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Polyphosphate Kinase 1 Is a Pathogenesis Determinant in *Campylobacter jejuni*▽†

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Campylobacter jejuni* is the leading cause of bacterial gastroenteritis in the developed world. Despite its prevalence, relatively little is known about *C. jejuni*'s precise pathogenesis mechanisms, particularly in comparison to other well-studied enteric organisms such as *Escherichia coli* and *Salmonella* spp. Altered expression of phosphate genes in a *C. jejuni* stringent response mutant, together with known correlations between the stringent response, polyphosphate (poly-P), and virulence in other bacteria, led us to investigate the role of poly-P in *C. jejuni* stress survival and pathogenesis. All sequenced *C. jejuni* strains harbor a conserved putative polyphosphate kinase 1 predicted to be principally responsible for poly-P synthesis. We generated a targeted *ppk1* deletion mutant (Δ*ppk1*) in *C. jejuni* strain 81-176 and found that Δ*ppk1*, as well as the Δ*spoT* stringent response mutant, exhibited low levels of poly-P at all growth stages. In contrast, wild-type *C. jejuni* poly-P levels increased significantly as the bacteria transitioned from log to stationary phase. Phenotypic analyses revealed that the Δ*ppk1* mutant was defective for survival during osmotic shock and low-nutrient stress. However, certain phenotypes associated with *ppk1* deletion in other bacteria (i.e., motility and oxidative stress) were unaffected in the *C. jejuni* Δ*ppk1* mutant, which also displayed an unexpected increase in biofilm formation. The *C. jejuni* Δ*ppk1* mutant was also defective for the virulence-associated phenotype of intraepithelial cell survival in a tissue culture infection model and exhibited a striking, dose-dependent chick colonization defect. These results indicate that poly-P utilization and accumulation contribute significantly to *C. jejuni* pathogenesis and affect its ability to adapt to specific stresses and stringencies. Furthermore, our study demonstrates that poly-P likely plays both similar and unique roles in *C. jejuni* compared to its roles in other bacteria and that poly-P metabolism is linked to stringent response mechanisms in *C. jejuni*.

Campylobacter jejuni is a gram-negative, microaerophilic bacterium belonging to the family *Campylobacteraceae* (73) and is now considered the leading cause of human gastroenteritis in the developed world (4, 69). *C. jejuni* lives harmlessly in the intestinal microflora of most mammals and birds, resulting in a commensal relationship (11). However, upon infecting a human host, *C. jejuni* invades the intestinal mucosa, interrupts intestinal integrity, and causes profuse watery and/or bloody diarrhea (13). Campylobacteriosis has been correlated with other medical sequelae, such as reactive arthritis, hemolytic-uremic syndrome, and inflammatory bowel disease; the most notable complication of infection is Guillain-Barré syndrome, an acute neuromuscular paralysis (34). *C. jejuni* is typically transmitted to humans via contact with infected animals or through undercooked food, unpasteurized milk, or contaminated water (3, 4, 25).

Despite the prevalence of *C. jejuni* infection, the molecular mechanisms *C. jejuni* uses to cause human disease, as well as to adapt to or survive stresses encountered during both in vivo colonization and ex vivo transmission, are not well understood, particularly in comparison to mechanisms used by other well-studied pathogens such as *Escherichia coli*, *Salmonella* spp., and *Helicobacter pylori.* For instance, although *C. jejuni* is not considered an obligate intracellular pathogen, its ability to invade and survive in intestinal epithelial cells correlates well with virulence (8–10, 16, 22, 41). Despite this, specific bacterial factors contributing to *C. jejuni* invasion and intracellular survival are poorly characterized. It is likewise perplexing how *C. jejuni* survives a multitude of environmental stresses given its fastidious laboratory growth requirements. Our current limited understanding of *C. jejuni* is largely due to its difficult culture conditions, significant interstrain virulence variability, and a historical intractability of *C. jejuni* to genetic manipulation. Moreover, the recent publication of three complete *C. jejuni* genome sequences revealed that *C. jejuni* lacks many virulence characteristics and factors found in other bacterial pathogens, such as pathogenicity islands and certain stress response factors such as RpoS (23, 31, 55). Nonetheless, the high worldwide prevalence of *C. jejuni* commensal and human infection suggests the presence of factors that allow this fastidious pathogen to navigate a multitude of environments during transmission, colonization, and virulence.

Several lines of evidence led us to hypothesize that polyphosphate (poly-P), unstudied in *C. jejuni* prior to the work described here, would prove to be a key factor impacting multiple aspects of the *C. jejuni* pathogenesis cycle. Poly-P is ubiquitous in nature and consists of phosphate residues linked by high-energy phosphoanhydride bonds such as those found in ATP. Analysis of published genome sequences revealed that *C. jejuni* harbors homologues of several genes predicted to participate

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in poly-P metabolism, including *ppk1*, *ppk2*, and *ppx* (23, 31, 55). The *ppk1* gene encodes a polyphosphate kinase, PPK1, that reversibly forms poly-P from the terminal γ-phosphate of ATP and is responsible for either all or the majority of poly-P formation in several bacteria, including *E. coli*, *H. pylori*, and *Pseudomonas aeruginosa* (1, 43, 70). It was recently shown that some bacteria, including *P. aeruginosa* and *C. jejuni*, also harbor a reversible PPK2 enzyme that preferentially synthesizes GTP from poly-P (35, 78). An exopolyphosphatase, encoded by the *ppx* gene, has been shown to be responsible for the conversion of poly-P into free phosphate residues in *E. coli* (2).

Poly-P has a multiplicity of functions within bacterial cells. Long chains of poly-P can serve as a phosphate reservoir, a cation chelator (30), a membrane channel for DNA entry (64), a capsular component (71, 72), a pH buffer (36, 57, 58), and likely an ATP substitute (12, 43). Furthermore, in *E. coli*, poly-P inhibits RNA degradation, promotes translation fidelity, and activates the Lon protease complex that degrades specific ribosomal proteins to meet nutritional requirements during starvation (45).

Important roles for poly-P formation in bacterial pathogenesis have been established in such pathogens as *E. coli*, *P. aeruginosa*, *Vibrio cholerae*, *Klebsiella aerogenes*, *S. enterica* serovar Typhimurium, *H. pylori*, and *Shigella flexneri*. In these organisms, poly-P formation was shown to be critical for attributes such as motility, quorum sensing, biofilm formation, resistance to oxidative, osmotic, heat, and alkaline stress, and stationary-phase survival (36, 40, 52, 58, 59, 62, 63, 70). Although the importance of poly-P in various bacterial phenotypes has been reported, the precise molecular mechanisms by which poly-P enacts specific functions, as well as the primary and secondary effects of poly-P accumulation, are still not understood in even the best-characterized bacterial species.

