Transducer-Like Protein in Campylobacter jejuni With a Role in Mediating Chemotaxis to Iron and Phosphate

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Chemotaxis-mediated motility enables Campylobacter jejuni to navigate through complex environmental gradients and colonize diverse niches. C. jejuni is known to possess several methyl accepting chemotaxis proteins (MCPs), also called transducer-like proteins (Tlps). While the role of some of the Tlps in chemotaxis has been identified, their regulation and role in virulence is still not very clear. Here, we investigated the contribution of Tlp2 to C. jejuni chemotaxis, stress survival and colonization of the chicken gastrointestinal tract. The Δtlp2 deletion mutant showed decreased chemotaxis toward aspartate, pyruvate, inorganic phosphate (Pi), and iron (FeSO4). Transcriptional analysis of tlp2 with a promoter fusion reporter assay revealed that the tlp2 promoter (Ptlp2) was induced by Pi and iron, both in the ferrous (Fe2+) and ferric form (Fe3+). RT-PCR analysis using overlapping primers indicated that the phoX gene, located immediately downstream of tlp2, is co-transcribed with tlp2. A transcription start site was identified at 53 bp upstream of the tlp2 start codon. The Δtlp2 mutant showed decreased colonization of the chicken gastrointestinal tract. Collectively, our findings revealed that the tlp2 plays a role in C. jejuni pathogenesis and colonization in the chicken host and its expression is regulated by iron.

Keywords: transducer like protein, chemotaxis, iron, regulation, promoter

INTRODUCTION

Foodborne gastrointestinal illness caused by a gram negative bacterium, Campylobacter jejuni, has seen a surge in incidence in the recent years (CDC, 2013). In the United States, Food and Drug Administration (FDA) has placed Campylobacter species in the list of “qualifying pathogens” capable of posing a serious public health risk (Food and Drug Administration and HHS, 2014). The prevalence and transmission of Campylobacter can be attributed to its widespread colonization in the gastrointestinal tract of farm animals, especially chickens (Hermans et al., 2012). It is well established that C. jejuni employs motility and chemotaxis to colonize the avian and mammalian gastrointestinal tract (Yao et al., 1994; Hendrixson and DiRita, 2004; Young et al., 2007; Hermans et al., 2011; Chandrashekhar et al., 2015, 2017). Directional motility in C. jejuni is mediated by
the chemotaxis system, composed of chemoreceptors and other core signal transduction proteins (Lertsethtakarn et al., 2011).

Transducer like proteins (TlpS) are the key components involved in sensing environmental signals through chemotaxis or energy taxis in C. jejuni. (Marchant et al., 2002; Vegge et al., 2009; Korolik, 2010; Tareen et al., 2010; Reuter and van Vliet, 2013; Rahman et al., 2014). Amino acids (aspartate, glutamate and serine), organic acid salts (succinate, isocitrate, and formate), bile and mucin are chemoattractants for C. jejuni (Hugdahl et al., 1988; Hartley-Tassell et al., 2010; Tareen et al., 2010). C. jejuni TlpS have been classified into three groups (A-C), based on sequence analysis and structural homology (Marchant et al., 2002; Chandrashekhar et al., 2017). The C. jejuni Tlp2 (CJJ81176_0180) is a group A transducer-like protein (Marchant et al., 2002) with transmembrane domains, a periplasmic ligand binding domain and a cytoplasmic signaling domain. BLAST analysis of the predicted amino acid sequence of Tlp2 shows greatest homology to C. jejuni Tlp3 and Tlp4 (60% identity). The cytoplasmic signaling domain is identical to Tlp3 but the periplasmic domain shows only 38% identity with Tlp3 (Rahman et al., 2014). An earlier study in C. jejuni NCTC11168 strain revealed that tlp2 deletion mutant exhibited no chemotaxis and invasion defects (Vegge et al., 2009). However, recent evidence indicates that tlp2 is one of the most abundantly expressed tlpS in mice infected with C. jejuni NCTC 11168-O (Day et al., 2012), thus emphasizing the significance of understanding the role of Tlp2 in C. jejuni pathophysiology. This warranted us to further investigate the role of C. jejuni Tlp2 in chemotaxis, virulence, and host colonization.

Iron is an essential nutrient and a cofactor for proteins involved in cellular metabolism, enzyme catalysis, and sensing extracellular and intracellular signals (Lill, 2009). The bioavailability of iron in the host and environment (10^{-18} – 10^{-24}M) being lower than the minimum requirement for bacterial growth (10^{-17}M), makes iron a key player in the host-pathogen interaction (Braun and Hantke, 2003). Chemotaxis toward iron has been studied in Shewanella oneidensis and the magnetotactic bacteria Geobacter metallireducens (Childers et al., 2002; Bencharit and Ward, 2005). In these bacteria, the chemotactic response to iron is due to the fact that it serves as an insoluble electron acceptor (Childers et al., 2002; Bencharit and Ward, 2005; Harris et al., 2010). Knowledge about the role of iron as an electron acceptor in C. jejuni, chemotaxis toward iron and/or regulation of Tlp genes by iron in C. jejuni is still scarce. However, a study in Helicobacter pylori indicated that tlpB, chemoreceptor for sensing bicarbonate and arginine, is induced by iron through a fur-independent mechanism (Ernst et al., 2005). Interestingly, a recent study in C. jejuni has identified that tlp genes (GJ0262c and GJ1110c) are regulated by iron and/or Ferric uptake regulator (Fur) protein (Butcher et al., 2012). The study also revealed that cj0145 (phoX), a gene located immediately downstream of tlp2, is induced in the presence of iron although the specific mechanism of regulation is still unexplored (Butcher et al., 2012).

Here we investigated the role tlp2 in C. jejuni chemotaxis, in-vitro virulence and colonization of the chicken gastrointestinal tract. We provide evidence that iron regulates chemotaxis in C. jejuni and tlp2 contributes to in-vivo colonization of the chicken gastrointestinal tract. The findings of this study not only highlight the significance of tlp2 in C. jejuni pathogenesis but also elaborate on the complex mechanism by which iron regulates the chemotaxis in C. jejuni through Tlp2.

**MATERIALS AND METHODS**

**Bacterial Strains, Media and Growth Conditions**

Bacterial strains and plasmids used in this study are described in Table 1. C. jejuni strains were grown on Mueller-Hinton media (MH; Oxoid, Hampshire, United Kingdom) under microaerophilic conditions (85% N2 (v/v), 10% CO2 (v/v) and 5% O2 (v/v)) in a DG250 Microaerophilic Workstation (Microbiology International, Frederick, Maryland, United States) at 42°C. E. coli DH5α was used for plasmid propagation and cloning purposes and was routinely cultured on Luria-Bertani (LB) medium at 37°C overnight. Growth media was supplemented with appropriate antibiotics; chloramphenicol (10 µg/ml for Campylobacter; 20 µg/ml for E. coli) and kanamycin (30 µg/ml for Campylobacter; 50 µg/ml for E. coli) as required.

