous gene *dgeo_0281* with 76.3% amino acid sequence identity, but no gene is equivalent to DrDps2. However, the putative Dps protein Dgeo_0257 of 200 aa-length is a hypothetic protein of *D. radiodurans* with 65.5% amino acid sequence identity when 40 aa is deleted in the N-terminal region of the orthologous protein DR0582 (Fig. 1 and Table 1). Dgeo_0257 has shown over 90% identity to match a DNA-binding protein with 194 aa-long from *D. apachensis*, *D. metallilatus*, *D. aerius*, and *D. phoenicis* genomes. *D. marmoris* contains two Dgeo_0257 like proteins: 205 and 210 aa-long with 71 % and 69 % identity, respectively (data not shown). However, Dgeo_0257 has not yet been characterized and revealed only 11.5% amino acid sequence identity with Dgeo_0281 as DgDps1. Therefore, gene disruption and protein purification experiments should be conducted to determine functional roles to confirm that Dgeo_0257 is a functional DNA-protecting Dps protein without a ferroxidase center.

Table 1
Comparison of Dps proteins between *D. radiodurans* and *D. geothermalis*.

Proteins	<i>D. radiodurans</i>	<i>D. geothermalis</i>	Identity	Note
Dps	DrDps	DgDps*		
Dps1	DR2263 (207 aa)	Dgeo_0281 (222 aa)	76.3%	
Dps2	DRB0092 (241 aa)	-	-	
Dps3	DR0582 (243 aa)	Dgeo_0257 (200 aa)**	65.5%	40 aa delete at N-terminal of DR0582
* Amino acid sequence identity between Dgeo_0281 and Dgeo_0257 was 11.5%.				
** Dgeo_0257 protein showed 90% identity to matched protein from <i>D. apachensis</i> and <i>D. phoenicis</i> . <i>D. marmoris</i> contains two Dgeo_0257 like proteins with 205 and 210 aa lengths of ca 70 % identity.				

Purification and conformation of Dgeo_0257 and Dgeo_0281 proteins

The Dgeo_0257 and Dgeo_0281 proteins were overexpressed in *E. coli* by attaching C-terminal histidine tags to elucidate the functional role of each protein. After the first stage of protein purification using a Ni-NTA column, analysis of the protein profile of the *dgeo_0257* or *dgeo_0281* gene with 6x His-tag was performed using SDS-PAGE. Both proteins were well induced by IPTG triggering at a concentration of 1 mM. The biochemical molecular weights of the monomers of Dgeo_0257 and Dgeo_0281 are approximately 23 kDa and 26 kDa. However, Dgeo_0281 was over 30 kDa in SDS-PAGE gel. The Dgeo_0257 and Dgeo_0281 proteins were purified at ca 90% purity per liter of culture in the first stage of one-step purification at 5 mg and 20 mg, respectively (Fig. S1).

Various sizes of protein forms were collected and confirmed using gel filtration. We expected that Dgeo_0257, a 23 kDa monomer with His-tag, would form a 12-mer or 24-mer polymer or a sphere of 240 kDa or 480 kDa like Dps1 and Dps 2 of *D. radiodurans*. The gel filtration analysis results showed that the intact protein size of Dgeo_0257 was ca 480 kDa representing the form of icositetrahedron compared to the dimer and monomer of Dgeo_0281 as DgDps1. The conformation did not affect the elution of the Dgeo_0281 protein in the presence of iron (Fig. S2). However, the elution fraction of Dgeo_0257 moved to the posterior region when 1 mM of ferrous iron was present. Adversely, the elution fraction moved to the anterior part when 1 mM of ferric iron was present. Therefore, Dgeo_0257 protein conformation is changed by the iron redox state from the dodecameric form when the iron is absent.

