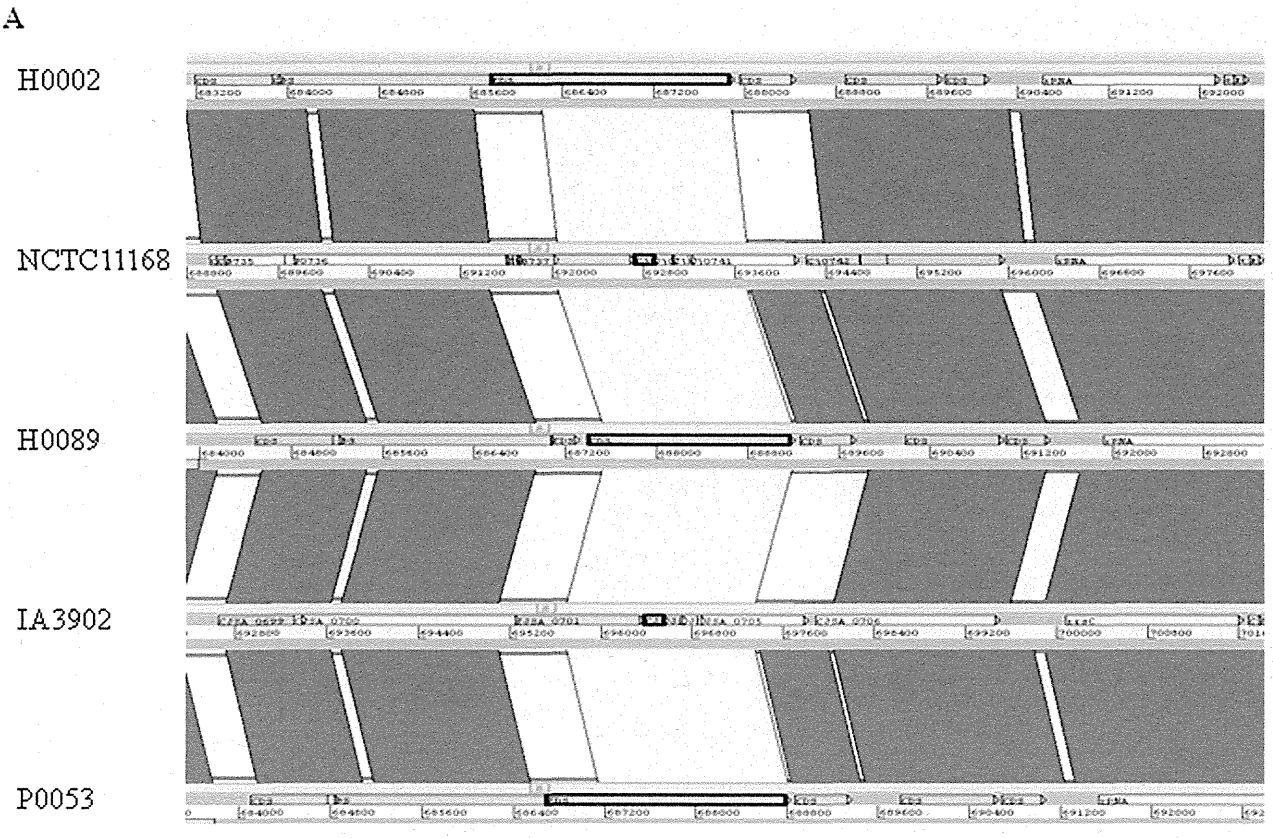
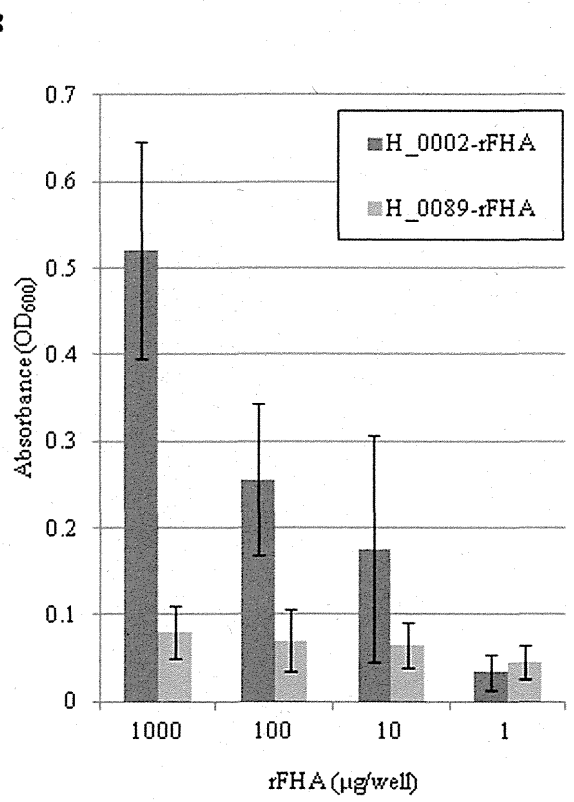

A
H0002
NCTC11168
H0089
IA3902
P0053


B
H_0002-rFHA
H_0089-rFHA
Absorbance (OD600)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1000
100
10
1
rFHA (μg/well)

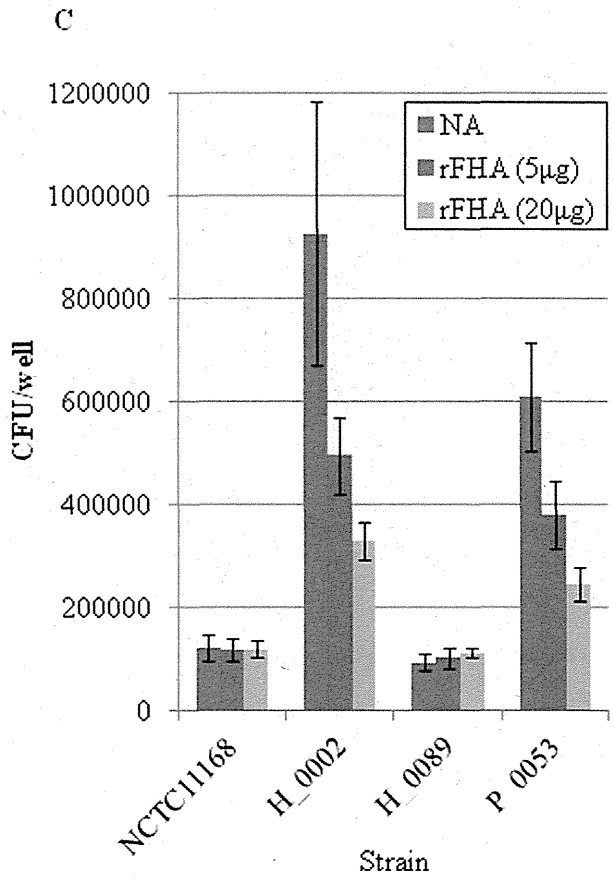

C
NA
rFHA (5μg)
rFHA (20μg)
CFU/well
1200000
1000000
800000
600000
400000
200000
0
NCTC11168
H_0002
H_0089
P_0053
Strain

Figure 6. Sequence variation and host cell adhesive property of the FHA protein in *C. jejuni*. (A) Focused genetic alignment for putative island-like structures in the genomes of *C. jejuni* ST-4526, NCTC11168, and IA3902. The putative filamentous hemagglutination domain protein (FHA) is indicated with a bold-edge. (B) The FHA protein shows affinity to heparan sulfate *in vitro*. The tissue culture plates were pre-coated with heparan sulfate (5 μg/well). After washing with PBS, the plates were co-incubated with 100 μg and serial dilutions of recombinant FHA (rFHA) from isolates H_0002 or H_0089. The bound rFHA were solubilized with 0.1% SDS, and the protein concentrations were measured chronometrically using a Bradford assay. (C) The rFHA blocks the adhesion of FHA-positive *C. jejuni* on Caco-2 cells. The cells were pre-treated with 0, 5, or 20 μg of rFHA derived from H_0002 for 30 min prior to *C. jejuni* infection. The numbers of cell-associated bacteria were counted after gentle washing with PBS.
doi:10.1371/journal.pone.0048394.g006

affinity to heparan than the rFHA from the H_0089 isolate (Fig. 6B), indicating that the N-terminus region of FHA protein was required for this binding affinity. To further support the idea that FHA mediated bacterial adhesion on Caco-2 cells (Fig. 5C), the cells were pre-treated with rFHA prior to infection with H_0002 and P_0053 isolates, and a significant reduction in their cell adhesion levels was observed (Fig. 6C). Thus, these data indicated that the ST-4526 isolates H_0002 and P_0053 expressed functional FHA, which functions as an adhesin.

