ATYPICAL ROLES FOR CAMPYLOBACTER JEJUNI AA-ABC TRANSPORTER COMPONENTS PAQP AND PAQQ IN BACTERIAL STRESS TOLERANCE AND PATHOGEN-HOST CELL DYNAMICS

by

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B.Sc. Honours, The University of British Columbia, 2006

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

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA
(VANCOUVER)

AUGUST, 2008

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ABSTRACT

Campylobacter jejuni is a human pathogen that causes severe diarrheal disease. However, our understanding of C. jejuni virulence mechanisms and survival during disease and transmission remains limited. Amino acid ATP Binding Cassette (AA-ABC) transporters in C. jejuni have been proposed as being important for bacterial physiology and pathogenesis. We have investigated a novel AA-ABC transporter system, encoded by *cj0467-9*, by generating targeted deletions of *cj0467* (membrane transport component) and *cj0469* (ATPase component) in *C. jejuni* 81-176. Analyses described herein have led us to designate these genes *paqP* and *paqQ*, respectively [pathogenesis-associated glutamine (q) ABC transporter permease (P) and ATPase (Q)]. We found that loss of either component resulted in amino acid uptake defects, most notably diminished glutamine uptake. Both Δ*paqP* and Δ*paqQ* mutants also exhibited a surprising but significant increase in short-term intracellular survival in macrophages and epithelial cells. Levels of resistance to a series of environmental and in vivo stresses were examined. Both mutants were hyper-resistant to aerobic and oxidative stress, and while Δ*paqP* was also hyper-resistant to heat and osmotic shock, Δ*paqQ* was more susceptible than wild-type to the latter two stresses. Annexin-V staining coupled with fluorescence microscopy revealed that macrophages infected with the Δ*paqP* and Δ*paqQ* mutants underwent a lower level of apoptosis than cells infected with wild-type bacteria. Macrophages infected with the mutant strains exhibited a transient decrease in ERK activation compared to wild type-infected macrophages, potentially explaining the reduced apoptosis phenotype. The Δ*paqP* mutant did not exhibit a defect for short or longer term mouse colonization, consistent with its increased stress survival and diminished host cell damage phenotypes. Collectively, these results demonstrate a unique correlation between an AA-ABC transporter with bacterial stress tolerance, intracellular survival, host cell damage, and host signal transduction in response to pathogen infection.
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<tbody>
<tr>
<td>ΔpaqP</td>
<td>C. jejuni pathogenesis associated glutamine transporter permease mutant</td>
</tr>
<tr>
<td>ΔpaqPc</td>
<td>C. jejuni pathogenesis associated glutamine transporter permease complement</td>
</tr>
<tr>
<td>ΔpaqQ</td>
<td>C. jejuni pathogenesis associated glutamine transporter ATPase mutant</td>
</tr>
<tr>
<td>ΔspoT</td>
<td>C. jejuni spoT mutant</td>
</tr>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP Binding Cassette</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CDT</td>
<td>Cytolethal Distending Toxin</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>Campylobacter jejuni</td>
</tr>
<tr>
<td>CFUs</td>
<td>colony forming units</td>
</tr>
<tr>
<td>Cm</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>CV</td>
<td>crystal violet</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified Eagle Media</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dH2O</td>
<td>distilled water</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GBS</td>
<td>Guillain-Barré syndrome</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma glutamyl transferase</td>
</tr>
<tr>
<td>gln</td>
<td>L-glutamine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>H. pylori</td>
<td>Helicobacter pylori</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxide</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>INT407</td>
<td>human intestinal 407</td>
</tr>
<tr>
<td>jnk</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipo-oligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipo-oligosaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Media</td>
</tr>
<tr>
<td>MH</td>
<td>Muller Hinton</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>NEAA</td>
<td>non-essential amino acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density at 600nm</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>r.p.m.</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline Tween-20</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>UI</td>
<td>uninfected</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
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ACKNOWLEDGEMENTS

I offer my enduring gratitude and sincere thanks to my supervisor, Dr. Erin Gaynor, for her constant encouragement and support. Her positive and cheerful attitude as well as helpful guidance throughout my studies have allowed me to work productively yet pleasantly in the lab during the past two years. I very much appreciate her incredible enthusiasm and patience in offering direction and insights at each stage of my project. This has truly been a wonderful learning experience for me.

I would also like to thank members of the Cvitkovitch lab from the University of Toronto for their kindness and hospitality during the 2 weeks when I was in Toronto performing the amino acid transport assay experiment. I am especially grateful to Kirsten Krastel and Dr. Dennis Cvitkovitch, not only for their extreme enthusiasm and hospitality, but also for helping me with the assays and data interpretations. I also thank Rhonda Hobbs and Dr. Stuart Thompson from the Medical College of Georgia for performing the in vivo mouse infections. I thank Dr. Michael Gold from our department for kindly providing the MAPK antibodies.

Sincere thanks to my thesis committee members, Dr. Bruce Vallance and Dr. Bill Mohn. A huge thank you to each and everyone in the Gaynor lab, past and present, for creating a positive, energetic and entertaining work environment, which made everyday at the lab fun and enjoyable. Particularly, I would like to thank Mizue Naito, my partner in fighting off the world of diarrheal diseases, for being a great friend in and out of the lab. I also thank Dr. Emilisa Fridrich, who I always share a good laugh with discussing various kinds of issues, lab related or not. Grad school has never been dull for a day with their presence.

I have to offer my deepest thanks to my family, particularly my mom and dad, as well as my friends, for their continuous support. Especially, I have to thank Dr. Min Liu, for being a great listener, and being like a sister anyone would only dream to have. Her compassion, wisdom advice and constant encouragement have helped me tremendously through the past few years of my life. I would also like to thank Karen Chang, Karen To and Mila Chou, my dearest and loyal friends, for being supportive since our undergraduate and high school years; I would not have made it through without them.

Finally, I would like to thank the Canadian Institutes of Health Research (CIHR) and Michael Smith Foundation for Health Research (MSFHR) for funding my research stipend, as well as CIHR, the MSFHR, and the Burroughs Wellcome Fund for supporting my research activities.
DEDICATION

To my uncle Feng - my inspirer in science, who is no longer with us.
1.0 INTRODUCTION
1.1 *Campylobacter jejuni* - History and Epidemiology

*Campylobacter jejuni* is a highly prevalent bacterial pathogen that causes human gastroenteritis (Young et al., 2007). *Campylobacter* spp. were first observed in 1886 by Escherich in the colons of neonates. However, it was not until 1968 that Dekeyser, Buzler and colleagues first isolated *Campylobacter* spp. from the feces of a human diarrheic patient (Butzler, 2004). By the mid-1970s, *Campylobacter* spp. were recognized as important human pathogens. Currently, *Campylobacter* spp. are ranked as the most common bacterial cause of diarrheal illnesses in developed countries (Blaser, 1997). With an estimated 400 million cases per year worldwide (Girard et al., 2006), *Campylobacter* spp. affect more individuals than *E. coli* O157, *Salmonella* spp., and *Shigella* spp. combined (Blaser et al., 1983). For instance, ~1% of the population in North America and Western Europe are infected each year, with approximately 3 million people infected in the United States alone (Mead et al., 1999). The genus *Campylobacter* contains 17 species, including *C. hyointestinalis, C. lanienae, C. sputorum, C. mucosalis, C. concisus, C. curvus, C. rectus, C. gracilis, C. showae, C. hominis, C. lari, C. insulaenigrae, C. canadensis, C. upsaliensis, C. helveticus, C. coli, and C. jejuni* (Debruyne et al., 2008). Although several of these cause diarrheal disease, *Campylobacter jejuni* is estimated to account for 90% of reported cases of diarrheal campylobacteriosis (Mead et al., 1999).

*C. jejuni* is a zoonotic organism that exists as a commensal in chickens and other avian species (Beery et al., 1988); hence, infection of chickens with *C. jejuni* typically does not lead to diarrhea. This commensal relationship is thought to account for the fact that the main source of human *C. jejuni* infection is via consumption of contaminated poultry or cross-contamination of other food with raw poultry juice. In addition, high carriage rates of *C. jejuni* have been identified in Canadian geese and migratory ducks, suggesting that wild birds may play a role in spreading *C. jejuni* in the environment (Pacha et al., 1988). Other routes of human *C. jejuni* infection are also of a primary zoonotic nature and include ingestion of water contaminated by
fecal runoff and ingestion of unpasteurized milk (Young et al., 2007; Blaser et al., 1983). The latter two also account for the majority of large-scale Campylobacteriosis outbreaks.

1.2 Campylobacteriosis

1.2.1 Symptoms

Upon infection, C. jejuni predominantly colonizes the jejunum, ileum and the colon in humans (Allos and Blaser, 1995). The infectious dose for C. jejuni can be quite low, with some strains colonizing with an approximate 200 bacteria/dose (Blaser, 1997). Clinical symptoms often appear within 4 days and are characterized by profuse, often bloody diarrhea, acute abdominal pain, fever, malaise, nausea, and vomiting (Allos et al., 1998; Blaser et al., 1983). The acute illness usually lasts for 2-5 days, and patients typically recover fully. However, approximately 25% of patients experience a relapse. Complications of infection can also include intestinal hemorrhage, haemolytic uraemic syndrome, or inflammation of abdominal lymph nodes, also known as mesenteric adenitis (Allos et al., 1998).

1.2.2 Medical Sequelae

Acute C. jejuni infections usually resolve by 10-14 days. In some cases, longer-term medical sequelae can occur, including reactive arthritis, Guillain-Barré Syndrome (GBS), which is the primary cause of acute ascending bilateral paralysis, and Miller Fisher Syndrome, a non-paralytic rare variant of GBS (Humphrey et al., 2007). GBS is an autoimmune disorder in which antibodies are produced against the myelin sheath that surrounds the axons of the peripheral nerves. This inhibits effective signal transmission resulting in acute paralysis. Although the exact cause of GBS still remains unknown, a
large majority of GBS cases have been associated with prior *C. jejuni* infection (13-39% of cases) (Yu *et al.*, 2006). *C. jejuni* lipo-oligosaccharide (LOS) closely resembles the peripheral nerve ganglioside, GM1 (Yu *et al.*, 2006), and during *C. jejuni* infection, antibodies produced against *C. jejuni* LOS may cross-react with GM1, thereby inducing GBS. GBS symptoms usually manifest 1-3 weeks after the resolution of enteritis symptoms (Butzler, 2004).

### 1.2.3 Treatment

Although acute *C. jejuni* infection is often self-limiting and typically resolves within 2 weeks, antimicrobial agents are sometimes required for more severe, complicated or systemic infection. Currently, macrolides such as erythromycin and fluororquinolones such as ciprofloxacin are the most common antibiotics prescribed for uncomplicated *C. jejuni* infection (Nachamkin, 2001). Furthermore, antibiotics have also been incorporated to feedings of chicks to lower *C. jejuni* colonization. Unfortunately, reports describing the emergence of antimicrobial-resistant *C. jejuni* have increased since the late 1980s (Moore *et al.*, 2001). Although a few candidate vaccines have been developed (Girard *et al.*, 2006), an effective vaccine is still unavailable. Vaccine development has been challenging due to the complex nature of *C. jejuni* surface antigens, including several polysaccharide structures conferring multiple serotype combinations.

### 1.3 Morphology and Characteristics

*C. jejuni* is a spiral, Gram-negative bacillus that is 0.2-0.9 μm wide, 0.5-5.0 μm long. It belongs to the epsilon class of proteobacteria, in the order of *Campylobacteriaceae*, together with the genera *Helicobacter*, *Wolinella*, *Sulfurospirillum*, *Acrobacter* and *Dehalospirillum*. These species typically have small genomes of 1.6-2.0 megabases (Young *et al.*, 2007; Thomas
et al., 1999). The complete genome sequences have been deduced for the *C. jejuni* strains NCTC 11168, RM1221, 81-176 and 269.97 isolated from human blood culture. The genome size may vary slightly depending on the strain; for instance, *C. jejuni* NCTC 11168 has 1633 protein encoding genes, and *C. jejuni* 81-176 has 1748 protein encoding genes according to the National Microbial Pathogen Data Resource Center (http://www.nmpdr.org/FIG/wiki/view.cgi/Main/Campylobacter). As a naturally competent species, *C. jejuni* is able to take up DNA from the environment. With a flagellum at each end, *C. jejuni* is highly motile and often exhibits rapid darting and spinning motions (Young et al., 2007).

*Campylobacter* is a difficult organism to culture, due to its unique growth requirements. As a fastidious microaerophilic organism, it survives optimally under 12% CO₂ and 6% O₂ conditions and requires complex nutrients for optimal survival (Gareaux et al., 2008; Park, 2002). Because *C. jejuni* is asaccharolytic and lacks a unidirectional enzyme, 6-phospho-fructokinase, in the glycolytic pathway, it cannot metabolize glucose and instead depends on amino acids for both carbon and energy sources (Guccione et al., 2008; Muller et al., 2005).

### 1.4 Metabolism

#### 1.4.1 Amino acid metabolism in *C. jejuni*

Amino acids (AAs) are essential nutrients required by *C. jejuni* for growth and survival (Guccione et al., 2008; Muller et al., 2005; Velayudhan and Kelly, 2002). As the most important carbon and energy resource, AAs thus contribute to the synthesis of numerous enzymes and metabolites including fatty acids, nucleic acids, proteins and other amino acids as well as organic acids, which can serve as metabolic substrates for the TCA cycle. Previous studies identified asparagine, aspartate, glutamine, glutamate, serine and proline as the major energy sources in *C. jejuni* strain NCTC 11168 (Guccione...
It is clear that as these AAs can be readily catabolized and act as precursors for the formation of organic acids such as pyruvate, fumarate, and succinate, which directly feed into the trichloracetic (TCA) cycle, serving as electron donors and/or acceptors essential for respiration. It was previously shown that glutamine, also the major nitrogen donor in \textit{C. jejuni}, is rapidly utilized at a significantly higher rate than glutamate (Westfall \textit{et al.}, 1986).

