Effect of human fecal extracts on *Campylobacter jejuni* gene expression and pathogenesis

by

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Abstract

Campylobacter jejuni (*C. jejuni*), a zoonotic commensal that is pathogenic in humans, is one of the most common bacterial causes of food borne illness worldwide. To assess how *C. jejuni* responds to the metabolome of a commensal host (chickens) versus a disease susceptible host (humans), differences in gene expression was evaluated after *C. jejuni* exposure to cell-free extracts prepared from chicken cecal and human fecal matter. RNA sequencing identified 12 genes with >2 fold difference in expression when *C. jejuni* was exposed to human fecal extracts in comparison to chicken cecal extract. 10 of these genes appear to be involved in iron uptake, of which 7 (*CJJ81176_1649* to *1655*) were part of one iron uptake system. This system likely acquires chelated iron not recognized by other iron uptake systems since measurement of total iron content showed that human fecal extracts contained ~4.5X more iron than chicken cecal extract. Homologs of the CJJ81176_1649 to 1655 proteins were identified in alpha, epsilon and gamma proteobacteria, and mapping of the homologous proteins to representative bacterial genomes showed that gene order and operon structure were well preserved for homologs of the entire *CJJ81176_1649* to *1655* gene cluster. The widespread prevalence of the entire gene cluster putatively suggests that the proteins encoded by *CJJ81176_1649* to *1655* represent a complete iron uptake system. The CJJ81176_1649 iron transporter and the p19 (CJJ81176_1650) periplasmic iron binding proteins have been previously characterized, but the downstream genes have not been directly studied and functions are predicted by homology. Deletion of *CJJ81176_1651* to 1655 and the *overlapping CJJ81176*_1656 gene in this study rendered *C. jejuni* more sensitive to iron depletion than wild type, comparable to that of the *p19* mutant. Furthermore, this iron uptake system appears to be involved in adaptation to low pH, but at the cost of increased sensitivity to hydrogen peroxide stress. This work demonstrates that the heretofore understudied, but widely conserved, *CJJ81176_1649* to *1656* iron uptake system may be involved in host colonization by uptake of chelated iron more abundantly present in the human intestinal environment than that of the chicken cecum.

Preface

This thesis represents work conducted at the University of British Columbia in the department of Microbiology and Immunology by Dr. Erin Gaynor and Martha Liu. Dr. Erin Gaynor initially conceptualized the project and provided project guidance. I designed the project, obtained ethics approval, performed extract preparation, completed *C. jejuni* testing, prepared purified RNA, and created and characterized the deletion and complementation mutants.

Ethics approval for collection of human fecal material was granted by the UBC Clinical Research Ethics Board (CREB) under application H14-00859 - "Effect of human intestinal metabolites on *Campylobacter jejuni* gene expression and pathogenesis". The chicken cecal samples were supplied by Dr. Neil Ambrose of Sunrise Poultry Processors Ltd, and human fecal samples were donated by volunteers. Chicken cecal sample collection and processing was performed by me with the help of the Gaynor Lab. RNA sequencing was performed by the Wellcome Trust Sanger Institute (WTSI) in Hinxton, Cambridge UK. The WTSI sequencing group prepared and sequenced the RNA samples. I analyzed the RNA sequencing data with the help and training of Dr. Christine Boinett in Dr. Julian Parkhill's research group at the WTSI. Inductively coupled plasma mass spectrometry (ICP-MS) was performed by Mariko Ikehata at the lab of Dr. Michael Murphy at UBC.

This research is currently not published; however a manuscript based on data and writing in the thesis is planned.

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List of abbreviations

bp	Base pairs
cfu	Colony forming units
Cia	Campylobacter invasion antigens
CJJ	*Campylobacter jejuni* subspecies *jejuni*. Acronym used as prefix to denote gene number/locus tag/protein (e.g. *CJJ81176_1649*)
Cm	Chloramphenicol
CmR	Chloramphenicol resistance cassette
CP	Chicken pooled cecal extract
DFO	Desferroxamine
H1 to H11	Human fecal sample identifiers
HCl	Hydrochloric acid
HP1	Human fecal extracts, pool 1
HP2	Human fecal extracts, pool 2
HP3	Human fecal extracts, pool 3
ICP-MS	Inductively coupled plasma mass spectrometry
Ig	Immunoglobulin
Km	Kanamycin
KmR	Kanamycin resistance cassette
LB	Lysogeny Broth - Luria
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
OD_{600}	Optical Density at 600nm
Padj	Adjusted p-value of the fold-change calculation as determined by DESeq2
PBMC	Peripheral blood mononuclear cells
pRRC	*C. jejuni* integrative plasmid containing a Chloramphenicol resistance marker
rpm	Revolutions per minute
SCFA	Short chain fatty acid
T	Trimethoprim
TCA	Tricarboxylic acid or citric acid
V	Vancomycin

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To Science, for being so darn intriguing and confounding both at the same time.

And to my family, for trying so hard to understand what I studied.

1 Introduction

1.1 *Campylobacter jejuni*

1.1.1 General background

Campylobacter spp. are Gram-negative helical epsilon proteobacteria and are considered to be one of the most common causes of food-borne gastroenteritis worldwide in both developed and developing nations [1, 2]. In 2010, *Campylobacter* species caused an estimated 96 million illnesses worldwide [2]. The majority of human *Campylobacter* infections are caused by two *Campylobacter* species: *C. jejuni* (88%) and *C. coli* (9%) [3]. In Canada, *C. jejuni* was ranked as the top bacterial cause of foodborne illness between 2000 and 2010 [4]. In the United States, *C. jejuni* caused 1,088 hospitalizations and 9 deaths in 2014 alone [3].

C. jejuni zoonotically colonizes the digestive tracts of livestock, domestic and wild animals without causing disease [5, 6]. The incidence of *C. jejuni* colonization in livestock animals such as poultry, pigs and cattle can be as high as 90% [7, 8]. The incidence in domestic pets such as dogs and cats is generally lower and ranges between 4% - 25% [6, 9, 10]. The prevalence of *C. jejuni* in multiple environments may be caused by spreading through wild birds such as crows, pigeons, ducks and other small birds where incidence rates for *C. jejuni* has been observed to be as high as 40% [9, 11].

C. jejuni infection in humans is often associated with sporadic cases or localized outbreaks caused by ingestion of contaminated meat, particularly chicken, drinking contaminated unpasteurized milk or water, and improper animal handling [1, 12, 13]. Accidental ingestion with as little as a few hundred bacterial cells can cause severely debilitating disease in humans ranging from mild to bloody diarrhea, nausea, and vomiting [14]. The disease is generally self-limiting and does not require treatment other than rehydration; however immunocompromised people, people over the age of 60, and those with other illnesses may require hospitalization [15]. People showing severe symptoms are generally treated

with fluoroquinolone (e.g. ciprofloxacin) or macrolide (e.g. erythromycin) antibiotics. However, increasing rates of resistance to these antibiotics are being reported and represents a major public health concern [16].

1.1.2 *C. jejuni* growth and stress response

C. jejuni is thermotolerant and grows optimally between 37°C and 42°C in a capnophilic environment consisting of > 6% CO_2, and is commonly grown microaerobically (12% CO_2, 6% O_2) in the laboratory. These growth conditions are found in the microaerobic/anaerobic intestinal environment of *C. jejuni* hosts, where body temperatures range from 37°C in humans, ~38°C to 40°C in cows, cats, dogs, and pigs, and 41° to 43°C in chickens [17].

During transition from host to host, *C. jejuni* encounters cooler, aerobic environmental conditions and variable osmotic pressures. While *C. jejuni* does not grow under aerobic conditions or at temperatures lower than 30°C, it is capable of maintaining measureable viability for up to several weeks [18-20]. *C. jejuni* is sensitive to high osmotic pressure, and shows reduced growth rate in media containing 1% w/v sodium chloride (NaCl) and does not grow at all in media containing > 2% w/v NaCl [19, 21, 22]. Upon infection of a new host, *C. jejuni* must survive the acidic stomach transit and high bile concentrations of the stomach and small intestine. *C. jejuni* survives for a short time (< 60 minutes) in media at pH <3, maintains viability in media at pH 4, and grows in media at pH >5 [20, 23]. *C. jejuni* is also able to survive in media containing up to 5% w/v bile, or containing up to 1% of the bile salt sodium deoxycholate [24, 25]. These studies indicated that while *C. jejuni* growth is limited outside of the host intestinal environment, it is able to tolerate and survive in a wide variety of stresses encountered during host transition.

Transcriptomic characterization of *C. jejuni* responses to heat shock, acid, oxidative, osmotic and nitrosative stresses have shown moderate overlap in gene regulation. *C. jejuni* responded to heat shock at 42°C by changing the expression of a large number of genes, the most highly and rapidly upregulated

of which included genes encoding chaperones and heat shock proteins such as *groEL*, *groES*, *dnaK*, *dnaJ*, and *hspR* [26]. *C. jejuni* exposed to acid stress showed increased expression of heat shock genes such as *hrcA*, *dnaK*, g*roES*, and g*roEL*, oxidative response genes such as *katA* and *perR*, and genes involved in iron acquisition [27]. In two studies, the most highly upregulated genes upon acid stress were iron uptake genes, particularly of the iron uptake system encoding the p19 periplasmic protein [28, 29]. *C. jejuni* grown in the absence and presence of iron and then exposed to hydrogen peroxide stress showed differential expression of 25 and 26 genes respectively, 11 of which were common in both iron conditions [30]. Interestingly, oxidative stress genes such as *ahpC*, *katA* and *sodB*, the heat shock responsive gene *grpE*, and the iron responsive gene *p19* only showed comparatively higher expression under hydrogen peroxide stress when iron was present, however this was expected to be a result of already high expression of these genes when cells are grown under iron restricted conditions. *C. jejuni* exposed to high osmotic stress increased expression of heat shock (e.g. *hrcA*, *dnaK*, *groEL*) and oxidative stress genes (*sodB* and *katA*), as well as various other genes involved in amino acid and ATP synthesis [21]. *C. jejuni* exposed to nitric oxide stress led to increased expression of genes in the nitric oxide regulon (e.g. *nssR, cgb,* and *ctb*), heat shock genes such as *hrcA*, *grpE*, and *dnaK*, oxidative stress genes such as *trxA* and *trxB*, and multiple iron responsive genes, the most highly upregulated of which was *p19* [31]. These studies demonstrated that *C. jejuni* relies heavily on the heat shock, oxidative, and iron responsive response systems in order to adapt to different types of stresses during host transition.

1.1.3 Biofilm formation

Despite the sensitivity of *C. jejuni* to oxygenated environmental conditions, it is still commonly found in watersheds, on meat, and on the surfaces of produce [32, 33]. The prevalence of *C. jejuni* is in part attributed to its ability to form and survive within biofilms, which are able to protect *C. jejuni* from environmental stresses. In the lab, *C. jejuni* is able to form monospecies biofilms at the liquid air interface on multiple types of surfaces such as animal tissue, plastic, glass and stainless steel [34-36].

Some studies have shown that biofilm formation in *C. jejuni* is enhanced under aerobic or low nutrient stress [35, 37, 38], which supports biofilm formation as a stress survival mechanism. *C. jejuni* monospecies biofilms are chemically simple and unstructured [34], and consist of a mixture of extracellular DNA, proteins, lipids, and polysaccharides [39-41]. In nature, however, biofilms often consist of a mix of bacteria. Multiple studies have found that *C. jejuni* is better able to survive environmental stresses when grown in biofilms with other bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella enterica* [34, 42]. In these systems, scattered clumps of *C. jejuni* could be found within the biofilm structure, but *C. jejuni* was never the major constituent of the biofilm. It is likely that *C. jejuni* benefits from the structured shelter of the biofilm matrix and reduced oxygen environment created by these other aerobic bacteria [42]. As a result, *C. jejuni* residing in biofilms may be better able to survive environmental stresses and therefore enhance its chances of host infection and colonization.

1.1.4 *C. jejuni* metabolism

C. jejuni typically relies on uptake of amino acids, short chain fatty acids (SCFA), and citric acid (TCA) cycle intermediates as nutrients for growth. Most acutely, *C. jejuni* is known to use the amino acids serine, aspartate, glutamate, and proline, and the SCFAs acetate and lactate as sole carbon sources [43, 44]. The metabolism of such amino acids and SCFAs generates pyruvate, fumarate and oxaloacetate that are fed into the TCA cycle for energy production. *C. jejuni* can also directly import TCA cycle intermediates such as 2-oxoglutarate, succinate, fumarate, and malate using the KgtP, DcuA and DcuB transporters [43, 44]. *C. jejuni* is considered largely asaccharolytic because it does not encode transporters for common sugars like glucose and galactose, nor does it possess key glycolytic enzymes for carbohydrate metabolism [43]. However, approximately half of *C. jejuni* isolates are able to chemotax towards, bind, transport, and utilize fucose, a sugar found in food, mucin and cells, which may provide a growth and colonization advantage [45-47].

C. jejuni requires trace metals and micronutrients such as iron, molybdate, tungstate, copper, zinc, cobalt and nickel for cellular metabolism and host colonization. Iron is required for formation of iron-sulfur complexes, which are necessary for the function of key enzymes in *C. jejuni* growth [43]. Molybdate and tungstate are required for several key enzymes in *C. jejuni* respiration, including nitrate reductase, sulphite oxidase, SN oxide reductase, and formate dehydrogenase [48]. Bacteria also possesses many other metalloproteins which require magnesium, copper, zinc, nickel and other trace metals for protein stability or catalytic activity; however, these metalloproteins are not well characterized in *C. jejuni* [43, 49, 50].

1.2 *C. jejuni* host colonization

1.2.1 Colonization in chickens

The most common reservoir of *Campylobacter* is in poultry, particularly chickens. Chicks are hatched free of *Campylobacter* and remain *Campylobacter* free for the first few weeks likely due to the presence of maternal anti-*Campylobacter* antibodies [51, 52]. Chickens are exposed to *Campylobacter* through numerous avenues including contact with contaminated livestock, humans, wild birds, and pests, ingesting contaminated water or feed, or being exposed to contaminated bedding, water and air [53, 54]. Upon infection, *C. jejuni* colonize chickens ceca to concentrations as high as 10^{10} cfu/g cecal material without causing pathology [5, 55]. However, chickens do appear to mount an anti-*Campylobacter* immune response, and culturable *Campylobacter* has been found in various chicken organs including the spleen, lymph nodes, and liver without obvious damage to the host [55, 56]. Furthermore, the strength of the chicken immune response to *C. jejuni* does not appear to impact cecal colonization [55]. The high colonization rates and high tolerance that chickens have for *C. jejuni* make them one of the most common sources for human infection.

1.2.2 Pathogenesis in humans

Human infection can occur after ingestion of a few hundred *C. jejuni* cells. In one study, 5 out of 10 volunteers were infected after ingestion of 800 cells, and 6 out of 10 were infected after ingestion of 8000 cells [14]. However, the infective dose can vary depending on the *C. jejuni* strain. Additional experimental infection in human volunteers showed that increasing the infectious dose results in higher incidence of colonization, reduced time for disease progression, and increasing disease severity [57]. While some people remain asymptomatic upon exposure to *Campylobacter* colonization, many infected people show symptoms of Campylobacteriosis which include mild to severe diarrhea, bloody stool, nausea, vomiting, and malaise which can start as early as 17 hours after ingestion and last from days to weeks [4, 57, 58]. During this time, the intestinal tract, particularly the ileum and colon, is colonized by *Campylobacter* and there is an increase in inflammatory cytokines (particularly Interferon-γ) produced by peripheral blood mononuclear cells (PBMCs) [59]. After infection, humans develop an antibody mediated immune response where there is an increase in serum *C. jejuni* specific IgG, IgM, and IgA as early as a week after infection [57, 58]. However, any protective immunity to *C. jejuni* is short lived as re-challenge with the same strain of *C. jejuni* as early as 1-2 months after the initial infection often resulted in reinfection and disease progression [58, 59].

Long term sequelae have also been linked to *C. jejuni* infection. *C. jejuni* infection may lead to development of chronic inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, as well as colorectal cancer [60]. While the root cause is still unknown, these effects may be a result of dysregulation of the intestinal microbiome and host immune responses. *C. jejuni* has also been linked to the development of autoimmune inflammatory demyelination diseases such as Guillain-Barrè and Miller-Fisher syndromes, where antibodies against *C. jejuni* lipooligosaccharides also cross-react with and attack the structurally similar moieties present on human gangliosides [61]. Because of these

possible sequelae experimental infection of *C. jejuni* in humans has been rare and uses strains that do not synthesize the ganglioside mimics [62].

