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Helicobacter hepaticus Dps protein plays an important role in protecting DNA from oxidative damage

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Abstract
The ferritin-like DNA-binding protein from starved cells (Dps) family proteins are present in a number of pathogenic bacteria. Dps in the enterohepatic pathogen, *Helicobacter hepaticus* is characterized and a *H. hepaticus* dps mutant was generated by insertional mutagenesis. While the wild type *H. hepaticus* cells were able to survive in an atmosphere containing up to 6.0% O_2, the dps mutant failed to grow in 3.0% O_2, and it was also more sensitive to oxidative reagents like H_2O_2, cumene hydroperoxide and *t*-butyl hydroperoxide. Upon air exposure, the dps$^-$ cells had more damaged DNA than the wild type; they became coccoid or lysed and they contained ~6-fold higher amount of 8-oxoguanine (8-oxoG) DNA lesions than wild type cells. Purified *H. hepaticus* Dps was shown to be able to bind both iron and DNA. The iron-loaded form of Dps protein had much greater DNA binding ability than the native Dps or the iron-free Dps.

Keywords: *Oxidative stress, dps, Helicobacter*, iron binding, DNA protection

Introduction

Helicobacter hepaticus is a member of enterohepatic *Helicobacter* species (EHS). It was first isolated from livers of several strains of inbred mice at the US National Cancer Institute in 1992 [1]. Later it was confirmed that the bacterium was associated with chronic active hepatitis and hepatocellular carcinoma (HCC) in mice [2]. However, the primary habitat of *H. hepaticus* is the lower intestinal tract. In mice with immune system deficiencies, severe enteritis developed, leading to weight loss, rectal prolapse and colon cancer [3–6]. Though closely related to other *Helicobacter* species, such as *H. pylori*, which is known to commonly infect humans, there is no direct evidence to show that *H. hepaticus* can cause disease in humans. However, Huang et al. [7] reported that *Helicobacter* genus specific DNA was found in human liver samples from patients with primary liver carcinoma but not in the control liver samples. Currently, *H. hepaticus* infected mice serve as an ideal animal model to study the mechanisms involved in the pathogenesis of human diseases like HCC [8] and inflammatory bowel diseases (IBD) [5], which have large impacts on public health. The whole genome sequence of *H. hepaticus* has been published in Ref. [9].

Bacteria have evolved efficient protective mechanisms to enable them to survive nutrient limitation and stress conditions in the environment. Oxidative stress is caused by reactive oxygen species (ROS), such as $OH^{\cdot}$, $O_2^{\cdot -}$, and H_2O_2, released from phagocytic host cells when the bacteria invade the host, or produced within the bacteria from O_2-dependent metabolism. Elevated levels of ROS were detected in the gastric mucosa of *H. pylori* infected patients [10] and these reactive chemicals were reported to stimulate cell

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proliferation and tumor initiation in the animal model [11]. At least two cellular systems have been recognized to reduce or eliminate oxidative stress in organisms. One is a regulated response, in which antioxidant enzymes are expressed to detoxify those stress agents [12]. Another system employs DNA repairing endonucleases to repair the damaged nucleic acids [13]. Superoxide dismutase (SOD) and *Escherichia coli* exonuclease III are representative enzymes involved in the above two responses. As yet, no studies to investigate the roles of anti-oxidative stress genes in *H. hepaticus* have been reported, and only a few targeted (antibiotic insertion) mutant strains of *H. hepaticus* have thus far been described in Ref. [14,15].

In nutrient-depleted habitats, such as the stationary growth phase of bacteria, the cells oftentimes lack the energy required for *de novo* protein or enzyme synthesis [16]. Hence, their ability to cope with oxidative stress is compromised under this situation. A class of non-specific DNA-binding proteins, DNA-binding protein in stationary phase (Dps) was expressed during exponential growth phase, at which time it protects the (*E. coli*) cells from oxidative stress. Dps accumulates so that it is the most abundant *E. coli* protein in stationary phase. At that phase, it is proposed to provide non-enzymatic protection for chromosomal DNA [17]. The *E. coli* Dps contains a ferritin-like spherical oligomeric structure assembled from 12 identical subunits [18]. It is recognized that Dps protects DNA from oxidative damage via the following mechanisms. First, through direct Dps–DNA interaction, the protein protects the DNA by forming a highly ordered and stable nucleoprotein complex called a biocrystal [19]. In addition, the protein sequesters intracellular free ferrous ions, thus curtailing the production of $OH^{\cdot}$ through reaction of Fenton chemistry [20]. So far, the Dps family proteins have been reported to be present in many prokaryotes, including *Listeria innocua* [21], *Streptococcus mutants* [22], *Mycobacterium smegmatis* [23], *Porphyromonas gingivalis* [24], *H. pylori* [25], and *Campylobacter jejuni* [26]. Dps proteins are all related to the ferritin–bacterioferritin–rubrerythrin superfamily [27]. Where tested, all Dps proteins have been reported to bind ferrous iron ions but most lack the ability to bind DNA [25,26,28]. Only the *E. coli* and *Mycobacterium* Dps have been shown to bind both [23,29]. Nonetheless, they all appear to play an important role in DNA protection under oxidative stress conditions. It is not known if the iron-binding is related to DNA binding ability.

A hypothetical *dps* gene (HH0210) is present in the *H. hepaticus* genome sequence [9]. To study the role of Dps in oxidative stress resistance in *H. hepaticus*, we made a dps mutant by knocking out the gene using insertional mutagenesis. The oxidative stress resistance phenotypes between wild type and the mutant were compared. The DNA and iron binding abilities were assessed with purified Dps protein. A connection is made between iron binding and DNA binding, so that the Dps–DNA adduct is expected to form under conditions when oxidative stress and free iron levels are greatest.

