Campylobacter jejuni metabolism in survival and host cell interactions

by

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B.Sc.H., University of Toronto, 2007

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Abstract

*Campylobacter jejuni* is a leading cause of foodborne bacterial gastroenteritis in both developed and developing nations. Although *C. jejuni* is a common environmentally acquired pathogen, it is quite fastidious, rapidly losing viability in aerobic conditions. Genome sequence analyses have failed to identify classical virulence factors, making the pathogenic success of *C. jejuni* a mystery. Mutational analysis described herein identified novel metabolic factors that are important for infection of human epithelial cells as well as generation of oxidative stress in *C. jejuni* during aerobic incubation.

Investigation of a novel operon, *fdhTU*, induced during *C. jejuni* epithelial infection, showed that FdhTU positively regulates formate dehydrogenase. Subsequent analyses found that *fdhTU* and formate dehydrogenase are important for recovery of *C. jejuni* from epithelial cells. Further work showed that intracellular *C. jejuni* are undergoing oxidative stress, and that neutralization of oxidative stress with sulfite or catalase could significantly enhance recovery of *C. jejuni* following epithelial cell infection.

Analyses of other respiratory dehydrogenases failed to identify other systems important for recovery of *C. jejuni* from epithelial cells, but did identify a role for gluconate dehydrogenase in reducing necrosis in T84 epithelial cells in a reactive oxygen species- and calpain-dependent manner. In addition to the importance for epithelial cell infection, metabolic features were also found to be involved in causing oxidative stress in *C. jejuni* under aerobic conditions. *C. jejuni* was found to produce H$_2$O$_2$ when incubated in aerobic but not microaerobic conditions at 37°C but not 4°C, with formate dehydrogenase and sulfite oxidoreductase dependent respiration important for H$_2$O$_2$ production. Sulfite and cysteine could reduce *C. jejuni* loss of viability in aerobic conditions in a manner dependent on the sulfur assimilation pathway protein Atps. Atps was identified as important for aerobic survival, H$_2$O$_2$ resistance, and in reducing H$_2$O$_2$ produced by formate dehydrogenase dependent respiration. Characterization of the role of multiple respiratory systems in *C. jejuni*, a bacterial model that shares little with other
common pathogenic bacteria, has identified a central role of respiration in epithelial cell infection and environmental survival.
Preface

All chapters are based on experimental design from Mark Pryjma and Professor Dr. Erin Gaynor. All experiments were performed by Mark Pryjma in the laboratory of Dr. Erin Gaynor (Department of Microbiology and Immunology, UBC, Vancouver BC) unless otherwise stated.

Data presented in chapter 2 have been published (Pryjma et al. 2012. “FdhTU-modulated formate dehydrogenase expression and electron donor availability enhance recovery of Campylobacter jejuni following host cell infection”. J. Bacteriol. 194(15):3803-13). Microarray analysis of differential gene regulation in an fdhU background described in chapter 2 was performed by Steven Huynh and Dr. Craig Parker at the United States Department of Agriculture (USDA) Western Research Center in Albany, CA. Measurement of oxygen consumption with a Clark type electrode was performed by Mark Pryjma in the lab of Dr. Lindsay Eltis (Department of Microbiology and Immunology, UBC, Vancouver BC) with help from Jenna Capyk. The Olympus Fluoview FV1000 laser scanning confocal microscope from Dr. Robert Nabi’s lab (Department of Cellular & Physiological Sciences UBC, Vancouver BC) was used with equipment training conducted by Pascal St. Pierre.

Data presented in chapter 3 are in late stages of preparation for publication (Title pending). All experiments were performed by Mark Pryjma. Where appropriate the Varioskan Flash luminometer plate reader from Dr. Steven Hallam’s lab (Department of Microbiology and Immunology, UBC, Vancouver BC), and the Olympus Fluoview FV1000 laser scanning confocal microscope from Dr. Robert Nabi’s lab.

Data presented in chapter 4 are in late stages of preparation for publication (Title pending). All experiments were performed by Mark Pryjma. Where appropriate, the Varioskan Flash luminometer plate reader from Dr. Steven Hallam’s lab was utilized.
In addition, Mark Pryjma was an author on the following additional publications during his Ph.D. studies:


Svensson, S.L., Pryjma, M., Gaynor, E.C. “Flagella-mediated adhesion and extracellular DNA release contribute to biofilm formation and stress tolerance of Campylobacter jejuni”. In revision, PLOS One.

Frirdich E., et al. Title in progress (Campylobacter jejuni transition to coccoid morphology). In preparation.
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<tbody>
<tr>
<td>5-ASA</td>
<td>5-Amino sialic acid</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4- benzenesulfonyl fluoride hydrochloride</td>
</tr>
<tr>
<td>AhpC</td>
<td>Alkylhydroperoxidase</td>
</tr>
<tr>
<td>ALLN</td>
<td>Ac-LnL-CHO, MG-101, N-Acetyl-L-leucyl-L-leucyl-L-norleucinal</td>
</tr>
<tr>
<td>APS</td>
<td>Adenine phosphosulfate</td>
</tr>
<tr>
<td>Apsk</td>
<td>Adenine phosphosulfate kinase</td>
</tr>
<tr>
<td>ArgT</td>
<td>lysine/arginine/ornithine transport protein</td>
</tr>
<tr>
<td>AtpF</td>
<td>ATP synthetase F subunit</td>
</tr>
<tr>
<td>Atps</td>
<td>ATP sulfurylase</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>1,2-Bis(2-aminophenoxy)ethane- ( N,N,N',N' )-tetraacetic acid tetrakis(acetoxymethyl ester)</td>
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<tr>
<td>Caco2</td>
<td>Colorectal adenocarcinoma cells</td>
</tr>
<tr>
<td>CadF</td>
<td>Fibronectin binding protein</td>
</tr>
<tr>
<td>Campto</td>
<td>camptothecin</td>
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<tr>
<td>CcoNPQRS</td>
<td>cytochrome c oxidase terminal oxidase</td>
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<tr>
<td>CCV</td>
<td><em>Campylobacter</em> containing vesicle</td>
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<td>Cdc42</td>
<td>Cell division control protein 42</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>CosR</td>
<td><em>Campylobacter</em> oxidative stress regulator</td>
</tr>
<tr>
<td>CprRS</td>
<td><em>Campylobacter</em> planktonic regulator</td>
</tr>
<tr>
<td>CydAB</td>
<td>Cytochrome bd oxidase</td>
</tr>
<tr>
<td>CYLD</td>
<td>Cylindromatosis protein</td>
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<tr>
<td>CysM</td>
<td>Cysteine synthase</td>
</tr>
<tr>
<td>CysTWAM</td>
<td>Cysteine importer</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Delbacco's modified essential media</td>
</tr>
<tr>
<td>Dock180</td>
<td>Dedicator of cytokinesis 180</td>
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<tr>
<td>Dps</td>
<td>DNA-binding proteins from starved cells protein</td>
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<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>F12</td>
<td>Ham's F12 nutrient mixture</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fdh</td>
<td>Formate dehydrogenase</td>
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<tr>
<td>FdhT</td>
<td>Fdh transporter</td>
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<tr>
<td>FdhU</td>
<td>Fdh regulator</td>
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<tr>
<td>FlaA</td>
<td>Flagellin A</td>
</tr>
<tr>
<td>FlaB</td>
<td>Flagellin B</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GBS</td>
<td>Guillain–Barré syndrome</td>
</tr>
<tr>
<td>Gdh</td>
<td>Gluconate dehydrogenase</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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Glut-1  Glucose transporter member 1
GM130  130kDa Golgi matrix protein
HrcA  Heat regulation at CIRCE regulator
HspR  Heat shock protein repressor
HtrA  High temperature requirement
HydAB  Hydrogenase
IBD  Inflammatory bowel disease
IBS  Irritable bowel syndrome
IL-8  Interleukin-8
INT407  Human intestinal cell line
KatA  Catalase
LAMP1  Lysosomal-associated membrane protein 1
LB  Luria broth
LDH  Lactate dehydrogenase
LysR  LysA regulator
Mdh  Malate dehydrogenase
MDL-28170  \( N\)-[(1S)-1-[[1-formyl-2-phenylethyl]amino]carbonyl]-2-methylpropyl]-carbamic acid, Phenylmethyl ester
MEM  Minimal essential media
MH-TV  Muller-Hinton broth with trimethoprim and vancomycin
MLKL  Mixed lineage kinase domain-like protein
MsrAB  Methionine sulphoxide reductases
NADH  Nicotinamide adenine dinucleotide
NADPH  Nicotinamide adenine dinucleotide phosphate
NFκB  nuclear factor κ-light-chain-enhancer of activated B cells
NMR  Nuclear magnetic resonance
NOX  NADPH oxidase
Nuo  NADH:Ubiquinone oxidoreductase
OOR  Oxaloacetate oxido-reductase
PAP  Phospho-adenosine phosphate
PAPS  Phospho-adenosine phosphosulfate
Papsr  Phospho-adenosine phosphosulfate reductase
PBS  Phosphate buffered saline
PD-15060  (2Z)-3-(4-iodophenyl)-2-mercapto-2-propenoic acid, 3-(4-iodophenyl)-2-mercapto-(2Z)-2-propenoic acid
PEG6000  Polyethylene glycol 6000
PEG8000  Polyethylene glycol 8000
PerR  Peroxide stress regulator
PFA  Paraformaldehyde
PFOR  Pyruvate flavin oxidoreductase
PI3K  Phosphoinositide 3-kinase
Ppi  Polyphosphate
PutA  Proline dehydrogenase
PutP  Proline uptake transporter
RacRS  Reduced ability to colonize regulator
Rip1  Receptor interacting protein 1
Rip3  Receptor interacting protein 3
RNS  Reactive nitrogen species
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RpoA</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription-quantitative PCR</td>
</tr>
<tr>
<td>Se</td>
<td>Selenium</td>
</tr>
<tr>
<td>SirA</td>
<td>Sporulation inhibitor of replication family protein</td>
</tr>
<tr>
<td>SodB</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SorAB</td>
<td>Sulfite oxidoreductase</td>
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<tr>
<td>SpoT</td>
<td>Stringent response regulator</td>
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<td>Src family kinase</td>
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<td>SuperScript III</td>
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<td>T84</td>
<td>Metastatic colon tumor cell line</td>
</tr>
<tr>
<td>TE Buffer</td>
<td>Tris EDTA buffer</td>
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<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
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<td>Tumor necrosis factor</td>
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<td>Z-VAD-FMK</td>
<td>N-Benzylxycarbonyl-Val-Ala-Asp-fluoromethyl ketone</td>
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Chapter 1:

General introduction

1.1: Characteristics, prevalence and treatment of *Campylobacter jejuni*

*Campylobacter jejuni* is a helical, motile, Gram-negative bacterium. It was first described as a pediatric infectious disease as early as the 1970’s (37, 126), and since then has been established as a major emerging pathogen and a leading source of bacterial gastroenteritis in many areas. Up to 1% of the population of the developed world contracts a new *C. jejuni* infection each year (26, 130, 269). *C. jejuni* belongs to the diverse *Campylobacteraceae* family which includes the human pathogens *C. coli* and *C. concisus*, as well as the important animal pathogen *C. fetus* (188). *C. jejuni* is a member of the epsilon class of Proteobacteria along with *Helicobacter pylori*, a broad colonizer of human gastric linings and a causative agent of gastric ulcers and gastric cancer (2, 174), and extremophiles, such as *Sulfurovum lithotrophicum* and *Nitratiruptor tergarcus* that live in deep sea hydrothermal vents (200). It has been hypothesized that many of the atypical metabolic pathways present in *C. jejuni* may be due to an evolutionary lineage originating in extremophiles.

1.1.1: Disease caused by *C. jejuni*

Over the last 30 years, *C. jejuni* has emerged as a major source of morbidity in the developed world (306). The most common disease associated with *C. jejuni* infection is watery to bloody diarrhea lasting 1-10 days (36, 47). Post-infectious sequelae associated with infection can also occur and result in minor to severe pathologies. *C. jejuni* is the most common cause of Guillain–Barré Syndrome (GBS), causing about 40% of cases annually. GBS is an acute ascending bilateral paralysis that can lead to severe respiratory problems, and in 3.9% of afflicted individuals results in death (104, 114, 280, 287). In addition, *C. jejuni* is now known to be a source of inflammatory bowel disease relapse (31, 194) as well
as a persistent cause of reactive arthritis (243). Of increasing concern is the spread of newly identified strains of *C. jejuni* associated with abortions in livestock. Traditionally, *C. jejuni* infections in livestock were asymptomatic; the reason for this apparent change of pathogenicity in livestock is unknown. These strains cause significant economic loss and may become a potential source of *C. jejuni*-associated septicemia, a complication that has been rare to this point (300).

### 1.1.2: Epidemiology of *C. jejuni*

In the United States and Canada, *C. jejuni* is thought to infect 1% of the population each year (197) with numbers higher in Europe (274, 288). However, due to under-reporting of infection, the actual rate of infection is thought to be much higher (197). Despite a basal rate of infection in many areas of the world, there are frequent outbreaks of *C. jejuni* (1, 94), with one of the best publicized being the *C. jejuni* and *Escherichia coli* O157:H7 Walkerton outbreak in Ontario, Canada (42). Infection with *C. jejuni* also usually has a seasonal distribution: the greatest incidence occurs in the summer months in warmer climates and peaks in winter in northern climates (178). Of the individuals infected, children around age 4 are a peak infected group, as are adults around 20-29 years old (204). Pregnant women infected with *C. jejuni* can develop bacteremia and other complications leading to abortion. However, secondary complications during *C. jejuni* infection in the fetus such as GBS and reactive arthritis are believed to be non-existent (252). Nonetheless, infection with *C. jejuni* during childbirth is a concern, as the infant can passively acquire *C. jejuni* during the birthing process and develop bacteremia, neonatal enteritis, or meningitis (252). *C. jejuni* is also a major risk factor in adult immunocompromised patients (222), such as people with HIV (144). Fortunately highly active antiretroviral therapy HIV treatment appears to reduce patient susceptibility to *C. jejuni* bacteremia to a non-significant level (65).
1.1.3: Sources of *C. jejuni* infection and treatment

*C. jejuni* human infection is rarely associated with human-to-human transmission and is instead transmitted from environmental reservoirs and food sources, primarily through the consumption of contaminated poultry that has been undercooked (306). *C. jejuni* is highly prevalent in chicken flocks; up to 100% of broiler chickens can be contaminated in the same flock (239). Generally, once one chicken is colonized with *C. jejuni*, the rest of the flock quickly becomes colonized due to coprophagy. Colonization typically lasts for the entire lifespan of the chicken (239). These factors may account for why *C. jejuni* is so widespread. Antibiotic treatment can reduce flock colonization; however, this has led to the emergence of antibiotic resistant *C. jejuni* strains in flocks and is not a permanent solution to reducing *C. jejuni* colonization (176, 179, 266). *C. jejuni* infection can also arise from other sources such as ducks, cattle, pigs, rodents and dogs (3, 59, 202), and from the ingestion of improperly pasteurized milk (118) or contaminated water (296). Once in the environment, *C. jejuni* can survive under cold damp conditions, and on refrigerated meat for long periods of time until ingestion (296).

*C. jejuni* infection usually results in a self-limiting disease. Treatment includes fluid replacement and rest, with antibiotics rarely being recommended and usually reserved for extreme cases, such as patients suffering from bacteremia or for those that are immunocompromised (136). Antibiotic treatment can reduce mean infection time slightly, but is more effective when taken early during infection (273).

1.2: General characteristics of *C. jejuni* that contribute to pathogenesis

The determination of the genome sequences of *C. jejuni* strain 11168 (217), and the strain used in our laboratory and described in this thesis, 81-176 (227), the latter of which is associated with a greater intensity of disease, led researchers to mine for novel virulence factors. Genomic analyses of sequenced *C. jejuni* strains revealed minimal evidence of classical virulence factors such as type III secretion.
systems, or toxins that could be directly linked to pathogenesis. Strain 81-176 was found to harbor a plasmid containing a putative type IV secretion system (16). The type IV secretion system is not uniformly distributed amongst C. jejuni strains, and its low correlation with disease brings into question its requirement for virulence. C. jejuni produces a cytolethal distending toxin which, when purified, induced double stranded breaks in human DNA (119). However, despite strong activity of the purified toxin against tissue cultured epithelial cells in vitro, cytolethal distending toxin has yet to be shown to be associated with C. jejuni pathogenesis in vivo (190). Despite a lack of classic virulence factors, analysis of the C. jejuni genome has revealed many factors vital to C. jejuni pathogenesis which will be discussed below.

1.2.1: Flagella, motility and chemotaxis

C. jejuni is highly motile and expresses a single flagellum at each pole. Motility is one of the most important C. jejuni colonization factors, as non-motile strains cannot colonize chickens or suckling mice (189, 198, 205). Furthermore, unbiased genetic screens for factors important for colonization and infection have consistently returned genes involved in flagellar biosynthesis and motility (93, 117). The flagella of C. jejuni harbor similarities to flagella of other bacteria (156). The two main flagellar structural components are FlaA and FlaB, with FlaA being the major component (156). In humans, Toll-Like Receptor 5 (TLR5) is involved in recognition of bacterial flagella during immune surveillance. The TLR5 recognition domain of C. jejuni FlaA contains mutations making it non stimulatory to TLR5 (10), although the effect of this on C. jejuni host infection is unknown. Chemotaxis has also emerged as a major colonization factor. C. jejuni possesses seven integral membrane and three soluble methyl accepting chemotaxis receptors that are involved in taxis to a variety of stimuli (156). Stimuli include fucose, pyruvate, fumarate, aspartate, and formate, with aspartate and formate chemotaxis being important for host colonization (90, 92, 102, 270, 285).
1.2.2: Surface polysaccharides

*Clostridium jejuni* is a highly glycosylated bacterium due to the number of different polysaccharide moieties decorating its surface; these include: capsular polysaccharide, lipooligosaccharide, *O*-linked and *N*-linked glycoproteins. The *C. jejuni* capsular polysaccharide has been shown to be important for resisting complement and antimicrobial peptide killing, preventing excessive cytokine production from dendritic cells, and enhancing host cell invasion (15, 132, 237), yet its influence in human colonization and disease is still unknown. The lipid A of *C. jejuni* is decorated by a short sugar chain or lipooligosaccharide. Studies of lipooligosaccharide truncation mutants have revealed the importance of the full-length lipooligosaccharide in resistance to antimicrobial peptides, human serum and bile, and in host cell invasion and colonization of chicks and mice (113, 199). *N*-linked glycosylation of proteins by PglB is also important for protection of these proteins from host proteases (7), and thereby maintaining bacterial fitness during infection (7). The role of *O*-linked polysaccharides in virulence has not been explored in depth in *C. jejuni*; however, *O*-linked glycosylation of FlaA and the major outer membrane protein (MOMP) has been associated with chick colonization and adherence to human epithelial cells (168).

1.3: Host cell invasion and intracellular survival in *C. jejuni* infection

*C. jejuni* is primarily an extracellular pathogen, but it is able to invade host epithelial cells and survive intracellularly. *C. jejuni* invasion and intracellular survival correlate with disease-associated phenotypes such as enhanced IL-8 induction, destruction of tight junctions/barrier functions, and induction of epithelial cell death (19, 95, 123). These observations have resulted in a large number of studies assessing the dynamics of *C. jejuni*-host cell interactions. It is unknown why *C. jejuni* adapted to an intracellular lifecycle but may be related to *C. jejuni*'s acquiring adaptations to infect amoeba's when in the environment (35, 211).
1.3.1: CadF-mediated invasion of epithelial cells through membrane ruffling

The current model describing *C. jejuni* host cell invasion is described below. Invasion is thought to begin with the cholesterol-dependent binding of *C. jejuni* to the surface of host epithelial cells at lipid rafts rich in caveolin-1, with depletion of host membrane cholesterol or disruption of caveolin-1 significantly inhibiting invasion (292). The *C. jejuni* surface protein fibronectin binding protein (CadF) is involved in adherence to epithelial cell surfaces through fibronectin binding and is also important for invasion (138, 139, 191, 203, 209). CadF binding and clustering of fibronectin in lipid rafts causes activation of alpha1-beta5-integrin, a protein involved in membrane ruffling. Activation of integrin causes phosphorylation of both focal adhesion kinase (FAK) and src family kinase (Src) which initiate a series of intracellular signaling events. Signaling results in the activation of platelet derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) stimulating phosphoinositide 3-kinase (PI3K). PI3K activation activates and recruits the vav family of guanine nucleotide exchange factors 2 protein (Vav2) and then cell division control protein 42 (Cdc42) to the site of invasion, resulting in downstream remodeling of host actin and microtubules, membrane ruffling, and the engulfment of *C. jejuni* by the host cell (Figure 1.1)(203, 209). An alternative pathway for induction of membrane ruffling during invasion has also been proposed. In this pathway, signaling through integrin and FAK instead activates paxillin and the dedicator of cytokinesis 180 (dock180)/ T-cell lymphoma invasion and metastasis 1 (Tiam-1)/ Engulfment and Cell Motility (Elmo) complex. This complex catalyzes the conversion of Rac1-GDP to Rac1-GTP. Activated Rac1-GTP triggers cytoskeletal rearrangements, initiating ruffling in the host cell membrane and bacterial engulfment (Figure 1.1) (203, 209). It is unknown if both pathways occur simultaneously and if they are differentially induced in different host cell types.
Figure 1.1. Signaling cascade activated by *C. jejuni* cell surface binding

Fibronectin-dependent signaling through integrin after *C. jejuni* binding causes activation of two pathways necessary for *C. jejuni* host epithelial cell invasion. In pathway 1, FAK and Paxallin dependent signaling causes the downstream conversion of inactive Rac1-GDP to active Rac1-GTP. This is required for actin and microtubule rearrangements resulting in membrane ruffling and invasion. In pathway 2, Src kinase and EGFR signaling results in downstream activation of Vav2 and Cdc42 which facilitates actin and microtubule rearrangements leading to membrane ruffling and invasion (60, 145).

1.3.2: Intracellular localization of *C. jejuni* in host epithelial cells

Little is known about the fate of *C. jejuni* following invasion. It can invade epithelial cells and monocytes and persist intracellularly for several days, with the length of intracellular survival depending on host cell type (64, 205, 206, 250). After invasion, the numbers of intracellular bacteria decrease over time, suggesting that the intracellular compartment is not a replicative niche. *C. jejuni* resides in the endocytic pathway immediately after invasion, as assessed by co-localization with the early endocytic marker dextran, and then begins to diverge from lysosomal trafficking (292). The early endocytic markers EEA-1, Rab4 and Rab5 are initially transiently co-localized with *C. jejuni*. These markers are lost 120 min post-infection. After this point only general details are known as to how *C. jejuni* trafficks within infected cells (292). The endocytic marker Rab7 then transiently co-localizes with *C. jejuni*, followed by the late lysosomal marker LAMP-1 (Figure 1.2a). Neither the lysosomal markers cathepsin nor phagocytosed
bovine serum albumin co-localized with *C. jejuni* during late points of infection (292), suggesting that live *C. jejuni* do not reside in a lysosomal compartment and must diverge from lysosomal trafficking to survive. Co-localization studies with other organelles determined that *C. jejuni* resides in a compartment that is near, but not within, the Golgi apparatus (Figure 1.2b). More detailed information about *C. jejuni* intracellular trafficking is not known, except that co-localization near the Golgi apparatus is dependent on proper microtubule dynamics (292).

**Figure 1.2. Intracellular lifecycle of *C. jejuni* in epithelial cells**


1.4: The electron transport chain and respiratory dehydrogenases

Analyses of published *C. jejuni* genome sequences revealed that a large proportion of the genome encodes for metabolic genes. This is intriguing in light of the emergence of “metabolic virulence” as being important for pathogenesis in other bacteria. More specifically, differential utilization of metabolites has been shown to confer a competitive growth advantage for some bacteria in the
intestinal tract by enabling them to utilize a greater range of nutrients (4, 298). Studies on the central metabolism of *C. jejuni* have yielded much insight into how it colonizes its hosts (21, 99, 127, 213, 271, 293).

1.4.1: *C. jejuni* has an intact but atypical electron transport chain

*C. jejuni* normally resides deep in colonic crypts and, as such, has adapted its core metabolism to persisting in this niche. *C. jejuni* relies on amino acids as a primary energy, carbon, and nitrogen source instead of carbohydrates (260). *C. jejuni* lacks the 6-phosphofructokinase enzyme that is required for the phosphorylation of fructose-6-phosphate to fructose-1,6-diphosphate of the glycolytic pathway, thus preventing utilization of glucose for energy generation (260). *C. jejuni* is still able to utilize pyruvate, likely generated through amino acid catabolism, using a pyruvate flavodoxin oxidoreductase (PFOR). Two of the major electron donors, PFOR and oxaloacetate oxidoreductase (OOR), generate energy by reducing the electron carrier flavodoxin (FldA) instead of NAD⁺ like in *E. coli* (50, 103, 259) (Figure 1.3). *C. jejuni* oxidizes flavodoxin with an atypical electron transport chain (ETC) Complex I/NADH dehydrogenase (259, 294). Reduced FldA is able to reduce the electron carrier proteins Cj1574c and Cj1575c, which act as the entry point of electrons into the ETC Complex I (Figure 1.3)(294). Electrons can also enter the ETC through the non-reversible Complex II (succinate dehydrogenase) and reversible fumarate reductase that can drive inter-conversion of succinate to fumarate. The other major members of the *C. jejuni* ETC, Complex III and cytochrome c, are well conserved compared to those of *E. coli* and other well-known model bacterial species and will not be addressed further here. *C. jejuni* possesses two functional terminal oxidases, the cyanide sensitive CcoNPQRS system and the cyanide resistant CydAB system (115). The CydAB system has been shown to have low affinity to oxygen and is not involved with the translocation of hydrogen ions across the inner bacterial membrane and ATP generation, unlike CcoNPQRS which is the major ETC hydrogen ion pump in *C. jejuni* (115). The affinity of CydAB for oxygen is such that it is not active at micro-aerobic oxygen concentrations, causing it to only
be active at higher oxygen tensions. CcoNPQRS, on the other hand, has a high affinity for oxygen and is saturated at lower oxygen tensions (115).