**Generation and Complementation of tlp2 Mutant**

Recombinant DNA techniques were performed as per standard procedures (Sambrook et al., 1989). C. jejuni tlp2 mutant was created by double crossover allelic exchange method as previously described (Rajashekara et al., 2009). Oligonucleotides used in the present study were synthesized from Integrated DNA Technologies (Skokie, IL, United States) and are listed in Table 2. Briefly, the gene of interest (tlp2) plus ~ 1 kb flanking DNA was amplified by PCR from C. jejuni strain 81–176 genome. The purified PCR products were ligated into zeocin-resistant pZErO-1 (zero background cloning vector) (Invitrogen, Carlsbad, CA, United States), and the ligation product was transformed into Library Efficiency DH5α E. coli competent cells (Invitrogen) to generate the plasmid pZErO1-tp2. The whole plasmid except the target gene was amplified by inverse PCR. Purified inverse PCR products were ligated either to a kanamycin resistant cassette (from pUC4K) or a chloramphenicol resistance cassette (from pUC4C), and the resulting suicide vector was electroporated into C. jejuni. Transformants were selected on MH agar supplemented with chloramphenicol or kanamycin. Individual clones were confirmed for deletion of the target gene by PCR. The tlp2 mutant with kanamycin resistance was used in all the assays; except for reporter studies, in which case tlp2 mutant with chloramphenicol resistance was used as reporter plasmid carries kanamycin resistance.

The complemented strain was created by amplifying coding regions of tlp2 along with its potential promoter region by PCR using primers indicated in Table 2. The resulting fragment was cloned into SalI-KpnI digested pRY112 (Yao et al., 1993) and the complementation plasmid was introduced into the

**Table 1.** Bacterial strains and plasmids used in this study are derivatives of strain 81–176 (WT) (Korlath et al., 1985) and NCTC 11168. C. jejuni strains were grown on Mueller-Hinton media (MH; Oxoid, Hampshire, United Kingdom) under microaerophilic conditions [(85% N2 (v/v), 10% CO2 (v/v) and 5% O2 (v/v))] in a DG250 Microaerophilic Workstation (Microbiology International, Frederick, Maryland, United States) at 42°C. E. coli DH5α was used for plasmid propagation and cloning purposes and was routinely cultured on Luria-Bertani (LB) medium at 37°C overnight. Growth media was supplemented with appropriate antibiotics; chloramphenicol (10 µg/ml for Campylobacter; 20 µg/ml for E. coli) and kanamycin (30 µg/ml for Campylobacter; 50 µg/ml for E. coli) as required.

**Table 2.** Bacterial strains and plasmids used in this study are derivatives of strain 81–176 (WT) (Korlath et al., 1985) and NCTC 11168. C. jejuni strains were grown on Mueller-Hinton media (MH; Oxoid, Hampshire, United Kingdom) under microaerophilic conditions [(85% N2 (v/v), 10% CO2 (v/v) and 5% O2 (v/v))] in a DG250 Microaerophilic Workstation (Microbiology International, Frederick, Maryland, United States) at 42°C. E. coli DH5α was used for plasmid propagation and cloning purposes and was routinely cultured on Luria-Bertani (LB) medium at 37°C overnight. Growth media was supplemented with appropriate antibiotics; chloramphenicol (10 µg/ml for Campylobacter; 20 µg/ml for E. coli) and kanamycin (30 µg/ml for Campylobacter; 50 µg/ml for E. coli) as required.


### TABLE 1 | Bacterial strains and plasmids used in this study.

| Strains | Relevant description | Source/Reference |
|---------|----------------------|------------------|
| C. jejuni 81–176 WT | Wild type strain of C. jejuni | Dr. Qijing Zhang |
| Δtlp2 | C. jejuni 81–176 derivative with deletion in tlp2 gene; tlp2::kan | This study |
| Δtlp2-cm | C. jejuni 81–176 derivative with deletion in tlp2 gene; tlp2::cm | This study |
| tlp2 comp | C. jejuni 81–176 tlp2 mutant complemented with wild type copy of tlp2 on pRY112 | This study |
| C. jejuni NCTC1168Δfur | C. jejuni NCTC1168 derivative with deletion in fur gene; fur::tst | Dr. Jun Lin |
| WT Ptlp2-pMW10 | C. jejuni 81–176 WT reporter strain carrying Ptlp2-pMW10 | This study |
| Δfur Ptlp2-pMW10 | C. jejuni 81–176 Δfur mutant carrying Ptlp2-pMW10 | This study |
| WT Ptlp2-pMW10 | C. jejuni 81–176 WT reporter strain carrying Ptlp2::phoX-pMW10 | This study |
| E. coli DH5α | E. coli strain used for cloning | Invitrogen |

### Plasmids

| Plasmids | |
|----------|----------------------|
| pZero1-1 | Cloning vector for making suicide vector; Zeo |
| pUC4K | Source plasmid for kanamycin resistance gene; Kan |
| pUC4C | Source plasmid for chloramphenicol resistance gene; Cm |
| pMW10 | Promoter shuttle vector; pMW10, Kan |
| pRY112 | E.coli-Campylobacter shuttle vector for complementation; Cm |
| pRK2013 | Helper plasmid for complementation; Kan |
| pZero1-1-kan | pZero1-1 containing the upstream and downstream sequences of tlp2; Zeo |
| pZero1-Δtlp2-kan | pZero1-tp2 with tlp2 gene replaced by the pUC4K kan gene through inverse PCR; Zeo, Kan |
| pZero1-Δtlp2-cm | pZero1-tp2 where kan was replaced by the chloramphenicol gene; Zeo, Cm |
| Ptlp2-pMW10 | pMW10 carrying the tlp2 promoter; Kan |
| Ptlp2::phoX-pMW10 | pMW10 carrying the intergenic region between tlp2 and phoX; Kan |

Cm, chloramphenicol resistance; Kan, kanamycin resistance; Zeo, Zeocin resistance.

Δtlp2 deletion mutant by biparental conjugation as described (Miller et al., 2000). Transconjugants were selected on MH agar supplemented with kanamycin and chloramphenicol and the resulting complementation strain was designated tlp2 comp as listed in Table 1.

