POLYPHOSPHATE KINASE 1 (PPK1) IS A PATHOGENESIS DETERMINANT IN *CAMPYLOBACTER JEJUNI*

by

Heather L. Candon
B.Sc., The University of Waterloo, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Microbiology)

THE UNIVERSITY OF BRITISH COLUMBIA

July, 2007

© Heather L. Candon, 2007
ABSTRACT

Campylobacter jejuni (C. jejuni) is the leading cause of bacterial gastroenteritis in the developed world. Despite the prevalence of C. jejuni as a human pathogen, relatively little is known about its precise pathogenesis mechanisms, particularly in comparison to other well-studied enteric pathogens like E. coli and Salmonella spp. Altered expression of phosphate genes in a C. jejuni stringent response mutant, together with known correlations between the stringent response, polyphosphate (poly P), and virulence in other pathogens, led us to investigate the role of poly P in C. jejuni physiology and pathogenesis. All sequenced C. jejuni strains harbour a conserved putative polyphosphate kinase (PPK1) predicted to be principally responsible for poly P synthesis. We generated a targeted ppkl deletion mutant (Δppkl) in C. jejuni strain 81-176 and found that this mutant, as well as the ΔspoT stringent response mutant, exhibited low levels of poly P at all growth stages. In contrast, wild-type C. jejuni poly P levels increased significantly as the bacteria transitioned from log to stationary phase. Phenotypic analyses revealed that the Δppkl mutant was defective for survival during osmotic shock and low-nutrient stress. However, certain phenotypes associated with ppkl deletion in other bacteria (i.e., motility, oxidative stress) were unaffected in the C. jejuni mutant, which also displayed a surprising increase in biofilm formation. The C. jejuni Δppkl mutant was also defective for the virulence-associated phenotype of intra-epithelial cell survival in a tissue culture infection model and exhibited a striking defect in dose-dependent chick colonization. These results indicate that poly P utilization and accumulation contribute significantly to C. jejuni pathogenesis and affect its ability to adapt to specific stresses and stringencies. Furthermore, our study demonstrates that poly P likely plays both similar and unique roles in C. jejuni compared to other bacteria, and that poly P metabolism is linked with stringent response mechanisms in C. jejuni.
1.0. INTRODUCTION

1.1. Campylobacter jejuni: Background

1.2. C. jejuni characteristics

1.3. Epidemiology

1.4. Campylobacteriosis

1.4.1. Disease symptoms

1.4.2. Medical sequelae

1.4.3. Treatment and control of infection

1.5. Transmission and prevention

1.6. Molecular mechanisms of C. jejuni pathogenesis

1.6.1. Virulence factors

1.6.1.1. Motility

1.6.1.2. Chemotaxis

1.6.1.3. C. jejuni-host cell association and invasion

1.6.1.4. pVIR

1.6.1.5. Sensing and regulation

1.6.1.6. Toxin production

1.6.1.7. Lipopolysaccharide and capsule

1.6.1.8. Biofilms

1.6.1.9. C. jejuni stringent response

1.7. Bacterial polyphosphate metabolism

1.7.1. Interaction of poly P and stringent response in E. coli
1.8. Hypothesis ................................................................. 17

2.0. MATERIALS AND METHODS ........................................ 18

2.1. Bacterial strains and growth conditions .......................... 18
2.2. Construction of \( C. \) jejuni 81-176 \( ppkl \) targeted deletion mutant .......... 18
2.3. Complementation of \( ppkl \) deletion mutant ....................... 19
2.4. Poly P Glassmilk Extraction ...................................... 20
2.5. Measurement of poly P levels by a Toluidine Blue O assay ....... 20
2.6. Nutrient down-shift survival assay ............................... 21
2.7. Osmotic stress survival assay .................................... 21
2.8. Static biofilm formation assay .................................. 22
2.9. INT407 cell infection assay for invasion and intracellular survival .... 22
2.10. Chick colonization assays ....................................... 22
2.11. Additional phenotypic assays for which the \( \Delta ppkl \) mutant was not different from wild-type ......................... 24

3.0. RESULTS .................................................................. 26

3.1. Introduction and rationale ........................................... 26
3.2. Poly P interacts with the stringent response in \( C. \) jejuni .......... 26
3.3. The \( \Delta ppkl \) is defective for poly P accumulation ............... 29
3.4. Poly P is critical for \( C. \) jejuni survival during nutritional down-shift .... 33
3.5. Poly P accumulation is required for \( C. \) jejuni to survive osmotic shock .... 35
3.6. The \( \Delta ppkl \) mutant displays accelerated static biofilm formation .... 37
3.7. The \( \Delta ppkl \) mutant is defective for prolonged intracellular survival .... 39
3.8. The \( \Delta ppkl \) mutant has reduced colonization capacity in a chick model .... 41
3.9. Phenotypes for which the \( \Delta ppkl \) mutant was not different from wild-type 43

4.0. DISCUSSION AND CONCLUSIONS ............................... 49

4.1. Future directions ....................................................... 55

Bibliography .................................................................. 57

Appendices .................................................................. 70

Appendix A .................................................................. 70
Appendix B .................................................................. 70
Appendix C ........................................................................................................70
Appendix D ........................................................................................................70
LIST OF TABLES

Table 1  Phenotypes that were indistinguishable between wild-type and Δppkl ....43
<table>
<thead>
<tr>
<th>Figure 1</th>
<th>At later growth stages, phosphate transport and metabolism genes are disregulated in the ΔspoT mutant versus wild-type C. jejuni.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2</td>
<td>Poly P levels in the ΔspoT mutant remain lower than wild-type C. jejuni.</td>
</tr>
<tr>
<td>Figure 3</td>
<td>C. jejuni ppkl and generation of a single insert Δppkl disruption strain.</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Poly P accumulates in wild-type and ppkl* strains at later growth stages but remains at low levels in the Δppkl mutant.</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Poly P is important for C. jejuni survival during nutritional downshift.</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Poly P is required for C. jejuni osmotic stress survival.</td>
</tr>
<tr>
<td>Figure 7</td>
<td>The Δppkl mutant exhibits increased static biofilm formation.</td>
</tr>
<tr>
<td>Figure 8</td>
<td>The Δppkl mutant is defective for long-term intracellular survival in an epithelial cell model of infection.</td>
</tr>
<tr>
<td>Figure 9</td>
<td>The Δppkl mutant exhibited a 7 day, dose dependent defect for chick caecal colonization.</td>
</tr>
<tr>
<td>Figure 10</td>
<td>The Δppkl mutant did not have a motility defect in 0.3% and 0.5% agar.</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Wild-type C. jejuni and the Δppkl mutant displayed similar oxidative stress sensitivity.</td>
</tr>
<tr>
<td>Figure 12</td>
<td>The Δppkl mutant was equally sensitive to the organic acids propionic acid and acetic acid as wild-type.</td>
</tr>
<tr>
<td>Figure 13</td>
<td>The Δppkl mutant was not different from wild-type C. jejuni in low iron conditions.</td>
</tr>
<tr>
<td>Figure 14</td>
<td>The Δppkl mutant and wild-type C. jejuni grow identically in 0.16 M NaCl in MH broth.</td>
</tr>
<tr>
<td>Figure 15</td>
<td>The Δppkl mutant and wild-type C. jejuni grow identically in MH broth.</td>
</tr>
<tr>
<td>Figure 16</td>
<td>The Δppkl mutant and wild-type C. jejuni grow identically under normal atmospheric conditions.</td>
</tr>
<tr>
<td>Figure 17</td>
<td>The Δppkl mutant and wild-type C. jejuni grow identically under anaerobic atmospheric conditions.</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

\( \Delta \text{ppkl} \) — polyphosphate kinase gene 1 deletion
\( \Delta \text{spoT} \) — spoT gene deletion
A\text{530} — Absorbance at 530 nm
A\text{630} — Absorbance at 630 nm
ATP — adenosine 5'-triphosphate
BHI — brain heart infusion
bp — base pair
C. coli — Campylobacter coli
C. jejuni — Campylobacter jejuni
CAT — chloramphenicol acetyltransferase
CFUs — colony forming units
Cm — chloramphenicol
Cm\text{R} — chloramphenicol resistance
dH\text{2}O — distilled water
DNA — deoxyribonucleic acid
E. coli — Escherichia coli
GBS — Guillain-Barré syndrome
GDP — guanosine diphosphate
GITC — guanidine isothiocyanate
GTP — guanosine triphosphate
H. pylori — Helicobacter pylori
INT407 — human intestinal 407
LB — Luria-Bertani
M — molar
MEM — Minimal Essential Media
MH — Mueller-Hinton
mM — millimolar
MOI — multiplicity of infection
MOPS — morpholine propane sulfonic acid
nmol — nanomolar
OD\text{560} — optical density at 560 nm
OD\text{600} — optical density at 600 nm
P. aeruginosa — Pseudomonas aeruginosa
Poly P — polyphosphate
ppGpp — guanosine tetra-phosphate
ppkl — polyphosphate kinase gene 1
PPK1 — polyphosphate kinase protein 1
ppkl* — Complemented of ppkl deletion mutant
ppk2 — polyphosphate kinase gene 2
PPK2 — polyphosphate kinase protein 2
PPX — exopolyphosphatase
ppx — exopolyphosphatase gene
PVC — polyvinyl chloride microtitre
r.p.m — revolutions per minute
SR — stringent response
TBO — Toluidine Blue O
V. cholerae — Vibrio cholerae
ACKNOWLEDGEMENTS

This thesis is the culmination of three years of work in the Department of Microbiology and Immunology at the University of British Columbia, which would not have been possible without the help, advice and support of many.

I would like to express my sincere appreciation and thanks to my advisor and mentor, Dr. Erin Gaynor, who encouraged and supported me throughout the duration of my research. Your constructive guidance, knowledge, insight, understanding and enthusiasm are very much appreciated. I have gained so much by being able to work with you.

Thank you to my thesis committee Dr. George Spiegelman, Dr. Bill Mohn and Dr. Jim Kronstad. Also, my appreciation to all the members of the Gaynor laboratory, past and present, who made my graduate school experience so enjoyable; particularly Sarah Svensson, who is a good friend, and who was always willing to share a laugh and discuss the peripheral issues. Also, Cres Fraley for insightful discussion and Dr. Brenda Allen at VIDO for performing the chick colonization assays.

To my Waterloo friends for the soul food— Kevin Hofstee, Colleen Bator, Kirsty Rennie, Kathleen Waller, Laura Brown, and Jean Yoon. Particularly, a heartfelt thanks to Nicky Potopsingh, my dearest and closest friend. Despite the distance between us, our regular conversations and discussions are a source of great fortitude, happiness and solace for me.

Deepest thanks to my family for their unwavering support and strength. Mom and Dad, you always made my education a priority and raised me to strive for happiness above all else. Thank you for the infinite words of wisdom, consoling phone calls, and for believing in me every step of the way.

To my fiancé Jason George, thank you for your incredible patience and understanding. You have supported me on this long journey as I have forged forward to accomplish my goals. Words cannot describe how grateful I am to have you by my side. Your love and support carried me through some of the roughest times and decisions.
DEDICATION

To my Father – my faithful friend and supporter.
1.0. INTRODUCTION

1.1. CAMPYLOBACTER JEJUNI: BACKGROUND

_Campylobacter jejuni_ (C. jejuni) is a Gram-negative, highly motile, microaerophilic, fastidious bacterium, and is now considered the leading cause of human gastroenteritis in the developed world (Altekruse et al., 1999; Skirrow, 1994; Smibert et al., 1974). The genus name _Campylobacter_ is derived from the Greek word meaning ‘curved rods’. Although _Campylobacter_ was first observed in 1886 by Escherich in the colons of neonates, it was not until the mid-1970s that _Campylobacter_ was recognized as an important human pathogen (Kist, 1986). First classified as ‘Vibrio fetus’ based on morphology and occurrence in abortive farm animals, _Campylobacter fetus_ was later renamed based on biochemical tests and DNA content (Sebald and Veron, 1963; Smith and Taylor, 1919; Veron, 1973). The first selective isolation of _Campylobacter_ spp. was accomplished in 1968 from the feces of a human diarrheic patient (Dekeyser et al., 1972). The genus _Campylobacter_ belongs to the class ε-proteobacteria and is a member of the recently defined family _Campylobacteraceae_ (Vandamme, 2000). Along with _Campylobacter_, the _Campylobacteraceae_ family contains the genera _Helicobacter_, _Arcobacter_, _Wolinella_, and _Flexispira_. At present, the genus _Campylobacter_ contains 14 species including _C. jejuni_, _C. coli_, _C. fetus_, _C. lari_, _C. hyointestinalis_, _C. upsaliensis_, _C. helveticus_, _C. mucosalis_, _C. concisus_, _C. curvus_, _C. rectus_, _C. showae_, _C. sputorum_, and _C. gracilis_ (Vandamme, 2000). Although several _Campylobacter_ species have been shown to cause gastroenteritis in humans, _C. jejuni_ is the species most frequently implicated in infections (Butzler, 2004).