![Flux of electrons from pyruvate flavin oxidoreductase (PFOR) to Complex I NADH dehydrogenase (Nuo/Comp. I)](image)

**Figure 1.3. Flux of electrons from pyruvate flavin oxidoreductase (PFOR) to Complex I NADH dehydrogenase (Nuo/Comp. I)**

PFOR and oxaloacetate oxidoreductase (OOR; not shown) reduce flavodoxin (FldA) as an electron carrier for electrons entering the electron transport chain (ETC) which in turn reduces the Cj1574c/Cj1575c electron carrier protein complex. Reduced Cj1574c/Cj1575c reduces the Nuo/Complex1. This cycle is important for entry of electrons into the ETC by certain components of the citric acid cycle. Electrons can also enter the ETC through other enzyme complexes.

**1.4.2: *C. jejuni* electron donors and acceptors**

*C. jejuni* is known to have a branched ETC able to use a wide variety of electron donors and acceptors. The *C. jejuni* genome sequence predicts the existence of genes that encode terminal reductases that use alternative electron acceptors, including fumarate, nitrate, nitrite, dimethyl sulfoxide and trimethylamine N-oxide (Figure 1.4.a) which may be involved in anaerobic respiration when in intestinal crypts (246). However, *C. jejuni* supplemented with any of these terminal electron acceptors still does not allow anaerobic growth, with O₂ still being required (246). The use of tetrathionate by *C. jejuni* TsdA as an electron acceptor has been identified (162), which is interesting as it has been reported that *Salmonella* spp. may use tetrathionate to gain a competitive advantage over other gut bacteria during inflammation (162, 298). It remains to be seen if the same is true for *C. jejuni*. *C. jejuni* also encodes numerous respiratory dehydrogenases and a well-studied energy conservation pathway (generation of
proton motive force without using molecules that can be used as a carbon source; Figure 1.4.b).

Formate, gluconate, sulfite, hydrogen gas, and lactate can all act as efficient electron donors in this pathway (196, 213, 229, 275, 293). C. jejuni respiratory dehydrogenases that have been identified to date have lower than expected amino acid sequence similarity to homologs in typical enteric pathogens, so it is expected that additional dehydrogenases will be annotated in the future.

The presence of different electron donors and acceptors can impact the ability of C. jejuni to invade epithelial cells and colonize commensal chicken species. The intestines of most animals are colonized with a wide variety of commensal organisms that utilize the available host nutrients. For C. jejuni to colonize or infect a host in such a competitive environment, it likely relies in part on metabolic enzymes to give it a competitive advantage. Studies conducted on respiratory enzymes, such as hydrogenase and gluconate dehydrogenase (213, 293), have found that many of them are required for animal colonization. In addition, respiratory systems like formate dehydrogenase (Fdh) and sulfite oxidoreductase (Sor) have been found to be important for host cell infection (229, 271). A complete list of how C. jejuni respiratory dehydrogenases affect colonization is included in Table 1.1.
Figure 1.4. Electron acceptors and donors of the respiratory chain in *C. jejuni*

A) The known *C. jejuni* electron acceptor systems and where electrons from the electron transport chain (ETC) feed them. Nitrate reductase (NapAB) and nitrite reductase (NrfA) can accept electrons from menaquinone (MK). TMAO/DMSO oxidoreductase (TorA), thiosulphate dehydrogenase (TsdA), and the cytochrome peroxidases (Cj0020/Cj0358) accept electrons through cytochrome c. For simplicity, the two terminal oxidases (CydAB and CcNOQP) are depicted as a single complex. B) The known *C. jejuni* respiratory dehydrogenases and how they feed into the ETC. Formate dehydrogenase (Fdh), malate dehydrogenase (Mdh), hydrogenase (Hyd), and lactate dehydrogenase (LDH) all directly reduce MK. Sulfite oxidoreductase (Sor) and gluconate dehydrogenase (Gdh) bypass MK and reduce cytochrome c. The reactions and redox-active metallocentres of C) formate dehydrogenase subunit FdhA and D) sulfite oxidoreductase subunit SorA are depicted with the electron acceptor and the prosthetic groups catalyzing the reaction. (85, 93, 99, 162, 196, 213, 225, 246, 275, 293, 294).

Table 1.1: Host infection and colonization phenotypes of respiratory dehydrogenase mutants from previous work

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue culture mutant defects</th>
<th>Animal colonization mutant defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfite oxidoreductase</td>
<td>Defective for adherence and invasion (271)</td>
<td>No data</td>
</tr>
<tr>
<td>Gluconate dehydrogenase</td>
<td>No data</td>
<td>Defective for chick intestine colonization (213)</td>
</tr>
<tr>
<td>Proline dehydrogenase</td>
<td>No data</td>
<td>Defective for mouse intestine colonization (99)</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>No data</td>
<td>Defective for chick intestinal colonization. Unable to invade deeper tissues in mice. (21, 293)</td>
</tr>
<tr>
<td>Hydrogenase</td>
<td>Defective for adherence and invasion (127)</td>
<td>Defective for chick colonization (293)</td>
</tr>
</tbody>
</table>

1.5: *C. jejuni* regulators of metabolism

As in all bacteria, regulation of metabolism is controlled through a variety of systems that either repress or activate transcription of metabolic genes. *C. jejuni* lacks such key factors as the Cyclic AMP Receptor Protein (CRP) for regulating glucose uptake and catabolism (since *C. jejuni* does not use glucose as a carbon or energy source); however, it does have a diverse set of regulatory proteins that control gene transcription. *C. jejuni* possesses a homologue of the Carbon Starvation Regulator (CsrA); however, it is
not known what metabolic features it controls (67). Several metabolic genes are controlled by the two-
component response regulators reduced ability to colonize regulator (RacRS) and Campylobacter
planktonic regulator CprRS (Svensson, Gaynor, et al, unpublished data). The heat shock regulators heat
shock protein repressor (HspR) and heat regulation at CIRCE regulator (HrcA) have also been linked to
the regulation of succinate dehydrogenase and aspartate aminolyase (100). An important regulator of C.
jejuni metabolism, as well as controlling other genes associated with C. jejuni stress survival, is the
stringent response regulator (SpoT) (80). SpoT was found to regulate many different metabolic
components including lactate dehydrogenase, nitrate reductases, and many components in the NAD(P)H
dehydrogenase/Complex I proteins (80). Many regulators that are involved in regulation of oxidative
stress are also important in regulation of metabolism. This includes LysA regulator (LysR), which
regulates succinate dehydrogenase, fumarate reductase, Sor and aspartate aminolyase (55), and the
peroxide stress regulator (PerR), a master regulator of oxidative stress responses, and key metabolic
genes like the iron sulfur containing oxaloacetate oxidoreductase (214).

Many C. jejuni enzymes require trace cofactors that must be imported in small amounts for proper
enzyme function. The molybdenum regulator (ModE) is vital for the import of both tungsten and
molybdenum via regulation of the tungsten uptake transporters (TupABC) and molybdenum transporter
(ModABC) complexes, respectively (272). Tungsten and molybdenum are important for the proper
function of enzymes important for host cell infection and host intestinal colonization, like Fdh, Sor, and
nitric oxide reductase. Zinc import via the zinc-uptake importer (ZnuA) is important for growth in low
zinc media, as well as chick colonization (48). Transcription of znuA is zinc dependent, but no regulator
has been identified to date (48). Given that regulation of uptake of specific metal cofactors can be
directly related to regulation of the enzymes for which they are cofactors, it is important to understand
cofactor uptake and incorporation to understand enzyme activity.
1.6: Host cell death pathways

The first attempt at classifying human cell death pathways was accomplished by Schweichel and Merker, who categorized cell death into four classes: apoptotic, pyroptotic, autophagic, and necrotic (236). Programmed cell death studies have historically focused in a large part on the apoptotic pathway. Apoptosis is a programmed cell death pathway carried out by the caspase family of proteins (23, 148, 151). Apoptosis results in non-inflammatory death associated with cell blebbing and shrinking, and condensation and fragmentation of chromatin (23, 148, 151). Recently, apoptosis has been subdivided into another class of cell death, pyroptosis, that undergoes many of the same markers as apoptosis. Unlike apoptosis, pyroptosis involves the maturation of pro-IL-1β by the pyroptosis executioner caspase, caspase-1, resulting in ‘apoptosis’ with a pro-inflammatory profile(70). The well described necrotic pathway was assumed to be an accidental process arising when damage to the cell is so severe that cellular processes stop before initiation of a programmed death cascade. However, in 1988 the notion of there being no program associated with necrosis was challenged when it was observed that different cell types could succumb to the same trigger, tumor necrosis factor (TNF), with cellular morphologies distinctive of apoptosis or necrosis (152). Recent work has found that necrosis is not accidental in all cases, and can be a programmed event with a complex and precise signaling cascade.

1.6.1: Induction of necrosis through the necrosome

In light of the view that necrosis can be either un-programmed or programmed, the term necroptosis was adopted for programmed necrosis. Necroptosis refers to the mode of cell death in which death receptors on the membrane are initiated, leading to induction of death (88, 283). When the TNF receptor (TNFR) binds to TNF-alpha, it causes assembly and activation of complex I containing poly-ubiquinated Receptor-interacting protein 1 (RIP1) and leads to NFκB activation and pro-survival signaling, so host cell death does not occur (88, 283) (Figure 1.5). However, under certain conditions, RIP1 can be deubiquinated by cylindromatosis (CYLD) which causes the transition of the TNFR complex I
to TNFR complex II (Figure 1.5). In complex II, the TNFR complex becomes associated with the apoptosis initiator protease caspase 8 (casp-8) which becomes activated (88, 283). Active casp-8 cleaves and inactivates de-ubiquinated RIP1 resulting in inhibition of necrosis and induction of apoptosis. However, in some cases casp-8 and apoptosis can be inhibited (88, 283). Casp-8 inhibition leads to a lack of cleavage of RIP1 which can then oligomerize with RIP3 to form the necrosome complex (complex IIb)(Figure 1.5)(88, 283). It is not fully understood how necrosome oligomerization leads to necrosis activation; however, many have hypothesized that the necrosome causes induction of mitochondrial free radicals, or an influx in cytoplasmic calcium levels (88, 283). There is growing evidence that RIP1 is not necessary for induction of necrosis, and the only essential component of necroptosis is RIP3 (257). This was observed in a study which found that necroptosis can occur when RIP3 oligomerizes with the mixed lineage kinase domain-like protein (MLKL) independent of RIP1. For example, it has been found that TLRs can activate the RIP3/MLKL complex and induce necrosis in a manner independent of RIP1(122). Since the necroptosis field is in its infancy, it is not completely known what the essential steps are.
Figure 1.5. The different complexes associated with the tumor necrosis factor receptor (TNFR)

Each complex depending on the conditions associates with a different set of proteins resulting in different outcomes. Complex 1: Interaction of ubiquinated (Ub) RIP1 with TNFR causes NFκB signaling followed by inflammation and pro-survival signals. Complex 2: If Rip1 is de-ubiquinated it cannot interact with TNFR when TNFR is activated. Caspase 8 (Casp8) will be recruited which degrades de-ubiquinated RIP1. Caspase 8 activation leads to apoptosis and inhibits necrosis. Complex IIb: If Rip1 is deubiquinated and caspase 8 is inhibited, the Rip1/Rip3/MLKL necrosome will form and necrosis will be induced.

1.6.2: Initiation of mitochondrial oxidative stress and its role in necrosis

Although induction of reactive oxygen species (ROS) in programmed necrosis is not a prerequisite, it is documented to be associated with necrosis under many different conditions. A wide range of work has documented that quenching of mitochondrial ROS can reduce necrosis (69). However, it is not completely known how ROS lead to necrosis. Production of ROS by the mitochondria has been suggested to be a result of TNF-alpha treatment leading to necrosis (244). Production of ROS in the mitochondria can also lead to changes in the ultrastructure of both the endoplasmic reticulum and mitochondria, which can further damage normal cellular functions resulting in ATP depletion and increases in cytosolic calcium levels (66, 244). NADPH oxidase (NOX) could be directly activated by forming a complex with the necrosome components TNFRSF1A-associated via death domain protein (TRADD), RIP1 and Rac1 during necrosis (135, 304). ROS generated from NOX proteins may cause lipid
peroxidation in the mitochondria which interferes with the respiratory components and leads to a rapid
depletion of ATP which is characteristic of necrosis (284). Such ROS mediated damage to the
mitochondria can also enhance production of mitochondrial ROS production, as well as reactive nitrogen
species (RNS) which may cause an oxidative damage positive feedback loop (228). It should be noted
that ROS production is not an essential step in necrosis or necroptosis, and its role in the progression
towards cell death is likely cell line and death signal dependent (135). However, the role of ROS in
necrosis is important in microbial pathogenesis, and ROS mediated host cell death may facilitate
bacterial dissemination. For example, TNF related ROS production during *Mycobacterium tuberculosis*
infection is associated with the eventual induction of necrosis and dissemination in the zebrafish model
of infection (236).

1.6.3: Calcium, calpain and cellular modification

Like the caspase proteins in apoptosis, necrosis has its own set of effector proteins that carry out some
of the steps in the pathway leading to necrosis (253). This family of proteins are the calcium activated
cysteine proteases called calpains. It is not known what comprises the exact steps that link upstream
signals, such as mitochondrial ROS- and TNF- induced necrosis to the activation of calpain; however, a
strong link to calcium levels has been observed. Calpains contain a calcium binding domain that, upon
the binding of calcium, induces a structural change that causes auto-cleavage and subsequent activation
(253). It is not known how the rise in cytoplasmic calcium occurs; however, it has been hypothesized
that calcium stored in the endoplasmic reticulum is released when the ER is damaged by mitochondrial
(or other) ROS (116). Another hypothesis is that ATP depletion resulting from mitochondrial
dysfunction prevents the activity of the ATP-dependent calcium transporters like sarcoendoplasmic reticulum
calcium transport ATPase (SERCA) which transports calcium to the sarcoplasmic reticulum from the
cytoplasm (89).
Once calcium reaches a certain critical threshold, the auto-cleavage of the calpain inhibitory domain occurs and domain II active site is activated (253). The two main calpains in humans are m-calpain and μ-calpain, which are ubiquitously expressed in many different human tissues and named after their activities in micromolar (µ) and millimolar (m) concentrations of calcium (253). Activity can be controlled by the calpain inhibitor calpastatin, which contains multiple copies of the calpain inhibitor domain and is able to inhibit multiple copies of active calpain simultaneously (253). Calpastatin expression is transcriptionally regulated by a variety of signaling pathways, such as NFκB, and has been found to be up-regulated in *Mycoplasma hyorhinis* infected cells to inhibit necrosis (58). It can also be up-regulated via TLR2 through the MEK1/ELK1 pathway in a NFκB independent manner (277).

Once calpain becomes active, it is responsible for the downstream cleavage of several proteins. Although several known calpain-specific cleavage sites have been determined, there are no specific recognition sequences, and the nature of calpain recognition is unknown. Determination of a defined cleavage sequence has also been complicated by the fact that calpain activation leads to disruption of lysosomes, resulting in the leakage of lysosomal products into the cytoplasm, such as cathepsins, which cause peptide cleavage in many different proteins. The known targets of calpain are diverse and include signaling molecules, membrane proteins, intracellular enzymes, and structural proteins (161). Amongst these are spectrin, paxillin, vinculin, talin and alpha-actinin proteins, which are all structural genes (27, 160, 186, 248). It is thought that cleavage of these structures causes inhibition of the structural integrity of the cell, resulting in the characteristic features of necrosis like cell rounding. One of the hallmarks of necrosis is the loss of membrane integrity and the release of cytoplasmic contents. This process was initially thought to be an all-or-nothing event, but there is evidence that it is a stepwise process, with smaller pores opening first followed by larger ones (161). This pore opening seems to coincide with the cleavage of cytoskeletal components (160). However, a direct link between the two has yet to be found. A schematic representation of the steps involved in the necrosis progression is shown in Figure 1.6.
Figure 1.6. Steps associated with the induction and progression of necrosis

Necrosis is thought to initiate with the induction of mitochondrial dysfunction, possibly due to damage by NADPH oxidase (NOX) generated reactive oxygen species (ROS). This leads to increased cytosolic calcium followed by calpain activation. Activation of calpain results in terminal death processes, such as lysosome rupture and cytoskeleton cleavage. Dashed arrows refer to steps that are not essential, but are induced in many forms of programmed necrosis. Solid arrows refer to steps thought to be essential in activation of calpain-dependent necrosis.

1.7: Generation of ROS in bacteria

Tolerance to oxygen varies amongst bacteria. Some, like *C. jejuni*, are microaerophilic, growing optimally at sub-atmospheric concentrations of oxygen. Others, such as *E. coli*, are fully aerobic, growing optimally under atmospheric conditions (~21% oxygen). However, hyperoxia can be toxic to all organisms and can induce growth defects and mutations (28). Toxicity arises from the production of the reactive oxygen species (ROS) such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) that are primarily created adventitiously by the one-electron reduction of oxygen in the active sites of redox-active enzymes (Figure 1.7a)(111). ROS are bio-reactive molecules that are extremely toxic to cells by preferentially damaging enzyme iron sulfur clusters and other metalloproteins, inhibiting metabolic functions as well.
as damaging protein thiols (109). Since the generation of O$_2^-$ and H$_2$O$_2$ production is dependent on oxygen, higher oxygen tensions result in faster rates of ROS production (Figure 1.7b) (111). Within bacteria, O$_2^-$ is dismutated to H$_2$O$_2$ and O$_2$ by superoxide dismutase (SodB) (111); however, H$_2$O$_2$ can also be generated directly by respiratory enzymes by the transfer of a second electron to O$_2^-$ if the O$_2^-$ does not dissociate from the enzyme active site rapidly enough (Figure 1.7c).

Attempts to determine the physiologically relevant ROS-generating enzymes in *E. coli* originally focused on components of the respiratory chain due to the association of mitochondrial complex I with ROS production (147). The rate of production of ROS from respiratory enzymes is dependent on solvent exposure, the redox potential, and the electron residency time on the redox center (182). In *E. coli*, an increase in H$_2$O$_2$ occurs when *E. coli* transitions from an anaerobic to an aerobic environment; however, H$_2$O$_2$ production quickly slows down during prolonged aerobic incubation (124). *In vitro* studies looking at membrane vesicles determined that fumarate reductase, sulfite reductase, and NADH dehydrogenase II (but not NADH dehydrogenase I) could all reduce O$_2$ to O$_2^-$, generating significant ROS species (110, 181). However, deletion of these ROS-generating enzymes did not significantly reduce the rate of ROS production under aerobic conditions, suggesting that non-respiratory dehydrogenases are the actual source of ROS production (245). This is due to the fact that respiratory dehydrogenases that are predisposed to ROS formation are down-regulated under higher oxygen tensions. An example of this is *E. coli* fumarate reductase that is repressed shortly after exposure to higher oxygen concentrations (218). Non-respiratory sources of ROS have since been identified in *E. coli* and include l-aspartate oxidase (NadB) which is involved in aspartate turnover, as well as glutamate synthase, a flavin-containing protein producing glutamate from glutamine and α-ketoglutarate (81, 143). Presumably, ROS production is not deleterious if the rate of production does not exceed the capacity of ROS-scavenging systems. *C. jejuni* is a microaerophilic bacterium and may not have adapted to rapidly down-regulate
ROS producing enzymes when transitioning to aerobic environments. No work on the origin of ROS in *C. jejuni* has been published to date.

Figure 1.7. ROS production in bacteria

A) Representation of how ROS are produced. A reduced substrate can reduce the active site (AS) of a protein containing a metal center or flavin. In rare cases, if the electron is unable to leave the active site of the enzyme, then the reduced active site (Re AS) can reduce O$_2$ to produce an ROS. B) The rate of change of O$_2$ concentration with respect to time is dependent on the concentration of O$_2$ and reduced electron donor (ED$^{RD}$). As the concentration of O$_2$ increases, the rate of O$_2$ production will increase. C) The Fenton reaction involved in producing hydroxyl radicals (OH$^-$) from O$_2$. ED$^{RD}$, SOD (superoxide dismutase), KatA (catalase).

1.8: *C. jejuni* oxidative stress resistance

During aerobic incubation, as well as during exposure to ROS produced by neutrophils and other immune cells during infection, *C. jejuni* must deal with significant oxidative stress. It has been hypothesized that ROS-mediated loss of viability in *C. jejuni* is due to the destruction of the iron sulfur clusters in Por and Oor enzymes which shuts down the citric acid cycle (131). In addition, the reaction of
H$_2$O$_2$ with iron sulfur clusters leads to the production of hydroxyl radicals (Figure 1.7b) which can react with and cleave DNA (109) and ROS stress can induce lipid peroxidation which disrupts membrane homeostasis and can induce DNA mutations (109, 111). To counteract the effects of ROS, C. jejuni has several ROS detoxification systems. These include single copies of genes encoding superoxide dismutase (sodB)(220, 231), catalase (katA)(84) and alkylhydroperoxidase (ahpC)(18) which are all involved in reducing ROS to non-toxic products. In addition, C. jejuni contains two thiol peroxidases, thiol peroxidase (Tpx) and the bacterioferritin containing protein (BCP), that have been shown to have peroxidase activity (13). To minimize the toxic effect of ROS species, C. jejuni contains iron binding proteins Cft and Dps which sequester free cytosolic iron preventing hydroxyl radical formation(112, 289). C. jejuni also encodes several systems capable of repairing oxidative damage, such as methionine sulfoxide reductases (MsrA/MsrB), which repair methionine oxidation (14), and the high temperature requirement (HtrA) chaperone, which assists in degrading proteins denatured by ROS (34). C. jejuni also has systems that help resist damage from oxidative stress, such as DNA-binding proteins from starved cells protein (Dps)(112) and the Campylobacter ferritin-encoding gene protein (Cft) (289), which bind free intracellular iron, and hemerythrin A (HerA)(131) which has a yet unknown role in resisting oxidative stress. C. jejuni lacks known glutathione synthesis genes that are present in several aerobic organisms such as E. coli (9) and help scavenge trace ROS. Regulation of the oxidative stress response is also very important for survival in the environment, and C. jejuni contains several regulators important for aerotolerance. The PerR (214, 282), LysR (55), Cj1556(87), and the Campylobacter oxidative stress regulator CosR (106, 107) all control expression of important antioxidant genes such as katA and ahpC. Even with functioning catalases and peroxidases, C. jejuni can rapidly lose viability in an aerobic environment. Therefore, it is likely that the C. jejuni scavenging enzymes are detoxifying ROS at a rate that is less than the rate of ROS production, reflecting C. jejuni’s need for environments with lower oxygen tensions for growth.
1.9: Sulfur assimilation pathway

Sulfur homeostasis plays an important role in aerobic survival. Sulfur-containing compounds like glutathione and mycothiol have been shown to be important ROS scavengers in bacterial systems like *E. coli* and *Mycobacterium*, respectively (61). In cysteine-limited media, the sulfur assimilation pathway can incorporate sulfate through a series of steps into ω-acetylserine to generate cysteine. This pathway is well conserved in prokaryotes and eukaryotes (91; Figure 1.8). The sulfur assimilation pathway is energetically demanding, requiring 2 ATPs and 6 electrons to reduce sulfate to sulfide. As such, the pathway is highly regulated in many organisms (91, 180, 221). In *M. tuberculosis*, the sulfur assimilation pathway has been found to be up-regulated by H$_2$O$_2$, as well as menadione (an antibiotic that induces ROS production), and is thought to reflect a need for sulfur assimilation during macrophage infection and transition to latent phase macrophage infection (91). No studies have examined the role of the sulfur assimilation pathway in *C. jejuni*. The *C. jejuni* genome was searched for possible homologs of sulfur assimilation proteins previously (6). Homologues of ATP sulfurylase (Atps), and adenosine phospho-sulfate (APS) kinase were identified (Figure 1.8); however, *C. jejuni* lacks obvious homologues of phospho-adenosine-phosphosulfate (PAPS) reductase, and sulfite reductase (Figure 1.8). It is unknown if PAPS reductase and sulfite reductase homologs exist in the *C. jejuni* genome, but they would be needed for *de novo* cysteine synthesis as sulfide that is generated by the activity of these enzymes serves as the substrate for CysM (78). CysM can also incorporate thiosulfate into ω-acetyl-serine to generate cysteine independent of sulfide, thus sulfite reductase may not be needed to generate cysteine (201).
**Figure 1.8. The sulfur assimilation pathway.**

The pathway for the incorporation of inorganic sulfur into cysteine. Enzymes involved in the pathway are boxed. CysDN (ATP sulfurylase), CysC (APS kinase), CysH (PAPS reductase), CysJI (sulfite reductase), and CysM/CysK (Cysteine synthase). Abbreviations: APS (γ, PAPS, PAP and 0-acetyl-serine. Enzymes for which no C. jejuni homologs have been identified are denoted with a ‘?’. Solid arrows indicate intermediate steps in the cysteine synthesis pathway and the dashed lines represent the activity of sulfotransferases that transfer the sulfur group from intermediates to a specific target. Examples of the end products of these sulfotransferases include the NodRm-IV nodulation factor of Rhizobium meliloti, and sulfolipid-1 of M. tuberculosis (193, 232).