### Chemotaxis Assay

To quantify chemotaxis, we adapted a modified capillary chemotaxis assay that quantitatively measures bacterial tactic responses (Mazumder et al., 1999; Cerda et al., 2003). The assay was previously used for quantifying chemotaxis in subsurface microaerophilic bacteria including *Campylobacter* (Mazumder et al., 1999; Chandrashekar et al., 2015) and other *Epsilonproteobacteria*, such as *H. pylori* (Cerda et al., 2003, 2011). Briefly, *C. jejuni* wild type (WT), Δtlp2 mutant and the complemented strains were grown microaerobically at 42°C for 18 h on MH agar and resuspended in chemotaxis buffer (Phosphate Buffered Saline, PBS or Normal Saline, pH 7.4) and OD$_{600}$ was adjusted to 0.5. A 100 μl volume of a solution of the compounds [All compounds at 0.1M except Pi (Inorganic Ventures, Christiansburg, VA, United States) at 1 mM and FeSO$_4$ (Sigma) at 0.1 mM] to be tested for chemotaxis response (buffer alone served as control) was aspirated through a 22 G stainless-steel needle (0.254 mm diameter × 20 mm long) into a 1 ml tuberculin syringe. The 0.1 M concentration of the compounds was selected based on previous studies and a series of preliminary experiments that showed that 100 mM resulted in the strongest chemotaxis response (Vegge et al., 2009; Tareen et al., 2010). A 100 μl of the OD$_{600}$ adjusted bacterial suspension was drawn into a 200 μl disposable pipette tip and the needle-syringe system was fitted to the pipette tip in such a way that the needle was immersed into the bacterial suspension. The system was positioned horizontally and incubated at 42°C for 1 h. The needle-syringe system was then separated from the bacterial suspension containing pipette tip and contents of the syringe were 10-fold serially diluted in chemotaxis buffer, plated onto MH agar plates and incubated at 42°C under microaerophilic conditions to determine colony-forming units (CFUs). Relative Chemotaxis Ratio (RCR) toward a test compound was ascertained as a ratio between the numbers of bacteria entering the test needle-syringes to those in the control needle-syringes. A test compound was considered as an attractant if the RCR was ≥ 2 (Mazumder et al., 1999). Results were expressed as the mean of three independent assays. A mutant was considered deficient in chemotaxis toward a substrate if both the corresponding RCR value was significantly < 2 (P < 0.05) and the CFU of the mutants were significantly lower (P < 0.05) than those of the wildtype. A *C. jejuni* 81–176 cheY mutant which is incapable of directional movement (negative control) (Yao et al., 1997) and 0.1% porcine gastric mucin (positive control; Sigma) were also used to evaluate the integrity of the assay. To test the response to repellents, *C. jejuni* cultures were mixed with a repellent and the bacteria that entered the syringe, which in this instance contained only buffer, to escape the repellent were quantified as described above. To account for any methodological bias, capillary chemotaxis results were further verified by using the disk method (Vegge et al., 2009) for selected compounds.
TABLE 2 | Oligonucleotide primers used in this study.

| Name                              | Sequence                                |
|-----------------------------------|-----------------------------------------|
| Primers for gene deletion         |                                         |
| tlp 2 F                           | ATATATGTAACCTGTGACTAGTATTTTGTTTC        |
| tlp 2 R                           | AATTAATCGGCACTAACCCTTGTGGTACATAAAGC     |
| tlp 2 F inv                       | ATATATGTAACCTGTGACTAGTATTTTGTTTC       |
| tlp 2 R inv                       | AATTAATCGGCACTAACCCTTGTGGTACATAAAGC     |
| Complementation primers           |                                         |
| tlp 2 comp F                      | AATTAATCGGCACTAACCCTTGTGGTACATAAAGC    |
| tlp 2 comp R                      | AATTAATCGGCACTAACCCTTGTGGTACATAAAGC    |
| Primers for promoter fusion studies and primer extension assay | |
| tlp2_PF_F                         | ACA TTG ACA TCC CGG GTA TTT GCA GC      |
| tlp2_PF_R                         | AAT CAG TGA GAT CTT CAA TAT TAC GC     |
| Cjj81-180_PE_temp_F               | GGG GCC AAA ATA ACA TTG ACA TCT AGA G   |
| Cjj81-180_PE_temp_R               | GCA TCT TGA CTA TCT AAT GCT GTC GTA AGA |
| Cjj81-180_PE_R1                   | ACC TAA AAT TAT CAA ACA CAC TAC TGC G  |
| Cjj81-180_PE_R2                   | TAA TTT ATT TCA GCA TTC ACA ACT TCA TG |
| Cjj81-181_PE_temp_F               | AAA CTG CAG GTA TCA CTC AAA TCA ATG    |
| Cjj81-181_PE_temp_R               | ACC TAG CAA ATT TCA TCT ATC CTT AAG C  |
| Cjj81-181_PE_R1                   | TTT GTA CAA AAA AAG CCA CCA TAG AAC C  |
| Cjj81-181_PE_R2                   | TTT GTA CAA AAA AAG CCA CCA TAG AAC C  |

Determination of the tlp2 Transcriptional Levels With Reporter Gene Assays

The partial coding region of tlp2 and the upstream region was amplified with tlp2-PF_F (SmaI) and tlp2-PF_R (BglIII) primers and cloned into pMW10, a shuttle vector for E. coli and C. jejuni, containing a promoterless lacZ gene (Wosten et al., 1998). The plasmid was mobilized into C. jejuni WT and Δtlp2::Cm strains by electroporation. The Δtlp2::Cm strain was used for reporter studies since pMW10 carries kanamycin resistance. The Δtlp2::Cm was generated as described above. β-Galactosidase assay was performed with C. jejuni strains harboring the tlp2 promoter (Ptlp2)-lacZ transcriptional fusion construct, as described previously (Wosten et al., 1998). To examine the effect of Pi and iron on tlp2 transcription, reporter strains were incubated in MOPS and MEMs (Life technologies, Invitrogen) supplemented with Pi and FeSO4 or FeCl3 (Iron), respectively.

Additionally, reporter fusions were also created for the intergenic region between tlp2 (Cjj81176_0180) and phoX (Cjj81176_181) to determine any potential promoter in the intergenic region. The intergenic region was amplified with specific primers listed in Table 2 and cloned into pMW10 using the BamHI-XbaI sites. Reporter gene assays were carried out as described above.

Reporter gene assays were also carried out in the C. jejuni 81–176 Δfur mutant. C. jejuni 81–176 Δfur mutant was created by natural transformation of WT containing the (Ptlp2)-lacZ transcriptional fusion construct with genomic DNA from C. jejuni NCTC11168 Δfur mutant as described previously (Jeon et al., 2008; Gangiah et al., 2009). Briefly, 1 ml of C. jejuni WT reporter strain was resuspended to an OD600 of 0.5. Approximately, 5 µg of genomic DNA from C. jejuni NCTC11168 Δfur mutant was added and incubated for 4 h microaerobically. The bacteria were plated on MH plates supplemented with appropriate antibiotics and incubated microaerobically at 42°C for 48 h. The deletion of the fur gene in 81–176 was confirmed by PCR.

