**Campylobacter jejuni pdxA Affects Flagellum-Mediated Motility to Alter Host Colonization**

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**Abstract**

Vitamin B6 (pyridoxal-5'-phosphate, PLP) is linked to a variety of biological functions in prokaryotes. Here, we report that the pdxA (putative 4-hydroxy-L-threonine phosphate dehydrogenase) gene plays a pivotal role in the PLP-dependent regulation of flagellar motility, thereby altering host colonization in a leading foodborne pathogen, *Campylobacter jejuni*. A. *C. jejuni* pdxA mutant failed to produce PLP and exhibited a coincident loss of flagellar motility. Mass spectrometric analyses showed a 3-fold reduction in the main flagellar glycan pseudaminic acid (Pse) associated with the disruption of pdxA. The *C. jejuni* pdxA mutant also exhibited reduced growth rates compared with the WT strain. Comparative metabolomic analyses revealed differences in respiratory/energy metabolism between WT *C. jejuni* and the pdxA mutant, providing a possible explanation for the differential growth fitness between the two strains. Consistent with the lack of flagellar motility, the pdxA mutant showed impaired motility-mediated responses (bacterial adhesion, ERK1/2 activation, and IL-8 production) in INT407 cells and reduced colonization of chickens compared with the WT strain. Overall, this study demonstrated that the *pdxA* gene affects the PLP-mediated flagellum motility function, mainly through alteration of Pse modification, and the disruption of this gene also alters the respiratory/energy metabolisms to potentially affect host colonization. Our data therefore present novel implications regarding the utility of PLP and its dependent enzymes as potent target(s) for the control of this pathogen in the poultry host.

**Introduction**

*Campylobacter jejuni* is a Gram-negative, spiral-shaped, micro-aerophilic bacterium that causes foodborne diarrheal illness worldwide [1,2]. Recent epidemiological and biochemical studies have shown that *Campylobacter* infection is also implicated in neuropathies, including Guillain-Barré syndrome (GBS), through the production of autoantibodies induced by bacterial lipooligosaccharides [3,4]. The chicken is the predominant natural host for this pathogen, through which *Campylobacter* can be transmitted to humans [5,6,7]. Thus, control of this pathogen in poultry habitats is associated with the global public health benefit of preventing human campylobacteriosis. However, how to control *Campylobacter* remains unresolved, mainly due to our lack of understanding regarding how this pathogen colonizes chickens and establishes persistent infections and how it is involved in human virulence [7]. An increased understanding of the molecular biology of *Campylobacter* would therefore provide valuable information for the development of therapeutic strategies and vaccines targeting this pathogen.

The differential expression of metabolic gene products in relation to pathogenesis has largely been left unexplored. However, the role of gene regulation in this phenomenon is now receiving more attention, as the metabolism of bacterial pathogens may hold important clues for understanding their life cycles and host defense mechanisms [8,9]. Vitamin B6 (pyridoxal-5'-phosphate, PLP) is an essential metabolic cofactor with numerous functions in more than one hundred enzymatic reactions in humans [10,11]. Among prokaryotes, the biosynthesis of PLP has been intensively studied, mainly in *Escherichia coli*, revealing the involvement of two pathways with seven enzymatic steps [12,15]. For a number of years, it was tacitly assumed that such pathways are ubiquitous in all organisms. However, the biological importance of vitamin B6 for bacterial pathogenesis has only recently been thoroughly investigated in other microorganisms, including *Mycobacterium tuberculosis* [14], *Bacillus subtilis* [15], and the *Campylobacter*-related microorganism *Helicobacter pylori* [16]. In *H. pylori*, a study involving a *pdxA* mutant recently demonstrated an essential role of the *pdxA* gene in flagellation, likely through inactivation of the flagellin glycosylation process (decoration with pseudaminic acid) [16]. There is no evidence regarding how the *pdxA* gene affects the process of flagellin glycosylation in *C. jejuni* similarly. However, a previous biochemical analysis showed that a UDP-4-keto-6-deoxy-GlnNAc aminotransferase (Gj1294) derived
from *C. jejuni* generates UDP-4-amino-4,6-dideoxy-alNAc with the catalytic support of PLP as a co-factor under *in vitro* conditions [17], which led us to the assumption that PLP biosynthesis also affects flagellation and certain types of metabolism in this pathogen, thereby altering bacterial fitness and *in vivo* colonization, for which flagellar motility is a prerequisite [18].

Given this background, we studied the PLP biosynthesis pathway in *C. jejuni* through *in silico* prediction and mutagenesis analyses. Biochemical and phenotypic analyses showed that the lack of the *pdxA* gene abolished PLP production and impaired the ability of *C. jejuni* to form flagella. We then focused on this mutant to characterize its biological effect(s) on host colonization through biochemical, metabolomic, and host infection approaches.

**Materials and Methods**

**Bacterial strains and media**

The bacterial strains and plasmids used in this study are listed in Table 1. *C. jejuni* strain 81–176 [19] was grown using routine methods in Mueller-Hinton (MH) broth or on MH agar (Becton-Dickinson, Franklin Lakes, NJ, USA) at 37°C for 24 h in a humidified CO₂ AnaeroPack-Microaero gas system (Mitsubishi Gas Chemicals, Tokyo, Japan). The media were supplemented with chloramphenicol (Cm) (20 μg ml⁻¹) or kanamycin (Km) (30 μg ml⁻¹), as appropriate. The *E. coli* DH5α strain, which was used as the host for subcloning and routine DNA manipulation, was grown in LB agar or LB broth unless otherwise indicated.

