The *Campylobacter jejuni* Dps protein binds DNA in the presence of iron or hydrogen peroxide

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Abstract

Iron is an essential co-factor for many enzymes, however, this metal can lead to the formation of reactive oxygen species. Ferritin proteins bind and oxidize Fe$^{2+}$ to Fe$^{3+}$, storing this metal in a non-reactive form. In some organisms, a particular subfamily of ferritins, namely Dps, have the ability to bind DNA. Here we show that the *Campylobacter jejuni* Dps has DNA binding activity which is uniquely activated by Fe$^{2+}$ or H$_2$O$_2$ under pH below neutral. The Dps-DNA binding activity correlated with the ability of Dps to self-aggregate. Dps-DNA interaction was inhibited by NaCl and Mg$^{2+}$ suggesting the formation of ionic interactions between Dps and DNA. Alkylation of cysteines affected DNA binding in the presence of H$_2$O$_2$ but not in the presence of Fe$^{2+}$. Substitution of the all cysteines in *C. jejuni* Dps to serines did not affect DNA binding excluding the participation of cysteine in H$_2$O$_2$ sensing. Dps was able to protect DNA *in vitro* from enzymatic cleavage and damage by hydroxyl radicals. A *C. jejuni* *dps* mutant was less resistant to H$_2$O$_2$ *in vivo*. The concerted activation of Dps-DNA binding in response to low pH, H$_2$O$_2$ and Fe$^{2+}$ may protect *C. jejuni* DNA during host colonization.
Introduction

_Campylobacter jejuni_ is a commensal organism of chickens and is the most common cause of human gastrointestinal disease in developed countries (1). _C. jejuni_ infection is characterized by fever, abdominal cramps and watery or bloody diarrhea and is typically self-limiting, however, in some cases _C. jejuni_ infection can lead to development of the neurologic disease Guillain-Barré syndrome (2,3)

Iron is an essential co-factor for many enzymes, required for both survival and pathogenicity of most bacteria, however, this metal can also lead to the formation of reactive oxygen species under oxidizing conditions (4). In the ferrous form (Fe^{2+}), iron can react with hydrogen peroxide to produce very reactive and toxic hydroxyl radicals through Fenton reaction (H_{2}O_{2} + Fe^{2+} → OH^{-} + OH^{•} + Fe^{3+}). In order to avoid the toxic effects of free Fe^{2+} organisms belonging to all domains of life store iron inside a ferritin family protein (5). Ferritins bind and oxidize Fe^{2+} to Fe^{3+} storing iron in an non-reactive iron-oxide form Fe_{2}O_{3} trapped in the protein cage (6).

One particular subfamily of ferritins is the Dps (DNA binding protein from starved cells), first characterized in _E. coli_ as a DNA binding protein induced in the stationary phase or under stress (7). Since this pioneer study, many different Dps proteins have been characterized. These proteins are structurally conserved and widely distributed in prokaryotes (8,9). Unlike typical ferritins that have 24 subunits and 432 symmetry, Dps proteins assemble in a quasi-spherical dodecamer with 23 symmetry and can store ~500 iron atoms (4,10). Furthermore, while in typical ferritins each subunit carries its own
ferroxidase center, the ferroxidase centers of Dps are located between the subunits were two Fe^{2+} ions binds to form a binuclear iron center (11,4).

A number of *in vivo* studies have demonstrated that Dps is involved in resistance to a variety of stressful conditions including reactive oxygen species, UV, ionizing radiation and thermal and acidic stress (7,12-21). Furthermore, Dps is able to protect DNA from oxidative damage *in vitro* (7,22-26). In all Dps proteins studied, DNA protection is exerted through the removal of free Fe^{2+} from the solution by reducing the formation of reactive oxygen species through Fenton chemistry. Furthermore, some Dps proteins can also physically protect DNA through the formation of non-specific protein-DNA complexes (7,22,27-30).

The mechanism of DNA binding by Dps proteins is not completely understood (9). A initial DNA binding event is followed by condensation into highly ordered DNA-protein structures also known as biocrystals (9,22,27). Apparently, the ability of Dps to condensate the DNA is related to its capacity to self-aggregate under certain conditions (24).

The N-terminal of the *E. coli* Dps has been implicated in interaction with DNA. This portion of *E. coli* Dps is very flexible and rich in positively charged lysine residues (10,24). However, different mechanisms for Dps-DNA interaction have been proposed. In *Deinococcus radiodurans* Dps1 the N-terminal region has been implicated in DNA binding through a mechanism involving the occupancy of a metal binding site located within this region (31,32). In *Mycobacterium smegmatis* Dps has a positively charged C-terminal that has been implicated in DNA binding (30). On the other hand, the *Helicobacter pylori* Dps (NapA) can bind DNA despite the absence of positively charged N or C-terminal
extensions; in this case it has been postulated that the protein’s highly positive surface may be involved in DNA-binding (22,33).

In *C. jejuni*, the gene encoding the Dps protein is required for resistance to H$_2$O$_2$ stress (17), biofilm formation and colonization of poultry and piglets (34,35). Previous studies failed to detect *C. jejuni* Dps-DNA binding activity (17). Here we show that *C. jejuni* Dps does have DNA binding activity, that it is stimulated by the presence of Fe$^{2+}$ or H$_2$O$_2$ and that *C. jejuni* Dps was able to protect DNA from oxidative damage and enzymatic degradation *in vitro* as well as able to enhanced cellular resistance to H$_2$O$_2$ *in vivo*. 
Materials and Methods

Bacterial strains, plasmids and oligonucleotides

The bacterial strains, plasmids and oligonucleotides used in PCR reactions are listed in Table 1.