Experimental procedures

H. hepaticus *growth conditions*

Published genome sequence strain of *H. hepaticus*, ATCC 51449 was used in this study. *H. hepaticus* was cultured on Brucella agar (DIFCO) plates supplemented with 10% defibrinated sheep blood in 37°C incubator. The optimal gas components for the growth is 1% O_2, 5% CO_2, balance of gas is nitrogen. Different oxygen levels (3 and 6%) were also applied in oxidative stress resistance assays. Chloramphenicol (20 μg/ml) was added to the medium for culturing the *H. hepaticus* mutant.

dps Mutant construction

Based on the *H. hepaticus* genome sequence [9], PCR primers dpsF1 (5′ CCTCATTCTCCCTATCCTC-ATCG 3′) and dpsB1 (5′ AAAACCAGGTGCCTT-ACCGCTTGG 3′) were designed to amplify a 941 bp fragment from wild type *H. hepaticus* genome. The dps PCR amplicon was then ligated into pGEMT vector (Promega) and the construct was used to transform *E. coli* DH5α through electrotransformation. The cloned plasmid was then extracted from culture and a chloramphenicol cassette (Cm) was inserted into the unique *Hind*III restriction site within *dps* gene. The recombinant plasmid pGEMT:*dps*:Cm was introduced into *H. hepaticus* by electrotransformation as described previously [14]. Allelic exchange occurred leading to the formation of dps mutant. The mutant was selected on blood agar plates supplemented with chloramphenicol (20 μg/ml). Genomic DNA from the mutant clones were used for PCR examination with primers dpsF1/dpsB1 and the disruption of the *dps* gene was confirmed by an 800 bp increment of PCR amplicon due to the insertion of the antibiotic cassette inside the gene.

Gel electrophoresis and protein identification using N-terminal sequencing

Bacteria were harvested from the plates and resuspended in 1 × PBS buffer (20 mM sodium phosphate and 150 mM NaCl, pH 8.0). After washing one time with the buffer, cells were resuspended in the same buffer and were broken by two passages through a French pressure cell at 138,000 kPa (SLM instruments, Inc.). The cell lysates were centrifuged (8000 rpm for 10 min) and the supernatant was transferred to a clean tube. The protein concentration

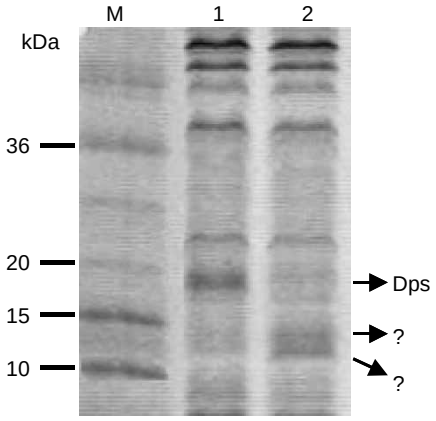

Figure 1. Protein profile of wild type and dps mutant cells. The 12.5% polyacrylamide gel was stained with Coomassie brilliant blue. Lanes: 1, wild type cell extract; and 2, dps mutant cell extract; M, protein standard (invitrogen). The arrows indicate the Dps band (lane 1) which is missing in the mutant and two up-expressed proteins (lane 2) with unknown identities.

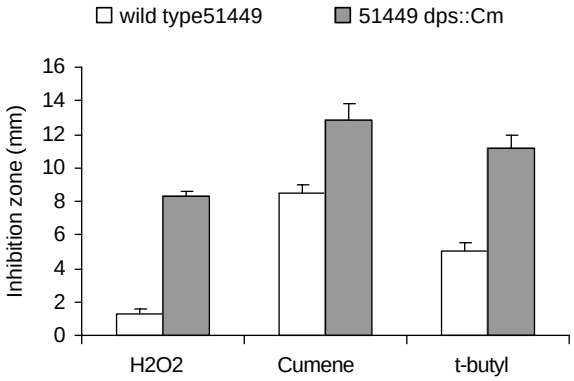

Figure 2. Paper disk assay for oxidative stress reagents: hydrogen peroxide, 1 M; cumene hydroperoxide, 0.2 M; *t*-butyl hydroperoxide, 0.2 M. Zones of inhibition were measured around the disks infiltrated with 10 µl of the indicated reagents. Water as a control did not yield any zones of growth inhibition. Six replicate experiments were performed for each testing reagent. According to Student's *t*-test, all of the mutant readings are significantly different from those of the parent strain at the 99% level of confidence.

of cell crude extract was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). Seven micrograms of cell extract was mixed with the SDS buffer and incubated at 90°C for 5 min. Proteins were then separated on the 12.5% SDS-PAGE gel by electrophoresis for 1.5 h at 100 V.

The suspected Dps protein band, which was observable in wild type strain ATCC 51449 extracts resolved on SDS-PAGE gels, but was missing for the mutant strain was subjected to N-terminal sequencing after transferring and excising from a PVDF membrane [30] (Protein sequencing lab, Georgia State University, GA, USA) (Figure 1).

For the 5.0% non-denaturing protein gel, SDS and β-mercaptoethanol were not used in the gel casting or loading buffer and the samples were not heated before electrophoresis. Native gel electrophoresis took about 7–8 h under constant voltage of 150 V.

Disk assay for oxidative stress sensitivity

The dps mutant was tested for its sensitivity towards different oxidative stress reagents in comparison to the wild type. The BA plates were streaked uniformly with 0.1 ml of liquid culture at OD_{600} of 0.8. Sterile 7.5 mm filter paper disks infiltrated with 10 µl of the stress reagents (1.0 M H_2O_2, 0.2 M cumene hydroperoxide, or 0.2 M *t*-butyl hydroperoxide) were placed onto the plates. The cells were cultured under microaerophilic conditions (1% O_2, 5% CO_2, balance N_2) for 72 h before the zones of inhibition were measured (Figure 2).

Oxygen sensitivity assay

The mutant was evaluated for O_2 sensitivity when growing on the BA plates in a variety of O_2 levels (1, 3 and 6%). To do this, both wild type and mutant culture suspension (0.1 ml) at OD_{600} of 0.8 were evenly spread onto the BA plates, which were subsequently cultured for 72 h under different O_2 partial pressures. The cells from a whole plate were harvested by suspending them in 1 ml of PBS and the OD_{600} was taken (or calculated from dilutions) as a measurement of comparative growth yield (Table I).

