

Polyphosphate and associated enzymes as global regulators of stress response and virulence in *Campylobacter jejuni*

Anand Kumar, Dharanesh Gangaiah, Jordi B Torrelles, Gireesh Rajashekara

Anand Kumar, Gireesh Rajashekara, 170 Food Animal Health Research Program and Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Wooster, OH 44691, United States

Anand Kumar, Biosecurity and Public Health, Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, United States

Dharanesh Gangaiah, Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, United States

Jordi B Torrelles, Department of Microbial Infection and Immunity, Center for Microbial Interface Biology, the Ohio State University, Columbus, OH 43210, United States

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Correspondence to: Gireesh Rajashekara, DVM, PhD, 170 Food Animal Health Research Program and Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, 1680 Madison Avenue, Wooster, OH

44691, United States. rajashekara.2@osu.edu
Telephone: +1-330-2633745
Fax: +1-330-2633677

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Abstract

Campylobacter jejuni (*C. jejuni*), a Gram-negative microaerophilic bacterium, is a predominant cause of bacterial foodborne gastroenteritis in humans worldwide. Despite its importance as a major foodborne pathogen, our understanding of the molecular mechanisms underlying *C. jejuni* stress survival and pathogenesis is limited. Inorganic polyphosphate (poly P) has been shown to play significant roles in bacterial resistance to stress and virulence in many pathogenic bacteria. *C. jejuni* contains the complete repertoire of enzymes required for poly P metabolism. Recent work in our laboratory and others have demonstrated that poly P controls a plethora of *C. jejuni* properties that impact its ability to survive in the environment as well as to colonize/infect mammalian hosts. This review article summarizes the current literature on the role of poly P in *C. jejuni* stress survival and virulence and discusses on how poly P-related enzymes can be exploited for therapeutic/prevention purposes. Additionally, the review article identifies potential areas for future investigation that would enhance our understanding of the role of poly P in *C. jejuni* and other bacteria, which ultimately would facilitate design of effective therapeutic/preventive strategies to reduce not only the burden of *C. jejuni*-caused foodborne infections but also of other bacterial infections in humans.

Key words: *Campylobacter jejuni*; Stress tolerance; Inorganic polyphosphate; Virulence; Colonization/ infection

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Core tip: Recent studies show that inorganic polyphosphate (poly P) plays several important roles in the biology of *Campylobacter jejuni* (*C. jejuni*), a major cause of bacterial foodborne gastroenteritis in humans. This review summarizes the latest findings on the role of poly P in *C. jejuni* stress survival and virulence, provides directions for future investigation, and discusses the potential of polyphosphate kinase enzymes as drug/vaccine targets to control *C. jejuni* infections in humans.

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INTRODUCTION

Campylobacter (Greek word “*Kampylos*” means curved) species are curved to spiral-shaped, non-spore forming, Gram-negative bacteria that contain a single flagellum at one or both ends. Most of the *Campylobacter* species are microaerophilic and use a respiratory type of metabolism; however, some species prefer to grow anaerobically. *Campylobacter* species are chemoorganotrophs - they primarily depend on amino acids or Krebs’ cycle intermediates for energy. Based on their optimal growth temperature, *Campylobacter* species are classified into thermophilic and non-thermophilic species. The thermophilic *Campylobacter* species grow optimally at 42 °C [*Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*)], while the non-thermophilic species grow optimally at the range between 30-37 °C (*Campylobacter fetus*). The genus *Campylobacter* consists of 34 species and 14 subspecies, which are human and animal pathogens (<http://www.bacterio.net/campylobacter.html>, last accessed 17 March 2016). Several of these species cause a plethora of clinical manifestations in humans (Table 1); however, *C. jejuni* and *C. coli* are the predominant species associated with human disease^[1].

C. jejuni is one of the major causes of bacterial foodborne diarrheal disease in the United States and worldwide^[2]. According to Foodborne Diseases Active Surveillance Network, *Campylobacter* spp. account for 9% of total foodborne illnesses, 15% of hospitalizations and 0.1% of deaths from foodborne illnesses each year^[3]. In immunocompetent patients,

C. jejuni infection typically manifests as a self-limiting acute gastroenteritis characterized by severe watery and sometimes bloody diarrhea, fever, nausea, and vomiting. However, in immunocompromised patients, *C. jejuni* can cause a severe, life-threatening disease, often requiring hospitalization and antibiotic treatment. In addition to causing gastroenteritis, *C. jejuni* is also associated with post-infection complications such as Guillain-Barre syndrome, which is a rare neuromuscular disease that is thought to occur in 1 in 1000 individuals infected with *C. jejuni*^[4]. Other sequelae such as Reiter’s syndrome, inflammatory bowel syndrome, and immunoproliferative small intestinal disease also significantly add to the burden of *C. jejuni* infection^[5-7].

Unlike in humans, *C. jejuni* lives as a commensal in the gut of a variety of domestic and wild animals and birds^[4]. Epidemiological studies show that the majority (50%-80%) of human infections are acquired by consumption of contaminated poultry and poultry products and that nearly 90% of the poultry flocks in the United States are colonized with *C. jejuni*, suggesting that controlling *C. jejuni* colonization in poultry is an effective strategy to control human infections^[4]. Rarely, contaminated raw milk, water, and vegetables also serve as sources of human infection^[1,4]. Although transmission through ingestion of contaminated food and water is the major route, person-to-person contact, as well as contact with pets or their feces is not uncommon^[1,4]. Although *C. jejuni* is a major public health concern worldwide, the genetic determinants that contribute to its ability to survive in different host and non-host environments, to colonize poultry and other domestic animals, and to cause disease in humans are relatively poorly understood.