1.2. _C. JEJUNI_ CHARACTERISTICS

_C. jejuni_ is a spiral-bacillus shaped bacterium that displays rapid darting motility via two polar flagella. However, upon late stationary phase growth or prolonged exposure to oxygen, cells transition morphologically from spirals to cocci where they maintain a viable but non-
Culturable state before becoming inviable (Rollins and Colwell, 1986). *C. jejuni* is moderately thermophilic, microaerophilic, capnophilic, and demonstrates fastidious laboratory growth requirements. Growth is optimal at 37°C and 42°C, where the ideal atmosphere is 5% O₂, 10% CO₂, and 85% N₂ (Nachamkin and Barbagallo, 1990; Shane and Montrose, 1985).

*Campylobacter* spp. can vary in length from 0.5 to 8 μm and 0.2 to 0.5 μm in width (Sebald and Veron, 1963). *C. jejuni*, along with other *Campylobacteraceae*, has a relatively small genome (~1.6 Mbp) with a low guanine and cytosine DNA content ranging from 29 to 37 mol% (Parkhill *et al.*, 2000). The small genome size is perhaps reflected in this organism’s requirement for complex growth media and the inability to ferment or oxidize carbohydrates, but instead acquire energy from amino acids or tricarboxylic acid cycle intermediates (Griffiths and Park, 1990; Kettle, 1997; Vandamme, 2000).

1.3. Epidemiology

*Campylobacter* infection is the most common cause of bacterial gastroenteritis in developed countries, affecting more individuals than *E. coli* O157:H7, *Salmonella* spp., and *Shigella* spp. combined (Altekruse *et al.*, 1999; Blaser *et al.*, 1983; Blaser, 1997; WHO, 2000). Current reports suggest that campylobacteriosis accounts for 5-15% of all diarrheal illnesses worldwide, affecting ~1% of the Canadian, U.S., U.K., and Australian populations annually (Adak *et al.*, 2005; NIAID, 2005; Yohannes *et al.*, 2004). *C. jejuni* is estimated to account for 90% of reported campylobacteriosis cases, 17% of all hospitalizations related to food-borne illnesses in North America, and 5% of food-related deaths (Mead *et al.*, 1999). Human *Campylobacter* isolates collected from Canadian patients from 1995 to 1999 were 99.4% *C. jejuni* or *C. coli* (HealthCanada, 2003). Canadian incidence rates for 2000 were approximately 40 per 100,000 population (CDC, 2003). In developing countries, where infection often goes...
unreported, the incidence of *C. jejuni* infection is even higher and represents a major cause of gastroenteritis for travellers from developed countries (Griffiths and Park, 1990; Tauxe, 1992).

Interestingly, the incidence of *C. jejuni* infection in the U.S. is considerably higher during the summer months, with an estimated 80% of cases occurring between May and August (Allos and Blaser, 1995; Allos, 2001; Ketley, 1997). In developing nations infection is endemic and outbreaks are not seasonal (Allos, 2001). *C. jejuni* can affect all ages, yet infants and young adults are reported to have the highest incidence rate of infection (Allos and Blaser, 1995). In developing countries, *C. jejuni* predominantly affects young children, while teenagers and adults are usually asymptomatic carriers (Lindblom *et al.*, 1995). While in industrialized countries infections occur primarily in < 1 year of age and during young adulthood at 15-44 years of age, asymptomatic carriage in any age group is rare (Allos, 2001). Interestingly, the demographics of *C. jejuni* infection is unlike any other enteric pathogen, in that males are more likely to experience infection than women and the reason for this preponderance is unknown (Friedman, 2001). Death as a result of *C. jejuni* infection is unusual at 0.05 per 1000 infections (Allos, 2001).

### 1.4. *CAMPYLOBACTERIOSIS*

#### 1.4.1. Disease symptoms

*C. jejuni* lives harmlessly in the intestinal microflora of most mammals and birds (Beery *et al.*, 1988). However, upon infecting a human host, *C. jejuni* invades the intestinal mucosa, interrupts intestinal integrity, and causes profuse watery and/or bloody diarrhea (Butzler and Skirrow, 1979). In humans, the jejunum and ileum are colonized initially followed by infection of the colon (Allos and Blaser, 1995). Experimentation in humans revealed that the infectious dose is as low as 500-800 organisms and infection rate increases with increasing dosage (Black *et al.*, 1988).
Clinical manifestations of campylobacteriosis can vary between individuals from asymptomatic to severe gastroenteritis (Blaser, 1997; Butzler and Skirrow, 1979). This dichotomy may be due to variations in strain virulence or host immune response to infection (Ketley, 1997). The incubation period following *C. jejuni* ingestion is between 1-7 days and is inversely related to dose of infection (Allos *et al.*, 1998; Blaser, 1997). Symptoms are usually self-limiting, lasting for several days and ranging from abdominal cramps, watery and frequently bloody diarrhea, fever, nausea and vomiting. Isolation of *C. jejuni* from stool is necessary to differentiate sickness from other enteric pathogens (Allos *et al.*, 1998). Continued shedding in feces lasts approximately 16 days, and relapse occurs in 20% of campylobacteriosis cases (Fields and Swerdlow, 1999).

**1.4.2. Medical sequelae**

In rare cases, campylobacteriosis has been correlated with a number of other medical sequelae such as reactive arthritis, hemolytic uremic syndrome, and inflammatory bowel disease (Butzler and Skirrow, 1979). Most patients develop inflammation of the ileum or the jejunum, but some patients, particularly young adults, may develop peritonitis from acute appendicitis (Butzler and Skirrow, 1979; Crushell *et al.*, 2004). Direct spread of *C. jejuni* from the gastrointestinal tract has been linked to complications such as cholecystitis, pancreatitis, and gastrointestinal hemorrhage (Allos, 2001). Infections outside of the gastrointestinal tract can include meningitis, endocarditis, septic arthritis, osteomyelitis, bacteremia and neonatal sepsis (Allos, 2001). Although bacteremia is rare, it is observed in 1% of patients and is more likely to occur in infants, the elderly or immunocompromised hosts (Butzler, 2004).

The most notable complication of *C. jejuni* infection is Guillain-Barré syndrome (GBS), an acute neuromuscular flaccid paralysis (Crushell *et al.*, 2004; Hughes, 2004). It is estimated that 30-40% of GBS patients experienced prior *C. jejuni* infections (Allos, 1998; Butzler, 2004).
GBS symptoms usually manifest 1-3 weeks after convalescence of enteritis symptoms (Butzler, 2004). O-side chain serotyping studies have demonstrated that specific serotypes of *C. jejuni* are associated with GBS (Konkel et al., 2001). *C. jejuni*-related GBS cases are currently thought to result from an autoimmune humoral response whereby antibodies produced against *C. jejuni* lipo-oligosaccharides cross-react with nearly identical structures on peripheral nerve gangliosides (Nachamkin et al., 1998; Nachamkin, 2002). *C. jejuni* has also been linked to cases of Miller Fisher syndrome, a rare variant of GBS characterized by onset of paralysis of the extraocular muscles and an absence of neurological reflexes (Endtz et al., 2000).

### 1.4.3. Treatment and control of infection

Acute *C. jejuni* infection is usually self-limiting and resolves with in 1-2 weeks; however, severe, complicated, or systemic infections of immunocompromised hosts generally require antibiotic therapy to quell infection (Nachamkin et al., 2002). The majority of strains are naturally resistant to antibiotics commonly used against other Gram-negative bacteria; these include trimethoprim, rifampicin and the penicillin family. Antibiotic therapy for *C. jejuni* infections consists of macrolide or fluoroquinolone treatment with either erythromycin or ciprofloxacin, respectively (Allos, 1998, 2001; Butzler and Skirrow, 1979; Butzler, 2004). Chemotheraputics are also thought to help prevent GBS in healthy individuals. Currently, there is no effective vaccine for the prevention or control of campylobacteriosis, and supportive treatment involves hydration and maintenance of electrolyte balance.

At present, there is rapid and widespread emergence of fluoroquinolone-resistant strains, and this is significantly limiting the utility of these drugs. Reports indicate that 90% of *C. jejuni* isolates from Thailand and other countries are found to be fluoroquinolone-resistant (Nachamkin et al., 2002). This increase in resistance coincides with countries introducing fluoroquinolones into veterinary medicine and the poultry industry for use in food animals (Endtz et al., 1991).
Erythromycin is considered the drug of choice in treating *C. jejuni* as resistant rates remain low (Allos, 2001).

1.5. TRANSMISSION AND PREVENTION

*Campylobacter* spp. are naturally zoonotic and reside commensally in the intestinal mucosa of a wide variety of wild and domestic animals. *C. jejuni* colonizes the ceca of birds in extremely high numbers (Newell, 2000); consequently, consumption and handling of meat from broiler chickens is a considerable risk factor in transmission (Allos, 2001; Friedman, 2001). *C. jejuni* is such a prevalent commensal among poultry flocks that up to 98% of commercial chicken and turkey products contain live *C. jejuni* (Newell, 2000; Willis and Murray, 1997). It has been estimated that a single drop of raw chicken juice may contain hundreds of live organisms (Hood *et al.*, 1988).

Other modes of transmission include contact with infected animals or through undercooked food, unpasteurized milk or contaminated water, as was in the case in Walkerton, Ontario, where fecal run-off contaminated clean water supplies leading to a community-wide outbreak of campylobacteriosis (Altekruse *et al.*, 1999; Friedman, 2001). Shedding from avian hosts can contaminate waterways and recreational swimming pools. *C. jejuni* is able to survive for weeks in environmental waters and is capable of colonizing hosts at temperatures of 37°C or 42°C; a temperature variability that allows for infecting a range of vertebrate hosts (Bereswill and Kist, 2003). Person-to-person spread via fecal-oral transmission is rare, but has occurred among young children with diarrhea in child-care centers (Goossens *et al.*, 1995).

Prevention of *C. jejuni* infections in humans can be accomplished by reducing risk factors for acquisition. Strategies for the control and prevention of poultry colonization at the farm level are considered an important approach to the reduction or elimination of campylobacteriosis in humans. Such strategies include decreasing animals’ consumption of antibiotics, disinfection of
animal food and water, treatment of manure, and isolation of sick animals (Allos, 2001).

Interestingly, studies have found that treatment of carcass wash water with active chlorine, organic acids, sodium chloride, and tri-sodium phosphate seems to reduce *C. jejuni* contamination (Altekruse *et al.*, 1999). However, proper food preparation, thoroughly cooking poultry and attention to hand hygiene appear to be the most important actions in preventing *C. jejuni* human infection (Allos, 2001).

1.6. **MOLECULAR MECHANISMS OF *C. JEJUNI* PATHOGENESIS**

1.6.1. **Virulence factors**

Despite the prevalence of *C. jejuni* infection, the molecular mechanisms by which *C. jejuni* causes human disease as well as to adapt to or survive stresses encountered during both *in vivo* colonization and *ex vivo* transmission are not well understood, particularly in comparison to other well-studied pathogens such as *Escherichia coli*, *Salmonella* spp., and *Helicobacter pylori*. Our current limited understanding of *C. jejuni* is largely due to its fastidious growth requirements, interstrain virulence variability, and intractability to genetic manipulation. Moreover, the *C. jejuni* NCTC11168 genome sequence, published in 2000, revealed that *C. jejuni* lacks many virulence characteristics and factors found in other bacterial pathogens, such as pathogenicity islands, type III secretion systems and certain stress response factors like RpoS (Parkhill *et al.*, 2000).

1.6.1.1. **Motility**

As mentioned previously, *C. jejuni* possesses two polar flagella and exhibits a characteristic rapid darting motility. The sequenced genome of *C. jejuni* NCTC 11168 highlighted the importance of motility in pathogenesis as a large portion of the genome encodes factors related to flagellum biosynthesis and chemotaxis (Parkhill *et al.*, 2000). The *C. jejuni*
flagellum consists of a basal body, hook, and filament; where the filament is comprised of two adjacent proteins FlaA and FlaB (Crushell et al., 2004). The flaA gene is expressed at a higher level than flaB (Ketley, 1997). A functional Flaa protein is required for C. jejuni motility and invasion and translocation of polarized cell monolayers in vitro (Fields and Swerdlow, 1999; Grant et al., 1993; Wassenaar et al., 1991). A controversial study in humans highlighted the importance of C. jejuni motility in vivo; volunteers were challenged with a mixture of a motile and non-motile phase-variant of C. jejuni and only motile strains were recovered from fecal samples (Black et al., 1988). Motility is thought to help the bacteria penetrate the mucosal layer of the intestinal epithelium and ultimately invade host cells (Wassenaar and Blaser, 1999).

