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Importance of Polyphosphate Kinase 1 for *Campylobacter jejuni* Viable-but-Nonculturable Cell Formation, Natural Transformation, and Antimicrobial Resistance[▽]

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Campylobacter jejuni*, a gram-negative, microaerophilic bacterium, is a predominant cause of bacterial gastroenteritis in humans. Although considered fragile and fastidious and lacking many classical stress response mechanisms, *C. jejuni* exhibits a remarkable capacity for survival and adaptation, successfully infecting humans and persisting in the environment. Consequently, understanding the physiological and genetic properties that allow *C. jejuni* to survive and adapt to various stress conditions is crucial for therapeutic interventions. Of importance is polyphosphate (poly-P) kinase 1 (PPK1), which is a key enzyme mediating the synthesis of poly-P, an essential molecule for survival, mediating stress responses, host colonization, and virulence in many bacteria. Therefore, we investigated the role of PPK1 in *C. jejuni* pathogenesis, stress survival, and adaptation. Our findings demonstrate that a *C. jejuni* Δ*ppk1* mutant was deficient in poly-P accumulation, which was associated with a decreased ability to form viable-but-nonculturable cells under acid stress. The Δ*ppk1* mutant also showed a decreased frequency of natural transformation and an increased susceptibility to various antimicrobials. Furthermore, the Δ*ppk1* mutant was characterized by a dose-dependent deficiency in chicken colonization. Complementation of the Δ*ppk*1 mutant with the wild-type copy of *ppk1* restored the deficient phenotypes to levels similar to those of the wild type. Our results suggest that poly-P plays an important role in stress survival and adaptation and might contribute to genome plasticity and the spread and development of antimicrobial resistance in *C. jejuni*. These findings highlight the potential of PPK1 as a novel target for therapeutic interventions.

Campylobacter jejuni, a gram-negative, microaerophilic bacterium, occurs as a commensal among the intestinal microflora of various animals, especially chickens and cattle (6, 73). However, *C. jejuni* can infect human hosts, invading the intestinal mucosa and causing watery and/or bloody diarrhea (9). *C. jejuni* is transmitted to humans primarily through the consumption of contaminated chicken products, raw milk, or water (2, 3). Currently, *C. jejuni* is considered a leading bacterial cause of human food-borne gastroenteritis (3, 61) and has also been associated with a plethora of symptoms, including acute neuromuscular paralysis (Guillain-Barré syndrome) (26). Since an appropriate vaccine for human campylobacteriosis has yet to be introduced, it has been suggested that *C. jejuni* infections might be alternatively controlled by reducing colonization in food animals (73). Consequently, determining the physiological and genetic properties that allow the survival of *C. jejuni* and its colonization of animal hosts, pathogenicity, and adaptation to various stresses is of critical importance.

The mechanisms underlying *C. jejuni* adaptation and survival under stresses imposed by its environment and host are not well understood. High variability between different *C. jejuni* strains and the unavailability of appropriate genetic tools and animal models have contributed to the lack of knowledge regarding its stress tolerance and pathogenicity. However, it is suggested that the capacity of *C. jejuni* to form viable-but-nonculturable (VBNC) cells under stress (14) and its readiness for natural transformation (68) and acquiring resistance to antibiotics (39) are among the strategies that promote stress adaptation and survival. Although little is known about the genetics underlying these processes, recent advances in *C. jejuni* genomics show that this bacterium carries several important genes that might play key roles in mediating stress adaptation and survival. Of particular interest are genes encoding polyphosphate (poly-P) kinases, *ppk1* (CJJ81176_1361) and *ppk2* (CJJ81176_0633), that were predicted to be involved in the metabolism of poly-P (22, 25, 47), an intracellular granule that impacts several physiological properties in many bacterial species, including pathogenicity, host colonization, adaptation to different environments, and survival (28, 31, 46).

Poly-P kinase 1 (PPK1) is encoded by *ppk1*, which mediates the synthesis of all or most of the poly-P in the cell (33), while *ppk2* encodes an enzyme (PPK2) that synthesizes GTP from poly-P (27). Both *ppk* genes have been associated with the metabolism of poly-P, which consists of phosphate residues that are linked by high-energy phosphoanhydride bonds and is widely distributed in bacterial species (60). Previous reports showed that poly-P plays important roles in bacterial survival and stress tolerance, including ATP production (8), entry of DNA through membrane channels (13, 54), capsule composition (67), maintaining nutritional requirements during starvation (34), motility, biofilm formation, and resistance to oxida-

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant description	Source or reference
Strains		
C. jejuni		
81-176	Wild type	Qijing Zhang
Δ*ppk1*/DG001	81-176 derivative with deletion in *ppk1* gene; *ppk1*::Kan	This study
Δ*ppk1*c/DG002	DG001 harboring pDG3, Cm	This study
81-176 (*dcuA*::*tetO*)	Carries tetracycline resistance marker for natural transformation; Tet	Qijing Zhang
E. coli DH5α	Used for cloning	Invitrogen
Plasmids		
pZErO-1	Cloning vector for making suicide vector; Zeo	Invitrogen
pRY111	*E. coli*-*Campylobacter* shuttle vector for complementation	71
pRK2013	Helper plasmid for conjugation	1
pUC4K	Source for kanamycin	Amersham
pDG1	pZErO-1 containing *ppk1* region plus 1 kb upstream and downstream of 81-176; Zeo	This study
pDG2	A portion of *ppk1* replaced with kanamycin resistance region from pUC4K in pDG1; Zeo Kan	This study
pDG3	pRY111 containing *ppk1* coding region and the upstream promoter sequence for complementation; Cm	This study

[a] Kan, kanamycin; Cm, chloramphenicol; Zeo, zeocin; *tetO*, tetracycline resistance gene.

tive, osmotic, heat, acid and alkaline stresses, and stationary-phase survival (28, 31, 46, 48, 50, 52, 65). Because of their importance in many bacterial species, it is not surprising to assume a role for PPK and poly-P in *C. jejuni* survival, colonization, and stress tolerance (8).

Interestingly, PPK1 has been shown to be important for *C. jejuni* stress responses and pathogenicity (10). However, the role of *ppk1* in key metabolic and physiological responses of *C. jejuni* still needs further analysis. For instance, it has been proposed that during starvation, poly-P might act as a reservoir for phosphorus and energy (7). Subsequently, poly-P would be crucial for maintaining viability/metabolism in stressed cells. This has been observed in *H. pylori*, where the occurrence of poly-P correlated with culturability and structurally intact cells (45). Poly-P-containing nonculturable *H. pylori* showed a capacity for ATP and mRNA synthesis after a nutrient stimulus (45). Consequently, poly-P might be an important factor for the formation of VBNC cells by stressed bacteria, including *C. jejuni*. Furthermore, natural transformation is perhaps one of the most important mechanisms in the adaptation of *C. jejuni*, and poly-P has been reported to play a role in the entry of DNA through membrane channels (13, 54). It follows that poly-P might be important for natural transformation, adaptation, and acquisition of antibiotic resistance genes in *C. jejuni*. Poly-P can further impact the survival and adaptation in *C. jejuni* by modulating antibiotic resistance properties. For example, poly-P interacted with *Escherichia coli* ribosomes (42), which are known targets of several antibiotics. These observations suggest that *ppk1* might be linked to important physiology and functions such as VBNC cell formation, natural transformation, and antimicrobial resistance in *C. jejuni*. Therefore, in the present study, we determined the contribution of PPK1 to *C. jejuni* stress responses and adaptation, including the ability to form VBNC cells under acid stress, natural transformation, and antimicrobial resistance. Furthermore, we assessed the impact of *ppk1* deletion on in vivo chicken colonization. Our findings highlight the importance of PPK1 in *C. jejuni* survival, adaptation to different environmental stresses, and in vivo colonization. These findings also indicate the suitability of PPK1 as a potential target for controlling the proliferation of this pathogen.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the bacterial strains and plasmids used in this study are described in Table 1. *C. jejuni* 81-176 is a highly invasive strain originally isolated from diarrheic patients (32). *C. jejuni* strains were cultured on Mueller-Hinton (MH) medium under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) at 42°C for 24 h. For isolation of *C. jejuni* from chicken feces and organs, MH agar plates were supplemented with *Campylobacter* selective supplement (SR117E; Oxoid, Lenexa, KS). *E. coli* strain DH5α was used for plasmid propagation and cloning purposes. *E. coli* strains were grown on Luria-Bertani medium at 37°C overnight. Chloramphenicol (20 μg/ml for *E. coli* and 10 μg/ml for *Campylobacter*), kanamycin (30 μg/ml), and zeocin (50 μg/ml) were added to the media where necessary.