DNA damage evaluation by fragmentation assay

Wild type and dps mutant cells were harvested at the late log phase from BA plates and suspended in 1 × PBS to an OD_{600} of 0.5. Samples were prepared in triplicate at 0, 5 or 10 h exposure to air before further treatment for electrophoresis (Figure 3). We followed the procedure formerly described by Zirkle et al. [31] with minor modification. Briefly, the harvested cells were washed, suspended in TE buffer (50 mM Tris–HCl, 5 mM EDTA, pH 8.0), and then mixed with 1.0% low melting point agarose. After solidification, the gel plugs were placed in a lysing solution containing 0.5% (w/v) sarkosyl and 0.5 mg/ml proteinase K and incubated overnight at

Table I. Growth under various O_2 concentrations.

Strain	Growth* at O_2 concentration of		
	1%	3%	6%
Wild type 51449	8.3 ± 0.7	3.0 ± 0.3	2.9 ± 0.3
51449 dps::Cm	2.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1

*The cells were grown on the plates for 72 h, harvested and suspended in PBS (1 ml/plate), and the OD_{600} was measured. The results are the average of three experiments with standard deviation. The growth of the mutant was significantly less than the wild type in all cases at 99% level of confidence by Student's *t*-test.

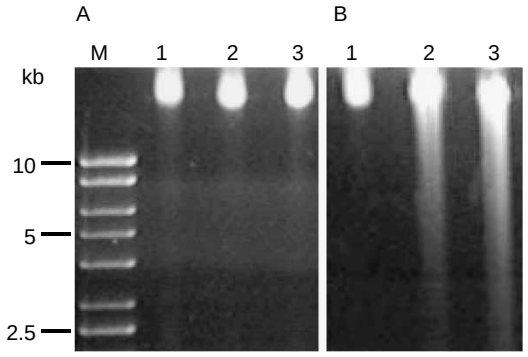

Figure 3. DNA fragmentation assay. Wild type and dps mutant cells grown to late log phase were harvested in PBS buffer and three different gel plugs were prepared for either wild type or mutant genomic DNA based on their exposure time to air. (A) wild type samples; and (B) mutant samples. Lanes: 1, samples of 0 min aeration; 2, samples of 5 h aeration; and 3, samples of 10 h aeration.

room temperature. The gel plugs were then washed with cold TE buffer and submerged in a 0.8% regular agarose gel. Samples were then subjected to electrophoresis and the gel was stained with EtBr (0.5 μg/ml) and visualized under UV light.

Fluorescent staining of cells and quantification of 8-oxoG

8-Oxoguanine (8-oxoG) is a common form of DNA modification under oxidative stress. Here, we used the protocol of Chen et al. [32] with some modifications to detect 8-oxoG. Late log phase cells were suspended in 30% methanol and adjusted to OD_{600} of 0.5. Twenty microliters of the suspension was distributed onto a gelatin-coated glass slide, air dried, and then covered with 100 μl of 25 mM Tris/HCl (pH 8.0), 50 mM glucose, 10 mM EDTA containing 2 mg lysozyme per ml for 5 min. The slides were then treated separately with methanol and acetone for 1 min followed by air-drying. PBS containing 0.05% (v/v) Tween 20 and 2.0% (w/v) BSA was then applied to the slides as a blocking buffer. After about 20 min, fluorescein-5-isothiocyanate (FITC)-conjugated avidin (1:200 dilution in PBS), which binds specifically and directly to 8-oxoG was added, and the slide incubated for 1 h in a moist chamber. After extensively washing the slide with PBS, the slide was then stained with propidium iodide (PI; 12.5% μg/ml) and mounted. The slides were examined with a Leica DM IRB fluorescence microscope with the images recorded by use of a Hamamatsu C4742-95 digital camera. The PI and FITC images adjustment was standardized and ratio of FITC signal over PI signal calculated (Table II). Eight different regions on the slide were chosen for each sample and an average ratio was calculated to evaluate the content of 8-oxoG in DNA.

Table II. Cell morphology and the level of 8-oxo guanine in *H. hepaticus* cells.

Strains	Abnormal cell morphology⋆ (% damaged cells)	8-OxoG level† (FITC/PI intensity ratio)
WT 51449	<5	0.34 ± 0.03
51449 dps::Cm	~ 48	2.10 ± 0.05

⋆ Cell morphology was examined under fluorescent microscope, and the numbers are estimated percentage of damaged cells including coccoid and broken cells in the whole cell population.
† 8-OxoG level is expressed as mean intensity ratios of avidin–FITC/PI with standard deviation. According to statistical analysis with Student's *t*-test, the data of the mutant strain is significantly different from that of the wild type ($p < 0.01$).

Construction of plasmid for overproduction of H. hepaticus *Dps*

A 461 bp DNA fragment containing the *dps* gene was PCR amplified using primer pair dps-pET-F (5′ GACAGTTCATATGAGCAAAGTTGTAGAT 3′), dps-pET-R (5′ ATATATTCTCGA GTTAGGC-AAGTGTGGCTTT 3′) and *H. hepaticus* ATCC 51449 genomic DNA as template. The PCR product was digested with *Nde* I and *Xho* I and cloned into vector pET-21a (Novagen) treated with the same restriction enzymes. The recombinant plasmid was then transformed into *E. coli* BL21 Origami competent cells (Novagen).

Overexpression and purification of Dps

E. coli BL21 Origami cells containing pET-HHdps were grown at 37°C in 500 ml Luria–Bertani medium supplemented with 100 μg/ml ampicillin to the OD_{600} of 0.5. Induction of Dps expression was achieved by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium, followed by extended incubation for 2 h. Cells were then harvested by centrifugation at 5000 rpm for 15 min, washed one time in cold buffer A (20 mM NaCl, 10 mM Tris–Cl, pH 8.0), and suspended in 5 ml of the same buffer. The cells were then broken by two passages through a French pressure cell and cell debris was removed by centrifugation at 10,000 rpm. To further remove some residual insoluble proteins, the supernatant was subjected to a high spin of 45,000 rpm for another 2.5 h. The supernatant (~5 ml) obtained was then applied to a HiTrap Q HP anion exchange column (Amersham Biosciences), which had been equilibrated with buffer A. Proteins were eluted out by use of a NaCl gradient which was generated by mixing buffer A with buffer B (1 M NaCl, 10 mM Tris–Cl, pH 8.0) in the mixer of the FPLC system (Amersham Biosciences). The eluted fractions were resolved on the SDS-PAGE gel and two partially purified fractions were pooled and further purified by gel filtration chromatography using a Sephacryl S-200 column

(Amersham Biosciences). Proteins were eluted with buffer A and the purified Dps was mixed into 20% glycerol and stored at −80°C. The concentration of the protein was determined with the Bradford assay method (Pierce).