Our interest in poly-P was further manifested by its connection to the stringent response (SR). In the SR, RelA and/or SpoT enzymes activated during stress synthesize guanosine tetraphosphate (ppGpp), an alarmone that binds RNA polymerase to alter transcription and allow the organism to cope with the stress condition. We recently showed that the *C. jejuni* SR is important for several pathogenesis attributes, including survival during the transmission-related stresses of nutrient limitation, aerobiosis, and rifampin exposure (29). The SR was also implicated in virulence: *C. jejuni*'s *spoT* gene encoding a bifunctional ppGpp synthetase/hydrolase was upregulated in the presence of intestinal epithelial cells, and deletion of *spoT* resulted in significant epithelial cell invasion and intracellular survival defects (29). These responses occur in the absence of RpoS, which mediates many SR-related phenotypes in other bacteria. Poly-P has been linked to SR mechanisms in *E. coli*, where SR mutants with diminished ppGpp levels also harbor lower levels of poly-P (44, 60). Furthermore, our microarray analysis of the *C. jejuni* Δ*spoT* mutant revealed increased expression of genes regulating inorganic phosphate uptake during stationary phase concurrent with upregulation of genes involved in heat shock (29), suggesting that poly-P may play a role in *C. jejuni* stress survival.

To test the hypothesis that poly-P plays an important role in *C. jejuni* stress survival and pathogenesis, the *ppk1* gene was disrupted in the highly invasive *C. jejuni* strain 81-176. Analyses of the Δ*ppk1* mutant strain, which was defective for poly-P accumulation, identified roles for poly-P in numerous aspects of pathogenesis, including the transmission-related stresses of osmotic shock and low-nutrient-stress survival. PPK1 is also now only the third *C. jejuni* factor found to be involved in prolonged survival of *C. jejuni* inside human epithelial cells, an important but underexplored aspect of *C. jejuni* virulence. Our studies also indicate that poly-P metabolism in *C. jejuni* likely intersects with SR mechanisms, as seen in *E. coli*. However, contrary to observations in other Δ*ppk1* mutant bacteria, which are generally defective for forming biofilms (18, 63, 67), the *C. jejuni* Δ*ppk1* mutant exhibited an unexpected increase in biofilm formation. The *C. jejuni* Δ*ppk1* mutant also displayed a striking, dose-dependent chick colonization defect. These findings identify poly-P as an important new factor involved in *C. jejuni* transmission, colonization, and pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All studies were performed with the highly invasive *Campylobacter jejuni* strain 81-176 originally isolated from a diarrheic patient (42). The Δ*spoT* mutant of this strain was previously described (29). *C. jejuni* was routinely cultured on Mueller-Hinton (MH; Oxoid, Ltd., Hampshire, England) agar plates, and growth/survival curves were determined in MH broth unless otherwise stated (Oxoid). *C. jejuni* was always grown in medium supplemented with 10 μg of vancomycin/ml and 5 μg of trimethoprim/ml; chloramphenicol was added at 20 μg/ml when required. All bacteria were enumerated on MH agar plates by serial 10-fold dilutions unless otherwise stated. Plates were incubated in a tri-gas incubator (Heraeus) in 6% O_2 and 12% CO_2 at 37°C. Broth culture growth and survival curves were determined by using the Oxoid CampyGen system to produce a microaerobic atmosphere of 6% O_2 and 12% CO_2, and cells were shaken at 200 rpm at 37°C. *E. coli* strain DH5α was grown on Luria-Bertani agar or broth, with the addition of 30 μg of chloramphenicol/ml as needed, at 37°C under normal atmospheric conditions. Karmali agar was used for growth of *C. jejuni* for the chick colonization studies.

Construction of *C. jejuni* 81-176 *ppk1* targeted deletion mutant and complemented strains. A polyphosphate kinase (*ppk1*) gene with 30.1% identity and 50.7% similarity to the *ppk1* gene in *E. coli* was identified in the *C. jejuni* 81-176, 11168, and RM1221 genomes using the BLAST features of CampyDB (http://campy.bham.ac.uk [17]). The *ppk1* gene was PCR amplified from *C. jejuni* chromosomal DNA prepared by using the QIAGEN DNeasy kit (QIAGEN, Inc., Valencia, CA), with the primers PPK1-2 Fp (GCAAATATTTACACCAAGAAAAAGAAC) and PPK1-2 Rp (ATCTGCACTCGATATAAAATAATTTGG), yielding a 1,550-bp fragment. The amplified product was cloned into the pGEM-T vector (Promega, Nepean, CA), which is a suicide plasmid in *C. jejuni.* Two EcoRI sites located within the *ppk1* gene were used to remove 1,048 bp of coding sequence. A chloramphenicol acetyltransferase cassette (*cat*) was excised from pRY109 (77) with EcoRI and ligated into the EcoRI-digested pGEM-*ppk1* vector to create the *ppk1* knockout construct. Insertion of the *cat* cassette was confirmed by restriction digestion and sequencing (Nucleic Acid Protein Service Unit, Vancouver, CA). This construct was used to naturally transform *C. jejuni* 81-176 (74). Recombinants were recovered on MH agar plates supplemented with chloramphenicol. Insertional inactivation of the *ppk1* gene via *cat* cassette insertion was verified by PCR using PPK1-1 Fp (TGCCCTTAGCGTTATAAAAAGTATAAA) and PPK1-1 Rp (AATTTTCGGTCATTTTTGATAGTGTAG) primers that are external to the *ppk1* gene and the region originally amplified. Southern analysis also verified a single chromosomal insertion of the *cat* cassette into the *ppk1* gene.

Generation of a reconstituted wild-type strain of *C. jejuni*, designated *ppk1**, was achieved via natural transformation of the Δ*ppk1* mutant with the *ppk1* gene in pGEM-T. The naturally transformed Δ*ppk1* mutant was spread onto MH plates, harvested after 2 h, and serial 10-fold dilutions plated on MH plates supplemented with 0.17 M NaCl and incubated for 48 h at 37°C in a tri-gas incubator. Individual colonies were selected and purified on MH agar containing 10 μg of vancomycin/ml and 5 μg of trimethoprim/ml. Colonies were tested for sensitivity to chloramphenicol by plating on MH agar containing 20 μg of chloramphenicol/ml. Colonies representing putative reconstituted wild-type strains were confirmed by using PCR with the PPK1-1 primer set and sequence analysis of 18 bona fide recombinants, represented by *ppk1**.