Media and growth conditions

*E. coli* was grown in LB medium at 37°C. *C. jejuni* was grown at 42°C in Columbia agar supplemented with 5% defibrinated horse blood, vancomycin (10 µg.ml⁻¹), polymyxin B (0.3 µg.ml⁻¹) and trimethoprim (5 µg.ml⁻¹), in microaerobic conditions (10% CO₂, 5% O₂ in N₂).

Cloning and molecular biology methods

Agarose gel electrophoresis, bacterial transformation and cloning were performed using standard procedures as described (36). Isolation of plasmid DNA were performed using QIApre MidiKit (Quiagen). Enzymes were obtained from New England Biolabs (NEB) and used according to the manufacturers’ instructions. DNA sequencing was performed using dye-labeled terminators in an automated DNA sequencer at the Australian Genome Research Facility.

Cloning, expression and purification of *C. jejuni Dps*

The gene coding *C. jejuni* Dps (Cj1534c) was amplified by PCR using Phusion Polymerase (NEB) and a boiled culture of *C. jejuni* 11168-O as template. The primers used were: *Cj1534c NdeI* and *Cj1534c BamHI* (Table 1). The amplified product was ligated into
the vector pCR-BluntII-TOPO (Invitrogen). The resultant plasmid was named pLHPCRcj1534c; the DNA insert was completely sequenced to confirm its integrity. The \textit{dps} gene was then sub-cloned into the expression vector pET19b using the \textit{NdeI-BamHI} sites, producing the plasmids pLHPETcj1534c that expressed \textit{C. jejuni} Dps with an N-terminal His tag.

\textit{E. coli} BL21 (\lambda DE3) carrying pLHPETcj1534c was grown overnight in 10 ml LB containing ampicillin 100 \( \mu \text{g.ml}^{-1} \). This culture was used to inoculate 500 ml of LB containing ampicillin at 100 \( \mu \text{g.ml}^{-1} \). Cells were grown at 37 °C with shaking at 180 rpm and 0.3 mM IPTG was added after 2 hours. Cells were collected after 4 hours by centrifugation, the pellet was resuspended in 15 ml of buffer 1 (50 mM Tris-HCl pH 8, 0.1 M NaCl, 10% glycerol) and the cells were lysed by sonication on ice. The extract was clarified by centrifugation at 30,000 x g for 30 minutes at 4 °C. The supernatant was applied into a 1 ml Talon column (Sigma). The column was equilibrated with 5 ml buffer 1 before sample loading. The Dps protein was eluted using a stepwise gradient of buffer 1 containing 10, 50, 300 and 500 mM of imidazole. Fractions containing Dps were pooled and dialyzed in 50 mM Tris-HCl pH 8, 0.1 M NaCl, 50% glycerol. Dps was stored in aliquots at -80 °C until use.

\textit{Site directed mutagenesis}

The \textit{C. jejuni} \textit{dps} gene carrying the C54S-C68S-C85S mutations and flanked by the \textit{NdeI} and \textit{BamHI} restriction sites were synthesized and cloned into pIDTsmartKm vector by Integrated DNA Tecnologies (IDT). The mutant genes were sub-cloned into the expression vector pET19b using the \textit{NdeI-BamHI} sites, producing the plasmid pLHPETcj1534c-C-S.
(C54S-C68S-C85S). The final construct was verified by DNA sequencing. The expression and purification of the variant protein was performed as described for the wild-type Dps.

**Construction of the C. jejuni isogenic dps mutant strain**

The entire pLHPCRej1534c plasmid was amplified by inverted PCR using Expand Long Template PCR System (Roche) and the *cj1534c* InvF and *Cj1534c* InvR primers. The chloramphenicol resistance gene from pC46 was PCR-amplified using using Phusion Polymerase (NEB) and the CATF and CATR primers (Table 1). Both PCR products were purified, digested with *BglII* and ligated. One plasmid containing the chloramphenicol cassette, inserted into the *cj1534c* gene, in the same transcriptional orientation (verified by DNA sequencing), was named pLHPCRej1543cCAT. This plasmid was purified and electro-transformed into *C. jejuni* 11168-O and colonies resistant to chloramphenicol were selected. One colony, named LFH2, was selected for further characterization. The insertion of the chloramphenicol cassette into the *cj1534c* gene by double-crossover recombination was confirmed by PCR using *Cj1534c* NdeI, *Cj1534c* BamHI, CATF and CATR primers.

**Protein analysis**

Protein electrophoresis was carried out by SDS-PAGE (37) and gels were stained with Comassie Blue. For molecular weight determination of the Dps in its native state, samples were loaded on regular SDS-PAGE without prior boiling. Protein concentrations were determined by the Bradford assay (Biorad) using bovine serum albumin as a standard (38).

**DNA binding analysis using agarose gel electrophoresis**
Purified *C. jejuni* Dps was diluted in Tris-HCl 50 mM pH 6.8 to 0.15 µM (final dodecamer concentration) containing 2 nM of pGEM (Promega). For the experiments in the presence of Fe$^{2+}$, pre-weighted FeCl$_2$ was freshly dissolved (within less than 1 minute before the experiment) in Tris-HCl 50 mM pH 6.8 and added to the final concentration of 50 µM (unless stated otherwise). For the experiments in the presence of H$_2$O$_2$ a freshly prepared solution of H$_2$O$_2$ in Tris-HCl 50 mM pH 6.8 was added to the final concentration of 10 mM (unless stated otherwise). When indicated, DTT 2 mM (final concentration) was incubated with Dps for 30 minutes before the addition of DNA. The Dps samples treated with iodoacetamide were first reduced in the presence of DTT 2 mM for 10 minutes followed by addition of iodoacetamide 10 mM for one hour at room temperature. Iodoacetamide and DTT were removed using a desalting column (Biorad).