ST-4526 Increases Antibiotic Resistance and Reduces DNA Uptake/Recombination

The single nucleotide polymorphism (SNP) analyses with NCTC11168 additionally revealed unique genetic variations in the genes for antibiotic resistance, DNA uptake/recombination, and flagellar motility in the ST-4526 (Table 3, Table S7).

(i) Antibiotic resistance. The ST-4526 isolates exhibited SNPs within DNA topoisomerase genes, including *gyrA* at Thr86Ile; *gyrB* at 5 positions, including Thr225Ile, Asp309Glu, Ile318Val, Ser667Asn, and Ser677Gly; and *topA* at 5 positions, including Ala360Ser, Val401Ala, Thr489Ile, Ser607Gly, and Ala212Val (Table S7). The single base mutation in *gyrA* at Thr86 plays a role in increasing the resistance of nalidixic acid (NA) and fluoroquinolones in *Campylobacter* [44], [45]. Accordingly, a disk diffusion test confirmed the resistance of the ST-4526 isolates to NA and fluoroquinolones (CPFX and LVFX) (Table 2).

(ii) DNA uptake and transformation efficiency. *C. jejuni* takes up exogenous DNA via the type II secretion system (T2SS), which is likely to affect genomic plasticity in this pathogen [36]. The Bi-BLAST analyses revealed a reduced amino acid similarity in the Cj1474c gene product (CtsD, a putative type II secretion system D protein) compared with that in NCTC11168 (% similarity at 65.5–66.3%) (Table S6). In addition, many variations were observed in the restriction-modification (R-M) system genes (Cj0690c, 39 SNPs, Cj0031, and 17 SNPs) (Table 3, Table S7), which are also involved in a barrier against foreign DNA and bacteriophages [46], [47]. We thus reasoned that ST-4526 might lose the ability for uptake of exogenous nucleotides, thereby reducing recombination. The ST-4526 isolates showed a more reduced (2.0–3.0%) uptake of exogenous ^{32}P-labeled plasmid DNA than the NCTC11168 strain (Fig. 7A). Similarly, ST-4526 also exhibited reduced transformation efficiency compared with NCTC11168 (5.0–6.1% relative efficiency, Fig. 7B). Taken together, our data indicated that ST-4526 exhibited a loss-of-function for the DNA uptake and recombination.

(iii) Flagellar-mediated motility. A total of 4 genes for flagellar-mediated motility, which is prerequisite for establishing host colonization during an early course of infection [48], [49], [50], also showed sequence variations between ST-4526 and NCTC11168, with 22 SNPs in Cj1729c (*flgE2*), 20 SNPs in Cj1313 (*pseH*), 18 SNPs in Cj1312 (*pseG*), and 9 SNPs in Cj1317 (*pseI*) (Table 3, Table S7). The soft agar motility assay, however, showed that the ST-4526 isolates were motile, with no significant difference with NCTC11168 (Fig. S5). Thus, we showed that the

Table 3. Genetic variations between the ST-4526 isolates and NCTC11168, determined by SNPs analyses.

Functional class	Gene (NCTC)*	Gene symbol	Description	No. variation*
Antibiotic resistance	Cj0003	*gyrB*	DNA gyrase B	5
	Cj1686c	*topA*	DNA topoisomerase I	5
	Cj1027c	*gyrA*	DNA gyrase A	1
DNA replication, recombination, repair	Cj0690c	–	putative restriction/modification (R/M) enzyme	39
	Cj1051c	*cjeI*	Restriction endonuclease S subunits (type I R/M)	21
	Cj0031	–	putative type II R/M enzyme	17
	Cj0139	–	putative endonuclease	9
	Cj1052c	*mutS*	recombination and DNA strand exchange inhibitor protein	6
	Cj0634	*dprA*	DNA processing protein A	5
	Cj1083c	–	putative nuclease	5
Flagellar-mediated motility	Cj1729c	*flgE2*	Flagellar basal body protein	22
	Cj1313	*pseH*	N-acetyltransferase specific for PseC product	20
	Cj1312	*pseG*	Nucleotidase specific for PseC product	18
	Cj1317	*pseI*	Pse synthetase	9

Representative SNPs in genes within the above functional categories were listed. For full SNP data, refer to Table S7.
*Gene orthologue in NCTC11168 was shown.
**No. unique variations found in the 3 ST-4526 isolates, with possible amino acid change, were shown.
doi:10.1371/journal.pone.0048394.t003

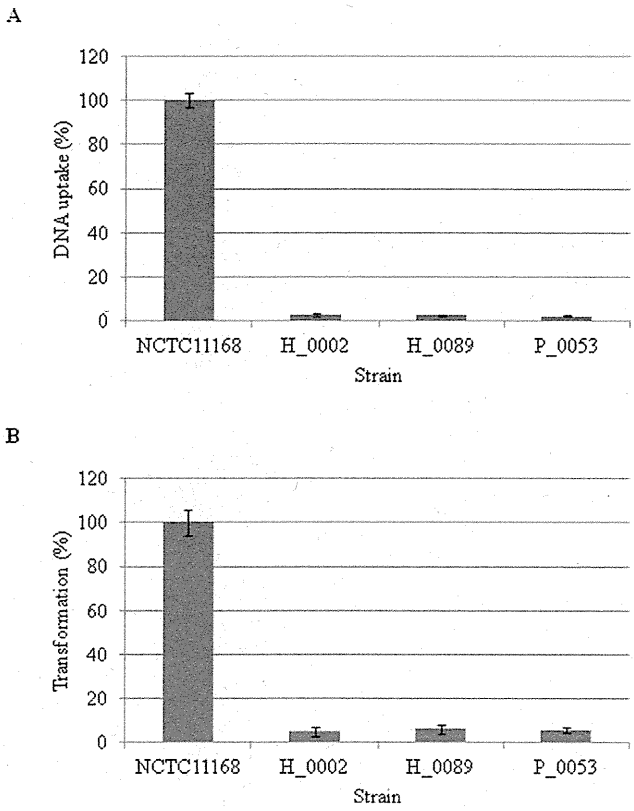


Figure 7. *C. jejuni* ST-4526 exhibits a reduced the ability for DNA uptake and natural transformation. (A) The bacterial cells were co-incubated with P^{32}-labeled pRY108 DNA, and the intrabacterial radioactivity was measured using a scintillation counter. The means in the NCTC11168 strain were set to 100% for the comparative analysis. (B) The natural transformation efficiency was assayed. The appearance of the Cm-resistant clones was normalized to the total number of colonies on the plates without antibiotics, and the test strains were further normalized to the means in the NCTC11168 strain, which were set to 100%.
doi:10.1371/journal.pone.0048394.g007

nucleotide variations did not affect the motility phenotype in ST-4526.