Extraction of poly-P with Glassmilk. Extraction of poly-P from *C. jejuni* cells and binding to Glassmilk (qBiogene) was performed essentially as described by

Ault-Riche et al. (6). *C. jejuni* cultures were grown in MH broth to mid-log phase (hereafter, mid-log phase means an optical density at 600 nm [OD_{600}] of ~0.2 to 0.5) and then diluted to an OD_{600} of 0.05 to initiate the time course experiment. Cells were harvested after 2, 10, and 24 h by pelleting 1 ml in a table-top centrifuge at 6,000 rpm for 10 min. All pellets were processed directly. To each pellet was added 500 μl of guanidine isothiocyanate (GITC) lysis buffer (4 M GITC, 500 mM Tris-HCl [pH 7.0]) prewarmed at 95°C. Tubes were vortex mixed, incubated for 5 min in a 95°C heating block, and sonicated briefly; a 10-μl sample was removed for total protein estimation. To each poly-P assay tube, 30 μl of 10% sodium dodecyl sulfate, 500 μl of 95% ethanol, and 5 μl of Glassmilk were added. Tubes were vortex mixed and centrifuged briefly to pellet glass, which was then resuspended in 500 μl of ice-cold new wash buffer (5 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM EDTA, 50% ethanol) by vortexing and repelleted; washing was repeated two additional times. The washed pellet was resuspended in 50 μl of 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 20 μg each of DNase and RNase per ml, followed by incubation at 37°C for 10 min. The pellet was washed with 150 μl of 4 M GITC lysis buffer and 150 μl of 95% ethanol and then twice in new wash buffer. Poly-P was eluted from the Glassmilk pellet with 50 μl of 50 mM Tris-HCl (pH 8.0) at 95°C for 2 min, followed by two additional elutions.

Measurement of poly-P levels by a TBO assay. A standard curve was determined by the addition of 100 μl of a known concentration of phosphorus standard (Sigma-Aldrich, Oakville, CA) sample that had been serially diluted (1:10) to 900 μl (64, 32, 16, 8, and 4 nmol of poly-P) of a toluidine blue O (TBO; Sigma-Aldrich, Oakville, CA) dye solution consisting of 6 mg of TBO/liter in 40 mM acetic acid. After the addition to TBO, all samples (standard curve and *C. jejuni* time course samples) were incubated at room temperature for 15 min. Absorbance at 630-nm and 530-nm levels were assessed spectrophotometrically and used to generate an A_{530}/A_{630} ratio. Poly-P binding to TBO results in a shift in TBO absorbance from 630 to 530 nm; the 530-nm/630-nm ratio thus reflects the amount of poly-P in a given sample. The levels of poly-P from each experimental sample were then determined by direct comparison to the standard curve. Poly-P levels were expressed in nanomoles of poly-P and per milligram of total cellular protein, as measured via Bradford analysis (Pierce Scientific).

Static biofilm formation. Biofilm formation was assayed as described previously (54). Briefly, cells were grown microaerobically in MH broth at 37°C to mid-log phase overnight and diluted to an OD_{600} of 0.20 and 0.02. Aliquots (100 μl) were added to 96-well polyvinyl chloride plates, followed by incubation at 37°C for 48 h. Static biofilm formation as measured by surface-associated bacteria was assessed by adding 25 μl of a 1% crystal violet solution in ethanol, followed by incubation for 15 min at room temperature, and three washes with distilled H_2O (54). To quantify biofilm formation, 150 μl of 80% dimethyl sulfoxide (DMSO) was added to each polyvinyl chloride (PVC) microtiter well; then each well was covered and incubated 24 h at room temperature. A 1:10 dilution in 80% DMSO of each well was measured at OD_{570} to indirectly quantify the amount of biofilm stained by crystal violet.

Nutrient downshift survival. *C. jejuni* were grown microaerobically at 37°C in MH broth to mid-log phase overnight, collected by centrifugation at 6,000 rpm for 10 min, and washed twice with minimal essential medium (MEM) or MOPS-MGS with Earle's salts and L-glutamine (Difco) or MOPS-MGS (50). Bacteria were resuspended in MEM and diluted to an OD_{600} of 0.05. Cultures were placed under microaerobic conditions at 37°C with shaking at 200 rpm. CFU were measured over time by plating on MH agar plates.

Osmotic stress survival. *C. jejuni* strains were grown to mid-log phase, serially diluted (1:10), and spotted onto MH agar plates containing 0.17 M NaCl to assess single-colony growth. Survival during osmotic stress was also tested in shaking liquid cultures by growing bacterial strains to mid-log phase in MH and diluting them to an OD_{600} of 0.05 in MH broth with or without 0.25 M NaCl. CFU were assayed by serial dilution and plating on MH agar.

INT407 cell infection assay for invasion and intracellular survival. INT407 cells were seeded to semiconfluence (~1×10^5 cells/well) and confluence (~5.5×10^5 cells/well) in 24-well plates approximately 16 h prior to infection assays. *C. jejuni* strains were inoculated at an OD_{600} of 0.001 into MH broth culture and grown overnight to mid-log phase. Bacteria were pelleted at 6,000 rpm for 10 min, washed twice with MEM, and diluted to an OD_{600} of 0.002 in MEM. Bacterial suspensions in MEM (1 ml) were used to infect tissue culture cells at multiplicities of infection (MOIs) of ~100 and ~20 (~10^7 bacteria/ml). All experiments were performed in triplicate. After 3 h of infection in a 5.0% CO_2 incubator, bacteria in MEM were removed from the wells, and cells were washed twice with MEM. To all wells, 1 ml of MEM containing 150 μg of gentamicin/ml was added to kill the remaining extracellular bacteria. After 2 h, wells were washed twice with MEM. To assay invasion, 1 ml of distilled H_2O was added to some of the wells, and INT407 cells were disrupted by lysis with a 27G syringe. Invaded bacteria were assayed by serial dilution and plating on MH agar.

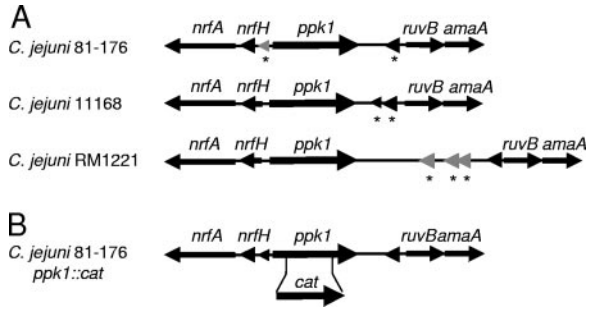

FIG. 1. *C. jejuni ppk1* and generation of a single insert Δ*ppk1* disruption strain. (A) Genomic location of the *ppk1* gene is conserved among the sequenced and annotated strains *C. jejuni* 81-176, *C. jejuni* 11168 and *C. jejuni* RM1221. MUMer alignment was performed by using the CampyDB gene viewer (17). Hypothetical proteins are represented by an asterisk (*), and genes with no predicted orthologues are colored gray. (B) The approximate site of the *cat*-marked insertion-deletion mutation generated in *C. jejuni* 81-176 *ppk1* is shown. The resultant mutant strain was designated Δ*ppk1*.