C. jejuni is unique among the enteric bacterial pathogens in that it lacks many classical stress response and virulence mechanisms. More specifically, *C. jejuni* lacks the stationary phase sigma factor RpoS, the heat shock sigma factor RpoH, the cold shock protein CspA, the oxidative stress response genes SoxRS, OxyR, SodA and KatG, the osmoprotectants ProU, OtsAB and BetAB, and the leucine-responsive global regulator Lrp, which are all essential for stress tolerance in other enteric pathogens^[8]. These findings are consistent with the unusual sensitivity of *C. jejuni* to various environmental stresses *i.e.*, *C. jejuni* is unable to grow in the presence of oxygen, has a narrow growth temperature range, is normally incapable of multiplication outside the host, does not survive well on dry surfaces, cannot withstand high temperature, and is more sensitive to osmotic and low pH stresses^[8]. *C. jejuni* also lacks many classical virulence mechanisms, including a type III secretion system and exotoxins, which play important roles in the pathogenesis of other enteric bacteria^[9]. Several *C. jejuni* strains contain type IV and type VI secretion systems but the absence of these systems in many pathogenic *C. jejuni* strains questions

Table 1 Comprehensive summary of *Campylobacter* infections in humans

Type	Clinical form/presentation	Risk factors	Target site	Symptoms	Species associated
(A) Intestinal	Periodontal diseases	Oral bleeding, increased vascular permeability ^[70] , and pregnancy ^[71] ?	Oral cavity	Bleeding, tenderness, and tooth loss	<i>C. rectus</i> ^[70] , <i>C. gracilis</i> , <i>C. showae</i> ^[72] and <i>C. concisus</i> ^[73]
	Esophageal diseases (gastroesophageal reflux disease, Barrett's esophagus, and esophageal adenocarcinoma)	Mucosal damage due to stomach contents regurgitation ^[1]	Esophagus	Heart burn, regurgitation, bloating, bad breath, nausea, and abdominal pain	<i>C. concisus</i> ^[74]
	Self-limited gastroenteritis, most common form	All factors as described in review	Jejunum and ileum	Diarrhea, fever, and abdominal pain	<i>C. jejuni</i> and <i>C. coli</i> ^[1,75]
	Post-infectious functional gastrointestinal disorder, irritable bowel syndrome, and functional dyspepsia	Infection with <i>C. jejuni</i> and other species ^[1]		Diarrhea, constipation or both, and abdominal pain	<i>C. jejuni</i> , <i>C. coli</i> and <i>C. concisus</i> ^[76]
	Inflammatory bowel disease, Crohn's diseases (CD), and ulcerative colitis (UC)	Gut dysbiosis ^[77]	CD-any part of intestine UC- colon	Diarrhea, fever and fatigue, abdominal pain, weight loss, and reduced appetite	<i>C. concisus</i> , <i>C. showae</i> , <i>C. hominis</i> , <i>C. rectus</i> , and <i>C. ureolyticus</i> ^[78]
	Colorectal cancer	Gut dysbiosis ^[79]	Colon	Diarrhea, constipation, abdominal pain, weight loss, and rectal bleeding	<i>C. showae</i> ^[80]
	Cholecystitis	Gall stones ^[1]	Gall bladder	Pain and tenderness in right abdomen, nausea, vomiting, and fever	<i>C. jejuni</i> ^[81]
(B) Extra-intestinal	Guillain-Barre syndrome	<i>C. jejuni</i> infection ^[82]	Nervous system	Progressive symmetric weakness in limbs, below or lack of reflex (hyporeflexia)	<i>C. jejuni</i> ^[1,82]
	Miller Fisher syndrome	<i>C. jejuni</i> ^[83]	Nervous system	Oculo-motor weakness	<i>C. jejuni</i> ^[83]
	Reactive arthritis	Infection with enteric bacterial pathogens, including <i>Campylobacter</i> ^[84]	Joints, eyes, and genitourinary tract	Pain and stiffness of joints, swollen toes, eye inflammation, and urinary problems	<i>C. jejuni</i> and <i>C. coli</i> ^[1,84]
	Cardiovascular complications	Immuno-compromised condition, and bacteremia ^[1]	Cardiovascular system	Chest pain leading to arrhythmia, dilated cardiomyopathy, and sudden death due to congestive heart failure	<i>C. jejuni</i> and <i>C. fetus</i> ^[85,86]
	Meningitis	Immuno-compromised condition ^[1,87]	Meninges of brain and spinal cord	Headache with nausea or vomiting, seizures, sensitive to light, and loss of appetite	<i>C. jejuni</i> and <i>C. fetus</i> ^[88,89]
	Abscesses of breast, brain, vertebra, and liver	Secondary bacterial infection	Breast, brain, vertebra, and liver	Varies with target organ	<i>C. rectus</i> ^[90] , <i>C. curvus</i> , <i>C. gracilis</i> and <i>C. showae</i> ^[91]
	Reproductive complication	Bowel infection, and periodontal disease ^[92]	Uterus, and placenta	Preterm birth, low birth weight, and intra-uterine growth restriction	<i>C. jejuni</i> , <i>C. coli</i> , <i>C. fetus</i> and <i>C. upsaliensis</i> ^[92]

Campylobacter infection types, clinical presentations, risk factors, target organ or tissues involved, symptoms and associated *Campylobacter* species are described.

their requirement for virulence^[10,11]. Nevertheless, the increasing incidence of *C. jejuni* infections in humans suggests that this organism may have evolved alternative mechanisms for stress survival and virulence. One such mechanism that has been relatively well characterized by several recent studies and plays important roles in *C. jejuni* survival and virulence involves inorganic polyphosphate (poly P). In this review, we summarize recent data on the role of poly P and related enzymes in *C. jejuni* biology, with particular focus on stress survival and virulence.

INORGANIC POLYPHOSPHATE

Even after the discovery of the subatomic particle (the God particle), the origin of the universe is still a

debated topic. However, there is almost a universal agreement that phosphate has played a key role in the origin of life on earth. Poly P is a linear polymer of ten to hundreds of phosphate residues linked by high-energy phosphoanhydride bonds. Poly P granules were first seen in bacteria as "Volutin granules" or "Metachromatic granules", named after their tendency to stain pink with basic blue dyes^[12]. Indeed, the presence of metachromatic granules was used as a diagnostic feature for pathogenic bacteria such as *Corynebacterium diphtheriae*. Later with the advent of electron microscopy, these granules were identified in nonpathogenic bacteria, which refuted the idea of using poly P granules as a marker for pathogenic bacteria^[12]. Recent studies have shown that poly P is essential for numerous cellular functions in bacteria,

