Functional Analysis of Inorganic Polyphosphate and its Associated Enzymes in *Campylobacter jejuni*

Dissertation

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Campylobacter jejuni, a Gram-negative microaerophilic bacterium, is a predominant cause of bacterial foodborne gastroenteritis in humans. Despite its importance as a major foodborne pathogen, our understanding of the molecular mechanisms underlying C. jejuni pathogenesis and survival under stress is limited. Of particular importance is the inorganic polyphosphate (poly P) which plays an important role in bacterial survival, stress responses, host colonization and virulence. Polyphosphate kinase 1 (PPK1) is the principal enzyme involved in poly P synthesis; while polyphosphate kinase 2 (PPK2) utilizes poly P to generate GTP. Exopolyphosphatases (PPX) degrade poly P to inorganic phosphate. The genome of C. jejuni possesses homologs of ppk1, ppk2 and 2 ppx/gppa genes. However, to our knowledge, there have been no studies addressing the role of poly P and associated enzymes in C. jejuni patho-physiology. In the present study, we hypothesize that poly P and its associated enzymes might play an important role in C. jejuni biology. Findings from this study will have implications for developing strategies to control Campylobacter through identification of new targets for vaccines and antimicrobials. The work in this dissertation is broadly divided into three studies.
In the first study, we investigated the role of PPK1 in *C. jejuni* pathogenesis, stress survival and adaptation using a deletion mutagenesis approach. Our findings demonstrate that *C. jejuni Δppk1* mutant was deficient in poly P accumulation, which was associated with decreased ability to form viable but non-culturable cells (VBNC) under acid stress. The Δppk1 mutant also showed decreased frequency of natural transformation and increased susceptibility to various antimicrobials. Furthermore, the Δppk1 mutant was characterized by a dose-dependent deficiency in chicken colonization. Complementation of the Δppk1 mutant with the wildtype copy of ppk1 restored the deficient phenotypes to levels similar to those of the wildtype. Our results suggest that poly P plays an important role in stress survival and adaptation and might contribute to genome plasticity, and spread and development of antimicrobial resistance in *C. jejuni*.

In the second study, we determined the role of PPK2 in *C. jejuni* pathophysiology using a deletion mutant of ppk2. Here, we demonstrate for the first time that the deletion of ppk2 in *C. jejuni* resulted in a significant decrease in poly P-dependent GTP synthesis, while displaying an increased intracellular ATP:GTP ratio. The Δppk2 mutant exhibited a significant survival defect under osmotic, nutrient, aerobic, and antimicrobial stresses and displayed an enhanced ability to form static biofilms. However, the Δppk2 mutant was not defective in poly P and ppGpp synthesis suggesting that PPK2-mediated stress tolerance is not ppGpp-mediated. Importantly, the Δppk2 mutant was significantly attenuated in invasion and intracellular survival within human intestinal epithelial cells as well as chicken colonization. Taken together, we have
highlighted the role of PPK2 as a novel pathogenicity determinant that is critical for *C. jejuni* survival, adaptation, and persistence in the host environments.

In the final study, we defined the role of PPX/GPPA enzymes in *C. jejuni* response to stress and virulence using deletion mutants of *ppx1/gppa* and *ppx2/gppa*. Here, we provide evidence that both PPX1/GPPA and PPX2/GPPA are important for *C. jejuni* motility and survival under nutrient limitation. However, PPX/GPPA enzymes appeared to play no role in *C. jejuni* biofilm formation, and tolerance to osmotic and oxidative stresses. Despite their enhanced capacity to accumulate poly P, ∆*ppx1/gppa* and ∆*ppx2/gppa* mutants exhibited similar growth kinetics compared to that of wildtype in rich medium. Additionally, our findings also suggest that PPX1/GPPA might be more important for *C. jejuni* functions than PPX2/GPPA as the former was relatively more defective in the tested phenotypes than the latter. In summary, our preliminary analysis suggests an important role for PPX/GPPA enzymes in *C. jejuni* biology.
Dedicated to my beloved, late parents and family
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CHAPTER I

Introduction and Literature Review

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Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, OH 44691.
1.1 A brief history of *Campylobacter*

*Campylobacter* was first described by a German bacteriologist Theodor Escherich in 1880. He identified “spiral, curved, non-culturable bacteria” in the intestines and stool specimens of neonates as well as kittens that had died of diarrheal infections (Escherich, 1886). In 1909, two veterinary surgeons McFadyean and Stockman described similar spiral shaped bacteria from infectious abortions in sheep and pregnant cattle (McFadyean and Stockman, 1913). Ten years later, Smith and Taylor identified a bacterium from the aborted bovine fetuses which was identical to vibrio-like organism reported by McFadyean and Stockman (Smith and Taylor, 1919). Due to their comma-shaped morphology, Smith and Taylor proposed the name “*Vibrio fetus*” for these organisms. Several years later, a new “vibrio” organism called “*Vibrio jejuni*” was associated with enteritis in calves and cattle (Jones *et al.*, 1931). During mid-1940s, Doyle isolated a similar vibrio-like organism called “*Vibrio coli*” from swine with diarrhea (Doyle, 1944).

Though well known as veterinary pathogens since 1880, campylobacters were not associated with human infections until 1938. In May 1938, the first documented incident of human *Campylobacter* infection was reported which involved a milk borne outbreak that affected 355 inmates of two adjacent state institutions in Illinois. Levy isolated organisms similar to *V. jejuni* from the blood cultures of these patients (Levy, 1946). In 1947, first isolation of “*V. fetus*” was reported in France from a woman who had suffered from septic abortion (Vinzent *et al.*, 1947). Later on, development of selective media for growth and the availability of sophisticated methods for creating microaerobic
environments led to better isolation and identification of campylobacters (Skirrow, 1977; Allos and Taylor, 1998). Recent reports show that Campylobacter jejuni is one of the leading causes of foodborne diarrhea in the developed world affecting approximately 1% of the United States, Australian and United Kingdom population (Friedman et al., 2000).

1.2 Toxonomy

The taxonomic classification of Campylobacter has undergone many major changes since its first milestone grouping by Elizabeth King in 1957. She made a systematic study of vibrio isolates and classified them into 2 groups: Vibrio fetus and thermotolerant vibrios (V. jejuni and V. coli) based on the difference in growth temperature (King, 1957). Vibrio fetus group was able to grow at 25 and 37 ºC but not at 42 ºC, while the thermotolerant vibrios which she called “related vibrios” were able to grow at 37 and 42 ºC but not at 25 ºC. At this time, sporadic abortions in sheep and cattle were becoming more appreciated. In 1959, Florent identified two different varities of V. fetus: V. fetus var. intestinalis and V. fetus var. veneralis based on their difference in biochemical and pathogenic properties (Butzler, 1984). In 1963, Sebald and Veron for the first time proposed the genus Campylobacter which included V. fetus and V. bubulus (now C. bubulus) (Sebald and Veron, 1963). In 1973, Veron and Chetalain published the first comprehensive study on Campylobacter taxonomy (Veron and Chetalain, 1973). Under this scheme, King’s V. jejuni and V. coli were renamed as Campylobacter jejuni and Campylobacter coli, respectively. Similarly, V. fetus var. intestinalis and V. fetus var. veneralis were renamed as C. fetus var. intestinalis and C. fetus var. veneralis,
respectively. This classification was later accepted by the International Committee of Systematic Bacteriology (now International Committee on Systematics of Prokaryotes). Eventually, Campylobacter taxonomy underwent several minor revisions with the inclusion of a large number of new species and subspecies, and the ever-increasing sophistication of methods used to classify bacteria.

At present, Campylobacter together with other genera Arcobacter, Sulfurospirillum and Dehalospirillum is included in the family Campylobacteraceae. The family Campylobacteraceae is a part of the rRNA super family IV or Epsilonproteobacteria. The genus Campylobacter contains 17 species and 6 subspecies (Debruyne et al., 2008; On, 2005). Campylobacter fetus is the type species of the genus. C. jejuni and C. coli are considered to be the most important species causing human infections. 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). Active surveillance data from Foodborne Diseases Active Surveillance Network estimated that Campylobacter spp. account for 9% (0.8 million) of the total foodborne illnesses, 15% of hospitalizations and 0.1% of deaths from foodborne illnesses each year (Scallan et al., 2011). Campylobacter spp. rank 3rd among the bacterial causes of foodborne illnesses, next to nontyphoidal Salmonella spp. (11%; 1 million) and Clostridium perfringens (10%; 1 million).

1.3 Campylobacter jejuni

1.3.1 Morphology
The term *Campylobacter* is derived from a Greek word “Kampylos” which means curved. *C. jejuni* is a Gram-negative, curved, 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. *C. jejuni* is highly motile and exhibits a characteristic darting motility by means of a single or occasionally multiple unsheathed polar flagella present at one or both ends of the cell (Ursing et al., 1994). The spiral shaped morphology is one of the important features in identification of *C. jejuni* in a clinical context. This morphology is assumed to be an adaptation of *C. jejuni* to move in viscous environments such as that found in the intestine. Under unfavorable conditions, *C. jejuni* has been proposed to enter a morphological, physiological and metabolic alteration called viable but noculturable (VBNC) state (Moran and Upton, 1987; Ng et al., 1985). *C. jejuni* cells show altered morphology from spiral to coccoid, reduced metabolic activity and altered cell wall structure during VBNC state.

1.3.2 Biochemical properties

*C. jejuni* does not ferment carbohydrates and usually obtain energy from amino acids and tricarboxylic acid (TCA) cycle intermediates (Blaser, 1986; Penner, 1988; Skirrow and Benjamin, 1980). *C. jejuni* is oxidase- and catalase-positive, and can reduce fumerate, nitrate as well as selenite (Forbes et al., 1998; Skirrow and Benjamin, 1980; Vandamme, 2000). It is negative for indole, methyl red and acetoin production, and cannot hydrolyze urea or grow in the presence of 3.5% NaCl. Only *C. jejuni* hydrolyzes hippurate and the ability to hydrolyze hippurate can be used to distinguish *C. jejuni* from
other *Campylobacter* species such as *C. coli* and *C. lari*. However, hippurate-negative *C. jejuni* have also been reported (Blaser, 2000; Doyle, 1990).

### 1.3.3 Growth requirements

*C. jejuni* is relatively slow growing and fastidious requiring specialized growth conditions. *C. jejuni* is strictly microaerophilic which means it requires low level of oxygen for growth (Doyle, 1990; Rowe and Madden, 2000). It grows best in an atmosphere containing 3 to 8% oxygen and 5 to 15% CO₂. An atmosphere of 5% O₂, 10% CO₂ and 85% N₂ is used for routine culturing of *C. jejuni* in the laboratory. Microaerophilic nature of this bacterium is believed to be due to its possession of several oxygen-sensitive enzymes in its metabolic pathways (Kelly *et al.*., 2001). Though *C. jejuni* can grow at 37 ºC, it grows optimally at 42 ºC (Blaser, 2000; Doyle, 1990). Unlike other foodborne pathogens, *C. jejuni* has a restricted growth temperature range of 30 to 47 ºC which restricts *C. jejuni* from multiplying outside the host environment (Doyle and Roman, 1982a). *C. jejuni* grows best at pH 6.0-8.0 with optimum growth at pH 6.5-7.5.

### 1.3.4 Genetic properties

*C. jejuni* has a small circular chromosome of 1.6-1.7 Mbp, with a low G+C content of around 30% (Newnham *et al.*, 1996; Taylor *et al.*, 1992). *C. jejuni* strain NCTC 11168 was the first strain to be sequenced. The *C. jejuni* strain NCTC 11168 encodes 1,643 proteins, 54 stable RNA species and 29 homopolymeric tracts (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000). *Campylobacter* genome is unusual in the sense that it contains a large number of homopolymeric tracts. Homopolymeric tracts have the
potential to cause phase variation, gene duplications and deletions, frameshift and point mutations and might play an important role in C. jejuni adaptation and persistence in the host as well as environment (Gilbert et al., 2002; Guerry et al., 2002; Karlyshev et al., 2002). Nearly 95% of the genome codes for proteins of which functional information is available for only 77.8% of the sequences (Gundogdu et al., 2007; Parkhill et al., 2000). In addition, C. jejuni genome contains no insertional sequences and few repeating elements. Another important feature of C. jejuni is its ability to take up DNA from its surrounding which is referred to as natural competence (Wilson et al., 2003).

1.3.5 Epidemiology

1.3.5.1 Incidence

C. jejuni is the most common cause of bacterial gastroenteritis in many developed countries and is the second most common cause in the United States (Butzler, 2004). Approximately 2.5 million cases of campylobacteriosis occur annually in the United States. Campylobacter infections accounts for 5% of the total estimated food-related deaths and 17% of hospitalizations resulting from foodborne infections (Mead et al., 1999). The annual economic cost of Campylobacter infections in the United States is estimated to be over 8 billion (Buzby et al., 1997). In immune-compromised patients, the incidence of Campylobacter infection is reported to be 40-100 times more than the immune-competent population (Sorvillo et al., 1991). In developed countries, the incidence is more common in children less than 1 year. C. jejuni infections are reported to be more in males than in females except for the age group of 20-29 years in which case
it is opposite (Olson et al., 2008). Majority of Campylobacter infections occur as sporadic cases not as part of large outbreaks and are more commonly reported in spring and summer, and least during winter months (Olson et al., 2008). Campylobacter infections cause approximately 124 deaths every year in the United States (Mead et al., 1999).

In developing countries, campylobacteriosis is a pediatric disease affecting children under 2 years of age (Coker et al., 2002). Often, Campylobacter-associated mortality is significant in this age group. C. jejuni infections decline with age and rarely occur in adults due to immunity acquired early in life from frequent exposure. Unlike developed countries, no seasonal occurrence has been reported in developing countries. Campylobacter is frequently isolated from patients with polymicrobial infections as well as healthy children, which is uncommon in developed countries.

1.3.5.2 Sources and transmission of infection

Campylobacteriosis is mainly a foodborne disease and often foods from animal origin are believed to be the predominant sources of human infections (Allos, 2001; Altekruse et al., 1999). Majority of human Campylobacter infections (approx. 70%) originate by consumption of undercooked poultry and other food products that are cross contaminated with raw poultry during food preparation (Allos, 2001; Altekruse et al., 1999; Altekruse and Tollefson, 2003). Campylobacter species, particularly C. jejuni are found as commensals in the gut of wide variety of animals and birds, notably poultry (Allos, 2001). C. jejuni is highly prevalent in the poultry population and nearly 90% of
the poultry flocks in the United States are colonized with *C. jejuni* (Stern *et al*., 2001). The chicken gut, particularly ceca, can carry a high load of *C. jejuni* (>10⁷ CFU per gram of feces; Mead *et al*., 1995). Therefore, owing to low infection dose in humans (approx. 500 bacteria) (Skirrow, 1982; Skirrow, 1990), it is not surprising that majority of human infections are acquired by handling or consumption of contaminated/undercooked poultry and poultry products.

Consumption of barbecued pork or sausage, unpasteurized milk and contaminated water and travelling abroad are among the other risk factors for campylobacteriosis in humans (Altekruse *et al*., 1999). Unpasteurized milk has become an important source of human *Campylobacter* infections in the United States and most commonly these infections occur as outbreaks (Allos, 2001; Skirrow, 1982; Skirrow, 1990). Campylobacters probably get into milk by fecal contamination or occasionally by secretion into milk in case of *Campylobacter* mastitis (Allos, 2001; Skirrow, 1990). Untreated water is also most commonly reported as a source of *Campylobacter* outbreaks in humans (Friedman *et al*., 2000; Skirrow, 1982). Errors in chlorination of municipal water systems, contamination of non-clorinated ground water and consumption of untreated natural water from lakes, ponds, rivers and streams which might have been contaminated with feces from wild birds are among some of the risk factors for waterborne *Campylobacter* outbreaks.

Campylobacteriosis is a zoonosis and the transmission of *Campylobacter* species from animals to humans occurs usually by indirect transmission *via* contaminated meat
and meat products (Skirrow, 1982; Skirrow, 1990). Humans acquire infection mainly by handling or consumption of raw or undercooked meat and meat products. Indirect transmission via non-meat food products such as mushrooms and other vegetables can also occur (Allos and Taylor, 1998). Direct transmission which is mainly occupational has also been reported (Butzler 2004, Skirrow, 1982). Farmers, veterinarians, abattoir workers and poultry processors are more likely to acquire Campylobacter infections via direct contact with infected animals. Contact with infected household pets and sand in the bathing beaches that has been contaminated with feces from wild animals and birds has also been described as a risk factor for human campylobacteriosis (Butzler, 2004; Allos and Taylor, 1998). Person to person transmission has been reported infrequently in young children (Butzler, 2004; Butzler and Skirrow, 1979). Vertical transmission from infected mothers to their neonates has also been described (Butzler and Skirrow, 1979). Perinatal transmission from a mother may also occur following exposure in utero, during passage through the birth canal or during the first days of life (Butzler, 2004).

1.4 Clinical manifestations

C. jejuni causes a spectrum of diseases in humans including gastroenteritis, septicemia, meningitis, abortion, reactive arthritis and polyneuropathies. The clinical outcome of C. jejuni disease depends on several factors such as the dose, genetic makeup of C. jejuni strain, and immune status and intestinal microbiota of the host (Young and Mansfield, 2005). However, self-limiting acute gastroenteritis is the most common manifestation of C. jejuni infection in the developed countries. The incubation period
ranges from 1 to 7 days (Allos, 2001). Typical symptoms of acute gastrointestinal disease include headache, fever, abdominal pain, myalgia, watery or bloody diarrhea and rarely vomiting. Regardless of the presence of blood in stools, leukocytes and erythrocytes are present in the feces from majority of cases. Young adults are the most commonly affected group. The intestinal illness lasts for less than a week without antibiotic treatment.

In developing countries, the disease can range from inflammatory diarrhea to non-inflammatory diarrhea and an asymptomatic carrier state (Young and Mansfield, 2005). Symptomatic illness mainly characterized by non-inflammatory diarrhea is seen only in infants and young children. *C. jejuni*-associated gastroenteritis is rarely seen in older children and adults, and asymptomatic shedding of *C. jejuni* can be observed in this group (Pazzaglia et al., 1991).

Though self-limiting in the majority of the population, complications including bacteremia and extra-intestinal manifestations such as pancreatitis, cholecystitis, hepatitis, peritonitis and meningitis have also been associated with *C. jejuni* infections (Allos, 2001). Other post-infection complications include Guillain-Barre Syndrome (GBS) (Nachamkin et al., 1998), Miller Fisher Syndrome (MFS) (Ang et al., 2002a and 2002b), reactive arthritis (Hannu et al., 2002), immunoproliferative small intestinal disease (Lecuit et al., 2004), inflammatory bowel disease (Kalischuk and Buret, 2010) and convulsions (Broides et al., 2010).

GBS is the most common and serious post-infection complication of *C. jejuni* infection (Nachamkin et al., 1998). It is an autoimmune disorder arising from cross-
reaction of *C. jejuni* lipooligosaccharide (LOS) antibodies with human peripheral nerve cell surface gangliosides. The disease begins 1-3 weeks after the onset of diarrheal illness and is mainly characterized by the neurological symptoms such as paralysis, pain, muscle weakness, abnormal sensations, including tingling, numbness and vibrations, abnormal facial and eye movements, and respiratory insufficiency warranting the use of ventilators. The disease is usually self-limiting; however, 15-20% of the patients suffer severe neurological defects (Nachamkin *et al*., 1998). GBS is estimated to occur in 1 in 1000 *C. jejuni* infections and *C. jejuni* has been shown to be the trigger in 30% of the GBS cases (Allos, 1997). Infections with cytomegalovirus, Epstein-Barr virus, *Mycoplasma pneumonia* and *Hemophilus influenzae* have also been associated with GBS (Tam *et al*., 2007; Kist and Bereswill, 2001).

MFS is another complication of *C. jejuni* infection and is believed to be a variant of GBS (Ang *et al*., 2002a and 2002b). MFS is characterized by the loss of coordination, paralysis of eye muscles and loss of tendon reflexes.

**1.5 Pathogenesis of *C. jejuni***

Despite being recognized as an important cause of foodborne illness for the last 3 decades, our understanding of *C. jejuni* pathogenesis mechanisms is limited. However, the availability of genome sequence and the recent development of new genetic tools have revealed a plethora of findings on *Campylobacter* pathogenesis. *C. jejuni* enters the host *via* ingestion of contaminated food or water. After passing through the acidity of stomach, *C. jejuni* colonizes the mucus blanket covering the epithelium of distal ileum
and colon (Ketley, 1997). Motility and corkscrew shaped morphology facilitate *C. jejuni* penetration in the mucus barrier. After successful colonization, *C. jejuni* damages the epithelial cells by invasion and toxin production, resulting in inflammation and diarrhea (Park, 2002).

### 1.5.1 *C. jejuni* virulence factors and infection

#### 1.5.1.1 Flagella

Flagella are one of the most important virulence factors involved in *C. jejuni* pathogenesis. *C. jejuni* contains a single or occasionally multiple unsheathed flagella at one or both poles of the bacterial cell. Flagellum imparts motility to *C. jejuni*, and is a strong immunogen as well as an adhesin. It is subject to phase (Caldwell *et al.*, 1985) and antigenic (Harris *et al.*, 1987) variation. *C. jejuni* flagellum is composed of major flagellin FlaA and minor flagellin FlaB, which are regulated by $\sigma^{28}$ promoter and $\sigma^{54}$-dependent promoter, respectively (Guerry *et al.*, 1991; Hendrixson and DiRita, 2003; Nuijten *et al.*, 1990; Poly *et al.*, 2007). The *fla* regulon in *C. jejuni* is regulated by a two-component system FlgS/FlgR, which is at the top of the *Campylobacter* flagellum hierarchy (Wosten *et al.*, 2004).

The flagella and flagellar motility are vital for many aspects of *C. jejuni* biology such as host colonization, secretion of virulence proteins, and host-cell adherence and invasion. Flagella and corkscrew shape of *C. jejuni* are thought to contribute to its ability to remain motile in the viscous environments such as that found in the intestine (Ferrero and Lee, 1988). An intact and functional flagellum is a prerequisite for *C. jejuni*
colonization of both human as well as animal hosts (Guerry et al., 2006; Wassenaar et al., 1991). Flagella have also been shown to be important for C. jejuni ability to adhere and invade host cells (Guerry et al., 2006; Grant et al., 1993; Nachamkin et al., 1993). In addition to motility, the flagellum also serves as a specialized secretion system for many non-flagellar proteins, some of which are crucial for C. jejuni pathogenesis (Konkel et al., 2004; Guerry et al., 2007). C. jejuni flagella are also important for chemotaxis, autoagglutination, microcolony formation, and evasion of host immune responses and bacteriophages (Guerry, 2007; Guerry et al., 2006; van Alphen et al., 2008b; Anderson-Nissen et al., 2005; Coward et al., 2006).

1.5.1.2 Chemotaxis

C. jejuni has the ability to move towards a variety of substances or away from harmful environments with the help of polar flagella. Two types of directed motility have been identified in C. jejuni, namely the classical metabolism-independent chemotaxis that responds to environmental stimuli and metabolism-dependent energy taxis which drives C. jejuni towards conditions favoring energy generation. Several chemical attractants and repellents have been identified for C. jejuni (Hugdahl et al., 1988). Homologs of all the fundamental components of chemotaxis pathway are found in C. jejuni including CheA, CheW and CheY, 10 putative chemoreceptors and two aerotaxis receptors (Marchant et al., 2002; Parkhill et al., 2000).

Chemotactic motility is a prerequisite for C. jejuni colonization and pathogenicity. Mutants defective in chemotaxis showed altered ability to adhere and invade in vitro
(Yao et al., 1997; Golden and Acheson, 2002), were unable to colonize mice (Takata et al., 1992; Yao et al., 1997; Chang and Miller, 2006) and chicken (Hartley-Tassell et al., 2010), and cause disease in the ferret model (Yao et al., 1997). A mutant of chemosensory receptor Tlp9 (Cj1189c or CetB) was deficient in its ability to invade human tissue culture cells (Golden and Acheson, 2002). Cj0262c and Cj0019 which encode chemoreceptors Tlp4 and Tlp10, respectively, were necessary for wildtype colonization of chicken gastrointestinal tract (Hendrixson and DiRita, 2004). Another study showed that deletion of genes encoding chemosensors Tlp1, Tlp2, Tlp3, DocB and DocC did not affect the chemotaxis of mutants compared to their respective parental strains (Vegge et al., 2009). However, mutants of tlp1, tlp3, docB and docC showed 10-fold reduction in the ability to invade human colon cancer cell line Colo 205 and chicken embryo cells. In addition, L-asparagine, formate, D-lactate and chicken mucus were identified as new attractants for C. jejuni (Vegge et al., 2009). Furthermore, a tlp1 mutant was defective in chicken colonization; however, it showed hyper-adherence to Caco-2 cells (Hartley-Tassell et al., 2010). The Tlp1 was also required for sensing aspartate in C. jejuni (Hartley-Tassell et al., 2010).

Similarly, energy taxis is important for C. jejuni virulence. C. jejuni energy taxis involves the sensory proteins CetA and CetB and mutants lacking cetA and cetB are deficient in energy taxis (Hendrixson et al., 2001). Inactivation of cetA and cetB did not affect chicken colonization; however, the cetA mutant showed reduction in invasion of cultured human epithelial cells (Elliot et al., 2009). A recent study showed that energy
taxis is the primary mechanism that drives *C. jejuni* towards optimal chemical/nutrient conditions for energy generation and colonization (Vegge et al., 2009).

1.5.1.3 Capsule and lipooligosaccharide

Like any other enteric pathogen, *C. jejuni* contains 2 surface structures, lipooligosaccharide (LOS) and capsular polysaccharide (CPS) (Linton et al., 2001). Another polysaccharide that may be involved in biofilm formation has also been identified in *C. jejuni* (Kalmokoff et al., 2006; Joshua et al., 2006). Carbamoylphosphate synthase was required for the biosynthesis of this polysaccharide (McLennan et al., 2008).

LOS plays multiple roles in *C. jejuni* pathogenesis, including immune avoidance, serum resistance, and adherence to and invasion of INT 407 cells (Hughes, 2004; Fry et al., 2000). *C. jejuni* LOS structures resemble human neuronal gangliosides. Thus, the antibodies elicited against LOS structures cross react with human neuronal gangliosides causing autoimmune diseases such as GBS and MFS (Ang et al., 2002a and 2002b; Yuki and Koga, 2006). The type of sequelae (GBS or MFS) is determined by the particular ganglioside mimicked by LOS (Yuki, 2007). A recent study showed that LOS modulates biofilm formation, serum sensitivity, resistance to antimicrobials, intracellular survival and is also required for host colonization (Naito et al., 2010).

The *C. jejuni* CPS, previously referred to as high molecular weight lipopolysaccharide (HMW LPS), plays an essential role in *C. jejuni* pathogenesis. Capsule has been demonstrated to play a role in serum resistance (Bacon et al., 2001), epithelial cell adherence (Bachtiar et al., 2007) and invasion (Bacon et al., 2001), and
diarrheal disease in a ferret diarrheal model (Bacon et al., 2001). However, capsule was found to be not required for colonization of chicken gut (Bachtiar et al., 2007). Though not important for colonization in chickens, the role of capsule in C. jejuni survival outside the natural host cannot be underestimated (Jilbauer et al., 2008). Historically, CPS represented major antigens in the Penner serotyping scheme (Karlyshev et al., 2000). CPS is considered to be highly variable and the variability has been attributed to phase variation of structural genes and O-methyl phosphoramide modification (Karlyshev et al., 2000; Szymanski et al., 2003; Karlyshev et al., 2005). The high variability of CPS possibly indicates its potential role in evasion of host immune responses. However, further studies are required to explore the role of C. jejuni capsule in immune evasion.

1.5.1.4 Protein glycosylation

During the last decade, general protein glycosylation has been identified as an important factor in C. jejuni pathogenesis (Karlyshev et al., 2004; Szymanski et al., 1999; Szymanski et al., 2002). C. jejuni is unique in several aspects with regard to protein glycosylation. First, both O-linked glycosylation which modifies serine or threonine residues and N-linked glycosylation which modifies asparagine residues have been reported in C. jejuni. Second, post-translational modification in bacteria was first identified in C. jejuni flagellin (Logan et al., 1989). Third, N-linked glycosylation in bacteria was also first reported in C. jejuni (Szymanski et al., 1999; Szymanski et al., 2003).
O-linked glycosylation modifies flagellin A and B on the flagellar filament and is necessary for proper assembly of flagellar filaments (Goon et al., 2003). The O-linked glycan consists of monosaccharides or disaccharides and different modifications such as acetamidino, dihydroxypropionyl and deoxypentose have been reported (Szymanski et al., 2003). Due to heavy glycosylation, flagellum is linked to antigenic variation (Chou et al., 2005; Guerry et al., 2006) and hence plays a potential role in immune evasion (Kelly et al., 2006; Szymanski et al., 2003). In addition, flagellar glycosylation is essential for motility, host cell adherence and invasion as well as virulence in ferrets (Guerry et al., 2006).

N-linked glycosylation synthesizes bacillosamine-containing heptasaccharide, which has been shown to modify over 65 secreted Campylobacter proteins and it is predicted to modify up to 150 proteins (Nothaft and Szymanski, 2010; Szymanski et al., 1999; Szymanski et al., 2003). C. jejuni N-linked glycosylation is highly conserved compared to other surface structures such as flagellum, LOS and capsule (Dorrel et al., 2001; Szymanski et al., 1999; Szymanski et al., 2003). N-linked glycosylation is encoded by pgl locus which contains 12 genes (Linton et al., 2005; Szymanski, 1999). N-linked-glycan consists of a heptasaccharide and most proteins modified by this system are predicted to be periplasmic and membrane proteins (Nothaft and Szymanski, 2010; Young et al., 2002). N-linked glycosylation is required for immunogenicity of several glycoproteins, natural competence, host cell adherence and invasion, as well as colonization in murine and avian infection models (Hendrixson and DiRita, 2004;
Karlyshev et al., 2004; Larsen et al., 2004; Szymanski et al., 2002; Kakuda and DiRita, 2006; Nothaft and Szymanski, 2010).

1.5.1.5 Secretion

The contribution of protein secretion systems to C. jejuni pathogenesis is poorly understood relative to other bacterial pathogens. Genome sequence analysis reveals the presence of both Sec-dependent and twin-arginine translocation (TAT) secretion systems in C. jejuni (Parkhill et al., 2000; Fouts et al., 2005). Sec-dependent secretion probably plays a role in secreting the periplasmic and membrane proteins similar to other Gram-negative bacteria. Several studies have been undertaken to understand the role of TAT system and TAT-dependent substrates in C. jejuni metabolism and pathogenesis. Deletion of C. jejuni tatC was shown to abolish the secretion of TAT-dependent proteins such as alkaline phosphatase PhoA<sup>cj</sup> and nitrate reductase protein NapA (van Mourik et al., 2008). In addition, TAT system is important for motility and flagellation, antimicrobial resistance, biofilm formation, stress tolerance and colonization of day-old chicks (Rajashekara et al., 2009). A recent study also identified the role of TAT system in the assembly of C. jejuni electron transport chains and molybdoenzyme-mediated periplasmic nitrosative stress resistance (Hitchcock et al., 2010).

Type V secretion system, also called as autotransport system, involves proteins which direct their own secretion to the cell surface or beyond (Henderson et al., 2004). Two genes encoding autotransporter proteins, CapA and CapB were identified by <i>in silico</i> analysis and these genes contain homopolymeric tracts, suggesting a possible role
in phase variation (Parkhill et al., 2000). CapA was shown to play a role in \textit{C. jejuni} pathogenesis, including adhesion and chicken colonization (Ashgar et al., 2007).

Random transposon mutagenesis identified several genes involved in \textit{C. jejuni} transformation and many of these proteins were homologous to those involved in type II secretion system, type IV pili and natural competence in other bacteria (Wiesner et al., 2003). Many proteins unique to \textit{C. jejuni} that have not been shown to be involved in natural transformation in other bacteria were also identified. However, all the components of the typical type II secretion system have not been identified in \textit{C. jejuni}.

A plasmid-encoded (pVir) Type IV secretion system has also been reported in a small subset of \textit{C. jejuni} isolates (Bacon et al., 2000; Larsen et al., 2004). Type IV secretion system in other bacteria is important for DNA export, conjugation and protein secretion. Type IV secretion genes are important for \textit{C. jejuni} adherence to and invasion of INT407 cells, virulence in ferret diarrheal model and natural competence (Bacon et al., 2002).

A type III-like secretion system called flagellar export apparatus has also been reported in \textit{C. jejuni}. Several important proteins such as \textit{Campylobacter} invasion antigens (Cia), FlaC and FspA were found to be secreted through flagellar apparatus. Cia proteins were first identified by Konkel et al. (1993) as important for invasion into INT407 cells. The CiaB was shown to be necessary for the secretion of other eight Cia proteins (CiaA-H) (Konkel et al., 1999a; Konkel et al., 2004). CiaB is required for invasion of cultured epithelial cells (Konkel et al., 1999b; Konkel et al., 2004) and chicken colonization
(Ziprin et al., 2001). Recently, CiaB expression but not secretion was shown to be induced by exposure to bile salt deoxycholate (Malik-Kale et al., 2008). Flagellar protein FlaC, which has some homology to FlaA and FlaB, is required for adherence to and invasion of HEp-2 cells (Song et al., 2004). In addition, the small protein FspA was shown to induce apoptosis in INT407 cells (Poly et al., 2007).

1.5.1.6 Bile tolerance

Bile is a yellow/green secretion stored in gall bladder and contains mainly bile salts, cholesterol, phospholipids and the pigment biliverdin (Begley et al., 2005). Bile is a surface active agent and possesses antimicrobial activity. Bile also induces secondary structure formation in RNA, DNA damage and protein denaturation and misfolding (Begley et al., 2005). *C. jejuni* resists the deleterious effects of bile with the help of multidrug efflux pump encoded by *cmeABC* operon (Lin et al., 2002). The CmeABC multidrug efflux pump is essential for growth on bile salt containing media (Lin et al., 2005b), resistance to various bile salts such as cholic acid, taurocholic acid and chenodeoxycholate, and chicken colonization (Lin et al., 2003; Lin and Martinez, 2006). The CmeR is a transcriptional regulator that represses the *cmeABC* operon (Lin et al., 2005a). The response regulator CbrR modulates bile resistance and is essential for chicken colonization in *C. jejuni* (Raphael et al., 2005). Bile salts also have been shown to affect the invasiveness by stimulating the synthesis of Cia proteins (Rivera-Amill et al., 2001), up-regulate *flaA* σ^28^ promoter (Allen and Griffiths et al., 2001), elongation factor, ferritin, chaperones and ATP synthase (Fox et al., 2007).
1.5.1.7 Iron acquisition

Iron is an essential nutrient for bacterial growth and survival as it is involved in multiple biochemical reactions (Andrews et al., 2003). *C. jejuni* acquires iron from the extracellular environment using scavenging molecules called siderophores (Park and Richardson, 1995). *C. jejuni* is capable of utilizing siderophores from other bacteria including the indigenous microflora in the gut (Field et al., 1986). However, only some strains are capable of synthesizing their own siderophores (Field et al., 1986). *C. jejuni* has been shown to utilize iron from several sources such as siderophores ferrichrome and enterochelin, haem compounds such as haemin, hemoglobin, haemin-haemopexin and hemoglobin-haptoglobin, Fe$^{3+}$, and Fe$^{2+}$ (Van Vliet et al., 2002). Ferric iron-siderophore complex is transduced into cytoplasm via TonB/ExbB/ExbD complex (Van Vliet et al., 2002) and a tonB mutant was unable to utilize siderophores such as haemin, ferrichrome and enterochelin (Guerry et al., 1997). In the host, majority of the iron is found complexed with transferrin and lactoferrin and free iron is almost nonexistent (Ratledge and Dover, 2000). Recently, *C. jejuni* has been shown to utilize iron from lactoferrin and transferrin (Miller et al., 2008).

Several iron uptake systems have been identified in *C. jejuni* such as haemin/hemoglobin uptake system ChuABCD (Ridley et al., 2006), an enterochelin transport system CeuBCDE (Richardson and Park, 1995) and a ferric siderophore uptake system CfhuABD (Galindo et al., 2001). Several individual genes such as cfrA, feoB, Cj1658, p19 (Van Vliet et al., 2002), cfrB (Xu et al., 2010), Cj1613c (chuZ, Ridley et al.,
and a ferric binding protein (cFbpA, Tom-Yew et al., 2005) have also been demonstrated to be involved in iron uptake. Both CfrA and CfrB have been identified as receptors for the *E. coli* siderophore, enterobactin (Palyada et al., 2004; Xu et al., 2010). FeoB is an iron specific transport protein, and a *feoB* mutant accumulated 50% less iron than the wildtype strain and was also compromised in its ability to colonize and survive within rabbit ileal loop (Naikare et al., 2006). Genes encoding iron storage proteins such as ferritin Cft (Cj0612c) and a putative bacterioferritin (Cj1534c) protein have also been identified in *C. jejuni* (Wai et al., 1996). A *cft* mutant was unable to grow under iron-limited conditions and was also more sensitive to oxidative stress (Wai et al., 1996).