1.4.2 Glutamine metabolism and pathogenesis

Aside from being primary energy sources for growth and survival, AAs and their derivate also contribute to pathogenesis in several bacterial pathogens (Guccione \textit{et al.}, 2008; Barnes \textit{et al.}, 2007; Shibayama \textit{et al.}, 2007; Tullius \textit{et al.}, 2003; Smirnova \textit{et al.}, 2001). Recently, an enzyme involved in glutamine metabolism was associated with bacterial stress responses and host-pathogen interactions in both \textit{Helicobacter pylori} and \textit{C. jejuni} (Barnes \textit{et al.}, 2007; Shibayama \textit{et al.}, 2007). Gamma-glutamyl transpeptidase (GGT) in \textit{H. pylori} is involved in the hydrolysis of extracellular glutamine or glutathione (GSH) into glutamate for subsequent uptake and is involved in the induction of host cell apoptosis and \textit{in vivo} colonization (Shibayama \textit{et al.}, 2007). In \textit{C. jejuni}, GGT is involved in \textit{H}_2\textit{O}_2 resistance, host cell apoptosis, intracellular survival, and colonization of chicks and mice (Barnes \textit{et al.}, 2007; Hofreuter \textit{et al.}, 2006). GSH also functions as an antioxidant that protects \textit{E. coli} from oxidative damage and osmotic shock (Smirnova \textit{et al.}, 2001). In addition, glutamine synthetase, GlnA, in \textit{Mycobacterium tuberculosis}, has also been shown to be essential for virulence, as a \textit{Δ}glnA mutant is avirulent \textit{in vivo} and unable to survive within human macrophages \textit{in vitro} (Tullius \textit{et al.}, 2003). These studies highlight the importance of AA homeostasis and its potential role in bacterial
pathogenesis. However, details describing the molecular mechanisms of how AA metabolism is linked to pathogenesis remain largely unknown and await investigation.

1.4.3 Glutamine contributes to host cell survival

While little is known about glutamine metabolism in bacteria and how it contributes to pathogenesis, the effect of glutamine on signaling processes in eukaryotic cells has been characterized. Intracellular glutamine modulates heat shock protein (Hsp) expression and reactive oxygen species (ROS) levels in cells such as enterocytes, or intestinal epithelial cells, and promotes lymphocyte cell proliferation as well as suppressing cell death (Phanvijhitsiri et al., 2006; Wischmeyer et al., 2003; Chang et al., 2002; Chang et al., 1999). Moreover, apoptosis in intestinal epithelial cells is prevented by the presence of intracellular L-glutamine (Larson et al., 2007; Nakamura and Hagen, 2002). Specifically, a study showed that L-glutamine is able to inhibit H. pylori-induced apoptosis in enterocytes, suggesting an anti-cytotoxic activity of glutamine in the stomach (Nakamura and Hagen, 2002).

1.5 ATP BINDING CASSETTE (ABC) TRANSPORTERS

1.5.1 AA-ABC Transporter Structures

To effectively utilize AAs for energy production, AAs must be actively transported from the external environment into the C. jejuni cytosol across the cytoplasmic membrane barrier. This process is predominantly mediated by ABC (ATP Binding Cassette) transporters, a superfamily composed of proteins found in both prokaryotes and eukaryotes. ABC uptake transporters are typically comprised of three functional domains: a permease, an ATPase, and a substrate-binding protein (Fig. 1.1).
While the permease and ATPase comprise the core of an ABC transporter and, in bacteria, are typically encoded by genes in the same operon, the substrate-binding domain is usually encoded by a gene encoded elsewhere on the genome (Muller et al., 2005; Hekstra and Tommassen, 1993). In bacteria, a functional transporter usually contains a homodimer of a single transmembrane polypeptide or a heterodimer of two distinct polypeptides (Saurin et al., 1999). Each of the hydrophobic permease components typically forms 6 putative alpha-helical integral transmembrane polypeptide segments, which shuttle the substrate across the bacterial membrane. The hydrophilic ATPase component is typically formed by two monomers and resides in either the periplasm or the cytoplasm. The catalytic pocket for ATP hydrolysis to provide energy for transfer of the substrate into the bacterial cytoplasm is only produced by cooperation between the two monomers. In contrast to the permease domain, the ATPases show a high degree of sequence similarity and identity across the ABC family, implying a conserved structure and function for these domains (Garmory and Titball, 2004; Jones and George, 1999).

Specifically, these proteins contain three highly conserved motifs: (1) Walker A, a glycine-rich loop; (2) Walker B, a hydrophobic domain, at the ATP binding site, and (3) C-motif, (LSGGWW/R/KWR), also known as the peptide linker, which is found in all ABC transporters. This motif is located immediately N-terminal to the Walker B motif and is the site of mutations which severely impair function in many ABC transporters.

It is also interesting to note that the peripheral substrate binding protein and ATP binding protein can be promiscuous and therefore free to interact with other transporter systems. Previous studies have shown that in the absence of a binding protein, certain transporter systems may couple with other periplasmic-binding proteins or ATP binding proteins to achieve high efficiency uptake (Forward et al., 1997; Schlosser et al., 1997;
Wilken et al., 1996; Hekstra and Tommassen, 1993), suggesting that these ABC transporter components are not always confined exclusively to a single system.
Figure 1.1. Schematic diagram of a bacterial ATP Binding Casette (ABC) transporter. An ABC transporter is composed of two alpha-helical transmembrane proteins and an ATP-binding protein, which are encoded by genes in the same operon. A periplasmic substrate binding protein encoded by a gene outside of this operon is often coupled to this system.
1.5.2 ABC Transporter Functions

ABC transporters play an essential role in most bacteria due to their ability to rapidly mediate nutrient uptake for energy and survival (Higgins, 1992). Through ATP-dependent active transport, nutrients such as sugars or amino acids can be accumulated within bacteria against a concentration gradient (Dippel and Boos, 2005). Although the exact mechanism remains unclear, it is proposed that once loaded with a specific substrate, the substrate binding protein is able to dock on the external domain of the permease protein to initiate ATP binding to the ATPase. This triggers simultaneous conformational change between the permease and ATPase to open the channel, and ATP hydrolysis to facilitate active transport of the substrate into the cytoplasm against the concentration gradient. In addition to importing nutrients for consumption, ABC transporters may also export substances from the bacterial cytoplasm (Nikaido, 2002). Previous studies have shown that ABC transporters participate in virulence protein secretion (Holland et al., 2005) as well as the efflux of hydrophobic compounds, some of which exert antimicrobial activities (Quinn et al., 2007; Elkins and Beenken, 2005). Bacterial ABC transporters have also been proposed to regulate other processes, such as modifying signal transduction pathways (Matsuo et al., 2003). For instance, the oligopeptide permease of the *Bacillus Subtilis* ABC transporter stimulates competence development and the initiation of sporulation by importing signaling peptides. Therefore, ABC transporters have been shown to affect bacterial virulence by several different means.
1.5.3 AA-ABC transporters serve as pathogenesis determinants in *C. jejuni*

It is estimated that *C. jejuni* harbours 30 complete ABC transporter systems, both importers and exporters (Kelly, 2008). Although it is difficult to deduce the substrates for each transporter system, it is predicted that most of these systems are responsible for AA and organic acid transport, since *C. jejuni* utilizes AAs as a primary carbon source (Guccione et al., 2008). Several AA-ABC transporter system components already identified in *C. jejuni* include a glutamate/aspartate-binding protein (Peb1) (Leon-Kempis Mdel et al., 2006), a cysteine-binding protein (CjaA) (Muller et al., 2005), and a serine transporter protein (SdaC) (Velayudhan et al., 2004).

Peb1, CjaA and SdaC all participate in *C. jejuni* virulence (Leon-Kempis Mdel et al., 2006; Muller et al., 2005; Velayudhan et al., 2004). Peb1 is currently the best characterized AA-ABC transporter component and has been suspected to function as a surface antigen and adhesin. It contains two predicted sequences for signal peptidases I and II binding, and therefore can serve as both a surface exposed adhesin and a periplasmic solute. Absence of Peb1 attenuates infection both in vitro and in vivo (Muller et al., 2007; Leon-Kempis Mdel et al., 2006; Muller et al., 2005). Similarly, CjaA may act as a surface antigen involved in host cell adhesion (Muller et al., 2005). SdaC participates in serine uptake and is also important for virulence in vivo (Velayudhan et al., 2004).

1.5.4 AA-ABC transporters serve as pathogenesis determinants in other pathogens

ABC transporters in other bacterial pathogens act as virulence determinants. For instance, the ATP binding protein (GlnQ) of a glutamine transporter in Group B Streptococcus is involved in host cell surface adhesion and in vivo virulence (Tamura et al., 2002). A putative polypeptide ABC transporter system in *Salmonella*, encoded by the
yefABEF operon, also serves as a virulence factor, since deletion of the ATPase component inhibits the ability of these bacteria to proliferate within host cells (Eswarappa et al., 2008). Several ABC transporter components in Brucella are also important for virulence both in vitro and in vivo (Castaneda-Roldan et al., 2006; Rosinha et al., 2002).

1.6 PHYSIOLOGY AND STRESS RESPONSE

C. jejuni needs to traverse unfavourable external environments and overcome various challenges posed during the transmission process to establish infection and cause disease. Several stress response proteins enable C. jejuni to survive and respond to external stimuli such as oxidative, aerobic, osmotic, heat and acid stresses.

1.6.1 Heat stress

C. jejuni replicates within a temperature range of approximately 32°C-47°C (Garenaux et al., 2008; Park, 2002). However, its optimal temperature is between 37°C-42°C, which spans the average body temperature of humans (37°C) and avian species (42°C). C. jejuni is often acquired through ingestion of undercooked poultry, where it has likely survived both heating and cooling processes during handling. C. jejuni harbours several heat shock proteins (Hsps) which are often induced as a result of a thermal stress response (Murphy et al., 2006). DnaJ, DnaK, GroEL, and ClpB are the major Hsps associated with thermo-tolerance in C. jejuni. Expression of some Hsps can also increase in response to other stresses such as aerobic and oxidative or osmotic stresses (Murphy et al., 2006). For instance, the endoprotease HtrA and the acyltransferase HtrB are Hsps of C. jejuni induced during aerobic, osmotic and oxidative shocks in addition to heat stress (Phongsisay et al., 2007; Brondsted et al., 2005). Other stress regulators such as RacRS, a two-component signal transduction regulator system, and orthologues of HrcA and
HspR, have also been proposed to associate with thermo-regulation (Alter and Scherer, 2006). More recently, polyphosphate (poly P) and the two-component system sensor kinase Cj1226c, have also been identified to participate in hyper-osmotic stress resistance (Candon et al., 2007; Svensson and Gaynor, manuscript submitted).

1.6.2 Aerobic stress

As a capnophilic and microaerophilic organism, C. jejuni is sensitive to high levels of oxygen and has an optimal growth environment of 12% CO₂ and 6% O₂ (Murphy et al., 2006). Hence, it is generally sensitive to the level of oxygen in the atmosphere, which is approximately 21%. However, C. jejuni uses several strategies to tolerate aerobic stress (Jones et al., 1993). In addition to inducing heat shock proteins such as HtrA and HtrB as described above, C. jejuni utilizes the stringent response to counter high O₂/low CO₂ stress (Gaynor et al., 2005) and also produces proteins to facilitate the removal of reactive oxygen species (ROS), which are generated as a result of aerobic metabolism.

1.6.3 Oxidative (reactive oxygen species) stress

As previously mentioned, C. jejuni encounters ROS stresses as a result of aerobic respiration. However, C. jejuni is also challenged by high levels of oxidative stress inside hosts and inside epithelial cells and macrophages, since ROS such as superoxide, hydrogen peroxide and other organic hydroperoxides are frequently produced by host cells as a defense mechanism to cause bacterial cell injury (van Vliet et al., 2002; Day et al., 2000).

C. jejuni expresses several oxidative stress response enzymes to remove ROS. Superoxide dismutase (SodB), catalase (KatA) and alkyl hydroxide reductase (AhpC) are
the three major proteins identified in *C. jejuni* to inactivate ROS. The iron-containing SodB is proposed to provide the first line of defense during exposure of *C. jejuni* to air since it has been shown to degrade superoxide anions and oxygen radicals such as dioxygen into hydrogen peroxide and oxygen (Purdy *et al.*, 1999; Pesci *et al.*, 1994; Purdy and Park, 1994). KatA is essential for hydrogen peroxide resistance as it converts peroxide into water and oxygen (Day *et al.*, 2000; Mongkolsuk *et al.*, 1998). The iron-regulated AhpC is important specifically for resistance to toxic hydroperoxide intermediates such as cumene and tert-butyl hydroperoxide, but not inorganic peroxides such as hydrogen peroxide (Stead and Park, 2000; Baillon *et al.*, 1999).