Preparation of apo and Fe-loaded Dps proteins

Apo-Dps protein was prepared according to the procedure described by Yamamoto et al. [33]. To make apo-Dps, native Dps protein was dialyzed against 500 volumes of 20 mM morpholinepropane-sulfonic acid (MOPS)–NaOH buffer (pH 7.0) containing 1 mM 2, 2′-dipyridyl and 0.3% sodium dithionite at 4°C. The dialysis buffer was changed twice over 48 h. The apo-Dps protein was then dialyzed against 0.1 mM MOPS–NaOH (pH 7.0) and concentrated by an YM-50 (Millipore Corp.) centricon and used for the subsequent assays.

To make Fe-loaded Dps protein, ferrous ammonium sulfate (Sigma) was added to 100 μg apo-Dps preparation/ml in 0.1 mM MOPS–NaOH buffer (pH 7.0) at a final concentration of 1 mM. The mixture was kept in a nitrogen atmosphere and incubated at room temperature for 1 h. The Fe-loaded Dps was then purified on a PD-10 column (Amersham Pharmacia Biotech) equilibrated with 20 mM MOPS–NaOH buffer (pH 7.0). The eluted protein was concentrated by an YM-50 (Millipore Corp.) centricon and used for all assays.

Ferene stain

Apo-Dps (7 μg), native Dps (7 μg), and Fe-loaded Dps (7 μg) proteins were resolved on 5.0% non-denaturing polyacrylamide gels. Electrophoresis was conducted at a constant voltage of 150 V for 8 h in a −4°C cold room. The gels were stained with Coomassie brilliant blue or 1 mM 3-(2-pyridyl)-5,6-bis(2-[5-furyl sulfonic acid])-1,2,4-triazine (Ferene S; Sigma Chemical Co., St Louis, Mo, USA) and 15 mM thioglycolic acid (Sigma Chemical Co.) in 2% (v/v) acetic acid [33].

DNA binding assay

The DNA binding assay was done as described by Almiron et al. [34]. The DNA substrate used in this assay was the plasmid pGEMT (Promega) containing a fragment of the *H. hepaticus* DNA (pGEMT-HH *mdaB*). Thirty micrograms of apo-Dps, native Dps, Fe-loaded Dps, or BSA was added to 200 ng of pGEMT-HH*mdaB* plasmid DNA in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and incubated at 37°C for 30 min. The reaction mixture was then run on 1.0% Tris–HCl agarose gel. DNA was detected by staining with ethidium bromide and was observed under UV light.

Results

dps Mutant construction

In this study, a dps mutant was constructed to investigate the physiological role of this gene in oxidative stress resistance. The *dps* gene of the type strain ATCC51449 was disrupted by insertion of a chloramphenicol resistance cassette [35] within the *dps* gene followed by transformation into *H. hepaticus* cells. PCR analysis verified the correct insertion of the antibiotic cassette in the gene (not shown). The dps mutant could grow under a microaerophilic condition (1% O_2, 5% CO_2 and balance N_2) on BA plates in the 37°C incubator.

Crude extracts from wild type and the dps mutant cells were resolved on the SDS-PAGE gel to examine the protein profiles. A band of 18 kDa that is present in the wild type strain and is missing in the mutant was confirmed to be Dps protein by N-terminal sequencing (Figure 1). The 10 amino acid residues identified from the N-terminus of this protein was MSKVVD-NLKQ, which matches exactly to the predicted N-terminal amino acid sequence of Dps in the *H. hepaticus* genome database (http://www.tigr.org). Two other proteins with sizes of about 10 kDa were shown to be up-regulated in the mutant compared to the wild type; perhaps these are other oxidative stress related proteins that are up-expressed due to the increased stress from the mutation (i.e. the loss of Dps). The identity of the two bands has so far not been determined.

dps Mutant is more sensitive to oxidative stress

To examine the phenotypes of the dps mutant in relation to oxidative stress resistance, we first conducted a series of paper disk assays with 1.0 M H_2O_2, 0.2 M cumene hydroperoxide and 0.2 M *t*-butyl hydroperoxide (Figure 2). Inhibition zones were measured around the disks that were previously saturated with the reagents. The dps mutant was highly sensitive to H_2O_2, displaying a 5-fold greater inhibition zone than the wild type. The mutant also showed moderate sensitivity to organic hydroperoxides. According to Student *t*-distribution test, the results of the mutant for all three reagents tested are significantly different from those of wild type at 99% level of confidence.

An oxygen sensitivity assay was conducted next by culturing both the wild type and the mutant cells under different oxygen levels (1, 3 and 6%) for 72 h. We found that 1% oxygen level is optimal for the growth of wild type cells although it could still grow well at a decreased rate in 6% O_2 (Table I). For the dps mutant, the extent of growth was much less compared to wild type even under the optimal growth condition (1% O_2). No significant growth was observed at 3 or 6% O_2. The result indicates that oxygen had a toxic

effect on the mutant. This is in agreement with the data of the disk assay. We also found that *H. hepaticus* had a strict requirement for oxygen supplementation indicating the microaerophilic nature of this organism [1,2].

dps Mutant cells contain more damaged DNA

An excess amount of intracellular free iron along with molecular oxygen will catalyze the generation of ROS which attack important cellular components, such as proteins, lipid, and DNA, subsequently causing cell death. Hence, more DNA damage would be expected in mutant cells than in the wild type due to the loss of the DNA protection by Dps. To test this hypothesis, we exposed the *H. hepaticus* wild type or dps mutant cells to air for 0, 5 or 10 h and examined the extent of DNA fragmentation within cells by agarose gel electrophoresis. The results showed no or very little amount of DNA smear (i.e. fragmentation) for the three wild type samples on the gel indicating that their DNA had been well protected by Dps and other oxidative stress resistance proteins (Figure 3). A significant amount of the DNA fragmentation was observed for the mutant cells after 5 or 10 h of exposure to air, indicating a role of Dps for DNA protection under oxidative stress.