Samples for assaying intracellular survival were covered with MEM with 3% fetal bovine serum and the addition of 10 μg of gentamicin/ml to halt bacterial growth if cell lysis occurred. After 23 to 24 h of incubation, intracellular survival was assayed as described for invasion samples.

Chick colonization assays. Colonization of 1-day-old chicks was performed essentially as described previously (14). Briefly, broiler chicks were obtained from a local hatchery in Saskatchewan on the day of hatch. Five chicks were euthanized, and their cecal contents cultured for *Campylobacter.* The remaining birds were randomly assigned into groups of 10 birds and provided with feed and water ad libitum. Birds were cared for in accordance to guidelines of the Canadian Council for Animal Care. Birds were orally challenged with the indicated strain (wild type or Δ*ppk1* mutant) and dose (1.5×10^5, 1.5×10^6, and 1.5×10^7 CFU) of *C. jejuni* in 0.5 ml of MH broth. Inocula for challenge experiments were produced by harvesting cells grown for 18 h under microaerophilic conditions at 37°C into cold MH broth, diluting them to the indicated concentration in MH broth, and maintaining them on ice until immediately before use. Viable cell counts were determined by plating serial dilutions onto MH agar (Becton Dickinson). Birds were maintained for 7 days after challenge and then euthanized by cervical dislocation. Ceca were aseptically collected for qualitative and quantitative assessment of colonization. Colonization of the birds was measured by culturing the cecal contents, after appropriate dilutions were made in MH broth, on Karmali agar (Bacto) under microaerophilic conditions at 42°C.

RESULTS

The Δ*ppk1* and Δ*spoT* mutants are defective for poly-P accumulation. All three published *C. jejuni* strain sequences contain a highly conserved *ppk1* gene (Fig. 1A), encoding a putative PPK1 with significant homology to PPK1 enzymes in other bacteria (23, 31, 55). The predicted *C. jejuni* amino acid sequences exhibit 45.4, 36.2, and 30.1% identity to PPK1 in *H. pylori*, *P. aeruginosa*, and *E. coli*, respectively. Moreover, two highly conserved histidine residues, required for PPK1 activity in *E. coli*, are also present at amino acid positions 427 and 580 in the *C. jejuni* 81-176 strain. In all three strains, *ppk1* appears to be a single-gene operon, and microarray data from multiple *C. jejuni* gene expression experiments with strains 81-176 and 11168 indicate that *ppk1* is transcribed independently of neighboring genes (23, 28, 29, 31, 48, 55). To explore the role of poly-P in *C. jejuni* 81-176, ~50% of the *ppk1* gene was deleted, including the codons for the conserved His residues, and replaced with a chloramphenicol acetyltransferase (*cat*) cassette conferring chloramphenicol resistance (Fig. 1B). Sequencing and Southern analyses demonstrated a single *cat* insert in the

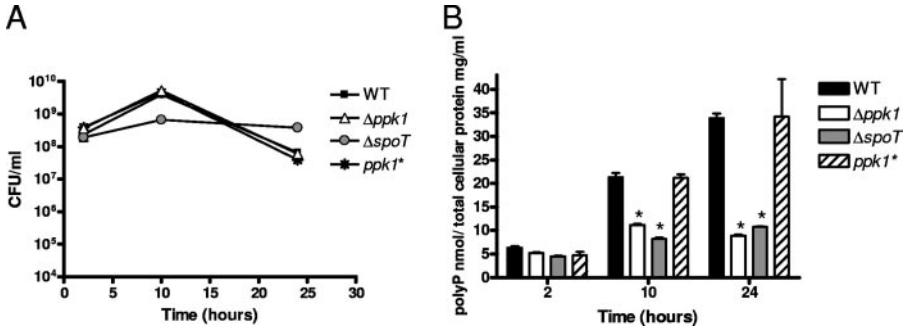

FIG. 2. Poly-P accumulates in wild-type and *ppk1** strains at later growth stages but remains at low levels in Δ*ppk1* and Δ*spoT* strains. Wild-type (WT) *C. jejuni* 81-176, Δ*ppk1* mutant, the complemented strain *ppk1**, and the Δ*spoT* mutant were grown microaerobically in shaking broth culture to early log phase and diluted to an OD_{600} of 0.05. Cultures (1 ml) were harvested at 2, 10, and 24 h and assayed for CFU/ml (A) and poly-P levels versus total cellular protein (B). Triplicate samples were harvested and assayed for each time point. Statistical significance ($P < 0.05$) is represented by an asterisk.

ppk1 gene (data not shown). To ensure that observed phenotypes were attributed to *ppk1*, a reconstituted wild-type strain was generated by homologous recombination of a wild-type copy of *ppk1* into the Δ*ppk1* strain *ppk1*::*cat* locus as described in Materials and Methods. The resulting reconstituted wild-type strain was designated the *ppk1** strain.

Growth rates (Fig. 2A) and poly-P levels (Fig. 2B) for wild-type *C. jejuni* 81-176, the Δ*ppk1* mutant, the Δ*spoT* mutant, and the *ppk1** strain were assessed in shaking MH broth cultures harvested at various growth stages. Poly-P was extracted after cell lysis by binding to Glassmilk and measured by using the metachromatic dye TBO as described in Materials and Methods. Poly-P levels in *C. jejuni* samples are expressed as nanomoles of poly-P and normalized to milligrams of total cellular protein.

We found that wild-type, Δ*ppk1*, and *ppk1** strains exhibited identical growth profiles under normal laboratory culture conditions by both OD_{600} (not shown) and CFU/ml (Fig. 2A) analyses, whereas the Δ*spoT* mutant, as previously reported (29), exhibited some growth differences from the wild type. In wild-type *C. jejuni*, the poly-P levels were significantly higher than those of the Δ*ppk1* and Δ*spoT* mutants, with levels peaking in stationary phase at 33.8 nmol of poly-P/mg of total protein (Fig. 2B). The Δ*ppk1* mutant exhibited significantly lower levels of poly-P at both 10 and 24 h ($P < 0.05$), with the largest difference (~3.8-fold) observed at the 24-h stationary-phase time point. The Δ*spoT* mutant exhibited poly-P levels that were also significantly lower than those of the wild type at both 10 and 24 h ($P < 0.05$), with the largest difference (~3.2-fold) again observed in the stationary phase. The complemented *ppk1** strain displayed levels of poly-P similar to that of the wild type at all time points assayed. Together, these data confirm that the *ppk1* gene is important for poly-P synthesis in *C. jejuni* and demonstrate a connection between the SR and poly-P metabolism.