### 1.6.4 Osmotic stress

Sensitivity to hyper-osmotic stress often arises during transmission processes such as food processing, desiccation and salt water survival. *C. jejuni* exhibits a lower tolerance to osmotic stress compared to other food-borne bacterial pathogens (Alter and Scherer, 2006). Factors contributing to osmotic stress responses have not been investigated in detail, and only three genes to date have been identified as being associated with hyper-osmotic stress resistance: htrB (Phongsisay *et al.*, 2007), *ppk1* (encodes polyphosphate kinase 1) (Candon *et al.*, 2007) and *cj1226c* (encodes a two-component system sensor kinase) (Svensson and Gaynor, manuscript submitted). Each of these genes is important for survival in hyper-osmotic conditions.
1.7 Host-pathogen interactions

1.7.1 Host epithelial cell colonization, invasion, and intracellular survival.

To establish an infection, *C. jejuni* must first colonize the intestinal epithelium. This involves traversing the mucus layer of the gastrointestinal tract and attaching to intestinal epithelial cells. *C. jejuni* harbours several surface adhesion factors that enable attachment to host cell surface receptors, leading to colonization. FlaA (flagellin), Pebl (an ABC transporter periplasmic substrate binding protein component that also serves as a surface adhesin) (Pei *et al.*, 1998), CadF (*Campylobacter* adhesion to fibronectin) (Konkel *et al.*, 2005), and JlpA (a lipoprotein) (Jin *et al.*, 2001) interact with host cell surface proteins during infection. Lipooligosaccharide (LOS), another major surface molecule found on the outer leaflet of the *Cj* outer membrane, has an endotoxic property and is involved in host cell adhesion (Fry *et al.*, 2000).

Although *C. jejuni* is often considered an extracellular pathogen, it may invade host cells via a caveolae and microtubule dependent but actin-independent process following initial colonization (Watson and Galan, 2008; Oelschlaeger *et al.*, 1993). Both transcellular and paracellular translocation are evident during *Cj* infection (Monteville and Konkel, 2002; Bras and Ketley, 1999; Oelschlaeger *et al.*, 1993; Konkel *et al.*, 1992), and the detailed mechanism of the internalization process remains an area of debate. Once *C. jejuni* invades host cells, it is able to survive within the cells. Although the intracellular fate of *C. jejuni* remains unclear, this bacterial pathogen utilizes mechanisms that allow it to survive within host cells (Candon *et al.*, 2007; Mihaljevic *et al.*, 2007; Naikare *et al.*, 2006; Gaynor *et al.*, 2005; Day *et al.*, 2000; Oelschlaeger *et al.*, 1993; Kiehlbauch *et al.*, 1985). While an early study suggested that *C. jejuni* survives in a vacuole mononuclear phagocytes (Kiehlbauch *et al.*, 1985), a more recent study using
intestinal epithelial cells showed that *C. jejuni* sustains intracellular survival by preventing fusion of this vacuole, subsequently named the *Campylobacter*-containing vacuole (CCV), with the lysosome (Watson and Galan, 2008).

While *C. jejuni* can survive intracellularly within intestinal epithelial cells, survival within macrophages has continued to remain controversial. Using electron microscopy and colony-forming unit (CFU) assays, previous studies have shown that following phagocytosis, *C. jejuni* is capable of both short- and long-term intracellular survival within cultured J77A.1 murine macrophages, 28SC human monocytes, and peritoneal macrophages from BALB/c mice (up to $10^5$ CFU by day 6) (Hickey et al., 2005; Day et al., 2000; Kiehlbauch et al., 1985). However, other studies using C57BL/6 murine bone marrow derived macrophages and macrophages derived from human peripheral monocytes have made contradicting observations, suggesting *C. jejuni* is rapidly killed in 24 hours following phagocytosis by these macrophages as it is being delivered to the lysosome (Watson and Galan, 2008; Wassenaar et al., 1997; Myszewski and Stern, 1991).

*C. jejuni* interactions with dendritic cells (DCs) have not been investigated in detail. DCs are important in both the innate and adaptive immune responses to microbial pathogens. DCs play an important role during infection of enteric pathogens such as *Salmonella enteric* serovar Typhimurium, *H. pylori* and *Shigella flexineri*, where release of various cytokines is induced (Kranzer et al., 2004; Edgeworth et al., 2002; Marriott et al., 1999; Medzhitov and Janeway, 1997). To date, only one study has described *C. jejuni* interaction with DCs, where it was found that 99% of internalized *C. jejuni* were killed within 24 hr, and no cytotoxicity effect was induced (Hu et al., 2006).
1.7.2 Toxin

*Campylobacter* produces several virulence factors that alter host cellular activities, one of which is a multi-subunit toxin termed cytolethal distending toxin (CDT). CDT is also produced by several other bacterial pathogens such as *Salmonella enterica* serovar Typhi, *Shigella dysenteriae*, *E. coli*, *Actinobacillus actinomycetemcomitans*, and enterohepatic *Helicobacter* spp. (Smith and Bayles, 2006). It is encoded by 3 highly conserved genes, *cdtA*, *cdtB*, *cdtC*: CdtA and CdtC function as dimeric subunits forming a complex with CdtB, and CdtB functions as a DNase I like protein that triggers DNA double-strand breaks (Lara-Tejero and Galan, 2000). Using purified CDT protein, the CDT was shown to cause apoptosis and cell death in eukaryotic cells, prevent dephosphorylation of the cyclin B1/cdc2 protein kinase complex, and elicit eukaryotic cell cycle arrest in the G2/M transition phase prior to mitosis (Lara-Tejero and Galan, 2000).

1.7.3 Apoptosis

Apoptosis, or programmed cell death, is characterized by chromatin condensation and DNA fragmentation. Since CDT is produced by *C. jejuni*, and *C. jejuni* causes apoptosis in host cells, it has been postulated that CDT is the primary factor responsible for inducing apoptosis in *C. jejuni*-infected cells (Ceelen *et al.*, 2006; Smith and Bayles, 2006). However, a recent study suggested that apoptosis is not dependent upon the presence of CDT, since infection of T84 intestinal epithelial cells with a CDT mutant did not yield a reduced cytotoxic effect compared to WT infection (Kalischuk *et al.*, 2007). Additionally, it was proposed that *C. jejuni* may also cause necrotic cell death via a CDT-independent pathway (Kalischuk *et al.*, 2007). Necrotic cells, unlike apoptotic cells, rapidly lose plasma membrane integrity after the death stimulus.
Although *C. jejuni* CDT has been shown to induce apoptosis in the human monocytic cell line THP-1 (Hickey and Guerry, 2005 IAI), another study showed that a proteinase K- and heat-stable component of *C. jejuni* is also capable of stimulating apoptosis (Siegesmund et al., 2004). Experiments by the same group further suggested that Cia (*Campylobacter* invasion antigens) proteins contribute to apoptosis in THP-1 macrophages (Siegesmund et al., 2004). Detailed mechanisms underlying apoptosis in both epithelial cells and macrophages during *C. jejuni* infection remain to be investigated.

1.8 HOST CELL SIGNALING

1.8.1 MAPK signaling pathway

One feature of *C. jejuni* pathogenesis is the stimulation of host cell signal transduction events to trigger an inflammatory response in addition to apoptosis (Borrmann et al., 2007; Hu et al., 2006; Hickey et al., 2005; MacCallum et al., 2005; Watson and Galan, 2005; Siegesmund et al., 2004). The MAPK (mitogen-activated protein kinase) family plays an important role in mediating signal transduction and is activated by a wide range of environmental stimuli via different cell surface receptors such as toll-like receptors (TLR), G-protein coupled receptors (GPCR), integrin, receptor tyrosine kinases (RTK), and calcium ion channels (Schorey and Cooper, 2003). There are three major MAPK families in mammals, the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK) and the p38 kinases, all of which lead to subsequent gene expression changes important for regulating diverse cellular activities such as proliferation, differentiation and apoptosis in response to external stimuli (Shan et al., 2007; Galindo et al., 2004).
1.8.2 MAPK dependent signaling activation in *C. jejuni* infected cells

*C. jejuni* infection in INT407 human epithelial cells as well as Caco-2 and T84 human intestinal epithelial cells has been shown to trigger ERK1/2, JNK and p38 kinase phosphorylation (Chen *et al.*, 2006; Hu *et al.*, 2006; MacCallum *et al.*, 2005; Watson and Galan, 2005). Furthermore, several lines of evidence from work on other organisms have shown that pathogen-induced ERK activation in macrophages is important for apoptosis induction (Fettucciari *et al.*, 2003; Tang *et al.*, 1998; Xia *et al.*, 1995), and lipopolysaccharide (LPS) has been identified as a virulence factor responsible for MAPK stimulation in monocytes and macrophages (Thomas *et al.*, 2006; MacCallum *et al.*, 2005; Guha and Mackman, 2001; Ruckdeschel *et al.*, 1997).

1.8.3 Inflammatory response

*Campylobacter* infection often stimulates a series of immune responses in host cells, resulting in inflammation. Activation of a proinflammatory cytokine, interleukin (IL)-8, is a hallmark of *C. jejuni* pathogenesis. IL-8 is important for the recruitment of other immune cells including DCs, macrophages and neutrophils, which interact with *C. jejuni* to cause the host mucosal inflammatory response, which is critical for the generation of diarrhea. IL-8 production in host epithelial cells may be stimulated via a CDT-dependent or -independent process. While CDT contributes to IL-8 secretion in the INT407 cell line (Hickey *et al.*, 2000), it was found that both IL-8 and NF-κB (nuclear factor-κB) activation are initiated through an ERK-dependent MAPK signaling pathway in cultured T84 epithelial cells 8 hours post-infection (Watson and Galan, 2005). Other pro-inflammatory cytokines such as IL-1α/β, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) are also induced in monocytic and epithelial cells (Young *et al.*, 2007; Hickey *et
al., 2005; Siegesmund et al., 2004; Jones et al., 2003), and IL-1β, IL-6, IL-8, IL-10, IL-12, interferon-gamma (IFN-γ) and TNF-α, are also activated in infected DCs (Hu et al., 2006).

1.9 **OBJECTIVE AND HYPOTHESIS**

In a previous study, we found that transcription of the putative *C. jejuni* AA-ABC transporter system encoded by *cj0467-9* was induced during *in vitro* infection of INT407 cells (Gaynor et al., 2005), suggesting a role for these genes in pathogenesis. Our objective in this study was to investigate the biological function(s) of this system. We hypothesize that if the *cj0467-9* encoded AA-ABC transporter expression level is increased during cellular infection, then absence of this system will alter *C. jejuni* physiology and pathogenesis.
2.0 MATERIALS AND METHODS
2.1 BACTERIAL STRAINS, CELL LINES, MEDIA AND GROWTH CONDITIONS

Campylobacter jejuni 81-176 wild type (WT) and mutants were cultured in Mueller Hinton (MH) broth (Oxoid Ltd, Hampshire, England) or agar supplemented with 10μg/ml of vancomycin and 5 μg/ml of trimethoprim (MHTV) in a microaerobic and capnophilic (hereafter referred to as microaerobic for simplicity) environment at 37°C. Microaerobic environments were generated using Oxoid CampyGen gas packs in an enclosed container or a tri-gas incubator with 6% O_2 and 12% CO_2. C. jejuni 81-176 mutants ΔpaqP (cj0467) and ΔpaqQ (cj0469) were cultured in the same condition with the addition of 50 μg/ml of kanamycin (kan). For overnight broth cultures, freshly growing plates of bacteria were inoculated into MH broth to a starting optical density at 600 nm (OD_{600}) of approximately 0.004. The flasks were placed in an enclosed jar under microaerobic conditions and grown at 37°C shaking at 200 r.p.m. All E. coli DH5α strains were grown in Luria Bertani (LB) agar or broth at 37°C. RAW264.7 and INT407 cells were cultured at 37°C in humidified air with 5% CO_2. RAW264.7 cells were maintained in Dulbecco modified Eagle Media (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco) while INT407 were maintained in Minimal Essential Media (MEM) (Gibco) supplemented with 10% FBS and Caco-2 cells were maintained in DMEM with 1% non-essential amino acid (NEAA) and 10% FBS.

2.2 CONSTRUCTION OF CAMPYLOBACTER JEJUNI 81-176 ΔPAQP AND ΔPAQQ TARGETED DELETION MUTANTS

To construct the ΔpaqP (cj0467) and ΔpaqQ (cj0469) mutant strains in C. jejuni 81-176, the target genes were amplified by PCR from C. jejuni chromosomal DNA prepared using a DNA extraction kit (Promega, Nepean, CA), with primers paqPF1 (5'-TCTAGAGAAGATGGGAGAAATTTTG-3') and paqPR1 (5'-
TCTAGAACCCACAAAAAGCCAT-3'), as well as paqF (5’-
TCTAGATCCTTGCAGAGTATTC-3’) and paqQR1 (5’-
TCTAGATACCAACTGAGCTAAACC-3’), yielding 1.3 kb and 1.2 kb fragments, respectively,
with XbaI sites at the flanking regions. The PCR products were purified (Qiagen, Mississauga,
ON) and cloned into the pGEM®-T vector (Promega); these constructs were designated pGEM-
paqP and pGEM-paqQ. Restriction digestion was performed using the enzymes HindIII for
paqP and MscI with BamHI for paqQ, to remove approximately 400 bp from paqP and 290 bp
from paqQ. A non-polar aphA-3 cassette encoding kanamycin resistance (KanR) was digested
from the pUC18K2 plasmid (Menard et al., 1993) using the enzymes HindIII or MscI and
BamHI, and ligated to the digested pGEM-paqP and pGEM-paqQ vectors, creating suicide
vectors carrying the ΔpaqP::aphA-3 and ΔpaqQ::aphA-3 deletion constructs. Following
selection and amplification in E. coli DH5α, the mutagenic plasmids were purified from DH5α
using a Qiagen Midi-prep kit and transformed into C. jejuni 81-176 WT by natural
transformation or by electroporation as previously described (Candon et al., 2007). As pGEM®-T
is a suicide vector in C. jejuni, colonies recovered from MHTV+ kan plates should represent
stable chromosomal integrants resulting from double cross-over homologous recombination.
Genomic DNA from several mutant clones was prepared using the Wizard Genomic DNA kit
(Promega). The resulting mutants were designated ΔpaqP and ΔpaqQ. Insertional inactivation of
the paqP and paqQ genes via KanR cassette insertion was verified by PCR and sequencing
analysis as well as Southern blot analysis.