8-OxoG is an accepted marker for oxidative DNA damage. We applied FITC–avidin/PI fluorescence staining method to evaluate the amount of 8-oxoG DNA lesions in *H. hepaticus* dps mutant compared to wild type cells. Since FITC–avidin specifically labeled 8-oxoG DNA lesions and the PI labels all DNA, the ratio of FITC to PI intensity reflects the level of DNA damage in the cells [32]. In this experiment, *H. hepaticus* wild type or mutant cells were first cultured in 1% O_2, 5% CO_2 (the balance N_2) at 37°C for 72 h and then exposed to air for 10 h before the staining. As a result, the majority of the wild type cells had normal morphology (bacillary form), and the cells were intact and motile. In contrast, a large portion of the mutant cell population was irregular in shape and composed of coccoid forms and broken cell pieces (Table II). The result indicated that mutant cells were approaching death, presumably due to oxidative damage. The FITC and PI luminosity were measured from eight different sets of images and the mean ratio of FITC/PI was calculated accordingly. The FITC/PI ratio for the mutant was significantly higher (6-fold) than that for the wild type (Table II), demonstrating that the mutant cells contain much higher level of 8-oxoG DNA lesions. This observation is in agreement with the result of the DNA fragmentation assay.

Purified Dps protein binds iron

To determine whether Dps in *H. hepaticus* has the iron binding activity, we over-expressed the protein in the *E. coli* host strain and purified it by using anion ion exchange and gel filtration techniques (Figure 4). Iron free apo-Dps was prepared using native Dps as the starting material and then subjecting to dialysis in a buffer containing the iron chelating agent 2, 2′-dipyridyl. Fe-loaded Dps was prepared by incubating the apo-Dps protein with ferrous ammonium sulfate as described in Materials and Methods. The protein was resolved on non-denaturing PAGE gel and the iron associated with protein was detected by Ferene S staining [36]. The result showed that both the native Dps and the iron-loaded Dps were stained with Ferene S (Figure 5), but the iron-loaded Dps was stained at least 2-fold stronger as determined by densitometry than that of the native Dps. Apo-Dps was not stained at all with Ferene S, as expected. All three forms of

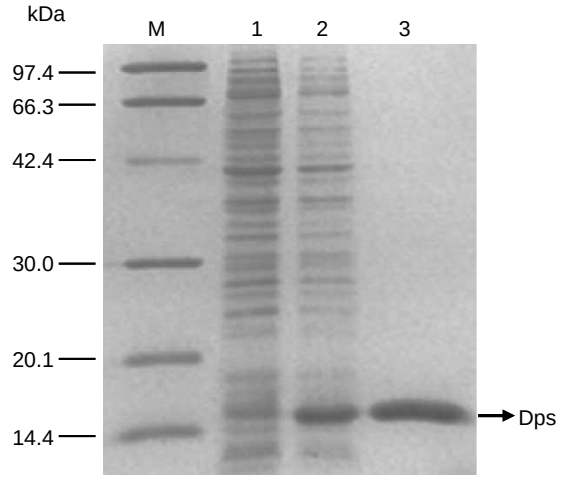

Figure 4. Over-expression and purification of *H. hepaticus* Dps. The following samples were resolved on the 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue. Lanes: 1, cell extract of non-induced *E. coli* BL21 Origami (7 μg); 2, cell extract of IPTG-induced *E. coli* cells (7 μg); and 3, purified Dps protein (7 μg); M, protein standard (Bio-Rad).

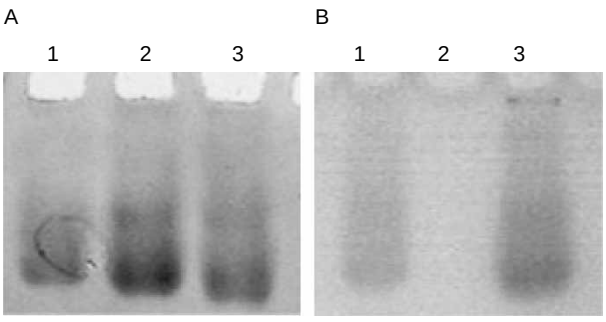

Figure 5. Non-denaturing PAGE examining the iron-binding ability of the *H. hepaticus* Dps protein. (A) Non-denaturing PAGE stained with Coomassie brilliant blue; and (B) non-denaturing PAGE stained with Ferene S. Lanes: 1, native *H. hepaticus* Dps (7 μg); 2, apo *H. hepaticus* Dps (7 μg); and 3, Fe-loaded *H. hepaticus* Dps (7 μg).

Dps protein were stained well with Coomassie blue on the native protein gel. The smearing of the protein bands on the gel are probably due to the disassociation of the intact Dps molecules into peptides with various numbers of subunits which have different migrating rates on the gel.

Purified Dps protein binds DNA

Some Dps proteins have been shown to associate with DNA, forming a stable nucleoprotein complex [23,24,29]. The complex is considered a significant factor in protecting the DNA from oxidative damage. To investigate the DNA binding activity of *H. hepaticus* Dps, we performed a DNA binding assay by incubating purified Dps (apo, native and iron saturated forms) with the plasmid DNA and resolved the reaction mixture on an agarose gel. We found that all three forms of Dps protein can bind to DNA and the binding capacity increased in the order as apo-Dps, native Dps and Fe-loaded Dps as shown in Figure 6. DNA was totally retained in the well of the agarose gel after incubating with Fe-loaded Dps. We attribute this to the biocrystal formation [29]. The results indicate that *H. hepaticus* Dps can bind DNA and the incorporated iron ions in Dps enhance the binding efficiency of the protein to DNA. Incubation with the control protein BSA did not change the electrophoresis pattern of plasmid on the gel, indicating that BSA was not associated with DNA.