DNA-binding and protection properties of DgDps proteins

In general, bacterial Dps proteins have non-specific DNA-binding activity (Almirón et al., 1992; Calhoun & Kwon, 2010). To confirm this phenomenon, we prepared three intergenic regions with long-distance divergent promoters and three ORF regions in Dgeo_2112 as a hypothetical protein with a 250–350 bp-long DNA fragment, and followed by EMSA detection with 6% native gel (Table S2). Both Dgeo_0257 and Dgeo_0281 showed distinct DNA-binding affinity with DNA fragments. The DNA-protein complex was in the super-shift position for Dgeo_0257. However, the DNA-protein complex was in the lower position for Dgeo_0281. We identified the unique DNA binding behavior of two Dps putative proteins. Dgeo_0257 showed DNA binding activity over 5-fold higher than Dgeo_0281 (Fig. 2). Furthermore, Dgeo_0257 protein required 10 μ M protein concentration for a complete shift-up migration, but Dgeo_0281 required more than 60 μ M protein concentration for full DNA binding. Interestingly, the DNA-binding behavior of Dgeo_0257 and Dgeo_0281 is different between the promoter region and the ORF region. Treatment with ferrous iron resulted in the complete release of the promoter region DNA from the protein-DNA complexes. However, the ORF region DNA was still bound to Dgeo_0257 (partially) and Dgeo_0281 (wholly) with 0.2 mM and 1.0 mM ferrous ions, respectively (Fig. 2).

To examine the DNA protection activity of Dps, we found that DNA disappears in the presence of an iron ion of 0.5 mM. This phenomenon is because unprotected naked DNA is damaged and degraded due to

oxidative stress through Fenton reactions by ferrous ions or ferrous ions and hydrogen peroxide mixtures. However, the DNAs were well protected from oxidative stress with the addition and increase of Dgeo_0257 and Dgeo_0281 proteins (Fig. 3). Furthermore, DNA-protein complexes were located in the lower position in the ORF DNA region where ferrous ions existed. Therefore, these two Dps proteins of *D. geothermalis* are DNA-binding proteins with non-specific DNA sequence targets with distinct patterns together with ferrous ions in both promoter and ORF regions and can be considered to have DNA protection against oxidative stress reactions.

Metal ion sensing on protein behavior of a novel Dps (DgDps3) and DgDps1

Various metal ions were added to the Dps and DNA complex formation reactions to determine the metal ion effect on the Dps-DNA binding activity. As a result of increased ferric ion concentrations, the DNA-protein complex did not maintain a complex form, and free DNAs were then released from Dps-DNA complexes at more than 0.5 mM and 1 mM Fe^{3+} , respectively (Fig. 4A). Ferric ions also showed similar DNA releasing activity to ferrous ions at more than 2-3-fold higher concentrations (Fig. S3A). Free DNA was degraded by a high concentration of ferrous ions (Lane 8–10 in Fig. S3A). Interestingly, as the concentration of manganese and zinc ions was increased, the two Dps proteins were much better bound to DNA, which stabilized the DNA binding even more (Fig. 4BC). Surprisingly, in the presence of more than 2 mM Mn, a novel Dgeo_0281 protein-DNA complex was observed (Fig. 4B). The reaction to manganese is similar to that reported by previous research groups (Nguyen & Grove, 2012; Reon et al., 2012). However, the zinc effect on DNA-binding stabilization is a finding in this study. Lead, copper, and cobalt did not affect DNA-binding ability (Fig. S3BCD). However, DNA-binding activity varied with chromate and cesium depending on metal ion concentration. At a particular concentration, Dgeo_0257 and Dgeo_0281 protein showed a fluctuated pattern depending on cesium and chromate concentrations, respectively (Fig. S3EF). Although the amino acid sequence equivalence of the novel Dps protein Dgeo_0257 (DgDps3) and DgDps1 (Dgeo_0281) was very low, both proteins have the same behavior in DNA-binding capabilities by metal ion sensing with differential conformation and DNA-binding affinity.

Physiological properties of Dps disrupted mutants

In our previous study, disrupted mutant strains of putative Dps *dgeo_0257* and *dgeo_0281* ($\Delta dgeo_0257$ and $\Delta dgeo_0281$) were developed by homologous recombination and kanamycin selection (Lee *et al.*, 2019). In the absence of oxidative stress, two mutant strains showed an almost identical growth pattern with 1 hour delayed to the wild-type strain (Fig. S4A). In the late exponential growth of O.D₆₀₀ 1.47, each strain showed different growth inhibition by treatment with 100 mM H_2O_2 (Fig. 5A). The viability test was performed to confirm the oxidative stress response among the WT, $\Delta dgeo_0257$, and $\Delta dgeo_0281$ mutants by treatment with H_2O_2 concentrations of 80, 100, and 120 mM, respectively. The $\Delta dgeo_0281$ mutant strain showed a slight decrease in viability at 80 mM, and cells did not grow above 100 mM H_2O_2 . The $\Delta dgeo_0257$ mutant strain endured well to 100 mM of slightly higher H_2O_2 concentrations but did