Uptake of iron should be tightly regulated as excess iron in combination with oxygen can generate toxic reactive oxygen species (ROS) resulting in cell damage (Andrews et al., 2003). Fur (Ferric Uptake Regulator) and PerR (Peroxide stress Regulator) proteins regulate the response of *C. jejuni* to changes in environmental iron concentrations (Van Vliet et al., 1998; Van Vliet et al., 1999). Fur is a repressor of iron regulated genes which responds to increased intracellular iron concentration. Transcriptome analysis identified 53 genes that were Fur-regulated (Palyada et al., 2004). A *fur* mutant was also shown to be significantly impaired in its ability to colonize chickens, suggesting the importance of Fur regulation in vivo (Palyada et al., 2004). *C. jejuni* was still able to repress intracellular iron concentration in a *fur* mutant, suggesting the existence of another regulator. Interestingly, oxidative defence proteins catalase (KatA) and alkyl hydroperoxide reductase (AhpC) were found to maintain iron regulation. Another repressor PerR was shown to regulate the expression of KatA and
ahpC in the presence of iron (Van Vliet et al., 1999). Thus, iron regulation is mediated by two repressors Fur and PerR. In *C. jejuni*, PerR is believed to be the functional and nonhomologous substitution for the OxyR protein which is a regulator of oxidative stress in many bacteria (Van Vliet et al., 1999).

1.5.1.8 Penetration of the mucus layer

Mucus layer is the first barrier *C. jejuni* encounters in the gut. *C. jejuni* effectively penetrates this barrier by its darting motility, spiral shaped morphology and the presence of putative mucin-degrading enzymes. *C. jejuni* freely moves in parallel strands along the mucus strands without apparent attachment to the microvillus (Lee et al., 1986; McSweegan et al., 1987). In addition, *C. jejuni* has been shown to swim at high speeds in environments of high rather than low viscosity (Ferrero and Lee, 1988).

Intestinal mucus is also an important determinant of *C. jejuni* pathogenesis. Mucin, a glycoprotein of high molecular weight, is the main component of the intestinal mucus and is also a potent chemoattractant for *C. jejuni* (Hughdahl et al., 1988). L-fucose is the principal chemoattractant in mucin. The chemoattraction of *C. jejuni* towards mucin and specifically L-fucose may be an important factor in intestinal colonization of *C. jejuni* in animals (Hughdahl et al., 1988). The use of mucus or high viscosity medium during infection has been shown to enhance adherence to and invasion of *C. jejuni* (McSweegan et al., 1987). MUC2, the most abundant secreted mucin in the human intestine, was shown to modulate colonization and pathogenicity determinants in *C. jejuni* (Tu et al., 2008). A recent study also highlighted that chicken mucus attenuated
*Campylobacter* binding and internalization into human intestinal epithelial cells *in vitro*, while human mucus enhanced the *Campylobacter* binding and internalization (Byrne *et al.*, 2007; Alemka *et al.*, 2010). Thus, difference in composition of human and chicken intestinal mucin is proposed to contribute to differential outcome of *Campylobacter* infection in chicken and humans. Another recent study showed that intestinal mucus protects *C. jejuni* in the ceca of colonized broiler chickens from bactericidal effects of medium chain fatty acids (Hermans *et al.*, 2010).

1.5.1.9 Adherence mechanisms

Adhesion is a fundamental step in the pathogenesis of any bacterial pathogen. To colonize host, many Gram-negative and Gram-positive bacteria typically need surface appendages called pili. However, such pilus or pilus-like structures have not been identified in *Campylobacter* (Wiesner *et al.*, 2003) and the genome annotations of several *C. jejuni* strains do no reveal any genes that encode pilus or pilus-like structures (Fouts *et al.*, 2005; Parkhill *et al.*, 2000).

Despite the absence of typical adhesins, several surface structures such as outer membrane proteins (Peb1, CadF and Jlp1), lipooligosaccharides and major outer membrane protein (MOMP) have been shown to be important for *C. jejuni* adhesion to host cells. Peb1 is a periplasmic protein encoded by *peb1A* locus. The *peb1A* mutant showed significantly decreased adherence and invasion of HeLa cells, and reduced colonization of mouse models (Pie *et al.*, 1998). Recent studies have shown that Peb1 is a conserved aspartate/glutamate binding protein that belongs to the family of bacterial ATP
transporters (Leon-Kempis Mdel et al., 2006; Muller et al., 2007). CadF specifically binds to fibronectin on the host cell (Konkel et al., 1997; Monteville et al., 2003) and is important for maximal adhesion and invasion of \textit{C. jejuni in vitro}. CadF has also been shown to be essential for chicken colonization and a \textit{cadF} mutant showed significant reduction in chicken colonization (Monteville et al., 2003). JlpA is a surface lipoprotein shown to be important for HEp-2 cell binding (Jin et al., 2001). JlpA specifically binds to Hsp90α and activates NF-kB and p38 mitogen-activated protein (MAP) kinase, both of which contribute to proinflammatory responses (Jin et al., 2003). JlpA has been predicted to mediate some of the inflammation that is observed during \textit{C. jejuni} pathogenesis. Capsular polysaccharide (Bachtiar et al., 2007), lipooligosaccharides (Walker, 1986) and MOMP (Moser and Schroder, 1997) have also been shown to serve as adhesins for \textit{C. jejuni}. Several other virulence factors have been implicated in mediating \textit{C. jejuni} adherence in the last 25 years and have been listed in Haddad et al. (2010).

1.5.1.10 Invasion mechanisms

Several studies including examination of intestinal biopsies of humans (van spreeuwel et al., 1985), \textit{in vivo} studies in infected primates (Russel et al., 1993) and other animal models (Babakhani and Joens, 1993; Newell and Pearson, 1984; Yao et al., 1997), along with the \textit{in vitro} experiments in human epithelial cells (de Melo et al., 1989; Konkel and Joens, 1989) have shown that \textit{C. jejuni} can invade intestinal epithelial cells. \textit{C. jejuni} strain 81-176 invades INT 407 or Caco-2 cells in about 2 hours and often an invasion efficiency of 1-2% was observed (Hu and Kopecko, 1999). Normally, one to
three bacteria were found internalized per cell even with the most invasive strains (Biswas et al., 2000). Invasion into intestinal epithelial cells is important for *C. jejuni* pathogenesis. Specifically, *C. jejuni* factors such as motility, glycosylation and capsular synthesis have been shown to be important for invasion and mutants of genes involved in these pathways were defective in their ability to adhere to and invade host cells, as well as colonize animals (Szymanski et al., 2002; Grant et al., 1993; Yao et al., 1994; Bacon et al., 2001; Hendrixson and DiRita, 2004; Kakuda and DiRita, 2006; Marooka et al., 1985; Karlyshev et al., 2004; Watson et al., 2007). *C. jejuni* invasion also plays an important role in production of proinflammatory cytokines such as IL-8 via MAP kinase activation (Hickey et al., 2000; Watson and Galan, 2005).

Flagella are one of the key factors in the uptake of *C. jejuni* into epithelial cells and *C. jejuni* that lack flagella showed reduced invasion of epithelial cells (Szymanski et al., 1995; Grant et al., 1993; Yao et al., 1994; van Alphen et al., 2008b; Wassenaar et al., 1991). CadF, an adhesin that specifically binds to fibronectin, is also required for *C. jejuni* invasion. CadF triggers signaling processes leading to activation of small GTPases Rac1 and CDC42 and internalization of *C. jejuni* (Krause-Gruszczyńska et al., 2007). *C. jejuni* invasion is unique in that it enters the cell with its tip first followed by flagellar end (Krause-Gruszczyńska et al., 2007). *C. jejuni* capsular polysaccharide (Karlyshev and Wren, 2001), sialylation of LOS outer core (Louwen et al., 2008) and *Campylobacter* invasive antigens (Cia; Konkel et al., 2004; van Alphen et al., 2008b; Goon et al., 2006) also are important for *C. jejuni* invasion. Gamma-glutamyl-transpeptidase (Barnes et al., 2007), cytolethal distending toxin (Fox et al., 2004) and pVir type IV secretion system
homologue (Bacon et al., 2000) were also found to be involved in *C. jejuni* invasion. However, the underlying mechanisms for most of these molecules remain to be explored.

A number of host-related factors have been associated with *C. jejuni* invasion. *C. jejuni* enters epithelial cells *via* the local depolymerization of cortical actin filaments and the formation of microtubule-based membrane projections (Krause-Gruszczynska et al., 2007; Watson and Galan, 2008; Konkel et al., 1992a; Hu and Kopecko, 1999). However, the involvement of actin cytoskeleton in *C. jejuni* internalization is not always found (Watson and Galan, 2008). Caveolin-1 was also required for efficient internalization of *C. jejuni* (Krause-Gruszczynska et al., 2007; Wooldridge et al., 1996; Hu et al., 2006). However, the entry process was found to be independent of dynein (Watson and Galan, 2008), which suggests that *C. jejuni* uptake is unlikely to occur *via* caveolea-mediated endocytosis. Rather, caveolin-1 might be important for activation of tyrosine kinases and small Rho GTPases Rac 1 and Cdc42 which could drive the cytoskeletal rearrangements (Krause-Gruszczynska et al., 2007; Wooldridge et al., 1996; Hu et al., 2006). *C. jejuni* infection was also associated with phosphorylation of at least nine proteins including phosphoinositol-3-kinase and heterotrimeric G proteins (Wooldridge et al., 1996; Biswas et al., 2004); however, the exact role of these proteins in *C. jejuni* uptake needs further investigation. Induction of Ca$^{2+}$ release from host intracellular stores during *C. jejuni* internalization was also reported (Hu et al., 2005), suggesting a role for Ca$^{2+}$ release in *C. jejuni* invasion.
Recently, a novel and efficient mechanism for *C. jejuni* invasion has been identified (van Alphen *et al.*, 2008a). Invasion with a highly invasive strain of *C. jejuni* yielded on average 10-15 intracellular bacteria. According to this mechanism, *C. jejuni* first moves towards the subcellular space of epithelial cells (subvasion) and then invades the cell at the base.

**1.5.1.11 Toxin production**

*C. jejuni* produces two types of toxins, enterotoxins and cytotoxins (Pickett, 2000). However, cytolethal distending toxin (CDT), a cytotoxin, is the most well characterized toxin of *C. jejuni*. CDT protein is encoded by three adjacent genes, *cdtA*, *cdtB* and *cdtC* (AbuOun *et al.*, 2005). CdtB is thought to be the toxic component, and CdtA and CdtC mediate internalization of toxin into host cells (Lara-Tejero and Galan, 2001; Young *et al.*, 2007). CdtB possesses activity similar to the enzyme deoxyribonuclease (DNaseI). Following translocation into the host cells, CdtB localizes in the nucleus and causes cell cycle arrest at the G2/M phase through blocking of CDC2 kinase involved in the entry into mitosis (Lara-Tejero and Galan, 2001). The toxin results in distension of affected cells in 3-4 days followed by cell death (Wassenaar, 1997), which gives the name cytolethal distending toxin.

CDT is considered to be an important factor in the pathogenesis of *C. jejuni* infection. CDT has been shown to induce cell distension in cell lines such as HeLa cells, Chinese hamster ovary (CHO) cells, and Caco-2 cells, which is characterized by elongation, swelling and eventually cell death (Whitehouse *et al.*, 1998). The presence of
CDT was associated with increased adherence to, invasion of and cytotoxicity towards HeLa cells (Jain et al., 2008). In addition, treatment of mice with supernatant containing *C. jejuni* CDT resulted in significant pathological changes in the colon. *C. jejuni* mutants lacking CDT were unable to produce gastroenteritis in NF-κB deficient mice compared to wildtype (Fox et al., 2004). However, the mutant strain showed similar colonization levels to that of wildtype. Chickens that lacked CDT colonized similar to the wildtype strain (Biswas et al., 2006). A mechanism for toxin pathogenesis is that the damage caused to epithelial cells from CDT results in altered absorptive capacity of the intestine followed by watery diarrhea (Pickett, 2000). CDT might also play a role in immune modulation (Purdy et al., 2000). CDT has been demonstrated to induce secretion of IL-8 in polarized human epithelial cells and thereby induces inflammation of the intestine (Hickey et al., 2000; Zheng et al., 2008). However, such inflammation of the intestinal epithelium was absent in chickens. CDT lacked antigenicity in young chicken model, while it was immunogenic in humans (AbuOun et al., 2005). Recently, CDT has been demonstrated to have role in carcinogenesis *in vivo* (Ge et al., 2008; Kalischuk et al., 2007).

### 1.5.1.12 Intracellular survival mechanisms

Following invasion, *C. jejuni* containing vacuoles move along the microtubules via the molecular motor, dynein, towards the perinuclear region (Hu and Kopecko, 1999). Thus, *C. jejuni* deviates from the canonical endocytic pathway avoiding delivery into lysosomes (Watson and Galan, 2008). The bacteria remain confined to vacuoles which
are functionally separated from described endocytic pathways (Konkel et al., 1992a; Russel et al., 1993; Watson and Galan, 2008). Previously, several studies showed that C. jejuni loses viability within the intestinal epithelial cells over a period of 24 hours (Candon et al., 2007; Day et al., 2000). A recent study showed that there is no significant decrease in C. jejuni viability within epithelial cells after 24 hours (Watson and Galan, 2008). However, these cells were not culturable under standard growth conditions and needed special conditions such as limiting oxygen for growth. Once inside the epithelial cells, C. jejuni might become oxygen sensitive or alter its respiration mode and thus can no longer be cultured in the presence of oxygen (Watson and Galan, 2008).

The intracellular fate of C. jejuni in phagocytic cells such as macrophages is different from that observed in intestinal epithelial cells. In macrophages, C. jejuni containing vacuoles are delivered to lysosomes and destroyed (Wassenaar et al., 1997). Superoxide dismutase, catalase and polyphosphate kinase did not protect bacteria against macrophage killing (Wassenaar et al., 1997). C. jejuni is susceptible to nitric oxide and reactive nitrogen species generated in the stomach from dietary nitrate as well as that generated by nitric oxide synthases in macrophages (Iovine et al., 2008). A single-domain hemoglobin protein Cgb was shown to provide resistance to nitric oxide and nitrosative stress in C. jejuni (Elvers et al., 2004). NssR, a transcriptional regulator, was later shown to regulate nitrosative stress-responsive expression of Cgb in C. jejuni (Elvers et al., 2005). C. jejuni also encounters reactive oxygen species inside the macrophages and neutrophils. However, C. jejuni encodes several proteins to counteract reactive oxygen species generated by macrophages and neutrophils. Catalase encoded by
katA breaks down hydrogen peroxide to H$_2$O and O$_2$ (Grant and Park, 1995) and is important for C. jejuni persistence and growth in macrophages, but not in intestinal epithelial cells (Day Jr. et al., 2000). C. jejuni also encodes a superoxide dismutase gene sodB that catalyzes the breakdown of superoxide to H$_2$O and O$_2$ (Purdy and Park, 1994) and has been shown to be important for Campylobacter survival and colonization in chickens (Purdy et al., 1999).

1.5.1.13 Trans-epithelial translocation mechanisms

The mechanisms underlying C. jejuni translocation across the host intestinal epithelium is not completely understood. Konkel and coworkers (1992b) reported that C. jejuni is actively translocated across the Caco-2 epithelial cell monolayer. This study also showed that C. jejuni translocation occurs both through and between the cells. Bras and Ketley (1999) showed that C. jejuni are translocated through the cytoplasm of invaded cells (transcellular) rather than via intercellular spaces (paracellular). Other studies showed that C. jejuni translocation also occurs via paracellular route by disruption of the cellular tight junctions (MacCallum et al., 2005b; Chen et al., 2006).

Flagella and de novo protein synthesis were found to be required for C. jejuni translocation across the epithelial monolayer (Bras and Ketley, 1999). The flaA mutant was unable to translocate across epithelial cells (Grant et al., 1993). Harvey et al. (1999) found no correlation between invasiveness of C. jejuni and its ability to translocate across the epithelial layer. Van Deun et al. (2008) showed that butyrate, a metabolite from commensal bacteria, protects Caco-2 cells from C. jejuni invasion and translocation. In
contrast, a recent study reported the role of *C. jejuni* in inducing transcellular translocation of non-invasive commensal bacteria in the intestine, suggesting the role of commensal bacteria in inducing inflammation in campylobacteriosis (Kalischuk *et al.*, 2009; Kalischuk *et al.*, 2010).

Translocation is important for *C. jejuni* pathogenesis as it leads to tissue damage and inflammation (Wine *et al.*, 2008; Wooldridge and Ketley, 1997). *C. jejuni* translocation alters the absorptive function and trans-epithelial electrical resistance of intestinal epithelium as well as results in rearrangement of tight junction protein occludin leading to diarrhea (MacCallum *et al.*, 2005b; Chen *et al.*, 2006). Following translocation, *C. jejuni* continues to proliferate in the lamina propria, eliciting an inflammatory response that disrupts the normal functioning of intestinal epithelium causing diarrhea (MacCallum *et al.*, 2005b).

1.5.1.14 Mechanisms of *Campylobacter* diarrhea

The most common outcome of *C. jejuni* infection is diarrhea, which could be watery or bloody (Janssen *et al.*, 2008). Following are some of the possible mechanisms for the induction of diarrhea in *C. jejuni* infection:

A. *C. jejuni* adherence/colonization in the intestine: Adherence of *C. jejuni* alters the status of normal intestinal microflora, decreases the trans-epithelial electrical resistance and disrupts the cellular tight junctions, resulting in malabsorption and diarrhea (Chen *et al.*, 2006; MacCallum *et al.*, 2005b). Following adherence, *C. jejuni* colonization of the intestine via formation of microcolonies and biofilms disturbs the normal intestinal
microflora (Haddock et al., 2010). *C. jejuni* colonization also activates NF-κB, MAP kinases and IL-8 production leading to malabsorption and diarrhea (Chen et al., 2006; Fields and Thompson, 2008; Hanning et al., 2008; Joshua et al., 2006).

B. **Production of toxins**: CDT mediated-developmental arrest and cell death results in malabsorption and diarrhea (Lara-Tejero and Galan, 2001).

C. **C. jejuni invasion and proliferation within epithelial cells**: *C. jejuni* invasion and proliferation within epithelial cells results in cell damage followed by diarrhea (Hu and Kopecko, 2000; Wooldridge and Ketley, 1997)

D. **C. jejuni translocation across the epithelial layer**: *C. jejuni* translocation, both by transcellular and paracellular routes, alters the absorptive function and trans-epithelial electrical resistance of intestinal epithelium as well as results in rearrangement of tight junction protein occludin leading to diarrhea (MacCallum et al., 2005b; Chen et al., 2006).

E. **Inflammatory response resulting from above events**: Inflammatory response resulting from adherence, colonization, toxin production and invasion results in inflammatory diarrhea (Hu and Hickey, 2005; Mamelli et al., 2006; Smith et al., 2008; Zheng et al., 2008).

1.5.2 **C. jejuni factors required for chicken colonization**

The development of new genetic tools has led to better understanding of the *C. jejuni* traits that are important for chick colonization. Signature-tagged mutagenesis
identified two methyl-accepting chemotaxis proteins and several other proteins of flagellar and chemotaxis machinery that are important for chick colonization (Hendrixson and DiRita, 2004). In addition, response regulator CbrR which regulates deoxycholate resistance (Raphael et al., 2005), and two component systems such as RacRS (Bras et al., 1999) and DccRS (MacKichan et al., 2004) and CprS sensor kinase (Svensson et al., 2009) were also found essential for efficient wildtype chick colonization. Several other genes such as those involved in glycosylation (Hendrixson and DiRita, 2004; Kakuda and DiRita, 2006; Karlyshev et al., 2004; Naito et al., 2010), adhesion and invasion (Ziprin et al., 2001; Ziprin et al., 1999), antimicrobial resistance (Lin et al., 2003; Luo et al., 2003), metabolism (Palyada et al., 2004; Velayudhan et al., 2004; Woodall et al., 2005) and secretion (Rajashekara et al., 2009) also play a key role in chick colonization.

1.5.3 Host factors

In addition to bacterial factors, host factors also play an important role in determining the severity and outcome of C. jejuni disease (Zilbauer et al., 2008). Given the fact that C. jejuni enteritis is a self-limiting disease, the role of host immune responses in clearing the infection cannot be underestimated. The intestinal epithelial layer plays a crucial role as a host defense barrier to C. jejuni infections. It serves as a physical barrier and protects the underlying mucosa from external environment (Zilbauer et al., 2008). It also plays a key role in microbial sensing and mounting appropriate innate immune responses (Zilbauer et al., 2008). The intestinal epithelial cells have been shown to produce chemokines, cytokines and antimicrobial peptides in response to C. jejuni
infections (Chen et al., 2006; Johanesen and Dwinell, 2006; Hickey et al., 1999; Hickey et al., 2000; Zilbauer et al., 2005).

1.5.3.1 Nuclear factor (NF) –κB

NF-κB, a transcription factor, plays a key role in early host immunity against infections via regulating the expression of several innate immune genes (Elewaut et al., 1999). Several studies have shown the activation of NF-κB in response to *C. jejuni* infection (Chen et al., 2006; Johanesen and Dwinell, 2006; Zilbauer et al., 2005). In addition, *C. jejuni* adhesin JlpA was specifically shown to activate NF-κB and p38 MAP kinase pathways via interaction with epithelial Hsp90 (Jin et al., 2003).

1.5.3.2 IL-8

IL-8 is a cytokine that recruits immune cells such as neutrophils to the site of local inflammation. Several studies have reported the induction of IL-8 in response to *C. jejuni*-mediated enteritis (Hickey et al., 1999; Hickey et al., 2000). Specifically, *C. jejuni* adherence and invasion of epithelial cells, and the presence of CDT have been shown to mediate the production of IL-8 (Hickey et al., 2000). IL-8 production in *C. jejuni* infection is believed to occur via activation of ERK and p38 mitogen-activated protein (MAP) kinase pathways (Watson and Galan, 2005; MacCallum et al., 2005a).

1.5.3.3 Epithelial β-defensins

Endogenous antimicrobial peptides play an important role in mucosal innate defense against microbial infections (Ganz, 2003). Endogenous antimicrobial peptides
such as human β-defensins have been shown to modulate *C. jejuni* infection via their bactericidal activity (Jilbauer *et al.*, 2005). *C. jejuni* treated with β-defensins suffered a significant damage after 30 minutes of treatment with these peptides. Recent studies suggest that chicken intestinal epithelial cells also express β-defensins (Byrne *et al.*, 2007; van Dijk *et al.*, 2007).

**1.6 Survival and adaptation mechanisms in *C. jejuni***

**1.6.1 Stress response and resistance in *C. jejuni***

*C. jejuni* encounters a number of unfavorable conditions in the transmission chain from animals and humans as well as in foods and other non-food environments. The environment between animal and human host, where they are exposed to oxygen, temperature changes, desiccation, UV light and other stress factors, poses the greatest challenge to *C. jejuni*. Unlike other enteric pathogens, *C. jejuni* is relatively fragile and highly sensitive to environmental stresses (Park, 2002). *C. jejuni* possesses uniquely fastidious growth requirements and unable to multiply outside the host. For example, *C. jejuni* is microaerophilic requiring 5% oxygen for its optimum growth and unable to grow in the presence of normal oxygen conditions. In addition, *C. jejuni* has a restricted growth temperature range and grows optimally at 42 °C. *C. jejuni* cannot grow at 30 °C and below or 47 °C and above. These properties place severe limitations on its ability to multiply outside the host and hence, *C. jejuni* is unable to multiply on food and non-food environments. Campylobacters are also relatively highly susceptible to a variety of environmental conditions compared to other enteric bacteria. Campylobacters are more
sensitive to desiccation, osmotic stress, low pH and high temperature compared to other foodborne pathogens (Park, 2002). These features reflect the absence of several classical stress response mechanisms that are important for mediating stress tolerance in other enteric bacteria such as *Salmonella* spp. and *Escherichia coli*. More specifically, *C. jejuni* lacks oxidative stress response genes SoxRS, OxyR, SodA and KatG, osmoprotectants ProU, OtsAB and BetAB, stationary phase sigma factor RpoS, heat shock regulating alternative sigma factor RpoH, major cold shock protein CspA and Leucine-responsive global regulator Lrp which are essential for stress resistance in the model organism *E. coli* (Park, 2002). Despite lack of several classical stress response mechanisms, *C. jejuni* remains one of the most frequent bacterial causes of foodborne diarrhea in humans. This suggests that *C. jejuni* might have evolved alternative mechanisms to circumvent a range of stresses imposed by the host and environment.

1.6.1.1 Stationary phase survival

Bacteria enter a stage of apparent growth cessation called stationary phase when conditions no longer support rapid growth in standard growth media. In many bacterial species, entry into stationary phase is typically characterized by profound structural and physiological changes that result in increased resistance to variety of stress conditions (Park, 2002). RpoS (RNA polymerase sigma 38 factor) regulates many of these changes and homologues of *rpoS* are widely present in many bacterial species including *Enterobacteriaceae, Pseudomonas* and *Vibrio* species (Park, 2002). Unlike other bacterial pathogens, *C. jejuni* lacks RpoS-dependent stationary phase stress response and
stationary phase *C. jejuni* showed increased sensitivity to stress conditions such as oxidative stress and mild heat stress compared to those in exponential phase (Kelly *et al.*, 2001). This was further confirmed by the fact that homologues of *rpoS* are absent in *C. jejuni* genome (Parkhill *et al.*, 2000). However, *C. jejuni* is believed to undergo selected physiological changes such as modulation of membrane fatty acid composition and pressure resistance (Martinez-Rodriguez *et al.*, 2004; Martinez-Rodriguez and Mackey, 2005b) upon entry into stationary phase. In addition, emergence of variants with survival advantages such as small increase in resistance to aeration, peroxide challenge and heat upon prolonged ageing of stationary phase cells has been reported (Martinez-Rodriguez *et al.*, 2004). Alternatively, SpoT-mediated stringent response (Gaynor *et al.*, 2005), polyphosphate accumulation (Gaynor *et al.*, 2007), increased motility, altered substrate utilization and switching (Wright *et al.*, 2009), and activation of DccRS two component system (Wosten *et al.*, 2010) might play a role in stationary phase survival of *C. jejuni*.

### 1.6.1.2 High temperature stress

*C. jejuni* replicates within a narrow temperature range of 30-47 °C, but grows optimally at 42 °C (Stintzi, 2003). *C. jejuni* growth decreases rapidly at 30 °C and below. While at temperatures above 42 °C it exhibits heat sensitivity with a decimal reduction time of one minute at 55 °C.

*C. jejuni* is very sensitive to heat treatment and is readily inactivated by pasteurization treatments and domestic cooking processes (Park, 2002). *C. jejuni* lacks the RpoS-mediated stationary phase thermotolerance (Kelly, 2001). However, *C. jejuni*
has a classic heat shock response and at least 24 proteins were found to be synthesized following heat shock (43-48 ºC). Some of these proteins were cloned and characterized as GroESL (Thies et al., 1999c; Wu et al., 1994), DnaJ (Konkel et al., 1998), DnaK (Thies et al., 1999b) and Lon proteases (Thies et al., 1999a; Thies et al., 1998). A dnaJ mutant showed severely retarded growth at 46 ºC and also was unable to colonize chickens, suggesting that DnaJ is important for both thermotolerance as well as chicken colonization in C. jejuni (Konkel et al., 1998). A whole genome microarray analysis revealed that 336 genes were altered in their expression in response to temperature upshift from 37 to 42 ºC (Stintzi et al., 2003). Fifty eight percent of these genes were up-regulated, while rest of them were down-regulated. These data suggest that C. jejuni adapts to temperature changes by altering its transcriptome. Extracellular proteins produced by C. jejuni have also been shown to confer thermotolerance (Murphy et al., 2003a).

Heat shock response in C. jejuni is regulated by three systems, namely RacRS regulon and orthologues of HrcA and HspR. However, the classical heat shock regulator σ32 is absent in C. jejuni. A C. jejuni racR mutant showed reduced ability to colonize chicken intestinal tract and a temperature-dependent change in protein profile and growth characteristics, suggesting that RacRS two component system is important for temperature-dependent adaptive responses in C. jejuni (Bras et al., 1999). Similarly, an hspR mutant exhibited reduced motility and growth, increased sensitivity to high temperatures, and impaired adherence to and invasion of epithelial cells, suggesting a role for HspR in C. jejuni thermoregulation as well as virulence (Anderson et al., 2005). The
hspR mutant showed up-regulation of 13 genes including the \textit{hrcA-grpE-dnaK, groESL} and \textit{cbpA-hspR} operons, while many genes of flagellar apparatus were down-regulated. HrcA also has been shown to regulate \textit{C. jejuni} virulence (Stintzi \textit{et al.}, 2005) and thermitolerlance (Holmes \textit{et al.}, 2010).

1.6.1.3 Low temperature stress

Campylobacters cannot grow at temperatures below 30 °C and consequently do not replicate during handling and storage at room temperature. Generally, \textit{C. jejuni} survives better at 4 °C than at 25 °C. \textit{C. jejuni} survived only for few days at 20 °C in surface water and microcosm water, while survival was prolonged to several weeks at 4 °C. Analysis of \textit{C. jejuni} genome sequence revealed the absence of classical cold shock proteins found in other bacteria, which partly explains the failure of \textit{C. jejuni} to grow at temperatures below 30 °C (Hazeleger \textit{et al.}, 1998). Although campylobacters can be isolated from frozen meats and poultry products (Fernandez and Pison, 1996), viability is rapidly lost at lower temperatures and freeze-thawing cycles significantly reduce \textit{C. jejuni} survival (Humphrey and Cruickshank, 1985). Several factors such as ice nucleation, dehydration and oxidative stress (Stead and Park, 2000; Garenaux \textit{et al.}, 2009) have been implicated in freeze-induced injury of \textit{C. jejuni} cells.

Despite a narrow growth temperature range of 30-47 °C, \textit{C. jejuni} exhibits remarkable ability to survive at low temperatures. In a VBNC state, \textit{C. jejuni} were shown to be viable for up to 4 months at 4 °C (Rollins and Colwell, 1986) and remained viable for up to 7 months based on signs of cellular integrity, respiratory activity and intact
DNA content (Lazaro et al., 1999). Cold-exposed stationary phase *C. jejuni* were more resistant to high hydrostatic pressure than those exposed to acid and heat stress (Sagarzazu et al., 2010). Similarly, cold exposed *C. jejuni* showed marginal difference in heat sensitivity compared to cold-exposed *E. coli* strains which showed significant sensitivity to heat exposure, suggesting that *C. jejuni* responds to low temperatures differently from that of *E. coli* (Hughes et al., 2009). Human isolates exhibited prolonged survival at 4 °C than the chicken isolates (Chan et al., 2001). Analysis of transcript profiles revealed that genes encoding proteins involved in flagellar assembly and metabolism were up-regulated at 5° C compared to 25 °C, suggesting that *C. jejuni* needs more energy for survival during low temperatures (Moen et al., 2005). A recent study showed that polynucleotide phosphorylase is required for long term survival of *C. jejuni* at lower temperatures (Haddad et al., 2009). However, further studies are needed to better understand the mechanistic aspects of low temperature survival in *C. jejuni*.

Several studies have reported the survival of *C. jejuni* on raw and cooked poultry and other meat samples during refrigeration and freezing. Banduri and Cottrell (2004) reported that refrigeration at 4 °C for 3-7 days resulted in reduction of 0.34-0.81 log_{10} CFU in ground chicken and 0.31-0.63 log_{10} CFU in chicken skin, while freezing at -20 °C resulted in a reduction of 0.56-1.57 log_{10} CFU in ground meat and 1.38-3.39 log_{10} CFU on chicken skin over 2 weeks period. These data suggest that refrigeration and chilling are not substitutes for safe handling and proper cooking. Sampers et al. (2010) reported that campylobacters were still detectable in naturally-contaminated chicken skin and minced meat after 84 days under freezing at -22 °C. In addition, this study showed that no
significant reduction in *C. jejuni* content was observed at 4 °C for 14 days, while freezing (-22 °C) for 14 days reduced the *C. jejuni* numbers by 1-log. Low concentration of salt (1.5%) did not appear to have any effect on *C. jejuni* survival at both 4 °C and -22 °C. Frozen chicken skin (both -20 and -70 °C) containing artificially inoculated *C. jejuni* retained significant viability and *C. jejuni* quickly replicated following thawing to levels which exceeded the permitted bacterial level on raw food products (Lee *et al.*, 1998). Lee *et al.* (1998) also reported differential *Campylobacter* content in products with and without skin upon storage at -20 °C, suggesting that chicken skin might protect campylobacters from freezing. Similarly, Dykes and Moorehead (2001) opined that subcutaneous fat might provide protection to *C. jejuni* in beef trimmings upon freezing (-20 °C). A recent study reports that rapid chilling of poultry, particularly at 4 °C storage enhanced *Campylobacter* survival (El-Shibiny *et al.*, 2009).

1.6.1.4 pH stress

*C. jejuni* grows and survives optimally at pH 6.5-7.5, but it can grow and survive between pH 4.0 and 9.0 (Chaveerach *et al.*, 2003). Organic acids such as acetic acid, formic acid, lactic acid and ascorbic acid have been shown to inhibit *Campylobacter* growth. In addition, *C. jejuni* can enter a state of VBNC upon exposure to acidic environment (Chaveerach *et al.*, 2003; Birk *et al.*, 2010). *C. jejuni* survival under acidic condition depends on temperature (Kelana and Griffiths, 2003a) as well as salt (NaCl) concentration (Kelana and Griffiths, 2003b). No viable *C. jejuni* cells were detected at pH 4.0 and 8.5 after 2 and 4 days of incubation. *C. jejuni* survived optimally at pH above 4.5
and below 7.5 at all temperatures tested (4, 22 and 30 °C). *C. jejuni* survived best between pH 6.5-7.0 at 4 °C over 14 days. It was concluded that *C. jejuni* grows better under acidic conditions than alkali conditions at all temperatures. At pH values of 6.5-7.7, *C. jejuni* showed the highest salt tolerance (NaCl) of 1.7% at 40 °C and 1.5% at 42 °C.

Adaptation to changing environmental pH is important for *C. jejuni* pathogenesis. Owing to low infection dose of 500-800 CFU, *C. jejuni* might have mechanisms to detect and respond to acidic environments found in stomach and intestine (Reid *et al.*, 2008a and 2008b). *C. jejuni* strain CI120 has been shown to possess an adaptive tolerance response (ATR) that increases its ability to survive at low pH (Murphy *et al.*, 2003b; Murphy *et al.*, 2005). Activation of ATR relies on protein synthesis, suggesting that pH stimulated proteins are involved in *C. jejuni* survival under low pH. An unidentified protein secreted by *C. jejuni* has been implicated to provide some resistance to acid stress (Murphy *et al.*, 2003a). In addition, the fact that *C. jejuni* is capable of colonizing both large and small intestines suggest that *C. jejuni* is not only capable of adaptation to mildly acidic conditions such as that found in the intestine but also can adapt to highly acidic pH conditions such as that found in stomach. Gene expression profile at mildly acidic pH conditions (7.0, 6.5, 6.0 and 5.5) indicated that gene expression was highest when *C. jejuni* was exposed to pH 5.5 and adaptation to acidic condition was characterized by down-regulation of 82 genes and up-regulation of 27 genes (Reid *et al.*, 2008b). Similarly, *C. jejuni* was shown to modulate its gene expression in response to *in vitro* and *in vivo* acid shock (such as that found in stomach) (Reid *et al.*, 2008a). Another study
reported that *C. jejuni* formed an ATR in response to acid stress, which allowed for *C. jejuni* survival in acidic broth of pH 4.5 (Ma et al., 2009). This study also showed that cells exposed to acidic conditions were resistant to further challenges of lethal acidic conditions over 200 minutes. A recent study showed that coincubation with amoeba increased the acid tolerance of *C. jejuni* (Axelsson-Olsson et al., 2010).

**1.6.1.5 Nutrient stress**

**The effect of carbon on *C. jejuni* metabolism and survival:** *C. jejuni* is commonly found in the lower avian cecum where amino acids are abundant and microbial biosynthesis occurs (Beery et al., 1988). *C. jejuni* is capnophilic (requires CO$_2$ for its growth) and asaccharolytic (does not utilize sugars and rather amino acids are utilized as carbon and energy source) (Kelly, 2001; Leach et al., 1997; Velayudhan et al., 2004). In addition, *C. jejuni* is capable of utilizing anaerobic fermentation byproducts from the gut such as small organic acids, pyruvate and TCA cycle metabolic intermediates as carbon and energy sources. *C. jejuni* utilizes L-serine and L-aspartate as preferred carbon sources and usually does not grow where bio-available nitrogen sources (amino acids or ammonia) are limiting (Leach et al., 1997; Wright et al., 2009).

Bacteria respond to nutrient limitation and other environmental stresses by eliciting what is called as stringent response (Cashel et al., 1996). Stringent response is a global stress response that alters bacterial gene expression to enable survival under diverse stress conditions. Stringent response is activated by environmental stimuli such as nutrient limitation, which results in the synthesis of guanosine pentaphosphate (pppGpp).
pppGpp is then hydrolyzed to guanosine tetraphosphate which 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). In most Gram negative bacteria, stringent response is regulated by two proteins RelA and SpoT (Cashel et al., 1996; Mittenhuber, 2001). RelA mediates the synthesis of pppGpp, while SpoT degrades (p)ppGpp. SpoT also has a limited capacity to synthesize pppGpp.

Unlike other bacteria, *C. jejuni* genome harbors a single gene that encodes SpoT that does both functions of synthesis and degradation of pppGpp (Parkhill et al., 2000; Gaynor et al., 2005). Similar to other bacteria, *C. jejuni* mounts a SpoT-dependent stringent response to nutrient limitation through (p)ppGpp synthesis (Gaynor et al., 2005). SpoT-mediated stringent response is required for *C. jejuni* stationary phase survival, growth and survival under low CO$_2$/high O$_2$ conditions, and rifampicin resistance. In addition, stringent response is important for *C. jejuni* virulence-related phenotypes such as adherence, invasion and intracellular survival. SpoT was also shown to modulate biofilm formation and a spoT mutant exhibited enhanced biofilm formation (MacKichan et al., 2008). Microarray analysis of widtype and the spoT mutant revealed a strong correlation between gene expression profiles and the phenotypes observed. Thus, *C. jejuni* utilizes a SpoT-mediated stringent response to adapt to varying stress conditions including carbon limiting nutrient stress both inside and outside the host.