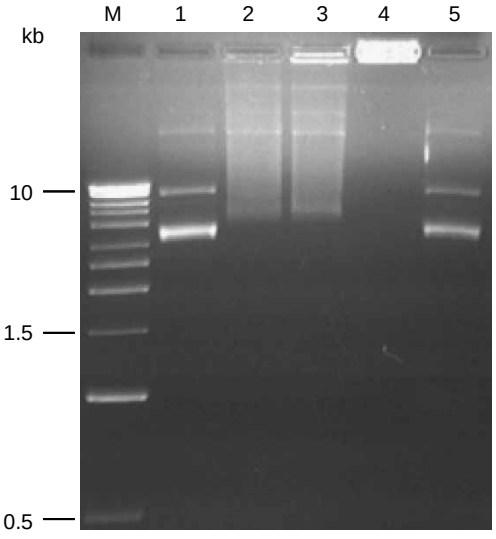

Figure 6. DNA-binding activity of *H. hepaticus* Dps. Purified Dps or BSA control proteins were incubated with pGEMT plasmid DNA at 37°C for 30 min before subjection to agarose gel electrophoresis. DNA on the gel was stained with ethidium bromide. Lanes: 1, DNA (200 ng) alone; 2, apo-Dps (30 μg) and DNA (200 ng); 3, native Dps (30 μg) and DNA (200 ng); 4, Fe-loaded Dps (30 μg) and DNA (200 ng); and 5, BSA (30 μg) and DNA (200 ng).

Discussion

In this study, we created a gene-disruption knockout of the *dps* gene in *H. hepaticus* and studied the resulting phenotypic changes in oxidative stress resistance. The data showed that the dps mutant is more sensitive to high O_2 concentrations and to various oxidative stress reagents compared to the wild type. A chloramphenicol cassette originated from *Campylobacter coli* [35] was used to disrupt the *dps* gene, which may have a polar effect on the downstream gene (3,4-dihydroxy-2-butanone 4-phosphate synthase). To verify that the observed phenotype changes are due to *dps* gene disruption, a genetic complementation assay is required. However, such complementation approaches are currently unavailable for *H. hepaticus*. In addition, the downstream gene would seem not to be involved in an oxidative stress response based on its known function, synthesis of riboflavin [37]. Therefore, the observed phenotype changes in oxidative stress resistance can most likely be attributed to the disruption of the *dps* gene.

We also found that purified *H. hepaticus* Dps could bind both iron and DNA *in vitro*, demonstrating the *H. hepaticus* protein contains both of the characteristics ascribed to Dps from a few sources previously. The attributes of the pure *H. hepaticus* protein are consistent with its ability to serve two roles, one direct (binding DNA) and one indirect (sequestering iron) in combating oxidative stress DNA damage in *H. hepaticus*.

The primary amino acid sequence of *H. hepaticus* Dps has significant homology to other Dps family proteins, which have been well characterized, including *E. coli* Dps (22%) [34], *Agrobacterium tumefaciens* (24%) [28], *Borrelia burgdorferi* (30%) [7], *L. innocua* ferritin (Fri) (32.3%) [21], *Bacillus subtilis* MrgA (34.6%) [38], *C. jejuni* Dps (43.3%) [26], and *H. pylori* NapA (59.4%) [25], etc. Those are ferritin like proteins with a dodecameric structure and those were reported to have an iron binding ability and provide protection for DNA under oxidative stress environment. No significant matching was found between *H. hepaticus* Dps and other iron binding proteins, like ferritin or bacterioferritin.

So far, all Dps family proteins studied have been reported to contain iron when the metal is provided [22,25,26,39]. Our results supported the same conclusion for the *H. hepaticus* Dps. The result in Figure 5 suggests that the Dps protein still oligomerizes in the absence of iron (apo-Dps moves at the same position as Fe-loaded Dps on the native gel). Similar results were observed for the Dps homologues in other organisms [26,33]. Ferritin is generally considered an iron storing and releasing protein with its major functional role in maintaining iron homeostasis. Such a role does not require rapid iron sensing while Dps is considered to quickly sequester fluxes of free iron in order to prevent generation of

oxygen-related radicals during peroxide stress conditions. For *E. coli* Dps, it has been reported that 12 ferroxidase sites in each molecule bind and oxidize from Fe^{2+} ions to Fe^{3+} along with the reduction of H_2O_2. By doing this, it nullifies the toxic combination of Fe^{2+} and H_2O_2 in Fenton reaction [33,39]. Similar ferroxidase sites were also reported in *L. innocua* Fri which uses O_2 instead of H_2O_2 as the main oxidant [21,40]. From the crystal structure of *L. innocua* Fri, the amino acid residues making up the ferroxidase sites (H31, H43, D47, D58 and E62) have been identified [41]. Those residue are well conserved in *H. hepaticus* Dps (H26, H38, E42, D53 and E57). We assume that those residues probably contribute to the iron binding activity for *H. hepaticus* Dps.

