FOODBORNE PATHOGENS AND DISEASE
Volume 8, Number 12, 2011

DOI: 10.1089/fpd.2011.0892

# A *Campylobacter jejuni* Dps Homolog Has a Role in Intracellular Survival and in the Development of Campylobacterosis in Neonate Piglets

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## Abstract

Iron acquisition is an absolute requirement by most microorganisms for host survival. In this work, we investigated the *Campylobacter jejuni* iron binding Dps protein for a potential role in virulence. *In vitro* assays using J774A.1 macrophage-like cells demonstrated a 2.5 log reduction in *C. jejuni* survival of the Dps mutant and a reduction of four logs in invasion of HEp-2 epithelial cells compared to the wild-type strain. To examine the role of the *dps* gene in host pathogenesis, the piglet model was used in *C. jejuni* challenge studies. *In vivo* inoculation studies of newborn piglets with wild-type *C. jejuni* demonstrated an 11-fold upregulation of the *dps* gene and intestinal lesion production typical of campylobacteriosis in humans. In contrast, piglets inoculated with the *dps* mutant were not colonized and remained normal throughout the study period. Mucosal lesion production was restored in piglets inoculated with the complemented Dps mutant strain. Based on these results, we conclude that the *C. jejuni* Dps homolog is a virulence factor in the production of campylobacteriosis, and warrants further investigation.

## Introduction

Campylobacteriosis caused by *Campylobacter jejuni* is the leading cause of bacterial gastroenteritis in the United States, causing an estimated 2.4 million cases annually (Altekruse *et al.*, 1999; Mead *et al.*, 1999; Samuel *et al.*, 2004). The infectious dose is highly variable, ranging from 500 to $10^6$ organisms (Steele and McDermott, 1978). Once ingested, a short incubation period of 24–72 h occurs, followed by an onset of symptoms marked with severe acute watery diarrhea occasionally with blood, variable fever, myalgia, and headache.

Studies on the Dps protein of *Helicobacter pylori*, a close relative of *C. jejuni*, have shown many functions linked to stress survival both *in vitro* and *in vivo*. These functions include iron binding (Tonello *et al.*, 1999), DNA binding (Ceci *et al.*, 2007), oxidative stress survival (Cooksley *et al.*, 2003), and immune modulation (Amedei *et al.*, 2006; Codolo *et al.*, 2008; Del Prete *et al.*, 2008).

In a previous study, a Dps homolog in *C. jejuni* was shown to bind iron up to 40 atoms per Dps monomer (Ishikawa *et al.*, 2003). Additionally, the authors showed a role for *C. jejuni* Dps in hydrogen peroxide stress resistance, as well as evidence for constitutive expression under several *in vitro* conditions. Lastly, researchers have shown that *C. jejuni* Dps can also bind to the myelin sheath and nodes of Ranvier of rat nerves, resulting in paranodal myelin detachment and axonal degeneration, suggesting a possible role for this protein in the development of Guillain-Barre syndrome in individuals (Piao *et al.*, 2009, 2010).

In this work, we expand the initial investigations on the *C. jejuni Dps* gene, and provide evidence that the Dps protein is a significant factor in *C. jejuni* colonization and survival. *In vitro* work using a J774A.1 macrophage-like and a HEp-2 epithelial cell line indicate a role for the Dps protein in extended intramacrophage survival and invasion of epithelial cells. Additionally, we show transcriptional upregulation of *dps* during infection of piglets, and a loss of virulence in the piglet model after challenge with a Dps deficient mutant.