1.2.3 Virulence factors

Despite extensive study, one of the most enigmatic questions is still why *C. jejuni* causes disease in humans but not in other animals (e.g. chickens). *C. jejuni* possesses a multitude of factors which allow host colonization such as a polar flagella, secreted Campylobacter invasion antigens (Cia), helical cell shape, and cell surface adhesins, [63, 64]. The polar flagella enable motility, chemotaxis, and secretion of Cia into host cells. Multiple Cia proteins (e.g. CiaB, CiaC, CiaD, and CiaI) have been identified that are involved in host cell invasion and intracellular survival [65-69]. The helical shape allows efficient burrowing through viscous mucosa [70]. Cell surface adhesins such as CadF and FlpA bind fibronectin and allow attachment and invasion of host cells [65, 71]. Compromising these factors reduces the ability of *C. jejuni* to colonize intestinal epithelial cells *in vitro* and live chicks *in vivo*, however none have been directly correlated with onset or severity of diarrheal symptoms in humans.

C. jejuni also has a cytolethal distending toxin system (CdtA, CdtB, and CdtC) that impacts host cell division [72]. However, the complete CdtABC system is not present in all *C. jejuni* strains [73-75] with prevalence ranging from 39% to 89% depending on the species from which the *C. jejuni* was isolated. Furthermore, the presence of CdtABC did not correlate well with disease severity in humans [73].

The relatively small ~1.6 Mb sequenced genome lacks many of the virulence traits that are known to be important for bacterial pathogenesis such as pathogenicity islands and Type III secretion systems. However the best annotated *C. jejuni* genome (that for strain NCTC 11168) still contains a large number of unannotated genes (782/1572; 50% of the genome), and 192 genes (12% of the genome) are still designated with “hypothetical protein” with no known homology. Therefore, additional as yet unidentified virulence factors may be present that contribute to human pathogenesis.

1.3 Fecal microbiome and metabolome

1.3.1 Human large intestine and chicken cecal microbiome

The healthy human gut microbiome is mainly composed of two bacterial phyla, the Bacteroidetes and Firmicutes [76]. While the phylum level composition and abundance is relatively stable between individuals, the genus and species level compositions are highly variable both over time and across different human populations [77]. Efforts to identify a core human microbiome have been unsuccessful due to this high variability; however, some bacterial species, such as *Escherichia coli*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *Bacteroides uniformis* are commonly found in the majority of human samples [77].

Similar to the human gut microbiome, the cecal microbiome of boiler hens consist mostly of members from the Firmicutes, Bacteroidetes, and Proteobacteria phyla, and members of the genera *Clostridia*, *Lactobacillus*, *Faecalibacterium*, *Ruminococcus*, and *Bacteroides* are commonly identified [78-81]. However, the cecal microbiome composition and abundance at the genus level are also variable within and between studies. The chicken cecal microbiome is affected by multiple factors, including but not limited to chicken age, genotype, geographic location, feed composition (protein, fat and fiber levels), farming practice (cage vs. free-range, and clean vs. reused litter), and *C. jejuni* infection [78, 79, 81, 82].

While the bacterial genus and species identities of microbiota differ widely between populations , it has been suggested that the core functional profile of the microbiome remains relatively consistent between individuals of the same species [77]. In silico functional characterization of biochemical pathways putatively encoded by the human gut microbiome reveals that it is likely able to perform a large array of metabolic processes [83]. These bioinformatic studies suggest that the human microbiome encodes multiple pathways for amino acid metabolism, carbohydrate metabolism, lipid metabolism, and short chain fatty acid (SCFA) production (e.g. formate, acetate, propionate, butyrate etc.). The

microbiome also encodes numerous likely biosynthesis pathways for secondary metabolite and vitamin production, as well as degradation pathways for cellulose, bile, and halogenated aromatic compounds [83]. The intermediates and products of these metabolic processes would contribute to the diversity of extracellular metabolites that are utilized both by the microbiota as well as the host.

Similar biochemical pathways have also been identified through functional characterization of the chicken microbiome in comparison to the human microbiome [81, 84]. However, due to the different intestinal structure (ceca vs. large intestine), digesta transit (shorter transit time in chickens), and different diet between human and chickens, chicken microbiota are believed to be more adapted to utilize simple sugars and peptides, and to produce greater concentrations of SCFAs than the human microbiome [85]. These differences in the functional profile of the chicken and human microbiomes may contribute to differences in levels of intestinal metabolites.

1.3.2 Intestinal metabolome

Host intestinal systems are a complex mixture of food and food breakdown products, digestive products, the microbiome, and host defense systems. In addition, the microbiome also produces compounds which allow signalling and modulation of the community structure [86]. Thus, the non-cellular milieu of intestinal contents consists of a complex mix of thousands of metabolites [87-89]. These include metabolites from multiple classes such as lipids, amino acids, nucleotides, peptides, vitamins, carbohydrates and a large number of unnamed and unidentified molecules [90]. Some metabolites that are associated with bacterial metabolism include SCFAs (e.g. acetate, propionate, butyrate, and valerate), organic acids (e.g. benzoate, hippurate, phenylacetate, and phenylpropionate), and vitamins (B1, B2, B5, B8, B9, B12, niacin, and vitamin K) [91]. Metabolites associated with bacteria mediated transformation and breakdown include bile salt (e.g. cholate, deoxycholate, and hyocholate), polyphenol (e.g. hydroxycinnamic acids and flavonoids), lipids (e.g. glycerol), and various amino acids

[91]. Numerous fecal metabolomics studies comparing metabolite profiles after antibiotic treatment, and in association with intestinal pathologies such as inflammatory bowel disease, Chrone's disease, and colorectal cancer have shown that the fecal metabolome is a dynamic system that may respond to and contribute to intestinal health [88, 91-94]. The impact this complex mixture of metabolites has on members of the commensal microbiome or pathogens has been poorly studied. In one recent study, exposure of *Salmonella* to mouse and human intestinal metabolites was shown to cause an increase in the expression of genes related to metabolism, motility and chemotaxis, and a reduction in expression of genes involved in host cell invasion relative to broth-grown bacteria [87]. This study highlighted that the intestinal metabolites modulate bacterial gene expression, and that bacteria change patterns of gene expression to better adapt to host environments.

1.4 Objective of the present study

The sites of the highest levels of *C. jejuni* colonization in chicken and humans are the ceca and the human large intestine, respectively. The purpose of this study is to expose *C. jejuni* to sterile extract isolated from both of these locations in order to compare *C. jejuni* responses to the chemical composition of human vs. chicken intestinal environments. Human fecal extracts will be used as a proxy for the human large intestine metabolome in this study.

It is hypothesized that *C. jejuni* exposed to human fecal extracts will respond by altering expression of genes specifically required for human colonization or pathogenesis. In order to do this sterile, cell-free extracts from adult human feces and chicken cecal material will be collected, and *C. jejuni* physiological responses and gene expression differences will be measured after exposure to the extracts from the zoonotic host (chickens) versus the disease susceptible host (humans). The objective is to identify genes which have not yet been associated with *C. jejuni* colonization or pathogenesis in humans. Results showed that one poorly characterized, but highly conserved, iron uptake system was

more highly expressed when *C. jejuni* was exposed to media containing extracts versus media alone, and especially in human fecal extracts in comparison to chicken cecal extract. A deletion mutation was created of the putative inner membrane and periplasmic components of this iron uptake system, and *C. jejuni* growth, iron uptake, stress response, and antibiotic resistance was characterized.

2 Methods

2.1 Ethics statement

Written and informed consent was obtained from all human fecal sample donors as described in the ethics application H13-00859, which was approved by the University of British Columbia Clinical Research Ethics Board.

2.2 Bacterial strains and growth conditions

Campylobacter jejuni jejuni strain 81176, originally isolated from a Campylobacteriosis outbreak in 1985 [13], was grown in Mueller-Hinton (MH; Oxoid) broth or agar (1.5% w/v). MH was supplemented with antibiotics where noted and appropriate: vancomycin (V; 10 μg/mL), trimethoprim (T; 5 μg/mL), kanamycin (Km; 50 μg/mL), and chloramphenicol (Cm; 20 μg/mL). Agar plates and standing cultures were incubated at 38°C under microaerobic and capnophilic conditions (12% CO_2 and 6% O_2 in N_2) in a Sanyo tri-gas incubator (hereafter this growth condition - 12% CO_2 and 6% O_2 in N_2 - is referred to only as "microaerobic" for simplicity). Shaking broth cultures were incubated microaerobically in airtight containers with the Oxoid CampyGen Atmosphere Generation System at 38°C and shaken at 200 rpm. Growth rate experiments were performed by inoculation of log phase cells from 15 – 18 hour overnight shaken cultures into fresh media at an OD_{600} of 0.005 unless otherwise stated. Cell growth was assessed by making 10X serial dilutions in MH, drop plating 10 μL onto MH plates, incubating the plates microaerobically at 38 °C for 22 to 30 hours, and enumerating the number of colonies at the dilution containing ~10 to 100 colonies. The limit of detection for this plate count method is 10^3 cfu/mL.

Escherichia coli strain DH5α (F– Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsd*R17 (rK–, mK+) *phoA sup*E44 λ– *thi*-1 *gyrA96 relA1*; Invitrogen) was used for cloning. *E. coli* was grown aerobically at 37°C and shaken at 200rpm in lysogeny broth - Luria (LB; Sigma), or incubated aerobically at 37°C on

LB agar (1.5% w/v) supplemented with chloramphenicol (20 µg/mL) and kanamycin (50 µg/mL) for selection.

Deletion and complementation mutants of the *CJJ81176_1651* to *1656* genes in *C. jejuni* 81176 are prepared as detailed below. A *Δp19* deletion and complement ($p19^C$) were obtained from Dr. Anson Chan in Dr. Michael Murphy's lab at the University of British Columbia [49].

2.3 Chicken cecal sample collection

Chicken gut pouches were obtained from Sunrise Poultry Processors Ltd in Surrey, British Columbia in Jan-2015. The samples were taken straight off the processing line where the unbroken gut sacks (crop to anus) were removed intact from the recently slaughtered chickens. The gut sacks were severed between the gizzard and the duodenum, and the portion containing the ceca was packed in individual bags and transported immediately to UBC on ice. Cecal material was extracted by making an incision at the distal tip of each cecal pouch and transferring the cecal material directly into pre-weighed 50 mL tubes. The material from both cecal pouches per chicken was pooled and weighed, and extract was prepared as per Section 2.5 within 20 hours receiving the samples.

2.4 Human fecal sample collection

Human fecal samples were collected from 11 volunteers between Nov-2014 to Apr-2015. All volunteers were healthy adults aged between 22 and 51 (Figure 1A). There was a total of 6 males and 5 females. Fecal samples were collected using containers provided by the specimen collection system (Fisher Scientific) and refrigerated within 20 minutes of collection. Each sample was given a study identifier (H1 to H11). Extract was prepared as per Section 2.5 within 8 hours of receiving the fecal samples. Fecal material was transferred into pre-weighed 50 mL tubes prior to extract preparation.

2.5 Extract preparation

Chicken cecal and human fecal samples were weighed and diluted using sterile water at a ratio of 1:1 to 1:1.5 (w:v) depending on dryness and solidity of material in order to ensure consistency of final homogenate. 8-15 glass beads (4 mm diameter) were added to the material and the mixture was homogenized by vortexing at maximum speed. The homogenate was mixed by inversion at 4°C for 30 minutes. The tubes were centrifuged at 10,000g for 30 minutes and the supernatant was transferred to a new tube in order to separate out solids. Centrifuging was repeated 3X to 6X as necessary to remove solid materials and mucus. The resulting supernatant was filtered sequentially through 5 µm, 0.45 µm containing a 1 mm pre-filter, and 0.2 µm filters to sterilize. The sterile extract was frozen in 1 mL aliquots in a -20°C freezer. Samples were pooled as noted in Figure 1C. Some solid precipitate was observed in the human extracts after freeze thaw so the human fecal pools were filtered again through 0.2 µM filters. The pH of the extracts was measured using a pH meter (SB20, VWR). Sterility of pooled extracts were confirmed by no observable bacterial or fungal growth after inoculation of a drop of extract onto the surface of MH and LB plates and incubating them microaerobically at 38°C and aerobically at 37°C (respectively). Pooled extract was stored frozen at -20°C.

2.6 Biofilm assay

Overnight log phase *C. jejuni* were inoculated at an OD of 0.02 into 1 mL of MH-TV or MH-TV containing 10% extract in borosilicate glass tubes. The cultures were incubated microaerobically at 38°C without agitation. At 12, 24 and 36 hours the tubes were removed from incubation and 20 µL of culture 2 mm below the surface was diluted and plated for planktonic cell count. The cultures were then stained by addition of 250 µL crystal violet (1% in ethanol) and incubated at room temperature for 10 to 15 minutes. The tubes were rinsed with H_2O, dried overnight, and destained by adding 1.5 mL of destaining solution (30% methanol and 10% acetic acid in ethanol), vortexing the tube, and incubating for 24 hours.

The total amount of biofilm was quantified by measuring absorbance at 570nm using the Varioscan Flash Spectrophotometer (Thermo Scientific).

2.7 RNA extraction and sequencing

C. jejuni from 15 – 18 hour overnight cultures were inoculated into fresh MH-TV broth at an OD of 0.04 and incubated microaerobically shaking at 38°C for 4-5 hours in order to obtain optimal log growth. MH-TV + 30% extract was prepared by combining 0.5 mL of 2X MH-TV, 0.3 mL of extract (CP, HP1, HP2, or HP3), and 0.2 mL of sterile ultrapure H_2O (0.5 mL for the control MH-TV condition). The standardized *C. jejuni* was inoculated into 24 well plates containing 1mL of either the control MH-TV or MH-TV containing extract at an OD of 0.25 for the 20 minute conditions and 0.06 for the 5 hour conditions in order to ensure comparable numbers of cells during harvest and RNA preparation. RNA was extracted from a total of 20 different conditions: *C. jejuni* 81176 exposed to 5 different media conditions (MH-TV, MH-TV + 30% CP, MH-TV + 30% HP1, MH-TV + 30% HP2, and MH-TV + 30% HP3), collected at 2 different incubation times (20 minute and 5 hour), and performed in duplicate on two different days. RNA from all samples was extracted by adding 0.1 mL of 10X stop solution (5% phenol in ethanol) to the 1 mL cultures to stop transcription. The cells were pelleted by centrifuging at 11,000 rpm for 5-10 minutes, washed once with 1X stop solution, and resuspended in 50 µL of 0.4 mg/mL lysozyme in TE (10 mM Tris pH8, 1 mM EDTA). The mixture was incubated for 5 minutes before 950 µL of TRizol reagent (Ambion) was added and the mixture was vortexed at maximum speed for 1 minute. 200 µL of molecular biology grade chloroform (Fisher) was added to the mixtures, manually shaken for 15 seconds, incubated at room temperature for 2 minutes, and then spun at 12000 rpm for 15 minutes at 4°C. The aqueous phase was added to an equal volume of 70% EtOH and vortexed briefly before transferring the samples into RNeasy Mini columns (Qiagen). 350 µL of the RW1 buffer was used to wash each column prior to on on-column DNAse treatment (Qiagen) with 80 µL of RNAse-free DNAse in RDD buffer for 30 minutes to 1 hr. Following the incubation, 350 µL of the RW1 buffer was added to the

column and spun at 12K rpm for 15 s. Manufacturer's directions were used for the remaining steps as per the RNeasy Mini kit. RNA purity was verified by gel electrophoresis and measurement of A_{260}/A_{280} using the ND-1000 NanoDrop Spectrophotometer.

2.8 RNA sequencing data analysis

RNA samples were shipped to the Wellcome Trust Sanger Institute (WTSI) on dry ice. Sample preparation, RNA sequencing, mapping and annotation were performed at WTSI. Briefly, RNA purity was re-verified using the Agilent Bioanalyzer and samples were prepared for high throughput sequencing using the Illumina TruSeq sample preparation kit. The samples were run on the Illumina HiSeq 2500 system with >600 fold coverage. The raw Illumina reads were mapped and assembled onto the *C. jejuni* 81176 genome using the WTSI automated mapping and assembly pipeline. The resulting data reported the number of reads per gene for each of the 20 conditions. The mapped and annotated data was analyzed for fold change using the DESeq2 package in Rstudio. The raw read data was adjusted using the rlog transformation in order to compare genes with low read count. Differential gene expression was determined at 20 minute and 5 hour exposures for *C. jejuni* exposed to MH + human extract (HP1, HP2, and HP3) versus MH + chicken extract (CP) (i.e. human vs. chicken), and MH + extract (HP1, HP2, HP3, CP) versus MH alone (i.e. extract vs. MH) using the DESeq command.