not grow at 120 mM (Fig. 5B). The WT and two Dps gene disrupted mutants were not grown at O.D₆₀₀ 0.5 with 100 mM H₂O₂ treatment (Fig. S4B). Thus, both *dps* genes participated in anti-oxidation responses.

Expression levels of two Dps genes in a growth phase-dependent manner

Experiments were performed to compare the expression levels of both *dps* genes *dgeo_0281* (DgDps1) and *dgeo_0257* (DgDps3) in the wild-type strain and proceed with $\Delta dgeo_0257$ and $\Delta dgeo_0281$ mutants. Control was based on the expression level of *dgeo_0257* at O.D₆₀₀ 2.0 of the wild-type strain. In the early stages of growth of wild-type *D. geothermalis*, DgDps1 (*dgeo_0281*) is predominant with over 150-fold, and as the growth phase stage progresses, the expression level of DgDps 1 is drastically decreased. Even if hydrogen peroxide is presented, it did not affect the expression of DgDps1 during the growth phases (Fig. 6A). The expression level of DgDps3 (*dgeo_0257*) is gradually increased when the cells reached the stationary phase with 2.46-fold. Expression of DgDps3 reached maximal level after treatment with 50 mM H₂O₂ with 11.63-fold at O.D₆₀₀ 4.0 (Fig. 6B). Therefore, it is expected that the DgDps1 plays a significant role in the early exponential growth phase without oxidative stress. And DgDps3 plays preventing DNA damage under oxidative stress, especially the late exponential growth phase and growth phase-dependent slightly induction without oxidative stress in *D. geothermalis*.

The $\Delta dgeo_0257$ mutant showed a half maximum expression level of DgDps1 with 60.69-fold at the beginning of growth and gradually became extinct on the growth progress without oxidative stress. However, the expression level of DgDps1 was increased by two times at both O.D₆₀₀ 4.0 and 8.0 under oxidative stress conditions (Fig. 6C). Therefore, DgDps1 took over the role of DgDps3 during the growth phase due to the absence of *dgeo_0257* involved in DNA protection. The $\Delta dgeo_0281$ mutant shows higher expression with more than 10-fold DgDps3 compared to the wild-type at O.D₆₀₀ 2.0. Thus, we inferred that expression of DgDps3 increased to compensate for the absence of *dgeo_0281* till O.D₆₀₀ 4.0 (Fig. 6D). DgDps1 and DgDps3 dependently correlate between DNA-protecting proteins to prevent DNA damage and ROS-scavenging proteins to survive under oxidative stress conditions, resulting in the absence of the one gene substituted by the expression of the other gene during growth phases. Nevertheless, unlike other bacteria like *E. coli*, both DgDps proteins do not act as dominant chromosomal stabilizers at expression levels in *D. geothermalis*.

Discussion

Dps protein, a ferritin and bacterioferritin-like subfamily protein, has multifaceted roles in four major categories: 1) dodecameric assembly conformation structure for iron storage and homeostasis, 2) DNA protection roles from oxidative damage, 3) bacterial genome packaging as nucleoid protein, and 4) enzymatic activity (Andrews, 2010; Chiancone & Ceci, 2010; Zeth, 2012). It can oxidize iron to prevent oxidative free radicals or form a protein-DNA complex to protect DNA physically (Haikarainen & Papageorgiou, 2010). In addition, Dps can bind DNA without sequence specificity (Calhoun & Kwon, 2010). However, in Chip-seq analysis, the Dps protein of *E. coli* was reported to combine genomic DNA in