Construction of the *C. jejuni ppk1* mutant and the complemented strain. Genomic DNA was extracted from *C. jejuni* strain 81-176 with a Masterpure DNA purification kit (Epicentre, Madison, WI) according to the manufacturer's instructions. Oligonucleotides were designed from the published genome sequence of *C. jejuni* strain 81-176 (GenBank accession no. NC_008787) with Vector NTI software and commercially synthesized by Integrated DNA Technologies (Skokie, IL). All of the oligonucleotides used in this study are listed in Table 2. Appropriate restriction sites were included in the primers to facilitate digestion and ligation into cloning vectors. Restriction enzymes were purchased from Promega (Madison, WI). To generate the deletion mutant, *ppk1* was amplified by using the PPK1 F and PPK1 R primers from the *C. jejuni* genomic DNA along with approximately 1 kb of DNA sequences upstream and downstream of the target gene. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA). Purified PCR products were ligated into zeocin-resistant pZErO-1 (Invitrogen, Carlsbad, CA), a zero background cloning vector, with a Fast-Link DNA ligation kit (Epicentre) and transformed to Library Efficiency DH5α *E. coli* competent cells (Invitrogen) to generate the plasmid pDG1. Plasmid (pDG1) DNA was isolated with a QIAprep spin mini prep kit (Qiagen), and the whole plasmid, except the target gene, was amplified by inverse PCR with the PPK1 INV F and PPK1 INV R primers. Purified inverse PCR products were ligated to a kanamycin resistant cassette from pUC4K, and the resulting suicide vector was named pDG2. pDG2 was electroporated into *C. jejuni* as described by Wilson et al. (69). Briefly, *C. jejuni* strains were grown on MH agar plates at 42°C for 24 h under microaerophilic conditions and the cells were harvested in 1 ml sterile, chilled, ice-cold wash buffer (15% glycerol, 272 mM sucrose) and pelleted (13,000 rpm for 5 min at

TABLE 2. Primers used in this study

Name	Sequence (5′–3′)[a]	Use
PPK1 F	ATAAAAGGTACCATTTAGCCATAAAACTCCCG	Amplification of 81-176 *ppk1* region
PPK1 R	AAAAAACTGCAGGCGATAGGTTTGAACCTTTA	
PPK1 INV F	ATAAAAGGATCCTACAGATCAAAGTCGTGCAA	Amplification of pDG1 except *ppk1*
PPK1 INV R	AAAAAAGGATCCGAACATTGGTCTAAAACACG	
PPK1 COMP F	AAATAACTGCAGGTCCTAAACCACTTTGCGCT	Amplification of *ppk1* gene for complementation
PPK1 COMP R	AAATAAGGTACCCCTCGCTTCCACCAGTTTTA	
CsrA F	TTATCGGAGAAGGTATAG	Real-time quantitative PCR amplification of cDNA from *ppk2*, *csrA*, *spoT*, *phosR*, *cmeC*, *pstS*, *pstC*, and CJJ81176_0750 from 81-176 strain
CsrA R	TTTCTAAGTATCATAAGGG	
SpoT F	GTAACCACTCGCACAATATC	
SpoT R	GATGTCGCAGTTTATTCTCC	
PhosR F	GCAAACATAATCATCACAACCAC	
PhosR R	GAGAGCAAGGATACAAAGAAGC	
CmeC F	GCTGCTGCTCAATTAGGTATAG	
CmeC R	GCTTCATAATCATACTCACTTGC	
PstS F	CCTTATACAAACTGGAATCAAATC	
PstS R	GACACATCACTCATTACAAGC	
PstC F	CGCTTATGCTTTAGGTATGAC	
PstC R	GCTGCCATCACCACTATC	
CJJ81176_0750 F	GGTCTTGTTGCCTTATTG	
CJJ81176_0750 R	GTATCGCTATGTTCTATGC	
PPK2 F	ATCTAATACTCCAACTTGTC	
PPK2 R	TTCTTCTTCTCCACTACG	

[a] Underlining indicates restriction sites added to the 5′ end of each primer.

4°C). The cells were then washed three times in ice-cold wash buffer and resuspended in 500 μl of ice-cold wash buffer, and 50 μl of cell suspension was used for electroporation. One microgram of pDG2 plasmid DNA was added to 50 μl of the cell suspension and mixed thoroughly by pipetting. The cell-DNA mixture was transferred into a prechilled 0.2-cm-wide cuvette and electroporated at 2.5 kV, 200 Ω, and 25 μF with a Gene Pulser Xcell (Bio-Rad, Hercules, CA). One hundred fifty microliters of SOC medium (Invitrogen) was added to stabilize the cells, and the suspension was spotted onto a nonselective MH agar plate and incubated overnight at 42°C. Following incubation, the cells were harvested, plated onto MH agar with kanamycin (30 μg/ml), and incubated at 42°C for 2 to 3 days under microaerophilic conditions. The kanamycin-resistant colonies were patched and streak purified, and the *ppk1* deletion was confirmed by PCR with the PPK1 F and PPK1 R primers. One of the PCR-confirmed mutant clones (DG001) was selected for further studies.

For construction of the complemented strain (the Δ*ppk1*c mutant), the *ppk1* gene, along with the potential upstream promoter sequence, was amplified from strain 81-176 chromosomal DNA with the PPK1 COMP F and PPK1 COMP R primers. Appropriate restriction sites for PstI and KpnI were incorporated in the oligonucleotides to facilitate ligation into digested pRY111, an *E. coli*-*Campylobacter* shuttle vector (71). The resulting complementation plasmid (pDG3) was introduced into the *C. jejuni* Δ*ppk1* mutant by conjugation as described previously (44). The resulting complemented strain (the Δ*ppk1*c mutant) was designated DG002.

TEM. Transmission electron microscopy (TEM) procedures were used as described previously (23, 46). Briefly, bacterial cells grown to the mid-log phase were adjusted to an optical density at 600 nm (OD_{600}) of 0.5 and fixed in 2% paraformaldehyde–2.5% glutaraldehyde, embedded in 1% agar, and postfixed in 1% osmium tetroxide. The samples were then dehydrated in a graded ethanol series and embedded in Spurr's resin (Ted Pella Inc., Redding, CA), and 80- to 90-nm sections were cut with a diamond microtome knife. The specimens were then stained with 2% uranyl acetate and lead citrate and examined under a Hitachi 7500 transmission electron microscope (Pleasanton, CA) at 80 kV at the Molecular and Cellular Imaging Center (Ohio Agricultural Research and Development Center [OARDC]; http://www.oardc.ohio-state.edu/mcic).

Induction and enumeration of VBNC cells of *C. jejuni*. *C. jejuni* VBNC cells were induced as described by Chaveerach et al. (14). Briefly, 1 ml of a culture grown overnight containing 5×10^8 bacterial cells/ml (OD_{600} of 0.5) was added to 4 ml of MH broth with the pH adjusted to 4.0 with formic acid. The cultures were incubated under microaerophilic conditions at 42°C for 3 h. The induction of VBNC cells was confirmed by determining the culturable cell counts by the spread plate method and viable cell counts by fluorescence microscopy with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)–4′,6-diamidino-2-phenylindole (DAPI) staining.

To determine culturable cell counts, 100-μl volumes of culture at 0, 0.5, 1, 2, and 3 h posttreatment with formic acid were serially diluted (10-fold) and plated onto MH agar in triplicate. The plates were incubated at 42°C under microaerophilic conditions, and the number of CFU per milliliter (culturable count) was calculated.

At the aforementioned time intervals, 1 ml of culture was stained with CTC-DAPI for actively respiring and total *C. jejuni* cell counts as described by Cappelier et al. (12). Briefly, CTC (Polysciences, Inc., Warrington, PA) was added to a final concentration of 5 mM to 1 ml of formic acid-treated cells and incubated in the dark for 1 h at room temperature. The cells were then counterstained with 5 μg/ml of DAPI for 30 min. The cells were subsequently pelleted by centrifuging at 13,000 rpm for 4 min, and the pellet was resuspended in 100 μl of phosphate-buffered saline (PBS) and fixed with formaldehyde. Twenty microliters of the cell suspension was placed on a clean, oil-free slide, and an 18-mm coverslip was placed on the sample and sealed with nail polish. The slides were observed with a Zeiss (Thornwood, NY) Axiophot upright fluorescence microscope equipped with AxioCam HRc and Axiovision 2.05 software. The cells were subjected to fluorescent light with an excitation filter of 405 nm and a 455-nm dichroic mirror, which facilitates the simultaneous visualization of both DAPI (blue) and CTC (red). Viable cells convert CTC into insoluble formazan crystals, which accumulate in the cell and appear red under fluorescence microscopy, while both viable and dead cells are stained by DAPI. Ten fields were counted for each sample, with an average of 90 bacteria in each field. The percentage of viable cells was calculated as follows: % viability = (viable cell count/total cell count) ×100. Total cell counts were determined by adding the viable (red) and dead (blue) cell counts. The results represent the mean of three independent experiments. We also recorded the OD_{600} at the specified time points after CTC staining in order to observe the temporal change in intensity of tetrazolium reduction and further confirm the viability results generated from the fluorescence microscopy counts.

Flow cytometry. Flow cytometry analysis of the bacterial samples was performed after formic acid treatment to determine possible change in cell morphology and granularity (the shifts in forward scatter [FSC] and side scatter [SSC]) with a FACScalibur (BD Biosciences, San Jose, CA) as described previously (66). Briefly, 100 μl of the bacterial samples at 0, 0.5, 1, 2, and 3 h posttreatment were diluted to 1 ml with 1× PBS (pH 7.4) and the samples were analyzed at an excitation wavelength of 488 nm. All parameters were read as

logarithmic values, and 100,000 events were recorded for each sample. The assay was repeated two times.

Natural transformation frequency. Natural transformation was performed as described previously (29). Briefly, cultures of the *C. jejuni* wild-type, Δ*ppk1*, and Δ*ppk1*c strains grown overnight on MH agar plates were resuspended in fresh MH broth to an OD_{600} of 0.05. The bacterial suspension (0.5 ml) was then incubated in sterile tubes at 42°C with shaking at 200 rpm under microaerophilic conditions for 3 h. After adding 1 μg of donor DNA from the *dcuA*::*tetO* mutant of *C. jejuni* 81-176 (tetracycline resistant), the bacterial suspension was incubated for another 4 h under the conditions described above. Transformants and total bacterial counts were obtained by plating on MH agar with or without tetracycline (5 μg/ml), respectively. Transformation frequency was expressed as the number of transformants from 1 μg of DNA divided by the total bacterial count. The experiment was repeated three times with triplicate transformation reactions each time.

Antibiotic susceptibility testing. The susceptibility of the wild-type, Δ*ppk1*, and Δ*ppk1*c strains to various antimicrobials was determined by the standard microtiter broth dilution method as described previously (38, 49). The bacterial cultures were grown to mid-log phase and adjusted to an OD_{600} of 0.05 in MH broth. One hundred microliters of culture was then added to serially diluted (twofold) antimicrobials on a 96-well microtiter plate, mixed by pipetting, and incubated at 42°C under microaerophilic conditions for 2 days. The MIC was determined by recording the lowest concentration of an antimicrobial showing complete inhibition of bacterial growth. The antimicrobials and compounds used in this study were ciprofloxacin, erythromycin, tetracycline, rifampin (rifampicin), polymyxin B, cefotaxime, deoxycholic acid, taurocholic acid (Sigma Chemical Co., St. Louis, MO), sodium dodecyl sulfate (SDS; EM Science, Gibbstown, NJ), cholic acid (ACROS Organics, Belgium), and ethidium bromide (Amresco, Solon, OH). The susceptibility testing was repeated three times, and the mean MIC (μg/ml) was calculated for each antimicrobial.