## Materials and Methods

### *Culture of bacterial strains*

All strains used in the study are provided in Table 1. Unless stated otherwise, all *Campylobacter* strains were routinely cultured on Mueller Hinton agar (BD, Sparks, MD) supplemented with 5% citrated bovine blood (Cleveland Scientific, Bath, OH) (MHB) and incubated under a normal atmosphere

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Table 1. Strains and Plasmids Used in This Study

| *Strain or plasmid* | *Relevant characteristics* | *Source* |
|---|---|---|
| Strains | | |
| *Escherichia coli* | | |
| DH5α | *endA1 hsdR17* ($r_k^-$ $m_k^-$) *supE44 thi-1 recA1 gryA relA1 Δ(lacZYA-argF)U169 deoR* [( 80*dlac*Δ(*lacZ*Δ M15)] | Invitrogen |
| *Campylobacter jejuni* | | |
| NCTC11168 | *C. jejuni* NCTC 11168 | NCTC |
| JRT10 | NCTC 11168 *1534c::cm*$^r$ | This study |
| JRT101 | NCTC 11168 *1534c::cm*$^r$ (pJRT101) | This study |
| Plasmids | | |
| pHSS19 | Nonreplicating suicide vector derived from pUC19 | Nickoloff and Reynolds (1991) |
| pSJB49 | pHSS19 carrying cm$^r$ | This study |
| pJRT10 | pSJB49 carrying *Cj1534c*::cm$^r$ | This study |
| pRY107 | Complementation vector; kan$^r$ | Yao *et al*. (1993) |
| pJRT101 | pRY107 carrying *Cj1534c*; kan$^r$ | This study |

cm$^r$, chloramphenicol resistance gene; kan$^r$, kanamycin resistance gene.

supplemented with 10% $CO_2$ at 42°C. *Escherichia coli* DH5α was routinely cultured on Luria Bertani agar (BD) under a normal atmosphere at 37°C. As appropriate, the medium was supplemented with antibiotics at the following concentrations: chloramphenicol 30 μg/mL, kanamycin 50 μg/mL, and ampicillin 100 μg/mL.

### Mutation and complementation

*C. jejuni* chromosomal DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI). Primers Cj1534KOF1 and Cj1534KOR1 (Table 2) were used to amplify a 982-base pair (bp) fragment containing the initial 96 bp of the *dps* gene and the flanking DNA. Primers Cj1534KOF2 and Cj1534KOR2 (Table 2) were used to amplify a second 843-bp fragment containing the terminal 110 bp of the *dps* gene and flanking DNA. The resultant inserts were digested with *Pst*I–*Xba*I and *Bam*HI–*Eco*RI, respectively. The two fragments were consecutively cloned into pSJB49, a pHSS19 derivative that contains the *Campylobacter* chloramphenicol acetyltransferase (*cat*) gene cloned between the *Bam*HI and *Xba*I sites (Nickoloff and Reynolds, 1991). Polymerase chain reaction (PCR) and sequencing of the cloned products were used to confirm the construct. Once confirmed, the construct was introduced into *C. jejuni* via electroporation (1.25 kV, 600Ω and 25 μF). Electroporated cells were plated on MHB and incubated for 16h. After incubation, cells were harvested and mutants were selected from growth on MHB supplemented with chloramphenicol (30 μg/mL), and confirmed via PCR.

Table 2. Primers Used in This Study

| *Primer* | *DNA sequence (5′ to 3′)* |
|---|---|
| Cj1534KOF1 | GGATTCAAAACTGCAGCAAGAAGGTG |
| Cj1534KOR1 | TTGCAAACCTTCTAGATTCCAGTGAT |
| Cj1534KOF2 | AAAAAGAAAGGGATCCTACAACAGCT |
| Cj1534KOR2 | TTTTAAGGTAGAATTCACATAAGTAT |
| Cj1534TCF1 | TTCTTAATCAGAATTCATTAAAAAAG |
| Cj1534TCR1 | TTTCAATTTTATCGATTAAAAAAGGA |
| Cj0402RTP1 | CGATGGAACGGATAATCACC |
| Cj0402RTP2 | AATACCTGCATTTCCAAGAGC |
| Cj1534RTP1 | AAAAAGAAAGTGATACTACAACAGCT |
| Cj1534RTP2 | AAGCACCTTGTAAAGTAGCGCCTATC |
| IpxA*C.jejuni* | ACAACTTGGTGACGATGTTGTA |
| IpxARKK2m | CAATCATGDGCDATATGASAATAHGCCAT |