**1.10: Rationale and aims**

The initial objective for this thesis was to identify new factors important for C. jejuni infection of human epithelial cells through analysis of uncharacterized C. jejuni genes that were previously identified as up-regulated during interaction with INT407 cells (80). The resulting mutational and phenotypic analyses, comprising Aim 1 (see below) and described in Chapter 2, revealed the importance of two novel proteins, FdhT and FdhU, as regulators of fdh gene expression and Fdh activity. This work also demonstrated an importance for FdhTU and the Fdh complex in the interaction of C. jejuni with epithelial cells - most notably, for recovery of C. jejuni following cell infection. It also led to the unexpected observation that supplementation of post-cell infection recovery plates with sulfite not only
abrogated apparent invasion and intracellular survival defects for fdhTU and fdhA mutants, but also dramatically enhanced recovery of wild-type C. jejuni. From this preliminary analysis, I hypothesized that metabolic enzymes, specifically respiratory dehydrogenases, play a crucial role in C. jejuni interaction with epithelial cells and in aerobic survival dynamics. The research described herein has three specific aims. As described above, **Aim I (Chapter 2)** was to determine the role of fdhTU in host cell infection by mutational analysis, and assess its role in adherence, invasion and recovery from host epithelial cells. In addition, microarray analysis was undertaken to determine genes differentially regulated in a ΔfdhU strain. The role of the FdhTU-regulated operon Fdh in epithelial cells was assessed as was performed for FdhTU. To expand on the work presented in Chapter 2, I then hypothesized that metabolic enzymes, specifically respiratory dehydrogenases, play a crucial role in C. jejuni interaction and recovery from epithelial cells. As such, **Aim II (Chapter 3)** was to study the role of Sor in enhanced recovery of C. jejuni from epithelial cells when plating on sulfite, and determine the role of sulfite in enhancing recovery of C. jejuni from epithelial cells. Related to this, I wished to determine if other respiratory dehydrogenases are involved in the recovery of C. jejuni from the intracellular niche, host cell infection, and in the induction of host cell necrosis by mutational analysis and quantification of host cell death during infection. Pursuing Aim II also resulted in the identification of several host factors important to the progression of necrosis during C. jejuni infection by analyzing host cell death in the presence of different chemical inhibitors. Finally, **Aim III (Chapter 4)** sought to identify factors that affect the environmental survival of C. jejuni. This builds on the previous identification of respiratory dehydrogenase redox cofactors as the site of ROS generation in aerobic conditions (110, 182, 245). Production of ROS by C. jejuni redox centers may explain the loss of viability observed under atmospheric oxygen concentrations. As described in Chapter 4, I showed the production of H₂O₂ by C. jejuni in aerobic conditions under different conditions to explore factors likely involved in why C. jejuni is not aero-tolerant. Furthermore, I investigated the relationship of several respiratory dehydrogenases with H₂O₂
production by *C. jejuni*. In addition, the role of two sulfur metabolites, sulfite and cysteine, in enhancing aerobic survival was investigated by mutational analysis of the sulfur assimilation pathway. The role of Atps in aerobic survival, sensitivity to ROS, and the rate at which it detoxifies or accumulates H$_2$O$_2$ was investigated to determine if there is a relation between sulfur homeostasis and aerobic survival in *C. jejuni.*
Chapter 2:

FdhTU-modulated formate dehydrogenase expression and electron donor availability enhance recovery of *Campylobacter jejuni* following host cell infection

2.1: Introduction and synopsis

A microarray-based screen to identify *C. jejuni* genes with enhanced expression during cell infection previously identified SpoT, PaqPQ, and the two-component response regulator system CprRS as important for various aspects of the pathogen-host cell interaction (80, 157, 265). Also up-regulated were two other uncharacterized genes, designated *Cj1500* and *Cj1501* in the first sequenced *C. jejuni* strain, 11168, and *CJ81176_1492* and *CJ81176_1493* in the virulent, invasive strain 81-176 used in our laboratory. I designated these genes *fdhT* (*CJ81176_1492*) and *fdhU* (*CJ81176_1493*) based on data presented in this chapter describing their function as a putative regulator and transmembrane protein required for Fdh activity. As will be described, I found that *fdhT*, *fdhU*, and *fdhA* were important for *C. jejuni* recovery following infection of human epithelial cells, but not for adherence, invasion, or intracellular survival. Microarray, RT-qPCR, biochemistry, and double mutant analyses suggest that the effect of FdhU on Fdh activity was responsible for FdhU effects on host cell interactions.

Supplementation of growth media used for post-cell infection recovery with sulfite rescued the defects observed for Δ*fdhU* and Δ*fdhA* mutants and enhanced recovery of wild-type (WT) *C. jejuni*. These findings established roles for each of these genes in an underexplored aspect of the pathogen-host cell interaction.
2.2: Materials and methods

2.2.1: Bacterial strains and growth conditions

All experiments were performed using the C. jejuni strain 81-176 background. All strains were grown in/on Mueller Hinton (MH) broth (Oxoid Ltd.) or agar plates at 38°C in a standard C. jejuni growth atmosphere of 6% O₂ and 12% CO₂ generated using a Sanyo tri-gas incubator (for plate growth) or using the Oxoid CampyGen system (for shaking broth cultures). All media used to culture C. jejuni were supplemented with 10 mg/mL vancomycin (Toku-E) and 5 mg/mL trimethoprim (Sigma) and is referred to hereafter as MH media. Where appropriate, MH media was supplemented with 50µg/mL kanamycin (Toku-E), 30µg/mL chloramphenicol (Sigma), or 20 mM sodium sulfite (Sigma). All genetic manipulations were performed in E. coli DH5α cells grown on LB plates or broth (Sigma) supplemented with 100 µg/mL ampicillin (Sigma), 25 µg/mL kanamycin, or 30 µg/mL chloramphenicol.

2.2.2: Construction of ΔfdhU, ΔfdhT, ΔfdhA, and ΔfdhUΔfdhA mutants and ΔfdhUc and ΔfdhTc complemented strains

All enzymes used for generation of C. jejuni mutant and complemented strains were purchased from New England Biolabs. Primers used are listed in Appendix 1. PCR amplification of fdhT, fdhU, and fdhA was performed with fdhT-Fw + fdhT-Rv, fdhU-FW + fdhU-Rv, or fdhA-Fw + fdhA-Rv primers, respectively, using iProof DNA polymerase. Purified PCR fragments were A tailed using Taq DNA polymerase and ligated into the pGem vector (plasmids used in this study are listed in Appendix 2). Generation of unique internal restriction sites in fdhU was performed by inverse PCR using primers fdhU-iPCR-Fw + fdhU-iPCR-Rv to introduce XbaI sites. The fdhT gene has an endogenous XbaI site, and fdhA has an endogenous PstI site. The non-polar aphA-3 cassette encoding a kanamycin resistance gene (212) was digested out of plasmid pUC18K-2 using XbaI and ligated into XbaI-digested fdhU inverse PCR product and pGem-fdhT. fdhA was disrupted with the aphA-3 kanamycin resistance cassette or the CAT chloramphenicol.
resistance cassette. For generation of the \textit{aphA-3} disruption construct, pGem-\textit{fdhA} was digested with PstI, and XbaI-digested \textit{aphA-3} was ligated into pGem-\textit{fdhA} as above. For generation of the \textit{CAT} disruption construct, pGem-\textit{fdhA} was digested with PstI and treated with the Klenow fragment to blunt-end the DNA. The \textit{CAT} cassette was digested out of plasmid pRY109 (303) with SmaI and ligated into pGem-\textit{fdhA}. All ligations were transformed into DH5α, colonies were screened by PCR, and plasmids from positive clones were purified. \textit{C. jejuni} was naturally transformed by double recombination with each plasmid and plated on MH agar supplemented with kanamycin or chloramphenicol for 48h to recover colonies. PCR and sequencing confirmed correct insertion in the chromosome by homologous recombination. All experiments were conducted with the \textit{aphA-3}-disrupted strains except for double mutant analysis which required the use of \textit{CAT}-disrupted \textit{ΔfdhA}. The \textit{ΔfdhUΔfdhA} mutant was constructed by isolating genomic DNA from the \textit{CAT}-disrupted \textit{ΔfdhA} strain, transformation into the \textit{aphA-3}-disrupted \textit{ΔfdhU} strain, and selection on chloramphenicol. Presence of both mutations was confirmed by PCR and sequencing. All \textit{C. jejuni} strains used in this study are listed in Appendix 3.

Complementation was achieved by amplification of \textit{fdhT} and \textit{fdhU} using primers \textit{fdhU-C-Fw + fdhU-C-Rv} and \textit{fdhT-C-Fw + fdhT-C-Rv}, respectively, which also introduced a PstI site into the 5’ end and a MfeI site to the 3’ end of each gene. PCR products were digested with PstI and MfeI and purified. The genomic integrative plasmid \textit{pRRC} (125) was digested with MfeI and XbaI and ligated with the digested \textit{fdhU-C} and \textit{fdhT-C} fragments. Insert expression was driven off the pRRC promoter. Plasmids were transformed into DH5α and selected on chloramphenicol. Colonies were screened by PCR, and plasmids from positive clones were purified and used to transform \textit{C. jejuni ΔfdhU} and \textit{ΔfdhT} mutants by natural transformation double recombination. Insertion of \textit{fdhT} or \textit{fdhU} in the rRNA spacer regions was confirmed by PCR using primers ak233, ak234, ak235 and ak237.
2.2.3: Generation of cDNA for microarray and RT-PCR analyses

Overnight *C. jejuni* cultures were diluted to an O.D. sub 600 of 0.05. Bacteria were harvested either at mid-log phase (for RT-PCR) or after 3, 6, 9, and 12 hours (for microarray and RT-qPCR) and immediately placed into 10X stop solution (95% ethanol plus 5% phenol) on ice prior to centrifugation and flash-freezing in a dry ice-ethanol bath. RNA was prepared as previously described (80). cDNA generation was performed using SuperScript III (SSIII; Invitrogen) as per the manufacturer’s instructions and purified with a PCR clean up kit (Zymo Research). RNA purity was confirmed by PCR and concentration was assessed using a ND-1000 spectrophotometer (Wilmington, DE).

2.2.4: Transcript analysis of cDNA

Establishment of *fdhT* and *fdhU* co-transcription was performed using cDNA generated from log-phase WT *C. jejuni* as above. PCR with Taq polymerase was performed using combinations of primers A, B, C, and D, and bands were resolved by gel electrophoresis. Quantitative PCR of cDNA was performed with the SYBR green (Biorad) q-PCR system using primers *fdhT*-q-Fw + *fdhT*-q-Rv, *fdhA*-q-Fw + *fdhA*-q-Rv, and *rpoA*-q-Fw + *rpoA*-q-Rv as per the manufacturer’s instructions. Reactions were run with 4ng cDNA, 0.3µM each primer, and 50% SYBR green mix per reaction. Increases in SYBR green fluorescence were measured using a Biorad CFX96 C1000 real time system thermocycler. The fold differences in amplifications between samples were calculated using the comparative threshold cycle (ΔΔCT) method as previously described (215).

2.2.5: Construction and analysis of the *C. jejuni* DNA microarray

Construction of the DNA microarray was performed essentially as previously described (170). In addition, ORFs specific to strain 81-176 were included on the array using primers from Operon Technologies (Alameda, CA) designed with ArrayDesigner 2.0 (Premier Biosoft, Palo Alto, CA). All PCR products were purified with a Qiagen 8000 robot and the QIAquick 96-well Biorobot kit (Qiagen). Purified amplicons were spotted in duplicate onto Ultra-GAPS glass slides (Corning Inc., Corning, NY).
using an OmniGrid Accent (GeneMachines, Ann Arbor, MI). After printing, the microarrays were immediately UV cross-linked at 300 mJ using a Stratalinker UV Cross-linker 1800 (Stratagene, La Jolla, CA) and stored in a desiccator. Prior to use, microarrays were blocked with Pronto! prehybridization solution (Corning Inc.) used according to the manufacturer’s specifications.

Gene expression comparison was performed indirectly by comparing expression profiles of C. jejuni WT bacteria ΔfdhU and ΔfdhUΔ strains separately on different slides (170). Comparison between strains was done for cultures grown in shaking broth for 3h, 6h, 9h, and 12h in MH broth at 38°C. C. jejuni WT, ΔfdhU and ΔfdhUΔ cDNA was labeled with Cy3-dUTP and mixed with Cy5-dUTP labeled reference genomic DNA from strain C. jejuni 81-176 before being hybridized to the cDNA array. Arrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA). The array was done with two technical replicates per array for each time point. GenePix 4.0 software was used to process spot and background intensity, and data normalization was performed to compensate for differences in the amount of template amount or unequal Cy3 or Cy5 dye incorporation as previously described (170). GeneSpring 7.3 software (Silicon Genetics, Palo Alto, CA) was used to analyze normalized data and a parametric statistical t test was used to determine the significance of the centered data at a P value of <0.05, adjusting the individual P value with the Benjamini-Hochberg false discovery rate multiple test correction in the GeneSpring analysis package.

2.2.6: Gentamicin assay for adherence and invasion of host epithelial cells

Cells from the human intestinal cell line Caco2 were acquired from the ATCC and passaged in Minimum Essential Media (MEM; Gibco) supplemented with 20% fetal bovine serum (Gibco) and 1X Penicillin-Streptomycin (Pen-Strep; Gibco). Caco2 cells were grown in a humidified air incubator at 37°C with 5% CO₂. Assessment of C. jejuni adherence and invasion into Caco2 cells was performed essentially as previously described (80). Caco2 cells were grown to semi-confluence and seeded into 24 well plates without Pen-Strep at 10⁵ cells per well and incubated for 24h. Mid-log phase C. jejuni grown with
shaking overnight in MH broth were centrifuged and suspended in MEM at an O.D.\textsubscript{600} of 0.002 (~$10^7$ C. \textit{jejuni}/mL). 1mL of this suspension (MOI ~100) was used to inoculate Caco2 cells. To assay ‘adherence and invasion,’ 3h after inoculation cells were washed two times with 1mL MEM before addition of 1mL distilled water. Caco2 cells were disrupted by syringe lysis to recover \textit{C. jejuni} and enumerated by plating on MH plates under standard \textit{C. jejuni} growth conditions. Where noted, strains were also plated on MH + 20mM sodium sulfite. To assess ‘invasion,’ 3h following inoculation fresh MEM containing 150 µg/mL gentamicin was added to each well for 2h to kill extracellular bacteria. The Caco2 cells were washed three times with MEM and lysed and plated as above. To asses ‘intracellular survival,’ 2h post gentamicin treatment the media was removed and replaced with MEM containing 3% FBS and 10µg/mL gentamicin. The cells were incubated for 3h and Caco2 cells were washed three times with MEM and lysed and plated as above. All experiments utilizing the gentamicin protection assay were repeated a minimum of three times, with each strain assayed in triplicate in each experimental trial. Data shown are from a representative experiment with similar findings consistently observed in each trial.

2.2.7: Processing of \textit{C. jejuni} for confocal microscopy

Caco2 cells were grown to semi-confluence and seeded onto glass coverslips (Fisher) at 1.5x$10^5$ cells per well for 24h. Caco2 cells were inoculated with mid log phase \textit{C. jejuni} as above at an MOI of 10 in MEM. Infection and gentamicin treatment were performed as above. At the ‘adherence and invasion’ and ‘invasion’ time points, monolayers were washed 2 times with PBS before fixation with 4% paraformaldehyde (Canemco) in PBS. When noted, monolayers were treated with 0.1% Triton-X100 (Fisher) to permeabilize cells. For immunofluorescence, samples were blocked in 10% goat serum with 1% Bovine serum albumin in PBS, washed 3 times with PBS, incubated with a 1:200 dilution of an anti-\textit{C. jejuni} rabbit IgG antibody (US. Biological) in PBS, washed 3 times with PBS, incubated with a 1:500 dilution of anti-rabbit goat IgG antibody conjugated to Alexa 568 (Invitrogen) in PBS, and washed 3 times with PBS. All samples for microscopy were mounted using Prolong Gold Antifade with DAPI (Invitrogen).
Imaging was performed with an Olympus Fluoview FV1000 laser scanning confocal microscope using the FV10-ASW 2.0 Viewer software to adjust images. For enumeration of GFP-labeled bacteria, 6 independent fields of view for each strain were counted. For permeabilization studies to differentiate between internalized vs. extracellular bacteria, 3 fields of view containing ~50 Caco2 cells apiece were visualized for each strain, with 3 independent experimental replicates. As almost no bacteria were observed to react with the antibody without cell permeabilization for any strain (making statistical comparisons between mutant and WT difficult), and given the data shown in the sulfite recovery experiment, precise numerical analyses for this experiment were not performed.

2.2.8: Measurement of respiration rates by oxygen uptake

Assays for formate oxidation were performed as previously described (196) by measuring oxygen depletion using a Clarke-type oxygen electrode (model 5301; Yellow Springs Instruments), an O₂ meter, and a microcomputer. Calibration was performed with oxygen-saturated PBS as an upper baseline and sodium dithionate in PBS as a bottom baseline. Cell-free extracts were generated from 100 mL of C. jejuni grown for 16h in shaking liquid culture. Bacteria were harvested at 10,000 rpm for 10 min and re-suspended in PBS. The bacteria were washed three times with PBS, re-suspended in 5 mL PBS, and sonicated 6 times for 15s. Unlysed cells and cellular debris were removed by centrifugation at 10,000 rpm for 15 min. The supernatant was removed and used as the cell-free extract and adjusted with PBS to bring all samples to the same protein concentration. A total of 1 mL of cell-free extract was added to the electrode and equilibrated at room temperature, after which formate dissolved in PBS was injected through the central pore to a final concentration of 12.5 mM. These experiments were repeated twice with identical results; the data shown are representative results from one experimental trial.
2.3: Results

2.3.1: *fdhT* and *fdhU* are co-transcribed and selectively conserved in a range of bacterial species

The genomic organization of *fdhT* and *fdhU* (*CJJ81176_1492* and *CJJ81176_1493*) is shown in Figure 2.1A. To test if *fdhT* and *fdhU* are co-transcribed, RT-PCR was performed on RNA isolated from *C. jejuni* strain 81-176 using primers annealing to specific regions within the predicted *fdhT* and *fdhU* coding regions (Figure 2.1A). When reverse transcriptase was included in the reactions, amplicons of the expected size, as determined by positive control PCR reactions using genomic DNA, were observed for primer sets both within *fdhT* (A and B) and *fdhU* (C and D) and also for primers spanning *fdhT* and *fdhU* (A and D) (Figure 2.1B). Amplicons were not observed using any of the *fdhT* or *fdhU* primers in combination with primers annealing to neighboring genes (not shown), or from control reactions without reverse transcriptase. This demonstrated that *fdhT* and *fdhU* are transcribed as a single operon.
Figure 2.1. The genomic organization and co-transcription of *fdhT* and *fdhU*.

A) Genomic organization of *fdhT* and *fdhU* (CIJ81176_1492 and CIJ81176_1493 in *C. jejuni* strain 81-176). Approximate sites of primer annealing for RT-PCR are shown as arrows labeled A, B, C, and D. Gene lengths are not to scale. B) PCR on *C. jejuni* cDNA with reverse transcriptase SuperScript III (+SSIII), without SuperScript III (-SSIII), or using genomic DNA (gDNA). Primer sequences are in Appendix 1.

*In silico* analyses of *FdhU* indicated that the entire predicted protein comprises a conserved domain found in the SirA_YedF_YeeD superfamily of transcription regulators. SirA regulators in *Salmonella* spp. and *Vibrio cholerae* contain N-terminal phosphorylation and C-terminal DNA binding domains and are ~220 residues in length, while *FdhU* is 76 residues. *FdhU* homology extends from aa42 to aa118 of SirA, lacking the canonical SirA DNA binding domain but harboring an N-terminal CPxP motif thought to stabilize the first helix of the protein (173). *FdhU* is closer in size and overall similarity (24% identity) to the ~81 residue *E. coli* SirA/YhhP/TusA proteins, one of which has been shown to be involved in tRNA modification (108). NMR studies of YhhP/TusA also indicate potential mRNA interaction domains (129). *In silico* analyses of *FdhT* predict a 402 residue, 10-transmembrane domain, inner membrane protein, and a conserved domain of unknown function found in the YeeE/YedE family of proteins which likewise have unknown function (76, 173).
Although the *in silico* analyses of FdhT and FdhU described above revealed some conserved domains found in previously identified proteins, BLAST analyses revealed significant homology to a number of uncharacterized gene pairs in a variety of other bacterial species. Representative examples are shown in Table 2.1. Among the other Campylobacters, FdhT and FdhU were highly conserved in *C. coli* and *C. fetus* (88% and 65% identity for FdhT, 92% and 73% identity for FdhU, respectively) species but were absent from *C. concisus* and *C. curvus*. Within other epsilon-proteobacteria, homologs were not found in *Wolinella* and *Sulfurospirillum* species but were present in *Arcobacter butzleri* (48% identity for FdhT, 66% identity for FdhU). Among the Helicobacters, homologs were absent from *H. pylori* but did occur in *H. hepaticus* and *H. mustelae* (48% and 47% identity for FdhT, 68% and 57% identity for FdhU, respectively). Homologs were also found in many other Gram-negative pathogens, including *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Shigella flexneri*, and the Gram-positive *Lactobacillus oris* and *Thermincola potens* (Table 2.1), as well as in many other species (data not shown).

In all observed cases, *fdhT* and *fdhU* were adjacent on the chromosome. The conservation of these two co-occurring genes in multiple different bacterial species suggests FdhT and FdhU function in the same pathway or functional unit.

### Table 2.1: FdhT and FdhU are conserved in a variety of bacterial species. Shown are select homologs derived from BLAST searches of FdhT and FdhU against other bacterial genomes.

<table>
<thead>
<tr>
<th>Species</th>
<th>FdhT Identity</th>
<th>Similarity</th>
<th>Coverage</th>
<th>E Value</th>
<th>Accession Number</th>
<th>FdhU Identity</th>
<th>Similarity</th>
<th>Coverage</th>
<th>E Value</th>
<th>Accession Number</th>
</tr>
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<tr>
<td><em>Campylobacter coli</em></td>
<td>88%</td>
<td>94%</td>
<td>99%</td>
<td>0.00</td>
<td>ZP_07401456.1</td>
<td>92%</td>
<td>99%</td>
<td>100%</td>
<td>3.00E-50</td>
<td>ZP_07401457.1</td>
</tr>
<tr>
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<td>78%</td>
<td>98%</td>
<td>0.00</td>
<td>YP_891776.1</td>
<td>73%</td>
<td>92%</td>
<td>98%</td>
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<td>YP_891777.1</td>
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<td>5.00E-135</td>
<td>NP_860829.1</td>
<td>68%</td>
<td>86%</td>
<td>96%</td>
<td>2.00E-34</td>
<td>NP_860830.1</td>
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<tr>
<td><em>Arcobacter butzleri</em></td>
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<td>65%</td>
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<td>ZP_07890639.1</td>
<td>66%</td>
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<td>ZP_07890640.1</td>
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<td>99%</td>
<td>1.00E-118</td>
<td>NP_00351653.1</td>
<td>57%</td>
<td>75%</td>
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<tr>
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<td>NP_003531661.1</td>
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<td>1.00E-122</td>
<td>NP_003365568.1</td>
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<td>84%</td>
<td>93%</td>
<td>2.00E-33</td>
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<td><em>Pseudomonas aeruginosa</em></td>
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<td>51%</td>
<td>72%</td>
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<td>3.00E-26</td>
<td>NP_252322.1</td>
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<tr>
<td><em>Escherichia coli</em></td>
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<td>98%</td>
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<td>NP_416439.1</td>
<td>66%</td>
<td>84%</td>
<td>93%</td>
<td>8.00E-35</td>
<td>NP_416440.1</td>
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<td>48%</td>
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<td>2.00E-27</td>
<td>ZP_07730371.1</td>
</tr>
<tr>
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<td>98%</td>
<td>4.00E-118</td>
<td>NP_667871.1</td>
<td>64%</td>
<td>84%</td>
<td>93%</td>
<td>3.00E-33</td>
<td>NP_667872.1</td>
</tr>
<tr>
<td><em>Thermincola potens</em></td>
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<td>56%</td>
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<td>45%</td>
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<td>100%</td>
<td>6.00E-22</td>
<td>NP_03641386.1</td>
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<tr>
<td><em>Campylobacter jejuni</em></td>
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<td>98%</td>
<td>8.00E-113</td>
<td>ZP_0227198</td>
<td>45%</td>
<td>68%</td>
<td>100%</td>
<td>6.00E-22</td>
<td>ZP_0227199</td>
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</tbody>
</table>
2.3.2: ΔfdhU and ΔfdhT mutants exhibit apparent host cell adherence and/or invasion defects by colony-forming unit (CFU) enumeration but not by direct microscopic counts of intracellular bacteria

To assess the importance of fdhT and fdhU in C. jejuni infection of epithelial cells, ΔfdhT and ΔfdhU mutant and complemented (ΔfdhT<sup>C</sup> and ΔfdhU<sup>C</sup>) strains were constructed in the invasive C. jejuni strain 81-176 as described in section 2.2.2. Both mutants exhibited WT behavior for motility, growth, and biofilm formation (data not shown). Roles for fdhT and fdhU in adherence and invasion in intestinal epithelial cells were initially investigated with gentamicin protection assays followed by host cell lysis and colony forming unit (CFU) enumeration of recovered C. jejuni. Caco2 epithelial cells were infected with WT, ΔfdhT, and ΔfdhU strains for 3 h, after which bacteria from the ‘adherence and invasion’ time point were harvested. Following a subsequent 2 h gentamicin treatment, cells were washed, and bacteria from the ‘invasion’ time point were harvested. Significant defects were observed for both mutants at both time points (Figure 2.2A). Complementation (ΔfdhT<sup>C</sup> and ΔfdhU<sup>C</sup>) rescued these defects in both mutant strains (Figure 2.2A).