1.6.1.2. Chemotaxis

Chemotaxis is the movement of an organism toward or away from a chemical stimulus. C. jejuni has chemotactic ability and can move up and down a chemical gradient (Ketley, 1997). Chemotaxis is an important virulence determinant in C. jejuni. Studies investigating colonization capacity of a C. jejuni chemotactic mutant found that this function was required for colonizing the intestine of mice (Takata et al., 1992). A number of genes have been identified as being involved in C. jejuni chemotaxis, one in particular being cheY. A C. jejuni chemotactic mutant in the regulatory gene, cheY, resulted in reduced colonization and disease in an animal model of infection (Yao et al., 1997). Conceivably, the reduced virulence of the cheY mutant was due to an inability to move toward the chemoattractant mucin (Hugdahl et al., 1988).

1.6.1.3. C. jejuni-host cell association and invasion

In the human host, C. jejuni causes disease by penetrating the intestinal mucosa, and adhering to and invading intestinal epithelial cells. C. jejuni can interact with host cells directly by microtubule-dependent invasion, translocation, or toxin-mediated cell cycle arrest (Bereswill
and Kist, 2003; Hu and Kopecko, 1999; Ketley, 1997; Kopecko et al., 2001; Lara-Tejero and Galan, 2000). Furthermore, host cell damage can occur indirectly by *C. jejuni* eliciting a host immune response (Bereswill and Kist, 2003). *C. jejuni* has been shown to induce interleukin-8 release from intestinal epithelial cells (Hickey et al., 1999). Human colonic epithelial cells exposed to *C. jejuni* show altered gene expression, suggesting that *C. jejuni* induces a host-specific response (Rinella et al., 2006). Damage to the intestinal mucosa and epithelial cell lining can cause persistent inflammation following resolution of infection (Everest et al., 1993; Russell et al., 1993).

Several reports have shown that adhesions are critical for *C. jejuni* colonization. The fibronectin binding protein, CadF, is highly conserved among all *C. jejuni* isolates and was shown to be involved in host cell adherence and invasion (Konkel et al., 1997; Konkel et al., 1999). PEB1 is an outer membrane adhesion protein, which is a homolog of Gram-negative amino acid transport systems. A PEB1 *C. jejuni* mutant displayed decreased adherence and invasion *in vitro* and reduced colonization *in vivo* (Pei et al., 1998). Other adhesion proteins include JlpA (*jejuni* lipoprotein A), major outer membrane protein (MOMP), P95, flagellum, and potentially the lipopolysaccharide and capsule (Jin et al., 2001; Kelle et al., 1998; McSweegan and Walker, 1986; Moser et al., 1992; Moser et al., 1997). The flagella are needed for motility in reaching host cells and establishing contact (Wassenaar et al., 1991). *C. jejuni* does not appear to produce pili or fimbriae that would assist in colonization (Gaynor et al., 2001; Parkhill et al., 2000).

*C. jejuni* is often considered an extracellular pathogen, yet it can invade and translocate through polarized monolayers. Invasion and translocation of intestinal epithelial cells *in vitro* correlates strongly with *C. jejuni* virulence *in vivo* (Bacon et al., 2000; Everest et al., 1992; Konkel et al., 1992). Interestingly, 86% of *C. jejuni* colitis-causing strains were able to translocate across polarized Caco-2 cell monolayers versus 48% of clinical isolates from non-
inflammatory disease patients (Everest et al., 1992). Upon internalization *C. jejuni* is often found in vacuoles and in the cytoplasm of invaded epithelial cells and has been shown to survive for up to 36 hours in epithelial cells and 6-7 days in macrophages (Day et al., 2000; Konkel et al., 1992; Russell et al., 1993; Wassenaar et al., 1997). Superoxide dismutase (SodB) and catalase activity (KatA) are known to participate in intramacrophage survival. To date, only the *C. jejuni* stringent response (SpoT) and Fe\(^{2+}\) iron transporter (FeoB) are known to be involved in long-term survival within epithelial cells (Gaynor et al., 2005; Naikare et al., 2006). Intracellular survival of *C. jejuni* is thought to be important for disease, immune evasion, relapse, and long-term persistence (De Melo et al., 1989; Russell et al., 1993).

### 1.6.1.4. pVIR

The presence of a pVIR plasmid in a subset of *C. jejuni* strains appears to correlate with invasiveness *in vitro*. pVIR is a 37.5 Kb plasmid that contains 54 open reading frames with many genes orthologous to components of a putative type IV secretion system (Bacon et al., 2000; Bacon et al., 2001). Mutations in two putative type IV secretion genes on the pVir plasmid in *C. jejuni* strain 81-176 resulted in decreased invasion of human intestinal epithelial cells *in vitro*, in addition to a reduction in natural transformation frequency (Bacon et al., 2000). The presence of the pVIR plasmid in strains of *C. jejuni* may increase an individuals risk for developing bloody stool; however, the correlation between pVIR and virulence *in vivo* remains somewhat unclear (Louwen et al., 2006; Tracz et al., 2005).

### 1.6.1.5. Sensing and Regulation

Although *C. jejuni* has fastidious *in vitro* nutritional and atmospheric CO\(_2\) and O\(_2\) requirements, it can survive for long periods in sub-optimal environments, including those outside of its natural zoonotic host. The mechanisms by which *C. jejuni* navigates its complex
stress survival and pathogenesis cycle despite lacking several hallmark stress genes such as the stationary phase sigma factor RpoS, and other stress factors such as CspA and RpoH is intriguing (Park, 2002). Interestingly, the *C. jejuni* genome contains relatively few regulatory systems to survive and adapt to *ex vivo* and *in vivo* environments. The majority of regulatory proteins that have been characterized in *C. jejuni* include those involved in managing chemotaxis, motility, and heat-shock. Other regulators involved in stress survival include SpoT, required for low nutrient and high oxygen stress (Gaynor *et al.*, 2005), NssR, involved in nitrosative stress survival (Elvers *et al.*, 2005), HspR, response to heatshock (Andersen *et al.*, 2005), PerR, required for oxidative stress (van Vliet *et al.*, 1999), and Fur, for response to low iron conditions (Holmes *et al.*, 2005). The *C. jejuni* genome contains only three predicted RNA polymerase sigma factors: $\sigma^{70}$, $\sigma^{54}$, $\sigma^{28}$ (Eppinger *et al.*, 2004).

One mechanism prokaryotes use to adapt to changing environments is two-component transduction systems, a family of proteins that are widely conserved among bacteria (Stock *et al.*, 2000). An extracellular stimulus interacts with the transmembrane sensor histidine kinase, which alters the cytoplasmic response regulator; thus, resulting in coordinated expression of multiple genes in response to environmental conditions. *C. jejuni* encodes seven histidine kinase and twelve response regulators. Characterized systems include RacRS, which was required for temperature-dependent growth and colonization of chicks (Markas *et al.*, 1999), DccRS, was required for optimal *in vivo* colonization (MacKichan *et al.*, 2004), FlgRS, which regulates the fla regulon (Wosten *et al.*, 2004), and finally PhoSR, which is involved in regulating the phosphate regulon in *C. jejuni* (Wosten *et al.*, 2006).

### 1.6.1.6. Toxin production

*C. jejuni* produces a cytolethal distending toxin (Cdt), an exotoxin that causes cell cycle arrest in the G1 or G2 phases (Ketley, 1997). Cdt is thought to be a significant contributor to *C.
jejuni pathogenesis. The cdt genes are commonly present in C. jejuni, although expression varies between strains (Bang et al., 2001). There are three adjacent genes required for Cdt production and activity, CdtA, CdtB and CdtC (Pickett et al., 1994; Pickett et al., 1996). CdtB has been shown to act as a DNase, where CdtA and CdtC interact to form a tripartite complex necessary for CdtB delivery into the cell (Lara-Tejero and Galan, 2001; Lee et al., 2003). C. jejuni human disease has been thought to be a direct result of the bacteria-host cell interaction, where Cdt induces host cell death and inflammatory response (Newell, 2001).

1.6.1.7. Lipooligosaccharide and Capsule

The C. jejuni strain NCTC 11168 genome sequence revealed many hypervariable regions related to lipooligosaccharide and surface carbohydrate biosynthesis (Parkhill et al., 2000). C. jejuni has multiple surface structures that contain carbohydrates, such as lipooligosaccharide, N-linked glycoproteins, capsular polysaccharides, and O-linked glycosylated flagellar proteins (Szymanski et al., 2003; Thibault et al., 2001). Multiple studies have shown that C. jejuni surface carbohydrates and glycosylation pathways play important roles in virulence and stress survival of this organism (Bacon et al., 2001; Guerry et al., 2000; Karlyshev et al., 2005; Szymanski et al., 2003).

1.6.1.8. Biofilms

A biofilm is a multicellular layer of bacteria embedded within extracellular polymeric substances that are attached to surfaces (Donlan, 2002). Some speculate that, despite its fastidious growth requirements in vitro, the prevalence of C. jejuni in the environment, and subsequent transmission to humans, is likely due to its ability to form biofilms on a variety of abiotic surfaces (Alter and Scherer, 2006; Reeser et al., 2007; Rollins and Colwell, 1986). Recent reports indicate that P. aeruginosa and H. pylori form biofilms during infection, thus
biofilms not only contribute to survival but also pathogenesis in some organisms (Carron et al., 2006; Garcia-Medina et al., 2005). However, the precise role of biofilm formation in C. jejuni in vivo host commensalisms or human infection is still unknown.

Various environmental stress conditions such as temperature fluctuation, oxygen tension, and nutritional status directly influence C. jejuni biofilm formation, and both flagella and quorum sensing are required for optimal biofilm formation under laboratory conditions (Reeser et al., 2007). Proteomic analyses of C. jejuni grown planktonically versus in a biofilm demonstrated that biofilm-grown cells exhibited increased protein expression levels involved in motility complex, the filament cap, basal body and the chemotactic protein, CheA. Moreover, enhanced protein expression was noted for general and oxidative stress responses, the adhesions, and proteins involved in biosynthesis and energy generation (Kalmokoff et al., 2006).

1.6.1.9. C. jejuni stringent response

The stringent response (SR) is a global stress response system that alters gene expression pathways to facilitate survival during times of stress and stringency, such as nutrient or carbon starvation. In many bacteria, extracellular stress results in a marked increase in the amount of uncharged tRNA molecules that accumulate in the cell. This activates RelA and/or SpoT enzymes to synthesize guanosine tetra-phosphate (ppGpp), the main effector molecule of the SR. ppGpp binds to the β-subunit of RNA polymerase, altering transcription pathways and promoter specificity to allow the cells to survive the stress condition (Chatterji and Ojha, 2001). In general, Gram-negative bacteria have been thought to possess both RelA and SpoT and Gram-positive bacteria a single bifunctional RelA/SpoT (Mittenhuber, 2001). However, recent work from our group and others has shown that C. jejuni, as well as other ε- and α-proteobacteria, contain a single bifunctional RelA/SpoT enzyme, in contrast to γ-proteobacteria, which harbor two separate proteins (Gaynor et al., 2005). Analysis of a C. jejuni spoT (ΔspoT) mutant revealed that
the SR was an important pathogenesis factor for specific stress and virulence-related conditions such as stationary phase survival and host cell infection. Microarray analysis of the C. jejuni ΔspoT SR mutant revealed increased expression of genes regulating inorganic phosphate uptake during stationary phase concurrent with up-regulation of genes involved in heat shock (Fig. 1), suggesting that poly P may play a role in C. jejuni stress survival (Gaynor et al., 2005). Moreover, poly P has been linked to stringent response (SR) mechanisms in the enteric pathogen E. coli (Kuroda et al., 1997; Rao et al., 1998).
Figure 1. At later growth stages, heat shock and phosphate transport and metabolism genes are disregulated in the ΔspoT mutant versus wild-type C. jejuni. A C. jejuni DNA microarray demonstrates gene expression profiles for the ppa/adk, pst operons, and C. jejuni heatshock genes as previously described (Gaynor et al., 2005). Samples were collected for RNA microarray analysis at 2, 6, 10, 24 hours. Time is represented by the angled black triangle above the expression profiles.
1.7. **Bacterial polyphosphate metabolism**

Polyphosphate (Poly P) is ubiquitous in nature and consists of phosphate residues linked by high-energy phosphoanhydride bonds as in ATP. The *ppk1* gene is principally responsible for poly P formation in bacteria such as *E. coli* and encodes a polyphosphate kinase that reversibly forms poly P from the terminal γ-phosphate of ATP (Ahn and Kornberg, 1990; Kornberg et al., 1999). Moreover, some bacteria also contain a homolog of *ppk1*, known as *ppk2*, which was first identified in *Pseudomonas aeruginosa* and uses poly P as a donor to convert GDP to GTP but can also function in poly P synthesis (Ishige et al., 2002). Interestingly, not all organisms possess *ppk2*, for example the highly characterized organism *E. coli* contains only *ppk1* (Zhang et al., 2002). *C. jejuni*, however, contains both *ppk1* and *ppk2* genes (Zhang et al., 2002). An exopolyphosphatase, encoded by the *ppx* gene, is responsible for the degradation of poly P into phosphate residues in *E. coli* (Akiyama et al., 1992). Poly P has a multiplicity of functions within bacterial cells. Long chains of poly P can serve as a phosphate reservoir, a chelator of cations (Harold, 1966), a membrane channel for DNA entry (Reusch and Sadoff, 1988), a capsular component (Tinsley et al., 1993; Tinsley and Gotschlich, 1995), a pH buffer (Jahid et al., 2006; Pick and Weiss, 1991; Price-Carter et al., 2005) and likely an ATP substitute. Furthermore, in *E. coli* poly P inhibits RNA degradation, promotes translation fidelity, and activates the Lon-protease complex that degrades specific ribosomal proteins to meet nutritional requirements during starvation (Kuroda et al., 2001).