The mutation was complemented *in trans* using the replicative plasmid pRY107 (Yao *et al.*, 1993). Briefly, primers Cj1534TCF1 and Cj1534TCF2 (Table 2) were used to amplify the entire *dps* gene, plus 126 bases upstream and 60 bases downstream of the gene. The insert was digested with *Eco*RI–*Cla*I, and cloned into pRY107, creating pJRT101. The insertion was confirmed by PCR and sequencing. This plasmid was then introduced into *C. jejuni* via electroporation using previously described settings. Electroporated cells were plated on MHB for 16 h at 42°C. After incubation, cells were harvested and transformants were selected from growth on MHB supplemented with kanamycin (50 μg/mL). PCR was used to confirm the presence of the plasmid.

### RNA isolation

For plate growth, *C. jejuni* was grown on MHB plates for 72 h under routine culture conditions. Postincubation, cells were harvested in phosphate-buffered saline (PBS) and combined with an equal volume of RNA protect (Qiagen, Valencia, CA). For pig samples, total colonic contents, including mucosal scrapings and fecal material, were harvested from piglets 4 days postchallenge with wild-type *C. jejuni* NCTC11168 and resuspended in a 50/50 PBS-RNA protect solution. Colon contents were then centrifuged (1000 *g*, 10 min) to pellet large debris. The supernatant was then filtered through a 0.8-μm filter, and centrifuged (10,000 *g*, 15 min) to pellet the filtered *C. jejuni*. Pelleted material containing *C. jejuni* was retained and stored on ice until RNA extraction was performed. RNA from harvested samples was isolated using RNeasy Mini Kits (Qiagen) and E.Z.N.A. RNase Free DNase I kit (Omega, Norcross, GA) as per manufacturers' instructions. Total RNA collected was quantified spectrophotomically and screened by PCR to ensure that no contaminating DNA was present in the extracted material.

### *Reverse transcription and real-time PCR*

Reverse transcription (RT) of 250 ng total isolated RNA was carried out in 10 $\mu$L reactions using qScript cDNA SuperMix (Quanta, Gaithersberg, MD) as per manufacturer's instructions. Reaction conditions were as follows: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. All cDNA samples were stored at 4°C until processed. Gene amplification and real-time analysis were carried out using a Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, CA). One microliter of cDNA and 800 nM concentrations of primers Cj1534RTP1 and Cj1534RTP2 (Table 2) were used in 20 $\mu$L reactions using PerfeCta SYBR Green FastMix (Quanta). Reactions were carried out as follows: 5 min at 95°C, followed by 40 cycles of 30 sec at 95°C, and 1 min at 57.5°C. To determine PCR efficiencies, standard curves were generated using 10-fold serial dilutions of genomic DNA and their respective threshold cycles. To account for variances in total RNA used and RT efficiency, the gene *Cj0402* (primers Cj402RTP1 and Cj402RTP2) (Table 2) was used as an internal control, as its expression was found to be constant during piglet colonization (Joens, unpublished data). All results were analyzed using the Pfaffl method (Pfaffl, 2001). Each sample was analyzed in triplicate, with the averages presented.

### C. jejuni *growth curves*

Mueller Hinton broth was inoculated with *C. jejuni* to a final $OD_{600}$ of 0.01, and incubated under standard conditions with aeration (150 RPM). Enumeration was performed at 3 h intervals, by plating 10-fold serial dilutions. Titers obtained were used to generate growth curves, and subsequently the doubling time for each strain in this study. Each strain was tested in three independent experiments.