2.3 COMPLEMENTATION OF ΔPAQP DELETION MUTANT

Generation of a re-constituted WT strain of C. jejuni, designated ΔpaqPc, was achieved
by natural transformation of the ΔpaqP mutant with the pRRC-paqP plasmid, carrying a
chloramphenicol resistance gene. To create the pRRC-paqP construct, the paqP fragment was obtained from pGEM-paqP by XbaI digestion. This fragment was subsequently inserted into the pRRC vector carrying a chloramphenicol resistant cassette, resulting in the pRRC-paqP construct (paqP::CAT). The pRRC delivery vector contains the C. jejuni 16S and 28S rRNA genes (Karlyshev and Wren, 2005) and provides a means to express genes, driven by the CAT promoter, at a heterologous chromosomal location in C. jejuni. This vector was delivered to the ΔpaqP strain via natural transformation, as previously described (Candon et al., 2007). Colonies carrying the paqP::CAT fragment were tested for sensitivity to chloramphenicol by plating on MH agar plates containing 20 μg/ml of chloramphenicol and 50 μg/ml of kanamycin. Sequencing and PCR amplification using the paqP primer set confirmed the selected colonies to be reconstituted WT strains carrying the recombinant paqP gene.

2.4 RNA EXTRACTION AND REVERSE TRANSCRIPT-PCR (RT-PCR) ANALYSIS

C. jejuni: RNA isolation was performed as previously described (Gaynor et al., 2005). Reverse transcription of the purified RNA was performed using Super Script II Mix and Random Primer (Invitrogen, Burlington, ON) followed by purification using the Qiagen PCR purification kit. The purified cDNA products were PCR amplified for nssR using primers nssRF1 (5’-AGAACTTTTATCTAGTGTAGG-3’) and nssRR1 (5’-CGTCTTAAATCTAATGC-3’), and tuf, using primers tufF1 (5’-GCCTGCTATTACTATTGCTAC -3’) and tufR1 (5’-TCGAAGTCAGTGTGGAG-3’). RNA was confirmed as DNA-free by RT-PCR.

2.5 SOUTHERN BLOT HYBRIDIZATION

Genomic DNA was isolated using a DNA isolation kit as described above. For each Southern blot, 100 ng of DNA from each strain was digested by EcoRV, which cuts in the middle of the KanR cassette but does not cut either paqP or paqQ. DNA was separated on a
0.75% agarose gel and directly blotted onto PVDF membranes using 0.25 N NaOH and 0.75 M NaCl as the transfer solution according to the manufacturer’s instruction in the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany). All probes were non-radioactively labeled using this kit. The *paqP* fragment was obtained by digesting the pGEM-*paqP* plasmid with *Xba*I while the *paqQ* fragment was obtained by digesting the pGEM-*paqQ* plasmid with *Xba*I and *Msc*I. The resulting fragments were denatured by heating at 99°C for 10 min and placed on ice. Hexanucleotides, DIG and Klenow were added to the denatured fragment and incubated overnight at 37°C as described in the DIG-kit. The blot was hybridized with these probes and visualized using enhanced chemiluminescence (ECL) (Perkin Elmer, Waltham, MA). Insertion of the Kan<sup>R</sup> cassette into *paqP* and *paqQ* should result in the generation of two smaller probe-reactive fragments (due to *EcoRV* digestion of the Kan<sup>R</sup> cassette) rather than the larger WT-sized fragment. In the case of Δ*paqQ*, only one smaller fragment is readily visible at the exposure shown.

2.6 **AMINO ACID TRANSPORT ASSAYS**

*C. jejuni* cells were grown for 15 hr to mid-log growth phase in 15 ml of MH broth. Cultures were harvested by centrifugation, washed twice in M9 minimal media (90.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 22.0 mM KH<sub>2</sub>PO<sub>4</sub>, 8.56 mM NaCl, 18.7 mM NH<sub>4</sub>Cl, 2 mM MgSO<sub>4</sub>, 22.2 mM glucose, 10 μM CaCl<sub>2</sub>, pH7.4), and re-suspended in the same medium, to an approximate OD<sub>600</sub> of 1.0. The re-suspension was kept on ice for no longer than 4 hr. An aliquot of 150 μl of the cell suspension was added to 1.5 ml of M9 minimal medium containing 0.5% (v/v) lactic acid (Sigma-Aldrich, Oakville, ON), and was allowed to equilibrate by incubating at 37°C for 3 min. The assay was then initiated by the addition of 5 μM of the [<sup>14</sup>Cl]-labelled amino acid (6.9- 9.36 GBq mmol<sup>-1</sup>) (Perkin Elmer). Samples (0.1 ml) were withdrawn at the indicated time points,
collected by vacuum filtration through 0.22 μm membrane filters (Millipore Corp., Billerica, MA), and washed twice with M9 minimal media. Sample filters were then immersed in Filter-Count scintillation cocktail (Fisher Scientific, Ontario, Canada) and counted in a Beckman Coulter™ scintillation counter. Transporter activity was expressed as nMoles of amino acid transported, mg dry cells⁻¹, min⁻¹

2.7 CELL INFECTION ASSAY FOR COLONIZATION, INVASION, INTRACELLULAR SURVIVAL AND GENTAMICIN MIC DETERMINATION

INT407 cells (a human intestinal epithelial cell line), RAW264.7 cells (a murine macrophage cell line) or Caco-2 cells (human intestinal epithelial cell lines) were seeded into 24 well tissue culture plates at semi-confluency (~ 5x10⁵ cells/ml) and allowed to grow for approximately 20 hr prior to infection. Mid-log phase WT and C. jejuni mutants from overnight shaking cultures were added to pre-warmed MEM or DMEM, which was used to infect the cells at an MOI~ 200 for 3 hr. The cells were then washed 3 times with PBS to remove any unbound bacteria. To assay adhesion, 1 ml of 1% Triton X-100 in PBS was added to some of the wells for 5 min to disrupt the cells; the samples were plated on MH agar, and grown for 48 hr at 37°C in a microaerobic condition. To assay invasion, fresh media supplemented with 10% FBS and 150 μg/ml of gentamicin were added to the cells and incubated at 37°C for 2 hr before washing and treating with Triton as described. Next, to assay short-term intracellular survival, fresh media supplemented with 10% FBS containing 10 μg /ml gentamicin for an additional 4 hr before washing and treating with Triton X-100 (the 9 hr post-infection time point). Finally, to assay long-term intracellular survival, cells were left in 10 μg/ml gentamicin for an additional 15 hr (or 24 hr after initial infection) before being subjected to washes and Triton X-100 treatment.

Gentamicin susceptibility of each strain was tested by determining the minimum inhibitory concentration (MIC) using an E-test® strip (AB Biodisc).
2.8 IN VIVO COLONIZATION USING A MOUSE MODEL

The in vivo study was performed by Rhonda Hobbs from our collaborator, Dr. Stuart Thompson's laboratory in the Medical College of Georgia. BALB/cByJ mice from Jackson Laboratories (Bar Harbor, ME) were housed at the animal care centre at the Medical College of Georgia, with seven mice per experimental group. Each mouse was infected with $5 \times 10^9$ CFU WT or ΔpaqP C. jejuni via oral gavage as previously described (Pajaniappan et al., 2008). C. jejuni shed in fecal pellets from each mouse at 7, 14, 19, 28 and 35 days post-infection, were homogenized and enumerated on MH agar containing 5% (v/v) sheep's blood and 20 µg/ml cefoperazone, 10 µg/ml vancomycin and 2 µg/ml amphotericin B (CVA). The level of detection was $1 \times 10^2$ CFU/g fecal pellet. All animal treatments were carried out in accordance with NIH guidelines for the care and use of laboratory animals, using procedures approved by the Medical College of Georgia Institutional Care and Use Committee.

2.9 OXIDATIVE, AEROTOLERANCE, HEAT STRESS AND OSMOTIC STRESS SURVIVAL ASSAYS

To assay oxidative stress, C. jejuni from an overnight culture in MH broth was inoculated into fresh MH broth to an OD$_{600}$~0.6; 1 ml of this culture was subsequently added to each well of a 24-well plate. Tert-butyl hydroperoxide, also known as t-BOOH (Sigma Aldrich) and hydrogen peroxide, H$_2$O$_2$, or paraquat (Sigma Aldrich) were made in MH broth at various concentrations. 1 ml of the oxidative agent was added to C. jejuni cultures and incubated for 30 min at 37°C in microaerobic conditions before harvesting for CFU enumeration. Aerotolerance was examined by diluting C. jejuni grown to mid-log phase in MH broth to an initial OD$_{600}$ ~0.004 in fresh MH broth and incubated in shaking culture at 200 r.p.m and 37°C in atmospheric conditions. To assess limited CO$_2$ stress and heat stress, mid-log phase bacteria were serially diluted, spotted onto MH agar plates, and incubated at 37°C in a 5% CO$_2$ incubator, or 45°C in
microaerobic conditions. Osmotic stress was examined by spotting the dilutions onto MH agar plates supplemented with 0.17 M NaCl and incubated at 37°C in a microaerobic environment.

2.10 **CELLULAR APOPTOSIS AND VIABILITY DETECTION**

*Annexin-V staining.* RAW264.7 cells were plated at semi-confluency on cover-slips in 24-well plates and cultured for 24 hr before infecting with *C. jejuni* followed by gentamicin treatment as described above. At the end of 9 hr infection (with gentamicin treatment as described previously in intracellular survival assay), cells were washed 3 times with PBS and stained with Annexin-V fluorescein and propidium iodide according to manufacturer’s instructions (Roche Applied Sciences). Annexin-V labeling was visualized using a Nikon eclipse TE2000 microscope (Nikon Instruments Inc., Mississauga, ON) fitted with appropriate filter sets for detecting fluorescence. The total cell number was approximated by counting cells at 6 different fields under DIC (Differential Interference Contrast). Percentage of cell death was calculated by dividing the number of annexin-stained cells by total number of cells.

*DAPI staining and immunofluorescence.* Following infection, RAW264.7 cells were washed and fixed as described by Guttman et al. (Guttman et al., 2007a). Cellular nuclei were labeled with 4′,6-Diamidino-2-phenylindole (DAPI) and mounted using Vectashield (Vector Labs, Burlington, ON). Relative cell viability was fluorescently quantified by counting DAPI stained cell nuclei in multiple randomly selected fields in a similar manner as previously described by Barnes et al. (Barnes et al., 2007). Cells exhibit fragmented and condensed nuclei or have lifted off the plate are considered non-viable therefore were not enumerated for viability.

2.11 **CELLLYSATE PREPARATION AND WESTERN BLOTTING**

RAW264.7 cells were grown on 150 mm tissue culture dishes and infected at an MOI ~ 200 for 9 hr. Cells were washed 3 times with PBS containing 1mM CaCl₂ and 1mM MgCl₂
followed by treatment with RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid, 1% SDS) for 10 min on ice. Western blotting was performed according to Guttman et. al. (Guttman et al., 2007b). Briefly, equal amounts of total proteins were loaded and separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Bio-Rad Laboratories, Mississauga, ON). Membranes were blocked with 4% skim milk and washed in Tris-buffered saline with 0.1% Tween-20 (TBST) 3 times for 5 min each. Primary mouse anti-phospho-ERK1/2 antibody (Cell Signaling Technology, Beverly, MA) was used at a dilution of 1:2000 and rabbit anti-ERK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:2000 dilution (0.1μg/ml). Rabbit anti-phospho-JNK (Thr183/Tyr185) antibody (Cell Signaling Technology) and rabbit anti-JNK1 antibody (Santa Cruz Biotechnology) were used at a 1:1000 dilution. After washing, a horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) was used at 1:5000 for labeling. Signals were detected by ECL (Perkin Elmer).

2.12 Additional phenotypic assays

Microaerobic growth in 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 0.01 and placed under microaerobic conditions at 37°C, shaking at 200 r.p.m.; serial dilutions of cultures were made and colony forming units (CFUs) were measured over time by plating on MH agar plates.

Minimal media survival. Strains were grown as described above and diluted to an OD$_{600}$ of 0.02. Cultures were in MEM at 37°C shaking at 200 r.p.m. under microaerobic condition and CFUs were measured over time by plating serially diluted cultures onto MH agar plates.
**Anaerobic survival.** Log phase bacteria grown as described above were diluted to an OD_{600} of 0.01. Cultures were placed under anaerobic conditions using an Anaero-GasPak (Oxoid), at 37°C, shaking at 200 r.p.m. CFUs were measured as described.

**Motility.** Log phase *C. jejuni* were diluted to OD_{600} ~ 0.02 and 1µl stabbed of each strain into MH agar plates that contained 0.4% agar. Migration of the cells from the point of inoculation was analyzed following 24 hr of incubation at 37°C under microaerobic conditions.

**Low iron conditions.** Log phase bacteria were diluted to an OD_{600} of 0.1, and 100 µl of each strain was evenly spread on a MH agar plate. Disks containing desferal (Fe^{3+} chelator) and dipyridyl (Fe^{2+} chelator) at 40 mM were placed on the cell lawns and incubated at 37°C for 24 hr before measuring the zone of inhibition.

**Antimicrobial susceptibility test.** Log phase bacteria were diluted to OD_{600} of 0.1, and 100 µL of each strain was evenly spread on a MH agar plate. Disks containing erythromycin, nalidixic acid and amikacin were placed on the cell lawns and incubated at 37°C for 24 hr before measuring the zone of inhibition.

**Heat Shock.** *C. jejuni* from an overnight culture was inoculated into MH media to an OD_{600} of 0.2 and placed at 55°C for 5 min or 15 min before cooling on ice. Serial dilutions of cultures were made and spotted onto agar plates. CFUs were measured followed by 48 hr of incubation under 37°C microaerobic conditions.