RNA Extraction and Reverse Transcriptase Overlapping PCR

Briefly, C. jejuni WT grown overnight in MH agar plate was scraped and resuspended to an OD600 of 0.05 in MEM-α or MH broth and grown up to mid log phase (6 h), respectively. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and quantified using NanoDrop ND-2000c spectrophotometer (Life technologies, Invitrogen) and NanoDrop ND-2000c spectrophotometer (Wilmington, DE, United States). cDNA synthesized using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen), was used as a template for PCR with a set of overlapping primers for the tlp2, phoX, and the 135 base pairs intergenic region between tlp2 and phoX. (Table 2).

Primer Extension Assay

Primer extension assay was performed as described previously (Kim et al., 2011). Briefly, C. jejuni WT strain was grown for 6 h (mid-log phase) with shaking in MH broth at 42°C and harvested by centrifugation at 10,000 × g for 5 min. Total RNA was purified with TRIzol (Invitrogen) according to the manufacturer’s instructions. Purified RNA was resuspended in sterile distilled RNase-free water, and the RNA concentration was determined by measuring the OD of the solution at 260 and 280 nm using NanoVue (GE Healthcare). A portion (10 pmol) of the PE_R primer was labeled with 32P at the 5’ end by 10 U
of T4 polynucleotide kinase (Invitrogen) and 80 µCi of \( [\gamma^{32}\text{P}] \) dATP for 30 min at 37°C. The labeling mixture was heated at 70°C for 10 min and purified with MicroSpin G-25 columns (GE Healthcare). The \( \gamma^{32}\text{P} \)-end-labeled primer (0.5 pmol) was co-precipitated with 15 µg of total RNA by the addition of sodium acetate and absolute ethanol. The pellet was washed with 75% ethanol, dried at room temperature, and resuspended in 20 µl of 250 mM KCl, 2 mM Tris (pH 7.9), and 0.2 mM EDTA. The mixture was heated to 65°C and then was allowed to cool to room temperature for 1 h. After annealing, 50 µl of reaction solution containing 5 µg of actinomycin D, 700 µM deoxynucleoside triphosphates, 10 mM MgCl₂, 5 mM DTT, 20 mM Tris (pH 7.6), 30 U of RNasin (Promega), and 150 U of Superscript® III reverse transcriptase (Invitrogen) was added. The mixture was incubated at 42°C for 70 min and treated with 15 µl of absolute ethanol and then washed with 75% ethanol. Sample was resuspended with 6 µl of formamide dye and 4 µl of Tris-EDTA (pH 8.0) buffer and then denatured at 90°C for 3 min. The samples were resolved on 6% polyacrylamide-8M urea gels, and the reverse transcription signals were analyzed by using BAS 2500 (Fuji Film). Primers, CJ81176_180_PE and CJ81176_181_PE (Table 2) were used for sequencing the upstream regions of tlp2 and phoX, for transcription start site with a SequiTherm EXCELII DNA sequencing system (Epicenter).

Alkaline Phosphatase Assay

PhoX activity was determined as described previously (Drozd et al., 2011). Briefly, WT, \( \Delta\text{tlp2} \), and the \( \text{tlp2 comp} \) strains were grown overnight on MH plates with appropriate antibiotics. The cultures were gently scraped, washed and resuspended in MEM and incubated at 42°C microaerobically with shaking for 2 h. Cultures were then centrifuged for 10 min at 7,000 × g and supernatant was removed. Cells were gently washed with 50 mM MOPS buffer (pH 7.4) (Sigma) and incubated with shaking at 42°C for 2 h following which, OD₆₀₀ readings were taken. Cells were pelleted and resuspended in PNPP buffer containing 2 mM p-nitrophenyl phosphate (PNPP; Sigma) and incubated at 37°C. OD measurements at 550 nm and 420 nm were taken, and the phosphatase activity was calculated as described previously (Wosten et al., 2006). The assay was performed a total of three times with duplicate samples in each assay. Additionally, effect of iron on PhoX activity was assessed by supplementation of FeSO₄ at 40 µM concentration in MOPS buffer.

Nutrient Downshift Assay

The role of tlp2 in \( C. \text{jejuni} \) survival under nutrient downshift was assessed using MEM-\( \alpha \) as described previously (Gangaiah et al., 2010). Briefly, mid-log-phase cultures of WT, \( \Delta\text{tlp2} \), and the \( \text{tlp2 comp} \) strains were pelleted, washed twice and resuspended in MEM-\( \alpha \) with OD₆₀₀ adjusted to 0.05. The bacterial suspensions were incubated microaerobically at 42°C with shaking. Samples were taken over time, serially diluted (10-fold) in MEM-\( \alpha \) media and plated on MH agar for determining CFU. The experiment was performed three times and the average for each time point was taken.

Quantitative Reverse Transcriptase PCR (qRT-PCR) Analysis of Phosphate Uptake Genes

The \( C. \text{jejuni} \) WT and \( \Delta\text{tlp2} \) cultures were assessed for changes in expression of phosphate uptake genes (\( \text{phosR, pstC, and pstS} \)) (Wosten et al., 2006). Briefly, \( C. \text{jejuni} \) WT and \( \Delta\text{tlp2} \) strains were grown to mid-log phase in MEM-\( \alpha \) microaerobically, with shaking at 37°C. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and cDNA was synthesized using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). RNA and cDNA concentrations and purity were determined using NanoDrop ND-2000c spectrophotometer (Wilmington, DE, United States). Quantitative RT-PCR was performed with a SensiMixPlus SYBR RT-PCR kit (Quantace, Norwood, MA, United States) in a Mastercycler ep realplex2 thermal cycler (Eppendorf, Westbury, NY, United States). Gene specific primers (Table 2) used in this analysis have been described previously (Drozd et al., 2014). The relative levels of expression of target genes were normalized to 16S rRNA gene expression of the same strain. The relative fold changes in gene expression was calculated using the comparative threshold cycle (CT) method to yield fold-difference in transcript level compared to WT (Livak and Schmittgen, 2001). The qRT-PCR was performed a total of three times with duplicate samples in each assay.