A defect in CFU recovery from cells prior to addition of gentamicin may result solely from an adherence defect, which in turn will influence CFU recovery of invaded bacteria. To investigate whether the above findings specifically reflected adherence defects, WT, ΔfdhT, and ΔfdhU strains were transformed with a plasmid carrying the green fluorescence protein (GFP) expressed from the strong constitutive atpF<sup>+</sup> promoter (166) to allow visualization by confocal microscopy. Caco2 cells were grown on glass coverslips and infected with C. jejuni as above; however, rather than harvesting for CFU enumeration, slides were washed and processed for microscopy as described in section 2.2.7. Direct counting of C. jejuni associated with Caco2 cells showed no significant differences between WT and the ΔfdhT and ΔfdhU mutant strains at either the ‘adherence and invasion’ or ‘invasion’ time points (Figure 2.2B). This indicated that ΔfdhT and ΔfdhU were not defective for cell association.
Figure 2.2. The effect of FdhU and FdhT on intracellular survival and association with Caco2 cells

A) Viability of intracellular *C. jejuni* WT, ΔfdhU, ΔfdhT, ΔfdhUC and ΔfdhTC strains was assessed in Caco2 cells at the ‘Adherence and Invasion’ or ‘Invasion’ timepoints by CFU enumeration. B) Enumeration of GFP expressing *C. jejuni* WT, ΔfdhU and ΔfdhT strains associated with Caco2 cells. NS denotes no statistically significant differences between indicated strains. Statistically significant differences (P<0.05) are denoted by an asterisk (*).

To further investigate if ΔfdhT and ΔfdhU were capable of invasion of Caco2 cells, cells grown on coverslips were infected with *C. jejuni* expressing GFP as above. While processing for confocal
microscopy, cells were either permeabilized with Triton-X100 or left unpermeabilized. Both permeabilized and unpermeabilized samples were then incubated with a rabbit anti-\textit{C. jejuni} antibody followed by a goat anti-rabbit antibody conjugated to Alexa568. In unpermeabilized samples, only extracellular (adhered) bacteria will react with the antibody (leading to yellow or red fluorescence depending on the level of GFP also being expressed), whereas all bacteria (adhered and invaded) will fluoresce yellow/red in permeabilized samples. Confocal microscopy (a representative image is shown in Figure 3; see section 2.2.7) showed that similar levels of WT, Δ\textit{fdhT} and Δ\textit{fdhU} strains had invaded the Caco2 cells (Figure 2.3). Furthermore, the majority of bacteria from all three strains were internalized even prior to gentamicin treatment (Figure 2.3). This indicates that Δ\textit{fdhT} and Δ\textit{fdhU} are capable of proper adherence and invasion of host cells and suggests that the CFU data represent either a rapid decline in intracellular viability or a defect in recovery following host cell infection.
A  Adherence and Invasion (3h post infection)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ΔfdhU</th>
<th>ΔfdhT</th>
</tr>
</thead>
<tbody>
<tr>
<td>-triton-X100</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>+triton-X100</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

31.7µm

B  Invasion (3h infection followed by 2h gentamicin treatment)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ΔfdhU</th>
<th>ΔfdhT</th>
</tr>
</thead>
<tbody>
<tr>
<td>-triton-X100</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>+triton-X100</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

31.7µm
Figure 2.3. The role of FdhU and FdhT in invasion of Caco2 cells

*C. jejuni* WT, Δ*fdhU*, and Δ*fdhT* strains expressing GFP (green) were used to infect Caco2 cells in a gentamicin protection assay. The ‘Adherence and Invasion’ time point is shown in A), and the ‘Invasion’ time point in B). To differentiate between extracellular and intracellular bacteria, *C. jejuni* were either permeabilized with Triton-X100 (+TritonX-100) or not permeabilized (-TritonX-100), then labeled with an anti-*C. jejuni* antibody and visualized using a secondary antibody labeled to Alexa-568 (red). Caco-2 cell nuclei were stained with DAPI (blue).

2.3.3: Transcript analysis reveals down-regulation of genes required for Fdh activity in the Δ*fdhU* mutant strain

The presence of a putative mRNA binding domain found in the YedF family of proteins suggested that FdhU might influence mRNA levels of genes that in turn modulate cell infection phenotypes. RNA from WT, Δ*fdhU*, and Δ*fdhU*C strains was harvested from broth cultures over a growth time course to assess global mRNA changes in the Δ*fdhU* mutant via microarray analyses. Only two genes, *fdhA* and *fdhB*, encoding subunits of the Fdh complex, were significantly (>2-fold) dis-regulated compared to WT. Both genes were down-regulated, with *fdhA* and *fdhB* displaying 3.4- and 10.5-fold lower levels of mRNA, respectively, in Δ*fdhU* compared to WT (*p<0.001* for both genes) at the 3 h time point. More modest down-regulation was observed at later time points, and WT expression levels were restored in the complemented strain (data not shown).

Microarray results for *fdhA* were confirmed by RT-qPCR using *rpoA* as an internal control as previously described (235). RT-qPCR revealed an 8.7-fold reduction of *fdhA* mRNA in the Δ*fdhU* mutant and a 9.2 fold-reduction in the Δ*fdhT* mutant (Figure 2.4). *fdhA* expression was restored to WT levels in the complemented strains Δ*fdhU*C and Δ*fdhT*C (Figure 2.4). RT-qPCR also showed that FdhU affects its own transcript levels, with the Δ*fdhU* mutant exhibiting 1.7-fold higher mRNA levels of *fdhT* than WT, and supplementation restoring WT mRNA levels. An *fdhT* promoter-luciferase reporter construct also displayed 3-fold higher activity in an Δ*fdhU* background compared to WT over 9 h of growth (data not shown). RT-qPCR investigating *fdhU* mRNA levels in Δ*fdhT* confirmed that the kanamycin resistance
cassette used to disrupt fdhT did not interfere with transcription of fdhU and in fact showed a modest increase in fdhU mRNA levels compared to WT (data not shown).

![Graph showing gene expression levels](image)

**Figure 2.4. Regulation of fdhA, fdhT and fdhU operons in ΔfdhU and ΔfdhT mutant strains**

The mRNA abundance of fdhA (grey bars) and fdhT (white bars) was assessed in various mutant and complemented strains as compared to WT. Statistically significant differences (P<0.05) are denoted by an asterisk (*) above the line connecting the indicated strains.

### 2.3.4: fdhU is required for respiration-dependent oxygen consumption using formate as an electron donor

To test if disruption of fdhU impacted Fdh function, respiration was measured using formate as an electron donor. An ΔfdhA mutant constructed as described in Methods was used as a control. WT, ΔfdhU, ΔfdhUC and ΔfdhA strains grown to mid-log phase were used to prepare cell-free extracts, and respiration-dependent oxygen consumption was measured using a Clark-type electrode. In WT and ΔfdhUC strains, the addition of 12.5mM sodium formate resulted in an immediate decrease in soluble oxygen (Figure 2.5). In contrast, ΔfdhU and ΔfdhA displayed no decrease in oxygen levels. This indicates that disruption of fdhU leads to a severe defect in Fdh activity.
Figure 2.5. Respiration-dependent oxygen consumption in the presence of formate in WT, ΔfdhU, ΔfdhU<sup>C</sup> and ΔfdhA strains

Respiration-dependent oxygen consumption was measured in A) WT, B) ΔfdhU, C) ΔfdhU<sup>C</sup> and D) ΔfdhA strains by formate-linked oxygen depletion using a Clark-type oxygen electrode. Arrows denote the addition of sodium formate to a total concentration of 12.5 mM.

2.3.5: The ΔfdhA mutant displays similar cell infection phenotypes as ΔfdhU

As most of the genes exhibiting >2-fold down-regulation in ΔfdhU were Fdh-related, I hypothesized that the dramatic effect of FdhU on Fdh activity (Figure 2.5) might also account for the apparent adherence and/or invasion defects observed by CFU enumeration of the ΔfdhU mutant following host cell infection. To test this, Caco2 cells were infected with WT, ΔfdhU, ΔfdhA and ΔfdhUΔfdhA double mutant strains and subjected to a gentamicin protection assay followed by CFU enumeration of bacteria as above. All three mutants were defective at both the ‘adherence and invasion’ and ‘invasion’ time points (Figure 2.6A). Although the double mutant was slightly more defective than either single mutant at the ‘adherence and invasion’ time point, differences between the strains were not statistically significant. The nearly identical and likewise non-statistically significant defects at the ‘invasion’ time point for ΔfdhU, ΔfdhA and ΔfdhUΔfdhA in particular suggest that FdhU and FdhA function in the same pathway.

An ΔfdhA strain expressing GFP was also constructed and used to infect Caco2 cells for analysis by confocal microscopy (Figure 2.6B). Equivalent numbers of WT and ΔfdhA bacteria were associated with Caco2 cells at both time points, suggesting no defect in cell association.
Figure 2.6. The intracellular survival and association with Caco2 cells in C. jejuni WT, ΔfdhU, ΔfdhA and ΔfdhUΔfdhA strains

A) C. jejuni strains were used to infect Caco-2 cells and viability of WT, ΔfdhU, ΔfdhA and ΔfdhUΔfdhA strains was assessed at the ‘Adherence and Invasion’ or ‘Invasion’ timepoints using the gentamicin protection assay by CFU enumeration. B) Enumeration of GFP-expressing WT and ΔfdhA C. jejuni associated with Caco-2 cells. Statistically significant differences (P<0.05) are denoted by an asterisk (*). NS denotes no statistically significant differences between indicated strains (P>0.05).

2.3.6: Supplementation of recovery plates with sulfite enhances CFU counts of C. jejuni following host cell infection and leads to equivalent recovery of WT, ΔfdhU and ΔfdhA strains

Finally, I wished to reconcile whether the apparent CFU-based adherence and/or invasion defects of ΔfdhT, ΔfdhU, and ΔfdhA mutant strains following infection of Caco2 cells reflected a decline in intracellular survival or a decreased ability to resume growth after liberation from the intracellular
environment. I hypothesized that mutants defective for Fdh activity may be equally viable as WT in the intracellular environment, but may require post-infection supplementation with an alternative electron donor to enhance recovery in vitro. As sulfite is one of the only known C. jejuni electron donors that is not likely to feed into other metabolic pathways, and as C. jejuni’s sulfite utilization system was previously suggested as potentially important in low-oxygen conditions as would be encountered inside cells (196), I decided to test this hypothesis using recovery plates supplemented with 20 mM sodium sulfite. Caco2 cells were infected with WT, ΔfdhU and ΔfdhA strains and assayed for CFU recovery using a gentamicin protection assay as above. In addition to ‘adherence and invasion’ and ‘invasion’ time points, I also assessed an ‘intracellular survival’ time point (3 h infection, 2 h gentamicin treatment, 3 h additional incubation with fresh tissue culture media). Recovered C. jejuni were enumerated by CFU counts on MH agar plates +/- 20mM sodium sulfite. On plates supplemented with sulfite, no significant differences in CFU recovery were observed between WT, ΔfdhU, and ΔfdhA strains (Figure 2.7A). This was in contrast to plating on MH media alone which, as expected, showed a significantly lower recovery of ΔfdhU and ΔfdhA compared to WT. Unexpectedly, recovery of WT C. jejuni on sulfite-supplemented plates was 5-fold higher at the ‘invasion’ time point and 11-fold higher at the ‘intracellular survival’ time point compared to plating on unsupplemented MH media, with even more dramatic differences seen for the mutant strains (Figure 2.7A,B). Enhanced recovery of WT on supplemented versus unsupplemented plates was not observed for the inoculum or at the ‘adherence and invasion’ time point, nor were the mutant inoculum enhanced for recovery on supplemented plates. Plating C. jejuni on MH media supplemented with 10mM sodium formate yielded no significant differences in recovery of any strain compared to MH media (data not shown). Collectively, this suggests that each apparent defect for the ΔfdhU and ΔfdhA mutants observed by CFU enumeration on MH media was due to post-infection recovery, and not to defects in adherence, invasion, or intracellular survival. Enhanced recovery of all
strains on sulfite-supplemented plates further suggests that \textit{C. jejuni} undergoes metabolic changes inside host cells.

![Recovery of viable WT, \(\Delta fdhU\) and \(\Delta fdhA\) strains from Caco2 cells on plates supplemented with the alternative electron donor sodium sulfite](image)

**Figure 2.7. Intracellular recovery of viable WT, \(\Delta fdhU\) and \(\Delta fdhA\) strains from Caco2 cells on plates supplemented with the alternative electron donor sodium sulfite**

A) Viability of intracellular \textit{C. jejuni} WT, \(\Delta fdhU\), and \(\Delta fdhA\) strains at the ‘Adherence and Invasion’, ‘Invasion’, and ‘Intracellular Survival’ timepoints was determined by CFU enumeration by plating on MH agar plates or MH agar plates supplemented with 20mM sodium sulfite. Relevant statistically significant differences (P<0.05) are denoted by an asterisk (*). B) Shown are the fold differences for select strains between CFU recovered on sulfite-supplemented plates (“+S”) compared to non-supplemented plates.

**2.4: Discussion**

It has been established that \textit{C. jejuni} can invade epithelial cells and persist in an intracellular compartment (51, 140, 169, 238, 292, 306). To date, however, it is poorly understood how \textit{C. jejuni}
survives intracellularly, and what genes and factors are important for the transition back to an extracellular lifestyle. Work by our group and others has identified roles for various regulators in adherence, invasion, and/or intracellular survival (11, 68, 80, 87, 169), indicating the importance of transcript modulation during these transitions. I also previously found, via a microarray-based screen, that several genes up-regulated in *C. jejuni* during infection of host cells were required for various aspects of the pathogen-host cell interaction (80, 157, 265). An operon I have designated *fdhTU* was also identified in that screen, leading to the hypothesis that FdhT and FdhU may be important for an aspect of bacterial physiology involved in epithelial cell interactions.

Precise roles for FdhT and FdhU could not be assigned from *in silico* analyses. However, amino acid sequence similarity suggested that FdhT is an inner membrane transport protein, and the presence of a CPxP motif potentially involved in mRNA interactions (129) and modest similarity to a protein involved in tRNA modification (108) suggested that FdhU may play a regulatory role. Indeed, significantly reduced levels of *fdhA* and *fdhB* mRNA were observed in the Δ*fdhU* strain by both microarray and RT-qPCR analysis. Correspondingly, FdhU was shown to be crucial for Fdh activity, as *fdhU* deletion caused a severe defect in oxygen-dependent respiration with formate as an electron donor.

Fdh activity is tightly regulated in other bacteria. In *E. coli*, fdh operons are differentially regulated by anaerobiosis, pH, nitrate, and formate levels (63, 187, 290). Fdh gene expression is regulated by H₂ in *Methanococcus maripaludis* (299) and by formate in *Desulfovibrio vulgaris* (307). In *C. jejuni*, *fdh* genes have been shown to be up-regulated in an animal model of infection and down-regulated in gastric fluid (234, 263), but specific inducing/repressing signals have not been determined. Transcriptional regulators binding directly upstream of Fdh operons have also been identified in other bacteria (210, 290), but homologs are not present in *C. jejuni*. An independent concurrent study by Shaw and colleagues using two different *C. jejuni* strains demonstrates a requirement for selenium in *C. jejuni* Fdh activity, confirms the importance of FdhTU for Fdh function, and suggests that FdhTU modulates Fdh – and possibly its
expression – via import of selenium which is then, via the Sel pathway, converted to selenocysteine which is required for FdhA synthesis (247). In some organisms, selenium availability has been postulated to affect mRNA stability (175); future work will determine if this is the case in C. jejuni and also shed light on specific mechanisms by which FdhU affects transcript levels of Fdh-related genes. The conservation of FdhTU among a variety of bacterial species, as well as its absence from others, may also reflect a novel metabolic regulatory mechanism required for navigation through specific niches.

Disruption of fdhT, fdhU, and fdhA and assessments of Caco2 cell adherence and invasion by gentamicin protection assays initially yielded contradictory CFU versus microscopy findings which were reconciled by supplementing post-infection recovery plates with sulfite. For instance, each mutant strain appeared defective for adherence and/or invasion when assessed by CFU recovery on MH media. However, similar assays with GFP-expressing strains followed instead by confocal microscopy showed no difference in the number of cell-associated C. jejuni between WT and mutant strains. Immunofluorescent staining of extracellular versus intracellular C. jejuni further showed that ΔfdhU and ΔfdhT could invade Caco2 cells at WT levels. This experiment also suggested that negligible numbers of cell-associated C. jejuni were extracellular. While these findings differ from numerous previous reports showing higher numbers of adhered vs. invaded C. jejuni using the classic gentamicin protection assay, they are in agreement with more recent microscopy-based work from other groups suggesting that even at time points traditionally assayed to represent “adherence”, the majority of recovered C. jejuni have invaded and/or subvaded (residing beneath host cells)cells (279, 292). Nonetheless, it should also be noted that it is possible that some extracellular bacteria are removed during the processing steps for confocal microscopy (i.e., fixation, antibody washes, etc.), and that the methodologies cannot discriminate between invaded and subvaded bacteria. However, it is also possible that the majority of C. jejuni are internalized at the “adherence” time point but then lose viability or culturability by the traditional “invasion” time point. Double mutant analysis together with data showing defective Fdh activity in the ΔfdhU mutant suggest
that loss of Fdh function likely accounts for the ΔfdhU CFU recovery phenotype, although additional
effects from disregulation of other genes cannot be completely discounted. My findings showing equal
recovery of fdh mutant and WT strains on sulfite-supplemented plates ultimately led us to conclude that
the mutants in fact have no obvious defects in adherence, invasion, or intracellular survival, but rather a
defect compared to WT for post-cell infection recovery on unsupplemented MH agar.
In addition to equalizing CFU recovery for the fdh mutants compared to WT, I found that sulfite
supplementation dramatically enhanced recovery of all assayed C. jejuni strains following liberation
from the intracellular environment. This suggests that C. jejuni undergoes metabolic changes when it is
intracellular, consistent with previous work demonstrating that anaerobic incubation of C. jejuni upon
liberation from epithelial cells can also enhance CFU recovery (292). C. jejuni has a branched respiration
system and is able to use a wide range of compounds as electron donors and acceptors (85, 98, 213,
275, 293). Furthermore, formate, sulfite, and hydrogen gas are abundant as byproducts of commensal
bacterial fermentative metabolism, from normal metabolism of amino acids, and as food additives (43,
56, 278). This suggests that both the metabolic potential and the role(s) of various dehydrogenases for
C. jejuni in vivo may be very complex. Shifts in metabolic potential during intracellular survival have
been observed in other bacteria [i.e., as reviewed in (57, 62)]. Specific examples include S. flexneri and
S. enteric serovar Typhimurium, which down-regulate the Krebs cycle (165) and up-regulate the arginine
importer ArgT (46), respectively. Numerous changes also occur in intracellular M. tuberculosis, including
enhanced lipid metabolism, cell wall synthesis, and iron uptake, and down-regulation of ATP synthesis
systems (195, 241). Future work in chapter 3 will elucidate why sulfite supplementation yielded
significant increases in C. jejuni culturability post-cell infection, and whether this effect is specific to
sulfite. My data indicate that enumeration of intracellular C. jejuni via CFU counts following gentamicin
protection assays may result in an underestimation of viable intracellular organisms.
In summary, I have identified a novel operon required for proper expression and function of Fdh and demonstrated the importance of both FdhTU and Fdh in recovery following epithelial cell infection. To the best of my knowledge, these are the first proteins shown to impact this aspect of the *C. jejuni*-host cell interaction. My findings likely have implications for *C. jejuni*-host cell interactions *in vivo* as well, elucidation of which will be the focus of later chapters in this thesis, and further support the need to better understand survival and metabolic strategies of bacterial pathogens, particularly for organisms such as *C. jejuni* which lack canonical virulence factors. This generally emerging theme also applies to other pathogens, as increased understanding of metabolic changes during intracellular existence may yield insights into new treatment strategies.
Chapter 3:

The role of sulfite in enhanced recovery of *C. jejuni* from host cells and the importance of gluconate dehydrogenase in inducing enhanced host cell necrosis.

3.1: Introduction and synopsis

I previously found in chapter 2 that upon liberation of *C. jejuni* from host epithelial cells at the invasion or intracellular survival time-points, the numbers of recovered *C. jejuni* could be enhanced by plating *C. jejuni* for CFU on media supplemented with sulfite. In addition, I found that Fdh is important for recovery of *C. jejuni* from host cells. As such, I was left with two remaining questions: (1) how does sulfite enhance recovery of *C. jejuni* from epithelial cells, and (2) are other respiratory dehydrogenases important for host cell infection, recovery from host cells, or other pathogenesis-related phenotypes.

Analyses herein determined that enhanced recovery of intracellular *C. jejuni* on sulfite is independent of the use of sulfite as an electron donor by Sor, the sole *C. jejuni* sulfite oxidoreductase that interacts with the ETC (196). Instead, intracellular *C. jejuni* appeared to be under oxidative stress produced by host cells, and the enhanced recovery of *C. jejuni* from host cells when plated on sulfite was due to the ROS scavenging activity of sulfite. With the finding that ΔfdhA is defective in host cell recovery, and not adherence or invasion as previously believed, I decided to re-visit roles for this in other dehydrogenase systems. In addition to assessing the nature of epithelial cell infection defects, I also assessed pathogen related phenotypes such as cytokine transcription and necrosis. The mutant strains Δgdh, ΔputA, Δmdh, and ΔhydB were found to be defective for intracellular survival in Caco2 intestinal epithelial cells as assessed by CFU enumeration after gentamicin treatment, but not significantly defective for adherence or proper intracellular trafficking as assessed by confocal microscopy. All strains could be partially
enhanced for recovery from epithelial cells by plating on sulfite, but none could be rescued to WT+sulfite levels, suggesting that defects are not strictly recovery related.

Analysis of pathogenesis-related phenotypes in infected T84 intestinal epithelial cells showed that the Δgdh mutant caused reduced TNF-α/IL-8 transcription and enhanced host cell death as compared to WT. Host cell death induced by both WT and Δgdh shared features consistent with necrosis. Inhibition of death pores and calpain activation significantly inhibited killing of host cells by WT and Δgdh strains. Activation of necrosis and calpain is likely linked to toxicity from ROS, as a mitochondrial scavenger of ROS species as well as inhibition of NADPH oxidase (NOX) family of proteins significantly reduced host cell death by WT and Δgdh C. jejuni. A better understanding of the steps leading to induction of necrosis by C. jejuni may help elucidate how C. jejuni initiates gastrointestinal distress. In addition, discovering why Δgdh causes enhanced induction of necrosis may reveal new information on how bacterial metabolism can impact pathogenesis as well as severity of disease.

3.2: Materials and methods

3.2.1: Reagents

Necrostatin-1 and Z-VAD-FMK (N-Benzylxoy carbonyl-Val-Ala-Asp-fluoromethyl ketone) were purchased from ENZO Life Science. Bovine catalase, camptothecin, ALLN (Ac-LL- Norleucinal) and allopurinol were purchased from Sigma, MDL-28170 (N-[(1S)-1-[[[(1-formyl-2-phenylethyl)amino]carbonyl]-2-methylpropyl]-carbamic acid, phenylmethyl ester), PD150606 ((2Z)-3-(4-iodophenyl)-2-mercaptop-2-Propenoic acid, 3-(4-iodophenyl)-2-mercaptop-(Z)-2-propenoic acid), mito-tempo, AEBSF (4-benzenesulfonyl fluoride hydrochloride), and apocynin were purchased from Santa Cruz Biotech.

3.2.2: Bacterial growth conditions

All experiments were performed using the C. jejuni strain 81-176. Strains were grown in Mueller-Hinton (MH) media (Oxoid, Ltd) on agar plates at 38°C in a Sanyo Tri-Gas Incubator at 6% O₂ and 12% CO₂ or in
shaking broth at 38°C using the Oxoid CampyGen system. All MH media were supplemented with 10µg/ml of vancomycin (Toku-E) and 5µg/ml trimethoprim. When indicated, media was supplemented with 50µg/ml kanamycin or 25µg/ml chloramphenicol (Sigma). All DNA manipulations prior to C. jejuni were done in E. coli DH5α on LB media (Sigma) plates or broth supplemented with 100µg/ml ampicillin (Sigma), 25µg/ml kanamycin, or 30µg/ml chloramphenicol.