a non-random manner (Antipov et al., 2017). DgDps1 (Dgeo_0281) and Dgeo_0257 also showed different DNA-binding specificity in the selected promoter and ORF regions (Fig. 2,4). The self-assembly characteristics and DNA-binding properties of Dps in solution are extensively agglomerated according to co-crystallization and general defense mechanism (Ceci et al., 2004). *E. coli* Dps has nucleoid clumping activity in *Staphylococcus aureus* resulting in H₂O₂ resistance and enzymatic activity (Ushijima et al., 2016). Dps has a ferroxidase activity that can catalyze oxidation of the ferrous irons (Fe²⁺) to the ferric state (Fe³⁺). In this process, H₂O₂ is reduced during oxidation of ferrous and Dps can detoxify H₂O₂ and protect DNA. Thus, in general, Dps is highly induced by direct treatment with H₂O₂ or relies on the growth phase in species specificity. Since *E. coli* Dps was first reported, various Dps has been reported in more than ten bacterial genera: *Bacillus*, *Campylobacter*, *Lactobacillus*, *Helicobacter*, *Mycobacterium*, *Agrobacterium*, *Streptococcus*, *Pseudomonas*, *Vibrio* together with *Deinococcus*, and several archaeal Dps in *Sulfolobus solfataricus*, *Pyrococcus furiosus*, and *Halobacterium salinarum* (Tonello et al., 1999; Wiedenheft et al., 2005; Haikarainen & Papageorgiou, 2010; Ramsay et al., 2006; Huergo et al., 2013; Xia et al., 2017).

Dps expression in *E. coli* depends heavily on the growth phase (Azam & Ishihama, 1999; Calhoun & Kwon, 2010; Sato et al., 2013). During exponential growth, Dps is up-regulated by OxyR regulator, induced by hydrogen peroxide stress, and activates σ^{70} -RNA polymerase. When exposed to hydrogen peroxide during the stationary period, Dps is induced by RpoS, encoded by σ^s and expressed as a dominant protein. When cells are not in oxidative stress, Dps is down-regulated by nucleoid-associated proteins, Fis and H-NS, which prevent initiation of *dps* transcription by combining adjacent areas within the core *dps* promoter (Grainger et al., 2008; Calhoun & Kwon, 2010). However, the expression of *dps* was repressed by the OxyR containing one cysteine residue in the case of *D. radiodurans* (Chen et al., 2008).

VC0139 protein was strongly induced by exposure to hydrogen peroxide in a Gram-negative bacterium *Vibrio cholerae* (Xia et al., 2017). This protein encodes a Dps homologous protein and has ROS resistance. OxyR regulates Dps during the exponential phase and by RpoS during the stationary phase. This Dps protein can be expressed relatively without H₂O₂. However, Dps is dramatically induced in the exponential growth phase but less induced in the stationary growth phase under H₂O₂. The involvement of Dps in resistance to various environmental stresses has also been reported. For example, exposure of the Δ *dps* strain to high iron levels during starvation resulted in a less viable Δ *dps* strain compared with wild-type. These data suggest that Dps is vital for the survival of bacteria in starved cells due to its reaction to ROS and iron toxicity tolerance. As with *V. cholerae*, Dps is related to colonization but does not involve toxic gene expression (Xia et al., 2017).

Interestingly, *Campylobacter jejuni* Dps has unusually high-temperature tolerance and activates DNA binding by ferrous ions. However, this DNA interaction has been inhibited by NaCl and Mg²⁺ (Huergo et al., 2013; Sanchuki et al., 2015). In the hyperthermophilic Crenarchaeon *S. solfataricus*, Dps protein was directly induced by H₂O₂ treatment and ferrous ion depletion (Wiedenheft et al., 2005).

Dps protein forms a dimer or dodecamer structure and binds to DNA (Grove & Wilkinson, 2005). In DrDps1 of *D. radiodurans*, oligomeric states change depending on the growth phase and presence of DNA. This suggests that DrDps1 oligomeric forms and functional roles may adapt to environmental changes (Santos et al., 2015). On the other hand, the DrDps2 oligomeric form maintains a dodecameric structure without affecting the surrounding environment. DrDps2 is thought to be more selective about iron depending on the cell environment and involved in intracellular metal storage (Reon et al., 2012; Santos et al., 2015).