In this study, we showed that *H. hepaticus* Dps can bind DNA. Dps proteins from a few other bacterial species such as *E. coli* and *M. smegmatis* were also reported to have DNA binding capacity. These proteins were shown to interact with DNA and condense it [23,39]. By doing so, the DNA is "sheltered" from the attack of the free oxidative radicals. So far, the underlying mechanism for the protein-DNA interaction has not been well determined but it has been considered that a mobile and lysine rich N-terminus is important for DNA binding in *E. coli* [29]. The *H. hepaticus* Dps protein N-terminus is lysine rich with three lysine residues at Lys-3, Lys-9 and Lys-21. By doing a second structural search using Swissprot (SSpro), we found that these lysine residues were included in the first alpha helix in the N-terminus. It has been reported that the presence of a lysine-rich N-terminal helix in *Lactococcus lactis* Dps is required for DNA binding [42]. Presumably, the first alpha helix structure containing the lysine residues contributes to DNA binding in *H. hepaticus* Dps. More interestingly, we find that the incorporated iron in Dps promotes DNA binding so that the binding capacity increases in the order of apo-Dps, native Dps and iron loaded Dps proteins. A study on *P. gingivalis* Dps yielded a similar result regarding DNA binding ability [24]. They showed that the iron-loaded recombinant Dps exhibited strong DNA binding while the recombinant Dps without additional iron loading had almost no DNA-binding ability. They did not perform the assay on the apo-Dps version (i.e. complete removal of iron by chelator). However, in our assay, the apo-Dps of *H. hepaticus* also showed a weak DNA-binding ability. Several Dps family proteins are reported not to have the DNA binding capacity, such as *H. pylori* NapA [25], *S. mutans* Dpr [33], and *A. tumefaciens* Dps [28], etc. However, in a recent study, it was found that *H. pylori* NapA co-localizes with the cellular DNA, suggesting that it can interact with DNA *in vivo* [43].

Since *H. hepaticus* Dps binds iron, which in turn promotes DNA binding, we propose that this mechanism ensures that DNA protection is heightened under conditions where oxidative stress is the most severe. That Dps DNA binding has a protective role is supported by the direct evidence from DNA fragmentation assays. Fluorescent staining examination to view cell morphology, and assays of 8-oxoG levels of the mutant after oxidative stress challenge yielded results supporting DNA protective roles. *H. hepaticus* Dps is phylogenetically most related to *H. pylori* NapA. Initial characterization of a *H. pylori* napA mutant [44] showed that it is not sensitive to organic hydroperoxides, but significantly sensitive to oxygen compared to the wild type. This indicates that *H. pylori* NapA is involved in oxidative stress defense, although its role is minor compared to AhpC, the alkyhydroperoxide reductase. The result of Cooksley [43] also showed that an *H. pylori* napA mutant survives less well than wild type upon exposure to oxidative stress (H_2O_2). In conclusion, both *H. hepaticus* Dps and *H. pylori* NapA play an important role in survival during oxidative stress by sequestering the intracellular free iron and binding DNA.

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References

[1] Ward JM, Fox JG, Anver MR, Haines DC, George CV, Collins Jr, MJ, Gorelick PL, Nagashima K, Gonda MA, Gilden RV, et al. Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel *Helicobacter* species. J Natl Cancer Inst 1994;86(16):1222–1227.

[2] Fox JG, Dewhirst FE, Tully JG, Paster BJ, Yan L, Taylor NS, Collins Jr, MJ, Gorelick PL, Ward JM. *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. J Clin Microbiol 1994;32(5):1238–1245.

[3] Burich A, Hershberg R, Waggie K, Zeng W, Brabb T, Westrich G, Viney JL, Maggio-Price L. Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. Am J Physiol Gastrointest Liver Physiol 2001;281(3):G764–G778.

[4] Chin EY, Dangler CA, Fox JG, Schauer DB. *Helicobacter hepaticus* infection triggers inflammatory bowel disease in T cell receptor alpha beta mutant mice. Comp Med 2000;50(6):586–594.

[5] Maggio-Price L, Bielefeldt-Ohmann H, Treuting P, Iritani BM, Zeng W, Nicks A, Tsang M, Shows D, Morrissey P, Viney JL. Dual infection with *Helicobacter bilis* and *Helicobacter hepaticus* in *p*-glycoprotein-deficient mdr1a-/-mice results in colitis that progresses to dysplasia. Am J Pathol 2005;166(6):1793–1806.

[6] Erdman SE, Poutahidis T, Tomczak M, Rogers AB, Cormier K, Plank B, Horwitz BH, Fox JG. CD4+ CD25+ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice. Am J Pathol 2003;162(2):691–702.

[7] Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O, Ketchum KA, Dodson R, Hickey EK,

et al. Genomic sequence of a Lyme disease Spirochaete, *Borrelia burgdorferi*. Nature 1997;390(6660):580–586.

[8] Rogers AB, Fox JG. Inflammation and cancer. I. Rodent models of infectious gastrointestinal and liver cancer. Am J Physiol Gastrointest Liver Physiol 2004;286(3):G361–G366.

[9] Suerbaum S, Josenhans C, Sterzenbach T, Drescher B, Brandt P, Bell M, Droge M, Fartmann B, Fischer HP, Ge Z, et al. The complete genome sequence of the carcinogenic bacterium *Helicobacter hepaticus*. Proc Natl Acad Sci USA 2003;100(13):7901–7906.

[10] Davies GR, Banatvala N, Collins CE, Sheaff MT, Abdi Y, Clements L, Rampton DS. Relationship between infective load of *Helicobacter pylori* and reactive oxygen metabolite production in antral mucosa. Scand J Gastroenterol 1994;29(5):419–424.

[11] Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med 1995;18(4):775–794.

[12] Storz G, Imlay JA. Oxidative stress. Curr Opin Microbiol 1999;2(2):188–194.

[13] Zhang QM, Takemoto T, Mito S, Yonei S. Induction of repair capacity for oxidatively damaged DNA as a component of peroxide stress response in *Escherichia coli*. J Radiat Res 1996;37(3):171–176, Tokyo.

[14] Mehta NS, Benoit S, Mysore JV, Sousa RS, Maier RJ. *Helicobacter hepaticus* hydrogenase mutants are deficient in hydrogen-supported amino acid uptake and in causing liver lesions in A/J mice. Infect Immun 2005;73(9):5311–5318.

[15] Ge Z, Feng Y, Whary MT, Nambiar PR, Xu S, Ng V, Taylor NS, Fox JG. Cytolethal distending toxin is essential for *Helicobacter hepaticus* colonization in outbreed Swiss Webster mice. Infect Immun 2005;73(6):3559–3567.

[16] Martinez A, Kolter R. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J Bacteriol 1997;179(16):5188–5194.

[17] Altuvia S, Almiron M, Huisman G, Kolter R, Storz G. The dps promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. Mol Microbiol 1994;13(2):265–272.