Discussion

Genetic attribution of bacterial genotypes has become a major tool in the investigation of the epidemiology of campylobacteriosis and has implicated retail chicken meat as the major source of human infection in several countries [51], [52]. In this study, we analyzed the population genetics of *C. jejuni* from different sources and areas in Japan using a MLST approach. We also attempted to understand the molecular basis underlying the unique and widespread predominance of ST-4526 in Japan using comparative genomic analyses. We identified several genomic traits beneficial for host adaptation, which seemingly accounted for the thriving of this genotype.

Many campylobacters are commensals that pass through a wide range of warm-blooded hosts and insects. Within farm animals, certain MLST lineages were identified in chickens and cattle, with different frequencies, whereas several CCs (i.e. CC-21) were identified at high frequencies in both sources [53]. CC-21 and -45 were the most frequent CCs in humans [54], [55], whereas CC-21, CC-45, and CC-574 were predominant in the poultry population in Great Britain from 2003 to 2006 [56]. In addition, Kwan et al. [57] reported the predominance of CC-61, CC-21, and CC-42 among cattle isolates in the United Kingdom. Our data are consistent with the finding for host assignment discussed above, regardless of geographic distance. In a recent large-scale MLST data analysis, Sheppard et al. [58] demonstrated that host association is more robust than geographic associations.

The results of the ClonalFrame analysis also revealed the presence of the rare genotype ST-58. In this study, however, this genotype was detected in 8 cattle isolates derived from the same location (Oita prefecture), although they were collected at different periods. Therefore, we could not rule out that this genotype might be adapted to the cattle population at a particular geographical location (farms). The additional collection of cattle isolates from different locations, including this area, would help to ascertain whether ST-58 also is a cattle-adaptive genotype.

There is an increasing evidence for the local and international transmission of host-associated lineages between food animal species [58]. In particular, CC-21, a widely distributed group, showed high genomic similarity regardless of origin [59]. We observed the frequent distribution of ST-4526 within CC-21 between the human and poultry populations of our collection. This genotype has not been previously reported from other countries; therefore, we examined the evolutionary linkage of this genotype within CC-21, which revealed a distinct lineage of this genotype from frequent genotypes worldwide. It remains unknown whether such phylogenetic distances represent bacterial traits for adaptation or host colonization. Lang et al. [60] showed that host species was a significant factor in explaining the genetic variation and macrogeography between Western Europe and the USA, and geographic variation of *C. jejuni* genotypes have been previously reported in Great Britain [56] and the USA [61]. The evolutionary linkage data herein thus suggested that ST-4526 genotype might be evolved in the Asian geography for adaptation. Comparative genomics between the closely related STs in goeBURST analysis (i.e. ST-4526 vs ST-883) would clarify whether the housekeeping genes-based clonal analysis might represent bacterial fitness for environmental survival or host infection, host specificity.

The availability of genome sequences facilitates a qualitative advance in our understanding of the epidemiology, ecology, and molecular biology of various organisms. To characterize the genomic features that account for the thriving of ST-4526, a comparative genomic analysis was performed for three ST-4526 isolates (H_0002, H_0089, and P_0053). This genotype showed (asialylated) LOS forms distinct from those of NCTC11168, although the latter showed forms similar to the widespread genotypes within CC-21. Habib et al. (2008) [62] showed a strong (85%) correlation between CC-21 and the sialylated class C LOS, indicating a potential role for class C LOS in the evolution of CC-21 that might be of particular importance in the poultry meat-related transmission of *C. jejuni* to humans.

We also observed variations in island-like structures, which included putative adhesin, in the genome of *C. jejuni*; i.e., fig|6666666.15583.peg.771 to fig|6666666.15583.peg.779 in H_0002 (Table S3), which showed less similarity to NCTC11168 (corresponded to the locus Cj0736-Cj743) (Table S6). The amino acid alignment revealed the truncation of the putative filamentous hemagglutination domain protein (FHA) among the *C. jejuni* strains. The recombinant FHA protein showed dose-dependent binding affinity to heparan sulfate, which competitively inhibited bacterial adhesion, suggesting a putative role for this gene product as an adhesin. Future studies will be focused on the in-depth biochemical characterization of this putative adhesin protein (i.e.,

determination of the active domain for cell adhesion, with screening of the host cell surface carbohydrates as interactive partners), which will be helpful for clarifying the biological role of this putative molecule during infection.

The SNP analyses in combination with a Bi-BLAST search revealed several genetic variations unique to the ST-4526 isolates; the polymorphisms in the topoisomerase genes (especially *gyrA*), could explain the increased resistance to NA and CPFX/LVFX, which is likely to confer an advantage for the spread and stability of this pathogen in poultry hosts as previously reported [63], [64]. This SNP was also identified in the genes for DNA uptake and recombination, and phenotypic or biochemical assays confirmed these reduced abilities. The genetic exchange of this pathogen was previously demonstrated in the chicken intestinal environment [65]. Nevertheless, the observed phenotypes in ST-4526 could thus explain the inability of this genotype to cause frequent genomic alterations, i.e., expanding the ability for clonal spread in human and poultry populations in Japan. It remains unknown how ST-4526 acquired such phenotypes; one possible explanation is that ST-4526 might evolve during the colonization of the ancestor in the poultry host, as passage in the chicken gut could alter the genetic and phenotypic traits of this pathogen [66]. With consideration for unique phylogeny, future studies will attempt to clarify the potential role of farm animals in the genomic evolution in this pathogen.

The ST-4526 isolates were commonly serotyped to O:2. A previous study showed that serotypes O:2, O:4, O:37, and O:8 shared more than 45.7% of a total of 601 poultry isolates in Japan collected from 2001 and 2006 [22]. The predominance of these genotypes in both the human and poultry populations reported herein reinforced the need for continued research on the potential importance of poultry as reservoirs for these human pathogens. We are currently collecting human isolates at different periods to determine whether the thriving of the ST-4526 is temporal or continuous with geographical significance.

In summary, this study is the first to show the population structure of *C. jejuni* from different sources in Japan, which deciphered the potent thriving of the ST-4526 among human and poultry populations. The results of genomic and phenotypic analyses provided possible reasons for the wide spread of this genotype. Our continuous examination of the molecular basis underlying the genotypic alterations and phylogeography of this pathogen will provide further insight into the link between the evolution and ecological features of this pathogen.

