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

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

**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.
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 Kreb’s 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_ 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.htm, 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[^5]. 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[^6-7].

Unlike in humans, _C. jejuni_ lives as a commensal in the gut of a variety of domestic and wild animals and birds[^1]. 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[^8]. Rarely, contaminated raw milk, water, and vegetables also serve as sources of human infection[^14]. 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 leuclidean-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

**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.

Kumar A et al. Poly P as a global regulator of stress response and virulence in C. jejuni

Table 1  Comprehensive summary of Campylobacter infections in humans

<table>
<thead>
<tr>
<th>Type</th>
<th>Clinical form/presentation</th>
<th>Risk factors</th>
<th>Target site</th>
<th>Symptoms</th>
<th>Species associated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(gastroesophageal reflux disease, Barrett’s esophagus, and esophageal adenocarcinoma)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Self-limited gastroenteritis, most common form</td>
<td>All factors as described in review Infection with C. jejuni and other species[21]</td>
<td>Jejunum and ileum</td>
<td>Diarrhea, fever, and abdominal pain</td>
<td>C. jejuni and C. coli[14,20]</td>
</tr>
<tr>
<td></td>
<td>Post-infectious functional gastrointestinal disorder, irritable bowel syndrome, and functional dyspepsia</td>
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<tr>
<td></td>
<td>Inflammatory bowel disease, Crohn’s diseases (CD), and ulcerative colitis (UC)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diarrhea, constipation, abdominal pain, weight loss, and rectal bleeding</td>
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<tr>
<td>(B) Extra-intestinal</td>
<td>Guillian-Barre syndrome</td>
<td>C. jejuni infection[26]</td>
<td>Nervous system</td>
<td>Progressive symmetric weakness in limbs, below or lack of reflex (hyporeflexia)</td>
<td>C. jejuni[14,25]</td>
</tr>
<tr>
<td></td>
<td>Abscesses of breast, brain, vertebra, and liver</td>
<td>Secondary bacterial infection</td>
<td>Breast, brain, vertebra, and liver</td>
<td>Varies with target organ</td>
<td>C. rectus[14,30], C. curvus, C. gracilis and C. showae[14,30]</td>
</tr>
</tbody>
</table>

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 diptheriae. 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,
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 (pppGpp) (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 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...
these granules are indeed poly P granules.

In another study, the role of PPK2 in poly P metabolism was investigated. 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. Unlike studies in other bacteria, which showed that PPK2 possesses poly P-synthetic activity, 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.

Malde et al. 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. However, whether PPX/GPPA enzymes possess PPX activity needs to be further experimentally confirmed.

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

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>PPK1</th>
<th>PPK2</th>
<th>PPX1</th>
<th>PPX2</th>
<th>PPK1-PPX2</th>
<th>PhoAcj</th>
<th>SpoT</th>
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<tbody>
<tr>
<td>Poly P metabolism</td>
<td>↑</td>
<td>-</td>
<td>-</td>
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<td>↑</td>
<td>↑</td>
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<tr>
<td>Poly P synthesis/accumulation</td>
<td>↑</td>
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<td>NT</td>
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<tr>
<td>Poly P-dependent GTP synthesis</td>
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<tr>
<td>Poly P degradation</td>
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<tr>
<td>Maintenance of ATP:GTP ratio</td>
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<td>ppGpp metabolism</td>
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<td>ppGpp synthesis</td>
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<td>↑</td>
<td>NT</td>
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<tr>
<td>Stress survival</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
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<tr>
<td>Aerobic stress survival</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
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<tr>
<td>VNBNC formation</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>Natural transformation</td>
<td>↑</td>
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<td>NT</td>
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<td>NT</td>
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<tr>
<td>Antimicrobial resistance</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>↓</td>
<td>R</td>
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<tr>
<td>Virulence-related</td>
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<tr>
<td>Motility</td>
<td>-</td>
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<td>↑</td>
<td>↑</td>
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<td>NT</td>
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<tr>
<td>Biofilm formation</td>
<td>↓</td>
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<td>↑</td>
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<td>↑</td>
<td>NT</td>
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<tr>
<td>Resistance to complement-mediated killing</td>
<td>NT</td>
<td>NT</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<td>NT</td>
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<tr>
<td>Adherence</td>
<td>NT</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>Invasion</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Intraepithelial survival</td>
<td>↑</td>
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<td>↑</td>
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<tr>
<td>Chicken colonization</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>↑</td>
<td>NT</td>
</tr>
</tbody>
</table>

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.