3.2.3: Construction of strains

All enzymes used for generation of C. jejuni mutant and complemented strains were purchased from New England Biolabs. Primers used are listed in Appendix 1. PCR amplification of sorA, gdh, mdh, putA and hydA was performed with sorA-Fw + sorA-Rv, gdh-FW + gdh-Rv, mdh-Fw + mdh-Rv, putA-Fw + putA-Rv and hydA-Fw + hydA-Rv primers, respectively, using iProof DNA polymerase. Purified PCR fragments were A tailed using Taq DNA polymerase and ligated into the pGem vector. Generation of unique internal Smal restriction sites in sorA, gdh, mdh, putA and hydA was performed by inverse PCR using primers sorA-iPCR-Fw + sorA-iPCR-Rv, gdh-iPCR-Fw + gdh-iPCR-Rv, mdh-iPCR-Fw + mdh-iPCR-Rv, putA-iPCR-Fw + putA-iPCR-Rv, and hydA-iPCR-Fw + hydA-iPCR-Rv respectively. The non-polar aphA-3 cassette encoding a kanamycin resistance gene (2) was digested out of plasmid pUC18K-2 using Smal and ligated into Smal-digested sorA, gdh, mdh, putA and hydA inverse PCR products. All ligations were transformed into DH5α, colonies were screened by PCR, and plasmids from positive clones were purified. C. jejuni was naturally transformed by double recombination with each plasmid and plated on MH agar supplemented with kanamycin or chloramphenicol for 48h to recover colonies. PCR and sequencing confirmed correct insertion in the chromosome by homologous recombination.

Complementation was achieved by amplification of gdh and mdh using primers gdh-C-Fw + gdh-C-Rv and mdh-C-Fw + mdh-C-Rv which introduced an XbaI site into the 5’ end and an MfeI site to the 3’ end of each gene. PCR products were digested with XbaI and MfeI and purified with the DNA clean and
concentrator kit (Zymo Research). The genomic integrative plasmid pRRC (1) was digested with MfeI and XbaI and ligated with the digested gdh-C and mdh-C fragments. Insert expression was driven off the pRRC promoter. Plasmids were transformed into DH5α and selected on kanamycin. Colonies were screened by PCR, and plasmids from positive clones were purified and used to transform Δgdh and Δmdh by natural transformation double recombination. All strains are listed in Appendix 3.

3.2.4: Passaging of Caco2 and T84 epithelial cells

The human colonic cell lines Caco2 and INT-407 was passaged and maintained in minimal essential media (MEM; Gibco) supplemented with 10% FBS 1% penicillin-streptomycin (Pen-Strep; Gibco) in a humidified air incubator at 37°C with 5% CO₂. 24h before experiments, Caco2 or INT-407 cells were harvested and seeded into 24 well plates at 10⁵ cells per well. The human colonic cell line T84 was passaged and maintained in in DMEM/F12 media (Gibco) supplemented with 10% FBS with 1% penicillin-streptomycin (Gibco). 24h before experiments, T84 cells were harvested and seeded into 24 well plates at 10⁵ cells per well.

3.2.5: Infection assay of epithelial cells

Assessment of C. jejuni adherence and invasion into Caco2 cells was performed essentially as previously described in Chapter 2. Mid-log phase C. jejuni grown shaking overnight in MH broth were centrifuged and suspended in MEM at an OD of 0.002 (~10⁷ C. jejuni/mL). 1mL of this suspension (MOI ~100) was used to inoculate Caco2 cells. To assay ‘adherence and invasion,’ 3h after inoculation cells were washed two times with 1mL MEM before addition of 1mL distilled water. Caco2 cells were disrupted by syringe lysis to recover C. jejuni and enumerated by plating on MH plates under standard C. jejuni growth conditions. Where noted, strains were also plated on MH + 20mM sodium sulfite. To assess ‘invasion,’ 3h following inoculation fresh MEM containing 150 µg/mL gentamicin was added to each well for 2h to kill extracellular bacteria. The Caco2 cells were washed three times with MEM and lysed and plated as
above. To assess ‘intracellular survival,’ 2h post gentamicin treatment the media was removed and replaced with MEM containing 3% FBS and 10µg/mL gentamicin. The cells were incubated for 3h and Caco2 cells were washed three times with MEM and lysed and plated as above. All experiments utilizing the gentamicin protection assay were repeated a minimum of three times, with each strain assayed in triplicate in each experimental trial. Data shown are from a representative experiment with similar findings consistently observed in each trial.

To assess adherence or GM130 localization of *C. jejuni* into Caco2 cells or INT-407 cells by confocal microscopy, cells were grown to semi-confluence and seeded onto glass coverslips (Fisher) at 1.5x10^5 cells per well for 24h. To assess adherence of *C. jejuni* to Caco2 cells, Caco2 cells were infected with mid-log phase *C. jejuni* strains expressing pRY112- atpF::GFP plasmid (12, 229) at an MOI of 10 in MEM. Infection and gentamicin treatment were performed as above. After co-incubation for 3h, monolayers were washed 2 times with PBS before fixation with 4% paraformaldehyde (Canemco) in PBS. To assess co-localization of *C. jejuni* with GM130, INT-407 cells were inoculated with mid-log phase *C. jejuni* at an MOI of 10 in MEM for 1h followed by replacement of media with MEM containing 150µg/ml gentamicin for 5h. Cells were washed twice with PBS and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton-X100 (Fisher) and blocked in 10% goat serum with 1% Bovine serum albumin in PBS, primary labeling was done with a 1:200 dilution of an anti-*C. jejuni* rabbit IgG antibody (US. Biological) and 1:200 dilution of anti-GM130 mouse IgG antibody (BD Bioscience) in PBS. Secondary labeling was done with a 1:500 dilution of anti-rabbit goat IgG antibody conjugated to Alexa 568 (Invitrogen) in PBS and 1:500 dilution of anti-mouse goat IgG antibody conjugated to Alexa 488 in PBS. All samples for microscopy were mounted using Prolong Gold Antifade with DAPI (Invitrogen). Imaging was performed with an Olympus Fluoview FV1000 laser scanning confocal microscope using the FV10-ASW 2.0 Viewer software to adjust images. For enumeration of GFP-labeled bacteria and GM130 co-localization, 6
independent fields of view for each strain were counted, and each experiment was repeated three times.

3.2.6: Assessment of intracellular transcription of *C. jejuni* genes

Caco2 cells were seeded into fifteen 70ml tissue culture flasks and allowed to grow until ~70% confluent. Caco2 cells were co-incubated with 20ml mid log phase *C. jejuni* at an OD=0.002. ‘Adherence and invasion’, ‘invasion’, and ‘intracellular survival’ were assessed as above. At the designated time-points, flasks were washed twice with warm PBS and lysed with 5ml lysis stop solution (1%triton X-100, 1% phenol, 19% ethanol in PBS) for 45min. The resulting lysate was removed and centrifuged at high speed to pellet intracellular *C. jejuni*, and the resulting pellet was washed twice in lysis stop solution. Extraction of *C. jejuni* RNA was done as previously (229). Pellets were re-suspended in 200ul TE buffer containing 0.4mg/ml lysozyme for 5min. To this, 950ul Trizol was added and vortexed on high speed before addition of 200ul chloroform. The solution was centrifuged at 10000rpm for 1min and the top aqueous fraction was removed to a new tube containing equal volumes of 70% ethanol. The resulting extract was added directly to the RNeasy spin column as per the manufacturer’s instructions. cDNA generation was performed using SuperScript III (SSIII; Invitrogen) as per the manufacturer’s instructions and purified with a PCR clean up kit (Zymo Research). RNA purity was confirmed by PCR and concentration was assessed using a ND-1000 spectrophotometer (Wilmington, DE). Quantitative PCR of cDNA was performed with the SYBR green (Biorad) q-PCR system using primers katA-qPCR-FW + kata-qPCR-RV, sodB-qPCR-FW + sodB-qPCR-RV, dps-qPCR-FW + dps-qPCR-RV as per the manufacturer’s instructions. Reactions were run with 8ng cDNA, 0.3µM each primer, and 50% SYBR green mix per reaction. Increases in SYBR green fluorescence were measured using a Biorad CFX96 C1000 real time system thermocycler. The fold differences in amplifications between samples were calculated using the comparative threshold cycle (ΔΔCt) method as previously described using *rpoA* as an internal control (229).
3.2.7: Assessment of extracellular H$_2$O$_2$ and intracellular ROS production from host cells

The accumulation of extracellular H$_2$O$_2$ in Caco2 cells was assessed with the Amplex Red reagent (Invitrogen). Infection of Caco2 cells was done as above with modification. MEM with glucose was found to produce a high background signal due to the non-enzymatic reaction between glucose and media salts to produce H$_2$O$_2$. To avoid this, all experiments were performed in RPMI media with no glucose. Amplex Red buffer and reagent was prepared in RPMI media without glucose as per the manufacturer’s instructions, and C. jejuni was added when appropriate to a final OD of 0.002. At the specified time points, 50µl of supernatant was removed and the fluorescence was read in a Varioskan Flash luminometer plate reader at 530nm excitation and 560nm emission. Concentrations of H$_2$O$_2$ were calculated by comparing to a standard curve of known H$_2$O$_2$ concentrations ranging from 1µM to 5µM.

3.2.8: Assessment of IL-8 and TNF-α expression from infected T84 cells

To assess transcription levels of IL-8 and TNF-α, 10$^5$ T84 cells were infected with C. jejuni for 6h. The infected cells were washed twice with PBS, and RNA was collected using the RNeasy spin kit. Preparation of cDNA was done using the enhanced Avian First Strand Kit (Sigma) as per the manufacturer’s instructions. The cDNA was diluted 1:5 in water, and quantitative PCR of cDNA was performed with the SYBR green (Biorad) q-PCR system using primers TNF-alpha-qPCR-FW + TNF-alpha-qPCR-RV and IL-8-qPCR-FW + IL-8-qPCR-RV as per the manufacturer’s instructions. Reactions were run with 4µl cDNA, 0.3µM each primer, and 50% SYBR green mix per reaction. Increases in SYBR green fluorescence were measured using a Biorad CFX96 C1000 real time system thermocycler. The fold differences in amplifications between samples were calculated using the comparative threshold cycle (ΔΔCt) method as previously described using GAPDH as an internal control (229).
3.2.9: Assessment of epithelial cell death by LDH release assay

To assess epithelial cell death, T84 cells were infected by *C. jejuni* at an MOI of 100 as above without gentamicin treatment using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega). At designated time points the media above infected cells was pipetted vigorously and 50µl of media was added to 50µl LDH assay reagent and incubated at room temperature for 30 min before measuring fluorescence. The fluorescence was assessed in a Varioskan Flash luminometer plate reader at 530nm excitation and 620nm emission and % lysis of infected cells was calculated by %lysis=(fluorescence of infected cell - fluorescence of uninfected cell)/ fluorescence of total cell lysate*100. A negative %lysis reflects a sample that has lower LDH release than the control uninfected sample and was expressed as BD for below detection.

3.3: Results

3.3.1: Enhanced recovery of *C. jejuni* from tissue culture cells on sulfite recovery media is due to neutralization of H$_2$O$_2$ produced by the host cells

In a previous study (and as described in chapter 2), I had reported that when enumerating *C. jejuni* from host cells after gentamicin protection assays, a significantly higher number of bacteria could be recovered if the plating media were supplemented with sulfite. The initial hypothesis was that a metabolic switch in *C. jejuni* had occurred when the bacteria were intracellular, causing preferential usage by *C. jejuni* of sulfite or other energy conservation system substrates. If intracellular *C. jejuni* were preferentially using sulfite or other electron donor substrates, they could require them in the media to resume growth post-infection. However, supplementation of plating media with other electron donors such as proline, gluconate, formate, or malate did not enhance recovery (data not shown). To determine if sulfite is important due to its use as an electron donor, I investigated if a ΔsorA mutant had enhanced recovery when plated on sulfite-containing media, since a ΔsorA mutant is unable to use sulfite as an
electron donor for the ETC (196). Consistent with previous reports, ΔsorA had a defect in recovery from host epithelial cells (Figure 3.1A). Unlike the ΔfdhA data reported in chapter 2, supplementation of plating media with sulfite did not allow recovery of the ΔsorA strain up to WT+sulfite levels. However, supplementation of enumeration plates still caused a significant increase in the amount of recoverable ΔsorA mutant bacteria suggesting that enhanced recovery with sulfite is independent of it being a metabolite. Previous reports had suggested that a ΔsorA strain had an adherence defect for epithelial cells (271), thus I hypothesized that the lower amounts of epithelial cell-associated C. jejuni may account for why a ΔsorA mutant could not be recovered to WT + sulfite levels. However, contrary to previous reports which suggested that ΔsorA had an adherence defect, there was no observable difference between WT and ΔsorA for adherence to Caco2 cells as assessed by confocal microscopy (Figure 3.1B). In addition, when assessing co-localization with the host marker GM130, a marker for invasion and intracellular trafficking, no observable differences were observed between WT and ΔsorA (Figure 3.1C). This suggests that ΔsorA is defective for intracellular survival rather than adherence, and that sulfite supplementation could enhance recovery of C. jejuni in a manner that is independent of Sor activity.
Figure 3.1. Intracellular survival, association and GM130 colocalization of ΔsorA in Caco2 cells and the effect of SorA on recovery from host cells when plated on sulfite

A) *C. jejuni* intracellular viability in Caco2 cells for WT and ΔsorA strains was assessed at the ‘Adherence and Invasion’, ‘Invasion’, and ‘Intracellular Survival’ timepoints using a gentamicin protection assay. Viability was calculated by CFU enumeration on MH media plates with or without supplementation with 20mM sulfite. B) Association of GFP-expressing *C. jejuni* WT and ΔsorA with Caco2 cells C) Colocalization of WT and ΔsorA with the Golgi marker GM130 in INT407 cells. All experiments were done in triplicate and figures depict one experiment with three technical replicates for A) or six fields of view for B) and C). The asterisk (*) denotes a statistically significant difference (*p* <0.05).
Sulfite is capable of scavenging \( \text{H}_2\text{O}_2 \) which may be important for recovery of *C. jejuni* if *C. jejuni* is under oxidative stress when intracellular, or under oxidative stress when transitioning to an extracellular environment. To determine if intracellular *C. jejuni* were undergoing oxidative stress, RT-qPCR was performed to determine if the transcription of *C. jejuni* oxidative stress regulated genes were up-regulated during infection of Caco2 cells. The genes *dps*, *katA* and *sodB* were all found to be initially down-regulated when *C. jejuni* infected Caco2 cells at the ‘adherence and invasion’ time-point; however, transcription progressively increased through the ‘invasion’ and ‘intracellular survival’ time-points, suggesting an increase in oxidative stress as the bacteria remain intracellular (Figure 3.2A). To determine if host cells are generating ROS during *C. jejuni* infection, the concentration of \( \text{H}_2\text{O}_2 \) was measured in the media above cells. I observed a significant increase in \( \text{H}_2\text{O}_2 \) production during infection with *C. jejuni* (Figure 3.2B). Sulfite is able to react with \( \text{H}_2\text{O}_2 \) in a neutralization reaction yielding water and sulfate, and catalase directly neutralizes \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) and oxygen. I hypothesized that intracellular *C. jejuni* may be under oxidative stress which sulfite is able to alleviate. To test this, I supplemented host cell lysates containing the liberated *C. jejuni* with 100U/ml catalase, 10mM sulfite, or both, to determine if neutralization of ROS in the lysate is sufficient to enhance recovery of *C. jejuni* from epithelial cells. Lysates supplemented with sulfite, catalase, or both significantly enhanced recovery of *C. jejuni* from host cells (Figure 3.2C). There was no significant difference in recovery of *C. jejuni* between samples supplemented with sulfite, catalase, or both, suggesting that there was no additive effect of supplementing recovery media with both catalase and sulfite.
Figure 3.2. The expression of C. jejuni oxidative stress genes in intracellular C. jejuni, the production of H$_2$O$_2$ by Caco2 cells infected with C. jejuni and the effect of H$_2$O$_2$ scavengers on recovery of C. jejuni from Caco2 cells

A) Transcription levels of sodB, katA and dps in intracellular C. jejuni at the ‘Adherence and Invasion’, ‘Invasion’, and ‘Intracellular Survival’ timepoints were compared to transcription levels of C. jejuni growing in the supernatant above Caco2 cells for 3h. B) The production of H$_2$O$_2$ from uninfected or C. jejuni infected Caco2 cells. C) The recovery of WT C. jejuni from Caco2 cells at the ‘Adherence and Invasion’, ‘Invasion’, and ‘Intracellular Survival’ timepoints using a gentamicin protection assay with supplementation of Caco2 cell lysates with 10mM sulfite, 100U/ml catalase or both when appropriate. Viable C. jejuni were assessed by CFU enumeration. All experiments were done in triplicate and figures depict one experiment with three technical replicates. The asterisk (*) denotes a statistically significant difference ($p <0.05$) and BD denotes values below detection.
3.3.2: Inactivation of gluconate dehydrogenase increased non-apoptotic lysis of host cells

Given that that Δ*fdhA* had a recovery defect from epithelial cells after infection, and a Δ*sorA* mutant had a possible intracellular survival defect, I decided to investigate if other respiratory dehydrogenases had epithelial cell infection-related defects. I constructed Δ*gdh* (gluconate dehydrogenase), Δ*putA* (proline dehydrogenase), Δ*mdh* (malate dehydrogenase) and Δ*hydB* (hydrogenase) mutants and assessed if they had defects in infection as assessed by CFU enumeration, and/or adherence and intracellular trafficking defects as assessed by confocal microscopy analyses. I found that Δ*gdh* (Figure 3.3a-b), Δ*mdh* (Figure 3.4a), Δ*putA* (Figure 3.4d), and Δ*hydB* (Figure 3.4g) all had recovery defects from Caco2 cells as assessed by CFU enumeration after gentamicin treatment. Each mutant could be significantly enhanced for recovery on media supplemented with sulfite, but not to WT+sulfite levels. The recovery defects were partially complemented in Δ*gdh* (Figure 3.3a) and Δ*mdh* (data not shown) strains at the invasion timepoint. However, complementation of Δ*gdh* was not observed at the short term survival timepoint which may reflect the fact that the pRRC promoter driving gdh expression was not active while intracellular. There was no observable defect for adherence to host cells for Δ*gdh* (Figure 3.3c), Δ*mdh* (Figure 3.4b), Δ*putA* (Figure 3.4e), or Δ*hydB* (Figure 3.4h) as assessed by microscopic enumeration of GFP-expressing *C. jejuni* associated with host cells. Determination of proper intracellular trafficking as assessed by microscopic analysis of co-localization of *C. jejuni* with the Golgi marker GM130 revealed no obvious intracellular trafficking defect for Δ*gdh* (Figure 3.3d), Δ*mdh* (Figure 3.4c), Δ*putA* (Figure 3.4f), or Δ*hydB* (Figure 3.4i) strains.
Figure 3.3. Intracellular survival, association, and GM130 colocalization of Δgdh in Caco2 cells

A) *C. jejuni* WT, Δgdh, Δgdh-Complement (Δgdh-C) strains were used to infect Caco2 cells and viability was assessed at the ‘Adherence and Invasion’, ‘Invasion’, and ‘Intracellular Survival’ timepoints using a gentamicin protection assay by CFU enumeration. B) *C. jejuni* WT and Δgdh were used to infect Caco2 cells as in A), but CFU enumeration was performed by plating on MH media with or without supplementation with 20 mM sulfite. C) Association of GFP-expressing WT and Δgdh with Caco2 cells. D) Colocalization of WT and Δgdh with the Golgi marker GM130 in INT407 cells. All experiments were done in triplicate and figures depict one experiment with three technical replicates for A) and B) or six fields of view for C) and D). The asterisk (*) denotes a statistically significant difference (p <0.05).
Figure 3.4. Intracellular survival, association and GM130 colocalization of Δmdh, ΔputA, and ΔhydB in Caco2 cells

C. jejuni WT and strains A) Δmdh, D) ΔputA, or G) ΔhydB were used to infect Caco2 cells and viability was assessed at the ‘Adherence and Invasion’, ‘Invasion’, and ‘Intracellular Survival’ timepoints using a gentamicin protection assay by CFU enumeration on MH agar or MH agar with 20 mM sulfite. Association of GFP-expressing C. jejuni with Caco2 epithelial cells was assessed for B) Δmdh, E) ΔputA, or H) ΔhydB. The % colocalization of C. jejuni with the Golgi marker GM130 in INT407 cells was assessed for C) Δmdh, F) ΔputA, or I) ΔhydB. All experiments were done in triplicate and figures depict one experiment with three technical replicates for A), D) and G) or six fields of view for B), C), E), F), H) and J). The asterisk (*) denotes a statistically significant difference (p<0.05).

3.3.3: A Δgdh mutant induces lower transcription of IL-8 and TNF-α, but enhances host cell death in T84 cells

The Caco2 cell line is useful for assessing intracellular survival defects and host cell interactions; however, it expresses inflammatory cytokines poorly in response to C. jejuni, and does not undergo programmed cell death pathways upon C. jejuni infection (data not shown). Thus, to look at host cell death pathway activation in response to C. jejuni infection, the T84 intestinal epithelial cell line was utilized. A previous report found that co-incubation of T84 cells with C. jejuni causes non-apoptotic host cell death consistent with necrosis when gentamicin is not added to the media (123). Host cell death was not different from WT when infected with ΔputA and ΔhydB, and reduced when infected with Δmdh. The latter likely a reflection of the fact that Δmdh grows slower than WT in both MH-TV media and in the supernatant above infected epithelial cells (data not shown). Conversely, infection with Δgdh enhanced induction of host cell death in T84 cells (Figure 3.5a). Enhanced host cell death from Δgdh was not due to apoptosis, as the pan-caspase inhibitor Z-VAD-FMK did not reduce host cell death during infection by Δgdh (Figure 3.5b). To ensure that C. jejuni were not inducing death by depleting the media, and thus T84 cells, of oxygen or nutrients, transcription of the hypoxia regulated genes VEGF and Glut-1 were investigated. There was no significant up-regulation of these genes when infected with WT or Δgdh C. jejuni compared to uninfected cells, suggesting T84 cells were not undergoing oxygen or
nutrient limitation (Figure 3.5c). Production of inflammatory cytokines by *C. jejuni* infected cells is a marker of infection and plays an important role in pathogenesis (17, 105, 146). The transcription of pro-inflammatory cytokines TNF-α and IL-8 were assessed in T84 cells infected with *C. jejuni*. Although both WT and Δgdh induced transcription of TNF-α and IL-8 above uninfected levels, transcription was reduced in Δgdh infected cells compared to WT (Figure 3.6).
Figure 3.5. The role of Δgdh on induction of host cell death in C. jejuni infected T84 cells and the regulation of hypoxia regulated genes during C. jejuni infection

A) The %lysis in T84 cells infected with WT, Δgdh, Δgdh-C, ΔputA, ΔhydB, and Δmdh strains was calculated 6h or 12h post infection. B) T84 cells were infected with WT or Δgdh in the presence of 125µM caspase inhibitor Z-VAD-FMK. As a positive control for apoptosis cells were incubated with 10µM camptothecin (Campt). Cell death was assessed at 6h or 12h post infection C) The fold difference in transcription of the hypoxia induced genes vegf and glut-1 as compared to uninfected samples were assessed when T84 cells were infected by WT, Δgdh or Δgdh-C for 9h. All experiments were done in triplicate and figures depict one experiment with three technical replicates. The asterisk (*) denotes a statistically significant difference (p <0.05) and BD denotes below detection.
Figure 3.6. The differential transcription of the genes encoding TNF-α and IL-8 in T84 cells infected with WT, Δgdh and Δgdh-C C. jejuni

The fold difference in transcription of tnf-α and il-8 in T84 cells infected with C. jejuni for 6h was assessed as compared to uninfected cells. All experiments were done in triplicate and figures depict one experiment with three technical replicates. The asterisk (*) denotes a statistically significant difference (p <0.05).

3.3.4: Host cell death initiated by WT and Δgdh C. jejuni shows characteristics of programmed necrosis

Necrotic cell death in T84 cells was previously described, but a programmed pathway to necrosis was not (123). To investigate how a Δgdh mutant induces enhanced necrosis, several different mediators of necrosis were investigated. The terminal stage of programmed necrosis involves opening of ‘death pores’ that allow water to enter the cytoplasm causing a swelling morphology. Death pores can be blocked by small molecules such as polyethylene glycols, or lysis can be inhibited by altering the osmolarity of the media with glycine or sucrose which can prevent water influx into the epithelial cells. Co-incubation of C. jejuni-infected T84 cells with glycine, sucrose or PEG8000 significantly reduced host cell death in both WT and Δgdh backgrounds (Figure 3.7a). There was no toxicity in C. jejuni or in T84
cells with glycine, sucrose and PEG8000 co-incubation alone, nor did they inhibit the ability of *C. jejuni* to invade host cells (data not shown).

Opening of death pores in programmed necrosis is thought to be initiated by cytoskeletal cleavage by the calpain family of calcium activated cysteine proteases (160). A rise in cytoplasmic calcium causes activation of the calcium binding site leading to autocleavage and activation of calpain protease activity. Proteolytic cleavage of a variety of cellular proteins by calpain ensues, as well as rupture of lysosomes which releases cathepsin B and other proteases. Calpain activity can be chemically inhibited in two ways: with calcium binding domain inhibitors (PD150606), or with calpain protease binding site inhibitors (MDL2810 and ALLN). Co-incubation of *C. jejuni*-infected T84 cells with PD150606 and MDL2810 caused a significant reduction in the amount of host cell death in both WT- and Δgdh-infected cells (Figure 3.7b). The inhibitor ALLN, however, did not cause a reduction in host cell death, with microscopic analysis of infected cells showing a distinct apoptotic morphology (data not shown).