Survival under nutrient downshift. The role of PPK1 in *C. jejuni* survival under nutrient downshift was assessed as described previously (10, 49). Briefly, mid-log-phase cultures of the wild-type, Δ*ppk1*, and Δ*ppk1*c strains were pelleted and washed twice with minimal essential medium (MEM). The pellets were resuspended in MEM, and the OD_{600} was adjusted to 0.05. The suspensions were then incubated under microaerophilic conditions at 42°C with shaking at 200 rpm. One hundred-microliter volumes of the culture at different time points were serially diluted (10-fold) in MEM and plated onto MH agar in triplicate. The plates were incubated under microaerophilic conditions, and the number of CFU/ml was calculated. The experiment was repeated three times.

Reverse transcription (RT)-PCR analysis. To study differential gene expression in the wild-type and mutant strains with and without formic acid treatment, we targeted key genes involved in phosphate uptake (*phosR*, CJJ81176_0899; *pstS*, CJJ81176_0642; *pstC*, CJJ81176_0643; and the periplasmic substrate binding protein-encoding gene, CJJ81176_0750) (53, 70), the stringent response (*spoT*, CJJ81176_1288) (24), and multidrug resistance (*cmeC*, CJJ81176_0388) (38), as well as posttranscriptional global regulator *csrA* (CJJ81176_1121) (20). The real-time quantitative PCR primers used in this study were designed with Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA). The wild-type and Δ*ppk1* mutant strains were grown to mid-log phase at 42°C in MH broth under microaerophilic conditions, and 1 ml of culture with an OD_{600} of 0.5 was added to 4 ml of MH broth with or without pH adjustment to 4.0 with formic acid and incubated under microaerophilic conditions at 42°C for 30 min. Formic acid-treated, as well as untreated, wild-type and mutant strains were used for total RNA extraction with an RNeasy Mini kit (Qiagen), and cDNA was synthesized with SuperScript III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's protocol. Quantitative RT-PCR was performed with a SensiMixPlus SYBR RT-PCR kit (Quantace, Norwood, MA) in a Mastercycler ep realplex^2 thermal cycler (Eppendorf, Westbury, NY). The relative expression of different genes in the wild-type and mutant strains was normalized with the *rpoA* (CJJ81176_1582) gene amplified from the corresponding sample. The difference in the expression of the genes was determined by the threshold cycle (C_T) method, and the assay was repeated three times with two replicates each time for each sample.

Since PPK2 is also involved in the metabolism of poly-P (27), we performed quantitative RT-PCR analysis of *ppk2* expression in the wild-type and Δ*ppk1* mutant strains as described above.

Chicken colonization studies. Day-old broiler chicks were obtained from a local hatching facility at the Food Animal Health Research Program (OARDC, Wooster, OH), and the chicks were confirmed to be negative for *Campylobacter* by culturing of cloacal swabs on MH agar with *Campylobacter* selective supplement (SR117E; Oxoid). The chicks were divided randomly into six groups with five chicks in each group. Groups 1, 2, and 3 were inoculated orally with 10^3, 10^4, and 10^5 CFU/chick of the wild-type bacterium, respectively, while groups 4, 5, and 6 received 10^3, 10^4, and 10^5 CFU/chick of the Δ*ppk1* mutant in 200 μl of 1× PBS (pH 7.4), respectively. The chicks were given feed and water ad libitum and cared for in accordance with the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care. The chicks from each group were euthanized on day 8 postinoculation. Cecal contents, feces, and bursae were collected aseptically, weighed, and homogenized in 1× PBS (pH 7.4). The suspensions were serially diluted (10-fold) and plated onto MH agar with *Campylobacter* selective supplement. The plates were incubated at 42°C under microaerophilic conditions, and the number of CFU per gram of cecal contents, feces, or bursas was calculated. The data represent the average from five chicks.

Statistical analysis. All of the data generated in this study were analyzed by one-way analysis of variance, followed by Tukey's honestly significant difference test and were expressed as mean ± SE (standard error). A *P* value of <0.01 or 0.05 (α level) was considered statistically significant for all of our assays.

RESULTS

The Δ*ppk1* mutant is defective in the accumulation of poly-P granules. The ability to accumulate poly-P has been demonstrated in several bacteria, and PPK mutants have been shown to have low levels of poly-P (4, 10, 46, 65). TEM of the *C. jejuni* wild-type and Δ*ppk1* mutant strains revealed the presence of large putative poly-P granules in the wild-type bacterial sections, while such granules were either absent or present as relatively smaller and indistinct granules in the Δ*ppk1* mutant (Fig. 1A). Specifically, large putative poly-P granules were found in 30% or more of the examined wild-type sections, and these granules were absent in the majority of Δ*ppk1* mutant sections, with only 3% or less of the sections showing comparatively small and indistinct granules. Our results corroborate the previous finding showing decreased levels of poly-P in the *ppk1* mutant strain compared to the wild type with the toluidine blue O staining assay (10).

We also monitored the growth of the wild-type, Δ*ppk1*, and Δ*ppk1*c strains in MH broth by plating 100 μl of the culture at 0, 10, 20, 30, 40, 50, 60, and 70 h after inoculation to determine if these strains have any growth defects under standard incubation conditions. All of the strains exhibited similar growth patterns in MH broth (Fig. 1B).

Role of poly-P in the formation of VBNC cells of *C. jejuni*. Since VBNC cell formation has been suggested as a mechanism for *C. jejuni* survival under environmental stress, we investigated the contribution of poly-P to VBNC cell formation. VBNC cells were induced with formic acid as described previously (11, 14), and direct culturable, total, and viable cell counts were determined at different time points. At 0 h, the wild-type and Δ*ppk1* and Δ*ppk1*c mutant strains showed a high number of culturable cells (5×10^8 CFU/ml). However, the culturability of these strains decreased drastically (5×10^2 CFU/ml) at 30 min posttreatment with acid, which was not significantly different among the three strains. Interestingly, cell culturability was completely lost at 1 h in all three strains (Fig. 2A). Despite the loss of culturability, CTC-DAPI staining showed the presence of viable cells (i.e., stained with CTC) of the wild-type, Δ*ppk1*, and Δ*ppk1*c strains after formic acid treatment, suggesting the formation of VBNC cells. The wild-type strain had a significantly ($P < 0.01$) higher number of VBNC cells than did the Δ*ppk1* mutant strain (Fig. 2B). The number of viable cells of the wild-type strain decreased gradually over time, while the number of these cells of the Δ*ppk1* mutant drastically dropped at 1, 2, and 3 h posttreatment

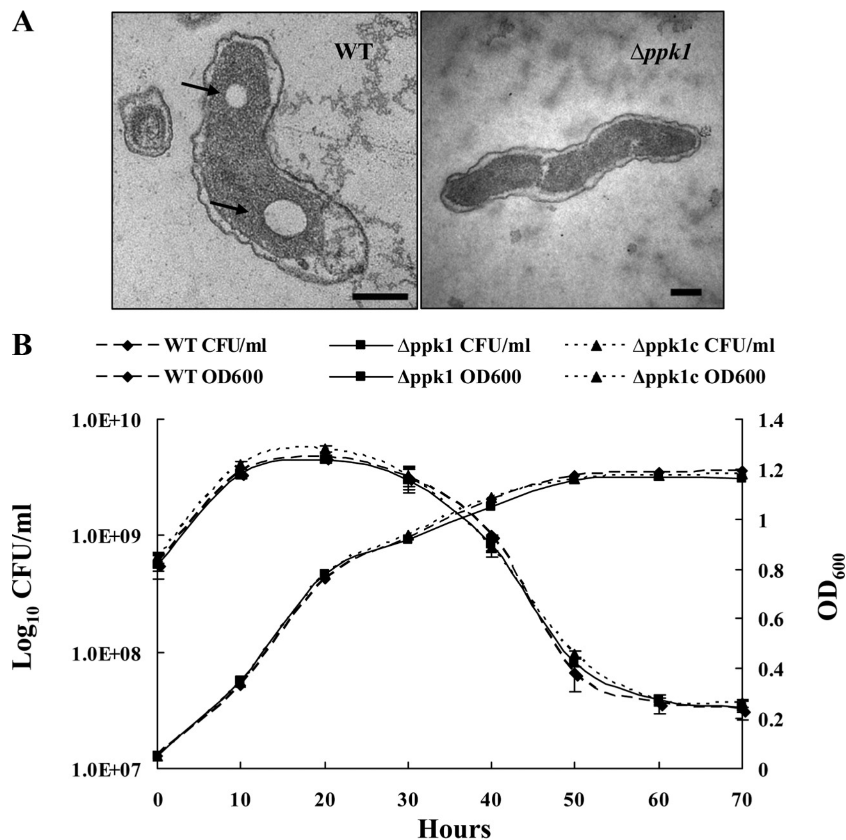

FIG. 1. (A) Transmission electron micrographs of the wild-type and Δ*ppk1* mutant *C. jejuni* strains. The TEM sections show large, distinct poly-P granules (arrows) in the wild type but not in the Δ*ppk1* mutant, as observed with other bacteria (46, 65). Representative images are shown. Bars, 200 nm. (B) Growth curves of the wild-type, Δ*ppk1*, and Δ*ppk1*c strains. The growth curves were assessed by growing the bacteria in MH broth and direct plating on MH agar in triplicate or by measuring the OD_{600} at different time points. Data represent the mean ± SE of two independent experiments. WT, wild type.