The essential role of poly P formation in bacterial pathogenesis has been established in such pathogens as *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Klebsiella aerogenes*, *Salmonella enterica* serovar Typhimurium, *Helicobacter pylori*, and *Shigella flexneri*. In these organisms, poly P formation was shown to be critical for various virulence attributes, including motility, quorum sensing, biofilm formation, and supportive resistance to oxidative, osmotic,
heat, alkaline stress, 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 et al., 2000a; Rashid et al., 2000b; Tan et al., 2005). The importance of poly P in various bacterial phenotypes has been reported, yet the molecular mechanisms and primary and secondary effects of poly P synthesis are still not understood.

1.7.1. Interaction of poly P and the stringent response in E. coli

Functional links between the SR and poly P accumulation have been demonstrated in E. coli (Ault-Riche et al., 1998; Kuroda et al., 1997; Kuroda et al., 1999; Rao et al., 1998). In that organism, the SR regulatory molecule ppGpp inhibits poly P hydrolysis by blocking the activity of the PPX exopolyphosphatase, which hydrolyzes poly P into inorganic phosphate monomers. In E. coli SR mutants lacking ppGpp, PPX remains active, resulting in diminished levels of poly P (Kuroda et al., 1997). In E. coli, ppk1 and ppx are in an operon; thus levels of PPK1 and PPX are also transcriptionally co-regulated. In contrast, ppk1 and ppx in C. jejuni and in several other bacteria (Mullan et al., 2002b) are not found in an operon and thus may not be transcriptionally linked.

1.8. Hypothesis

Our preliminary data involving microarray analysis of the C. jejuni ΔspoT mutant, together with work in other organisms, suggested that poly P utilization and accumulation, thus far unstudied in C. jejuni, plays an important role in C. jejuni physiology and pathogenesis and potentially interacts with stringent response mechanisms.
2.0. MATERIALS AND METHODS

2.1. BACTERIAL STRAINS AND GROWTH CONDITIONS

All studies were performed using the highly invasive strain, *Campylobacter jejuni* 81-176 originally isolated from a diarrheic patient (Korlath *et al.*, 1985). The Δspo*T* mutant of this strain was previously described (Gaynor *et al.*, 2005). *C. jejuni* was routinely cultured on Mueller-Hinton (MH) (Oxoid Ltd, Hampshire, England) agar plates, and growth/survival curves were conducted in MH broth unless otherwise stated (Oxoid Ltd, Hampshire, England). *C. jejuni* was always grown in media supplemented with 10 μg/ml vancomycin and 5 μg/ml trimethoprim; chloramphenicol (Cm) was added at 20 μg/ml when required. All bacteria were enumerated on MH agar plates by performing serial 10-fold dilutions, unless otherwise stated. Plates were incubated in a tri-gas incubator (Heraeus), under the following conditions: 6% O₂, 12% CO₂, at 37°C. Broth culture growth/survival curves were performed using the Oxoid CampyGen system to produce a microaerobic atmosphere of 6% O₂, and 12% CO₂, and cells were shaken at 200 r.p.m, at 37°C. *E. coli* strain DH5α was grown on Luria-Bertani (LB) agar or broth, with the addition of 30 μg/ml of Cm as needed, at 37°C under normal atmospheric conditions. Karmali agar was used for growth of *C. jejuni* for the chick colonization studies.

2.2. CONSTRUCTION OF *C. JEJUNI* 81-176 *ppk1* TARGETED DELETION MUTANT

A polyphosphate kinase (*ppk1*) gene with 30.1% identity and 50.7% similarity to the *ppk1* gene in *E. coli* was identified in the *C. jejuni* genome using the Blast features of CampyDB (http://campy.bham.ac.uk/ (Chaudhuri and Pallen, 2006). The *ppk1* gene was PCR-amplified from *C. jejuni* chromosomal DNA prepared using the Qiagen DNeasy kit (Qiagen Inc., Valencia, CA), with primers PPK1-2 Fp (GCAAATATTTACACCAAGAAAAAGAAC) and PPK1-2 Rp (ATCTGCACTCGATATAAAATAATTTGG), yielding a 1550 bp fragment. The amplified product was cloned into the pGEM-T vector (Promega, Nepean, CA.), which is a suicide plasmid
in *C. jejuni*. Two EcoRI sites located within the *ppkl* gene were used to remove a 1048 bp fragment. A chloramphenicol acetyltransferase cassette (*cat*) was excised from pRY109 (Yao *et al.*, 1993) with EcoRI and ligated into the EcoRI-digested pGEM-*ppkl* vector to create the *ppkl::cat* knockout construct. *Cat* cassette insertion was confirmed by restriction digestion and sequencing (Nucleic Acid Protein Service Unit, Vancouver, CA.). This construct was used to naturally transform *C. jejuni* 81-176 (van Vliet *et al.*, 1998). Transformants were recovered on MH agar plates supplemented with Cm. Clones with proper insertion of the *cat* cassette into the *C. jejuni ppkl* gene resulted from a double recombination event with the described construct.

Insertional inactivation of the *ppkl* gene via *cat* cassette (∆*ppkl*) insertion was verified by PCR using PPK1-1 Fp (TGCCCTTAGCGTTATAAAAAGTATAAA) and PPK1-1 Rp (AATTTTCGGTCATTTTTGATAGTGTAG) primers that are external to the *ppkl* gene and the region originally amplified (Appendix). Sequencing also verified a single chromosomal insertion of the *cat* cassette into the *ppkl* gene (Appendix).

### 2.3. COMPLEMENTATION OF ∆*ppkl* DELETION MUTANT

Generation of a re-constituted wild-type strain of *C. jejuni*, designated *ppkl* *, was achieved via natural transformation of the intact full length ∆*ppkl* mutant with the *ppkl* gene in pGEM-T. Serial 10-fold dilutions of naturally transformed ∆*ppkl* mutant were spread on MH plates, harvested after 2 hours, plated on MH plates supplemented with 0.17 M NaCl, and incubated for 48 hours at 37°C in a tri-gas incubator. Individual colonies were selected and purified on MH agar containing 10 µg/ml of vancomycin and 5 µg/ml trimethoprim. Colonies were tested for sensitivity to chloramphenicol by plating on MH agar plates containing 20 µg/mL of chloramphenicol. Colonies representing putative re-constituted wild-type strains were confirmed using PCR with the PPK1-1 primer set and sequence analysis of 18 bona fide recombinants, represented by *ppkl* *. 

19
2.4. POLY P GLASSMILK EXTRACTION

Extraction of poly P from *C. jejuni* cells and binding to glassmilk was performed as described by Ault-Riché *et al.*, 1998. *C. jejuni* cultures were grown in MH broth to mid-log phase, and then diluted to 0.05 OD_{600} to initiate the time course experiment. Cells were harvested after 2, 10 and 24 hours by pelleting 1 mL in a table top centrifuge, at 6000 r.p.m. for 10 minutes. All pellets were processed immediately for poly P assays. To each pellet 500 μL of prewarmed at 95°C 4 M guanidine isothiocyanate (GITC)-500 mM Tris-HCl, pH 7.0 (GITC lysis buffer) was added. Tubes were vortexed, incubated for 5 minutes in a 95°C heating block, and sonicated briefly; a 10 μL sample was removed for total protein estimation using a Coomassie Plus Protein Assay Reagent (Pierce) with bovine serum albumin as the standard. Each sample and all bovine serum albumin standards were treated with Compat-Able Protein Assay Kit (Pierce). To each tube 30 μL of 10% sodium dodecyl sulfate, 500 μL of 95% ethanol, and 5 μL of Glassmilk. Tubes were vortexed and centrifuged briefly to pellet the glassmilk, which was then resuspended in 500 μL of ice cold New Wash Buffer (5 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM EDTA, 50% ethanol) by vortexing and re-pelleted; washing was repeating two additional times. The washed pellet was resuspended in 50 μL of 50 mM Tris-HCl (pH 7.4)-10 mM MgCl₂-20 μg each of DNase and RNase per ml and incubated at 37°C for 10 minutes. The pellet was washed with 150 μL of 4 M GITC lysis buffer and 150 μL of 95% ethanol and then twice in New Wash Buffer. Poly P was eluted from the Glassmilk pellet with 50 μL of 50 mM Tris-HCl (pH 8.0) at 95°C for 2 minutes, followed by two additional elutions.

2.5. MEASUREMENT OF POLY P LEVELS BY A TOLUIDINE BLUE O ASSAY

A standard curve was determined by the addition of 100 μL of a known concentration of Phosphorus Standard (Sigma-Aldrich, Oakville, CA.) sample that had been serially diluted (1:10)
in 900 μL (64, 32, 16, 8, 4 nmol poly P) of a Toluidine Blue O (TBO) (Sigma-Aldrich, Oakville, CA.) dye solution consisting of 6 mg/mL of TBO in 40 mM of acetic acid. After the addition to TBO, all samples (standard curve and C. jejuni time course samples) were incubated at room temperature for 15 minutes. Absorbance at 630 nm and 530 nm levels were assessed spectrophotometrically and used to generate an A₅₃₀/A₆₃₀ ratio; poly P binding to TBO results in a shift in TBO absorbance from 630 nm to 530 nm. All C. jejuni extracted samples of poly P were measured identically to the standard curve, and poly P levels were expressed in nmol of poly P and per milligrams of total cellular protein, as measured via Bradford analysis (Pierce Scientific).

2.6. Nutrient Downshift Survival

C. jejuni were grown microaerobically at 37°C in MH broth to mid-log phase overnight, collected by centrifugation at 6000 r.p.m. for 10 minutes, and washed twice with Minimal Essential Media (MEM) with Earle’s salts and L-Glutamine (Difco, USA) or in MOPS-MGS media. Bacteria were re-suspended in MEM or MOPS-MGS and diluted to an OD₆₀₀ of 0.05. Cultures were placed under microaerobic conditions at 37°C, shaking at 200 r.p.m. Colony forming units (CFUs) were measured over time by plating on MH agar plates.

2.7. Osmotic Stress Survival

C. jejuni strains were grown to mid-log phase, serially diluted (1:10), and spotted onto MH agar plates containing 0.17 M NaCl to assess single colony growth. Survival during osmotic stress was also tested in shaking liquid cultures by growing bacterial strains to mid-log phase in MH and diluting to 0.05 OD₆₀₀ in MH broth +/- 0.25 M NaCl and/or Brain Heart Infusion broth. CFUs were assayed.
2.8. STATIC BIOFILM FORMATION

Biofilm formation was assayed as described previously (O'Toole, 1998). Briefly, cells were grown microaerobically in MH broth at 37°C to log phase overnight and diluted to OD$_{600}$ of 0.20 and 0.02. 100 μL aliquots were added to 96-well polyvinyl chloride plates and incubated at 37°C. Static biofilm formation as measured by surface-associated bacteria was assessed by adding 25 μL of a 1% crystal violet solution in ethanol, incubation for 15 minutes at room temperature, and washing 3x with distilled water (Kolter, 2000). To quantify biofilm formation, 150 μL of 80% dimethyl sulfoxide was added to each PVC well, covered, and incubated 24 hours at room temperature. A 1:10 dilution in 80% DMSO of each well was measured at OD$_{560}$ to quantify the amount of biofilm stained by crystal violet.

