CHAPTER I

Introduction

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Campylobacter jejuni Biology

As a human pathogen, *Campylobacter jejuni* is an accidental tourist. The microbe has reservoirs in a variety of animals and in water [1-3] (Fig 1). In the developed world, where waterborne infection is much less likely, animals are the primary source of human infection and disease (termed campylobacteriosis). Frequently, perhaps most prevalently, disease arises after consummation of chicken products contaminated during processing. *C. jejuni* is commonly considered a commensal of chickens and other avian species. Although experimental infection of chickens with *C. jejuni* can lead to diarrhea [4, 5], this is not typical, and it appears that the human response to *C. jejuni* infection is more symptomatic than the chicken response. This situation is similar to that with a better-characterized pathogen, enterohemorrhagic *E. coli*, a common inhabitant of cattle that causes human disease through ingestion of ground beef and other foods that become contaminated through contact with cattle feces. In both cases, effective control measures for human populations may be more properly directed at reducing colonization of the natural host.

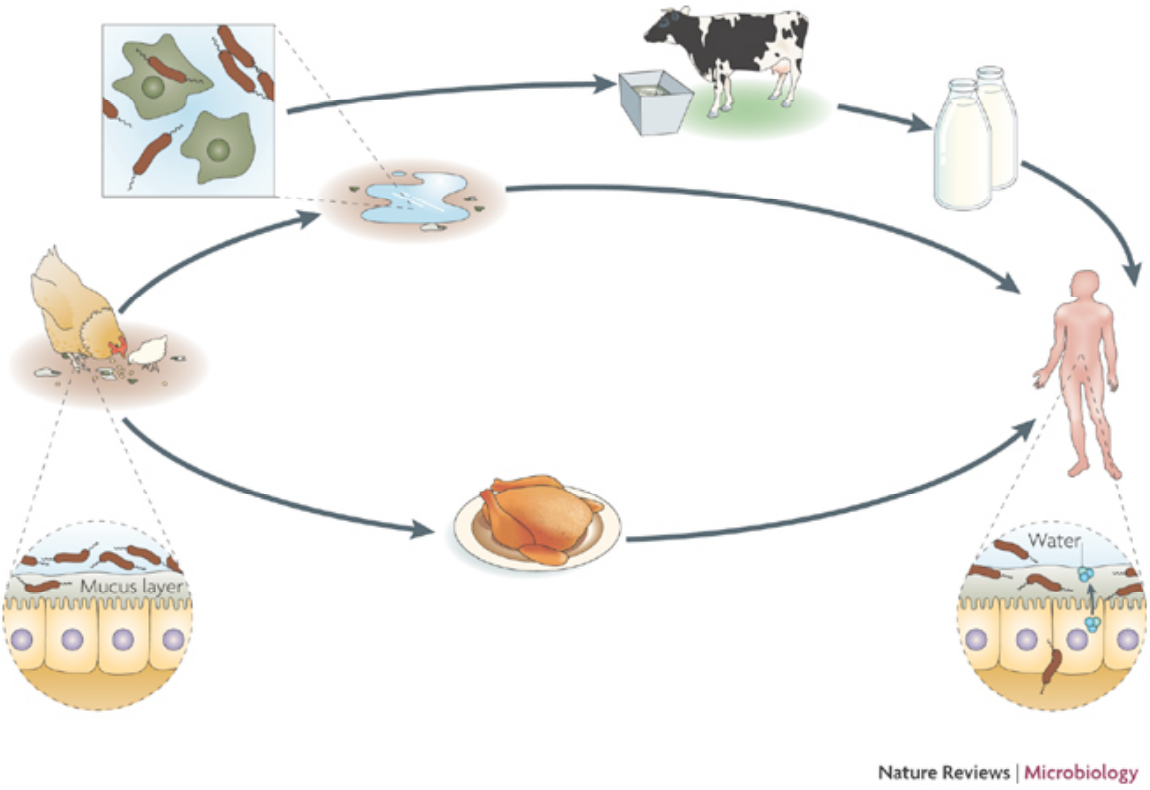

Figure 1. Sources and Outcomes of *C. jejuni* Infection Several environmental reservoirs can lead to human infection by *C. jejuni*. It colonizes the chicken gastrointestinal tract in high numbers, primarily in the mucosal layer, and is passed between chicks within a flock through the fecal–oral route. *C. jejuni* can enter the water supply, where it can associate with protozoans, such as freshwater amoebae, and possibly form biofilms. *C. jejuni* can infect humans directly through the drinking water or through the consumption of contaminated animal products, such as unpasteurized milk or meat, particularly poultry. In humans, *C. jejuni* can invade the intestinal epithelial layer, resulting in inflammation and diarrhea.

C. jejuni belongs to the epsilon class of proteobacteria, in the order *Campylobacteriales*; this order includes two other genera, *Helicobacter* and *Wolinella*. Like *C. jejuni*, members of these genera have small genomes (1.6 – 2.0 Mbp) and can establish long-term associations with hosts, sometimes with pathogenic consequences. *Helicobacter* includes the species *H. pylori*, which may causes gastric ulcers and is clearly pathogenic, but can be carried asymptomatically in humans for decades. *Wolinella* has a single species, *W. succinogenes*, which colonizes cattle intestine as a commensal. Thus, these related organisms all appear to be host-adapted and can establish and maintain their niches without generating a response sufficient for clearance.

Specific bacterial factors or regulation of these bacterial factors can often dictate the outcome of infection for many pathogenic organisms. A number of *Campylobacter* factors have been shown to be necessary for virulence in humans or for colonization of chickens. The following body of work is dedicated to the exploration of factors that dictate the outcome of infection in specific hosts. Here, we review *Campylobacter* factors that are crucial for interactions with its various hosts.

Flagellar Motility

C. jejuni flagella and flagellar motility are vital to many aspects of *C. jejuni* biology, including host colonization, virulence in ferret models, secretion and host cell invasion. Consequently, regulation of flagella biogenesis and motility is an active area of research. Various studies have elucidated a flagellar regulatory hierarchy that includes σ^{54} (*rpoN*) and σ^{28} (*fliA*) as the flagellar sigma factors and the two-component system FlgRS (which is phase variable)[6-11]. Homologues of the flagellar master regulators FlhC and FlhD, critical for flagellar gene expression in other species, have not been

identified in the *C. jejuni* genome [12]. Two proteins (FlgP and FlgQ) required for flagellar motility have recently been identified, but their roles are unclear; no homologues of these are found in *E. coli* and only uncharacterized homologues have been identified in other species [13]. The GTPase activity of FlhF is required for proper assembly of flagella, independent of the σ^{54} –dependent regulon. Mutations in the FlhF protein resulted in mis-localized flagella in *C. jejuni*, highlighting the complex nature of polar-flagellar regulation in this system [14].

Chemotaxis likely plays an important role for *C. jejuni* in both commensal and pathogenic lifestyles. According to genome sequence analysis, *C. jejuni* encodes most features of the *E. coli* chemotaxis system [12, 15]. *C. jejuni* displays chemotactic motility towards amino acids found in high levels in the chick GI tract and to components of mucus [16]. Mutants lacking either Cj0019c (DocB) and Cj0262c, which are both methyl-accepting chemotaxis receptors, show decreased chick colonization, but the attractants to which these proteins respond are not known [17]. Strains either lacking or over-expressing CheY, the response regulator that controls rotation of the flagella, show decreased virulence in the ferret model [18]. In addition, *C. jejuni* lacks a homologue of the phosphatase CheZ and possesses a homologue of the poorly understood protein CheV [12] [15], first discovered in *Bacillus subtilis* [19] and absent in *E. coli*. CheV has an N-terminal CheW-like domain and a C-terminal CheY-like domain and may act as a phosphate sink for the chemotaxis signal transduction machinery [15, 20]. If CheV acts as a phosphate sink in *C. jejuni*, this might ameliorate the effect of the absence of a CheZ phosphatase on phosphate flow through this signal transduction pathway [12, 15]. Sequence and genetic analyses indicate that *C. jejuni* transduces an energy taxis (or

aerotaxis) signal using two proteins, CetA and CetB, in place of the single protein Aer of *E. coli* and other species [21, 22]. It is apparent that *C. jejuni* combines elements of both the *E. coli* and *B. subtilis* chemotaxis signaling paradigms, as well as some proteins found in neither organism. While understanding of these model systems aids our study of *C. jejuni* chemotaxis, much remains to be understood in terms of signal transduction mechanisms that control *C. jejuni* motility.

Adherence Mechanisms

To colonize hosts, microbes typically require adherence factors, such as the pili found on many Gram negative and Gram positive species. Genome annotations of several *C. jejuni* strains do not include obvious pilus or pilus-like open reading frames (ORFs) [12, 23]. A multi-protein type II-like secretion system similar to that associated with pilus assembly in *Vibrio cholerae* and *Neisseria gonorrhoeae* was identified in *C. jejuni*, with no obvious surface appendages [24].

Notwithstanding the lack of identifiable adherence organelles, several proteins contribute to *C. jejuni* adherence to eukaryotic cells. CadF binds specifically to fibronectin, located basolaterally on epithelial cells *in situ* [25-27]. The fibronectin-binding domain of CadF consists of amino acids 134-137 (FRLS), which represents a novel fibronectin-binding motif [28]. CadF is required for maximal binding and invasion by *C. jejuni in vitro*, and *cadF* mutants are greatly reduced in chick colonization compared to wild-type [26, 29]. CadF is similar to *E. coli* OmpA and forms membrane channels, but the purpose of this activity, if any, in CadF function has not been established [30]. Processing of the C-terminal motif of CadF may provide antigenic

variation for *C. jejuni* while retaining fibronectin binding, aiding in immune evasion and persistence in hosts [31].

Similar to CadF, the Fibrinogen-like protein A (FlpA)(Cj1279c) contains a fibrinogen-binding domain [32, 33]. FlpA and CadF promote binding to INT407 cells and fibrinogen cooperatively, and both are required for intimate attachment [33]. Further, *cadF* mutants poorly colonize broiler chickens, highlighting the necessity for bacterial-host interactions for efficient colonization [32].

Another characterized adhesin, JlpA, is a surface exposed lipoprotein critical for HEp-2 cell binding [34]. JlpA binds to Hsp90α, activating NF-KB and p38 MAP kinase, both of which contribute to pro-inflammatory responses [35]. This suggests that some of the inflammation observed in *C. jejuni* pathogenesis may be related to JlpA-dependent adherence.

Another lipoprotein, CapA, was implicated as a possible adhesin [36]. CapA is an autotransporter with homology to an autotransporter adhesin, and CapA-deficient mutants have decreased adherence to Caco-2 cells, as well as decreased colonization and persistence in a chick model [36]. Again, this highlights the importance of bacterial adherence factors in colonization of hosts.

Some putative adhesins of *C. jejuni* are intriguingly localized in the periplasm. The Peb1 adhesin, also known as CBF1, is one of these. Although critical for adherence to HeLa cells, Peb1 is periplasmic, and shares homology to periplasmic-binding proteins of amino-acid ABC transporters [37-40]. In fact, Peb1 binds both aspartate and glutamate with high affinity, and *peb1*-deficient mutants cannot grow if these amino acids are the major carbon source [40]. Peb1 contains a predicted signal peptidase II

recognition site, a common motif in surface-localized lipoproteins [39]. This, as well as the observation of Peb1 in culture supernatants, suggests that some Peb1 is surface accessible, despite the failure of fractionation techniques to demonstrate this [39, 40]. Mutants that lack *peb1* colonize mice poorly, but this could be attributed to loss of either the adhesion or amino acid transport functions, or both [39, 40]. Another periplasmic protein, the glycoprotein Cj1496c, with homology to a magnesium transporter, is also required for wild-type levels of adherence [41]. The mechanism by which these periplasmic proteins contribute to host cell adherence by *C. jejuni* remains unclear.

Invasion

C. jejuni is an invasive organism and both *in vivo* and *in vitro* studies have indicated that invasion is a putative virulence trait [42-44]. Because a suitable animal model that mimics human infections is lacking, invasion has been studied in cell culture [45, 46]. The ability of *C. jejuni* to colonize the gastrointestinal tract of chickens has also been attributed to a strain's ability to bind to and invade eukaryotic cells [47, 48].

While *C. jejuni* invasion into eukaryotic cells (INT407 and Caco-2 cells) has been shown to be strain dependent [43, 46, 48, 49], the mechanisms behind invasiveness variability have not been fully reported. Transposon mutagenesis of hyper-invasive strains of *C. jejuni* identified 26 mutants with reduced invasive potential [50]. One gene identified in the screen, *cipA* (cj0685) was previously associated with invasiveness of HEp-2 cells and is annotated as a sugar transferase [50]. Other bacterial factors identified for effective *C. jejuni* invasion have been attributed to metabolism as well as motility; including flagellar biosynthesis, regulation, and rotation [50] [17, 51].

A small percentage of *C. jejuni* strains carry a putative virulence plasmid, pVir. The pVir plasmid encodes several genes homologous to a type IV secretion system in *H. pylori*, including *virB11*, a putative ATPase [52]. VirB11 is necessary for efficient invasion of INT407 cells, but the mechanism behind this phenotype is unknown [52]. Detection of *virB11* in *C. jejuni* from several sources, including human, bovine, and chicken reveal its ubiquitous nature, but the rapid loss of the gene from *C. jejuni* isolates from chickens from day 21 days old (30% prevalence) to 56 days old (3% prevalence), questions the necessity of *virB11* during chick colonization [53-55].

C. jejuni strains from both human and chicken origins passaged through the chicken gastrointestinal tract and isolated from systemic organs of chickens were more invasive in human intestinal cell lines [48]. This suggest a dynamic adaptation or selection for *C. jejuni* invasive potential in the gastrointestinal tract of chicks, again highlighting the importance of motility and invasiveness for colonization of the chick intestines.

Host factors contribute to *C. jejuni's* ability to invade epithelial cells. Addition of peptic oligosaccharides to Caco-2 cells significantly inhibited *C. jejuni* invasion, probably by affecting the efficacy of adherence [56]. Further, *C. jejuni* invasion of cultured intestinal cells is significantly inhibited by the presence of chicken intestinal mucus, but not intestinal mucus from humans [57]. Specific factors of the mucus that either contribute or inhibit *C. jejuni* invasion of epithelial cells have not been reported. Previous studies in gnotobiotic pig polymicrobial infections with *C. jejuni* and *Trichuris suis* resulted in marked increase of *C. jejuni* invasion into epithelial cells and macrophages, suggesting that dis-regulation of the host immune system, or intestinal

damage, may contribute to *C. jejuni* pathogenesis [58, 59]. Treatment of pig intestinal cells with recombinant IL-4 results in an increase in *C. jejuni* invasion, mostly likely due to damage to the paracellular junctions [60]. These studies demonstrate that the state of the host environment, including presence of peptic oligosaccharides, mucus composition, and immune state contribute to *C. jejuni's* invasive potential, and thus, virulence in a host.

Most work to date has focused on the entry of *C. jejuni* into host cells. Studies of survival of *C. jejuni* intracellularly have suggested that *C. jejuni* could be classified as a facultative intracellular pathogen. *C. jejuni* was able to survive in both phagocytes (72 hours) and in intestinal epithelial cells in cell culture (96 hours) [61]. Significant intracellular survival in Caco2 and murine macrophages was also demonstrated [62, 63]. Adherence, invasiveness, and survival in pig intestinal cells were demonstrated to depend on exposure to oxidative stress, heat shock and nutrient limitation prior to being assayed [63].

C. jejuni has been shown to disrupt intestinal epithelial integrity by targeting tight junctions in cell culture intestinal cell models [64, 65]. Both wild-type and invasion-deficient *C. jejuni* were able to promote translocation of non-invasive *E. coli* mediated through lipid rafts of T84 cells in cell culture [66]. Further, translocation of non-invasive intestinal bacteria to systemic sights increased with *C. jejuni* infection in mice, indicating that *C. jejuni* could be acting as a stressor to induce translocation [66].

Cytolethal Distending Toxin

Cytolethal distending toxin (CDT), is common in most strains of *C. jejuni* and is encoded by the *cdt* operon, which consists of three genes, *cdtA, cdtB*, and *cdtC* [67, 68]. The presence of *cdtB* aides in *C. jejuni* adherence, invasion and cytotoxicity of HeLa cells. The DNase activity of the CdtB subunit leads to cell death through cell cycle arrest at the G2/M stage [69-72]. The active AB2 holotoxin is a tripartite complex of CdtA, CdtB, and CdtC [73]. However, one study indicated that CdtB and CdtC combined have some cytotoxicity in the absence of CdtA [74].

