Functional Characterization of Exopolyphosphatase/ Guanosine Pentaphosphate Phosphohydrolase (PPX/GPPA) Enzymes of Campylobacter jejuni

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Anand Kumar, BVSc&AH, MVSc

Graduate Program in Veterinary Preventive Medicine

The Ohio State University

2012

Master's Examination Committee:

Dr. Gireesh Rajashekara, Advisor
Dr. Yehia M. Saif
Dr. Daral J. Jackwood
Dr. Jordi B. Torrelles
Abstract

*Campylobacter* are small, curved, flagellated, non-spore forming, Gram-negative rods. Of 17 species within the genus *Campylobacter*, *C. jejuni* and *C. coli* are of most important public health concern. *Campylobacter* are considered to be most common causative agents of foodborne bacterial gastroenteritis and nearly, 90% of human *Campylobacter* infections are attributed to *C. jejuni*. The high prevalence of *C. jejuni* and its rare potential fatal sequelaes like Guillain Barre Syndrome, Miller Fisher Syndrome, and Reactive Arthritis have attracted considerable research interest. Efforts to reduce *C. jejuni* infection in humans are directly linked to the better understanding of its physio-biology which inturn explore the pathogenesis mechanism in host. Despite the availability of genomic information of different strains of *C. jejuni* and advanced genetic tools, the complete understanding of the virulence mechanism is still an ongoing effort. Hence, any research diverted towards better understanding of host-microbe interaction is of immense importance.

In-organic polyphosphate (poly-P), homeostasis is known to be essential for the virulence related phenotypic traits in pathogenic bacteria including *C. jejuni*. Although many enzymes are explored and implicated for their role in poly-P metabolism, the exopolypophosphatases (PPX) that hydrolyze poly-P is a less studied group of enzymes. More importantly, *C. jejuni* encodes two dual-function exopolyposphatases
(PPX1/GPPA and PPX2/GPPA) which in addition to poly-P hydrolysis also hydrolyze guanosine pentaphosphate (pppGpp) to guanosine tetraphosphate (ppGpp), an intracellular signaling molecule or an alarmone in bacteria. Therefore, we hypothesize that PPX/GPPA enzymes might play a key role in poly-P metabolism and thus significantly impact C. jejuni’s ability to persist in both the host and environment.

C. jejuni 81-176 encodes two potential dual-function exopolyphosphatases ppx1/gppa (CJJ81176_0377) and ppx2/gppa (CJJ81176_1251) and we generated deletion mutants (Δppx1, Δppx2 and dkppx) and complement strains (Cppx1, Cppx2) to better understand the PPX/GPPA role in C. jejuni pathophysiology. The mutants (Δppx1, Δppx2 and dkppx) exhibited defect in motility, invasion and intracellular survival, survival under nutrient limited condition. Furthermore they showed increased capacity to accumulate poly-P, however only Δppx1 and dkppx showed decreased accumulation of ppGpp compared to wildtype. Strikingly, the deletions of either ppx1 or ppx2 genes did not affect the biofilm formation, survival under osmotic and oxidative stresses; but on contrary deletion of both the genes (dkppx) did affect amount of biofilm formation and osmotic stress tolerance. Bactericidal assay to determine the role of PPX1/GPPA and PPX2/GPPA for resistant to complement mediated killing indicated that both Δppx1, and Δppx2 mutant were resistant to human complement mediated killing however, dkppx mutant was sensitive. In contrary, the chicken serum did not have any effect on the survival of Δppx1, Δppx2, and dkppx mutants. Interestingly, the expression of ppk1 and spoT genes, which are known to be interconnected with poly-P and ppGpp homeostasis were up regulated in the both Δppx1 and Δppx2 mutants compared to wildtype
suggesting a compensatory role in poly-P and ppGpp homeostasis. In all the cases complemented strains (Cppx1, Cppx2) restored the phenotypic characters similar to wildtype.

Our future studies will decipher the direct or indirect role of ppx/gppa genes in poly-P metabolism, investigate mechanism behind resistant of Δppx1, Δppx2 mutants to human complement factors, and study the functional redundancy of ppx/gppa genes in C. jejuni. In conclusion, we report that C. jejuni PPX1/GPPA protein has bifunctional activity capable of both poly-P and pppGpp hydrolysis, whereas PPX2/GPPA protein has monofunctional activity and mainly involved in poly-P hydrolysis. Thus, theses enzymes play critical role in poly-P metabolism and ppGpp homeostasis. Beside their role in stress related phenotypic traits they do have role in complement mediated resistance in human serum. Our study expands the multi-factorial regulation of poly-P and ppGpp metabolism in C. jejuni, may serve as unique model for other bacteria as well.
Acknowledgments

It’s my pleasure to thank my advisor, Dr. Rajashekara for his kind guidance, valuable criticism and constant motivation throughout my master program. His mentorship has a great value in framing my career and creating interest in science. I am also great full to him for providing an opportunity to continue my PhD in his lab. I would like to thank my advisory committee members, Dr. Saif, Dr. Jackwood, and Dr. Torrelles for their kind hearted guidance and close supervisions during my graduate student.

I would also like extend my thanks to Dr. Rajashekara lab members- Zhe Liu, Issmat I. Kassem, Mary Drozd, Yasser Sanad, Kshipra Chandrashekhara, Isaac Kashoma, Ruby Pina Mimbela, Xiulan Xu, and Annalalai Thavamathi for their constant assistance and timely help in my research work. My heart-felt gratitude to office associates Hannah Gehman, Robin Weimer, Ann Sanders and Graduate Program Coordinator Janelle Henderson for their kind support in academic issues. I would like to humbly acknowledge all professors, research scientists, post-doctoral researchers, and graduate students at FAHRP, Wooster, The Ohio State University, for their personal and intellectual support. I would have been all alone without my friend- very special thanks to Dharnesh and family for their emotional support and homely hospitality during their stay at Wooster. I would like to take this opportunity to thank Basavaraj and family for their cordial hospitality.
Special thanks to all my friends- Jagadeesh, Amimo, Kang, Daniel Thomas and Gaochang, who made my stay at Wooster enjoyable.

Reaching this stage in my life would have been impossible for me without the kind blessings of GOD, and unconditional love, support and sacrifice of my parents, brothers and their families. At the last but not the least, my heartfelt thanks and affection to my fiancée Meenakshi for her constant emotional support and for making me to believe in a wonderful saying- “We come (begin) to love not by finding a perfect person, but by learning to see an imperfect person perfectly”.

At last, I would like thank all those other known and unknown persons, who helped me directly or indirectly during my graduate studies.

Anand Kumar
Vita

August 1999 ........................................ Pre-University College, Gulbarga, India

1999-2004 .................................. Bachelor of Veterinary Science & Animal

    Husbandry, University of Agricultural

    Sciences, Dharwad, India

2004-2006 ................................... Master of Veterinary Science in Animal

    Biotechnology, GB Pant University of

    Agriculture and Technology, Pantnagar, India

2006-2007 ................................... Senior Research Fellow, Institute of Animal

    Health and Veterinary Biologicals,

    Bangalore, India

2007-2010--------------------------Veterinary Officer, Department of

    Animal Husbandry, Government of

    Karnataka, India

2010-present-------------------------Graduate Research Associate

    Food Animal Health Research Program

    Department of Veterinary Preventive Medicine

    The Ohio State University
Fields of Study

Major Field: Veterinary Preventive Medicine

Molecular Pathogenesis of *Campylobacter jejuni*
# Table of Contents

Abstract ........................................................................................................................................... ii

Acknowledgments ......................................................................................................................... v

Vita .................................................................................................................................................. vii

List of Tables .................................................................................................................................. x

List of Figures ................................................................................................................................. xi

Chapter 1 Review of Literature ..................................................................................................... 1

Chapter 2 Introduction .................................................................................................................... 20

Chapter 3 Materials and Methods ................................................................................................ 23

Chapter 4 Results ............................................................................................................................ 33

Chapter 5 Discussions .................................................................................................................... 41

Bibliography ................................................................................................................................... 66
List of Tables

Table 3.1 List of primers used in this study ................................................................. 48

Table 3.2. Bacterial strains and plasmids used in this study ................................. 49
List of Figures

Figure 1. Important routes of C. jejuni transmission in humans................................. 2
Figure 2. Inhibitory actions of pppGpp and ppGpp on PPX enzymes............................ 15
Figure 3. Lon protease roles in stringent and stress response.................................. 16
Figure 4. Phylogenetic tree of C. jejuni PPX/GPPA enzymes ................................. 50
Figure 5. Structure-based sequence alignment of PPX/GPAA domains of C. jejuni and related bacteria ................................................................. 51-52
Figure 6. Predicted three-dimensional structure of C. jejuni PPX/GPPA enzymes .... 53
Figure 7. Genetic organization of ppx1/gppa and ppx2/gppa genes in C. jejuni genome, and approximate location of antibiotic markers in the deletion mutants ............... 54
Figure 8. Growth kinetics of C. jejuni ppx/gppa mutants ........................................ 55
Figure 9. Intracellular poly-P levels in C.jejuni Δppx mutants................................. 56
Figure 10. Intracellular (p)ppGpp levels in C.jejuni Δppx mutants.............................. 57
Figure 11. Motility of C. jejuni Δppx mutants ......................................................... 58
Figure 12. Biofilm formation by C. jejuni Δppx/gppa mutants................................. 59
Figure 13. Nutrient downshift assay of C. jejuni Δppx mutants ............................... 60
Figure 14. Sensitivity of Δppx/gppa mutants to oxidative stress ............................. 61
Figure 15. Sensitivity of Δppx/gppa mutants to oxidative stress ............................. 62
Figure 16. The ∆ppx mutants’ Intracellular invasion & Intracellular survival assay……63
Figure 17. Complement dependent killing of C. jejuni ∆ppx/gppa mutants…………..64
Figure 18. Quantitative RT-PCR analysis of the WT and C. jejuni ∆ppx mutants……..65
Chapter 1: Review of Literature

1.1 Overview

The term *Campylobacter* is coined from a Greek word “Kampylos” means curved. The genus *Campylobacter* consists of 17 species and 6 subspecies (On, 2005; Debruyne *et al*., 2008) with *Campylobacter fetus* as a type species. *Campylobacter* species readily colonize in the gut of domestic, feral and wide variety of animals and birds, notably poultry (Allos, 2001). Infection of *Campylobacter* ‘Campylobacteriosis’, is a food and water-borne, regarded as a leading cause of bacterial induced acute gastroenteritis worldwide. *C. jejuni* and *C. coli* are considered to be the most common species causing human infections and are ubiquitously found in nature; environment, wild birds, and mammals.

*C. jejuni* is a curved, Gram-negative, motile, non-spore forming bacteria with size ranging from 0.2 to 0.8 μm in width and 0.5 to 5.0 μm in length, belongs to the epsilon class of proteobacteria, in the order *Campylobacteriales*. *C. jejuni* is considered to be a commensal organism of avian species especially, chickens. Thermophilic *Campylobacter* species grows optimally at temperature near 42°C (Park SF, 2002), the higher metabolic temperature found in poultry species may predispose poultry to be a prominent reservoir for the thermotolerant *Campylobacter*. Although the experimental infection with *C. jejuni* in chickens can lead to diarrhea (Ruiz-Palacios *et al*., 1981; Sanyal, S. C. *et al*., 1984),
this is not typical in natural conditions, and it appears that the human response to C. jejuni infection is more symptomatic than that of the chicken. As a human pathogen, C. jejuni accounts for nearly 90% of the total human Campylobacter infections, while C. coli infections constitute 3-4% of cases (Frost et al., 1999). Reports show that C. jejuni affect approximately 1% of population in the developed countries like, United States, Australia and United Kingdom (Friedman et al., 2000).