DNA binding reactions were performed in a final volume of 20 µl for 10 minutes at 25°C, the reaction was mixed (at 10:2 ratio) with glycerol 50% and bromophenol blue 0.01% and loaded into a 1% agarose gel prepared in TAE buffer (pH 8) containing 0.1 µl.ml$^{-1}$ of ethidium bromide. After electrophoresis for 20 minutes at 120V at room temperature, DNA was visualized after UV exposure using a gel doc system (Biorad).

**DNA protection assays**

Oxidative stress: DNA protection assay against hydroxyl radicals were performed using similar conditions as described for the DNA binding assay. A fresh solution of FeCl$_2$ was added to final concentration of 50 µM to the solution containing Dps and pGEM (Promega). After 10 minutes at 25°C, the reactions received H$_2$O$_2$ to 10 mM final concentration in order to generate hydroxyl radicals through the Fenton chemistry.
Reactions were quenched after 30 minutes with the addition of the iron chelator bipyridyl to 5 mM. The integrity of the DNA was analyzed by 1% agarose gel electrophoresis.

Nuclease cleavage: Purified *C. jejuni* Dps 0.15 μM (final dodecamer concentration) and 100 ng of pGEM (Promega) were incubated in 50 mM Tris-HCl pH 6.8, 0.25 mM MgCl₂ and 0.01 mM CaCl₂ in 20 μl reactions. When indicated FeCl₂ or H₂O₂ were present at final concentration of 50 μM and 10 mM, respectively. After 10 minutes at 25°C, 0.01 U of DNase I was added, reactions were quenched after 15 minutes at 25°C with EDTA 10 mM, the integrity of the DNA was analyzed by 1% agarose gel electrophoresis.

*C. jejuni* and *E. coli* resistance to H₂O₂

*C. jejuni*, cultivated overnight at 42°C in Columbia agar, was collected and suspended in LB to an OD₆₀₀nm = 0.25. *E. coli* BL21 cells carrying the control pET19b plasmid or the plasmid expressing Dps (pLHPETcj1534c) were cultivated in LB to an OD₆₀₀nm = 0.5. 1 mM IPTG was added to the cultures, after 4 hours shaking at 37°C cells were collected and diluted in LB to an OD₆₀₀nm = 0.35. Both *C. jejuni* and *E. coli* received H₂O₂ 5 mM, cells were incubated in LB under air at 37°C for 30 minutes. Viable cells were determined by serial dilution CFU counting in LB agar for *E. coli* or Columbia agar for *C. jejuni* strains.

Iron uptake experiments

Purified Dps was diluted to 0.15 μM or 0.3 μM (final dodecamer concentration) in 400 μl Tris-HCl 50 mM pH 6.8. Freshly prepared FeCl₂ was added to 100 μM final concentration and the formation of the iron core was monitored at 305 nm under air in a 0.5
cm quartz cuvette. When indicated 1 mM of H$_2$O$_2$ was added to final concentration just before the addition of iron. Control experiments without the addition of Dps showed negligible changes in absorbance at during the assay time course.

*Surface Plasmon Resonance detection (SPR)*

SPR experiments were performed using a BIAcore T100 biosensor system (GE-heathcare) at 25 °C in Tris-HCl pH 6.8 at a flow rate of 30 µl per minute. Purified His-Dps was diluted to 0.15 µM (final dodecamer concentration) in Tris-HCl pH 6.8 and loaded on flow cell 2 (FC2) of a Ni$^{2+}$ NTA sensor chip with 5 minutes contact time. FC3 and FC4 received His-Dps that were pre-incubated for 10 minutes at 25 °C with FeCl$_2$ 50 µM or H$_2$O$_2$ 10 mM, respectively at the same final concentration and immobilized for the same contact time as the untreated Dps. FC1 had no protein loaded and was used as reference.

Serial dilutions of purified pGEM (Promega) or a PCR product (the *cat* gene PCR-amplified using CATF and CATR primers and pC46 as template, 756 bp) were prepared in Tris-HCl pH 6.8 to 50, 25, 12.5 and 6.25 ng/ml. The DNA dilutions were loaded to the sensor chip using a multi-cycle kinetics, i.e. after the injection of each dilution the chip was regenerated with EDTA. Subsequently, the chip was re-loaded with Ni$^{2+}$ and His-Dps before the injection of the next DNA dilution. To validate the reproducibility of the SPR response two cycles of the 12.5 ng/ml dilution were recorded in each experiment. The specificity of the DNA binding was recorded as the response signal difference between each Dps loaded FC and the reference FC1. A 10 minute dissociation time was allowed after the addition of each concentration of analyte. SPR signals were analyzed using the Biacore Evaluation software to determine K$_d$. 
Results

The *C. jejuni* Dps protein is a dodecamer and has ferroxidase activity under atmospheric oxygen conditions

The *C. jejuni* Dps protein was purified and analysed using SDS-PAGE. Dps prepared under standard conditions, migrated though the polyacrylamide matrix with the expected molecular weight for a monomer of ~20 kDa (Fig. 1A, lane 2). However, when Dps was loaded on SDS-PAGE without prior denaturation by boiling, the protein migrated at ~250 kDa, suggesting the formation of dodecamers (Fig. 1A, lane 1). Analysis of the crystal structure of *C. jejuni* Dps (PDB: 3KWO) confirmed the dodecameric assembly (Fig. S1A). Other characteristic Dps features such as the presence of acidic residues at iron pore entrance (Fig. S1B) and conserved residues at the inter-subunit ferroxidase center (Fig. S2A, S2C) are conserved in the *C. jejuni* Dps.