(Table 3). In the wild-type strain at 1 h, 96% of the cells were viable but none were culturable. In contrast, cells of the mutant strain showed only 36% viability while none were culturable. A similar trend was seen at 2 h with 94% of the cells maintaining viability in the wild type compared to 4% in the Δ*ppk1* mutant strain. At 3 h posttreatment, there was a significant decline in the viable cell counts of the wild-type strain (42%) but still the viable counts were significantly higher than that of the Δ*ppk1* mutant (3%). Complementation of the mutant with the wild-type copy of *ppk1* restored its viability to levels similar to those of the wild type. We also recorded the OD_{600} at specified time points after CTC staining, and we found that the viability followed a trend similar to that observed by fluorescence microscopy (Fig. 2A). The difference between the viable counts of the wild-type and Δ*ppk1* mutant strains was significant ($P < 0.01$) at 1, 2, and 3 h posttreatment with acid.

We performed fluorescence-activated cell sorter analysis to determine if bacterial cells undergo changes in morphology and granularity after formic acid treatment. Our results showed a change in cell size (FSC) and granularity (SSC) in the mutant strain compared to the wild-type strain from 1 to 3 h posttreatment. Separation of the total (P1) cell population based on their size and granularity demonstrated that there was a significant ($P < 0.01$) drop in P1 gated cells of the Δ*ppk1* mutant from 1 to 3 h posttreatment with formic acid compared to those of the wild-type strain (Fig. 3). Complementation of the mutant with the wild-type copy of *ppk1* restored cell size and granularity to wild-type levels.

The Δ*ppk1* mutant exhibits a decrease in transformation frequency. To determine if *ppk1* has a role in natural transformation, we assessed the transformation frequency in the wild-type, Δ*ppk1*, and Δ*ppk1*c strains. Since the *C. jejuni* 81-176 strain used in the present study is pTet (confers tetracycline resistance) plasmid cured, we used genomic DNA from a *dcuA*::*tetO* mutant of the 81-176 strain (tetracycline resistant) as the donor for determining the transformation frequency. The Δ*ppk1* mutant showed a significant decrease (18-fold, $P < 0.01$) in natural transformation frequency compared to the wild-type strain (Fig. 4). Complementation of the mutant with the wild-type copy of *ppk1* restored the transformation frequency to wild-type levels.

The Δ*ppk1* mutant is sensitive to various antimicrobials and bile acids. In order to determine the contribution of *ppk1* to antimicrobial resistance, we tested the susceptibility of the 81-176 wild-type, Δ*ppk1*, and Δ*ppk1*c strains to various antimicrobials and compounds. Our results showed that the Δ*ppk1* mutant strain was more susceptible to several antimicrobials (Table 4) than was the wild-type strain. For example, the Δ*ppk1* mutant showed a significant increase in susceptibility to erythromycin (128-fold, $P < 0.01$), cefotaxime (66-fold, $P <$

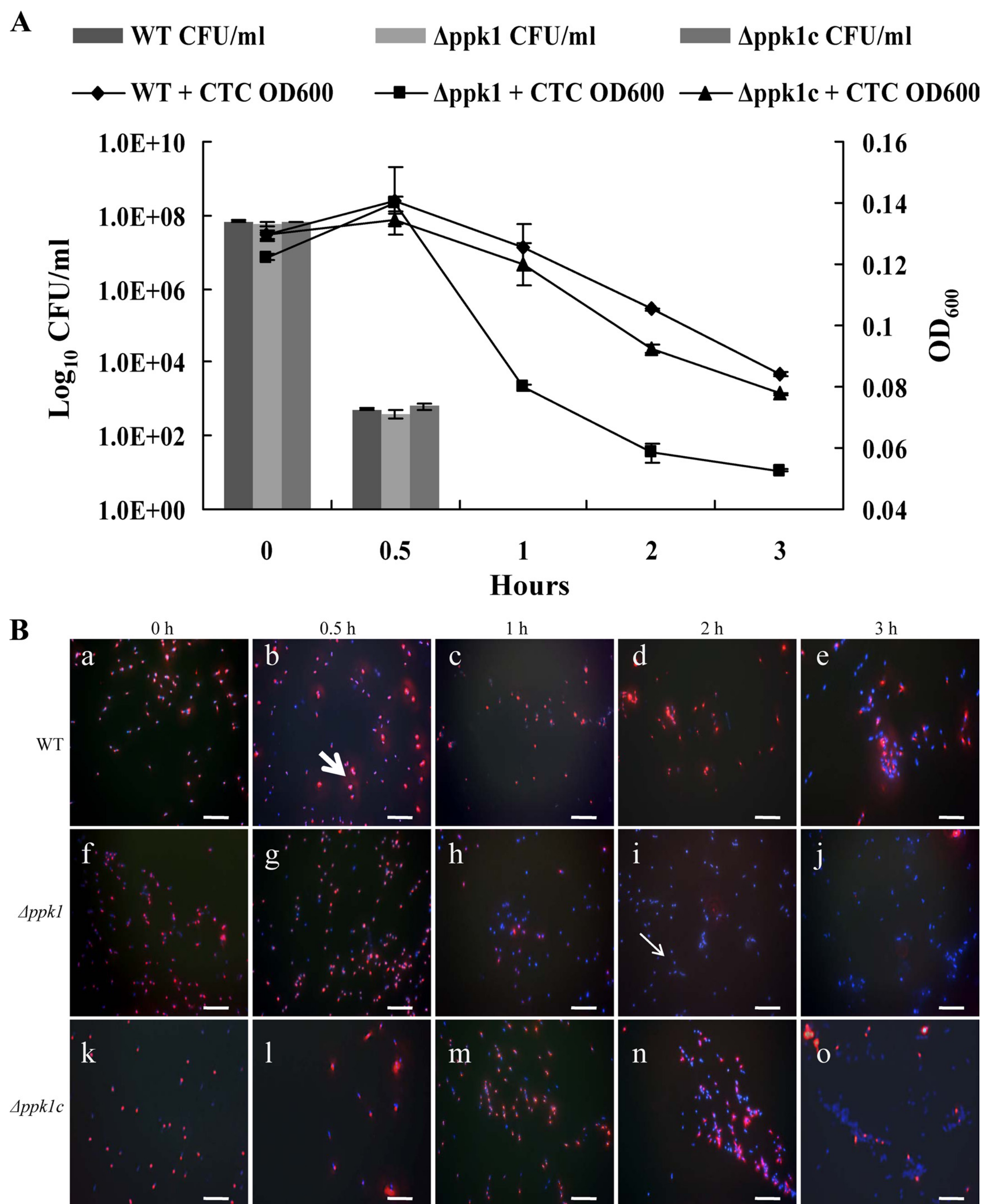

FIG. 2. Effect of *ppk1* deletion on the formation of VBNC cells of *C. jejuni*. (A) Culturability of the wild-type, Δ*ppk1*, and Δ*ppk1*c strains after formic acid treatment as determined by CFU enumeration and OD_{600} measurements after CTC staining. The data represent the mean ± SE of three independent experiments. (B) Fluorescence microscopy images of CTC-DAPI-stained *C. jejuni* cells showing viable (CTC-stained red cells, thick arrow) and dead (DAPI-stained blue cells, thin arrow) cells at different time points after formic acid treatment. Microscopy images a, b, c, d, and e (wild-type strain); f, g, h, i, and j (Δ*ppk1* mutant strain); and k, l, m, n, and o (complemented strain) are the representative images of three independent experiments treated with formic acid for 0, 0.5, 1, 2, and 3 h, respectively. Note the drop in viable cells (red cells) of the Δ*ppk1* mutant at 1, 2, and 3 h posttreatment compared to the wild-type strain. Bars, 10 μm. WT, wild type.

0.01), and ciprofloxacin (16-fold, $P < 0.05$). The Δ*ppk1* mutant strain was also sensitive to rifampin (twofold), polymyxin B (fourfold), and tetracycline (eightfold). Interestingly, the Δ*ppk1* mutant showed susceptibility to cholic acid (8-fold), taurocholic acid (8-fold), and deoxycholic acid (16-fold). In addition, the Δ*ppk1* mutant demonstrated 31-fold and 4-fold increases in sensitivity to ethidium bromide and SDS, respectively. Complementation of the mutant with the wild-type copy

TABLE 3. Percentages of viable cells as determined by CTC-DAPI staining[a]

Strain	% of viable cells after:				
	0 h	0.5 h	1 h	2 h	3 h
Wild type	100	98.4	96.7	94.5	42.4
Δ*ppk1* mutant	100	97.7	30.5	3.5	2.8
Δ*ppk1*c mutant	100	98.9	95.2	46.9	27.6

[a] A total of 10 fields were counted for each sample, with an average of 90 bacteria in each field. The percentage of viable cells was calculated as follows: % viability = (viable cell count/total cell count) ×100. Total cell counts were determined by adding viable (red) and dead (blue) cell counts. The results represent the mean of three independent experiments.

of *ppk1* either partially or completely restored resistance to wild-type levels.

Transcription of the *ppk2*, *phosR*, *pstS*, *pstC*, *cmeC*, *csrA*, *spoT*, and CJJ81176_0750 genes was affected in the Δ*ppk1* mutant. In order to understand the mechanism behind PPK1-mediated stress responses and adaptation, quantitative RT-PCR analysis was performed to examine the expression of phosphate regulon genes (*phosR*, *pstS*, *pstC*, and the periplasmic substrate binding protein-encoding gene, CJJ81176_0750), PPK2 (*ppk2*), the multidrug resistance efflux pump gene (*cmeC*), the global posttranscriptional regulator (*csrA*), and the stringent response regulator (*spoT*). Interestingly, the aforementioned genes were found to be significantly ($P <$ 0.01) upregulated (fivefold or more) in the Δ*ppk1* mutant relative to the wild-type strain (Fig. 5). Of particular interest, *pstC*, CJJ81176_0750, *pstS*, *phosR*, and *ppk2* were upregulated 48-fold, 40-fold, 30-fold, 9-fold, and 9-fold, respectively. Gene expression was also analyzed after formic acid treatment and during the induction of VBNC cells. Our results showed that, with the exception of *ppk2*, the expression of the aforementioned genes was not affected in the acid-treated Δ*ppk1* mutant. The *ppk2* gene was significantly downregulated (fivefold) ($P < 0.05$) in the Δ*ppk1* mutant after acid treatment. However,

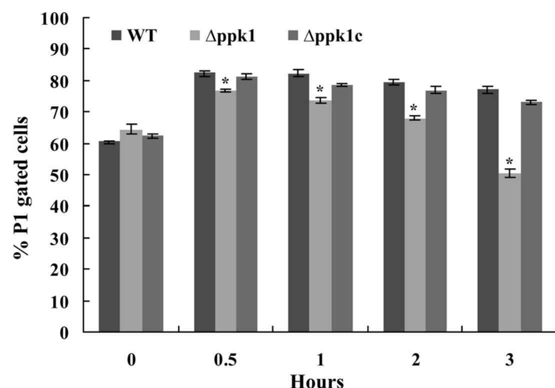

FIG. 3. Two-parameter FSC/SSC flow cytometry analysis of *C. jejuni* strains with and without formic acid treatment. FSC represents cell size, while reverse scatter is for cell granularity. A gate (P1) was set on FSC versus SSC to select the cell population for determining the change in cell size/granularity. The percent changes in P1-gated cells of the wild-type and Δ*ppk1* and Δ*ppk1*c mutant strains at different time points after formic acid treatment are shown. The bars represent the mean ± SE of two independent experiments. *, $P < 0.01$. WT, wild type.