The role of CDT in *C. jejuni* pathogenesis is unclear, but its mechanism of action is well understood (Fig 2). CdtB is the toxic component, as microinjection or transfection of this subunit alone into host cells leads to similar effects observed with the full holotoxin [71]. CdtB shares similarity with a family of DNAase I-like proteins. CdtB localizes to the nucleus of host cells, causes DNA damage, and phosphorylation of the histone protein H2AX, causing the recruitment of DNA-repair protein Rad50 to double-stand breaks [74]. CdtB has weak DNase activity *in vitro*, and studies that attempted to determine whether DNA damage *in vivo* is a result of CdtB activity have been conflicting [71, 75-78].

The functions of CdtA and CdtC are unclear, but one or both may mediate toxin binding to host cells as they share some similarity to the B chain of ricin toxin, which is responsible for receptor-mediated endocytosis of ricin [76]. As *H. ducreyi* CDT is taken up into cells by clathrin-coated pits, it seems likely that the CdtA and CdtC mediated binding and internationalization through this pathway [79].

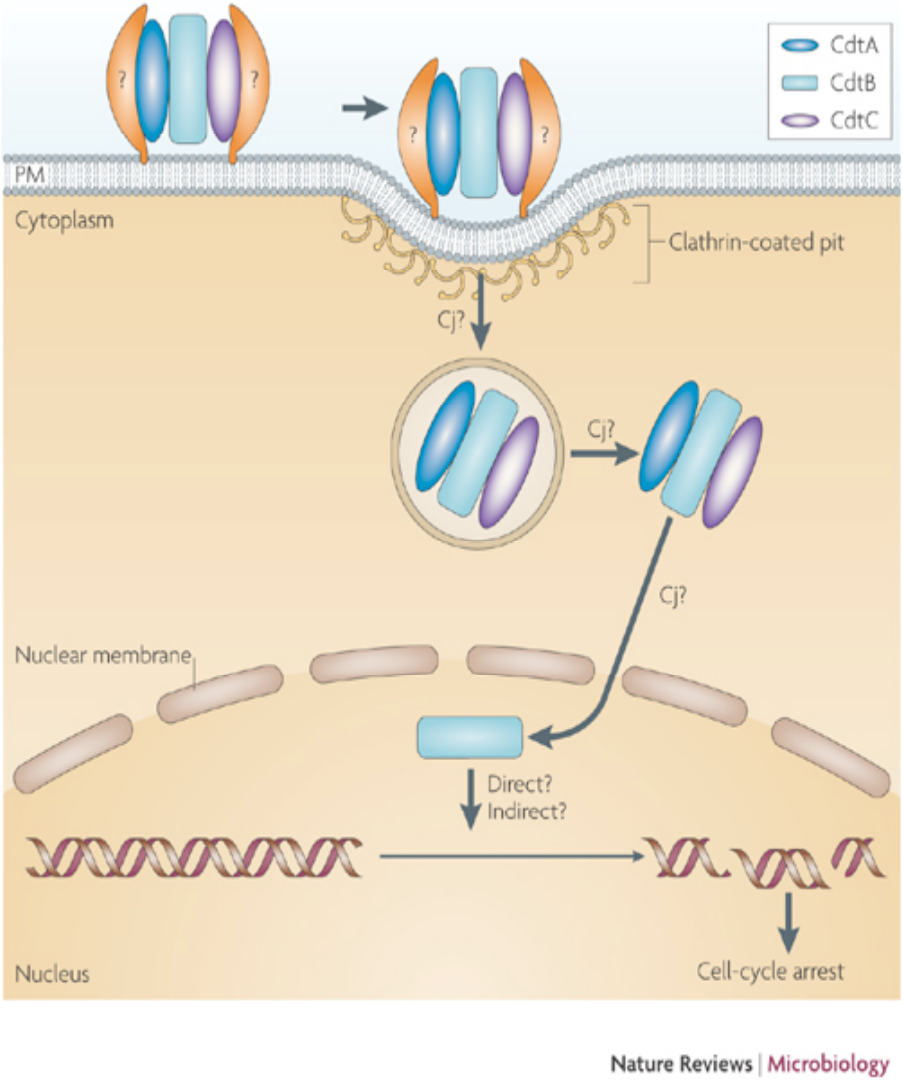

Figure 2. Uptake and Activity of Cytolethal Distending Toxin. The cytolethal distending toxin (CDT) holotoxin consists of three subunits, CdtA, CdtB and CdtC. CdtA and CdtC are thought to bind to an unknown receptor on the host cell surface. CDT is taken up into host cells by way of clathrin-coated pits. Following internalization, nuclear localization signals on CdtB probably lead to its active transport into the nucleus through the classical nuclear-import cycle. Once in the nucleus, the toxin leads to double-strand DNA breaks and cell-cycle arrest.

Bacterial assembly and secretion of the holotoxin is poorly understood. However, the CDT proteins were found to be associated with outer-membrane vesicles, which had toxic effects on tissue culture cells, suggesting that the release of outer membrane vesicles function to deliver CDT to the extracellular environment and host tissue [80].

The fact that *C. jejuni*, which establishes long-term asymptomatic associations with many hosts, has retained CDT is intriguing. CDT is responsible for some of the secretion of IL-8, a hallmark of *C. jejuni* pathogenesis. *In vitro, C. jejuni* CDT induces apoptosis in monocytic cell lines [81]. In chickens, which are the a natural host of *C. jejuni*, CDT is expressed by bacteria in the ceca, although colonized chicks do not generate CDT—neutralizing antibodies, and mutants that lack CDT colonize chicks with wild-type efficiency [82, 83].

Cia Protein Secretion

Secretion mechanisms of *C. jejuni* are poorly characterized relative to other pathogens. *C. jejuni* secretes a protein called CiaB, required for invasion of culture epithelial cells [84]. Mutants lacking *ciaB* exhibit reduced chick colonization levels, again indicating that invasion may be a factor necessary for colonization of chicks [85].

The mechanism of CiaB secretion and its role in invasion has been likened to type III secretion systems, whose effectors are injected directly into host cells [84, 86]. However, *C. jejuni* does not encode a type III secretion system. Rather, CiaB, and other secreted Cia proteins (CiaA-H) require a functional flagellum export apparatus for their secretion, similar to secretion of some proteins from *Yersinia* [87, 88]. CiaC (Cj1242) is secreted by the flagellar apparatus, and mutation of *ciaC* results in reduced host cell

invasion [89]. In addition to the Cia proteins and FlaA, the flagellar export apparatus secretes FlaC, which is also required for invasion [90]. These findings highlight the Importance of the flagellar apparatus for not only motility, but secretion of virulence factors involved in *C. jejuni* invasion.

Protein Glycosylation

C. jejuni expresses two protein glycosylation systems: one modifies serine or threonine residues on flagellin (O-linked glycosylation), and the other modifies asparagine residues on many proteins (*N*-linked glycosylation) (Fig 3). Prior to discovery of the *N*-linked modification system in *C. jejuni*, *N*-glycosylation had been observed exclusively in eukaryotes and archea [91]

Proteins of the O-glycosylation system, as well as many of their biochemical functions and a hypothetical biosynthetic pathway, have been elucidated through a combination of sequence analysis, targeted mutation and chemical analysis [92-94]. The flagellin in *C. jejuni* strain 81-176 is glycosylated with pseudaminic acid at up to 19 sites, accounting for approximately 10% of its observed mass [92]. The flagellin of *C. coli* strain VC167 is modified with legionaminic acid, and the genes for the biosynthesis of this glycan are shared by many strains of *C. jejuni* (not including strain 81-176), indicating that this modification may also occur in these strains [95]. A specific recognition sequence for O-glycosylation has not been identified, and addition of the glycan is thought to require surface exposure and hydrophobicity [92]. However, recent work demonstrated that glycosylation of flagellin subunits occurs prior to secretion [96]. O-glycosylation of flagellin is necessary for proper assembly of the flagellar filament,

leading to the hypothesis that the O-glycan may play a role in interactions of flagellin subunits with one another or with other elements of the flagellar apparatus [96, 97]. Indeed, many proteins of the O-glycosylation machinery are localized to the poles of *C. jejuni* cells, consistent with the location of the flagellar export apparatus suggesting the two systems act in concert to assembly a functional flagellin on the *C. jejuni* surface [96]. However, glycosylation of the flagellin subunits does not require the flagellar export apparatus, demonstrating modularity of the modification system from flagellar assembly [96]. Defects in O-glycosylation result in loss of motility, decreased adherence and invasion of host cells, and decreased virulence in ferrets [93]. It is unknown whether O-glycosylation plays any role in immune avoidance or host cell interactions.

C. jejuni strains associated with livestock contain a cluster of five genes (Cj1321 to Cj1325/6) adjacent to structural flagellin genes [98]. This cluster is not found in *C. jejuni* 81-176 [98]. Deletion of Cj1324 resulted in an alteration of the flagellin-associated glycan, loss of aggregation, and significant decrease in hydrophobicity [99]. Similarly, changes in flagellin glycosylation through mutation of the *pse* locus affect *C. jejuni* autoagglutination and virulence [93]. *C. jejuni* adherence and invasion of INT407 is slightly affected by mutation of *pseA*, while mutations in the Cj1321-Cj1325/6 locus did not have an affect on invasion or adherence using HEp2, CKC, and HD11 cells [93, 99]. It would be interesting to see whether mutations of both flagellin-associated glycan loci would have more significant effects on chick colonization and host cell association. Deletion of the Cj1321-Cj1325/6 locus results in reduced colonization of the chick ceca [99]. The reason for this attenuation could be due to hydrophobicity, change in biofilm formation, (which has been shown to be important in chick colonization),

autoagglutination, or interactions with the host [99-101]. Alternatively, the role of the Cj1321-Cj1325/6 loci could be a matter of species-specificity.

Unlike other surface carbohydrate structures of *C. jejuni* (such as LOS, the capsule and the O-linked glycan), the *N*-linked glycan is conserved in all *C. jejuni* strains so far examined, as well as in *Campylobacter coli* [91, 102, 103]. The conservation of *N*-glycosylation compared with the variability of these other surface carbohydrate traits suggests that *N*-glycosylation may have a more fundamental role in the biology of *C. jejuni*. The *N*-glycosylation system, encoded by the *pgl* genes, has been extensively studied since its discovery, both for a better understanding of its role in *C. jejuni* pathogenicity and for its potential importance in biotechnological applications [91, 104-109]. The *N*-linked glycan assembled by the Pgl system consists of a heptasaccharide, unlike the tetradecasaccharide transferred by the eukaryotic N-glycosylation machinery [110, 111]. In contrast to the O-glycosylation system, a consensus sequence element (sequon), for N-glycosylation has been identified (D/E-X1-N-X2-S/T, where X1 and X2 can be any amino acid except proline) [108, 112]. The glycosylation sequon is necessary, but not sufficient, for glycosylation, indicating that other sequences or factors, such as tertiary or quaternary structure, also play a role [108]. The specific effect of a sequon mutation was tested with the periplasmic protein Cj1496c [41]. A strain lacking *cj1496c* is defective for both chick colonization and adherence to INT 407 human intestinal epithelial cells *in vitro*. However, a strain expressing a Cj1496 sequon mutant, which expresses wild-type levels of protein that cannot be glycosylated, colonizes chicks like wild-type and is not defective for INT 407 association [41]. In contrast, VirB10, a competence protein that is *N*-glycosylated by the Pgl system, may require glycosylation

for its function. A mutant allele lacking one of its two functional glycosylation sequons (*virB10N87A*) was unable to complement a *virB10* mutant to wild-type levels of competence [113].

The role of *N*-linked glycosylation in *C. jejuni* biology is not clear. Strains with *pgl* mutations exhibit reduced adherence and invasion in the INT 407 intestinal cell line, and defects in colonization in mouse and chick models and natural competence [17, 41, 114-116]. *N*-glycosylation changes the immunoreactivity of at least some glycosylated proteins, which suggests the possibility that *N*-linked glycosylation may play a role in evading the immune system [91]. Although most proteins modified by the Pgl system are predicted to be periplasmic, rather than surface exposed, proteins modified by the N-linked glycosylation machinery bound the human Macrophage Galactose-type lectin (MGL) [111, 117]. MGL is a type of innate immune receptor that recognize carbohydrates and acts in cell-cell communication, providing signals that modulate immune function [118]. *C. jejuni* strains lacking *N*-linked glycosylation (through a Δ*pglA* or Δ*pglB* mutation) did not react with MGL, and induced higher IL-6 production by dendritic cells compared to the wild-type strain [117]. This suggests that *C. jejuni* may use the *N*-glycosylation system to modulate the immune system. Although lectin binding studies have identified numerous glycosylated proteins and provided insight for possible immune modulation, no obvious hypothesis for the role of *N*-linked glycosylation has arisen from the knowledge of which proteins are glycosylated [108, 111, 112, 119].

The identification of free oligosaccharides derived from the *N*-glycosylation pathway adds to the complexity of the *C. jejuni* glycome [120]. Free oligosaccharide

released into the periplasmic space requires a functional PglB oligotransferase, and is dependent on the growth phase and osmolarity of the environment [121]. The presence of free glycan could represent a default state where protein acceptors are lacking in the periplasmic space, or could be a mechanism to cope with environmental salt stress. The significance of *N*-glycosylation for *C. jejuni* biology is still incompletely understood.

In summary, there is much more to discover about these two post-translational modification systems. While extensive knowledge about the mechanism O-linked and N-linked glycosylation has greatly expanded in recent years, there are many gaps in our understanding of these systems, including the biological function of this glycan. Conversely, while less is known about the mechanisms of O-glycosylation, its role in *Campylobacter* biology, particularly its importance in flagella assembly and, consequently, host cell interactions, is better appreciated.

ZnuA

Zinc is an essential divalent metal for eukaryotic and prokaryotic cells, since zinc is a component of metalloproteins and serves as an enzymatic cofactor and structural element. Although zinc is essential, excess zinc is toxic to all cell-types. The intracellular levels of zinc must be tightly regulated, utilizing transporters and regulation of gene expression based on intracellular zinc concentrations to avoid metal depletion or toxicity.