**Carbon metabolic pathways:** There are two carbon metabolic pathways in *C. jejuni*, namely glycolytic pathway and anaplerotic pathway (Hughes et al., 1998; Kelly, 2001;
Parkhill et al., 2000). Glycolytic pathway is used to generate ATP from ADP and phosphate, while anaplerotic pathway replaces carbon compounds used to produce other cell constituents. Glycolytic pathway in *C. jejuni* operates in reverse of most other bacteria due to the absence of 6-phosphofructokinase (Kelly, 2001; Parkhill et al., 2000; Velayudhan, 2004). *C. jejuni* possesses all the necessary enzymes for gluconeogenesis.

**Sources of amino acids for metabolism:** Nutrient deficiency has been shown to be the highest cause of stress on an *in vitro* culture of *C. jejuni* compared to temperature and oxygen stresses (Mihaljevic et al., 2007). Stress was quantified by monitoring survival and virulence properties. *C. jejuni* utilizes amino acids that are incorporated into the TCA cycle. *C. jejuni* preferentially utilizes certain amino acids such as L-serine, L-glutamine, L-glutamate, L-proline, L-aspartate and L-asparagine. Wright et al. (2009) showed that *C. jejuni* growing in Brain-Heart Infusion (BHI) broth under standard growth conditions utilized L-aspartate, L-serine, L-asparagine and L-glutamate most rapidly and L-aspartate, L-asparagine, and L-serine were completely depleted by mid stationary phase (28 hours). L-glutamine was less rapidly utilized and was depleted completely in the decline phase (54 hours). L-proline is a less preferred substrate for *C. jejuni* and is not completely exhausted over the growth cycle. These findings indicate that *C. jejuni* is metabolically active throughout the stationary phase and can respond to altered nutrient availability to enable its survival (Guccione et al., 2008; Leach et al., 1997; Wright et al., 2009).
Nitrogen and amino acid usage: Nitrogen containing compounds play an important role in *C. jejuni* metabolism. At least one amino acid was found to be required for growth of 44% (Tenover and Patton, 1987) and 58% (Tenover *et al.* 1985) of the strains tested. While rest of the strains did not require any amino acids for their growth, suggesting that these strains might assimilate fixed forms of nitrogen into amino acids. Under oxygen limiting conditions, *C. jejuni* reduces nitrate and nitrite to ammonia and the produced ammonia is probably recycled back into amino acid biosynthesis cycle (Sellars *et al.*, 2002). However, additional studies are required to understand nitrogen assimilation mechanisms in *C. jejuni*.

Several amino acids have been shown to be important for *in vivo* growth of *C. jejuni*. L-serine is critical for *C. jejuni* colonization of the avian gut (Velayudhan *et al.*, 2004), while the ability to catabolize other amino acids is important for *C. jejuni* persistence in the avian gut (Guccione *et al.*, 2008). Strains capable of metabolizing glutamine and glutathione were better at colonizing the mouse intestine, while strains capable of utilizing asparagine were better at colonizing mouse livers (Hofreuter *et al.*, 2008).

1.6.1.6 Osmotic stress

*C. jejuni* is relatively more sensitive to osmotic stress compared to other enteric bacteria and this reflects the limited capacity of *C. jejuni* to respond to osmotic stress (Doyle and Roman, 1982b). Other bacteria respond to osmotic stress by synthesis or transport of compatible solutes such as proline, glycine betaine and K⁺ (Kempf and
Bremer, 1998). *E. coli* responds to osmotic stress by producing glycine betaine via the Bet system and also can import this solute by ProP/ProU transport system. *C. jejuni* genome contains an orthologue of ProP, a low affinity transporter for proline and glycine betaine, and two genes that encode K⁺ transport system (Park, 2005). However, orthologues of Bet system and high affinity transporters such as ProU system for betaine transport are absent in *C. jejuni* (Parkhill *et al*., 2000). In addition, *C. jejuni* also lacks the capacity to synthesize compatible solutes as it lacks the biosynthetic pathways to synthesize such solutes (Lamark *et al*., 1991; Strom and Kaasen, 1991).

Sodium chloride, an osmotic agent that absorbs water out of bacteria through osmotic pressure, is routinely used as a food preservative. *C. jejuni* can grow in the presence of 0.5-1.5% sodium chloride at 42 °C and concentrations above 2% decrease *C. jejuni* culturability (Doyle and Roman, 1982b). However, *C. jejuni* was shown to withstand higher concentrations of NaCl by decreasing the incubation temperature (Doyle and Roman, 1982a; Abram and Potter, 1984; Kelana and Griffiths, 2003a). For example, *C. jejuni* was shown to survive longer at 4.5% NaCl at lower temperatures.

Water has emerged as an important source of *Campylobacter* infections in humans and *C. jejuni* has been shown to survive for extended periods of time in water (Park, 2005). *C. jejuni* can be exposed to hypoosmotic stress in water, but little is known about the mechanisms by which *C. jejuni* counteracts hypoosmotic stress in water (Park, 2005). Bacterial response to hypoosmotic environments involves both solute and water efflux via mechanosensitive and stretch activated channels (Berrier *et al*., 1996). *E. coli*
possesses 3 types of mechanosensitive channels, namely MscM, MscS and MscL (Berrier et al., 1996). *C. jejuni* genome encodes a protein with some similarity to MscL channel of *H. pylori* (Park, 2005; Kloda and Matinac, 2001); however, experimental evidence is lacking as to its importance in *C. jejuni* osmoregulation. Certain bacteria also utilize aquaporins to counteract hypoosmotic stress, but homologues of such proteins are absent in *C. jejuni* (Park, 2005). *C. jejuni* has been shown to lose culturability significantly upon exposure to low osmolality and such environments have been shown to trigger *C. jejuni* into VBNC state (Reezal et al., 1998).

1.6.1.7 Oxidative stress

As a microaerophile, *C. jejuni* is unavoidably exposed to atmospheric oxygen in the environment during transmission and infection. Exposure to atmospheric oxygen leads to formation of reactive oxygen intermediates (ROIs) such as superoxide radicals which cause lethal damage to nucleic acids, proteins and membranes (Imlay, 2003). However, *C. jejuni* produces inducible anti-oxidant proteins to neutralize highly reactive oxygen species. Superoxide dismutase (SodB), alkyl hydroperoxide reductase (AhpC), catalase (KatA), ferritin (Cft), ferredoxin (FdxA), DNA-binding protein from starved cells (Dps) and twin arginine translocation system (TatC) play important roles in the oxidative defense of *C. jejuni*. SodB counteracts the deleterious effects of superoxides and thus plays a key role in the defense against oxidative stress and aerotolerance (Purdy et al., 1999). A *sodB* mutant showed reduced ability to survive in air, in food, during freezing, macrophages, INT407 cells and also colonized chickens poorly (Stead and Park,
Similarly, an ahpC mutant showed increased sensitivity to oxidative stresses caused by cumene hydroperoxide and exposure to atmospheric oxygen (Baillon et al., 1999). Catalase encoded by katA breaks down hydrogen peroxide to H₂O and O₂ (Grant and park, 1995) and is important for C. jejuni persistence and growth in macrophages, but not in intestinal epithelial cells (Day Jr. et al., 2000). Both cft and dps mutants were sensitive to H₂O₂ (Wai et al., 1996; Ishikawa et al., 2003) and an fdxA mutant exhibited significantly reduced aerotolerance but resistance to H₂O₂ and cumene hydroperoxide were unaffected (van Vliet et al., 2001). A tatC mutant also was significantly defective in survival in the presence of hydrogen peroxide and paraquat (Rajashekar et al., 2009).

The oxidative stress defense mechanisms in many Gram-negative bacteria are regulated by superoxide-sensing and peroxide-sensing regulators SoxRS and OxyR, respectively. However, homologues of these regulators are absent in the C. jejuni genome (Parkhill et al., 2000). Alternatively, peroxide-sensing regulator perR regulates oxidative stress defense in C. jejuni (van Vliet et al., 1999). PerR regulates both KatA and AhpC and inactivation of perR resulted in constitutive expression of katA and ahpC and increased resistance to peroxide stress. Though Fur is primarily involved in regulation of genes involved in iron metabolism (Palyada et al., 2004), it also regulates several potential proteins involved in oxidative defense such as KatA, FdxA and TrxB (Palyada et al., 2004; Holmes et al., 2005). In addition, there is clear evidence that iron homeostasis and oxidative defense are linked in C. jejuni and intracellular iron level is a key factor in C. jejuni response to oxidative stress (Palyada et al., 2008).
1.6.1.8 Hydrostatic pressure stress

High hydrostatic pressure processing (HPP) is commonly used to inactivate pathogens in food. *C. jejuni* has been shown to be relatively more sensitive to HPP compared to other foodborne pathogens and a pressure treatment of 400 MPa was required to obtain bactericidal effect (Shigehisa *et al.*, 1991; Solomon and Hoover, 2004). Sensitivity of *C. jejuni* to HPP was shown to depend on several factors such as strain, medium, growth phase and temperature of incubation (Martinez-Rodriguez, 2005a and 2005b; Lori *et al.*, 2007; Bieche *et al.*, 2009). Sagarzazu *et al.* (2009) showed that acid and heat treated *C. jejuni* were more sensitive to HPP treatment, while cold exposure increased the resistance of *C. jejuni* to HPP treatment. Proteomic analysis of HPP treated *C. jejuni* by two-dimensional gel electrophoresis revealed differential expression of several genes, suggesting that *C. jejuni* can adapt to high pressure treatment by altering its proteome (Bieche *et al.*, 2010).

1.6.1.9 UV Irradiation stress

In the environment, *C. jejuni* is commonly exposed to UV radiation in the form of sunlight (Murphy *et al.*, 2006). *C. jejuni* was shown to be more sensitive to UV than *E. coli* (Butler *et al.*, 1987). Obiri-Danso *et al.* (2001) showed that natural populations of *C. jejuni* were more sensitive to UV than the culture collection strains. Adaptive tolerance response-induced expression of DNA repair protein RecA was thought to provide some resistance to UV radiation in *C. jejuni* (Murphy *et al.*, 2006).

1.6.2 Viable but nonculturable (VBNC) cell state
Campylobacter can enter a viable but nonculturable state in response to environmental stress or upon entry into stationary phase. The VBNC state in many bacterial pathogens is considered to play an important role in human health (Chaveerach et al., 2003). By definition, VBNC cells are live bacterial cells which can no longer be cultured using conventional methods. VBNC form of Campylobacter was first reported by Rollins and Colwell (1986) and since then VBNC state has remained a contentious issue with many microbiologists supporting this state and many others dismissing the existence of this state (Park, 2002). During transition into VBNC state, C. jejuni transforms from motile spiral shape to a coccoid form (Rollins and Colwell, 1986). VBNC state can be induced upon exposure to oxygen, changes in temperature and starvation and the induction process is believed to be relatively easy owing to fastidious nature of C. jejuni (Park, 2002).

Experimentally, VBNC state was induced using various stress conditions such as low temperature (4 ºC) (Tangwatcharin et al., 2006; Lazaro et al., 1999), high temperature (60 ºC) (Tangwatcharin et al., 2006), aquatic microcosms (Baffone et al., 2006) as well as formic acid exposure (Chaveerach et al., 2003). The viability of VBNC state is largely believed to be due to the capacity of this form to modify chemical indicators of viability (Cappelier et al., 1997; Tholazan et al., 1999) or to the longevity of certain macromolecules following loss of culturability (Lazaro et al., 1999). Various methods such as use of Acrindine Orange dye which intercalates with DNA making elongated cells visible or tetrazolium salts such as 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) which result in accumulation of insoluble formazan crystals (Kogure et
al., 1979; Rodriguez et al., 1992) or CTC-specific fluorescent antibody (Pyle et al., 1995; Hegarty et al., 1999) have been employed to determine the viable state of VBNC cells. Tangwatcharin et al. (2006) induced VBNC by exposing C. jejuni to 4 and 60 °C in 25 days and 15 minutes, respectively. They confirmed the VBNC induction by direct viable count techniques and live staining. Transmission electron microscopy of C. jejuni VBNC cells revealed loss of outer membrane. Differential synthesis of protein was also observed in VBNC cells induced at 60 °C. Similarly, Chaveerach et al. (2003) used formic acid to induce VBNC cells in C. jejuni and VBNC cells were induced in 30 minutes after formic acid treatment. Organic acids such as formic acid, acetic acid and lactic acid are extensively used in poultry industry as a way to reduce Campylobacter through drinking water or on carcasses (Chaveerach et al., 2003; Byrd et al., 2001; Dickens et al., 1997).

Some authors showed that VBNC cells can be resuscitated in animal models such as chicks and mice (Saha et al., 1991; Jones et al., 1991; Pearson et al., 1993; Stern et al., 1994; Tholozan et al., 1999), embryonated eggs (Capellier et al., 1999), aquatic environments (Thomas et al., 2002) and after acid treatment (Chaveerach et al., 2003). In support of this, Baffone et al. (2006) demonstrated that VBNC state can be induced in aqueous microcosms and the induced VBNC cells can be resuscitated in a mouse model, although a relatively high titer of respiring bacteria in the VBNC state was found important for resuscitation and subsequent colonization of intestine. On the contrary, other authors demonstrated that nonculturable cells were unable to infect chicks, mice and human volunteers (Beumer et al., 1992; Medema et al., 1992; Ziprin et al., 2003; Ziprin and Harvey, 2004). This inconsistency in results is believed to be due to strain
variations, differences in exposure challenge levels and routes, differences in methods used to determine nonculturability, or varying conditions under which nonculturatable cells were formed (Verhoeff-Bakkenes et al., 2007; Jones et al., 1991; Medema et al., 1992; van de Giessen et al., 1996). For example, nonculturatable cells were formed at 4 °C in all studies that showed that nonculturatable cells were infective; while in experiments using higher temperature for formation of nonculturatable cells, no infectivity was observed (Verhoeff-Bakkenes et al., 2007). In addition, nonculturatable cells formed at different temperatures showed physiological differences (Hazeleger et al., 1995). For instance, nonculturatable cells formed at 4 °C showed ATP ratio and membrane fatty acid composition comparable to culturable cells. While nonculturatable cells formed at 25 °C showed ATP ratio and membrane fatty acid composition clearly different from culturable cells indicating significant degeneration. Furthermore, Tholozan et al. (1999) showed that only a limited number of strains are able to induce VBNC formation in C. jejuni.

Hazeleger et al. (1995) showed that VBNC state is not associated with any differences in protein profile or protein synthesis compared to culturable state; while, Lazaro et al. (1999) showed that there is a significant difference in the protein profile between culturable and nonculturatable states as determined by two dimensional gel electrophoresis. Another study showed that culturability and adhesion/invasion are linearly related, suggesting that nonculturatable bacteria may represent low risk with respect to campylobacteriosis (Verhoeff-Bakkenes et al., 2007). A recent study demonstrated that C. jejuni can survive in VBNC state following exposure to starvation (Klancnik et al., 2009). Upon starvation, C. jejuni compromises its virulence and
infection ability; however, these pathogenic properties were not completely abolished indicating that nonculturable state can be a potential public health hazard. Despite a contentious issue, VBNC state can play an important role in \textit{C. jejuni} survival upon exposure to stress conditions (Hazeleger \textit{et al.}, 1995; Klancnik \textit{et al.}, 2009). However, further studies are required to explore more on the significance of VBNC state in \textit{C. jejuni}.

1.6.3 Genetic variation in \textit{C. jejuni}

\textit{C. jejuni} strains exhibit high genetic variation, which can be partly due to genetic exchange between the strains as well as mechanisms that occur within the genome (Young \textit{et al.}, 2007). \textit{C. jejuni} is naturally competent, which means it can take up DNA from its surroundings (Wilson \textit{et al.}, 2003). It is regarded as an important mechanism for generating genetic diversity through horizontal gene transfer (de Boer \textit{et al.}, 2002; Dorrell \textit{et al.}, 2001; Fouts \textit{et al.}, 2005; Kim \textit{et al.}, 2006; Wilson \textit{et al.}, 2003). In addition to conjugation, natural transformation also plays a significant role in the transfer of antibiotic resistance determinants in \textit{C. jejuni} (Kim \textit{et al.}, 2008; Kim \textit{et al.}, 2006). Several factors have been identified to contribute to \textit{C. jejuni} natural transformation, including RecA involved in DNA recombination (Guerry \textit{et al.}, 1994), virB10 encoded by the pVir plasmid (Bacon \textit{et al.}, 2000; Bacon \textit{et al.}, 2002), ComEA involved in DNA binding (Jeon and Zhang, 2007), proteins of the type II secretion pathway (Wiesner \textit{et al.}, 2003), the N-linked protein glycosylation pathway (Larsen \textit{et al.}, 2004), Cj1211 (Jeon \textit{et al.}, 2008), lipooligosaccharide and capsular polysaccharide (Jeon \textit{et al.}, 2009). Recently,
nucleases encoded by the integrated elements CJIE1 (Gaasbeek et al., 2009), CJIE2 and CJIE4 (Gaasbeek et al., 2010) have been shown to inhibit natural transformation of C. jejuni.

C. jejuni genome contains several hypervariable regions that contain repetitive sequences of a single nucleotide called as homopolymeric tracts (Parkhill et al., 2000). These homopolymeric tracts are relatively unstable due to their inherent susceptibility to a process called slipped-strand mispairing which occurs during DNA replication (Newell et al., 2000). Slipped-strand mispairing results in insertion/deletion events producing a frame shift to achieve a rapid change in the translation of the corresponding gene product and thus accounts for phase variation (Newell et al., 2000; Parkhill et al., 2000; Linton et al., 2000; Gilbert et al., 2002; Guerry et al., 2002; Karlyshev et al., 2000). Phase variation, which is defined as the random and reversible switching on/off of gene expression, is a frequent phenomenon in C. jejuni and is believed largely due to lack of DNA repair function in this bacterium (Allos, 2001; Lastovica and Skirrow, 2000; Parkhill et al., 2000; Wassenaar et al., 2002). Phase variation is a common mechanism for regulation of C. jejuni LOS biosynthesis genes (Gilbert et al., 2002; Linton et al., 2000), capsular polysaccharide genes (Szymanski et al., 2003) and flagellar genes (Karlyshev et al., 2002; van Alphen et al., 2008b; Hendrixson, 2006; Hendrixson, 2008).

1.6.4 Antimicrobial resistance

Gastroenteritis caused by C. jejuni is usually self-limiting requiring no antibiotic treatment; however, antibiotic treatment is needed in severe and prolonged cases,
septicemia or extra-intestinal complications. Often, fluoroquinolone (FQ) and macrolide antibiotics are used for treatment of *C. jejuni* infections when clinical treatment is warranted. Macrolide antibiotic, erythromycin is the first choice of treatment for confirmed *Campylobacter* infections. Fluoroquinolone antibiotic, ciprofloxacin is used in enteritis of unknown origin such as Traveller’s diarrhea (Al-Abri *et al*., 2005). Development of resistance to both macrolides and fluoroquinolones in *Campylobacter* is increasing and has emerged as a major public health problem worldwide (Engberg *et al*., 2001; Gibreel and Taylor, 2006; Luangtongkum *et al*., 2009). This is most likely due to antibiotic use in livestock (Angulo *et al*., 2004), clonal dissemination of resistant strains (Bae *et al*., 2007) and use of antibiotics in human medicine (Engberg *et al*., 2004).

*C. jejuni* genome harbors 12 antibiotic resistance genes: three in multidrug efflux pump CmeABC, another three predicted to encode for an efflux pump CmeDEF, two additional putative efflux pumps Cj0035c/Cj1687, a putative periplasmic β-lactamase Cj0299, a putative acetyltransferase Cj1715, and two hypothetical proteins Cj1296/Cj1297 (Gundogdu *et al*., 2007). Several mechanisms of antimicrobial resistance have been identified in *Campylobacter* (Luangtongkum *et al*., 2009). Target mutations and drug efflux are among the most common mechanism for fluoroquinolone and macrolide resistance. Natural transformation and conjugation play a role in spread of antibiotic resistance genes between strains in *C. jejuni* (Jeon and Zhang, 2007; Jeon *et al*., 2008; Kim *et al*., 2008).
Point mutations in the quinolone resistance determining region of GyrA alters the affinity of fluoroquinolones to DNA gyrase and results in resistance to this class of antibiotics (Engberg et al., 2001). Similarly, mutations in the 23S rRNA subunit results in resistance to macrolide antibiotics (Gibreel et al., 2005). Mfd, a transcription-repair coupling factor, modulates the spontaneous mutation rates in Campylobacter and an mfd mutant showed 100-fold decrease in the frequency of spontaneous ciprofloxacin resistant mutants, while overexpression of mfd increased the frequency of spontaneous resistant mutants (Han et al., 2008). In addition, FQ-resistant Campylobacter was shown to emerge rapidly from FQ-susceptible population when exposed to FQ antibiotics (Luo et al., 2003; Han et al., 2008). In the absence of antibiotic selection, spontaneous mutations also affect the fitness of Campylobacter as FQ-resistant Campylobacter out compete the FQ-susceptible Campylobacter (Luo et al., 2005). On the other hand, the spontaneous mutation rate is less with macrolides which require long-term exposure for development of macrolide-resistant mutants (Lin et al., 2007; Luangtongkum et al., 2009). This possibly explains the low prevalence of macrolide resistance in Campylobacter compared to FQ resistance.

Drug efflux is another mechanism of FQ and macrolide resistance in Campylobacter. The extrusion of structurally diverse antimicrobials and toxic compounds in Campylobacter is mediated by RND-type efflux pump called CmeABC (Lin et al., 2002; Lin et al., 2003; Pumbwe and Piddock, 2002; Luo et al., 2003). In addition to mediating antimicrobial resistance, CmeABC is also important for intestinal colonization of the host (Lin et al., 2003). CmeABC efflux pump is regulated by a repressor called
CmeR and bile salts induce expression of CmeABC efflux pump by inhibiting the binding of CmeR (Lin et al., 2005a and 2005b). *C. jejuni* LOS, but not CPS, reduces the permeability to hydrophobic antibiotics such as erythromycin (Jeon et al., 2009). CmeG, an efflux transporter, also contributes to antimicrobial resistance and oxidative defense in *C. jejuni* (Jeon et al., 2010).

**1.6.5 Biofilm formation**

Biofilms are matrix enclosed bacterial communities adherent to each other or surfaces or interfaces. Biofilm formation is now recognized as a well-known mode of bacterial survival under stressful environmental conditions such as UV radiation, desiccation and predation (Elasri and Miller, 1999; Matz et al., 2005; Chang et al., 2007). In addition, bacteria in biofilms are known to be >1000-fold resistant to disinfectants and antimicrobials compared to their planktonic counterparts (Fux et al., 2005).

*C. jejuni* is capable of forming monospecies biofilm (Joshua et al., 2006; Kalmokoff et al., 2006) and also can colonize a preexisting biofilm (Hanning et al., 2008). Biofilm formation has been demonstrated under laboratory conditions and in poultry environments such as watering supplies and plumbing systems of poultry houses as well as poultry processing plants (Bull et al., 2006; Pearson et al., 1993; Zimmer et al., 2003). *C. jejuni* strains also have been shown to form biofilms on stainless steel, polyvinyl chloride, nitrocellulose membranes, glass filter fibers and glass (Kalmokoff et al., 2006). *C. jejuni* forms three types of biofilms, classical biofilm on glass surfaces at the liquid-gas interface, a pellicle also at the liquid-gas interface and finally unattached
aggregates in liquid culture (Joshua et al., 2006). Flagella were shown to be required for C. jejuni attachment on glass surfaces. Growth in nutrient rich media or high osmolarity is known to inhibit biofilm formation, while high temperatures and microaerobic environments enhance biofilm formation (Reeser et al., 2007).

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. Both flagella and cell signalling are important for biofilm formation as flaAB and luxS mutants showed significantly reduced ability to form biofilms compared to their parental strains (Reeser et al., 2007). Global posttranscriptional regulator CsrA (Fields and Thompson, 2008), twin-arginine translocation system component TatC (Rajashekara et al., 2009) and sensor kinase CprS (Svensson et al., 2009) are also important for biofilm formation in C. jejuni. Similarly, LOS and SpoT modulate biofilm formation and lack of C. jejuni LOS and SpoT resulted in enhanced biofilm formation (MacKichan et al., 2008; Naito et al., 2010). C. jejuni biofilm formation is increased under aerobic conditions, suggesting that biofilm formation is an essential adaptation mechanism for C. jejuni to survive high oxygen levels in the environment during transmission (Reuter et al., 2010). Using in vitro-infected human intestinal tissue, C. jejuni was shown to form microcolonies before forming biofilms (Haddock et al., 2010). A recent study also showed that C. jejuni can form biofilms in association with other bacteria under microaerobic conditions in controlled mixed-microbial populations (Teh et al., 2010).
1.6.6 Metabolic adaptation

*C. jejuni* is a fastidious bacterium with limited capacity for biosynthesis and requires complex media for growth (Kelly, 2001). *C. jejuni* is unable to catabolize glucose and other hexose sugars due to the absence of the key glycolytic enzyme 6-phosphofructokinase (Parkhill *et al*., 2000; Velayudhan and Kelly, 2002). Amino acids and organic acids serve as major carbon sources for *C. jejuni* (Smibert, 1984; Velayudhan and Kelly, 2002). In complex medium under microaerobic conditions, serine, aspartate, glutamate and proline are among the most heavily used amino acids (Leach *et al*., 1997; Guccione *et al*., 2008). These amino acids also support growth as sole carbon sources in minimal medium. Glutamine and asparagine can also be catabolized by some *C. jejuni* strains (Hofreuter *et al*., 2008). *C. jejuni* strains also utilize organic acids such as pyruvate, fumarate and lactate as carbon sources (Velayudhan and Kelly, 2002; Hinton, 2006). As a microaerophile, *C. jejuni* cannot grow fermentatively and relies mainly on oxidative phosphorylation for its energy requirements. *C. jejuni* has a branched electron transport chain with a range of electron acceptors including oxygen and other alternative electron acceptors (Sellars *et al*., 2002; Guccione *et al*., 2010; Thomas *et al*., 2010). Several studies have shown the importance of electron transport chain to *C. jejuni* colonization *in vivo* in the chicken gut (Weingarten *et al*., 2008; Weerakoon and Olson, 2008; Weerakoon *et al*., 2009).

*C. jejuni* encounters a wide variety of environmental niches ranging from surface water to the gut of animals and humans. Adaptation to these varying niches is key to *C.
*jejuni* persistence in the environment as well as host colonization. For example, microarray analysis of *C. jejuni* grown under two different temperatures (37 and 42 °C) revealed major differences in gene expression (Stintzi, 2003). This reflects the capability of *C. jejuni* to adapt to two hosts with different body temperatures, human and chicken. Similarly, *C. jejuni* can switch to alternative electron acceptors such as fumarate and nitrite (Sellars *et al.*, 2002) as well as alternative amino acids as preferred carbon source in response to limited oxygen environment (Guccione *et al.*, 2008; Wright *et al.*, 2009).

The change in metabolic state in different environmental niches might contribute to altered virulence properties in *C. jejuni*. More specifically, compared to human intestine, the chicken cecum is rich in amino acids such as serine, proline, aspartate and glutamate, which are preferentially metabolized by *C. jejuni*. In addition, *C. jejuni* exhibits differential carbon source utilization when grown at different temperatures, 37 and 42 °C (Line *et al.*, 2009). Thus, change in metabolic state as well as body temperature might contribute to altered virulence of *C. jejuni* in the chicken host. Unlike humans, *C. jejuni* apparently do not adhere to or invade the intestinal tissue and thus causes no intestinal pathology in chickens. A recent study showed that *C. jejuni* becomes active during stationary phase and responds to changing conditions by altering gene expression to increase motility, switching to alternative amino acid substrates and scavenging to use molecules such as acetate which were previously excreted (Wright *et al.*, 2009). *C. jejuni* adaptation to varying environments is driven by two-component systems as well as post-transcriptional regulators (reviewed in 1.6.7) that control the expression of distinct
metabolic regulons (Bras et al., 1999; Svensson et al., 2009; MacKichan et al., 2004; Wosten et al., 2004).

1.6.7 Gene regulation and environmental stimuli

Despite its small genome, C. jejuni employs a range of regulatory systems to enable its adaptation and survival under variety of environmental conditions and stresses (Park, 2000). C. jejuni possesses a number of regulatory systems which allow a rapid response to changes in environment through adjustment of the transcriptome. Slippage synthesis, alternative sigma factors, gene organization into operons and regulons, activator/repressor proteins, two component regulation systems, small noncoding RNAs and global regulatory proteins are among the common gene regulation mechanisms in C. jejuni (Finlay and Falkow, 1997; Helmann, 2002; Nuijten et al., 1995; Park, 2000; Parkhill et al., 2000). The role of slippage synthesis in gene regulation has been discussed elsewhere in this dissertation.

The use of alternative sigma factors is another common mechanism of gene regulation in bacteria. Sigma factor binds to the core polymerase forming the RNA polymerase holoenzyme and binding of sigma factor to core polymerase confers binding specificity to the RNA polymerase holoenzyme. C. jejuni genome is predicted to contain 3 sigma factors, namely σ^{28}, σ^{54} and σ^{70} which are encoded by fliA, rpoN and rpoD, respectively (Parkhill et al., 2000; Jagannathan et al., 2001). However, heat shock and stationary phase sigma factors are absent in C. jejuni genome. The σ^{28} promoter regulates the expression of flaA and is up-regulated by chemotactic relevant intestinal stimuli such
as pH, bovine bile, deoxycholate and L-fucose and down-regulated by viscosity (Allen and Griffiths, 2001). The $\sigma^{54}$ regulates the expression of flaB, the hook protein gene flaE and genes responsible for flagellar assembly and nitrogen metabolism (Guerry et al., 1991; Hendrixson and DiRita, 2003; Luneberg et al., 1998; Park, 2000). The regulation of $\sigma^{54}$ is affected by environmental stimuli such as pH, temperature, inorganic salts and divalent cations (Alm et al., 1993). Like in other bacteria, $\sigma^{70}$ regulates housekeeping genes in C. jejuni (Helmann, 2002; Park, 2000). However, $\sigma^{70}$ promoter in C. jejuni exhibits a strong periodic signal instead of a -35 box (Peterson et al., 2003).

Operon model is one more mechanism for gene regulation which involves clustering of genes with related function allowing coordinated expression (Helmann, 2002). Several genes including genes encoding proteins involved in glycosylation, LOS biosynthesis, CPS biosynthesis and CmeABC efflux pump are organized into operons in C. jejuni (Parkhill et al., 2000; Wren et al., 2001; Lin et al., 2002). Operons are under the control of regulatory proteins which specifically interact with binding sites within or near the promoter sequence (Helmann, 2002). Activators enhance the interaction of RNA polymerase and a particular promoter which results in increased expression of one or more genes (Helmann, 2002; Palyada et al., 2004). While repressors act negatively by binding to specific sites called operators (Helmann, 2002; Palyada et al., 2009). The expression of several genes or operons can be regulated by the same protein to form a regulon (Helmann, 2002; Holmes et al., 2010). The expression of several operons and regulons can be under the control of the same stimulus or signal forming a stimulon (Helmann, 2002; Holmes et al., 2010).
Two-component systems (TCSs) also play an important role in regulation of gene expression in bacteria. TCSs typically contain a histidine kinase that is responsible for sensing a specific environmental stimulus and a response regulator that mediates the cellular response, mostly through altered gene expression (Helmann, 2002). Several TCSs have been identified each of which are involved in regulating the expression of a specific gene or a subset of genes in C. jejuni. The FlgS/FlgR system regulates flagellar motility via activating the fla regulon (Wosten et al., 2004) and recently it has been linked to flagellar export pathway (Joslin and Hendrixson, 2009). The FlgR response regulator also contains a homopolymeric tract and thus is subject to phase variation (Hendrixson, 2006). The RacR/RacS system is required for temperature dependent growth as well as colonization of chickens (Bras et al., 1999). The PhosS/PhosR regulates pho regulon and is essential for sensing phosphate limitation in C. jejuni (Wosten et al., 2006). The DccRS is required for optimal in vivo chicken colonization but not for in vitro growth (MacKichan et al., 2004) and is activated in the late stationary phase (Wosten et al., 2010). The CprRS regulates biofilm formation, chicken colonization, and stress tolerance in C. jejuni (Svensson et al., 2009).

Global regulatory proteins are also important for regulation of gene expression in bacteria. C. jejuni is predicted to encode global regulatory proteins such as LysR (Cj1000) and AraC (Cj1042c) based on their homology to proteins in E. coli (Park, 2000). However, experimental confirmation of this data has not been reported in C. jejuni. CsrA, a post-transcriptional global regulator regulates biofilm formation, oxidative stress resistance and adherence to and invasion of IN407 cells in C. jejuni (Fields and
The DksA-like protein, a post-transcriptional regulator, regulates genes involved in multiple metabolic functions and pathogenesis (Yun et al., 2008). In addition, small noncoding RNAs also have been recently shown to play a central role in gene regulation in bacteria (Narberhaus and Vogel, 2009; Gripenland et al., 2010). A similar role for small noncoding RNAs in C. jejuni gene regulation cannot be underestimated.

Bacteria also use quorum sensing to regulate a diverse array of physiological activities. Quorum sensing is a cell density-dependent phenomenon where in bacteria produce and release signaling molecules called autoinducers (AI) which increase in concentration as a function of cell density. The detection of minimum threshold concentration of the signaling molecule results in altered gene expression. The most well characterized quorum sensing system in bacteria involves the production of AI-2 (Bassler et al., 1994) and LuxS is the critical enzyme involved in the biosynthesis of AI-2. Quorum sensing regulates factors involved in diverse functions including biofilm formation and pathogenesis. Like in other bacteria, exponentially growing C. jejuni produces the functional AI-2 and the mutation of luxS abolished AI-2 production (Elvers and Park, 2002). Growth rate, oxidative stress resistance, and the ability to invade intestinal epithelial cells were not affected by luxS mutation; however, luxS mutant exhibited a significantly decreased motility (Elvers and Park, 2002). Similarly, luxS mutation in C. jejuni strain 81116 also resulted in reduced motility and fla transcription, suggesting a role for LuxS in regulating C. jejuni motility (Jeon et al., 2003). Microarray analysis of luxS mutant in C. jejuni strain 81-176 revealed a central role for LuxS in
regulation of gene expression particularly of those involved in *C. jejuni* motility and metabolism (He *et al*., 2008). A recent study showed that AI-2 does not play a role in cell-to-cell communication in *C. jejuni* strain NCTC 11168 during exponential growth *in vitro* and the effects of luxS mutation on the transcriptome are related to the consequential loss of function in the activated methyl cycle (Holmes *et al*., 2009).

### 1.7 Inorganic polyphosphate

#### 1.7.1 Introduction

Inorganic polyphosphate (poly P) is a linear polymer of tens or hundreds of orthophosphate (P_1) residues linked together by high energy phosphoanhydride bonds (Kornberg *et al*., 1999). Poly P is produced from P_1 by dehydration at high temperature and likely has been present since the prebiotic times. It has been speculated to have contributed to the origin of species and possibly was a source of energy in the pre-ATP world. Poly P is found in every cell that has been studied in nature thus far (bacteria, archaea, fungi, protozoa, plant and animal) (Kulaev, 1979; Wood and Clark, 1988; Kulaev and Vegabov, 1983). It is stable over a wide range of temperatures, pH and oxidants. It serves as a source of ATP for ~ 500 reactions (Rao *et al*., 2009). For example, poly P is involved in phosphorylating sugars, nucleosides and proteins and also serves as means of activating the precursors of fatty acids, phospholipids, polypeptides and nucleic acids. Poly P was first reported in yeast (Liebermann, 1890; Ogawa *et al*., 2000) and later in bacteria as metachromatic granules (also called as volutin granules) (Pallerla *et al*., 2005). The term “metachromatic” originates from their tendency to stain pink with basic
blue dyes. Historically, 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 as poly P granules.

**1.7.2 Polyphosphate distribution and location**

Poly P is involved in numerous and varied biological functions depending on its location and concentration. 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). In alga *Chlamydomonas reinhardtii*, poly P is found in the vacuoles along with Ca$^{2+}$ and Mg$^{2+}$ (Komine *et al*., 2000). In unicellular eukaryotes as well as in bacteria, Poly P is found in acidocalcisomes which are vacuoles containing poly P, pyrophosphate and divalent metal ions. Acidocalcisomes have been well documented in unicellular eukaryotes such as trypanosomatid and apicomplexan parasites, algae and slime molds (Docampo *et al*., 1995; Moreno and Zhong, 1996). Acidocalcisomes have also been reported in bacteria such as *Agrobacterium tumefaciens* and *Rhodospirillum rubrum* (Seufferheld *et al*., 2003). In humans, granules similar to acidocalcisomes called platelet-dense granules have been found (Ruiz *et al*., 2004). In *Neurospora crassa*, poly P occurs with basic aminoacids in 1:1 ratio, along with polycationic spermidine and Mg$^{2+}$ (Cramer and Davis, 1984; Vaughn and Davis, 1981). Poly P has been shown to help relieve the osmotic burden in these vacuoles, allowing the vacuoles to remain small during cytoplasmic streaming. In *Saccharomyces cerevisiae*, poly P is widely distributed.
in every compartment including cytosol, vacuoles, nuclei and mitochondria (Lichko et al., 2006; Wurst et al., 1995; Trilisenko et al., 2002). Nearly, 80-90% of the total poly P in *S. cerevisiae* is found in the cytosol, while 15% occurs in the vacuoles. Mitochondrial fraction of *S. cerevisiae* contained short chain poly P (<15 P\(_i\) residues). Poly P has also been reported in all higher eukaryotes tested (Kumble and Kornberg, 1995). In higher eukaryotes, poly P is present in tissues such as kidney, liver, lungs, brain, and heart, and subcellular compartments such as mitochondria.