Supporting Information

Figure S1 Japan geography for the source of *C. jejuni* isolates used in this study.
(TIF)

Figure S2 The goeBURST analysis of *C. jejuni* CC-21. A total of 547 STs belonging to the CC-21 were used to analyze possible patterns of their evolutionary descent.
(TIF)

Figure S3 Multiple alignment of putative filamentous hemagglutination domain protein (FHA) in *C. jejuni* ST-4526 representative isolates (H_0002, H_0089, P_0053), and those from 12 other *C. jejuni* strains (CF93-6, 1213, LMG23263, 1997-14, 1854, S3, 84-25, NCTC11168, IA3902, DFVF1099, M1, and 81116). The conserved domain Architecture Retrieval Tool (CDART) (http://www.ncbi.nlm.nih.gov/Structure/exington/lexington.cgi) predicts the putative hemagglutination activity domain at 22 to 135 aa from the N-terminus of the H_0002 isolate (shown in bold).
(TIF)

Figure S4 Cloning and expression of the recombinant filamentous hemagglutination domain protein (rFHA) from *C. jejuni* H_0002 and H_0089. The putative FHA-encoding gene was PCR-amplified (section A), and ligated into pBAD202 D-Topo vector. Expression was induced under the control of L-arabinose in *E. coli* LMG194 strain. Both the purified rFHA proteins were loaded onto 10% acrylamide gel, visualized by CBB stain. M, molecular markers (section A, λ/*Hin*dIII; section B, Bio-Rad Precision plus Protein standard Dual color).
(TIF)

Figure S5 Motility of *C. jejuni* ST-4526 isolates on soft agar plates. 5 μl aliquots of *C. jejuni* ST-4526 isolates (H_0002, H_0089, P_0053) and NCTC11168 cultures were spotted on 0.4% soft agar plates. At 24 h post incubation, the diameters of halo around the spot were measured. The data showed means ± standard deviation (SD) from three independent testing.
(TIF)

Table S1 *Campylobacter jejuni* isolates used in this study. *Novel STs found in this study were shown in bold. **UA, unassigned to any CCs. ***Isolate ID, deposited onto the *Campylobacter* MLST database (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db = pubmlst_campylobacter_isolates&page = query).
(XLS)

Table S2 Summary of the MLST alleles statistics for the *C. jejuni* dataset in Japan. Seven alleles obtained from a total of 212 *C. jejuni* isolates (= 62STs) were statistically analyzed by START2 program, providing the allele frequency, and polymorphisms. **dN/dS*: Mean non-synomous substitutions per non-sinoumous site (*dN*)/mean synonymous substitutions per suynomous site (*dS*). **Sites are categorized by the degeneracy of the codon they belong to, rather than that of the individual site.
(XLSX)

Table S3 Genomic annotation of *C. jejuni* H_0002 (Table S3), P_0053 (Table S4), and H_0089 (Table S5).
(XLS)

Table S4 Genomic annotation of *C. jejuni* H_0002 (Table S3), P_0053 (Table S4), and H_0089 (Table S5).
(XLSX)

Table S5 Genomic annotation of *C. jejuni* H_0002 (Table S3), P_0053 (Table S4), and H_0089 (Table S5).
(XLSX)

Table S6 Bidirectional BLAST (Bi-BLAST) comparisons for *C. jejuni* H_0002, H_0089, P_0053, IA3902 toward NCTC11168 strain.
(XLSX)

Table S7 Single nucleotide polymorphism (SNP) analysis for the ST-4526 isolates to the NCTC11168 strain. Contig sequences of three ST-4526 isolates (H_0002, H_0089, P_0053) were used to search SNPs unique to ST-4526 by comparison with the genome of NCTC11168 strain using CLC Genomic workbench software.
(XLSX)

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Author Contributions

Conceived and designed the experiments: HA HB. Performed the experiments: HA HB TE. Analyzed the data: HA HB. Contributed reagents/materials/analysis tools: SKS TFM SY SI. Wrote the paper: HA HB.

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

Hiroshi Asakura[1]*, Noritaka Hashii[2], Masashi Uema[1], Nana Kawasaki[2], Yoshiko Sugita-Konishi[3], Shizunobu Igimi[1], Shigeki Yamamoto[1¤]

1 Division of Biomedical Food Research, National Institute of Health Sciences, Setagaya-ku, Tokyo, Japan, **2** Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Setagaya-ku, Tokyo, Japan, **3** Department of Food and Life Sciences, Azabu University, Fuchinobe Chuo-ku, Sagamihara, Kanagawa, Japan

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 *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 flagellar 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.

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* E-mail: hasakura@nihs.go.jp

¤ Current address: Department of Fisheries, Tokai University, Orido, Shimizu-shi, Shizuoka, Japan

Introduction

Campylobacter jejuni is a Gram-negative, spiral-shaped, microaerophilic 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 [12,13]. 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 (Cj1294) 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 CO_2 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 Cm-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-BI or pdxA-s-BI and pdxA-as primers (Table S1). After *Bam*HI 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 *Bam*HI 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 *pdxAJ* locus and the upstream region predicted by the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html) to contain −35 and −10 promoter binding sites were amplified *via* PCR using the pdxA-CF and pdxA-CR primers (Table S1). The resultant PCR fragments were cloned into the *Xba*I/*Eco*RI sites of the pRY108 plasmid [21], yielding pRY-pdxA-Km (Table 1). This plasmid was introduced into the *pdxA* mutant strain through 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 OD_{600} 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).

Table 1. Bacterial strains and plasmids used in this study.

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	Cm- 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::*pdxAJ* for complementation	This study

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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 points, 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 sialic 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 45% 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-MS/MS/MS/MS (MS^n) with IT-MS.

Detection of metabolic compounds

(i) Sample preparation. A total of 3.2–3.4×10^8 *C. jejuni* WT or *pdxA* mutant cells grown in MH 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 together 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 3.0×10^7 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* (CJJ81176_1253) and *pdxJ* (CJJ81176_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 microbial 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 ml^{-1}) in the *pdxA* mutant than the WT strain (34.55 ± 7.61 μg 10 ml^{-1}), and complementation of the *pdxA* gene in the *pdxA* mutant restored PLP production (33.85 ± 7.45 μg 10 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 flagellins 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/CJJ81176_1311) gene product, UDP-4-keto-deoxy-GlcNAc transaminase, is reported to require PLP to generate UDP-4-amino-4,6-dideoxy-GalNAc, a spectrometric analintermediate 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, pyridoxamine-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*.

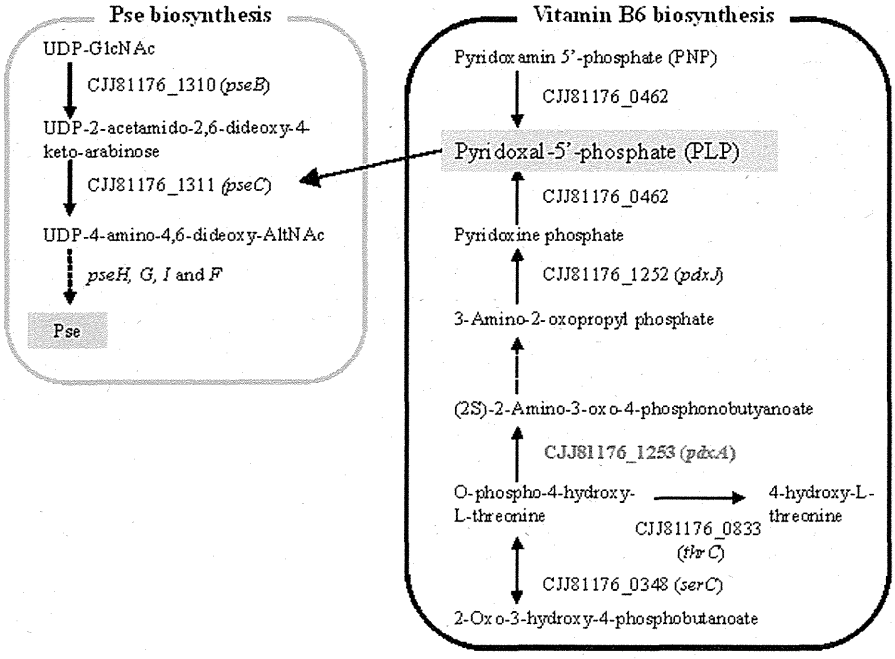
The *C. jejuni pdxA* mutant exhibits altered respiratory/energy metabolism

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 1.6×10^8 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 *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*