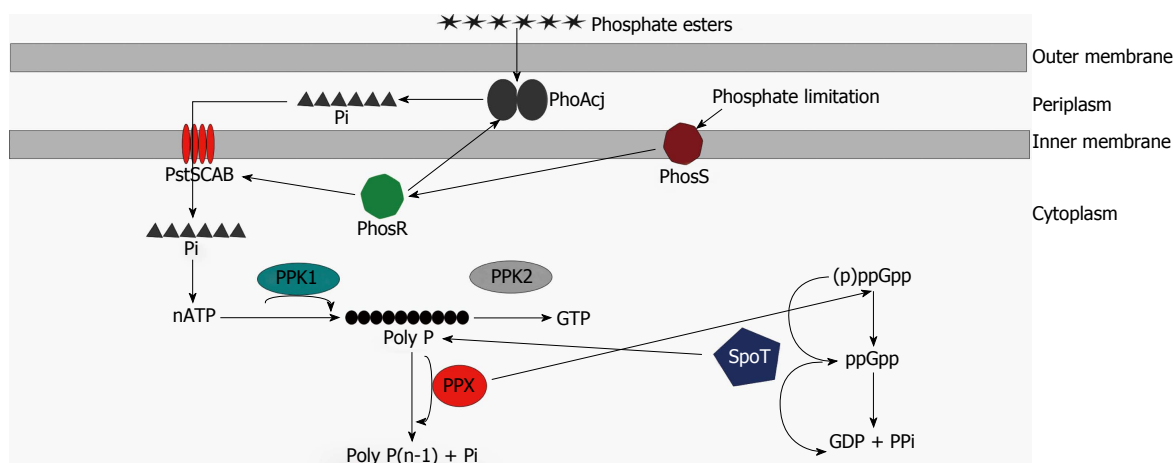


Figure 1 Model of poly P metabolism in *Campylobacter jejuni*. Phosphate esters are hydrolyzed to inorganic phosphate (Pi) by alkaline phosphatase (PhoAcj) in the periplasm. Phosphate uptake proteins and alkaline phosphatase are directly regulated by the PhoS/PhosR two-component system. Pi is transported across the inner membrane via the high affinity phosphate transport system PstSCAB. ATP generated from Pi is utilized for poly P synthesis by PPK1. PPK2 utilizes poly P to generate GTP, while PPX hydrolyzes poly P back to Pi. PPX also affects conversion of (p)ppGpp to ppGpp. SpoT is a bifunctional enzyme involved in both ppGpp synthesis as well as ppGpp hydrolysis. SpoT is also linked to poly P metabolism and a *spoT* mutant has reduced ability to accumulate poly P.

such as energy source, phosphate reservoir, cation sequestration, buffering role against alkali, participation in membrane transport, cell envelope formation and function, regulator of enzyme activities, gene activity control and development, chromatin destabilization, DNA replication and phage production, sporulation and germination, bacterial virulence/pathogenesis, and regulator of stress and survival^[13]. Additionally, bacteria capable of storing large amounts of intracellular poly P are used in the biological treatment of wastewater^[14].

GENERAL ASPECTS OF POLY P METABOLISM IN BACTERIA

Polyphosphate kinase 1 (PPK1) is the principal enzyme responsible for poly P synthesis in many bacteria^[15,16]. PPK1 is highly conserved; homologs of this enzyme have been found in over 100 bacterial species, including 20 major human and animal pathogens. PPK1 is perhaps the most known of all poly P-related enzymes for its role in bacterial survival under conditions of stress, virulence, and host colonization^[13]. Many bacterial species contain another enzyme, PPK2, which preferentially mediates poly P-driven generation of guanosine triphosphate (GTP)^[17,18], a molecule known to have important roles in cell signaling and DNA, RNA, protein, and polysaccharide synthesis^[19,20]. Similar to PPK1, PPK2 is also widely conserved in bacteria, including major human pathogens such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* among others^[17]. Although relatively less studied compared to PPK1, few recent studies have shown a role for PPK2 in bacterial survival and virulence^[18]. The third family of enzymes involved in poly P metabolism includes exopolyphosphatases, which degrade poly

P to inorganic phosphate^[13,21]. Many bacteria contain 2 types of exopolyphosphatases^[22], one that only hydrolyzes poly P (hereafter referred to as PPX) and the other that, in addition to hydrolyzing poly P, also hydrolyzes guanosine pentaphosphate (pppGpp) to guanosine tetraphosphate (ppGpp) (hereafter referred to as PPX/GPPA)^[13,23]. ppGpp is a signaling molecule that plays an important role in bacterial stringent response induced by starvation^[13,23]. Of all the poly P-metabolizing enzymes, PPX enzymes are the least understood with regards to their role in bacterial survival and virulence.

POLY P METABOLISM IN *C. JEJUNI*

C. jejuni contains all the enzymes involved in poly P metabolism- PPK1, PPK2, and 2 PPX/GPPA enzymes (Figure 1). Two independent studies have evaluated the contribution of PPK1 to poly P metabolism in *C. jejuni*^[24,25]. Using a toluidine blue O assay, both studies demonstrated that the *C. jejuni* wildtype strain accumulated more poly P in stationary phase than in mid-log and transition phases and that deletion of *ppk1* significantly reduced poly P accumulation (Figure 1)^[24,25]. The *C. jejuni ppk1* mutant retained a modest ability to synthesize poly P^[24,25], thus, the alternative enzyme that contributes to residual poly P levels in the *ppk1* mutant remains to be identified. Using electron microscopy, the authors further demonstrated that the *C. jejuni* wildtype strain contained several poly P-like granules and that the *ppk1* mutant contained fewer of these granules^[25]. More recently, a detailed ultrastructure analysis of *C. jejuni* at 5 nm resolutions also showed the presence of orange, poly P storage granules in *C. jejuni*^[26]. Although the two studies suggested that the observed granules are poly P granules, additional work is required to confirm that