2.9. INT407 CELL INFECTION ASSAY FOR INVASION AND INTRACELLULAR SURVIVAL

Human intestinal 407 (INT407) cells were seeded to semi-confluence (~1 x 10$^5$ cells/well) and confluence (~5.5 x 10$^5$ cells/well) in 24 well plates approximately 16 hours prior to infection assays. $C.\ jejuni$ strains were inoculated at OD$_{600}$=0.001 into MH broth culture and grown overnight to mid-log phase. Bacteria were pelleted at 6000 r.p.m. for 10 minutes, washed twice with MEM, and diluted to 0.002 OD$_{600}$ in MEM. Bacterial suspensions in MEM (1 mL) were used to infect tissue culture cells at a multiplicity of infection (MOI) of ~100 and ~20, respectively (~10$^7$ bacteria/ ml). All experiments were performed in triplicate. Following three hours of infection in a 5.0% CO$_2$ incubator, bacteria in MEM were removed from the wells and cells were washed 2x with MEM. To all wells, 1 mL of MEM containing 150 μg/mL of gentamicin was added to kill remaining extracellular bacteria. After two hours, wells were washed 2x with MEM. To assay invasion, 1 mL of dH$_2$O was added to some of the wells, and INT407 cells were disrupted by lysis with a 27G syringe. Invaded bacteria were assayed by serial dilution and plating on MH agar. Samples for assaying intracellular survival were covered with
MEM with 3% Fetal Bovine Serum and the addition of 10 μg/mL of gentamicin to halt bacterial growth if cell lysis occurred. After 23-24 hours of incubation, intracellular survival was assayed as described for invasion samples.

2.10. CHICK COLONIZATION ASSAYS

Colonization of one-day-old chicks was performed essentially as described previously (Carrillo et al., 2004). Briefly, broiler chicks were obtained from a local hatchery in Saskatchewan on the day of hatch. Five chicks were euthanized and their cecal contents cultured for Campylobacter. The remaining birds were randomly assigned into groups of 10 birds and provided with feed and water ad libitum. Birds were cared for in accordance to guidelines of the Canadian Council for Animal Care. Birds were orally challenged with the indicated strain (wild-type or Δppkl mutant) and dose (1.5x10⁵, 1.5x10⁶, and 1.5x10⁷ CFUs) of C. jejuni in 0.5 ml of MH broth, based on previous experiments to determine the optimal dose of our wild-type strain. Inocula for challenge experiments were produced by harvesting cells grown for 18 h under microaerophilic conditions at 37°C into cold MH broth, diluting to the indicated concentration in MH broth, and maintaining on ice until immediately before use. Viable cell counts were determined by plating serial dilutions onto MH agar (Becton, Dickinson and Company, USA). Birds were maintained for seven days after challenge and then were euthanized by cervical dislocation. Caeca were aseptically collected for qualitative as well as quantitative assessment of colonization. Colonization of the birds was monitored by culturing cecal contents, after appropriate dilutions were made in MH broth, on Karmali agar (Bacto, USA) under microaerophilic conditions at 42°C.
2.11. ADDITIONAL PHENOTYPIC ASSAYS FOR WHICH THE Δappk1 MUTANT WAS NOT DIFFERENT FROM WILD-TYPE

**Motility**— *C. jejuni* strains were grown microaerobically in MH broth at 37°C to log phase overnight and OD$_{600}$ were read. In vitro motility assays were preformed by diluting cells to OD$_{600}$ of 0.02 or 0.1 and stabbing the appropriate volume of each strain into MH agar plates that contained 0.3% or 0.5% agar, respectively. Migration of the cells from the point of inoculation was analyzed following 24 hours of incubation at 37°C.

**Oxidative sensitivity**— Cells were grown microaerobically in MH broth at 37°C to log phase overnight and diluted to OD$_{600}$ of 0.05 and 100 µl of each strain was evenly spread on a MH agar plate. Whatman filter paper disks were autoclaved and moistened with 10 µl of 3% H$_2$O$_2$. Disks were placed on the cell lawns and incubated at 37°C for 24 hours prior to analysis of growth inhibition. The zone of growth-inhibition surrounding the disk was measured to determine sensitivity.

**Survival in organic acids**— *C. jejuni* were grown microaerobically at 37°C in MH broth to mid-log phase overnight, collected by centrifugation at 6000 r.p.m. for 10 minutes. Bacteria were re-suspended in MH broth with the appropriate organic acid and diluted to an OD$_{600}$ of 0.05 in MH broth containing either 9mM propionic acid or 22mM acetic acid. Cultures were placed under microaerobic conditions at 37°C, shaking at 200 r.p.m. CFUs for survival in organic acids were measured over time by plating on MH agar plates.

**Low iron conditions**— *C. jejuni* strains were grown microaerobically in MH broth at 37°C to log phase overnight and diluted to OD$_{600}$ of 0.05 and 100 µl of each strain was evenly spread on a MH agar plate. Disks containing sterile desferal (Fe$^{3+}$) and dipyridyl (Fe$^{2+}$) at 40 mM concentrations were placed on the cell lawns and incubated at 37°C for 24 hours prior to analysis of growth inhibition. The zone of growth-inhibition surrounding the disk was measured to determine sensitivity.
MH broth — *C. jejuni* were grown microaerobically at 37°C in MH broth to mid-log phase overnight. Bacteria were diluted to an OD_{600} of 0.05. Cultures were placed under microaerobic conditions at 37°C, shaking at 200 r.p.m. CFUs were measured over time by plating on MH agar plates.

Aerobic survival — *C. jejuni* was grown as described above and diluted to an OD_{600} of 0.05. Cultures were placed under aerobic atmospheric conditions at 37°C, shaking at 200 r.p.m. CFUs were measured over time by plating on MH agar plates.

Anaerobic survival — Strains were grown as described above and diluted to an OD_{600} of 0.05. Cultures were placed under anaerobic conditions, using an Anaero-GasPak from Oxoid, at 37°C, shaking at 200 r.p.m. CFUs were measured over time by plating on MH agar plates.

Osmotic stress — Survival during osmotic stress was also tested in shaking liquid cultures by growing bacterial strains to mid-log phase in MH and diluting to 0.05 OD_{600} in MH broth +/- 0.16 M or +/- 0.3 M NaCl. CFUs were assayed over time.

Calcofluor white — To assay calcofluor white fluorescence, bacteria were grown overnight in shaking broth cultures to mid-log phase. Cells were diluted to OD_{600} of 0.05. Bacteria were streaked out on Brain Heart Infusion broth plates (Difco) containing 0.02% calcofluor white. Plates were grown under 37°C microaerobic conditions for 24 hours and transferred to 42°C anaerobic conditions for an additional 24 hours. All calcofluor white incubations were preformed in the dark. Calcofluor white reactivity was visualized as fluorescence under long-wave UV light.
3.0. RESULTS

3.1. INTRODUCTION AND RATIONALE

Poly P's importance in basic processes and bacterial pathogenesis has been established in organisms such as *E. coli*, *P. aeruginosa*, and *V. cholerae*. Tangible links have also been drawn between the SR and poly P in several of the abovementioned pathogens. A wild-type versus ΔspoT microarray experiment (Gaynor et al., 2005) led us to query a possible involvement of poly P in *C. jejuni* pathogenesis and a putative connection with the SR (Fig. 1). *C. jejuni* harbours homologs of several genes potentially involved in poly P metabolism, one of which, *ppkl* (polyphosphate kinase), was predicted to be responsible for the bulk of poly P production. To test this, the *ppkl* gene was disrupted in the highly invasive *C. jejuni* strain 81-176. The *ppkl* deletion mutant (Δ*ppkl*) was subjected to specific stresses to compare its growth, survival, invasiveness and commensal colonization to that of wild-type.

3.2. POLY P INTERACTS WITH THE STRINGENT RESPONSE IN *C. JEJUNI*

Poly P levels in wild-type and the ΔspoT mutant strains were determined in shaking broth cultures harvested at various stages of growth (Fig. 2A, B). Poly P was extracted following cell lysis by binding to glassmilk and measured using the metachromatic dye, TBO. Poly P binds TBO, resulting in an absorbance shift from 630 nm to 530 nm; the 530 nm/630 nm ratio thus reflects the amount of poly P in a given sample (Mullan et al., 2002a). A standard curve was determined by the addition of a known concentration of phosphorus standard to TBO and plotting phosphorus concentrations versus the absorbance ratio. Poly P levels in *C. jejuni* samples are expressed as nmol of poly P and normalized to milligrams of total cellular protein.

In wild-type *C. jejuni*, poly P levels were significantly higher than those of the ΔspoT, with levels peaking in stationary phase at 33.8 nmol poly P/mg of total protein. The ΔspoT mutant exhibited poly P levels that were also significantly lower than wild-type at both 10 and 24
hours ($p$-value < 0.05), with the largest difference (~3.2-fold) again observed in stationary phase. Furthermore, wild-type, $\Delta spoT$ exhibited similar growth profiles by CFU/ml (Fig. 2A) analyses.
Figure 2. Poly P levels in the ΔspoT mutant remain lower than wild-type C. jejuni. (A) Wild-type (WT) C. jejuni 81-176 (black squares), and the ΔspoT mutant (open circle) were grown microaerobically in shaking broth culture to early log phase and diluted to 0.05 OD_{600} per ml. Cultures (1ml) were harvested at 2, 10 and 24 hours and assayed for CFU/ml, and (B) intracellular poly P levels versus total cellular protein. Triplicate samples were harvested and assayed for each time point. Statistical significance (student's t-test p-value < 0.05) is represented by an asterisk (*). n=3
3.3. The \( \Delta ppk1 \) mutant is defective for poly P accumulation

All three sequenced \( C. jejuni \) strains harbour a \( ppk1 \) gene (Fig. 3A) encoding a putative polyphosphate kinase (PPK1) with significant sequence identity to that in other bacteria. The predicted \( C. jejuni \) amino acid sequence exhibits 45.4%, 36.2%, and 30.1% identity to PPK1 in \( Helicobacter pylori, P. aeruginosa, \) and \( E. coli \), respectively. Moreover, two highly conserved histidine residues, required for PPK1 activity in \( E. coli \), are also conserved at H427 and H580 in the \( C. jejuni \) 81-176 strain. In 81-176, \( ppk1 \) appears to be a single-gene operon, and microarray data from multiple gene expression experiments indicate that \( ppk1 \) is transcribed independently of neighbouring genes (Fouts et al., 2005; Gaynor et al., 2004; Gaynor et al., 2005; Hofreuter et al., 2006; MacKichan et al., 2004; Parkhill et al., 2000). To explore the role of poly P in \( C. jejuni \) 81-176, \( \sim50\% \) of the \( ppk1 \) gene was deleted, including the codons for the conserved His residues, and replaced with a \( cat \) cassette (Fig. 3B). PCR and sequence analysis demonstrated a single \( \text{Cm}^R \) insert in the \( ppk1 \) gene (Appendix A, B). To ensure that observed phenotypes were attributed to \( ppk1 \), a reconstituted wild-type strain was generated by homologous recombination of a wild-type copy of \( ppk1 \) into the \( \Delta ppk1 \) strain \( ppk1::cat \) locus as described in Experimental Procedures. The resulting reconstituted wild-type strain was designated \( ppk1^* \) and was verified by PCR and sequence analysis (Appendix C, D).
Figure 3. *C. jejuni* ppk1 and generation of a single insert Δppk1 disruption strain. (A) Genomic location of the ppk1 gene is conserved among the sequenced and annotated strains *C. jejuni* 81-176, *C. jejuni* 11168 and *C. jejuni* RM1221. MUMer alignment was performed using CampyDB gene viewer (Chaudhuri and Pallen, 2006). The diagram shows the region encompassing bases of 1265972-1286763 *C. jejuni* 81-176, and the equivalent region encompassing bases of 12872174-1302173 of *C. jejuni* NCTC11168 and 1434899-1457437 *C. jejuni* RM1221. Hypothetical proteins are represented by an asterisk (*), and genes with no predicted orthologues are coloured grey. (B) The approximate site of the CmR-marked insertion-deletion mutation generated in *C. jejuni* 81-176 ppk1 is shown. The resultant mutant strain was designated Δppk1.
Poly P levels in wild-type, the Δppk1 mutant, and ppk1* strains were determined in shaking broth cultures harvested at various stages of growth. Poly P was extracted following cell lysis by binding to glassmilk and measured using TBO. The standard curve was used to determine levels of poly P accumulation. Poly P levels in C. jejuni samples are expressed as nmol of poly P and normalized to milligrams of total cellular protein.