2.9 Inductively coupled mass spectrometry (ICP-MS)

0.5 mL of each CP, HP1, HP2 and HP3 were added in duplicate into microcentrifuge tubes and dried overnight using a SpeedVac Concentrator (DNA 120; Thermo) at low power. Samples were tested at Dr. Michael Murphy's lab (UBC, Microbiology and Immunology) using ICP-MS. Briefly samples were digested in 1% nitric acid and heated in a closed Savillex vessel using a hot plate. 100ppb scandium (Sc) and indium (In) were added as internal standards. ICP-MS was conducted using the Perkin Elmer NexIon^{TM} 300D ICP-MS instrument.

2.10 Assessment of protein domains

The Simple Modular Architecture Research Tool (SMART) in Genomic mode was used to determine protein domains, signal peptides, and PFAM domains for the amino acid sequences of CJJ81176_1649 to 1656 (http://smart.embl.de/) in Sep-2016. SMART uses SignalP v4.0 for detection of the presence of signal peptides, and TMHMM v2.0 for detection of transmembrane domains [95].

2.11 Homology screening

Homologs of *CJJ81176_1649* to *1656* were identified using the NCBI online database with the blastn and blastp suites. The list of homologs returned all *Campylobacter* results. To identify homologs in non-*Campylobacter* organisms, the "*Campylobacter* (taxid: 194)" group was excluded. The resulting lists were screened by assessment of the resulting score and homolog coverage. Where results showed multiple species for one genus, one organism was selected for additional inspection. The genome for the representative strains per species were downloaded from NCBI between Sep-2016 and Oct-2016 and visualized using Artemis. The genomic regions for homologs of *CJJ81176_1649* to *1656* were identified by mapping the amino acid sequences from BLAST results to the selected organism and further inspection of the surrounding regions. Percent amino acid identity was determined by comparing the CJJ81176_1649 to 1656 protein sequences with the corresponding amino acid sequence for each organism using BLAST Needleman-Wunsch Global Align.

2.12 Creating the *Δ1651-1656* deletion mutants

The *Cjj81176_1651* to *1656* deletion *(Δ1651-1656*) was prepared using a modified Gibson assembly protocol with the NEBuilder HiFi DNA Assembly kit (NEB). 676 bases upstream of *CJJ81176_1651* and 535 bases downstream of *CJJ81176_1656* was PCR amplified using IProof DNA polymerase (BioRad) and primers ML1651u5' and ML1651gu3', and ML1655gd5' and ML1655d3' as shown in Table 1. Primers ML1651gu3' and ML1655gd5' were designed to have a complementary

overhang to allow attachment of the Km antibiotic cassette. The Km resistance cassette was amplified from the plasmid pRRK (obtained from J. Ketley) with IProof polymerase and primers MLkanRgu5' and MLkanRgd3'. The three PCR fragments were purified using the DNA Clean & Concentrator Kit (Zymo Research) and annealed as per manufacturer's directions (NEBuilder). The resulting annealed fragment was PCR amplified using primers ML1651u5' and ML1655d3' and purified. *C. jejuni* was naturally transformed using the PCR fragment. Briefly, 30 µL of the PCR DNA was mixed with a loop of *C. jejuni* cells from an overnight plate and incubated overnight on an MH plate before being spread plated onto MH-Km plates for selection. The genomic deletion was confirmed by PCR using primers ML1651u5' and ML1655d3'. The deletion strain was stored at -80°C in MH + 40% glycerol.

Table 1. List of primers used in this study.

Primer ID	Description	Primer Sequence	Restriction Site
For Deletion Mutant			
ML1651u5'	Upstream of *CJJ81176-1651*	GAACCGATTTTATGGCTTGG	N/A
ML1651gu3'	Upstream of *CJJ81176-1651* with sticky ends to attach KmR	GTCGACCTCGACTAGAACACTCGGAAAATCCGAGTGT AAAATCATTTTGGCGTGCCTGTG	N/A
MLkanRgu5'	KmR with upstream sticky end	GATTTTCCGAGTGTTCTAGTCGAGGTCGACGGTATCG ATAAGCTTGATATCGAATTCCTG	N/A
MLkanRgd3'	KmR with downstream sticky end	ATGGCACTTGAAAGGGAACTAGTGGATCCCGGCCTCA GGCACGCAAGCTTTTTAGACATC	N/A
ML1655gd5'	Downstream of *CJJ81176_1655* with sticky ends to attach KmR	GGGATCCACTAGTTCCCTTTCAAGTGCCATTGGGGAA ATATATGGAGTGCCTGTGCTTAG	N/A
ML1655d3'	Downstream of *CJJ81176-1655*	CTCACTCTTACGCAAGCTAAG	N/A
For Complement			
ML165116565'	Upstream of gene *CJJ81176-1651*	CATTCTAGAGCCATGTTGATGAAGAAACAG	*XbaI*
ML165116563'	Downstream of gene *CJJ81176-1656*	CATTCTAGAGATGGAAGCTATGAGCTTTATGG	*XbaI*

2.13 Creating the *1651-1656^{C}* complement

The *CJJ81176_1651* to *CJJ81176_1656* gene region was PCR amplified using IProof High Fidelity DNA Polymerase (BioRad) from *C. jejuni* 81176 using primers ML165116565' and ML165116563' (Table 1). The PCR product and plasmid pRRC, obtained from [96], was restriction digested using *XbaI* (NEB) and ligated overnight at 16°C using T4 DNA Ligase (NEB). The ligated product was incubated on ice with chemically competent *E. coli* DH5α for 20 minutes, heat shocked for 45s at 42°C, incubated at 37°C with LB for 1 hour, and then spread onto LB-Cm agar plate for colony selection after 24 hour incubation at 37°C. The presence of plasmid in the *E. coli* colonies was confirmed by colony PCR using primers ML165116565' and ML165116563', and the insertion was confirmed by sequencing. Plasmids were amplified from *E. coli* by inoculating 200 mL of LB with the transformed *E. coli* cells and incubating shaken at 37°C overnight. The plasmids were purified using the HiPure Plasmid Filter Midiprep Kit (Invitrogen) as per manufacturer's instructions. The plasmids were transformed into *C. jejuni Δ1651-1656* by incubating the cells with 15 µL of plasmid overnight on an MH plate incubated microaerobically at 38°C, then spread plating onto MH-Cm plates and incubating microaerobically at 38°C for 3 – 5 days. Complements were confirmed by preparing genomic DNA using the Promega DNA extraction kit and sequencing using primers ML165116565' and ML165116563'. Complements were stored at -80°C in MH + 40% glycerol.

2.14 Iron depletion and supplementation

Log phase 81176, *Δ1651-1656*, *1651-1656^{C}*, *Δ p19*, and *p19^{C}* from 15-18 hour overnight cultures were inoculated at 0.005 OD into 3 mL of control MH or MH containing 15 µM and 20 µM of the ferric iron chelator desferroxamine mesylate (DFO) or 100 µM of the supplement iron (III) citrate (Sigma). The cultures were incubated shaking microaerobically at 38°C. Cells were enumerated as described in Section 2.2 at 0, 12 and 24 hours in order to measure growth.

2.15 Hydrogen peroxide testing

Log phase 81176, *Δ1651-1656*, *1651-1656^{C}*, *Δ p19*, and *p19^{C}* from 15-18 hour overnight cultures were inoculated at 0.005 OD into 3 mL of MH and incubated shaking microaerobically at 38°C. After 6 hours growth, 3.7 µL of 3% H_2O_2 (Sigma) was added into the tubes for a final concentration of 1 mM H_2O_2 and returned to shaking incubation. Growth and survival were enumerated as described in Section 2.2 at 0, 6, 9, and 12 hours.

2.16 Acid stress testing

Acidic MH was made by adding 1 mL of 1 N HCl into 20 mL of MH, and the pH was measured using a pH meter (SB20, VWR). Log phase 81176, *Δ1651-1656*, *1651-1656^{C}*, *Δ p19*, and *p19^{C}* from 15-18 hour overnight cultures were inoculated at 0.005 OD into 3 mL of MH at pH 5.07 and incubated shaking microaerobically at 38°C. Growth was quantified as described in Section 2.2 at 0, 12, and 24 hours by plating for cell count.

2.17 Antibiotic screening

Initial screening for minimum inhibitory concentration (MIC) was performed by preparing doubling dilutions of streptomycin, dihydrostreptomycin, apramycin, chloramphenicol, ampicillin, polymyxin B, ciprofloxacin, and erythromycin from 0.125 µg/mL to 128 µg/mL and adding 100 µL into a 96 well plate. 100 µL of log phase *C. jejuni* 81176 and *Δ1651-1656* diluted to an OD_{600} of 0.04 was added to each plate and incubated for 48 hours at 38°C. The MIC was noted as the lowest antibiotic concentration at which no cell growth was visible.

Additional growth measurements in the presence of streptomycin was performed for wildtype 81176, *Δ1651-1656*, *1651-1656^{C}*, *Δ p19*, and *p19^{C}*. Streptomycin was prepared in MH and MH + 100 µM iron(III) citrate at 32 µM, 16 µM, 8 µM, 4 µM, 2 µM, 1 µM, 0.5 µM, 0.25 µM, 0.125 µM, and 0.0625 µM. 100 µL of each dilution was added to the wells of a 96 well plate. Log phase 81176, *Δ1651-1656*, *1651-*

1656^{C}, *Δ p19*, and *p19*^{C} from 15-18 hour overnight cultures were diluted in MH to an OD of 0.04 and 100 µL of the diluted cultures was added into the antibiotic plates. The final cell OD_{600} was 0.02 and the antibiotic concentrations were half of the prepared value. The plates were incubated at 38°C for 48 hours. The wells were mixed by pipetting and measured using the Varioscan Flash spectrophotometer OD_{600}.

2.18 Statistics

All data were analyzed and graphed using Microsoft Excel 2010 and Graphpad Prism 7. Statistical differences were calculated using Analysis of Variance (ANOVA) or the student's T test as indicated.

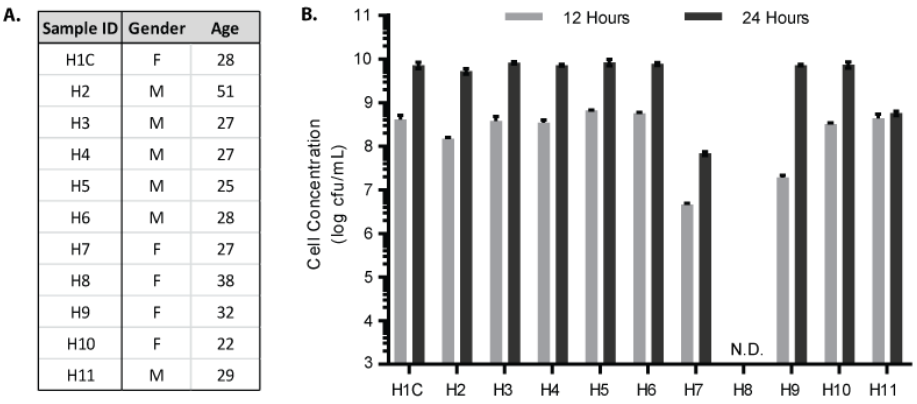
3 Results

3.1 *C. jejuni* response to human fecal and chicken cecal extracts

Sterile fecal extracts from 11 healthy human volunteers, 5 female and 6 male, were collected, homogenized with H_2O, filter sterilized, and stored frozen at -20°C between Nov-2014 and Apr-2015 (Figure 1A). These samples were sequentially designated H1C, H2-H11. Media (MH-TV) + 30% extract was selected as the *C. jejuni* exposure condition based on preliminary optimization screening studies. This condition represented the highest percentage of extract that could be supplemented into MH media without impeding *C. jejuni* logarithmic growth (data not shown). Growth of *C. jejuni* exposed to MH + 30% of each human fecal extracts was measured at 12 and 24 hours to assess consistency of *C. jejuni* viability in the different human fecal extracts (Figure 1B). *C. jejuni* showed comparable growth in the presence of extracts H1 – H6 and in H10. Growth in the presence of H9 and H11 appeared slower after 12 hours and 24 hours, respectively, however showed comparable levels of growth to most extracts at other time points. Extract H7 was found to visibly hinder *C. jejuni* growth both at 12 and 24 hours of incubation, and extract H8 completely killed *C. jejuni* after a 12 hour exposure. The cause of the reduced viability in these two extracts is unknown, but they were excluded from the remainder of the testing. The remaining 9 human extract samples were combined into 3 fecal extract pools consisting of an equal volume of extracts from 3 donors per pool (HP1, HP2, and HP3; Figure 1C). Chicken cecal extract was prepared from cecal material collected from 35 boiler hens in Jan-2015, and samples were pooled to create the chicken pooled extract (CP). The pH of CP, HP1, HP2, and HP3 pooled extracts were measured to be 7.59, 7.55, 7.60, and 7.44 respectively. Logarithmic growth of *C. jejuni* in MH media containing no extract, 30% chicken extract, and 30% pooled human extract (HP1, HP2, and HP3) was comparable for the first 12 hours after inoculation, with doubling times for all conditions ranging between 1.9 and 2.2 hours (Figure 1D). After 12 hours, *C. jejuni* in MH showed a typical drop in viability.

A.

Sample ID	Gender	Age
H1C	F	28
H2	M	51
H3	M	27
H4	M	27
H5	M	25
H6	M	28
H7	F	27
H8	F	38
H9	F	32
H10	F	22
H11	M	29



C.

Pool ID	Individual Samples			pH
CP	35 chicken cecal extracts			7.59
HP1	H2	H3	H5	7.55
HP2	H1C	H4	H6	7.60
HP3	H9	H10	H11	7.44

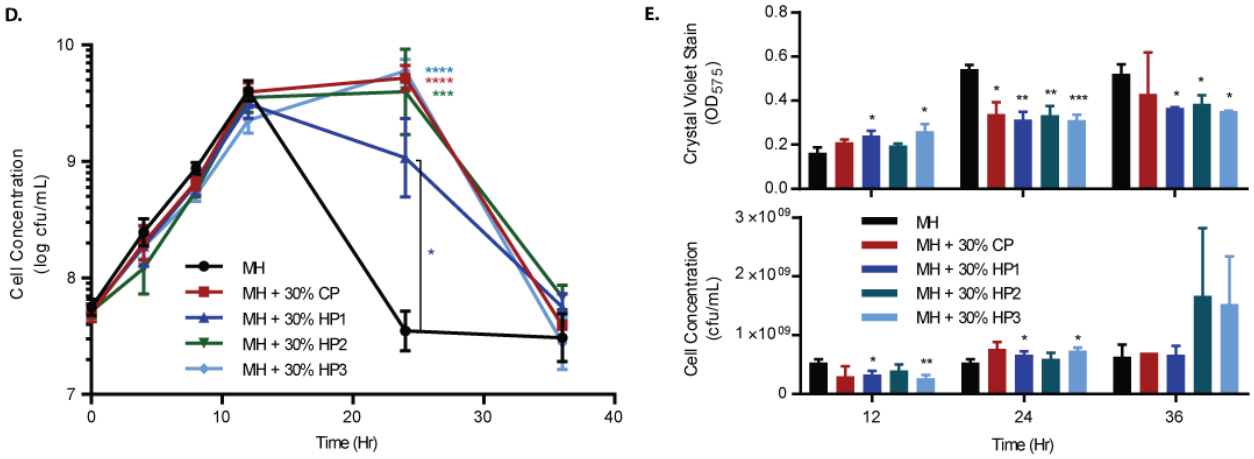


Figure 1. Response of *C. jejuni* to extracts

C. jejuni 81176 was exposed to human fecal and chicken cecal extract to evaluate viability, growth rate, and biofilm formation. (A) Sample ID (H= human, 1 – 11 = sequential collection number, C = third collection from this volunteer) with gender (F= female, M=male) and age information. (B) *C. jejuni* was screened for viability and growth in MH + 30% extract prepared from each human fecal sample. N.D. = Not Detected. Error bars represent standard deviation of 3 replicates. (C) The 35 chicken extracts were made into one pool and the 9 human extracts that best supported *C. jejuni* growth were assigned into 3 pools. (D) *C. jejuni* growth curve when exposed to MH, MH + 30% of the chicken pooled extract (CP), each of the 3 human pooled extracts (HP1, HP2, and HP3). (E) *C. jejuni* biofilm formation after 12, 24, and 36 hours as measured by the crystal violet assay (top) compared to the viable planktonic cell count in solution (bottom). Error bars represent standard deviation of 3 replicates. Statistical analysis for growth and biofilm formation was performed using the student's t-test with Welch's correction and compares MH + extract conditions (CP = red, HP1 = dark blue, HP2 = green, or HP3 = light blue) to the MH control (black) for each time point. P-value < 0.05 = *, p-value < 0.005 = **, p-value < 0.0005 = ***, p-value < 0.0001 = ****.