### 1.7.3 Polyphosphate concentration

Poly P occurs in varying concentrations in different species. For instance, poly P level in *E. coli* ranges from 0.1 to 150 nmol/mg of protein, while *P. aeruginosa* contains levels between 150-325 nmol of poly P/mg of protein (Ishige et al., 1998; Schroder and Muller, 1999). These levels are elevated during stress condition. In poly P accumulating bacteria used for enhanced biological phosphorus removal from waste water, poly P can accumulate up to 48% of the total dry weight (McMahon et al., 2002; Seviour et al., 2003). In unicellular eukaryotes such as *S. cerevisiae*, the total cellular poly P concentration is 120 mM, while under phosphate limiting conditions the levels can go up to 20% of its total dry weight (Wurst et al., 1995). In multicellular eukaryotes, poly P is found in varying levels depending on the type of tissue (Kumble and Kornberg, 1995). For example, rat liver cells contain 89 µmol of poly P in the nucleus, while it was 12, 11 and 4 µmol in the cytosol, mitochondria and microsome, respectively.

### 1.7.4 Enzymes of polyphosphate metabolism

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**Polyphosphate kinase 1 (PPK1):** PPK1 is the principal enzyme responsible for synthesis of poly P in many 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. 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 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. Studies with EcPPK1 showed that autophosphorylation occurs at histidine residues H435 and H454 (Kumble *et al*., 1996); however, recent studies suggest only H435 histidine residue is involved in autophosphorylation (Rao *et al*., 2009).

Studies in several 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 ATP production (Brown and Kornberg, 2008), entry of DNA through membrane channels (Castuma *et al*., 1995; Reusch and Sadoff, 1988), capsule composition (Tinsley *et al*., 1993), maintaining nutritional requirements during starvation (Kuroda *et al*., 1997), Fidelity of DNA replication (McInerney *et al*., 2006; Stumpf and Foster, 2005), activation of Lon protease (Kuroda *et al*., 2001; Nomura *et al*., 2004), growth, motility, biofilm formation, quorum sensing, bacterial signaling, antibiotic resistance, stationary-phase survival, resistance to nutrient, oxidative, osmotic, heat, desiccation, acid and alkaline stresses, competitive fitness, invasion and intracellular
survival and host colonization (Jahid et al., 2006; Kim et al., 2002; Ogawa et al., 2000; Price-Carter et al., 2005; Rao and Kornberg, 1996; Rashid and Kornberg, 2000; Rashid et al., 2000; Tan et al., 2005; Tunpiboonsak et al., 2010; Silby et al., 2009; Richards et al., 2008; Sureka et al., 2007; Fraley et al., 2007; McMeechan et al., 2006; Ayraud et al., 2005; Rao et al., 2009).

Polyphosphate kinase 2 (PPK2): Many bacterial species contain another enzyme, PPK2, which preferentially mediates poly P-driven generation of GTP (Ishige et al., 2002; Sureka et al., 2009), a molecule known to have important roles in cell signaling and DNA, RNA, protein, and polysaccharide synthesis (Chakrabarty, 2002; Sundin et al., 1996). 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 are distinct from PPK1 in several aspects. The *P. aeruginosa* PPK2 paralog PA0141 utilizes poly P to generate GTP which is 75-fold greater than poly P synthesis from GTP or ATP (Ishige et al., 2002). The PA0141 was shown to be important for the synthesis of alginate which is required for virulence in *P. aeruginosa*. Contrary to PA0141, *P. aeruginosa* PPK2 paralogs PA2428 and PA3455 preferentially catalyze the synthesis of ATP/ADP from poly P and do not contribute to poly P synthesis (Nocek et al., 2008). However, in *Corynebacterium glutamicum*, which lacks PPK1, PPK2 catalyzes poly P synthesis from ATP or GTP (Lindner et al., 2007). In mycobacteria, 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).
The *P. aeruginosa* PPK2 (PA0141) and *M. tuberculosis* PPK2 (rv3232c) preferentially catalyze the synthesis of GTP using poly P as phosphate donor (Ishige *et al*., 2002; Nocek *et al*., 2008). The PA0141 also synthesizes poly P from GTP but at a 75-times lower rate than GTP synthesis from poly P, while rv3232c does not contribute to poly P synthesis. Contrary to PA0141 and rv3232c, *P. aeruginosa* PPK2 paralogs PA2428 and PA3455 primarily catalyze the synthesis of ATP/ADP from poly P and do not mediate poly P synthesis from ATP/GTP (Nocek *et al*., 2008).

PPK2 class of enzymes belongs 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 are essential for binding nucleoside triphosphates, and Mg\(^{2+}\) cation, respectively (Leipe *et al*., 2003). Nocek *et al*., (2008) classified PPK2 enzymes into 2 groups, those containing 1 or 2-fused PPK2 domains. Proteins with a 1-PPK2 domain catalyze polyP-dependent phosphorylation of ADP to ATP, while proteins containing 2-fused PPK2 domains phosphorylate AMP to ADP.

**Exopolyphosphatases (PPXs):** Poly P is degraded to inorganic phosphate by endo-(PPN) and exopolyphosphatases (PPX) (Rao *et al*., 2009). PPN is widely distributed in animal cells and is more abundant in yeast (Kumble and Kornberg, 1996). PPN cleaves long chains of poly P to generate shorter chains. PPX enzymes release orthophosphate (Pi) from the linear poly P chain containing three or more phosphoanhydride bonds. *E.
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 as long as 750 Pi residues in a processive manner and requires Mg$^{2+}$ and KCl for its maximum activity. E. coli PPX1 contains two functional domains, N-terminal catalytic domain and C-terminal domain that is responsible for poly P binding and processivity. 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 domains I and domain II.

PPX2/GPPA is a bifunctional enzyme possessing both exopolyphosphatase and guanosine pentaphosphate phosphorylase activities (Kristensen et al., 2004; Rao et al., 2009). This enzyme plays an important role in bacterial stringent response induced by starvation. E. coli PPX2/GPPA liberates Pi by processive hydrolysis of phosphoanhydride bonds of poly P chains as long as 750 Pi residues. PPX2/GPPA hydrolyses guanosine pentaphosphate (pppGpp) to ppGpp and the latter molecule functions as 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 that modulate stringent response. The crystal structure of Aquifex aeolicus GPPA/PPX2 revealed four domains (I, II, III and IV) and the active site is located at the interface between domains I and domain II (Kristensen et al., 2004 and 2008).
Corynebacterium glutamicum contains 2 genes encoding exopolyphosphateses, *ppx1* and *ppx2* (Lindner et al., 2009). PPX2 was shown to be the primary exopolyphosphatase in *C. glutamicum*. *C. glutamicum* PPX2 was shown to be active as monomer and requires 2 mM Mg$^{+2}$ and Mn$^{+2}$ for maximum activity. However, the enzyme was inhibited by higher concentrations of Mg$^{+2}$, Mn$^{+2}$ and Ca$^{+2}$. Moreover, PPX2 enzyme was shown to be active with short-chain polyphosphates, even accepting pyrophosphates, and was inhibited by nucleoside triphosphates. PPX has been shown to be important for avoidance of complement-mediated killing in *Neisseria meningitides* (Zhang et al., 2010).

**Poly P metabolizing enzymes in eukaryotes:** Poly P metabolizing enzymes are largely uncharacterized in eukaryotes. However, a PPK1 homologue (DdPPK1) has been identified in the social slime mold *Dictyostelium discoideum* and is believed to be acquired by horizontal transfer (Eichinger et al., 2005; Kornberg et al., 1999; Zhang et al., 2005; Zhang et al., 2007; Rao et al., 2009). Similar to PPK1 in *E. coli*, DdPPK1 contains the conserved residues for ATP binding and autophosphorylation. However, DdPPK1 contains a unique N-terminal extension of 370 amino acids with no homology with any known proteins. This N-terminal domain is necessary for enzymatic activity, cellular localization and physiological functions. DdPPK1 produces heterogeneous products upon degradation of poly P chain and usually these products are shorter than those seen in *E. coli*. Mutants of DdPPK1 showed defects in development, sporulation, and predation, and cytokinesis and cell division. Another enzyme capable of forming an actin-like filament concurrently with the synthesis of poly P has been found in *D.*
Dicoideum. PPK1 activity with 34% homology to E. coli PPK1 has also been reported in Candida humicola (McGrath et al., 2005). Accumulation of a large amount of poly P has also been reported in S. cerevisiae; however, no PPK homologs or poly P synthesizing activity has been detected (Kornberg et al., 1999; Ogawa et al., 2000).

1.7.5 Functions of polyphosphate

Poly P is vital for several cellular functions in bacteria 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 (Rao et al., 2009; Brown and Kornberg, 2004 and 2008; Seufferheld et al., 2008; Kornberg et al., 1999). In addition, Polyphosphate has been suggested to have role in biological and chemical evolution (Brown and Kornberg, 2004; Brown and Kornberg, 2008). Moreover, 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, as a food additive and antimicrobial and as a source of high phosphate to substitute costly ATP (Rao et al., 2009).

Poly P also mediates diverse functions in eukaryotes such as energy and phosphate source, cation sequestration and cell envelope formation, gene activity control (Kornberg et al., 1999; Kumble and Kornberg, 1996; Kumble and Kornberg, 1995; Rao
et al., 2009), development, sporulation and predation in *D. discoideum* (Zhang et al., 2005), 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 (Wang et al., 2003; Shiba 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).

1.7.6 Role of polyphosphate in bacterial survival and stress response

1.7.6.1 Basic metabolism

Poly P plays multiple roles in bacterial growth and survival including basic metabolism and maintenance of cellular structure (Kornberg et al., 1999). Poly P is known to serve as a source of ATP for over 500 known chemical reactions. Poly P is believed to mediate its functions by altering the structure of DNA, RNA and protein.

Poly P physically and functionally interacts with RNA polymerase *in vitro* and was also found to be tightly bound to histone-like proteins (HUs) *in vitro*, suggesting a potential role for poly P in gene regulation in bacteria (Kusano and Ishihama, 1997). Upon entry into stationary phase, RNA polymerase exhibits altered promoter selectivity and consequent change in gene selection and overall transcription. In the exponential phase, RNA polymerase contains $\sigma^{70}$, while RpoS is the primary $\sigma$-factor in stationary phase. Poly P was shown to be tightly coupled to stationary phase RNA polymerase (Kusano and Ishihama, 1997). It was also found that the effect of poly P on RNA
polymerase holoenzyme depends on the type of σ-factor (σ^70 or RpoS) in the holoenzyme. Salt concentration was found to affect the influence of poly P on RNA polymerase. At low salt concentrations, the transcription of both holoenzymes was inhibited by polyphosphate \textit{in vitro}. While at high salt concentrations, σ70 was not functional and RpoS remained active in the presence of poly P. These data suggest that poly P which is induced to high levels in stationary phase might participate in gene regulation in stationary phase. In addition, a recent study also showed that poly P binds to principal sigma factor of RNA polymerase during starvation response (Yang \textit{et al.}, 2010).

Poly P also interacts with ribosomal proteins and promotes translational fidelity \textit{in vitro} and \textit{in vivo}. McInerney \textit{et al.} (2006) also showed that poly P makes contact with ribosomes and low poly P levels resulted in low translation efficiency as well as fidelity. Poly P interacts with ribosomes and mediates ribosomal binding to Lon protease (Kuroda \textit{et al.}, 2001).

PPK is also a part of RNA degradosome which contains four main functional enzymes, RNase E, exoribonuclease PNPase, RNA helicase RhlB and enolase (Blum \textit{et al.}, 1997). The absence of PPK resulted in increased mRNA stability and \textit{in vitro} assays have showed that PPK binds with RNA which was prevented with ATP. Poly P was shown to inhibit the degradosome and the inhibition was prevented by ADP which is required for the degradation of poly P and regeneration of ATP. These data suggest that PPK serves a member of RNA degradosome by removing inhibitory polyphosphates and
NDPs and regenerating ATP. Thus, as a component of degradosome, PPK might play an essential role in posttranscriptional regulation of gene expression in bacteria.

1.7.6.2 Stress response and poly P levels

Several previous studies have shown that poly P plays an essential role in stress tolerance in bacteria (Adult-Riche et al., 1998; Rao and Kornberg, 1996). Upon entry into stress response, poly P levels increase initially and slowly decrease to basal level. Poly P levels increase in response to variety of stress conditions such as carbon, nitrogen, phosphate and amino acid limitations, pH change, osmotic stress, temperature change and oxidative stress (Adult-Riche et al., 1998). In E. coli, the fluctuations in poly P levels are dynamic and range from 100-1000 fold (Rao et al., 1998). The type of stress also decides the time frame for changes in poly P levels. For example, carbon and amino acid limitation results in 30-fold increase in poly P levels with in first 2 hours. Phosphate and amino acid limitation causes 500-fold increase in poly P levels within 3 hours.

1.7.6.3 Poly P and cell survival

Deletion of ppk resulted in significant decrease in cell survival in stationary phase (Crooke et al., 1994; Rao and Kornberg, 1996). However, a subpopulation of the bacteria with adaptive mutations survived longer stationary phase exposure. Upon exposure to environmental stresses, the mutated cells also showed less viability compared to the wild type strain (Rao and Kornberg, 1996). Thus, bacteria are exposed to multiple stress conditions in their environment and ppk plays an important role in bacterial survival.
under such conditions. However, the mechanisms behind how poly P contributes to cell survival are not known.

1.7.6.4 Sensing and responding to minor environmental changes

Bacteria are exposed to constant changes in their environmental parameters such as pH, osmolarity, nutrient availability and temperature. It is suggested that poly P containing membrane channels might play a role in responding to such minor environmental changes (Brown and Kornberg, 2008). The membrane channel containing polyhydroxybutyrate, calcium, and poly P complex is necessary for competence and acquisition of new genes and also exhibits a strong selection for cations (Pavlov et al., 2005). This complex can also form large, weakly selective pores with high conductance that promote genetic transformability or competence.

Motility (swimming, swarming and twitching) is also important for seeking nutrients, avoiding toxins, avoiding stressful environments, finding a suitable place for aggregation and biofilm formation as well as host colonization (Brown and Kornberg, 2004). Poly P plays a vital role in bacterial motility and a ppk1 mutant was defective in motility in many bacterial species (Rashid et al., 2000; Rashid and Kornberg, 2000; Shi et al., 2004; Zhang et al., 2005; Tan et al., 2005; Fraley et al., 2007). In addition, bacteria use quorum sensing to adapt to minor changes in their environment. Poly P has been shown to be essential for quorum sensing in *P. aeruginosa* as ppk1 mutant was defective in production of AI-1 and AI-2 autoinducers (Rashid et al., 2000; Fraley et al., 2007). *P. aeruginosa* ppk1 mutant was also defective in secretion of quorum sensing-dependent
extracellular virulence factors as well as biofilm formation. Quorum sensing sets the stage for bacterial aggregation and biofilm formation. Biofilm formation is an important mechanism that bacteria employ to survive under stressful environments and a ppk1 mutant was defective in biofilm formation in many bacteria species (Rashid et al., 2000; Shi et al., 2004; Fraley et al., 2007).

1.7.6.5 General stress response

General stress response is essential for bacterial survival in stationary phase as well as under a variety of stress conditions (Hengge-Aronis, 2002). RpoS (σS) subunit of RNA polymerase is the dominating σ-factor in stationary phase and it regulates general stress response in many Gram-negative bacteria including E. coli. RpoS-mediated general stress response is characterized by reprogramming of global gene expression pattern and over 500 genes are known to be affected in E. coli (over 10% of the E. coli genome) (Weber et al., 2005). Poly P was shown to support resistance and survival of stationary phase E. coli (Rao and Kornberg, 1996; Rao and Kornberg, 1999). A ppk1 mutant was significantly defective in growth in carbon-limiting medium, resistance to heat, oxidative, and osmotic stresses as well as long time survival. Poly P was shown to regulate the induction, maintenance and action of RpoS and RpoS-controlled genes such as katE, suggesting a vital role for poly P in regulating RpoS-mediated stationary phase/stress survival in bacteria (Shiba et al., 1997).

1.7.6.6 Stringent response
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., 2001).

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).

1.7.6.7 Poly P and Lon protease

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., 1999; 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 protein 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.

1.7.6.8 Oxidative, UV radiation and nutrient stress resistance

Poly P is associated with oxidative stress resistance in bacteria and SOD-deficient *E. coli* mutant showed transient accumulation of poly P which correlated with resistance to H$_2$O$_2$ (Al-Magrebi and Benov, 2001). The protection against oxidative stress-induced DNA damage by catalase and other DNA repair enzymes is controlled by RpoS. Poly P regulates RpoS and RpoS-regulated genes and thus might contribute to oxidative stress
resistance (Al-Magrebi and Benov, 2001). The induction of RecA protein which is essential for repair and maintenance of DNA is regulated by poly P (Tsutsumi et al., 2000). Poly P modulates DNA repair by regulating the activity and fidelity of DNA polymerases (Stumpf and Foster, 2005). Oxidative stress can result in protein unfolding and aggregation. Chaperones such as DnaK and GroEL play an important role in protein stabilization by promoting proper folding (Seufferheld et al., 2008). Poly P has been suggested to serve as a source of ATP required for the functioning of these chaperones and itself can act as a chemical chaperone promoting protein stabilization and proteolysis of denatured proteins (Seufferheld et al., 2008).

Bacteria are also exposed to UV radiation stress in the environment and biofilm formation is one way bacteria protect themselves from UV exposure. Poly P is important for biofilm formation and a ppk mutant was deficient in biofilm formation, quorum sensing, and resistance to UV-A exposure (Fraley et al., 2007; Rashid et al., 2000). UV exposure also induces mutations in bacteria and poly P has been shown to regulate DNA protective mechanisms as discussed elsewhere (Stumpf and Foster, 2005).

Bacteria respond to nutrient limitation by eliciting general stress response, stringent response, and Lon protease activation. Under phosphate limitation, bacteria express the two component system PhoP/PhoR which regulates the transcription of multiple genes involved in phosphate scavenging (Ghorbel et al., 2006a). Poly P stores have been shown to play a role in the ability of Vibrio cholera to overcome environmental stresses under phosphate limitation (Jahid et al., 2006). In addition, poly P
has been shown to serve as internal source of ATP under Pi starvation and oxidative stress in *Streptomyces lividans* (Ghorbel *et al.*, 2006a). This study also showed that both cell growth and energy charge were low under these circumstances. Thus, ATP generated from poly P can be used to activate metabolic pathways that enhance carbon and phosphate uptake, which is translated to antibiotic synthesis and an increase in oxidative stress (Ghorbel *et al.*, 2006b).

**1.7.6.9 Osmotic stress resistance**

Poly P also plays an important role in osmotic stress resistance in bacteria. It has been suggested that poly P might play a regulatory role in the bacterial response to high osmolarity. Poly P is directly involved in regulating gene expression (Kim *et al.*, 2007; Kusano and Ishihama, 1997; Rao and Kornberg, 1996; Rao *et al.*, 2009). In addition, poly P plays a role in the promoter selectivity control of RNA polymerase in *E. coli* grown in high concentrations of salts in stationary phase (Kusano and Ishihama, 1997). At high osmolality, poly P might assist RNA polymerase in differentiating between stationary phase and vegetative promoters. High osmotic stress is also associated with desiccation. Several studies have shown that bacteria when exposed to high salinity, UV radiation, and desiccation, synthesize extracellular polymers which enhance biofilm formation and prevent water loss by a mechanism that seals the cell (Ortega-Morales *et al.*, 2001; Raguenes *et al.*, 2004). The absence of poly P was shown to result in impaired production of extracellular polymers making cells sensitive to desiccation stress and UV damage and impaired biofilm formation in *P. aeruginosa*. 
1.7.6.10 Heavy metal stress

Poly P plays an important role in detoxification of metal cations in many bacterial species (Keasling and Hupf, 1996; Keyhani et al., 1996). The hydrolysis of poly P in heavy metal stress has also been documented in many bacteria (Keasling, 1997). This aspect has been exploited in phytoremediation of heavy metals such as mercury (Keasling, 1998). In arsenic-rich aquatic systems, *Herminiimonas arsenicoxydans* was shown to accumulate poly P in acidocalcisome-like granules in addition to expressing genes encoding inorganic phosphate transport and phosphate specific transport systems (Muller et al., 2007). Exposure to high concentrations of arsenic can result in generation of ROS and nitrogen reactive species which can damage DNA, RNA and proteins (Lantz and Hays, 2006). *H. arsenicoxydans* utilizes catalases, DNA repair proteins, and Pol IV, which are all regulated by poly P, to mediate resistance to ROS and nitrogen reactive species (Al-Maghrebi and Benov, 2001; Foster, 2007; Stumpf and Foster, 2005). It was also shown that extracellular polymers that constitute biofilms protect *H. arsenicoxydans* from high arsenic levels (Muller et al., 2007) and poly P might modulate the synthesis of such polysaccharides (Fraley et al., 2007).

1.7.6.11 SOS response

Bacteria are exposed to several physical and chemical agents such as UV radiation and oxidative stress that cause damage to DNA. Bacteria respond to DNA damage using DNA repair mechanisms to restore normal cellular functions and enable survival. SOS response is the best characterized DNA repair mechanism in bacteria and is
characterized by the induction of more than 40 genes (Courcelle et al., 2001). Genes encoding the specialized DNA polymerases Pol II, Pol IV, and Pol V are also among the genes induced in response to DNA damage (Goodman, 2002). Bacteria also respond to DNA damage by adaptive mutation. Adaptive mutation involves the accumulation of mutations that provide growth advantage to non-growing bacteria under non-lethal stress. Adaptive mutation results from error-prone DNA polymerases. Poly P has been shown to directly or indirectly regulate activity or fidelity of DNA polymerase IV during adaptive mutagenesis (Stumpf and Foster, 2005). Tsutsumi et al. (2000) showed that poly P regulates the expression of SOS genes such as recA and umuDC in E. coli. Lack of poly P resulted in reduced induction of SOS genes and high sensitivity to mitomycin C or UV which are indicators of DNA damage repair, suggesting an important role for poly P in SOS response in bacteria. A recent study also showed that poly P accumulated in E. coli cells with defective DNA metabolism, suggesting a role for poly P in general DNA stress (Amado and Kuzminov, 2009).

1.7.6.12 Sporulation

Sporulation Spores are the reproductive structures that are resistant to environmental stress. Deletion of ppk1 in Myxococcus xanthus resulted in delayed fruiting body formation, fewer spores and delayed germination (Zhang et al., 2005a). In lower eukaryotes, PPK1 has been shown to be important for spore formation, cytokinesis and normal development (Zhang et al., 2005b and 2007).

1.7.6.13 Adaptive evolution
Adaptive evolution denotes the natural selection of strains that have survival advantages. This kind of natural selection driven evolution occurs over several generations through accumulation of mutations in response to a nonlethal stress condition which gives survival advantages. Stress can cause error-prone DNA replication followed by genetic variation and survival of only certain strains. PPK1 has been previously shown to regulate error-prone DNA replication through regulating DNA polymerase activity or fidelity which leads to adaptive mutation in \textit{E. coli} (Stumpf and Foster, 2005).

1.7.6.14 Stress response in relation to virulence

The above described stress responses might also affect the ability of the bacteria to cause disease in the host. In addition to stresses imposed by the environment, bacteria are also exposed to multiple stresses \textit{in vivo} (during adherence, invasion and inside the host cell) as well as during interaction with other bacteria. The deletion of \textit{ppk1} has resulted in loss of virulence in several bacterial pathogens (Rashid \textit{et al.}, 2000; Ayraud \textit{et al.}, 2003; Tinsley and Gotschlich, 1995; Kim \textit{et al.}, 2002; Rao \textit{et al.}, 2009). Thus, PPK1-mediated stress response might also affect the virulence of pathogens, in addition to PPK1 being directly involved in virulence.

1.7.7 Poly P and bacterial virulence

\textbf{Virulence:} Bacterial virulence factors are generally expressed in stationary phase and the role of poly P and PPK1 for stationary phase survival of \textit{E. coli} has been previously reported (Kornberg \textit{et al.}, 1999; Rao and Kornberg, 1996). The \textit{Salmonella ppk1} mutant exhibited defect in long term survival (Kim \textit{et al.}, 2002). Mutants of \textit{ppk1} in other
bacteria have revealed defects in growth, motility, biofilm formation, quorum sensing, stress resistance, stationary phase fitness and resistance to antibiotics (Fraley et al., 2007; Rashid et al., 2000; Ogawa et al., 2000; Rashid and Kornberg, 2000; Shi et al., 2000; Schurig-Briccio et al., 2009). 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), prey-predator relationship in M. xanthus (Zhang et al., 2005a), invasion and intracellular survival within epithelial cells in Salmonella and Shigella (Kim et al., 2002), natural competence in E. coli (Castuma et al., 1995), lytic growth of P1 and fd phages (Li et al., 2007) and host colonization (Fraley et al., 2007). The AlgR2, a regulatory protein, has been shown to regulate alginate synthesis along with ppGpp and poly P accumulation (Kim et al., 1998). PPK2 was also suggested to play a role in alginate synthesis as PPK2 mediates poly P-driven GTP synthesis and GTP is a precursor for alginate synthesis (Rao et al., 2009; Ishige et al., 2002; Kim et al., 1998).

**Chromosome condensation:** 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. These defects in turn might have contributed to sensitivity to dessication stress and carbenicillin in the P. aeruginosa ppk1 mutant. Histone-like (HU) proteins, which are known to regulate gene expression and contribute to bending and alteration of DNA at specific sites, are believed to be involved in nucleoid
stability and integrity (Rao et al., 2009). HUs from *P. aeruginosa* and *E. coli* have been shown to strongly bind to poly P *in vitro* (Ishige and Kornberg, unpublished results quoted in Rao et al., 2009). HUs 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*. These findings suggest a role for poly P in regulation of gene expression possibly by displacing HUs from DNA.

**Poly P and regulation of gene expression**: 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 up-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).

1.7.8 PPK1 and PPK2 as therapeutic targets

PPKs serve as attractive targets for therapeutic intervention of microbial diseases for several reasons (Brown and Kornberg, 2008; Rao et al., 2009). First, PPKs are conserved across several bacterial species. Consequently, the successful chemical compound will be effective against multiple bacterial species. Second, both PPK1 and
PPK2 are essential for survival and virulence in many bacterial pathogens. Thus, inhibition of PPK activity might compromise bacterial survival (both inside and outside the host) and virulence. In addition, anti-PPK therapy might also alter the bacterial cell surface and increase the surface exposure of antigens that were otherwise hidden. This will make bacteria more vulnerable to host defenses such as complement, antibodies, low iron, lysozyme, and acidic pH in the vacuoles. Furthermore, since ppk1 mutant is sensitive to several antibiotics, targeting PPK1 might make the bacteria more sensitive to conventional antibiotics. Thus, anti-PPK therapy might be an important strategy for eradicating antibiotic-resistant bacteria. Finally, no homologs of PPKs have been identified in higher eukaryotes so far, making the toxicity of inhibitory drugs less likely.

1.8 Research Statement

*Campylobacter jejuni*, a Gram-negative microaerophile, is one of the major causes of bacterial foodborne diarrheal disease in the industrialized nations, affecting up to 1% of the population of North America, Australia, Europe, and New Zealand (Adak *et al.*, 2005; NIAID, 2005; Yohannes, 2004). The infection manifests as acute gastroenteritis characterized by severe watery to bloody diarrhea, fever, nausea and vomiting. *C. jejuni* infections are also correlated with post-infection complications such as Guillain-Barre syndrome which is thought to occur in 1 in 1000 individuals infected with *C. jejuni* (Jacobs *et al.*, 2008). Despite a pathogen in humans, *C. jejuni* lives as a commensal in the gut of wide variety of birds and animals. Nearly 90% of the poultry products in the United States are contaminated with *C. jejuni* and thus poultry and poultry
products are considered to be the primary source of sporadic *C. jejuni* infections (Jacobs-Reitsma, 2008). *C. jejuni* is microaerophilic and exhibits unique sensitivity to varying environmental stresses (Murphy *et al.*, 2005; Park, 2002). Despite its high prevalence and fastidious attributes, the genetic determinants involved in human disease as well as adaptation to and survival under stresses encountered both within and outside the host are poorly understood.

As a foodborne pathogen, *C. jejuni* is exposed to a variety of unfavorable environments during its infection cycle, both inside and outside the host. *C. jejuni* is unique among the enteric bacterial pathogens with respect to its unusual sensitivity to varying environmental stresses and fastidious growth requirements. For example, *C. jejuni* is unable to grow in the presence of oxygen, normally incapable of multiplication outside the host and possesses a restricted growth temperature range. Similarly, *C. jejuni* do not survive well on dry surfaces, cannot withstand high temperatures despite being a thermophile and more sensitive to osmotic and low pH stresses. These features reflect the absence of several classical stress response mechanisms that are important for mediating stress tolerance in other enteric bacteria such as *Salmonella* spp. and *Escherichia coli*. Despite lack of several classical stress response mechanisms, *C. jejuni* remains one of the most frequent bacterial causes of foodborne diarrhea in humans. This suggests that *C. jejuni* might have evolved alternative mechanisms to circumvent a range of stresses imposed by the host and environment.
Several studies have identified inorganic polyphosphate (poly P) as important not only for physiology but also for pathogenesis/virulence as well as stress tolerance in many bacterial pathogens (Rao et al., 2009; Seufferheld et al., 2008; Brown and Kornberg, 2008). Poly P is a linear chain of hundreds of orthophosphate residues joined together by high energy phosphoanhydride bonds. Polyphosphate kinase 1 (PPK1) is the principal enzyme that catalyzes the polymerization of inorganic phosphate into long chains of poly P. Polyphosphate kinase 2 (PPK2) mediates utilization of poly P to synthesize GTP, while exopolyphosphatases (PPXs) facilitate poly P degradation into inorganic phosphate. Poly P mediates multiple functions in bacteria including energy and phosphate source, motility, biofilm formation, quorum sensing, stress tolerance, inhibition of RNA degradation, activation of Lon protease, membrane channel formation, fidelity of DNA replication and protein translation, and virulence (Rao et al., 2009). Poly P has also been implicated in regulation of gene expression in E. coli and P. aeruginosa and thus can serve as a global regulator of virulence and stress-related functions in bacteria (Rao et al., 2009). Interestingly, PPK enzymes are absent in higher eukaryotes and are conserved among many bacterial pathogens, making them attractive targets for vaccine and drug development (Brown and Kornberg, 2008; Rao et al., 2009).

The genome sequence of Campylobacter revealed homologs of several genes encoding proteins involved of poly P metabolism (Parkhill et al., 2000). However, no studies have looked at the contribution of poly P associated enzymes to C. jejuni colonization/pathogenesis and stress survival as well as their role in the regulation of other C. jejuni genes. Understanding the role of poly P associated enzymes in C. jejuni
biology might lead to identification of novel control strategies to reduce *C. jejuni* prevalence in livestock species as well as the incidence of disease in humans.

The overall goal of this work was to define the role of poly P and its associated enzymes in *C. jejuni* patho-physiology.

**Specific objectives:**

The specific objectives of this work were:

- To determine the contribution of PPK1 to *C. jejuni* biology, including survival and adaptation mechanisms as well as host colonization.

- To define the role of PPK2 to *C. jejuni* physiology, stress tolerance, pathogenesis and host colonization.

- To understand the contribution of exopolyposphatases to *C. jejuni* pathophysiology.
CHAPTER II

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

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2.1 ABSTRACT

_Campylobacter jejuni_, a Gram-negative, microaerophilic bacterium, is a predominant cause of bacterial gastroenteritis in humans. Although considered fragile, fastidious and lacking many classical stress response mechanisms, _C. jejuni_ exhibits a remarkable capacity for survival and adaptation, successfully infecting humans and persisting in the environment. Consequently, understanding the physiological and genetic properties that allow _C. jejuni_ to survive and adapt to various stress conditions is crucial for therapeutic interventions. Of importance is polyphosphate kinase 1 (PPK1), which is a key enzyme mediating the synthesis of polyphosphate (poly P), an essential molecule for survival, mediating stress responses, host colonization and virulence in many bacteria. Therefore, we investigated the role of PPK1 in _C. jejuni_ pathogenesis, stress survival and adaptation. Our findings demonstrate that _C. jejuni_ Δppk1 mutant was deficient in poly P accumulation, which was associated with decreased ability to form viable but non-culturable cells (VBNC) under acid stress. The Δppk1 mutant also showed decreased frequency of natural transformation and increased susceptibility to various antimicrobials. Furthermore, the Δppk1 mutant was characterized by a dose-dependent deficiency in chicken colonization. Complementation of the Δppk1 mutant with the wildtype copy of ppk1 restored the deficient phenotypes to levels similar to those of the wildtype. Our results suggest that poly P plays an important role in stress survival and adaptation and might contribute to genome plasticity, and spread and development of antimicrobial resistance in _C. jejuni_. These findings highlight the potential of PPK1 as a novel target for therapeutic interventions.
2.2 INTRODUCTION

_Campylobacter jejuni_, a Gram-negative, microaerophilic bacterium, occurs as a commensal among the intestinal microflora of various animals, especially chicken and cattle (Beery _et al._, 1988; Young _et al._, 2007). However, _C. jejuni_ can infect human hosts, invading the intestinal mucosa and causing watery and/or bloody diarrhea (Butzler and Skirrow, 1979). _C. jejuni_ is transmitted to humans primarily through the consumption of contaminated chicken products, raw milk, or water (Allos, 2001; Altekruse _et al._, 1999). Currently, _C. jejuni_ is considered a leading bacterial cause of human foodborne gastroenteritis (Altekruse _et al._, 1999; Skirrow, 1994) and has also been associated with a plethora of symptoms, including acute neuromuscular paralysis (Guillain-Barre syndrome) (Hughes, 2004). Since an appropriate vaccine for human campylobacteriosis has yet to be introduced, it has been suggested that _C. jejuni_ infections might be alternatively controlled by reducing colonization in food-animals (Young _et al._, 2007). Consequently, determining the physiological and genetic properties that allow the survival of _C. jejuni_ and its colonization of animal hosts, pathogenicity, and adaptation to various stresses is of critical importance.

The mechanisms underlying _C. jejuni_ adaptation and survival under stresses imposed by environment and host are not well understood. High variability between different _C. jejuni_ strains and the unavailability of appropriate genetic tools and animal models have contributed to the lack of knowledge regarding its stress-tolerance and pathogenicity. However, it is suggested that the capacity of _C. jejuni_ to form viable but
non-culturab cells (VBNC) under stress (Chaveerach et al., 2003) and its readiness for natural transformation (Wang and Taylor, 1990) and acquiring resistance to antibiotics (Luangtongkum et al., 2009) are among strategies that promote stress adaptation and survival. Although little is known about the genetics underlying these processes, recent advances in C. jejuni genomics show that this bacterium carries several important genes that might play key roles in mediating stress adaptation and survival. Of particular interest are genes encoding polyphosphate kinases, \textit{ppk1} (CJJ81176_1361) and \textit{ppk2} (CJJ81176_0633) that were predicted to be involved in the metabolism of polyphosphate (poly P) (Fouts et al., 2005; Hofreuter et al., 2006; Parkhill et al., 2000), an intracellular granule that impacts several physiological properties in many bacterial species, including pathogenicity, host colonization, adaptation to different environments, and survival (Jahid et al., 2006; Kim et al., 2002; Ogawa et al., 2000).

Polyphosphate kinase 1 (PPK1) is encoded by \textit{ppk1}, which mediates the synthesis of all or the majority of poly P in the cell (Kornberg et al., 1999), while \textit{ppk2} encodes an enzyme (PPK2) that synthesizes GTP from poly P (Ishige et al., 2002). Both \textit{ppk} genes have been associated with the metabolism of poly P, which consists of phosphate residues that are linked by high energy phosphoanhydride bonds and is widely distributed in bacterial species (Shiba et al., 2000). Previous reports showed that poly P plays important roles in bacterial survival and stress-tolerance, including ATP production (Brown and Kornberg, 2008), entry of DNA through membrane channels (Castuma et al., 1995; Reusch and Sadoff, 1988), capsule composition (Tinsley et al., 1993), maintaining nutritional requirements during starvation (Kuroda et al., 1997), motility, biofilm
formation, and resistance to oxidative, osmotic, heat, acid and alkaline stresses, and stationary-phase survival (Jahid et al., 2006; Kim et al., 2002; Ogawa et al., 2000; Price-Carter et al., 2005; Rao and Kornberg, 1996; Rashid and Kornberg, 2000; Tan et al., 2005). Because of their importance in many bacterial species, it is not surprising to assume a role for PPK and poly P in survival, colonization, and stress-tolerance of *C. jejuni* (Brown and Kornberg, 2008).