In the developed world, where waterborne infection is less likely, animals are the primary source of human infection. Nearly 90% of the United States poultry flocks are colonized with C. jejuni (Stern et al., 2001) besides, can carry a load >10^7 CFU per gram of feces of C. jejuni. (Mead et al., 1995). Due to high prevalence and the low infection dose in humans’ i.e.as low as 500 bacteria (Skirrow, 1982; Skirrow, 1990), it is not surprising that the most of human campylobacteriosis are acquired by handling or consumption of contaminated/undercooked poultry and poultry products. However, consumption of unpasteurized milk, contaminated fruits and vegetables, environmental water sources are also frequently attributed in human infections.

Figure 1 Important routes of C. jejuni transmission in humans.

Source: Dasti I.J et al., 2009
Following the exposure, *C. jejuni* withstand the acidity of stomach and can colonize the lower intestinal tract (ileum, jejunum, and colon) without causing any symptoms (Ketley, 1997). The symptomatic cases are often self-limiting, represented with fever, vomiting, headache, watery or bloody diarrhea with abdominal pain. *C. jejuni* motility and corkscrew shaped morphology aids in successful colonization at the intestinal epithelial cells, subsequently damage caused by invasion and toxin production results in inflammation and diarrhea (Park, 2002). In extraintestinal manifestations, and post-infection sequelae by *Campylobacter* are attributed to autoimmune disorders like Guillian-Barre syndrome (GBS), Miller Fishers syndrome, and reactive arthritis. The most common and obvious answer to autoimmune disorder is the ‘Molecular mimicry’, occurs due to shared terminal molecules of *C. jejuni* antigens with proteins on human peripheral nerves (Komagamine and Yuki, 2006).

The basic mechanism of the different outcomes of *C. jejuni* infection in humans versus chickens is not clearly understood. This can be partly attributed to the lack of a good small animal model that reproduces the human disease. Although, ferret model simulates the human symptoms of *C. jejuni* infection (Fox A.G. et al., 1987), the high cost and lack of good knockout technology to study host factors that are involved in disease limits the usage of this model. As a natural host, chicken is a good model for studying basic aspects of host colonization and is potentially a good target for anti-*Campylobacter* strategies. In addition to natural and experimental models of animal
infection, human intestinal epithelial cell lines, such as the INT 407 cell line, have also been used in *C. jejuni* studies.

In contrast to other well-known enteropathogens such as *Salmonella*, *Shigella*, and *E. coli* species, *C. jejuni* is quite fastidious, with a complex metabolism requiring microaerophilic and capnophilic growth conditions. Furthermore, *C. jejuni* is asscchrolytic, lacking capacity to both transport and catabolize the most carbohydrates, and instead relies on aminoacids such as serine, glutamine, and aspartate as primary carbon and energy source (Kelly *et al.*, 2008). It also lacks many “classical” virulence factors, such as exotoxins and type III secretions systems that are hallmark of enteropathogens. The “invasiveness” of *C. jejuni* strains is often used as a measure of *Campylobacter* virulence, to date bacterial factors implicated in host cell invasion and disease pathogenesis are capsular polysaccharide (CPS), flagellar apparatus, cytolethal distending toxins (CDT), and post-translational glycosylation. The host factors are nuclear factor (NF)-κB, IL-8, and epithelial β-defensins (Zilbauer M. *et al.*, 2008).

1.2 In-organic polyphosphate (poly-P)

1.2.1 Introduction: In-organic polyphosphate is a linear polymer of ten to hundred phosphate residue linked by high energy phosphoanhydride bonds as found in ATP. Primitive earth under high pressures and desiccation resulted in phosphate rocks, some of them as poly-P. Yet, indisputably origin of life is a phosphate world, the unique chemical and physical properties of poly-P and its ubiquity must have played key roles in the origin and survival of species (Rao *et al.*, 2009). Poly-P granules in bacteria are also called “Volutin granules” or “Metachromatic granules” due to their tendency to stain pink
with basic blue dyes (Pallerla et al., 2005). Over a century ago, the presence of metachromatic granules was used as a diagnostic feature for medically important bacteria such as *Corynebacterium diptheriae*. Recently, with the advent of electron microscopy, these granules were identified in other nonpathogens like *Corynebacterium glutamicum* thereby refuting the presence of poly-P granules as characteristic of diagnosis of disease state alone (Pallerla et al., 2005).

1.2.2 Poly-P analysis methods: These methods are important to give better understanding of poly-P structure and its role. Below mentioned analysis methods help us to reveal the poly-P chain length specification of enzymes, localization inside the cell, complex structure formed with other proteins etc.

1.2.2.1 Enzymatic method—Requires the action of a yeast exopolypolyphosphatase (scPPX1) to hydrolyze the Poly P to inorganic phosphate (P$_i$). The released P$_i$ measured either by thin-layer chromatography (TLC) or by phosphoimager (Rao N et al., 1998). Another enzymatic assay uses the reverse reaction of Poly-P kinase 1 (PPK1) to measure conversion of ADP to ATP by Poly P, using a specific and sensitive luciferase method (Ault et al., 1998). Chain-length specificity of these enzymatic assays are below 60 P$_i$.

1.2.2.2 Ion Exchange Method—Chromatography with an online hydroxide eluent generator was used to analyze Poly-P up to 50 residues (Ohtomo et al., 2004). Combination of above mentioned methods are used to measure both long- and short-chain Poly-P.
1.2.2.3 **Metachromatic Staining** – Here, Toluidine blue dye is used to determine broad chain-length specificity but is relatively insensitive. This method was used to measure poly-P synthesizing activity in crude extracts (Mullan A. *et al.*, 2002).

1.2.2.4 **$^{31}$P-NMR Spectroscopy** - This method is used to measure Poly-P in intact cells, the relative Poly-P content is estimated as the intensity ratio of the “core Poly P” signal to the intracellular $P_i$ (Zakrzewska J *et al.*, 2005 and Zivic M *et al.*, 2007). A bioreactor coupled with $^{31}$P NMR spectroscopy was used to analyze both intra- and extracellular Poly- P (Gonzalez B *et al.*, 2000).

1.2.2.5 **Electron Ionization Mass Spectrometry** - The high specificity of electron ionization mass spectrometry (ESI-MS) allows the detection of different Poly-P species without prior separation by ion exchange chromatography or capillary electrophoresis and the detection limit ranges from ≈1 to 10 ng/ml.

1.2.2.6 **Cryoelectron Tomography and Spectroscopy Imaging** - This method is applied for characterizing intact subcellular poly-P granules inside the bacterial cell using combination of techniques. Cryoelectron microscopy and tomography is used to study the biogenesis and morphology of these bodies (resolution >10 nm), whereas spectroscopic imaging provides the direct identification of their chemical composition (Comolli LR *et al.*, 2006). This method can also distinguish between carbon-rich and $P_i$-rich bodies.

1.2.2.7 **In Vivo Protein Affinity Labeling** - This method offers visualization of Poly-P in intact cells at the electron microscopy level with much higher specificity and resolution. Here, the poly P-binding domain (PPBD) of *E. coli* PPX1 protein is exploited to directly demonstrate the poly- P localization inside the bacterial cell (Saito K *et al.*, 2005). The
samples are labeled with epitope-tagged PPBD, followed by the detection of the tag by an immunocytochemical procedure.

1.2.3 Cellular location of Poly-P: (a) Prokaryotes- It has been reported to occur as electron-dense volutin granules in bacteria (Meyer, 1904). Poly-P also exists as poly-(R)-3-hydroxybutyrate complex in the outer membranes of *Hemophilus influenza* and *E. coli* (Zakharian and Reusch, 2007). Ca$^{2+}$-Poly-P rich granules are also seen in *Agrobacterium tumefaciens* and *Rhodospirillum rubrum* (Seufferheld et al., 2003).

(b) Eukaryotes - Acidocalcisomes are vacuoles containing poly-P, pyrophosphate and divalent metal ions, commonly documented in the unicellular eukaryotes such as trypanosomatid and apicomplexan parasites, algae and slime molds (Docampo et al., 1995; Moreno and Zhong, 1996). In alga *Chlymydomonas reinhardtii*, poly-P is found in the vacuoles along with Ca$^{2+}$ and Mg$^{2+}$ (Komine et al., 2000). In *Saccharomyces cerevisiae*, poly-P is widely distributed in every compartment including cytosol, vacuoles, nuclei and mitochondria (Wurst et al., 1995; Trilisenko et al., 2002; Lichko et al., 2006). Nearly, 80-90% of the total poly-P in *S. cerevisiae* is found in the cytosol, while 15% occurs in the vacuoles. Poly-P has also been reported in all higher eukaryotes so far investigated (Kumble and Kornberg, 1995), and commonly seen in the tissues like kidney, liver, lungs, brain, and heart, and subcellular compartments such as mitochondria.

1.2.4 Functions of Poly-P: In prokaryotes, poly- P is essential for numerous cellular functions such as ATP and energy source, phosphate reservoir, cations sequestration and storage, buffer 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 (Kornberg et al., 1999; Brown and Kornberg, 2004 and 2008; Seufferheld et al., 2008; Rao et al., 2009).

In eukaryotes beside above mentioned functions, poly-P also mediates cytokinesis and cell division (Zhang et al., 2007), blood clotting (Smith et al., 2006), bone and calcium metabolism (Hacchou et al., 2007; Morimoto et al., 2010), signaling, cell growth, proliferation and apoptosis (Shiba et al., 2003; Wang et al., 2003; Hernandez-Ruis et al., 2006), mitochondrial ion transport and respiratory chain activity (Kulakovskaya et al., 2010) and yolk mobilization and embryogenesis (Gomes et al., 2010).

1.2.5 Industrial applications of Poly-P: Additionally, poly-P has several industrial applications such as microbial remediation of phosphate and toxic metals in waste water treatment, the use of poly-P in the biomining of copper, the use of plants in phytoremediation. Poly-P as a food additive is widely used, especially in the meat and dairy industry to enhance flavor, water binding, color retention, and emulsification, while retarding oxidative rancidity. Poly-P is also used as antimicrobial, and in general Gram positive bacteria are found to be more sensitive than the Gram negative bacteria. The antimicrobial effect is concentration dependent, higher concentration is bactericidal, whereas sublethal concentration retards the cell growth (Maier SK, et al., 1999). Generally, the ability of poly-P to chelate cations is regarded as the cause of antimicrobial effect, and it was shown that the membrane permeabilizer like poly-P
increased the susceptibility to a range of antibiotics (Denny B, *et al.*, 2003). In biotechnology, it is supplied as a cheap source of high-energy phosphate to substitute for costly ATP (Rao N, *et al.*, 2009).

**1.2.6 Poly-P homeostasis**

**1.2.6.1 Polyphosphate kinase 1 (PPK1):** PPK1 is the principal enzyme responsible for synthesis of poly-P in most bacteria (Ahn and Kornberg, 1990; Akiyama *et al.*, 1992). PPK1 catalyzes the polymerization of the terminal phosphate of ATP into a poly-P chain. This reaction is reversible but favors synthesis of poly-P. PPK1 is highly conserved and PPK1 homologs have been found in over >100 bacterial species including 20 major pathogens. *E. coli* PPK1 (EcPPK1) is a homotetramer of 80 KDa subunits attached to the inner cell membrane. The crystal structure of EcPPK1 revealed that its active site is located in a highly conserved structural tunnel with a unique ATP binding pocket (Zhu *et al.*, 2005). The first step in poly-P synthesis is auto-phosphorylation of the enzyme. The recent studies suggest that histidine residue H435 is involved in autophosphorylation (Rao *et al.*, 2009).