C. jejuni viability in MH containing both chicken and human fecal extracts remained significantly higher at 24 hours of growth before a drop in viability was observed at 36 hours.

Biofilm formation in 10% extract was comparable to that seen in 30% extract (optimization data not shown), thus to conserve the limited volume of extract, 10% extract was used for the biofilm assay. Biofilm formation for *C. jejuni* exposed to 10% chicken cecal or human fecal extracts was comparable to or slightly higher than the control MH condition after 12 hours incubation, however was notably lower after 24 hours (Figure 1E). After 36 hours, biofilm formation was still lower for *C. jejuni* exposed to human fecal extracts. The reduction in biofilm formation for *C. jejuni* exposed to extract was not caused by differences in cell density, in fact, at 36 hours there was a higher number of planktonic cells in the cells exposed to HP2 and HP3.

3.2 RNA sequence results

RNA was collected from *C. jejuni* exposed to MH only, MH + 30% CP, MH + 30% HP1, MH + 30% HP2, and MH + 30% HP3 after 20 minutes of exposure and after 5 hours of exposure for a total of 10 different conditions as shown in Table 2. This was performed twice to obtain duplicate replicates. The purpose of the short 20 minute exposure was to determine immediate, transient changes in transcriptional profile, and the long 5 hour exposure was to determine the adaptive homeostatic transcriptional profile. The differences in gene expression were calculated by grouping comparable treatments prior to assessment of fold change as shown in Table 2. For example, duplicates of *C. jejuni* exposed to MH + HP1, HP2 and HP3 extracts at 20 minutes (n=6) was used as one group for fold change comparative analysis. Grouping similar treatment conditions allows greater statistical power to find differences in gene expression between biological conditions by increasing the n-number per comparative and taking into account the variation within each assessment group. Genes with fold change > 2 and a p-value <0.05 are shown for the two different evaluation conditions: 1) human fecal

Table 2. Grouping of conditions for calculation of fold change

Exposure Time	Exposure Condition	Comparative Condition Groups	
		Extract vs. Media	Human vs. Chicken
20 Minutes	MH only	"Media"	*Not Used*
	MH only		
	MH + 30% CP	"Extract"	"Chicken"
	MH + 30% CP		
	MH + 30% HP1		"Human"
	MH + 30% HP1		
	MH + 30% HP2		
	MH + 30% HP2		
	MH + 30% HP3		
	MH + 30% HP3		
5 Hours	MH only	"Media"	*Not Used*
	MH only		
	MH + 30% CP	"Extract"	"Chicken"
	MH + 30% CP		
	MH + 30% HP1		"Human"
	MH + 30% HP1		
	MH + 30% HP2		
	MH + 30% HP2		
	MH + 30% HP3		
	MH + 30% HP3		

extracts versus chicken cecal extract (i.e. human vs. chicken) in Table 3, and 2) MH containing extract vs. MH alone (i.e. extract vs. media) in Table 4.

Comparison of *C. jejuni* exposed to human fecal extracts versus chicken cecal extract showed 2 genes with higher expression for *C. jejuni* exposed to human fecal extracts after 20 minutes, and 12 genes with higher expression after 5 hours (Table 3). There were no genes showing reduced expression in media with human compared to chicken extracts. The 2 genes with higher expression in human extracts after 20 minutes (*fdhT*: 2.42 fold, and *fdhU*: 2.88 fold) were even more elevated at 5 hours (6.44 and 3.35 fold higher respectively). FdhTU is involved in formate dehydrogenase activity and contributes to the invasion and intracellular survival of *C. jejuni* in intestinal epithelial cells [97, 98]. The remaining 10 genes that were more highly expressed after 5 hours appeared to be involved in iron uptake and/or

utilization. *cfbpA*, *ceuB*, and *chuC*, which were 2.78, 2.93, and 3.36 fold higher, are involved in uptake of chelated iron from transferrin, enterochelin, and haem respectively [99]. The remaining 7 genes (*CJJ81176_1649*, *p19*, *CJJ81176_1651*, *CJJ81176_1652*, *CJJ81176_1653*, *CJJ81176_1654*, and *CJJ81176_1655*) showed 2.51 to 2.85 fold higher expression and were consecutively organized. These genes encode a poorly studied iron uptake system that may recognize and transport iron bound by rhodotorulic acid, a fungal siderophore [99].

Table 3. *C. jejuni* genes showing > 2 fold difference in expression after 20 minute and 5 hour exposure to media containing human fecal extract versus media containing chicken cecal extract

Locus Tag	Gene Name	Gene Description	20 Minute		5 Hour	
			FC*	Padj**	FC*	Padj**
CJJ81176_0211	*cfbpA*	iron ABC transporter, periplasmic iron-binding protein			**2.78**	1.6E-02
CJJ81176_1351	*ceuB*	enterochelin ABC transporter, permease protein			**2.93**	1.6E-02
CJJ81176_1492	*fdhT*	membrane protein, putative	**2.88**	8.3E-03	**6.44**	1.3E-10
CJJ81176_1493	*fdgU*	conserved hypothetical protein	**2.42**	3.0E-02	**3.35**	9.0E-05
CJJ81176_1603	*chuC*	hemin ABC transporter, ATP-binding protein, putative			**3.36**	2.6E-02
CJJ81176_1649	*CJJ81176_1649*	iron permease			**2.71**	3.4E-03
CJJ81176_1650	*p19*	iron transporter			**2.85**	2.0E-03
CJJ81176_1651	*CJJ81176_1651*	membrane protein			**2.64**	7.9E-03
CJJ81176_1652	*CJJ81176_1652*	ABC transporter permease			**2.71**	7.9E-03
CJJ81176_1653	*CJJ81176_1653*	membrane protein			**2.68**	7.9E-03
CJJ81176_1654	*CJJ81176_1654*	GTPase			**2.54**	1.6E-02
CJJ81176_1655	*CJJ81176_1655*	thioredoxin			**2.51**	3.6E-02

*** FC = Fold change**

**** Padj = Adjusted p-value as output from the DESeq2 software**

Comparison of *C. jejuni* exposed to media with extracts (both human and chicken) vs. media alone showed 23 genes with higher expression and 18 genes with lower expression (Table 4). *CJJ81176_0438* and *0439* displayed the highest increase in gene expression both at 20 minutes (4.50 and 4.42 fold higher) and 5 hours (11.83 and 12.08 fold higher). These genes encode oxidoreductases, and the homologous genes in *C. jejuni* strain 11168 (*cj0414* and *cj0415*) were shown to be necessary for gluconate dehydrogenase (GADH) activity and optimal chick colonization [100]. Other genes that

Table 4. *C. jejuni* genes showing > 2 fold difference in expression after 20 minute and 5 hour exposure to media containing extract versus media only

Locus Tag	Gene Name	Gene Description	20 Minute		5 Hour	
			FC*	Padj**	FC*	Padj**
Genes with Increased Expression						
CJJ81176_0122	*aspA*	aspartate ammonia-lyase	**3.22**	6.1E-08	**2.53**	4.6E-05
CJJ81176_0123	*dcuA*	anaerobic C4-dicarboxylate membrane transporter DcuA	**2.94**	2.8E-09	**2.48**	1.3E-06
CJJ81176_0204	*CJJ81176_0204*	hypothetical protein	**2.87**	1.8E-12	**4.44**	4.0E-26
CJJ81176_0438	*CJJ81176_0438*	putative oxidoreductase subunit	**4.50**	2.0E-54	**11.83**	8.6E-149
CJJ81176_0439	*CJJ81176_0439*	oxidoreductase, putative	**4.42**	4.5E-28	**12.08**	2.7E-80
CJJ81176_0440	*CJJ81176_0440*	conserved hypothetical protein	**2.43**	1.7E-11	**2.15**	1.1E-08
CJJ81176_0697	*dcuB*	anaerobic C4-dicarboxylate membrane transporter DcuB	**2.09**	3.5E-08	**3.05**	4.1E-19
CJJ81176_0884	*CJJ81176_0884*	cytochrome c family protein, degenerate	**2.06**	2.5E-08	**2.94**	5.3E-19
CJJ81176_0885	*CJJ81176_0885*	cytochrome C	**2.39**	5.3E-10	**4.12**	3.2E-27
CJJ81176_1005	*CJJ81176_1005*	membrane protein, putative	**2.07**	1.1E-03		
CJJ81176_1389	*CJJ81176_1389*	DNA-binding protein	**2.08**	2.8E-05		
CJJ81176_1390	*CJJ81176_1390*	reactive intermediate/imine deaminase	**2.38**	3.9E-02		
CJJ81176_1391	*CJJ81176_1391*	C4-dicarboxylate ABC transporter	**2.47**	1.3E-02		
CJJ81176_1392	*metC*	cystathionine beta-lyase	**2.46**	6.8E-03		
CJJ81176_1393	*purB-2*	adenylosuccinate lyase	**2.19**	2.6E-02		
CJJ81176_1570	*CJJ81176_1570*	hypothetical protein			**2.39**	8.6E-03
CJJ81176_1649	*CJJ81176_1649*	iron permease			**3.23**	1.2E-04
CJJ81176_1650	*p19*	iron transporter			**3.80**	1.0E-05
CJJ81176_1651	*CJJ81176_1651*	membrane protein			**2.94**	9.1E-04
CJJ81176_1652	*CJJ81176_1652*	ABC transporter permease			**3.53**	6.0E-05
CJJ81176_1653	*CJJ81176_1653*	membrane protein			**3.94**	5.6E-06
CJJ81176_1654	*CJJ81176_1654*	GTPase			**4.28**	1.1E-06
CJJ81176_1655	*CJJ81176_1655*	thioredoxin			**4.17**	4.8E-06
Genes with Decreased Expression						
CJJ81176_0033	*gltB*	glutamate synthase			**-2.07**	5.1E-04
CJJ81176_0035	*gltD*	glutamate synthase subunit beta			**-2.12**	8.3E-31
CJJ81176_0109	*CJJ81176_0109*	methyl-accepting chemotaxis protein			**-2.37**	1.1E-03
CJJ81176_0266	*herA*	hemerythrin	**-2.09**	2.1E-02		
CJJ81176_0315	*peb3*	major antigenic peptide PEB3	**-3.02**	2.9E-06	**-6.21**	2.3E-17
CJJ81176_0580	*CJJ81176_0580*	C4-dicarboxylate ABC transporter			**-3.42**	5.8E-21
CJJ81176_0581	*CJJ81176_0581*	amidohydrolase			**-2.01**	3.0E-07
CJJ81176_0685	*CJJ81176_0685*	Major facilitator superfamily transporter			**-2.93**	1.8E-26
CJJ81176_0912	*CJJ81176_0912*	amino acid carrier protein			**-2.50**	1.9E-08
CJJ81176_0941	*CJJ81176_0941*	sodium:alanine symporter	**-2.46**	7.1E-07		
CJJ81176_0942	*CJJ81176_0942*	sodium:alanine symporter	**-2.56**	9.8E-07		
CJJ81176_1006	*CJJ81176_1006*	hypothetical protein			**-2.31**	1.4E-03
CJJ81176_1184	*CJJ81176_1184*	hypothetical protein			**-2.47**	3.6E-04
CJJ81176_1185	*CJJ81176_1185*	hypothetical protein			**-2.61**	9.4E-04
CJJ81176_1356	*CJJ81176_1356*	plasmid stabilization system protein, RelE/ParE family	**-2.18**	2.1E-02	**-2.05**	5.0E-02
CJJ81176_1386	*CJJ81176_1386*	conserved hypothetical protein	**-2.57**	4.4E-02		
CJJ81176_1656	*CJJ81176_1656*	thioredoxin	**-2.00**	2.9E-02		
CJJ81176_1657	*CJJ81176_1657*	hypothetical protein	**-2.65**	5.5E-31	**-2.70**	1.7E-32

*** FC = Fold change**

**** Padj = Adjusted p-value as output from the DESeq2 software**

showed higher expression either transiently and/or adaptively, encoded products responsible for uptake and utilization of food intermediates (*aspA*, *dcuA*, *dcuB*, *CJJ81176_1389* to *1393*), production of energy (*CJJ81176_0884* and *0885*), and again the *CJJ81176_1649* to *1655* iron uptake system. Genes with reduced expression in the presence of extracts included *peb3*, which encodes the major antigenic peptide, ion transporters (*CJJ81176_1685, 0941, 0942*), and glutamate synthase genes (*gltB* and *gltD*). These changes suggest that *C. jejuni* is responding to metabolites present in extracts in order to reduce antigenicity, and to defend against increased osmotic stress. There were also multiple hypothetical proteins with no predicted function showing reduced expression in the presence of extracts. Interestingly *CJJ81176_1656*, a thioredoxin which overlaps with *CJJ81176_1655*, showed 2 fold lower expression after exposure to extracts. Since the upstream *CJJ81176_1649* to *1655* genes showed 2.94 to 4.28 fold higher expression, this suggests that *CJJ81176_1656* is regulated differently than the upstream genes despite having overlapping open reading frames (ORFs).

Since *CJJ81176_1649* to *1655* was more highly expressed in extracts vs. MH, and even more highly elevated in human fecal extracts in comparison to chicken cecal extract, the focus of the remainder of this thesis is to further characterize this iron uptake system.

3.3 *CJJ81176_1649* to *1656*

The gene architecture of *CJJ81176_1649* to *1656*, as well as the genes upstream and downstream of this cluster, is shown in Figure 2. The amino acid sequences of the proteins encoded in this cluster were analyzed using the EMBL software Simple Modular Architecture Research Tool (SMART) to locate conserved domains and features Figure 2B. CJJ81176_1649, an FTR1 family iron permease, encodes an N-terminal signal peptide, 7 transmembrane regions, and the FTR1 iron transport domain. P19 has been previously characterized as a copper binding periplasmic iron transport protein [49]. CJJ81176_1651 to 1654 have not yet been studied in detail but are hypothesized to encode the putative inner membrane

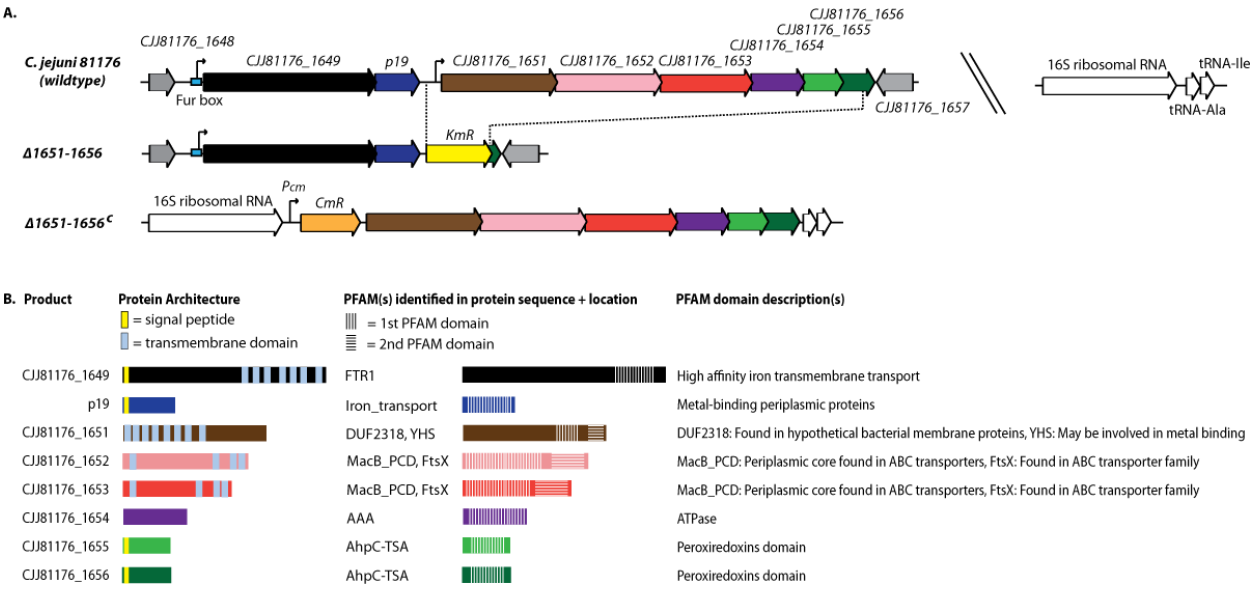


Figure 2. The *CJJ81176_1649* to *1656* gene cluster.