The ZnuABC system is a well-characterized high affinity ABC-zinc uptake system found in several bacterial species *including E. coli, Synecosystis, Bacillus, Haemophilus*, and *Campylobacter* species [122-125]. ZnuA is a metallochaperone,

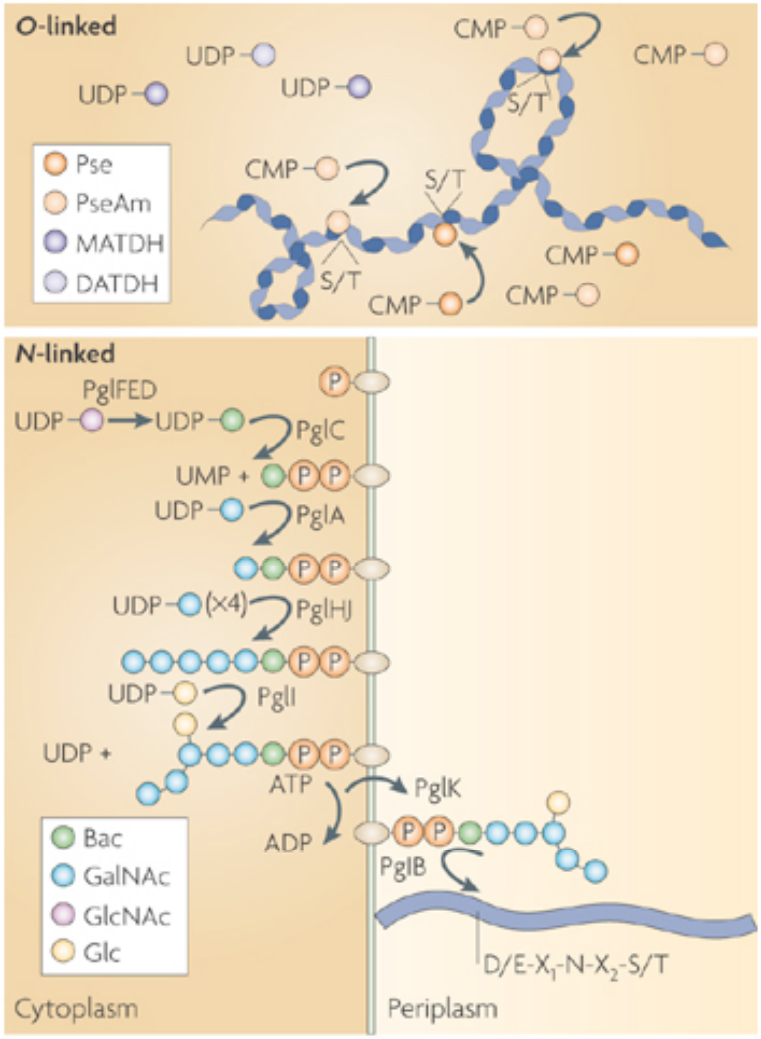

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Figure 3. *O*- and *N*-Linked Protein Glycosylation. The O-linked-glycosylation system in *C. jejuni* modifies flagellin, and the glycan is linked to flagellin through a serine or threonine residue. The *N*-linked-glycosylation system of *C. jejuni* (Pgl) is a general glycosylation system that modifies asparagine residues on many proteins. Glycan assembly occurs in the cytoplasmic face of the inner membrane, where nucleotide-activated sugars are sequentially added to undecaprenylpyrophosphate. The *N*-glycan heptasaccharide is then flipped across the membrane and added as a block to target proteins in the periplasm. Bac, bacillosamine; CMP, cytosine monophosphate; DATDH, diacetamido-trideoxyhexose; GalNAc,N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; HexNAc, N-acetylhexosamine; MATDH, monoacetamido-trideoxyhexose; Pse, pseudaminic acid; PseAm, 5-acetamidino analogue of Pse; UDP, uridine diphosphate; UMP, uridine monophosphate. This figure is modified with permission from [126] © (2005) MacMillan Publishers Ltd.

ZnuB is a membrane permease, and ZnuC is an ATPase component [127, 128]. Together, the ZnuABC proteins transport zinc from the periplasmic space to the cytoplasm in low zinc conditions. The *znuABC* genes are regulated by the DNA-binding protein, Zur. In the presence of zinc, Zur forms a dimer and binds a palindromic DNA sequence, inhibiting expression of the *znuABC* genes [129]. *C. jejuni* does not contain a Zur protein and mechanisms of regulation of the *znuABC* operon is still unknown.

ZnuA from *Synechocystis* was previously crystallized and found to have a novel three-histidine zinc-chelating site. This site might favor binding of zinc over other metals such as manganese [123]. Unlike *E. coli* ZnuA, the structure of ZnuA S*ynechocystis* revealed a flexible loop near the entrance to the metal binding site that is rich in histidine and acidic amino acids [123, 130]. *C. jejuni* ZnuA contains a homologous His-rich sequence to *Synechocystis*. Interestingly, this domain has been reported in a number of eukaryotic zinc transporters [131]. The Synechocystis ZnuA loop binds several zinc ions, with a lower affinity to the main, high-affinity site. Zinc binding to the high-affinity site is unaffected by the presence of the loop, suggesting that the loop may not function in zinc sequestration [132]. It is possible that the His-rich loop could be sensing zinc levels, and acts allosterically to inhibit zinc uptake in high zinc concentrations. While a ZnuA-variant devoid of the His-rich region in *S. Typhimurium* behaved similar to wild-type in growth and infection studies, there was a significant attenuation when the ZnuA-variant lacking the His-region was inserted in a *zinT* mutant [133]. ZinT participates in ZnuABC- transport of zinc, but is dispensable for the function ZnuABC [133]. It is possible that ZnuA and ZinT play similar roles in *S. Typhimurium* and some *E. coli* strains [133, 134]. *C. jejuni* does not contain a *zinT* homologue.

Outcomes of Infection in Humans versus Chickens

Several environmental reservoirs may lead to human infection by *C. jejuni. C. jejuni* colonizes the gastrointestinal tract of chickens in high numbers, primarily in the mucosal layer, and is passed between chicks within a flock through the fecal-oral route. It can enter the water supply and there may associate with protozoans, such as freshwater amoeba. Campylobacter survival and replication was longer in co-culture with four amoebozoas of the genus *Acanthamoeba,* indicating that these organisms may have an important role in the survival of *C. jejuni* in natural water sources [2]. *C. jejuni* can infect humans directly though drinking water or through consumption of contaminated animal products such as unpasteurized milk or meat, particularly poultry [135] (Fig 1).

An intriguing trait of *C. jejuni* is that it colonizes some animals, such as chickens, asymptomatically while in others, such as humans, it causes profuse diarrhea. This raises the question of what factors dictate benign colonization versus symptomatic infection. Several bacterial factors have been identified as potential virulence factors, such as motility and cytolethal distending toxin (CDT). However, such virulence factors cannot explain how *C. jejuni* causes different disease outcomes in different hosts, as these factors are commonly necessary for benign colonization as well. Increasing evidence suggests that the outcome is not only dependent on *C. jejuni*, but on the host response as well.

The physiology of the two model hosts, the chicken and humans, are vastly different. The body temperature of a chicken is 42°C while the average human body temperature is 37°C. Among other differences, such as the digestive systems and

composition of the hosts' microbiota, the immune systems of these two hosts have significant differences. The mesenteric lymph system in humans is part of the secondary lymph system, that drains from the intestines. It is a specialized system composed of lymphatic vesicles and lymph nodes, where antigen presentation and tolerance to the normal microbiota or pro-inflammatory activation to pathogens occurs. Alternatively, chickens do not have a secondary lymph system. Their primary organs, the bursa and spleen, are the locations of antigen presentation and cell proliferation.

The cells that compose the immune system in the two hosts varies as well. In mammals, the neutrophil is the primary responder to invading pathogens. They are highly phagocytic, short-lived cells with internal granules that can be released to kill bacteria extracellularly or intracellularly, using reactive oxygen species. The avian innate immune system contains similar cells, termed heterophils. Like the neutrophil, the heterophil is the first responder to invading pathogens and contain granules that can be released to kill bacteria intracellularly or extraceullarly. However, the heterophil does not rely on reactive oxygen species for bactericidal activity and is less phagocytic [136].

There are a number of similarities between the human and avian systems. Both recognize pathogens through toll-like receptors (TLRs), which activates the pro-inflammatory pathway. Recent work has characterized a number of chicken TLRs homologous to the human TLRs. Further, production of antibodies, such as systemic IgG and IgM, and secretory IgA at mucosal surfaces are similar.

The following section is dedicated to reviewing the current knowledge of *C. jejuni* interactions within two hosts (humans and chickens) during infection (Fig 4).

Infection In Humans

Campylobacter infection commonly presents as an acute gastroenteritis, characterized by inflammation, abdominal pain, fever and diarrhea, with an infectious dose as low as 500-800 bacteria [137-139]. The incubation period preceding development of acute diarrhea is two to five days. Symptoms may last for up to two weeks, but the disease is typically resolved in one week. Epidemiology studies of *Campylobacter* indicate that there are two disease manifestations, depending on socio-economic status [3, 140]. In the developed world, campylobacteriosis manifests as bloody diarrhea with mucus and is usually self-limiting. In the developing world, watery diarrhea predominates, and infection is more frequent among children, which may naturally vaccinate them against becoming infected as adults. The reason for this disparity of outcomes is not clear but may perhaps reflect different levels of, and Th1/Th2 biases of, pre-existing immunity arising from differing natural immune stimulants in these environments.

C. jejuni must bypass the mechanical and immunologic barriers of the gastrointestinal tract to establish an infection. The mucus layer of the GI epithelium serves as the first line of defense, but several traits contribute to the ability of *C. jejuni* to penetrate this barrier. These include the motility and corkscrew morphology of *C. jejuni*, and the relatively short O side chain of its LOS, which is proposed to reduce non-specific binding to the mucin glycoproteins [141]. Once *C. jejuni* passes through the mucus layer, it can interact with the underlying epithelial cells using the various mechanisms discussed above.

Cia protein synthesis is stimulated by the bile component deoxycholate, but Cia secretion is not [86]. This observation led to the suggestion that Cia production may be stimulated early in colonization, in the small intestine, but that secretion occurs only upon adherence at the site of long-term colonization [86]

Campylobacter apparently invades intestinal epithelial cells, as intracellular bacteria have been observed in patients [142] and invasion can be reproduced in cell lines *in vitro*. The mechanism underlying this invasion is being dissected experimentally, and complete understanding is complicated by differences between strains. It is clear that all strains require microtubule polymerization for maximal invasion, although some also require microfilament polymerization [26, 88, 142-144]. This is quite different from the microfilament-dependent mechanism of entry many other invasive bacteria, in which disruption and subversion of actin-based processes has been well described [145, 146]. Scanning electron microscopy has captured epithelial cell membrane pseudopods extending towards and enveloping *C. jejuni*, and immunofluorescence experiments indicate that these pseudopods contain microtubules [88, 143]. Once internalized, *C. jejuni*-containing vacuoles appear to move along microtubules to the perinuclear region of the cell via interactions with dynein[147]. The fate of these internalized bacteria and their role in pathogenesis, possibly in immune evasion or establishment of a protected reservoir, has not yet been determined.

The responses of intestinal epithelial cells to *C. jejuni* are generally characterized by induction of cytokines, such as IL-8, a pro-inflammatory cytokine that is a hallmark of campylobacteriosis, although this response is not universal for all *C. jejuni* strains [148]. *C. jejuni* infection of two polarized intestinal epithelial lines, Caco-2 and T84, as well as

of human intestinal tissue explants, results in activation of MAP kinase family proteins ERK and p38; for T84 cells, ERK activation is essential for stimulation of the pro-inflammatory cytokine IL-8 [65, 149]. CDT contributes to IL-8 secretion in the INT 407 intestinal cell line [148]. Thus, although *C. jejuni* has invasion mechanisms to breach the physical barrier presented by the intestinal epithelium, these cells can in turn signal for recruitment of inflammatory cells to the site of infection.

Human Immune Responses to *C. jejuni* Infection

As with many other pathogens, the Toll-like receptor (TLRs)-dependent innate immune response presumably represents the first immunologic challenge *C. jejuni* must overcome during infection. TLRs are a family of membrane-spanning receptors that recognize conserved microbial patterns and mediate cytosolic signaling. Bacteria are primarily sensed by TLR2/1/16 (detect lipoproteins), TLR4 (detects LPS), TLR5 (detects flagellin) and TLR9 (detects DNA).

Campylobacter jejuni initiates expression of MyD88 and TRIF-dependent immune regulation through activation of human TLR2 and TLR4 [150, 151]. *Campylobacter* evades stimulation of TLR-5 by its flagellin owing to key alterations in the flagellin primary structure relative to TLR-5 stimulatory flagellins [149, 152, 153]. *C. jejuni* also does not efficiently stimulate TLR-9, which recognizes unmethylated CpG dinucleotides, owing to the AT rich nature of the genome [154]. However, the intracellular pathogen recognition receptor NOD1 does serve a critical role in immune stimulation by *C. jejuni* [155].

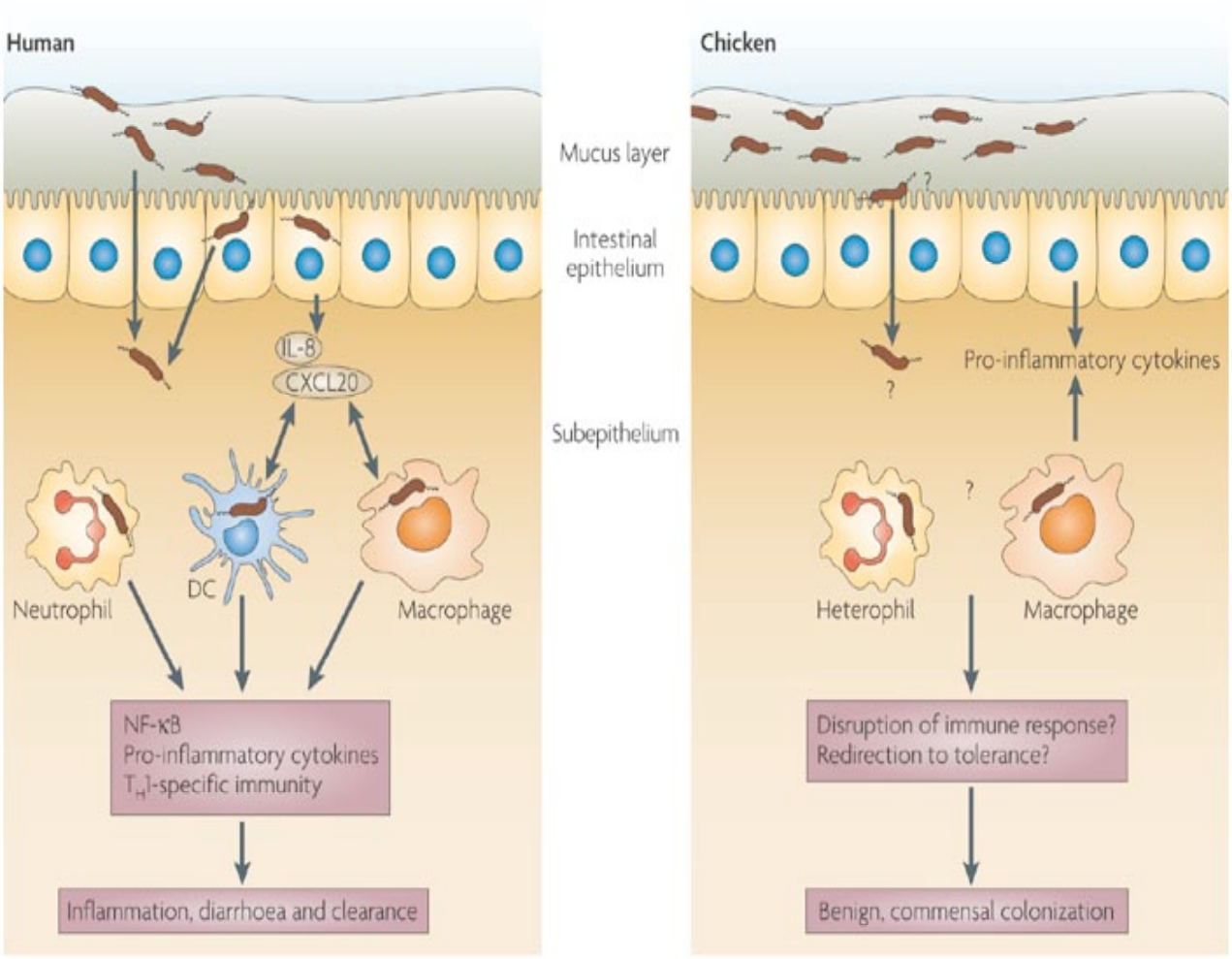

Figure 4. Molecular and Cellular Features of the Innate Immune Response to *C. jejuni* in Humans and Chickens. *C. jejuni* circumvents the mucus layer in humans and interacts with the intestinal epithelial cells causing interleukin (IL)-8 production. *C. jejuni* binds to, and is internalized by, epithelial cells. The induction of IL-8 causes the recruitment of dendritic cells (DC), macrophages and neutrophils, which interact with *C. jejuni*. These interactions result in a massive pro-inflammatory response and increases in the corresponding cytokines. By contrast, *C. jejuni* resides primarily in the mucosal layer in chicken intestines. In vitro evidence shows that *C. jejuni* can stimulate the production of IL-1β, IL-6 and intracellular nitric oxide synthase from epithelial cells and macrophages, but the ensuing host response does not typically lead to inflammatory diarrhea in chickens. Unknown factors either dampen the immune response or redirect it towards tolerance. Heterophils and macrophages might also have a role in the establishment of *C. jejuni* colonization in chickens, but epithelial cell invasion is not typically reported.