A



B

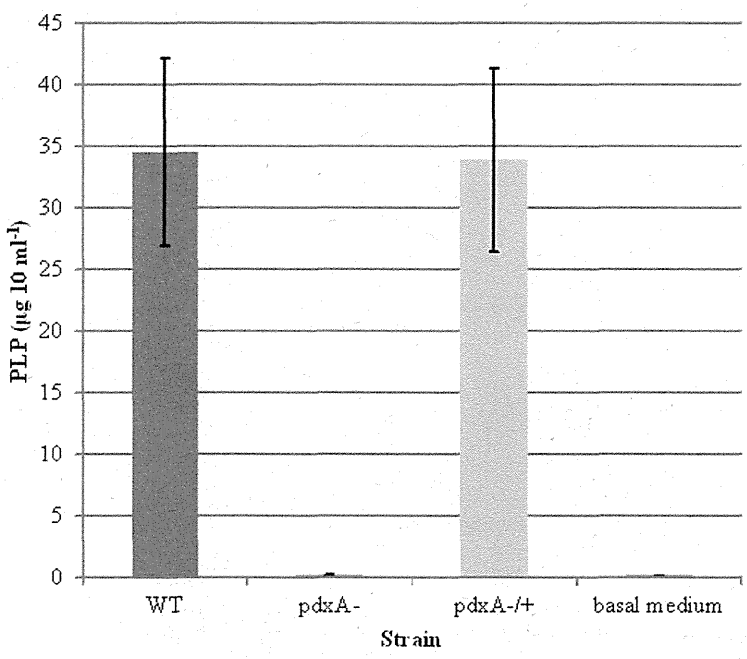


Figure 1. Inactivation of the *pdxA* gene impairs the biosynthesis of vitamin B6 (PLP) in *C. jejuni*. (A) A scheme for the PLP production pathway (right box) in *C. jejuni* in relation to Pse biosynthesis (left box) is illustrated based on *in silico* pathway analysis performed using PATRIC (http://patricbrc.vbi.vt.edu/portal/portal/patric/Home). (B) The *pdxA* mutant produced no PLP. The *C. jejuni* 81–176 WT, *pdxA* mutant, and the complemented strains were grown in 10ml of MH broth to an OD_{600} 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.
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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

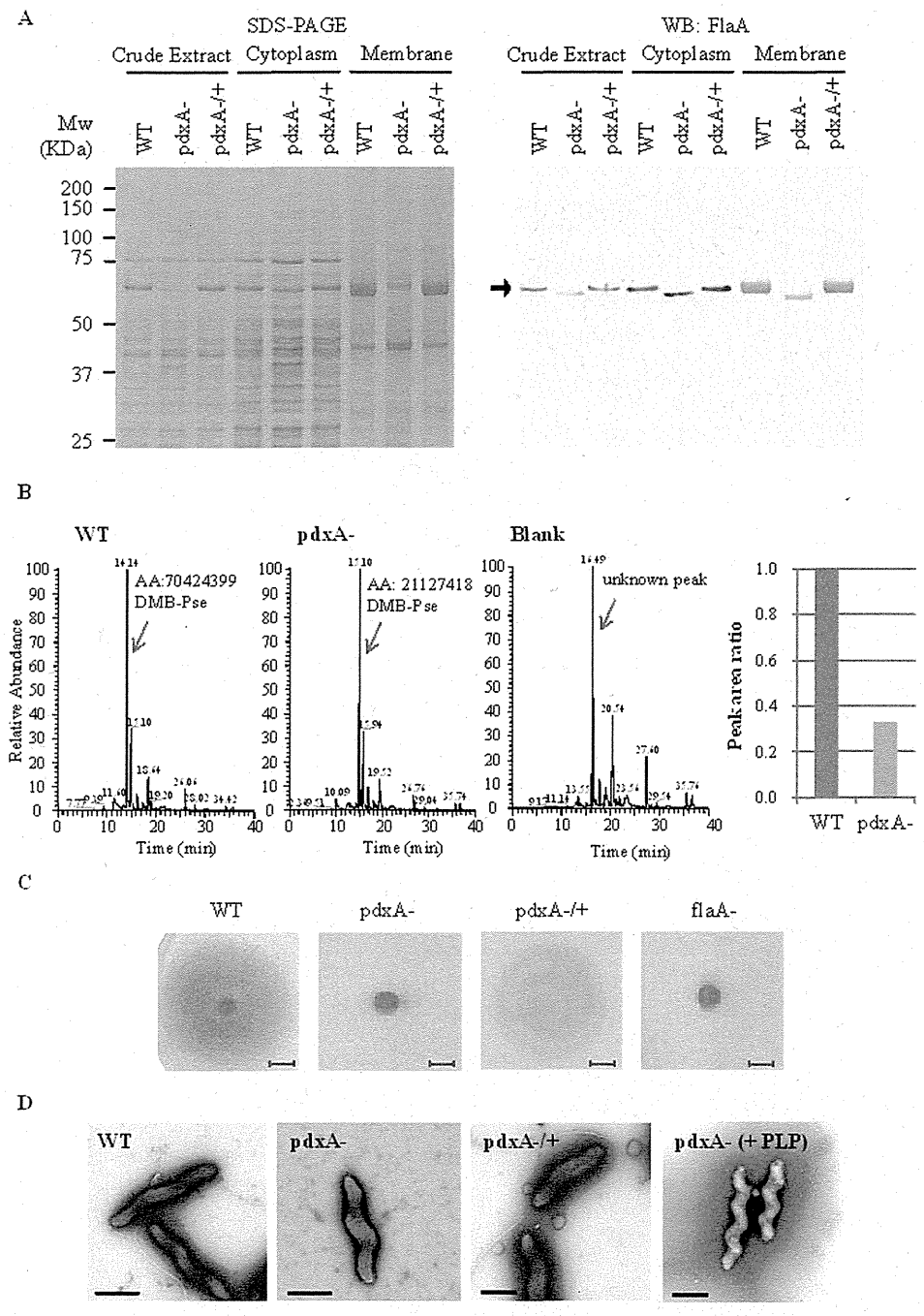

Figure 2. The *pdxA* mutant shows impaired pseudaminic acid (Pse) production and flagellar motility. (A) The *pdxA* mutant shows less glycosylation of FlaA. SDS-PAGE and western blotting were conducted to detect the *C. jejuni* FlaA protein. Crude extracts and subcellular (cytoplasmic and membrane) fractions were extracted from *C. jejuni* and visualized using CBB staining in an SDS-polyacrylamide gel (left panel). Western blot analyses were simultaneously performed to detect the FlaA protein (arrow, right panel). (B) The *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 *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 *pdxA* gene impairs motility of *C. jejuni*. The WT, *pdxA* mutant, *pdxA*-complemented (*pdxA*−/+), and *flaA* mutant (flaA-) strains were spotted and incubated onto 0.4% soft agar. Scale bars represent 3 mm. The motility of *pdxA* mutant was also assayed in the supplementation of 10 mg l^{-1} of PLP (pdxA− + PLP). (D) The *pdxA* mutant is aflagellated. Electron micrographs of the *C. jejuni* WT, *pdxA* mutant with or without supplementation of PLP (10 mg l^{-1}), *pdxA*- complemented strains. The scale bars represent 1 μm.
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induced significantly less IL-8 production in the INT407 cells compared with the WT strain at 4 h *p.i.* ($p<0.05$, 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 *pdxA* gene is a prerequisite for cell adhesion, with the mutant