The genomic architecture of the *CJJ81176_1649* to *1656* genes and the domain homologies of the encoded protein products are shown to scale. (A) Top: the wildtype *CJJ81176_1649* to *1656* locus including the upstream and downstream gene as well as the 16s rRNA region. Middle: the mutant *Δ1651-1656* was made by deletion of all *CJJ81176_1651* to *1656* genes and insertion of a kanamycin resistance cassette (KmR). Bottom: the entire *CJJ81176_1651* to *1656* gene cluster was inserted into a non-coding region between the 16s rRNA and the tRNA-Ala to generate the complement *1651-1656*^{C}. The complement also contained a chloramphenicol resistance marker (CmR) under its native promoter (Pcm) (B) The presence of predicted signal peptides (yellow box), transmembrane domains (light blue box), and PFAM domains (vertical and horizontal white stripes), as well as the predicted PFAM domain functions are outlined for each gene in the *CJJ81176_1649* to *1656* cluster.

portion of this iron uptake system [99]. CJJ81176_1651 was not predicted to have a signal peptide but possesses multiple N-terminal transmembrane domains, and a C-terminal YHS domain which may be involved in metal binding and is most commonly associated with copper. CJJ81176_1652 and 1653 are predicted permeases that contain 4 predicted transmembrane regions and the MacB_PCD and FtsX PFAM domains which are found in the periplasmic ABC transporters. CJJ81176_1654 is a cytoplasmic ATPase, which hydrolyzes ATP to generate energy for this iron uptake system. Finally, CJJ81176_1655 and 1656 are thioredoxins, and the presence of N-terminal signal peptides suggest that they are

transported into the periplasm. *CJJ81176_1649* and *p19* have been previously studied [49], therefore the main focus of this study was to characterize the remainder of the gene cluster, *CJJ81176_1651* to *1656*. A deletion of the *CJJ81176_1651* to *1656* region in the wild type 81176 strain background was created by deletion/insertion with a Kanamycin (Km) resistance gene and was designated as *Δ1651-1656* (Figure 2A). A complemented strain, marked with a Chloramphenicol (Cm) resistance gene and designated as *1651-1656*^{C}, was made by recombination of the *CJJ81176_1651* to *1656* genes into an rRNA spacer region of the *C. jejuni* 81176 *Δ1651-1656* deletion strain. The *p19* deletion mutant (designated as *Δp19*) and complement (designated as *p19*^{C}) were obtained from Dr. Anson Chan and Dr. Michael Murphy [49], and were tested side by side with *Δ1651-1656* to compare observed responses.

3.4 Homologs of *CJJ81176_1651* to *1655* are widely distributed among multiple classes of proteobacteria

Homologs of the *Cjj81176_1649* to *1655* genes were previously reported to be conserved within in the *Campylobacter* genus and in a few non-*Campylobacter* species such as *Yersinia pestis* and some *E. coli* plasmids [101]. A BLAST search using the amino acid sequences of CJJ81176_1649 to 1655 showed that homologs of these proteins are more widely conserved than previously reported and is found in multiple members of alpha, gamma, and epsilon proteobacteria (Figure 3A and Appendix A). A list of 13 different genera from the 3 different bacterial classes identified to contain homologs *C. jejuni CJJ81176_1649* to *1656* is presented in Figure 3. The list is not exhaustive, and, with the exception of the *Campylobacter* genus, shows only a single representative species per genus. However, it should be noted that homologs were observed to be present in many, if not most, of the species in each bacterial genus. A thorough bioinformatics approach will be required in order to characterize the distribution of this iron uptake system in all bacteria.

Further inspection showed that the amino acid identities for protein homologs of CJJ81176_1649 to 1655 in different proteobacteria classes varied between 25% and 100% (Figure 3B). The periplasmic iron binding protein P19 and the ATPase CJJ81176_1654 appeared to be the most well conserved between different proteobacteria with >50% amino acid identity. This suggests that the structures or active sites on p19 and the ATPase are more important for protein function. Mapping the genomic locations of the identified homologous proteins revealed that the order, length, orientation and gaps between genes in the cluster were also conserved between different proteobacteria classes. Interestingly, homologs were not genomically coded for *Escherichia*, *Klebsiella*, or *Shigella*, however plasmids containing the homologs are frequently associated with these organisms. Furthermore, homologs of the CJJ81176_1649 to 1655 proteins and corresponding gene cluster were found encoded in the genome of *S. enterica* T000240, but not in other *Salmonella* species, due to integration of a plasmid into the T000240 genome [102]. The highly conserved nature of this iron uptake system found in the genome and plasmids of multiple classes of proteobacteria suggest that the genes work in combination for iron transport and that they form one interdependent system.

The last gene in the cluster, *CJJ81176_1656*, had homologs in epsilonproteobacteria but not alpha or gamma proteobacteria. *C. fetus* and *R.rubrum* did not appear to encode an eighth gene, whereas gammaproteobacteria appeared to encode either a cytochrome or a hypothetical protein as the last gene in the cluster. This suggested that the last gene, a thioredoxin for most epsilonproteobacteria, may not be critical for the function of this iron uptake system, or is perhaps more important for the epsilon class than the other proteobacteria shown.

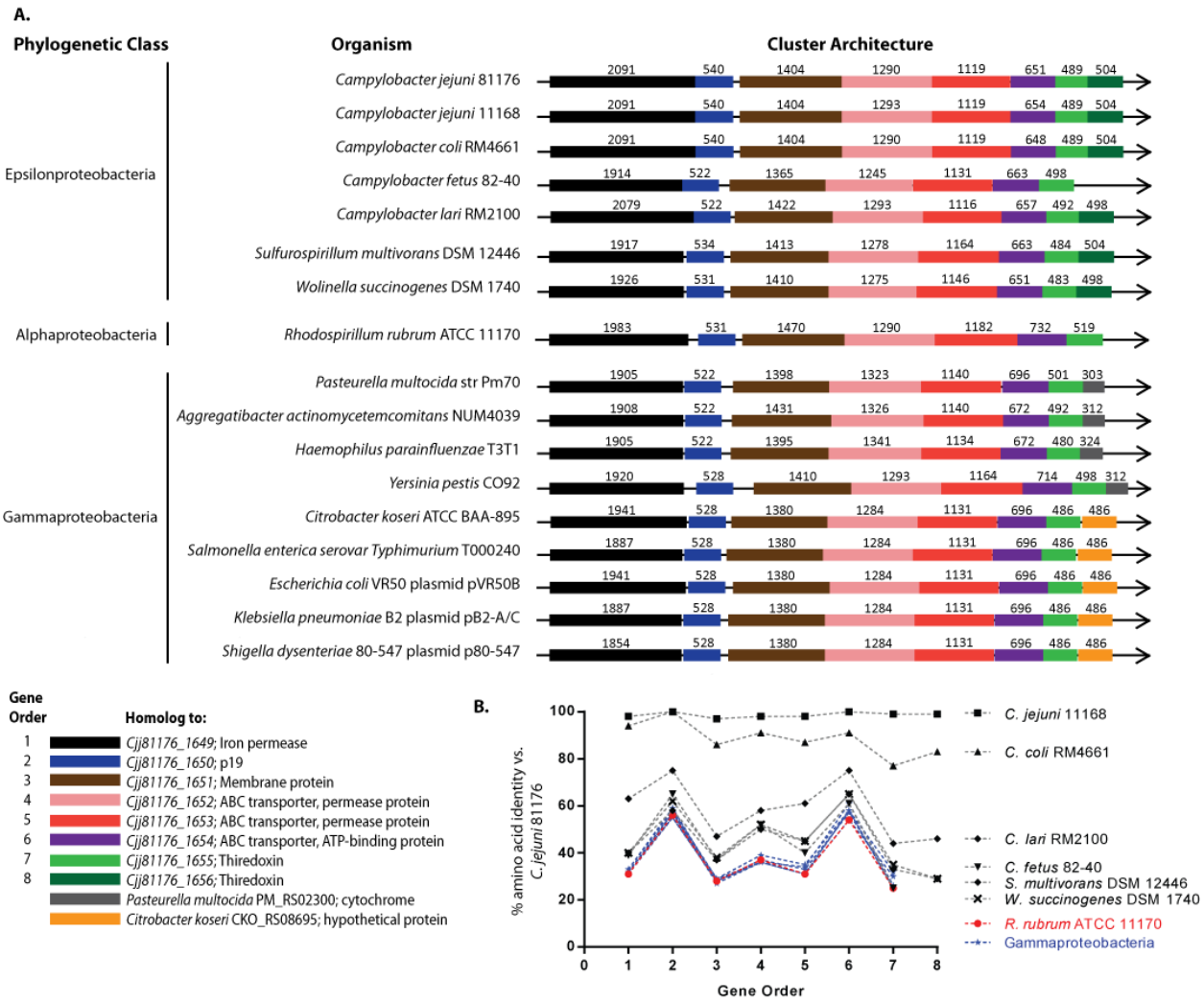


Figure 3. Homologs of the *CJJ81176_1649* to *1656* genes in Proteobacteria

Homologs of the *CJJ81176_1649* to *1656* genes were found in multiple members of the epsilon, alpha, and gamma proteobacteria. (A) The homologous gene clusters found in representative organisms from each genus are shown to scale. The key is shown below and to the left of the cluster diagrams. Genes with the same color represent homologs. (B) The % amino acid identity for each gene in comparison to CJJ81176_1649 to 1656. Black = epsilonproteobacteria, red = alphaproteobacteria, and blue = gammaproteobacteria. The % amino acid identities for all 9 gammaproteobacteria homologs are graphed but they overlap.

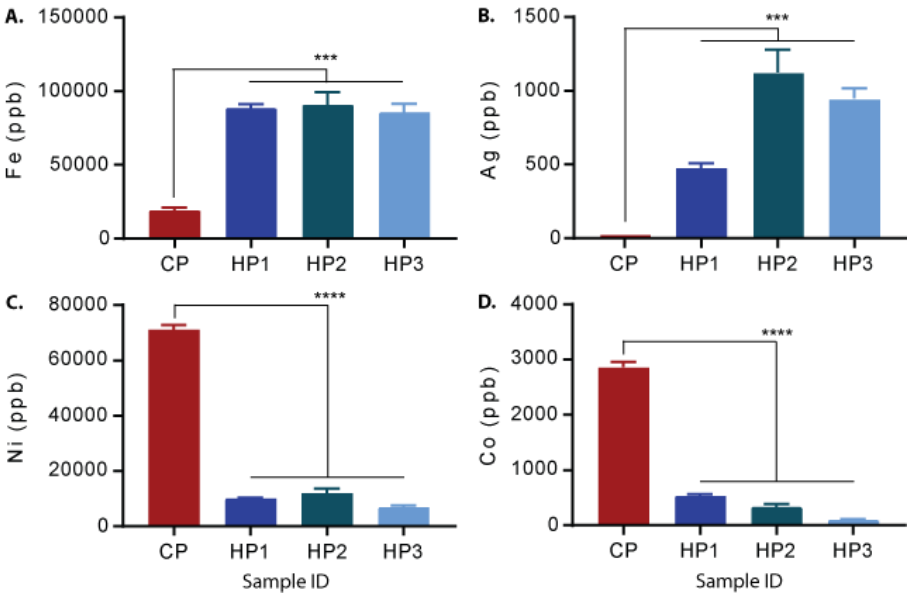


Figure 4. ICP-MS results

The amount of iron (A), silver (B), nickel (C), and cobalt (D) measured in parts per billion (ppb) present in the pooled chicken extract (CP) and the pooled human fecal extracts (HP1, HP2, and HP3). Error bars represent the standard deviation of 2 duplicate tests. The significance was calculated by ANOVA. P-value < 0.001 = ***, p-value < 0.0001 = ****.

3.5 Human fecal extracts contain more iron than chicken cecal extract

The increased expression of *CJJ81176_1649* to *1655* iron uptake genes when *C. jejuni* was exposed to human fecal extracts versus chicken cecal extract suggested that 1) there was less total iron in the human extracts, and/or that 2) there was more total iron, but it was sequestered by a chelator recognized specifically by this iron uptake system. Inductively coupled plasma pass spectrometry (ICP-MS) was used to test the total transition metals including iron concentration present in the extracts alone. The chicken pooled cecal extract and 3 human pooled fecal extracts were measured in duplicate. An analysis of variance showed that the amount of multiple metals in chicken cecal and human fecal extracts was significantly different: $F(3, 4) = 82.61$, $p = 0.0005$ for iron, $F(3, 4) = 66.07$, $p = 0.0007$ for silver, $F(3, 4) = 1206$, $p < 0.0001$ for nickel, and $F(3, 4) = 1064$, $p < 0.0001$ for cobalt. Interestingly, the human pooled fecal extracts contained more than 4 times more iron than the chicken cecal extract

(Figure 4A). This suggested that the *Cjj81176_1649* to *1656* system is involved in transport of chelated iron more abundantly found in human intestinal systems. Human fecal extracts also contained significant higher concentrations of silver (Ag) and lower cobalt (Co) and nickel (Ni) than chicken cecal extract (Figure 4B – D). These differences are likely caused by differences in diet and environmental exposure between chicken and humans, however the impact this that different concentrations of these trace elements would have on the intestinal microbiome and *C. jejuni* physiology is currently unknown.

3.6 *CJJ81176_1651 to 1656* are involved in iron acquisition

To test if *CJJ81176_1651* to *1656* is involved in iron acquisition, *Δ1651-1656* was grown in iron supplemented and iron depleted media to compare the growth kinetics with that of the *Δp19* mutant, which is involved in iron uptake [49]. The *Δp19* and *Δ1651-1656* mutants both exhibited slower growth rates in log phase than wildtype 81176 or the corresponding complements in MH medium (Figure 5A), but were able to achieve comparable maximum cell density after 32 hours. The $p19^{C}$ and $1651\text{-}1656^{C}$ complements showed wildtype growth kinetics in MH. The growth rate for each mutant could be restored to wildtype and complement levels by supplementation of MH medium with 100 µM iron(III) citrate (Figure 5B).

Depletion of iron with 15 µM and 20 µM desferroxamine (DFO), an iron chelator, resulted in reduced cell growth for the *Δp19* and *Δ1651-1656* mutants compared to wildtype and complements (Figure 5C and D). Furthermore, after 24 hours of incubation in iron depleted media, *Δp19* and *Δ1651-1656* showed reduced viability in comparison to wildtype and complemented strains. These growth kinetics suggest that the *Δ1651-1656* mutants were compromised in their ability to uptake and/or utilize iron present in the media. The iron sensitive growth of the *Δ1651-1656* deletion strain is consistent with previously reported *Δp19* results showing that *p19* is required for optimal growth and survival upon iron restriction [49].