### *Intra-macrophage survival assays*

Intra-macrophage survival assays were performed using a modification of a method described elsewhere (Day *et al.*, 2000). Briefly, 24-well polystyrene plates were seeded with $2\times10^5$ J774A.1 murine macrophage-like cells and incubated for 24 h in 5% $CO_2$ at 37°C. The resulting monolayers were washed twice with Dulbecco's modified Eagle's medium (DMEM; Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) (DMEM + 10% FBS) and inoculated with $1\times10^7$ colony forming units (CFU) *C. jejuni* in DMEM + 10% FBS. Cells were incubated as above for 21, 45, or 69 h. After incubation, cells were rinsed once with DMEM + 10% FBS and incubated as above an additional 3 h in DMEM + 10% FBS and 250 $\mu$g/mL gentamicin. Postincubation, cells were rinsed three times with PBS, and lysed with 0.5% sodium deoxycholate. Ten-fold serial dilutions were performed on the lysates and the lysates plated to determine surviving bacteria. Assays were repeated in three independent experiments.

### *HEp-2 epithelial cell attachment and invasion assay*

The attachment assay was performed as described by Monteville *et al.* (2003). Briefly, 24-well polystyrene plates were seeded with $2\times10^5$ HEp-2 cells and incubated for 24 h in 5% $CO_2$ at 37°C. The resulting monolayers were washed twice with Eagle's minimum essential media (EMEM; Cellgro, Herndon, VA) supplemented with 10% FBS (EMEM + 10% FBS) and inoculated with $1\times10^7$ CFU *C. jejuni* in EMEM + 10% FBS. Cells were incubated 3 h as above, rinsed three times with EMEM + 10% FBS, and incubated an additional 3 h in EMEM + 10% FBS. For invasion assays, cells were incubated 3 h, rinsed three times with EMEM + 10% FBS, and incubated an additional 3 h in EMEM + 10% FBS and 250 $\mu$g/mL gentamicin. After incubation, cells were rinsed three times with PBS, and then lysed with 0.5% sodium deoxycholate. Lysates were serially diluted, plated on MHB agar, and incubated under standard conditions. The assay was repeated in three independent experiments.

### *Piglet model*

Piglets were challenged with *C. jejuni* as described previously (Babakhani *et al.*, 1993). Briefly, colostrum-deprived piglets were obtained at parturition and housed in *Campylobacter*-free facilities. Piglets were fed a diet of 40 mL Similac® (Abbott Laboratories, Abbott Park, IL) every 4 h. At 3 days of age, piglets were challenged via oral gavage with $\sim3\times10^{10}$ CFU *C. jejuni* resuspended in Similac. Four days postchallenge, piglets were euthanized and subjected to postmortem examination. Lesions were recorded and tissue samples taken and prepared for histological examination as described by Babakhani *et al.* (1993).

### *Detection of fecal shedding of* C. jejuni

To determine shedding patterns of the various strains used, daily fecal swabs were collected from all pigs postchallenge at 24 h intervals throughout the study and assayed for *C. jejuni*. Briefly, fecal swabs were transported to the laboratory in Cary-Blair transport media (Copan, Murrieta, CA). Swabs were placed in 20 mL of Bolton broth supplemented with 20 $\mu$g/mL sodium cefoperazone, 26.4 $\mu$g/mL trimethoprim, 20 $\mu$g/mL vancomycin, and 10 $\mu$g/mL amphotericin B for 24 h at 42°C under standard conditions. Postincubation, enrichments were assayed for the presence of *C. jejuni* via PCR using the *C. jejuni* primers IpxA and IpxARKK2m (Table 2) (Klena *et al.*, 2004).

### *Statistical analysis*

Attachment and invasion data were analyzed using univariable factorial analysis: strain vs. repeats. Macrophage survival data were analyzed using repeated measure analysis using strain and repeats as between factors, and the time variable served as the within factor. Macrophage data were further analyzed with the Wilks lambda test.

### *Animal care and use*

All animal work was approved and overseen by the Institutional Animal Care and Use Committee at the University of Arizona, under protocol number 06-037.