The primary response needed to clear *C. jejuni* is polarized towards cell-mediated immunity (or Th1) presumably involving dendritic cells (DCs) and macrophages, as opposed to antibody-mediated immunity [156, 157]. Fox *et al* demonstrated *in vivo* that the clearance of *C. jejuni cdtB* mutant was mediated by a Th1 dependent IgG2a response. [156]. Several *in vitro* studies have contributed to understanding the interaction of *Campylobacter* with these immune cells, but their specific contributions to *C. jejuni* clearance *in vivo* remain undetermined. Exposure of the T84 epithelial cell line to *C. jejuni* results in increased expression of CXCL20, a cytokine that has been implicated in recruitment of DCs [153]. On the basis of *in vitro* studies, DCs likely encounter and internalize *C. jejuni* rapidly [158]. This results in NF-KB activation and secretion of several cytokines and tumor necrosis factor-α, two traits of maturing DCs [158]. Much of the DC maturation response to *C. jejuni* is attributed to bacterial LOS [158].

The role of monocytes and macrophages in *C. jejuni* infection is unclear because results vary with different cell lines or primary cells. NF-KB, as well as the pro-inflammatory cytokine IL-1β, are induced by THP-1 monocyte cells stimulated with *C. jejuni*, providing evidence that monocytes could serve a role in inflammation during *C. jejuni* infection [159, 160]. At the same time, a significant proportion of monocytic cells infected with *C. jejuni* undergo apoptosis [81, 160]. Confounding the issue, one report showed that *C. jejuni* is killed by macrophages derived from human monocytes, whereas other groups have found that clinical isolates of *C. jejuni* survived for several days in murine peritoneal macrophages and the J774A.1 macrophage cell line [61, 81, 161, 162]. Differences in these observations may be due to strain variation or, perhaps more likely, to the use of different macrophage or macrophage-like cell lines.

Although the multiple levels of variation in *C. jejuni* surface structures noted above may help it evade the antibody response, an adaptive immune response has been demonstrated during *C. jejuni* infection. Antibodies to a number of bacterial components have been observed in human sera, including those to flagella, major outer-membrane protein (MOMP), outer membrane proteins [163] and LOS [164, 165]. The unusual structure of the LOS layer of *C. jejuni* has focused investigation into its potential role in virulence. The absence of NeuNAc from the LOS core decreases its immunogenicity [166]. CDT has also been implicated as a major antigen for antibody production and neutralizing antibodies directed against CDT are elicited during human infection; additionally, pooled anti-sera from infected patients neutralized the toxin [82].

Infections in children younger than 6 months of age resulted in low levels of specific IgA, IgG and IgM, possibly owing to the presence of maternal antibodies. On the other hand, 80-90% of patients infected with culturable *C. jejuni* showed specific serum immunoglobulins against *C. jejuni* [167]. In that study, elevated levels of IgG persisted after clearance, but elevated IgA was detected only from the onset of symptoms until the clearance of *C. jejuni* [167].

A number of *C. jejuni* proteins elicit a humoral immune response, including flagellin, CadF, Outer membrane protein, Peb1, JlpA and CjaA [168]. CadF, the outer membrane adhesin required for fibrinogen binding, may be involved in humoral avoidance and virulence in *C. jejuni.* While the C-terminal domain of CadF contains the fibrinogen-binding domain, alterations in the N-terminal length abolished immunogenicity [31]. Variations in CadF length corresponded with strain virulence variability, and may represent an important virulence determinant in *C. jejuni.*

JlpA, a surface-exposed, fibrinogen-binding lipoprotein, is arguably antigenic [34, 168]. Previous studies indicated that JlpA is not immunogenic [34], while others show patient sera reactivity to a 42kDa band consistent with JlpA antigen [168-170]. JlpA is *N*-glycosylated at two residues, and while all forms are present, the double-glycosylated variant is more abundant at the cell surface than the single, or un-glycosylated variants [168]. JlpA promotes adherence to epithelial cells, and the role of glycosylation of JlpA in cell adherence has not been investigated [34].

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Infection in Chickens

A better understanding of the responses of chickens to *C. jejuni* infection, which typically does not lead to the same symptoms and pathological inflammatory response seen in humans, might reveal ways to target the natural source of human infections. *C. jejuni* can colonize chickens to very high numbers, up to 10^{10} colony-forming units (cfu) per gram of infected intestine, with the primary site of colonization being the deep crypts of the cecum where *C. jejuni* is found in the mucus layer close to the epithelial cells [171]. Avian ceca are large, blind-ended diverticula of the colon originating just distal to the ileal junction. Dietary cellulose is broken down in the ceca and fermentation products such as lactic acid and short chain fatty acids are abundant, probably from the metabolic action of the microbiota. This environment is, therefore, likely similar to that of the human colon. A slight inhibition of human epithelial cell invasion by *C. jejuni* in the presence of chicken intestinal mucus has been observed, prompting the suggestion that the mucus might contribute to the asymptomatic nature of chick infection [57]**.**

Various studies that have exploited genetic screens and/or targeted mutagenesis of candidate genes have led to a growing understanding of which *C. jejuni* traits play an important role in the chicken colonization. A common emerging theme from this work is the importance of flagella and flagellar-motility. Signature-tagged mutagenesis of *C. jejuni* in a chick model of infection resulted in identification of two methyl-accepting chemotaxis receptors (MCPs) and other elements of flagellar and chemotactic machinery as important for wild-type chick colonization [17]. Additionally, mutants in the genes that encode the flagellins and flagellar biosynthesis regulators FlgR, σ^{54}, and σ^{28} all display defects in chick colonization [10, 11, 17, 172, 173]. Other regulators not associated with flagellar motility have also proven important for efficient chick colonization. These include CbrR, which regulates deoxycholate resistance and contains two response-regulator domains and a GGDEF domain, thereby implicating this protein in cyclic-di-GMP regulation [174].

One differential trait of chickens compared with humans and other mammals that could contribute to different outcomes of infection with *C. jejuni* is body temperature. Chickens have a body temperature ranging from 41 – 45 ˚C as opposed to the 37 ˚C for humans, making temperature a potential signal for host-specific infection. Transcription profiles of *C. jejuni* cultures shifted from 37 ˚C to 42 ˚C show evidence of potential alterations in membrane structure, by the upregulation of genes for transport and binding proteins, as well as cell wall and envelope constituents [175]. A regulatory system that might contribute to survival at the higher temperature is discussed below. RacRS is a two component system required for wild-type chick colonization, and mutants that lack it have a growth defect at 42 °C [176]. The RacRS system can act as both an activator and

repressor to regulate gene expression, sometimes in a temperature-dependent manner [177]. Mutants in the DccRS two component system, for which an activating signal is unknown, are also poor colonizers of chicks compared to wild type [178]. DccRS-regulated genes that have been identified have no known or predicted functions, but one appears essential for growth and mutants in two others each lead to chick colonization defects [178].

Several genes beyond motility and gene regulation are also required for chick colonization. These include genes that encode enzymes responsible for N-glycosylation of several proteins, as well as various adherence and invasion factors such as *cadF* and *ciaB* [17, 41, 115] [29, 85]. Finally, antimicrobial resistance mechanisms and elements of metabolism relating to low iron, low oxygen (but not anaerobic) and high serine/amino acid environments may have significant impacts on chick colonization [179-184].

The chicken innate immune response to *C. jejuni* infection has been investigated using both epithelial and macrophage cell lines, although the epithelial line used in this study was a chicken kidney cell line as opposed to an intestinal line [185]. Elevated production of IL-1β, IL-6 and inducible Nitric Oxide Synthase (iNOS) [186] were observed from both cell types, indicating that *C. jejuni* can stimulate innate responses by the chick immune system. The microbial ligands and host receptors by which this occurs has only begun to be characterized.

A systematic comparison of *C. jejuni* activation of human and chicken TLRs was recently performed. Human and chicken TLR2 and TLR4, which detect lipoprotein and LPS respectively, were similarly activated by *C. jejuni* [187]. However, a major difference between human and chicken cell stimulation was the absence of *C. jejuni*-

induced IFN-β up-regulation through TLR4/MD-2, which indicates an inability to signal through a MyD88-independent TRIF pathway [187]. IFN-β is a potent inducer of systemic inflammation, and may represent a mechanism by which *C. jejuni* is able to retain non-inflammatory colonization of the chicken gastrointestinal tract. Similar to the inability to stimulate human TLR5, *C. jejuni* flagellin does not activate chicken TLR5 [187]. TLR21, recently identified as a human TLR9 homologue in chickens, (which recognized CpG DNA), was activated by chromosomal DNA of *Campylobacter* [187]. In contrast, human TLR9 is not activated in response to *C. jejuni* DNA. This divergent recognition could represent another determinant of *C. jejuni* colonization versus infection in humans and chickens; however much more work is needed to characterize the functions of TLR9 and TLR21.

Young chicks are exposed to *C. jejuni* during the period when their innate intestinal immune system is developing. Within the first two weeks of life exposure to feed and low doses of bacteria can cause recruitment of heterophils and lymphocytes, as well as the release of inflammatory cytokines Il-1β, IL-8 and K203[188]. Maternal antibodies that recognize *C. jejuni* may also be present in newborn chicks until 2 weeks, providing possible protection from *C. jejuni* colonization [189]. Such antibodies, which recognize surface components including LOS, MOMP and flagellin, lead to complement-mediated killing of *C. jejuni*, in a strain specific manner [189, 190]. Successful early colonization by *C. jejuni* must therefore bypass the immature innate immune response and the presence of maternal antibodies.

By 2 weeks of age, maternal antibodies are no longer present, and by 3 weeks the chick produces its own antibodies in response to *C. jejuni*, which tend to react primarily

to flagellin[181, 191]. Although CDT is expressed by *C. jejuni* in chicks, neutralizing antibodies against CDT are not produced in chicks, unlike humans [82]. This may point to a mechanistic difference in the way *C. jejuni* antigens are recognized by the two hosts. Although the capsule that is expressed in many pathogens is strongly antigenic, the chicken humoral response towards polysaccharides is weak [189]. This is likely due to an incomplete response by chickens to T-cell independent type 2 (TI-2) antigens (usually polysaccharides) in general [192]. TI-2 antigens activate B cells independently of T cells, presumably due to their ability to cross-link cell surface immunoglobulins. This ineffective TI-2 response may contribute to *C. jejuni* colonization in chicks.

Vaccination of chicks to limit *C. jejuni* colonization in the ceca is an attractive method for limiting transmission to humans. Expression of *C. jejuni* proteins, Peb1a, GlnH, ChuA and CjaA, in a *S. Typhimurium* vector have provided protection against *C. jejuni* challenge [193-196]. Immunization with CjaA, an N-glycosylated inner membrane amino acid binding lipoprotein, elicits both serum IgY and mucosal IgA reactivity against *C. jejuni* and provides a 1.4 fold to 6-fold reduction in *C. jejuni* colonization after challenge depending on time of challenge and vaccine administration [193, 194, 197].

Animal Models of *Campylobacter jejuni* Colonization and Virulence

Although campylobacteriosis is a common problem throughout the world, less is known about its pathogenic mechanisms than is known of many other bacteria with lower rates of morbidity. This is partially due to the difficulty of studying a small animal model of infection. Ferrets, non-human primates, and weaning piglets develop symptoms of campylobacteriosis upon infection with *C. jejuni*, but they are expensive, and difficult to obtain and handle [198]. Thus, none of these animals provides an ideal model system. The use of day-of-hatch chicks as a model of infection is widely used to study bacterial factors that contribute to colonization in this natural host.

An intriguing trait of *C. jejuni* is that it colonizes some animals, such as chickens, asymptomatically while in others- humans- it causes profuse diarrhea. This raises the question of what factors dictate benign colonization versus symptomatic infection. Several bacterial factors have been identified as potential virulence factors, such as motility and cytolethal distending toxin (CDT). However, such virulence factors cannot explain how *C. jejuni* causes different disease outcomes in different hosts, as these factors are commonly necessary for benign colonization as well. Increasing evidence suggests that the outcome is not only dependent on *C. jejuni*, but on the host response as well. Recent work has taken advantage of gene-deleted mice, as well as germ-free facilities, and nematode co-infections to advance our understanding of host influences on *C. jejuni* outcomes of infection.

Murine Models of Infection

The appeal of using mouse models comes from the vast amount of knowledge of host genetics that can be exploited in analyzing host-microbe interactions. Generally, two types of mouse models are used to study bacterial pathogenesis, conventional and germ-free. The presence or absence of a microbiota affects bacterial-host interactions. The microbiota down-regulate the inflammatory response and compete with pathogenic bacteria for space and nutrients. A number of germ-free species have been used to study *C. jejuni* pathogenesis, including dogs, pigs, and mice [59, 199] [200]. *C. jejuni* colonization of conventional mice is not stable and does not produce signs of disease, although conventional mice are used to assess colonization [163]. However, germ-free and limited flora mice are colonized well with *C. jejuni* and, when combined with gene-deleted or immuno-compromised mouse backgrounds, produce clinical signs of campylobacteriosis analogous to human infection. Specific deletions in genes encoding key components of the immune system, deregulating signaling pathways or removing key inflammatory mediators have been useful in understanding how the host immune system plays a role in disease manifestation and control of specific bacterial populations.

Murine Colonization Models Not Leading to Disease

C. jejuni is able to asymptomatically colonize a number of animals naturally, and research into factors that influence colonization could aid in understanding how *C. jejuni* colonization could lead to disease versus benign colonization. Research into murine models has focused on the observed inability of *C. jejuni* to colonize conventional mice. Experiments investigating the importance of normal flora have been carried out in both

germ-free and limited flora environments. As noted above, *C. jejuni* is able to colonize germ-free BALB/c mice throughout the intestinal tract three days-post challenge [200]. Infected germ-free immuno-competent mice do not exhibit clinical signs of disease, but upon dissection, show marked congestion and redness of the ceca [200]. The ability of *C. jejuni* to produce a stable colonization in germ-free mice could suggest that competition may be a major determinant in the ability to colonize a host.

Although competition may be a major factor in limiting *C. jejuni* colonization of conventional mice, it is not the sole cause as demonstrated by experiments using mice with limited flora. Utilization of mice colonized by a limited flora represent another means by which to investigate the importance of competition with respect to *C. jejuni* colonization in the mouse. Two limited flora murine models have been used for this purpose, and have resulted in different outcomes. A common method of developing a limited flora model is treatment of the animals with streptomycin, as it clears the intestines of a majority of normal flora in conventional mice. Streptomycin treatment enables colonization by a number of organisms, including *P. aeruginosa, E. coli, V. cholerae* and *S. typhimurium* [201-204]. *C. jejuni* infection of streptomycin-treated mice resulted in irregular colonization of the gastrointestinal tract after three days, similar to the results seen with conventional mice, suggesting that the normal flora of mice do not have a significant impact on *C. jejuni* colonization [200]. However, mice containing a defined, limited flora infected with *C. jejuni* were colonized by *C. jejuni* for 28 days, but eventually *C. jejuni* was not recoverable from the animals [205]. The inconsistent results observed from limited flora and streptomycin-treatment experiments suggest that competition by the microbiota is not the limiting factor in *C. jejuni* colonization of the

mice. Differences in the residing flora between streptomycin-treated and limited-flora mice could be affecting the outcome of *C. jejuni* colonization. Studies examining specific bacteria species that affect *C. jejuni* colonization have yet to be undertaken. However, taken together, the available evidence suggests that the presence of other bacteria, while important, may not be the sole factor inhibiting *C. jejuni* colonization of conventional mice.

The host immune response plays a major role in the outcome of infection or colonization. T-cells, a type of lymphocyte, are key mediators in regulating the innate and adaptive immune responses. During an infection, T cells can be activated into two pathways, Th1 or Th2. The two immune responses act to eliminate different types of pathogens, and can be distinguished by the various cytokines that are elicited during an infection. Generally, the Th1 response is responsible for eliminating viruses and intracellular bacteria while the Th2 response is active against extracellular pathogens such as parasites or extracellular bacteria. The presence of a microbiota affects the T-cell response. The default pathway for gut T-cells is the Th2 pathway, meaning that in the absence of stimuli, T cells will differentiate into Th2 cells. However, the presence of a microbiota stimulates the Th1 pathway. This stimulation forms a balance between the Th1/Th2 pathways that is required to maintain a healthy gut epithelium and response to pathogens (as reviewed by Edelman 2008)[206].