**pH sensitivity.** *C. jejuni* from an overnight culture was diluted to OD_{600} of 0.0005 and 0.002, and serial dilutions were inoculated onto MH agar plates at different pHs: pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. CFUs were measured after 48 hours of incubation under 37°C microaerobic conditions.

**Nutrient Deprivation assay.** Overnight *C. jejuni* cultures were resuspended to OD_{600} of 0.015 in 10ml MEM supplemented with 1% non-essential amino acids and incubated in 37°C shaking
cultures in the absence or presence of 40 mM L-glutamine. Bacterial growth was assessed at various time intervals by optical density at 600 nm analyses and CFU enumeration.

**Glutamine rescue assay.** RAW264.7 and INT407 cells were grown to semi-confluency on cover-slips in 24 well plates as described above. Log-phase *C. jejuni* were diluted to OD$_{600}$~ 0.02 in DMEM or MEM with 10% FBS in the absence or presence of 20mM L-glutamine. The cells were infected with *C. jejuni* (2 ml per well) for 9 hours. At the end of the infection, cells were washed with three times with PBS, fixed with 3% paraformaldehyde for 15 minutes at room temperature, washed once with PBS, and permealized with 0.2% Triton X-100 for 5 minutes at room temperature. Finally, the cells were washed and stained with DAPI for visualization of cellular nuclei on the microscope.

**Gamma-glutamyl transferase (GGT) assay.** The GGT assay protocol was adapted from Barnes *et al.* (Barnes *et al.*, 2007). Briefly, log-phase *C. jejuni* from overnight cultures were centrifuged at 10,000xg for 5 min. Pellets were resuspended in 3 ml of 1M Tris-EDTA buffer (pH 8) and cells were disrupted using an ultra-sonicator (Ultrasonic Processor SL, Misonix Incorporated) at setting 3 for 10 times at 10 sec each. Lysed bacteria were centrifuged at 13,000 r.p.m, for 5 min at 4°C and supernatants were collected for a Bradford assay to determine protein concentration. GGT reagent (Pointe Scientific, Inc. Canton, MI) was reconstituted in 10 ml dH$_2$O according to the instruction protocol. Bacterial lysates were diluted by 1/10 in dH$_2$O and 10 µl was added to 190 µl pre-warmed GGT reagent in a 96 well microtire plate. The assay was incubated at 37°C and absorbance was measured at 405 nm at various time points.

**Protein secretion profile.** 100 ml of log-phase *C. jejuni* from overnight culture was centrifuged at 10,000 x g for 5 min. The supernatant was filtered using 0.22 µm filter, and a 1/10 volume of 100% trichloracetic acid (TCA) (w/v) (Sigma Aldrich) was added to the supernatant for overnight precipitation at 4°C. The suspension was centrifuged at 9800xg for 30 min and the
pellets were re-suspended in 200 μl of 1X SDS-PAGE sample buffer and subjected to 16.5% SDS-PAGE. Briefly, the gel was fixed for 1 hour in fixing solution (40: 5: 55 solution of 95% ethanol/acetic acid/ distilled water (v/v)), treated with oxidizing solution (0.7% periodic acid in fixing solution (v/v)) for 5 min, followed by three washes with dH2O, 10 min each. Next, the gel was stained with fresh silver staining reagent (20 mM NaOH, 0.67% AgNO3, 1.33% NH4OH in dH2O (v/v)) with gentle shaking. Finally, the gel was washed with distilled water 3 times for 10 minutes, and developing solution (Bio-Rad Silver Stain Developer) was added to the gel for 10 minutes before adding the stop solution (5% acetic acid and 10% ammonium pesulfate in dH2O).
3.0 Results
3.1 Overview

AA-ABC transporter systems in *C. jejuni* and other pathogenic organisms act as virulence factors during cellular infections (Leon-Kempis MDEL et al., 2006; Muller et al., 2005; Tamura et al., 2002). In a previous study, we observed increased transcription of *cj0467-9*, which encodes a putative AA-ABC transporter, during *in vitro* infection of INT407 cells, suggesting a role for these genes in pathogenesis. This led us to explore the role of the Cj0467-9 AA-ABC transporter system and its connection with host cells during the infection process. As both the integral membrane protein, or permease, and ATP binding protein/ATPase components are frequently required to produce a functional AA-ABC transporter (Nikaido, 2002), we generated non-polar mutations in both the Cj0467 (PaqP) permease and the Cj0469 (PaqQ) ATPase, using a highly invasive *C. jejuni* 81-176 strain, to ensure a comprehensive analysis of the system’s functions (rationale for the “Paq” designation is described below). Both mutants were tested for their tolerance in environmental stresses which can be often encountered *in vitro* or *in vivo*, amino acid transport to explore the system’s biochemical function, and its roles in the pathogen-host cell interaction.
3.2 \textit{cj0467-9 encodes a putative amino acid (AA) ABC transporter}

The putative AA-ABC transporter system encoded by \textit{cj0467-9} is highly conserved and occurs in the same genomic context among \textit{C. jejuni} strains, as described in CampyDB (http://www.xbase.bham.ac.uk/campydb). For simplicity, we have referred to the \textit{cj0467-9} gene numbers as annotated for strain NCTC11168, although our studies were performed using the invasive strain 81-176. \textit{Cj0467-9} is annotated as Cjj0492-4 in the 81-176 strain (campyDB). \textit{cj0467} and \textit{cj0468} are predicted to encode integral membrane proteins, with \textit{Cj0467} exhibiting 33\% identity to the \textit{E. coli} glutamine ABC transporter permease \textit{GlnP}, and \textit{Cj0468} exhibiting 26\% identity to the \textit{E. coli} GltJ permease (campyDB). \textit{cj0469} is predicted to encode an ATP binding protein with 55\% identity to the \textit{Bacillus subtilis} glutamine ABC transporter ATP binding protein \textit{GlnQ} (campyDB). National Centre for Biotechnology Information BLAST and Archaeal and Bacterial ABC transporter database searches also showed that \textit{Cj0467} and \textit{Cj0468} are similar to \textit{GlnP} (approximately 43\% identity), while \textit{Cj0469} is highly similar to \textit{GlnQ} (approximately 60\% identity) in \textit{Streptococcus pneumoniae}, \textit{Pseudomonas spp.}, and \textit{Helicobacter spp.} \textit{Cj0467} and \textit{Cj0469} also exhibit homology to other likely \textit{C. jejuni} AA-ABC transport components, including two uncharacterized proteins putatively annotated as \textit{GlnP} (Cj0940c) and \textit{GlnQ} (Cj0902) based on slightly higher initial BLAST search homologies (Cj0940c: 37.5\% identity to \textit{E. coli} \textit{GlnP}; Cj0902: 56.2\% identity to \textit{Bacillus stearothermophilus} \textit{GlnQ}). To avoid confusion with these previously annotated genes, and as our data (described below) indicate that \textit{Cj0467} and \textit{Cj0469} participate not only in glutamine transport but also in other important biological processes, we have designated them PaqP and PaqQ, respectively [pathogenesis-associated glutamine (q) ABC transporter permease (P) and ATPase (Q)].
3.3 Construction of targeted, non-polar ΔpaqP and ΔpaqQ disruption strains

To investigate the role of this AA-ABC transporter system in C. jejuni, paqP and paqQ were individually disrupted using a non-polar kanamycin resistance (KanR) cassette, aphA-3 (Figure 3.1A). PCR, sequencing, and Southern blot analyses confirmed that the resultant mutants, hereafter referred to as ΔpaqP and ΔpaqQ, harboured disruptions in the appropriate genes (Figure 3.1B, and data not shown). To verify that KanR insertion into both paqP and paqQ was non-polar, RT-PCR was used to confirm that nssR and tuf were transcribed in both mutant strains (Figure 3.1C), and that paqQ was transcribed in the ΔpaqP strain (data not shown).
Figure 3.1. Generation of non-polar, single insert ΔpaqP and ΔpaqQ disruption strains. (A) Genomic organization of the cj0467, cj0468 and cj0469 genes, encoding a putative AA-ABC transporter system. The aphA-3 cassette encoding kanamycin resistance was used to create insertion-deletions in cj0467 (paqP) and cj0469 (paqQ) genes by double crossover homologous recombination. The resultant mutant strains are designated ΔpaqP and ΔpaqQ. (B) Southern blots of EcoRV-digested genomic DNA using paqP and paqQ probes confirm that the target gene in each mutant was disrupted. (C) Reverse transcription PCR (RT-PCR) was performed to assay transcription of genes upstream of ΔpaqP (nssR) and downstream of ΔpaqQ (tuf) in the deletion strains.
3.4 The ΔpaqP and ΔpaqQ C. jejuni mutants are defective for L-glutamine uptake and moderately defective for uptake of other amino acids

To investigate the functionality of PaqP and PaqQ, transport assays were performed using different radioactively labeled AAs. Based on strong homology to GlnP and GlnQ and bioinformatics analyses described above, together with a previous report suggesting that this AA-ABC system may participate in cysteine transport (Muller et al., 2005), WT C. jejuni and the two mutants were grown to mid-log phase and assayed for rates of uptake of [\(^{14}\text{C}\)]-L-glutamine, [\(^{14}\text{C}\)]-L-glutamate, [\(^{14}\text{C}\)]-L-aspartate and [\(^{14}\text{C}\)]-L-cysteine. Both mutants exhibited a significant defect for glutamine uptake: at 1 min, glutamine transport levels of both ΔpaqP (∼1.97 nmol/min/mg dry weight) and ΔpaqQ (∼1.66 nmol/min/mg dry weight) mutants were <50% that of the WT strain (∼4.12 nmol/min/mg dry weight) (Figure 3.2A). An intermediate defect was observed for glutamate uptake (Figure 3.2B), while much smaller decreases in cysteine and aspartate uptake were observed (Figure 3.2C, 3.2D). This suggests that this AA-ABC transporter system has high affinity for glutamine but also appears to transport other amino acids to a lesser extent. The complemented ΔpaqPc strain partially restored glutamine uptake, as a two tailed t-test did not show a statistically significant difference between WT and ΔpaqPc (Figure 3.2E).
Figure 3.2. ΔpaqP and ΔpaqQ mutants exhibit reduced levels of glutamine and other amino acid uptake relative to WT. C. jejuni 81-176 WT (square, solid line), ΔpaqP (circle, dashed line) and ΔpaqQ (triangle, dotted line) mutants were grown microaerobically, shaking in MH broth, for 15hr to early log phase (≈ 0.3 OD_{600}/ml), then harvested and assayed for high affinity transport of (A) [¹⁴C] L- glutamine, (B) [¹⁴C] L-glutamate, (C) [¹⁴C] L- cysteine, or (D) [¹⁴C] L-aspartate at a final concentration of 5 μM. Samples were taken either every 30 sec, 1 min or 2 min. (E) ΔpaqP and ΔpaqQ both showed statistically significant differences in glutamine uptake compared to WT, while the ΔpaqP complemented strain ΔpaqPc showed no statistically significant difference in glutamine uptake compared to WT. Rate of uptakes were determined from three separate biological replicate cultures, with duplicate samples harvested for each strain at each time point. The asterisk (*) represents statistical significance (p < 0.05) using a two-tailed t-test. Each experiment was performed in triplicates (n=3) and the result is a representation of three separate experiments.
3.5  *C. jejuni ΔpaqP* and *ΔpaqQ* exhibit increased short-term intracellular survival in RAW264.7 macrophages, INT407 epithelial cells, and Caco-2 intestinal epithelial cells

In a previous microarray analysis, we observed an increase in levels of *cj0467-9* mRNA in *C. jejuni* during INT407 cell infections (Gaynor et al., 2005). Thus, we were interested in exploring whether this system might influence the interaction of *C. jejuni* with host cells. RAW264.7 murine macrophages as well as INT407 and Caco-2 human epithelial cells were infected with WT, ΔpaqP, and ΔpaqQ strains and assayed for adherence, invasion, and intracellular survival using a gentamicin protection assay. No significant differences in cell adherence, invasion or long-term intracellular survival at 24 hr were observed for the mutants compared to the WT strain (Figure 3.3). However, short-term intracellular survival (9 hr post-infection) of both ΔpaqP and ΔpaqQ mutants was up to 20-fold higher than WT in RAW264.7 cells (Figure 3.3A) and approximately 10-fold higher than WT in INT407 cells (Figure 3.3B). Human intestinal Caco-2 epithelial cells were also tested, and similar results were observed (Figure 3.3C). Both WT and mutant strains were equally susceptible to gentamicin by E-test strip analyses (MIC = 1.5 μg/ml), suggesting that the elevated short-term intracellular survival level of the mutants was not due to differences in gentamicin resistance. The level of intracellular survival at 9 hours post-infection was also examined for the ΔpaqPc complement strain, and was found to be similar to the ΔpaqP mutant (data not shown).
Figure 3.3. \( \Delta paqP \) and \( \Delta paqQ \) mutants exhibit a statistically significant increase in short-term intracellular survival in macrophages and epithelial cells 9 hr post-infection. WT (filled), \( \Delta paqP \) (striped) and \( \Delta paqQ \) (white) \textit{C. jejuni} were grown overnight in MH shaking broth to log phase. (A) A murine macrophage cell line, RAW264.7, and (B) a human epithelial cell line, INT407, and (C) Caco-2, a human intestinal epithelial cell line, were infected with bacteria at an MOI \( \sim 200 \). After 3 hr, the cells were washed with PBS and treated with 150 \( \mu \)g/ml of gentamicin for an additional 2 hr to kill extracellular bacteria before washing the cells and adding fresh media with 10 \( \mu \)g/ml gentamicin; after an additional 4 hr, intracellular bacteria were recovered and plated for CFU enumeration. All experiments included triplicate infections for each strain for each time point. The asterisk (*) represents statistical significance \((p < 0.05)\) using a two-tailed t-test. This result is a representative experiment of three separate repeats.
3.6 No significant differences between the $\Delta paqP$ mutant and WT *C. jejuni* colonization were observed *in vivo* using a mouse infection model.