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)
			WT	pdxA−	
Increased					
UDP-glucose/galactose	565.05	8.22	2.25E-04	7.99E-04	3.55
Azelaic 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	9.78	2.25E-04	5.55E-04	2.46
β-Alanine	90.06	6.96	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 >2.0-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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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 ($2.14\pm2.92\times10^4$ CFU/g at day 7) and 4.6×10^4-fold ($6.00\pm7.48\times10^3$ CFU/g at day 28) decreases in the colonization of chicken cecum tissues compared with the parental strain ($2.14\pm2.12\times10^6$ CFU/g at day 7 and $2.74\pm2.55\times10^8$ 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

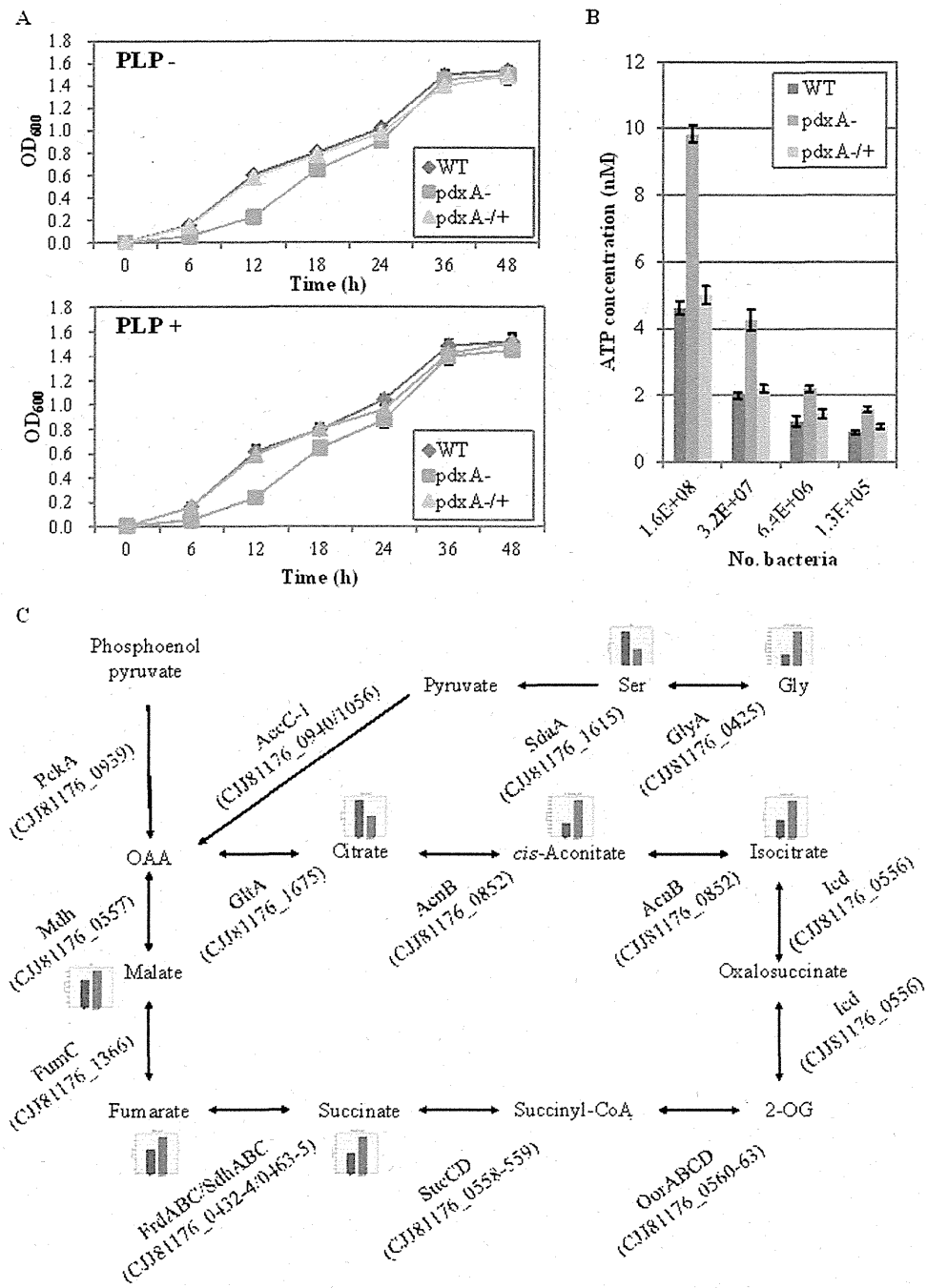

Figure 3. The *C. jejuni pdxA* mutant shows altered growth kinetics and respiratory/energy metabolism. (A) Growth curves of *C. jejuni* 81–176 WT, pdxA−, and the complemented mutant strains in MH broth not supplemented (left panel) or supplemented (right panel) with PLP (10 mg l^{-1}). (B) Intracellular 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.
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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 CO_2 and H_2O 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.