To assess the contributions of T-cells to *C. jejuni* colonization in mice, germ-free athymic and euthymic mice were infected with a mouse-adapted strain of *C. jejuni* [207]. Athymic mice lack a functional thymus, and therefore lack T-cells. Both the euthymic and athymic strains were stably colonized with C. *jejuni*, with the majority of bacteria

recovered from the cecum. Systemic spread of *C. jejuni* was more pronounced in athymic mice. Athymic mice also showed decreased IgA production, compared to euthymic mice upon *C. jejuni* infection. IgA induction occurs through both T-cell independent and T-cell dependent mechanisms in the gut, and confers protection against bacteria at the mucosal surfaces [208]. In mice deficient in IgA production, an increased amount of symbiotic bacteria penetrate the epithelium, which has led to the hypothesis that IgA traps the bacteria to the mucus layer [208, 209]. The role of IgA synthesis in the athymic mouse model has not been examined.

Epithelial cells in the gut monitor the bacterial population though a number of molecular sensors. Toll-Like receptors (TLR), proteins on the surface of epithelial cells, recognize pathogen associated molecular patterns (PAMPS), such as flagella, LPS, RNA, etc. Upon binding to TLRs, a signal transduction protein, MyD88 (myeloid differentiation factor 88) relays this activation through NF-κB, which causes the up-regulation of several inflammatory cytokines, including IL-8. MyD88 is required for signaling through most Toll-like receptors. Mice deficient in MyD88 and therefore unable to signal through many TLRs, were stably colonized with *C. jejuni* in the intestines after 3 weeks [210] with oral or IP inoculation with no pronounced clinical signs or histopathology. *C. jejuni* was detected in different tissues such as the spleen, liver and mesenteric lymph nodes. Further, mutations in *pglF*, part of the *N*-glycosylation system, and Cj1418, involved in synthesis of extracellular polysaccharide capsule, were attenuated for colonization in this model [210], demonstrating that this model could be used to identify and study bacterial colonization factors, or factors that are necessary for survival within the host that do not lead to pathology.

Nramp1 (natural resistance associated macrophage protein one), regulates intracellular pathogen proliferation and inflammatory responses by regulating intracellular cation levels [211]. Nramp1-deficient mice are susceptible to infections by intracellular pathogens such as *Salmonella, Leishmania*, and *Mycobacterium* species. Nramp1-deficient mice infected with *C. jejuni* are unable to clear the infection in the cecum and liver as rapidly as their wild-type counterpart [212]. Further, systemic spread of *C. jejuni* to the liver, spleen and mesenteric lymph nodes was more pronounced in Nramp1- deficient mice. By day 30, the livers of Nramp1 +/+ (which did not shed *C. jejuni* in their feces) appeared normal, while Nramp1-/- mice (which shed *C. jejuni* in feces) had large livers and lymphocytic infiltrate in the livers. This suggests that in mice, Nramp1 mediated killing of *C. jejuni* by phagocytes is critical for control of *C. jejuni* colonization as well as spread to systemic sites.

Similar to Nramp1, NOD1/2 proteins are involved in the intracellular detection and control of bacteria. Nod proteins function similarly to TLRs, recognizing conserved PAMPS, but unlike TLRs, they are located in the cytosol. *C. jejuni* infection of NOD1/2 -/- mice caused persistent colonization, compared to wild-type mice [58]. This suggests that intracellular *C. jejuni* in mammalian hosts is critical for persistence in mice. However, the invasive nature of *C. jejuni* and its intracellular lifestyle during infection has never been documented. Alternatively, the NOD1/2 proteins could be sensing intracellular peptidoglycan that has been delivered by a secretion system, similar to that of *Helicobacter pylori*, where delivery of peptidoglycan was shown to be dependent on the type IV secretion system encoded by the *cag* locus [213]. Infection of NOD1 -/- mice resulted in an increase susceptibility to *H. pylori* infection [213]. Although several

strains of *C. jejuni* harbor orthologues of the *cag* locus on the pVir plasmid, a type IV secretion system has not been identified in *C. jejuni*. Therefore, the mechanism of NOD1/2 detection of *C. jejuni* and its role during an infection are yet to be determined.

The murine models reviewed here represent unique tools for exploring *C. jejuni*-host interactions. Because little to no pathology is reported in the intestines of *C. jejuni* infected mice in these models, examining virulence factors in terms of human infections is not recommended. However, these models highlight the specific roles that the microbiota and host immune response play during an infection. The presence of a microbiota, through competition or stimulation of the host immune system can affect the outcome of *C. jejuni* colonization. The athymic mouse infections demonstrate a role of T-cells in controlling systemic spread and colonization outcome while the NOD1/2 -/- and Nramp1 -/- models suggest a specific intracellular lifestyle of C. *jejuni* necessary for persistence. Indeed, although disease does not occur in these models, valuable information concerning factors governing *C. jejuni*-host-interactions can be gathered from these studies as well as bacterial factors that contribute to non-symptomatic colonization.

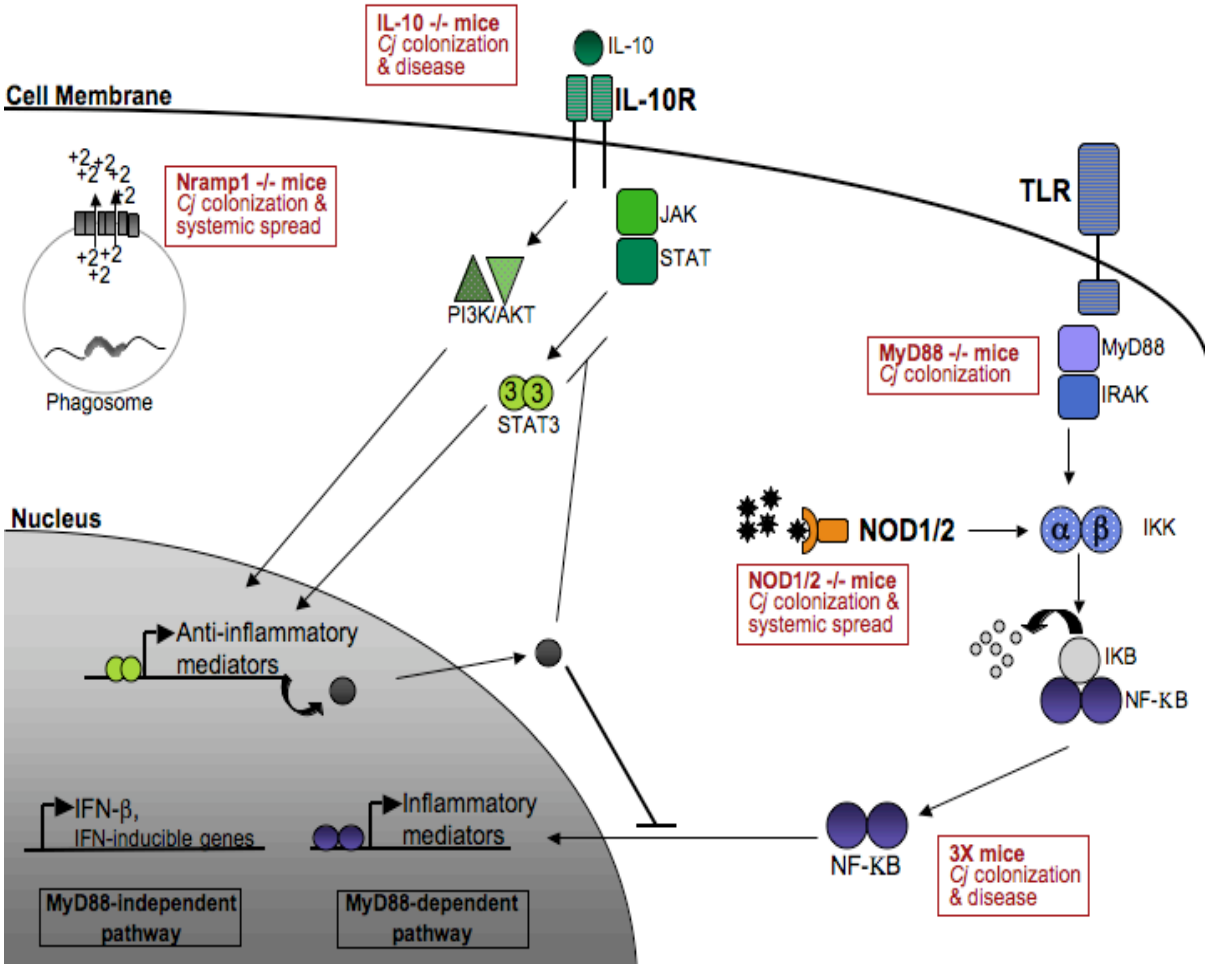

Figure 5. Signaling Cascades and Outcomes of Infection. TLR MyD88-dependent signaling (blue pathway) results in the activation of NF-KB, translocation to the nucleus (grey), and transcription of inflammatory mediators. NOD1/2 proteins (orange) recognize conserved bacterial molecular patterns (black stars) in the cytoplasm of cells, and stimulate activation of NF-KB. IL-10 stimulation (green pathway) causes activation of the Jak/STAT pathway and PI3K/AKT pathways, which lead to transcription of anti-inflammatory mediators. These mediators go on to inhibit both NF-KB translocation to the nucleus and activation of the Jak/STAT pathway. Nramp1 regulates intracellular bacterial proliferation by pumping out divalent cations (+2) such as Fe, Cu, Mn, and Zn from phagosomes. Outcomes of *C. jejuni* (*Cj*) infections of genetically altered mice in these pathways are given (red boxes).

Murine Disease Models

Severe combined immune deficient (SCID) and specific gene-deleted mice have been used to develop murine virulence models for a number of bacterial species. SCID mice rely on their functional innate immune response to control bacterial infections, and represent a means to examine the specific roles the this arm of the immune system plays in combating pathogens. Alternatively, specific gene deletions in components of immune response pathways of mice demonstrate the role for specific immune responses in *C. jejuni* pathogenesis and show how disruption of a single pathway can dramatically alter the outcome of disease.

SCID mice contain a recessive mutation of Prkdc (protein kinase, DNA activated, catalytic peptide), involved in DNA repair. The humoral and cellular immune systems do not develop, resulting in a lack of T-lymphocytes, B-lymphocytes, and a complement system. However, SCID mice contain a functional innate immune response and can combat most bacterial infections with macrophages, neutrophils and natural killer cells. SCID-Beige and C.B-17-SCID-Beige specific-pathogen-free mice were all heavily colonized with *C. jejuni* [214]. C.B-17-SCID-Beige mice inoculated with clinical isolates of *C. jejuni* were heavily colonized for five months. Ten percent of these mice had clinical signs of disease, with histopathological lesions consistent with reports of those seen in human patients [214]. Further, systemic spread of *C. jejuni* to the spleen and liver was evident in SCID mice after 2 and 6 days [215]. This systemic spread was dependent on CDT, as a *cdtB* mutant had reduced systemic spread with no change in colonization of the intestines [215]. These data from infected SCID mice agreed with that generated from athymic mice in suggesting that the host humoral immune response is

important for controlling *C. jejuni* systemic spread as well as clearing the infection in the intestines. The low rate of lesion generation in this model does not make it ideal for studying bacterial factors that contribute to severe disease. But, it does give an opportunity to examine the role of the innate immune response in controlling systemic spread and disease manifestation during *C. jejuni* infections.

To ascertain the role of the microbiota in *C. jejuni* pathogenesis in SCID mice, both SCID and wild-type mice were conventionalized with limited flora (LF) and infected with *C. jejuni*. *C. jejuni* was consistently recovered from the LF-SCID mice at high levels (10^8 cfu/g of stool), similar to LF- wild-type mice [205]. However, *C. jejuni* persistently colonized the LF-SCID mice for up to 28 days, while the LF-wild-type mice had a significant reduction in *C. jejuni* recovery. The LF-SCID mice remained colonized with *C. jejuni* after one year, which was recovered from the stomach, duodenum, small intestines, cecum, and large intestines. After seven days, severe inflammation occurred predominantly in the cecum. Non-chemotactic mutants (*motB, cheA*, and *fliI)* of *C. jejuni* were unable to colonize, validating the model as a means to identify bacterial factors necessary for colonization [205]. This study suggested that the presence of microbiota does not affect *C. jejuni* colonization, as LF-SCID mice were colonized up to a year with *C. jejuni,* similar to the 5 month colonization of specific pathogen free SCID mice. However, the LF-SCID infected mice developed lesions more severe and more consistent than those in SCID infected mice. These differences could be due to the use of different *C. jejuni* strains, age of the mice, or the differing microbiota. An experiment looking at the direct comparison of *C. jejuni* infections of LF and conventional SCID mice has not been reported.

NF-κB is one of the key transcription factors involved in the response to stress, such as bacterial infection. Activation of the NF-κB signaling can occur through several mechanisms, including stimulation of a number of cell-surface receptors, including TLRs. 3X mice (on a C57BL/129 background) are used to examine the role of inflammatory homeostasis through NF-kB signaling specifically during a bacterial infection. 3X mice lack the p50 subunit and are heterozygous for the p65 subunit of the NF-κB complex. These subunits are necessary for regulating intestinal homeostasis during acute inflammation. C. *jejuni* infected 3X mice developed persistent colonization accompanied by inflammation [156]. Inflammation was CDT dependent, as a *C. jejuni cdtB* deletion mutant resulted in persistent colonization with no inflammation. The 3X mice infected with either *C. jejuni* or the *cdtB* mutant had significantly impaired IgG subclass humoral responses to *C. jejuni* compared to C57BL/129 (wild-type) infected mice. The C56BL/129 wild-type mice were able to clear *C. jejuni* infections after 60 days, which was associated with induction of Th1-associated IgG levels. The inability to control the pro-inflammatory response, subsequent inflammatory gastroenteritis and impaired antibody production seen in this model suggests that both the innate and humoral immune responses respond poorly during the infection. However, the *C. jejuni cdtB* mutant stimulated significantly less inflammation than wild-type *C. jejuni*, implicating CDT in eliciting the inflammatory response during infection. Further, the lack of gastroenteritis elicited by the *cdtB* mutant validates the use of the 3X mouse model, demonstrating that active bacterial factors contribute to the inflammatory response observed in wild-type *C. jejuni* infections, and not just the genetic background

of the mouse. This model could be used to identify bacterial factors that stimulate an inflammatory response.

IL-10 is an anti-inflammatory cytokine that signals through the JAK/Stat pathway to down-regulate inflammatory genes as well as through inhibiting NF-κB signaling. IL-10 is necessary for the resolution of an inflammatory response. IL-10 -/- mice were colonized with *C. jejuni* for 35 days and developed an inflammatory reaction upon infection with *C. jejuni*. In this study, IL-10 +/+ mice also remained colonized with *C. jejuni* for 35 days post inoculation, but at lower levels than those seen in IL-10 -/- mice [216]. The clinical signs and histopathological lesions observed in the IL-10 -/- mice resembled those of human cases, with inflammation in the lamina propria dominated by polymorphonuclear cell infiltrate. The majority of IL-10 -/- infected mice produce Th1 associated IgG2b responses to *C. jejuni* [151, 216]. However, there was no correlation between the level of colonization after 30 days by *C. jejuni* and specific immunoglobulins [216]. Various strains of *C. jejuni* were examined for their ability to cause disease symptoms in IL-10 -/-mice, with variable levels reported [151]. The results from these studies are consistent with those from the 3X (NF-κB deficient) mouse model, in demonstrating that *C. jejuni* stimulates an inflammatory reaction during infection. Control of the NF-κB signaling through anti-inflammatory mediators such as IL-10, are necessary for the control of this inflammatory response. CDT was found to be necessary for inflammation in the 3X mouse model, but whether it is necessary for inflammation seen in IL-10 -/- mice has not been determined.

Other Models of Infection

A number of *in vivo* models have been described for *C. jejuni* including non-human primate, ferret, pig, rodent, rabbit and chicken models and are reviewed elsewhere [44]. In recent years, new models have been reported, or have been expanded to explore various aspects of *C. jejuni* pathogenesis. This section is committed to examining these models and how they contribute to our understanding of the host response in *C. jejuni* infections.