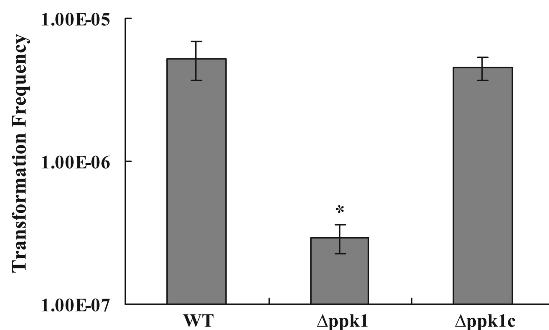

FIG. 4. Effect of *ppk1* deletion on *C. jejuni* natural transformation. The donor DNA contained a tetracycline resistance (*tetO*) marker. The data represent the mean transformation frequency ± SE of three independent experiments with triplicate transformation reactions in each experiment. *, $P < 0.01$. WT, wild type.

the expression of *ppk2* and the aforementioned genes was not affected in the wild type before and after acid treatment.

Poly-P is required for dose-dependent chicken colonization. We assessed the ability of the Δ*ppk1* mutant to colonize the cecum and bursa and its shedding in the feces of 2-day-old chicks. The dose dependency of colonization was studied by inoculating the chicks with three doses of inoculum separately, 10^3, 10^4, and 10^5 CFU/chick ($n = 5$). Our results demonstrated that the Δ*ppk1* mutant was significantly defective ($P < 0.05$) in chicken colonization at all inoculum doses for all organs and feces compared to the wild type (Fig. 6). Chicks infected with 10^3 or 10^4 CFU/chick of the Δ*ppk1* mutant had no detectable bacteria in any of the samples tested. However, chicks infected with 10^5 CFU/chick of the Δ*ppk1* mutant were colonized with approximately 4 logs, 3 logs, and 2 logs fewer bacteria in the cecal contents, feces, and bursae, respectively, compared to the wild-type strain.

DISCUSSION

To better understand the role of poly-P in stress survival and environmental adaptation mechanisms in an important enteric pathogen, *C. jejuni*, we constructed a deletion mutant by targeting PPK1 (Δ*ppk1*), an enzyme that mediates poly-P synthe-

TABLE 4. Susceptibility of *C. jejuni* to antimicrobials, bile acids, and other compounds

Agent	Inhibitory concn(μg/ml)		
	Wild type	Δ*ppk1* mutant (fold difference)[a]	Δ*ppk1*c mutant
Ciprofloxacin	0.26	0.016 (16)	0.13
Erythromycin	0.5	0.003 (128)	0.12
Cefotaxime	2.0	0.03 (66)	1.0
Rifampin	103	51.5 (2)	51.5
Polymyxin	4.0	1.0 (4)	1.0
Tetracycline	2.06	0.26 (8)	2.06
Ethidium bromide	1.25	0.04 (31)	1.25
SDS	375	93.75 (4)	375
Cholic acid	5,156	645 (8)	5,156
Taurocholic acid	36,000	4,500 (8)	36,000
Deoxycholic acid	16,500	1,031 (16)	8,250

[a] Fold difference in susceptibility between the wild type and the Δ*ppk1* mutant.

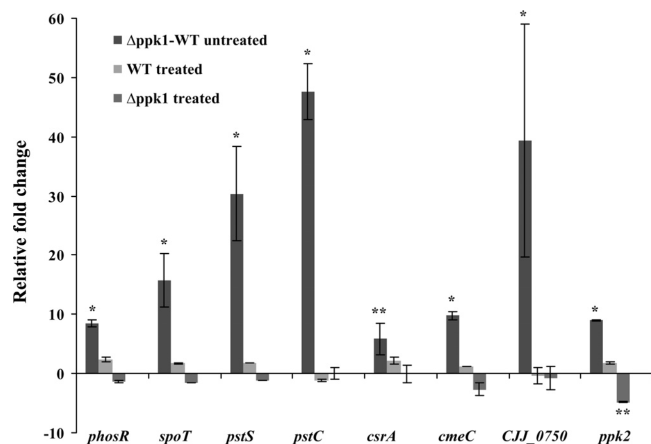

FIG. 5. Histogram showing the relative change in the expression of target genes before and after formic acid treatment. The relative change ($2^{-\Delta\Delta C_T}$) in gene expression was calculated from the $\Delta\Delta C_T$ value after normalization. Δ*ppk1*-WT (wild type) untreated indicates the fold change in gene expression of the Δ*ppk1* mutant strain relative to the wild-type strain. WT treated indicates the fold change in gene expression in the wild-type strain treated with formic acid relative to the untreated wild type. Δ*ppk1* treated indicates the relative fold change in gene expression of the Δ*ppk1* mutant treated with formic acid relative to the untreated Δ*ppk1* mutant. Genes with a twofold or greater ($P < 0.01$ or 0.05) relative change in expression were considered upregulated or downregulated. Each bar represents the mean ± SE of the relative fold change in expression from three independent experiments with triplicate reactions for each sample. *, $P < 0.01$; **, $P < 0.05$.

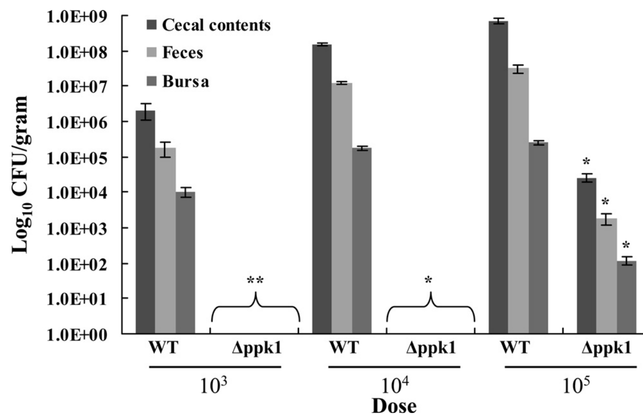

FIG. 6. Effect of *ppk1* deletion on chicken colonization. Chicks were randomly assigned to six groups of five. The first three groups were inoculated with 10^3, 10^4, and 10^5 CFU/chick of the wild-type strain, while the other three groups received 10^3, 10^4, and 10^5 CFU/chick of the Δ*ppk1* mutant strain. At 8 days postinoculation, the chicks were euthanized and the numbers of CFU/gram of cecal contents, feces, and bursae were calculated. Each bar represents the mean ± SE of five chicks. *, $P < 0.01$; **, $P < 0.05$. WT, wild type.

sis in the cell. Although the *C. jejuni* Δ*ppk1* mutant showed growth patterns similar to those of the wild-type strain, as expected, the mutant was deficient in the accumulation of poly-P and exhibited defects in key survival and adaptation responses. Significantly, the Δ*ppk1* mutant showed reduced abilities to form VBNC cells under stress, acquire DNA through natural transformation, and resist antimicrobials. The impairment of these physiological and stress responses, along with other observed deficiencies, confirmed that poly-P plays an essential role in the survival and adaptation of *C. jejuni*.

The absence of *ppk* genes was associated with defects in growth, responses to stress and starvation, and viability in several bacterial species (31, 40, 45, 58). This is also true of *C. jejuni* survival and stress tolerance, as our results showed that the Δ*ppk1* mutant, after losing culturability (Fig. 2A), exhibited reduced viability (Table 3) and an inability to form VBNC cells under acid stress (Fig. 2B). Furthermore, flow cytometry analysis showed a significant change in cell size and granularity of the Δ*ppk1* mutant during acid treatment (Fig. 3), probably indicating an increase in dead cells (35) and corroborating the inability to form VBNC cells, which have been reported to occur in spiral (noncoccoidal) forms in *C. jejuni* (19). Poly-P particles have been found in starved and morphologically altered *Vibrio parahaemolyticus* (15), while Nilsson et al. (45) showed that poly-P accumulated in structurally intact coccoid forms of starved *H. pylori*, probably for storing energy during starvation. Despite this, no direct association between poly-P and PPK1 and the formation of VBNC cells has been reported previously. This is important, as VBNC cell formation has been proposed as a strategy for surviving environmental stresses in many bacterial species, including enteric pathogens such as *C. jejuni* (55, 56). While usually undetectable by standard culturing techniques, in many cases, VBNC cells were reported to maintain pathogenicity, further increasing the public health importance of these forms. Although VBNC cell forms have been reported for *C. jejuni* strains that were exposed to starvation and other stresses (14, 37, 55), the significance of these forms in the epidemiology of *C. jejuni* has been a subject of debate (43, 57, 63), probably due to variations in the animal model, dose of VBNC cells, and strains used (14). However, it appears that VBNC *C. jejuni* cells were successfully resuscitated after passage in embryonated eggs (12, 14) and the strains recovered their capacity to adhere to HeLa cells, an indication of regained pathogenicity (12).