Invasion and Intracellular Survival Assays

Invasion and intracellular survival of \( C. \text{jejuni} \) WT and \( \Delta\text{tlp2} \) mutant in INT 407 cell line (human embryonic intestine cells, ATCC CCL 6) was assessed as described previously (Kassem et al., 2012). Briefly, mid-log phase grown bacterial cells were collected by centrifugation (5,000 × g, 10 min), washed twice with MEM containing 1% (v/v) FBS and resuspended in MEM. INT 407 cells (1.4 × 10⁵ per well) in MEM with 10% (v/v) fetal bovine serum (FBS) were seeded in 24-well tissue culture plate and incubated for 18 h at 37°C with 5% CO₂. INT 407 cells were infected with multiplicity of infection (MOI) 100 for invasion and intracellular survival assays and incubated for 3 h at 37°C. Following 3 h of incubation with bacteria; cells were treated with gentamicin (150 µg/ml) and incubated for additional 2 h. After 2 h of incubation, the infected cells were rinsed three times with MEM, lysed with 0.1% (v/v) Triton-X 100, serially diluted in MEM and plated on MH agar. The percent invasion was calculated as follows: (no. of CFU recovered after lysis of INT 407 cells/CFU added to each well) × 100.

To assess survival of \( C. \text{jejuni} \) WT and \( \Delta\text{tlp2} \) mutant in INT 407 cell line, following 2 h of gentamicin treatment, the infected cells were washed with MEM three times and covered with MEM containing gentamicin (10 µg/ml) and incubated for 24 h at 37°C. After 24 h of incubation, infected cells were washed with MEM, lysed and plated as described above. In parallel, we also cultured the supernatant of gentamicin treated monolayers to ensure the quality of the gentamicin protection assay.
**Chicken Colonization Assay**

Chicken colonization study was performed as described previously (Gangaiah et al., 2009). Briefly, 3 day-old specific pathogen free chickens (n = 6 for each group) were obtained from a local hatching facility (Food Animal Health Research Program, OARDC, Wooster, OH, United States). *Campylobacter* free chickens were inoculated orally with 10⁴ CFU of the *C. jejuni* WT and Δtlp2 mutant strain in 200 µl of PBS (pH 7.4). Chickens were euthanized after 7 days post-inoculation and ceca, duodenum, jejunum, liver, spleen and bursa were collected aseptically, weighed, homogenized, serially diluted in PBS (pH 7.4) and plated on appropriate MH agar containing *Campylobacter* selective supplement with or without kanamycin to determine colony forming units (CFU). Plates were incubated at 42°C microaerobically and CFUs per gram of tissues were determined.

**Statistical Analysis**

Statistical significance of data generated in this study was determined using two tailed Student’s t-test. Results of the promoter fusion assay were statistically analyzed using one way Anova with Dunnett’s multiple comparison posttests. Data from the chicken colonization experiment was analyzed using the Mann Whitney test. P ≤ 0.01 or 0.05 (α level) was considered statistically significant.

**RESULTS**

**The Δtlp2 Mutant Is Defective in Chemotaxis Toward Aspartate, Pyruvate, Pi and Iron**

To assess the role of tlp2 in *C. jejuni* chemotaxis, a deletion mutant was constructed with the coding region of tlp2 being replaced with kanamycin resistance gene. Syringe capillary chemotaxis assays were performed to determine the chemotactic activity of *C. jejuni* WT, the Δtlp2 mutant and the complemented strains toward different substrates (Table 3). Substrates with RCR values > 2 and < 0.1 were considered as chemo attractants and repellants, respectively, for WT *C. jejuni* (Hugdahl et al., 1988; Cerda et al., 2003; Chandrashekhar et al., 2015). In addition, capillary assay showed strong chemotaxis of *C. jejuni* toward 0.1% porcine gastric mucin (RCR = 9.0), while a non-motile cheY mutant had an RCR below the detection limit (~0) for some of the known attractants (Chandrashekhar et al., 2015). Compared to the WT, the Δtlp2 mutant was defective in chemotaxis toward aspartate (P = 0.0292) and pyruvate (P = 0.0010) with RCR values < 2 (Figure 1) (RCR values: aspartate: 3.81 for the WT and 1.45 for the Δtlp2 mutant; pyruvate: 2.96 for the WT and 0.33 for the Δtlp2 mutant) (Cerda et al., 2003). Even though the tlp2 mutant showed RCR values less than 2 for isocitrate, succinate and propionate; they were not statistically significant (Table 3). Interestingly, Δtlp2 mutant also showed a chemotaxis defect toward Pi and iron (FeSO₄), compared to WT (Figure 1). We observed that FeSO₄, but FeCl₃·6H₂O (ferric iron source) and (NH₄)₂SO₄ (sulfate source) were not a chemoattractant for *C. jejuni* 81–176 and were therefore not tested (NT) for chemotaxis with the mutant. The RCR of these compounds are lesser than 2 for the tlp2 mutant, however, not significant statistically P > 0.05.

**TABLE 3** | RCR values for the WT and Δtlp2 mutant for all compounds tested.

| Chemicals tested | WT  | Δtlp2 |
|------------------|-----|-------|
| Aspartate        | 3.81 ± 0.32 | 1.45 ± 0.35 |
| L-glutamine      | 4.19 ± 0.77  | 2.93 ± 0.38  |
| L-serine         | 2.04 ± 0.46  | 1.99 ± 0.82  |
| Fumarate         | 9.32 ± 1.45  | 2.52 ± 0.79  |
| Isocitrate       | 2.51 ± 0.41  | 1.5 ± 0.66   |
| Formate          | 4.41 ± 0.13  | 2.36 ± 1.05  |
| Succinate        | 3.73 ± 1.2   | 1.2 ± 0.70   |
| Pyruvate         | 2.96 ± 0.566 | 0.33 ± 0.15  |
| Propionate       | 2.97 ± 1.51  | 1.35 ± 0.30  |
| Inorganic phosphate | 2.15 ± 0.46  | 0.4 ± 0.15   |
| Deoxycholic acid | <0.1         | <0.1         |
| Cholic acid      | <0.1         | <0.1         |
| FeSO₄            | 3.40 ± 0.58  | 0.66 ± 0.10  |
| FeCl₃·6H₂O        | 1.4 ± 0.35   | NT           |
| (NH₄)₂SO₄        | 0.1          | NT           |

The results show the means and standard errors of three independent experiments. An RCR value of 2 or above indicates chemotaxis toward the test chemical (Mazumder et al., 1999). The RCR value of the WT strain was <2.0, hence the compounds were not chemoattractants for *C. jejuni* 81–176 and were therefore not tested (NT) for chemotaxis with the mutant. The RCR of these compounds are lesser than 2 for the tlp2 mutant, however, not significant statistically P > 0.05.
using the disk method for selected substrates such as aspartate and pyruvate (data not shown).