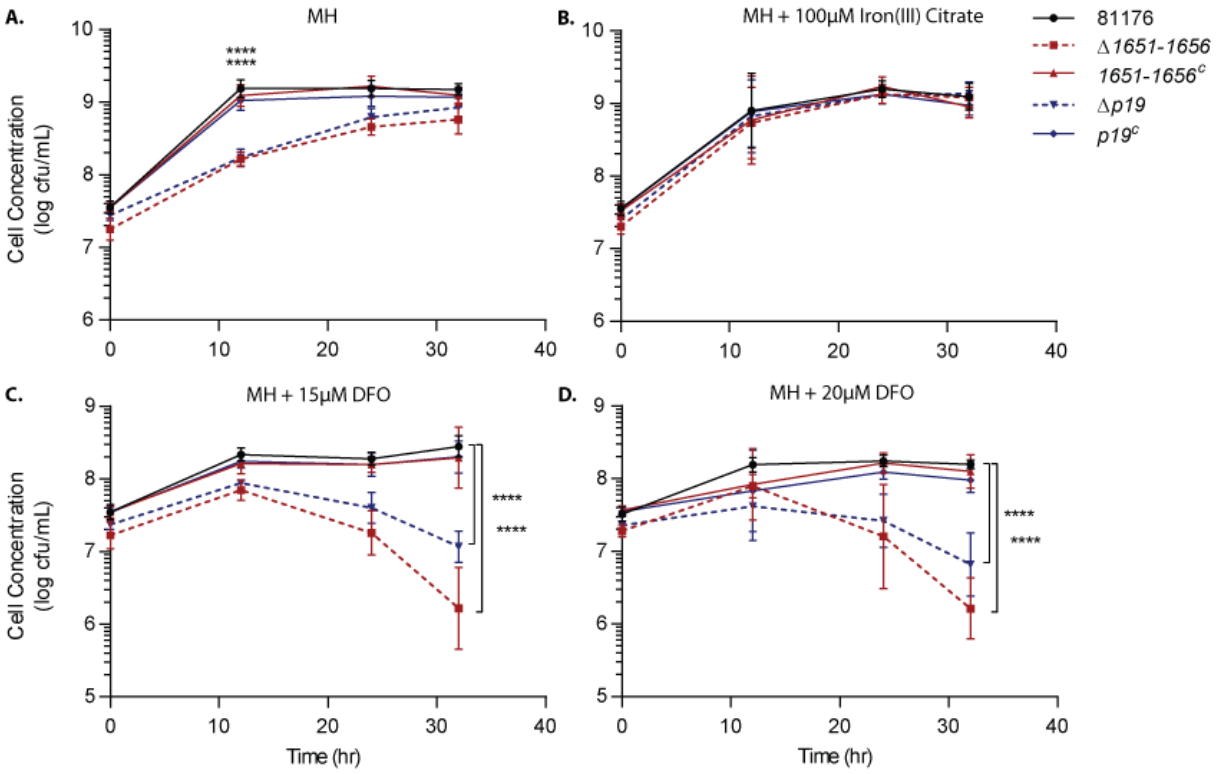


Figure 5. Growth in iron supplemented and iron depleted media

Growth of *C. jejuni* 81176 wildtype, *Δ1651-1656*, *1651-1656*^{C}, *Δp19*, and *p19*^{C} was measured in MH medium (A), iron supplemented MH containing 100 µM iron(III) citrate (B), and iron depleted MH containing 15 µM (C) and 20 µM desferroxamine (DFO) (D). Growth was tested by plate count at 0, 12, 24 and 32 hours after inoculation. Error bars represent standard deviation of 3 replicates. Statistical comparison of *Δ1651-1656* and *Δp19* vs. the wildtype control was performed using the student's t-test with Welch's correction where indicated. P-value < 0.0001 = ****.

3.7 The *Δ1651-1656* and *Δp19* mutant strains are more sensitive to low pH but are more resistant to hydrogen peroxide stresses

To determine whether *p19* and *CJJ81176_1651* to *1656* is directly involved in resistance to different types of environmental stress, the mutants were exposed to low pH (pH 5.07) and hydrogen peroxide (1.0 mM) stresses. The *Δp19* and *Δ1651-1656* mutants showed reduced growth in MH at pH 5.07 in comparison to MH at neutral pH, while the wildtype and complements were unaffected by low pH (Figure 6A, B and C). This reduced tolerance was observed in both regular MH media and MH containing 100 µM iron(III) citrate, which shows that the difference in response was not a result of

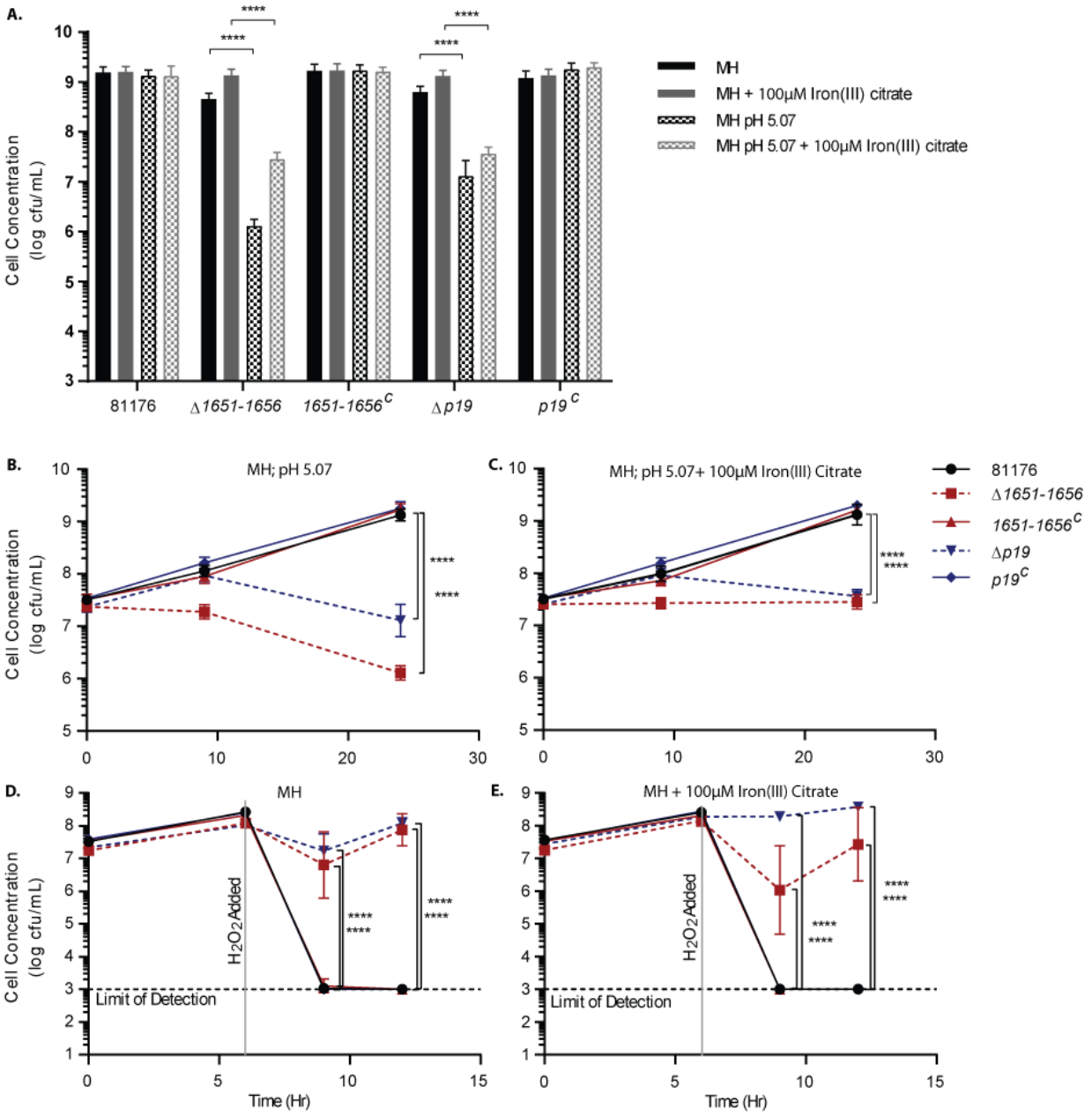


Figure 6. *C. jejuni* survival in low pH and H_2O_2 stress in MH and iron supplemented MH

The growth and survival of *C. jejuni* 81176 wildtype, *Δ1651-1656*, *1651-1656*^{C}, *Δp19*, and *p19*^{C} during exposure to acid (pH = 5.07) (A, B and C) and H_2O_2 (1mM) (D and E) stresses. (A) Comparison of *C. jejuni* growth with and without acidic stress after 24 hour incubation. (B and C) *C. jejuni* growth profile in pH 5.07 in unsupplemented MH (B) and MH supplemented with iron (III) citrate (C). (D and E) *C. jejuni* survival after exposure to hydrogen peroxide stress after 6 hours growth in MH alone (D) or MH supplemented with iron (III) citrate. The limit of detection for plate count measurements is 10^3. Error bars represent the standard deviation of 3 replicates. Statistical comparison of *Δ1651-1656* and *Δp19* to the wildtype 81176 was performed using the student's t-test with Welch's correction. P-value < 0.0001 = ****.

slower growth rate due to iron limitation. Interestingly, *Δp19* appeared to be able to tolerate the low pH up until 9 hours after exposure in both MH and iron supplemented MH but showed reduced cell growth after long term exposure. The reduced growth of *Δp19* and *Δ1651-1656* to low pH media shows that this system is involved in acid tolerance.

Exposure of *Δp19* and *Δ1651-1656* to 1 mM hydrogen peroxide showed that the mutants were more resistant to oxidative stress than wildtype or complements (Figure 6C and D). *Δp19* and *Δ1651-1656* remained viable 3 hours after addition of 1 mM H_2O_2 into log phase growing cells and had even higher cell concentration after 6 hours, whereas the cell counts for wildtype and corresponding complements dropped below the level of detection and remained undetectable for the remainder of the experiment. The resistance to oxidative cell death was observed for both cells grown in either unsupplemented MH or MH supplemented with 100 µM iron (III) citrate, which suggests that resistance to H_2O_2 is not caused by a slower growth rate or iron availability.

3.8 The *Δ1651-1656* and *Δp19* mutants lose streptomycin tolerance under iron limiting conditions

The *Δ1651-1656* mutant was screened for susceptibility to multiple classes of antimicrobial compounds, including aminoglycoside, amphenicol, beta lactam, cationic peptide, fluoroquinolone, and macrolide antibiotics (Figure 7A). Only 2-fold or no difference in minimum inhibitory concentration (MIC) was observed for all classes except aminoglycoside antibiotics. For instance, the MIC of *Δ1651-1656* was 8 times lower than that of the wildtype for streptomycin (2 µg/mL vs.16 µg/mL respectively), and 4 times lower than that of the wildtype for the chemically similar dihydrostreptomycin (2 µg/mL vs. 8 µg/mL).

Measurement of cell density showed that wildtype *C. jejuni* and complemented strains exhibited a bimodal growth phenotype in the presence of doubling dilutions of streptomycin in both MH alone and MH supplemented with 100 µM iron(III) citrate (Figure 7B). Growth of wildtype and complements

A.

Class	Antibiotic	MIC (ug/mL)	
		81176	*Δ1651-1656*
Aminoglycoside	Streptomycin	16	2
	Dihydrostreptomycin	8	2
	Apramycin	16	8
Amphenicol	Chloramphenicol	2	2
Beta lactam	Ampicillin	4	2
Cationic peptide	Polymyxin B	8	4
Fluoroquinolone	Ciprofloxacin	0.13	0.13
Macrolide	Erythromycin	0.5	0.5

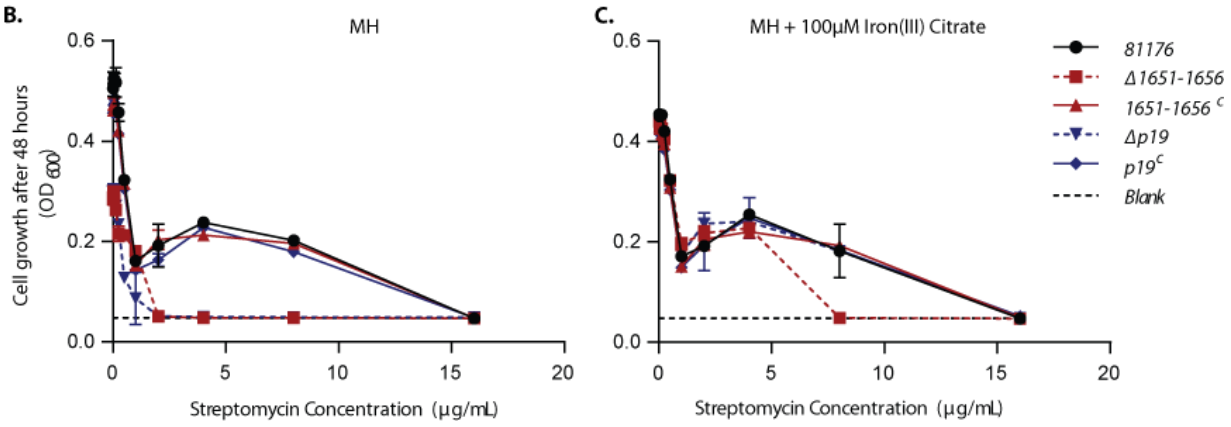


Figure 7. Antibiotic susceptibility testing

Antibiotic susceptibility of *C. jejuni* wildtype, deletion and complemented strains was measured by assessment of growth in the presence of doubling dilutions of antibiotics. (A) The minimum inhibitory concentration observed for *C. jejuni* 81176 and *Δ1651-1656* grown in multiple classes of antibiotics. Cell growth in doubling dilutions of streptomycin was measured for *C. jejuni* 81176 wildtype, *Δ1651-1656*, *1651-1656*^{C}, *Δp19*, and *p19*^{C} when grown in MH (B) and MH supplemented with 100 μM iron(III) citrate. Error bars represent the standard deviation of 3 replicates.

exhibited a dramatic reduction as streptomycin concentration increased from 0.13 μg/mL to 1 μg/mL, however, *C. jejuni* still grew as streptomycin concentrations increased from 1 μg/mL to 8 μg/mL. There even appeared to be more growth at 4 μg/mL in comparison to 1 μg/mL streptomycin in both iron limiting and iron supplemented media. This tolerance was not observed for *Δp19* or *Δ1651-1656*, which demonstrated a continuous reduction in growth as streptomycin concentration increased from 0.13 μg/mL to 2 μg/mL. Growth of *Δp19* and *Δ1651-1656* was not observed in MH containing ≥2 μg/mL

streptomycin. However, supplementation of MH with 100 µM iron(III) citrate restored the tolerance of *Δp19* and *Δ1651-1656* to 2-8 µg/mL streptomycin. These results suggest that the *CJJ81176_1649* to *1656* iron uptake system may be involved in *C. jejuni* tolerance of aminoglycoside antibiotic stress, especially streptomycin, under low iron conditions.

4 Discussion

In order to infect any host, *C. jejuni* must survive in the intestinal environment, which consists of food breakdown products, the resident microbiome, and a variety of host defenses. These are complex systems, and there have been many attempts to study the impact of individual components (e.g. bile, pH, oxidative stresses, etc.) on *C. jejuni* responses [25, 27, 31]. These studies have given us an insight on how *C. jejuni* copes with various stresses in isolation, but the results for individual stressors are difficult to translate for a complex intestinal system. There have also been attempts to study *C. jejuni* gene expression inside the chicken intestinal environment in comparison to *in-vitro* lab grown cells [103]. However, the genes showing the greatest change were responsible for adaptation to the vastly different oxidative environments. The recent success in mapping *Salmonella* gene expression changes during exposure to human intestinal metabolites [87] inspired us to use fecal extract as a method to directly compare the chemical composition of the human vs. chicken intestinal environments in a laboratory controlled setting. In this way, multiple exposure conditions could be controlled such as media richness, temperature, pH, and oxygen concentration, as well as *C. jejuni* growth phase and length of exposure to extract.

A review of extraction methods showed that aqueous solutions (H_2O, PBS, and saline), organic solvents (acetonitrile and ethyl acetate), and alcohol solutions (ethanol and methanol) have been used to extract sterile metabolites from feces [66, 67, 74]. Numerous aqueous and organic solvents and extraction techniques were screened in order to find a suitable extract condition. Initial trials showed that organic solvents extracted non-polar materials and oils from feces which did not dissolve readily in medium (data not shown). One additional consideration was that exposure to then removal of the organic and alcohol solutions from the extracts using the speedvac may denature active compounds. Therefore, aqueous solutions were chosen in order to preserve the activity of as many proteins and enzymes in the fecal extracts as possible. Fecal extraction was tested with PBS during initial trials, but

the salts present in PBS caused slower cell growth during initial viability testing in comparison to media alone (data not shown). This was not completely unexpected, since previous research showed that *C. jejuni* is sensitive to high osmotic stress [19, 21]. Therefore, sterile H_2O was chosen as the solvent for fecal extraction. In doing so, it is important to point out that H_2O extracts aqueous compounds from fecal material and that non-polar, organic, or hydrophobic compounds may not be represented. Since mucus and mucin homogenize readily in H_2O, but notably not in alcohols or organic solvents with lower density, the samples were very viscous and difficult to filter sterilize.