PPK1 is responsible for most of the poly-P synthesis in bacterial cells (8), which was also inferred from our electron microscopy images that showed deficient accumulation of poly-P granules in the *C. jejuni* Δ*ppk1* mutant (Fig. 1A). Analysis of *ppk* mutants of other bacteria has also revealed defective poly-P granules (46, 65). Since poly-P acts as an energy store allowing cells to tolerate stress (45), we propose that poly-P- and PPK1-deficient bacteria lose their ability to enter a VBNC state, which impacts their capacity to reduce metabolism and potentially tolerate and adapt to environmental stresses and the possibility for recovery when conditions are favorable (41). With the exception of *ppk2*, which was downregulated under formic acid treatment, our quantitative RT-PCR data did not show any significant changes in the expression of selected genes involved in phosphate uptake and stress tolerance in the Δ*ppk1* mutant (Fig. 5). Since *ppk2* mediates a poly-P-driven reverse reaction, synthesizing GTP from GDP in a greater magnitude (75-fold) than the forward reaction (i.e., poly-P synthesis from GTP) (51), the downregulation of *ppk2* can be interpreted as an additional effort by the cell to decrease energy (GTP/ATP) expenditure and maintain its already decreased poly-P reserves, which might be needed to form VBNC cells and resist stress. Unlike PPK1, PPK2 is stimulated and

becomes stable in the presence of poly-P (51). As a result, the absence of or decrease in poly-P accumulation (Fig. 1A) might have prompted the overexpression of *ppk2* in the non-acid-treated Δ*ppk1* mutant compared to the wild type (Fig. 5), possibly compensating for the deficiency in poly-P-dependent PPK2 stability and partially maintaining its functions. Interestingly, the upregulation of *ppk2* in the non-acid-treated Δ*ppk1* mutant was accompanied by overexpression of the genes related to phosphate regulation and uptake, possibly for maintaining intracellular phosphate levels (51). This does not contradict the gene expression profiles of the acid-treated Δ*ppk1* mutant, since VBNC cell formation might require a certain decrease in some metabolic and cellular processes, which would conserve energy and phosphate utilization and consequently limit the need for the overexpression of the aforementioned genes. However, it should be emphasized that the precise mechanism(s) by which poly-P influences the formation of VBNC cells requires further investigation.

An important mechanism for adaptation to environmental changes and antibiotic stress is the acquisition of important genetic material through natural transformation (16, 29, 30). Natural transformation plays an important role in mediating genetic diversity and the acquisition of antibiotic resistance in *C. jejuni* while also facilitating genetic manipulations in this naturally competent bacterium (17, 29, 68). Interestingly, poly-P is involved in the formation of cell membrane channels that allow DNA uptake (13, 54). However, to our knowledge, the impact of poly-P and its associated enzyme (PPK1) on natural transformation has not been investigated before. Our results show that the Δ*ppk1* mutant was significantly deficient in the frequency of natural transformation (Fig. 4) compared to the wild-type and complemented strains. While it is true that the Δ*ppk1* mutant showed an increased susceptibility to tetracycline (Table 4), it should be emphasized that the transformation experiments with the tetracycline gene occurred under nonselective conditions. Therefore, the acquisition of the tetracycline resistance gene by the mutant should have not been affected by its susceptibility to tetracycline. We suggest that PPK1 and poly-P possibly impact genetic diversity, acquisition of antibiotic resistance genes, and adaptation in *C. jejuni* by indirectly impacting the entry of DNA from the environment. This is important, as *ppk*-deficient bacteria, especially those with relatively smaller genomes, like *C. jejuni*, might be incapable of accessing collective gene pools ("supragenome"), which greatly restricts natural transformation and adaptation to environments (5).

Poly-P increases the activity of the stationary-phase RNA polymerase RpoS (59), possibly leading to adaptive mutations (21), including those that induce resistance to antibiotics (59). However, certain stress response factors, such as RpoS, are not found in *C. jejuni*. The Δ*ppk1* mutant was significantly more susceptible to a variety of antibiotics and other antimicrobials than was the wild-type strain (Table 4). It follows that poly-P and its associated enzyme (PPK1) can affect the antibiotic resistance of *C. jejuni* through various other pathways that are evidently RpoS independent. For example, poly-P accumulation regulates the synthesis of guanosine tetraphosphate (ppGpp) (59), an important molecule in stringent responses, which at high levels, in turn, induces the accumulation of poly-P (34). Gaynor et al. (24) showed that the stringent response mediated the survival of *C. jejuni* under exposure to rifampin, probably through the activation of RelA and/or SpoT, enzymes that affect the synthesis of ppGpp, which alters transcription of genes that facilitate survival under stress. This was further confirmed by our observation that the Δ*ppk1* mutant indeed showed an increased susceptibility to rifampin (Table 4). However, *spoT* was upregulated in the Δ*ppk1* mutant (Fig. 5), probably in an attempt to induce ppGpp-dependent accumulation of poly-P (through the inhibition of exopolyphosphatase) (34) in the deficient mutant, which also highlights the importance of previously suggested interactions of poly-P with stringent response factors (36). The resistance of *C. jejuni* to macrolides (erythromycin) and fluoroquinolones (ciprofloxacin) is mediated through changes in the ribosome and mutations in DNA gyrase (encoded by *gyr*A), respectively (18). Interestingly, an *E. coli ppk* mutant showed a 40% decrease in induced mutagenesis to nalidixic acid resistance (64), which is a result of base mutations in DNA gyrase genes (72), while poly-P has been reported to interact with ribosomes, supporting translation fidelity in *E. coli* (42). Furthermore, *ppk* deletion mutations in *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Dublin showed an increased susceptibility to polymyxin B (31), corroborating our observations that the *C. jejuni* Δ*ppk1* mutant was susceptible to polymyxin (Table 4) and emphasizing the role of poly-P in mediating antibiotic resistance. Surprisingly, *cmeC*, a component of a multidrug efflux pump that mediates resistance to antibiotics and other important compounds such as bile, which induces virulence gene expression (38), was upregulated in the Δ*ppk1* mutant under no stress (Fig. 5), possibly in response to poly-P deficiency. However, overexpression of *cmeC* did not appear to facilitate the tolerance of the Δ*ppk1* mutant when it was challenged with antibiotics or bile. Although our results indicate a role for PPK1 and poly-P in mediating the resistance of *C. jejuni* to different antibacterial compounds, the precise mechanism of the observed susceptibility and/or resistance remains largely unknown and requires further investigation. However, this might emphasize the importance of poly-P and its associated enzymes as potential targets for antibiotics and/or other compounds.

Differential-expression analysis of *C. jejuni* genes involved primarily in phosphate uptake, transport, and metabolism showed that *ppk2* (regulates the reversible synthesis of poly-P from GTP or ATP), *phosS*/*phosR* (a two-component system that activates the transcription of the phosphate regulon), *pstSC* (involved in phosphate transport), and a gene for a periplasmic solute-binding protein (CJJ81176_0750) that is part of an operon encoding a putative ABC transporter for phosphate uptake (20, 53, 70) were expectedly upregulated under normal conditions in the Δ*ppk1* mutant compared to the wild type (Fig. 5). It has been previously suggested that the Pho regulon is associated with stress responses (36), as the Pho regulon was suppressed in *E. coli* mutant strains (e.g., *relA* and *spoT* mutants) that were deficient in ppGpp accumulation (62). Consequently, direct and/or indirect interactions have been proposed to occur between pathways involved in phosphate regulation, poly-P synthesis, and the stringent response (36). In *C. jejuni*, the *phosS*/*phosR* two-component system triggers transcription of the phosphate regulon, including CJJ81176_0750 and the *pstS* and *pstC* genes, both of which are also transcribed

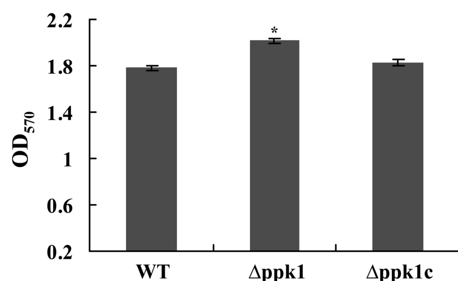

FIG. 7. Relative amounts of biofilm formation in the wild-type, Δ*ppk1*, and Δ*ppk1*c strains. Static biofilm formation in borosilicate tubes was assessed by inoculating 100 μl of culture with an OD_{600} of 0.05 into 1 ml of MH broth and incubating it at 42°C under microaerophilic conditions for 2 days without shaking. Biofilms were visualized by staining with 250 μl of 1% crystal violet for 15 min. Biofilms were quantified by measuring the absorbance at 570 nm after dissolving them in 1 ml of dimethyl sulfoxide for 24 to 48 h. Each bar represents the mean ± SE of two independent experiments. *, $P < 0.01$. WT, wild type.