## Results

### *Construction and analysis of a dps mutant in* C. jejuni

To determine the role of the Dps protein in pathogenesis, a *dps* mutant was constructed by allelic exchange in the sequenced strain NCTC11168 of *C. jejuni.* The *C. jejuni* NCTC11168 *Dps* mutant was complemented *in trans* using the vector pRY107. PCR and sequencing of the insertion product

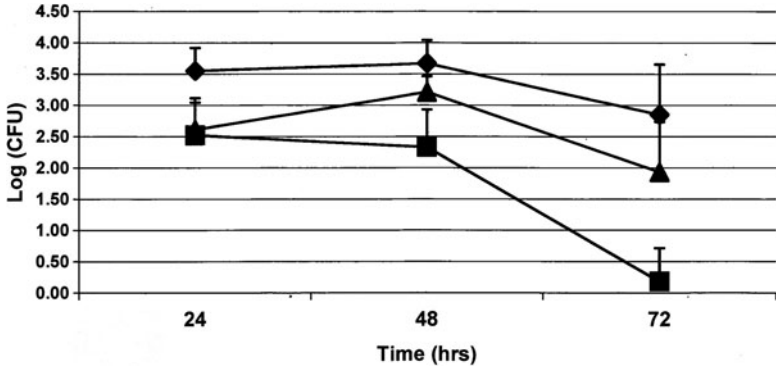


**FIG. 1.** Intra-macrophage survival assay. Macrophage survival assays were performed using J774A.1 murine macrophage cell line and *Campylobacter jejuni* strains NCTC11168 (diamond), NCTC11168Δ*dps* (square), or NCTC11168Δ*dps* (pJRT101) (triangle) at 24, 48, and 72 h time points. All studies were repeated in three independent experiments, with the average log of viable bacteria that survived within cultured J774A.1 cells (CFU/well of 24-well plate) presented. Error bars indicate 1 standard deviation. CFU, colony forming units.

confirmed the genetic manipulations. Additionally, to demonstrate that observed effects were the result of *dps* mutation and not changes in growth rates from the mutation, growth curves were generated and the doubling time of the cells calculated. No difference in doubling times was observed.

### *Dps effects on* in vitro *intra-macrophage survival*

To assess the effects of Dps loss for *Campylobacter* survival in macrophages, *in vitro* J774A.1 intra-macrophage survival assays were performed using *C. jejuni* strain NCTC11168, NCTC11168Δ*dps* mutant, and complemented NCTC11168Δ*dps* (pJRT101). When compared to the wild type, the loss of the Dps protein resulted in a significant decrease ($p \leq 0.05$) at all three time points (24, 48, 72 h). Complementation of the mutant partially restored wild-type function (Fig. 1).

### *Dps effects on* in vitro *attachment and invasion*

To determine if the Dps protein is involved in attachment and invasion of epithelial cells, assays were performed with HEp-2 cells and a wild-type NCTC11168, NCTC11168Δ*dps* mutant, or complemented NCTC11168Δ*dps* (pJRT101) *C. jejuni*. Results demonstrated a significant ($p \leq 0.05$) one-log decrease in attachment to the HEp-2 cells by the Dps mutant compared to the wild-type NCTC11168 strain. Complementation of the Dps mutant restored wild-type levels of attachment (Fig. 2). Examination of viable *C. jejuni* after exposure to gentamicin demonstrated a significant ($p \leq 0.05$) decrease in cell invasion by 4 logs with the Dps mutant compared to the wild-type strain. However, the complemented mutant failed to restore the invasive ability of the strain (Fig. 2).

### *Dps-deficient* C. jejuni *is avirulent in the porcine model of infection*

To assess the phenotypic effect of the Dps mutation on pathogenesis, piglets were intragastrically inoculated with wild-type NCTC11168, NCTC11168Δ *dps,* or NCTC11168Δ *dps* (pJRT101) *C. jejuni* and monitored daily for fecal shedding of the bacterium. All piglets infected with the wild-type strain NCTC11168 *C. jejuni* were positive for shedding at all time points throughout the study, whereas shedding of the *C. jejuni* Dps mutant was not detected at any time points during the study. Piglets infected with the Dps complemented strain showed sporadic shedding, positive in half of the samples taken.