To evaluate whether the enhanced *in vitro* intracellular survival of the AA-ABC mutants might also reflect a survival difference *in vivo*, we infected BALB/cByJ mice with WT and $\Delta paqP$ mutant strains according to a previously established mouse colonization model (Pajaniappan *et al.*, 2008; Pei *et al.*, 1998). Colonization was monitored for seven to 35 days. $\Delta paqP$ mutant *C. jejuni* colonization levels did not significantly differ from the WT at any given time point (Figure 3.4). It should be noted that although WT *C. jejuni* colonized the mouse intestinal tract at a significant and high level up to 28 days, this and other tractable animal models do not consistently trigger inflammation and thus can only assay colonization, not virulence (Chang and Miller, 2006; Hendrixson and DiRita, 2004).
Figure 3.4. ΔpaqP is not defective for mouse colonization in vivo. WT (solid circle) and the ΔpaqP mutant (open circle) C. jejuni colonized BALB/cByJ mice at a similar level from day 7 up to day 35 post-infection. The dashed line indicates the level of detection, which was $1 \times 10^2$ CFU/g fecal pellet. Values on the x-axis represent mice with no detectable colonization. This experiment was performed by Rhonda Hobbs from the Thompson Lab in the Medical College of Georgia.
3.7 $\Delta paqP$ and $\Delta paqQ$ *C. jejuni* mutants exhibit increased resistance to limited CO$_2$ and aerobic conditions.

To survive successfully throughout the pathogenesis cycle, *C. jejuni* must overcome a multitude of environmental stresses in both extracellular and intracellular environments (Mihaljevic et al., 2007). As a microaerophilic organism, *C. jejuni* requires elevated levels of CO$_2$ for normal growth and is sensitive to atmospheric levels of O$_2$. To examine whether mutation of this AA-ABC transporter influenced the growth of *C. jejuni* under sub-optimal gas conditions, mid-log phase bacteria were serially diluted, spotted onto MH agar, and allowed to grow in a 5% (vs. ideal 12%) CO$_2$ environment. Under these conditions, both the $\Delta paqP$ and $\Delta paqQ$ mutants grew better than the WT strain (Figure 3.5A). To specifically evaluate aerobic sensitivity, WT and mutant *C. jejuni* were grown microaerobically in broth culture, shifted to normal aerobic atmospheric conditions, and assayed for survival after 4-6 hrs by CFU enumeration. Consistent with the CO$_2$ growth observations, both mutants exhibited sustained survival under aerobic conditions compared to the WT strain (Figure 3.5B).
Figure 3.5. \( \Delta\text{paqP} \) and \( \Delta\text{paqQ} \) are more resistant to aerobic stresses than WT.

(A) Log phase cultures grown microaerobically were serially diluted, plated and incubated in a 5\% CO\(_2\) incubator at 37\(^\circ\)C overnight. (B) Log phase cultures WT (solid), \( \Delta\text{paqP} \) (striped), and \( \Delta\text{paqQ} \) (white), grown microaerobically, were shifted to normal atmospheric oxygen conditions for 4 and 6 hr before being serially diluted, plated and incubated under microaerobic conditions for 2 days for CFU enumeration. Error bar represents assay replicates (n=3) of bacteria recovered at each time point. This result is a representative experiment of three separate repeats. The asterisk (*) indicates a statistically significant difference between the mutants and WT (p<0.05).
3.8 ΔpaqP and ΔpaqQ C. jejuni mutants exhibit an increase in resistance to organic hydroperoxide tBOOH, but not inorganic peroxides H₂O₂ and paraquat.

O₂- and CO₂-related stress responses are often closely associated with other oxidative stress responses in C. jejuni (Alter and Scherer, 2006; Baillon et al., 1999). We thus investigated whether loss of this AA-ABC transporter system in C. jejuni might also influence responses to other oxygen derivatives such as reactive oxygen species (ROS). WT, ΔpaqP and ΔpaqQ mutant C. jejuni were treated for 30 min with hydrogen peroxide (H₂O₂), an inorganic peroxide frequently generated by macrophages as a defense mechanism to eradicate intramacrophage pathogens (Alter and Scherer, 2006), or tert-butyl hydroperoxide (t-BOOH), an organic peroxide also generated by host cells (Baillon et al., 1999). While neither mutant exhibited altered sensitivity to H₂O₂ or paraquat (Figure 3.6A, 3.6B), both displayed increased resistance to t-BOOH (Figure 3.6C). ΔpaqP in particular was found to exhibit higher t-BOOH resistance than WT at a statistically significant level. Although ΔpaqQ did not exhibit a statistically significant difference relative to WT, experimental repeats consistently showed a higher t-BOOH tolerance of ΔpaqQ relative to WT. Together, our data indicate that disruption of this AA-ABC system impacts aerobic and ROS sensitivity in C. jejuni.
Figure 3.6. The ΔpaqP mutant exhibits a significant increase in resistance to tert-butyl hydroperoxide (t-BOOH) but not to hydrogen peroxide (H₂O₂) or paraquat. WT (square), ΔpaqP (circle) and ΔpaqQ (triangle) were treated with (A) H₂O₂, (B) paraquat, or (C) t-BOOH at various concentrations and incubated microaerobically at 37°C for 30 minutes before being harvested for CFU enumeration. All samples were taken in triplicates at each concentration. The asterisk (*) represents statistical significance (p-value < 0.05) using a two-tailed t-test.
3.9 Tolerance to heat and osmotic stress is differentially altered in $\Delta paqP$ and $\Delta paqQ$ mutants

To examine if the $\Delta paqP$ and $\Delta paqQ$ mutants exhibit other stress tolerance alterations, the mutants were also subjected to heat and osmotic stress assays. To test heat tolerance, mid-log phase bacteria were diluted to $OD_{600} \sim 0.2$ in MH broth, and serial dilutions were spotted onto MH agar and grown under microaerobic conditions at 45°C, a mild heat stress condition for C. jejuni (Phongsisay et al., 2007; Brondsted et al., 2005; Park, 2002; Konkel et al., 1998). As a control, cultures were harvested and plated at 37°C (Figure 3.7A). While $\Delta paqP$ exhibited an approximate 10-fold increase in heat stress resistance compared to WT, $\Delta paqQ$ was approximately 10-fold more sensitive to heat stress than WT (Figure 3.7B). Similar results were obtained when the strains were subjected to growth under osmotic stress using MH agar with 0.17 M NaCl: the $\Delta paqP$ mutant exhibited increased resistance to osmotic stress compared to WT, while the $\Delta paqQ$ mutant was more sensitive to NaCl (Figure 3.7C).
Figure 3.7. The ΔpaqP mutant is more resistant to heat and osmotic stress while the ΔpaqQ mutant is more sensitive than WT. Overnight bacterial cultures were serially diluted from an initial concentration of OD$_{600}$ ~0.2. (equivalent to approximately 1x10$^9$ cfu/ml) and grown microaerobically for 2 days at (A) 37°C and (B) 45°C on MH agar to assess the response to heat stress, or at (C) 37°C on MH agar supplemented with 0.17M NaCl to assess the response to osmotic stress. This result is a representation of more than three separate repeats.
3.10 Macrophages infected with *C. jejuni ΔpaqP* and *ΔpaqQ* exhibit reduced apoptosis and ERK activation compared to macrophages infected with WT bacteria.

The observations described above suggested that the enhanced intracellular survival of the ΔpaqP and ΔpaqQ mutants can at least partly be attributed to enhanced or altered bacterial stress tolerance. As host cell death results in detachment of the cells from the tissue culture surface and a resultant loss of *C. jejuni* inside those cells from the intracellular survival assay counts, we hypothesized that the observed increase in intracellular survival of the mutants might also reflect increased host cell survival. To investigate this, we separately monitored levels of host cell apoptosis and cell viability using Annexin-V Fluor and DAPI staining, followed by visualization using fluorescent microscopy. Cells were also stained with anti-*Campylobacter jejuni* antibody to ensure infection (data not shown). Cell viability levels were assessed by counting the number of cell nuclei remaining on the cover-slip that were neither condensed nor fragmented following infection. Consistent with previous findings in THP-1 human macrophages (Siegesmund *et al.*, 2004), apoptosis was induced in WT *C. jejuni*-infected RAW264.7 cells (Figure 3.8A). However, apoptosis was significantly reduced in ΔpaqP- and ΔpaqQ-infected cells compared to WT-infected cells (Figure 3.8A), by 66% and 55%, respectively (Figure 3.8B). The ratio of viable cells in WT-infected RAW264.7 cells relative to uninfected cells was approximately 30% less than that of the ΔpaqP- and ΔpaqQ- infected cells (Figure 3.8C).

Finally, to explore if reduced levels of apoptosis observed in ΔpaqP- and ΔpaqQ-infected RAW264.7 macrophages correlated with altered host cell signal transduction pathways, we examined phosphorylation of several MAP kinases known or suspected to be induced upon *C. jejuni* infection (Chen *et al.*, 2006; MacCallum *et al.*, 2005; Watson and Galan, 2005). We found that ERK phosphorylation levels in RAW264.7 macrophages infected with either ΔpaqP or ΔpaqQ were severely diminished compared to WT-infected cells at 9 hr post-infection (Figure 3.8D). In contrast, JNK and p38 phosphorylation levels were similar in macrophages infected
with WT and mutant strains, as were p-ERK levels at time points where the mutants did not exhibit invasion or intracellular survival differences from WT (Figure 3.8D, and data not shown). Levels of phospho-ERK INT407 or Caco-2 epithelial cells infected with either ΔpaqP or ΔpaqQ were also similar to those observed for WT-infected cells (Figure 3.9). These data indicate that this AA-ABC transporter system participates in ERK activation in C. jejuni-infected macrophages.
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**B**

- **Annexin-V stained cells**
  - WT: **3.0%**
  - ΔpaqP: **1.0%**
  - ΔpaqQ: **0.0%**

**C**

- **Number of DAPI stained nuclei**
  - UI: 200
  - WT: 250
  - ΔpaqP: 200
  - ΔpaqQ: 250

**D**

- **kDa**
  - 60
  - 50
  - 40

- **Protein Analysis**
  - p-ERK1/2
  - ERK
  - p-JNK2/3
  - p-JNK1
  - JNK

---

54
Figure 3.8. $\Delta$paqP and $\Delta$paqQ infected cells showed decreased levels of apoptosis and ERK activation compared to WT infected cells. (A) C. jejuni infected RAW264.7 cells were stained with Annexin-V fluorescein to visualize apoptotic cells at 9 hr post-infection with gentamcin treatment as mentioned in materials and methods. Frames shown are one representative view of several different frames. Scale bar= 5 µM. (B) Percentage of Annexin-V stained cells per total number of cells at 10X magnification. Error bar represents counts from several random fields. (C) C. jejuni infected RAW264.7 cells were stained with DAPI and cellular viability was assessed by counting cellular nuclei per random field. Error bar represents numbers of DAPI counts from a multiple random fields. (D) Phospho-ERK levels were induced at the 9 hr time point in WT but not $\Delta$paqP and $\Delta$paqQ-infected RAW264.7 cells. ERK1/2 phosphorylation (42 and 44 kDa) was detected by Western blot using anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody. The membrane was stripped and re-probed with anti-ERK antibody to assess total ERK protein levels as a control. JNK phosphorylations (46k Da and 54 kDa) were detected using Western blot using anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody. The membrane was stripped and re-probed with anti-JNK1 antibody to assess total JNK protein level as a control. This is one representative experiment of three separate repeats.
Figure 3.9. ΔpaqP and ΔpaqQ infected Caco-2 and INT407 epithelial cells show similar p-ERK and p-JNK levels as WT infected cells. INT407 and Caco-2 cells were infected with WT and mutant *C. jejuni* for 9 hr before being harvested for cell lysate preparation.
3.11 A summary of phenotypes for which ΔpaqP and ΔpaqQ mutants were not significantly different from the WT strain.