In this study, the protein form of Dgeo_0281, known as DgDps1, the homologous protein of DrDps1, was not identified in the dodecameric form. The maximum combining in gel filtration and EMSA test was the dimer structure. The DNA binding affinity was K_d 30 μ M, half the maximum of the non-specific DNA sequence. Interestingly, the novel Dps protein (Dgeo_0257, called DgDps3) showed higher DNA-affinity than DgDps1 (Dgeo_0281) in just 10 μ M. EMSA showed that the DgDps3-DNA complex was located near the upper pocket, unlike the DgDps1 found below. In the presence of metal ions together with DNA and protein, EMSA showed that those two DgDps proteins carried out the identical electrophoretic shift with DNA. Therefore, further analysis of protein structure conversion by metal ion and DNA-binding is needed.

Ferritin is a 24-mer protein with an inner diameter of 8 nm and stores 24,000 Fe atoms *in vivo* (Lawson et al., 1991; Harrison & Arosio, 1996; Yokoyama et al., 2012). However, the inner diameter of 5 nm Dps protein provides storage space for less than 500 iron atoms (Tonello et al., 1999; Ramsay et al., 2006). Thus, DNA-binding Dps proteins can store iron in a bioavailable form and protect cells against oxidative stress. The oxidative protection is achieved by binding Fe^{2+} ions and preventing the Fenton reaction-catalyzed formation of toxic hydroxyl radicals or binding DNA to block them from oxidative radicals (Imlay et al., 2013). Despite knowledge of the basic mechanisms of iron entry and oxidation, many essential questions regarding iron core formation and the iron release mechanism in response to cellular needs still need to be elucidated (Papinutto et al., 2002). Zeth *et al.* recently studied the metal position of three ions, Co^{2+} , Zn^{2+} , and La^{3+} , and the translocation pathway in *Listeria innocua* Dps protein (Zeth et al., 2019). In addition, negatively charged residues inside the cavity cause dynamic change to Dps conformation. Thus, the Dps cage may be modified for different metal-holding specificities depending on the bioremediation of metal ions.

The ability of microorganisms to resist the toxic effects of metals is often related to their ability to transform metals into less soluble and less toxic chemical states with specific proteins (Brim et al., 2003). Dps of *E. coli*, involved in copper homeostasis, are good examples (Thieme & Grass, 2010). In the *D. radiodurans* experiment, Hg (II) and Hg (0) identified the ability to reduce toxicity in the cell (Brim et al., 2003). Fe (III), U (VI), Tc (VII), and Cr (VI) were also reduced under anaerobic and aerobic conditions (Fredrickson et al., 2000).

The original DR2263 (DrDps1) of *D. radiodurans* has a long N-terminal extension involved in DNA-binding and binding of several metals, such as iron and zinc (Romão et al., 2006; Nguyen & Grove, 2012; Ushijima et al., 2016). Due to their ability to act as protein cages for iron and various other metals, Dps-like proteins

have recently become of considerable interest in nanotechnology. Furthermore, Dps can aggregate and remove metal ions harmful to bacteria and humans; therefore, the primary goal of our research is the industrial application of Dps. In addition to Fe (II) and Fe (III), Cr (VI), Dps is known to collect Hg (II), U (VI), and Cs (Brim et al., 2003). In this regard, we have established a vital research theme to ensure that Dgeo_0257 and Dgeo_0281 can react with these heavy and harmful metal ions. The various metal ions listed above and the interaction between the two proteins allowed us to compare the properties of Dgeo_0257 and Dgeo_0281. A better understanding of their functions and mechanisms can develop new biotechnology and nanotechnology (Haikarainen & Papageorgiou, 2010; Gerber et al., 2015).

We previously analyzed a transcriptomic analysis to define the functional role of putative Dps protein Dgeo_0257 using RNA-Seq technology (NCBI GEO accession number GSE151903; Lee et al., 2020). Tables S3 and S4 show a list of genes that have been over 3-fold up-regulated and 0.3 down-regulated. Interestingly, the 17 proteins/enzymes and IS*Dge5* transposases have also been up-regulated and 14 genes have been down-regulated. These results led us to believe that the putative Dps protein Dgeo_0257 was related to specific gene regulation together with DNA stabilization.