At necropsy, all piglets infected with *C. jejuni* strain NCTC11168 demonstrated mucosal lesions of catarrhal inflammation accompanied by hyperemia and petechial hemorrhage. Piglets infected with the NCTC11168 Dps mutant were normal at necropsy. Piglets infected with the NCTC11168 complemented Dps mutant had intestinal lesions that were indistinguishable from piglets infected with the wild-type strain.

Histologically, piglets infected with the wild-type and complemented strains had marked blunting of intestinal villi and mildly increased lymphocytes plus scattered neutrophils infiltrating into the lamina propria. Additionally, some fibrin was present on the intestinal mucosal surface with focal areas of epithelial erosion (Table 3). Piglets infected with the Dps mutant or negative controls lacked microscopic lesions.

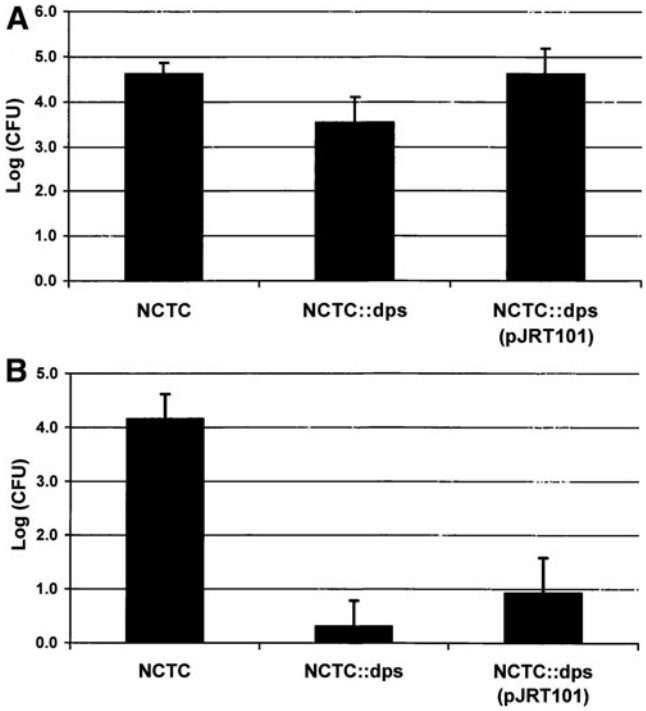


**FIG. 2.** Attachment and invasion assay. Effects of the loss of Dps on attachment and invasion of cultured Hep-2 cells. Attachment **(A)** and invasion **(B)** assays were repeated in three independent experiments with the average log of viable bacteria that attached or invaded cells (CFU/well of 24-well plate) presented. Error bars indicate 1 standard deviation.

Table 3. Microscopic Lesion Development in Piglets Infected with *Campylobacter jejuni*

| *Strain* | *Congested mucosa* | *Epithelial cell erosion* | *Villous degeneration* |
|---|---|---|---|
| NCTC11168 | 2/4 | 3/4 | 2/4 |
| NCTC11168Δ*dps* | 0/4 | 0/4 | 0/4 |
| NCTC11168Δ*dps*(pJRT101) | 2/3 | 2/3 | 3/3 |

Piglets were intragastrically inoculated with $\sim 3 \times 10^{10}$ colony forming units of wild-type NCTC11168, NCTC11168Δ*dps* mutant, or complemented NCTC11168Δ*dps* (pJRT101) *Campylobacter jejuni.* Results indicate number of piglets with congested mucosa, epithelial cell erosion, or villous degeneration.