### PPKS: ROLE IN C. JEEJUNI 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. In other organisms, ppk1 mutants are defective in stationary phase growth and survival. 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. 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.

#### 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 (spot7) mutant, which is defective in eliciting a stringent response, was compromised in its ability to survive under low nutrient stress[29]. 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 ppk1 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 PPX 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[29].

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[35,37]. 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 PP1-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 PP1 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, Chandrashekar et al[32] demonstrated that several flagella-associated genes
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. Two independent groups demonstrated that the ppk1 mutant formed higher amount of biofilms than the wildtype. This finding is in contrast to other organisms, where PPK1 is required for biofilm formation. In another study, Gangiah *et al.* demonstrated that ppk2 mutant also formed higher amount of biofilms than its parent. Drozd *et al.* 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. 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. The quorum sensing molecule Autoinducer-2, which is required for biofilm formation, was increased in both mutants.

The authors evaluated the mutants for altered expression of genes involved in biofilm formation and found that several genes (*pgiH, kpsM, cj0688, neuB1, neuB1, filS*, and *maf5*) involved in biofilm formation were altered in ppk1 and ppk2 mutants.

### PPKs: Role in adherence, invasion and intracellular survival

Adherence, invasion, and intracellular survival are also essential virulence mechanisms for *C. jejuni*. Recent studies demonstrated that both PPK1 and PPK2 are important for invasion and intracellular survival within INT-407 human intestinal epithelial cells. Pina-Mimbela *et al.* 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. The authors of this study went one step further to evaluate which fractions were associated with invasion and intracellular survival. They found that outer membrane proteins were uniquely associated with invasion, whereas outer membrane proteins, lipids, and lipoglycans all were associated with intracellular survival.

### PPKs: Role in chicken colonization

Two independent studies evaluated the role of PPK1 in *C. jejuni* colonization in day-old chicks (Table 3). Candon *et al.* 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. In another study, Gangiah *et al.* evaluated the role of PPK1 in colonization of ceca and bursa as well as *C. jejuni* load in feces using low inoculation doses. 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. While the wildtype colonized to an average of about 1.0 × 10⁶ CFU/g of ceca and about 1.0 × 10⁵ CFU/g of bursa, and showed a *C. jejuni* load of about 1.0 × 10⁶ CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10⁴ CFU/chick. Similarly, while the wildtype colonized to an average of about 1.0 × 10⁶ CFU/g of ceca and about 1.0 × 10⁵ CFU/g of bursa, and showed a *C. jejuni* load of about 1.0 × 10⁶ CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10⁴ CFU/chick.
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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 10^6 CFU/chick[20]. Except for 10^6 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[22] 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 × 10^6 CFU/g of ceca and about 1.0 × 10^5 CFU/g of bursa, and showed a C. jejuni load of about 1.0 × 10^6 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^5 CFU/chick, except for 1 chick[27]. Similarly, while the wildtype colonized to an average of about 4.0 × 10^5 CFU/g of ceca and about 1.0 × 10^5 CFU/g of bursa, and showed a C. jejuni load of about 1.0 × 10^5 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^5 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[25]; transcriptome and outer membrane proteome analyses showed that these genes did not appear to be regulated by PPK1 and PPK2[23-25,45]. 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[27]. 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 P PX/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(63) 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 PK1, PK2, P PX/GPPA, SpoT, and PhoAcj enzymes overlap between each other as shown in Table 2(24,25,27,29,34,63). For example, osmotic shock survival and low nutrient stress survival phenotypes were common to PK1, PK2, P PX/GPPA, and PhoAcj enzymes. Similarly, intraepithelial survival phenotype was common to all enzymes and, except for PK1, invasion was also common to all the enzymes. These findings suggest that (p)ppGpp and poly P metabolism are linked 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 RELATED ENZYMES

**Source**: www.wjgnet.com

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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; thus, the identified drugs will be effective against many bacterial species. Second, homologs of PPKs are absent in higher eukaryotes; thus, it is less likely that the drugs will be toxic to host cells. Third, a recent study by Pina-Mimbela et al. 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; 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, 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. 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. Both PPK1 and PPK2 are necessary for C. jejuni colonization in chickens. 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. 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 PPK 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 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. 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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