Interestingly, PPK1 has been shown to be important for *C. jejuni* stress responses and pathogenicity (Candon et al., 2007). However, the role of *ppk1* in key metabolic- and physiological responses of *C. jejuni* still needs further analysis. For instance, it has been proposed that during starvation, poly P might act as a reservoir for phosphorus and energy (Bode et al., 1993). Subsequently, poly P would be crucial for maintaining viability/metabolism in stressed cells. This has been observed in *H. pylori*, where the occurrence of poly P correlated with culturability and structurally intact cells (Nilsson et al., 2002). Poly P containing non-culturable *H. pylori* showed capacity for ATP and mRNA synthesis after nutrient stimulus (Nilsson et al., 2002). Consequently, poly P might be an important factor for the formation of VBNC in stressed bacteria, including *C. jejuni*. Furthermore, natural transformation is perhaps one of the most important mechanisms in the adaptation of *C. jejuni*, and poly P has been reported to play a role in the entry of DNA through membrane channels (Castuma et al., 1995; Reusch and Sadoff, 1988). It follows that poly P might be important for natural transformation, adaptation, and acquisition of antibiotic resistance genes in *C. jejuni*. Poly P can further impact survival and adaptation in *C. jejuni* by modulating antibiotic resistance properties. For
example, poly P interacted with *E. coli* ribosomes (McInerney *et al.*, 2006), which are known targets for several antibiotics. These observations suggest that *ppk1* might be linked to important physiology and functions such as VBNC, natural transformation and antimicrobial resistance in *C. jejuni*. Therefore, in the present study, we determined the contribution of PPK1 to *C. jejuni* stress responses and adaptation, including the ability to form VBNC under acid stress, natural transformation, and antimicrobial resistance. Furthermore, we assessed the impact of *ppk1* deletion on *in vivo* chicken colonization. Our findings highlight the importance of PPK1 in *C. jejuni* survival, adaptation to different environmental stresses and *in vivo* colonization. These findings also indicate the suitability of PPK1 as a potential target for controlling the proliferation of this pathogen.

### 2.3 MATERIALS AND METHODS

**Bacterial strains and growth conditions**

All bacterial strains and plasmids used in this study are described in **Table 2.1**. *C. jejuni* 81-176 is a highly invasive strain originally isolated from diarrheic patients (Korlath *et al.*, 1985). *C. jejuni* strains were cultured on Mueller-Hinton (MH) medium under microaerophilic (85% N₂, 10% CO₂ and 5% O₂) conditions at 42 °C for 24 h. For isolation of *C. jejuni* from chicken feces and organs, MH agar plates were supplemented with *Campylobacter* selective supplement (SR117E, Oxoid, Lenexa, KS). *E. coli* strain DH5α was used for plasmid propagation and cloning purposes. *E. coli* strains were grown on Luria-Bertani (LB) medium at 37 °C overnight. Chloramphenicol (20 µg/ml for *E. coli*...
and 10 µg/ml for *Campylobacter*), kanamycin (30 µg/ml) and zeocin (50 µg/ml) were added to the media, where necessary.

**Construction of *C. jejuni ppk1* mutant and the complemented strain**

Genomic DNA was extracted from *C. jejuni* strain 81-176 using Masterpure® DNA purification kit (Epicentre, Madison, WI) according to the manufacturer’s instructions. Oligonucleotides were designed from the published genome sequence of *C. jejuni* strain 81-176 (GenBank accession no. NC_008787) using Vector NTI® software and commercially synthesized by Integrated DNA Technologies (Skokie, IL). All the oligonucleotides used in this study are listed in Table 2.2. Appropriate restriction sites were included in the primers to facilitate digestion and ligation into cloning vectors. Restriction enzymes were purchased from Promega (Madison, WI). To generate the deletion mutant, *ppk1* was amplified using PPK1 F and PPK1 R primers from *C. jejuni* genomic DNA along with approximately 1-kb of DNA sequences upstream and downstream of the target gene. The PCR products were purified using QIAquick® PCR purification kit (Qiagen, Valencia, CA). Purified PCR products were ligated into zeocin resistant pZErO-1 (Invitrogen, Carlsbad, CA), a zero background cloning vector, using Fast-Link DNA ligation kit (Epicentre) and transformed to Library Efficiency®DH5α™ *E. coli* competent cells (Invitrogen) to generate the plasmid pDG1. Plasmid (pDG1) DNA was isolated using QIAprep® spin mini prep kit (Qiagen) and the whole plasmid except the target gene was amplified by inverse PCR using PPK1 INV F and PPK1 INV R primers. Purified inverse PCR products were ligated to kanamycin resistant cassette from
pUC4K and the resulting suicide vector was named pDG2. pDG2 was electroporated into *C. jejuni* following the method described by Wilson *et al.* (2003). Briefly, *C. jejuni* strains were grown on MH agar plates at 42 °C for 24 h under microaerophilic conditions and the cells were harvested in 1 ml sterile, chilled ice-cold wash buffer (15% glycerol and 272 mM sucrose) and pelleted (13,000 rpm for 5 min at 4 °C). The cells were then washed three times using ice-cold wash buffer and resuspended in 500 µl of ice-cold wash buffer and 50 µl of cell suspension was used for electroporation. One microgram of pDG2 plasmid DNA was added into 50 µl of the cell suspension and mixed thoroughly by pipetting. The cell-DNA mixture was transferred into a pre-chilled 0.2 cm wide cuvette and electroporated at 2.5 Kv, 200 Ω, 25 µF using Gene Pulser Xcell (Bio-rad, Hercules, CA). One hundred and fifty microliters of SOC medium was added to stabilize the cells and the suspension was spotted onto non-selective MH agar plate and incubated overnight at 42 °C. Following incubation, the cells were harvested and plated onto MH agar with kanamycin (30 µg/ml) and incubated at 42 °C for 2-3 days under microaerophilic conditions. The kanamycin resistant colonies were patched, streak purified and confirmed for *ppk1* deletion by PCR using PPK1 F and PPK1 R primers. One of the PCR confirmed mutant clones (DG001) was selected for further studies.

For constructing the complemented strain Δ*ppk1c*, the *ppk1* gene along with the potential upstream promoter sequence was amplified from 81-176 chromosomal DNA using PPK1 COMP F and PPK1 COMP R primers. Appropriate restriction sites for *PstI* and *KpnI* were incorporated in the oligonucleotides to facilitate ligation into digested pRY111, an *E. coli-Campylobacter* shuttle vector (Yao *et al.*, 1993). The resulting
complementation plasmid (pDG3) was introduced into *C. jejuni* \(\Delta ppk1\) by conjugation as described previously (Miller *et al*., 2000). The resulting complemented strain was designated DG002 or \(\Delta ppk1c\).

**Transmission Electron Microscopy**

Transmission electron microscopy (TEM) procedures were used as described previously (Fraley *et al*., 2007; Ogawa *et al*., 2000). Briefly, mid log-phase grown bacterial cells were adjusted to an OD\(_{600}\) of 0.5 and fixed in 2% paraformaldehyde/2.5% gluteraldehyde, embedded in 1% agar, and post-fixed in 1% osmium tetroxide. The samples were then dehydrated in graded ethanol series, embedded in Spurr’s resin (Ted Pella Inc., Redding, CA) and 80-90 nm sections were cut with a diamond microtome knife. The specimens were then stained with 2% uranyl acetate and lead citrate and examined under a Hitachi-7500 transmission electron microscope (Pleasanton, CA) at 80 kV at the Molecular and Cellular Imaging Center (OARDC; http://www.oardc.ohio-state.edu/mcic).

**Induction and enumeration of viable but non-culturable cells in *C. jejuni***

Viable but non-culturable cells (VBNC) in *C. jejuni* were induced following the method of Chaveerach *et al*. (2003). Briefly, 1 ml of overnight grown culture containing \(5\times10^8\) bacterial cells/ml (OD\(_{600}\) of 0.5) was added to 4 ml of MH broth with pH adjusted to 4.0 using formic acid. The cultures were incubated under microaerophilic conditions at 42 °C for 3 h. The induction of VBNC was confirmed by determining the culturable cell
counts by spread plate method and viable cell counts by fluorescent microscopy using CTC-DAPI staining.

For determining the culturable cell counts, 100 µl of culture at 0, 0.5, 1, 2 and 3 h post-treatment with formic acid was serially diluted (10-fold) and plated onto MH agar in triplicate. The plates were incubated at 42 ºC under microaerophilic conditions and CFU/ml was calculated (culturable counts).

At aforementioned time intervals, 1 ml of culture was stained with CTC-DAPI for actively respiring and total C. jejuni cell counts as described by Cappelier et al. (1999). Briefly, 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC: Polysciences, Inc., Warrington, USA) was added to a final concentration of 5 mM into 1 ml of formic acid treated cells and incubated in dark for 1 h at room temperature. The cells were then counterstained with 5 µg/ml of DAPI (4', 6-diamidino-2-phenylindole) for 30 min. The cells were subsequently pelleted by centrifuging at 13,000 rpm for 4 min and the pellet was resuspended in 100 µl of PBS and fixed using formaldehyde. Twenty microliters of the cell suspension was placed on a clean, oil-free slide and an 18 mm cover slip was placed on the sample and sealed using nail polish. The slides were observed using Zeiss Axiophot upright fluorescent microscope equipped with AxioCam HRc and Axiovision 2.05 software (Thornwood, NY). The cells were subjected to fluorescent light with an excitation filter of 405 nm and a 455 nm dichroic mirror which facilitates simultaneous visualization of both DAPI (blue) and CTC (red). Viable cells convert CTC into insoluble formazan crystals which accumulate in the cell, appearing red under fluorescent
microscopy; while both viable and dead cells are stained by DAPI. A total of 10 fields were counted for each sample with an average of 90 bacteria in each field. The percentage of viable cells was calculated as follows: 

\[ \% \text{ viability} = \left( \frac{\text{viable cell count}}{\text{total cell count}} \right) \times 100. \]

Total cell counts were determined by adding viable (red) and dead (blue) cell counts. The results represent the mean of three independent experiments. We also recorded the OD\textsubscript{600} at the specified time points after CTC staining in order to observe the temporal change in intensity of tetrazolium reduction and further confirm the viability results generated from the fluorescent microscopy counts.

**Flow cytometry**

Flow cytometry analysis of the bacterial samples was performed after formic acid treatment to determine possible change in cell morphology and granularity (the shifts in forward scatter, FSC and side scatter, SSC) using FACScalibur (BD biosciences, San Jose, CA) as described previously (Tholozan et al., 1999). Briefly, 100 µl of the bacterial samples at 0, 0.5, 1, 2 and 3 h post-treatment were diluted to 1 ml using 1X PBS (pH 7.4) and the samples were analyzed using an excitation wavelength of 488 nm. All parameters were read as logarithmic values and 100,000 events were recorded for each sample. The assay was repeated two times.

**Natural transformation frequency**

Natural transformation was performed as described previously (Jeon et al., 2008). Briefly, overnight grown cultures of *C. jejuni* wildtype, Δppk1 and Δppk1c strains grown on MH agar plates were resuspended in fresh MH broth to OD\textsubscript{600} of 0.05. The bacterial
suspension (0.5 ml) was then incubated in sterile tubes at 42° C with shaking at 200 rpm under microaerophilic conditions for 3 h. After adding 1 μg of donor DNA from the dcuA::tetO mutant of C. jejuni 81-176 (tetracycline resistant), the bacterial suspension was incubated for another 4 h under the same conditions described above. Transformants and total bacterial counts were obtained by plating on MH agar with or without tetracycline (5 μg/ml), respectively. Transformation frequency was expressed as the number of transformants from 1 μg of DNA divided by total bacterial count. The experiment was repeated three times with triplicate transformation reactions each time.

**Antibiotic susceptibility testing**

The susceptibility of the wildtype, Δppk1 and Δppk1c strains to various antimicrobials was determined by standard microtiter broth dilution method as described previously (Lin et al., 2002; Rajashekara et al., 2009). The bacterial cultures were grown to mid log-phase and adjusted to an OD₆₀₀ of 0.05 in MH broth. One hundred microliters of culture was then added to serially diluted (2-fold) antimicrobials on a 96-well microtiter plate, mixed by pipetting and incubated at 42° C under microaerophilic conditions for 2 days. The MIC was determined by recording the lowest concentration of an antimicrobial showing complete inhibition of bacterial growth. The following antimicrobials and compounds were used in this study: ciprofloxacin, erythromycin, tetracycline, rifampicin, polymyxin B, cefotaxime, deoxycholic acid, taurocholic acid (Sigma Chemicals Co., St. Louis, MO), SDS (EM science, Gibbstown, NJ), cholic acid (ACROS organics, Belgium), and ethidium bromide (Amresco, Solon, OH).
susceptibility testing was repeated three times and the mean MIC (µg/ml) was calculated for each antimicrobial.

**Survival under nutrient downshift**

The role of PPK1 in *C. jejuni* survival under nutrient downshift was assessed as described previously (Candon *et al.*, 2007; Rajashekara *et al.*, 2009). Briefly, mid-log phase cultures of the wildtype, ∆ppk1 and ∆ppk1c strains were pelleted and washed twice with minimal essential medium (MEM). The pellets were resuspended in MEM and the OD$_{600}$ was adjusted to 0.05. The suspensions were then incubated under microaerophilic conditions at 42° C with shaking at 200 rpm. One hundred microliters of the culture at different time points were serially diluted (10-fold) in MEM and plated onto MH agar in triplicates. The plates were incubated under microaerophilic conditions and CFU/ml was calculated. The experiment was repeated three times.

**RT-PCR analysis**

For studying the differential gene expression in the wildtype and mutant strains with and without formic acid treatment, we targeted key genes involved in phosphate uptake (*phosR*, CJJ81176_0899; *pstS*, CJJ81176_0642; *pstC*, CJJ81176_0643 and periplasmic substrate binding protein, CJJ81176_0750) (Reid *et al.*, 2008; Wosten *et al.*, 2006), stringent response (*spoT*, CJJ81176_1288; Gaynor *et al.*, 2005), and multidrug resistance (*cmeC*, CJJ81176_0388; Lin *et al.*, 2002) as well as post transcriptional global regulator *csrA* (CJJ81176_1121; Fields and Thompson, 2008). The real-time quantitative PCR primers used in this study were designed using Beacon Designer 7.0 software (Palo
Alto, CA). The wildtype and Δppk1 strains were grown to mid-log phase at 42 °C in MH broth under microaerophilic conditions and 1 ml of 0.5 OD_{600} culture was added to 4 ml of MH broth with or without pH adjusted to 4.0 using formic acid and incubated under microaerophilic conditions at 42° C for 30 min. Formic acid treated as well as untreated wildtype and mutant strains were used for total RNA extraction using RNeasy Mini Kit (Qiagen) and cDNA was synthesized using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer’s protocol. Quantitative RT-PCR was performed using SensiMixPlus® SYBR RT-PCR Kit (Quantace, Norwood, MA) in a Mastercycler® ep realplex² thermal cycler (Eppendorf, Westbury, NY). The relative expression of different genes in the wildtype and mutant strains were normalized with rpoA (CJJ81176_1582) gene amplified from the corresponding sample. The difference in expression of the genes was determined by threshold cycle (CT) method and the assay was repeated three times with two replicates each time for each sample.

Since PPK2 is also involved in metabolism of polyphosphate (Ishige et al., 2002), we performed quantitative RT-PCR analysis of ppk2 expression in the wildtype and Δppk1 strains as described above.

**Chicken colonization studies**

Day-old broiler chicks were obtained from a local hatching facility at Food Animal Health Research Program (OARDC, Wooster, OH), and the chicks were confirmed negative for *Campylobacter* by culturing the cloacal swabs on MH agar with *Campylobacter* selective supplement (SR117E, Oxoid). The chicks were divided
randomly into 6 groups; 1, 2, 3, 4, 5, and 6 with 5 chicks in each group. Groups 1, 2 and 3 were inoculated orally with $10^3$, $10^4$ and $10^5$ CFU/chick of the wildtype bacterium, respectively; while groups 4, 5 and 6 received $10^3$, $10^4$ and $10^5$ CFU/chick of the Δppk1 mutant in 200 µl of 1X PBS (pH 7.4), respectively. The chicks were given *ad libitum* feed and water and cared-for following the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care. The chicks from each group were euthanized on day 8 post-inoculation. Cecal contents, feces and bursa were collected aseptically, weighed and homogenized in 1X PBS (pH 7.4). The suspensions were serially diluted (10-fold) and plated onto MH agar with *Campylobacter* selective supplement. The plates were incubated at 42°C under microaerophilic conditions and the CFU per gram of cecal contents, feces or bursa was calculated. The data represents the average from five chicks.

**Statistical analysis**

All data generated in this study were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey’s HSD (Honestly Significant Difference) test and were expressed as mean±SE (standard error). $P<0.01$ or 0.05 ($\alpha$ level) was considered statistically significant for all our assays.

**2.4 RESULTS**

Δppk1 mutant is defective in accumulation of poly P granules
The ability to accumulate poly P has been demonstrated in several bacteria, and mutants of polyphosphate kinase have been shown to have low levels of poly P (Ayraud et al., 2005; Candon et al., 2007; Ogawa et al., 2000; Tan et al., 2005). Transmission electron microscopy of C. jejuni wildtype and Δppk1 revealed the presence of large putative poly P granules in the wildtype bacterial sections, while such granules were either absent or present as relatively smaller and indistinct granules in Δppk1 (Figure 2.1). Large putative poly P granules were found in 30% or more of the examined wildtype sections and these granules were absent in majority of Δppk1 sections with only 3% or less of the sections showing comparatively small and indistinct granules. Our results corroborate the previous finding showing decreased levels of poly P in the ppk1 mutant strain compared to the wildtype using the toluidine blue O staining assay (Candon et al., 2007).

We also monitored the growth of the wildtype, Δppk1 and Δppk1c in MH broth by plating 100 µl of the culture at 0, 10, 20, 30, 40, 50, 60, and 70 h after inoculation to determine if these strains have any growth defects under standard incubation conditions. All strains exhibited similar growth patterns in MH broth (Figure 2.2).

**Role of polyphosphate in the formation of viable but non-culturable cells (VBNC) in C. jejuni**

Since VBNC formation has been suggested as a mechanism for C. jejuni survival under environmental stress, we investigated the contribution of polyphosphate to VBNC formation. VBNC were induced using formic acid as described previously (Cappelier et
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al., 1997; Chaveerach et al., 2003) and direct culturable, total and viable cell counts were
determined at different time points. At 0 h, the wildtype, Δppk1 and Δppk1c strains
showed a high number of culturable cells (5×10^8 CFU/ml). However, the culturability of
these strains decreased drastically (5×10^2 CFU/ml) at 30 min post-treatment with acid,
which was not significantly different among the three strains. Interestingly, cell
culturability was completely lost at 1 h in all three strains (Figure 2.3). Despite the loss
of culturability, CTC-DAPI staining showed the presence of viable cells (i.e. stained with
CTC) in the wildtype, Δppk1 and Δppk1c strains after formic acid treatment, suggesting
the formation of VBNC. The wildtype strain had a significantly (P<0.01) higher number
of VBNC than Δppk1 (Figure 2.4). The number of viable cells decreased gradually over
time in the wildtype strain, while the number of these cells drastically dropped in the
Δppk1 mutant at 1, 2 and 3 h post-treatment (Table 2.3). In the wildtype strain, at 1 hour,
96% of the cells were viable but none were culturable. In contrast, the mutant strain
showed only 36% viability while none were culturable. A similar trend was seen at 2 h
with 94% of cells maintaining viability in the wildtype compared to 4% in Δppk1. At 3 h
post-treatment, there was a significant decline in the viable cell counts in the wildtype
strain (42%) but still the viable counts were significantly higher than that of the Δppk1
mutant (3%). Complementation of the mutant with the wildtype copy of ppk1 restored
viability to levels similar to those of the wildtype. We also recorded the OD_{600} at
specified time points after CTC staining, and we found that the viability followed a
similar trend to that observed with the fluorescent microscopy (Figure 2.3). The
difference in the viable counts between the wildtype and Δppk1 was significant ($P<0.01$) at 1, 2 and 3 h post-treatment with acid.

We performed FACS analysis to determine if bacterial cells undergo changes in morphology and granularity after formic acid treatment. Our results showed a change in cell size (FSC) and granularity (SSC) in the mutant strain compared to the wildtype strain from 1 to 3 h post-treatment. Separation of the total (P1) cell population based on their size and granularity demonstrated that there was a significant ($P<0.01$) drop in P1 gated cells in Δppk1 from 1 to 3 h post-treatment with formic acid as compared to the wildtype strain (Figure 2.5). Complementation of the mutant with the wildtype copy of ppk1 restored the cell size and granularity to the wildtype levels.

The Δppk1 mutant exhibits a decrease in transformation frequency

To determine if ppk1 has a role in natural transformation, we assessed the transformation frequency in the wildtype, Δppk1 and Δppk1c. Since C. jejuni 81-176 strain used in the present study is pTet (confers tetracycline resistance) plasmid-cured, we used genomic DNA from dcuA::tetO mutant of 81-176 strain (tetracycline resistant) as the donor for determining the transformation frequency. The Δppk1 mutant showed a significant decrease (18-fold, $P<0.01$) in natural transformation frequency compared to the wildtype strain (Figure 2.6). Complementation of the mutant with the wildtype copy of ppk1 restored the transformation frequency to the wildtype levels.

The Δppk1 mutant is sensitive to various antimicrobials and bile acids
In order to determine the contribution of \( ppk1 \) to antimicrobial resistance, we tested the susceptibility of 81-176 wildtype, \( \Delta ppk1 \) and \( \Delta ppk1c \) to various antimicrobials and compounds. Our results showed that \( \Delta ppk1 \) was more susceptible to several antimicrobials (Table 2.4) compared to the wildtype strain. For example, the \( \Delta ppk1 \) mutant showed a significant increase in susceptibility to erythromycin (128-fold, \( P < 0.01 \)), cefotaxime (66-fold, \( P < 0.01 \)) and ciprofloxacin (16-fold, \( P < 0.05 \)). The \( \Delta ppk1 \) was also sensitive to rifampin (2-fold), polymyxin B (4-fold) and tetracycline (8-fold). Interestingly, the \( \Delta ppk1 \) mutant showed susceptibility to cholic acid (8-fold), taurocholic acid (8-fold) and deoxycholic acid (16-fold). In addition, the \( \Delta ppk1 \) mutant demonstrated a 31-fold and 4-fold increase in sensitivity to ethidium bromide and SDS, respectively. Complementation of the mutant with the wildtype copy of \( ppk1 \) either partially or completely restored the resistance to the wildtype levels.

**Transcription of \( ppk2, \) phosR, \( pstS, \) pstC, cmeC, csrA, spoT and CJJ81176_0750 genes was affected in the \( \Delta ppk1 \) mutant**

In order to understand the mechanism behind PPK1 mediated stress responses and adaptation, quantitative RT-PCR analysis was performed to examine the expression of phosphate regulon genes (phosR, pstS, pstC, and periplasmic substrate binding protein, CJJ81176_0750), polyphosphate kinase 2 (ppk2), multidrug resistance efflux pump gene (cmeC), global post-transcriptional regulator (csrA), and stringent response regulator (spoT). Interestingly, the aforementioned genes were found to be significantly (\( P < 0.01 \)) up-regulated (5- or more fold) in \( \Delta ppk1 \) mutant relative to the wildtype strain (Figure
2.7). Of particular interest, pstC, CJJ81176_0750, pstS, phosR and ppk2 were up-regulated 48-fold, 40-fold, 30-fold, 9-fold and 9-fold, respectively. Gene expression was also analyzed after formic acid treatment and during the induction of VBNC. Our results showed that, with the exception of ppk2, the expression of the aforementioned genes was not affected in acid-treated Δppk1 mutant. The ppk2 gene was significantly down-regulated (5-fold) \( (P<0.05) \) in Δppk1 mutant after acid treatment. However, the expression of ppk2 and the aforementioned genes was not affected in the wildtype before and after acid treatment.

**Poly P is required for dose-dependent chicken colonization**

We assessed the ability of Δppk1 to colonize cecum and bursa and its shedding in feces of 2 day-old chicks. The dose dependency of colonization was studied by inoculating the chicks with three doses of inoculum separately; \( 10^3 \), \( 10^4 \) and \( 10^5 \) CFU/chick (n=5). Our results demonstrated that the Δppk1 mutant was significantly defective \( (P<0.05) \) in chicken colonization at all inoculum doses for all organs and feces as compared to the wildtype (Figure 2.8). Chicks infected with \( 10^3 \) or \( 10^4 \) CFU/chick of Δppk1 mutant had no detectable bacteria in all the samples tested. However, chicks infected with \( 10^5 \) CFU/chick of Δppk1 mutant colonized with approximately 4 logs, 3 logs, and 2 logs fewer bacteria in the cecal contents, feces and bursa, respectively, compared to the wildtype strain.

**2.5 DISCUSSION**
To better understand the role of poly P in stress survival and environmental adaptation mechanisms in an important enteric pathogen, *C. jejuni*, we constructed a deletion mutant targeting polyphosphate kinase 1 (Δppk1), an enzyme that mediates poly P synthesis in the cell. Although *C. jejuni* Δppk1 mutant showed growth patterns similar to the wildtype strain, as expected, the mutant was deficient in the accumulation of poly P and exhibited defect in key survival and adaptation responses. Significantly, the Δppk1 mutant showed a reduced ability to form VBNC under stress, acquire DNA through natural transformation, and resist antimicrobials. The impairment of these physiological and stress responses along with other observed deficiencies confirmed that poly P plays an essential role in the survival and adaptation of *C. jejuni*.

The absence of *ppk* genes was associated with defects in growth, responses to stress and starvation, and viability in several bacterial species (Kim *et al.*, 2002; Nilsson *et al.*, 2002). This is also true in terms of *C. jejuni* survival and stress-tolerance, as our results showed that the Δppk1 mutant, after losing culturability (Figure 2.3), exhibited reduced viability (Table 2.3) and inability to form VBNC under acid stress (Figure 2.4). Furthermore, flow cytometry analysis showed a significant change in cell size and granularity of Δppk1 mutant during acid treatment (Figure 2.5), probably indicating an increase in dead cells (Kusters *et al.*, 1997) and corroborating the inability to form VBNC, which have been reported to occur in spiral (non-coccolidal) forms in *C. jejuni* (Federighi *et al.*, 1998). Polyphosphate particles have been found in starved and morphologically altered *V. parahaemolyticus* (Chen *et al.*, 2009), while Nilsson *et al.* (2002) showed that poly P accumulated in structurally intact cocccoid forms of starved *H.*
\textit{pylori}, probably for storing energy during starvation. Despite this, no direct association between poly P and PPK1 and the formation of VBNC has been reported previously. This is important as VBNC formation has been proposed as a strategy for surviving environmental stresses in many bacterial species, including enteric pathogens such as \textit{C. jejuni} (Rollins and Colwell, 1986; Roszak et al., 1984). While usually undetectable using standard culturing techniques, in many cases, VBNC were reported to maintain pathogenicity, further increasing the public health importance of these forms. Although VBNC forms have been reported for \textit{C. jejuni} strains that were exposed to starvation and other stresses (Chaveerach et al., 2003; Lazaro et al., 1999; Rollins and Colwell, 1986), the significance of these forms in the epidemiology of \textit{C. jejuni} has been a subject for debate (Medema \textit{et al.}, 1998; Saha \textit{et al.}, 1991; Stern \textit{et al.}, 1994), probably due to variations in the animal model, dose of VBNC, and strains used (Chaveerach \textit{et al.}, 2003). However, it appears that viable but non-culturable \textit{C. jejuni} were successfully resuscitated after passage in embryonated-eggs (Cappelier \textit{et al.}, 1999; Chaveerach \textit{et al.}, 2003) and the strains recovered their capacity to adhere to HeLa cells, an indication of regained pathogenicity (Cappelier \textit{et al.}, 1999).

PPK1 is responsible for the majority of poly P synthesis in bacterial cells (Brown and Kornberg, 2008), which was also inferred from our electron microscopy images that showed deficient accumulation of poly P granules in the \textit{C. jejuni} \textit{Δppk1} mutant (Figure 2.1). Analysis of \textit{ppk} mutants in other bacteria have also resulted in defective poly P granules (Ogawa \textit{et al.}, 2000; Tan \textit{et al.}, 2005). Since poly P acts as an energy store allowing cells to tolerate stress (Nilsson \textit{et al.}, 2002), we propose that poly P and PPK1
deficient bacteria lose their ability to enter a VBNC state, which impacts their capacity to reduce metabolism and potentially tolerate and adapt to environmental stresses and the possibility for recovery when conditions are favorable (McDougal et al., 1998). With the exception of *ppk2* that was down-regulated under formic acid treatment, our quantitative RT-PCR data did not show any significant changes in the expression of selected genes, involved in phosphate uptake and stress-tolerance, in the Δppk1 mutant (Figure 2.7). Since *ppk2* mediates a poly P-driven reverse reaction, synthesizing GTP from GDP in a greater magnitude (75-fold) than the forward reaction (*i.e.* poly P synthesis from GTP) (Rao et al., 2009), the down-regulation of *ppk2* can be interpreted as an additional effort by the cell to decrease energy (GTP/ATP) expenditure and maintain its already-decreased poly P reserves, which might be needed to form VBNC and resist stress. Unlike PPK1, PPK2 is stimulated and becomes stable in the presence of poly P (Rao et al., 2009). As a result, the absence or decrease in poly P accumulation (Figure 2.1) might have prompted the over-expression of *ppk2* in acid-untreated Δppk1 compared to the wildtype (Figure 2.7), possibly compensating for the deficiency in poly P-dependent PPK2 stability and partially maintaining its functions. Interestingly, the up-regulation of *ppk2* in acid-untreated Δppk1 mutant was accompanied by the over-expression of the genes related to phosphate regulation and uptake, possibly for maintaining intracellular phosphate levels (Rao et al., 2009). This does not contradict with the gene expression profiles that were observed in acid-treated Δppk1 mutant, since VBNC formation might require a certain decrease in some metabolic and cellular processes, which would conserve energy and phosphate utilization and consequently,
limit the need for the over-expression of the aforementioned genes. However, it should be emphasized that the precise mechanism(s) by which poly P influences the formation of VBNC requires further investigation.

An important mechanism for adaptation to environmental changes and antibiotic stress is the acquisition of important genetic material through natural transformation (Claverys et al., 2000; Jeon et al., 2008; Johnsborg et al., 2007). Natural transformation plays an important role in mediating genetic diversity and the acquisition of antibiotic resistance in *C. jejuni*, while also facilitating genetic manipulations in this naturally competent bacterium (de Boer et al., 2002; Jeon et al., 2008, Wang and Taylor, 1990). Interestingly, poly P is involved in the formation of cell membrane channels that allow DNA uptake (Castuma et al., 1995; Reusch and Sadoff, 1998). However, to our knowledge, the impact of poly P and its associated enzymes (PPK1) on natural transformation have not been investigated before. Our results show that the Δppk1 mutant was significantly deficient in the frequency of natural transformation (Figure 2.6) compared to the wildtype and complemented strains. While it is true that the Δppk1 mutant showed an increased susceptibility to tetracycline (Table 2.4), it should be emphasized that the transformation experiments with the tetracycline gene occur under non-selective conditions. Therefore, the acquisition of the tetracycline resistance gene by the mutant should have not been affected by its susceptibility to tetracycline. We suggest that PPK1 and poly P possibly impact genetic diversity, acquisition of antibiotic resistance genes and adaptation in *C. jejuni* by indirectly impacting the entry of DNA from the environment. This is important as *ppk* deficient bacteria, especially those with
relatively smaller genomes like *C. jejuni*, might be incapable of accessing collective gene pools (“supragenome”), which greatly restricts natural transformation and adaptation to environments (Baltrus *et al.*, 2007).

Poly P increases the activity of the stationary-phase RNA polymerase RpoS (Shiba *et al.*, 1997), possibly leading to adaptive mutations (Foster, 2007), including those that induce resistance to antibiotics (Shiba *et al.*, 1997). However, certain stress response factors such as RpoS are not found in *C. jejuni*. The ∆ppk1 mutant was significantly more susceptible to a variety of antibiotics and other antimicrobials as compared to the wildtype strain (*Table 2.4*). It follows that polyphosphate and its associated enzymes (PPK1) can affect antibiotic resistance in *C. jejuni* through various other pathways that are evidently RpoS-independent. For example, poly P accumulation regulates the synthesis of guanosine tetraphosphate (ppGpp) (Shiba *et al.*, 1997), an important molecule in stringent responses, which at high levels induces in turn the accumulation of poly P (Kuroda *et al.*, 1997). Gaynor *et al.* (2005) showed that the stringent response mediated the survival of *C. jejuni* under exposure to rifampin, probably through the activation of RelA and/or SpoT enzymes that synthesize ppGpp, which alters transcription and facilitate survival under stress. This was further confirmed by our observation that ∆ppk1 mutant indeed showed an increased susceptibility to rifampin (*Table 2.4*). However, *spoT* was up-regulated in ∆ppk1 mutant (*Figure 2.7*), probably in an attempt to induce ppGpp-dependent accumulation of poly P (through the inhibition of exopolyphosphatase) (Kuroda *et al.*, 1997) in the deficient mutant, which also highlights the importance of previously suggested interactions of poly P with
stringent response factors (Lamarche et al., 2008). The resistance of *C. jejuni* to macrolides (erythromycin) and fluoroquinolones (ciprofloxacin) is mediated through changes in the ribosome and mutations in DNA gyrase (encoded by *gyrA*), respectively (Engberg et al., 2001). Interestingly, an *E. coli* ppk mutant showed a 40% decrease in induced mutagenesis to nalidixic acid resistance (Stumpf and Foster, 2005), which is a result of base mutations in DNA gyrase genes (Yoshida et al., 1988), while poly P has been reported to interact with ribosomes, supporting translation fidelity in *E. coli* (McInerney et al., 2006). Furthermore, *ppk* deletion mutations in *S. typhimurium* and *S. dublin* showed an increased susceptibility to polymyxin B (Kim et al., 2002), corroborating our observations that the *C. jejuni Δppk1* was susceptible to polymyxin (Table 2.4) and emphasizing the role of poly P in mediating antibiotic resistance. Surprisingly, *cmeC*, a component of a multidrug efflux pump that mediates resistance to antibiotics and other important compounds such as bile, which induces virulence gene expression (Lin et al., 2002), was up-regulated in Δppk1 mutant under no stress (Figure 2.7), possibly in response to polyphosphate deficiency. However, the over-expression of *cmeC* did not appear to facilitate the tolerance of Δppk1 when challenged with antibiotics or bile. Although our results indicate a role for PPK1 and poly P in mediating the resistance of *C. jejuni* to different antibacterial compounds, yet the precise mechanism of the observed susceptibility and/or resistance remains largely unknown and requires further investigation. However, this might emphasize the importance of polyphosphate and its associated enzymes as potential targets for antibiotics and/or other compounds.
Differential expression analysis of *C. jejuni* genes involved primarily in phosphate uptake, transport, and metabolism showed that *ppk2* (regulates the reversible synthesis of poly P from GTP or ATP), *phosS/phosR* (a two-component system that activates the transcription of the phosphate regulon), *pstSC* (involved in phosphate transport) and a periplasmic solute-binding protein (CJJ81176_0750) that is part of an operon encoding a putative ABC transporter for phosphate uptake (Fields and Thompson, 2008; Reid *et al*., 2008; Wosten *et al*., 2006) were expectedly up-regulated under normal conditions in ∆*ppk1* compared to the wildtype (Figure 2.7). It has been previously suggested that the Pho regulon is associated with stress responses (Lamarche *et al*., 2008), as Pho regulon was suppressed in *E. coli* mutant strains (*e.g.* relA and spoT mutants) that were deficient in ppGpp accumulation (Spira *et al*., 1995). Consequently, direct and/or indirect interactions have been proposed to occur between pathways involved in the phosphate regulation, poly P synthesis, and the stringent response (Lamarche *et al*., 2008). In *C. jejuni*, the two component system *phosS/phosR* triggers the transcription of phosphate regulon, including CJJ81176_0750, and *pstS* and *pstC*, both of which are also transcribed during phosphate limitation (Wosten *et al*., 2006). Consequently, the up-regulation of *ppk2* and other genes that are involved in phosphate uptake and transport in ∆*ppk1* appears to constitute a compensatory response by the bacterium in an attempt to accumulate poly P. These observations further highlight the role of poly P and its associated metabolic enzymes in adaptation and survival responses of *C. jejuni*.
Consistent with previous findings, *C. jejuni* motility and resistance to oxidative stress were not impacted in the \(\Delta ppk1\) mutant (data not shown, Candon *et al.*, 2007), which surprisingly showed enhanced biofilm formation (Figure 2.9). Interestingly, *csrA* (putative global post-transcriptional regulator) that contributes to biofilm formation and oxidative resistance in *C. jejuni* (Fields and Thompson, 2008) was up-regulated in \(\Delta ppk1\) (Figure 2.7), possibly explaining the tolerance of this mutant to oxidative stress and its enhanced capacity for biofilm formation. Furthermore, our results indicated that the \(\Delta ppk1\) mutant was susceptible to osmotic stress and nutrient downshift (Figures 2.10 and 2.11) and exhibited a dose-dependent chicken colonization deficiency (Figure 2.8), while the thermal stress response and resistance to heavy metals were not impacted (data not shown). With the exception of thermal stress and resistance to heavy metals, the tolerance to osmotic and nutrient stresses was previously investigated and our results were consistent with the earlier report (Candon *et al.*, 2007). Similar to the *ppk1* mutant, a *C. jejuni spoT* mutant was deficient in poly P accumulation during growth in a rich medium despite the presence of an intact *ppk1* gene (Candon *et al.*, 2007). In our \(\Delta ppk1\) mutant, poly P did not accumulate despite the over-expression of *spoT* and the phosphate regulon genes, further confirming that a functional PPK1 is required for poly P accumulation. Additionally, this suggested that *spoT* might affect the activity of *ppk1*, which could also be inferred from *E. coli spoT* mutants that failed to accumulate ppGpp and pppGpp as well as poly P under nutrient deprivation (Kuroda *et al.*, 1997).