### C. jejuni dps *is transcriptionally upregulated in piglets*

RNA isolated from the intestine of piglets infected with wild-type NCTC11168 was subjected to RT real-time PCR to assess if *dps* transcription is increased in the *in vivo* piglet model as compared to *in vitro* growth. Results of this work yielded an 11.2-fold increase in the expression of *dps* in the piglet model.

## Discussion

In this work, we examined the role of the Dps protein in cell survival and *C. jejuni* piglet pathogenesis. Beyond simply binding iron, the Dps protein has been shown to have additional roles in the cell, including protection from oxidative stress. This protection is provided through utilizing hydrogen peroxide (either generated from the Fenton reaction or exposure from the environment) for oxidation of iron or reducing the formation of hydroxyl radicals (Bellapadrona *et al.*, 2010). Ishikawa *et al.* (2003) partially demonstrated this occurring in *C. jejuni*, when they demonstrated that a Dps-deficient mutant had an increased sensitivity to hydrogen peroxide *in vitro*. This work provides further evidence of this function in *C. jejuni*, as the Dps-deficient strain was significantly attenuated in its ability to survive in J774A.1 murine-like macrophages compared to the wild-type strain.

An additional role that has been attributed to the Dps protein is the ability to function as an adhesion, where it has been specifically shown to bind sulfated carbohydrates *in vitro* (Namavar *et al.*, 1998). This ability to bind select carbohydrates may explain the one log decrease in attachment observed in this work. In addition to the decrease in attachment, a 4 log decrease in invasion was also observed. These results are possibly due to a synergistic effect of the aforementioned decrease in attachment, and an overall decrease in stress tolerance, making the strain less viable intracellularly.

Genes (as well as subsequent proteins) that are biologically relevant under certain conditions are typically upregulated during exposure to those given conditions. To determine a potential role *in vivo* for the Dps protein, RT real-time experiments using *C. jejuni* RNA isolated from infected piglets were performed to determine if upregulation of the *dps* gene was occurring. Results of these experiments demonstrated an 11.2-fold increase in *dps* expression in the host, indicating a potential role in colonization and pathogenesis.

Piglet challenge studies confirmed that *C. jejuni* Dps has a role in piglet colonization and *Campylobacter* pathogenesis. Overall results of the piglet studies indicate that the C. *jejuni* Dps mutant is severely attenuated, causing no gross or histological lesions. Comparatively, wild-type and complemented strains produced strong inflammatory responses accompanied by intestinal cell degradation and diarrhea. The lack of lesions appears to be from an inability to effectively colonize the piglets, as no *C. jejuni* was detected in fecal swabs taken from piglets challenged with the Dps mutant, whereas all wild-type challenged piglets were positive at all time points. How the loss of the Dps protein is causing this has not been definitively proven, but several contributing factors may exist. First, considering the loss of attachment and invasion *in vitro*, the loss of Dps may prevent the organism from escaping the lumen of the intestine into the epithelial cells, resulting in a washout of the organism within the first 24 h post-challenge. Second, introduction into a host will expose an organism to numerous stressors, including prolonged exposure to acidic environments, bile salts, and reactive oxygen species, among others. The loss of the Dps protein may have an effect of rendering the strain unfit to survive these stresses, and unable to colonize the host.

In conclusion, continuing the initial investigations of Ishikawa *et al.* (2003), we have demonstrated a role for the Dps protein in the pathogenesis of campylobacteriosis. Considering the myriad of functions that have been attributed to the *dps* gene, particularly in the closely related *H. pylori*, further research on its role in *C. jejuni* infections is definitely required.

## Acknowledgments

The authors wish to thank Dr. Mohammad Torabi for his help with the statistical analysis and Dr. Stephen Billington for his assistance with the genetic manipulations. These studies were supported by the Food Safety Research Response Network, a Coordinated Agricultural Project, funded through the National Research Initiative (now National Institute for Food and Agriculture, Agricultural and Food Research Initiative) of the USDA Cooperative State Research, Education and Extension Service, Grant number #2005-35212-15287.

## Disclosure Statement

No competing financial interests exist.

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