Several investigations in many bacterial species have shown that PPK1 plays an important role in bacterial survival under conditions of stress, virulence, and host colonization. Poly-P is important for growth, motility, biofilm formation, antibiotic resistance, stationary-phase survival, resistance to nutrient, oxidative, osmotic, invasion and intracellular survival and host colonization (Rao and Kornberg, 1996; Rashid and Kornberg, 2000; Ayraud *et al.*, 2005; Candon *et al.*, 2007; Gangaiah *et al.*, 2009; Rao *et al.*, 2009; Tunpiboonsak *et al.*, 2010).
1.2.6.2 Polyphosphate kinase 2 (PPK2): PPK2 is another major enzyme, which preferentially mediates poly-P driven generation of GTP (Ishige et al., 2002; Sureka et al., 2009). PPK2 is widely conserved in bacteria including major human pathogens such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Vibrio cholera* (Ishige et al., 2002). PPK2 class of enzymes belong to the large superfamily of P loop kinases, which catalyze the hydrolysis or binding of nucleoside triphosphates (Leipe et al., 2003). The hallmark of P loop kinases is the presence of Walker A (GXXXXGK), and Walker B (hhhhD, where h is a hydrophobic residue) motifs as well as a lid module (Rx(2-3)R). Walker A and Walker B motifs are essential for binding nucleoside triphosphates, and Mg$^{2+}$ cation, respectively (Leipe et al., 2003). PPK2 enzymes are classified into 2 groups, those containing 1 or 2-fused PPK2 domains. Proteins with a 1-PPK2 domain can also catalyze poly-P dependent phosphorylation of ADP to ATP, while proteins containing 2-fused PPK2 domains phosphorylate AMP to ADP (Nocek et al. 2008).

PPK2 class of enzymes are distinct from PPK1 in several aspects; PPK2 utilizes poly-P to generate GTP at a rate 75-fold greater than that of poly-P synthesis from GTP or ATP (Ishige et al., 2002). The other feature is the relative selectivity of guanosine and adenosine nucleotides as donors and acceptors; PPK2 uses GTP and ATP equally well in poly-P synthesis, but PPK1 is strictly specific for ATP. For poly-P synthesis activity, Mn$^{2+}$ is preferred over the Mg$^{2+}$ by PPK2, whereas reverse is true for PPK1. In *M.tuberculosis*, PPK2 was shown to be important for survival under stress conditions such as heat, acid and hypoxic stresses, regulating intracellular nucleotide pool as well as intracellular survival in macrophages (Sureka et al., 2009).
1.2.6.3 Polyphosphatases (PPN/Xs): Poly-P is degraded to inorganic phosphate by two class of enzymes; endo- (PPN) and exopolypophosphatases (PPX) (Rao et al., 2009).

1.2.6.3.1 Endopolyphosphatases (PPN): PPN are the least studied of the poly-P metabolizing enzymes and have been reported in species of *Penicillium* and *Aspergillus* and in *S. cerevisiae* (Krishna P.S. 1952; Mattenheimer, H. et al., 1956). They are widely distributed in animal cells and is more abundant in yeast (Kumble and Kornberg, 1996), however, remarkably little or none detected in *E. coli* due to abundant exopolypophosphatase activities (Akiyama, M et al., 1993). PPN also called poly-P depolymerases or polyphosphorylases, catalyze the non-processive cleavage of poly-P to release intermediate-size chains during the course of the reaction. An endopolyphosphatase attacks long chain poly-P (P$_{700}$) internal phosphaanhydride bonds, hydrolyzing poly-P molecules into oligophosphates of smaller size (P$_{60-3}$), which are further processed by exopolypophosphatases.

In yeast, mutant lacking *ppn1* shows limited growth and fails to survive in a minimal medium in the stationary phase. The loss of viability of the yeast *ppn1* mutant may be the result of an accumulation of large amounts of poly-P as long chains. The powerful chelating capacity of this poly-P might sequester divalent metal cations like Ca$^{2+}$ Mg$^{2+}$ and Mn$^{2+}$ that would damage essential cellular functions (Dunn, T et al., 1994). In yeast, PPN1 enzyme shown to be a homotetramer of a 35-kDa (352 amino acids) subunit, derived from protease action on a 78-kDa polypeptide of vacuolar origin (674 amino acids) to generate 35-kDa subunit (Kumble K, Kornberg A. 1996; Shi X, Kornberg A. 2004). For the PPN activity to manifest in yeast, it is necessary that strains
should possess vacuolar proteases, indicating a need for proteolytic processing of the pre-PPN1 enzymes.

1.2.6.3.2 Exopolyphosphatases (PPX): Exopolyphosphatases, PPXs hydrolyze the terminal phosphate from linear long chain Poly-P (P$_{60}$) to release short chain containing three or more phosphoanhydride bonds. They are classified into two types; (a) the cytoplasmic PPX1 from S. cerevisiae (scPPX1) and PPX from fungi and protozoa belonged DHH phosphoesterase family. The DHH family are named after the characteristic triplet motif, Asp- His-His, that contributes to the active site of each member. Although, these two PPX types have most of things in common but differ in their molecular weight and ATP hydrolyzing ability; scPPX1 cannot hydrolyze ATP (Kulakovskaya, T. V et al., 1997 and Guranowski, A et al., 1998).

Beside above classification, structurally two PPXs have been characterized: the stringent response-related exopolyphosphatase/ guanosine pentaphosphate phosphohydrolase (PPX/GPPA), a monomeric protein that folds into two domains and is thought to be principally a GPPA; and the PPX related with hydrolysis of linear poly-P particularly, P$_{60-3}$, a dimer with four domains in each subunit (Kristensen, O et al., 2004).

E. coli contains 2 PPXs, PPX1 and GPPA (PPX2) (Rangarajan et al., 2006). E. coli PPX1 is an enzyme of 513 amino acids with a molecular mass of 58.1 kDa. PPX1 enzyme releases orthophosphate (Pi) from the linear poly-P chain residues in a processive manner and requires Mg$_{2+}$ and KCl for its maximum activity. The action of PPX is directed towards the ends of poly-P chain, from which it removes orthophosphate processively (Masahiro et al., 1993). The mechanism that enables PPX to distinguish the
ends of a long chain poly-P from those of a short one is obscure and intriguing. As for the cellular salvage and disposal of short chains are concerned, they may be used as primers for polyphosphate kinase or degraded by numerous other phosphatases (Ahn et al., 1990).

The crystal structure of *E. coli* PPX1 revealed four domains (I, II, III and IV) and the putative PPX site is located at the interface between domain I and domain II. PPX1 contains two functional domains, N-terminal catalytic domain and C-terminal domain that is responsible for poly-P binding and processivity. Overproduction of PPX results in several striking phenotypes in *E. coli* (Crooke et al., 1994; Rao N et al., 1996 and Shiba et al., 1997); decreased long-term survival in stationary phase; increased sensitivity to oxidative, osmotic, and thermal stresses; and defects in adaptive growth in minimal medium after a shift from rich medium. These phenotypes are likely due to the decreased expression of the rpoS gene, which encodes the principal stationary-phase sigma factor, sS, or RpoS (Shiba et al., 1997). *Corynebacterium glutamicum* contains 2 genes encoding exopolyphosphatases, *ppx1* and *ppx2* (Lindner et al., 2009). PPX2 was shown to be the primary exopolyphosphatase in *C. glutamicum* and was shown to be active as monomer requiring Mg$^{2+}$ and Mn$^{2+}$ for maximum activity. However, the enzyme was inhibited by higher concentrations of Mg$^{2+}$, Mn$^{2+}$ and Ca$^{2+}$. Furthermore, PPX2 enzymes also shown to be active with short-chain polyphosphates, even accepting pyrophosphates, and was inhibited by nucleoside triphosphates. Interestingly, PPX enzymes have been shown to be important for avoiding complement-mediated killing in *Neissariameningitidis* (Zhang et al., 2010).
PPX2/GPPA is a bifunctional enzyme possessing both exopolyphosphatase and guanosine pentaphosphate phosphohydrolase activities (Kristensen et al., 2004; Rao et al., 2009). *E. coli* PPX2/GPPA is a bifunctional enzyme, liberates Pi by processively hydrolyzing poly-P chains and also hydrolyzes guanosine pentaphosphate (pppGpp) to generate ppGpp, an intracellular second messenger. PPX2/GPPA enzymes are the members of the sugar kinase/actin/hsp-70 superfamily and are distinct in sequence and structure from the functionally related RelA/SpoT enzymes which modulate stringent response. The poly-P homeostasis and stringent response are closely connected with each other (Kuroda et al., 1997; Kristensen et al., 2008), and it has been shown that pppGpp and ppGpp signaling molecules interconnects the ppGpp and poly-P homeostasis. In amino acids limitation condition, uncharged tRNA binds non-enzymatically to the acceptor site of an elongating ribosome activating the RelA protein to synthesize the pppGpp (Potrykus et al., 2008). Accumulation of pppGpp inhibits the PPX mediated poly-P degradation activity (Kuroda et al., 1997) thereby increased level of poly-P. Poly-P and pppGpp also induces the expression of RNA polymerase sigma factor, RpoS (Rao NN et al., 1996 and Shiba T et al., 1997), which inturn directs the transcription of > 50 key genes involved in shifting normal growth and metabolism to persistent state (Hengge-Aronis R et al., 2002). Hence, both ppGpp and poly-P accumulate in response to nutrient limitation and play essential roles in bacterial survival under diverse stress conditions.
Under amino acid starvation, both general stress response and stringent response are elicited, and the Lon protease degrades unnecessary proteins to salvage amino acids. Ribosomal proteins are among the commonly degraded proteins under amino acid starvation. Bacteria use these amino acids to synthesize new proteins required for survival in the minimal medium (Yen et al., 1980). Poly-P has been shown to regulate the Lon protease-mediated protein degradation in *E. coli* (Kuroda et al., 2001; Nomura et al., 2004; Kuroda, 2006). Free ribosomal proteins were preferentially degraded by poly-P-Lon protease complex compared to Lon protease alone. Poly-P was found to facilitate binding of Lon protease and the ribosomal protein. The DNA binding site for Lon protease is the same as that for poly-P and Lon protease has more affinity for poly-P than DNA. Thus, poly-P not only regulates Lon protease-mediated protein degradation, but also influences Lon protease-DNA binding activities.

Figure 2 Inhibitory actions of ppGpp and pppGpp on PPX enzymes.

Source: Kornber A. *et al.*, 1999
Although different suggestions were presented in these studies concerning the bifunctional aspects of PPX/GPPA; one study suggested two separate active sites (Rangarajan et al., 2006), and the other was in favor of a common active site (Alvarado et al., 2006). Kristensen O et al., 2004 and 2008 crystallized the PPX/GPPA enzymes of A. aeolicus and E. coli and showed the existence of common active site. A simple hinge movement of domain I and II is sufficient to bring the E. coli PPX enzyme into an “Open State” that is compatible with both poly-P and pppGpp hydrolysis at a common active site. This revealed a structural flexibility that has previously been described as a

Annu. Rev. Biochem. 78:605–47

Figure 3 Lon protease roles in stringent and stress response.
"butterfly-like" cleft opening around the active site in other actin-like superfamily proteins. A calcium ion is observed at the center of this region in crystals, substantiating that PPX/GPPA enzymes use metal ions for catalysis (Kristensen et al., 2008).

1.3 Poly-P role in bacterial pathogenesis

Bacterial virulence factors are generally expressed in stationary phase, and poly-P and its metabolizing enzymes are essential for stationary phase survival (Rao and Kornberg, 1996; Kornberg et al., 1999; Condon et al., 2007; Gangaiah et al., 2009 and 2010). Microarray analysis of ppk1 mutants of stationary phase P. aeruginosa and E. coli revealed up-regulation of over 250 genes and down-regulation of more than 450 genes (unpublished data quoted in Rao et al., 2009). Genes involved in quorum sensing, iron starvation response system, and type III secretion system were down-regulated, while genes involved in amino acid and polyamine metabolism, nitrogen metabolism and transport, and biogenesis of type IV fimbriae were found to be up-regulated in P. aeruginosa. Similarly, PPK2 was also shown to regulate the expression of over 20 genes in P. aeruginosa. As a component of degradosome, PPK1 might also play a role in posttranscriptional regulation in bacteria (Blum et al., 1997).