The formation of the iron oxide core in *C. jejuni* Dps proteins was investigated by monitoring the absorbance at 305 nm after the addition of Fe$^{2+}$ under atmospheric oxygen conditions. Control experiments showed negligible changes in absorbance of a FeCl$_2$ solution in absence of Dps. However, when Dps was incubated with FeCl$_2$ there was a rapid increase in the absorbance at 305 nm and this increase was Dps concentration dependent (Fig. 1B). Addition of 1 mM H$_2$O$_2$ did not change the initial velocity of iron core formation (Fig. 1B). It worth mentioning that purified *C. jejuni* Dps did not show absorbance peak in the region between 290 to 320 nm suggesting that the purified protein does not carry significant amounts of oxidized iron. These data support that *C. jejuni* Dps
acts as an iron-storage protein that can use O$_2$ as iron oxidant. Furthermore, the Dps ferroxidase activity is not affected by H$_2$O$_2$ under atmospheric oxygen conditions.

*C. jejuni Dps protein binds DNA in the presence of Fe$^{2+}$ or H$_2$O$_2$*

The ability of Dps to interact with supercoiled plasmid DNA was analyzed by electrophoretic mobility shift assays. When *C. jejuni* Dps was incubated with DNA, there were no changes in DNA mobility through agarose gel (Fig. 2A, lane 3). However, when Fe$^{2+}$ or H$_2$O$_2$ were present, the DNA mobility shifted with the appearance of two DNA bands; a minor band with decreased mobility (Fig. 2A, indicated by arrow II) and a major band that was too large to enter the gel (Fig. 2A, indicated by arrow I). The appearance of these slower migrating bands was directly proportional to the amount of Dps added (data not shown), indicating that they represent formation of Dps-DNA complexes in concentration dependent manner.

Samples containing 15 µg of purified Dps were incubated under the same conditions and resolved using the same gel shown in Fig. 2A, a part of the gel was separated and stained with coomasie blue to demonstrate presence and mobility of Dps (Fig. 2B). The addition of either Fe$^{2+}$ or H$_2$O$_2$ shifted the migration behavior of purified Dps so dramatically that part of the protein could not enter the gel, suggesting the formation of protein self-aggregates. The formation Dps self-aggregates is well documented in *E. coli* and *H. pylori* and it correlates with the ability of these proteins to interact with DNA *in vitro* (22-24).

As *C. jejuni* Dps carries two surface exposed cysteine residues per subunit (Fig. S2B and S2C), we suspected that Dps self-aggregation in the presence of Fe$^{2+}$ or H$_2$O$_2$
could be caused by the formation of disulfide bounds between different Dps dodecamers. However, the SDS-PAGE profiles of Dps treated with Fe$^{2+}$ or H$_2$O$_2$ were identical to the untreated Dps both in the presence or absence of β-mercaptoethanol (data not shown). Hence, Dps self-aggregation does not occur by disulfide bound formation and is readily reversed in the presence of SDS.

The capacity of both Fe$^{2+}$ and H$_2$O$_2$ to activate Dps-DNA binding was dose dependent. Concentrations of H$_2$O$_2$ as low as 10 µM were able to promote DNA binding (Fig S3), hence, it is likely that H$_2$O$_2$ affects Dps activity under relevant physiological conditions. Increasing concentrations of iron favored Dps-DNA interaction, a saturation point was observed when an iron:Dps dodecamer ratio of approximately 25:1 was achieved (Fig. S4, lane 5). Interestingly, this saturation point is just above the 12 iron:Dps ratio which is the expected ratio required to occupy all the 12 Fe$^{2+}$ binding sites present within the Dps dodecamer. Other divalent metals tested, including Mn$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$ were not able to induce Dps DNA binding (data not shown).

*Effects of pH, salts and Mg$^{2+}$ on the C. jejuni Dps-DNA interaction*

The DNA binding activity of Dps was further tested in a range of different buffer conditions. Higher pH decreased the Dps-DNA interaction for both Fe$^{2+}$ and H$_2$O$_2$ treatments until no DNA binding was observed at pH 8.8 (Fig. 3, compare lanes 3 and 6 to 5 and 8). The H$_2$O$_2$-induced DNA binding activity of Dps seems to be more sensitive to higher pH than that induced by Fe$^{2+}$, at pH 8 there was some DNA binding to Dps treated
with Fe$^{2+}$ while no DNA binding was detected with for Dps treated with H$_2$O$_2$ (Fig. 3, lanes 4 and 7).

The presence of NaCl or MgCl$_2$ decreased the ability of Dps to interact with DNA. In the presence of 0.1 M NaCl or 5 mM MgCl$_2$, the Fe$^{2+}$ induced Dps-DNA complex formation decreased slightly (Fig. 3, lanes 9 and 15) but was significantly affected in the presence of H$_2$O$_2$ (Fig. 3, lanes 12 and 17). Throughout, Dps-DNA interactions were still observed for both Fe$^{2+}$ and H$_2$O$_2$ treatments under NaCl and MgCl$_2$ concentrations up to 1 M and 25 mM, respectively (Fig. 3).

Effects of iodoacetamide, DTT and presence of cysteine residues on Dps-DNA interaction

The DNA binding activity in presence of H$_2$O$_2$ has never been reported for Dps proteins making of C. jejuni Dps unique. H$_2$O$_2$ is too simple structurally to be specifically recognized by a protein, however, this molecule can catalyze oxidation of a variety of protein groups (39,40). Oxidation of cysteine residues and formation of disulfide bridges have been implicated in H$_2$O$_2$ sensing in both prokaryotes and eukaryotes (40-42,42).