Treatment of *C. jejuni*-infected T84 cells with both ALLN and the pan-caspase inhibitor Z-VAD-FMK caused a significant reduction in host cell death in both WT- and Δgdh-infected cells suggesting that ALLN caused a switch from programmed necrosis to apoptosis (Figure 3.7c). ALLN may have a secondary target besides calpain that is causing this switch to apoptosis. Activation of calpain can be induced by influx of calcium into the cytoplasm and inhibited by the intracellular calcium chelator BAPTA-AM or the extracellular calcium chelator EGTA. Co-incubation of BAPTA-AM with *C. jejuni*-infected T84 cells caused an increase in T84 cell death that could be partially inhibited by the apoptosis inhibitor Z-VAD-FMK (data not shown). I concluded that intracellular calcium chelation with BAPTA-AM was causing a switch to apoptosis when infected with *C. jejuni*. However, even with Z-VAD-FMK incubation I could not significantly reduce host cell death below *C. jejuni*-only infection levels. As such, the role of calcium in host cell death when infected with *C. jejuni* remains inconclusive.
Figure 3.7. The effect of death pore blockers and calpain inhibition on induction of host cell death in T84 cells infected with WT and Δgdh C. jejuni and the role of calpain inhibitor ALLN in apoptosis in infected cells.

A) T84 cells infected with C. jejuni were co-incubated with death pore blockers 200 mM sucrose, 100 mM glycine, 30 mM PEG6000, or 30 mM PEG8000 and %lysis was assessed. B) T84 cells infected with C. jejuni were co-incubated with 125 µM MDL28170 or 70 µM PD150606 and %lysis was assessed. C) T84 cells infected with C. jejuni were co-incubated with 100 µM ALLN and 125µM caspase inhibitor Z-VAD-FMK when applicable and %lysis was assessed. All experiments were done in triplicate and figures depict one experiment with three technical replicates. The asterisk (*) denotes a statistically significant difference (p <0.05) and BD denotes below detection.
3.3.5: Induction of programmed necrosis is dependent on mitochondrial ROS but independent of Rip1

No work to date has focused on the upstream signals induced by *C. jejuni* that lead to necrosis. Work in other systems has implicated TNFR-mediated Rip1 signaling in complex IIb of necroptosis (Figure 1.5) with induction of excessive mitochondrial O$_{2}^-$ species in the initiation of necrosis (236, 291). I found that inhibition of Rip1 activity with necrostatin-1 could not inhibit host cell death by either WT or Δgdh strains, suggesting host cell death is independent of the necrosome (Figure 3.8a). However, quenching of mitochondrial O$_{2}^-$ with the mitochondrial targeting ROS scavenger Mito-Tempo caused a significant reduction in host cell death in both WT- and Δgdh-infected T84 cells, suggesting that *C. jejuni* is inducing mitochondrial oxidative stress and death (Figure 3.8a). Mitochondrial dysfunction may account for the depletion of ATP in T84 cells infected with *C. jejuni* reported by the Buret group (123). Mitochondrial O$_{2}^-$ can be induced in infected cells primarily by two separate complexes, the NADPH reductase complex NOX, or by Xanthine Oxidase, XO-1 (154, 226). Co-incubation of *C. jejuni*-infected T84 cells with the XO-1 inhibitor allopurinol caused no significant reduction in host cell death. However, significant inhibition of cell death was observed when *C. jejuni*-infected T84 cells were co-incubated with the NOX inhibitors apocynin and AEBSF Hydrochloride for both WT and Δgdh strains (Figure 3.8b). Apocynan inhibits the active site of NOX, whereas AEBSF HCl is a serine protease inhibitor which inhibits the assembly of the active NOX complex. This suggests that ROS production from the NOX family of proteins is one of the main initiators leading to host cell death upon *C. jejuni* infection.
Figure 3.8. The effect of inhibition of the necrosome and mitochondrial ROS production on induction of cell death in T84 cells infected with WT and Δgdh C. jejuni

A) T84 cells infected with WT or Δgdh C. jejuni were co-incubated with 75µM necrostatin-1 or 200µM MitoTempo and %lysis was assessed. B) T84 cells infected with C. jejuni were co-incubated with 1mM allopurinol, 1mM apocynin or 500µM AEBSF hypochloride and %lysis was assessed. All experiments were done in triplicate and figures depict one experiment with three technical replicates. The asterisk (*) denotes a statistically significant difference (p <0.05) and BD denotes below detection.

3.4: Discussion

Previous studies have found that global transcriptional changes occur in C. jejuni when it transitions from an extracellular to intracellular niche (80, 159). In chapter 2, it was discovered that supplementation of recovery media with sulfite could enhance the amount of C. jejuni recovered from Caco2 epithelial cells. It was hypothesized that intracellular C. jejuni may up-regulate certain metabolic systems, such as sulfite oxidoreductase, and the metabolites used by these enzymes may be important when transitioning to an extracellular environment. This is consistent with semi-quantitative mass spectrometry results by another group that showed that protein levels of SorA were relatively well-expressed in intracellular C. jejuni compared to other respiratory proteins (159). However, the enhanced recovery of C. jejuni from Caco2 cells on media containing sulfite was not due to its use as an electron donor, as a ΔsorA mutant was also enhanced for recovery from Caco2 cells when plated on sulfite. The
activity of Sor was required for recovery from epithelial cells in the absence of sulfite supplementation. Previously, lower recovery of a $\Delta$sor mutant from Caco2 epithelial cells was attributed to a perceived adherence defect preventing proper invasion(196). However, in this chapter I showed that Sor activity is not required for adherence and is likely involved in some aspect of intracellular survival. This is based on observations that show a $\Delta$sorA mutant is able to adhere and invade to WT levels, but could not be enhanced to WT levels when plated on sulfite, ruling out a defect in recovery. However, I cannot rule out the possibility that a $\Delta$sorA mutant may be recovered to WT levels if recovery plates are supplemented with some other unknown metabolite besides sulfite. Transcriptional analysis of oxidative stress-regulated genes sodB, katA and dps in intracellular C. jejuni accompanied by measurements of $H_2O_2$ produced by infected Caco2 cells suggested that intracellular C. jejuni were under oxidative stress at the ‘invasion’, and ‘intracellular survival’ time-points. This is consistent with the observation that catalase or sulfite addition to host cell lysates could enhance recovery of intracellular C. jejuni. This indicates that adaption to ROS is a significant burden needed to be overcome by C. jejuni when intracellular or when transitioning back to an extracellular environment. Presumably, intracellular C. jejuni would be subjected to ROS species when in an infected host, so it is unknown why C. jejuni would initially down-regulate ROS scavenging systems. The bulk of the ROS species generated in the intestine during C. jejuni infection are likely to be generated by activated neutrophils. Host cytoplasmic superoxide dismutases, catalases, and glutathione in the infected epithelial cells may initially detoxify the ROS produced by neutrophils before they reach intracellular C. jejuni, thus limiting the need for high transcription levels of intracellular oxygen scavengers. Enhanced production of $H_2O_2$ is observed by Caco2 cells during prolonged C. jejuni infection. The enhanced transcription of C. jejuni ROS scavenging enzymes later in infection may be reflective of this enhanced ROS production by the host cells.

A $\Delta$fdhA mutant was previously shown to have an infection defect in epithelial cells by several groups. However, I found that supplementation of recovery media with sulfite could enhance recovery of $\Delta$fdhA,
and mutants in its putative regulator \( \Delta fdhTU \), up to WT+sulfite levels. Adherence, invasion, and intracellular survival defects have been reported in a wide range of \( C. jejuni \) genes. I wished to discover if other respiratory dehydrogenase mutants besides \( \Delta fdhA \) were defective for infection of epithelial cells, and if any recovery defects could be abolished by addition of sulfite to recovery media. I found that \( \Delta sorA, \Delta gdh, \Delta putA, \Delta mdh \), and \( \Delta hydB \) are all defective for infection of Caco2 epithelial cells, but not for adherence, invasion, or intracellular trafficking. In addition, these strains could be partially rescued by plating on sulfite-containing media, but not to WT+sulfite levels which suggests they have an intracellular survival defect. As is the case with \( \Delta sorA \) it is not known if \( \Delta sorA, \Delta gdh, \Delta putA, \Delta mdh \), and \( \Delta hydB \) have a genuine intracellular survival defect or are just defective for recovery. A previous study employed a propidium iodide based live/dead assay to determine the number of live intracellular \( C. jejuni \) (292). Such a method may be useful here to better distinguish if intracellular mutants are indeed not viable, but propidium iodide based assessment of viability have been found to overestimate dead \( C. jejuni \) due to non-specific uptake of the dye. A better live/dead assay methodology has not been described for \( C. jejuni \).

Metabolic virulence has recently emerged as an exciting new area of study, and defining what metabolites bacteria exploit during infection has yielded information on new and exciting biosynthetic pathways. In \( S. flexneri \), glucose and glycerol seem to be the primary carbon sources depending on strain (83, 165), and \( Salmonella spp. \) preferentially utilizes glucose as its main carbon source, although it is able to utilize other compounds (83). \( M. tuberculosis \) utilizes fatty acids, glycerol and cholesterol during infection (57, 216, 281), and accumulation of cholesterol in \( Mycobacterium leprae \)-infected macrophages is important for intracellular survival (177). Mass spectrometry assessing protein levels has yielded some insight into gene expression and metabolism in intracellular \( C. jejuni \). However, these studies have shown a generally lower abundance of all proteins, making it difficult to define which systems are important. Mutational analysis of metabolic systems is still the best method to investigate if
certain pathways are important for \textit{C. jejuni} infection. My findings that \(\Delta\text{sorA}, \Delta\text{gdh}, \text{and } \Delta\text{hydB} \) are defective for intracellular survival suggests the importance of respiratory dehydrogenases in infection, while the importance of \textit{mdh} in intracellular survival suggests a fully functional Krebs cycle is important as well. It is possible that utilizing respiratory dehydrogenases may allow \textit{C. jejuni} to conserve scavenged amino acids for protein turnover instead of for ATP generation. In this study, \textit{putA} was found to be important for intracellular survival, and a previous study found genes involved in aspartate, lysine and arginine utilization were also important for epithelial cell infection (209). The need for amino acids in intracellular \textit{C. jejuni} is not surprising given it primarily utilizes amino acids as the major carbon and energy source. The intracellular pathogen \textit{Legionella pneumophila} also primarily utilizes amino acids as carbon and energy sources while intracellular and detects amino acid availability to modulate virulence factors through regulators SpoT and ArgR (73). It is possible that amino acid levels in intracellular \textit{C. jejuni} may modify its virulence program or its ability to survive inside cells. \textit{C. jejuni} does not replicate when intracellular, but rather seems to simply persist much like \textit{Chlamydia} elementary bodies, \textit{Staphylococcus aureus}, and latent \textit{M. tuberculosis} (24, 29, 79, 101, 163, 242). Mutation of respiratory elements may break metabolic homeostasis and lead to detection and enhanced clearance by the invaded cells. Given that my studies indicate that intracellular \textit{C. jejuni} are undergoing oxidative stress, a role of metabolism in resisting oxidative stress may be important. A recent study suggested that electron acceptors and proper flux through the ETC in \textit{C. jejuni} is important for resisting oxidative stress (72). The importance of these different systems in intracellular survival and in resisting host-generated ROS when \textit{C. jejuni} is intracellular is still unknown. Regardless, this study provides a preliminary analysis of the respiratory systems used by \textit{C. jejuni} when intracellular, but no data to date has established what the primary energy source is for \textit{C. jejuni} in an intracellular environment and what the metabolic hierarchy is.
This study also found that there are significant differences between tissue culture cell lines in terms of the fate of intracellular *C. jejuni*. Enhanced recovery of *C. jejuni* on media containing sulfite was evident when infecting Caco2 cells; however, equal recovery of *C. jejuni* on media with or without sulfite was achieved when harvesting *C. jejuni* from T84 cells (data not shown). In contrast, *C. jejuni* Δgdh was defective for intracellular survival in Caco2 cells, but was not defective for survival in T84 cells (data not shown). The rate of death of intracellular *C. jejuni* is also more rapid in Caco2 cells compared to T84 cells suggesting Caco2 cells are more efficient at clearing infection after gentamicin treatment of the media.

This may be related to the fact that T84 cells produce reduced H₂O₂ compared to Caco2 cells when infected with *C. jejuni* (Data not shown). It is tempting to think the enhanced H₂O₂ mediated killing of intracellular *C. jejuni* in Caco2 cells may be related to the reason they do not undergo necrosis during prolonged infection. The reasons for these differences are unknown, but tissue culture cells are derived from different progenitor sources. The intestinal epithelia are also composed of different cell types, and the differences I observed in tissue culture cell types during infection may reflect differences in the fate of different cell types in the host intestinal tract. It would be interesting to study, in future work beyond the scope of this thesis, if *C. jejuni* induces different pathologies in different cells during human infection. However, given that the carcinoma cell lines used in this study likely contain mutations that provide pro-survival signals, the differences observed between cell lines may be reflective of different mutations acquired during passaging. Reproducing these findings in human biopsies would give greater clarity to the nature of host cell death induced by *C. jejuni*.

The metabolism of bacteria, besides being important for intracellular survival, can also be very important for differential induction of pathogenesis in infected cells (29, 57, 73). Analysis of Δgdh, ΔputA, Δmdh, and ΔhydB revealed that Δgdh causes enhanced induction of host cell death and reduced cytokine transcription. A previous study in *Listeria monocytogenes* showed that a Δgdh mutant induced enhanced IFN-beta and possibly activation of the host surveillance system (45). It was previously
unknown how *C. jejuni* induces T84 epithelial cell death, but analysis of T84 cells infected with *C. jejuni* showed a progressive release of LDH, indicative of membrane rupture which could be inhibited with pore blockers as well as by calpain inhibition. This work provides the first evidence that *C. jejuni* induces programmed necrotic death in T84 cells. Induction of necrosis through calpain activation has been reported during infection with other bacteria such as *S. aureus, S. flexneri, and Enteropathogenic E. coli (EPEC)* (22, 52, 254). The consequences of activating calpain are unknown; however, calpain activation is essential in Coxsackie virus escape from the cytoplasm and *S.aureus* dissemination (33, 255). Given that *C. jejuni* infection of T84 cells has been shown to result in occludin cytoskeletal depletion, and given that occludin is cleaved by calpain, it is tempting to speculate that necrotic pathways may also increase epithelial barrier permeability leading to *C. jejuni* transmigration to deeper tissues (38, 40). Evidence that mitochondrial ROS production in *C. jejuni*-infected T84 cells leads to necrosis is also novel. It is not well defined what links upstream signals in necrosis to calpain activation, but a rise in intracellular calcium are strongly linked to calpain activation. Further studies are needed to link cytosolic calcium levels to necrosis. Induction of ROS and mitochondrial ROS by *C. jejuni* independent of RIP1 is also interesting, as the up-stream signal leading to ROS production is unknown. *C. jejuni* is known to activate TLRs which have been associated with ROS production and necrosis activation through the common adaptors MLKL and RIP3 (Figure 1.5) (122). Further analysis is needed to determine if these components are involved in *C. jejuni*-dependent necrosis. A study involving *Salmonella spp.*-infected macrophages found that enhanced host cell death through pyroptosis was enhanced in *Salmonella spp.* harboring TCA cycle mutants. The accumulation of citrate was hypothesized to cause increased inflammasome activation by an increase in mitochondrial ROS production in a yet not understood manner (301). It is tempting to postulate that T84 cells infected by Δ*gdh* may be undergoing an analogous fate.

Calpain activation and necrosis may have a vital role in onset of pathogenesis, however, induction of necrosis may have evolved to facilitate *C. jejuni* egress from infected cells. Many bacterial species
initiate apoptosis or necrosis in infected cells to escape the intracellular niche they are inhabiting. For example, *M. tuberculosis* induction of necrosis is needed to exit macrophages (258) and *L. pneumophila* will initiate pore formation and necrosis in infected epithelial cells when the bacterial replication phase is terminated (8).

The finding that a Δ*gdh* mutant causes altered pathology in infected T84 epithelial cells was unexpected but reflects the complicated interplay between host and pathogens. Despite finding key elements that contribute to induction of necrosis in T84 cells, the reason Δ*gdh* induces enhanced cell death still awaits discovery. One hypothesis relates to the fact that gluconate is an intermediate in the pentose phosphate pathway (PPP), which is important for production of purines, but is also a major source of NADPH in human cells (261). Cellular NADPH is vital for many different processes, including generation of O$_2^-$ from the NADPH oxidases (or H$_2$O$_2$ in the case of NOX4) as well as supplying reducing power for glutathione reductase (150, 305). In this regard, the PPP is important for controlling redox state and ROS levels inside human cells. Infection with Δ*gdh* may result in higher host NADPH levels as compared to infection with WT *C. jejuni* due to decreased consumption of host gluconate. The elevated NADPH levels in a Δ*gdh*-infected host cell may lead to increased NOX activity resulting in higher ROS burden. This higher oxidative stress burden may account for the enhanced host cell death seen during infection with a Δ*gdh* mutant. However, a larger pool of NADPH has also been associated with a larger pool of reduced glutathione which is protective against oxidative stress (53). Measurements of O$_2^-$, reduced glutathione and NADPH levels in Δ*gdh* versus WT infected cells may yield information on how Δ*gdh* and the PPP affects host cell death.

In this study, and thesis chapter, I found that there is a complicated interaction between *C. jejuni* metabolism and host cell infection as well as host cell death. Given that *C. jejuni* invasion of epithelial
cells is one of the only ways to assess virulence in the absence of a readily testable disease model, it is worth further investigating the interaction of *C. jejuni* metabolic mutants with epithelial cells.
Chapter 4:

*Campylobacter jejuni* produces formate dehydrogenase- and sulfite oxidoreductase-dependent H$_2$O$_2$ in aerobic conditions, and the sulfur assimilation pathway is important for aero-tolerance

4.1: Introduction and synopsis

*C. jejuni* is a microaerophilic bacterium that grows at oxygen levels of 5-15% (133). More particularly, *C. jejuni* rapidly loses viability under aerobic culture conditions in the lab, yet is able to persist under atmospheric conditions (~21% O$_2$), often surviving on contaminated poultry and in water for weeks before human consumption (41, 54, 208). How it is able to do so is not well understood and is an important question to answer in order to decrease rates of *C. jejuni* human infection. Research on *C. jejuni* oxidative stress has focused on identifying the *C. jejuni* genes involved in regulating the oxidative stress response, ROS detoxification and oxidative stress repair. The rates of ROS production under microaerophilic and aerobic conditions and the primary sites of ROS formation have not been previously described. Here we report that *C. jejuni* produces H$_2$O$_2$ after exposure to aerobic conditions at 37°C, but not at 4°C, and dependent on Fdh and Sor, the enzymes responsible for using formate and sulfite, respectively, as electron donors for the electron transport chain. Further analysis found that sulfite and cysteine could enhance *C. jejuni* aero-tolerance and that this was dependent on the activity of both Sor and ATP sulfurylase (Atps). An Δatps mutant, the gene responsible for the first step of the sulfur assimilation pathway, was found to have a significant defect in aero-tolerance and H$_2$O$_2$ resistance, and had decreased recovery after intracellular intestinal epithelial cell infection. These results are the first to demonstrate that sulfur homeostasis is important for the aerobic survival of *C. jejuni*. 
4.2: Materials and methods

4.2.1: Bacterial growth conditions

All experiments were performed using the *C. jejuni* strain 81-176 (141). *C. jejuni* strains were grown in Mueller-Hinton (MH) media (Oxoid, Ltd) on agar plates at 38°C in a Sanyo Tri-Gas Incubator at 6% O₂ and 12% CO₂ or in shaking broth at 38°C using the Oxoid CampyGen system unless otherwise specified. All MH media was supplemented with 10 µg/ml of vancomycin (V) and 5 µg/ml trimethoprim (T) (MH-TV). Media was supplemented with 50 µg/ml kanamycin, 25 µg/ml chloramphenicol, 10 mM sodium sulfite, cysteine, methionine, glutamine, proline, gluconate, or sodium formate (Sigma) as indicated. *E. coli* DH5α used for DNA manipulations were grown on LB (Sigma) plates or broth supplemented with 100 µg/ml ampicillin (Sigma), 25 µg/ml kanamycin, or 30 µg/ml chloramphenicol as required.

4.2.2: Generation of deletion strains

All enzymes used for DNA manipulations were purchased from New England Biolabs. Primer sequences are listed in Appendix 1. Construction of the ΔfdhA mutant is described in chapter 2 and construction of ΔsorA is described in chapter 3. Deletion mutations in genes *cj0200*, *cj0358*, *cydA*, *cysM*, and *atps* were carried out by first PCR amplification of the genes from *C. jejuni* 81-176 genomic DNA with primers *cj0020*-Fw + *cj0020*-Rv, *cj0358*-FW + *cj0358*-Rv, *cydA*-Fw + *cydA*-Rv, *cysM*-Fw + *cysM*-Rv and *atps*-Fw + *atps*-Rv primers, respectively. Purified PCR fragments were A tailed and ligated into pGem (Promega). Generation of unique internal Smal restriction sites in *cj0020*, *cj0358*, *cydA*, *cysM* and *atps* was performed by inverse PCR using primers *cj0020*-iPCR-Fw + *cj0020*-iPCR-Rv, *cj0358*-iPCR-Fw + *cj0358*-iPCR-Rv, *cydA*-iPCR-Fw + *cydA*-iPCR-Rv, *cysM*-iPCR-Fw + *cysM*-iPCR-Rv, and *atps*-iPCR-Fw + *atps*-iPCR-Rv, respectively. The non-polar *aphA*-3 cassette encoding a kanamycin resistance gene (2) was digested out of plasmid pUC18K-2 using *SmaI* and ligated into *SmaI*-digested *cj0020*, *cydA*, and *cysM* inverse PCR products. The chloramphenicol resistance cassette (CAT) was digested out of plasmid pRY109 (4) with
Smal and ligated into Smal digested cj0358 and atps inverse PCR products. All ligations were transformed into DH5α, colonies were screened by PCR, and plasmids from positive clones were purified. Plasmids were introduced into C. jejuni by natural transformation double recombination and plated on MH agar supplemented with kanamycin or chloramphenicol for 48h to recover colonies. PCR and sequencing confirmed correct insertion in the chromosome by homologous recombination.

Complementation was achieved by amplification of atps using primers atps-C-Fw + atps-C-Rv to introduce an XbaI site at the 5’ end and an MfeI site at the 3’ end of the gene. PCR products were digested with XbaI and MfeI and ligated to the similarly digested genomic integrative plasmid pRRK (1). Insert expression was driven off the pRRK promoter. Plasmids were transformed into DH5α and selected on kanamycin. Colonies were screened by PCR, and plasmids from positive clones were purified and used to transform Δatps by natural transformation double recombination. Insertion of atps in the rRNA spacer regions was confirmed by PCR using primers ak233, ak234, ak235 and DL3.

4.2.3: Assessment of C. jejuni viability in the presence of metabolites or H2O2

An 18h culture of C. jejuni was diluted to an OD of 0.01 in MH-TV media supplemented with sulfite, cysteine, methionine, glutamine, proline, gluconate or formate at 10 mM, or MH-TV alone. Cultures were incubated aerobically at 38°C with shaking. At each timepoint, culture aliquots were removed and serially plated for colony forming units (CFUs) under standard growth conditions. To assess viability in H2O2, an 18 h broth culture of C. jejuni was resuspended at an OD of 0.01 in MH-TV media containing H2O2 and incubated at 38°C for 1 h microaerobically. CFUs were determined by serial dilution.

4.2.4: Determination of H2O2 concentration

The concentration of H2O2 in solution was determined using the Amplex Red assay kit (Invitrogen). Since H2O2 can form non-enzymatically in the presence of glucose and high salt concentrations, RPMI media lacking glucose (RPMI-G) was used. Prior to the experiment, Amplex Red assay buffer was prepared in
RPMI-G. An 18 h culture of *C. jejuni* was washed two times in RPMI-G and diluted in fresh RPMI-G to a final OD of 0.2. Cultures were supplemented with 20 mM sodium sulfite or sodium formate where indicated. Fifty µl of bacterial culture in RPMI-G at an OD of 0.2 was added to each well of a black 96 well plate (Greiner Bio-One) followed by 50 µl of Amplex Red assay buffer. The plates were incubated under conditions specific to each experiment and at each timepoint were measured with a Varioskan Flash luminometer plate reader at 530nm excitation and 560nm emission. Concentrations of H$_2$O$_2$ were determined by comparison to a standard curve of known H$_2$O$_2$ ranging from 0-10µM. The values are presented as $\Delta$[H$_2$O$_2$] which represents the concentration of H$_2$O$_2$ minus the blank values, the latter of which are assays incubated without *C. jejuni*. Aqueous media contain a basal amount of H$_2$O$_2$ which will be detoxified by *C. jejuni*. Detection of basal levels of H$_2$O$_2$ in the blank can result in the $\Delta$[H$_2$O$_2$] being negative during the early stages of aerobic incubation with *C. jejuni*.

4.2.5: Caco2 epithelial cell infection

The human colonic cell line Caco2 was passaged and maintained in minimal essential media (MEM; Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin (Pen-Strep; Gibco) in a humidified air incubator at 38°C with 5% CO$_2$. 24 h prior to the experiment, Caco2 cells were harvested and seeded into 24 well plates at $10^5$ cells per well. Assessment of *C. jejuni* adherence, invasion, and short term survival was performed as previously described (229). Enumeration of *C. jejuni* for CFUs was done on both MH-TV agar and MH-TV agar supplemented with 20 mM sulfite.