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

2.6 ACKNOWLEDGEMENTS

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<tr>
<td>pZErO-1</td>
<td>Cloning vector for making suicide vector; Zeo</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRY111</td>
<td><em>E. coli-Campylobacter</em> shuttle vector for complementation</td>
<td>71</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for conjugation</td>
<td>1</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source for kanamycin</td>
<td>Amersham</td>
</tr>
<tr>
<td>pDG1</td>
<td>pZErO-1 containing <em>ppk1</em> region plus 1Kb upstream and downstream of 81-176; Zeo</td>
<td>This study</td>
</tr>
<tr>
<td>pDG2</td>
<td>A portion of <em>ppk1</em> replaced with kanamycin resistance region from pUC4K in pDG1; Zeo, Kan</td>
<td>This study</td>
</tr>
<tr>
<td>pDG3</td>
<td>pRY111 containing <em>ppk1</em> coding region and the upstream promoter sequence for complementation; Cm</td>
<td>This study</td>
</tr>
</tbody>
</table>

Kan, kanamycin; Cm, Chloramphenicol; Zeo, zeocin; *tetO*, tetracycline

**Table 2.1.** Bacterial strains and plasmids used in this study
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPK1 F</td>
<td>ATAAAAGgtaccATTTAGCCATAAAAACCTCCCG</td>
<td>To amplify 81-176 ppk1 region</td>
</tr>
<tr>
<td>PPK1 R</td>
<td>AAAAAAActgcagGTGATAGTGTGAAGCCACCTTTA</td>
<td></td>
</tr>
<tr>
<td>PPK1 INV F</td>
<td>ATAAAAGgtacATGCAGTCAAAATGTCGTCGCA</td>
<td>To amplify pDG1 except ppk1</td>
</tr>
<tr>
<td>PPK1 INV R</td>
<td>AAAAAAActgcagGAACATTTGGCTCTAAAAACCG</td>
<td></td>
</tr>
<tr>
<td>PPK1 COMP F</td>
<td>AAATAActgcagGTTCCTAAAACACTTTTGCGCT</td>
<td>To amplify ppk1 gene for complementation</td>
</tr>
<tr>
<td>PPK1 COMP R</td>
<td>AAATAAGgtaccCTCGTTCCACCCAGTTTTA</td>
<td></td>
</tr>
<tr>
<td>CsrA F</td>
<td>TTATCGGAGAAGGTATAG</td>
<td></td>
</tr>
<tr>
<td>CsrA R</td>
<td>TTITCAAGTAGATAAAGGG</td>
<td></td>
</tr>
<tr>
<td>SpoT F</td>
<td>GAAAAAActgcagGTGATAGTGTGAAGCCACCTTTA</td>
<td></td>
</tr>
<tr>
<td>SpoT R</td>
<td>GATGTCGAGTTATTTATCCTCC</td>
<td></td>
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<tr>
<td>PhosR F</td>
<td>GCAAACATAATCTACACAACCAC</td>
<td></td>
</tr>
<tr>
<td>PhosR R</td>
<td>GAGAGCAGGAATACAAAGAAC</td>
<td></td>
</tr>
<tr>
<td>CmeC F</td>
<td>GCTGCTGCTCAATTAGGTATAG</td>
<td></td>
</tr>
<tr>
<td>CmeC R</td>
<td>GCTTCATAACTCATACTCATTTTC</td>
<td>Primers for real-time quantitative PCR</td>
</tr>
<tr>
<td>PstS F</td>
<td>CTTTATAAAATCTGGAATCACAATC</td>
<td>amplification of cDNA from ppk2, csrA, spoT,</td>
</tr>
<tr>
<td>PstS R</td>
<td>GACACATCACTCATAAAGC</td>
<td>phosR, cmeC, pstS, pstC and CJJ81176_0750 from</td>
</tr>
<tr>
<td>PstC F</td>
<td>GCTTCATACCTTACCTTAC</td>
<td>81-176 strain</td>
</tr>
<tr>
<td>PstC R</td>
<td>GCTGCCATACCACTTAC</td>
<td></td>
</tr>
<tr>
<td>CJJ81176_0750 F</td>
<td>GGTCTTCTGCTGTTCTTGG</td>
<td></td>
</tr>
<tr>
<td>CJJ81176_0750 R</td>
<td>GTATCGCTATGCTTCTATGC</td>
<td></td>
</tr>
<tr>
<td>PPK2 F</td>
<td>ATCTAATCTCATAACTTGG</td>
<td></td>
</tr>
<tr>
<td>PPK2 R</td>
<td>TTCTTCTTCTCCTAGC</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.2.** Primers used in this study
Strains | Time (hours) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT</td>
<td>100</td>
</tr>
<tr>
<td>Δppk1</td>
<td>100</td>
</tr>
<tr>
<td>Δppk1c</td>
<td>100</td>
</tr>
</tbody>
</table>

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

**Table 2.3.** Percentage of viable cells as determined by CTC-DAPI staining\(^a\)
<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>WT</th>
<th>Δppk1</th>
<th>Δppk1c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>(Fold difference&lt;sup&gt;a&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.26</td>
<td>0.016 (16)</td>
<td>0.13</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.5</td>
<td>0.003 (128)</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>2.0</td>
<td>0.03 (66)</td>
<td>1.0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>103</td>
<td>51.5 (2)</td>
<td>51.5</td>
</tr>
<tr>
<td>Polymyxin</td>
<td>4.0</td>
<td>1.0 (4)</td>
<td>1.0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2.06</td>
<td>0.26 (8)</td>
<td>2.06</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>1.25</td>
<td>0.04 (31)</td>
<td>1.25</td>
</tr>
<tr>
<td>SDS</td>
<td>375</td>
<td>93.75 (4)</td>
<td>375</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>5,156</td>
<td>645 (8)</td>
<td>5,156</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>36,000</td>
<td>4,500 (8)</td>
<td>36,000</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>16,500</td>
<td>1,031 (16)</td>
<td>8,250</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fold difference in susceptibility between WT and Δppk1

Table 2.4. Susceptibility of *C. jejuni* to antimicrobials, bile acids and other compounds
Figure 2.1. Transmission electron micrographs of the wildtype *C. jejuni* and Δppk1 strains. The TEM sections show large distinct poly P granules (Arrow) in wildtype but not in Δppk1 mutant as observed with other bacteria (46, 65) (Representative images are shown). Scale bar is 200 nm in length.
Figure 2.2. Growth curves for the wildtype, ΔppkI and ΔppkIc strains. The growth curves were assessed by growing the bacteria in MH broth and direct plating on MH agar in triplicate or by measuring the OD$_{600}$ at different time points. Data represents the mean±SE of 2 independent experiments.
Figure 2.3. Effect of ppk1 deletion on the formation of viable but non-culturable cells in *C. jejuni*. Culturability of the wildtype, ∆ppk1 and ∆ppk1c strains after formic acid treatment as determined by CFU enumeration and OD_{600} measurements after CTC staining. The data represents the mean±SE of three independent experiments.
Figure 2.4. Effect of *ppk1* deletion on the formation of viable but non-culturible cells in *C. jejuni*. Fluorescent microscopy images stained with CTC-DAPI of *C. jejuni* cells showing viable (CTC stained red cells, thick arrow) and dead cells (DAPI stained blue cells, thin arrow) at different time points after formic acid treatment. Microscopy images a, b, c, d and e (wildtype strain); f, g, h, i and j (∆*ppk1* strain) and k, l, m, n and o (∆*ppk1c* strain) are the representative images of three independent experiments treated with formic acid for 0, 0.5, 1, 2 and 3 h, respectively. Note the drop in viable cells (Red cells) in ∆*ppk1* at 1, 2 and 3 h post-treatment compared to the wildtype strain. The scale bar is 10 µm in length.
Figure 2.5. Two-parameter forward/side scatter (FSC/SSC) flow cytometry analysis of *C. jejuni* strains with and without formic acid treatment. Forward scatter represents cell size, while reverse scatter is for cell granularity. A gate (P1) was set on the FSC vs SSC to select the cell population for determining the change in cell size/granularity. Percent change in P1 gated cells in the wildtype, Δppk1 and Δppk1c strains at different time points after formic acid treatment is shown. The bars represent the mean±SE of two independent experiments. * P<0.01 and ** P<0.05.
Figure 2.6. Effect of \textit{ppk1} deletion on \textit{C. jejuni} natural transformation. The donor DNA contained a tetracycline resistance (\textit{tetO}) marker. The data represents the mean transformation frequency±SE of three independent experiments with triplicate transformation reactions in each experiment. * $P<0.01$ and ** $P<0.05$. 
Figure 2.7. Histogram showing the fold change in expression of target genes before and after formic acid treatment. Relative fold change \( (2^{-\Delta\Delta CT}) \) in gene expression was calculated from \( \Delta\Delta CT \) value after normalization. The \( \Delta ppk1 \)-wildtype untreated indicates the fold change in gene expression in \( \Delta ppk1 \) mutant relative to the wildtype strain. Wildtype treated indicates the fold change in gene expression in the wildtype strain treated with formic acid relative to the wildtype untreated. \( \Delta ppk1 \) treated indicates the relative fold change in gene expression in \( \Delta ppk1 \) mutant treated with formic acid relative to untreated \( \Delta ppk1 \) mutant. The genes with 2 or more-fold \( (P<0.01 \text{ or } 0.05) \) relative change in expression were considered up-regulated or down-regulated. Each bar represents mean±SE of relative fold change in expression from three independent experiments with triplicate reactions for each sample. * \( P<0.01 \) and ** \( P<0.05 \).
Figure 2.8. Effect of *ppk1* deletion on chicken colonization. The chicks were randomly assigned into six groups of five chicks each. First three groups were inoculated with $10^3$, $10^4$ and $10^5$ CFU/chick of the wildtype strain, while the other three groups received $10^3$, $10^4$ and $10^5$ CFU/chick of *Δppk1* strain. Eight days post-inoculation, the chicks were euthanized and CFU/gram of cecal contents, feces and bursa was calculated. Each bar represents mean±SE of five chicks. * $P<0.01$ and ** $P<0.05$. 
Figure 2.9. Relative amount of biofilm formation in the wildtype, Δppk1 and Δppk1c strains. The static biofilm formation in borosilicate tubes was assessed by inoculating 100 µl of 0.05 OD$_{600}$ culture into 1 ml of MH broth and incubating at 42$^{\circ}$ C under microaerophilic conditions for 2 days without shaking. The biofilms were visualized by staining with 250 µl of 1% crystal violet for 15 min. The biofilms were quantified by measuring the absorbance at 570 nm after dissolving in 1 ml of DMSO for 24-48 h. Each bar represents the mean±SE of two independent experiments. * P<0.01 and ** P<0.05.
Figure 2.10. Survival of the wildtype, Δppk1 and Δppk1c in MH broth with 0.25 M NaCl. Bacterial strains were grown into mid log-phase and adjusted to an OD$_{600}$ of 0.05 in MH broth with and without 0.25 M NaCl and incubated under microaerophilic conditions at 42° C for 70 h with shaking at 200 rpm. At different time points samples were serially diluted (10-fold) and plated onto MH agar in triplicates. The plates were incubated under microaerophilic conditions and the number of CFU/ml was calculated. The assay was repeated three times and the mean CFU/ml was reported.
Figure 2.11. *C. jejuni* survival under nutritional downshift in MEM. Each data point represents the mean±SE of three independent experiments. * P<0.01 and ** P<0.05.
CHAPTER III

Polyphosphate Kinase 2: A Novel Determinant of Stress Responses and Pathogenesis in *Campylobacter jejuni*

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\(^2\)Center for Microbial Interface Biology, Division of Infectious Diseases, Department of Internal Medicine, The Ohio State University, Columbus, OH 43210.

3.1 ABSTRACT

Inorganic polyphosphate (poly P) plays an important role in stress tolerance and virulence in many bacteria. PPK1 is the principal enzyme involved in poly P synthesis, while PPK2 uses poly P to generate GTP, a signaling molecule that serves as an alternative energy source and a precursor for various physiological processes. Campylobacter jejuni, an important cause of foodborne gastroenteritis in humans, possesses homologs of both ppk1 and ppk2. ppk1 has been previously shown to impact the pathobiology of C. jejuni. Here, we demonstrate for the first time that the deletion of ppk2 in C. jejuni resulted in a significant decrease in poly P-dependent GTP synthesis, while displaying an increased intracellular ATP:GTP ratio. The Δppk2 mutant exhibited a significant survival defect under osmotic, nutrient, aerobic, and antimicrobial stresses and displayed an enhanced ability to form static biofilms. However, the Δppk2 mutant was not defective in poly P and ppGpp synthesis suggesting that PPK2-mediated stress tolerance is not ppGpp-mediated. Importantly, the Δppk2 mutant was significantly attenuated in invasion and intracellular survival within human intestinal epithelial cells as well as chicken colonization. Taken together, we have highlighted the role of PPK2 as a novel pathogenicity determinant that is critical for C. jejuni survival, adaptation, and persistence in the host environments. PPK2 is absent in humans and animals; therefore, can serve as a novel target for therapeutic intervention of C. jejuni infections.
3.2 INTRODUCTION

_Campylobacter jejuni_, a Gram-negative microaerophilic bacterium, frequently causes gastroenteritis in humans and accounts for up to 15% of all diarrheal cases worldwide (Allos, 2001; Altekruse et al., 1999). Human infections with _C. jejuni_ are characterized by a rapid onset of fever, diarrhea, abdominal pain and vomiting. Although self-limiting in the majority of the population, _C. jejuni_ infections are also associated with Guillain-Barré Syndrome (Hughes, 2004), Reiter’s syndrome (Ponka et al., 1981), inflammatory bowel syndrome (Kalischuk and Buret, 2010) and immunoproliferative small intestinal disease (Lecuit et al., 2004). _C. jejuni_ is a zoonotic pathogen that exists as a commensal in the gastrointestinal tract of chickens and mammals (Beery et al., 1988; Young et al., 2007). Human infections are primarily acquired through consumption of contaminated chicken and other livestock meat, contaminated water, and unpasteurized milk (Allos, 2001). Despite its public health significance, relatively little is known about the molecular mechanisms contributing to _C. jejuni_ stress tolerance, host colonization, and pathogenesis.

Inorganic polyphosphate (poly P), a phosphate polymer, plays an important role in bacterial survival, stress tolerance and virulence in many bacterial species (Rao et al., 2009). This is not surprising since poly P is involved in several housekeeping functions such as reservoir for phosphate and energy, chelator of metals, component of membrane channel for DNA entry, component of bacterial capsule, and buffer against alkali (Kornberg, 1995). Additionally, poly P is essential in several pathogenic bacteria for
stress and virulence-related functions (Rao and Kornberg, 1996; Rao et al., 2009; Seufferheld et al., 2008). Several specialized enzymes are involved in poly P metabolism. Polyphosphate kinase 1 (PPK1) is responsible for reversible synthesis of the majority of poly P in the cell (Kornberg et al., 1999; Tan et al., 2005). The ppk1 deletion mutants in many bacterial pathogens show diverse phenotypes including defects in stress responses, motility and virulence (Candon et al., 2007; Gangaiah et al., 2009; Jahid et al., 2006; Kim et al., 2002; Ogawa et al., 2000; Price-Carter et al., 2005; Rao et al., 2009; Rashid and Kornberg, 2000; Tan et al., 2005). Many bacterial species contain another enzyme, PPK2, which preferentially mediates poly P-driven generation of GTP (Ishige et al., 2002; Sureka et al., 2009), a molecule known to have important roles in cell signaling as well as DNA, RNA, protein, and polysaccharide synthesis (Chakrabarty, 2002; Sundin et al., 1996). In addition, PPK2 is an important virulence factor as it regulates intracellular survival in Mycobacterium (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). However, with the exception of a recent report on PPK2 from Mycobacteria (Sureka et al., 2009), previous studies have focused on only characterizing the structure and enzymatic activity of PPK2 (Ishige et al., 2002; Lindner et al., 2007; Nocek et al., 2008) and little effort has been made towards understanding the role of PPK2 in stress responses and pathogenesis.

The genome of C. jejuni possesses homologs of both ppk1 and ppk2 genes. Previously, we and others have demonstrated the role of PPK1 in C. jejuni stress survival, adaptation and in vivo colonization (Candon et al., 2007; Gangaiah et al., 2009).
However, to our knowledge, there have been no studies addressing the role of PPK2 in *C. jejuni* physiology, stress tolerance and *in vivo* pathogenesis. Here, we examined the role of PPK2 in *C. jejuni* patho-physiology by generating a *ppk2* mutant in a highly virulent strain of *C. jejuni*. We report that the Δ*ppk2* mutant displayed deficiency in several survival- and virulence-related phenotypes, which emphasizes a role for PPK2 in mediating stress tolerance and pathogenesis in *C. jejuni*.

### 3.3 MATERIALS AND METHODS

**Bacterial strains, media and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 3.2. *C. jejuni* strain 81-176 (WT), a highly invasive strain originally isolated from an outbreak associated with raw milk (Korlath *et al.*, 1985), was used to generate the *ppk2* deletion mutant. *C. jejuni* strains were routinely grown on Mueller-Hinton broth (MH; Oxoid) microaerobically [(85% N₂ (v/v), 10% CO₂ (v/v) and 5% O₂ (v/v))] in a DG250 Microaerophilic Workstation (Microbiology International) at 42 °C. MH agar plates were supplemented with *Campylobacter* selective supplement (SR117E, Oxoid) when isolating *C. jejuni* from chicken feces and organs. For growth curve and stress survival assays, *C. jejuni* was grown microaerobically in MH broth with appropriate antibiotics at 37 °C with shaking at 200 rpm. *E. coli* DH5α was used for plasmid propagation and cloning purposes and was routinely cultured on Luria-Bertani (LB) medium at 37 °C overnight. Growth media was supplemented with appropriate antibiotics, chloramphenicol (20
144 µg/ml for *E. coli*; 10 µg/ml for *Campylobacter*, kanamycin (30 µg/ml) and zeocin (50 µg/ml), where necessary.

**General cloning techniques**

Cloning and other molecular biology techniques were performed according to Sambrook and Russel (2001). Oligonucleotides were designed using Vector NTI® software (Invitrogen) and commercially synthesized by Integrated DNA Technologies. All the oligonucleotides used in the present study are listed in Table 3.3. Masterpure® DNA purification kit and Fast-Link DNA ligation kit were purchased from Epicentre. Restriction enzymes were purchased from Promega. QIAquick® PCR purification kit and QIAprep® spin mini prep kit for plasmid isolation were purchased from Qiagen. Zero background cloning vector pZErO-1 and *E. coli* DH5α competent cells were purchased from Invitrogen.

**Targeted deletion of *ppk2* in *C. jejuni***

BLAST search using *ppk2* sequence from other *C. jejuni* strains showed that *C. jejuni* 81-176 harbored CJJ81176_0632 (encoding 162 aa) and CJJ81176_0633 (137 aa) which together had a 98-100% sequence similarity with Cj0604 (NCTC 11168), C8J_0566 (81116) and CJE0707 (RM 1221), each of which encode a putative PPK2 with 293 aa in length. The CJJ81176_0633 is annotated as *ppk2* while CJJ81176_0632 is annotated as a hypothetical protein in the NCBI genome database. Based on the BLAST analysis and size agreement with the PPK2 homologs in other bacteria (Nocek et al., 2008) including other *C. jejuni* strains, which all harbored the larger approximately 293
aa protein, these two genes seem to encode PPK2 in 81-176. Consequently, in this study, we deleted most of the coding sequence (95%) of both CJJ81176_0632 and 0633 together in the same mutant to ensure that the PPK2 function is completely abolished.

Deletion of ppk2 (0632-0633) was achieved by double crossover homologous recombination using a suicide vector containing approximately 1 kb of homologous sequences on either side of ppk2 gene as described previously (Gangaiah et al., 2009). Briefly, ppk2 along with 1 kb flanking region on either side of the target gene was amplified by PCR using PPK2 F and PPK2 R primers from C. jejuni 81-176 genomic DNA. The amplified PCR product was ligated into pZErO-1 to generate plasmid pDG4. Inverse PCR was performed on pDG4 using PPK2 INV F and PPK2 INV R primers to delete majority of the ppk2 coding sequence. Kanamycin cassette from pUC4K was then cloned into inverse PCR product, the resulting suicide vector designated, pDG5, was electroporated into C. jejuni 81-176 as described (Wilson et al., 2003). Recombinants were selected on MH agar plates containing kanamycin, kanamycin resistant colonies were streak purified and one such mutant designated DG003/Δppk2 was used for further studies. The deletion of the ppk2 gene was confirmed by PCR.

Complementation of the Δppk2 mutant

The ppk2 coding sequence (0632-0633 included) along with the potential promoter region was amplified by PCR using PPK2 COMP F and PPK2 COMP R primers. The amplified PCR product was ligated to pRY111, an E. coli-Campylobacter shuttle vector (Yao et al., 1993), and the resulting complementation plasmid pDG6 was
introduced into the Δppk2 mutant by triparental conjugation as described previously (Miller et al., 2000). Transconjugants were selected on MH agar plate containing kanamycin and chloramphenicol and one such transconjugant designated DG004/Δppk2c was used in complementation studies to confirm the specific effects of ppk2 deletion except for invasion, intracellular survival and chicken colonization studies in which case it is difficult to maintain the selective pressure.

**Growth curve assay**

Mid-log phase grown cultures of *C. jejuni* were diluted to an OD<sub>600</sub> of 0.05 in MH broth and incubated microaerobically for 60 h at 42°C with shaking at 200 rpm. For assessing the growth and culturability, serial 10-fold dilutions of the cultures at different time points were plated on MH agar and CFU were determined.

**Growth kinetics after pyruvate kinase inactivation**

Growth kinetics of *C. jejuni* after inactivation of pyruvate kinase by Tween 20 was performed as described (Sundin et al., 1996). Briefly, mid-log phase grown cultures were diluted to an OD<sub>600</sub> of 0.05 in MH broth containing 0.1% (v/v) Tween 20 and incubated microaerobically at 42°C for 60 h. At different time points, serial ten-fold dilutions of cultures were plated on MH agar and CFU were determined.

**Assay for PPK2 as a poly P-dependent GTP/ATP generator**

Preparation of crude lysate for PPK2 activity was performed as described previously (Ishige et al., 2002). Briefly, mid-log phase grown cultures of *C. jejuni* were
diluted to an OD$_{600}$ of 0.05 in MH broth and incubated microaerobically for 16 h at 42°C with shaking at 200 rpm. Cells were harvested by centrifugation at 5,000 g for 5 min and resuspended in TED buffer (50 mM Tris HCl, pH 8.0/0.5 mM EDTA/1 mM DTT). Cells were sonicated and centrifuged at 10,000 g for 10 min and the crude lysate was assayed for PPK2 activity. Poly P-dependent synthesis of ATP/GTP was determined by using a modified enzyme-coupled assay with hexokinase and glucose-6-phosphate dehydrogenase (Nocek et al., 2008). Assay mixture (100 µl) contained 10 mM MnCl$_2$ (for GTP)/MgCl$_2$ (for ATP), 80 mM (NH$_4$)$_2$SO$_4$, 50 mM Tris pH 8.0, 5 mM GDP/ADP, 1.5 mM NADP, 1 mM glucose, 3 U yeast hexokinase, 1.5 U *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase, 1-2 mg crude lysate and 5 mM sodium polyphosphate (P$_{12-13}$, added at last). Hexokinase converts GTP/ATP generated by PPK2 to GDP/ADP and glucose-6-phosphate using glucose as the phosphate acceptor and the glucose-6-phosphate will be converted to 6-phosphogluconate by NADP-dependent glucose-6-phosphate dehydrogenase. During this reaction, NADP will be converted to NADPH which was measured spectrophotometrically at 340 nm (ε$_{340}$ nm = 6.22 mM$^{-1}$ cm$^{-1}$). NADPH formed was measured up to 10 min and the enzyme activity was expressed as Vmax as described before (Nocek et al., 2008). The enzyme kinetics was analyzed by nonlinear curve fitting using GraphPad Prism 5.0 software.

Further, the amount of GTP and ATP in the above PPK2 reaction products was calculated using TLC. Briefly, 10 µl of the reaction mixture, normalized by volume, was loaded on to TLC plate and run using saturated ammonium sulfate, 3 M sodium acetate and 2-Propanol (80:6:2, v/v/v) as a solvent system. Nucleotides were visualized using UV
light, developed by ninhydrin and quantified by densitometry using Image J Software from NIH. Quantifications are presented as fold increase or decrease with respect to WT strain.

**ppGpp isolation and detection**

ppGpp was assayed as described previously (Gaynor et al., 2005). Briefly, the bacterial strains were grown to early exponential phase (OD$_{600}$~0.3). Cultures were diluted to OD$_{600}$ of 0.1 in MH broth and incubated for 2 h. Following incubation, the OD$_{600}$ was adjusted to ~0.25; cells were pelleted, washed twice in MOPS-MGS (50 mM MOPS, 55 mM mannitol, 1 mM MgSO$_4$, 0.25 mM CaCl$_2$, 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 × 10$^{12}$ Bq) was added to cells and incubated for 1 and 3 h at 37°C microaerobically. Labeled cells were harvested, washed and treated with lysozyme in 10 mM Tris (pH 8.0) for 20 min. The cells were lysed using 1% SDS (w/v) and 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.

**Quantification of poly P**

Poly P was extracted using glassmilk and quantified using toluidine blue O as described earlier (Candon et al., 2007). Poly P was quantified from mid-log, late-log and mid-stationary phase cultures by measuring the ratio of 530 to 630 nm
spectrophotometrically using appropriate concentrations of phosphorous standard (Sigma).

**Survival under nutrient stress**

To determine survival under nutrient stress, mid-log phase grown bacterial cultures were pelleted, washed twice with MEM and resuspended in MEM as described previously (Candon *et al.*, 2007; Gangaiah *et al.*, 2009). The OD$_{600}$ was adjusted to 0.05 and the cultures were incubated microaerobically at 42°C for 60 h with shaking at 200 rpm. One hundred microliters of culture at different time points was serially diluted (10-fold), plated on MH agar and CFU were determined.

**Osmotic stress survival**

Osmotic stress tolerance was determined as described previously (Candon *et al.*, 2007; Gangaiah *et al.*, 2009). To assess the osmotic stress survival in liquid culture, bacterial strains were grown to mid-log phase, adjusted to an OD$_{600}$ of 0.05 in MH broth with and without 0.25 M NaCl and incubated microaerobically at 42°C for 60 h with shaking at 200 rpm. One hundred microliters of the culture at different time points was serially diluted (10-fold) in MH broth containing 0.25 M NaCl and plated on MH agar. The plates were incubated microaerobically and CFU were determined. To determine osmotic stress tolerance on solid media, mid log-phase grown WT, Δppk2, and Δppk2c cultures were serially diluted (10-fold), 10 µl of diluted culture was spotted on MH agar containing 0.17 M NaCl and incubated microaerobically at 42°C for 2 days.
Aerobic survival

Aerobic survival of *C. jejuni* was assessed by exposing the bacteria to aerobic conditions and determining their culturability at different time points. Briefly, *C. jejuni* was grown to mid-log phase and adjusted to an OD<sub>600</sub> of 0.05. The samples were incubated at 42°C under aerobic conditions with shaking for 60 h and 100 μl of ten fold serial dilutions of the culture was plated on MH agar at different time points and CFU were determined.

Induction and enumeration of viable but non-culturable (VBNC) cells in *C. jejuni*

*C. jejuni* has been previously shown to form VBNC cells in response to unfavorable environments (Rollins and Colwell, 1986; Tholozan *et al.*, 1999). VBNC cells in *C. jejuni* were induced and enumerated as described previously (Chaveerach *et al.*, 2003; Gangaiah *et al.*, 2009). Briefly, 1 ml of overnight grown culture containing 5×10<sup>8</sup> CFU was added to 4 ml of MH broth with pH adjusted to 4.0 using formic acid and incubated microaerobically at 42 °C for 3 h. VBNC formation was confirmed by determining the total culturable counts and viability. Total culturable counts were determined by plating the formic acid treated bacteria at different times for enumerating CFU. Viability was determined by measuring OD<sub>600</sub> after CTC staining. Briefly, the bacterial samples were stained by adding 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC: Polysciences) to a final concentration of 5 mM for 1 h at room temperature in dark. Cells were pelleted, pellet was resuspended in 500 μl of PBS and viability was determined by measuring CTC reduction spectrophotometrically at 600 nm.
Biofilm formation

Static biofilm formation was assessed in borosilicate tubes as described previously (Candon et al., 2007; Gangaiah et al., 2009; McLennan et al., 2008) by inoculating 100 µl of 0.05 OD$_{600}$ culture into MH broth and incubating at 42°C microaerobically for 2 days without shaking. Biofilms were visualized by staining with 250 µl of 1% (w/v) crystal violet for 15 min, and quantified by measuring the absorbance at 570 nm after dissolving in 1 ml DMSO for 48 h.

Antimicrobial susceptibility testing

The susceptibility of the WT, Δppk2 and Δppk2c strains to different antimicrobials was determined by microtiter broth dilution using polypropylene plates as described previously (Gangaiah et al., 2009; Lin et al., 2002). Briefly, 100 µl of mid-log phase grown cultures adjusted to an OD$_{600}$ of 0.05 in MH broth was added to microtitre plate containing serially (2-fold) diluted antimicrobials. Plates were incubated at 42°C microaerobically at 200 rpm for 2 days without shaking, and minimal inhibitory concentration (MIC, µg/ml) was determined by recording the lowest concentration of an antimicrobial showing complete inhibition of visible bacterial growth.

Susceptibility to azithromycin, ciprofloxacin, erythromycin, tetracycline, florfenicol, nalidixic acid, telithromycin, clindamycin, and gentamicin was determined by using Sensititre® susceptibility plates for *Campylobacter* (TREK Diagnostic). Briefly, one hundred microliters of log-phase grown cultures adjusted to an OD$_{600}$ of 0.05 in MH broth was added to each well in the Sensititre® susceptibility plate and the wells were
covered using the perforated adhesive seal. Plates were incubated microaerobically at 42 °C for 24 h and MIC was recorded. Results were read following the manufacturer’s instructions and interpreted according to MIC interpretive guidelines by Clinical Laboratory Standards Institute. The susceptibility testing was repeated 3 times and mean MIC was calculated.

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed targeting key genes involved in phosphate uptake (phosR, pstS, pstC, and periplasmic substrate binding protein, CJJ81176_0750) (Reid et al., 2008a; Reid et al., 2008b; Wosten et al., 2006), stringent response (spoT) (Gaynor et al., 2005), and multidrug resistance (cmeC) (Lin et al., 2002), post transcriptional global regulator (csrA) (Fields and Thompson, 2008) and poly P synthesis (ppk1) (Candon et al., 2007; Gangaiah et al., 2009). Total RNA was extracted from log-phase grown bacterial cultures using RNeasy Mini Kit (Qiagen). The RNA concentration and purity was determined using NanoDrop ND-1000 spectrophotometer. cDNA synthesis was carried out using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). Gene specific primers were designed to amplify the abovementioned genes along with rpoA (internal control) using Beacon Designer 7.0. The rpoA has recently been shown to be the most suitable internal control for qRT-PCR analysis of stress responses and growth phase effects in C. jejuni (Ritz et al., 2009). qPCR was performed using SensiMixPlus® SYBR RT-PCR Kit (Quantace) in a realplex² mastercycler (Eppendorf). The relative levels of expression of genes were normalized with rpoA
amplified from the corresponding sample. The difference in expression of the genes was calculated using the comparative threshold cycle (CT) method to yield fold-difference in transcript levels.

**Adherence, invasion and intracellular survival in INT407 cells**

Adherence, invasion and intracellular survival assays were performed as described previously (Gaynor *et al.*, 2005; Svensson *et al.*, 2009). Each well of a 24-well tissue culture plate was seeded with $1.4 \times 10^5$ INT 407 cells in MEM with 10% (v/v) fetal bovine serum (FBS) and incubated for 18 h at 37°C with 5% CO$_2$. *C. jejuni* strains were grown to mid-log phase in MH broth microaerobically, the cells were pelleted at 5,000 g for 10 min, washed twice with MEM containing 1% (v/v) FBS, resuspended in MEM to an OD$_{600}$ of 0.02 and used for infection. INT407 cells were infected with different multiplicities of infection (MOI), 100:1 for adherence and intracellular survival assays and 0.01:1, 0.1:1, 1:1, 10:1 and 100:1 for invasion assay. For infection, 1 ml of bacterial cell suspension was pipetted on to INT 407 cells, centrifuged at 1000 g for 3 min and incubated for 3 h. For determining adherence, cells were rinsed with MEM three times, lysed with 0.1% (v/v) Triton-X 100 and diluted serially in MEM and plated on MH agar in duplicate to determine CFU. For determining invasion, after 3 h of incubation with bacteria, cells were treated with gentamicin (150 µg/ml) and incubated for additional 2 h. After 2 h of incubation, the infected cells were rinsed with MEM three times, lysed with 0.1% (v/v) Triton-X 100, serially diluted in MEM and plated on MH agar in duplicate to determine CFU. To assess intracellular survival, following 2 h gentamicin treatment, the
infected cells were washed with MEM three times and covered with MEM containing gentamicin (10 µg/ml) and incubated for 24 h. After 24 h of incubation, the infected cells were washed with MEM, lysed with 0.1% (v/v) Triton-X 100, serially diluted in MEM and plated on MH agar in duplicate to determine CFU.

**Chicken colonization studies**

Chicken colonization studies were performed as described previously (Candon *et al.*, 2007; Gangaiah *et al.*, 2009). Colonization experiments were conducted according to the guidelines of AAALAC. Briefly, day-old broiler chicks (n=5 for each group) from a local hatching facility (Food Animal Health Research Program, OARDC, Wooster, OH) were inoculated orally with $10^3$, $10^4$ and $10^5$ CFU of the *C. jejuni* WT and ∆ppk2 mutant strains in 200 µl of PBS (pH 7.4). Eight days post-inoculation, the chicks were euthanized, cecal contents, feces and bursa were collected aseptically, weighed, homogenized, diluted in PBS (pH 7.4) and plated on MH agar containing *Campylobacter* selective supplement. Plates were incubated at 42°C microaerobically and CFU per gram of tissues were determined.

**Statistical analysis**

Statistical significance of data generated in this study was determined using one-way analysis of variance (ANOVA) followed by Tukey’s HSD (Honestly Significant Difference) test or Student’s t-test (paired 2-tailed). $P \leq 0.01$ or 0.05 ($\alpha$ level) was considered statistically significant.
3.4 RESULTS

The Δppk2 mutant is impaired in GTP synthesis and exhibits increased intracellular ATP:GTP ratio

PPK2 from *P. aeruginosa* and *Mycobacterium* species mediate preferential generation of GTP using poly P as a phosphate donor (Ishige *et al.*, 2002; Sureka *et al.*, 2009). Therefore, we assessed the ability of *C. jejuni* Δppk2 mutant to generate GTP from poly P. The *C. jejuni* Δppk2 mutant was significantly (*P*≤0.05) defective in poly P-dependent GTP generation compared to WT (Figure 3.1), while complementation restored the ability of the Δppk2 mutant to generate GTP to levels comparable to WT (Figure 3.1). Further, consistent with the enzyme assay, thin layer chromatography (TLC) analysis of the reaction mixture revealed 1.2 fold lower GTP level in the Δppk2 mutant compared to WT. The *C. jejuni* Δppk2 mutant, however, showed no general growth defect in rich medium (Figure 3.18). This observation led us to hypothesize that the *C. jejuni* Δppk2 mutant possesses an alternative source for GTP required to support normal growth. *P. aeruginosa* possesses the alternative NTP-generating enzymes, nucleoside diphosphate kinase (NDK) and pyruvate kinase (PK) (Sundin *et al.*, 1996). PK generates NTPs using phosphoenolpyruvate as a phosphate donor, while NDK synthesizes NTPs using ATP as a phosphate donor. PK is sensitive to Tween 20, while NDK is not. Therefore, we monitored the growth of the Δppk2 mutant in MH broth containing 0.1% (v/v) Tween 20. The presence of Tween 20 did not affect the growth of the *C. jejuni* Δppk2 mutant in rich medium (data not shown).
Since PPK2 can mediate poly P-driven ATP synthesis in other bacteria (Nocek et al., 2008), we investigated whether \textit{C. jejuni} PPK2 has a role in ATP synthesis. Interestingly, the \( \Delta \text{ppk2} \) mutant displayed a significant increase \((P \leq 0.05)\) in the ability to generate poly P-dependent ATP compared to WT (Figure 3.2). Consistent with our enzyme assay, densitometry analysis measuring ‘de novo’ ATP production by thin layer chromatography (TLC) showed that the \( \Delta \text{ppk2} \) mutant had 1.6 fold higher ATP levels compared to WT.

**The \( \Delta \text{ppk2} \) mutant and parental strain exhibit similar ppGpp and poly P levels**

Since GTP serves as the precursor for ppGpp synthesis (Kim et al., 1998), we asked whether PPK2 has a role in ppGpp accumulation. Surprisingly, though the \( \Delta \text{ppk2} \) mutant displayed slightly elevated ppGpp accumulation compared to WT (Figure 3.3), there was no significant difference in the ppGpp accumulation either after 1 hr or 3 hr labeling. Additionally, densitometry analysis also revealed no statistically significant difference in the amount of ppGpp between the WT and \( \Delta \text{ppk2} \) mutant.