**Construction of *C. jejuni* mutants and complementation of the *pdxA* mutant**

The 81–176 mutant, in which most of the *pdxA* or *flaA* genes were replaced with a cat cassette (encoding a Km-resistance protein), was constructed as described previously [20]. To construct a *pdxA* mutant, a 500-bp fragment upstream of the 5’ end and a 500-bp fragment downstream of the 3’ end of the *pdxA* locus were amplified from the wild-type (WT) strain via PCR using either *pdxA*-s and *pdxA*-as-Bl or *pdxA*-s-Bl and *pdxA*-as primers (Table S1). After BamHI digestion, the two fragments were ligated and cloned into pGEM-T vector (Promega, Madison, WI, USA). A cat gene from the plasmid pRY109 [21] was then inserted into the BamHI site in the pGEM-T plasmid, and this allelic exchange plasmid (pGEM-*pdxA*-Cm, Table 1) was introduced into the genome of strain 81–176 through natural transformation [22]. Successful transformants were selected on MH agar supplemented with 5% horse blood and Cm (20 μg ml⁻¹). Allelic replacement was confirmed via nucleotide sequencing. Disruption of the *flaA* gene was performed in the same manner (the oligonucleotide primers used in these procedures are listed in Table S1). The *pdxA* mutant strain was confirmed via natural transformation, and the transformants were recovered on MH agar containing Km (10 μg ml⁻¹) and Cm (20 μg ml⁻¹). The construction of this *pdxA*⁻/+ mutant strain was confirmed via PCR using the *pdxA*-conF and *pdxA*-conR primers (Table S1).

**Quantification of PLP**

Bacteria were grown microaerobically in 10 ml of MH broth to mid-logarithmic phase (an OD₆₀₀ of 0.6), and crude homogenates were prepared in 20 mM Tris-HCl (pH 7.4) via bead crushing. After centrifugation for 10 min at 7,000 rpm at 4°C, the PLP contents in 50 μg of protein of the lysate and serial dilutions of the lysate were measured using a vitamin B6 ELISA kit (Uscn Life Science, Houston, TX, USA) according to the manufacturer’s instructions. Fresh MH broth was also tested for the measurement of PLP.

**Protein fractionation, SDS-PAGE, and immunoblotting**

Membrane and cytoplasmic proteins from *C. jejuni* were isolated as described previously [23]. These protein samples were then separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and stained with CBB (Coomassie Brilliant Blue) to visualize the protein profiles. The proteins on the gel were simultaneously transferred to a PVDF membrane (Merck-Millipore, Billerica, MA, USA), and the FlaA protein was detected using a rabbit polyclonal antibody generated against *C. jejuni* flagellin [24] and an HRP-conjugated goat anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, UK). The blots were developed using the ECL detection system (GE Healthcare).

| Name          | Description                                                                 | Source/Reference |
|---------------|------------------------------------------------------------------------------|------------------|
| **Bacterial strain** |                                                                               |                  |
| WT            | *C. jejuni* wild-type (WT) strain 81–176                                      | [19]             |
| pdxA⁻         | *C. jejuni* 81–176 *pdxA* (CJJ81176_1253) mutant                             | This study       |
| flaA⁻         | *C. jejuni* 81–176 *flaA* (CJJ81176_1339) mutant                             | This study       |
| pdxA⁻/+       | *pdxA*- strain complemented with pRY-*pdxA*-Km                              | This study       |
| DH5α          | *E. coli* strain for DNA manipulation                                         | Sigma-Aldrich    |
| **Plasmid**   |                                                                               |                  |
| pRY108, pRY109| Km- or Km-resistant *C. jejuni*/E. coli shuttle vector                         | [21]             |
| pGEM-*pdxA*-Cm| pGEM:*pdxA*-cat for homologous recombination                                  | This study       |
| pGEM-*flaA*-Cm| pGEM:*flaA*-cat for homologous recombination                                  | This study       |
| pRY-*pdxA*-Km | pRY109:*pdxA*-J for complementation                                           | This study       |

Table 1. Bacterial strains and plasmids used in this study.
Motility and growth assays

The motility of the WT, *pdxA* mutant, and complemented *C. jejuni* strains was assayed on 0.4% soft MH agar plates as previously described [25]. To measure bacterial growth, 1.4–1.9×10^6 cells of *C. jejuni* that were microaerobically grown in MH broth to an OD_{600} of 1.2–1.3 at 37°C, were incubated in 10 ml of fresh MH broth supplemented with or without PLP (10 mg l^{-1}) with agitation (120 rpm) for 0, 12, 24, 36, 48, 72 h. At each time point, turbidity of the medium was measured at 600 nm.