Table 2 Phenotypes associated with enzymes of poly P and ppGpp metabolism

Phenotype	PPK1	PPK2	PPX1	PPX2	PPX1-PPX2	PhoAcj	SpoT
Poly P metabolism							
Poly P synthesis/accumulation	↑	-	-	-	-	↑	↑
Poly P-dependent GTP synthesis	-	↑	NT	NT	NT	NT	NT
Poly P degradation	-	-	↑	↑	↑	NT	NT
Maintenance of ATP:GTP ratio	NT	↑	NT	NT	NT	NT	NT
ppGpp metabolism							
ppGpp synthesis	NT	-	↑	-	↑	NT	↑
Stress survival							
Stationary phase survival and growth	-	-	-	-	-	-	↑
Survival under low CO ₂	NT	NT	NT	NT	NT	NT	↑
Osmotic shock survival	↑	↑	↑	↑	↑	↑	NT
Low nutrient stress survival	↑	↑	↑	↑	↑	↑	NT
Aerobic stress survival	-	↑	NT	NT	NT	NT	↑
VBNC formation	↑	↑	NT	NT	NT	NT	NT
Natural transformation	↑	NT	NT	NT	NT	NT	NT
Antimicrobial resistance	↑	↑	NT	NT	NT	↓	R
Virulence-related							
Motility	-	-	↑	↑	↑	NT	NT
Biofilm formation	↓	↓	↑	↑	↑	↓	NT
Resistance to complement-mediated killing	NT	NT	↑	↑	↑	NT	NT
Adherence	NT	-	NT	NT	NT	NT	↑
Invasion	-	↑	↑	↑	↑	↑	↑
Intraepithelial survival	↑	↑	↑	↑	↑	↑	↑
Chicken colonization	↑	↑	NT	NT	NT	↑	NT

NT: Not tested; R: Tested only for Rifampicin resistance; -: Phenotype absent; ↑: Phenotype is positively regulated *i.e.*, deletion of the gene reduces the phenotype when compared to wildtype; ↓: Phenotype is negatively regulated *i.e.*, deletion of the gene enhances the phenotype when compared to wildtype.

these granules are indeed poly P granules.

In another study, the role of PPK2 in poly P metabolism was investigated^[27]. The authors of this study demonstrated that a *ppk2* mutant was deficient in poly P-dependent GTP synthesis. The authors also showed that the ratio of ATP:GTP was altered in the *ppk2* mutant, thus suggesting that PPK2 is required for poly P-dependent GTP synthesis as well as for maintenance of nucleotide balance in the cell (Figure 1). GTP is a signaling molecule that modulates many physiological functions in bacteria^[19,20]. Unlike studies in other bacteria, which showed that PPK2 possesses poly P-synthetic activity^[28], the authors demonstrated that poly P levels were unaltered in the *C. jejuni ppk2* mutant when compared to wildtype, suggesting that PPK2 does not appear to contribute to poly P synthesis in *C. jejuni*^[27].

Malde *et al.*^[29] evaluated the contributions of PPX/GPPA enzymes to poly P metabolism in *C. jejuni*. They demonstrated that both *ppx1/gppa* and *ppx2/gppa* mutants accumulated more poly P than the wildtype strain and that the *ppx1/gppa* mutant accumulated relatively more poly P than the *ppx2/gppa* mutant when compared to wildtype. Based on these data, the authors suggested that PPX1/GPPA and PPX2/GPPA are involved in poly P degradation and that PPX1/GPPA is probably the primary enzyme that contributes to poly P degradation in *C. jejuni* (Figure 1)^[29]. However, whether PPX/GPPA enzymes possess PPX activity needs to be further experimentally confirmed.

PPKS: ROLE IN *C. JEJUNI* SURVIVAL AND VIRULENCE

Mutations in *ppk1* and *ppk2* genes have resulted in a variety of phenotypic changes in *C. jejuni*. Although the mechanisms underlying these phenotypes are poorly understood, the phenotypes as such are significant and relevant to *C. jejuni* pathogenesis. In the following sections, we will review our latest understanding of the role of PPKs in these phenotypes, which are summarized in Table 2.

PPKs: Role in growth and stationary phase survival

Several studies evaluated the effect of *ppk1* and *ppk2* mutations on *C. jejuni* growth and demonstrated that these mutations do not affect *C. jejuni* growth^[24,25,27]. In other organisms, *ppk1* mutants are defective in stationary phase growth and survival^[30,31]. The *C. jejuni* wildtype strain accumulated more poly P in stationary phase than in mid-log and transition phases and more genes were differentially expressed in the *ppk1* mutant in stationary phase when compared to mid-log phase^[24,25,32]. However, the *C. jejuni ppk1* mutant grew and survived similar to wildtype in stationary phase. A possible explanation for this difference is that *C. jejuni* lacks RNA polymerase, sigma S (RpoS), which regulates stationary phase survival and itself is modulated by poly P in other organisms^[8,33].

PPKs: Role in stress survival and adaptation

C. jejuni encounters a number of unfavorable

environments both in the external environment during transmission as well as in the host during colonization/infection^[8]. Poly P is known for its role in modulating stress tolerance in bacteria^[13]. Two independent studies by Candon *et al.*^[24] and Gangaiah *et al.*^[25] demonstrated that the *ppk1* mutant had a reduced ability to survive under low nutrient stress and osmotic shock. Similarly, in another study, Gangaiah *et al.*^[27] showed that the *ppk2* mutant, in addition to displaying a reduced ability to survive under low nutrient stress and osmotic shock, also had reduced ability to survive under aerobic stress. Bacteria generally respond to nutrient stress by eliciting a stringent response regulated by ppGpp and/or a general stress response regulated by RpoS. A *C. jejuni* bifunctional (p)ppGpp synthase/hydrolase (*spoT*) mutant, which is defective in eliciting a stringent response, was compromised in its ability to survive under low nutrient stress^[34]. Thus, to rule out the possible role of ppGpp in PPK1-mediated nutrient stress survival phenotype, it would be interesting to determine if ppGpp levels are altered in the *ppk1* mutant. Transcriptome analysis revealed that, despite *C. jejuni* lacking RpoS, homologs of several genes involved in general stress response were downregulated in *ppk1* and *ppk2* mutants; this might partly explain the stress survival deficiencies in the *ppk* mutants^[32]. In *E. coli*, poly P also affects stress tolerance by several other mechanisms- poly P is thought to be involved in sensing minor changes at the cell surface, serves as a source of energy during times of stress, and regulates Lon protease, which degrades nonessential proteins providing free amino acids for survival during stress^[13,35-38]. Whether such mechanisms also play a role in poly P-mediated stress resistance in *C. jejuni* is worth investigating.