A mouse intranasal challenge model has been adapted for intestinal pathogens including *Vibrio cholerae* and *Shigella felxneri* [217, 218]. Infection with *C. jejuni* in the mouse intranasal challenge model has been contradictory. In one study, upon intranasal inoculation, *C. jejuni* was able to kill mice from a number of different backgrounds within 6 days of inoculation, with systemic spread to the lungs, blood, large intestines, small intestines, liver, mesenteric lymph nodes, and spleen [186]. A second study examining the same strain of *C. jejuni* in the mouse lung model did not result in death or health deterioration in any of the animals for 6 days [219]. In the latter study, *C. jejuni* elicited a strong inflammatory reaction, with predominantly polymorphonuclear cells and macrophages in the lung. Further, very few organisms were recovered in the lung, spleen, liver, intestines and blood. Although the lung infection model demonstrates the possible systemic nature of C. *jejuni*, the lack of reproducibility of the model along with the fact that *C. jejuni* is a fastidious organism, requiring low oxygen environments for survival, make this model un-desirable for examination of *C. jejuni* pathogenesis.

Nematode infections drive a type-2 cytokine response (Th2), while repressing the type-1 immune response (Th1) [220]. Germ-free piglets co-infected with the nematode,

Trichuris suis and *C. jejuni* exhibited pathologic lesions, with significant hemorrhaging and inflammatory cell infiltration, which led to severe diarrhea [59]. Germ-free piglets infected with *T. suis* or *C. jejuni* alone had mild clinical signs and pathology. Pigs inoculated with C. *jejuni* alone or *C. jejuni* and *T. suis* had recoverable *C. jejuni* in their feces after 27 days [59]. However, quantification of *C. jejuni* colonization of intestinal tissue directly were not reported, so it is unknown whether the co-infection model results in variable colonization levels. In a separate study by Babakhani *et al*, conventional newborn piglets experienced diarrhea and upon histopathologic examination, had edema, hyperemia and lesions between days 1 and 6 after *C. jejuni* infection [221]. Although both studies demonstrated clinical signs of campylobacteriosis in *C. jejuni* infected pigs, the conventional newborn pigs in the Babakhani study had significantly more pathology. Differences in disease manifestation of the two models may be attributed to a higher inoculation dose in the study by Babakhani *et al*, *C. jejuni* strain differences, the age of the pigs, or the presence of microbiota. The presence of a microbiota is able to skew the Th1/Th2 balance in the gut toward the Th1 pathway [222, 223]. It has been consistently shown that the Th1 immune response is necessary for the control and clearance of *C. jejuni* infection [58, 158, 224]. Further research is needed in these models, to characterize how Th1/Th2 immune skewing and subsequent innate and adaptive immune responses dictate the outcome of an infection. Taken together, these results suggest that the regulation of the immune response due to the presence of other organisms affects the outcome of infection by *C. jejuni* and the use of nematodes for this purpose could be a useful tool in converting a number of colonization models into disease models.

Colonization vs. Disease; A Model

The ability of *C. jejuni* to colonize chicks at a high level without causing any signs of disease is a prime example of how *C. jejuni* is able to causes different disease outcomes in various hosts. As reviewed here, *C. jejuni* is able to invade and cause an up-regulation of pro-inflammatory cytokines in both chickens and mammals, and yet the disease manifestations are starkly different. Understanding the mechanism of *C. jejuni* establishing a colonization state would give insight on how *C. jejuni* causes disease and not benign colonization in humans.

Traditional commensals and their hosts have evolved such that the introduction of commensal bacteria does not cause an inflammatory response [225]. Commensals inhabiting the gastrointestinal tract are taken up by dendritic cells and are restricted to the mesenteric lymph system. In mouse models, secretory IgA (sIgA) is produced against the commensal antigens, and secreted into the mucus of intestines. This is done without systemic priming, causing the host to be ignorant of the commensal bacteria in the lumen of its intestines [225]. Intestinal dendritic cells are at the center of the immune response in the intestines. They specialize in antigen capture and presentation to T cells, and therefore dictate the T cell response. The T cell response then dictates the outcome of infection; inflammation or tolerance. Dendritic cells are responsible for recognizing pathogens versus commensal bacteria and dictating the appropriate response. Commensal activation of dendritic cells causes their maturation and induction of Th1-mediated response [226] [227] [223].

In chickens, *C. jejuni* deviates from this traditional view of commensal biology, as colonization of chickens results in the production of systemic immunolgobulins (IgG

and IgM) as well as mucosal IgA. Studies of Th1 versus Th2 skewing in chickens has not been examined. However, interactions of *C. jejuni* in other animals, as discussed, suggests that T cell polarization to Th1 is a key factor driving disease versus asymptomatic colonization and may influence *C. jejuni* colonization in chicks. Chicks are exposed to *C. jejuni* from hatch, and the day-of-hatch chick colonization model is the standard model for examining bacterial-host interactions. The lack of developed T-cells in young chicks, as well as exposure at a young age may cause the immune system to become tolerant of C. *jejuni* in broiler flocks. Research into the modulation of the immune response in chickens, skewing to a Th2-associated or Th1-associated immune response, is needed to further understand how the immune responses to *C. jejuni* in the various hosts dictate the outcome of colonization versus disease.

C. jejuni colonization of humans has been reported in Bangladeshi patients where *C. jejuni* is a common enteric pathogen in the area, but is often asymptomatic in adults [228]. Bangladeshi children commonly experience symptomatic *C. jejuni* enteritis with symptoms ranging from inflammatory diarrhea, mild secretory diarrhea, or asymptomatic carriage [3]. The early exposure and development of immunity, and thereby tolerance, in children exposed to *C. jejuni* in these endemic areas could explain the asymptomatic carriage in adults. Other factors that could contribute to differences in disease manifestation could be strain differences of the presence of other infections. A high prevalence of concurrent bacterial or pre-existing nematode infections in these areas could influence the disease manifestations due to Th1/Th2 immune skewing. The piglet co-infection model with *T. suis* and *C. jejuni* demonstrates that immune modulation in the intestines may be a major contributor to the pathogenesis of *C. jejuni*. However, those

studies demonstrated co-infection of *T. suis* and *C. jejuni* resulted in more severe symptoms in the pig, in opposition to the decreased severity of disease manifestations in humans in endemic areas. However, multiple factors could be influencing the different outcomes of infection, including duration of exposure to nematode infections, duration and timing of exposure to *C. jejuni*, presence and composition of the microbiota, as well as strain differences and host differences. Nonetheless, it is clear that immune modulation in the host plays an integral role in the outcome of *C. jejuni* infections and future research is needed to ascertain how influential immune modulation is in *C. jejuni* infection manifestations.

One cannot disregard the contributions that *C. jejuni* ultimately brings to the disease. A point of variation in disease manifestation could be in strain differences. These differences could lie in the carriage of a known virulence plasmid (pVir) or phase variation at a number of loci (i.e. LOS, flagella). For example, various strains of *C. jejuni* were examined for their ability to cause disease symptoms in IL-10 -/- mice, with variable levels reported [151]. The mice that were given strains that produced little to no disease produced high Th2 associated IgG1 and IgA responses. These results suggest that variability in how well any particular *C. jejuni* strain elicits a Th2 associated immune response could be responsible for the variability of strains to cause disease versus colonization in a host.

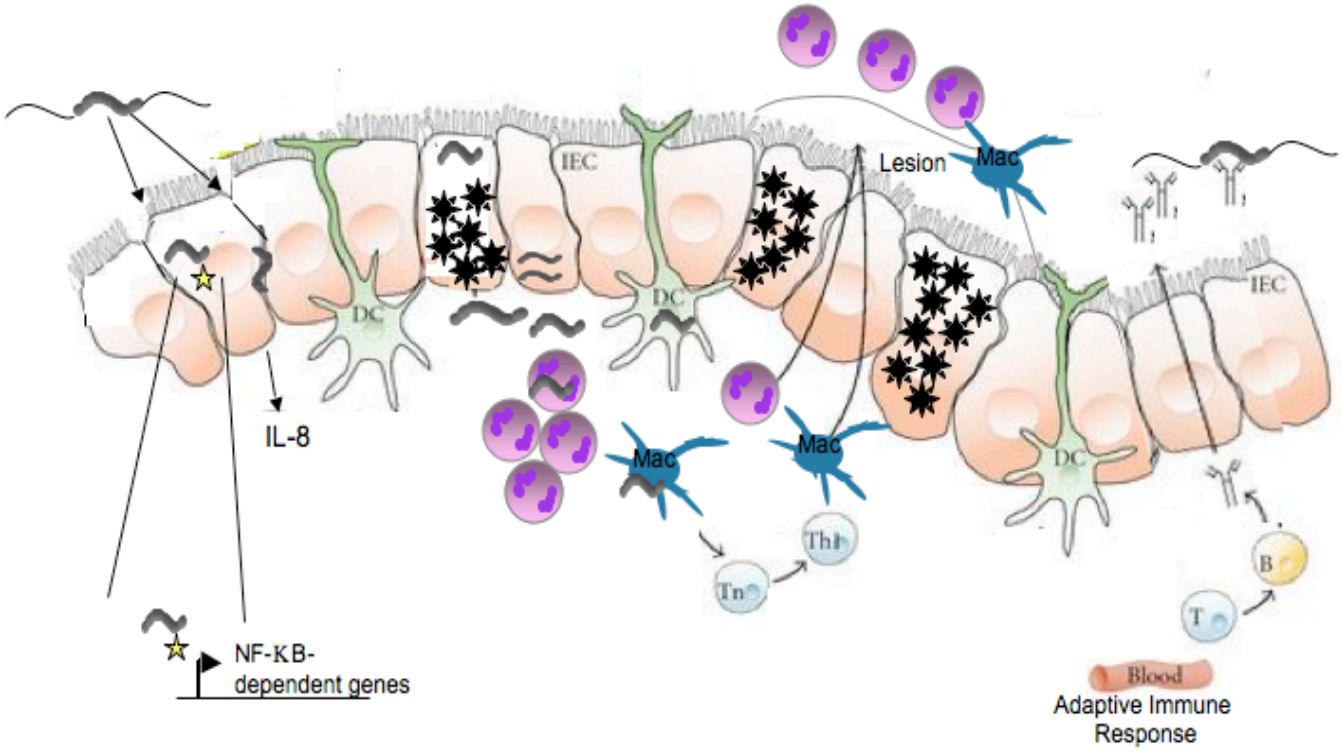

Figure 6. Model of *C. jejuni* Infection in Humans. A model of *C. jejuni* infection in humans based on animal model studies and histopathologic examination. *C. jejuni* (grey), invades the intestinal epithelium. Production of CDT (star) causes activation of NF-KB-dependent genes and leaves to apoptosis of cells (black stars). This damage causes the secretion of IL-8. Immune cells are recruited to the area, including neutrophils (pink), and macrophages (blue), and *C. jejuni* is taken up and killed. Lesions in the lumen form due to cell damage and death (black stars). Helper T cells (Th) are induced to form Th1 cells (light blue). Eventually, B cells cause the production of *C. jejuni* specific antibodies, including sIgA at the mucosal layer.

Zinc Homeostasis

Zinc is an essential trace element. Over 300 enzymes or proteins have been identified that require zinc for function in eukaryotic cells [229, 230]. Zinc has two broad functions; as a cofactor for enzyme catalytic functions, and as the structural factor for folding of domains involved in protein-protein and protein-DNA binding interactions.

Zinc supplementation is a common method for treating diarrheal infections in underdeveloped countries due to its inhibitory effects on bacteria, as well as its ability to boost the Th1 immune response of the host [231]. High concentrations of zinc are inhibitory. Zinc is known to inhibit the respiratory electron transport systems of bacteria as well as interfere with binding to other non-zinc metalloproteins [232, 233]. Zinc resistance in bacteria can be attributed to exclusion of the metal by a permeability barrier, exclusion by active export from the cell, intracellular physical sequestration of metal by binding proteins, or extracellular sequestration (reviewed by Choudhury)[234].

The host limits available zinc for intracellular and extracellular bacteria usage through a number of mechanisms. Zinc is the second most abundant metal in cells, and although intracellular concentrations are estimated to be in the millimolar range, available zinc may be inaccessible to bacterial pathogens due protein binding to zinc [235, 236] [237]. The free concentration of zinc in the cytoplasm is kept well below the picomoloar range [129]. During bacterial infection in mammals, calprotectin is released by neutrophils, restricting the availability of metals, including zinc and mangansese [238]. Further, zinc levels in the serum are decreased after exposure to lipopolysaccharide and

accumulate in the liver [239]. These examples demonstrate the necessity for bacterial zinc uptake systems.

The requirement for zinc in prokaryotic cells is unquestionable, but compared to the uses of zinc in eukaryotic cells, relatively little is known. About 20-30 zinc-binding proteins have been identified in *E. coli*, including RNA polymerase subunits and ribosomal protein subunits [240]. Further investigation into this binding as revealed that zinc binding to these subunits serves as a storage mechanism for some prokaryotic cells such as *Bacillus, Streptococcus*, *and E. coli* spp. [241]. Under normal conditions, ribosomal proteins contain functional zinc binding sites. Under zinc-limiting conditions, transcription of ribosomal proteins without zinc binding properties proceeds as a result of Zur regulation. These non-zinc-binding proteins replace the zinc-binding ribosomal proteins. The zinc is released into the cytoplasm and is incorporated into other zinc-requiring proteins [241]. In this manner, the ribosomal proteins serve as a storage mechanism for zinc under normal conditions, until the zinc is needed for other proteins under zinc-limiting conditions.

Zinc homeostasis in all cells must be tightly regulated and several zinc-responsive transcription factors and transporters have been shown to mediate homeostasis. The metallothionein family of proteins binds and sequesters zinc, primarily in higher eukaryotic systems. The metallothionein SmtA has been well studied in *Synechococcus* and has also been identified in a number of bacteria including *Synechococcus* spp, *Pseudomonas* spp, *Anabaena* spp, and *E. coli* [242, 243]. SmtA binds zinc in elevated zinc environments, sequestering the zinc ions away. Deletion of *smtA* in *Synechococcus* leads to decreased zinc resistance [244]. A metallothionein that binds and sequesters zinc

in low zinc environments, similar to the way that iron siderophores may function has not been identified.

However, bacterial species have evolved to survive within zinc-depleted environments, through the use of zinc-specific regulators and transporters, such as Zur and ZnuABC respectively. Several bacterial species have been shown to require a functional high affinity ZnuABC transport system for colonization and/or virulence, including *C. jejuni, E. coli, Salmonella*, *Neisseria, Pasteurella*, *Haemophilus*, and *Bacillus* species [122, 125] [245] [127, 128].

Intestinal absorption of dietary zinc is highly regulated, and dependent on several transporters. The jejunum has the highest rate of zinc absorption in humans, but absorption occurs throughout the small intestines [246]. The rate of absorption is dependent on the concentration s of zinc available, as well as the presence of glucose and sodium [246]. Expression of zinc transporters and intracellular vesicle sequestration proteins, including metallothionein, in the small intestines in rodent models increased with zinc supplementation [247, 248]. This regulation contributes to zinc homeostasis through regulation of zinc absorption and storage [248].

Metallothioneins are a family of intracellular, cysteine-rich proteins with structural characteristics for strong metal-binding capacities. In mammals, metallothioneins primarily exist in Zn^{+2}-binding isoforms, however the proteins can bind a number of divalent cations and displace Zn^{+2} atoms including Cu^{+2}, Cd^{+2}, Pb^{+2}, Ag^{+2}, Hg^{+2}, and Bi^{+2} [249, 250]. Metallothionein production is induced by the presence of several metals, and inflammatory stimuli in the liver, while in the gut and pancreas, metallothioneins are induced based on Zn^{+2} levels [251, 252]. Dramatic induction of

metallothionein occurs due to tissue injury, infection and inflammation and could be beneficial to the host by lowering plasma zinc which modulates leukocyte function, increasing the pool of intracellular zinc aiding in metabolic processes, and sequestering zinc away [253]. The use of metallothionein knockout mice demonstrate that metallothioneins are not necessary for normal growth and development, but they function to limit metal toxicity, mediate zinc homeostasis, and metabolic rate [254-257].

The intestinal tract of higher eukaryotes is thought to contain. 10^{12} bacteria per gram of contents. The sheer number of bacteria using and storing zinc must affect the amount of zinc readily available. Rats fed a zinc-limiting diet and kept in germ-free conditions had a lower requirement for zinc than conventional animals [258]. The presence of a microbiota could affect zinc levels in the intestines by use or though alterations of zinc metabolism. The presence of a microbiota has been shown to affect a number of metabolic processes, including the uptake of calcium and magnesium [259]. Germ-free animals tend to have lower amounts of transthyretin (also known as pre-albumin), which is used as a measurement of nutrition. A decrease in the nutritional state and absorption of nutrients suggests that the microbiota dramatically shape the digestive functions of the host.