Detection of pseudaminic acid (Pse)

(i) Derivatization of Pse. Pse was released from the 30 μg of cytosolic protein fractions from the WT and *pdxA* mutant strains grown in MH broth to OD_{600} of 0.55–0.60, using the GlycoProfile™ β-elimination kit (Sigma-Aldrich) according to the manufacturer’s instructions. The released Pse was labeled with 1,2-diamino-4,5- methylenedioxybenzene (DMB) using a silicic acid fluorescence-labeling kit (TaKaRa, Shiga, Japan), and the reaction mixture was applied to a solid-phase extraction cartridge (Envi-Carb C, Supelco, Bellefonte, PA, USA). After washing with 2.5 ml of 5 mM ammonium acetate (pH 9.6), the labeled Pse was eluted using 3 ml of 43% acetonitrile/5 mM ammonium acetate (pH 9.6) and freeze-dried. Fresh MH broth was also subjected to the above sample preparation to observe the effect of background growth medium.

(ii) Liquid chromatography/mass spectrometry (LC/MS). Chromatographic separation of DMB-labeled Pse was performed using the Paradigm MS4 HPLC system (Michrom BioResource, Auburn, CA, USA). The separated DMB-labeled Pse was applied to a C18 trap column (L-column Micro, CERI) and eluted using 0.1% formic acid/2% acetonitrile (buffer A) and 0.1% formic acid/90% acetonitrile (buffer B) with a linear gradient of 10–90% buffer B over 30 min at a flow rate of 300 μl min^{-1}. Mass spectrometric analysis of DMB-labeled Pse was performed using a Fourier transformation ion cyclotron resonance (FT-ICR)/ion trap (IT)-type mass spectrometer (LTQ-FT) (Thermo Electron, San Jose, CA, USA) equipped with a nanoelectrospray ion source (AMR, Tokyo, Japan). The presence of DMB-Pse was determined via sequential scans consisting of selected ion monitoring (SIM, m/z 441–461) using FT-ICR-MS and data-dependent MS/MS-M/MS/MS/MS (MS^4) with IT-MS.

Detection of metabolic compounds

(i) Sample preparation. A total of 3.2–3.4×10^6 *C. jejuni* WT or *pdxA* mutant cells grown in broth to an OD_{600} of 0.6 were trapped on a 0.4-μm filter membrane (Merck-Millipore), washed twice with 10 ml of water, and immersed in 2 ml of methanol containing 10 μM internal standard solution 1 (Human Metabolome Technologies (HMT), Yamagata, Japan). After sonication for 30 s, 1.6 ml of each suspension was mixed with 640 μl of water and 1.6 ml of chloroform, followed by centrifugation for 5 min at 2,300×g. The 750 μl of upper aqueous layer was filtered through a 5 KDa-cutoff filter (Millipore), lyophilized, and resuspended in 25 μl of water.

(ii) Capillary Electrophoresis-Time of Flight/mass spectrometry (CE-TOF/MS). Cationic metabolites were analyzed using a fused silica capillary tube (50 μm×80 cm) and Cation Buffer Solution (HMT) in a capillary electrophoresis system equipped with a Time-of-Flight mass spectrometer (CE-TOF/MS) and a CE-ESI-MS sprayer (Agilent Technologies, Santa Clara, CA, USA). Electrospray ionization-mass spectrometry (ESI-MS) was conducted in positive ion mode at 4,000 V. Anionic metabolites were analyzed using a fused silica capillary and Anion Buffer Solution (HMT). ESI-MS was conducted in negative ion mode at 3,500 V. In both modes, the spectrometer was scanned from m/z 50 to 1,000. The other conditions were followed the cation analysis methodology of Soga and Heiger [26].

(iii) Data analysis. Raw data were processed using the MasterHands program [27]. Signal peaks corresponding to the isotopomers of 108 compounds (including the intermediates of the glycolytic system, the intermediates of the TCA cycle, and amino acids; see Table S2 for more details) were extracted. Each obtained migration time (MT) was normalized using the values of the internal standards. The resultant relative area values were further normalized based on the sample amounts. We used duplicate sets of samples from two independent experiments.

ATP assay

To determine the intracellular ATP concentration of bacterial samples, the BacTiter-Glo Microbial Cell Viability assay kit (Promega) was used. After growing the bacteria in MH broth at 37°C under microaerobic conditions to an OD_{595} of 0.55–0.60, serial dilutions of all samples were prepared according to the manufacturer’s instructions. Following incubation of the samples at room temperature in a 96-well plate, luminescence was measured with an ATP standard using GloMax Multi system (Promega), according to the manufacturer’s instructions. Simultaneously, we measured the bacterial numbers in the originally cultured MH broth by plate count.

Cell adhesion assay, IL-8 measurements, and immunoblotting

INT407 cells were seeded into 24-well culture plates (TPP (3.0×10^5 cells well^{-1}) and incubated in RPMI1640 medium (Life Technologies, Carlsbad, CA, USA) for 20 h at 37°C in a humidified CO_2 incubator. The cells were then rinsed and inoculated with *C. jejuni* at a multiplicity of infection (m.o.i.) of 100. At 60 min post-infection, the cells were washed three times with PBS to remove non-adherent bacteria, followed by cell detachment using 0.1% saponin in PBS. Serial dilutions of the suspensions were plated onto MH agar to determine the numbers of viable, cell-associated bacteria. To measure IL-8 secretion from the INT407 cells after infection, INT407 cells were infected with *C. jejuni* at an m.o.i. of 100 for 0, 4, and 16 h, and the culture supernatants were used to measure IL-8 levels with a human IL-8 ELISA kit (Becton-Dickinson), according to the manufacturer’s instructions. ERK activation was examined via western blotting using tyrosine-phosphorylated and total ERK1/2 monoclonal antibodies (Cell Signaling Technology, Danvers, MA, USA).