**Iron Induces tlp2 Promoter (P_{tlp2}) Activity**

Decreased chemotaxis toward iron observed in the Δtlp2 mutant encouraged us to investigate the tlp2 expression under different growth conditions. The level of tlp2 transcription was quantified with β-galactosidase assays in presence of metal ions, such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} in MEM-α that does not contain these metals (van Vliet et al., 1998; Kim et al., 2011). Assay was performed in the presence of 20 μM CuCl₂, 40 μM FeSO₄, 40 μM FeCl₃, 40 μM MnCl₂, and 10 μM ZnCl₂. Since MEM-α media already has Ca^{2+} (1.8 mM) and Mg^{2+} (0.8 mM), we did not supplement the media with these two metal ions. Iron in both ferrous (FeSO₄) and ferric (FeCl₃) forms induced tlp2 expression at 40 μM concentrations (Figure 2A), whereas other metals had no effect on the level of tlp2 transcription (Supplementary Figure S1A). For Fe^{2+}, 40 μM was used based on the dose response assay (Supplementary Figure S1B) which showed best result at this concentration (Supplementary Figure S1B). Concentrations of iron as low as 5 μM FeSO₄ also significantly induced tlp2 expression (Supplementary Figure S1B).

Similarly, the activity of P_{tlp2} was investigated in the presence of Pi due to the observed chemotaxis defect toward Pi (Table 3 and Figure 1). MOPS buffer was used as a low phosphate medium for the incubation of the C. jejuni reporter strains (Gangaiah et al., 2009). The concentration of Pi added to MOPS

**FIGURE 2** | β-galactosidase activity of Campylobacter jejuni WT carrying P_{tlp2}-lacZ transcriptional fusion construct. (A) β-galactosidase activity in the absence (uninduced) and presence of 40 μM FeSO₄ or FeCl₃(Η₂Ο)₆ (induced) added to MEM-α. (B) β-galactosidase activity in the absence (uninduced) and presence of 2mM Pi (induced) added to MOPS. (C) β-galactosidase activity of the P_{tlp2}-lacZ fusion assays in the Δtlp2::Cm mutant in the presence or absence of 40 μM FeSO₄ in MEM-α and in the presence or absence of 2mM Pi in MOPS. (D) β-galactosidase activity of the P_{tlp2}-lacZ fusion in the Δfur mutant in MEM-α. The cells were incubated for 8 h before carrying out the assay. The results show the means and standard deviations of three independent experiments. *P < 0.05 where each group is compared with the WT reporter strain that is not induced (MEM-α or MOPS) and **P < 0.05 where each group is compared with the WT that is induced (with FeSO₄ or Pi).
buffer ranged from 1 to 3 mM but P_{tlp2} was most significantly induced in the presence of 2 and 3 mM of Pi (Figure 2B and Supplementary Figure S1C). Further, P_{tlp2} activity in the tlp2 deletion mutant was also studied to assess the effect of the gene product on its promoter activity. Since pMW10 shuttle vector has a kanamycin resistant cassette, we created a Δtlp2 mutant with a chloramphenicol resistant cassette. We found that the tlp2 expression was also induced in the Δtlp2 mutant in the presence of Pi and FeSO₄ similar to WT (Figure 2C). Even though the P_{tlp2} activity in Δtlp2 mutant was higher than the WT both in the presence or absence of Pi and Fe, the difference was not statistically significant. These results suggest that tlp2 transcription is independent of Tlp2 protein levels in the cell.

Ferric uptake regulator protein (Fur) plays an important role in C. jejuni iron homeostasis (van Vliet et al., 1998). In addition, iron and Fur are shown to regulate tlp genes (Cj0262c; Tlp4 and Cj1110c; Tlp8) in C. jejuni (Butcher et al., 2012). We, therefore, investigated if tlp2 expression is regulated by Fur. Interestingly, the P_{tlp2} activity was increased in a Δfur mutant of C. jejuni 81–176 in MEM-α (Figure 2D). These observations revealed a role for fur in the regulation of tlp2 expression. Since a fur mutation derepresses genes involved in iron acquisition in C. jejuni (Holmes et al., 2005), there will be over-accumulation of iron in the fur mutant. The increased levels of intracellular levels may increase the P_{tlp2} activity in the fur mutant.

The tlp and phoX Genes Are Co-transcribed

The tlp2 gene (CJ81176.180) is located upstream to phoX (CJ81176.181) in the same orientation with an intergenic region of 135 base pairs (Figure 3A). Additionally, a previous study indicated that phoX gene (CJ0145), located immediately downstream of tlp2, is induced by iron and was also enriched in the CjFur ChIP-chip assay (Butcher et al., 2012). As the Δtlp2 mutant is defective in chemotaxis toward iron (Fe) and Pi, and the tlp2 transcription is modulated by iron and Pi, we hypothesized that these two genes may be co-transcribed. To test this, total RNA was extracted from WT grown in MEM-α and analyzed by RT-PCR using primers designed to amplify a flanking region of the two genes. The results indicated that tlp2 and phoX are co-transcribed (Figure 3B). In addition, an amplicon was also observed when WT was grown in nutrient-rich MH broth (data not shown). These results implied that phoX is co-transcribed with tlp2 under the conditions tested in this study.

Further, an intergenic region between tlp2 and phoX was fused to the promoterless lacZ gene to confirm that the promoter activity observed was specific to P_{tlp2}. The reporter strains did not show any promoter activity; the promoter activity in the β-galactosidase assay was similar to that of the negative control (empty plasmid) (Figure 3C). Similar findings were observed when media were supplemented with iron or Pi (data not shown), confirming that there was no promoter in the intergenic region between tlp2 and phoX under these tested conditions.

Furthermore, a primer extension analysis revealed a single transcription start site (TS) upstream to the tlp2 gene (Figures 3D, F). The TS is located 53 bp upstream of the tlp2 start codon with a ribosomal binding site located 12 bp upstream from the start codon. The -10 region was identified with the first T of the TATA box located 59 bp upstream of the start codon. Consistent with the results above, no transcription start site was observed in the 135 bp intergenic region between tlp2 and phoX (Figure 3E). This result indicates that tlp2 and phoX genes constitute an operon, and the transcription of phoX is dependent on the tlp2 promoter and they are co-transcribed.

Alkaline Phosphatase Activity Is Increased in the Presence of Iron

A study investigating the regulatory potential of Fur of C. jejuni identified that phoX is activated by iron (Butcher et al., 2012). Therefore, the PhoX activity of C. jejuni WT was evaluated in MEM-α supplemented with 40 μM FeSO₄. The PhoX activity of the WT strain increased approximately four-fold in the presence of iron (Figure 4). Similarly, a higher PhoX activity was also observed in the Δtlp2 mutant in MEM-α in the presence of iron similar to the WT (Figure 4); however, this increase was not significant (P ≥ 0.09). In the complemented strain, the PhoX activity was similar to the WT with or without iron (Figure 4). These results suggest that iron upregulates the PhoX activity in C. jejuni and potentially intersects the phosphate utilization pathway of C. jejuni.