In addition, poly-P was shown to be important for competitive fitness of P. fluorescens (Silby et al., 2009), serum resistance of N. meningitidis (Tinsley and Gotschlich, 1995), invasion and intracellular survival within epithelial cells in Salmonella and Shigella (Kim et al., 2002), and host colonization in P. aeruginosa (Fraley et al., 2007). The Salmonella ppk1 mutant exhibited defects in long term survival (Kim et al., 2002). Mutants of ppk1 in other bacteria have revealed defects in growth, motility,
biofilm formation, quorum sensing, stress resistance, stationary phase fitness and resistance to antibiotics (Ogawa et al., 2000; Rashid et al., 2000; Rashid and Kornberg, 2000; Fraley et al., 2007; Schurig-Briccio et al., 2009; Gangaiah et al., 2009 and 2010).

Poly-P also plays a role in structure and function of bacterial chromosome and a ppk1 mutant exhibited compaction of nucleoid from 46% of the cell area to 26% in P. aeruginosa (Fraley et al., 2007). Nucleoid compaction is believed to have contributed to defect in motility, biofilm formation, quorum sensing, and virulence in P. aeruginosa ppk1 mutant. Histone-like (HU) proteins were found to bind poly-P more strongly than the linear double-stranded DNA and poly-P could displace HUs bound to DNA in vitro. This contribute to bending and alteration of DNA at specific sites, are believed to be involved in nucleoid stability and integrity (Rao et al., 2009). These findings suggest a role for poly-P in regulation of gene expression possibly by displacing HUs from DNA.

1.4 ppGpp role in bacterial pathogenesis

Stringent response is a global stress response that alters bacterial gene expression to enable survival under diverse stress conditions such as nutrient limitation, phosphate limitation, oxidation, heat, desiccation and hyper-osmolarity (Cashel et al., 1996). Stringent response is mediated by ppGpp which is synthesized from pppGpp by RelA or SpoT. ppGpp is thought to bind to RNA polymerase and alter the gene expression by affecting promoter specificity, transcription initiation and elongation (Cashel et al., 1996; Chatterji and Ojha, 2001). Both pppGpp and ppGpp repress many genes including those involved in ribosome synthesis and activate 50 or more genes responsible for coping with stress and starvation. ppGpp plays multiple roles in bacterial physiology and pathogenesis
including long term persistence, symbiosis, quorum sensing, production of antibiotics, biofilm formation, virulence, and sporulation (Dalebroux et al., 2010). ppGpp is also a positive effector of RpoS and RpoS-dependent genes and thus ppGpp regulates the induction of RpoS-dependent general stress response (Jishage et al., 2002).

It has been shown that both pppGpp and ppGpp affect poly P accumulation in E. coli in response to amino acid starvation (Kuroda et al., 1997; Rao et al., 1998). Mutants of E. coli and Salmonella deficient in synthesis of stringent factors relA and spoT were not only defective in (p)ppGpp synthesis but also poly-P accumulation (Kuroda and Ohtake, 2000). The stringent response nucleotides (pppGpp and ppGpp) had no effect on the activities of poly-P synthesizing enzyme PPK1. However, (p)ppGpp was inhibitory to PPX and thus affecting the dynamic balance between poly P synthesis by PPK1 and poly P degradation by PPX, accounting for elevated levels of poly P. The poly-P utilizing enzyme PPK2 also has been associated with ppGpp accumulation as PPK2 contributes to GTP synthesis and GTP is a precursor for ppGpp synthesis (Kim et al., 1998).
Chapter 2: Introduction

*Campylobacter jejuni* is a microaerophilic Gram-negative curved and non-spore forming bacterium that persistently colonize in the intestinal microflora of various livestock especially, poultry (Berry *et al.*, 1998). However, it can infect humans and infection by *C. jejuni* is considered to be a prevalent cause of bacterial mediated diarrheal disease worldwide (Adk *et al.*, 2005). Campylobacteriosis is often a self-limiting disease condition characterized by fever, abdominal pain, vomiting and sometimes bloody diarrhea in healthy persons. In addition, seldom post-infectious fatal complications like Guillian-Barre Syndrome, Miller Fisher Syndrome and reactive arthritis can also occur in 1 in 1000 cases (van Koningsveld *et al.*, 2001). Efforts to reduce campylobacteriosis in humans are directly linked to a better understanding of the pathobiology of *C. jejuni*, however the molecular bases of such virulence mechanisms are not fully elucidated yet.

In-organic polyphosphate (poly-P), a polymer of ten to hundred phosphate residues, linked by high energy phosphoanhydride bonds, is conserved in every cell in nature: bacterial, fungal, plant and animal (Kulaev *et al.*, 1983). It can serve as a source of energy for synthesis of sugars, nucleosides, and proteins; and activating precursor for fatty acids, phospholipids, polypeptides, and nucleic acids. Poly-P is also a key regulator of bacterial survival, stress response, host colonization and virulence in many pathogenic
bacteria including *C. jejuni* (Kornberg *et al*., 1999; Rao *et al*., 2009; Candon *et al*., 2007). Poly-P metabolism is governed by several specialized enzymes. Polyphosphate kinase-1 (PPK1) is responsible for the synthesis of long chain poly-P from ATP, although the reaction is reversible but it favors the synthesis of most of poly-P in the cell (Kornberg *et al*., 1999; Tan *et al*., 2005; Gangaiah *et al*., 2010); polyphosphate kinase-2 (PPK2), which utilizes poly-P to generate GTP at rate 75-fold greater than that of the poly-P synthesis from GTP (Ishige *et al*., 2002; Sureka *et al*., 2009); and the exopolyphosphatases (PPXs) that degrade poly-P into a smaller branch of inorganic phosphate. Exopolyphosphatases have been classified into two kinds (1) classical PPX enzymes: primarily monofunctional and have exopolyphosphatase activity. Besides, PPX’s essential role in poly-P homeostasis (Akiyama *et al*., 1993; Bolesch and Keasling, 2000; Rangarajan *et al*., 2006; Lindner *et al*., 2009), it has been shown to be required for motility, biofilm formation, and sporulation in *Bacillus cereus* (Shi *et al*., 2004) and also shown to be critical for resistance to complement-mediated killing in *Neisseria meningitidis* (Zhang *et al*., 2010). (2) PPX/GPPA enzymes: have both exopolyphosphatase and guanosine pentaphosphate (pppGpp) phosphohydrolase activities (Kristensen *et al*., 2004; Rangarajan *et al*., 2006; Rao *et al*., 2009) to generate Guanosine tetraphosphate (ppGpp). The ppGpp is an alarmone molecule synthesized under stringent response in plants and bacteria to initiate a global physiological change; the expression and activity of many virulence regulators are directly or indirectly regulated by ppGpp mediated global response (Dalebroux *et al*., 2010). The bacterial intracellular level of ppGpp is modulated by two classes of enzymes: monofunctional synthetase (Rel-A) and bifunctional synthetase/hydrolase (SpoT). In
addition, PPX2/GPPA enzymes also play an essential role in ppGpp metabolism thereby involved in bacterial stringent response induced by starvation (Keasling et al., 1993; Reizer et al., 1993; Kuroda et al., 1997; Kristensen et al., 2004 and 2008; Rao et al., 2009). In *C. jejuni*, SpoT is involved in stringent response, lack of SpoT function shown to affect several specific stresses, transmission and antibiotic resistance related phenotypes (Gaynor et al., 2005). Taken together, the complex metabolic network of the ppGpp and poly-P are very critical for pathogenic bacteria survival, replication, and transmission.

*C. jejuni* 81-176 encodes two potential dual-function exopolyphosphatases *ppx1/gppa* (CJJ81176_0377) and *ppx2/gppa* (CJJ81176_1251). The dual function PPX/GPPA enzymes have been shown in addition to poly-P hydrolysis, they can also generate guanosine tetraphosphate (ppGpp) (Rangarajan et al., 2006). However, to date no studies describe the role of these enzymes in poly-P hydrolysis, ppGpp synthesis as well as their pathophysiology in *C. jejuni*. Since poly-P homeostasis is critical, and the PPX/GPPA enzymes are involved in poly-P metabolism and possibly in ppGpp (alarmone molecule) synthesis, we hypothesize that PPX/GPPA enzymes may have an important role in *C. jejuni*’s pathobiology and persistence in the different environmental conditions. To test our hypothesis, we generated deletion mutants of *ppx1* and *ppx2* (Δ*ppx1*, and Δ*ppx2*) as well as double knock out (dkppx) and assessed their role in virulence and stress related phenotypic characters. Here, we report the key role of *C. jejuni* PPX/GPPA proteins and their role in transmission related phenotypic traits like, motility, osmotic stress survival, intracellular invasion and survival.
Chapter 3: Materials and Methods

3.1 Bacterial strains and growth conditions

All studies were performed with the highly invasive *Campylobacter jejuni* strain 81-176 originally isolated from a diarrheic patient (Korlath et al., 1985). The *C. jejuni* cultures were routinely grown on Muellers-Hinton (MH; Becton Dickinson and Company) medium under microaerobic conditions (85% N₂, 10% CO₂ and 5% O₂) in a DG250 Microaerophilic Workstation (Microbiology International). For the growth curve and stress survival assays, *C. jejuni* and generated strains were cultured on MH broth with specific antibiotics at 42° C with shaking at 200 g. All the bacterial strains were enumerated (CFU/ml) on MH agar plates by serial 10-fold dilutions. For the purpose of cloning and plasmid propagation, *E. coli* DH5α cells were used and routinely cultured on Luria-Bertani (LB; Becton Dickinson and Company) agar or broth at 37° C. The growth mediums were supplemented with specific antibiotics concentration, chloramphenicol (20 µg/ml for *E. coli*, 10 µg/ml for *C. jejuni*), zeocin (50 µg/ml) and kanamycin (30 µg/ml) wherever required.

3.2 Cloning techniques

Primers were designed using Vector NTI® software (Invitrogen) and commercially synthesized by Integrated DNA Technologies. All the primes used in the
present study are listed in the Table (3.1). QIAquick® PCR purification kit and spin mini prep kit for plasmid isolation were purchased from Qiagen. Masterpure® DNA purification kit and Fast-Link DNA ligation kit were purchased from Epicenter. Restriction enzymes were purchased from Promega. pZErO-1, the zero background cloning vector and E. coli DH5α competent cells were purchased from the Invitrogen. Cloning and other molecular biological techniques were performed according to Sambrook and Russel (2001).

3.3 Construction of C. jejuni 81-176 deletion mutants and complemented strains

The BLAST search using ppx/gppa gene as a reference sequence from Aquifex aeolicus showed that C. jejuni possesses two exopolyphosphatases ppx1/gppa (CJJ81176_0377) and ppx2/gppa (CJJ81176_1251) encoding 486 and 324 amino acids. In this study, we generated deletion mutants (Δppx1, Δppx2, and dkppx) by double crossover homologous recombination as described previously (Rajashekara et al., 2009). Briefly, to construct the mutant, the target gene was amplified from C. jejuni 81-176 genomic DNA with designated primers. The PCR product was cloned into pZEro-1 plasmid, the resulting construct was amplified by inverse PCR ensuring that most of the ppx1 and ppx2 coding sequence were deleted. The inverse PCR product was ligated to a kanamycin gene from pUC4K and the final suicide vector was introduced into C. jejuni 81-176 by electroporation as described (Wilson et al., 2003). To generate a double knockout (dkppx) mutant, ppx2/gppa suicide vector containing the chloramphenicol gene was electroporated into Δppx1 mutant. The MH agar plate containing kanamycin and
chloramphenicol resistant colonies were selected for the double knockout mutants. The deletion of target gene was confirmed by PCR.

For complementation, the target genes along the potential promoter regions were amplified from genomic DNA with a specific set of primers; the products were cloned into pRY111, an *E. coli-Campylobacter* shuttle vector (Yao *et al.*, 1993). The resulting constructs were introduced into the respective mutant by triparental conjugation. The complement strain for the *dkppx* mutant was not generated due to limited availability of replicating plasmids as well as antibiotic markers choice for use in *C. jejuni*. The complemented strains (*Cppx1* and *Cppx2*) were also confirmed by PCR. The list of all the bacterial stains and plasmids used in the study are listed in Table (3.2)

### 3.4 Growth curve assay

The growth curve assay was done as described previously (Gangaiah *et al.*, 2010). Cultures were to grown mid-log phase on MH plates and were washed, resuspended in MH broth to adjust to 0.05 OD<sub>600</sub>. The MH broth cultures were incubated microaerobically at 42°C for 60 h with shaking at 200 g. For assessing the growth, CFU was determined at different time points by plating 10-fold serially diluted cultures on MH agar.