4.1 *C. jejuni* response to extracts

For this study, healthy human volunteers were defined as people who did not have chronic gastrointestinal disorders (such as inflammatory bowel disease, Celiac disease, Crohn's disease, or intestinal cancer), who were not taking drugs that may impact the intestinal microbiome (such as antibiotics or immune modifying drugs), and who had not had an episode of diarrhea within 5 days prior to donation. These exclusionary criteria were implemented to reduce variables that would disrupt the intestinal microbiome, or introduce drugs or antibodies that may kill *C. jejuni* in extract. Despite these restrictions, *C. jejuni* growth was visibly reduced in the presence of extract H7 and completely killed upon incubation with extract H8. The cause is unknown; however, may have resulted from diet, presence of anti-*Campylobacter* antibodies due to previous exposure, intestinal dysregulation, or any number of other factors. For example wine has been shown to have anti-*Campylobacter* activity [104], and beer has been shown to have anti-microbial activity [105]. This study did not control the diet in human volunteers; however, diet criteria may need to be a consideration for future studies involving bacterial responses to fecal extract.

Previously published work on *Salmonella* exposed to fecal extracts showed that cells exposed to extracts had the same logarithmic growth rate compared to the control condition without extract, but that the final concentration of cells in extract were lower than the control [87]. In this study, exposure of

C. jejuni to extracts did not impact *C. jejuni* logarithmic growth rate or maximum cell density (Figure 1D), however it allowed for a longer growth stationary phase and impaired long-term biofilm formation (Fig. 1D and E). The difference between the responses to extracts for *S. enterica* in the Antunes et.al. study [87] and *C. jejuni* in this study may have been caused at least in part by the different solvents used for extract preparation (ethyl acetate vs. water), which would have extracted different subsets of metabolites. The prolonged cell viability of wild type *C. jejuni* during stationary growth in the presence of extracts versus MH alone in this study may likewise have been caused by any of multiple factors, including (1) introduction of additional nutrients present in the extracts, (2) cell survival signals present in the extracts, and/or (3) triggering of general stress response(s). A reduction in biofilm formation for *C. jejuni* exposed to chicken cecal and human fecal extracts was also observed after 24 and 36 hours. It is currently unknown whether this represents active repression of *C. jejuni* biofilm formation by metabolites in the extracts, or passive repression due to introduction of additional nutrients which increased the richness of the media. Various studies have shown that *C. jejuni* forms better biofilms under nutrient limitation, and that biofilm formation is less abundant in rich media such as Brucella or Bolton broth [106]. The reduced long term biofilm formation in the presence of extracts may also represent elevated biofilm dispersion. The total biofilm quantified for *C. jejuni* in extracts at 12 hours was comparable or higher than the MH control, and showed comparable or lower planktonic cell concentration but the trend was reversed after 24 hours.

4.2 *C. jejuni* RNA sequencing results and iron uptake

Iron is an essential micronutrient for survival, and many living organisms produce high affinity iron chelating proteins in order to bind and uptake iron, as well as to sequester it for personal use using chelator specific iron uptake systems. Animals produce and secrete high affinity iron binding proteins such as haemoglobin, transferrin, lactoferrin and ferritin, and bacteria and fungi produce siderophores such as enterobactin and rhodotorulic acid. *C. jejuni* does not encode nor secrete its own siderophores,

but possesses at least 5 different systems that recognize and uptake iron from chelators produced by other organisms or the environment [99]. These systems transport iron from enterobactin (*CfrA, ceuBCDE*), haem (*chuABCDZ*), lactoferrin/transferrin (*ctuA, cfbpABC, chaN*), rhodotorulic acid (*CJJ81176_1649 to 1655*), and ferrous ions (*feoB*) and are under the direct regulation of the Fur repressor [99, 107]. Interestingly of the 12 genes showing higher expression in human extracts vs. chicken extract, 10 were involved in iron transport: *cfbpA*, *ceuB*, *chuC*, and *CJJ81176_1649* to *1655*. *CfbpA* encodes the periplasmic iron binding protein for the ferri-transferrin uptake system, *ceuB* encodes the periplasmic permease for the ferri-enterochelin uptake system, and *chuC* encodes part of the ABC transporter system for the haem uptake system. None of the other components of these iron uptake systems showed higher expression in human fecal extracts vs. chicken cecal extract. The only iron uptake system where all putative members showed higher expression was the *CJJ81176_1649* to *1655* system, consisting of an iron transporter (CJJ81176_1649), the periplasmic protein (CJJ81176_1650; p19), the putative inner membrane transporter proteins (CJJ81176_1651 to 1654), and a periplasmic thioredoxin (CJJ81176_1655). The specificity of increased expression of the *CJJ81176_1649* to *1655*, suggested that rather than a global response to low iron availability, the CJJ81176_1649 to 1655 system was specifically upregulated to obtain iron from a source that was more abundant in human fecal versus chicken cecal extracts. This was supported by measurement of the total iron present in human fecal and chicken cecal extracts, which showed that rather than having less iron, there was ~4.5x more total iron present in human fecal extracts. Furthermore previous transcriptomic study which showed that *C. jejuni* under general iron limitation nonspecifically increased expression of 27 genes involved in iron uptake, which included the majority of known members of the haem uptake system (*chuABCDZ*), ferri-transferrins uptake system (*ctuA, cfbpA, cfbpC*), enterobactin uptake system (*cfrA and ceuE*), *Cj1658* to *Cj1663* (homologs of *CJJ81176_1649* to *CJJ81176_1654*), and outer membrane energy transduction systems for iron transport (*exbB1-exbD1*, *exbB2-esbD2, tonB1, TonB2, and tonB3*) [108].

It is possible that the extra iron may be chelated to exogenous siderophore(s) which are recognized and utilized by the *C. jejuni CJJ81176_1649* to *1655* iron uptake system. It was claimed in 2008 that the *CJJ81176_1649* to *1656* iron uptake system recognizes iron associated with rhodotorulic acid, a fungal siderophore, but the data for that assertion have not yet been published [109]. Furthermore, an iron competition study showed that *C. jejuni*, *C. coli* and *C. laridis* were unable to grow using rhodotorulic acid [110]. However, in the unlikely possibility that rhodotorulic acid is recognized, this would suggest that there is a large community of yeasts and other fungi within the host intestinal microbiome, particularly in humans, which may contribute to pathogen success.

Upon assessment of genes more highly expressed in the presence of extracts vs. media alone, *CJJ81176_1649* to *1655* genes again showed higher expression but not genes associated with other iron uptake systems. This selectively higher expression of *CJJ81176_1649* to *1655* suggests a Fur independent upregulation of this iron uptake system. Other genes showing higher expression in extract vs. media alone appeared to be mainly responsible for allowing transport of food intermediates into the cell, energy production, and metabolism, which is likely a response to the additional nutrients added by the extracts [111]. This transcriptional response also supports the hypothesis that the prolonged cell viability during logarithmic growth when *C. jejuni* was exposed to extracts vs. in MH alone was due to the introduction of additional nutrients from extract.

4.3 The CJJ81176_1649 to 1656 iron uptake system

Inspection of the *CJJ81176_1649* to *1655* genes revealed an additional thioredoxin-encoding gene, *CJJ81176_1656*, which overlapped with *CJJ81176_1655* by 35 base pairs. Unlike *CJJ81176_1649* to *1655*, *CJJ81176_1656* was not more highly expressed in human fecal extracts vs. chicken cecal extract, and even showed a slight reduction in expression in extract vs. media alone. The amino acid sequence of *CJJ81176_1656* has low similarity to that of the *CJJ81176_1655* thioredoxin so is unlikely a gene duplication of *CJJ81176_1655.* It is unknown how this gene benefits the CJJ81176_1649 to 1655 iron

uptake system or how it is regulated differently than the upstream genes given the overlapping open reading frames (ORF). However, thioredoxins are necessary for oxidative protein folding, which is critical for maintaining protein stability and function, so there may be an advantage to having 2 periplasmic thioredoxins [112].

While homologs of the *CJJ81176_1649* to *1656* gene cluster were previously known to be conserved in *C. jejuni*, other *Campylobacter* species [109], and in *Yersinia pestis*, there has been minimal in depth study of these genes [101]. This thesis represents the first study identifying a high prevalence of homologs of the entire *CJJ81176_1649* to *1655* iron uptake cluster in multiple classes of alpha, gamma and epsilon proteobacteria. *CJJ81176_1656*, the last gene in the cluster, is conserved in epsilonproteobacteria but not in alpha or gamma proteobacteria. The high conservation of the entire gene cluster, with the exception of the last gene, in multiple classes of proteobacteria suggests that *CJJ81176_1649* to *1655* represents a complete iron transport system. A model of the iron uptake system is shown in Figure 8 based on the protein domains identified in Section 3.3.

The bacteria encoding this iron uptake system are found in widely different niches, which hints that this iron uptake system is important for iron acquisition under a variety of different conditions. For example, the alpha-proteobacterium *R. rubrum* is a phototrophic bacterium found in aquatic environments, the epsilon-proteobacterium *S. multivorans* is an anaerobic bacterium found in soils and water that have been polluted with chlorinated compounds, and the gamma-proteobacterium *P. multocida* is a zoonotic bacterium that colonizes the respiratory tract of wild and domesticated animals [113-115]. Since data supporting that CJJ81176_1649 to 1656 recognizes and uptakes iron bound to rhodotorulic acid has not yet been published, it is currently difficult to assess why this iron uptake system is so widely conserved in bacteria living in these diverse environments.

The observation that *C. jejuni* increased expression of this entire set of genes upon exposure to fecal extract, and even more so for human fecal extracts vs. chicken cecal extract, suggests that this iron

uptake system may be important in host colonization. Furthermore, while it appears that the *CJJ81176_1649* to *1656* cluster is not commonly genomically encoded in sequenced *Escherichia*, *Salmonella*, *Klebsiella*, or *Shigella*, plasmids containing the entire iron uptake cluster has been associated with each of these pathogens. Since plasmid encoded complete iron uptake systems are relatively rare, the presence of this system may provide these pathogenic organisms an advantage for host colonization [116].

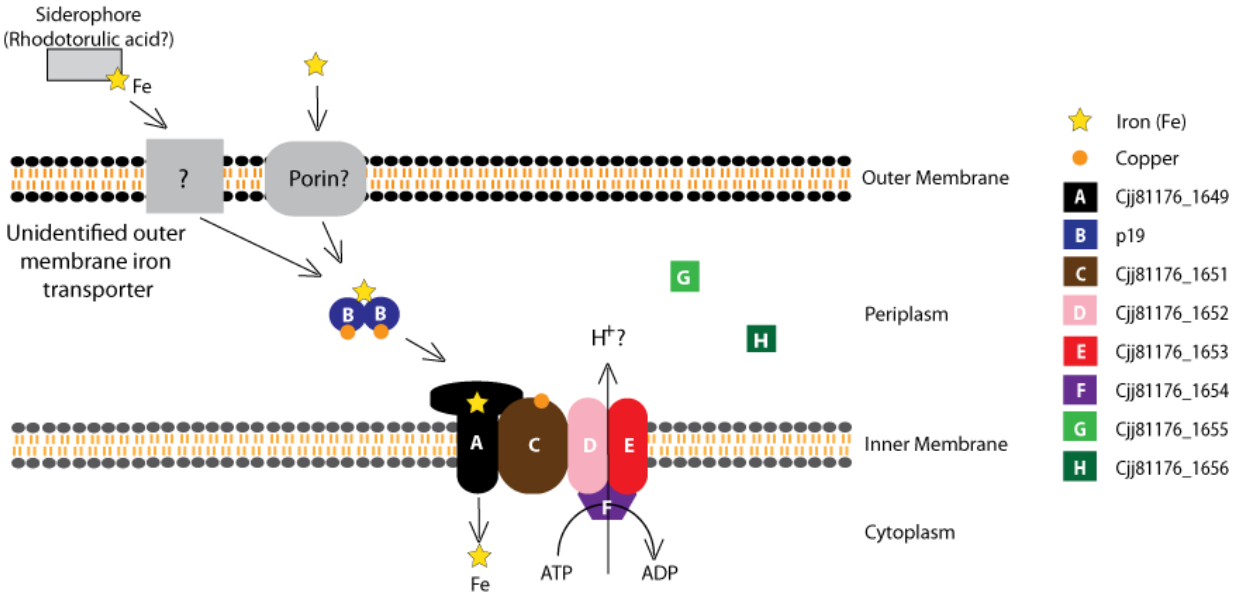


Figure 8. Putative model of the *CJJ81176_1649* to *1656* iron transport system

Iron is transported into the periplasmic space either by diffusion through outer membrane porins or through recognition of a siderophore via a yet unidentified outer membrane iron transporter. Iron inside the periplasmic space is recognized by the p19 homodimer, and pumped into the cytoplasm through CJJ81176_1649 coupled to the CJJ81176_1651-1654 inner membrane proteins. Copper ions bound to p19 and the YHS domain of the membrane protein CJJ81176_1651 likely aids in iron redox. The acid resistance provided by this iron uptake system could be caused by ATP dependent pumping of H^+ into the periplasmic space by the CJJ81176_1652 and 1653 permeases and the CJJ81176_1654 ATPase. The periplasmic thioredoxins prevent oxidative damage, are involved in protein refolding during iron transport, and may aid in iron redox.

Two transcriptomic studies have shown that p19 and the downstream genes of this iron uptake system (*CJJ81176_1651 to 1655*) have increased expression when *C. jejuni* are exposed to acid stress [28, 29]. Consistent with these observations, deletion of the *CJJ81176_1651* to *1656* genes resulted in a mutant that was unable to grow in media at a pH 5.07 even when iron was supplemented in the form of iron (III) citrate. While numerous transcriptomic and proteomic analyses have characterized the global changes in gene expression and changes in protein profiles when *C. jejuni* is exposed to low pH, mechanism(s) of acid tolerance in *C. jejuni* are still unknown. In contrast, *E. coli* has multiple well characterized mechanisms to adapt to acid stress, including strengthening the outer membrane, blocking outer membrane porins to prevent diffusion of H^+ ions, stabilizing cytoplasmic proteins, protecting DNA from damage, consuming H^+ in the cytoplasm, and transporting H^+ out of the cell using antiporters [117]. It is unknown how the *CJJ81176_1649* to *1656* membrane-associated iron transport system fits in with these previously described acid resistance mechanisms, however one possible method is through active ATP dependent transport of H^+ from the cytoplasm into the periplasmic space via the inner membrane permeases.

The transcriptomic profiles for *C. jejuni* upon exposure to either iron limitation or oxidative stress have consistently shown that they both induce a subset of genes related to both response systems, which suggests that they are closely linked together for stress tolerance [30, 118]. Therefore, it was surprising that deletion of either the periplasmic protein p19 or the putative inner membrane transporters and thioredoxins (*CJJ81176_1651* to *1656*) resulted in significantly increased tolerance to hydrogen peroxide stress. Perhaps the deletion of this iron uptake system caused cellular stress that resulted in elevated baseline expression of genes related to oxidative stress tolerance. This would be consistent with the previously reported elevation of oxidative stress genes (*katA, sodB, and ahpC)* in *C. jejuni* under iron limiting conditions [30]. However, the same resistance to hydrogen peroxide stress was observed even when the media was supplemented with iron (III) citrate, which should have alleviated

the low iron stress for deletion mutants since they are able to grow at wildtype rates. However, it may also be possible that the increased resistance to H_2O_2 stress is solely due to the deletion of the *CJJ81176_1655* and *CJJ81176_1656* periplasmic thioredoxins. In *E. coli,* deletion mutants of two thioredoxins (*trxA* and *trxC*) were more resistant to H_2O_2 stress than the wildtype control hypothetically due to higher sensitivity of the oxidative stress response system in mutants versus wildtype [119]. Another hypothesis is that deletion of iron binding proteins reduced conversion of hydrogen peroxide into free radicals via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$, and $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$) which may result in protein and DNA damage. In either case analysis of genes responsible for hydrogen peroxide resistance such as *katA* and *sodB* may reveal whether the deletion mutants have higher cytoplasmic resistance to sudden addition of hydrogen peroxide, or whether the presence of the *CJJ81176_1649* to *1655* system somehow renders the cells more sensitive to oxidative damage.

Lastly, it was observed that wildtype *C. jejuni* exhibited a bimodal streptomycin sensitivity phenotype. This bimodal antibiotic sensitivity has not been previously observed for *C. jejuni*, however, since most studies only assess MICs by visual observation, it is likely that this behavior is present but has been overlooked. Deletion of *p19* and *CJJ81176_1651* to *1656* showed that the growth tolerance for media containing 2 µg/mL to 8 µg/mL streptomycin was abolished only in the iron limited MH condition and not in MH supplemented with iron (III) citrate. It is unknown how this iron transport system may increase sensitivity of *C. jejuni* to streptomycin under iron limitation, but suggests that cellular iron concentration may play a role. In one study, iron restriction synergistically enhanced killing of *E. coli* with ampicillin, cefotaxime, chloramphenicol, methicillin and vancomycin antibiotics, and iron supplementation eliminated the synergistic effects [120]. The mechanism proposed by Wiuff et.al. was that iron limitation caused increased levels of iron sequestered per cell, which led to higher oxidative stress in *E. coli*, especially when treated with antibiotics. Whether or not this is the case in *C. jejuni* remains to be tested.