4.3: Results

4.3.1: *C. jejuni* produces enhanced H$_2$O$_2$ during incubation in aerobic but not microaerobic conditions, and at 37°C but not 4°C

*C. jejuni* is a microaerophilic bacterium that rapidly loses viability in atmospheric oxygen, suggesting the accumulation of lethal levels of ROS. The extracellular accumulation of H$_2$O$_2$ was measured in cultures of
C. jejuni incubated under aerobic (~21% O₂) and microaerobic (6% O₂) conditions (Figure 4.1A). Initial experiments in MH, the standard C. jejuni growth media used in our laboratory, resulted in a high level of non-specific H₂O₂ formation due to the reaction between glucose and salts in the media. Therefore, WT C. jejuni was grown in RPMI-G to prevent background H₂O₂ formation. C. jejuni does not use glucose as a carbon or energy source, thus media lacking glucose has no effect on its growth rate. Assessment of H₂O₂ concentrations with the Amplex Red H₂O₂ assay reagent showed a significant accumulation of H₂O₂ in the media over time under aerobic, but not microaerobic conditions (Figure 4.1A). As mentioned in section 4.2.4, a negative Δ[H₂O₂] reflects the fact that the concentration of H₂O₂ is lower in the sample than in the blank. This is due to detoxification of basal levels of H₂O₂ present in the media that forms non-enzymatically by C. jejuni peroxidases present in viable bacteria. The H₂O₂ production was slightly but significantly increased in a catalase (ΔkatA) mutant in comparison to WT grown aerobically, suggesting that catalase is partially involved in H₂O₂ detoxification during aerobic growth, although it likely is not the major scavenging enzyme due to the marginal increase in H₂O₂ production.

C. jejuni is often found on refrigerated meat and is able to survive for long periods of time under these conditions, with refrigeration having been reported to decrease the loss of C. jejuni viability (77). The rate of H₂O₂ production during C. jejuni aerobic incubation at 4°C was determined to ascertain whether reduced H₂O₂ production might be at least partially responsible for increased survival at 4°C. Aerobic incubation of C. jejuni at 4°C resulted in no detectable accumulation of H₂O₂ (Figure 4.1B), similar to C. jejuni grown microaerobically at 37°C (compare to Fig. 4.1A), and resulted in a slower loss of viability in comparison to 37°C (Figure 2C). The Amplex Red dye used to determine H₂O₂ concentrations reacted at 4°C equally as well as at 37°C (results not shown).
Figure 4.1. Temperature and O$_2$-dependence of H$_2$O$_2$ accumulation and cell viability in cultures of C. jejuni.

A) WT and a ΔkatA mutant were incubated at 37°C under aerobic (21% O$_2$) or microaerobic (6% O$_2$) conditions for 12 h and production of H$_2$O$_2$ was measured. B) WT was incubated aerobically at 37°C or at 4°C for 12 h and production of H$_2$O$_2$ was measured. C) C. jejuni WT was incubated in aerobic conditions at 37°C or 4°C and viability was assessed by enumerating CFUs at each timepoint. Experiments were
done in triplicate. Figures depict one representative experiment with three technical replicates. Asterisks (*) and (**) denote a statistically significant difference of $p < 0.05$ and $p < 0.001$, respectively.

### 4.3.2: Aerobic incubation of *C. jejuni* with formate enhanced H$_2$O$_2$ production dependent on Fdh and correlates with enhanced aero-tolerance of ΔfdhA

It was previously postulated that respiratory dehydrogenases can be a source of H$_2$O$_2$ during aerobic growth if the active site has a redox-active group, such as a flavin or metal ion capable of donating an electron to O$_2$ (245); this is consistent with FdhA which contains a solvent exposed iron sulfur cluster which has been associated with ROS formation (109). On the other hand, previous work from another group also suggested Fdh activity may be important for resistance to exogenous H$_2$O$_2$ (128). Incubation of *C. jejuni* with formate, the substrate for Fdh, caused significantly enhanced accumulation of H$_2$O$_2$ (Figure 4.2A). To determine if the higher rate of H$_2$O$_2$ associated with aerobic incubation with formate is dependent on Fdh, H$_2$O$_2$ production in an ΔfdhA mutant constructed previously (229) was examined. Fdh is a multi-subunit enzyme, and disruption of *fdhA* disrupts all Fdh activity. The accumulation of H$_2$O$_2$ with formate addition during aerobic incubation was significantly reduced in ΔfdhA as compared to WT (Figure 4.2A). H$_2$O$_2$ production was also significantly lower in an ΔfdhA strain than in WT (Figure 4.2A). This shows that the presence of Fdh is important for production of ROS during aerobic growth. To determine if the Fdh dependent H$_2$O$_2$ formation in aerobic conditions is associated with loss of viability, WT and ΔfdhA were incubated aerobically for 12h and viability was assessed by plating for CFUs (Figure 4.2B). The ΔfdhA mutant exhibited a slower loss of viability in oxygen compared to WT. This indicates that Fdh dependent generation of H$_2$O$_2$ is at least partially responsible for loss of *C. jejuni* viability under aerobic conditions.
Figure 4.2. Effect of formate and Fdh on *C. jejuni* H$_2$O$_2$ production and viability in aerobic conditions.

A) WT and ΔfdhA were incubated aerobically at 37°C with or without media supplementation with 10 mM formate and H$_2$O$_2$ was measured. B) WT and ΔfdhA were incubated in aerobic conditions at 37°C and viability was determined by CFU enumeration at each time-point. All experiments were done in triplicate and figures depict one experiment with three technical replicates. Asterisks (*) and (**) denote a statistically significant difference of $p < 0.05$ and $p < 0.001$, respectively.
4.3.3: Sulfite and cysteine supplementation can prevent the loss of *C. jejuni* viability under aerobic growth and is dependent on Sor activity

Previously we reported that supplementation of growth media with sulfite could enhance recovery of *C. jejuni* from host epithelial cells after host cell infections (229). Other groups have reported that sulfite, pyruvate, and bisulfate could also prevent the loss of viability associated with *C. jejuni* incubation in atmospheric oxygen (39, 137, 286). Those groups postulated that these compounds are antioxidants capable of detoxifying ROS produced by *C. jejuni*. While sulfite may be able to neutralize extracellular \( \text{H}_2\text{O}_2 \), it would be unable to neutralize intracellular \( \text{H}_2\text{O}_2 \) unless imported. To determine if any other metabolic additives could increase the aero-tolerance of *C. jejuni*, bacteria were grown in shaking broth at 37°C under aerobic conditions in the presence of glutamine, proline, methionine, gluconate, sulfite or cysteine. Both sulfite and cysteine prevented loss of viability associated with aerobic growth with a 3.9 log and 1.9 log fold increase in survival at 12 h, respectively (Figure 4.3A). Supplementation with glutamine, proline, methionine, or gluconate had no significant effect on aerobic survival (data not shown). Sulfite can be oxidized by Sor in *C. jejuni* (196), therefore the role of sulfite as a metabolite was investigated by constructing a \( \Delta\text{sorA} \) mutant to determine whether SorA is required to enhance *C. jejuni* aerobic survival. Sor is a multi-subunit enzyme and disruption of \( \text{sorA} \) eliminates all Sor activity(196). The aerobic survival of a \( \Delta\text{sorA} \) mutant was still enhanced in media supplemented with sulfite and cysteine; however, the enhanced survival was significantly diminished in comparison to WT (Figure 4.3B). The role of sulfite as an electron donor can therefore only partially explain sulfite-enhanced aerobic survival of *C. jejuni*.

*C. jejuni* possesses two terminal oxidases, CydAB and CcNOQP (115), which transfer electrons from the electron transport chain to oxygen to generate water. This lowers the concentration of dissolved oxygen in the media and should reduce the rate of \( \text{H}_2\text{O}_2 \) generation. In addition, *C. jejuni* possesses two putative cytochrome c peroxidases (Cj0020 and Cj0358) that use electrons from the electron transport chain
system to eliminate $\text{H}_2\text{O}_2$ by converting to water. To determine if the electrons liberated from sulfite by Sor are being used by the terminal oxidases and peroxidases, a mutant in the cydA terminal oxidase was constructed, as well as a $\Delta_{cj0020}\Delta_{cj0358}$ double mutant in which both predicted cytochrome c peroxidase-encoding genes were deleted. The terminal oxidase CcoNOQP is essential for growth, and a deletion was previously shown to be lethal in C. jejuni (115). Although $\Delta_{cydA}$ was found to have a defect in survival in aerobic conditions, it was rescued up to WT levels by sulfite and cysteine supplementation (Figure 4.3C). The $\Delta_{cj0020}\Delta_{cj0358}$ mutant also displayed enhanced survival in the presence of sulfite and cysteine similar to WT (Figure 4.3C). This suggests that the role of sulfite in preventing loss of viability in aerobic conditions is independent of these electron transport chain components, but is partially dependent on Sor.
Figure 4.3. The effect of SorA, CydA, and Cj0358/Cj0020 on C. jejuni aerobic viability with sulfite and cysteine media supplementation

A) Viability of C. jejuni in MH-TV media unsupplemented, or supplemented with 10 mM of sulfite or cysteine was assessed for cultures incubated at 37°C in aerobic conditions by CFU enumeration. B) The viability of WT and ΔsorA mutants, or C) WT, Δcj0020Δcj0358 double mutant or ΔcydA mutant was determined 12 h post inoculation in MH-TV media at 37°C in aerobic conditions unsupplemented (-) or supplemented with 10 mM sulfite (S) or cysteine (C) by CFUs enumeration. All experiments were done in triplicate and figures depict one experiment with three technical replicates. Asterisks (*) and (**) denote a statistically significant difference of \( p < 0.05 \) and \( p < 0.001 \), respectively.

4.3.4: \( \text{H}_2\text{O}_2 \) formation in the presence of sulfite is dependent on Sor

To determine whether sulfite is involved in suppressing \( \text{H}_2\text{O}_2 \) concentrations produced by C. jejuni, \( \text{H}_2\text{O}_2 \) formation in the WT and ΔsorA strains was measured over time in the presence of sulfite under aerobic and microaerobic conditions. Some C. jejuni respiratory dehydrogenases may be a source of ROS
formation but the same should not be true for SorAB if it is suppressing H₂O₂ formation. Sulfite supplementation increased the total production of H₂O₂ in WT under aerobic but not microaerobic conditions (Figure 4.4A). Sulfite is able to react with H₂O₂ directly to form sulfate and water, so the presence of sulfite in the media will reduce the H₂O₂ levels present in Figure 5. Therefore, the values of H₂O₂ shown in Figure 4.5 are likely an underrepresentation of the actual values. To determine if the enhanced H₂O₂ production with sulfite supplementation was dependent on Sor, rates of H₂O₂ formation in aerobic conditions were measured in a ΔsorA mutant (Figure 4.4B). The rate of H₂O₂ produced by a ΔsorA mutant was identical with or without sulfite supplementation (Figure 4.4B), suggesting that H₂O₂ formation in the presence of sulfite is dependent on Sor. Complementation of ΔsorA was attempted; however, repeated attempts to generate a PCR product containing 5’ elements of sorA were not achieved, possibly due to secondary structure formation in the DNA upstream.
Figure 4.4. Effect of sulfite and SorA on H$_2$O$_2$ production by *C. jejuni* in microaerobic or aerobic conditions

A) H$_2$O$_2$ production from WT with and without 10 mM sulfite in the media, assessed in aerobic (21% O$_2$) or microaerobic (6% O$_2$) conditions at 37°C. B) WT and the ΔsorA mutant were incubated under aerobic conditions at 37°C in the absence or presence of 10 mM sulfite and H$_2$O$_2$ was measured. All experiments were done in triplicate; figures depict one experiment with three technical replicates. An asterisk denotes a statistically significant difference of $p < 0.05$. 
4.3.5: The sulfur capture system is important for aerobic survival as well as enhanced aerobic survival with sulfite and cysteine

Sor plays an important role in enhancing *C. jejuni* aerobic survival in the presence of sulfite (Figure 4.3B). Although sulfite appeared to suppress H$_2$O$_2$ production in the absence of Sor (Fig. 4.4B), sulfite actually resulted in greater H$_2$O$_2$ production when Sor was present (Figure 4.4B). This suggests while sulfite can act as an ROS scavenger, it enhances H$_2$O$_2$ production in a Sor-dependent fashion. Enhanced aerobic survival in the presence of cysteine was also Sor-dependent, suggesting a potential role for sulfur cycling in aerobic survival. In other organisms, sulfate is incorporated and converted to cysteine via the sulfur assimilation pathway when environmental cysteine is unavailable (Figure 1.8). Sulfate and ATP are combined to form adenosine-phosphosulfate (APS) by the ATP sulfurylase (Atps) (Figure 1.8). APS can then be used to synthesize cysteine or other sulfur containing molecules that may act as antioxidants. Given that oxidative stress can induce cysteine bradytrophy (20), the role of sulfur assimilation in *C. jejuni* aerobic survival was examined. Previously *atps* has not been described in *C. jejuni*; however, an *atps* had previously been annotated in the genome of *C. jejuni* as well as in related species. Although *C. jejuni* Atps has not been characterized, BLAST analysis showed 20% amino acid sequence identity with the previously studied 180-residue Atps domain of *M. tuberculosis* CysDN (223). The N terminal 150 residues of *C. jejuni* Atps has little similarity to known protein domains and is functionally unknown. A Δ*atps* mutant (Cjj81176_1596) was constructed and displayed a 4-log fold defect for aerobic survival as compared to WT, which was partially rescued in a complemented strain Δ*atps*-C (Figure 4.5A). Addition of sulfite and cysteine only caused a very modest increase in aerobic survival of the Δ*atps* mutant (Figure 4.5A). Sulfate supplementation had no effect on *C. jejuni* aerobic survival (data not shown). In other organisms, the cysteine synthase CysM has been shown to catalyze the incorporation of sulfide into O-acetyl-serine to produce cysteine. A mutant was constructed in a *C. jejuni cysM* homolog
(Cjj81176_0912c). The ΔcysM mutant displayed a 1.4 log fold defect in aerobic survival compared to WT. Sulfite and cysteine addition enhanced ΔcysM aerobic survival to WT levels (Figure 4.5B).

Given that a Δatps mutant is more sensitive to air, we hypothesized that the mutant either had an enhanced rate of H$_2$O$_2$ production by respiratory dehydrogenases, or lacked peroxidase activity to degrade H$_2$O$_2$. A Δatps mutant was found to have significantly enhanced sensitivity to H$_2$O$_2$ in comparison to WT, which was partially restored in the complemented strain (Figure 4.5C). This suggested there is either a defect in H$_2$O$_2$ detoxification or an inability to repair damage induced by H$_2$O$_2$ stress, leading to a loss of viability. H$_2$O$_2$ detoxification in a Δatps mutant was measured as compared to WT. The half life of H$_2$O$_2$ detoxification was calculated using a first order exponential decay line of best fit. The WT and Δatps strains had non-significant differences in half lives of 0.846 min and 0.66 min respectively, suggesting an Δatps mutant is not defective in H$_2$O$_2$ detoxification (data not shown). To further explore the role of Δatps in ROS sensitivity, WT C. jejuni and a Δatps mutant were incubated in the presence or absence of formate. The formate-dependent production of H$_2$O$_2$ by C. jejuni under aerobic conditions (Figure 4.2) was measured in a Δatps mutant. Incubation of the Δatps mutant in the presence of formate resulted in a significantly increased level of H$_2$O$_2$ production in comparison to WT grown with formate that could be partially rescued by complementation (Figure 4.5D). This suggests that an Δatps mutant may not be defective for H$_2$O$_2$ detoxification, but does produce H$_2$O$_2$ more rapidly in aerobic conditions.
Viability of A) WT, Δatps mutant, and the Δatps complemented (Δatps-C) strains, and B) WT and ΔcysM mutant strains was assessed in MH-TV media unsupplemented (-), or supplemented with 10mM sulfite (S) or 10 mM cysteine (C) after incubation for 12h in aerobic conditions at 37°C. Viability was assessed by CFU enumeration. C) Viability of WT, Δatps, and Δatps-C C. jejuni strains was assessed after 1h microaerobic incubation at 37°C in MH-TV media with different concentrations of H₂O₂. Viability was assessed by CFU enumeration. D) Production of H₂O₂ was measured in WT, Δatps or Δatps-C strains with or without formate supplementation of the RPMI-G media in aerobic conditions at 37°C. All experiments were done in triplicate and figures depict one experiment with three technical replicates. Single (*) and double (**) asterisks denote statistically significant differences of p < 0.05 and p < 0.001, respectively.
4.3.6: The Δatps mutant is defective for infection of host cells

Generation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) from epithelial and immune cells during infection is one of the first line defenses. If Atps is important for resisting oxidative stress, it may also be important for resisting the effects ROS produced by host cells to prevent infection. Caco2 colonic epithelial cells were infected with the \textit{C. jejuni} WT, Δatps mutant, and the Δatps complemented strain, and CFU recovery following gentamicin treatment was assessed. The Δatps mutant displayed diminished recovery at the ‘adherence and invasion’, ‘invasion’, and ‘short term survival’ time-points that was restored to WT levels with the complemented strain (Figure 4.6). This suggests that sulfur assimilation is not only important for aerobic survival, but for survival within host cells. A Δatps mutant showed significant enhancement in recovery on MH-TV supplemented with sulfite, although not up to WT levels. This suggests that the enhanced recovery of \textit{C. jejuni} from host epithelial cells on sulfite is independent of Atps.
Figure 4.6. The effect of Atps on *C. jejuni* infection of Caco2 intestinal epithelial cells

The *C. jejuni* WT, Δatps and Δatps complemented (Δatps-C) strains were used to infect Caco2 cells and assayed at the ‘adherence and invasion’, ‘invasion’ or ‘short term intracellular survival’ using a gentamicin protection assay. CFUs were determined on MH-TV agar with and without 20 mM sulfite (S). All experiments were done in triplicate and figures depict one experiment with three technical replicates. Asterisks (*) and (**) denote a statistically significant difference of *p* <0.05 and *p* <0.001, respectively.

4.4: Discussion

*C. jejuni* must survive in the environment during transmission. An important aspect in understanding this survival is characterizing how *C. jejuni* responds to aerobic conditions. In *E. coli*, the rate of ROS production initially increases when *E. coli* transitions from anaerobic to aerobic conditions and then quickly slows during longer aerobic incubation as enzymes such as fumarate reductase that are predisposed to ROS formation are down-regulated (124, 218). Here, the transition of *C. jejuni* from a microaerobic to an aerobic environment was also shown to stimulate the production of ROS after a 6h
lag phase that correlates with a sharp decrease in viability. In addition, the viability of *C. jejuni* in aerobic conditions was found to be enhanced at 4°C versus 37°C, as shown previously (77). This potentially was due to the lower rate of H₂O₂ production at 4°C, and indicates that at lower temperatures in aerobic environments *C. jejuni* undergoes decreased oxidative stress in comparison to 37°C commensurate with the bacterium’s reduced metabolic rate at 4°C.

We postulated that homologs of the *E. coli* H₂O₂-producing enzymes may be sources of H₂O₂ production during aerobic incubation of *C. jejuni*. For example, a *C. jejuni* fumarate reductase (MfrA) mutant, despite having a defect in survival in H₂O₂, was shown previously to survive better than WT under aerobic conditions, suggesting it is a source of ROS (128). Similarly, deletion of Fdh reduced the overall production of H₂O₂ under aerobic conditions, with a concomitant increase in viability as compared to WT. Incubation of *C. jejuni* in the presence of formate, the substrate for Fdh, also enhanced aerobic H₂O₂ production in an Fdh-dependent manner. Production of H₂O₂ from enzyme-associated redox cofactors can occur when a solvent-exposed cofactor capable of single electron transfer to oxygen comes into contact with O₂. Reduced flavins are the most widely reported sources of ROS in many organisms (111), although iron sulfur clusters are also able to reduce oxygen (111). There have been no crystallographic studies on *C. jejuni* FdhA; however, analysis of *E. coli* FdhH, which shares 31% amino acid sequence identity with *C. jejuni* FdhA, contains an iron sulfur cluster at its active site (32).

Moreover, *C. jejuni* FdhA has been reported to contain an iron sulfur cluster (240). However, the iron sulfur ligands are poorly conserved between *C. jejuni* FdhA and *E. coli* FdhH, necessitating further work to determine if the iron sulfur group of FdhA is O₂-reactive. *C. jejuni* Fdh also contains a quinone binding site in FdhC which may be a site of ROS production based on studies in mitochondria (96), although such interpretations based on mitochondrial ROS generation should be taken with care. Nevertheless, no studies have linked Fdh to ROS production in bacteria, thus a direct role of the Fdh active site in H₂O₂ remains unproven.
Although reduction of $O_2$ at the FdhA and SorA active site is possible, the data presented here do not establish this. Rather the data show that $O_2$ reduction is dependent on the presence of Fdh and Sor. The increased production of $H_2O_2$ after 6h incubation with formate suggests that the enhanced production of $H_2O_2$ may be due to the upregulation of the $fdh$ operon. Previous studies found no evidence that $fdh$ is differentially regulated during $C. jejuni$ oxidative stress, suggesting it may not be differentially expressed in 20% oxygen; however, further work is needed to determine how $fdh$ is regulated in aerobic conditions(55, 214). Moreover, no work to date has assessed whether formate regulates $fdh$ transcription, although $E. coli$ $fdhGHIF$ is induced when media is supplemented with formate(290). These experiments are currently underway for $C. jejuni$. Regardless, upregulation of the $fdh$ operon in the presence of formate may be resulting in an increase in Fdh redox active prosthetic groups which are involved in reduction of oxygen. $E. coli$ FdhH is proposed to involve the oxidation of formate independent of oxygen transfer(25, 134). However, the limited amino acid similarity between FdhH and FdhA makes such interpretations about FdhA difficult. Varying the levels of Fdh by expressing $fdh$ under the control of different strength promoters, thus eliminating the effect of formate-induced upregulation of $fdh$, could help determine if Fdh alone, or Fdh dependent-respiration of formate is involved in ROS production. A positive correlation between the amount of Fdh produced and the amount of ROS produced in the absence of formate supplementation would argue that Fdh redox cofactors are reactive with $O_2$ and a direct site of ROS formation. An alternate hypothesis is that Fdh-dependent electron flux may increase the level of reduced redox centers such as in fumarate reductase, a known site of $O_2$ reduction(182) or in Fdh itself. Unpublished data found that a ΔcydA mutant produced less $H_2O_2$ when incubated with formate than WT+formate during the first 9h of aerobic incubation (data not shown). Fdh-dependent electron flux through the ETC may be causing enhanced reduction of CydA leading to greater $H_2O_2$ production (the role of CcoNOQP has not been investigated). Incubation of $C. jejuni$ with
sulfite may be causing analogous increased expression of Sor and increased reduction of ETC reductase redox centers which may be sites of H$_2$O$_2$ production as proposed for Fdh above.

Elevated respiration in the presence of formate will also cause downstream reduction of menaquinone and complex III, which may be potential sources of H$_2$O$_2$. Reduced menaquinone is a known source of H$_2$O$_2$ (142). As noted above, experiments are currently underway to determine the role of menaquinone and terminal oxidases in H$_2$O$_2$ production. The inhibitor HQNO which inhibits the interaction of menaquinone with complex III (196) can be used to determine if enzymes downstream of menaquinone may be a source of enhanced H$_2$O$_2$ production in the presence of formate. If reduced Fdh redox centers or menaquinone are the sites of O$_2$ reduction, then incubation with formate will increase the titre of reduced Fdh redox centers (as well as in menaquinone), thereby enhancing H$_2$O$_2$ production.

The presence of Fdh resulted in increased production of H$_2$O$_2$, and the presence of Fdh decreased C. jejuni aerobic survival, yet other metabolic additives have been reported to have the opposite effect and enhance survival, such as sulfite, bisulfite, pyruvate (mentioned in results) and iron ascorbate (120, 155). It is unknown if these compounds have direct anti-oxidant activity or have important metabolic functions. The two sulfur-containing metabolites, sulfite and cysteine, were able to enhance aerobic survival in a manner that is partially dependent on Sor. Despite this, it was found that H$_2$O$_2$ production was dependent on Sor under aerobic conditions when the media were supplemented with sulfite. Previous work established that E. coli sulfite reductase is a site of O$_2$ reduction (181); however, C. jejuni Sor is not a homolog of E. coli sulfite reductase and lacks the flavin group which was associated with O$_2$ reduction in E. coli. Interpretation about Sor based on E. coli sulfite reductase data should be done with care. Since sulfite enhanced H$_2$O$_2$ production under aerobiosis, it would be expected to decrease C. jejuni aerobic viability. However, the opposite was observed: sulfite enhanced aerobic survival. We hypothesized that the role of sulfite and cysteine in aerobic survival might be due to a role in sulfur
homeostasis instead of as anti-oxidants, as both compounds are intermediates in the sulfur assimilation pathway. The primary role of the sulfur assimilation pathway in many organisms is to convert sulfate into the sulfur-containing amino acid cysteine (Refer to Figure 1.8). The sulfur assimilation pathway is thus far not known to be involved in aerobic survival in other bacteria, but was found to be important for \textit{C. jejuni} aero-tolerance and partially responsible for the enhanced aerobic survival resulting from sulfite supplementation. To investigate the role of the sulfur assimilation pathway, the first enzyme in the pathway, \textit{AtpS}, and the last enzyme in the pathway, \textit{CysM}, were mutated. Although a \textit{\Delta cysM} mutant was slightly more sensitive to exposure to oxygen than WT, it was much more resistant to aerobic conditions than an \textit{\Delta atps} mutant. The \textit{\Delta cysM} mutant, unlike \textit{\Delta atps}, could be rescued to WT+sulfite or WT+cysteine levels of aerobic survival with sulfite or cysteine addition respectively; indicating that the \textit{\Delta atps} defect in aero-tolerance is not due to defects in cysteine synthesis. An \textit{\Delta atps} mutant was more sensitive to higher levels of \textit{H}_2\textit{O}_2 than WT, and the addition of formate to an \textit{\Delta atps} mutant, caused rapid \textit{H}_2\textit{O}_2 accumulation but did not have a defect in \textit{H}_2\textit{O}_2 detoxification compared to WT. The fact that the \textit{\Delta atps} mutant is defective for aero-tolerance implicates the sulfur assimilation pathway in oxidative stress management in \textit{C. jejuni}, although the exact role remains to be determined. These features are also important for host cell infection, as a \textit{\Delta atps} mutant was defective for recovery from Caco\textit{2} epithelial cells, although this defect was partially abrogated in the presence of sulfite. Previously, we showed that sulfite could enhance recovery of \textit{C. jejuni} from host epithelial cells (229); however, this is likely not due to a function of the sulfur assimilation pathway, as sulfite still enhanced recovery from epithelial cells in a \textit{\Delta atps} background as expected from data presented in chapter 3.