### 3.5 Extraction and Quantification of poly-P

The extraction of poly-P from *C. jejuni* strains were performed using Glassmilk method as previously described (Candon *et al.*, 2007; Gangaiah *et al.*, 2010). Briefly, *C. jejuni* cultures were grown in MH broth to mid-stationary phase to reach OD<sub>600</sub> of 0.3, cells were harvested followed by lysis with buffer (guanidine isothiocynate-GITC 4 mM,
500 mM Tris-HCl pH 7.0). The 10 ul of samples were removed for the total cellular protein estimation and the remaining samples were subjected for washing with buffer (5 mM Tris-HCl (pH-7.5), 50 mM NaCl, 5 mM EDTA, 50% ethanol). The washed pellets were suspended in resuspension buffer (50 mM Tris-HCl pH (7.4), 10 mM MgCl₂, and 20 ug each of DNase and RNase per ml). Finally poly-P was eluted by brief centrifugation at 10,000 g with elution buffer (50 mM Tris-HCl pH 8.0).

3.5.2 Quantification of poly-P by a TBO assay: Briefly, standard curve was determined by using known concentrations (128nM, 64nM, 32nM, 16nM, 8nM, 4nM) of phosphorus standard (Sigma) with toluidine blue O dye (TBO 6 mg/liter in 40mm acetic acid) (Sigma-Aldrich). The unknown poly-P samples of C. jejuni were incubated with TBO at room temperature followed by assessing absorbance at 630nm and 530 nm. The poly-P binding to TBO results in a shift in TBO absorbance from 630 nm to 530nm, hence the ratio of 530nm/630nm reflects the amount of poly-P in a given sample. The levels of poly-P in samples were determined by direct comparison with standard curve and were expressed as nmol/mg of total cellular protein. The total cellular protein was measured by Bradford analysis (BCA kit- Pierce Scientific).

3.6 ppGpp isolation and detection

ppGpp isolation and detection was assayed as described previously (Gaynor et al., 2005; Gangaih et al., 2010). Briefly, the bacterial strains were grown in MH agar for 16-18 h. Cultures were diluted to adjust OD₆₀₀ of 0.25; cells were pelleted, washed twice in MOPS-MGS (50 mM MOPS,55 mM mannitol, 1 mM MgSO₄, 0.25 mM CaCl₂, 19 mM glutamic acid, and 0.004 mM biotin) (Mendrygal and Gonzalez, 2000) and resuspended
in 250 μl MOPS-MGS. The $^{32}$P at 100 μCi ml-1 ($3.7 \times 10^{12}$ Bq) was added to cells and incubated for 3, 6, and 24 h at 37°C microaerobically. Labeled cells were harvested, washed and treated with lysozyme in 10 mM Tris (pH 8.0) for 20 min. ppGpp was extracted with equal volume of 2 M formic acid and placed on ice for 15 min. Samples were spun for 5 min at 10,000 g, and 3 μl of supernatant was spotted directly onto cellulose TLC plates, dried, and developed in 1.5 M KH$_2$PO$_4$ and visualized by autoradiography.

3.7 Motility assay

The motility assay was performed as described previously (Rajaskekara et al., 2009). Briefly, C. jejuni cells were grown overnight on MH plate microaerobilically at 42°C. The cells were washed with MH broth and OD$_{600}$ was adjusted to 0.05. The 2ul of the culture (≈ 5 x $10^7$ cells) was stabbed onto 0.4% semi-solid agar and incubated microaerobically at 42°C. After 24 hrs of incubation, the swarming zone around the stabbed area was measured (cm).

3.8 Biofilm assay

The static biofilm formation was assayed as described previously (Candon et al., 2007; Gangaiah et al., 2010). Briefly, cells were grown overnight microaerobically on MH plate at 42°C. The cells were harvested in MH broth and OD$_{600}$ was adjusted to 0.05. One hundred ul of OD adjusted culture was inoculated to 2 ml MH broth in borosilicate tubes and incubated at 42°C microaerobically for 48 h without shaking. The static biofilm formations were visualized by staining with 250 ul of 1% (w/v) crystal violet for 15 min. To quantify amount of biofilm formation, 2ml of dimethyl sulfoxide (DMSO) was added
and incubated at room temperature for 24 h. The absorbance was measured at 570 nm to quantify the amount of biofilm stained by crystal violet dye.

3.9 Nutrient down shift assay

Nutrient down shift assay was performed as described previously (Candon et al., 2007; Gangaiah et al., 2009). Briefly, *C. jejuni* cultures were grown on MH plate microaerobically at 42°C for 16-18 h. The cultures were washed twice by centrifuging at 10,000 g for 2 min with minimum essential media (MEM). Bacteria were resuspended in MEM to adjust OD$_{600}$ of 0.05 and placed under microaerobic conditions at 42°C with shaking at 200 rpm. CFU was determined by plating on MH agar at different time point intervals.

3.10 Osmotic stress assay

The ability of *C. jejuni*’s ppx mutants to tolerate osmotic stress was determined as described previously (Candon et al., 2007). Briefly, mid log-phase grown bacterial cultures were suspended in MH agar containing 0.17 M NaCl and OD$_{600}$ adjusted to 0.05. OD adjusted cultures were serially diluted (10-fold) and a 10 μl of cultures were spotted on MH agar containing 0.17 M NaCl. After incubation microaerobically at 42°C for 2 days, the growth of *C. jejuni* strains on MH agar containing 0.17 M NaCl was assessed.

To test survivability of ppx/gppa mutants to osmotic stress in a liquid media, cultures OD were adjusted to 0.05 in MH broth containing 0.25 M NaCl and incubated under microaerobic conditions at 42°C for 60 h with shaking at 200 g. The CFU was assessed at different time points by plating on MH agar.

3.11 Oxidative stress assay
The ability of *C. jejuni* *ppx* mutants to survive under oxidative stress was assessed as previously described (Tunpiboonsak *et al.*, 2010). Briefly, the bacterial strains (≈ 5 x 10⁷ cells) were spread for confluent growth on MH agar. At the center of MH agar, 6 mm diameter hole was made and well bottom was sealed with 50 ul of molten MH agar. The wells were filled with the 30 ul 0.3% H₂O₂ or 20 mM paraquat and the plates were incubated under microaerobic conditions at 42°C for 24 hours, the clear zones of sensitivity surrounding the wells were measured (mm).

### 3.12 Tissue culture infection model (INT407 cell invasion and intracellular survival assay)

The infection model for invasion and intracellular survival with INT407 cells were performed as mentioned previously (Gaynor *et al.*, 2005; Svensson *et al.*, 2009). INT407 cells (≈1.4 x 10⁵) in MEM with 10% (v/v) fetal bovine serum (FBS) were seeded to 24-well tissue culture plate and incubated at 37°C with 5% CO₂ for 18 h. The MH broth grown mid-log phase *C. jejuni* cultures were pelleted by centrifuging at 6000 g for 10 min and washed twice with MEM containing 1% (v/v) FBS, adjusted OD₆₀₀ of 0.02 was adjusted in MEM, and cultures were subsequently used to infect INT407 cells. Different multiplicities of infection (MOI) were followed to infect the cells in duplicate wells, for invasion 0.1:1, 1:1, 10:1, 100:1 MOIs were used and 100:1 MOI was used for intracellular survival assay. To assess invasion, INT407 cells were incubated with bacteria for 3 h and treated with gentamicin (150 ug/ml), incubated for additional 2 h. The infected cells were washed with MEM two times and lysed with 0.1% (v/v) Triton-x
100. One hundred ul of aliquot from each well was serially 10-fold diluted in MEM and plated on MH agar plate in duplicate to determine CFU.

In order to determine intracellular survival, all the above mentioned steps were followed till 2 h of gentamicin treatment. After that, the infected cells were washed two times with MEM and added with fresh MEM containing gentamicin (10 ug/ml) and incubated for additional 24 h. Following incubation, the infected cells were washed with MEM two times and lysed with 0.1% (v/v) Triton-x 100. 100 ul of aliquot from each well was serially 10-fold diluted in MEM and plated on MH agar plate in duplicate to determine CFU.

3.13 Serum bactericidal assay

This assay was performed as described previously (Blaser et al., 1984; Thormika et al., 2011). The commercially available pooled normal human serum (NHS) and pooled normal chicken serum (NCS) (Innovative Research) were used. Briefly, C. jejuni strains were harvested after 16-18 h of growth on MH plates, washed and resuspended to $10^6$ cells/ml in Medium 199 with Hank’s balanced salt solution (with 0.01% glutamine). From each dilution, 150 ul of cultures were transferred in duplicate to 96 well microtiter plates. To each well, 50 ul of either 100% NH/CS in Medium 199, 100% heat – inactivated (56°C for 30 min) NH/CS (HINH/CS) or Medium 199 alone was added to obtain final concentration of NH/CS and HINH/CS to 25% and the plate was incubated for 60 min under microaerobic conditions at 37°C for human serum, and 42°C for chicken serum. The 100 ul of aliquots from the each well was 10-fold serially diluted and plated to assess CFU. $\log_{10}$ killing was determined by subtracting the difference between the
numbers of CFU at HINHS/HINCS to NHS/NCS. Percent of cell survival was also
calculated by dividing the number of cells in control (NH/CS) to number of cell in heat
inactivated (HI-NH/CS).

3.14 Quantitative RT-PCR
Quantitative RT-PCR (qRT-PCR) was performed targeting key genes involved in poly-P
homeostasis; stringent response (spoT) (Gaynor et al., 2005), and poly-P metabolism
(ppk1 and ppk2) (Candon et al., 2007; Gangaiah et al., 2009 and 2010). The mid
stationary phase grown bacterial cultures were used for total RNA extraction using
RNeasy Mini Kit (Qiagen). The RNA concentration and purity was determined using
NanoDrop ND-1000 spectrophotometer and gel electrophoresis. Two hundred ng of
DNAse treated RNA was used for cDNA synthesis using SuperScript® III First-Strand
Synthesis SuperMix kit (Invitrogen). Following cDNA synthesis, cDNA concentration
was normalized to 200 ng and gene specific primers were used to amplify the spoT, ppk1
and ppk2 genes along with 16S-rRNA as internal control. The qRT-PCR was performed
using SensiMixPlus® SYBR RT-PCR Kit (Quantace) in a realplex2 mastercycler
(Eppendorf). The relative levels of expression of genes were normalized with 16S-rRNA
amplified from the corresponding sample. The difference in expression of the genes was
calculated using the comparative threshold cycle (ΔΔCt) method to yield fold-difference
in transcript levels.

3.15 Statistical analysis method
In this study, statistical significance of data was determined by using one-way
analysis of variance (ANOVA) followed by Tukey’s multiple comparison test or Student
t-test (paired 2-tailed). A P value of $P \leq 0.05$ or 0.001 (α level) was considered statistically significant for all the experiments. The error bar represents the mean ±SE from 3 or 2 independent experiments with duplicate sample.
Chapter 4: Results

4.1 The ppx/gppa genes of C. jejuni

The phylogenetic analysis using MEGA-5 (Center for Evolutionary Medicine and Informatics, Arizona, USA) showed that the PPX/GPPA domains of C. jejuni, A. aeolicus, H. pylori, S. epidermidis, C. glutamicum, A. butzleri JV22, Streptomyces spp AA4, S. erythraea NRRL2338 and E. coli PPX/GPPA proteins belong to same family of exopolyphosphatases as all share common evolutionary branch (Figure-4). In addition, C. jejuni ppx1/gppa clustered is with Helicobacter and Acrobacter where as ppx2/gppa with Corynebacterium species. ClustalW2, structure based sequence alignment also showed that the C. jejuni ppx1/gppa sequence has both the catalytic residue (E119) required for PPX activity and arginine residues (R22 and R267) necessary for ppGpp specificity, suggesting this enzyme might mediate both poly P and (p)ppGpp hydrolysis (bifunctional activity). On the contrary, C. jejuni PPX2/GPPA domain possess the catalytic residue (E119) and R22 but not R267 (Figure-5), indicating this enzyme is less likely a primary mediator of (p)ppGpp hydrolysis. In addition, predicted three dimensional structural arrangement of PPX1/GPPA domain of C. jejuni using A. aeolicus PPX/GPPA as reference sequence exhibited more alignment with the reference sequence.