Poly-P is critical for *C. jejuni* survival during nutritional downshift. Initial tests of the *C. jejuni* Δ*ppk1* mutant based on known Δ*ppk1* mutant defects in other bacteria (i.e., motility and oxidative shock survival) revealed that the *C. jejuni* Δ*ppk1* mutant was indistinguishable from the wild type for a number of predicted phenotypes and stress resistance characteristics (see Table S1 in the supplemental material).

In contrast, poly-P was found to be important for the ability of *C. jejuni* to survive low-nutrient stress. To test this, wild-type, Δ*ppk1*, and *ppk1** strains were shifted from rich MH broth to nutrient-poor MEM and assayed for CFU/ml over a 55-h time course (Fig. 3A). The Δ*ppk1* mutant exhibited survival defects during low-nutrient stress compared to the wild type at all of the time points assayed. The most pronounced effect of nutrient downshift was seen after 5 h, at which time

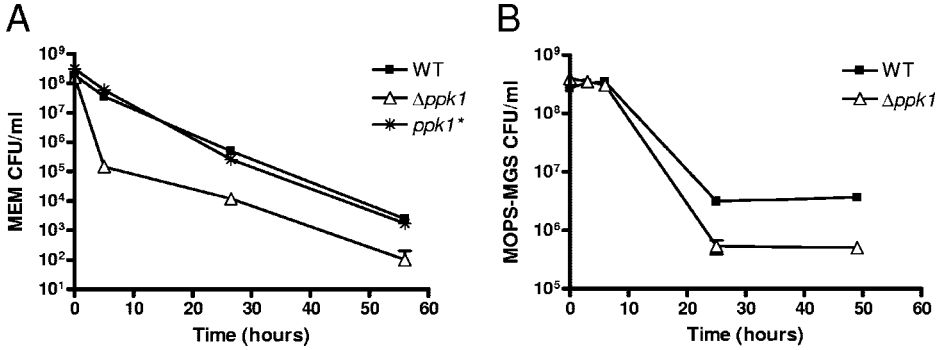

FIG. 3. Poly-P is important for *C. jejuni* survival during nutritional downshift. Wild type (WT), Δ*ppk1*, and *ppk1** strains were grown in MH broth to mid-log phase. Cells were subjected to nutritional downshift by centrifugation, followed by resuspension to an OD_{600} of 0.05 in minimum essential medium (A) or MOPS-MGS buffered medium (B).

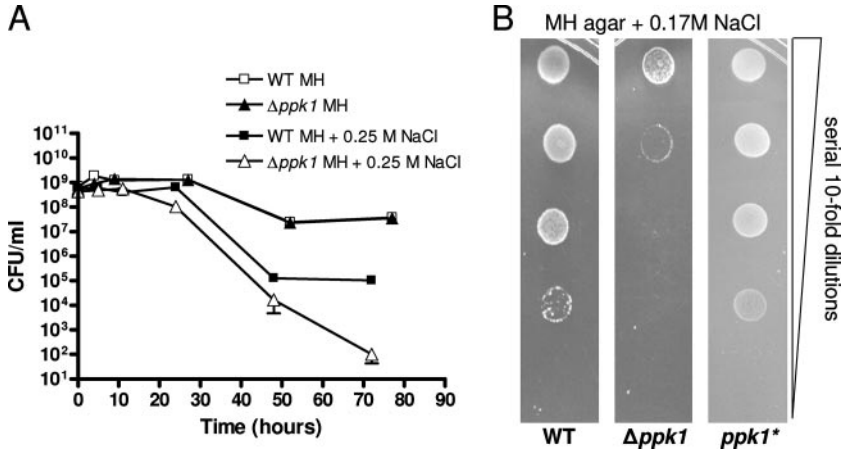

FIG. 4. Poly-P is required for *C. jejuni* osmotic stress survival. (A) *C. jejuni* 81-176 wild-type and Δ*ppk1* strains were grown to mid-log phase and shifted to MH broth with or without 0.25 M NaCl. Growth and/or survivability was monitored by CFU/ml plate counts. (B) Wild-type, Δ*ppk1*, and *ppk1** strains were grown to mid-log phase in MH broth and serially diluted (1:10) from 2×10^5 to 2×10^1 CFU/ml and spotted onto 0.17 M NaCl MH agar plates. Growth was assessed after 48 h under microaerobic conditions.

the culturability of the mutant dropped >250-fold relative to the wild-type and *ppk1** strains. Survivability was also assessed in another limited-nutrient medium, MOPS-MGS buffered medium (50) without phosphate (Fig. 3B). As expected, the Δ*ppk1* mutant showed a considerable defect in survivability at later time points assayed; however, the differences were not as pronounced as those in MEM. These data demonstrate that the Δ*ppk1* mutant exhibits nutrient downshift tolerance defects in two different minimal media.

Poly-P accumulation is required for *C. jejuni* to grow and survive during osmotic shock. To test survival under osmotic stress, strains were grown to mid-log phase and shifted to MH broth with or without NaCl added to 0.25 M, and the CFU/ml were enumerated at the time points indicated (Fig. 4A). During osmotic shock, both the wild-type and the Δ*ppk1* mutant strains ceased growth at 5 h. After 24 h, Δ*ppk1* CFU/ml were >10-fold lower than wild-type CFU/ml. By 72 h, the Δ*ppk1* mutant CFU/ml were >1,000-fold lower than the wild-type levels. Wild-type and Δ*ppk1* grew identically in MH broth without the addition of salt (Fig. 4A); levels less than 0.25 M NaCl did not inhibit the survival of wild-type or Δ*ppk1* strains (data not shown).

The ability of single bacteria to grow into colonies during continuous osmotic stress was tested by growing wild-type, Δ*ppk1*, and *ppk1** strains to mid-log phase in MH broth and then serially diluting and spotting the bacteria onto MH plates containing 0.17 M NaCl. Under these conditions, the Δ*ppk1* mutant was significantly defective for growth compared to wild-type and *ppk1** strains (Fig. 4B); all strains grew identically on MH agar without added salt (data not shown).