PPKs: Role in viable but nonculturable cell formation

C. jejuni is also capable of forming viable but nonculturable cell (VBNC) under stressful conditions; this phenotype is thought to provide a survival advantage to *C. jejuni* during times of stress^[39,40]. Two recent studies showed that both *ppk1* and *ppk2* mutants were compromised in their ability to form VBNCs after formic acid treatment^[25,27]. The reduced ability of the *ppk* mutants to form VBNCs could be due to the fact that these mutants were more sensitive to other stresses such as low nutrient stress and osmotic shock. Poly P can serve as a source of energy during times of stress^[13]; this could also contribute to the reduced ability of the *ppk1* mutant to form VBNCs. A *ppk2* mutant had similar poly P levels but was compromised in its ability to regulate nucleotide balance; thus, whether altered nucleotide balance is the cause of the *ppk2* mutant's reduced ability to form VBNCs needs to be further studied. One might also speculate that PPK enzymes, as global regulators, might modulate VBNC formation by affecting the expression of other genes involved in VBNC formation;

however, transcriptome analysis of *ppk* mutants did not appear to provide any additional insights into this mechanism. For more information on the role of poly P in VBNC formation in *C. jejuni*, refer to the review by Kassem *et al.*^[39].

PPKs: Role in antimicrobial resistance and natural transformation

C. jejuni is increasingly becoming resistant to several clinically-relevant antimicrobials^[41-43]. Two recent studies demonstrated that both *ppk1* and *ppk2* mutants were more susceptible to several antimicrobials (erythromycin, cefotaxime, ciprofloxacin, rifampin, polymyxin, and tetracycline) compared to their respective wildtypes^[25,27]. The stringent-response mediator ppGpp affects bacterial resistance to antimicrobials^[44]; studies looking at ppGpp levels in the *ppk1* mutant would provide some insights on this mechanism. Both *ppk1* and *ppk2* mutants had altered outer membrane profiles, suggesting that membrane permeability of these mutants may be compromised^[45]; where altered membrane permeability could be the cause of increased susceptibility of *ppk* mutants to antimicrobials. Stress resistance is an emerging mechanism of antimicrobial resistance in bacteria^[44]; thus, the susceptibility of *ppk* mutants to antimicrobials may also be explained by their general sensitivity to other stresses. In *E. coli*, poly P is known to regulate adaptive evolution and ribosome fidelity, both of which modulate resistance to antimicrobials^[46]. Whether these mechanisms play a role in PPK1-mediated resistance to antimicrobials in *C. jejuni* also need to be investigated.

C. jejuni is naturally competent (*i.e.* ability to take up DNA from its surroundings) and this feature impacts the organism's ability to adapt to different environments as well as to acquire antimicrobial resistance genes. Gangaiah *et al.*^[25] showed that a *ppk1* mutant was compromised in its ability to acquire foreign DNA for natural transformation. Poly P is a component of the membrane channels that mediate DNA uptake^[47,48]; the potential of such a mechanism for PPK1 to mediate natural competence in *C. jejuni* should be explored. Evidence suggests that outer membrane composition of the *ppk1* mutant is altered^[45]; whether this influences the ability of *C. jejuni* to take up DNA is also unknown.

PPKs: Role in motility and biofilm formation

Several recent studies have evaluated the role of PPKs in virulence-associated phenotypes such as motility, biofilm formation, adherence, invasion, and intracellular survival^[24,25,27,45]. Motility is an essential *C. jejuni* virulence mechanism^[49]. Three independent studies demonstrated that both PPK1 and PPK2 were not required for *C. jejuni* motility^[24,25,27]. This is in contrast to other organisms, where PPK1 is required for motility. Surprisingly, Chandrashekhar *et al.*^[32] demonstrated that several flagella-associated genes

Table 3 Summary of chicken colonization phenotypes of *ppk1* and *ppk2* mutants

Study	Organ/feces	Inoculation dose (CFU/chick)	Wildtype (CFU/g)	<i>ppk1</i> mutant (No. of chicks colonized)	<i>ppk2</i> mutant (No. of chicks colonized)
Candon <i>et al.</i> ^[24] 2007	Ceca	1.5 × 10 ⁵	1.8 × 10 ⁸	0/10 chicks	NS
		1.5 × 10 ⁶	1.8 × 10 ⁸	8/10 chicks	NS
		1.5 × 10 ⁷	1.8 × 10 ⁸	10/10 chicks at an average of 1.8 × 10 ⁸ CFU/g	NS
Gangaiah <i>et al.</i> ^[25] 2009 and 2010	Ceca	1.0 × 10 ³	1.0 × 10 ⁶	0/5 chicks	0/5 chicks
		1.0 × 10 ⁴	1.0 × 10 ⁸	0/5 chicks	0/5 chicks
		1.0 × 10 ⁵	8.0 × 10 ⁸	5/5 chicks at an average of 2.0 × 10 ⁴ CFU/g	5/5 chicks at an average of 9.0 × 10 ³ CFU/g
	Bursa	1.0 × 10 ³	1.0 × 10 ⁴	0/5 chicks	0/5 chicks
		1.0 × 10 ⁴	1.0 × 10 ⁵	0/5 chicks	0/5 chicks
		1.0 × 10 ⁵	1.5 × 10 ⁵	5/5 chicks at an average of 1.0 × 10 ² CFU/g	5/5 chicks at an average of 8.0 × 10 ² CFU/g
	Feces	1.0 × 10 ³	1.0 × 10 ⁵	0/5 chicks	0/5 chicks
		1.0 × 10 ⁴	1.0 × 10 ⁷	0/5 chicks	0/5 chicks
		1.0 × 10 ⁵	3.0 × 10 ⁷	5/5 chicks at an average of 1.5 × 10 ³ CFU/g	5/5 chicks at an average of 8.0 × 10 ³ CFU/g

NS: Not studied.

and flagellar glycosylation genes were downregulated in the *ppk* mutants; the authors hypothesized that the degree of downregulation in the *ppk* mutants may not have been enough to impair motility. Although motility was not affected by PPKs in *C. jejuni*, the potential role of PPKs on other phenotypes associated with flagella such as autoagglutination, secretion, and/or invasion needs further investigation.