Though GTP synthesis is the preferred function, PPK2 is also involved in poly P synthesis in other bacteria (Lindner et al., 2007). Therefore, we asked whether PPK2 has a role in poly P accumulation in \textit{C. jejuni}. The \( \Delta \text{ppk2} \) mutant exhibited similar poly P levels to that of WT (Figure 3.4). However, as previously reported (Candon et al., 2007; Gangaiah et al., 2009), the \( \text{ppk1} \) mutant showed a significant \((P \leq 0.01)\) defect in poly P accumulation (Figure 3.4) suggesting that \( \text{ppk1} \) but not \( \text{ppk2} \) is involved in poly P synthesis in \textit{C. jejuni}. 

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PPK2 is essential for *C. jejuni* nutrient and osmotic stress survival and aerotolerance

To assess the contribution of PPK2 to *C. jejuni* survival under nutrient stress, we monitored the survival of the Δppk2 mutant in MEM. The Δppk2 mutant was significantly (*P*≤0.01) impaired in survival in MEM compared to WT only during stationary-phase (Figure 3.5). After exposure to nutrient stress, the Δppk2 mutant had 3.5-log and 2.1-log fewer bacteria than the WT strain at 48 and 60 h, respectively, while complementation of the Δppk2 mutant restored the survival defect to levels comparable to WT (Figure 3.5). This finding is in contrast to the ppk1 mutant (Candon *et al.*, 2007; Gangaiah *et al.*, 2009) where a nutrient survival defect was evident at both log and stationary-phases of growth. For *P. aeruginosa*, it is shown that PPK2 is induced >100-fold when the culture approaches stationary phase as a consequence of the GTP requirement for different cellular processes during this growth stage (Rao *et al.*, 2009). Thus, the defect in GTP synthesis observed during stationary phase in the nutritionally limited media may explain the reduced ability of the Δppk2 mutant to survive in the stationary phase.

*C. jejuni* Δppk2 mutant was significantly (*P*≤0.01 or 0.05) defective in survival under osmotic stress both in liquid culture and on solid medium compared to the WT strain (Figure 3.6 and 3.7). After 0.25 M NaCl treatment in liquid culture, the Δppk2 mutant exhibited a 3- and 5-log reduction in bacterial numbers compared with WT at 12 and 24 h, respectively. While at 48 and 60 h, no mutant bacteria were recovered
compared to WT which showed $1.8 \times 10^4$ and $2.7 \times 10^2$ CFU of bacteria, respectively. Similarly, the Δppk2 mutant failed to grow on solid medium in the presence of 0.17 M NaCl. The Δppk2 mutant grew only when $5 \times 10^5$ CFU were spotted, while the WT and complemented strains grew even when 1000 fold less CFU were spotted (Figure 3.7, a difference of 1000-fold). Complementation of the Δppk2 mutant restored survival to levels comparable to WT (Figure 3.5 and 3.6).

The role of PPK2 in C. jejuni aerotolerance was assayed by monitoring the survival of the Δppk2 mutant under aerobic condition. Interestingly, the Δppk2 mutant had a significant ($P \leq 0.01$) survival defect at 12 and 24 h compared to WT (Figure 3.8). At 12 h, 3.2-log fewer bacteria were recovered from the Δppk2 mutant compared to WT. At 24 h, no bacteria were recovered from the Δppk2 mutant, while the WT showed $5 \times 10^7$ CFU of bacteria. Complementation of the Δppk2 mutant restored survival to levels comparable to WT (Figure 3.8). Unlike the Δppk2 mutant, the ppk1 mutant has no survival defect under aerobic conditions (Candon et al., 2007; Gangaiah et al., 2009).

The Δppk2 mutant exhibits reduced capacity to form VBNC cells

VBNC formation is an important mechanism used by C. jejuni to survive in the environment under different stress conditions (Rollins and Colwell, 1986; Tholozan et al., 1999]. The Δppk2 mutant showed reduced VBNC formation under formic acid stress compared to WT (Figure 3.9). Though no culturable bacteria were recovered from the WT and Δppk2 mutant at 1 h post-treatment, both strains retained viability until 3 h after formic acid treatment as determined by CTC staining. However, the Δppk2 mutant
showed significantly \((P \leq 0.05)\) reduced viability at 1, 2 and 3 h post-treatment compared to WT and complementation of the \(\Delta ppk2\) mutant restored the viability to levels comparable to WT (Figure 3.9).

**The \(\Delta ppk2\) mutant exhibits enhanced biofilm formation**

The *C. jejuni* \(\Delta ppk2\) mutant had no motility defect when tested on semisolid agar (Table 3.4). However, when grown in MH broth microaerobically at 37°C, the \(\Delta ppk2\) mutant formed more unattached aggregates at the bottom of the tube which suggested that the \(\Delta ppk2\) mutant may display enhanced biofilm formation (Joshua et al., 2006). Indeed, the \(\Delta ppk2\) mutant exhibited significantly \((P \leq 0.01)\) enhanced biofilm formation compared to WT after 48 h of static growth (Figure 3.10 and 3.11). Complementation of the \(\Delta ppk2\) mutant decreased biofilm formation similar to WT levels (Figure 3.10 and 3.11).

**The \(\Delta ppk2\) mutant shows increased susceptibility to antimicrobials**

Since poly P plays a role in mediating antimicrobial resistance in *C. jejuni* (Gangaiah et al., 2009), we asked whether *C. jejuni* PPK2 has a role in antimicrobial resistance. Though not significant, the \(\Delta ppk2\) mutant exhibited increased susceptibility to several antimicrobials compared to WT (Table 3.1). Specifically, the \(\Delta ppk2\) mutant showed increased susceptibility to both macrolides (erythromycin; 4-fold) and fluoroquinolones (ciprofloxacin; 2-fold) that are considered drugs of choice for treating *Campylobacter* infections in humans. A low MIC was observed for tetracycline in the WT. The WT strain used in this study is cured for pTet plasmid that carries *tet(O)* gene
which confers tetracycline resistance to C. jejuni (Bacon et al., 2000). This was further confirmed by PCR using tet(O) specific primers (data not shown). No difference in susceptibility was observed for other antibacterials and detergents including arsenical compounds (Table 3.1). Complementation of the Δppk2 mutant restored the susceptibility to levels comparable to WT (Table 3.1).

The expression of spoT, phosR, pstS, pstC, ppk1, csrA and cmeC was up-regulated in the Δppk2 mutant

To understand the mechanisms underlying PPK2-mediated phenotypes, we performed quantitative RT-PCR targeting genes encoding SpoT, a mediator of stringent response; PhosR, PstS and PstC which are involved in phosphate uptake; PPK1, a key protein in poly P synthesis; CsrA, a post-transcriptional regulator and CmeC, a component of multidrug efflux pump. The aforementioned genes were up-regulated (2-fold or more) in the Δppk2 mutant compared to WT (Figure 3.12). However, only spoT, ppk1, cmeC, pstC and csrA showed significant up-regulation (P≤0.05). The transcription of genes involved in oxidative stress resistance [CJJ81176_0356 and, CJJ81176_0298 (anti-oxidant AhpCTSA family protein)] and sodB was unaltered in the Δppk2 mutant compared to WT (data not shown) suggesting that PPK2 is only involved in regulation of a subset of genes.

The Δppk2 mutant is defective in invasion and intracellular survival in INT407 cells

To investigate if PPK2 is involved in virulence-associated phenotypes, we examined whether the Δppk2 mutant could adhere, invade and survive within INT407
human intestinal epithelial cells. Though the \( \Delta ppk2 \) mutant did not show a defect in adherence (data not shown), the mutant exhibited a significant \((P \leq 0.05)\) dose-dependent defect in invasion (Figure 3.13). Specifically, defect in invasion was more evident at a lower MOI (0.01:1, 0.1:1 and 1:1). The \( \Delta ppk2 \) mutant also exhibited a significant \((P \leq 0.01)\) defect in intracellular survival in INT 407 cells, while complementation restored the defect to WT values (Figure 3.14).

**The \( \Delta ppk2 \) mutant exhibited a dose-dependent chicken colonization defect**

To test the contribution of PPK2 to *C. jejuni* host colonization, we tested the ability of the \( \Delta ppk2 \) mutant to colonize day-old chicks. The \( \Delta ppk2 \) mutant exhibited a significant \((P \leq 0.01\) or 0.05) dose-dependent colonization defect compared to WT (Figure 3.15, 3.16 and 3.17). At \( 10^3, 10^4, \) and \( 10^5 \) inoculation doses, the \( \Delta ppk2 \) mutant had significantly \((P \leq 0.01\) or 0.05) fewer average CFU in the cecal contents, feces and bursa compared to WT. At \( 10^3 \) and \( 10^4 \) inoculation levels, no detectable bacteria were recovered from the \( \Delta ppk2 \) mutant strain in all chicks from all organs tested except from cecal contents in one chicken at \( 10^4 \) inoculation dose, while the WT strain colonized all organs in all chicks. These findings indicate that \( \Delta ppk2 \) mutant exhibits a significant colonization defect at lower levels of inoculum.

**3.5 DISCUSSION**

In the absence of classical stress response mechanisms which are crucial for the success of many foodborne pathogens, it is imperative for *C. jejuni* to use alternative mechanisms to survive in different environments. Poly P plays an important role in
mediating bacterial survival under various stresses and stringencies (Rao et al., 2009; Seufferheld et al., 2008). Here, we characterized the role of ppk2 which is involved in poly P-dependent GTP synthesis. Our data suggest that ppk2 might play a role in several phenotypes that are important for C. jejuni transmission, colonization, and persistence within and outside the host environment. Specifically, the Δppk2 mutant was sensitive to aerobic, osmotic, nutrient, and antimicrobial stresses (Figure 3.5, 3.6, 3.7, 3.8 and Table 3.1) as well as defective in chicken colonization (Figure 3.15, 3.16 and 3.17).

The C. jejuni 81-176 ppk2 is conserved among various C. jejuni strains and Campylobacter species with a sequence similarity ranging from 98-100% and 69-90%, respectively (data not shown). Based on the BLAST analysis and size agreement with the PPK2 homologs in other bacteria (Nocek et al., 2008) including other C. jejuni strains, which all harbored the larger approximately 293 aa protein, ORFs 0632 (162 aa) and 0633 (137 aa) together seem to encode PPK2 in 81-176. However, further analysis is needed to identify whether 0632 and 0633 encode unique proteins in 81-176.

The C. jejuni PPK2 is a 1-domain protein and has high sequence similarity to P. aeruginosa PPK2 paralogs, PA0141 (61%), PA2428 (56%), and PA3455 (35%); M. smegmatis PPK2, SMEG_0891 (55%) and M. tuberculosis PPK2, rv3232c (48%) (Figure 3.19 and 3.20). The predicted tertiary structure of C. jejuni PPK2 sequence revealed a higher similarity to the C-terminal domain of P. aeruginosa PPK2 paralog PA3455 (Figure 3.22). PPK2 class of enzymes belongs to the large superfamily of P loop kinases, which catalyze the hydrolysis or binding of nucleoside triphosphates (Leipe et
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). The Walker A, and Walker B and the lid module in *C. jejuni* PPK2 are highly conserved (Figure 3.20 and 3.21) as well as nine conserved residues within the catalytic site that are required for PPK2 activity in *P. aeruginosa* (Nocek *et al.*, 2008) are also present in *C. jejuni* PPK2 (Figure 3.20) suggesting that this protein has a similar catalytic mechanism.

The *P. aeruginosa* PPK2 (PA0141) and *M. tuberculosis* PPK2 (rv3232c) preferentially catalyze the synthesis of GTP using poly P as phosphate donor (Ishige *et al.*, 2002; Sureka *et al.*, 2009). The PA0141 also synthesizes poly P from GTP but at a 75-times lower rate than GTP synthesis from poly P, while rv3232c does not contribute to poly P synthesis. Contrary to PA0141 and rv3232c, *P. aeruginosa* PPK2 paralogs PA2428 and PA3455 primarily catalyze the synthesis of ATP/ADP from poly P and do not mediate poly P synthesis from ATP/GTP (Nocek *et al.*, 2008). Our results showed that the *C. jejuni Δppk2* mutant was significantly defective in poly P-dependent GTP generation, indicating that *C. jejuni* PPK2 catalyzes poly P-driven GTP synthesis (Figure 3.1) similar to PA0141 and rv3232c. However, the *C. jejuni Δppk2* mutant showed no defect in poly P-dependent ATP synthesis, rather it displayed increased ability to generate ATP resulting in increased intracellular ATP:GTP ratio (Figure 3.2), suggesting that PPK2 plays a role in regulating intracellular nucleotide pool in *C. jejuni*. The Mycobacterial rv3232c and SMEG_0891 modify NDK, which is involved in the transfer of phosphate from ATP to other NTPs with preference to GTP synthesis (Sureka *et al.*, 2009).
2009). In the *Mycobacterium ppk2* mutant, NDK lacked preferential synthesis of GTP suggesting that PPK2 modulates NDK activity. Interestingly, *C. jejuni* genome possesses a homolog of NDK with 48 and 47% sequence similarity to rv3232c and SMEG_0891, respectively. Thus, the possibility of a similar interaction of PPK2 to direct NDK towards GTP synthesis cannot be ruled out in *C. jejuni*.

The deletion of *ppk2* significantly reduced the *C. jejuni* survivability under nutrient, osmotic and aerobic stresses (Figure 3.5, 3.6, 3.7 and 3.8). *C. jejuni* responds to environmental stresses by eliciting a stringent response mediated by ppGpp, a signal molecule that regulates virulence and stringent response in *C. jejuni* and other bacteria (Gaynor et al., 2005). GTP is a precursor for ppGpp synthesis (Kim et al., 1998), surprisingly, though not statistically significant, the *C. jejuni Δppk2* mutant showed slightly elevated ppGpp levels compared to WT (Figure 3.3). NDK, in the absence of PPK2, might serve as an alternative source of GTP required for ppGpp synthesis as NDK can still contribute to residual levels of GTP. In addition, ppGpp regulates poly P levels in *E. coli* and high levels of ppGpp resulted in accumulation of large amounts of poly P as a result of inhibition of PPX, an enzyme involved in poly P degradation (Kuroda et al., 1997). As expected, our findings showed no altered levels of poly P in the Δppk2 mutant compared to WT (Figure 3.4), possibly a consequence of no change in ppGpp levels. Consistent with our finding, a recent study in *M. smegmatis* also showed that deletion of *ppk2* did not alter intracellular poly P levels compared to WT (Sureka et al., 2009). However, *spoT* was up-regulated in our *C. jejuni Δppk2* mutant when grown in rich media (Figure 3.12). Unlike in other bacteria, SpoT is critical for both ppGpp synthesis
as well as its hydrolysis in *C. jejuni* (Gaynor *et al*., 2005). In nutrient-rich conditions, SpoT hydrolyzes ppGpp to GTP and inorganic phosphate, keeping ppGpp levels low and preventing the cells from entering stress response and growth arrest (Raskin *et al*., 2007). Thus, the up-regulation of *spoT* in the *C. jejuni* ∆ppk2 mutant may be a compensatory response to keep ppGpp levels low preventing cells from entering stress response. Despite unaltered intracellular ppGpp and poly P levels, it is intriguing to note that the *C. jejuni* ∆ppk2 mutant showed significant defect in stress tolerance and pathogenicity, suggesting that these phenotypes probably are not mediated by a ppGpp-mediated stringent response. Alternatively, some of the phenotypes in the *C. jejuni* ∆ppk2 mutant may be due to the potential role of PPK2 in regulating other virulence and stress response-related genes (Chakrabarty, 2002; Pandit and Srinivasan, 2003; Rao *et al*., 2009; Sundin *et al*., 1996).

Adherence, invasion and intracellular survival are important virulence mechanisms in *C. jejuni* pathogenesis (Malik-Kale *et al*., 2009; Pogačar *et al*., 2009) as these attributes permit evasion of immune response, in addition to their role in cellular damage, relapse and persistence in the human host (Day *et al*., 2000; De Melo *et al*., 1989; Russel *et al*., 1993). The *C. jejuni* ∆ppk2 mutant was significantly defective in invasion (Figure 3.13) and intracellular survival (Figure 3.14) within human intestinal epithelial cells, suggesting an important role for PPK2 in *C. jejuni* pathogenesis. Our observation that the ∆ppk2 mutant is sensitive to a variety of *in vitro* stresses is consistent with the fact that *C. jejuni* must overcome nutrient and acid stresses within the intestinal
epithelial cells, providing an explanation for the intracellular survival defect in the Δppk2 mutant.

Our study describes for the first time the importance of PPK2 in vivo in bacteria. The C. jejuni Δppk2 mutant displayed a significant dose-dependent colonization defect in chickens (Figure 3.15, 3.16 and 3.17). Inactivation of ppk1 which encodes a poly P synthesizing enzyme (Candon et al., 2007; Gangaiah et al., 2009) and cprS which encodes a sensor kinase (Svensson et al., 2009) in C. jejuni also resulted in a dose-dependent colonization defect in chickens. An enhancement in biofilm formation was proposed as the possible explanation for dose-dependent chicken colonization defect in ppk1 and cprS mutants. Specifically, at lower doses, the mutant may be significantly susceptible to in vivo stresses; however, at higher doses, a dose-dependent hyperbiofilm phenotype was suggested to restore resistance of the mutant to in vivo stresses similar to WT. A similar mechanism might explain the dose dependent colonization defect in C. jejuni Δppk2 mutant as the Δppk2 mutant also exhibited hyperbiofilm phenotype similar to ppk1 and cprS mutants (Figure 3.10 and 3.11). However, it should be noted that the defect in chicken colonization by the Δppk2 mutant is multifactorial.

Similar to the ppk1 mutant, resistance to oxidative, heavy metal, heat, anaerobic, and acid (acetic, propionic, and hydrochloric acids) stresses were not affected in the Δppk2 mutant (Table 3.4). The Δppk2 mutant was also not defective in iron utilization and growth at non-permissive temperatures (4 and 25°C) (Table 3.4). Although C. jejuni PPK2 is structurally not related to PPK1, certain phenotypes are common to both PPK1
and PPK2, while others are not, suggesting that PPK1 and PPK2 contribute to overlapping but not redundant functions. Most importantly, the absence of either PPK1 or PPK2 compromises *C. jejuni* physiology and pathogenesis. Although PPK1 and PPK2 might work by distinct mechanisms, PPK1 may require direct or indirect participation of PPK2 for particular functions and vice-versa, which partially explains the overlapping phenotypes in these proteins. Our repeated efforts to generate a *ppk1* and *ppk2* double mutant to better understand the role of these genes in *C. jejuni* patho-physiology were unsuccessful, suggesting that either *ppk1* or *ppk2* is required for *C. jejuni* viability. Consistent with our finding, a recent report in *M. smegmatis* also showed that viable double knockouts lacking both *ppk1* and *ppk2* could not be obtained (Sureka *et al.*, 2009). Though, the exact mechanism by which poly P, PPK1 and PPK2 mediate stress response and virulence in *C. jejuni* is unknown, this study adds to our understanding of the contribution of poly P-associated proteins in *C. jejuni* adaptation and survival under different stringencies in the absence of RpoS-mediated classical stress response mechanisms.

### 3.6 ACKNOWLEDGEMENT

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<td>Azithromycin</td>
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<td>0.06 (2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.25</td>
<td>0.12 (2)</td>
<td>0.25</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.5</td>
<td>0.125 (4)</td>
<td>0.5</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2</td>
<td>0.5 (4)</td>
<td>1.0</td>
</tr>
<tr>
<td>Fluorfenicol</td>
<td>2</td>
<td>0.5 (4)</td>
<td>1.0</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>16</td>
<td>4 (4)</td>
<td>16</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>2</td>
<td>1 (2)</td>
<td>2</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.5</td>
<td>0.25 (2)</td>
<td>0.5</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.5</td>
<td>0.5 (-)</td>
<td>0.5</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1.6</td>
<td>1.6 (-)</td>
<td>1.6</td>
</tr>
<tr>
<td>Rifampin</td>
<td>100</td>
<td>100 (-)</td>
<td>100</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>3</td>
<td>3 (-)</td>
<td>3</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>0.625</td>
<td>0.625 (-)</td>
<td>0.625</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>6250</td>
<td>6250 (-)</td>
<td>6250</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>36,000</td>
<td>36,000 (-)</td>
<td>36,000</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>16,500</td>
<td>16,500 (-)</td>
<td>16,500</td>
</tr>
<tr>
<td>Arsenite</td>
<td>64</td>
<td>64 (-)</td>
<td>64</td>
</tr>
<tr>
<td>Arsenate</td>
<td>1024</td>
<td>1024 (-)</td>
<td>1024</td>
</tr>
<tr>
<td>Roxarsone</td>
<td>128</td>
<td>128 (-)</td>
<td>128</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fold difference between the WT and Δppk2 mutant

**Table 3.1.** Susceptibility of the Δppk2 mutant to various antimicrobials, bile acids and arsenical compounds
<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni 81-176 WT</td>
<td>WT strain of <em>Campylobacter jejuni</em></td>
<td>Qijing Zhang</td>
</tr>
<tr>
<td>Δppk2l DG003</td>
<td>C. jejuni 81-176 derivative with deletion in ppk2 gene; ppk2::Kan</td>
<td>This study</td>
</tr>
<tr>
<td>ppk2c/ DG004</td>
<td>DG003 harboring pDG6. Cm</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>E. coli strain used for cloning</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pZERO-1</td>
<td>Cloning vector for making suicide vector; Zeo</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRY111</td>
<td><em>E. coli-Campylobacter</em> shuttle vector for complementation</td>
<td>58</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for conjugation</td>
<td>12</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source for kanamycin</td>
<td>Amersham</td>
</tr>
<tr>
<td>pDG4</td>
<td>pZEro-O-1 containing ppk2 region plus 1 kb upstream and downstream sequences from 81-176; Zeo</td>
<td>This study</td>
</tr>
<tr>
<td>pDG5</td>
<td>Suicide vector with ppk2 replaced by kanamycin resistance region from pUC4K in pDG4; Zeo, Kan</td>
<td>This study</td>
</tr>
<tr>
<td>pDG6</td>
<td>pRY111 containing ppk2 coding region and the upstream promoter sequence for complementation; Cm</td>
<td>This study</td>
</tr>
</tbody>
</table>

Kan, kanamycin; Cm, Chloramphenicol; Zeo, zeocin

**Table 3.2.** Bacterial strains and plasmids used in this study.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primers used for construction of Δppk2 mutant and Δppk2c complemented strains</strong></td>
<td></td>
</tr>
<tr>
<td>PPK2 F</td>
<td>ATAAAAAGGTACCAGGTTGGATCTTAATGGC</td>
</tr>
<tr>
<td>PPK2 R</td>
<td>AAAAATCTGCAAGCGTTGGAATTTCTCTCTGGA</td>
</tr>
<tr>
<td>PPK2 INV F</td>
<td>ATAAAAAGATTCAGTTGCTAAGTGCC</td>
</tr>
<tr>
<td>PPK2 INV R</td>
<td>AAAAAGATTCAGTTGCTAAGTGCC</td>
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<tr>
<td>PPK2 COMP F</td>
<td>TAATAACTGAGACGCAAATCTTCTCTCTAA</td>
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<tr>
<td>PPK2 COMP R</td>
<td>ATATATGTTACCACGATTCTCTCTCTCTAA</td>
</tr>
<tr>
<td><strong>Primers used for quantitative RT-PCR</strong></td>
<td></td>
</tr>
<tr>
<td>CsrA F</td>
<td>TTTCTGAGGGAAGGTATAG</td>
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<tr>
<td>CsrA R</td>
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<tr>
<td>SpoT F</td>
<td>GTAACACACGACTGACACATG</td>
</tr>
<tr>
<td>SpoT R</td>
<td>GTAACACACGACTGACACATG</td>
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<tr>
<td>PhosR F</td>
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<tr>
<td>PhosR R</td>
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<tr>
<td>CmeC F</td>
<td>GCTGCTGCTCAATTAGTAG</td>
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<td>CmeC R</td>
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<td>PstS F</td>
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<tr>
<td>PstS R</td>
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<tr>
<td>PstC F</td>
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</tr>
<tr>
<td>PstC R</td>
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<td>CJ181176_0750 F</td>
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<td>CJ181176_0750 R</td>
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<td>PPK2-RT F</td>
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<td>PPK2-RT R</td>
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<td>PPK1-RT R</td>
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<tr>
<td>RpoA R</td>
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<td>CJJ_0298 F</td>
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<td>CJJ_0298 R</td>
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<tr>
<td>CJJ_0356 F</td>
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<td>SodB F</td>
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</tr>
<tr>
<td>SodB R</td>
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**Table 3.3.** Primers used in this study.
<table>
<thead>
<tr>
<th>Phenotypic assay</th>
<th>Method</th>
<th>WT</th>
<th>∆ppk2</th>
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<tr>
<td>Motility</td>
<td>Semisolid agar (0.4%) stab assay</td>
<td>28 mm</td>
<td>26 mm</td>
</tr>
<tr>
<td>Acid stress</td>
<td>CFU determination of acid exposed</td>
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<td>ND</td>
</tr>
<tr>
<td>Acetic acid</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Propionic acid</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Heat shock at 55° C for 1, 2, 3 and 5 min</td>
<td>CFU determination</td>
<td></td>
<td>ND</td>
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<tr>
<td>Anaerobic survival</td>
<td>CFU determination after anaerobic growth using BBL Gaspak Plus Anaerobic system</td>
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<td>ND</td>
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<tr>
<td>Survival at non-growth temperatures</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>4° C</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>25° C</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Growth difference between 37 and 42° C</td>
<td>CFU determination</td>
<td></td>
<td>ND</td>
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<tr>
<td>Growth after pyruvate kinase inactivation</td>
<td>CFU determination</td>
<td></td>
<td>ND</td>
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<tr>
<td>Oxidative stress</td>
<td>Disc (6 mm) diffusion assay</td>
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<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td>0.03% 3 mm 3 mm</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.1% 7.5 mm 7 mm</td>
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<tr>
<td></td>
<td>0.3% 12 mm 11.5 mm</td>
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<tr>
<td>Paraquat</td>
<td>1 mM 3 mm 3 mm</td>
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<tr>
<td></td>
<td>10 mM 16 mm 16 mm</td>
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<tr>
<td>Heavy metal tolerance</td>
<td>MIC determination by microdilution method</td>
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<tr>
<td>Arsenite</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>64 µg 64 µg</td>
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</tr>
<tr>
<td>Arsenate</td>
<td>1024 µg 1024 µg</td>
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</tr>
<tr>
<td>Roxarsone</td>
<td>Disc (6 mm) diffusion assay</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>128 µg 128 µg</td>
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<td></td>
</tr>
<tr>
<td>CuSO₄ (50 mM)</td>
<td>5 mm 4.5 mm</td>
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<td></td>
</tr>
<tr>
<td>ZnSO₄ (50 mM)</td>
<td>4.5 mm 4.5 mm</td>
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<tr>
<td>Iron chelation</td>
<td>Disc (6 mm) diffusion assay</td>
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<tr>
<td>Desferal (0.05 mM)</td>
<td>24 mm 22 mm</td>
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<td></td>
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<tr>
<td>Dipyridyl (0.05 mM)</td>
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<tr>
<td>Chemotaxis for L-fucose</td>
<td>By determining the zone of attraction on 0.4% PBS soft agar</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Adherence to INT407 cells</td>
<td>By CFU determination</td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

ND indicates no difference

**Table 3.4.** Phenotypes with no significant difference between the WT and the ∆ppk2 mutant.
Figure 3.1. The Δppk2 mutant is defective in poly P-dependent GTP synthesis. GTP synthetic activity was determined spectrophotometrically using a modified enzyme coupled assay. The assay was repeated 3 times with 3 replicates in each assay and the data was expressed as mean ± SE. * P≤0.05.
Figure 3.2. The Δppk2 mutant exhibits increased intracellular ATP:GTP ratio. Poly P-dependent ATP synthesis was determined spectrophotometrically using a modified enzyme coupled assay. ATP synthesis was measured for up to 10 min. Only data for 5 and 10 min are shown. The assay was repeated 3 times with 3 replicates in each assay and the data was expressed as mean ± SE. * \( P \leq 0.05 \).
Figure 3.3. The \( \Delta ppk2 \) mutant shows similar ppGpp levels to that of WT under nutrient downshift. The amount of ppGpp accumulation was assessed by growing the bacterial strains in MH broth microaerobically to early log phase (OD\(_{600}\) of 0.25) and labeling in nutrient poor medium (MOPS-MGS) with \(^{32}\)P. Nucleotides were resolved by TLC and visualized using autoradiography.
**Figure 3.4.** Poly P accumulation in the WT and Δppk2 mutant at different growth phases.

The amount of poly P in the cell was determined by toluidine blue O method. Each data point is the mean ± SE of 3 independent experiments. **P≤0.01.**
**Figure 3.5.** Survival of *C. jejuni* under nutrient downshift. Survival under nutrient stress was assessed by growing bacterial strains in MEM and determining the CFU at different time points. Each data point represents the mean ± SE of 3 independent experiments. *P ≤ 0.05 and **P ≤ 0.01.
Figure 3.6. Survival of *C. jejuni* under osmotic stress. Survival under osmotic stress in liquid culture was assessed in MH broth containing 0.25 M NaCl. Each data point represents the mean ± SE of 3 independent experiments. * $P \leq 0.05$ and ** $P \leq 0.01$. 
Figure 3.7. Survival of *C. jejuni* under osmotic stress in solid medium. Survival in solid medium was determined by spotting $5 \times 10^2$-$5 \times 10^5$ CFU onto MH agar containing 0.17 M NaCl.
Figure 3.8. Survival of *C. jejuni* under aerobic stress. Survival of the *C. jejuni* 81-176 WT and Δppk2 mutant was assessed by determining the CFU of aerobically grown bacteria at different time points. Each data point represents the mean ± SE of 3 independent experiments. * P≤0.05 and ** P≤0.01.
Figure 3.9. Culturability and viability of the *C. jejuni* strains after formic acid treatment. Culturability was determined by plating formic acid treated bacteria on MH agar at different time points. Viability was assessed by measuring OD$_{600}$ after CTC staining. Each data point represents the mean ± SE of 3 independent experiments. * $P \leq 0.05$. 
Figure 3.10. The Δppk2 mutant shows enhanced biofilm formation. Biofilm formation was visualized by staining with crystal violet.
Figure 3.11. Quantification of biofilm using DMSO. The amount of biofilm formed was dissolved in 1 ml DMSO for 48 h and quantified by measuring absorbance at 570 nm. Each bar represents the mean ± SE of 3 independent experiments. ** $P \leq 0.01$. 
Figure 3.12. Quantitative RT-PCR analysis of the WT and Δppk2 mutant. Fold differences in transcript levels were assessed from ΔΔCT after normalization using rpoA. Each bar represents the mean ± SE of relative fold change in expression from 3 independent experiments. * $P \leq 0.05$. 
Figure 3.13. The Δppk2 mutant displays a dose-dependent invasion defect in INT407 human intestinal epithelial cells. The data represents the average of 2 experiments with 3 replicates in each experiment. * $P \leq 0.05$. 
The Δppk2 mutant is defective in intracellular survival in INT407 cells. INT407 cells were infected with 100:1 MOI of bacteria. Intracellular survival of *C. jejuni* was determined after 24 h of incubation. The data represents the average of 2 independent experiments with 3 replicates in each experiment. **$P \leq 0.01$.**
Figure 3.15. The Δppk2 mutant exhibits a dose-dependent colonization defect in day-old chicks at $10^3$ inoculation dose. Eight days after inoculation, the chicks were sacrificed; cecum, feces and bursa were harvested and colonization level was assessed by determining the CFU/g of tissue. Each data point represents $\log_{10}$ CFU/g of tissue. Average CFU for each dose is denoted by a line. The dotted line indicates minimum detection limit of 50 CFU. ** $P<0.01$. 
Figure 3.16. The Δppk2 mutant exhibits a dose-dependent colonization defect in day-old chicks at $10^4$ inoculation dose. Eight days after inoculation, the chicks were sacrificed; cecum, feces and bursa were harvested and colonization level was assessed by determining the CFU/g of tissue. Each data point represents $\log_{10}$ CFU/g of tissue. Average CFU for each dose is denoted by a line. The dotted line indicates minimum detection limit of 50 CFU. * $P \leq 0.05$ and ** $P \leq 0.01$. 
Figure 3.17. The Δppk2 mutant exhibits a dose-dependent colonization defect in day-old chicks at $10^5$ inoculation dose. Eight days after inoculation, the chicks were sacrificed; cecum, feces and bursa were harvested and colonization level was assessed by determining the CFU/g of tissue. Each data point represents $\log_{10}$ CFU/g of tissue. Average CFU for each dose is denoted by a line. The dotted line indicates minimum detection limit of 50 CFU. * $P \leq 0.05$. 
Figure 3.18. Growth kinetics of the *C. jejuni* 81-176 WT and Δppk2 mutant as assessed by CFU determination. Each data point represents the mean ± SE of 3 independent experiments.
Figure 3.19. Phylogram of PPK2 from *C. jejuni* and its near neighbors. Branch lengths are indicated next to the protein name and are proportional to the predicted evolutionary change. Phylogram was constructed using ClustalW2.
**Figure 3.20.** Structure-based sequence alignment of PPK2 domains from *C. jejuni*, *P. aeruginosa*, *M. tuberculosis* and *M. smegmatis*. Strictly conserved residues are highlighted by white letters on grey background. The conserved motifs Walker A and

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Walker B are indicated by triangles and squares, respectively. Lid module is indicated by dashed line. *indicates residues critical for PPK2 catalysis. PA0141 and PA2428-\textit{P. aeruginosa} 1-domain PPK2 paralogs; PA3455-C- C-terminal domain of \textit{P. aeruginosa} 2-domain PPK2 paralog PA3455; PA3455-N- N-terminal domain of \textit{P. aeruginosa} 2-domain PPK2 paralog PA3455; PA3455-N- N-terminal domain of \textit{P. aeruginosa} 2-domain PPK2 paralog PA3455; CJJ81176_0632/633-\textit{C. jejuni} PPK2; rv3232c-\textit{M. tuberculosis} PPK2 and SMEG_0891-\textit{M. smegmatis} PPK2. Sequence alignment was performed using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html).
Figure 3.21. Predicted three-dimensional structure of *C. jejuni* PPK2. Three-dimensional structure was identified with vector alignment search tool (www.ncbi.nlm.nih.gov/Structure/VAST/vast.shtml) using *P. aeruginosa* PPK2 paralog PA3455 as reference. Walker A, Walker B and lid module are indicated by letters A, B and C in yellow, respectively. The region in pink or red indicates *C. jejuni* PPK2 residues identical to PA3455. The region in grey indicates unaligned sequences of *C. jejuni*. 
Figure 3.22. *C. jejuni* PPK2 superimposed on *P. aeruginosa* PPK2 paralog PA3455. Note that PA3455 has 4 domains (PA3455 is a 2-domain PPK2 and exists as a dimer). C and N indicate C- and N-terminal domains. *C. jejuni* PPK2 superimposes only with the C-terminal domain of PA3455. Superimposed structures were obtained using VAST and Cn3D structure and sequence alignment viewer.
CHAPTER IV

Preliminary Analysis of Exopolyphosphatase/Guanosine Pentaphosphate Phosphohydrolase Enzymes in *Campylobacter jejuni*

Dharanesh Gangaiah and Gireesh Rajashekara

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4.1 Abstract

The Gram-negative bacterium *Campylobacter jejuni* is one of the predominant causes of bacterial foodborne gastroenteritis in humans worldwide. Despite its significance as a major foodborne pathogen, our understanding of the molecular mechanisms underlying *C. jejuni* pathogenesis and survival under stress is limited. Inorganic polyphosphate (poly P), a biological macromolecule composed of multiple phosphate residues, plays important roles in bacterial survival under conditions of stress and virulence. Polyphosphate kinase 1 (PPK1) is the principal enzyme involved in poly P synthesis and polyphosphate kinase 2 (PPK2) utilizes poly P to generate GTP. While, exopolyphosphatases (PPX) degrade poly P to inorganic phosphate. The genome of *C. jejuni* possesses homologs of ppk1, ppk2 and 2 ppx/gppa genes. Previously, we have characterized the significance of PPK1 and PPK2 to *C. jejuni* biology. Here, we investigated the role of PPX/GPPA enzymes in *C. jejuni* response to stress and virulence using deletion mutants of ppx1/gppa and ppx2/gppa. Despite their defect in motility, Δppx1/gppa and Δppx2/gppa mutants showed similar capacity to form biofilm under static conditions. Additionally, *C. jejuni* Δppx/gppa mutants showed defect in survival under nutrient limitation. However, deletion of ppx/gppa genes did not affect *C. jejuni* ability to survive under osmotic and oxidative stresses. Despite their enhanced capacity to accumulate poly P, Δppx/gppa mutants exhibited similar growth kinetics compared to that of wildtype in rich medium. In summary, our preliminary analysis suggests an important role for PPX/GPPA enzymes in *C. jejuni* biology.
4.2 Introduction

*Campylobacter jejuni* is one of the most common causes of bacterial diarrhea in humans worldwide (Allos, 2001). *C. jejuni* causes self-limiting gastroenteritis which is typically characterized by symptoms such as headache, fever, abdominal pain, myalgia, watery or bloody diarrhea and rarely vomiting. Additionally, a post-infection complication such as GBS is also thought to occur in 1 in 1000 individuals infected with *C. jejuni* (Jacobs *et al.*, 2008). Despite a cause of serious intestinal disease in humans, *C. jejuni* harmlessly colonizes the gut of many wild and domestic animals and birds, particularly poultry. Nearly 90% of the commercial chicken carcasses are contaminated with *C. jejuni* in the United States (Jacobs-Reitsma, 2008). Consequently, poultry and poultry products are considered as the major food sources for human *C. jejuni* infection. Though *C. jejuni* infections are common in humans, relatively little is known about the pathogenic and stress survival mechanisms of *C. jejuni* compared to that for other enteric pathogens such as *E. coli* and *Salmonella*.