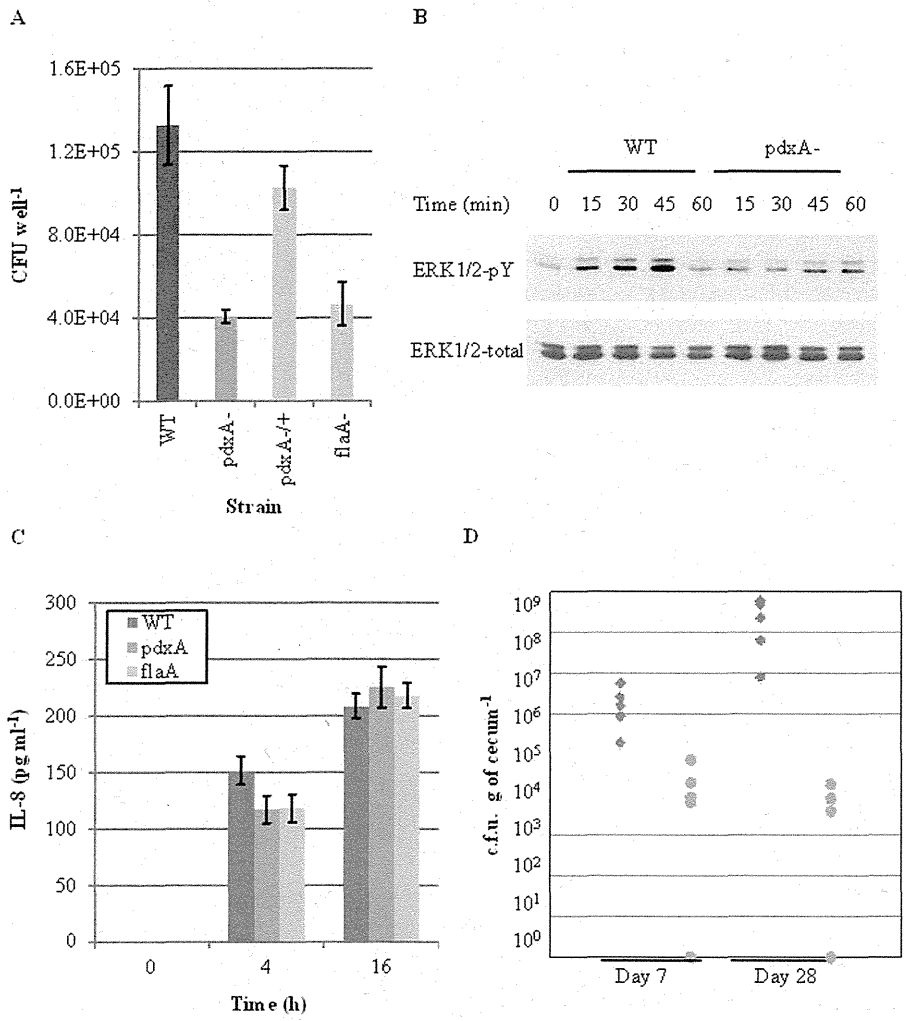

Figure 4. Deletion of the *pdxA* gene impairs *in vitro* cellular responses and *in vivo* colonization. (A) INT407 cells were infected for 1 h with the *C. jejuni* WT, pdxA−, pdxA−/+, and flaA− strains. The number of cell-adherent bacteria was measured by counting the plates after washing three times with PBS. (B) ERK1/2 activation upon infection. Western blotting was performed to detect the levels of phosphorylated and total ERK1/2 in the lysates from infected cells. (C) IL-8 production in INT407 cells was measured at 4 h and 16 h *p.i. via* ELISA. The data are presented in sections A and C as the mean values ± standard deviations from samples run in duplicate in at least three experiments. (D) Disruption of the *pdxA* gene reduces the colonization of the chicken cecum by *C. jejuni*. Groups of 14-day-old chickens (n = 10 per group) were orally inoculated with approximately 3×10^7 CFU of WT or *pdxA* mutant *C. jejuni*. At 1 week and 4 weeks *p.i.*, the ceca were aseptically removed from the infected animals (n = 5 for each time point) and homogenized. Serial dilutions of the suspensions were plated on mCCDA agar to count CFU numbers. The closed diamonds and open circles represent the numbers of WT and *pdxA* mutant CFUs recovered from the animals, respectively.
doi:10.1371/journal.pone.0070418.g004

(i) 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.

(ii) Citrate/*cis*-aconitate/isocitrate

These TCA intermediates are interconverted by aconitases [46], among which AcnB 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 decarboxylation 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 inactivated in this mutant, thereby causing the accumulation of asparagine, a precursor of β-alanine.

(v) Glycolate

The *pdxA* mutant displayed decreased production of glycolate (hydroxylacetic 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 MS^n 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)

Figure S3 MS^n spectra of DMB-labeled pseudaminic acid (Pse) from the 81–176 *pdxA* mutant. (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* 415.0) 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).
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Figure S4 STRING network analysis. Protein-protein network analysis was carried out using the STRING database (http://string.embl.de/). Protein entries from *C. jejuni* strain 81–176 or *H. pylori* strain G27 were used for the identification of putative protein-protein associations of PdxA to other bacterial proteins according to the guideline of the database.
(TIF)

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

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

Acknowledgments

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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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Research Article

Molecular Approach for Tracing Dissemination Routes of Shiga Toxin-Producing *Escherichia coli* O157 in Bovine Offal at Slaughter

Hiroshi Asakura,[1] Kazuya Masuda,[1] Shigeki Yamamoto,[2] and Shizunobu Igimi[1]

[1] *Division of Biomedical Food Research, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan*
[2] *Department of Fisheries, Tokai University, 3-20-1 Orido, Shimizu-ku, Shimizu-shi, Shizuoka 424-8610, Japan*

Correspondence should be addressed to Hiroshi Asakura; hasakura@nihs.go.jp

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Bovine offal is currently recognized as one of the sources of human Shiga toxin-producing *Escherichia coli* (STEC) infection in Japan. Here, the prevalence and genetic characterization of STEC O157 in bovine feces, offal, and carcasses at slaughtering were examined between July and October in 2006. STEC O157 was detected in 31 of 301 cattle feces (10.3%) delivered from 120 farms. Simultaneously, 60 bovine-originated offal (tongue, liver, and omasum) and carcasses were randomly selected and the detection of O157 STEC was examined as well. STEC O157 was isolated from 4 tongues (6.7%), 1 liver (1.7%), 3 omasa (5.0%), and 2 carcasses (3.3%), respectively. All the O157 isolates were positive for *eae* and *hlyA* genes, and 37 of 41 isolates (90.2%) exhibited *stx2c* genotype. PFGE analysis revealed the identical macrogenotypes of 4-tongue- and 1-liver-originated isolates and among 2 fecal isolates from animals slaughtered consecutively. Considering their continuous detection according to the slaughtering order, we concluded that these distributions of O157 in bovine offal and feces might be due to cross-contamination at (pre)slaughter. Our data thus reposes implication of better sanitary control in diapedesis from both upper and lower sites to prevent spread of this pathogen to bovine offal at slaughtering.

1. Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is one of the major foodborne pathogens that causes diarrheal illness in humans worldwide. Among a number of serotypes categorized in the STEC, O157 is the most major serotype associated with human infection [1]. Infection with STEC O157 can be achieved at least in part through the intake of contaminated foods, in which dairy products and meats contaminated with animal feces or intestinal contents during/after slaughtering are considered as the most common sources [2].

In Japan, bovine offal which include liver, heart, tongue and intestines are customarily eaten, a part of which are consumed raw and the contamination with pathogenic microorganisms in these meat products is therefore considered a high risk for human health. Indeed, as epidemiological records for foodborne O157 infections in Japan, 6 of 52 cases (11.5%) were associated with the bovine offal in years 2010-2011 [3]. More recently, we examined the prevalence of STEC in retail bovine offal products in Japan, revealing that 38 of 229 samples (16.6%) were positive for *stx* gene and four O157 and one O26 STEC were finally isolated from small intestine and omasum products [4]. However, the routes of contamination in these products remain unclear, especially the issue of how and whether cross-contamination might occur during slaughtering processes.

Given the background, here we examined the prevalence and genetic characterization of STEC O157 in bovine feces and offal at slaughterhouse in Japan.

2. Materials and Methods

2.1. Sampling, Isolation, and Identification of STEC O157. Fecal samples were collected with cotton swab from a total of

301 bovine from 120 farms between July and September, 2006, at a slaughterhouse in Japan. Simultaneously, 60 of these animals were randomly selected and 100 cm^2 surface areas of their offal (livers, tongues, and omasum) and carcasses were swabbed, thereby being subjected to the detection procedure of STEC O157 as well. The swab samples were incubated in 10 mL of novobiocin-supplemented mEC broth (Eiken Kagaku, Tokyo, Japan) at 42°C for 24 h. The cultures were then subjected to screening of O157 using Path-Stik *E. coli* O157 (Celsis, Cambridge, UK) and mini-VIDAS (bioMérieux-Vitek, France). The O157-positive culture samples were then plated on CT-SMAC (Eiken Kagaku, Tokyo, Japan), CHRO-Magar O157 (CHROMagar, Paris, France), and Rainbow agar O157 (Biolog, Hayward, CA, USA). After incubation at 37°C for 24 h, suspected colonies were biochemically and/or genetically identified to be STEC O157 with API-20 kit (bioMérieux), O157 PCR screening set (Takara Bio, Shiga, Japan), and NH immunochromatography (Nippon-Ham, Tokyo, Japan) accordingly. The above culturing flow for each sample was started immediately within the day of slaughter.

2.2. Genetic Characterization and Toxin Production of STEC O157 Isolates. To characterize virulence gene possession of these isolates, total DNA was extracted from bacterial isolates with DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The *eaeA* and *hlyA* (*ehxA*) genes were detected by PCR using primers as described previously [5, 6]. The *stx* genes were subtyped by PCR as described [4, 7]. Shiga toxin (Stx) production was assayed by VTEC-RPLA (Denka Seiken, Tokyo, Japan).

2.3. Pulsed-Field Gel Electrophoresis (PFGE). Representative O157 isolates were subjected to PFGE with *Xba*I endonuclease (New England BioLabs, Ipswich, MA, USA) using the CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) as described previously [8]. The gel images were obtained using ethidium bromide stain. The electrophoretic patterns from PFGE were compared based on band position using FingerPrinting II software (Bio-Rad Laboratories, Hercules, CA, USA) and derived using the Dice coefficient with a maximum position tolerance of 1%. The strains were clustered using the unweighted pair group (UPGMA) method with arithmetic averages according to the manufacturer's instructions.