Chicken colonization assay

Specific pathogen-free, 14-day-old white leghorn chickens (obtained from Nisseiken Co., Ltd., Japan) were orally challenged with 500 μl of MH broth containing approximately 3.0×10^10 WT or *pdxA* *C. jejuni* cells. The animals were euthanized at 7 and 28 days post-infection, and post-mortem cecal samples were collected after aseptic removal of the ceca. *C. jejuni* colonization of the cecum was examined by counting viable cells on mCCDA agar plates (Oxoid, Hampshire, UK). A control group was confirmed to be negative for Campylobacter. The above animal experiments were approved by the Committee for Animal Care and Use of the National Institute of Health Sciences, Japan.

Statistics and web tool

The PATRIC prediction system (http://patricbrc.vbi.vt.edu/portal/portal/patric/Home), which assesses metabolic pathways...
in various prokaryotes based on their genomic sequences, was used to illustrate the putative PLP and Pse biosynthesis pathways in the *C. jejuni* 81–176 strain. The results from the motility, growth, ATP activity, cell adhesion, IL-8 production, and chicken colonization assays were expressed as the mean ± standard deviations of at least three independent observations. The significant differences between the measurements obtained from the WT and mutant strains were determined using Student’s *t*-test. *P* values <0.05 were considered statistically significant.

**Results**

**Disruption of the pdxA gene abolishes PLP production in *C. jejuni***

To predict the PLP biosynthesis pathway in *C. jejuni* 81–176, we used the *in silico* pathway tool PATRIC (http://www.patricbrc.org/portal/portal/patric/Home). The result of this prediction illustrated that at least five genes might constitute two independent pathways for PLP biosynthesis in this pathogen (right box, Fig. 1A). Among these genes, *pdxA* (Cj11176_1253) and *pdxJ* (Cj11176_1252) are known to be involved in the *de novo* synthesis of PLP in *E. coli* [12] and are, indeed, conserved in the *C. jejuni* genome [28]. Recently, Stahl and Stintzi [29] reported that the *pdxA* gene (Cj1239 in the NCTC11168 strain) may be essential for microbrial growth. We therefore decided to use the *pdxA* gene to study the role of PLP biosynthesis in the biology of this pathogen and constructed an insertional *pdxA* mutant in *C. jejuni* strain 81–176. Biochemical assays collectively detected very less amounts of PLP (0.15 ± 0.10 μg 10−1 ml−1) in the *pdxA* mutant than the WT strain (34.55 ± 7.61 μg 10−1 ml−1), and complementation of the *pdxA* gene in the *pdxA* mutant restored PLP production (33.85 ± 7.45 μg 10−1 ml−1) (Fig. 1B). Thus, we could demonstrate that the *pdxA* gene is truly a prerequisite for the PLP biosynthetic metabolism of this pathogen.

**The pdxA mutant impairs Pse production, flagellin glycosylation, and flagellation**

*Campylobacter* Bagellins are decorated with O-linked glycans, which are derivatives of Pse synthesized through sequential enzymatic reactions (i.e., transamination, decarboxylation, and racemization) [30], and this type of glycomodification is a prerequisite for the biogenesis, transport, and assembly of functional flagellar filaments [31,32]. Among components of the Pse biosynthesis process, the *pseC* (Cj1294/Cj11176_1311) gene product, UDP-4-keto-deoxy-GlcNAc transaminase, is reported to require PLP to generate UDP-4-amino-4,6-dideoxy-GalNAc, a product, UDP-4-keto-deoxy-GlcNAc transaminase, is reported to require PLP to generate UDP-4-amino-4,6-dideoxy-GalNAc, a spectrometric anilineintermediate in the synthesis of Pse (left box, Fig. 1A) [17]. Immunoblot analyses showed the less glycosylation but expression of the flagellin A (FlaA) in cytoplasmic fraction of the *pdxA* mutant compared with that of WT strain (Fig. 2A), and the complementation of the *pdxA* gene restored the glycosylation of FlaA in the *pdxA* mutant (Fig. 2A). Having less detection of FlaA in the membrane fraction of the *pdxA* mutant than that of the WT strain (Fig. 2A), it could be considered that the less glycosylated FlaA was not transported to the surface of the *pdxA* mutant. In agreement, mass spectrometric analyses clearly showed that the *pdxA* mutant produced approximately 3-fold less Pse than the WT strain (Fig. 2B, Fig. S1, S2, S3), providing a link between *pdxA*, PLP, and Pse biosynthesis in this pathogen. In consistent with the fact that the glycosylation and surface expression of flagellar filaments are prerequisite for bacterial motility [31,32], phenotypic assays clearly demonstrated that the *pdxA* mutant was not motile, and the complementation of the *pdxA* gene restored motility (Fig. 2C). Furthermore, microscopic analyses consistently showed that the *pdxA* mutant did not generate any flagellar filaments and that complementation of the *pdxA* gene restored flagellation, likely to the same level as in the isogenic WT strain (Fig. 2D). Addition of PLP did not restore the flagellar production of the *pdxA* mutant (Fig. 2D). Together, we were able to demonstrate that disruption of the *pdxA* gene impaired the glycosylation of flagellin, thereby reducing bacterial motility.