We conducted a study assuming that DgDps1 has existing Dps characteristics and DgDps3 has similar Dps characteristics lacking ferroxidase active center. Of particular interest, both DgDps proteins showed similar metal ion-sensing. However, a novel DgDps3 has higher DNA-binding affinity and more metal ion sensitivity than DgDps1. Two DgDps proteins have an intracellular responsibility for DNA protection, and detoxification of harmful iron depends on growth phases. Especially, DgDps1 as a dominant at early exponential growth phase does not induce at the stationary growth phase. When H₂O₂ treated stress was present in the DgDps3 disrupted mutant, DgDps1 was gradually induced on growth progress. However, DgDps3 was sensible to oxidative stress by H₂O₂ treatment, and when dominantly expressed DgDps1 was absent, substitutional induced at early exponential growth phase (Fig. 6). Therefore, the novel DgDps3 (Dgeo_0257) is proposed as one of the Dps DNA-binding proteins in *D. geothermalis* and will play a different role than DgDps1 (Dgeo_0281). Further investigation into DgDps3 is required to explain this protein's function, structure, and network regulation through redox-sensing regulators in *D. geothermalis*.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

MKB, ES, and SJL designed the experiments, analyzed the data, and wrote the manuscript.

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Supporting Information

The Supporting Information for this article can be found online.

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Figures

Figure 1

A

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Dgeo_0257  --MTKRSKVFVPAVVTVATVGVAAGAAYVARHRKDDVKEFFVAQVLERPAGRMSYSDLAQ
DR0582      MTKRKS SSGVGVTAVLVTVATVGAAAGAAALARRRKGDIKEFVVANVLEKPAGRSSFTDLGQ
          * * * . . . : * * * * * . * * * * . * * . * * . * : * * * . * * : * * . *
Dgeo_0257  GLERGGVFLAQRAARAANTDANRAILTHIIGIERWGQNRLRVALGQRPFERDEHHYPKPG
DR0582      NLERGGTFLTGRAERAADT PENREVLAHIIGIERWGQSKRIQVALGQREFVRDEYRGYRPE
          . * * * * . * * : * * * * * . * * * * : * . * * * * * * * . * : * * * * * . * * * : * * . *
Dgeo_0257  AGATLRELQDLLSQTRAQTVDLARQLHAQPPQDDLTIEHNGLGPLTPKGWLRYLTHQHADL
DR0582      GGATLGQLRSLLSQTRARTVDLARQLHFNPPDDFVVEHNGLGPLTAGWLRYLTHQHADL
          . * * * * . * : . * * * * * . * * * * * * * . * * . * * . : * * * * * * * . * * * * * . * * * *
Dgeo_0257  ESRKLRGAKEYQALGEERLASQ-
DR0582      ESRKLRGAGGTAVHEEETQAPAS
          * * * * * . * * :

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B

N-terminal extension

```
DrDps1 -----MT KKSTKSEAASKT KKSQVPETGAQGVRAGGADHADA AHLGTVNN
Dgeo_0281 MTRATKASARTSGTRKKTKDDQAQPASAALEQTAEQAGPVPAQGDAKADAAHLDTVNN
          ..*.::*:.*:.:..** ** * :*****.***
                      helix A                                helix B
          _____|_____||_____|_____||_____
DrDps1 ALVNHYLEEKEFQTV AETLQRNLATTISLYLKFKKYHWDIRGRFFRDLHAYDEFIAEI
Dgeo_0281 QLVDHGYLTTEEFPQTISETLQRNLATTISLYLKFKKYHWDIRGRFFRDLHAYDEFIEEI
          **: * *:****.:*****
                                  helix C
          _____|_____|_____||_____
DrDps1 FPSIDPQERLVALGGSPAAPADLARISTVQVPQETVRDARTQVADLVQDLSRVGKGYS
Dgeo_0281 FPTIDPQERLVALGGSPAAPADIERSVVQVPTETVRDARTQVKDLVADLVGVGKYR
          ****:*****:****:*:*** ***** ** *:*****
                                  helix D
          _____|_____||_____
DrDps1 DDSQACDEANDPVTADMINGYAAITDKIRWMLQAIMDDRLED
Dgeo_0281 DDSQTVEANDPATADMINGYAAITDKIRWMLQAILDDRMM
          ****:*****:*****:***:*
```