Sequence alignments between C. jejuni, E. coli and H. pylori Dps revealed that the C. jejuni Dps has 3 unique cysteine residues (Fig. S2C). Two of these (C68 and C85) are surface exposed and located in flexible loops at dimer interface in the protein’s two-fold symmetry axis (Fig. S2B). Furthermore, the presence of nearby lysine residues could decrease the pKa of thiol rendering C68 and C85 very sensitive to H$_2$O$_2$ oxidation in similar fashion to that described for the archetypical E. coli OxyR H$_2$O$_2$ sensor transcriptional factor (43).
To verify if cysteine oxidation could be involved in H$_2$O$_2$ sensing, DNA binding experiments were performed using Dps that was pre-treated with iodoacetamide or DTT. In the presence of iodoacetamide or DTT, the H$_2$O$_2$ effect on the DNA binding activity of Dps was abolished (Fig. 4A), however, the iodoacetamide treatment did not affect the ability of Dps to binding DNA in the presence of Fe$^{2+}$ (Fig. 4A, lane 7), suggesting that Fe$^{2+}$ and H$_2$O$_2$ induce DNA binding through different mechanisms. The presence of DTT abolished the Dps-DNA interaction in the presence of Fe$^{2+}$ (Fig. 4A, lane 8). However, the migration of the control DNA was also affected (Fig. 4A, lane 4), probably as result of DNA nicking due to radicals that are known to form when Fe$^{2+}$, DTT and oxygen are combined (44).

The fact that the H$_2$O$_2$-induced DNA binding activity of C. jejuni Dps was abolished by iodoacetamide or DTT suggests that cysteine oxidation could be involved in H$_2$O$_2$ sensing. To verify this hypothesis a Dps variant where all the three cysteines were converted to serines (C54S-C68S-C85S) was prepared. This variant kept 75% of the iron oxidation activity (Fig. S5). The DNA binding activity of this variant (induced by Fe$^{2+}$ or H$_2$O$_2$) was comparable to the wild-type (Fig. 4B). As expected, iodoacetamide which is not reactive towards the serine, did not affect DNA binding for the C54S-C68S-C85S Dps variant (Fig. 4B).

These observations exclude a role of cysteine oxidation in H$_2$O$_2$ sensing by C. jejuni Dps. However, the fact that iodoacetamide blocked DNA binding induced by H$_2$O$_2$ suggest that cysteine alkylation may sterically affect interaction between the wild-type Dps and DNA. Hence, the Dps two-fold symmetry (Fig. S2B) is likely to be the site for DNA binding, at least for the H$_2$O$_2$ response.
Analysis of the Dps-DNA interaction using surface Plasmon resonance

We further analyzed the DNA binding activity of *C. jejuni* Dps using surface plasmon resonance which is more sensitive than the DNA shift assays. Purified Dps protein samples were either left untreated or were pre-treated with H$_2$O$_2$ or Fe$^{2+}$ and then mobilized in three different flow cells of a Ni$^{2+}$ NTA Biacore sensor chip, a fourth flow cell without any protein loaded was kept as reference. Different concentrations of supercoiled plasmid DNA was used as ligand.

When the experiments were performed at pH 8.8, no Dps-DNA interaction were detected, confirming the results of the electrophoresis mobility shift assays (data not shown). However, when buffer at pH 6.8 was used, Dps was able to interact with plasmid DNA (Fig. 5). Pre-treatment of Dps with H$_2$O$_2$ or Fe$^{2+}$ increased the affinity of binding to DNA by 8-9 orders of magnitude (Fig. 5). The Dps-DNA binding interaction was virtually irreversible in the presence of H$_2$O$_2$ and Fe$^{2+}$ such that no dissociation was observed after the removal of the ligand DNA (Fig. 5B and 5C). However, in the absence of H$_2$O$_2$ and Fe$^{2+}$, partial dissociation the Dps-DNA complex was observed within 30 seconds of the removal of the DNA (Fig. 5A).

The addition of DTT 1 mM resulted in partial dissociation of the Dps-DNA complex that were induced by Fe$^{2+}$ or H$_2$O$_2$ as analyzed by SPR (data not shown), this confirms the results obtained in the gel shift assays (Fig. 4A, lane 8). Hence, the Dps-DNA interaction is abolished in the presence of DTT, however, the molecular mechanism underling such response remains unknown.
The SPR data essentially confirmed the results obtained using gel shift. Furthermore, the higher sensitivity of the SPR allowed the detection of low affinity Dps-DNA interactions that could not be observed using gel shifts: the interaction between untreated Dps and DNA under pH 6.8 (Fig. 5A) but not at pH 8.8 (data not shown). Dps-DNA interaction was also detected by SPR when a linear relaxed PCR product DNA was used as ligand at pH 6.8. Hence, the Dps-DNA complex formation occurs independently of the DNA overall topological structure.

Dps protects DNA from DNase I and binds DNA under oxidative stress conditions

The ability of Dps to interact with DNA suggested that it could protect DNA from enzymatic and/or oxidative damage when the Fe\(^{2+}\) and H\(_2\)O\(_2\) levels are high. Hence, we evaluated the capacity of Dps to protect plasmid DNA from both DNase I and from hydroxyl radicals. Treatment of purified pGEM (Promega) plasmid DNA with DNase I resulted in nearly complete DNA degradation (Fig. 6A). However, when Dps and Fe\(^{2+}\) or H\(_2\)O\(_2\) were present, the DNA signal was still observed in the well of the gel after agarose gel electrophoresis (Fig. 6A), supporting the hypothesis that Dps can physically protected DNA.