33
compared to PPX2/GPPA of *C. jejuni* further support the above mentioned speculation (Figure-6A, 6B) that PPX2/GPPA is less likely to encode pppGpp hydrolytic function.

The genetic organizations of *ppx1/gppa* and *ppx2/gppa* genes are conserved across the different strains of *C. jejuni* (Figure-7) with amino acid sequence identity ranging from 97% to 100%. In addition, PPX/GPPA enzymes are also conserved among other sequenced *Campylobacter* species. (*PPX1/GPPA sequence identity 35% to 97%; PPX2/GPPA sequence identity 46% to 99%*)

**4.2 PPX/GPPA proteins have exopolypophosphatase activity**

In order to verify that the deletion of *ppx/gppa* genes did not affect the overall growth properties of the mutants’ we assessed the Δppx mutants growth curve by determining CFU at different time interval in MH broth. There was no significant difference between CFU forming ability of Δppx mutants (Δppx1, Δppx2, dkppx) and wildtype (Figure-8).

To investigate *C. jejuni* PPX/GPPA annotated protein’s exopolypophosphatase activity, we measured the intracellular poly-P levels in Δppx mutants (Δppx1, Δppx2 and dkppx). Poly-P was extracted by glass milk method and quantified by toluidine blue O dye as described in materials and methods. Our results indicated that the deletion of *ppx* genes alter the intracellular poly-P levels compared to wild type. The Δppx1, and dkppx mutants showed significant (p<0.05, p<0.001) increase in the poly-P level (45.5 nM and 48.5 nM/mg of total cellular protein (TCP), respectively) compared to wildtype (35 nM/mg TCP), however, the Δppx2 mutant also exhibited increased poly-P level (39.5 nM/mg TCP) but it was not significant (p>0.05) (Figure-9). The complemented strains
(Cppx1 and Cppx2) displayed level of poly-P (37 nM/mg TCP) similar to that of wildtype. Above findings confirm that the ppx1/gppa and ppx2/gppa genes of C. jejuni have exopolyphosphatase activity, this findings is consistent with previous published report showing accumulation of poly-P in Δppx mutants (Shin et al., 2004; Lindner et al., 2009). Furthermore, these data indicate that ppx1/gppa proteins have major function in poly-P degradation and both the genes may function synergistically in hydrolysis of poly-P as shown with dkppx mutant.

4.3 PPX/GPPA enzymes have binfunctional activity

Since, C. jejuni ppx/gppa domains have structural and conserved amino acid residue required for the bifunctional activity as seen in A. aeolicus, our obvious anticipation is, they may have role in ppGpp metabolism. To address our hypothesis, we determined the level of ppGpp in the Δppx mutants compared to wildtype using Thin Layer Chromatography (TLC) and the results were analyzed with densitometry. There was no difference in ppGpp accumulations at 3 h but difference was observed at 6 and 24 h (Figure-10A). The Δppx1, and dkppx mutant display decreased level of ppGpp (4% and 14% respectively), contrary, the level of ppGpp in Δppx2 mutant was slightly higher (2%) than the wildtype (Figure-10B). Surprisingly, the level of ppGpp accumulation in dkppx mutant showed 3.5 fold higher than the Δppx1 mutant suggesting complex network pathways are involved ppGpp metabolism. These results indicates that PPX1/GPPA has both poly-P and pppGpp hydrolysis activity whereas PPX2/GPPA has only poly-P
hydrolytic activity. This is consistent with the predicted 3-dimensional structure arrangement (Figure-6A, 6B).

**4.4 PPX/GPPA enzymes are essential for motility and biofilm formation in C. jejuni**

In addition to motility, the *C. jejuni* flagellum is also known to serve as a specialized secretion system for many non-flagellar proteins, some of which are crucial for pathogenesis (Konkel *et al*., 2004; Guerry *et al*., 2007). The Δppx mutant of *Bacillus cereus* displayed the phenotypic defect in motility and here we asked whether *C. jejuni ppx/gppa* genes have role in motility related phenotype. *C. jejuni Δppx* mutants motility was determined on semi-solid agar; the Δppx mutants displayed significant (p≤0.05, p≤0.001) defect in motility. The motility zone for Δppx1, Δppx2, and dkppx were 1.7 cm, 2.3 cm, and1.5 cm respectively compared to wild type (3.3 cm). The motility defects were partially restored in complemented strains Cppx1 (2.5 cm) and Cppx2 (2.7 cm) (Figure-11A, 11B).

Biofilms play an essential role in *C. jejuni* survival, virulence and host colonization (Hanning *et al*., 2009; Reuter *et al*., 2010; Rajashekara *et al*., 2009) and several factors have been identified to contribute to *C. jejuni* biofilm formation. Biofilm formation is important for survival and persistence of *C. jejuni* in the environment and plays important role in the transmission of *C. jejuni* between host and environment. In this study we investigated the role of PPX/GPPA enzymes in *C. jejuni* biofilm formation in borosilicate glass tubes and quantified by staining with 1% (w/v) crystal violet dye. The Δppx1, Δppx2 mutants showed no impairment in biofilm formation. The amount of biofilm formation was quantified by measuring the absorbance at OD 570nm. The Δppx1
(0.154), and Δppx2 (0.119) had no significant defect compared to wildtype (0.145). However, the deletion of both the genes, (dkppx) did significantly (p≤0.001) affect the amount of biofilm formation (OD 570nm = 0.033) (Figure-12A, 12B). Taken together, these findings indicate that ppx/gppa genes role in transmission related phenotypes like motility and biofilm formation in C. jejuni.

4.5 Deletion of ppx/gppa genes affected C. jejuni survival during nutritional downshift

Previous research has shown that poly-P is essential for the ability of C. jejuni to survive under low nutrient stress (Gangaiah et al., 2009.). In C. jejuni Δppx mutants the poly-P homeostasis is imbalanced, this led us to investigate the mutants’ ability to survive under nutrient limitation. To assess the ability of C. jejuni to withstand nutrient limitation stress, we monitored the survival of the Δppx mutants’ in minimum essential media (MEM) (without glutamine) at different time points. The Δppx1, Δppx2 and dkppx mutants were significantly (p≤0.05) defective in survival after 36 h. Trans-complementation of ppx1 and ppx2 restored the nutrient stress tolerant ability of mutants’ comparable to wildtype (Figure-13). These data demonstrate that, C. jejuni PPX/GPPA enzymes play an important role in nutrient starvation stress.

4.6 Impaired osmotic stress tolerance in C. jejuni dkppx mutant

Ability of C. jejuni’s ppx mutants to osmotic stress tolerance was determined in solid and liquid media using NaCl as osmotic stressor. In solid media, (MH agar containing 0.17M NaCl) osmotic stress response was determined by monitoring the
growth after spotting 10-fold serially diluted cultures of *C. jejuni* strains. There was no significant difference in the osmotic stress tolerance response in the Δppx1 and Δppx2 mutants compared to wildtype. On contrary, *dkppx* mutant at 10⁴ cells/ml dilution did exhibit significant (p≤0.05) sensitivity to osmotic stress in comparison to wildtype (Figure-14A, 14B). Furthermore, survivability of *dkppx* mutant under liquid (MH broth containing 0.25M NaCl) osmotic stress was significantly (p≤0.05) affected at 48 and 60 h time points as assessed by determining CFU at different time points. Since, the Δppx1 and Δppx2 mutant did not show defect in osmotic stress tolerance on solid media we did not test these strains under liquid condition. These results indicate that the *ppx/gppa* genes may have role in *C. jejuni* osmotic stress tolerance mechanism.

**4.7 PPX/GPPA enzymes do not affect the oxidative stress response**

The Δppx mutants were also assessed for their ability to resist oxidative stress. 0.3% H₂O₂ or 20 mM paraquat was used as oxidative stressors to determine oxidative stress sensitivity of the mutants. The Δppx mutants including *dkppx* showed no significant difference between zones of sensitivity to oxidative stress compared to wildtype (Figure-15A, 15B).

**4.8 The Δppx mutants’ exhibit dose-dependent defect for invasion and intracellular survival**

To assay the virulence associated phenotypes of invasion and intracellular survival, *C. jejuni* Δppx mutants and wildtype strains were allowed to infect INT407
human intestinal epithelial cells at different MOIs. The invasion profile of the Δppx mutants and wildtype were similar except at 1:1 and 0.1:1 MOIs. The Δppx2 and dkppx mutant exhibited invasion defect compared to wildtype at 1:1 and 0.1:1 MOIs. The intracellular survival was assessed using 100:1 MOI, although both Δppx2 and dkppx mutant were defective in intracellular survival but only dkppx mutant was significantly (p≤0.05) defective in comparison to wildtype (Figure-16A, 16B). These data suggest that both PPX/GPPA enzymes are critical for the invasion and intracellular survival.

4.9 The Δppx mutants were resistant to complemented mediated killing

The bactericidal activity of complement present in serum is an innate defense mechanism against intruding pathogenic bacteria. Since the lack of PPX, an exopolyphosphatase activity, in N. meningitidis increased its resistance to complement mediated killing (Zhang et al., 2010), we asked whether PPX/GPPA deletion may have similar role in C. jejuni. To assess the complement mediated killing we compared the growth and survival of mutant and wildtype strains in pooled normal human/chicken serum (NH/CS) and respective heat inactivated serum (HINH/CS). The Δppx1 and Δppx2 mutants showed significant (p≤0.05) resistance to complement mediated killing surprisingly, the deletion of both the genes (dkppx) in C. jejuni significantly (p≤0.05) increased the sensitivity to complement mediated killing (Figure-17A). Trans-complementation of ppx1 and ppx2 genes were restored the complement mediated killing compared to wildtype. Further, complement mediated killing at 37°C and 42°C was similar however, the killing efficiency was slightly pronounced at 37°C (data not shown).
Contrast to human serum, chicken serum had no effect on the killing of Δppx mutants (Figure-17B).

4.10 Up-regulation of spoT and ppk1 in Δppx mutants

We performed the real time quantitative PCR (qRT-PCR) targeting key known genes involved in poly-P homeostasis to better understand the mechanistic aspects of poly-P homeostasis in C. jejuni Δppx mutants. We targeted the spoT, ppk1 and ppk2 genes as they have a role in poly-P and ppGpp metabolism; spoT has a bifunctional synthetase/hydrolase activity for pppGpp; PPK1 is responsible for the synthesis of long chain poly-P from ATP; and PPK2 utilizes poly-P to generate GTP. The differential expression of genes were expressed as fold change as calculated by (∆∆Ct) method using 16S-rRNA as internal control. The spoT and ppk1 genes were up-regulated 2 fold or more in Δppx1, Δppx2, dkppx mutants but the expression of ppk2 gene remained similar in all the mutants’ in comparison to wildtype. However, only spoT expression found to be significantly (p≤0.05) up-regulated in Δppx1 mutant (Figure-18). These data suggest that the expression of ppk1 and spoT genes enhance the production of ppGpp to avoid the accumulation of poly-P, hence may have a compensatory role in poly-P homeostasis.
Chapter 5: Discussion

In contrast to other better understood enteric pathogens like *Salmonella*, *Shigella*, and *E. coli* species, *C. jejuni* lacks most of the classical virulence factors such as toxins and type III secretion system; even the recent sequencing of additional strains, including one known to be highly virulent and invasive (*C. jejuni* 81-176) did not uncover any of the classical virulence factors (Fout *et al*., 2005; Hofreuter *et al*., 2006 and Pearson *et al*., 2007). In addition, it’s relatively small size genome (1.64 Mb), *C. jejuni* has to exploit most of its genes to meet great challenge to survive and transmit between hosts. Hence, better understanding of the host-microbe interactions that affect the pathogenesis of *C. jejuni* is of an immense importance.