5 Conclusion and future directions

This work demonstrated that the *CJJ81176_1649* to *1656* system, which is found in multiple classes of proteobacteria, was the only *C. jejuni* iron uptake system where all putative members were more highly expressed upon exposure to human fecal and chicken cecal extract. Importantly, this system was even more highly expressed in human fecal extracts in comparison to chicken cecal extract, which suggested it may be especially important for human colonization. However, this iron uptake system has not been studied in detail partly because of limited knowledge of homologs in other organisms and because, despite being widely encoded in multiple plasmids, it is not encoded on the genomes of the more well-studied bacteria such as *Escherichia*, *Salmonella*, or *Shigella* spp. The deletion of *CJJ81176_1651* to *1656* in this study is the first time that the putative inner membrane transporters of this iron uptake system have been analyzed in any organism. Results showed that *CJJ81176_1651* to *1656* is required for optimal growth under iron limiting conditions comparable to what has been previously reported for *p19*, and that the CJJ81176_1649 to 1656 system is required for acid stress tolerance.

This study opens the door to further evaluation of this iron uptake system, and much more work is required to understand the structures, interactions, and mechanisms behind how iron is transported and how this system is involved in acid tolerance. One outstanding task is to experimentally identify what siderophore(s) are recognized by this iron uptake system, and/or test whether Rhodotorulic acid is the substrate. Individual gene deletions will also be needed to understand the role of each of the 8 members of this iron uptake cluster. A plan for making the individual deletions is included in Appendix B. Evaluation of specific domains, such as the YHS metal binding domain of the inner membrane protein CJJ81176_1651, will aid in understanding the processes involved in iron transport. Better understanding of this iron uptake system will increase our understanding of *C. jejuni* physiology, and will lead to an

overall increased knowledge for why this iron uptake system is so widely conserved in multiple classes of proteobacteria.

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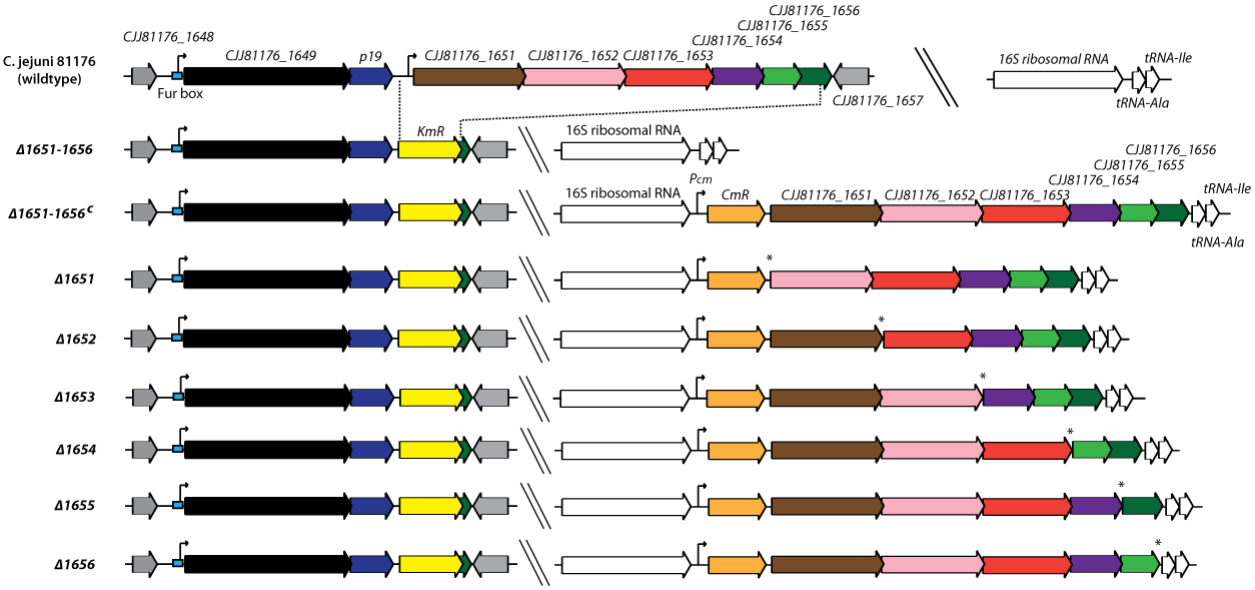
Appendices

Appendix A: Homolog list of the *Cjj81176_1649* to *1656* cluster in other proteobacteria

Class	Organism	Order in cluster	Gene ID	Annotated Protein Product	Gene Length (# base pairs)	% amino acid identity to *C. jejuni* 81176 proteins
Epsilonproteobacteria	*Campylobacter jejuni 81176*	1	*CJJ81176_1649*	FTR1 family iron permease	2091	Reference for 1st protein
		2	*CJJ81176_1650*	hypothetical protein	540	Reference for 2nd protein
		3	*CJJ81176_1651*	hypothetical protein	1404	Reference for 3rd protein
		4	*CJJ81176_1652*	ABC transporter, permease protein	1290	Reference for 4th protein
		5	*CJJ81176_1653*	ABC transporter, permease protein	1119	Reference for 5th protein
		6	*CJJ81176_1654*	ABC transporter, ATP-binding protein	651	Reference for 6th protein
		7	*CJJ81176_1655*	thiredoxin,-like protein	489	Reference for 7th protein
		8	*CJJ81176_1656*	thioredoxin family protein	504	Reference for 8th protein
	Campylobacter jejuni 11168	1	*Cj1658*	putative iron permease	2091	98
		2	*p19*	periplasmic protein p19	540	100
		3	*Cj1660*	putative integral membrane protein	1404	97
		4	*Cj1661*	possible ABC transport system permease	1293	98
		5	*Cj1662*	putative integral membrane protein	1119	98
		6	*Cj1663*	putative ABC transport system ATP-binding protein	654	100
		7	*Cj1664*	putative periplasmic thiredoxin	489	99
		8	*Cj1665*	putative lipoprotein thiredoxin	504	99
	Campylobacter coli RM4661	1	*YSS_RS00485*	iron permease	2091	94
		2	*YSS_RS00480*	iron transporter	540	100
		3	*YSS_RS00475*	membrane protein	1404	86
		4	*YSS_RS00470*	ABC transporter permease	1290	91
		5	*YSS_RS00465*	membrane protein	1119	87
		6	*YSS_RS00460*	GTPase	648	91
		7	*YSS_RS00455*	thioredoxin	489	77
		8	*YSS_RS00450*	thioredoxin	504	83
	Campylobacter fetus 82-40	1	*CFF8240_RS02595*	iron permease FTR1 family	1914	39
		2	*CFF8240_RS02600*	iron transporter	522	65
		3	*CFF8240_RS02605*	membrane protein	1365	37
		4	*CFF8240_RS02610*	ABC transporter permease	1245	52
		5	*CFF8240_RS02615*	multidrug ABC transporter substrate-binding protein	1131	40
		6	*CFF8240_RS02620*	GTPase	663	61
		7	*CFF8240_RS02625*	hypothetical protein	498	25
	Campylobacter lari RM2100	1	*CLA_RS05835*	iron permease	2079	63
		2	*CLA_RS05830*	ferrirhodotorulic acid transporter, periplasmic binding protein	522	75
		3	*CLA_RS05825*	hypothetical protein	1422	47
		4	*CLA_RS05820*	ABC transporter permease	1293	58
		5	*CLA_RS05815*	membrane protein	1116	61
		6	*CLA_RS05810*	ABC transporter ATP-binding protein	657	75
		7	*CLA_RS05805*	lipoprotein thioredoxin	492	44
		8	*CLA_RS05800*	thioredoxin	498	46
	Sulfurospirillum multivorans DSM 12446	1	*SMUL_2708*	Ftr1/P19 iron uptake system permease Ftrl	1917	40
		2	*SMUL_2709*	Ftr1/P19 iron uptake system periplasmic protein p19	534	58
		3	*SMUL_2710*	membrane protein	1413	37
		4	*SMUL_2711*	macrolide exporter ABC transport permease	1278	50
		5	*SMUL_2712*	macrolide exporter ABC transport permease	1164	45
		6	*SMUL_2713*	macrolide exporter ABC transport ATPase	663	65
		7	*SMUL_2714+2715*	thioredoxin	484	33
		8	*SMUL_2716*	putative lipoprotein thioredoxin	504	29
	Wolinella succinogenes DSM 1740	1	*WS1566*	conserved hypothetical protein-high-affinity Fe2+/Pb2+ permease	1926	40
		2	*WS1564*	periplasmic protein-probably involved in high-affinity Fe2+ transport	531	62
		3	*WS1562*	integral membrane protein	1410	38
		4	*WS1561*	ABC transport permease protein	1275	52
		5	*WS1560*	integral membrane protein-Permease Component	1146	45
		6	*WS1559*	ABC transport protein	651	65
		7	*WS1558*	thiredoxin	483	35
		8	*WS1557*	hypothetical protein	498	29
Alphaproteobacteria	*Rhodospirillum rubrum ATCC 11170*	1	*Rru_A2809*	iron permease FTR1	1983	31
		2	*Rru_A2808*	hypothetical protein	531	56
		3	*Rru_A2807*	hypothetical protein	1470	28
		4	*Rru_A2806*	hypothetical protein	1290	37
		5	*Rru_A2805*	hypothetical protein	1182	31
		6	*Rru_A2804*	ABC transporter	732	54
		7	*Rru_A2803*	thioredoxin	519	25

Class	Organism	Order in cluster	Gene ID	Annotated Protein Product	Gene Length (# base pairs)	% amino acid identity to *C. jejuni* 81176 proteins
Gammaproteobacteria	*Pasteurella multocida Pm70*	1	*PM_RS02335*	iron permease	1905	33
		2	*PM_RS02330*	iron transporter	522	59
		3	*PM_RS02325*	membrane protein	1398	28
		4	*PM_RS02320*	ABC transporter permease	1323	37
		5	*PM_RS02315*	membrane protein	1140	33
		6	*PM_RS02310*	ABC transporter ATP-binding protein	696	57
		7	*PM_RS02305*	protein ResA	501	26
		8	*PM_RS02300*	cytochrome553 (soluble cytochrome f)	303	None (Reference for 8th gene)
	Aggregatibacter actinomycetemcomitans NUM4039	1	*AANUM_1503*	high-affinity Fe2+/Pb2+ permease	1908	33
		2	*AANUM_1502*	periplasmic protein	522	58
		3	*AANUM_1501*	integral membrane protein	1431	29
		4	*AANUM_1500*	membrane protein	1326	36
		5	*AANUM_1499*	efflux ABC transporter, permease protein	1140	34
		6	*AANUM_1498*	macrolide export ATP-binding/permease protein MacB	672	61
		7	*AANUM_1497*	redoxin family protein	492	26
		8	*AANUM_1496*	cytochrome c-553	312	None (47% to *PM_RS02300*)
	Haemophilus parainfluenzae T3T1	1	*PARA_17390*	unnamed protein product	1905	32
		2	*PARA_17400*	unnamed protein product	522	57
		3	*PARA_17410*	unnamed protein product	1395	29
		4	*PARA_17420*	unnamed protein product	1341	39
		5	*PARA_17430*	unnamed protein product	1134	35
		6	*PARA_17440*	predicted transporter subunit: ATP-binding component of ABC superfamily	672	58
		7	*PARA_17450*	unnamed protein product	480	32
		8	*PARA_17460*	unnamed protein product	324	None (38% to *PM_RS02300*)
	Yersinia pestis CO92	1	*YPO1941*	hypothetical protein	1920	31
		2	*YPO1942*	hypothetical protein	528	57
		3	*YPO1943*	hypothetical protein	1410	27
		4	*YPO1944*	hypothetical protein	1293	36
		5	*YPO1945*	hypothetical protein	1164	31
		6	*YPO1946*	ABC transporter ATP-binding protein	714	54
		7	*YPO1947*	thioredoxin	498	30
		8	*YPO1948*	cytochrome	312	None (36% to *PM_RS02300*)
	Citrobacter koseri ATCC BAA-895	1	*CKO_RS08730*	iron permease	1941	32
		2	*CKO_RS08725*	iron transporter	528	55
		3	*CKO_RS08720*	hypothetical protein	1380	28
		4	*CKO_RS08715*	ABC transporter permease	1284	36
		5	*CKO_RS08710*	ABC transporter permease	1131	31
		6	*CKO_RS08705*	ABC transporter ATP-binding protein	696	58
		7	*CKO_RS08700*	thioredoxin	486	25
		8	*CKO_RS08695*	hypothetical protein	486	None (Reference for 8th gene)
	Salmonella enterica serovar Typhimurium T000240	1	*STMDT12_C39040*	high-affinity Fe2+/Pb2+ permease	1887	31
		2	*STMDT12_C39030*	hypothetical protein	528	55
		3	*STMDT12_C39020*	high affinity Fe+2 binding protein membrane component	1380	28
		4	*STMDT12_C39010*	high affinity Fe+2 binding protein permease component	1284	36
		5	*STMDT12_C39000*	hypothetical protein	1131	31
		6	*STMDT12_C38990*	putative ABC transporter system ATP-binding component	696	58
		7	*STMDT12_C38980*	hypothetical protein	486	25
		8	*STMDT12_C38970*	hypothetical protein	486	None (100% to *CKO_RS08695*)
	Escherichia coli VR50 plasmid pVR50B	1	*efeU*	ferrous iron permease	1941	32
		2	*tpd*	pathogen-specific membrane antigen	528	55
		3	*ECVR50_B068*	putative membrane protein	1380	28
		4	*ECVR50_B067*	putative integral membrane protein	1284	36
		5	*ECVR50_B066*	putative integral membrane protein	1131	31
		6	*ECVR50_B065*	ABC transporter ATP-binding protein	696	58
		7	*ECVR50_B064*	thioredoxin-family protein	486	25
		8	*ECVR50_B063*	Sigma-70, region 4	486	None (100% to *CKO_RS08695*)
	Klebsiella pneumoniae B2 plasmid pB2-A/C	1	*AOG75827.1*	hypothetical protein	1887	31
		2	*AOG75828.1*	hypothetical protein	528	55
		3	*AOG75829.1*	hypothetical protein	1380	28
		4	*AOG75830.1*	hypothetical protein	1284	36
		5	*AOG75831.1*	hypothetical protein	1131	31
		6	*AOG75832.1*	hypothetical protein	696	58
		7	*AOG75833.1*	hypothetical protein	486	25
		8	*AOG75834.1*	hypothetical protein	486	None (100% to *CKO_RS08695*)
	Shigella dysenteriae 80-547 plasmid p80-547	1	*AMQ11474.1*	high-affinity Fe2+/Pb2+ permease	1854	31
		2	*AMQ11349.1*	periplasmic protein p19 involved in high-affinity Fe2+ transport	528	55
		3	*AMQ11350.1*	Fe2+ ABC transporter2C substrate binding protein	1380	28
		4	*AMQ11351.1*	cell division protein FtsX	1284	36
		5	*AMQ11352.1*	Fe2+ ABC transporter2C permease protein 2	1131	31
		6	*AMQ11353.1*	Fe2+ ABC transporter2C ATP-binding subunit	696	58
		7	*AMQ11354.1*	putative periplasmic thioredoxin	486	25
		8	*AMQ11475.1*	hypothetical protein	486	None (100% to *CKO_RS08695*)

Appendix B: Proposed method for creating single deletion mutants of *CJJ81176_1651* to *1656* genes



This is the proposed method for creating individual deletions of *CJJ81176_1651* to *1656* genes. Deletion insertion of a resistance marker directly into the chromosomally encoded operon may disrupt downstream gene expression, so a selective complementation strategy was designed. Based on the results of this thesis, complementation of the whole *CJJ81176_1651* to *1656* cluster restores wildtype phenotype as shown in Section 3. Therefore, evaluation of complements missing one gene will show whether all components of this iron uptake system is required for function. Selective deletion can be performed by amplification and ligation of the required upstream and downstream genes, then inserting the single deletion complement into the rRNA region of the C. jejuni genome. The * indicates the original location of the deleted gene. KmR = Kanamycin resistance cassette. CmR = Chloramphenicol resistance cassette. Pcm¬ = Chloramphenicol resistance gene promoter.