Symbiotic Relationships

All organisms have evolved and survive interacting with any number of other organisms. The relationships between various species and the resulting outcomes of interactions are the subject of intense research; whether it be symbiotic, mutualistic, commensal, or parasitic. These relationships can be classified as obligate (necessary for the survival of one of the species involved) or facultative (not necessary for survival). With increased research into microbiota and its interactions with the host, it is necessary to explore these various relationships.

When two species live in a close association, it is referred to symbiosis. Previously, a symbiotic relationship referred to conditions where each species benefits from the interaction. However, this terminology has shifted and mutualism refers to the condition where both species benefit from the close association. Examples of mutualism can be seen throughout nature. The relationship between bacteria and ruminant animals (such as cows) is a common example of mutualism, because the animals benefits from cellulase produced by the bacteria that is necessary for digestion, while the bacteria benefit from nutrients supplied by the host and an environment to replicate in.

The term commensalism is derived from the English and Latin words meaning "sharing of food" or "sharing of a table." Commensalism is a relationship where one organism benefits while the other is unaffected (neither harmed or benefited). The microbiota of mammalian gastrointestinal tracts are considered commensal bacteria. However, as our understanding of the mechanisms behind the microbiota-host relationship increases, we find there exists a more mutualistic relationship. The

microbiota provide health benefits to the mammalian host, including increased digestive functions, exclusion and competition with harmful microbes, and development of the host immune system. The intestinal microbiota provide necessary signals and stimuli that are essential for the development of a healthy intestinal immune system and for maintaining the gut homeostasis mechanisms. While research demonstrates that the fecal microbiota of patients with irritable bowel syndrome or Crohn's disease is significantly altered, the contribution of the microbiota or the significance of these alterations to the altered gut homeostasis in these diseases is unknown [260].

The following section is dedicated to examining the relationship that exists between a mammalian host and its microbiota, highlighting the fact that the microbiota can be considered mutualistic with its host. While the microbiota, including *C. jejuni* in chickens, benefits from the host by taking up residence and utilizing the food in the intestines, the host benefits from the bacterial breakdown of the food, development of the immune system, and exclusion of potential pathogens by the resident microbiota.

Intestinal Microbiota and its Influences on Host-Bacterial Interactions

Understanding the ecology of the gastrointestinal tract of a host is crucial in trying to discern bacterial-host interactions. *C. jejuni* infection outcomes in the chicken versus humans could be due to physiological differences between the two hosts, including the residing microbiota.

Phylogenetic analysis of gut microbiota using 16S rDNA clone libraries has given insight into the composition of microbiota from different organisms. The predominant intestinal microbiota of humans belong to *Bacteroides, Streptococcus*, *Bifidobacterium*, and *Clostridium* clusters [261-263]. Conversely, species from *Clostridium, Sporomusa*, and enterics (including *Escherichia* and *Enterobacter*) made up the majority of the microbiota in broiler chickens, with less than 2% of the total from *Bacteroides*, and *Bifidobacterium* species [264]. The differences between the microbiota of chickens and humans are not surprising, due to the diets and lifestyles of the two hosts. However, two of the more abundant bacteria in both chickens and human fecal microbiota were C*lostridium leptum* and *Clostridium coccoides* [261-263].

The composition of the microbiota in the gastrointestinal tract changes based on numerous factors including age, disease, feed, introduction of new bacterial species, and environmental conditions. For example, the chicken cecal microbiota is predominantly *C. coccoides* group in 4-day-old chickens whereas in 19-day old chicken, the cecal microbiota evolved towards a more diverse microbiota with *Faecalibacterium* and *C. coccoides* groups as the predominant bacteria [265]. In return, the presence of and

composition of the microbiota in the host influences on the nutritional status of the host, immune status, and competes with incoming pathogens and other microbial species.

Nutrition

Interactions between gut bacteria and their hosts can influence the diet of the host. The presence of a microflora has been attributed to nutrient exchange for the host. The microflora deconjugates and dehydroxylates bile acids, reduces cholesterol to coprostamol and degrades mucus glycoproteins [266, 267]. The microbiota aide in extracting nutrients from the diet, as conventionally raised animals require 30% less caloric intake to maintain their body weight, compared to germ-free animals [268]. Microbial metabolism converts many dietary substances into nutrients that can be absorbed by the host, and the presence of microbes alters the intrinsic metabolic machinery of host cells, resulting in more efficient nutrient uptake. For example, microbial digestion of complex carbohydrate polymers to short-chain fatty acids benefits the host through the extraction of key nutrients from the polymers that the host would be otherwise unable to utilize. Vitamin synthesis by the microbiota has been recognized for many years as germ-free animals require vitamin K, and B complex vitamins in their diets [268, 269].

Synthesis of short-chain fatty acids benefits the host, as supplementation of diets with short-chain fatty acids reduces ileal atrophy and enhances functional adaptation following intestinal damage [270, 271]. Through these means, as well as others, commensal microbiota are important for cell proliferation, differentiation, and function [272]. The microbiota degrade mucous glycoproteins allowing more efficient nutrient

uptake, and due to this function, germ-free animals have enlarged ceca as well as an accumulation of mucus [273].

Campylobacter jejuni has long been considered a commensal bacterium in chicks, with commensalisms defined as a relationship that benefits one without necessarily being detrimental to the other in contrast to symbiotic relationships with are mutually beneficial for both organisms. Intestinal microbes that contribute to metabolic enhancement of the host is an example of a symbiotic relationship. Chickens have been found with and without *Campylobacter* species colonization and there does not seem to be a benefit for the chicken to have *Campylobacter* inhabitation in terms of nutrient acquisition.

The Immune System

The benefits of the microbiota to the host in terms of nutrition and health are undeniable. Not only do the bacteria influence the nutritional state of the host, but also influence the immune development and response to pathogenic organisms. The immune system is responsible for recognizing and responding to foreign and self -molecules. Unlike pathogens, which generally elicit a pro-inflammatory response leading to tissue damage, species of the normal microbiota have been shown to prevent disease during colonization [274].

Observations of the immune development of germ-free animals have revealed that the microbiota influence the development of the immune system of the gut as well as the overall health of the tissue. Intestinal epithelial cells as well as cells of the immune system have altered patterns of microvilli formation and decreased rates of cell turn-over in germ-free animals compared to conventional animals [275]. Further, localization of

TLRs and expression of defensins and other antimicrobial proteins is defective in germ-free animals [276]. These defects in innate immune function, as well as defects in the adaptive immune system, including reduced T cell and B cell trafficking, IgA production, and maturation of immunological sites such Peyers patches, lymphoid follicles, can lead to an increase in susceptibility to infection [277-279]. For example, germ-free animals are more susceptible to *Shigella flexneri*, Salmonella Typhimurium, and *Listeria monocytogenes* infection, as well as *Campylobacter jejuni* colonization [163, 200, 207, 280, 281]. These higher susceptibilities could be due to defects in immune response, but also could be due to competition with the microbiota for space and nutrients.

The gut bacteria also promote homeostasis within the gut. The use of bacterial species as probiotics is due to their ability to control intestinal inflammation. Normal microbiota interaction with regulatory T cells leads to their ability to reduce inflammation and disease, with the increased production of IL-10 [282]. IL-10 is the major anti-inflammatory cytokine produced primarily by monocytes, and down regulates expression of Th1 cytokines.

Given the high bacterial density of the microbiota and the close interactions of the host and microbiota at the epithelial layer, how does the immune system maintain a balance of immune readiness in the face of pathogenic bacteria, and immune tolerance in the face of a large number of friendly bacteria?. The microbiota and the immune system frequently interact with one another, and it comes down to whether the immune system is ignorant or tolerant of the bacteria. Simply, does the immune system never see the bacteria, or chose to ignore the microbiota? The systemic immune system is never primed to the existence of the bacteria in the intestines, in terms of the presence of

immunoglobulins against bacteria of the microbiota, suggesting ignorance [208, 277][225]. Indeed, a systemic IgG response is generated following intravenous inoculation of a commensal bacterium, *Enterobacter cloaca [225]*. This study demonstrates that the systemic immune response will mount a response to commensal bacteria if exposed.

Work by Machpherson *et al* have proposed that the commensal bacteria are taken up and recognized in the host, but the immune response to the commensal bacteria are kept within the mesenteric lymph system, keeping the systemic immune system ignorant of the bacterial species [225, 283]. Within the mesenteric lymph system, B cells are activated in response to antigen presentation of the bacterial antigens, and are converted to sIgA secreting plasma cells. The plasma cells return to the intestines to secrete bacterial specific sIgA into the mucus. The mucus, along with the sIgA molecules provide a barrier for the host, seemingly trapping the commensal bacteria away from the host. Evasion of IgA by specific members of the microbiota has been proposed as a strategy to attain dominance in the gut over the other microbiota species. Expression of IgA in mice leads to immuno-selection against *B. thetaiotaomicron*, altering the composition of the microbial community [284].

Bacteria alter their expression of immuno-dominant epitopes. *Campylobacter jejuni* contains several virulence factors that promote adherence to and invasion of epithelial cells (as reviewed here), as well as those that confer resistance to antimicrobial proteins and allow evasion of the host antibody response. *C. jejuni* is phase variable at several loci, including flagella, LOS, and outer membrane proteins, that would allow it to avoid the immune system of both the human and chicken hosts. In a single passage

through the chick intestinal tract, *C. jejuni* is able to phase vary at these potential virulence and immuno-dominant loci and can become increasingly motile [11, 48]. This leads to one hypothesis that *C. jejuni* persistence in the chicken intestinal tract is though immune evasion.

Microbiota Relationships

The use of microbiota to benefit the host has been explored though probiotics, or administration of live microorganisms. The best-characterized use of probiotics is attributed to reduced intestinal illnesses, such as rotovirus- and *Clostridium difficile*-associated diarrhea through competitive exclusion, alterations in the intestinal microbial community, and modification of host signaling and defenses [285] [286].

Lactobacillus and *Bifidobacteria*, common inhabitants of both mammalian and avian intestines, inhibit the growth of several *Campylobacter* strains in broth [145, 287]. Further, lactobacilli inhibited *C. jejuni* invasion of epithelial cells in cell culture though competitive exclusion and could be used as a therapeutic agent during *C. jejuni* infection [285].

Nonpathogenic yeast, such as *Saccharyomyces boulardii*, have been used as a probiotic to treat human patients with diarrhea [288]. Administration of S. *boulardii* to chickens decreased *Salmonella* colonization [289]. Although the mechanism for this is unknown, *Salmonella*'s predilection for binding to mannose sugars could explain this inhibition, as *S. boulardii* cells contain mannose in their cell wall and could bind Salmonella in the intestines, inhibiting attachment to epithelial cells [289].

Campylobacter colonization was not affected by *S. boulardii* administration, which could be explained by the fact that *Campylobacter* does not bind mannose [289].

Bacterial inhibition by the intestinal microbial occurs not only by competitive exclusion and stimulation of the immune system, but direct mechanisms as well, such as production of toxic conditions and compounds. Production of volatile fatty acids, low pH, and bacteriocins in the intestines of host inhibit pathogenic bacteria [290].

Bacteriocins are proteins that have anti-bacterial effects on bacteria other than the producing strain. Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lacti*s is used in foods. Due to the high rate of transmission of pathogenic bacteria from poultry products, several studies have investigate the use of bacteriocins in chicks for their antibacterial effects. *Fusobacterium mortiferum*, an intestinal bacterium isolated from chicken ceca, are able to synthesize bacteriocins *in vitro* and could play a role in survival in vivo [291]. An avian strain of *E. coli* expressing the bacteriocin microcin affected *S. typhimurium* survival in chickens [292]. Further, *Enterococcus faecium* isolated from the crop of chickens produces a bacteriocin that protects chicks from *S. pullorum* [293].

Antimicrobial peptides are small molecules produced by all eukaryotic organisms studied to date, and generally interact with negatively charged membranes [294, 295]. Antimicrobial peptides from chicken leukocytes inhibited both *L. monocytogenes* and *E. coli in vitro* [296]. Antimicrobial activity was demonstrated against *Candida albicans, S. enteritidis*, and C*ampylobacter jejuni* using two chicken and two turkey peptides [297]. The full extent of antimicrobial production and their role in the health of the chicken gastrointestinal tract is currently unknown.

Intestinal microbiota compete with each other for adherence sites as well as nutrients. However, bacteria can scavenge nutrients not only from the host, but from the intestinal microbiota as well. Growth of *C. jejuni in vivo* is thought to depend mainly on the availability of free amino acids, as it cannot utilize sugars as carbon sources due to the lack of the glycolytic enzyme phosphofructokinase [12, 171]. To meet its energy needs, *C. jejuni* uses oxidative phosphorylation, a highly branched electron transport chain that can extract carbon from amino acids, and can utilize hydrogen and formate as respiratory substrates [12, 183, 298-300]. A *C. jejuni* mutant lacking both the hydrogenase and formate reductases are impaired for chick colonization, suggesting that the use of these respiratory substrates is essential in the chick cecum [301]. This is supported by the fact that the microbiota of the chick cecum is predominantly anaerobic fermentative organisms, and would normally provide abundance hydrogen and formate for *C. jejuni* use [265, 302].

C. jejuni metabolic predilection aids in its colonization of the chick cecum and could be a determinant in host specificity. *In vitro*, *C. jejuni* preferentially utilizes serine, aspartate, glutamate, and proline, and requires genes for serine catabolism and amino acid transporters for colonization of the chick intestinal tract [17, 183, 303]. There are significant differences in the acquisition of nutrients between strains of *C. jejuni,* where many clinical isolates do not encode genes necessary to utilize asparagine, glutathione, and/or glutamine [304]. These metabolic differences between strains may result in differences of colonization in several hosts (such as humans versus chickens), as well as specific tissue colonization [304].

Summary

There are numerous factors that influence the outcome of a bacterial infection. To understand why a bacterium, such as *C. jejuni*, causes different outcomes of infection in various hosts, it is necessary to look at all the factors that influence this relationship.

Bacterial virulence factors, such as motility, toxins, immune suppressive or stimulatory agents play integral roles in eliciting a productive infection or colonization. However, bacterial replication is necessary for survival in a host, and therefore metabolism and acquisition of nutrients become putative colonization and virulence factors as well. Zinc is an essential element for most living cells. *C. jejuni* is no exception. *C. jejuni* uses a specific zinc transporter, ZnuABC to acquire zinc in limiting environments, such as within a host. A functional ZnuABC transporter is essential for colonization of chicks.

The host response to bacteria has a profound effect on the outcome of infection, as the immune response can either kill or aid in bacterial colonization. Mammals have evolved mechanisms to respond and kill pathogens while repressing this response to microbiota, allowing the establishment of an asymptomatic colonization. The host can also sequester specific key nutrients away from bacteria, which can cause some bacteria to invade the tissue in search of nutrients, leading to tissue damage and inflammation. However, sequestration of vital elements, such as iron, can inhibit infectious bacterial colonization.

The microbiota of vertebrates play key roles in bacteria-host relationships. Not only do the microbiota compete with incoming bacteria for space and nutrients, but their

stimulation of the immune response and influences on host nutrition and metabolism affect the host response to incoming bacteria.

It is with these factors in mind that the studies presented here were performed. Here, we investigate the factors and events that lead to *C. jejuni* colonization of the chicken, examining the roles that the microbiota, the host innate immune response, and a bacterial factor, ZnuA, all influence the outcome of infection. These studies were done with the hope of elucidating what factors may lead to disease in humans versus benign colonization in the chick.