Even though the main role of the sulfur assimilation pathway is to synthesize cysteine, intermediates in the pathway can also be used by sulfotransferases to generate other sulfo-compounds. Examples include: \textit{M. tuberculosis} sulfolipid-1 shown to be important for virulence, possibly by mediating resistance to antimicrobial peptides (82, 193), and oligosaccharide sulfation that is vital for the
production of nodulation factors in *Rhizobium* species. A sulfocompound synthesized by the activity of a
sulfotransferase may be important in mediating aerobic tolerance in *C. jejuni*. Extensive searches of the
*C. jejuni* genome did not identify homologs of known sulfotransferases, although *C. jejuni*
sulfotranferases may have low sequence similarity to known sulfotransferases. Future work will
determine how the sulfur assimilation pathway influences *C. jejuni* aero-tolerance and whether *C. jejuni*
produces any novel sulfur-containing compounds. It is unknown how sulfate and sulfite cross the *C.
jejuni* plasma membrane as both are charged and unable to cross lipid bilayers. In other bacteria, there
is a dedicated sulfate transporter that is absent in *C. jejuni* (5). Addition of sulfate to cultures did not
enhance survival of *C. jejuni* in aerobic conditions (data not shown). Previous work has demonstrated
that Sor is the only respiratory enzyme that can use sulfite as an electron donor to the electron
transport chain(196). However, *C. jejuni* also possesses another putative sulfite oxidoreductase that was
shown to have a role in nitrosative stress (97), although it is unknown if it is important for oxidative
stress. It is in the same operon as a putative transmembrane protein with homology to ferric reductase,
which may suggest that sulfite oxidation can be coupled to iron reduction in *C. jejuni*.

A major paradox in *C. jejuni* biology is why it loses viability rapidly in aerobic conditions, yet is able to
persist in the environment for long periods of time. To understand this phenomenon, it is important to
understand sources of oxidative stress in *C. jejuni* and how environmental conditions effect the
bacterium’s oxidative stress response. This study determined that Fdh and Sor are involved in \( \text{H}_2\text{O}_2 \)
production. The main source of formate and sulfite in *C. jejuni* are likely pyruvate formate lyase and
cysteine dioxygenase respectively and as such both compounds are likely to be encountered due to
endogenous production by *C. jejuni*. In addition, it was found that \( \text{H}_2\text{O}_2 \) production is temperature- and
oxygen tension-dependent, with production of \( \text{H}_2\text{O}_2 \) ceasing in microaerobic oxygen conditions and at
4°C and. This may account for the ability of *C. jejuni* to persist on refrigerated meat for extended periods
of time (296).
The role of sulfite in enhancing *C. jejuni* aerobic survival that we have described also brings up another important issue for the food industry: sulfite is a common food preservative as it produces sulfur dioxide which inhibits the oxidase activity of enzymes (183). However, sulfite may actually be enhancing the aerobic survival of *C. jejuni* and promote the transmission of *C. jejuni* to humans. In addition, recent reports have shown that neutrophils release high levels of sulfite during inflammation in humans (184). This may also augment *C. jejuni* aerobic survival during transmission. The wide use of sulfite and refrigeration in the food industry is unlikely to stop, and strategies to overcome these factors are needed to break transmission of *C. jejuni* to humans.
Chapter 5:

General discussion

5.1: Summary

In order to better understand factors that are important for *C. jejuni* interaction with human epithelial cells, a microarray experiment investigating *C. jejuni* genes up-regulated when interacting with human cells was analyzed (80). Mutational analysis of a subset of these genes revealed the importance of the *fdhTU* operon in epithelial cell infection. The operon, encoding a transmembrane importer (*fdhT*) and an RNA binding regulatory gene (*fdhU*) were found to be a novel regulator of Fdh. Bioinformatics analysis found the *fdhTU* operon is conserved across many different bacterial species. A concurrent study found the operon was important for selenium uptake, and confirmed the importance of FdhTU in *fdh* regulation (247). This is consistent with a previous study that found selenium was an important cofactor in Fdh activity (251).

During studies involving *fdhTU*, we found that both Fdh and media supplementation with sulfite are important for recovery of *C. jejuni* from Caco2 epithelial cells. To further investigate how sulfite is able to cause this to occur I analyzed the intracellular transcript levels of ROS stress-regulated genes, measured H$_2$O$_2$ production from Caco2 cells, and the effects of supplementation of host cell lysates with catalase. These experiments revealed that sulfite addition was likely detoxifying ROS, and that *C. jejuni* is diminished for oxidative stress resistance when transitioning from an intracellular to extracellular niche.

The finding that Fdh is important for recovery from epithelial cells led us to investigate the role of other respiratory dehydrogenases in host cell recovery. Multiple different dehydrogenase mutants were constructed and tested for epithelial cell adherence, invasion, intracellular survival and intracellular trafficking. ΔsorAB, Δgdh, ΔputA, Δmdh, and ΔhydAB were found to all be required for proper
intracellular survival of *C. jejuni* – but, unlike Fdh, likely not required for recovery following cell infection. Further analysis found that a \(\Delta gdh\) mutant induced reduced cytokine secretion and increased induction of host cell necrosis from T84 cells compared to WT infected cells. The induction of necrosis was subsequently found to be due to production of NADPH oxidase-dependent mitochondrial ROS and activation of calpain proteases. A specific role for \(\Delta gdh\) in enhanced induction of necrosis has yet to be determined.

Sulfite not only enhanced survival of *C. jejuni* from epithelial cells, but was also found to enhance survival under aerobic conditions which are normally toxic to *C. jejuni*. *C. jejuni* loss of viability in aerobic conditions correlated with production of \(H_2O_2\). In *E. coli*, ROS generation is due to accidental oxidation of respiratory dehydrogenases by \(O_2\) (245); Consistent with this, we identified Fdh and Sor enzyme complexes to be significant sources of \(H_2O_2\) production when *C. jejuni* were incubated aerobically. Enhanced survival in aerobic conditions was achieved when *C. jejuni* was supplemented with sulfite or cysteine, and enhanced survival was found to be dependent on the sulfur assimilation pathway gene ATP sulfurylase (\(atps\)). An \(\Delta atps\) mutant was found to be severely defective for survival in aerobic conditions, had reduced peroxidase activity and was defective for infection of epithelial cells.

### 5.2: FdhTU is important for regulation of *fdh* and recovery from host epithelial cells

Analysis of *C. jejuni* metabolism has revealed many metabolic systems important for infection and colonization (196, 213, 306). However, regulation of metabolism in *C. jejuni* is poorly understood. *C. jejuni* has relatively few regulatory systems, with only twelve response regulators of two component signaling systems (265) and relatively few other annotated regulators (306). Understanding how *C. jejuni* regulates metabolic potential is vital to understanding how it responds to environmental cues. Work within chapter 2 identified a novel regulator conserved in many bacterial species that is important for
regulation of \textit{fdh} in \textit{C. jejuni}. This work may shed light on metabolic regulation of Fdh in a wide range of bacteria.

Data presented in chapter 2 showed FdhTU is a regulator of \textit{fdh}; however, exactly how FdhTU regulates \textit{fdh} is still unknown. Formate dehydrogenase subunit A (FdhA) contains a selenocysteine residue that is important for enzyme function and is likely incorporated into protein through the SelABCD system (264, 309). In a study concurrent to ours that also assessed the function of FdhTU, it was found that supplementation of media with excess selenium dioxide (selenium redox state +VI) could restore Fdh activity in a $\Delta fdhU$ mutant (247). That study reported that \textit{fdhT} encodes a selenium importer and \textit{fdhU} is involved in regulating \textit{fdh} expression in the absence of selenium (247). Incorporation of selenium into selenocysteine requires a complex biochemical pathway that involves incorporation of selenocysteine at the UGA codon downstream of a SECIS sequences (264). Selenocyteine synthesis requires selenide (selenium redox state –II), which is converted to selenophosphate by selenide water dikinase (\textit{cjj81176_1496}) before incorporation into selenocysteine (207, 264). Direct binding of selenium to FdhT or FdhU has not been demonstrated, and the redox state of selenium recognized by the selenium importer FdhT or by the regulatory element FdhU is unknown. How \textit{C. jejuni} reduces selenium dioxide (IV) to selenide (-II) is also unknown, although glutathione reductase and thioredoxin reductase have been implicated in reduction of selenium in \textit{E. coli} (164, 267). \textit{C. jejuni} lacks a glutathione reductase, but does have a thioredoxin reductase, and it would be interesting to determine its role in selenium reduction (121).

Domain architecture of the regulator FdhU found no DNA-binding domain but did find significant amino acid sequence identity to the RNA-binding protein TusA (108, 229). Studies have not determined how FdhU binds to RNA, what the RNA binding consensus sequence is, or how FdhU binding to selenium affects RNA binding. Determination of these characteristics could shed light on how \textit{C. jejuni} and other
bacteria regulate cytoplasmic selenium levels. Given that the \textit{fdhTU} operon is conserved in Gram
negative and Gram positive bacteria knowing how FdhU affects RNA stability in other bacterial systems
may provide information about metabolic regulation in those systems. In \textit{C. jejuni} FdhTU is restricted to
regulation of Fdh, which is the only known selenium-containing enzyme (229, 247). However, in other
organisms, selenium containing proteins include glycine reductase, NiFeSe hydrogenase, and
heterodisulfide reductase (264). Future studies should focus on if FdhTU regulates these proteins in
other bacterial species.

How Fdh affects recovery of \textit{C. jejuni} from host epithelial cells remains to be determined. There were no
previous reports in \textit{C. jejuni} that linked any genes to extracellular recovery after tissue culture infection
prior to the work presented in chapter 2. Since plating \textit{ΔfdhA} and \textit{ΔfdhTU} mutants on sulfite-containing
media allows recovery of \textit{ΔfdhA}+sulfite and \textit{ΔfdhTU}+sulfite to WT+sulfite levels, and since sulfite is
involved in neutralization of H$_2$O$_2$ when exiting epithelial cells, it is tempting to postulate that \textit{ΔfdhA}
is defective in resisting oxidative stress. However, this is contrary to data in chapter 4, which suggests that
Fdh enhances H$_2$O$_2$ production. Generation of endogenous ROS species is different than neutralization
of exogenous ROS, and electron flux from Fdh may be required to resist some of the destructive effects
of excess ROS species. This is consistent with a report that found that a \textit{ΔfdhA} mutant is more
susceptible to exogenously added H$_2$O$_2$ (127). Generation of enhanced membrane polarity or enhanced
generation of ATP is unlikely to account for Fdh mediated resistance to H$_2$O$_2$, since H$_2$O$_2$ is non-polar and
easily diffuses through membranes, and supplementation of recovery plates with other metabolites like
proline, malate, gluconate and formate did not enhance recovery of \textit{C. jejuni} from epithelial cells. If Fdh
is involved in resisting oxidative stress, then one would expect mutants in ROS scavenging genes like
alkyl hydrogen peroxidase (\textit{ΔahpC}) or catalase (\textit{ΔkatA}) to have recovery defects coming out of
Caco2epithelial cells that can be abolished by sulfite or catalase supplementation to the lysate. Mutants
in \textit{ΔkatA} and \textit{ΔsodB} were found to be defective for survival in multiple cell lines which may be due to a
recovery defect (49, 209). Such studies have not been undertaken and would be interesting to investigate in the future.

5.3: *C. jejuni* metabolism in infection and induction of necrosis

*C. jejuni* lacks classic virulence factors and must utilize fundamental properties such as metabolic adaptations, surface sugar modification, and motility to infect human hosts (15, 86, 213, 271, 306). The recent discovery that *fdh* and serine utilization are up-regulated in the sheep abortion-causing ‘SA’ *C. jejuni* strain further points to nutrient utilization in disease pathology severity (300). Understanding metabolic features that allow *C. jejuni* to invade and persist in human hosts may shed light on how other pathogenic bacterium persist in human hosts. As part of the work presented in chapter 3, we found that metabolic systems are not only important for epithelial cell infection, but also can alter pathogenesis-associated phenotypes such as cytokine secretion and induction of necrosis. This is analogous to a studies in *Salmonella* spp. in which TCA cycle mutants accumulated citrate, which caused enhanced induction of NOD-like receptor family, pyrin domain containing protein 3 (NLRP3) and pyroptosis-mediated cell death (301), and in *Helicobacter pylori* in which gamma glutamyl transpeptidase depleted host glutathione, also inducing epithelial cell death (71). As the *C. jejuni* metabolic systems investigated here are conserved in other bacteria, we feel that research described here will be relevant to other bacterial systems as well.

Work presented in this thesis show that inhibition of calpain activation can cause inhibition of *C. jejuni* induced host cell death linking *C. jejuni* infection to programmed necrosis. Not discussed in detail in chapter 3 is that a major target of activated calpain are cytoskeletal components such as α-fodrin and actin (161). If calpain is activated it can lead to defects in epithelial barrier function due to degradation of tight junction proteins (149, 153, 219, 255). A previous study had shown that prolonged incubation of *C. jejuni* with T84 cells causes breakdown of occludin protein in tight junctions leading to barrier
dysfunction in polarized epithelial cells (38). Investigation of the role of calpain activation in C. jejuni mediated degradation of occludin and tight junction degradation would yield vital knowledge of how intestinal barrier function is compromised during C. jejuni pathogenesis.

We found that host cell death was dependent on the production of ROS that could be inhibited by NOX inhibitors or by mitochondrial ROS scavengers. The mechanism of C. jejuni-induced accumulation of ROS in epithelial mitochondria to lethal levels is unknown; however, a Δpgp1 mutant was found to also cause enhanced epithelial cell necrosis compared to WT (not shown). Pgp1 was previously found by our group to modify peptidoglycan, and Δpgp1 peptidoglycan causes enhanced activation of Nod1 (75), which hints at a possible link between Nod activation and necrosis. Nods have been implicated in ROS production and NOX stimulation (158), which may account for why Δpgp1 causes enhanced T84 death. Nod1 and Nod2 stimulation by C. jejuni is important for C. jejuni-induced inflammation (308), although TLR-2 and TLR-4 have also been found to be mediators of C. jejuni inflammation and contributors to ROS production (74, 233, 262). It is unknown if Nods interact with MLKL and RIP3 of the necrosome (for reference please see Figure 1.5) to induce necroptosis, but TLR-4 has been shown to directly stimulate necrosis by interfacing with MLKL and Rip3 independent of Rip1 and the TNF-α receptor (122, 172, 295, 302). It would be interesting to investigate if ROS production is linked to TLR and Nod signaling, and if RIP1-independent oligomerization of MLKL and RIP3 are important for necrosis induction when infected with C. jejuni.

Consistent with the theme of ROS inducing necrosis, the common clinical IBD drugs 5-aminosialic acid (5-ASA) and azathioprine were also found to inhibit C. jejuni-induced host cell death (data not shown) without inhibiting C. jejuni invasion or intracellular survival (data not shown). Azathioprine inhibits Rac1, an important element in NOX activation (30, 185, 268, 276), and 5-ASA is thought to suppress or scavenge ROS in an unknown way (44, 230). The inhibition of C. jejuni induced necrosis with 5-ASA and
azathioprine further suggests that ROS generation is an important factor involved in necrosis. This has secondary implications, as the role of 5-ASA and azathioprine are traditionally thought to inhibit T-cells to restore intestinal homeostasis. Inhibition of C. jejuni-mediated host cell death in a T-cell free system argues that 5-ASA and Azathioprine may be also acting directly on intestinal epithelial cells to restore epithelial homeostasis. C. jejuni has been associated in relapse of inflammatory bowel disease and post-infectious irritable bowel syndrome (31, 256); understanding how to restore homeostasis after infection may help prevent GI complications.

The finding that a Δgdh mutant causes altered pathology in infected T84 epithelial cells was unexpected but reflects the complicated interplay between host and pathogens. Despite finding key elements that contribute to induction of necrosis in T84 cells, the reason Δgdh induces enhanced cell death still awaits discovery. One hypothesis relates to the fact that gluconate is an intermediate in the pentose phosphate pathway (PPP), which is important for production of purines, but is also a major source of NADPH in human cells (261). Cellular NADPH is vital for many different processes, including generation of O₂⁻ from the NADPH oxidases (or H₂O₂ in the case of NOX4) as well as supplying reducing power for glutathione reductase (150, 305). In this regard, the PPP is important for controlling redox state and ROS levels inside human cells. Infection with Δgdh may result in higher host NADPH levels in the host as compared to infection with WT C. jejuni due to decreased consumption of host gluconate. The elevated NADPH levels in a Δgdh infected host cell may lead to increased NOX activity resulting in higher ROS burden. This higher oxidative stress burden may account for the enhanced host cell death seen during infection with a Δgdh mutant. However, a larger pool of NADPH has also been associated with a larger pool of reduced glutathione which is protective against oxidative stress (53). Measurements of O₂⁻, reduced glutathione and NADPH levels in Δgdh verses WT infected cells may yield information on how Δgdh and the PPP affects host cell death.
The characteristic pathology initiated by *C. jejuni* during infection is due to the massive influx of neutrophils; however, *C. jejuni* must first disrupt the intestinal wall barrier to destroy intestinal homeostasis (38, 224, 297). Alterations in epithelial cell barrier function have been noted after *C. jejuni* infection, but not all the cellular changes have been completely investigated (38, 297). Induction of calpains and necrosis may play an important role in disruption of the intestinal epithelium and migration of *C. jejuni* to deeper tissues to induce gastroenteritis. Antibiotic therapy for *C. jejuni* is only effective during the initial stages of infection and only slightly reduces mean disease duration, suggesting that *C. jejuni* may be inducing runaway inflammation that becomes partially independent of the bacterium (273). This inflammation in susceptible people can cause manifestation of Guillain–Barré syndrome or relapse of IBD (171). Understanding steps during *C. jejuni* infection that lead to inflammation may help develop treatments to restore intestinal homeostasis more rapidly, or develop intervention for individuals with IBD who are at risk of relapse from *C. jejuni*.

### 5.4: *C. jejuni* produces ROS species in aerobic conditions

Previous work on *C. jejuni* oxidative stress has focused on specific proteins *C. jejuni* uses for detoxification of reactive oxygen species, as well as how it repairs oxidative damage (14, 18, 84, 231). Despite multiple adaptations to cope with oxidative damage, *C. jejuni* rapidly loses viability in aerobic conditions, suggesting it is producing ROS faster than it can scavenge them under aerobic oxygen. To date, no studies have shown which enzymes are responsible for ROS generation in *C. jejuni*. Despite the susceptibility to aerobic conditions, *C. jejuni* is still a major contaminant of food and water in aerobic conditions. Understanding how *C. jejuni* survives in the environment and how it succumbs to oxygen toxicity may shed some insights on control strategies.

To better understand why *C. jejuni* loses viability in oxidative environments, we set out to understand how *C. jejuni* metabolism affects ROS production. We showed that H₂O₂ production was dependent on
Fdh and Sor and that ΔfdhA survives better in aerobic conditions compared to WT. The redox active sites responsible for H₂O₂ production are still unknown. One likely candidate, fumarate reductase (MfrA), was shown by another group to survive better than WT in aerobic conditions, which suggests it may be a source of ROS (128). To identify novel sources of ROS, screening of flavin-containing enzymes as sources of ROS production would be a good approach, as they are often a source of ROS in E. coli and other organisms. C. jejuni flavodoxin, for example, may be a good candidate for H₂O₂ production due to having a solvent exposed flavin (259).

As mentioned in chapter 4, FdhA and SorA active site redox centers may not be the direct sources of ROS, but rather, electron flux through these systems may be important for generation of ROS. Electrons from Fdh pass through menaquinone as well as the terminal oxidases CcoNOQP and CydAB, whereas electrons from Sor pass electrons directly to cytochrome c without interfacing with menaquinone (115, 196, 213). Reduced menaquinone can be directly oxidized by O₂ to generate O₂⁻; it is usually only produced in low oxygen tension with ubiquinone taking its place under aerobic conditions in E. coli (142). As such, menaquinone reduced by Fdh may be a source of ROS when formate is added to the media. Unfortunately, menaquinone is essential in C. jejuni making direct studies difficult, but inhibition of the menaquinone-cytochrome bc₁ complex with the inhibitor HQNO may shed light on components upstream of menaquinone in production of ROS when incubated with formate. The other possible explanation is that redox centers in a terminal oxidase may be the source of ROS, enhances ROS production. The role of CydAB in ROS generation was mentioned in the discussion of chapter 4 and further analysis is needed to determine if it is a source of ROS production. Other groups have suggested that terminal oxidases are not a source of ROS; however, exceptions to this have been observed (111). Given the fact that environmental survival in oxygen is a major stress C. jejuni must cope with between hosts, a better understanding of systems that cause generation of ROS in C. jejuni may help reduce carriage of the bacterium on contaminated consumables.
5.5: Role of sulfur assimilation in aero-tolerance

Sulfur containing compounds like glutathione, mycothiol and ergothioneine play a major role in ROS detoxification (61). Many new sulfur containing compounds have since been discovered, and functional characterization is being undertaken (61). *C. jejuni*, however, lacks any known classical sulfur containing antioxidant like glutathione. The importance of Atps and sulfur homeostasis in aerobic survival may point to the existence of an as yet unknown glutathione-like molecule. Discovering how *C. jejuni* assimilates sulfur, and discovering if *C. jejuni* produces any novel sulfur containing molecules may shed light on how it survives in the environment under aerobic conditions.

Data in chapter 4 determined that the sulfur assimilation pathway is important for survival of *C. jejuni* in aerobic conditions in a manner independent of *de novo* cysteine synthesis. Two major questions remain: does *C. jejuni* have a functional PAPS reductase and sulfite reductase, and does *C. jejuni* contain sulfotransferases that can transfer sulfate from PAPS to a recipient compound (please see Figure 1.8 for reference). Homologues in these proteins could not be found in the *C. jejuni* genome. A previous study attempting to make a *C. jejuni* minimal media investigated the role of cysteine auxotrophy. In that study, it was found that sulfide (*H*₂*S*) could be supplemented to allow *C. jejuni* to grow in the absence of cysteine, but sulfite and sulfate could not restore growth (6). This suggests that *C. jejuni* may be lacking the sulfite reductase, and PAPS reductase and may rely on sulfide and thiosulfate as sulfur donors for CysM.

APS and PAPS have been previously found to be capable of transferring sulfur groups to other molecules such as lipids and tyrosine residues (82, 193, 249). It is possible that the role of the sulfur assimilation pathway is in generation of a novel ROS scavenger that fulfills the same role as glutathione or mycothiol. However, due to no observable defect in *H*₂*O*₂ removal in an Δatps background, it is questionable if Atps is involved in *H*₂*O*₂ detoxification. It is possible that the pathway may be involved in some other aspect
of aero-tolerance that is unknown, such as iron sulfur cluster repair. SILAC analysis using ‘heavy’ sulfite isotopes could help identify molecules that are labeled with heavy isotopes of sulfite. Such an approach was used to identify sulfur containing molecules in *M. tuberculosis* (192).

Sulfite is a common additive to foods as a preservative and it is secreted by neutrophils during gastroenteritis (167, 184). *C. jejuni* may be exposed to sulfite during infection or transmission, and assimilating this sulfite may be important for assisting with aero-tolerance. Understanding the sulfur assimilation pathway is important, and understanding its role may help understand and find novel intervention strategies.

**5.6: Final thoughts**

*C. jejuni* is one of the leading causes of bacterial gastroenteritis worldwide and is a cause of significant disease both due to primary infection as well as secondary complications. Unfortunately, how it causes disease remains an enigma due to challenges with genetic manipulation, and remains difficult to treat clinically due to its propensity to rapidly mutate to generate antibiotic resistance. I feel within this work interesting revelations have been identified about the biology of *C. jejuni* that may be applicable to other bacterial systems. Future work will need to be conducted to fully explore the phenomena described within. I feel that as a result of this work multiple avenues for future studies have been opened.
References


261. **Stanton, R. C.** 2012. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. IUBMB Life **64:**362-9.


### Appendix 1: Primer list

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## Appendix 2: List of plasmids

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<tr>
<th>Plasmid</th>
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<td>pGEM</td>
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<td>pRRK</td>
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<td><em>C. jejuni</em> rRNA spacer integration vector, chlorR ampR</td>
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### Appendix 3: List of strains

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