The combination of Fe\(^{2+}\) and H\(_2\)O\(_2\) leads to the formation of hydroxyl radicals which, in turn, cause double stranded DNA breaks that can be accessed by the conversion of supercoiled circular plasmid DNA to linear DNA by electrophoresis (12). Indeed, when pGEM plasmid DNA was treated with Fe\(^{2+}\) and H\(_2\)O\(_2\) it was converted to a slower migrating band that corresponds to the molecular weight of linear plasmid DNA suggesting
double stranded DNA breaks (Fig. 6B). However, when Dps was present, all the DNA signal was retained in the well of the gel (Fig. 6B), suggesting the formation of protein-DNA complex under oxidative stress conditions that could potentially protected the DNA from degradation. Unfortunately we could not access the integrity of the DNA in isolation after Dps treatment because all attempts to dissociate the Dps-DNA complex using: organic solvent extraction, DTT, hot SDS or DNA clean-up kits were unsuccessful.

*Dps enhances resistance to H₂O₂ in vivo*

In order to analyze if Dps could protect *C. jejuni* against H₂O₂ in vivo a *C. jejuni dps* knock-out strain was constructed and its resistance to H₂O₂ was compared to the parental 11168-O strain. The parental strain viable cell count was reduced by 2 logs after challenge with H₂O₂ 5 mM whilst the *dps* mutant strain was much less resistant to H₂O₂ as a 6 logs reduction in cell count was observed (Table 2), confirming previous observations (17).

*E. coli* cells lacking Dps showed a significant change in protein expression profile as indicated by two dimension gel electrophoresis (7). Hence, the reduced resistance of the *C. jejuni dps* mutant to H₂O₂ could be a pleiotropic effect of the *dps* mutation. However, we noted that expression of the *C. jejuni* Dps protein in host *E. coli* cells was able to increase the resistance of *E. coli* to H₂O₂ stress, by one log (Table 2) suggesting that, at least in part, the H₂O₂ Dps-protective affected is direct.
Discussion

In this work we used gel shift, DNase I protection and SPR to show that *C. jejuni* Dps has the unique ability to sense the Fe\(^{2+}\) and H\(_2\)O\(_2\) levels and bind to DNA in response to increasing concentrations of Fe\(^{2+}\) or H\(_2\)O\(_2\). Furthermore, Dps-DNA interaction was maximized in pHs below 7. When low pH and Fe\(^{2+}\) or H\(_2\)O\(_2\) were combined the Dps-DNA interaction was very tight, with K\(_d\)s in the femtomolar range (Fig 5), the Dps-DNA interaction was virtually irreversible such that no dissociation was observed after the removal of the ligand DNA in SPR (Fig. 5B and 5C) and all attempts to dissociate the Dps-DNA complex formed *in vitro* were unsuccessful.

It is well established that Dps proteins confer resistance to reactive oxygen species. This is achieved by removal of Fe\(^{2+}\) from the cytoplasm and storing this metal in the oxidized form inside the protein cage thereby reducing Fenton chemistry (4). Here we show that the *C. jejuni* Dps is able to oxidize iron (Fig. 1B), however, unlike typical Dps proteins that preferentially use H\(_2\)O\(_2\), the *C. jejuni* Dps can efficiently use O\(_2\) as oxidant. Notably, ferrooxidase activity was also detected in aerated buffers without added H\(_2\)O\(_2\), and furthermore, addition of H\(_2\)O\(_2\) did not alter this activity (Fig. 1B).

Dps proteins from several organisms can physically interact with DNA (7,22,25,28,45). In this study we demonstrated that *C. jejuni* Dps can bind DNA in the presence of Fe\(^{2+}\) or H\(_2\)O\(_2\) using electrophoretic mobility shift and DNase I protection assays (Fig. 2A and 5A). A previous report failed to detect *C. jejuni* Dps-DNA interaction, probably because no Fe\(^{2+}\) or H\(_2\)O\(_2\) were used (17). We further analyzed the *C. jejuni* Dps-DNA interaction by SPR and we were able to conclusively demonstrate that Dps protein
was able to interact with DNA in the absence of Fe\textsuperscript{2+} or H\textsubscript{2}O\textsubscript{2}, however, much stronger binding was observed when Dps was pre-treated with Fe\textsuperscript{2+} or H\textsubscript{2}O\textsubscript{2} (Fig. 5) further validating the results obtained by electrophoretic mobility shift assays.

The presence of Fe\textsuperscript{2+} or H\textsubscript{2}O\textsubscript{2} could induce the Dps-DNA interaction by affecting the DNA, Dps or both. When DNA was pre-incubated with Fe\textsuperscript{2+} or H\textsubscript{2}O\textsubscript{2} and then purified, no interaction with Dps was detected by electrophoretic mobility shift assay in the absence of Fe\textsuperscript{2+} or H\textsubscript{2}O\textsubscript{2} (data not shown). During the SPR analysis, Dps was pre-incubated with Fe\textsuperscript{2+} or H\textsubscript{2}O\textsubscript{2} and these molecules were washed way before the addition of the DNA. The increase in binding affinity for the Fe\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2} Dps treated samples (Fig. 5) suggest that both Fe\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2} have an effect on the Dps structure. This is corroborated by the fact that Dps self-aggregated when treated with Fe\textsuperscript{2+} or H\textsubscript{2}O\textsubscript{2} (Fig. 2B). Indeed, the ability of the \textit{H. pylori} and \textit{E. coli} Dps proteins to bind DNA closely correlated with the capacity of Dps to form self-aggregates \textit{in vitro} (22,24).