The poly P assay confirmed that the ppk1 gene is responsible for the majority of C. jejuni poly P synthesis (Fig. 4B). Poly P levels were significantly higher in wild-type C. jejuni than those of the Δppk1 mutant. The Δppk1 mutant exhibited significantly lower levels of poly P at both 10 and 24 hours (p-value < 0.05), with the largest difference (~3.8 fold) observed at the 24-hour stationary phase time point. The complemented ppk1* strain displayed similar levels of poly P compared to wild-type at all time points assayed. Furthermore, wild-type, Δppk1, and ppk1* exhibited identical growth profiles by CFU/ml (Fig. 4A) analyses.
Figure 4. Poly P accumulates in wild-type and \textit{ppk}^* strains at later growth stages but remains at low levels in the \textit{Δppk1} mutant. Wild-type (WT) \textit{Cj} 81-176 (black squares), \textit{Δppk1} mutant (grey triangle), and the complemented strain \textit{ppk}^* (star) were grown microaerobically in shaking broth culture to early log phase and diluted to 0.05 OD$_{600}$ per ml. (A) Cultures (1ml) were harvested at 2, 10 and 24 hours and assayed for CFU/ml, and (B) intracellular poly P levels versus total cellular protein. Triplicate samples were harvested and assayed for each time point. Statistical significance (student's $t$-test $p$-value < 0.05) is represented by an asterisk (*). $n=5$. 

n=5.
3.4. POLY P IS CRITICAL FOR C. JEJUNI SURVIVAL DURING NUTRITIONAL DOWN-SHIFT

To simulate a nutritionally poor milieu, wild-type, Δppkl, and ppkl* strains were subjected to starvation conditions by a downshift from rich MH broth to nutrient-poor Minimum Essential Medium and assayed for CFUs/ml over a 9 hour (Fig. 5A) and 55 hour (Fig. 5B) time course. The Δppkl mutant exhibited defects in adaptation to survival during low nutrient stress versus wild-type in early adaptation time-points assayed from 5-10 hours. The most pronounced effect of nutrient down-shift was seen after 5 hours in Fig. 5B, at which time culturability of the mutant dropped ~250 fold relative to the wild-type and ppkl* strains. Survival was also assessed in another limited-nutrient media, MOPS-MGS buffered media (Mendrygal and Gonzalez, 2000) without phosphate (Fig. 5C). As expected, the Δppkl mutant showed a considerable defect in survival at later time points assayed; however, the differences were not as pronounced as those in MEM. These data demonstrate that the Δppkl strain exhibits nutrient down-shift tolerance defects in two different minimal media.
Figure 5. Poly P is important for *C. jejuni* survival during nutritional downshift. WT (black square), Δ*ppk1* (grey triangle), and *ppk1* * (star) were grown in MH broth to mid-log phase. Cells were subjected to nutritional downshift by centrifugation followed by resuspension to 0.05 OD$_{600}$ in (A and B) minimum essential media or (C) MOPS-buffered media. n=5.
3.5. POLY P ACCUMULATION IS REQUIRED FOR *C. JEJUNI* TO SURVIVE OSMOTIC SHOCK

To test survival under osmotic stress, strains were grown to mid-log phase, shifted to MH broth with or without NaCl added to 0.25 M, and CFU/mL were enumerated at time points indicated (Fig. 6A). During osmotic shock, both the wild-type and the Δppkl mutant strains ceased growth at 5 hours in MH broth. After 24 hours, Δppkl survival levels were >10-fold lower than wild-type. By 72 hours, the Δppkl mutant CFU/ml were >1000-fold lower than wild-type levels. Wild-type and Δppkl grow identically in MH broth without the addition of salt (Fig. 6A); levels less than 0.25 M NaCl did not inhibit survival of wild-type or Δppkl strains (data not shown). Moreover, a nutrient and salt rich media, Brain Heart Infusion broth, also decreased Δppkl mutant survival at later time points compared to wild-type.

The ability of single bacteria to grow into colonies during continuous osmotic stress was tested by growing wild-type, Δppkl, and ppkl* strains to mid-log phase in salt-free broth, then serially diluting and spotting the bacteria onto MH plates containing 0.17 M NaCl. Under these conditions, the Δppkl mutant was significantly defective for growth compared to wild-type and ppkl* strains (Fig. 6B); all strains grew identically on MH agar without added salt (data not shown).
Figure 6. Poly P is required for *C. jejuni* osmotic stress survival. *C. jejuni* 81-176 wild-type and Δppk1 strains were grown to mid-log phase and shifted to MH broth +/- 0.25 M NaCl (A). Growth/survival was monitored by CFU/ml plate counts. (B) Wild-type, Δppk1, and ppk1* strains were grown to mid-log phase in MH broth and serially diluted (1:10) from 2x10^5 – to 2x10^1 CFU/ml and spotted onto 0.17 M NaCl MH agar plates. Growth was assessed after days 48 hours under microaerobic conditions. n=4.
3.6. THE Δppk1 MUTANT DISPLAYS ACCELERATED STATIC BIOFILM FORMATION

Biofilm formation contributes to bacterial virulence, colonization, environmental survival, and antibiotic resistance. Poly P has been shown to be essential for biofilm formation in several bacteria, including *P. aeruginosa* (Rashid *et al.*, 2000b). The role of poly P in *C. jejuni* biofilm formation was assayed by growing standing broth cultures of *C. jejuni* wild-type, Δppk1, and ppk1* strains in polyvinyl chloride microtitre (PVC) plates from starting OD<sub>600</sub> levels of 0.02 and 0.20. Biofilm formation at the air-liquid interface and on the sides of the PVC plates wells were assayed by staining the wells with a crystal violet solution; in this assay, increased crystal violet staining correlates with increased biofilm production (O'Toole and Kolter, 1998). After two days, wild-type *C. jejuni* bacteria formed a faint biofilm at the air liquid interface for both starting doses, while the Δppk1 mutant demonstrated a statistically significant (student’s *t*-test *p*-value <0.05) increase in the amount of static biofilms at both the air-liquid interface as well as on the bottom and sides of the well compared to wild-type for both inoculating doses (Fig. 7A, B). The Δppk1 mutant also exhibited a dose-dependent biofilm phenotype: the higher inoculating dose of Δppk1 yielded a statistically significant (student’s *t*- test *p*-value <0.05) increase in biofilm formation compared to the lower inoculating dose of Δppk1, whereas a similar phenomenon was not observed for wild-type (Fig. 7B). After three days, wild-type and Δppk1 biofilm densities were approximately equal (data not shown). Consistent with our other observations, ppk1* exhibited wild-type biofilm formation levels (Fig. 7A, B).
Figure 7. The Δppk1 mutant exhibits increased static biofilm formation. 96-well PVC plates were inoculated with *C. jejuni* in MH broth at OD$_{600}$ 0.02 and 0.20. At 48 hours, the biofilms were qualitatively observed by crystal violet staining. (A) Representative wells from the OD$_{600}$ 0.02 inoculation are shown for wild-type, Δppk1, and ppk1*. (B) Biofilm formation was quantified by addition of 80% DMSO to each crystal violet stained well, followed by OD$_{570}$ absorbance measurements of solubilized crystal violet. Statistical significance (student’s t-test p-value < 0.05) is represented by an asterisk (*). n=4.
3.7. The Δppkl mutant is defective for prolonged intracellular survival

To assay the virulence-associated phenotypes of invasion and intracellular survival, wild-type, Δppkl, and ppkl* strains were allowed to infect INT407 cells at an MOI of ~20 or ~100, respectively, for three hours. Gentamicin was added to all wells to kill extracellular bacteria, after which cells were assayed for invasion as well as both short-term and long-term intracellular survival, after 2h and 18h IC (MOI ~20) or 2h and 19h IC (MOI ~100), respectively. Wild-type and the Δppkl mutant exhibited similar invasion survival profiles (Fig. 8A and B). However, the Δppkl mutant was reproducibly defective for longer-term (18h and 19h IC) intracellular survival, exhibiting a statistically significant (student's t-test p-value < 0.05), greater than 100-fold defect compared to the wild-type intracellular survival profile. The complemented ppkl* strain survival was similar to wild-type. All strains used survived equally well in MEM at the 3 hour time point, showed identical gentamicin susceptibility, and were fully resistant to dH₂O-syringe lysis (data not shown).
Figure 8. The Δppk1 mutant is defective for long-term intracellular survival in an epithelial cell model of infection. WT (black square), Δppk1 (grey triangle), and ppk1* (star) were grown to mid-log phase in MH broth. At the zero time point, semiconfluent or confluent monolayers of INT407 cells were inoculated with bacteria at an MOI ~100 (A) and ~20 (B), respectively. After 3 hours, the cells were washed, and gentamicin was added at 150 μg/ml to all wells for 2 hours to kill extracellular bacteria. Gentamicin was washed from the cells at 5 hours, and invaded intracellular (IC) bacteria were harvested and plated for enumeration (inv). To all remaining wells, fresh media containing 5 μg/ml of gentamicin and 3% fetal bovine serum were added. After an additional 2 hours (2h IC) or 18-19 hours (18h or 19h IC) of incubation, cells were washed, and surviving intracellular bacteria were harvested. All time points taken were performed in triplicate and error bars are shown. n=3.
3.8. The Δppk1 mutant exhibits a dose-dependent chick colonization defect

To assess the role of poly P in commensal colonization, 1-day-old chicks were infected with wild-type and Δppk1 strains at increasing inoculation levels (1.5×10^5, 1.5×10^6, and 1.5×10^7 CFUs/infection). Chicks were sacrificed after 7 days and caecal contents assayed for viable C. jejuni. Wild-type exhibited colonization levels greater than 1.79×10^8 CFU/g of caecal content at all inoculating doses, with nearly all infected chicks colonized to high levels (Fig. 9). However, the Δppk1 mutant colonization levels were dependent upon inoculating dose. Strikingly, at an inoculating dose of 1.5×10^5 CFU, no Δppk1 bacteria were recovered from any chick infected. The intermediate dose of the Δppk1 mutant (1.5×10^6 CFU/0.5 ml) resulted in colonization of 8 out of 10 chicks. The highest dose of the Δppk1 mutant (1.5×10^7 CFU/0.5 ml) yielded colonization of all chicks, at levels similar to wild-type C. jejuni, with a mean concentration of 6.79×10^8 CFU per g of caecal content.
Figure 9. The Δappk1 mutant exhibited a 7 day, dose dependent defect for chick caecal colonization. Chicks were challenged orally with C. jejuni 81-176 wild-type (black triangles) and the Δappk1 mutant (open inverted triangles), using doses of $1.5 \times 10^5$, $1.5 \times 10^6$, $1.5 \times 10^7$ CFU in 0.5 ml of broth. After seven days post infection, chicks were sacrificed, and bacterial colonization of caeca was determined by plating on Karmali agar. Levels of colonization at specific doses are expressed as CFU per gram of caecal content. The detection limited was 40 CFUs. Each data symbol represents CFUs recovered from an individual chick. The geometric mean of bacterial concentration recovered is represented by a bar for each dosage. n=1.
3.9. Phenotypes for which the Δppk1 mutant was not different from wild-type

Poly P specific phenotypes seen in other Δppk mutant organisms were tested in the C. jejuni Δppk mutant. A number of phenotypes that were present in other Δppk mutant organisms were not observed in the C. jejuni Δppk mutant. These phenotypes included: motility, oxidative stress, organic acid survival, survival under low iron conditions, and survival under normal atmospheric (aerobic) and anaerobic conditions, calcofluor white fluorescence, and serum sensitivity.

Table 1: Phenotypes that were indistinguishable between wild-type and Δppk1

<table>
<thead>
<tr>
<th>Phenotype Tested</th>
<th>Wild-type</th>
<th>Δppk1</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3% agar</td>
<td>Motile</td>
<td>Motile</td>
<td>Fig. 10</td>
</tr>
<tr>
<td>0.5% agar</td>
<td>Motile</td>
<td>Motile</td>
<td></td>
</tr>
<tr>
<td>Oxidative sensitivity</td>
<td>41 mm zone of inhibition</td>
<td>41 mm zone of inhibition</td>
<td>Fig. 11</td>
</tr>
<tr>
<td>Organic acids survival:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 mM propionic acid</td>
<td>No difference</td>
<td></td>
<td>Fig. 12</td>
</tr>
<tr>
<td>22 mM acetic acid</td>
<td>No difference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low iron conditions:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>desferal (Fe(^{3+}))</td>
<td>17 mm zone of inhibition</td>
<td>17 mm zone of inhibition</td>
<td>Fig. 13</td>
</tr>
<tr>
<td>dipyridyl (Fe(^{2+}))</td>
<td>29 mm zone of inhibition</td>
<td>29 mm zone of inhibition</td>
<td></td>
</tr>
<tr>
<td>MH Broth</td>
<td>No difference in growth and survival</td>
<td></td>
<td>Fig. 14</td>
</tr>
<tr>
<td>Aerobic Survival</td>
<td>No difference</td>
<td></td>
<td>Fig. 15</td>
</tr>
<tr>
<td>Calcofluor white fluorescence</td>
<td>No difference</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Phenotype Tested</td>
<td>Wild-type</td>
<td>Δppk1</td>
<td>Figure</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td>Anaerobic survival</td>
<td>No difference</td>
<td>No difference</td>
<td>Fig. 16</td>
</tr>
<tr>
<td>Low level osmotic shock:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.16 M NaCl</td>
<td>No difference</td>
<td></td>
<td>Fig. 17</td>
</tr>
<tr>
<td>Serum Sensitivity:</td>
<td>No difference</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>10% human serum, 80'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 10. The Δppk1 mutant did not have a motility defect in 0.3% and 0.5% agar.
Figure 11. Wild-type *C. jejuni* and the ∆ppk1 mutant displayed similar oxidative stress sensitivity.