Deletion of tlp2 Affected Nutrient Stress Survival

The effect of a tlp2 mutation on stress survival was monitored by comparing the growth of the Δtlp2 mutant strain to the WT C. jejuni in nutrient-limited conditions. The Δtlp2 mutant did not display any growth defect when grown in nutrient-rich MH broth (data not shown); however, the tlp2 mutant on transition from nutrient rich MH broth to nutrient deficient MEM (without glutamine) exhibited survival defects in the late stationary phase especially 36 h and onward. The survivability of the tlp2 mutant strain was decreased by one and more than two orders of magnitude at 36 and 60 h, respectively, as compared to the WT (P < 0.05) (Figure 5).

Deletion of tlp2 Affected Intracellular Survival in Intestinal Epithelial Cells

The consequence of tlp2 deletion on virulence-associated traits of C. jejuni was evaluated by the ability of Δtlp2 mutant to invade and survive within the human intestinal epithelial INT 407 cells (Candon et al., 2007). The Δtlp2 demonstrated similar invasion in INT 407 cells; however, the Δtlp2 mutant showed a higher intracellular survival, with almost 2 logs more bacteria recovered compared to the WT (Figures 6A,B).

The Δtlp2 Mutant Is Defective in Colonization of the Chicken Gastrointestinal Tract

To investigate the role of Tlp2 in colonization of C. jejuni, we investigated the colonization of Δtlp2 mutant and WT in different segments of the chicken intestine. The Δtlp2 mutant...
FIGURE 3 | (A) Genetic organization of tlp2. The tlp2 gene (cjj81176_180) is located upstream of the phoX gene (cjj81176_181) which encodes the alkaline phosphatase (PhoX) enzyme. The tlp2 and phoX genes are separated by a 135 bp intergenic region. (B) Reverse Transcriptase overlapping PCR showing co-transcription of tlp2 and phoX. Intergenic region was amplified with primer pair P1 F (forward) and R (reverse) in WT strain grown in MEM-α. Primers for the tlp2 (P2 F and R) and phoX (P3 F and R) genes were included as control regions as well (data not shown). (C) The P_{tlp2}-lacZ fusion showed no β-galactosidase activity compared to the P_{phoX}-lacZ fusion in the WT strain. Determination of the transcriptional start site for tlp2 (D) and phoX (E) by a primer extension assay. Only one transcriptional start site is seen upstream to tlp2, designated TS (Transcriptional Start) indicated with an arrowhead on the right and by the * in the sequence. No transcriptional start site was found in the region upstream to phoX (F). The −10 and −35 elements of the P_{tlp2} are underlined and the ribosomal binding site is indicated as RBS.
and WT were inoculated into 3-day old chicks orally ($10^4$ CFU/chicken), and bacterial burden was analyzed after 7 days of infection. Colonization of the C. jejuni WT strain in the chicken gastrointestinal tract (cecum and duodenum/jejunum) ranged from $4 \times 10^7$ to $2 \times 10^8$ CFU per gram of tissue; while in the Δtlp2 mutant varied from $2 \times 10^7$ to $8 \times 10^3$ CFU per gram of tissue. The Δtlp2 mutant showed a 4–5 logs decrease in cecal colonization compared to the WT (Figure 7A). The Δtlp2 mutant was not detected in the duodenum and colonization of the jejunum was also reduced by almost 4 logs (Figures 7B,C). However, the liver, spleen, and bursa showed no colonization by C. jejuni WT and Δtlp2 mutant. These findings suggest that the tlp2 is essential for achieving optimal colonization in the proximal and distal segments of the gastrointestinal tract, including the cecum.

**DISCUSSION**

In this study, we characterized the role of tlp2 in chemotaxis, stress survival, and colonization of the chicken gut. Our results indicated that tlp2 is involved in chemotaxis toward aspartate, pyruvate, Pi, and iron. Promoter fusion assays revealed that iron, in the ferrous and ferric form induces the tlp2 promoter activity. Iron is essential for C. jejuni colonization in the host as it is one of the limiting nutrients sequestered away from the pathogen by the host and the bioavailability of iron in the intestine is not very well understood (Naikare et al., 2006).

Predicted domain structure of the Tlp2 in the SMART database (Schultz et al., 1998) revealed a single periplasmic Cache_1 (Ca$^{2+}$ channels and chemotaxis receptors) domain (Anantharaman and Aravind, 2000) and a cytoplasmic MCP signaling domain. Cache domain is found in the extracellular or periplasmic portions of chemoreceptors from Gram-positive and Gram-negative bacteria, and is associated with sensing of small molecules (Anantharaman and Aravind, 2000). The Cache domains of Pseudomonas aeruginosa and Vibrio cholerae have been associated with chemotaxis toward amino acids (Nishiyama et al., 2012). The Cache domain is responsible for interaction with multiple ligands and thereby chemotaxis (Tasneem et al., 2005). Δtlp2 mutant shows decreased chemotaxis toward aspartate, pyruvate, iron and Pi. Additionally, Tlp2 shows 38% identity with the periplasmic region of the multiple ligand binding Tlp3 (Ccml) of C. jejuni, possessing a single cache domain which can potentially bind to multiple ligands with varying affinity (Rahman et al., 2014).

Studies in S. oneidensis and G. metallireducens report chemotaxis toward iron in the ferrous form (Childers et al., 2002; Bencharit and Ward, 2005). Iron is a redox active metal, and chemotaxis to iron suggests bacterial movement through reduced metal gradients toward potential electron acceptors (oxidized ferric form). Our observations in the WT (C. jejuni 81–176) strain show that it is chemotactic toward ferrous iron. The chemotactic response of C. jejuni toward iron (Fe$^{2+}$) can be explained as bacterial adaptation to the assimilatory requirement for iron, as it is an important constituent of iron sulfur proteins and other cellular processes (Bencharit and Ward, 2005). Comparably, the chemotactic response of H. pylori toward a metal ion (zinc) has been primarily attributed to the mechanism of nutrient acquisition by bacteria (Sanders et al., 2013).

A study in C. jejuni, employing CjFur ChIP-chip analysis, identified cj0145 (phoX) as a novel gene in the Fur regulon in C. jejuni, which is activated by iron (Butcher et al., 2012). Much in line with the study above, we found in our study that PhoX activity in the WT is significantly increased in the...
presence of iron. A recent study on the *Pseudomonas fluorescens* PhoX revealed that iron is a cofactor required for enzyme activity, additionally implying that the bioavailability of iron affects bacterial phosphate uptake (Yong et al., 2014). Although a similar mechanism for increased PhoX activity in *C. jejuni* in the presence of iron can be envisioned, a further biochemical investigation on *C. jejuni* PhoX is needed to identify the precise role of iron in its enzymatic activity.