In order to understand the role of the AA-ABC system in bacterial stress tolerance, several additional phenotypic tests related to physiological stresses were examined in the ΔpaqP and ΔpaqQ strains. These included bacterial growth under microaerobic conditions, survival in minimal media or minimal media supplemented with serum, survival under anaerobic and low iron conditions, as well as antimicrobial susceptibility, pH stresses, heat shock, motility and biofilm formation. To assess if the mutants exhibited any difference in their ability to survive in minimal media +/- additional glutamine, glutamine was added to MEM and survival was monitored over time. In addition, to test if glutamine supplementation rescued host cell viability during C. jejuni infection, a glutamine rescue assay was performed by supplementing the culture medium with excess glutamine during infection, and cells were stained with DAPI to assess host cell viability. Finally, levels of protein secretion and GGT activity in WT and mutants were also examined (Table 3.1).
# Table 3.1. Phenotypes that were indistinguishable between ΔpaqP, ΔpaqQ mutants and WT C. jejuni 81-176

<table>
<thead>
<tr>
<th>PHENOTYPE</th>
<th>COMMENTS</th>
<th>FIGURE/TABLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microaerobic growth (MH broth)</td>
<td>No statistical significant difference between WT and mutants</td>
<td>Figure 3.10A</td>
</tr>
<tr>
<td>Minimal media survival (MEM)</td>
<td>No statistical significant difference between WT and mutants</td>
<td>Figure 3.10B</td>
</tr>
<tr>
<td>Rich media survival (DMEM+10% heat-inactivated FBS)</td>
<td>No statistical significant difference between WT and mutants</td>
<td>Figure 3.10C</td>
</tr>
<tr>
<td>Anaerobic growth curve (MH broth)</td>
<td>No statistical significant difference between WT and mutants</td>
<td>Figure 3.11</td>
</tr>
<tr>
<td>Low iron conditions (desferal and dipyridyl)</td>
<td>No significant difference between WT and mutants</td>
<td>Table 3.2</td>
</tr>
<tr>
<td>Antimicrobial agents</td>
<td>No significant difference between WT and mutants</td>
<td>Table 3.2</td>
</tr>
<tr>
<td>pH sensitivity test (MH agar)</td>
<td>Lethal at pH4, pH5, pH9. Optimal growth at pH6 and pH7. No statistical significant difference between WT and mutants</td>
<td>Not shown</td>
</tr>
<tr>
<td>55°C Heat shock</td>
<td>5 minute treatment showed identical results from untreated samples shown in Figure 3.7A</td>
<td>Not shown</td>
</tr>
<tr>
<td>Motility</td>
<td>No difference between WT and mutants</td>
<td>Figure 3.12</td>
</tr>
<tr>
<td>GGT assay</td>
<td>No statistical significant difference between WT and mutants</td>
<td>Figure 3.13</td>
</tr>
<tr>
<td>Glutamine rescue assay</td>
<td>Supplementation of glutamine to RAW264.7 and INT407 cells during WT C. jejuni infection did not show any difference in DAPI staining from cells without glutamine supplementation.</td>
<td>Not shown</td>
</tr>
<tr>
<td>Protein secretion (Culture supernatant)</td>
<td>No significant difference between WT and mutants</td>
<td>Figure 3.14</td>
</tr>
<tr>
<td>Nutrient deprivation assay (Survival in MEM-/+ 40mM glutamine)</td>
<td>No significant difference between WT and mutants in optical density or CFU measurement.</td>
<td>Figure 3.15</td>
</tr>
</tbody>
</table>
Figure 3.10. ΔpaqP and ΔpaqQ mutants survive similarly to WT C. jejuni in rich or minimal media. (A) Shaking culture in MH broth at a starting optical density of OD₆₀₀ ~ 0.006 and (B) MEM at a starting optical density of OD₆₀₀ ~ 0.02 (C) DMEM with 10% heat-inactivated FBS at a starting optical density of OD₆₀₀ ~ 0.02.
Figure 3.11. \( \Delta paqP \) and \( \Delta paqQ \) mutants exhibit similar survival as the WT strain under anaerobic conditions over time. Log phase \( C. jejuni \) were diluted to OD\(_{600} \approx 0.03 \) in MH broth. Cultures were placed in a sealed jar with an Anaero-GasPak, and incubated at 37°C, shaking at 200 r.p.m. Serial dilutions of cultures were made at each time point and spotted onto agar plates for CFU enumeration.

Table 3.2. Antimicrobial Susceptibility test. Using diffusion discs, the zone of inhibition diameter was recorded (mm).

<table>
<thead>
<tr>
<th>Type</th>
<th>Antimicrobial agent</th>
<th>Amount</th>
<th>WT</th>
<th>( \Delta paqP )</th>
<th>( \Delta paqQ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron chelator</td>
<td>Dipyridyl</td>
<td>0.4µmol</td>
<td>10</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Iron chelator</td>
<td>Desferal</td>
<td>0.4µmol</td>
<td>R*</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td>Amikacin</td>
<td>30µg</td>
<td>27</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td>Nalidixic Acid</td>
<td>30µg</td>
<td>38</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Macrolide</td>
<td>Erythromycin</td>
<td>15µg</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

Resistant, no zone of inhibition.
3.12 **WT and mutant C. jejuni exhibit similar tolerance to heat shock and pH stresses.**

To test tolerance to heat shock, log phase bacteria were diluted in MH broth and heated at 55°C for 5 min or 15 min before harvesting the bacteria for serial dilutions and microaerobic growth for 2 days at 37°C for CFU enumeration. While no bacteria were recovered from the 15 min time point, the number of CFU recovered from the bacteria treated for 5 min was identical to the untreated sample (Figure 3.7A). No differences were observed among the strains. To assess tolerance to a range of pH (pH 4- pH 9), log phase bacteria were diluted and spotted onto MH agar at different pHs. All C. jejuni strains exhibited optimal growth under neutral, or slightly acidic environments (pH 6-7), and were sensitive to acidic (pH 4, 5) and basic (pH 9) conditions. No differences were observed between WT and mutants.
Figure 3.12. \( \Delta paqP \) and \( \Delta paqQ \) mutants do not exhibit a motility defect in 0.4\% agar. Log phase \( C. \) jejuni were diluted to \( OD_{600} \sim 0.02 \) and 1 \( \mu l \) of each strain stabbed into MH agar plates containing 0.4\% agar. Migration of the cells from the point of inoculation was analyzed following 24 hr of incubation at 37\(^\circ\)C under microaerobic conditions. The experiment was performed in 3 replicates.

Figure 3.13. Rates of GGT activity in \( \Delta paqP \) and \( \Delta paqQ \) mutants do not significantly deviate from WT \( C. \) jejuni. Absorbance was measured at 405nm at a 10 min interval. International unit (U/ml) measurements were obtained by multiplying the absorbance reading by 2.211 according to the manufacture's protocol. Protein concentrations (mg/ml) of each bacterial lysate sample were measured using a Bradford assay. The experiment was performed in replicates (n=10), and this is one representative experiment of three separate repeats.
Figure 3.14. $\Delta paqP$ and $\Delta paqQ$ mutants exhibit a similar protein secretion profile as WT C. jejuni. Culture supernatants were collected from overnight $C. jejuni$ cultures, and proteins were prepared for TCA precipitation and SDS-PAGE silver staining.
Figure 3.15. ΔpaqP and ΔpaqQ mutants and WT C. jejuni survive similarly in minimal essential media supplemented with L-glutamine. Log phase C. jejuni were grown in 10ml MEM with 1% NEAA supplement in the absence or presence of 40 mM L-glutamine. Bacteria were grown microaerobically under shaking conditions and cultures were harvested at each time point and diluted for (A) CFU enumeration or (B) Absorbance reading at 600nm. (C) Final cell yields after 24 hr microaerobic growth in MEM minimal medium only or with glutamine. Cells were measured at 600 nm. No significant difference was observed between the WT and mutant strains. The experiment was performed in triplicate and repeated 3 times.
4.0 DISCUSSION AND CONCLUSIONS
*C. jejuni* pathogenesis depends on the organism’s ability to survive diverse conditions encountered in the external environment, in the intestinal tract, and inside epithelial and immune system cells. External stresses frequently challenging *C. jejuni* include heat, osmotic, oxidative, and aerobic stress as well as nutrient limitation. Growing evidence also indicates that *C. jejuni* can survive intracellularly within host enterocytes and macrophages (Watson and Galan, 2008; Young *et al*., 2007; Day *et al*., 2000), suggesting that *C. jejuni* must also tolerate intracellular stresses for sustained survival. However, specific factors promoting intracellular bacterial survival and triggering host cell signal transduction pathways remain largely unknown.

In this study, we have investigated the biological and pathogenesis-related roles of a putative *C. jejuni* AA-ABC transporter originally annotated as Cj0467-9. As both the integral membrane protein, or permease, and ATP binding protein/ATPase components are frequently required to produce a functional AA-ABC transporter (Nikaido, 2002), we generated non-polar mutations in both the Cj0467 (PaqP) permease and the Cj0469 (PaqQ) ATPase to ensure a comprehensive analysis of the system’s functions. Bioinformatics analyses predicted this AA-ABC system to be a glutamine ABC transporter with high homology to glutamine ABC transporters in *Helicobacter*, *Streptococcus* and *Pseudomonas* spp.; a previous study also suggested that this system may be a putative cysteine ABC transporter coupled to CjaA, a cysteine substrate binding protein (Muller *et al*., 2005). Our amino acid transport assays indicate that the transporter does not exhibit specificity exclusively for a single amino acid, since both Δ*paqP* and Δ*paqQ* mutants were defective for the uptake of glutamine, glutamate, cysteine and asparate. Nevertheless, glutamine appeared to be a preferred substrate, since both mutants exhibited a more severe decrease in glutamine uptake compared to the other amino acids. We observed some residual glutamine transport in the Δ*paqP* and Δ*paqQ* mutants (Figure 3.2), and preliminary glutamine uptake competition assays performed on the Δ*paqP* and Δ*paqQ* mutants (unpublished observations) likewise indicate that there is likely a redundant mechanism for
glutamine transport in C. jejuni. This notion is supported by the previous annotation of \textit{cj}0940c and \textit{cj}0902 as \textit{glnP} and \textit{glnQ} which, like \textit{paqP} and \textit{paqQ}, are present in all sequenced strains of \textit{C. jejuni} (Pearson \textit{et al.}, 2007; Hofreuter \textit{et al.}, 2006; Parkhill \textit{et al.}, 2000)

Our previously published microarray analysis identifying a significant increase in levels of \textit{cj}0467-9 mRNA during INT407 cell infection (Gaynor \textit{et al.}, 2005) suggested a potential role for this AA-ABC transporter in \textit{C. jejuni} pathogenesis. Infection assays using INT407 cells, Caco-2 cells and RAW264.7 macrophages revealed no significant differences in adhesion, invasion, or long-term intracellular survival at 24 hr for the \textit{ΔpaqP} and \textit{ΔpaqQ} mutants compared to WT. Mouse colonization levels were likewise similar for \textit{C. jejuni} WT and mutant-infected animals, at both shorter- and longer-term time points. Surprisingly, however, an apparent increase in short-term intracellular survival was observed for the \textit{ΔpaqP} and \textit{ΔpaqQ} mutants in INT407, Caco-2 and RAW264.7 infected cells 9 hr post-infection. This is in contrast to observations for other genes identified in the aforementioned microarray cluster as up-regulated during cell infection: both \textit{ΔspoT} and pVir mutants are diminished for invasion (Gaynor \textit{et al.}, 2005; Bacon \textit{et al.}, 2002; Bacon \textit{et al.}, 2000), and \textit{ΔspoT} is also defective for intracellular survival (Gaynor \textit{et al.}, 2005). Our findings also deviate from previous studies on the \textit{C. jejuni} Peb1 asparatate/glutamate binding protein mutant (Leon-Kempis Mdel \textit{et al.}, 2006; Pei \textit{et al.}, 1998), as well as a Group B \textit{Streptococcus} glutamine ABC transporter \textit{ΔglnQ} mutant (Tamura \textit{et al.}, 2002), where significant infection defects were observed both \textit{in vitro} and \textit{in vivo}.

One initial hypothesis to explain the enhanced intracellular survival of the \textit{ΔpaqP} and \textit{ΔpaqQ} mutants was that they may be more resilient to stresses occurring inside cells. For instance, the intracellular environment will have different \textit{O}_2 and \textit{CO}_2 levels than the intestinal tract or the external environment. Furthermore, as a defense mechanism, host epithelial cells and macrophages produce oxygen derivatives and reactive oxygen species (ROS) to generate a
highly oxidative environment (Baillon et al., 1999; Purdy et al., 1999; Shepherd, 1986).

Consistent with this hypothesis, the ΔpaqP and ΔpaqQ mutants exhibited an increased ability to
grow in a sub-optimal CO₂ environment, as well as an increased resistance to aerobic and t-
BOOH organic peroxide stresses. Interestingly, it was recently shown that a gamma glutamyl
transpeptidase (GGT)-deficient C. jejuni mutant (Δggt) exhibited increased invasion efficiency
concomitant with increased H₂O₂ resistance (Barnes et al., 2007). GGT is involved in the
degradation of glutathione to amino acids or peptides in the gamma glutamyl cycle, and
 glutathione has been previously implicated in resistance to oxidant-mediated killing in
eukaryotes (Shi et al., 1993; Tate and Meister, 1981). Although a connection between our
observations and GGT is plausible, particularly given the involvement of related molecules (i.e.,
amino acids such as glutamine) in both pathways, several lines of evidence suggest that our AA-
ABC transporter system affects stress responses independent of GGT: our mutants were not
hyper-resistant to H₂O₂ (Figure 3.6), a Δggt mutant was previously found to be defective for
mouse colonization (Hofreuter et al., 2006), and there was no apparent alteration of GGT activity
in our mutant strains (Figure 3.14). The aerobic, CO₂, and oxidative stress phenotypes of ΔpaqP
and ΔpaqQ are also distinct from those observed in an ΔnssR mutant, even though nssR (cj0466),
which is involved in nitrosative stress response regulation (Elvers et al., 2005), is directly
upstream of and likely co-transcribed with paqP and paqQ. Although little is known about
aerotolerance and CO₂ stress survival in C. jejuni, the stringent response, which is modulated by
the spoT gene, has also been shown to be up-regulated during cell infections, and was found to
be important for both low CO₂ and high O₂ stress survival (Gaynor et al., 2005). C. jejuni also
harbours a multitude of oxidative stress regulators to target a specific type of ROS. For instance,
while H₂O₂ is readily degraded by catalase (Day et al., 2000), organic peroxides such as t-BOOH
are degraded by alkyl hydroperoxide reductase, known as AhpC (Klancnik et al., 2006). It is
possible that PaqP and PaqQ are involved in this organic peroxide degradation pathway through means yet to be identified, or that this AA-ABC transporter system participates in oxidative stress tolerance by a novel mechanism.