Biofilms play an important role in *C. jejuni* tolerance to environmental stresses, disinfectants, and antimicrobials, as well as facilitating colonization of animal and human hosts^[50-52]. Two independent groups demonstrated that the *ppk1* mutant formed higher amount of biofilms than the wildtype^[24,25]. This finding is in contrast to other organisms, where PPK1 is required for biofilm formation^[13]. In another study, Gangaiah *et al.*^[27] demonstrated that *ppk2* mutant also formed higher amount of biofilms than its parent. Drozd *et al.*^[53] went onto characterize how PPK enzymes modulate biofilm formation in *C. jejuni* and demonstrated that *ppk1* and *ppk2* mutants formed higher adherent colonies on day 1 and 2 compared to wildtype. This study suggested that, compared to mutant strains, the wildtype might spend more time in planktonic phase, delaying biofilm formation^[53]. The authors further demonstrated that *ppk1* and *ppk2* mutants had reduced calcofluor white reactivity, suggesting that polysaccharide structures might be altered in the mutant strains^[53]. The quorum sensing molecule Autoinducer-2, which is required for biofilm formation, was increased in both mutants^[53]. Finally, the authors evaluated the mutants for altered expression of genes involved in biofilm formation and found that several genes (*pglH*, *kpsM*, *cj0688*, *neuB1*, *neuB1*, *fliS*, and *maf5*) involved in biofilm formation were altered in *ppk1* and *ppk2* mutants^[53].

PPKs: Role in adherence, invasion and intracellular survival

Adherence, invasion, and intracellular survival are also essential virulence mechanisms for *C. jejuni*^[54]. Recent studies demonstrated that both PPK1 and PPK2 are important for invasion and intracellular survival

within INT-407 human intestinal epithelial cells^[24,25,27]. Pina-Mimbela *et al.*^[45] showed that *ppk1* and *ppk2* mutants had qualitative and quantitative differences in outer membrane composition compared to wildtype. They also demonstrated that the differences in outer membrane composition in the mutants were directly related to the ability of *C. jejuni* to invade and survive within INT-407 cells^[45]. The authors of this study went one step further to evaluate which fractions were associated with invasion and intracellular survival^[45]. They found that outer membrane proteins were uniquely associated with invasion, whereas outer membrane proteins, lipids, and lipoglycans all were associated with intracellular survival^[45].

PPKs: Role in chicken colonization

Two independent studies evaluated the role of PPK1 in *C. jejuni* colonization in day-old chicks (Table 3)^[24,25]. Candon *et al.*^[24] demonstrated that, after 7 days of oral inoculation, the wildtype colonized the ceca of chicks at a minimum average of 1.79 × 10⁸ CFU/g of cecal contents at all inoculation doses examined. The *ppk1* mutant colonized the ceca of all chicks at a rate similar to that of wildtype at a dosage of 1.5 × 10⁷ CFU/chick, of 8 out of 10 chicks at a dosage of 1.5 × 10⁶ CFU/chick, and of 0 out of 10 chicks at a dosage of 1.5 × 10⁵ CFU/chick^[24]. In another study, Gangaiah *et al.*^[25] evaluated the role of PPK1 in colonization of ceca and bursa as well as *C. jejuni* load in feces using low inoculation doses *i.e.*, 10³, 10⁴, and 10⁵ CFU/chick. They demonstrated that, after 8 d of oral inoculation, the *ppk1* mutant colonized the ceca and bursa and showed a *C. jejuni* load in feces of all chicks but at a significantly lower rate than the wildtype at a dosage of 10⁵ CFU/chick^[25]. While the wildtype colonized to an average of about 1.0 × 10⁸ CFU/g of ceca and about 1.0 × 10⁵ CFU/g of bursa, and showed a *C. jejuni* load of about 1.0 × 10⁷ CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10⁴ CFU/chick^[25]. Similarly, while the wildtype colonized to an average of about 1.0 × 10⁶ CFU/g of ceca and about 1.0 ×

10^4 CFU/g of bursa, and showed a *C. jejuni* load of about 1.0×10^5 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^3 CFU/chick^[25]. Except for 10^5 CFU/chick, the two studies used different dosages for inoculation. Both studies were done in the same strain background; thus, the reason for the difference in the results between the two studies is unclear.

Furthermore, Gangaiah *et al.*^[27] evaluated the role of PPK2 in colonization of ceca and bursa as well in *C. jejuni* load in feces using 10^3 , 10^4 , and 10^5 CFU/chick inoculation doses (Table 3). They demonstrated that, at 10^5 CFU/chick inoculation dose, the *ppk2* mutant colonized the ceca and bursa as well as showed a *C. jejuni* load in feces of all chicks but at a significantly lower rate than the wildtype^[27]. While the wildtype colonized to an average of about 1.0×10^8 CFU/g of ceca and about 1.0×10^5 CFU/g of bursa, and showed a *C. jejuni* load of about 1.0×10^7 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^4 CFU/chick, except for 1 chick^[27]. Similarly, while the wildtype colonized to an average of about 4.0×10^6 CFU/g of ceca and about 1.0×10^4 CFU/g of bursa, and showed a *C. jejuni* load of about 1.0×10^5 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^3 CFU/chick^[27].

It is intriguing that both *ppk1* and *ppk2* mutants behave similar with respect to chicken colonization. Several *C. jejuni* genes have been identified to be important for chicken colonization^[55]; transcriptome and outer membrane proteome analyses showed that these genes did not appear to be regulated by PPK1 and PPK2^[32,45]. Thus, the mechanisms underlying the contribution of poly P to chicken colonization are unclear. Both *ppk1* and *ppk2* mutants showed dose-dependent colonization defects in day-old chicks^[24,25,27]. The authors hypothesized that the dose-dependency may be related to the hyperbiofilm phenotype of *ppk1* and *ppk2* mutants^[24,25,27]. According to this hypothesis, at lower inoculation doses, the *ppk* mutants are more sensitive to *in vivo* stresses and thus, display no or reduced colonization. At higher doses, the *ppk* mutants form hyperbiofilms, conferring resistance to *in vivo* stresses and thus, similar colonization of the mutants as that of wildtype. Additional *in vivo* studies complementing the mutants to rule out the effect of secondary mutations on colonization are warranted.

PPX/GPPA ENZYMES: ROLE IN *C. JEJUNI* SURVIVAL AND VIRULENCE

Malde *et al.*^[29] demonstrated that mutations in *ppx/gppa* genes were associated with a variety of phenotypes in *C. jejuni*. In this study, it was shown that *ppx/gppa* mutants were deficient in survival under nutrient limitation and osmotic stress. The authors further showed that the nutrient survival defect in

the mutants could be complemented by amino acid supplementation. Based on these findings, it was hypothesized that the nutrient survival phenotype in the mutants is likely due to reduced ppGpp or increased poly P levels. Both *ppx/gppa* mutants had increased poly P levels; however, only *ppx1/gppa* mutant had reduced ppGpp levels when compared to wildtype but not *ppx2/gppa* mutant, which indeed accumulated more ppGpp than the wildtype^[29]. This suggests that the nutrient survival defect in the mutants is more likely due to increased poly P levels rather than due to reduced ppGpp levels.