**The pdxA mutant exhibits altered Pse biosynthetic metabolism**

Considering that PLP mediates a variety of enzymatic processes [33], a comparative metabolomic analysis was performed to characterize/confirm the types of metabolism that might be related to PLP activity. CE-TOF/MS (capillary electrophoresis time-of-flight/mass spectrometry) analysis detected 99 metabolic compounds extracted from the WT and *pdxA* mutant strains, among which the levels of 10 and 6 compounds were either increased or reduced >2-fold, respectively, in the *pdxA* mutant compared with the WT strain (Table 2 and more detailed information in Table S2). In support of the link between the presence of the *pdxA* gene and Pse production, the *pdxA* mutant exhibited greater amounts of UDP-glucose (a Pse precursor, Fig. 1A left panel) and a PLP precursor, pyridoxalamine-5'-phosphate (PNP, Fig. 1A right panel), which showed concentrations that were at 3.6-fold and 2.5-fold higher than were exhibited by the WT strain, respectively (Table 2). Thus, these data clearly indicated an essential role of the *pdxA* gene in Pse biosynthesis in *C. jejuni*.

**The *C. jejuni* pdxA mutant exhibits altered respiratory/energy metabolism**

As an additional characteristic, we notified that the *C. jejuni* pdxA mutant exhibited growth defect compared with the WT strain, but this mutant showed successive growth in the absence of PLP, indicating that the *pdxA* gene was not essential for the growth of this pathogen (Fig. 3A, left panel). Different from *H. pylori* *pdxA* mutant [16], the addition of PLP did not restore growth of the *C. jejuni* *pdxA* mutant (Fig. 3A). To investigate the metabolisms associated with the altered growth kinetics of the *C. jejuni* *pdxA* mutant, we thus focused indicators of significant alterations in energy/respiratory metabolisms because of the pivotal role of these metabolisms in the growth in this pathogen [34]. The metabolomic data showed that the *pdxA* mutant indeed produced greater amounts of ATP and GDP (by 2.7-fold each) and, hence, decreased amounts of NADP+ (0.5-fold) compared with the levels in the WT strain (Table 2). In agreement with these findings, the *pdxA* mutant exhibited an approximately 2.14-fold greater amount of ATP compared with the WT strain (when 1.6×109 cells were assayed; Fig. 3B). Energy metabolism is well known to depend on the respiratory cycle. Corroborating this fact, the *pdxA* mutant showed alterations in the concentrations of TCA cycle intermediates including α-cis-aconitic acid (2.7-fold), isocitrate (2.1-fold), succinate (2.1-fold), malate (1.5-fold), citrate (0.6-fold), and serine (0.5-fold), a major carbon source for the respiratory cycle in this pathogen [35] compared with the WT strain (Fig. 3C, Table 2, Table S2). Thus, we were able to show that the *C. jejuni* *pdxA* mutant exhibited altered growth and respiratory/energy metabolism.

**The pdxA mutant exhibits impaired *in vitro* cell adhesion and chicken colonization**

*C. jejuni* requires flagellum-mediated motility for establishing the early phase of infection both *in vitro* [36] and *in vivo* [37,38]. Accordingly, when INT407 cells were infected with the *pdxA*...
mutant, WT, and flaA mutant strains, the cell adhesion score of the pdxA mutant was found to be almost identical to that of the flaA mutant, exhibiting a 3-fold reduction compared with the WT strain at 1 h post infection (p.i.) (Fig. 4A). Following adherence, C. jejuni activates ERK1/2 MAPK signaling, which stimulates the production of interleukin (IL)-8 in INT407 cells [39]. In agreement with the above cell adhesion scores, the pdxA mutant caused delayed phosphorylation of ERK1/2 MAPK compared with the WT strain (Fig. 4B), which was similar to the flaA mutant [39]. Additionally, similar to the flaA mutant [39], the pdxA mutant

Figure 1. Inactivation of the pdxA gene impairs the biosynthesis of vitamin B6 (PLP) in C. jejuni. (A) A scheme for the PLP production pathway (right box) in C. jejuni in relation to Pse biosynthesis (left box) is illustrated based on in silico pathway analysis performed using PATRIC (http://patricbrc.vbi.vt.edu/portal/portal/patric/Home). (B) The pdxA mutant produced no PLP. The C. jejuni 81–176 WT, pdxA mutant, and the complemented strains were grown in 10 ml of MH broth to an OD600 of 0.60. The suspensions were then homogenized, serially diluted, and subjected to ELISA to quantify the amounts of PLP (μg 10 ml⁻¹). The data show the mean +/- standard deviations from three independent assays. doi:10.1371/journal.pone.0070418.g001
induced significantly less IL-8 production in the INT407 cells compared with the WT strain at 4 h p.i. \( (p<0.05, \text{Fig. 4C}) \). A prolonged period after infection (18 h p.i.) resulted in normalization of the IL-8 secretion observed in response to all of the bacterial strains tested (Fig. 4C). These data indicated that the \( \text{pdxA} \) gene is a prerequisite for cell adhesion, with the mutant