The sensing, uptake and utilization of inorganic phosphate in prokaryotes enables their ability to withstand conditions of phosphate deprivation. The Pi sensing or taxis has been studied in bacterial pathogens such as *Enterococcus cloacae* and *P. aeruginosa* under conditions of phosphate starvation, with two chemotactic transducers identified for Pi taxis in *P. aeruginosa*. The Pho regulon and the phosphate uptake system regulate Pi taxis in both bacteria (Kusaka et al., 1997; Wu et al., 2000).
C. jejuni being an enteric pathogen is subjected to its survival under low phosphate conditions in the chicken gastrointestinal tract. While the uptake and utilization of Pi in C. jejuni through the two-component PhoS/PhoS-R operon has been previously described (Wosten et al., 2006), nothing is known about Pi taxis in this microaerophile. In our study, C. jejuni WT is chemotactic toward Pi, whereas the Δtlp2 mutant displayed a decreased chemotaxis. The decreased cellular availability of Pi in the tlp2 mutant was accompanied by an upregulation of the phosR (response regulator of Pho regulon) and the genes for phosphate uptake (pstC and pstS) which is normally induced in response to Pi limitation (Supplementary Figure S2) (Wosten et al., 2006). Additionally, the tlp2 mutant’s decreased survival under nutrient mediated stress (Figure 5) can be attributed to the Pi limiting conditions created due to decreased Pi taxis. Earlier studies have indicated that survival under low-nutrient stress is regulated by PPK1 mediated synthesis of poly-P from Pi (Candon et al., 2007; Gangaiah et al., 2009).

PhoX hydrolyzes phospho-organic compounds to Pi, a preferred phosphate source and a building block for poly-P in C. jejuni (Candon et al., 2007; Drozd et al., 2011). PhoX in C. jejuni is activated by the PhosS-PhosR two component system, under phosphate limiting conditions (Wosten et al., 2006). However, what remains to be investigated is whether PhosR also regulates the tlp2 promoter activity in C. jejuni. The tlp2 gene is located upstream to phoX in C. jejuni, and our investigation of tlp2 transcriptional organization revealed that both genes are transcribed together from a single promoter (Ptlp2) located upstream to tlp2. These findings however, contradict a previous finding in C. jejuni 81116, where phoX was shown to be transcribed by a promoter located in the intergenic region of tlp2 and phoX, when C. jejuni was grown in a chemically defined medium (Wosten et al., 2006). However, we could not observe any promoter activities in the intergenic region using a primer extension assay under our experimental conditions (Figure 3). Further, both strains possess 135 bps intergenic region between tlp2 and phoX; however, showed 95.5% sequence similarity. Therefore, the disparity could be due to the different media and strains used in the two different studies.

The Δtlp2 mutant exhibited an increased intracellular survival in INT 407 cell monolayer than the WT strain. The group A Tlps 1, 4, 7 and 10 but not Tlp2, have been shown to play a role in C. jejuni invasion of human intestinal epithelial cells (Vegge et al., 2009; Hartley-Tassell et al., 2010; Tareen et al., 2010). C. jejuni is known to survive within epithelial cells and can be viable for up to 24 h (Watson and Galan, 2008). Studies have also indicated a role for iron acquisition in C. jejuni intracellular survival (Naikare et al., 2006). This therefore piqued our interest in identifying a role for a Tlp involved in chemotaxis toward iron, in C. jejuni survival within host cells. The results of our study showed that the deletion of tlp2 increased intracellular recovery of C. jejuni. This was in contrary to our belief that deletion of tlp2 would decrease the survival of C. jejuni in host cells, due to the decreased chemotaxis toward iron. It must however, be noted that intracellular C. jejuni undergo a metabolic reprogramming which affects their survival within epithelial cells (Svensson et al., 2009; Liu et al., 2012). The increased intracellular survival in the Δtlp2 mutant may indicate a dysregulation of cellular process which warrants further investigation.

The role of tlp2 in tissue specific colonization of the chicken gastrointestinal tract was investigated. Mutation in tlp2 resulted in a colonization defect in the cecum, with a more profound reduction seen in the duodenum and jejunum. Catabolism of amino acids such as aspartate and serine are essential for C. jejuni colonization of the avian gut (Guccione et al., 2008), as reflected by the tlp1 mutant (aspartate chemoreceptor), which was severely impaired in colonization of the chicken ceca (Hartley-Tassell et al., 2010). The tlp2 mutant demonstrated a decreased chemotaxis toward aspartate, which might explain the reduced colonization. The utilization of glutamine, glutathione and asparagine in C. jejuni 81–176 is associated to tissue-specific colonization of the murine intestine (Hofreuter et al., 2008). However, it is not known if the ability to metabolize these nutrients also supports tissue specific colonization in the chicken intestinal tract. Additionally, chemotaxis toward pyruvate and fumarate mediated by Tlp9 represents energy taxis in C. jejuni. Energy taxis is an essential driving force for C. jejuni for establishment during colonization of the host (Vegge et al., 2009). The chicken cecum represents an iron and phosphate limiting environment for C. jejuni and iron acquisition is known to be essential for C. jejuni colonization of the chicken (Naikare et al., 2006). It is not surprising to see that Δtlp2 mutant, defective in chemotaxis toward Pi and iron, is also defective in colonization of the chicken cecum, duodenum, and jejunum. These findings clearly indicate that tlp2 contributes to C. jejuni interaction with host cells, which is an important determinant for C. jejuni pathogenesis and colonization of the chicken gastrointestinal tract.

In summary, the present study identifies a role for tlp2 in C. jejuni chemotaxis, stress survival and colonization of the chicken gastrointestinal tract. Further, our findings indicate that iron regulates tlp2. The tlp2 mutant was also defective in chemotaxis to Pi and showed increased PhoX activity. This suggests a possible cross-talk between iron and phosphate regulatory pathways, which needs further investigation. In addition, the increased PhoX activity in the presence of iron seen in C. jejuni indicates that iron may reduce the bioavailability of phosphate. Our findings in this study suggest a basis for future biochemical characterization of PhoX in C. jejuni.

ETHICS STATEMENT

Animal experiments were conducted according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC), the Ohio State University. Chickens were housed at the Food Animal Health Research Program Animal Care Facility, which is fully accredited by AAALAC and the animals were supervised by a senior veterinarian. Infectious agents were administered using manual restraint for less than
one minute to minimize distress. Before necropsy, chickens were euthanized by carbon dioxide inhalation. This method is consistent with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

GR and KC designed the experiments. KC, SH, BJ, and SR performed the experiments and collected the data. KC, GR and VS analyzed the data. KC, GR, VS, and BJ wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02674/full#supplementary-material

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