Further, Malde *et al.*^[29] also demonstrated that *ppx/gppa* mutants were compromised in several virulence-associated phenotypes such as motility, biofilm formation, and invasion and intracellular survival within human intestinal epithelial cells. Unlike *ppk1* and *ppk2* mutants, which had similar motility as the wildtype, the *ppx/gppa* mutants were defective in motility compared to wildtype. The *ppx/gppa* mutants also had decreased ability to form biofilms. Poly P is essential for chelation of cations^[13], which are required for biofilm formation^[56]; whether such a mechanism is the cause of reduced biofilms in *ppx/gppa* mutants is an interesting question. The group further went on to test the contributions of PPX/GPPA enzymes to serum resistance and demonstrated that *ppx/gppa* mutants were resistant to human complement but not to chicken complement.

Although the contributions of PPX/GPPA enzymes to *C. jejuni* biology is well characterized at the phenotypic level using *in vitro* assays, it remains to be understood if these contributions impact colonization of *C. jejuni* in day-old chicks and infection in humans. The mechanisms underlying the PPX/GPPA-associated phenotypes are also largely unknown; a transcriptome analysis of the *ppx* mutants would provide some insights on this aspect. In other bacteria, it has been shown that excess poly P in the *ppx* mutants restricts growth and downregulates metabolism, which is thought to have caused the underlying phenotypes^[57]. The *C. jejuni* *ppx/gppa* mutants grew very similar to the wildtype strain, yet were compromised in several phenotypes (*i.e.*, motility, biofilm formation, nutrient stress survival, invasion and intracellular survival, and resistance to human complement-mediated killing), suggesting that growth restriction and metabolic downshift are less likely the reasons for PPX-dependent phenotypes in *C. jejuni*. Phenotypes associated with excess of poly P were modest compared to those associated with poly P deficiency, suggesting that lack of poly P impacts *C. jejuni* biology more than excess of poly P. It is also intriguing to note that the phenotypes of the double mutant lacking both *ppx1/gppa* and *ppx2/gppa* genes were more severe compared to those of the individual mutants, suggesting some degree of functional redundancy in these enzymes; the reason behind this functional redundancy is unclear. Overall, findings from the analysis of *ppx/*

gppa mutants suggest that poly P levels are tightly regulated and that dysregulation of poly P levels as seen in the *ppx/gppa* mutants compromises *C. jejuni*'s survival and virulence properties.

LINK BETWEEN POLY P AND (P)PPGPP METABOLISM IN *C. JEJUNI*

Malde *et al.*^[29] assessed the role of PPX/GPPA enzymes in ppGpp synthesis and demonstrated that PPX1/GPPA but not PPX2/GPPA is important for ppGpp synthesis. In another study, Candon *et al.*^[24] demonstrated that a *spoT* mutant, which is deficient in ppGpp synthesis, had significantly reduced poly P levels than its parent. These findings suggest that (p)ppGpp and poly P metabolism are linked in *C. jejuni* (Figure 1). Both, (p)ppGpp and poly P metabolism are known to be linked in *E. coli*; in this organism, ppGpp is known to modulate poly P levels by inhibiting PPX enzymes^[58,59]. Analogous to *E. coli*, the authors speculated that (p)ppGpp might modulate poly P levels in *C. jejuni* by inhibiting PPX enzymes, which would not only affect the dynamic balance between poly P degradation by PPX enzymes but also affects poly P synthesis by PPK1. However, this hypothesis remains to be experimentally confirmed. In *Pseudomonas aeruginosa*, PPK2 affects (p)ppGpp accumulation; this role of PPK2 is attributed to its ability to serve as a source of GTP, which is a precursor for (p)ppGpp synthesis^[60]. However, a *C. jejuni ppk2* mutant accumulated similar (p)ppGpp levels to that of wildtype, suggesting that PPK2 is less likely the link between poly P and (p)ppGpp metabolism in *C. jejuni*. Although PPK1 does not appear to have ppGpp synthetic/hydrolytic activity, it remains to be understood if (p)ppGpp levels are altered in the *ppk1* mutant.

POLY P AND PHOSPHATE METABOLISM

Inorganic phosphate (Pi) is an essential nutrient for many bacterial species. As most natural environments are limiting in Pi, phosphate esters are the preferred source of Pi for bacteria^[61]. Phosphate esters are broken down into Pi in the periplasm by alkaline phosphatase. PhoAcj is the only alkaline phosphatase in *C. jejuni*^[62]. Drozd *et al.*^[53] recently demonstrated that a *phoAcj* mutant had significantly reduced intracellular poly P levels compared to its parent, suggesting that PhoAcj is likely the primary source of Pi for poly P synthesis^[53,63]. The authors also demonstrated that the *phoAcj* mutant had a significantly reduced ability to survive under nutrient stress, to invade and survive within INT407 cells and to colonize day-old chicks^[63]. The phenotypes in the *phoAcj* mutant were in general less severe when compared to those in the *ppk1* mutant. This is

likely due to the fact that *phoAcj* mutant only had a modest defect in poly P accumulation compared to the *ppk1* mutant, which had a severe deficiency in poly P accumulation^[63]. Unlike *ppk1* mutant, the *phoAcj* mutant had enhanced resistance to antimicrobials, suggesting that this phenotype is less likely due to poly P deficiency^[63].