![Figure 2. The \( \text{pdxA} \) mutant shows impaired pseudaminic acid (Pse) production and flagellar motility.](image)

(A) The \( \text{pdxA} \) mutant shows less glycosylation of \( \text{FlaA} \). SDS-PAGE and western blotting were conducted to detect the \( \text{C. jejuni} \) \( \text{FlaA} \) protein. Crude extracts and subcellular (cytoplasmic and membrane) fractions were extracted from \( \text{C. jejuni} \) and visualized using CBB staining in an SDS-polyacrylamide gel (left panel). Western blot analyses were simultaneously performed to detect the \( \text{FlaA} \) protein (arrow, right panel). (B) The \( \text{pdxA} \) mutant shows reduced Pse production. The left panel shows an extracted ion chromatogram at \( m/z \) 441.0–461.0 obtained through SIM of DMB-labeled Pse from the WT and \( \text{pdxA} \) mutant strains (arrowed). The extracted ion chromatogram of blank sample (fresh MH broth) was simultaneously subjected to confirm the absence of Pse. AA, peak area in arbitrary units. Each ion signal is expressed as a relative percentage of the WT-derived sample (set to 100%) from two independent tests (right panel). MS\(^n\) data were shown in Fig. S1, S2, S3. (C) The disruption of the \( \text{pdxA} \) gene impairs motility of \( \text{C. jejuni} \). The WT, \( \text{pdxA} \)-mutant, \( \text{pdxA} \)-complemented \( (\text{pdxA}^+/-+) \), and \( \text{flaA} \)-mutant \( (\text{flaA}^-) \) strains were spotted and incubated onto 0.4% soft agar. Scale bars represent 3 mm. The motility of \( \text{pdxA} \) mutant was also assayed in the supplementation of 10 mg l\(^{-1}\) of PLP (\( \text{pdxA}^- + \text{PLP} \)). (D) The \( \text{pdxA} \) mutant is aflagellated. Electron micrographs of the \( \text{C. jejuni} \) WT, \( \text{pdxA} \)-mutant with or without supplementation of PLP (10 mg l\(^{-1}\)), \( \text{pdxA}^- \)-complemented strains. The scale bars represent 1 \( \mu \)m.

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exhibiting delayed activation of ERK1/2 signaling and impaired IL-8 production in intestinal epithelial cells. We also tested the colonization ability of the pdxA mutant in chickens because this host represents the most important reservoir of the pathogen for human infection [5] as well as the fact that the flagellin mutants exhibited less colonization in chicken [38]. At 7 and 28 days after infection, the pdxA mutant exhibited approximately 100-fold (2.14 ± 0.07log10 CFU/g at day 7) and 4.6 ± 0.7-fold (6.00 ± 7.48log10 CFU/g at day 28) decreases in the colonization of chicken cecum tissues compared with the parental strain (2.14 ± 2.12log10 CFU/g at day 7 and 2.74 ± 2.55log10 CFU/g at day 28, both of which were significantly different (p<0.05) from the pdxA mutant-infected animals) (Fig. 4D). Together, we were able to demonstrate that disruption of the pdxA gene impaired the colonization of chicken intestine by C. jejuni.

Discussion

Here, we examined the role of the PLP synthetic pathway in the biology of C. jejuni. Disruption of the pdxA gene clearly impaired PLP production. Mass spectrometric and biochemical analyses revealed a reduced production and glycosylation of flagellins in the pdxA mutant, which is likely to impair bacterial motility. Having the altered growth by disruption of the pdxA gene in this pathogen, we then performed comparative metabolomic approaches, further revealing the association of pdxA gene to energy/respiratory metabolisms. We finally showed that The pdxA mutant exhibited decreased cell adhesion-dependent responses in vitro and in vivo host colonization.

Based on the in silico pathway prediction for the PLP biogenesis, we selected the pdxA as a putative essential gene for the PLP production in this pathogen. A mutation of the pdxA gene impaired production of PLP in C. jejuni 81–176, supporting our prediction. The reduced Pse production in the pdxA mutant was also supportable to a previous study demonstrating the essentiality of PLP in the Pse production in C. jejuni [17]. The reduced production, but not complete loss of Pse in the pdxA mutant might be explained by the fact that small amounts of PLP (0.14±0.07 μg 10ml−1) were also detected from basal MH broth (Fig. 1B). Perhaps, the residual Pse peak in the pdxA mutant might be stem from residual PLP in the medium.

Since flagellin glycosylation is prerequisite for the biogenesis, transport, and assembly of flagellar filaments in this pathogen [31,32] and which thereby alters the motility and host colonization of this pathogen [37,38], it was plausible that the decreased Pse levels in the pdxA mutant, affected flagellar glycosylation, thereby altering transport of flagellin to the bacterial surface. Phenotypic and infection studies indeed showed impaired motility and host colonization of C. jejuni by disruption of the pdxA gene, supporting the idea that the reduced motility of the pdxA mutant was mainly due to the altered network between PLP and Pse.