The Δ*ppk1* mutant displays enhanced static biofilm formation. Biofilm formation contributes to bacterial virulence, colonization, environmental survival, and antibiotic resistance. Poly-P has been shown to be essential for biofilm formation in several bacteria, including *P. aeruginosa* (63). The role of poly-P in *C. jejuni* biofilm formation was assayed by growing standing broth cultures of *C. jejuni* wild-type, Δ*ppk1*, and *ppk1** strains in PVC plates from starting OD_{600} levels of 0.02 and 0.20. Biofilm formation at the air-liquid interface and on the sides of the PVC wells were assayed by staining the wells with a crystal violet solution; in this assay, increased crystal violet staining correlates with increased biofilm production (54; M. M. McLennan et al., unpublished data). After 2 days, wild-type *C. jejuni* formed a faint biofilm at the air-liquid interface for both starting doses, while the Δ*ppk1* mutant exhibited

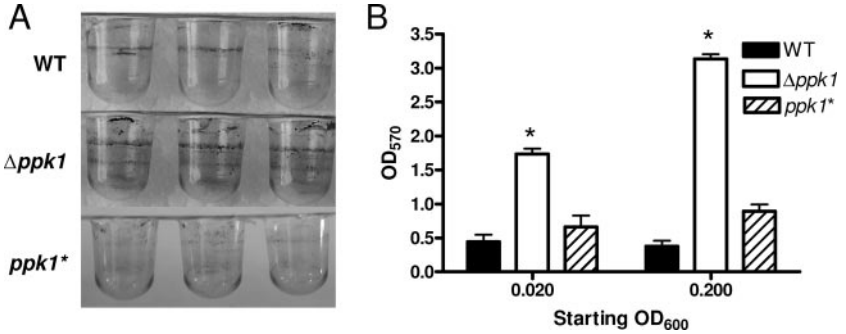

FIG. 5. The Δ*ppk1* mutant exhibits increased static biofilm formation. Microtiter PVC plates were inoculated with *C. jejuni* in MH broth at OD_{600}s of 0.02 and 0.20. At 48 h, biofilms were qualitatively observed by crystal violet staining. (A) Representative wells from the OD_{600} 0.02 inoculation are shown for wild-type, Δ*ppk1*, and *ppk1** strains. (B) Biofilm formation was quantified by the addition of 80% DMSO to each crystal violet-stained well, followed by OD_{570} absorbance measurements of solubilized crystal violet. Statistical significance ($P < 0.05$) is represented by an asterisk.

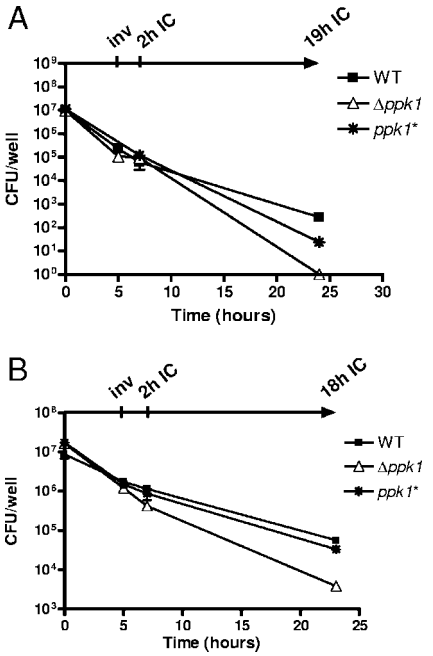

FIG. 6. The Δ*ppk1* mutant is defective for long-term intracellular survival in an epithelial cell model of infection. Wild-type (WT), Δ*ppk1*, and *ppk1** strains were grown to mid-log phase in MH broth. At the zero time point, semiconfluent or confluent monolayers of INT407 cells were inoculated with bacteria at MOIs of ~100 (A) and ~20 (B). After 3 h, the cells were washed, and gentamicin was added at 150 μg/ml to all wells for 2 h to kill extracellular bacteria. Gentamicin was washed from the cells at 5 h, and invaded intracellular bacteria were harvested and plated for enumeration (inv). To all remaining wells, fresh media containing 10 μg of gentamicin/ml and 3% fetal bovine serum were added. After an additional 2 h (2h IC) or 18 or 19 h (18h or 19h IC) of incubation, cells were washed, and surviving intracellular bacteria were harvested. For all time points, experiments were performed in triplicate, and error bars are shown.

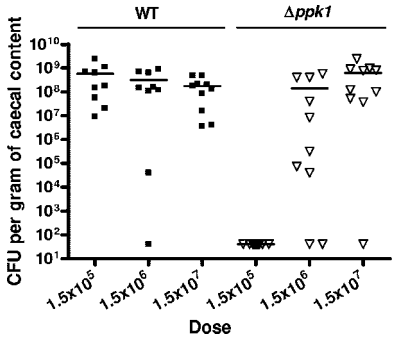

FIG. 7. The Δ*ppk1* mutant exhibits a dose-dependent defect for chick cecal colonization. Chicks were challenged orally with *C. jejuni* 81-176 wild type (■) and the Δ*ppk1* mutant (▽), using doses of 1.5×10^5, 1.5×10^6, and 1.5×10^7 CFU in 0.5 ml of broth. After 7 days postinfection, chicks were sacrificed, and bacterial colonization of ceca was determined by plating on Karmali agar. Levels of colonization at specific doses are expressed as CFU/g of cecal content. The detection limited was 40 CFU. Each data symbol represents CFU recovered from an individual chick. The geometric mean of bacterial concentration recovered is represented by a bar for each dosage.

statistically significant ($P < 0.05$) ~3-fold and ~6-fold increases in biofilm formation at the air-liquid interface and on the bottom and sides of the wells compared to wild-type at the lower and higher inoculating doses, respectively (Fig. 5). The Δ*ppk1* mutant also exhibited a dose-dependent biofilm phenotype: the higher inoculating dose of the Δ*ppk1* mutant yielded a statistically significant ($P < 0.05$) increase in biofilm formation compared to the lower inoculating dose of the Δ*ppk1* mutant, whereas a similar phenomenon was not observed for the wild type (Fig. 5B). Consistent with our other observations, the *ppk1** strain exhibited wild-type biofilm formation levels (Fig. 5).

***The* Δ*ppk1* mutant is defective for prolonged intracellular survival.** To assay the virulence-associated phenotypes of invasion and intracellular survival, wild-type, Δ*ppk1*, and *ppk1** strains were allowed to infect INT407 cells at an MOI of ~20 or ~100 for 3 h. Gentamicin was added to all wells to kill extracellular bacteria, after which cells were assayed for invasion, as well as for both short-term and long-term intracellular (IC) survival, at 2 and 18 h (MOI, ~20) or at 2 and 19 h (MOI, ~100) after invasion, respectively. The wild type and the Δ*ppk1* mutant exhibited similar invasion profiles (Fig. 6A, B). However, the Δ*ppk1* mutant was reproducibly defective for longer-term IC survival (18 and 19 h), exhibiting a statistically significant ($P < 0.05$) >100-fold defect compared to the wild-type IC survival profile. The complemented *ppk1** strain survival was similar to that of the wild type. All strains survived equally well in MEM at the 3-h time point, showed identical gentamicin susceptibilities, and were fully resistant to distilled H_2O-syringe lysis (data not shown).