Inorganic polyphosphate (poly P), a polymer of phosphate residues, is a regulator of bacterial tolerance to stresses and virulence in many pathogenic bacteria (Kornberg *et al.*, 1999; Rao *et al.*, 2009). Several enzymes are involved in the metabolism of poly P in bacteria. Polyphosphate kinase 1 (PPK1) catalyzes the reversible synthesis of poly P, while polyphosphate kinase 2 (PPK2) mediates the synthesis of GTP from poly P. PPK1 has been shown to be essential for diverse bacterial functions including stress survival and virulence (Rao *et al.*, 2009). Similarly, PPK2, which has been studied in a limited
number of bacteria, has also been shown to mediate stress responses and virulence (Sureka et al., 2009; Ishige et al., 2002; Kim et al., 2002).

Poly P is degraded to inorganic phosphate by exopolyphosphatases (PPXs) (Rao et al., 2009). Two types of PPX enzymes have been identified in bacteria, namely the classical PPX enzymes which possess primarily exopolyphosphatase activity and PPX/GPPA enzymes which have both exopolyphosphatase and guanosine pentaphosphatase phosphorylase activities (Kristensen et al., 2004; Rao et al., 2009; Rangarajan et al., 2006). In addition to poly P hydrolysis, PPX/GPPA enzymes hydrolyze guanosine pentaphosphate (pppGpp) to guanosine tetraphosphate (ppGpp). ppGpp is an intracellular second messenger that mediates stringent response in bacteria.

PPX enzymes play an important role in poly P homeostasis in several bacterial species (Lindner et al., 2009; Akiyama et al., 1993; Bolesch and Keasling, 2000; Rangarajan et al., 2006). In addition, PPX enzymes have been shown to mediate bacterial survival under conditions of stress as well as contribute to virulence. For example, PPX has been shown to be essential for motility, biofilm formation, and sporulation in Bacillus cereus (Shi et al., 2004). Similarly, PPX was shown to be important for resistance to complement-mediated killing in Neisseria meningitides (Zhang et al., 2010). Furthermore, PPX2/GPPA enzymes play a critical role in bacterial stringent response induced by starvation (Reizer et al., 1993; Kuroda et al., 1997; Rao et al., 2009; Keasling et al., 1993; Kristensen et al., 2004 and 2008).
C. jejuni genome harbors two genes that encode PPX/GPPA class of enzymes. However, to our knowledge, the role of these enzymes in C. jejuni patho-physiology has not been investigated previously. Here, we provide evidence that both PPX1/GPPA and PPX2/GPPA are important for C. jejuni motility and survival under nutrient limitation. However, PPX/GPPA enzymes were found to play no role in C. jejuni biofilm formation and tolerance to osmotic and oxidative stresses. Despite their enhanced capacity to accumulate poly P, ∆ppx1/gppa and ∆ppx2/gppa mutants exhibited similar growth kinetics compared to that of wildtype in rich medium. In summary, our preliminary analysis suggests an important role for PPX/GPPA enzymes in C. jejuni biology.

4.3 Materials and Methods

Bacterial strains, media and growth conditions

The strains and plasmids used in this study are listed in Table 4.1. C. jejuni strain 81-176 was used to generate ppx1/gppa and ppx2/gppa mutants. C. jejuni strains were grown on Mueller-Hinton broth (MH; Oxoid) microaerobically [(85% N₂ (v/v), 10% CO₂ (v/v) and 5% O₂ (v/v)] in a DG250 Microaerophilic Workstation (Microbiology International) at 42 °C. For growth curve and stress survival assays, C. jejuni was grown in MH broth with appropriate antibiotics at 37 °C with shaking at 200 rpm. E. coli DH5α was used for plasmid propagation and cloning purposes. E. coli was routinely cultured on Luria-Bertani (LB) medium at 37 °C overnight. Growth media was supplemented with appropriate antibiotics, chloramphenicol (20 µg/ml for E. coli; 10 µg/ml for Campylobacter), kanamycin (30 µg/ml) and zeocin (50 µg/ml), where necessary.
General cloning techniques

Cloning and other molecular biology techniques were performed according to Sambrook and Russel (2001). Oligonucleotides were designed using Vector NTI® software (Invitrogen, Carlsbad, CA) and commercially synthesized by Integrated DNA Technologies (Skokie, IL). All the oligonucleotides used in the present study are listed in Table 4.2. Masterpure® DNA purification kit and Fast-Link DNA ligation kit were purchased from Epicentre (Madison, WI). Restriction enzymes were purchased from Promega (Madison, WI). QIAquick® PCR purification kit and QIAprep® spin mini prep kit for plasmid isolation were purchased from Qiagen (Valencia, CA). Zero background cloning vector pZErO-1 and E. coli DH5α competent cells were purchased from Invitrogen.

Targeted deletion of ppx/gppa genes

Deletion of ppx1/gppa (CJJ81176_0377) and ppx2/gppa (CJJ81176_1251) was performed as described previously (Gangaiah et al., 2009). Briefly, the target genes along with 1 kb flanking regions were amplified by PCR from C. jejuni 81-176 genomic DNA using PPX1/GPPA_F or PPX2/GPPA_F and PPX1/GPPA_R or PPX2/GPPA_R primers. The PCR product was ligated into pZErO-1 and inverse PCR was performed on the resulting vector (pDG6/pDG8) using PPX1/GPPA-INV_F/PPX2/GPPA-INV_F and PPX1/GPPA-INV_R/PPX2/GPPA-INV_R primers to delete majority of the coding sequence. The inverse PCR product was then ligated with kanamycin cassette from pUC4K and the resulting suicide vector pDG7/pDG9 was electroporated into C. jejuni
81-176 as described (Wilson et al., 2003). Recombinants were selected on MH agar plates containing kanamycin, kanamycin resistant colonies were streak purified and one such mutant designated DG005 (\(\Delta ppx1/gppa\)) or DG006 (\(\Delta ppx2/gppa\)) was used for further studies. The deletion of the \(ppx1/gppa\) or \(ppx2/gppa\) gene was confirmed by PCR.

**Growth curve assay**

Growth kinetics of *C. jejuni* \(ppx/gppa\) mutants was assessed as described previously (Gangaiah et al., 2010). Briefly, mid-log phase grown cultures of *C. jejuni* were diluted to an \(OD_{600}\) of 0.05 in 5 ml of MH broth in borosilicate tubes and incubated microaerobically at 42 °C with shaking at 200 rpm. The growth was assessed by CFU determination at different time points.

**Motility assay**

In order to assess the motility of \(ppx/gppa\) mutants, a 2 µl of culture containing \(5 \times 10^7\) CFU/ml (\(OD_{600}\) of approx. 0.05) was stabbed onto 0.4% soft agar and incubated under microaerobic conditions at 42 °C. After 24 h of incubation, the swarming zone surrounding the stabbed area was measured.

**Biofilm formation**

Biofilm formation by *C. jejuni* \(ppx/gppa\) mutants was assessed as described previously (Candon et al., 2007; Gangaiah et al., 2009; McLennan et al., 2008). Briefly, 100 µl of 0.05 \(OD_{600}\) culture was inoculated into MH broth and incubated at 42 °C microaerobically without shaking. After 2 days of incubation, biofilm formation was
visualized by staining with 250 µl of 1% (w/v) crystal violet for 15 min. The amount of biofilm formed was quantified by measuring the absorbance at 570 nm after dissolving in 1 ml DMSO for 48 h.

**Stress survival assays**

We determined the ability of *C. jejuni ppx/gppa* mutants to survive under nutrient limitation as described previously (Candon *et al.*, 2007; Gangaiah *et al.*, 2009). Briefly, mid-log phase grown bacterial cultures were pelleted, washed twice with MEM and resuspended in MEM to an OD$_{600}$ of 0.05. After incubation microaerobically at 42 °C with shaking at 200 rpm, we assessed the survival of bacterial strains in MEM by CFU determination.

The ability of *ppx/gppa* mutants to tolerate osmotic stress was determined as described previously (Candon *et al.*, 2007; Gangaiah *et al.*, 2009). Briefly, mid log-phase bacterial cultures were serially diluted (10-fold) and a 10 µl of diluted culture was 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 0.17M NaCl was assessed.

The ability of *C. jejuni ppx/gppa* mutants to survive under oxidative stress was assessed by disk assay (Tunpiboonsak *et al.*, 2010). Briefly, the bacterial strains were spread for confluent growth on MH agar and a 6 mm disc soaked with 20 µl of 1% hydrogen peroxide was placed onto the center of plate. After the plates were incubated under microaerobic conditions at 42 °C for 24 hours, the clear zones of sensitivity surrounding the disc were measured.
Poly P assay

Poly P extraction and quantification was performed as described previously (Candon et al., 2007; Gangaiah et al., 2010). Briefly, poly P was extracted using glassmilk from stationary phase cultures and quantified using toluidine blue O method by measuring the ratio of 530 to 630 nm spectrophotometrically.

4.4 Results

PPX/GPPA phosphatases in C. jejuni

The BLAST analysis of C. jejuni 81-176 genome revealed the presence of two putative genes encoding PPX/GPPA family phosphatases. The PPX1/GPPA encoded by CJJ81176_0377 possesses 42, 28, 26 and 26% amino acid sequence similarity to Helicobacter pylori PPX/GPPA family phosphatase, Aquifex aeolicus exopolyphosphatase, Escherichia coli guanosine pentaphosphate hydrolase and Staphylococcus epidermidis PPX/GPPA family phosphatase, respectively. Similarly, PPX2/GPPA encoded by CJJ81176_1251 possesses 29 and 26% amino acid sequence similarity to exopolyphosphatase from Corynebacterium glutamicum and A. aeolicus, respectively. C. jejuni ppx/gppa genes are conserved among various C. jejuni strains (Fig. 4.1 and 4.2) and Campylobacter species with a sequence similarity ranging from 98-100% and 32-86%, respectively.

The crystal structure analysis of the PPX/GPPA from A. aeolicus revealed the presence of two domains with the active site located at the interface between the two
domains (Kristensen et al., 2004 and 2008). The predicted three dimensional structures of C. jejuni PPX/GPPA enzymes revealed two domains similar to A. aeolicus (Figure 4.13). The two domains of A. aeolicus PPX/GPPA can be flexibly moved with a single hinge providing the accessibility to the catalytic site as well as the dual substrate specificity. It is said that the dual specificity of this enzyme is determined by the distinct and different degrees of closure between the two domains of the enzyme. Alanine substitution mutagenesis revealed that E119 is critical for PPX activity. Though a similar mutagenesis study has not been conducted for (p)ppGpp hydrolytic activity, Kristensen et al. (2008) opines that this residue might be a common catalytic site for both PPX activity and pppGpp hydrolysis considering its position and easy accessibility to pppGpp (Kristensen et al., 2008). Additionally, arginine residues R22 and R267 were found to be crucial for guanosine pentaphosphate specificity. Sequence alignment of C. jejuni PPX/GPPA enzymes with PPX/GPPA enzymes from other bacteria revealed that the catalytic residue E119 is highly conserved (Figure 4.11, 4.12 and 4.13), suggesting that these proteins have a common catalytic mechanism. In addition, the arginine residues R22 and R267 that are crucial for guanosine pentaphosphate specificity are also conserved in C. jejuni PPX1/GPPA (Figure 4.11 and 4.13), suggesting a role for PPX1/GPPA in both poly P and (p)ppGpp hydrolysis. However, only R22 but not R267 is conserved in PPX2/GPPA (Figure 4.11).

PPX/GPPA enzymes are required for C. jejuni motility but not for biofilm formation.
We assessed if deletion of ppx/gppa genes affects the motility in *C. jejuni*. Both \( \Delta \text{ppx1/gppa} \) and \( \Delta \text{ppx2/gppa} \) mutants were significantly \( (P \leq 0.05) \) defective in their motility when grown on semi solid agar (Figure 4.3 and 4.4). The \( \Delta \text{ppx1/gppa} \) and \( \Delta \text{ppx2/gppa} \) mutants exhibited 1.5-fold and 1.35-fold defect in motility compared to wildtype strain, respectively. Previously, it has been shown that deletion of *ppx* in *Bacillus cereus* impaired its motility (Shi et al., 2004).

Despite the motility defect, quantification of biofilm using DMSO revealed that *C. jejuni* \( \Delta \text{ppx/gppa} \) mutants had similar amount of biofilm compared to wildtype (Figure 4.5 and 4.6). However, visually a thinner biofilm ring was observed in the \( \Delta \text{ppx/gppa} \) mutants at the liquid-air interface compared to wildtype, possibly indicating the decreased capacity of these mutants to survive under aerobic conditions. Several previous studies have shown the lack of correlation between *C. jejuni* motility and the ability to form biofilm (Candon et al., 2007; Gaynor, et al., 2005; McLennan et al., 2008; Gangaiah et al., 2009; Gangaiah et al., 2010). Reuters et al. (2010) showed that aflagellate strains also displayed increased biofilm formation under aerobic conditions, suggesting that flagella are not essential for *C. jejuni* biofilm formation; however, flagella may improve or facilitate initial attachment or biofilm structuring.

**C. jejuni \( \Delta \text{ppx1/gppa} \) and \( \Delta \text{ppx2/gppa} \) mutants are defective in survival under nutrient limitation**

We assessed the contribution of PPX/GPPA enzymes to *C. jejuni* survival under nutrient stress by monitoring the survival of \( \Delta \text{ppx1/gppa} \) and \( \Delta \text{ppx2/gppa} \) mutants in
minimal essential medium (MEM without glutamine). Both $\Delta ppx1/gppa$ and $\Delta ppx2/gppa$ mutants were significantly ($P \leq 0.05$) impaired in survival in MEM compared to wildtype (Figure 4.7). Following exposure to nutrient stress, the $\Delta ppx1/gppa$ mutant had 1.3-, 1.9-, 1.9-, 2.4- and 0.6-log fewer bacteria than the WT strain at 12, 24, 36, 48 and 60 h, respectively. Similarly, the $\Delta ppx2/gppa$ mutant exhibited 1.4-, 2.5-, 2.1-, 1.3- and 1.0-log fewer bacteria than the WT strain at 12, 24, 36, 48 and 60 h, respectively.

**C. jejuni $\Delta ppx1/gppa$ and $\Delta ppx2/gppa$ mutants exhibit enhanced intracellular poly P levels**

In order to determine if PPX/GPPA enzymes contribute to poly P hydrolysis in *C. jejuni*, we quantified the intracellular poly P levels in $\Delta ppx/gppa$ mutants using toluidine blue O method. Our results indicated that both $\Delta ppx1/gppa$ and $\Delta ppx2/gppa$ mutants displayed enhanced accumulation of poly P compared to wildtype strain (Figure 4.8). However, only $\Delta ppx1/gppa$ mutant showed statistically significant increase in poly P compared to wildtype ($P \leq 0.05$). The $\Delta ppx1/gppa$ and $\Delta ppx2/gppa$ mutants exhibited 1.45-fold and 1.2-fold increase in poly P accumulation compared to wildtype strain, respectively.

### 4.5 Discussion

A dynamic balance between poly P synthesis and its degradation is critical for normal functioning of bacterial cell (Kornberg *et al.*, 1999). PPK1 catalyzes the synthesis of poly P leading to its accumulation, while PPX enzyme along with PPK1 removes poly P to maintain the dynamic balance (Kornberg *et al.*, 1999). PPK1 reversibly catalyzes
poly P utilization to generate ATP although synthesis of poly P is favored. In addition to poly P hydrolysis, PPX enzymes also hydrolyze pppGpp to ppGpp and ppGpp is an intracellular second messenger that mediates stringent response in bacteria (Rao et al., 2009). The classical PPX enzymes primarily hydrolyze poly P to inorganic phosphate but are also thought to provide auxiliary pppGppase activity (Kuroda et al., 1997). PPX/GPPA enzymes hydrolyze both poly P and pppGpp and thus play an essential role in regulating both poly P- and ppGpp-dependent functions in bacteria. Here, we characterized two genes that encode PPX/GPPA enzymes in C. jejuni and our preliminary data suggests that these enzymes might play an essential role in C. jejuni survival under conditions of stress (Figure 4.7) as well as virulence (Figure 4.3 and 4.4).

Bacteria respond to stresses in a variety of ways and stringent response induced under nutrient limitation is one of the best studied stress responses (Cashel et al., 1996). 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. Stringent response is mediated by ppGpp which serves as a signaling molecule to coordinate changes in transcription, protein synthesis, and degradation. In E. coli, ppGpp is synthesized from pppGpp by the gppa gene product guanosine pentaphosphate phosphohydrolase (GPPA) (Keasling et al., 1993). pppGpp itself is synthesized by RelA or SpoT from ATP and GTP. Unlike other enteric bacteria, C. jejuni stringent response is mediated by a single enzyme SpoT and this enzyme is thought to mediate both pppGpp synthesis and hydrolysis (Gaynor et al., 2005). Like many other bacteria, C. jejuni has been shown to
accumulate ppGpp in response to nutrient limitation. *C. jejuni Δppx/gppa* mutants were defective in survival under nutrient stress (Figure 4.7) but not osmotic (Figure 4.9) and oxidative (Figure 4.10) stresses compared to wildtype, which suggests a possible role for PPX/GPPA enzymes in *C. jejuni* survival under nutrient stress probably by regulating ppGpp-mediated stringent response. However, the role of PPX/GPPA enzymes in ppGpp-mediated stringent response needs to be experimentally confirmed. Both ppGpp and pppGpp are 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.

Stringent response and the metabolism of poly P are closely connected to each other (Kristensen *et al.*, 2008; Kuroda *et al.*, 1997). It has been shown that both pppGpp and ppGpp affect poly P accumulation in *E. coli* in response to amino acid starvation by inhibiting PPX (Kuroda *et al.*, 1997; Rao *et al.*, 1998). *C. jejuni Δppx/gppa* mutants exhibited enhanced ability to accumulate poly P compared to wildtype (Figure 4.8), suggesting a role for PPX/GPPA enzymes in poly P degradation in *C. jejuni*. However, further confirmation of poly P hydrolytic activity of PPX/GPPA enzymes is required. The accumulated poly P subsequently associates with Lon protease causing degradation of free ribosomal proteins to compensate for the lack of amino acids (Kuroda *et al.*, 2001). Thus both ppGpp and poly P accumulate in response to nutrient limitation and play an essential role in bacterial survival under diverse stress conditions.
In addition to mediating stringent response, ppGpp also has been shown to mediate virulence and pathogenesis-related phenotypes such as long term persistence, symbiosis, motility, quorum sensing, production of antibiotics, biofilm formation, virulence and sporulation in bacteria (Dalebroux et al., 2010). For example, a ppGpp\(^0\) strain was defective in motility on 0.3% soft agar and electron microscopy analysis showed that ppGpp\(^0\) strain did not produce flagella (Magnusson et al., 2007). Consequently, the defective motility in *C. jejuni* ∆ppx/gppa mutants (Figure 4.3 and 4.4) might be related to the possible defect in pppGpp hydrolysis. Additionally, it was noted that *C. jejuni* ∆ppx1/gppa mutant exhibited more defect in survival under nutrient limitation (Figure 4.7) and motility (Figure 4.3 and 4.4), and accumulated more poly P (Figure 4.8) compared to ∆ppx2/gppa mutant, suggesting that PPX1 possibly is the major PPX/GPPA phosphatase in *C. jejuni*. Alignment of *C. jejuni* ppx1/gppa sequence (Figure 4.11) with similar enzymes from other bacteria indicated that both the catalytic residue (E119) required for PPX activity and arginine residues (R22 and R267) necessary for ppGpp specificity are conserved in *C. jejuni* PPX1/GPPA, suggesting that this enzyme might mediate both poly P and (p)ppGpp hydrolysis. On the contrary, the catalytic residue (E119) and only R22 but not R267 are conserved in *C. jejuni* PPX2/GPPA (Figure 4.11), suggesting that this enzyme is less likely a primary mediator of (p)ppGpp hydrolysis.

In summary, PPX/GPPA enzymes might play an essential role in poly P metabolism, survival under conditions of stress and virulence in *C. jejuni*. However, further experimental confirmation of pppGpp and poly P hydrolytic activities of
PPX/GPPA enzymes and complementation of defective phenotypes is required. In addition, generation of a double knockout mutant lacking both ppx1/gppa and ppx2/gppa genes will aid in better understanding of these genes in C. jejuni patho-physiology.

4.6 Acknowledgements

We would like to thank Donnese Franklin and Jasmine Cheeks for help with construction of mutants and phenotypic assays.
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<th>Strain/Plasmid</th>
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<td>WT strain of <em>Campylobacter jejuni</em></td>
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<td><em>E. coli</em> strain used for cloning</td>
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<td>Cloning vector for making suicide vector; Zeo</td>
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<td>pDG9</td>
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Kan, kanamycin; Zeo, zeocin

**Table 4.1.** Bacterial strains and plasmids used in this study
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**Table 4.2.** Primers used in this study
Figure 4.1. Genetic organization of \textit{ppx1/gppa} in \textit{C. jejuni} genome. \textbf{A}. Location of \textit{ppx1/gppa} is conserved among different \textit{C. jejuni} strains. \textbf{B}. Approximate location of kanamycin marked \textit{ppx1/gppa} deletion in \textit{C. jejuni} strain 81-176. The \textit{fdxB} encodes for ferredoxin.
Figure 4.2. Genetic organization of ppx2/gppa in C. jejuni genome. A. Location of ppx1/gppa is conserved among different C. jejuni strains. B. Approximate location of kanamycin marked ppx2/gppa deletion in C. jejuni strain 81-176. The pdxJ encodes for pyridoxine 5’-phosphate synthase and the pdxA encodes for 4-hydroxythreonine-4-phosphate dehydrogenase.
**Figure 4.3A.** Motility of *C. jejuni Δppx/gppa* mutants as determined on 0.4% soft agar.

These images are representative of 3 independent experiments.
Figure 4.3B. Quantification of motility defect in *C. jejuni* Δppx/gppa mutants. Motility was quantified by measuring the swarming zone (radius in mm) surrounding the stabbed area. Each bar represents the average from 3 independent experiments. * $P \leq 0.05$. 
Figure 4.4A. Biofilm formation by *C. jejuni* Δppx/gppa mutants. Biofilm formation was visualized by staining with 1% (w/v) crystal violet for 15 minutes. This image is representative of 3 independent experiments.
Figure 4.4B. Quantification of biofilm in Δppx/gppa mutants. The amount of biofilm was quantified by measuring the absorbance at 570 nm after dissolving in 1 ml DMSO for 48 h. Each bar represents the mean ± SE of 3 independent experiments.
**Figure 4.5.** The *C. jejuni* Δppx/gppa mutants are defective in survival under nutrient limitation. Sensitivity of *C. jejuni* ppx/gppa mutants to nutrient stress was assessed by monitoring their survival in minimal essential media at different time points. Each data point represents the mean ± SE of 3 independent experiments. * indicates the statistical significance (*P*≤0.05) for both Δppx1/gppa and Δppx2/gppa mutants.
Figure 4.6. Intracellular poly P levels in *C. jejuni* ∆ppx/gppa 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.
Figure 4.S1. Osmotic stress tolerance of *C. jejuni* Δppx/gppa mutants. Survival of *C. jejuni* ppx/gppa mutants under osmotic stress was determined by spotting 5×10²-5×10⁵ CFU onto MH agar containing 0.17 M NaCl. This image is representative of 3 independent experiments.
Figure 4.S2. Sensitivity of Δppx/gppa mutants to oxidative stress. Sensitivity to oxidative stress was determined by disc diffusion assay using 1% hydrogen peroxide. The value on the image indicates the zone of inhibition measured after 24 hours of incubation. These images are representative of 3 independent experiments.
Figure 4.S3A. Structure-based sequence alignment of PPX1/GPAA domains from *C. jejuni*, *H. pylori*, *A. aeolicus*, *S. epidermidis* and *E. coli*. “*” character indicates positions which have a single, fully conserved residue. “:” character indicates that the column is strongly conserved. “.” Character indicates that the column is weakly conserved. Catalytic residue required for phosphatase activity is highlighted by white letter on black background. The residues required for guanosine pentaphosphate specificity are highlighted by black letters on grey background. CJI81176_0377- *C. jejuni* PPX1/GPPA family phosphatase; HPG27_257- *H. pylori* PPX/GPPA family phosphatase; aq_891- *A. aeolicus* exopolyphosphatase; SE2033- *S. epidermidis* PPX/GPPA family phosphatase; CJI81176_1251- *C. jejuni* PPX2/GPPA phosphatase; cg0488- *C. glutamicum* exopolyphosphatase and ECS88_4200- *E. coli* Guanosine pentaphosphate hydrolase. Sequence alignment was performed using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html).
Figure 4.S3B. Phylogram of PPX/GPPA enzymes from *C. jejuni* and their near neighbors. Branch lengths are indicated next to the protein name and are proportional to the predicted evolutionary change. CJJ81176_0377 - *C. jejuni* PPX1/GPPA family phosphatase; HPG27_257 - *H. pylori* PPX/GPPA family phosphatase; CJJ81176_1251 - *C. jejuni* PPX2/GPPA phosphatase; aq_891 - *A. aeolicus* exopolyphosphatase; SE2033 - *S. epidemridis* PPX/GPPA family phosphatase; ECS88_4200 - *E. coli* Guanosine pentaphosphate hydrolase and cg0488 - *C. glutamicum* exopolyphosphatase. Phylogram was constructed using ClustalW2.
**Figure 4.S4.** Predicted three-dimensional structure of *C. jejuni* PPX/GPPA enzyme. Three-dimensional structure was identified with vector alignment search tool (www.ncbi.nlm.nih.gov/Structure/VAST/vast.shtml) using *A. aeolicus* PPX/GPPA as reference. The catalytic residue E119 is indicated by letter A and the residues R22 and R267 required for guanosine pentaphosphate specificity are indicated by letters B and C, respectively. 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.
CHAPTER IV

Summary and Conclusions
Summary and Conclusions

Poly P, a polymer of phosphate residues, plays a central role in bacterial tolerance to stresses and virulence in many bacterial pathogens. Polynphosphate kinase 1 (PPK1) is the principal enzyme involved in poly P synthesis; while polyphosphate kinase 2 (PPK2) utilizes poly P to generate GTP. Exopolyphosphatases (PPX) degrade poly P to inorganic phosphate. We used deletion mutagenesis approach to study the role of ppk1, ppk2 and ppx/gppa genes to various C. jejuni functions.

Deletion of ppk1 encoding polyphosphate kinase 1 compromised C. jejuni ability to accumulate poly P, survive under stress conditions and form viable-but-nonculturable cells under acid stress. In addition, the Δppk1 mutant also showed decreased frequency of natural transformation and increased susceptibility to various antimicrobials. Furthermore, the Δppk1 mutant was characterized by a dose-dependent deficiency in chicken colonization. Our results suggest that poly P plays an important role in stress survival and adaptation and might contribute to genome plasticity, and spread and development of antimicrobial resistance in C. jejuni.

Additionally, this study showed that PPK2 is important for poly P-dependent generation of GTP as well as maintaining intracellular nucleotide balance in C. jejuni. Deletion of ppk2 resulted in decreased ability of C. jejuni to tolerate osmotic, nutrient, aerobic, and antimicrobial stresses. However, the Δppk2 mutant was not defective in poly P and ppGpp synthesis suggesting that PPK2-mediated stress tolerance is not ppGpp-mediated. Importantly, the Δppk2 mutant was significantly attenuated in invasion and
intracellular survival within human intestinal epithelial cells as well as chicken colonization. In summary, we have highlighted the role of PPK2 as a novel pathogenicity determinant that is critical for \textit{C. jejuni} survival, adaptation, and persistence in the host environments.

Findings from this study also demonstrated that PPX2/GPPA enzymes play a role in \textit{C. jejuni} motility and nutrient stress tolerance. Despite their defect in motility, \textit{}\Delta ppx1/gppa \textit{and} \textit{\Delta ppx2/gppa} mutants showed similar capacity to form biofilm under static conditions. Additionally, quantification of intracellular poly P by toluidine blue O method revealed that \textit{\Delta ppx/gppa} mutants accumulated more poly P than the wildtype strain, suggesting a role for PPX/GPPA enzymes in poly P degradation. Our preliminary data suggests that PPX/GPPA enzymes might play an essential role in \textit{C. jejuni} survival under conditions of stress as well as virulence.

Though, the exact mechanism by which poly P, PPK1 and PPK2 mediate stress response and virulence in \textit{C. jejuni} is unknown, this study adds to our understanding of the contribution of poly P-associated proteins in \textit{C. jejuni} adaptation and survival under different stringencies in the absence of RpoS-mediated classical stress response mechanisms. Findings from this study and several previous studies have allowed us to develop a hypothetical model illustrating poly P and phosphate metabolism and their relation to stringent response factors in \textit{C. jejuni} (Figure 5.1). Briefly, phosphate esters are hydrolyzed to inorganic phosphate (Pi), which is the preferred source of phosphate for most bacteria, by alkaline phosphatase in the periplasm. Pi uptake into the cell is
mediated by phosphate uptake proteins (PstSCAB). ATP generated from Pi will be utilized for poly P synthesis by PPK1. PPK2 utilizes poly P to generate GTP, while PPX hydrolyzes poly P back to Pi. SpoT synthesizes pppGpp using GTP and ATP. pppGpp will be converted to ppGpp, a molecule that mediates stringent response in \textit{C. jejuni} and other bacteria. SpoT also mediates hydrolysis of ppGpp to GDP and inorganic phosphate. NDK, an enzyme involved in phosphate transfer from ATP to other NTPs, may interact with PPK2 to preferentially synthesize GTP. PPK1 may interact with SpoT to regulate its functions. Phosphate uptake proteins and alkaline phosphatase are directly regulated by PhosS/PhosR two-component system. Dashed lines and arrows indicate possible direct or indirect interaction of proteins. Question mark indicates pathways predicted based on the experimental evidence from other bacteria.

\textbf{Future directions}

Several lines of evidence suggest that poly P might be involved in global regulation of bacterial genes that provide survival advantages in the host as well as environment. First, poly P mediates numerous and diverse biological functions in many bacterial pathogens. Second, several previous studies have revealed differential expression of selected genes involved in stress response and virulence in \textit{ppk1} mutant. Third, poly P binds strongly to the principal sigma factor of RNA polymerase to mediate starvation/stress response in \textit{H. pylori}, modulates cAMP receptor protein, a global regulator, to mediate oxidative stress response in \textit{Salmonella} Typhimurium and shows a strong affinity to histone like proteins, suggesting a probable role for poly P in regulation
of gene expression. A recent comparative proteomic analysis by two-dimensional gel electrophoresis of \textit{Pseudomonas sp.} B4 showed that 81 proteins were differentially expressed in poly P-deficient compared to control cells. The most compelling evidence is provided by microarray analysis of \textit{ppk1} mutants in \textit{P. aeruginosa} and \textit{E. coli} which revealed up-regulation of over 250 genes and down-regulation of more than 450 genes (unpublished data quoted in Rao \textit{et al.}, 2009). Similarly, PPK2 was also shown to regulate the expression of over 20 genes in \textit{P. aeruginosa} (unpublished data quoted in Rao \textit{et al.}, 2009), suggesting a role for PPK2 in regulation of gene expression in bacteria. Therefore, studies involving high throughput assays such as microarray or deep RNA sequencing would reveal new insights into the mechanistic aspects of PPK1- and PPK2-mediated functions in \textit{C. jejuni}. Since GTP serves as an important molecule for the synthesis of polysaccharides, studies directed to understand the impact of PPK2 in the regulation of \textit{C. jejuni} glycoconjugates such as glycosylated proteins and lipids would reveal mechanisms behind PPK2 contribution to \textit{C. jejuni} physiology. Understanding the mechanisms underlying how PPX/GPPA enzymes regulate poly P homeostasis and stringent response would provide more insights into \textit{C. jejuni} survival under unfavorable conditions.
**Figure 5.1.** Hypothetical model illustrating poly P metabolism in *C. jejuni.*
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Appendix A: Effect of *ppk1* Mutation on *C. jejuni* Motility

Motility assay was performed by stabbing 2 µl of culture containing $5 \times 10^7$ CFU/ml (OD$_{600}$ of ~ 0.05) onto 0.4% soft agar and measuring the swarming zone after 24 h of incubation under microaerobic conditions at 42 ºC.
Appendix B: Motility Zone (inches) Assessed after 24 h of Incubation

<table>
<thead>
<tr>
<th>SI No.</th>
<th>WT</th>
<th>Δppk1</th>
<th>Δppk1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>3.5</td>
<td>2.5</td>
<td>3.25</td>
</tr>
<tr>
<td>Average</td>
<td>3.75±0.25</td>
<td>2.75±0.25</td>
<td>3.625±0.38</td>
</tr>
</tbody>
</table>

The data represents the average from 2 independent experiments.
**Appendix C**: The $\Delta ppk1$ Mutant Shows Enhanced Biofilm Formation

Biofilm formation was visualized by staining with 1% crystal violet in 95% ethanol.
Appendix D: Survival of \( \Delta ppk1 \) Mutant under Oxidative Stress

A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>( \Delta ppk1 )</th>
<th>( \Delta ppk1c )</th>
</tr>
</thead>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>( \Delta ppk1 )</th>
<th>( \Delta ppk1c )</th>
</tr>
</thead>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>( \Delta ppk1 )</th>
<th>( \Delta ppk1c )</th>
</tr>
</thead>
</table>
C. jejuni survival under oxidative stress was determined by plating 100 µl of bacterial culture containing 5×10^8 CFU/ml (OD_{600} of 0.5) on MH agar and placing a 6 mm disc soaked with 20 µl of paraquat or hydrogen peroxide onto the center of plate. The plates were incubated under microaerobic conditions at 42 ºC for 24 hours and the sensitivity zones were measured. **A.** Sensitivity of Δppk2 mutant to 5 mM paraquat. **B.** Sensitivity of Δppk2 mutant to 0.03% hydrogen peroxide. **C.** Sensitivity of Δppk2 mutant to 1% hydrogen peroxide.
Appendix E: Genetic Organization of *ppk2* in *C. jejuni* Genome

Genetic organization of *ppk2* in *C. jejuni* genome. **A.** Approximate location of *ppk2* in relation to *ppk1, ppx1* and *ppx2* in *C. jejuni* genome. **B.** Location of *ppk2* is conserved among different *C. jejuni* strains. **C.** Approximate location of kanamycin marked *ppk2* deletion in *C. jejuni* strain 81-176.
## Appendix F: Metabolic Profile of the Wildtype and Δppk2 Mutant

<table>
<thead>
<tr>
<th>AN MicroPlate™</th>
<th>Wildtype</th>
<th>Δppk2</th>
<th>GN2 MicroPlate™</th>
<th>Wildtype</th>
<th>Δppk2</th>
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</thead>
<tbody>
<tr>
<td>1. Acetic acid</td>
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<td>+</td>
<td>1. Pyruvic acid methyl ester</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2. Formic acid</td>
<td>+</td>
<td>+</td>
<td>2. Succinic acid mono-methyl ester</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3. Fumaric acid</td>
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<td>+</td>
<td>3. Cis-Aconitic acid</td>
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<td>+</td>
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<tr>
<td>4. Glyoxylic acid</td>
<td>+</td>
<td>+</td>
<td>4. Citric acid</td>
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</tr>
<tr>
<td>5. Hydroxybutyric acid</td>
<td>+</td>
<td>+</td>
<td>5. Acetic acid</td>
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<td>+</td>
</tr>
<tr>
<td>7. L-Lactic acid</td>
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<td>+</td>
<td>7. Hydroxybutyric acid</td>
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<td>+</td>
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<tr>
<td>8. D-Lactic acid methyl ester</td>
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<td>8. α-Ketoglutaric acid</td>
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<td>10. Succinic acid</td>
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<td>12. Pyruvic acid</td>
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<td>12. Succinamic acid</td>
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<td>13. Pyruvic acid methyl ester</td>
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<td>13. L-Asparagine</td>
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<td>+</td>
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<tr>
<td>15. Succinic acid</td>
<td>+</td>
<td>+</td>
<td>15. Glycyl-L-Aspartic acid</td>
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<td>+</td>
</tr>
<tr>
<td>16. Succinic acid mono-methyl ester</td>
<td>+</td>
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<td>16. L-Proline</td>
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<td>18. L-Asparagine</td>
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<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. L-Glutamic acid</td>
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<td></td>
</tr>
<tr>
<td>20. L-Glutamine</td>
<td>+</td>
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<tr>
<td>21. Glycyl-L-Aspartic acid</td>
<td>+</td>
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<td></td>
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<td>22. L-Valine plus L-aspartic acid</td>
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<td></td>
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<td>23. L-Serine</td>
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</tr>
<tr>
<td>24. L-Fucose</td>
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</tbody>
</table>
Substrate utilization was assayed using Biolog GN2 and AN (Biolog Inc., Hayward, CA) identification systems. Briefly, the bacterial strains were grown to mid-log phase in MH agar with 5% laked horse blood and adjusted to an OD$_{600}$ of 0.75. One hundred microliters of the culture was then added into each well in the AN/GN2 plate and incubated at 37° C for 24 h. The color change resulting from reduction of tetrazolium violet by bacterial activity was determined by measuring the optical density at 490 nm. The experiment was repeated three times with 2 replicates each time.