We identified a link between PLP and the Pse modification system in C. jejuni 81–176, in agreement with the previously reported essential role of the pdxA gene in flagellar glycosylation in a closely related pathogen, H. pylori [16]. Moreover, the less glycosylation of FlaA protein in the cytoplasm of pdxA mutant was in agreement with the previous report demonstrating that the C. jejuni pseC mutant expressed unglycosylated FlaA in the cytoplasm [40]. Unlike H. pylori, however, the C. jejuni pdxA mutant could grow without supplementation of PLP, and the addition of PLP did not restore the motility and growth of C. jejuni pdxA mutant. These suggest the distinct metabolic impacts of PLP to the growth and/or viability between H. pylori and C. jejuni. A protein-protein network prediction tool, STRING database (http://string.embl.de/) indeed shows differential networks of the pdxA gene between the two pathogens (Fig. S4).

Campylobacter exhibits unique nutritional requirements, and it has been thought to only utilize amino acids and TCA cycle

### Table 2. Representative metabolites that are altered between the C. jejuni WT and pdxA mutant strains.

| Compound name          | m/z  *1  | MT  *2  | Relative Area | Ratio  *3 (pdxA−/WT) |
|------------------------|---------|---------|---------------|----------------------|
| **Increased**          |         |         |               |                      |
| UDP-glucose/galactose  | 565.05  | 8.22    | 2.25E-04      | 7.99E-04             | 3.35 |
| Azealic acid           | 187.10  | 11.56   | 2.84E-04      | 9.31E-04             | 3.28 |
| 2-Amino-2-hydroxymethyl-1,3-propanediol | 122.08 | 7.83 | 7.14E-04 | 2.01E-03 | 2.82 |
| cis-Aconitic acid      | 173.01  | 26.16   | 3.36E-03      | 9.17E-03             | 2.73 |
| ATP                    | 505.99  | 11.16   | 4.37E-04      | 1.16E-03             | 2.66 |
| GDP                    | 442.02  | 10.11   | 1.91E-04      | 5.09E-04             | 2.66 |
| Pyridoxamine-5-phosphate (PNP) | 249.06 | 7.97 | 2.25E-04 | 5.55E-04 | 2.46 |
| β-Alanine              | 90.06   | 9.69    | 6.85E-04      | 1.65E-03             | 2.40 |
| Glycine                | 76.04   | 7.87    | 4.58E-03      | 1.04E-02             | 2.27 |
| Isocitrate             | 191.02  | 26.91   | 3.00E-03      | 6.39E-03             | 2.13 |
| **Decreased**          |         |         |               |                      |
| NADP                  | 742.07  | 8.92    | 5.3E-03       | 2.6E-03              | 0.49 |
| ppGpp_divalent         | 300.47  | 13.69   | 4.3E-03       | 2.0E-03              | 0.48 |
| Asparagine             | 133.06  | 9.80    | 1.3E-03       | 5.0E-04              | 0.40 |
| Agmatine               | 131.13  | 4.94    | 5.5E-03       | 1.1E-03              | 0.19 |

The detected metabolites exhibiting ≥2-fold differences between the WT and pdxA− strains are shown. Each mean represents average from two independent tests. Candidate compounds are identified based on the detection peak (m/z)*1 and migration time (MT)*2 through HTM database.*3 Relative mean of the pdxA−/WT ratio. Full lists are shown in Table S2.

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intermediates as carbon sources for energy production [41]. The TCA cycle is a sequential process involving enzymatic reactions in which a two-carbon acetyl unit is oxidized to CO2 and H2O to provide energy in the form of high-energy phosphate bonds. The different types of energy metabolism observed in the WT and pdxA mutant strains therefore suggested a possible link of PLP with these types of metabolism. Representative metabolites that were significantly altered by inactivation of the pdxA gene were thus discussed below.

Figure 3. The C. jejuni pdxA mutant shows altered growth kinetics and respiratory/energy metabolism. (A) Growth curves of C. jejuni 81–176 WT, pdxA –, and the complemented mutant strains in MH broth not supplemented (left panel) or supplemented (right panel) with PLP (10 mg l−1). (B) Intracellular ATP levels of C. jejuni 81–176 WT, pdxA –, and the complemented mutant strains. ATP contents of four serial dilutions of the bacteria (shown as CFU 100 µl−1) under investigation were measured. The results are shown as means ± SD of data from triplicate wells of a representative experiment. (C) Focused dynamics of the C. jejuni TCA-cycle pathway. The pathway, the relative mean concentrations of the related metabolites in the WT (blue bars) and the pdxA mutant (red bars) strains, and the genes associated with the enzymatic conversion of each metabolite were illustrated with the PATRIC pathway analysis program. doi:10.1371/journal.pone.0070418.g003
Serine/Glycine

This pathogen exhibits a complete TCA cycle [42], and serine is particularly useful as a nutritional substrate that can be catabolized for growth and colonization in the chicken gut [37,43,44]. The decreased serine level detected would appear to be connected to glycine metabolism because *E. coli* serine hydroxymethyltransferase (GlyA) catalyzes the reversible interconversion of the amino acids serine and glycine using one-carbon tetrahydrofolate and PLP [45]. Thus, it could be considered that the imbalance between serine and glycine in the *pdxA* mutant might associate with the altered functionality of GlyA due to the lack of PLP.