Figure 12. The ∆ppk1 mutant was equally sensitive to the organic acids propionic acid and acetic acid as wild-type. AA = acetic acid; PA = propionic acid.
Figure 13. The Δappk1 mutant was not different from wild-type C. jejuni low iron conditions.

Figure 14. The Δappk1 mutant and wild-type C. jejuni grew identically in MH broth.
Figure 15. The Δppk1 mutant and wild-type C. jejuni grow identically under normal atmospheric conditions.

Figure 16. The Δppk1 mutant and wild-type C. jejuni grow identically under anaerobic atmospheric conditions.
Figure 17. The Δppk1 mutant and wild-type C. jejuni grow identically in 0.16 M NaCl in MH broth.
4.0. DISCUSSION

In this study, we have established that poly P accumulation in C. jejuni is important for survival of low nutrient and osmotic shock stresses, biofilm formation, and transmission. Furthermore, C. jejuni poly P was also identified as crucial for the virulence-related phenotype of intracellular survival in a human epithelial cell culture model of infection and for low dose-dependent colonization of chicks. To our knowledge, this is the first study demonstrating the importance of poly P in C. jejuni biology and pathogenesis.

The poly P molecule has been hypothesized as playing multiple functions in the bacterial cell. Poly P accumulation in bacteria is generally characterized by low levels during exponential phase growth, followed by a significant increase during stationary phase or during the onset of stress (Fuhs, 1975; Kornberg, 1995). Our wild-type C. jejuni strain exhibited this phenomenon, where poly P levels peaked during stationary phase (Fig. 4). In contrast, the C. jejuni Δppk1 mutant was unable to attain these levels (Fig. 4). The low basal level of poly P observed could be due to other mechanisms involved in acquiring or synthesizing poly P. For instance, C. jejuni harbours homologs of genes involved in phosphate uptake and possible alternate means of poly P metabolism, such as a high affinity phosphate uptake (pst) operon and a gene encoding a conserved putative PPK2 enzyme (Fouts et al., 2005; Hofreuter et al., 2006; Parkhill et al., 2000; Zhang et al., 2002). The PPK2 enzyme was first identified in P. aeruginosa, where it was shown to generate poly P preferentially from GTP in a reversible reaction, as opposed to the PPK1 synthesis of poly P preferentially from ATP (Ishige et al., 2002; Zhang et al., 2002). However, similar poly P levels at the 2 h time point may also be due to subculture ‘carry-over’ from the mid-log phase inoculum (Fig. 2, 4).

Our data demonstrate that poly P is important for the transmission-related phenotypes of low-nutrient stress survival, osmotic stress survival, and biofilm formation. C. jejuni's ability to survive in nutritionally poor environments is particularly critical during conditions such as
waterborne transmission, which despite the organism’s fastidious laboratory culture requirements, is a major source of larger-scale C. jejuni outbreaks (Auld et al., 2004; Friedman, 2001; Schuster et al., 2005). Our low-nutrient nutrient stress observations (Fig. 5) are consistent with studies of ppkl mutants in E. coli and other organisms, which also exhibit reduced survival following starvation (Kim et al., 2002; McMeechan et al., 2007; Tan et al., 2005; Tinsley and Gotschlich, 1995). A mechanistic model to explain this has been developed from work in E. coli, where nutrient downshift causes an immediate upsurge in poly P, which in turn complexes with and activates the ATP-dependent Lon protease to selectively degrade free ribosomal proteins, liberating amino acids to meet the nutritional requirements of the cell (Kuroda et al., 1997). A similar phenomenon could be occurring in C. jejuni and would explain the decreased survival of the Δppkl mutant under nutrient deprivation conditions.

Osmotic shock experiments suggest that C. jejuni’s requirement for poly P in both growth and survival during osmotic stress is most acutely required when (a) the organism must grow from isolated single bacteria into colonies (Fig. 6B), and (b) during later growth stages (Fig. 6A), where poly P levels were shown to rise dramatically in wild-type but not the Δppkl mutant (Fig. 4B). A number of enteric pathogens lacking ppkl are also less tolerant of osmotic stress than the parental wild type strains; this includes E. coli, Salmonella spp., and V. cholerae (Jahid et al., 2006; McMeechan et al., 2007; Rao and Kornberg, 1996). The decreased salt stress survival of the Δppkl mutant in later growth stages and in isolated bacteria may also be explained by the discovery that poly P affects mRNA stability by means of the RNA degradosome (Blum et al., 1997); more recently, poly P was also found to interact with ribosomes in promoting translation fidelity (McInerney et al., 2006). Also, studies in Salmonella spp. indicate that poly P may play a role in ATP homeostasis, particularly in stationary phase (McMeechan et al., 2007).

In contrast to the osmotic shock and low nutrient data, and in marked contrast to poly P mutants in other bacteria, the C. jejuni Δppkl mutant appeared to accelerate abiotic surface
attachment and biofilm development versus wild-type (Fig. 7 A, B), with no obvious differences in planktonic growth rate (Fig. 4A, 14). The role of poly P in biofilm formation was first studied in *P. aeruginosa*, where a ΔppkI mutant was defective for motility, biofilm maturation and quorum sensing (Rashid *et al.*, 2000b). Likewise, poly P was also required by *V. cholerae*, *Bacillus cereus* and *Porphyromonas gingivalis* for motility and biofilm formation (Chen *et al.*, 2002; Rashid and Kornberg, 2000; Shi *et al.*, 2004). Biofilm formation is thought to protect bacteria from adverse environmental conditions and is considered an important virulence factor. Environmental biofilms have also been proposed as a likely mechanism by which *C. jejuni* survives hostile environments and overcomes its fastidious survival requirements, thereby contributing significantly to its worldwide prevalence (Dykes *et al.*, 2003; Kalmokoff *et al.*, 2006). We have recently found that the *C. jejuni* SR mutant also exhibits increased biofilm formation (McLennan, 2007). As with ΔppkI, this is contrary to observations in other bacteria, where loss of the SR typically leads to decreased biofilm formation. An ensuing hypothesis is that in *C. jejuni*, both the SR mutant and ΔppkI may be constantly stressed, resulting in activation of alternative stress response pathways that may be distinct from those found in γ-proteobacteria (see *rpoS* discussion below) and which in turn accelerate conversion to a protective biofilm state.

We have also identified clear roles for poly P in both virulence- and colonization-related attributes of *C. jejuni*. Intra-epithelial cell survival is thought to be important for *C. jejuni* immune and chemotherapeutic evasion, in addition to damage, relapse and persistence in the human host (Day *et al.*, 2000; De Melo *et al.*, 1989; Kiehlbauch *et al.*, 1985; Russell *et al.*, 1993). Although it represents an important virulence phenotype, little is known about this aspect of *C. jejuni* pathogenesis. PPK1 is now the third *C. jejuni* factor, in addition to SpoT and the ferrous iron Fe^{2+} transporter FeoB (Naikare *et al.*, 2006), shown to be required for extended intracellular survival in epithelial cells (Fig. 8 A, B). Previous cell biology-based work suggested
that *C. jejuni* resides in a vacuole or vacuole-like compartment following cell internalization (Hu and Kopecko, 1999; Hu et al., 2006). Notably, a requirement for SpoT, FeoB, and PPK1 in intracellular survival also supports this hypothesis. Vacuoles are typically low-nutrient, low-iron, environments (O'Riordan and Portnoy, 2002). The SR is induced in such an environment, which would also be expected to require FeoB for iron uptake when the extracellular concentration has been reduced. Poly P's importance in low nutrient survival is consistent with this and likely provides a mechanistic explanation for the Δppk1 intracellular survival defect. A *S. Typhimurium* Δppk1 mutant also displayed an invasion and long-term intracellular survival defect in HEp-2 epithelial cells (Kim et al., 2002). The only other study investigating a role for poly P in *Campylobacter* spp. showed that a *C. coli* UA585 Δppk1 mutant was equally sensitive to macrophage killing as wild-type, and that PPK1 was not involved in protection against oxygen radicals in macrophage cells (Wassenaar et al., 1997). However, poly P was shown to be important for macrophage survival of *S. Typhimurium* (Kim et al., 2002).

The importance of poly P accumulation *in vivo* has been shown in various pathogens, including *Salmonella* spp. and *P. aeruginosa*, and for colonization of certain strains of *H. pylori* (Ayraud et al., 2003; McMeechan et al., 2007; Rashid et al., 2000b; Tan et al., 2005). Chickens are a natural zoonotic reservoir for *C. jejuni*, and contamination of commercial broiler flocks is thought to account for the majority of human *C. jejuni* infections (Lee and Newell, 2006). Interestingly, the *C. jejuni* Δppk1 mutant exhibited a dose-dependent colonization defect in chicks, with no chicks colonized to any detectable level at a ~10^5 CFU inocula, whereas the same dose of wild-type colonized to 10^7-10^9 cfu/g cecal content (Fig. 9). Although 10^5 CFU is the lowest dose used in our studies, wild-type *C. jejuni* 81-176 colonizes chicks well at doses as low as 10^3 CFU (MacKichan et al., 2004); thus, this is a significant colonization defect for a fully motile *C. jejuni* mutant (Fig. 10). The Δppk1 mutant hyper-biofilm formation phenotype also appears to be dose-dependent (Fig. 7). One hypothesis to explain the dose-dependent
colonization for the Δppkl mutant is that at low doses, the mutant is primarily planktonic and significantly more susceptible than wild-type to in vivo stresses. As dose increases, biofilm formation is accelerated in the mutant versus wild-type, protecting Δppkl during the initial (or later) stages of colonization. Recent reports indicate that several bacteria, including P. aeruginosa and H. pylori, form biofilms during infection (Carron et al., 2006; Garcia-Medina et al., 2005). Although such studies have not yet been conducted for C. jejuni, it is interesting to note that the C. jejuni SR mutant, which also exhibits certain planktonic sensitivities yet forms highly exaggerated biofilms, is fully colonization-competent in both chicks and mice (Gaynor et al., 2005; McLennan, 2007).

Poly P clearly affects certain conserved phenotypes in all (or most) bacteria studied, while other phenotypes are much more species-specific. In most bacteria, poly P-deficient mutants are unable to express rpoS, and this has been proposed as the reason for certain Δppkl mutant phenotypes (Fraley et al., 2007; Shiba et al., 1997). One study has shown that poly P levels in P. aeruginosa were not involved in modulating rpoS expression levels (Bertani et al., 2003). However, this is not true for all pseudomonads, as the root colonizer P. chlororaphis poly P-depleted mutant demonstrated decreased rpoS expression at all growth phases (Kim et al., 2007). C. jejuni lacks rpoS, which may account for some of the surprising phenotypic differences between the C. jejuni Δppkl mutant and other Δppkl mutant organisms such as E. coli and P. aeruginosa. A double knockout of ppkl and ppk2 may be necessary to severely deplete cellular poly P. However, a single ppkl deletion sufficiently induced motility defects in both P. aeruginosa, and V. cholerae, each of which harbor both ppkl and ppk2 (Ogawa et al., 2000; Rashid and Kornberg, 2000), while the C. jejuni Δppkl mutant was fully motile. There is also conflicting evidence as to whether the phosphate (pho) regulon regulates ppkl expression in various bacteria (Gavigan et al., 1999). C. jejuni was recently shown to harbour a PhoSR two-component signal transduction system which, like PhoBR in E. coli, controls numerous...
phosphate acquisition genes via binding to promoter pho box regions (Wosten et al., 2006). The
*C. jejuni ppkl* gene does not appear to be under the molecular control of the pho regulon, as
neither a ‘traditional’ pho box (Wosten et al., 2006) nor the recently identified PhoSR consensus
binding sequence are found upstream of the *ppkl* gene, and *ppkl* was not reported as down-
regulated in a ΔphoR mutant.