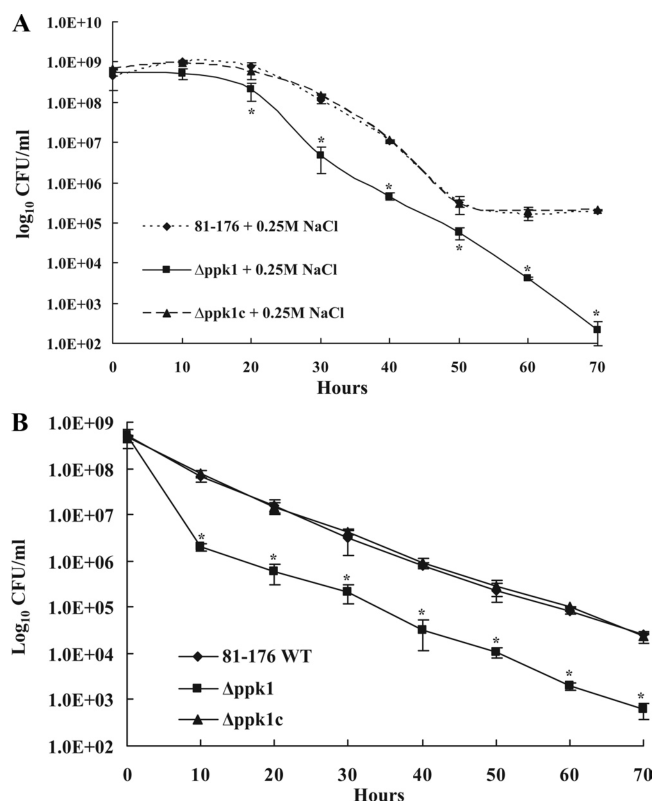

FIG. 8. (A) Survival of the wild-type, Δ*ppk1*, and Δ*ppk1*c strains in MH broth with 0.25 M NaCl. Bacterial strains were grown into mid-log phase and adjusted to an OD_{600} of 0.05 in MH broth with and without 0.25 M NaCl and incubated under microaerophilic conditions at 42°C for 70 h with shaking at 200 rpm. At different time points, samples were serially diluted (10-fold) and plated onto MH agar in triplicate. The plates were incubated under microaerophilic conditions, and the number of CFU/ml was calculated. The assay was repeated three times, and the mean number of CFU/ml was reported. (B) *C. jejuni* survival under nutritional downshift in MEM. Each data point represents the mean ± SE of three independent experiments. *, $P < 0.01$. WT, wild type.

during phosphate limitation (70). Consequently, the upregulation of *ppk2* and other genes that are involved in phosphate uptake and transport in the Δ*ppk1* mutant appears to constitute a compensatory response by the bacterium in an attempt to accumulate poly-P. These observations further highlight the role of poly-P and its associated metabolic enzymes in adaptation and survival responses of *C. jejuni*.

Consistent with previous findings, *C. jejuni* motility and resistance to oxidative stress were not impacted in the Δ*ppk1* mutant (data not shown; 10), which surprisingly showed enhanced biofilm formation (Fig. 7). Interestingly, *csrA* (putative global posttranscriptional regulator), which contributes to biofilm formation and oxidative resistance in *C. jejuni* (20), was upregulated in the Δ*ppk1* mutant (Fig. 5), possibly explaining the tolerance of this mutant to oxidative stress and its enhanced capacity for biofilm formation. Furthermore, our results indicated that the Δ*ppk1* mutant was susceptible to osmotic stress and nutrient downshift (Fig. 8A and B) and exhibited a dose-dependent chicken colonization deficiency (Fig. 6), while the thermal stress response and resistance to heavy metals were not impacted (data not shown). With the exception of thermal stress and resistance to heavy metals, the tolerance to osmotic and nutrient stresses was previously investigated, and our results were consistent with the earlier report (10). Similar to the *ppk1* mutant, a *C. jejuni spoT* mutant was deficient in poly-P accumulation during growth in a rich medium despite the presence of an intact *ppk1* gene (10). In our Δ*ppk1* mutant, poly-P did not accumulate despite the overexpression of *spoT* and the phosphate regulon genes, further confirming that functional PPK1 is required for poly-P accumulation. Additionally, this suggested that *spoT* might affect the activity of *ppk1*, which could also be inferred from *E. coli spoT* mutants that failed to accumulate ppGpp and pppGpp, as well as poly-P, under nutrient deprivation (34).

In summary, our observations confirm the importance of PPK1 in the adaptation, stress tolerance, and survival of *C. jejuni*. Furthermore, we elucidate for the first time the role of poly-P in influencing VBNC cell formation, natural transformation, and resistance to antimicrobial compounds and bile acids, while impacting the expression of genes that are important in these mechanisms. With limited options (e.g., absence of RpoS) for survival under various stresses, poly-P and its associated enzymes might be critical for *C. jejuni* survival, both in vivo and during transmission. In addition, poly-P might contribute to the plasticity of the *C. jejuni* genome, as well as the spread and development of antibiotic resistance, by mediating natural transformation and antimicrobial resistance. Further studies are warranted to understand the molecular mechanisms of poly-P regulation of stress responses and pathogenicity and the interplay of poly-P, phosphate regulation genes, SpoT, and ppGpp in *C. jejuni* pathophysiology.

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REFERENCES

1. **Akiba, M., J. Lin, Y. W. Barton, and Q. Zhang.** 2006. Interaction of CmeABC and CmeDEF in conferring antimicrobial resistance and maintaining cell viability in *Campylobacter jejuni*. J. Antimicrob. Chemother. **57:**52–60.
2. **Allos, B. M.** 2001. *Campylobacter jejuni* infections: update on emerging issues and trends. Clin. Infect. Dis. **32:**1201–1206.
3. **Altekruse, S. F., N. J. Stern, P. I. Fields, and D. L. Swerdlow.** 1999. *Campylobacter jejuni*: an emerging foodborne pathogen. Emerg. Infect. Dis. **5:**28–35.
4. **Ayraud, S., B. Janvier, A. Labigne, C. Ecobichon, C. Burucoa, and J. L. Fauchere.** 2005. Polyphosphate kinase: a new colonization factor of *Helicobacter pylori*. FEMS Microbiol. Lett. **243:**45–50.
5. **Baltrus, D. A., K. Guillemin, and P. C. Phillips.** 2008. Natural transformation increases the rate of adaptation in the human pathogen *Helicobacter pylori*. Evolution **62:**39–49.
6. **Beery, J. T., M. B. Hugdahl, and M. P. Doyle.** 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl. Environ. Microbiol. **54:**2365–2370.
7. **Bode, G., F. Mauch, H. Ditschuneit, and P. Malfertheiner.** 1993. Identification of structures containing polyphosphate in *Helicobacter pylori*. J. Gen. Microbiol. **139:**3029–3033.
8. **Brown, M. R., and A. Kornberg.** 2008. The long and short of it—polyphosphate, PPK and bacterial survival. Trends Biochem. Sci. **33:**284–290.
9. **Butzler, J. P., and M. B. Skirrow.** 1979. *Campylobacter* enteritis. Acta Paediatr. Belg. **32:**89–94.
10. **Candon, H. L., B. J. Allan, C. D. Fraley, and E. C. Gaynor.** 2007. Polyphosphate kinase 1 is a pathogenesis determinant in *Campylobacter jejuni*. J. Bacteriol. **189:**8099–8108.
11. **Cappelier, J. M., B. Lázaro, A. Rossero, A. Fernandez-Astorga, and M. Federighi.** 1997. Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable *Campylobacter jejuni* cells. Vet. Res. **28:** 547–555.
12. **Cappelier, J. M., C. Magras, J. L. Jouve, and M. Federighi.** 1999. Recovery of viable but non-culturable *Campylobacter jejuni* cells in two animal models. Food Microbiol. **16:**375–383.
13. **Castuma, C. E., R. Huang, A. Kornberg, and R. N. Reusch.** 1995. Inorganic polyphosphates in the acquisition of competence in *Escherichia coli*. J. Biol. Chem. **270:**12980–12983.
14. **Chaveerach, P., A. A. H. M. Huurne, L. J. A. Lipman, and F. Knapen.** 2003. Survival and resuscitation of ten strains of *Campylobacter jejuni* and *Campylobacter coli* under acid conditions. Appl. Environ. Microbiol. **69:**711–714.
15. **Chen, S. Y., W. N. Jane, Y. S. Chen, and H. C. Wong.** 2009. Morphological changes of *Vibrio parahaemolyticus* under cold and starvation stresses. Int. J. Food Microbiol. **129:**157–165.
16. **Claverys, J. P., M. Prudhomme, I. Mortier-Barriere, and B. Martin.** 2000. Adaptation to the environment: *Streptococcus pneumoniae*, a paradigm for recombination-mediated genetic plasticity? Mol. Microbiol. **35:**251–259.
17. **de Boer, P., J. A. Wagenaar, R. P. Achterberg, J. P. van Putten, L. M. Schouls, and B. Duim.** 2002. Generation of *Campylobacter jejuni* genetic diversity *in vivo*. Mol. Microbiol. **44:**351–359.
18. **Engberg, J., F. M. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin.** 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. Emerg. Infect. Dis. **7:**24–34.
19. **Federighi, M., J. L. Tholozan, J. M. Cappelier, J. P. Tissier, and J. L. Jouve.** 1998. Evidence of non-coccoid viable but non-culturable *Campylobacter jejuni* cells in microcosm water by direct viable count, CTC-DAPI double staining, and scanning electron microscopy. Food Microbiol. **15:**539–550.
20. **Fields, J. A., and S. A. Thompson.** 2008. *Campylobacter jejuni* CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. J. Bacteriol. **190:**3411–3416.
21. **Foster, P. L.** 2007. Stress-induced mutagenesis in bacteria. Crit. Rev. Biochem. Mol. Biol. **42:**373–397.
22. **Fouts, D. E., E. F. Mongodin, R. E. Mandrell, W. G. Miller, D. A. Rasko, J. Ravel, L. M. Brinkac, R. T. DeBoy, C. T. Parker, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, S. A. Sullivan, J. U. Shetty, M. A. Ayodeji, A. Shvartsbeyn, M. C. Schatz, J. H. Badger, C. M. Fraser, and K. E. Nelson.** 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. PLoS Biol. **3:**e15.
23. **Fraley, C. D., M. H. Rashid, S. S. Lee, R. Gottschalk, J. Harrison, P. J. Wood, M. R. Brown, and A. Kornberg.** 2007. A polyphosphate kinase 1 (*ppk1*) mutant of *Pseudomonas aeruginosa* exhibits multiple ultrastructural and functional defects. Proc. Natl. Acad. Sci. USA **104:**3526–3531.
24. **Gaynor, E. C., D. H. Wells, J. K. MacKichan, and S. Falkow.** 2005. The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. Mol. Microbiol. **56:**8–27.
25. **Hofreuter, D., J. Tsai, R. O. Watson, V. Novik, B. Altman, M. Benitez, C. Clark, C. Perbost, T. Jarvie, L. Du, and J. E. Galan.** 2006. Unique features of a highly pathogenic *Campylobacter jejuni* strain. Infect. Immun. **74:**4694–4707.
26. **Hughes, R.** 2004. *Campylobacter jejuni* in Guillain-Barré syndrome. Lancet Neurol. **3:**644.
27. **Ishige, K., H. Zhang, and A. Kornberg.** 2002. Polyphosphate kinase (PPK2), a potent, polyphosphate-driven generator of GTP. Proc. Natl. Acad. Sci. USA **99:**16684–16688.
28. **Jahid, I. K., A. J. Silva, and J. A. Benitez.** 2006. Polyphosphate stores enhance the ability of *Vibrio cholerae* to overcome environmental stresses in a low-phosphate environment. Appl. Environ. Microbiol. **72:**7043–7049.
29. **Jeon, B., W. Muraoka, O. Sahin, and Q. Zhang.** 2008. Role of Cj1211 in natural transformation and transfer of antibiotic resistance determinants in *Campylobacter jejuni*. Antimicrob. Agents Chemother. **52:**2699–2708.
30. **Johnsborg, O., L. Eldholm, and L. S. Håvarstein.** 2007. Natural genetic transformation: prevalence, mechanisms and function. Res. Microbiol. **158:** 767–778.
31. **Kim, K. S., N. N. Rao, C. D. Fraley, and A. Kornberg.** 2002. Inorganic polyphosphate is essential for long-term survival and virulence factors in *Shigella* and *Salmonella* spp. Proc. Natl. Acad. Sci. USA **99:**7675–7680.
32. **Korlath, J. A., M. T. Osterholm, L. A. Judy, J. C. Forfang, and R. A. Robinson.** 1985. A point-source outbreak of campylobacteriosis associated with consumption of raw milk. J. Infect. Dis. **152:**592–596.
33. **Kornberg, A., N. N. Rao, and D. Ault-Riche.** 1999. Inorganic polyphosphate: a molecule of many functions. Annu. Rev. Biochem. **68:**89–125.
34. **Kuroda, A., H. Murphy, M. Cashel, and A. Kornberg.** 1997. Guanosine tetra and pentaphosphate promote accumulation of inorganic polyphosphate in *Escherichia coli*. J. Biol. Chem. **272:**21240–21243.
35. **Kusters, J. G., M. M. Gerrits, J. A. G. Van Strijp, and C. M. J. E. Vandenbroucke-Gauls.** 1997. Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. Infect. Immun. **65:**3672–3679.
36. **Lamarche, M. G., B. L. Wanner, S. Cre′pin, and J. Harel.** 2008. The Pho regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. FEMS Microbiol. Rev. **32:**461–473.
37. **Lázaro, B., J. Carcamo, A. Audicana, I. Perales, and A. Fernandez-Astorga.** 1999. Viability of DNA maintenance in nonculturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. Appl. Environ. Microbiol. **65:**4677–4681.
38. **Lin, J., L. O. Michel, and Q. Zhang.** 2002. CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. Antimicrob. Agents Chemother. **46:**2124–2131.
39. **Luangtongkum, T., B. Jeon, J. Han, P. Plummer, C. M. Logue, and Q. Zhang.** 2009. Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. Future Microbiol. **4:**189–200.
40. **Manganelli, R.** 2007. Polyphosphate and stress response in mycobacteria. Mol. Microbiol. **65:**258–260.
41. **McDougald, D., S. A. Rice, D. Weichart, and S. Kjelleberg.** 1998. Nonculturability: adaptation or debilitation? FEMS Microbiol. Ecol. **25:**1–9.
42. **McInerney, P., T. Mizutani, and T. Shiba.** 2006. Inorganic polyphosphate interacts with ribosomes and promotes translation fidelity *in vitro* and *in vivo*. Mol. Microbiol. **60:**438–447.
43. **Medema, G. J., F. M. Schets, A. W. Van de Giessen, and A. Havelaar.** 1992. Lack of colonization of one day old chicks by viable, non-culturable *Campylobacter jejuni*. J. Appl. Bacteriol. **72:**512–516.
44. **Miller, W. G., A. H. Bates, S. T. Horn, M. T. Brandl, M. R. Wachtel, and R. E. Mandrel.** 2000. Detection on surfaces and in Caco-2 cells of *Campylobacter jejuni* cells transformed with new *gfp*, *yfp*, and *cfp* marker plasmids. Appl. Environ. Microbiol. **66:**5426–5436.
45. **Nilsson, H. O., J. Blom, W. A. Al-Soud, A. Ljungh, L. P. Andersen, and T. Wadstrom.** 2002. Effect of cold starvation, acid stress, and nutrients on metabolic activity of *Helicobacter pylori*. Appl. Environ. Microbiol. **68:**11–19.
46. **Ogawa, N., C. M. Tzeng, C. D. Fraley, and A. Kornberg.** 2000. Inorganic polyphosphate in *Vibrio cholerae*: genetic, biochemical, and physiologic features. J. Bacteriol. **182:**6687–6693.
47. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665–668.
48. **Price-Carter, M., T. G. Fazzio, E. I. Vallbona, and J. R. Roth.** 2005. Polyphosphate kinase protects *Salmonella enterica* from weak organic acid stress. J. Bacteriol. **187:**3088–3099.
49. **Rajashekara, G., M. Drozd, D. Gangaiah, B. Jeon, Z. Liu, and Q. Zhang.** 2009. Functional characterization of the twin-arginine translocation system in *Campylobacter jejuni*. Foodborne Pathog. Dis. **6:**935–945.
50. **Rao, N. N., and A. Kornberg.** 1996. Inorganic polyphosphate supports resistance and survival of stationary-phase *Escherichia coli*. J. Bacteriol. **178:** 1394–1400.
51. **Rao, N. N., M. R. Gómez-García, and A. Kornberg.** 2009. Inorganic polyphosphate: essential for growth and survival. Annu. Rev. Biochem. **78:**605–647.
52. **Rashid, M. H., and A. Kornberg.** 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **97:**4885–4890.
53. **Reid, A. N., R. Pandey, K. Palyada, L. Whitworth, E. Doukhanine, and A.**