3. Results and Discussion

3.1. Dynamics for the Prevalence of STEC O157 at Slaughter. The majority of primary STEC infections are considered to be food- or water-borne, in which bovine and its products are one of the main sources of infection [9]. Epidemiological studies have mount evidence for the high prevalence of STEC especially O157 serotype in cattle intestines [10–12]. Throughout the screening tests herein, STEC O157 was detected from 31 of 301 samples from bovines feces that were slaughtered between July and September in 2006 in Japan (Table 1), of which 6 isolates (isolates # 7–12) were originated from animal slaughtered at the same day, even though they were delivered from different farms (Table 1). 60 offal (tongues, livers, and omasum) and carcass samples were simultaneously subjected to the O157 detection tests, resulting in that the STEC O157 was isolated from 4 tongues (6.7%), 1 liver (1.7%), 3 omasa (5.0%), and 2 carcasses (Table 1). Among them, 4 tongues and 1 liver isolates (isolates number 1–5) were originated from samples consecutively slaughtered (Table 1). Thus, these data showed the prevalence of STEC O157 in bovine feces and offal at slaughter. The consecutive detection of O157 from fecal and offal samples (i.e., isolates #1–3, 5) suggested that these isolates might be originated from identical sources.

The prevalence date herein at 10.5% is likely to be similar to that in a previous nationwide study in Japan [13]. Comparatively, little is known about the prevalence of STEC O157 in bovine offal at slaughter worldwide. Such biased information is likely to depend on the intake custom, as the individuals who eat them raw are limited and very few in westerns. Our data herein thus provided the implication of these foods for microbial risks to human infection.

3.2. Genetic Characterization of STEC O157 Isolates. Genetic characterization assays showed that all isolates were positive for *eaeA* and *hlyA* (Table 1). The most frequent *stx* subtype was *stx2c* (37 of 41, 90.2%), followed by *stx1+stx2c* (isolates number 6 and #21, 4.9%), and *stx1* (isolate #12, 2.4%), *stx1+stx2* (isolate #41, 2.4%), respectively (Table 1). The high yields of *stx2c* among the bovine isolates herein are in agreement with the previous reports [13]. We confirmed the Stx production in all isolates by VTEC-RPLA. Thus, these data indicated the potent virulence of these isolates.

3.3. Macrogenotypes of Representative O157 Isolates. Having nonconsecutive isolation of O157 STEC from fecal samples originated from identical farm (i.e., isolates #8, 9, 27, and 28 from farm F) and almost consecutive detection from tongue/liver (i.e., isolates #1–4 and #5) and omasum/fecal samples (isolates #21-22 and 17–20), their genetic associations were examined by pulsed-field gel electrophoresis (PFGE). This approach then revealed the identical macrogenotypes among four tongue isolates and one liver isolate that were slaughtered almost consecutively (Figure 1). Likewise, two omasum isolates (isolates #21-22) also exhibited identical PFGE pattern with fecal isolates #17–20 that were obtained at the same date (24/Aug/2006) (Figure 1). These suggested that cross-contamination at slaughter might be a possible factor for this dissemination.

Moreover, four fecal isolates #8-9 and #27-28 exhibited identical PFGE patterns with close phylogenic lineages (Figure 1). Because these isolates were originated from animals fed at the same farm (farm F, Table 1), it could be considered that this O157 might be widely disseminated at that farm continuously. Indeed, a previous study demonstrated that a part of bovine animals shed high doses of O157 longitudinally (so-called "super shedders") [14]. A recently trialed vaccine against type III secreted proteins [15] might be effective for the reduction of such continuous spread of this pathogen at farms. In this relation, two beef carcass isolates #40 and 41 showed different PFGE genotypes although they were

Table 1: Summary of STEC O157 isolates obtained in this study.

Isolate number	Date of slaughter	Animal number*1	Place*2	Farm*2	Source	Virulence gene		
						stx	*eaeA*	*hlyA*
1	6/Jul/2006	206	i	A	Tongue	2c	+	+
2	6/Jul/2006	207	ii	B	Tongue	2c	+	+
3	6/Jul/2006	208	ii	B	Tongue	2c	+	+
4	6/Jul/2006	210	iii	C	Tongue	2c	+	+
5	6/Jul/2006	207	ii	B	Liver	2c	+	+
6	20/Jul/2006	842	iv	D	Omasum	1 + 2c	+	+
7	27/Jul/2006	1202	v	E	Feces	2c	+	+
8	27/Jul/2006	1207	v	F	Feces	2c	+	+
9	27/Jul/2006	1208	v	F	Feces	2c	+	+
10	27/Jul/2006	1242	iii	G	Feces	2c	+	+
11	27/Jul/2006	1257	i	H	Feces	2c	+	+
12	27/Jul/2006	1263	i	I	Feces	1		
13	28/Jul/2006	1315	vi	J	Feces	2c	+	+
14	28/Jul/2006	1316	vi	J	Feces	2c	+	+
15	28/Jul/2006	1318	vi	J	Feces	2c	+	+
16	23/Aug/2006	834	vii	K	Feces	2c	+	+
17	24/Aug/2006	852	iii	L	Feces	2c	+	+
18	24/Aug/2006	868	vii	M	Feces	2c	+	+
19	24/Aug/2006	869	vii	M	Feces	2c	+	+
20	24/Aug/2006	883	vii	N	Feces	1 + 2c	+	+
21	24/Aug/2006	851	iii	O	Omasum	2c	+	+
22	24/Aug/2006	853	iii	L	Omasum	2c	+	+
23	25/Aug/2006	936	viii	P	Feces	2c	+	+
24	25/Aug/2006	937	viii	P	Feces	2c	+	+
25	30/Aug/2006	1048	v	E	Feces	2c	+	+
26	30/Aug/2006	1050	v	Q	Feces	2c	+	+
27	30/Aug/2006	1057	v	F	Feces	2c	+	+
28	30/Aug/2006	1058	v	F	Feces	2c	+	+
29	30/Aug/2006	1068	vii	R	Feces	2c	+	+
30	9/Sep/2006	383	i	I	Feces	1		
31	9/Sep/2006	388	i	S	Feces	2c	+	+
32	9/Sep/2006	389	i	S	Feces	2c	+	+
33	9/Sep/2006	392	i	T	Feces	2c	+	+
34	9/Sep/2006	399	vii	U	Feces	2c	+	+
35	9/Sep/2006	400	vii	U	Feces	2c	+	+
36	9/Sep/2006	401	vii	U	Feces	2c	+	+
37	9/Sep/2006	424	ix	V	Feces	2c	+	+
38	14/Sep/2006	601	viii	P	Feces	2c	+	+
39	30/Sep/2006	1152	ix	W	Feces	2c	+	+
40	17/Aug/2006	635	vii	N	Carcass	2c	+	+
41	24/Aug/2006	890	vii	N	Carcass	1 + 2	+	+

*1Animals were numbered monthly according to the slaughtering order. *2The places (prefectures) and farms, where the animals were fed, were shown in Arabic numerals or alphabetic orders.

slaughtered almost consecutively (Figure 1). It is likely that the super shedders can have a disproportionate effect on the animal's hide and subsequent carcass contamination while low-shedding animals are also linked to the contamination on beef carcass [16]. Minimizing or eliminating the super shedding animals would thus contribute, at least in part, to the reduction of contamination with O157 STEC on beef carcasses.

As a molecular classification tool, we used PFGE to genetically discriminate representative STEC O157 isolates.