PHENOTYPIC OVERLAP BETWEEN POLY P AND RELATED ENZYMES

Several phenotypes of PPK1, PPK2, PPX/GPPA, SpoT, and PhoAcj enzymes overlap between each other as shown in Table 2^[24,25,27,29,34,63,64]. For example, osmotic shock survival and low nutrient stress survival phenotypes were common to PPK1, PPK2, PPX/GPPA, and PhoAcj enzymes. Similarly, intraepithelial survival phenotype was common to all enzymes and, except for PPK1, invasion was also common to all the enzymes. There are two possible explanations for this overlap; the enzymes likely affect the shared phenotypes independently, or the enzymes might impact the phenotypes through a common protein or molecule. As PPK2 is required for poly P-dependent GTP synthesis, it is conceivable that poly P deficiency in the *ppk1* mutant likely also affects GTP levels^[25,27]. Thus, the phenotypic overlap between PPK1 and PPK2 may be the consequence of low GTP levels in these mutants. Both *spoT* and *phoAcj* mutants had low poly P levels^[34,63]; thus, the phenotypic overlap between PPK1, PhoAcj, and SpoT may be due to low poly P in these mutants. The ability to form biofilms was upregulated in the *ppk1* mutant^[24,25], which has low poly P levels, and downregulated in *ppx/gppa* mutants^[29], which have high poly P levels; this suggests that the biofilm phenotype is directly associated with poly P. This is further supported by the fact that *spoT* mutant, which has low poly P, also formed more biofilms than its parent^[34]. However, the *ppk2* mutant, which has similar poly P levels as its parent, also formed more biofilms compared to its parent^[27]; suggesting that the effect of PPK2 on biofilm formation is independent of poly P. Further studies are required to precisely define phenotypic overlap between poly P and related enzymes. For example, if the biofilm phenotype of the *spoT* mutant is due to low poly P levels, supplementation with poly P might rescue the biofilm phenotype in this mutant. Similarly, if the phenotypic overlap between *ppk1* and *ppk2* mutants is due to altered GTP levels, supplementation with GTP might rescue the associated phenotypes in the *ppk1* mutant. Strains containing mutations in multiple of these enzymes accompanied by complementation may also provide insights as to whether these enzymes have a synergistic or antagonistic effect on the overlapping phenotypes.

PPKS AS PROMISING DRUG AND VACCINE TARGETS

PPK enzymes have the potential to be ideal drug targets for controlling *C. jejuni* and other bacterial infections. First, these enzymes are highly conserved across a broad array of bacterial species^[13]; thus, the identified drugs will be effective against many bacterial species. Second, homologs of PPKs are absent in higher eukaryotes^[13]; thus, it is less likely that the drugs will be toxic to host cells. Third, a recent study by Pina-Mimbela *et al.*^[45] showed that PPKs modulate outer membrane composition of *C. jejuni*; thus, anti-PPK drugs could expose surface antigens, which are otherwise hidden, and make *C. jejuni* vulnerable to host defense mechanisms. Fourth, deletion of *ppk1* and *ppk2* does not affect growth of bacteria *in vitro*^[24,25,27]; thus, there is less selection pressure on the bacteria to develop resistance to anti-PPK drugs. Lastly, *ppk* mutants are more sensitive to several conventional antibiotics^[24,25,27]; thus, anti-PPK drugs could be used in conjunction with existing antibiotics for drug-resistant strains.

Mutants of poly P enzymes could also be sought as potential live attenuated vaccine candidates. Strains with deleted virulence genes have been successfully used for controlling *Salmonella* colonization in poultry^[65,66]. To have a synergistic effect on virulence/colonization attenuation, mutants containing deletions in *ppk* genes and other established virulence determinants could be used. As discussed before, using *ppk* mutants as live attenuated vaccine candidates not only has the advantage of the strain being attenuated for colonization but also could expose previously hidden antigens, likely inducing a strong protective immunity.

Epidemiological studies have shown that nearly 50%-80% of the human infections originate from ingestion of poultry and poultry products^[4]. Both PPK1 and PPK2 are necessary for *C. jejuni* colonization in chickens^[4,24,25,27]. These data suggest that PPKs could at least be targeted for controlling *C. jejuni* colonization in chickens, which likely would aid in reducing human infections. A human challenge model is available to study campylobacteriosis using the *C. jejuni* strain CG8421, which lacks ganglioside mimicry, a mechanism known to cause Guillain Barre Syndrome^[67-69]. The potential of using PPKs as drug and vaccine targets for human infections warrants that the contributions of these enzymes to human infection be studied using the human challenge model of campylobacteriosis.

CLOSING REMARKS AND PERSPECTIVES

Recent studies have yielded several important insights into the role of poly P in *C. jejuni* biology. Poly P is associated with a plethora of *C. jejuni* phenotypes,

which not only impact how *C. jejuni* survives in the environment under different stress conditions but also impact how this organism colonizes poultry and other domestic animals and causes disease in humans. Transcriptome and outer membrane proteomics analyses of the *ppk1* and *ppk2* mutants have provided some valuable mechanistic insights with regards to poly P-associated phenotypes. Nevertheless, our understanding of poly P in *C. jejuni* is still in its first steps. For example, the signals that activate poly P-mediated response are largely unknown. Most bacteria, including *C. jejuni*, contain two PPX enzymes; the reason behind this redundancy is an open question. Although transcriptome and outer membrane proteomic studies have yielded valuable insights into poly P functions, the few genes or proteins differentially expressed in the *ppk* mutants compared to wildtype do not seem to explain the plethora of phenotypes arising from mutants of poly P-associated enzymes. This suggests that poly P may also mediate its functions by affecting its targets posttranscriptionally; global proteomic analyses of the *ppk1* and *ppk2* mutants will likely shed some light on this aspect. It is also not known how poly P is regulated; in other words, what are the upstream components that feed into poly P-mediated response? How poly P feeds into the global regulatory network also remains to be understood. Poly P and ppGpp are linked in *C. jejuni* and other bacteria but the precise mechanisms underlying their interaction are poorly understood.

Given that poly P-related enzymes affect numerous aspects of *C. jejuni* life, these enzymes have the potential to be promising drug and vaccine targets. Therefore, it would be worthwhile to determine the contributions of poly P-related enzymes in a human model of *Campylobacter* infection. Gaynor *et al.*^[34] demonstrated that *ppk1* was upregulated during *C. jejuni* infection of human intestinal epithelial cells. Thus, studies defining at which stage of human infection and chicken colonization the poly P-mediated response is activated would provide additional insights into its role in *C. jejuni* pathogenesis/colonization. The recent finding that Poly P enzymes modulate IL-8 production in INT-407 cells suggests that host immune response to the mutant strains may be critical to study^[45]. Such studies could facilitate development of *ppk* mutants alone or in combination with mutations in other established virulence genes as live attenuated vaccines for reducing *C. jejuni* colonization in poultry and one day for controlling human infections.

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