The Δ*ppk1* mutant exhibits a dose-dependent chick colonization defect. To assess the role of poly-P in commensal colonization, 1-day-old chicks were infected with wild-type and Δ*ppk1* strains at increasing inoculation levels (1.5×10^5, 1.5×10^6, and 1.5×10^7 CFU/infection). Chicks were sacrificed after 7 days, and cecal contents were assayed for viable *C. jejuni*. Wild-type *C. jejuni* colonized chicks at a minimum average of 1.79×10^8 CFU/g of cecal content at all inoculating doses, with nearly all infected chicks colonized to high levels (Fig. 7). In contrast, the Δ*ppk1* mutant colonization levels were dependent upon the inoculating dose. Strikingly, at an inoculating dose of 1.5×10^5 CFU, no Δ*ppk1* bacteria were recovered from any chick infected. The intermediate dose of the Δ*ppk1* mutant (1.5×10^6 CFU/0.5 ml) resulted in colonization of 8 of 10 chicks. The highest dose of the Δ*ppk1* mutant (1.5×10^7 CFU/0.5 ml) yielded colonization of all chicks, at levels similar to that for wild-type *C. jejuni*, with a mean concentration of 6.79×10^8 CFU per g of cecal content.

DISCUSSION

The poly-P molecule has been hypothesized as playing multiple diverse roles in the bacterial cell, including acting as a phosphate reservoir, an energy source, a regulatory molecule, a pH modulator, a metal chelator, and a structural component of transport channels (12, 43). Given *C. jejuni*'s relatively small genome (23, 31, 55), a molecule such as poly-P would be predicted to impact numerous aspects of *C. jejuni* biology, including those related to pathogenesis. In the present study, we have found that, similar to other bacteria, wild-type *C. jejuni* exhibited a significant, *ppk1*-dependent increase in

poly-P levels as the bacteria transitioned from exponential to stationary phase (Fig. 2). As discussed below, we have also found that poly-P accumulation in *C. jejuni* is important for numerous transmission-, colonization-, and virulence-related phenotypes. Although certain *C. jejuni* Δ*ppk1* phenotypes are consistent with those in other bacteria, interesting differences are also noted that may reflect genome dissimilarities and/or species-specific stresses. Finally, phenotype comparisons and poly-P assays suggest that poly-P and the SR likely intersect in *C. jejuni*; however, the two responses also impact distinct functions. To our knowledge, this is the first study demonstrating the importance of poly-P in *C. jejuni* biology and pathogenesis.

Our data demonstrate that poly-P is important for the transmission-related phenotypes of low-nutrient-stress survival, osmotic stress survival, and biofilm formation. *C. jejuni*'s ability to survive in nutritionally poor environments is particularly critical during conditions such as waterborne transmission, which despite the organism's fastidious laboratory culture requirements, is a major source of larger-scale *C. jejuni* outbreaks (5, 25, 66). Our low-nutrient-stress observations (Fig. 3) are consistent with studies of *ppk1* mutants in *E. coli* and other organisms, which also exhibit reduced survivability during starvation (40, 50, 70, 71). A mechanistic model to explain this has been developed from work in *E. coli*, where in wild-type bacteria a nutrient downshift causes an immediate upsurge in poly-P, which in turn complexes with and activates the ATP-dependent Lon protease to selectively degrade free ribosomal proteins, liberating amino acids to meet the nutritional requirements of the cell (44). *C. jejuni* harbors a putative but currently uncharacterized Lon protease (23, 31, 55); a similar link between poly-P and Lon in *C. jejuni* might explain the decreased survivability of the Δ*ppk1* mutant under nutrient deprivation conditions.

Resistance to high osmolarity is important for survival during food processing, in certain aquatic environments, and in fecal matter. Although a role for the heat shock and lipooligosaccharide gene *htrB* in osmotic shock survival has been proposed (56), little else is known about this phenomenon in *C. jejuni*. Our data indicate that *C. jejuni* requires poly-P for both growth and survival during osmotic stress, most acutely (i) when the organism must grow from isolated single bacteria into colonies (Fig. 4B) and (ii) during later growth stages in broth culture (Fig. 4A), where poly-P levels were shown to rise dramatically in wild-type but not the Δ*ppk1* mutant (Fig. 2B). A number of enteric pathogens lacking *ppk1* are also less tolerant to osmotic stress than the parental wild-type strains; these include *E. coli*, *Salmonella* spp., and *V. cholerae* (36, 49, 59). Although poly-P has been hypothesized to participate in osmotic shock protection by virtue of its polyanion structure (59), specific molecular mechanisms have not yet been elucidated.

In contrast to the osmotic-shock and low-nutrient data, and in marked contrast to poly-P mutants in other bacteria, the *C. jejuni* Δ*ppk1* mutant exhibited enhanced biofilm development versus the wild type (Fig. 5), with no obvious differences in planktonic growth rate (Fig. 2A). The role of poly-P in biofilm formation was first studied in *P. aeruginosa*, where a Δ*ppk1* mutant was defective for motility, biofilm maturation, and quorum sensing (63). Poly-P is likewise required by *V. cholerae*, *Bacillus cereus*, *P. aeruginosa*, and *Porphyromonas gingivalis* for motility and biofilm formation (18, 24, 61, 67). Biofilm formation is thought to protect bacteria from adverse environmental conditions and is considered an important virulence factor. Environmental biofilms have also been proposed as a likely mechanism by which *C. jejuni* survives hostile environments and overcomes its fastidious survival requirements, thereby contributing significantly to its worldwide prevalence (21, 37). We recently found that the *C. jejuni* SR mutant also exhibits increased biofilm formation (McLennan et al., unpublished). As with Δ*ppk1*, this is contrary to observations in other bacteria, where the loss of the SR typically leads to decreased biofilm formation. An ensuing hypothesis is that in *C. jejuni*, both the SR mutant and the Δ*ppk1* mutant may be constantly stressed, resulting in activation of alternative stress response pathways that may be distinct from those found in gammaproteobacteria (see the discussion of *rpoS* below) and which in turn accelerate conversion to a protective biofilm state.

We have also identified clear roles for poly-P in both virulence- and colonization-related attributes of *C. jejuni*. Intraepithelial cell survival is thought to be important for *C. jejuni* immune and chemotherapeutic evasion, in addition to damage, relapse, and persistence in the human host (19, 20, 38, 65). Although it represents an important virulence phenotype, very little is known about this aspect of *C. jejuni* pathogenesis. PPK1 is now the third *C. jejuni* factor, apart from SpoT and the ferrous iron Fe^{2+} transporter FeoB (51), shown to be required for extended intracellular survival in epithelial cells (Fig. 6). Previous cell biology-based work suggested that *C. jejuni* resides in a vacuole or vacuole-like compartment after cell internalization (32, 33), a hypothesis corroborated by a recent report describing a role for NOD1 in the innate immune response to *C. jejuni* cell infection (79). A requirement for SpoT, FeoB, and PPK1 in intracellular survival also supports this notion. Vacuoles are typically low-nutrient, low-iron environments (53). The SR is induced in such an environment, which would also be expected to require FeoB for reduced iron uptake. Poly-P's importance in low-nutrient survivability is consistent with this hypothesis and likely provides a mechanistic explanation for the Δ*ppk1* intracellular survival defect. An *S. enterica* serovar Typhimurium Δ*ppk1* mutant also displayed an invasion and long-term intracellular survival defect in HEp-2 epithelial cells (40). The only other study investigating a role for poly-P in *Campylobacter* spp. showed that a *C. coli* UA585 Δ*ppk1* mutant was as sensitive to macrophage killing as the wild type and that PPK1 was not involved in protection against oxygen radicals in macrophage cells (75). However, poly-P was shown to be important for macrophage survival of *S. enterica* serovar Typhimurium (40).