[18] Grant RA, Filman DJ, Finkel SE, Kolter R, Hogle JM. The crystal structure of Dps, a ferritin homology that binds and protects DNA. Nat Struct Biol 1998;5(4):294–303.

[19] Wolf SG, Frenkiel D, Arad T, Finkel SE, Kolter R, Minsky A. DNA protection by stress-induced biocrystallization. Nature 1999;400(6739):83–85.

[20] Buda F, Ensing B, Gribnau MC, Baerends EJ. O_2 evolution in the Fenton reaction. Chemistry 2003;9(14):3436–3444.

[21] Bozzi M, Mignogna G, Stefanini S, Barra D, Longhi C, Valenti P, Chiancone E. A novel non-heme iron-binding ferritin related to the DNA-binding proteins of the Dps family in *Listeria innocua*. J Biol Chem 1997;272(6):3259–3265.

[22] Yamamoto Y, Fukui K, Koujin N, Ohya H, Kimura K, Kamio Y. Regulation of the intracellular free iron pool by Dpr provides oxygen tolerance to *Streptococcus mutans*. J Bacteriol 2004;186(18):5997–6002.

[23] Gupta S, Chatterji D. Bimodal protection of DNA by *Mycobacterium smegmatis* DNA-binding protein from stationary phase cells. J Biol Chem 2003;278(7):5235–5241.

[24] Ueshima J, Shoji M, Ratnayake DB, Abe K, Yoshida S, Yamamoto K, Nakayama K. Purification, gene cloning, gene expression and mutants of Dps from the obligate anaerobe *Porphyromonas gingivalis*. Infect Immun 2003;71(3):1170–1178.

[25] Tonello F, Dundon WG, Satin B, Molinari M, Tognon G, Grandi G, Del Giudice G, Rappuoli R, Montecucco C. The *Helicobacter pylori* neutrophil-activating protein is an iron-binding protein with dodecameric structure. Mol Microbiol 1999;34(2):238–246.

[26] Ishikawa T, Mizunoe Y, Kawabata S, Takade A, Harada M, Wai SN, Yoshida S. The iron-binding protein Dps confers hydrogen peroxide stress resistance to *Campylobacter jejuni*. J Bacteriol 2003;185(3):1010–1017.

[27] Andrews SC. Iron storage in bacteria. Adv Microb Physiol 1998;40:281–351.

[28] Ceci P, Ilari A, Falvo E, Chiancone E. The Dps protein of *Agrobacterium tumefaciens* does not bind to DNA but protects it toward oxidative cleavage: X-ray crystal structure, iron binding and hydroxyl-radical scavenging properties. J Biol Chem 2003;278(22):20319–20326.

[29] Ceci P, Cellai S, Falvo E, Rivetti C, Rossi GL, Chiancone E. DNA condensation and self-aggregation of *Escherichia coli* Dps are coupled phenomena related to the properties of the N-terminus. Nucleic Acids Res 2004;32(19):5935–5944.

[30] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979;76(9):4350–4354.

[31] Zirkle RE, Krieg NR. Development of a method based on alkaline gel electrophoresis for estimation of oxidative damage to DNA in *Escherichia coli*. J Appl Bacteriol 1996;81(2):133–138.

[32] Chen SK, Tsai MH, Hwang JJ, Chang WP. Determination of 8-oxoguanine in individual cell nucleus of gamma-irradiated mammalian cells. Radiat Res 2001;155(6):832–836.

[33] Yamamoto Y, Poole LB, Hantgan RR, Kamio Y. An iron-binding protein, Dpr, from *Streptococcus mutans* prevents iron-dependent hydroxyl radical formation *in vitro*. J Bacteriol 2002;184(11):2931–2939.

[34] Almiron M, Link AJ, Furlong D, Kolter R. A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. Genes Dev 1992;6(12B):2646–2654.

[35] Wang Y, Taylor DE. Chloramphenicol resistance in *Campylobacter coli*: Nucleotide sequence, expression and cloning vector construction. Gene 1990;94(1):23–28.

[36] Chung MC. A specific iron stain for iron-binding proteins in polyacrylamide gels: Application to transferrin and lactoferrin. Anal Biochem 1985;148(2):498–502.

[37] Bacher A, Eberhardt S, Fischer M, Kis K, Richter G. Biosynthesis of vitamin B2 (riboflavin). Annu Rev Nutr 2000;20:153–167.

[38] Chen L, Helmann JD. *Bacillus subtilis* MrgA is a Dps(PexB) homologue: Evidence for metalloregulation of an oxidative-stress gene. Mol Microbiol 1995;18(2):295–300.

[39] Zhao G, Ceci P, Ilari A, Giangiacomo L, Laue TM, Chiancone E, Chasteen ND. Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. J Biol Chem 2002;277(31):27689–27696.

[40] Stefanini S, Cavallo S, Montagnini B, Chiancone E. Incorporation of iron by the unusual dodecameric ferritin from *Listeria innocua*. Biochem J 1999;338(Pt 1):71–75.

[41] Ilari A, Stefanini S, Chiancone E, Tsernoglou D. The dodecameric ferritin from *Listeria innocua* contains a novel intersubunit iron-binding site. Nat Struct Biol 2000;7(1):38–43.

[42] Stillman TJ, Upadhyay M, Norte VA, Sedelnikova SE, Carradus M, Tzokov S, Bullough PA, Shearman CA, Gasson MJ, Williams CH, et al. The crystal structures of *Lactococcus lactis* MG1363 Dps proteins reveal the presence of an N-terminal helix that is required for DNA binding. Mol Microbiol 2005;57(4):1101–1112.

[43] Cooksley C, Jenks PJ, Green A, Cockayne A, Logan RP, Hardie KR. NapA protects *Helicobacter pylori* from oxidative stress damage, and its production is influenced by the ferric uptake regulator. J Med Microbiol 2003;52(Pt 6):461–469.

[44] Olczak AA, Olson JW, Maier RJ. Oxidative-stress resistance mutants of *Helicobacter pylori*. J Bacteriol 2002;184(12):3186–3193.