High NaCl concentrations reduced the Dps-DNA interactions (Fig. 3) suggesting that ionic interactions could be involved in Dps-DNA binding. The predicted surface of the \textit{C. jejuni} Dps resembles the \textit{H. pylori} orthologue, i.e. it is highly positively charged due to the presence of lysine side chains (33), furthermore, both \textit{C. jejuni} and \textit{H. pylori} Dps do not carry the N-terminal extension present in the \textit{E. coli} Dps that has been implicated in DNA-binding (Fig. S2C). The positively charged \textit{C. jejuni} and \textit{H. pylori} Dps surfaces are likely to be responsible for the interaction with the negatively charged DNA (22). This model could explain why Mg\textsuperscript{2+} negatively affected Dps-DNA interaction (Fig. 3) as Mg\textsuperscript{2+} can bind to the DNA phosphates counterbalancing its intrinsic negative charge. The Dps-DNA binding is strongly influenced by the pH, such that DNA interaction was strong in pH
6.8 and not detected at pH 8.8 (Fig. 3). Similar results have been reported for *H. pylori* and *E. coli* Dps (20,22). We speculate that the rise on pH from 6.8 to 8.8 could result in deprotonation of the lysine side chains on the *C. jejuni* Dps surface reducing its overall positive charge, thereby reducing its ability to interact with DNA. Even though the ε-amino group of lysine has typical pKa in the range of 10, the presence of nearby lysine residues could decrease the pKa resulting in lysine de-protonation at pHs around 8.

The activation of Dps-DNA binding by Fe$^{2+}$ observed here has also been reported in *H. pylori* (16). Fe$^{2+}$ titration experiments suggest that Dps-DNA interaction is maximized when all the 12 Fe$^{2+}$ binding sites of Dps are occupied (Fig. S4). The binding of Fe$^{2+}$ could increase the overall positive charge of Dps protein, favoring interaction with the negatively charged DNA. Alternatively, Fe$^{2+}$ binding could affect the overall Dps structure enhancing the affinity for DNA.

The identification of H$_2$O$_2$ induced DNA binding of *C. jejuni* Dps is novel and it remains to be determined if this is a unique feature of the *C jejuni* Dps. In *E. coli*, Dps is induced in the presence of H$_2$O$_2$ by the transcriptional factor OxyR (46), once accumulated, Dps has default DNA binding activity (7). In contrast, *C. jejuni* Dps expression does not respond to H$_2$O$_2$ (17) but Dps DNA binding activity does (Fig. 2A). The final output is the same in both species, Dps will bind DNA whenever H$_2$O$_2$ accumulate. The fact that *C. jejuni* lacks an OxyR orthologue might explain why *C. jejuni* Dps has evolved to directly sense H$_2$O$_2$.

Even though we could not determine how H$_2$O$_2$ activates the *C. jejuni* Dps-DNA binding at the molecular level, our results excluded the participation of cysteine oxidation
in this process (Fig. 4B). The fact that iodoacetamide blocked the H$_2$O$_2$ induced (but not the Fe$^{2+}$ induced) Dps-DNA binding (Fig. 4A) indicate that these molecules may activate DNA binding through different mechanisms. Furthermore, it suggests the two-fold symmetry surface of Dps (Fig. S2B) as the site for DNA interaction in the presence of H$_2$O$_2$. Similarly, Dps surface has been considered to be the DNA binding site for Deinococcus radiodurans Dps-1 (32,47).

The sum of the ferroxidase and DNA binding activities of C. jejuni Dps are likely to confer the DNA protection against hydroxyl radicals observed in vitro (Fig. 5B), and to be responsible for the reduced survival of C. jejuni dps mutants to H$_2$O$_2$ in vivo (Table 2) and inside macrophages (34). A H$_2$O$_2$ burst is the primary host response against pathogens. The C. jejuni Dps binds DNA in response to H$_2$O$_2$ concentrations as low as 10 µM (Fig. S3), which is far below the amount generated inside macrophages.

During its life cycle, C. jejuni has to cope with the acidic environment found in its hosts digestive tract and inside phagolysosomes. Our data suggests that Dps could act to protect DNA under low pH; this hypothesis is supported by the fact that C. jejuni Dps expression is induced under acid stress (48) and during host colonization (35). Furthermore, it was shown that E. coli Dps contributes to acid tolerance by protecting DNA under low pH (20) and a dps gene isolated from a metagenome library conferred acid resistance to E. coli (49).

The concerted activation of C. jejuni Dps DNA binding in response to low pH, H$_2$O$_2$ and Fe$^{2+}$ would effectively protect DNA during host colonization and explaining, at least in part, the described inability of C. jejuni dps mutants to colonize poultry and piglets.
Other animal pathogens, such as *Haemophilus influenza* and *Salmonella enterica*, also rely on Dps for bacterial resistance during host colonization (21,50). The critical protective role played by bacterial Dps during host colonization indicate that Dps could be an interesting target for the development of vaccines and/or antimicrobial drugs.
Acknowledgments

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References


**Tables**

**Table 1 – Strains and plasmids and primers.**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/phenotype</th>
<th>Source / Reference</th>
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<td><em>C. jejuni</em></td>
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<td>11168-O</td>
<td>Wild type (human isolated strain) originally strain 5636/77</td>
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<td>LFH2</td>
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**Plasmids**

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<td>pLHPETcj1534c</td>
<td>Amp&lt;sup&gt;f&lt;/sup&gt; (pET19b) expresses N-terminal His-tagged <em>C. jejuni</em> Dps</td>
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pLHPETcj1534c-C-S | Amp\textsuperscript{f} (pET19b) expresses N-terminal His-tagged \textit{C. jejuni} Dps C54S-C68S-C85S | This work
---|---|---
pC46 | Cm\textsuperscript{f} | This work
pGEM | Amp\textsuperscript{f} Cloning vector | Promega
pCRbluTOPO | Km\textsuperscript{f} Cloning vector | Invitrogen
pLHPCRc1543cCAT | Cm\textsuperscript{f} pLHPCRc1534c carrying the \textit{cat} insertion \textit{Dps::cat} | This work