Previous studies have shown that the poly-P plays critical role in virulence related phenotypic traits in pathogenic bacteria including *C. jejuni* (Candon *et al*., 2007; Gangaiah *et al*., 2009 and 2010). In this work, we focused to expand our understanding of Poly-P homeostasis. Poly-P molecule has multiple diverse functions like, phosphate reservoir, source of energy, pH modulator, metal chelator, and structural component of membrane transport channels (Brown *et al*., 2004; Korneberg *et al*., 1999). Furthermore, the dynamic balance between poly-P synthesis and its breakdown is essential for normal functioning of bacterial cell (Korneberg *et al*., 1999). Perturbation in poly-P synthesizing
mechanism (PPKs) in C. jejuni has shown to be a novel determinant of stress response and pathogenesis (Gangaiah et al., 2009 and 2010). PPXs breakdown the linear poly-P containing three or more phosphoanhydride bonds to release the P_i. In M. tuberculosis deficiency of PPX lead to increased accumulation of poly-P and restricted the growth in auxenic cultures and in human macrophages (Mc Dermott W, 1959). In A. aeolicus, PPX/GPPA enzymes in addition to poly-P degradation, they also breakdown the pppGpp to ppGpp (Kristensen O et al., 2004 an 2006) thereby playing an important role in starvation induced stringent response. Hence, we hypothesized that PPX/GPPA proteins might play critical role poly-P breakdown and might also govern unique characteristics of C. jejuni pathobiology.

To explore the role of PPX/GPPA proteins in poly-P metabolism, we compared the intracellular poly-P level accumulation in Δppx to wild type. Our results suggests that PPX/GPPA proteins have a role in poly-P hydrolysis as increased accumulation of poly-P in Δppx mutants were seen and both enzymes act synergistically in poly-P degradation as shown with dkppx mutant (Figure-9). The ppx1/gppa and ppx2/gppa genes of C. jejuni have additive but not redundant effect in poly-P degradation. Furthermore, PPX1/GPPA has the major exopolyphosphatase activity compared to PPX2/GPPA. Our findings are in accordance with previous published data in C. glutamicum (Linder et al., 2009) except interchange in the role of PPX1 and PPX2 gene. In C. glutamicum, PPX1 and PPX2 share only 25% amino acids identity and deletion of ppx1 and ppx2 genes resulted in increase poly-P accumulation but less when either ppx1 or ppx2 was over-expressed. C. glutamicum, growth experiments, exopolyphosphatase activities and intracellular poly-P
concentration determination revealed that PPX2 is a major exopolyphosphatase, which is shown active with short chain poly-P. Hence, our obvious speculation was that PPX1/GPPA and PPX2/GPPA may have substrate specificity in *C. jejuni*. However, whether the PPX1 and PPX2 encoded by *C. glutamicum* are indeed differing with respect to substrate specificity remains to be studied. The occurrence of 2 or more exopolyphosphatase within a species is not restricted to *C. jejuni* but is also seen in *E. coli*, *V. cholera*, and *C. glutamicum*. Non-overlapping substrate specificity may be the reason for occurrence of two or more exopolyphosphatase within one species (Linder *et al.*, 2009).

We next addressed whether predicted PPX/GPPA proteins have bifunctional activity. Previous investigations of PPX/GPPA proteins in *A. aeolicus*, and *E. coli* have shown to possess the bifunctional activity (Kristensen O *et al.*, 2004). Except in the above mentioned study in bacteria no other study has proved the PPX/GPPA bifunctional enzyme activity. To our knowledge this is the first study in *C. jejuni* depicting its PPX/GPPA bifunctional role and its significance in numerous transmission and virulence related phenotypes. Densitometry data analysis for ppGpp indicates that Δppx1 and dkppx mutant shows 4%, and 14% decreased in the level ppGpp, respectively. The Δppx2 mutant showed 2% increase in the level of ppGpp in compared to wildtype (Figure-10). The 3-fold increased level of ppGpp in dkppx mutant in comparison to Δppx1 signifies ppGpp metabolism in *C. jejuni* is also regulated by some other factors other than PPXs. Gaynor *et al.*, 2005 showed that *C. jejuni* stringent response is mediated by a single enzyme SpoT and this enzyme is thought to mediate both pppGpp synthesis
and hydrolysis. Beside, our quantitative RT-PCR data support the up-regulation of SpoT as a compensatory mechanism in poly-P and ppGpp homeostasis (Figure-18). This can be explained by considering indirect effect of ppx2/gppa gene on mediators like SpoT in ppGpp metabolism. However, this is mere speculation yet needs to be experimentally supported.

*C. jejuni*’s ability to withstand the nutrient limited conditions in environment is critical for waterborne transmission. Poly-P homeostasis in *C. jejuni* is important for the low nutrient stress survival, osmotic stress tolerance, biofilm formation, VBNC state (Candon *et al*., 2007; Gangaiah *et al*., 2009). *C. jejuni* flagellar motility and biofilms play an essential role in many aspects of its biology such as host colonization, secretion of virulence proteins, and host-cell adherence and invasion (Ferrero and Lee, 1988; Hanning *et al*., 2009; Reuter *et al*., 2010; Rajashekara *et al*., 2009). Besides PPKs, PPXs also have been shown to be essential for the transmission related phenotype traits like biofilm formation, motility and sporulation (Shin *et al*., 2004). Our data indicate that PPX/GPPA enzymes are required for the motility and biofilm formation. Although the motility was affected in the mutants, pronounced effect was seen in *dkppx*, suggesting that *ppx1/gppa* and *ppx2/gppa* genes have synergistic action in motility. Contrary, the amount of biofilm formation was only affected in *dkppx* mutant indicating a complex interconnected network of gene regulation of biofilm formation in *C. jejuni* (Figure-11A, 11B and 12A, 12B). These findings are in agreement with the previous published report (Shi *et al*., 2004). In *B. cereus*, deletion of *ppx* gene was shown to affect motility, biofilm formation and sporulation. In addition, the Δ*ppx* mutant also displayed defective survival under
nutrient limited conditions, invasion and intracellular survival, and osmotic stresses (Figure-13, 14A, 14B and 16A, 16B).

The above described stress responses might also affect the ability of C. jejuni to cause disease in the host. In addition to stresses imposed by the environment, C. jejuni is also exposed to multiple stresses in vivo (like GIT mucosal barrier, local innate immune response during adherence, invasion and inside the host cell) as well as during interaction with other bacteria. Thus, PPX/GPPA-mediated stress response might also affect the virulence of C. jejuni.

Complement-mediated bacterial killing is a key component of the humoral arm of innate immunity necessary to control systemic infection from mucosal sources. Encapsulated bacteria counterattack the antibacterial effect of complement by preventing deposition of the membrane attack complex on bacterial membranes (Moffitt MC, Frank MM., 1994). Zhang et al., work in N. meningitidis showed that lack of PPX functions increased the resistance to complement-mediated killings by means of biochemical interactions with the complement. They also showed that the relative resistance of ppx mutant does not result from changes in structures such as capsule, lipopolysaccharides, and factor-H-binding protein, instead in modification of alternative pathway of complement activation. These findings made us to determine the contribution of PPX/GPPA genes in complement mediated killing. Our findings are in agreement with previous work of Zhang et al. (2010), as the deletion of genes (∆ppx1 and ∆ppx2) did confer the resistance to complement mediated killing. Strikingly, deletion of both the genes (dkppx) results in sensitivity to complement mediated killing (Figure-17A). We
speculate that the PPX genes may affect surface structures such as capsule and lipopolysaccharides hence, our future studies are focused on comparing the capsule and lipopolysacchride profiles in Δppx mutants.

Our investigation was further expanded to assess the chicken serum mediated killings. Surprisingly, there was no effect of chicken complement factors on ppx mutant killing (Figure-17B). This result poses numerous questions to address, may be ppx genes functions is affected by number of factors; temperature dependent, or host restricted expression of ppx genes, or host immune system tolerance etc. To our knowledge, this is the first study to report that ppx genes deletion confirms the resistant against complement mediated killing in human serum but not in chicken serum.

Deinococcus radiodurans, Clostridium acetobutylicum, and Vibrio cholerae seem to be relics of the coexistence of two putative ppx genes. If we take into account that poly-P confers improved fitness under environmental stress (Rao N. N et al., 1996), then the selective maintenance of ppx genes may be strongly associated with changing environments, while an immovable environment, such as a cell host, could cause gene loss, as suggested by the lack of ppx genes in Mycoplasma spp (Cardona ST et al., 2002). Our future studies will be focused on deciphering the direct or indirect role of ppx/gppa genes in poly-P metabolism, investigating mechanism behind resistance to human serum complement, and studying the relative importance of ppx/gppa genes in C. jejuni.

In conclusion, we report that C. jejuni PPX/GPPA proteins have exopolyphosphatase activity, PPX1/GPPA has bifunctional enzyme functions and plays critical role in poly-P metabolism and ppGpp homeostasis. Beside their role in stress
related phenotypic traits they do have role in complement mediated resistance in human serum. Our study expands the multi-factorial regulation of poly-P metabolism in C. jejuni, which may serve as unique model for other bacteria as well.
Table 3.1 List of primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPX1/GPPA_F</td>
<td>AAATTAGGTACCATATGAGTGAGGAGCTTTTCATATGTCCTACAAACGATCCTCTTC</td>
<td>Used for <em>ppx1/gppa</em> gene amplification along with 1Kb up and downstream.</td>
</tr>
<tr>
<td>PPX1/GPPA_R</td>
<td>AAATTACTGCAGGTTATCACAAACGATCCTCTTC</td>
<td></td>
</tr>
<tr>
<td>PPX1/INV_F</td>
<td>TTAATGGATCCTCTCTATGTGTTGATTTTAA</td>
<td>Used for <em>ppx1/gppa</em> gene deletion by inverse PCR.</td>
</tr>
<tr>
<td>PPX1/INV_R</td>
<td>AATTAAAGGTACCCTCTATGTTGATTTTAA</td>
<td></td>
</tr>
<tr>
<td>PPX1C_F</td>
<td>TTAATGGATCCTCTCTATGTTGATTTTAA</td>
<td>Used for <em>ppx1/gppa</em> gene amplification for complementation.</td>
</tr>
<tr>
<td>PPX1C_R</td>
<td>GATATGGATCCAGCTATTTATGAAATAG</td>
<td></td>
</tr>
<tr>
<td>PPX2/GPPA_F</td>
<td>AAATAGGTACCAACAAAGTTGATCCTTTTGGAA</td>
<td>Used for <em>ppx2/gppa</em> gene amplification along with 1Kb up and downstream.</td>
</tr>
<tr>
<td>PPX2/GPPA_R</td>
<td>AAAATTACTGAGGTAAAGGCTTTTGGATGATAAAA</td>
<td></td>
</tr>
<tr>
<td>PPX2/INV_F</td>
<td>TAATAAGGTACCTCAGTGATTGGAACCAAGATCTA</td>
<td>Used for <em>ppx2/gppa</em> gene deletion by inverse PCR.</td>
</tr>
<tr>
<td>PPX2/INV_R</td>
<td>AATTAAAGGTACCTCAGTGATTGGAACCAAGATCTA</td>
<td></td>
</tr>
<tr>
<td>PPX2C_F</td>
<td>ATGCCTGCAGCTCTTTTAATGGATTTTGGATGATAAAA</td>
<td>Used for <em>ppx2/gppa</em> gene amplification for complementation.</td>
</tr>
<tr>
<td>PPX2C_R</td>
<td>ATAAAGGTACCCCGTCTTTGAAGTGCTATTTA</td>
<td></td>
</tr>
<tr>
<td>SpoT F</td>
<td>GTAACCACCTCGCACAATATC</td>
<td>Used for quantitative RT-PCR.</td>
</tr>
<tr>
<td>SpoT R</td>
<td>GATGTCGAGTTATTCTCC</td>
<td></td>
</tr>
<tr>
<td>PPK1 F</td>
<td>TGAAGCAAGTGATAAGGAGGAG</td>
<td>Used for quantitative RT-PCR.</td>
</tr>
<tr>
<td>PPK1 R</td>
<td>ATATAGGAGTCATAAGTTCTAAGC</td>
<td></td>
</tr>
<tr>
<td>PPK2 F</td>
<td>ATCTAATACCAACTTTGTC</td>
<td>Used for quantitative RT-PCR.</td>
</tr>
<tr>
<td>PPK2 R</td>
<td>TTCTCTCTCCTACTACG</td>
<td></td>
</tr>
<tr>
<td>16s RNA_F</td>
<td>GTCTCTTGAGAAATCTAATG</td>
<td>Used as quantitative RT-PCR internal control.</td>
</tr>
<tr>
<td>16s RNA_R</td>
<td>GATTTCTGAGATATCTAC</td>
<td></td>
</tr>
</tbody>
</table>