Stintzi. 2008. Identification of *Campylobacter jejuni* genes contributing to acid adaptation by transcriptional profiling and genome-wide mutagenesis. Appl. Environ. Microbiol. **74:**1598–1612.

54. **Reusch, R. N., and H. L. Sadoff.** 1988. Putative structure and functions of a poly-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc. Natl. Acad. Sci. USA **85:**4176–4180.
55. **Rollins, D. M., and R. R. Colwell.** 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. **52:**531–538.
56. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. **30:**334–338.
57. **Saha, S. K., S. Saha, and S. C. Sanyal.** 1991. Recovery of injured *Campylobacter jejuni* cells after animal passage. Appl. Environ. Microbiol. **57:**388–3389.
58. **Seufferheld, M. J., H. M. Alvarez, and M. E. Farias.** 2008. Role of polyphosphates in microbial adaptation to extreme environments. Appl. Environ. Microbiol. **74:**5867–5874.
59. **Shiba, T., K. Tsutsumi, H. Yano, Y. Ihara, A. Kameda, K. Tanaka, H. Takahashi, M. Munekata, N. N. Rao, and A. Kornberg.** 1997. Inorganic polyphosphate and the induction of *rpoS* expression. Proc. Natl. Acad. Sci. USA **94:**11210–11215.
60. **Shiba, T., K. Tsutsumi, K. Ishige, and T. Noguchi.** 2000. Inorganic polyphosphate and polyphosphate kinase: their novel biological functions and applications. Biochemistry **65:**315–323.
61. **Skirrow, M. B.** 1994. Diseases due to *Campylobacter*, *Helicobacter*, and related bacteria. J. Comp. Pathol. **111:**113–149.
62. **Spira, B., N. Silberstein, and E. Yagil.** 1995. Guanosine 3′,5′-bispyrophosphate (ppGpp) synthesis in cells of *Escherichia coli* starved for P_i. J. Bacteriol. **177:**4053–4058.
63. **Stern, N. J., D. M. Jones, I. V. Wesley, and D. M. Rollins.** 1994. Colonization of chicks by non-culturable *Campylobacter* spp. Lett. Appl. Microbiol. **18:**333–336.
64. **Stumpf, J. D., and P. L. Foster.** 2005. Polyphosphate kinase regulates error-prone replication by DNA polymerase IV in *Escherichia coli*. Mol. Microbiol. **57:**751–761.
65. **Tan, S., C. D. Fraley, M. Zhang, D. Dailidiene, A. Kornberg, and D. E. Berg.** 2005. Diverse phenotypes resulting from polyphosphate kinase gene (*ppk1*) inactivation in different strains of *Helicobacter pylori*. J. Bacteriol. **187:**7687–7695.
66. **Tholozan, J. L., J. M. Cappelier, J. P. Tissier, G. Delattre, and M. Federighi.** 1999. Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. Appl. Environ. Microbiol. **65:**1110–1116.
67. **Tinsley, C. R., B. N. Manjula, and E. C. Gotschlich.** 1993. Purification and characterization of polyphosphate kinase from *Neisseria meningitidis*. Infect. Immun. **61:**3703–3710.
68. **Wang, Y., and D. E. Taylor.** 1990. Natural transformation in *Campylobacter* species. J. Bacteriol. **172:**949–955.
69. **Wilson, D. L., J. A. Bell, V. B. Young, S. R. Wilder, L. S. Mansfield, and J. E. Linz.** 2003. Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. Microbiology **149:**3603–3615.
70. **Wösten, M. M., C. T. Parker, A. van Mourik, M. R. Guilhabert, L. van Dijk, and J. P. van Putten.** 2006. The *Campylobacter jejuni* PhosS/PhosR operon represents a non-classical phosphate-sensitive two-component system. Mol. Microbiol. **62:**278–291.
71. **Yao, R., R. A. Alm, T. J. Trust, and P. Guerry.** 1993. Construction of new *Campylobacter* cloning vectors and a new mutational *cat* cassette. Gene **130:**127–130.
72. **Yoshida, H., T. Kojima, J. Yamagishi, and S. Nakamura.** 1988. Quinolone resistant mutations of the *gyrA* gene of *Escherichia coli*. Mol. Gen. Genet. **211:**1–7.
73. **Young, K. T., L. M. Davis, and V. J. DiRita.** 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. Nat. Rev. Microbiol. **5:**665–679.