C

```
Dgeo_0257 ---MTKRSKVFPVAVTVTAVGVGAAGAAVVARHRKDDVKEFFVAQVLERPAGRMSYSDLA
Dgeo_0281 MTRATKSASRTSGTRKTKDDQAPASAALEQTAPEQGAPGVPAQGDAKADAHLDTVNN
      * * : . . . . * : : : . * * . . :
Dgeo_0257 QGLERGCVFLAQRAARAANTDANRAILTHIIG--IERWGQNRRLVALGQRPFERDEHHFY
Dgeo_0281 QLVDHGYLTEEEFQTISETLQRNLATTISLYLKFKK[REDACTED]WDIRGFRFDDI[REDACTED]LAYDEFIEEI
      * * : : : : : : : : : : : : : : : :
Dgeo_0257 KPGAGATLRE LQDLLSQTRAQTVDLARQLHAQPP----QDDLTIEHNGLGPLTPKGWLRY
Dgeo_0281 FPTIDEQA[REDACTED]RLVALGGSPTIAAPADIERFSVQVQVPTETVRDARTQVKDLVADLIRVKGKYR
      * . . * * * . . * * : * * : : . * * *
Dgeo_0257 LTQHADLESRLRGAKEYQALGEERLASQ-----
Dgeo_0281 DDSQTVDEANDPATADMYNGYAATIDKIRMLQAILDDDRMN
      . : * : . * . * . :

```

Figure 1

Comparison of amino acid sequences between a novel Dps Dgeo_0257 of *D. geothermalis* and a hypothetic protein DR0582 of *D. radiodurans* with 65.5% identity (A), between DrDps1 and DgDps1 (Dgeo_0281) with 76.3% identity (B), and between DgDps1 and DgDps3 (Dgeo_0257) with 11.5% identity (C). The secondary structure of helix and ferroxidase active center with red box are marked in B & C panel.

The ferroxidase active center is missed in DgDps3 (C). Red words indicated the predicted DNA-binding domains (HTH) by prabi (<https://npsa-prabi.ibcp.fr/>).

Figure 2A

Figure 2B

Figure 2

EMSA assay of Dgeo_0257 (A) and Dgeo_0281 (B) on both promoter and ORF region increasing protein concentration. The ferrous ion concentration for Dgeo_0257 and Dgeo_0281 were treated with 0.2 mM and 1.0 mM at lanes 6-10, respectively. Lanes 1, free DNA; 2-5, increased amounts of purified proteins of Dgeo_0257 and Dgeo_0281 with 2.5-10 μ M and 15-60 μ M, respectively.

Figure 3

Figure 3

DNA-Dps complex formation and DNA protection analysis from iron treatment and Fenton reaction. Lanes 1, free DNA; 2, only 0.5 mM Fe²⁺, 3 & 7, both proteins added with different amounts; 4-5 & 8-9, increased Dgeo_0257 and Dgeo_0281 proteins with ferric ions of 0.5 mM; 6 & 10, Fenton reaction samples with 1.0 mM H₂O₂.

Figure 4

EMSA assay of Dgeo_0257 and Dgeo_0281 maintaining a protein concentration of 10 μ M and 30 μ M, respectively, while increasing metal ion concentration: A, ferric ion; B, manganese; C, zinc.

Figure 5

Figure 5

A, Comparison of growth pattern among wild-type, Δ dgeo_0257, and Δ dgeo_0281 mutants on TGY medium and treatment of 100 mM H₂O₂ at O.D₆₀₀ 1.47. Optical density was measured at 600 nm wavelength. B, Viability test by hydrogen peroxide treatment of 80, 100, and 120 mM among wild-type, Δ dgeo_0257, and Δ dgeo_0281 mutants.

Figure 6

Figure 6

Expression levels of both DgDps1 (dgeo_0281) (A & C), and a putative DgDps3 (dgeo_0257) (B & D) under 50 mM H₂O₂ present or absent condition in wild-type and in Δ dgeo_0257 and Δ dgeo_0281 mutants at three different growth phases, O.D₆₀₀ 2.0, 4.0, and 8.0, by qRT-PCR analysis, respectively. The probability t-test was considered significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

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