Previous studies in *Salmonella typhimurium* and *C. jejuni* suggested that cross-talk exists between oxidative and heat or osmotic stress responses (Phongsisay et al., 2007; Andersen et al., 2005; Brondsted et al., 2005; Rychlik and Barrow, 2005; Stead and Park, 2000; Lee et al., 1995). Since an increase in oxidative stress resistance was observed in both ΔpaqP and ΔpaqQ mutants, we suspected that these mutants may also exhibit an increased resistance to heat and salt treatments. As predicted, the ΔpaqP permease mutant likewise tolerated heat and osmotic stresses better than WT; however, the ΔpaqQ ATPase mutant was unexpectedly more sensitive than WT to both heat and osmotic stresses. Thus, although both components are important for amino acid uptake, and participate in host cell interactions and oxidative/aerobic stress responses, they appear to exhibit differential functions in thermoregulation and osmoregulation. Although similar observations have not previously been reported, and thus no literature is currently available to help account for this phenomenon, several hypotheses may explain why loss of a permease or ATPase could have different consequences on specific aspects of bacterial physiology. For instance, as soluble proteins, ATPases harbor a certain level of promiscuity (Wyckoff et al., 1999; Schlosser et al., 1997; Wilken et al., 1996; Hekstra and Tommassen, 1993). Thus, an ATPase may couple with other ABC transporter systems in the absence of a functional permease, while a system that has lost ATPase function but retains functional permease proteins may be at least partially rescued by another ATP binding protein. This hypothesis is also consistent with the high degree of homology (52.9% identity) observed between PaqQ and Cj0902/GlnQ. Alternatively, absence of an ATPase may result in reduced ATP consumption, causing a general disruption in energy balance. Another possible interpretation is that the PaqP permease and PaqQ ATPase may exert distinct effects on gating...
small solutes or metabolites important for balancing the membrane electropotential gradient, which has been associated with osmoregulation and shock resistance in *E.coli* (Berger and Heppel, 1974). Extensive future studies will be required to distinguish between these possibilities.

In addition to characterizing physiological differences between the *C. jejuni* mutants, we also investigated if this AA-ABC transporter system might alter host cell viability, thereby also affecting the number of viable bacteria (CFUs) recovered in the intracellular survival assay. Interestingly, RAW264.7 cells (but not INT407 cells) infected with mutants exhibited approximately 30% higher viability than cells infected with WT bacteria at the short-term intracellular survival time point. Consistent with the cell viability data, Δ*paqP* and Δ*paqQ*-infected RAW264.7 macrophages also displayed a significantly lower level of apoptosis, or programmed cell death, as assayed by Annexin-V staining. Several previous reports have also made tangible connections between host cell cytotoxicity and specific *C. jejuni* virulence factors, although the literature varies considerably depending on *C. jejuni* strain and host cell type used. For instance, one study reported that apoptosis of 28SC monocytes induced by infection with *C. jejuni* strain 81-176 was dependent on cytolethal distending toxin (CDT) (Hickey et al., 2005), a DNase-like molecule that causes host cell cycle arrest (Lara-Tejero and Galan, 2000; Whitehouse et al., 1998). However, another study reported that apoptosis of THP-1 monocytes by *C. jejuni* strain F38011 was CDT- and lipooligosaccharide (LOS)-independent [in contrast to LPS observations for other pathogens (Guiney, 2005; Navarre and Zychlinsky, 2000)] and instead involved secreted *C. jejuni* Cia proteins (Siegesmund et al., 2004; Konkel et al., 1999). A third study reported that T84 enterocytes underwent oncrosis (not apoptosis) in response to *C. jejuni* infection that was dependent on the *C. jejuni* strain used (i.e., more invasive strains were more oncotic) as well as the FlaAB flagellins, but was independent of CDT (Kalischuk et al., 2007). We have not observed any obvious differences in secreted protein profiles between our WT and Δ*paqP* or Δ*paqQ* mutant strains (Figure 3.15). Nonetheless, it will be very interesting to
explore, in future work, potential connections between our findings and those described in the above studies.

To explore mechanisms underlying the effect of this AA-ABC transporter system on macrophage apoptosis, we next investigated whether several host cell MAP kinase proteins exhibited altered phosphorylation profiles in WT compared to mutant-infected cells. Each MAPK signaling molecule investigated is essential for eukaryotic cell growth and survival (Shan et al., 2007; Schorey and Cooper, 2003). C. jejuni has been shown to activate these signaling pathways, which in turn leads to significant downstream effects, including cytokine production and host cell damage (Chen et al., 2006; MacCallum et al., 2005; Watson and Galan, 2005). Interestingly, deletion of paqP or paqQ in C. jejuni abrogated ERK phosphorylation in RAW264.7 cell infections at the same short-term intracellular survival time point assayed for cell viability (Figure 7C). In contrast, WT, ΔpaqP, and ΔpaqQ elicited similar ERK activation profiles during INT407 and Caco-2 epithelial cell infections, and during later infection timepoints in RAW264.7 cells (Figure 3.9 and data not shown). JNK and p38 kinase, which participate in different MAPK signaling pathways, also displayed no significant differences in phosphorylation between WT and mutant-infected RAW264.7 cells.

Our observation that this AA-ABC transporter system participates in ERK activation during macrophage infection was an unexpected and novel finding. It is also interesting to hypothesize that this may at least partially contribute to our observed PaqP- and PaqQ-induced macrophage apoptosis. The role of ERK activity in apoptosis has been controversial. Some studies have proposed that ERK is important for cell proliferation, survival and anti-apoptosis (Huang et al., 2007; Larson et al., 2007; Marin-Kuan et al., 2007). However, other studies have revealed that ERK activation contributes to cisplatin-induced apoptosis in HeLa and A549 cells and hydrochloric-inomenine-induced apoptosis in RAW264.7 cells (Xaus et al., 2001; Wang et al., 2000) as well as H2O2-induced apoptosis in intestinal epithelial cells (Zhou et al., 2005).
Additionally, ERK activation in macrophages has been suggested to participate in caspase-independent apoptotic signal transduction during Group B Streptococcus infection (Fettucciari et al., 2003). Activation of ERK and pro-inflammatory cytokines is also often stimulated by virulence determinants such as LPS via toll-like receptor (TLR) signaling (Thomas et al., 2006; Guha and Mackman, 2001). Future work will be required to determine if this is also the case during C. jejuni infection. However, to date there have been no connections made between C. jejuni and host cell TLR4 activation, and as noted above, at least one study suggested that C. jejuni-induced macrophage apoptosis may be LOS-independent (Siegesmund et al., 2004).

Interestingly, glutamine has also been implicated as an anti-apoptotic factor. For instance, glutamine supplementation has been shown to reduce apoptosis of human intestinal epithelial cells in a manner that may interface with the ERK activation pathway (Larson et al., 2007; Evans et al., 2005; Evans et al., 2003). Furthermore, glutamine depletion was found to elicit apoptosis in epithelial cells and immune cells such as T-cells and neutrophils (Chang et al., 2002). Thus, one working hypothesis to explain the increased host cell death observed for WT vs. ΔpaqP and ΔpaqQ mutant-infected cells is that to acquire nutrients, intracellular C. jejuni may hijack glutamine (and likely other amino acids) from host cells, and that this AA-ABC transporter system, which is up-regulated during cell infection, participates in this process. Preliminary experiments exploring glutamine supplementation during cell infections have not to date identified an effect on our C. jejuni-induced macrophage apoptosis (Figure 3.16). However, this hypothesis is consistent with our previous observations that C. jejuni requires the stringent response (spoT) and polyphosphate kinase 1 (ppkI) for both survival in low-nutrient environments and in an intracellular environment (Candon et al., 2007; Gaynor et al., 2005), as well as recent work suggesting that C. jejuni resides intracellularly in an anaerobic and likely nutrient-poor vacuole (Watson and Galan, 2008).
In summary, we have provided evidence that the AA-ABC transporter system components PaqP and PaqQ participate in several key aspects of *C. jejuni* stress survival and pathogenesis, some of which are novel not only for *C. jejuni* but for other bacteria as well. Our work has also created additional links between *C. jejuni* disease etiology, physiology, and basic metabolic processes (i.e., amino acid uptake), an increasingly emerging theme in the study of this prevalent pathogen. Future work aimed at addressing the hypotheses above as well as identifying potentially unpredicted functions for this system should yield additional insight into *C. jejuni* biology and pathogenesis and potentially uncover new roles for AA-ABC transporters in other bacteria as well.
5.0 Future Directions
We have shown that PaqP and PaqQ are involved in several key aspects of *C. jejuni* physiology and biology. For instance, we found that in the absence of PaqP or PaqQ, *C. jejuni* exhibited an increased tolerance to CO₂ and aerobic stress. This is in direct contrast to observations with the ΔspoT stringent response mutant, which exhibits an increase in sensitivity to aerobic and CO₂ stresses (Gaynor et al., 2005). Thus, one ensuing hypothesis is that diminished nutrient (i.e., glutamine) uptake may cause ΔpaqP and ΔpaqQ to up-regulate the stringent response in order to survive the unfavourable nutrient-limited condition. To test this, we will first examine levels of spoT transcription in the mutants vs. WT by Real-Time quantitative PCR. Second, we will assess levels of the stringent response effector molecule guanosine tetraphosphate (ppGpp), which is synthesized by SpoT during times of nutrient stress and binds RNA polymerase to elicit global transcriptional changes to the stress condition. This will be accomplished by labeling WT and mutant strains with ³²P and detecting ppGpp and its precursor pppGpp by Thin Layer Chromatography. If a deficiency in AA uptake in mutants defective for our AA-ABC transporter system activates the stringent response, then lack of PaqP or PaqQ should increase spoT transcription and/or ppGpp synthesis.

We have also demonstrated that PaqP and PaqQ impact intracellular survival, host cell death, and the ERK-MAPK signaling pathway. However, it is not completely understood whether PaqP and PaqQ induce macrophage apoptosis via ERK-MAPK dependent signaling independent of or dependent on other bacterial apoptosis-inducing factors, such as the CDT. Since the CDT has been postulated as a major inducer of apoptosis in *C. jejuni* infected cells (Hickey et al., 2000), one future question is whether PaqP and PaqQ enhance apoptosis in a CDT-dependent or -independent manner. One approach to explore this will be to generate ΔcdtBΔpaqP and ΔcdtBΔpaqQ double knockout mutants and compare levels of apoptosis in RAW264.7 macrophages infected with WT, ΔpaqP, ΔpaqQ, ΔcdtB, ΔcdtBΔpaqP, and
ΔcdtBΔpaqQ mutants. If CDT induces apoptosis in RAW264.7 cells in a PaqP- and PaqQ-dependent manner, or vice versa, levels of apoptosis induced by all mutant strains should be similar to each other. Conversely, if PaqP and PaqQ induce apoptosis in RAW264.7 cells in a CDT-independent manner, we would expect infection with ΔcdtBΔpaqP or ΔcdtBΔpaqQ double mutants to result in significantly reduced apoptosis compared to single mutant ΔpaqP, ΔpaqQ and ΔcdtB-infected cells. Regardless of the outcome, this study will introduce a novel paradigm further associating this ABC transporter with pathogenesis during bacterial infection.

To date, nothing has been explored regarding the correlation of PaqP and PaqQ with MAPK signaling pathways, nor is much understood about C. jejuni induction of MAP kinase activation in general. Hence, we are also interested in investigating the upstream molecular effects of PaqP and PaqQ on the activation of ERK signaling in macrophages during C. jejuni infection. One of our hypotheses is that this ABC transporter system, or another C. jejuni factor (i.e., an outer membrane protein or other cell surface component) that may be directly influenced by the transporter system, may interact with host cells and activate ERK by triggering a signal transduction cascade upon internalization (or while surviving intracellularly). An initial approach will include examining signaling molecules upstream of ERK in the MAPK pathway. MEK (Raf-MAPK/ERK kinase), immediately upstream of ERK, will be the first logical candidate to examine. This will be accomplished by assessing levels of phosphorylated MEK in WT and ΔpaqP and ΔpaqQ infected RAW264.7 cells by Western blot analysis. Next, to investigate if PaqP and PaqQ are involved in ERK-MAPK activation in a Ras-dependent manner similar to the receptor tyrosine kinases (RTKs), levels of Raf-1 activation will be examined. It is understood that RTK is activated by extracellular ligands, often growth factors (such as the epidermal growth factor), and undergoes autophosphorylation. The phosphorylated tyrosine kinase receptor then recruits RasGRP to activate Ras from Ras-GDP (an inactive form) into Ras-GTP (an active
Ras is a small G protein that recruits Raf-1, an intermediate signaling molecule that triggers a series of downstream cascades to activate ERK (Hagemann and Blank, 2001). If PaqP and PaqQ activate ERK via a Ras and Raf-1 dependent mechanism similar to the RTKs, it will be interesting to identify signaling molecules that may interact with PaqP and PaqQ to trigger subsequent signal cascades in the MAPK pathway. If PaqP and PaqQ are involved in the Ras/Raf-1 dependent pathway, we will next investigate whether PaqP and PaqQ are involved in the activation of RasGRP. In addition, we will use MEK/ERK inhibitor to investigate whether PaqP and PaqQ contribute to apoptosis in host cells in an ERK-dependent manner. It is expected that if PaqP or PaqQ contribute to apoptosis via ERK activation, we should observe a decrease in levels of apoptosis (or increased levels of cell viability) in the MEK/ERK inhibitor treated WT-infected cells.

Finally, we will examine an alternate putative glutamine ABC transporter permease GlnP and ATPase GlnQ to investigate whether the observed phenotypes in ΔpaqP and ΔpaqQ are due to shifts in AA (specifically glutamine) metabolism or by other potential effects of PaqP and PaqQ. It is hypothesized that if increased levels of intracellular survival and stress resistance are due to changes in glutamine metabolism, then ΔglnP and ΔglnQ mutants with reduced glutamine uptake will behave similarly to the ΔpaqP and ΔpaqQ strains. Furthermore, double knockout mutants ΔglnPΔpaqP and ΔglnQΔpaqQ should show an augmented phenotype in comparison to the single knockout mutants.

These future experiments will enhance our knowledge of PaqP and PaqQ and yield new ideas in understanding the role of ABC transporters in bacterial pathogenesis.
REFERENCES