Underline sequence indicates the restriction enzymes site
Table 3.2 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em> 81-176</td>
<td>Used as wild type strain of <em>Campylobacter jejuni</em></td>
<td>Dr. Qijing Zhang</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td><em>E. coli</em> strain used for cloning</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Δppx1</td>
<td><em>C. jejuni</em> 81-176 derivative with deletion in ppx1/gppa gene; ppx1/gppa::Kan</td>
<td>This study</td>
</tr>
<tr>
<td>Δppx2</td>
<td><em>C. jejuni</em> 81-176 derivative with deletion in ppx2/gppa gene; ppx2/gppa::Kan</td>
<td>This study</td>
</tr>
<tr>
<td>Δkppx</td>
<td><em>C. jejuni</em> 81-176 derivative with deletion of ppx1/gppa and ppx2/gppa; ppx1/gppa::Kan, ppx2/gppa::Cm</td>
<td>This study</td>
</tr>
<tr>
<td>Δppx1</td>
<td>Δppx1 with pRY111- containing ppx1 coding region and the upstream promoter sequence for complementation; Cm</td>
<td>This study</td>
</tr>
<tr>
<td>Δppx2</td>
<td>Δppx2 with pRY111- containing ppx2 coding region and the upstream promoter sequence for complementation; Cm</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRY111</td>
<td><em>E. coli-Campylobacter</em> shuttle vector for complementation</td>
<td>129</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for conjugation, Kan</td>
<td>3</td>
</tr>
<tr>
<td>pZErO-1</td>
<td>Cloning vector for making suicide vector; Zeo</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source for kanamycin gene</td>
<td>Amersham</td>
</tr>
<tr>
<td>pZErO-1-ppx1</td>
<td>pZErO-1 containing ppx1/gppa region plus 1 kb upstream and downstream sequences from 81-176; Zeo</td>
<td>This study</td>
</tr>
<tr>
<td>ppx1-suicide vector-</td>
<td>Suicide vector with ppx1/gppa replaced by kanamycin resistance region from pUC4K in; Zeo, Kan</td>
<td>This study</td>
</tr>
<tr>
<td>pZErO-1-ppx2</td>
<td>pZErO-1 containing ppx2/gppa region plus 1 kb upstream and downstream sequences from 81-176; Zeo</td>
<td>This study</td>
</tr>
<tr>
<td>ppx2-suicide vector-</td>
<td>Suicide vector with ppx2/gppa replaced by kanamycin resistance region from pUC4K in; Zeo, Kan</td>
<td>This study</td>
</tr>
<tr>
<td>pRY111-ppx1</td>
<td>pRY111 containing ppx1 coding and upstream sequence for complementation.</td>
<td>This study</td>
</tr>
<tr>
<td>pRY111-ppx2</td>
<td>pRY111 containing ppx2 coding and upstream sequence for complementation.</td>
<td>This study</td>
</tr>
</tbody>
</table>

Kan, kanamycin; Zeo, zeocin; Cm, chloramphenicol
Figure 4. Phylogenetic tree of *C. jejuni* PPX/GPPA enzymes. MEGA-5 software tool was used to construct phylogenetic tree. Branch lengths are indicated next to the protein name and are proportional to the predicted evolutionary change. HPG27_257- *H. pylori* PPX/GPPA family phosphatase; ZP_07891646- *A. butzleri* JV22 PPX/GPPA family phosphatase; CJJ81176_0377- *C. jejuni* PPX1/GPPA family phosphatase; ECS88_4200- *E. coli* Guanosine pentaphosphate hydrolase; ZP_07282576- *Streptomyces spp* AA4 PPX/GPPA family phosphatase; YP_001103109- *S. erythraea* NRRL2338 PPX/GPPA family phosphatase; aq_891- *A. aeolicus* exopolyphosphatase; SE2033- *S. epidermidis* PPX/GPPA family phosphatase; CJJ81176_1251- *C. jejuni* PPX2/GPPA phosphatase and cg0488- *C. glutamicum* exopolyphosphatase.
Figure 5. Structure-based sequence alignment of PPX/GPAA domains of *C. jejuni* and related bacteria. Sequence alignment was performed using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html). Where “*” indicates positions of characters which have a single, fully conserved residue. “▲” the column is strongly conserved, “◼” the column is weakly conserved. Catalytic residue required for phosphatase activity is highlighted by **black letter on sky-blue background** and the residues required for guanosine pentaphosphate specificity are highlighted by **black letters on yellow background**. CJJ81176_0377- *C. jejuni* PPX1/GPPA family phosphatase; ZP_07891646-*A. butzleri* JV22 PPX/GPPA family phosphatase; HPG27_257- *H. pylori* PPX/GPPA family phosphatase; ZP_07282576- *Streptomyces spp* AA4 PPX/GPPA family phosphatase; YP_001103109-*S. erythraea* NRRL2338 PPX/GPPA family phosphatase; aq_891- *A. aeolicus* exopolyphosphatase; SE2033- *S. epidermidis* PPX/GPPA family phosphatase; ECS88_4200- *E. coli* Guanosine pentaphosphate hydrolase; cg0488- *C. glutamicum* exopolyphosphatase and CJJ81176_1251- *C. jejuni* PPX2/GPPA phosphatase.
Figure 6. Predicted three-dimensional structure of C. jejuni PPX/GPPA enzymes. Three-dimensional structure was identified with CBLAST, 3-dimensional structure alignment search tool using A. aeolicus PPX/GPPA as reference (www.ncbi.nlm.nih.gov/Structure/CBLAST). The region in pink or red indicates C. jejuni PPX/GPPA residues identical to A. aeolicus PPX/GPPA, while the region with no homology is indicated in blue. The region in grey indicates unaligned sequences of C. jejuni PPX/GPPA. (A) Structural alignment of C. jejuni PPX1/GPPA. (B) Structural alignment of C. jejuni PPX2/GPPA.
Figure 7. Genetic organization of \(ppx1/gppa\) and \(ppx2/gppa\) genes in \(C.\) jejuni genome.

(A) Location of \(ppx/gppa\) genes are conserved among different sequenced \(C.\) jejuni strains. (B) Schematic representation of approximate location of kanamycin, and chloramphenicol antibiotic markers in deletion \(\Delta ppx\) mutants of \(C.\) jejuni strain 81-176. The \(fdxB\) encodes for ferredoxin, \(pdxJ\) encodes for pyridoxine 5’-phosphate synthase and the \(pdxA\) encodes for 4-hydroxythreonine-4-phosphate dehydrogenase.
Figure 8. Growth kinetics of *C. jejuni ppx/gppa* mutants. Mid-log phase grown cultures were used to adjust an OD$_{600}$ of 0.05 in 5 ml of MH broth and incubated microaerobically at 42 °C with shaking at 200 g. The growth was assessed by CFU determination at different time points.
Figure 9. Intracellular poly-P levels in *C. jejuni Δppx* mutants. Poly P was extracted using glass milk and quantified by toluidine blue O method. Each data point is the mean± SE of 3 independent experiments. * P ≤ 0.05 and ** P ≤ 0.001.
Figure 10. Intracellular (p)ppGpp levels in the C. jejuni Δpx mutants. The amount of ppGpp accumulation was assessed using early log phase culture and labeling with $^{32}$P. (A) Nucleotides were resolved at 3, 6 and 24 h by TLC and visualized using autoradiography. (B) Densitometry quantification of ppGpp and pppGpp at 24 h. Each bar represents the average from 2 independent experiments with duplicate sample.
Figure 11. Motility of *C. jejuni* Δppex mutants (A) Motility assay: The 2 µl of 0.05 OD$_{600}$ cultures were stabbed onto 0.4% soft agar and incubated under microaerobic conditions at 42 °C. The results were recorded at 24 h. (B) Quantification of motility defects: Motility was quantified by measuring the swarming zone (radius in cm) surrounding the stabbed area. Each bar represents the average from 3 independent experiments. * P ≤ 0.05 ** P ≤ 0.001.
Figure 12. (A) Biofilm formation by C. jejuni Δppx/gppa mutants. The biofilm formation was visualized by staining with 1% crystal violet for 15 mints. (B) Quantification of biofilm in Δppx/gppa mutants. The amount of biofilm was quantified by measuring the absorbance at 570 nm after dissolving in 2 ml DMSO for 48 h. Each image and bar represents the mean ± SE of 3 independent experiments with triplicate samples. * * P ≤ 0.001.
Figure 13. Nutrient downshift assay of C. jejuni Δppx mutants. Sensitivity of C. jejuni Δppx mutants to nutrient stress was assessed by monitoring their survival in minimal essential media without glutamine at different time points. Each data point represents the mean ± SE of 2 independent experiments with duplicate sample. * P ≤ 0.05.
Figure 14. Osmotic stress assay. The *dkppx* mutant shows decreased osmotic stress tolerance in (A) solid (MH agar with 0.17mM NaCl) as well as (B) liquid media (MH broth with 0.25 mM NaCl). In liquid media osmotic stress was determined by monitoring cells survival at different time points. The dotted line indicates the limit of detection. Each bar represents the mean ±SE from 3 independent experiments with duplicate sample. *P≤ 0.05
Figure 15. Sensitivity of Δppx/gppa mutants to oxidative stress. (A) Sensitivity to oxidative stress was determined by disc diffusion assay using 0.3% hydrogen peroxide or 20 mM paraquat. (B) Quantification of oxidative stress, the zone of inhibition diameter was measured after 24 hours of incubation and expressed in cm. Each bar represents the mean ±SE from 3 independent experiments with triplicate sample.
Figure 16. The ppx mutants display a dose dependent invasion defect (MOIs 1:1, 0.1:1 and 100:1) and intracellular survival defect (MOI 100:1) in INT 407 human intestinal epithelial cells. (A) Invasion assay (B) Intracellular survival assay. The dotted line indicates the limit of detection. Each bar represents the mean ±SE from 2 independent experiments with duplicate sample. *P≤ 0.05
Figure 17. Complement dependent killing of *C. jejuni* Δppx/gppa mutants to (A) human and (B) chicken serum. Log₁₀ killing was determined by subtracting the difference between number of CFU in heat inactivated serum (HINHS or HINCS) to normal serum (NHS or NCS). Bars represent the mean± SE of 3 independent experiments with duplicate sample.

* P ≤ 0.05 ** P ≤ 0.001.
Figure 18. Quantitative RT-PCR analysis of the wildtype and C. jejuni Δppx mutants.

Fold difference in transcript level was assessed from ΔΔCT after normalizing to 16sRNA. Each bar represents the mean ±SE of the relative fold change in expression from 3 independent experiments with duplicate sample. *P≤ 0.05
Bibliography


103. Saito, K., Ohtomo, R., Kuga-Vetake, Y., Aono, T., and Saito, M. 2005. Direct labeling of polyphosphate at the ultrastructural level in *Saccharomyces cerevisiae*
by using the affinity of the polyphosphate binding domain of *Escherichia coli* exopolyphosphatase. *Appl. Environ. Microbiol.* **71**:5692–701.