Functional and regulatory links between the SR and poly P accumulation have been
demonstrated in *E. coli* (Ault-Riche et al., 1998; Kuroda et al., 1997; Kuroda et al., 1999; Rao et
al., 1998). In that organism, ppGpp inhibits poly P hydrolysis by blocking the activity of the PPX
exopolyphosphatase. In *E. coli* SR mutants lacking ppGpp, PPX remains active, resulting in
diminished levels of poly P (Kuroda et al., 1997). Consistent with this, we observed diminished
levels of poly P in the *C. jejuni ΔspoT* mutant (Fig. 2B), suggesting that this mechanism of poly
P regulation may be conserved between *C. jejuni* and *E. coli*. However, in *E. coli*, *ppkl* and *ppx*
are in an operon; thus levels of PPK1 and PPX are also transcriptionally co-regulated. In
contrast, *ppkl* and *ppx* in *C. jejuni* and in several other bacteria (Mullan et al., 2002b) are not
found in an operon and thus may not be transcriptionally linked. Thus, as with the pho regulation
described above, this aspect of poly P modulation may also differ significantly from *E. coli*.

In summary, this study demonstrates the importance of poly P in *C. jejuni* transmission,
colonization, and infection of host cells, and has established that poly P likely interacts with SR
mechanisms in *C. jejuni*. Future work exploring the downstream molecular effects of poly P in
*C. jejuni* should lend significant additional insight into mechanisms allowing *C. jejuni* to remain
such a prevalent human pathogen.
4.1. Future Directions

It is now known that poly P plays multifactorial roles in *C. jejuni* pathogenesis and that it intersects with the SR. However, much more can be learned about poly P and its role in pathogenesis via further investigation of the genes responsible for its metabolism, the downstream effects it exerts on *C. jejuni*, and the connection between poly P and the SR. *C. jejuni* harbours two enzymes involved in poly P synthesis: PPK1, which generates poly P from ATP, and PPK2, which primarily converts GDP to GTP, but can also synthesize poly P (Ishige *et al.*, 2002; Zhang *et al.*, 2002). The *C. jejuni* Δppk1 mutant demonstrated a significant deficit in poly P accumulation, yet low basal levels poly P remained at all time-points tested. Thus, to completely abrogate poly P synthesis a double ppk1 and ppk2 mutant may be necessary to determine further phenotypes relating to virulence and survival that are controlled by poly P metabolism. *C. jejuni* appears to harbour a gene homologous to an exopolyphosphatase, known as ppx. The creation of a Δppx mutant strain will allow the examination of poly P overexpression in *C. jejuni* and may lead to novel observations regarding poly P metabolism and its role in pathogenesis. Conceivably, overproduction of poly P in *C. jejuni* may increase colonization capacity, as was seen in an poly P over-expressing strain of *Helicobacter pylori* (Ayraud *et al.*, 2003).

To date, nothing is known regarding the downstream molecular effects of poly P synthesis in *C. jejuni*. In other bacterial, poly P is known to play a role in ATP homeostasis, protein synthesis, and mRNA levels. To explore the downstream effects of altered poly P levels in *C. jejuni*, intracellular ATP levels over various growth phases in wild-type versus the Δppk1 mutant could be tested via an ATP bioluminescence assay, as used in assessing a *Salmonella* spp. Δppk1 mutant (McMeechan *et al.*, 2007). Moreover, polysome levels should be monitored in the Δppk1 mutant versus wild-type. Furthermore, transcriptional profiling through microarray analysis experiments may reveal global gene expression changes incurred as a result of deleting...
ppkl and would be valuable in analyzing gene expression differences between the Δppkl mutant and wild-type. No poly P-related microarray experiments have ever been published. Thus, these studies will likely yield novel information regarding C. jejuni and poly P in metabolism in other organisms.

We have demonstrated a connection between poly P accumulation and the SR in C. jejuni. In E. coli, nutrient downshift leads to the activation of the SR and the accumulation of ppGpp, which inhibits the hydrolysis of poly P; thus, poly P accumulates and complexes with and activates the ATP-dependent Lon protease to degrade free ribosomal proteins, meeting the nutritional requirements of the bacteria cell (Kuroda et al., 1997). Therefore, to elucidate the precise role in the E. coli proposed model of poly P metabolism a lon protease mutant would assist in determining the role of poly P in ribosomal protein degradation.

These future experiments will be very informative and yield new data regarding C. jejuni pathogenesis, connections with the SR, and poly P metabolism in other bacteria.
BIBLIOGRAPHY


68

APPENDICES

Appendix A

**Insertional inactivation of the ppkl gene via cat cassette insertion.** Insertion was verified by PCR using PPK1-comp Fp (CGGGATCCTGCCCTTAGCGTTATAAAAAGTATAAA) and PPK1-comp Rp (CGATCGATAATTTTCGGTCGGTCATTTTTGATAGTGTAG) primers that are external to the ppkl gene. WT = wild-type C. jejuni 81-176; Δppkl = inactivated ppkl gene with cat cassette insertion. The wild-type C. jejuni band generates a 2.3 Kb PCR product and the Δppkl mutant PCR product is 2.0 Kb.
Appendix B

Sequence analysis with the PPK1-1 Fp and subsequent ClustalW alignment of *C. jejuni* 81-176 *ppk1::cat* with *cat* cassette verified insertional inactivation of the *ppk1* gene.

<table>
<thead>
<tr>
<th>PPK1-1 cat</th>
<th>aaacaatatatcttttcatagcgttttaaatttgcgataaagccatcctgaacttgt</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPK1-1 cat</td>
<td>aaatttggaatgatttcagctgctgctgggtttcttttcaagttaattgcg</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>-----------------------------------------------gcggtttccttttcaagttaattgcg</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>gatatagattgaaaagtggatagatttatgatatagtggatagatttatgatataatgag</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>gatatagattgaaaagtggatagatttatgatatagtggatagatttatgatataatgag</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>ttatcaacaacctggaatttacggaggataaatgatgcaattcacaaagattgatataaa</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>ttatcaacaacctggaatttacggaggataaatgatgcaattcacaaagattgatataaa</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>tatgacggttaaactccaatattttctaatagtgatagatgatatatag</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>tatgacggttaaactccaatattttctaatagtgatagatgatatatag</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>ctcttttatatttctaatgtagatcatcatcaatccgacatgaaaaattcaggacccctt</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>ctcttttatatttctaatgtagatcatcatcaatccgacatgaaaaattcaggacccctt</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>aaatgaaaaaacggaaatagtggatgtgcttttttccaaaatgctgcctgccacatcaggcgtttttt</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>aaatgaaaaaacggaaatagtggatgtgcttttttccaaaatgctgcctgccacatcaggcgtttttt</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>aaaaaggaaccc- -----------------------------------------------</td>
</tr>
<tr>
<td>PPK1-1 cat</td>
<td>ttctcaggatataatagacgtttttgtgaaatggtgcctgcttcgcaaa</td>
</tr>
</tbody>
</table>
Appendix C
Colonies representing putative reconstituted wild-type strains were confirmed using PCR with the PPK1-1 primer set. WT = wild-type *C. jejuni* 81-176; Δppk1 = inactivated *ppk1* gene with *cat* cassette insertion; *ppk1* = reconstituted wild-type *C. jejuni*. Wild-type and *ppk1* generate a 2.6 Kb PCR product when amplified with PPK1-1 primer set. The *ppk1* mutant PCR is 2.4 Kb.
Appendix D

Sequence analysis with the PPK1-2 Fp and subsequent ClustalW alignment of *C. jejuni* 81-176 *ppkl* gene and the *ppkl* * recombinant sequence verified complementation.

**PPK1-2**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ppkl</em></td>
<td>GCCATACGCAAATTTTACCAACAGGAAGAATTTCGTTATTTTAATGAAATC</td>
<td>ABINNNTCTTGGG---TATTTTA---TGAATC</td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>ACAAGTGAATAGAAAAAGAAAAATTCTTTTCAAAAAACATATGAGAATTTTAGATGAAAAAT</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>TTAAAACAATTTGATAGAATTTTCTCTCTATATTTTTCTCCTTATATTTTTCTCTCTTTTTGTTTCAAATA</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>ACAAGTGAATAGAAAAAGAAAAATTCTTTTCAAAAAACATATGAGAATTTTAGATGAAAAAT</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>TTAAAACAATTTGATAGAATTTTCTCTCTATATTTTTCTCCTTATATTTTTCTCTCTTTTTGTTTCAAATA</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GCTGTTGATGCACTCACCCTTTTCCGATTTAAACATCTTTTCACTAAGCGGTT</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GCTGTTGATGCACTCACCCTTTTCCGATTTAAACATCTTTTCACTAAGCGGTT</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>AAAATTTGCGATAAAGCGCATCCTGAACTTGTAAAATTTGGAATGATTAGAATTCCAAGA</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>AAAATTTGCGATAAAGCGCATCCTGAACTTGTAAAATTTGGAATGATTAGAATTCCAAGA</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GTTTTACTCTCGTTTTTATGAAGTAAGTGCAAATATTTATGTTCCTATAGAAAGTATAGTC</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GTTTTACTCTCGTTTTTATGAAGTAAGTGCAAATATTTATGTTCCTATAGAAAGTATAGTC</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>CATCAACACGCAGAAAATAATTTTTCCAGGCTATAAACTCTTAGCTTCAGCAGCATTTAGA</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>CATCAACACGCAGAAAATAATTTTTCCAGGCTATAAACTCTTAGCTTCAGCAGCATTTAGA</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GT GACTAGAAATGCAGATATGGTAATAGAAGAGGAAGAAGCTGATGATT TATTATGATGATT</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GT GACTAGAAATGCAGATATGGTAATAGAAGAGGAAGAAGCTGATGATT TATTATGATGATT</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>TTAGAACAAGGCGTACGCTCAAGCCAAAGGAGCTTTTGTAAGATTGCAAATTTCAAAAA</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>TTAGAACAAGGCGTACGCTCAAGCCAAAGGAGCTTTTGTAAGATTGCAAATTTCAAAAA</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GATGCAGATGAGCAAATCGTAGAATTTCTTAATACTCACATGAAAATTTTTCATAAGAT</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GATGCAGATGAGCAAATCGTAGAATTTCTTAATACTCACATGAAAATTTTTCATAAGAT</td>
<td></td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GTTTATGAAATATTCTTTTACTCAAATCTCCTTCCCTGCTTTGGCAATCCGAGAAATAA</td>
<td>GATGCAGATGAGCAAATCGTAGAATTTCTTAATACTCACATGAAAATTTTTCATAAGAT</td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>GTTTATGAAATATTCTTTTACTCAAATCTCCTTCCCTGCTTTGGCAATCCGAGAAATAA</td>
<td>GATGCAGATGAGCAAATCGTAGAATTTCTTAATACTCACATGAAAATTTTTCATAAGAT</td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>ACCTTTACGCACTTTTAAAGCCCACTTTTACAGCCTAAACTTTACCACCTTTTTGATGAG</td>
<td>ACCTTTACGCACTTTTAAAGCCCACTTTTACAGCCTAAACTTTACCACCTTTTTGATGAG</td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>ACCTTTACGCACTTTTAAAGCCCACTTTTACAGCCTAAACTTTACCACCTTTTTGATGAG</td>
<td>ACCTTTACGCACTTTTAAAGCCCACTTTTACAGCCTAAACTTTACCACCTTTTTGATGAG</td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>AATTTATCTTTTATTGAGAAGAAAAGAGATAATACAATAAACAACCTTTTTGAA</td>
<td>AATTTATCTTTTATTGAGAAGAAAAGAGATAATACAATAAACAACCTTTTTGAA</td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>AATTTATCTTTTATTGAGAAGAAAAGAGATAATACAATAAACAACCTTTTTGAA</td>
<td>AATTTATCTTTTATTGAGAAGAAAAGAGATAATACAATAAACAACCTTTTTGAA</td>
</tr>
<tr>
<td><em>ppkl</em></td>
<td>AGTTTTGATCCAGTTTAAATTTTATCAAAGAGCAAGCAAGATCCTGAAAGTAATTTCC</td>
<td>73</td>
</tr>
</tbody>
</table>
ppk1* AGTTTTGATCCAGTTTATAATTATCTAAAGGAAGGCA-GCAAGATCCTGAAGTAATTCC

PPK1-2 ATTAGAATGACACTTTATAGGTTAAAAAAATTCCAAATAGTTCAAGCTTAAATTGAT

ppk1* ATTAGAATGACACTTTATAGTTGAAAAAAATTCCAAATAGTTCAAGCTTAAATTGAT

********** ********** ********** **********