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* Restriction sites are underlined
Table 2 – The *C. jejuni* Dps protein confers resistance to H$_2$O$_2$

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<td>5.0 x 10$^5$</td>
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</table>

The *C. jejuni* and *E. coli* strains were diluted in LB to an OD$_{600nm}$ = 0.25 and 0.35, respectively. Cells were incubated at 37°C in the absence (control) or presence of H$_2$O$_2$ 5 mM. After 30 minutes viable cells were determined by serial dilution CFU counting.
Fig. 1 – Analysis of the C. jejuni Dps protein.

(A) One mg of purified Dps was loaded into a 12 % SDS-PAGE before (Lane 1) or after incubation at 99oC for 10 minutes (Lane 2). The gel was stained with Coomassie blue. MW indicates the molecular mass marker in kDa. (B) Spectrophotometric kinetic curve of Fe2+ oxidation by C. jejuni Dps. Reactions were performed aerobically in Tris-HCl 50 mM pH 6.8 at 25 oC. Reactions started by the addition of FeCl2 100 mM, the formation of iron core was monitored by measuring absorbance at 305 nm in a 0.5 cm cuvette. Conditions were as follows: Squares, Dps 0.30 mM; circles, Dps 0.15 mM; Triangles, Dps 0.15 mM in the presence of H2O2 1 mM.
Fig. 2 – The DNA binding activity of C. jejuni Dps is promoted by either Fe2+ or H2O2.

(A) The DNA binding activity of C. jejuni Dps was analyzed by the capacity to retard the migration of supercoiled pGEM plasmid in 1% agarose gel. The DNA on the gel was stained with ethidium bromide. Binding reactions were performed in Tris-HCl 50mM pH 6.8, containing 50 mM of FeCl2 or 10 mM of H2O2 as indicated. Lanes: 1, DNA only; 2, Dps only; 3, Dps + DNA; 4, BSA + DNA. Two different forms of the Dps-DNA complexes are indicated by arrows I and II. (B) Fifteen mg of purified Dps was incubated in the same conditions and run in the same agarose gel as in (A), protein was stained with Coomassie blue. Lane 1, buffer only; Lane 2, FeCl2 50 mM; Lane 3, H2O2 10 mM.
Fig. 3 – DNA binding activity of C. jejuni Dps under different buffers conditions.

The DNA binding activity of C. jejuni Dps was analyzed by the capacity to retard the migration of supercoiled pGEM plasmid in 1% agarose gel. The DNA on the gel was stained with ethidium bromide. Binding reactions were performed in different conditions as indicated in the table. Lane 1 is a control without the addition of Dps.

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Fig. 4 – Effects of iodoacetamide, DTT and cysteines on Dps-DNA interaction.

The DNA binding activity of C. jejuni Dps was analyzed by the capacity to retard the migration of supercoiled pGEM plasmid in 1% agarose gel. The DNA on the gel was stained with ethidium bromide. (A) Reactions contained H2O2 or FeCl2 as indicated (except lanes 1 and 5 that are controls), BSA was used as a negative control. Proteins in lanes 3 and 7 were pre-treated with 10 mM of iodoacetamide for 30 minutes. Proteins in lanes 4 and 8 were pre-treated with DTT 2 mM for 30 minutes. (B) Reactions contained H2O2 10 mM. Lane 1, control DNA only; Lane 2, Dps C54S-C68S-C85S mutant; Lane 3, C54S-C68S-C85S mutant pre-treated with iodoacetamide.
Fig 5- Interaction between C. jejuni Dps and plasmid DNA at pH 6.8 observed by SPR.

The response difference between the treatment and the reference flow cell is shown (RU). The arrows indicate the addition (Time 0) and removal (Time 60s) of the ligand DNA. (A) Interaction between DNA and Dps without any pretreatment of the protein. (B) Interaction between DNA and the Dps pretreated with Fe2+. (C) Interactions between DNA and Dps pretreated with H2O2. Concentrations of DNA tested were 6.25ng/mL, 12.5ng/mL (in duplicate), 25ng/mL and 50ng/mL. The Kds were determined using the Biacore Evaluation software.
Fig. 5

A

![Graph A](image1)

Kd = 9.7 x 10^{-3} M

B

![Graph B](image2)

Kd = 3.5 x 10^{-12} M

C

![Graph C](image3)

Kd = 3.2 x 10^{-11} M
The C. jejuni Dps protein protects DNA from DNase I and hydroxyl radical.

(A) Reactions were performed in Tris-HCl 50 mM pH 6.8 containing MgCl2 0.25 mM and CaCl2 0.01 mM at 25 oC. The purified Dps protein was pre-treated with FeCl2 or H2O2 for 10 minutes followed by addition of DNase I 0.01 U. After 15 minutes reactions were quenched with EDTA 100 mM and analyzed on a agarose gel stained with ethidium bromide. (B) Supercoiled pGEM plasmid was incubated with BSA (lanes 1 and 3) or with Dps (lanes 2 and 4) in Tris-HCl 50 mM pH 6.8 for 30 minutes at 25 oC. Hydroxyl radical generation through Fenton reaction was achieved by adding FeCl2 100 mM and H2O2 10 mM (lanes 3 and 4). Reactions were quenched with the iron chelator bipyridyl 5mM and analyzed on an agarose gel stained with ethidium bromide.