Citrate/cis-aconitate/isocitrate

These TCA intermediates are interconverted by aconitases [46], among which AcnA functions as the major TCA cycle enzyme in *E. coli* [47,48]. Considering that *C. jejuni* 81–176 also harbors an *acnB* gene (CJJ81176_0852), the imbalance in these three TCA intermediates in the *pdxA* mutant might be due to reduced AcnB activity. AcnB forms an iron-sulfur cluster, thereby affecting its enzymatic activity [49]. Iron depletion has been shown to inhibit AcnB activity in *E. coli* [49], suggesting that the *pdxA* mutant might exhibit an altered iron metabolism and/or iron-sulfide cluster formation and, thus, reduced AcnB activity.

(iii) Agmatin

Agmatin is a decarboxylation product of arginine that is involved in the urea cycle, the synthesis of creatine, and the generation of nitric oxide in eukaryotes [50]. The unaltered levels of arginine between the WT and *pdxA* mutant strains suggested that arginine decarboxylase (SpeA) might also require PLP for its activation. In support of this concept, *E. coli* SpeA shows a PLP-binding affinity [51], and a recent structural analysis showed that...
C. jejuni SpeA contains potent PLP-binding residues, similar to those of E. coli [52].

(iv) β-alanine/asparagine

In contrast to the above three examples, the pdxA mutant exhibited an increased level of β-alanine, a precursor of coenzyme A (CoA), compared with the WT strain. β-alanine is mainly synthesized via the deamination of L-aspartate in E. coli [53]. In this regard, the decreased levels of asparagine observed in the pdxA mutant suggested that asparaginase (AnsB), which is capable of deaminating periplasmic asparagine to aspartate [54], might be inactive in this mutant, thereby causing the accumulation of asparagine, a precursor of β-alanine.

(v) Glycolate

The pdxA mutant displayed decreased production of glycolate (hydroxyacetic acid), one of the smallest alpha-hydroxy acids (AHA). This metabolite is synthesized from 3-hydroxypyruvate (3HP) through reaction with glycoaldehyde, followed by decarboxylation, which requires PLP in E. coli [55], providing a possible reason for the decreased glycolate detected in the pdxA mutant.

Further studies will be necessary to elucidate the molecular impacts of PLP activity on the infection process in this pathogen through in-depth functional and/or structural analyses of each enzymatic reaction. Nevertheless, the data obtained in the present study provide the first evidence that biologically links PLP to the respiratory/energy metabolism as well as the flagellar glycosylation system, affecting the host colonization of C. jejuni.

It is likely that a number of factors could contribute to the colonization of chickens by C. jejuni (i.e., flagellum-mediated motility, chemotaxis, amino acid metabolism, energy metabolism, and iron utilization) [18]. The in vivo growth of C. jejuni has been argued to depend mainly on the availability of free amino and keto acids scavenged from the host or the intestinal microbiota [56]. The data reported herein therefore suggest that in addition to the decreased motility of the pdxA mutant, the altered levels of respiratory/energy metabolism might also participate in the impaired colonization of the chicken gut by this mutant. In vivo metabolic profiling of this pathogen would improve our understanding of the molecular basis underlying its adaptation to and interaction with the host and microbiota during infection.

In summary, this is the first report to demonstrate a functional role of the pdxA gene in altering the motility of and colonization of chickens by a leading foodborne pathogen, Campylobacter jejuni, including the demonstration of a novel link between PLP and flagellar glycosylation. PLP-dependent enzymes are likely to represent approximately 4% of the enzymes present in mammals [57], which attracted our interest in the investigation of PLP functions in terms of potential drug targets. Indeed, certain PLP-dependent enzymes are increasingly being identified as potential drug targets for the treatment of protozoan diseases [58,59]. As poultry animals are the predominant reservoirs of this pathogen for human infection, our data reveal new prospects for potent targeting of PLP and its dependent enzymes to modulate the dynamics of and control this pathogen in livestock animals.

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Supporting Information

Figure S1 Mass spectrum of DMB-labeled pseudaminic acid (Pse) acquired from the arrowed peaks in an extracted ion chromatogram at m/z 441.2-461.2 obtained through SIM of DMB-labeled Pse from the C. jejuni 81–176 wild type (WT), pdxA mutant (pdxA-), and fresh MH broth (blank) samples shown in Fig. 2B. (TIF)

Figure S2 MSn spectra of DMB-labeled pseudaminic acid (Pse) from the 81–176 wild type (WT). (A) the MS/MS spectrum acquired from the molecular ion [M + H]+ (m/z 451.2) of peak (arrowed) in Fig. S1; (B) the MS/MS/MS spectrum acquired from the product ion (m/z 433.1) in the MS/MS; (C) the MS/MS/MS/MS spectrum acquired from the product ion (m/z 415.1) in the MS/MS/MS; (D) Fragmentation of DMB-labeled Pse. In addition to the DMB-labeled Pse, some ms/ms/peaks were also detected. To indicate the molecular mass of these peaks, green ticks were used (to distinguish from the mass peaks). (TIF)

Table S1 Oligonucleotide primers used in this study. (XLSX)

Table S2 Metabolic compounds in C. jejuni identified by CE-MS analysis. (XLS)

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Author Contributions

Conceived and designed the experiments: HA NH. Performed the experiments: HA NH MU. Analyzed the data: HA NH MU. Contributed reagents/materials/analysis tools: NK YSK SI SY. Wrote the paper: HA NH.

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