The importance of poly-P accumulation in vivo has been shown in various pathogens, including *Salmonella* spp. and *P. aeruginosa*, and for colonization of certain strains of *H. pylori* (7, 49, 63, 70). Chickens are a natural zoonotic reservoir for *C. jejuni*, and contamination of commercial broiler flocks is thought to account for the majority of human *C. jejuni* infections (47). Interestingly, the *C. jejuni* Δ*ppk1* mutant exhibited a dose-dependent colonization defect in chicks: no chicks were colonized to any detectable level at a $\sim 10^5$ CFU inoculum of Δ*ppk1* mutant, whereas the same dose of wild type colonized to 10^7 to 10^9 CFU/g of cecal content (Fig. 7). Although 10^5 CFU is the lowest inoculating dose used in our studies, wild-type *C.*

jejuni 81-176 colonizes chicks well at doses as low as 10^3 CFU (48); thus, this is a significant colonization defect for a fully motile *C. jejuni* mutant (see Table S1 in the supplemental material). The Δ*ppk1* mutant hyperbiofilm formation phenotype also appears to be dose dependent (Fig. 5). One hypothesis to explain the dose-dependent colonization for the Δ*ppk1* mutant is that at low doses, the mutant is primarily planktonic and significantly more susceptible than the wild type to in vivo stresses. As the dose increases, biofilm formation is enhanced in the mutant versus the wild type, protecting the Δ*ppk1* mutant during the initial (or later) stages of colonization. Recent reports indicate that several bacteria, including *P. aeruginosa* and *H. pylori*, form biofilms during infection (15, 26). Although such studies have not yet been conducted for *C. jejuni*, it is of note that we have found that the *C. jejuni* SR mutant, which also exhibits certain planktonic sensitivities yet forms highly exaggerated biofilms, is fully colonization competent in both chicks and mice (29; McLennan et al., unpublished). Future in vivo studies to address this directly should yield insight into an in vivo role for *C. jejuni* biofilms.

As described above, poly-P clearly affects certain conserved phenotypes in most bacteria studied, while other phenotypes are much more species specific. Specific differences between *C. jejuni* and other organisms may occur via a number of genetic and genomic mechanisms. For instance, in most bacteria, and especially other enteric pathogens, poly-P-deficient mutants are unable to express *rpoS*, and this has been proposed as the reason for certain *ppk1* mutant phenotypes (24, 39, 49, 68). *C. jejuni* lacks *rpoS*, which may account for some of the surprising phenotypic differences between the *C. jejuni* Δ*ppk1* mutant (Fig. 2 to 7 and see Table S1 in the supplemental material) and Δ*ppk1* mutants in *E. coli*, *Salmonella* spp., and *P. aeruginosa*. Alternatively, a double deletion of *ppk1* and *ppk2* may be necessary to completely deplete or severely limit poly-P in *C. jejuni*. However, a single *ppk1* deletion sufficiently induced motility defects in both *P. aeruginosa* and *V. cholerae*, each of which harbor both *ppk1* and *ppk2* (24, 52, 61), while the *C. jejuni* Δ*ppk1* single mutant was fully motile. *C. jejuni ppk1* gene regulation may also differ from that of other organisms. In *E. coli*, *ppk1* and the *ppx* exopolyphosphatase gene are in an operon; thus, the levels of PPK1 and PPX are also transcriptionally coregulated. In contrast, *ppk1* and *ppx* in *C. jejuni* are not found in an operon and thus may not be transcriptionally linked. There is also conflicting evidence as to whether the phosphate (pho) regulon regulates *ppk1* expression in various bacteria (27). *C. jejuni* was recently shown to harbor a PhoSR two-component signal transduction system which, like PhoBR in *E. coli*, controls numerous phosphate acquisition genes via binding to promoter pho box regions (76). The *C. jejuni ppk1* gene does not appear to be under the molecular control of the pho regulon, since neither a "traditional" pho box nor the recently identified PhoSR consensus binding sequence is found upstream of the *ppk1* gene, and *ppk1* was not reported as downregulated in a Δ*phoR* mutant (76).

Finally, previous work in *E. coli* has identified functional and regulatory links between the SR and poly-P accumulation (6, 44, 46, 60). In that organism, ppGpp inhibits poly-P hydrolysis by blocking the activity of PPX; in *E. coli* SR mutants lacking ppGpp, PPX remains active, resulting in diminished levels of poly-P (44). Consistent with this, and despite the above-mentioned lack of conserved *ppx*/*ppk* operon structure in *C. jejuni*, we also observed diminished levels of poly-P in the *C. jejuni* Δ*spoT* mutant (Fig. 2), suggesting that this mechanism of poly-P regulation may be conserved between *C. jejuni* and *E. coli*. Our phenotype data indicate that certain *C. jejuni* Δ*spoT* and Δ*ppk1* defects are similar (see above), while others are not (i.e., invasion and aerobic survival defects for Δ*spoT* but not Δ*ppk1*, osmotic shock and commensal colonization defects for Δ*ppk1* but not Δ*spoT*), indicating that these two stress responses provide both overlapping and complementary cellular functions. Further elucidation of how these multifunctional factors intersect as well as the precise nature of poly-P regulation in *C. jejuni* will provide many interesting avenues for future study and may serve as unique models for other bacteria as well.

In summary, the present study demonstrates the importance of poly-P in *C. jejuni* transmission, colonization, and infection of host cells and has established that poly-P likely interacts with SR mechanisms in *C. jejuni*. The phenotypic differences between the *C. jejuni* Δ*ppk1* mutant and Δ*ppk1* mutants in other organisms suggest that this work may also provide a model for further exploring novel, RpoS-independent roles for poly-P in *C. jejuni* and other bacterial species. Finally, the present study will serve as a platform for numerous future studies exploring the up- and downstream molecular events surrounding poly-P accumulation in *C. jejuni*, which should in turn lend significant additional insight into mechanisms allowing *C. jejuni* to remain such a prevalent human pathogen.

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