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CHAPTER II

Materials and Methods

Described here are the materials and methods for the experiments performed in the following body of work. The protocols for these experiments and others can also be found in the following publications;

Davis, L. and DiRita,V.(2008). *Experimental Chick Colonization by Campylobacter jejuni*, Current Protocols in Microbiology Unit 8A.1

Davis, L. Young, K, and DiRita,V. (2008). *Campylobacter jejuni* Genetic Manipulation and Molecular Biology, Current Protocols in Microbiology Unity 8A.2

Davis, L. and DiRita,V (2008). *Campylobacter jejuni Growth and Laboratory Maintenance*, Current Protocols in Microbiology Unit 8A.3

Bacterial Experiments

Bacterial Strains and Growth Conditions

The bacteria used in these studies are shown in Table 1. *Campylobacter jejuni* strains were grown on Mueller Hinton (MH) agar containing 10 μg ml^{-1} trimethoprim (TMP) under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2) in a tri-gas incubator at 37° C. A streptomycin resistant variant of *C. jejuni* 81-176 (81-176 Str^R) was used in the following studies as the parental wild-type strain [1]. *Salmonella enterica* serovar Typhimurium, strain SL1344 expressing GFP-Amp, was grown on Luria Broth (LB) agar containing 30ug ml^{-1} ampicillin at 37°C

Inoculations for animal infections and growth experiments were made from swabbing *C. jejuni* from 24-hour heavy-growth on MH agar plates and resuspending the bacteria in PBS. Growth experiments were carried out in 96 well plates in 200 μL of static MH broth under the same atmospheric conditions.

Strains and Plasmids.

Campylobacter jejuni strain 81-176 was isolated from a human clinical case of campylobacteriosis. DRH2.12 is a streptomycin resistant derivative of *C. jejuni* 81-176 [1]. The plasmid pBW210 is derived from pEco101 [2] and contains a C-terminal Flag sequence under a chloramphenicol acetyltransferase (*cat)* promoter [3-5]. All strains and plasmids used in this study are shown in Table 2.

Table 1. Strains and Plasmids Used

Strain or plasmid	Description	Source	Reference
Campylobacter jejuni			
81-176 Str^R	Streptomicin resistant variant of 81-176		Hendrixson, 2001
81-176	Clinical isolate		Korlath, 1985
81116	Lab strain	Gaynor, UBC	Pearson, 2007
11168-O	Original isolate of 11168, clinical isolate	Gaynor, UBC	
CR1 1-1	Chicken isolate, Bilbie Farms Michigan		
CR1 2-1	Chicken isolate, Bilbie Farms Michigan		
CR1 3-1	Chicken isolate, Bilbie Farms Michigan		
Δ*cdtB*	isogenic mutant of cdtB in 81-176	Fox, MIT	
81-176	Parental strain to *vir* mutants	Iovine, NYU	
Δ*virB11*	Transposon insertion in *virB11*	Iovine, NYU	
Δ*virD4*	Transposon insertion in *virD4*	Iovine, NYU	
TK429	81-176 Str^R *Δcj0143c*		
TK420	81-176 Str^R Δ*cj0143c* 3NQ		
TK211	81-176 Str^R *ΔpglB* non-motile variant		Kakuda, 2006
Escherichia coli			
DH5a			Hanahan, 1983
JM101			Figurski, 1979
DH5a/pRK212.1	Conjugative plasmid for plasmid DNA transfer into *Campylobacter*		Wiesener
Salmonella			
Salmonella enterica	serovar Typhimurium strain SL1344 expressing pGFP-AMP	O'Riordan, UM	
Plasmids			
pGEM-T	Cloning vector system		Promega
pECO101	pRY108 derivative with cat promoter in XhoI-BamHI site; Kn^R		Elliot, 2008
pBW210	pECO101 derivative with N-terminal Flag in fame with BamHI site; Kn^R		
pLD36	pBW210 with cj0143c coding sequence cloned into BamHI/XhoI site		
pLD37	pBW210 with cj0143c 3NQ coding sequence cloned into BamHI/XhoI site		

Bacterial Growth Conditions.

C. jejuni was grown at 37°C in a tri-gas incubator containing N2, O_2, CO_2 on Mueller Hinton (MH) containing 10ug/ml trimethoprim (TMP) plates. For growth curves, bacteria were grown overnight on solid MH media containing 10ug/ml TMP, collected and resuspended to an OD_{600} of 0.4. For 1mL of media, 4uL of 1:10 dilution of this suspension was added. Cultures were grown statically under atmospheric conditions described above. MH broth was utilized for growth curves unless otherwise stated. When necessary, media were supplemented with antibiotics at the following concentrations: chloramphenicol (15 μg ml^{-1}), kanamycin (50 μg ml-^{1}), trimpethoprim (10 μg ml^{-1}), streptomycin (2 mg ml^{-1}). All *C. jejuni* strains were stored in MH broth with 20% glycerol at -80°C.

E. coli strains were grown in Luria-Bertani (LB) broth or agar. When necessary, media were supplemented with antibiotics at the following concentrations: kanamycin (50μg ml^{-1}), chloramphenicol (30μg ml^{-1}), kanamycin (50μg ml^{-1}). All *E. coli* strains were stored at -80°C in LB broth with 20% glycerol.

When stated, specific media were used to test *C. jejuni* growth. These media include a Campylobacter Defined Medium developed by et al [6] and MH agar containing EDTA to test the contributions of metals to *C. jejuni* replication. A Cecal Content Medium was developed by collecting the cecal contents from 7 day old chicks, diluting to 10% (weight/vol) in PBS, and filter sterilizing.

Construction of DRH212 Δ*cj0143c*.

A chromosomal, in-frame deletion of *cj0143c* was constructed in a streptomycin resistant derivative of *C. jejuni* 81-176 (DRH212) using the method described by Hendrixson *et al* (Hendrixson, 2001). The *cj0143c* gene was amplified using primers 500bp upstream (5'-CTTTAGTGTTGGTATGCAAG-3') and downstream (5'-CTGCTAAGAATCTTCCAGCA-3') of the coding sequence by PCR with primers based on the genomic sequence of *C. jejuni* NCTC 11168. The product was cloned into pGEM-T vector. The resulting plasmid (pTK007) was then digested with PflMI and treated with T4 DNA polymerase to make blunt ends. A *cat-rpsL* cassette was then ligated into the gene and transformed into DH5α, resulting in the plasmid pTK059. pTK059 was electroporated into DRH212, constructing *cj0143c*::cat-rpsL (TK106).

The deletion of *cj0143c* was made by Pfu DNA polymerase-mediated mutagenesis using *cj0143c* on pTK007 to make pTK259, using the primers 5'TTAGGGATATTTTATACTTTCACACAAGCTACTGCTGATGCTTTTCTCATAA TTTATAA-3' and 5'-TTATAAATTATGAGAAAAGGCATCAGCAGTAGCTTGTGT GAAAGTATAAAATATCCCTAA- 3'. This suicide plasmid was electroporated into the *cat-rpsL* inactivated mutant TK106. Transformants were selected on 2mg of streptomycin ml^{-1} and screened for the loss of the *cat-rpsL* cassette by plating on MH agar with 10 μg chloramphenicol ml^{-1}. Deletion was verified by PCR and sequencing.

Construction of DRH212 *cj0143c*3NQ.

In order to study how glycosylation affects *cj0143c*, we performed site-directed mutagenesis. Three putative sequons were initially identified in the coding sequence of

cj0143c, at residues N28, N216, and N169 based on the initial definition of the sequon (N-X-S/T) [7]. An allele of *cj0143c* was constructed in which all three of these sites were replaced with glutamines. Introduction of point mutations in *cj0143c* was performed by site-directed mutagenesis using Pfu DNA polymerase as described above The primers 5'-GCTAAAAATTTAGAGCAAGAACAACAAACTAGCAGCAAT TTAGTTAGTG-3' and 5'-CACTAACTAAATTGCTGCTAGTTTGTTGTTCTTGCTC TAAATTTTTAGC-3'; 5'-CTTGGACATATTTTGCAAAACGCTATCAACTTACGC AAATTCCTGTAT-3' and 5'-CTACAGGAATTTGCGTAAGTTGATAGCGTTTTG CAAAATATGTCCAAG-3'; and 5'-GAAAAAGTTTTTACGGATAAATTTAAAC AACAATTTTCAAAACAA CAAGTTGTAAATATGC-3' and 5'-GCATATTTACA ACTTGAAGTTTTGAAAAT TGTTGTTTAAATTATCCGTAAAAACTTTTTC-3' , to alter residues N28, N216, and N169 respectively. This was then used in allelic replacement to alter *cj0143c* on the chromosome, creating the strain c*j0143c*3NQ. Subsequent to our mutagenesis experiment, the sequon was re-defined by Kowarik *et al* to be (D/E-X-N-Y-S/T, X,Y ≠ P)[8]. Based on this definition, N28 is the sole glycosylation sequon in *C. jejuni cj0143c*.

Both *Δcj0143c* and *cj0143c*3NQ grew similarly to wild-type in MH medium over time, suggesting that these mutations did not induce a growth defect in rich media. Although the *cj0143c*3NQ mutant contains extraneous mutations, because they do not interrupt the function of the protein, (see Results), we used the triple mutant in our studies.

Construction of Flag-tagged Proteins.

The coding sequence from the second codon up to, but not including, the last codon of *cj0143c* and *cj0143c3NQ* was amplified by PCR. These products were cloned into the BamHI site of pBW210. The plasmids were introduced into *E. coli* DH5α/pRK212.1 and conjugated into DRH212*, Δcj0143c*, *ΔpglB* and *cj0143c*3NQ strains.

Bacterial Fractionation.

C. jejuni was grown under microaerobic conditions at 37°C for 24 hours on MH agar containing 10ug/ml TMP. Bacteria were resuspended in MH Broth, and centrifuged at 10,000x g, 10min at 4°C. The pellet was resuspended in 10% (w/vol) sucrose and 30mM Tris/HCl (pH 7.4). 1mM EDTA was added, and the suspension was stirred for 10min at room temperature. The mixture was centrifuged at 10,000 xg, for 10 minutes at 4°C. The pellet was resuspended in cold 0.5mM $MgCl_2$ and stirred on ice for 10 minutes. This was then centrifuged at 10,000 xg, for 10 minutes at 4°C. The supernatant was used as the periplasmic fraction. The pellet was resuspended in 10mM HEPES (pH 7.3) and frozen at -80°C for 1 hour. The suspension was sonicated for 10 seconds, six times, or until the lysate cleared. To remove cell debris, the suspension was centrifuged at 10,000 xg, for 10 minutes at 4°C and the supernatant was centrifuged at 100,000 xg for 1 hour. The pellet of this was used as the membrane (insoluble) fraction and the supernatant as the cytoplasmic (soluble) fraction. Protein content was measured by BioRAD Protein Assay.

Immunoblotting with anti-FLAG antibody.

Western blots were performed as previously described [4]. Briefly, *C. jejuni* strains expressing pEco101-FLAG were resuspended to an OD_{600} of 0.8. One ml of the suspension was centrifuged and resuspended in 100μl of 2X loading buffer. The samples were boiled for 10min, and subjected to electrophoresis on 10% SDS-PAGE gel. The proteins samples were transferred to nitrocellulose paper and blocked with 5% milk in TTBS (20mM Tris 7.4/ 150mM NaCl/ 0.1% Tween 20).

Zinc Binding Assay

Strains expressing FLAG-tagged Cj0143c and FLAG-tagged Cj0143c3NQ were grown for 18 hours on MH agar plates containing 10 μg ml^{-1}TMP. Periplasmic fractions were collected from the two strains. EZ-view Red ANTI-FLAG M2 Affinity Gel beads (Sigma) were washed with TBS (50mM Tris HCl, 150mM NaCl, pH 7.4), according to the protocol supplied by the manufacturer. The bacterial periplasmic lysates were mixed with the ANTI-FLAG M2 Affinity Gel beads and incubated overnight at 4°C on a rocking shaker. The beads were washed 4X with TBS. The bead-protein complex was incubated with HBS (50mM HEPES-KOH pH 7.5, 0.2M NaCl) buffer only, 1mM $ZnSO_4$ or 1mM EDTA for 1 hour at 4°C on a rocking shaker. The samples were washed 4X and the protein eluted off the beads by incubation with 0.1M glycine HCl (pH 3.5) for 5 minutes at room temperature. Beads were centrifuged at 8,000 x g for 10 seconds. The supernatant was collected and neutralized with 0.5M Tris HCl, pH 7.4, 1.5M NaCl. The sample was divided in half, and total protein was determined by Bio-Rad protein assay. The metal content of the sample was determined by the PAR assay. A portion of the

samples was analyzed by electrophoresis on SDS-PAGE gels and stained with Coomassie Blue to monitor protein purification.

PAR assay

The PAR assay (described by Hunt *et al* [9] uses 4-(2-pyridylazo)resorcinol (PAR) to quantify the amount of zinc within a sample. To quantify the amount of zinc in purified protein, following purification, the protein samples were digested with 50μg/ml proteinase K in HBS for 30 min at 56°C. An equal volume of HBS with 0.2mM PAR was added to the digested sample and read on a plate reader at 490nm. The quantity of zinc was determined by comparison to a standard curve of $ZnSO_4$ and normalized to the amount of protein in each sample, as determined by BioRAD assay following purification. To quantify the amount of zinc within a non-proteinacous sample, equal amounts (vol/vol) of 0.2mM PAR and the sample in question were mixed and read by a spectrophotometer at OD 490 nm and compared to a standard curve.

Gentamicin Protection Assay

Gentamicin-protection assays were performed as previously described [4]. Briefly, INT407 cells (not the Caco2 contaminant) or primary chick intestinal cells were seeded into 24-well tissue culture plates, with 1x 10^5 cells per well. *C. jejuni* grown overnight on MH + TMP agar plates was resuspended in tissue culture media and used to infect the cells at an MOI of 200. The cells were centrifuged at 500xg for 5 minutes to optimize bacterial contact with the cells and incubated for 2 hours at 37°C in a 5% CO_2 incubator. Cells were then washed twice with PBS and half the wells were treated with

100μg ml^{-1} gentamicin to kill extracellular bacteria. The cells were incubated for 2 hours, and then lysed with 0.1% trypsin in PBS, and plated for quantification of *C. jejuni* on MH agar containing 10μg/ml TMP. Wells not treated with gentamicin represent total cell associated bacteria and wells treated with gentamicin represent intracellular bacteria

Animal Use and Experiments

Chicken Care

All animals were humanely treated and given access to food and water under conditions set forth by the University of Michigan Committee on the use and care of Animals (UCUCA). Conventional chicks were hatched in specific pathogen free environment and were given immediate access to water. Conventional chicks were fed a diet consisting of Mod Lab Chick Diet S-G # 5065.

To generate limited-flora chicks, one day prior to hatch, eggs were transferred to the Germ-Free Life Facility at the University of Michigan. To sterilize the surface of the eggs, they were dipped in a 10% solution of bleach for 30 seconds, followed with 3 washes in sterilized water. The eggs were then aseptically transferred to germ-free isolators. Humidity was maintained at 85% relative and the temperature was maintained at 90ºF-100ºF until hatch. The birds were fed autoclaved Mod LabDiet 5065 with Silicon Dioxide and Extra Vitamins. The feed for conventional and germ-free raised birds is of similar composition and contains similar quantities of zinc.

Chicken Colonization Assay

Chicken eggs (white leghorn, Charles River Laboratories) were ordered and placed in rotating incubators with temperature set to 100°F and 90% humidity. After 18 days, rotation was stopped and the eggs laid on their sides. Chicks began to hatch on day 21 of incubation.

The day-of-hatch chicks were orally inoculated with 10^4 cfu or 10^6 cfu *C. jejuni* or *S. typhimurium* diluted in PBS. Chicks inoculated with PBS served as a control. Groups of chickens were kept in separate cages. After 1, 4, 7 and 14 days post inoculation, the chicks were euthanized by overdose with Isoflurane and their ceca removed. Cecal contents were weighed, diluted and plated onto MH agar containing 10μg ml^{-1} TMP, 30μg ml^{-1} cefaperazone, and 2mg ml^{-1} streptomycin for 81-176 Str^r and incubated in microaerobic atmosphere, 37°C for 2 days. *C. jejuni* colonies were counted and cfu g^{-1} cecal contents reported.

Histology

Assessment of heterophil infiltrate was done by examining tissue sections from the ceca of uninfected and infected animals at days 1, 4, 7 and 14-post inoculation. Mid-cecal cross sections were immersed in 10% neutral buffered formalin, embedded in paraffin, cut in 5 micron sections, and stained with haematoxylin and eosin (H&E) using standard protocols. Heterophils were enumerated in a blind assessment using 10 random fields of view at 400x magnification for each chick.

Immunofluoresent Microscopy

Tissue from infected chicks were formalin-fixed and paraffin-embedded as described above and deparaffinized in xylene. The antigen retrieval methods of boiling and proteinase K treatment were used to increase antibody binding. The sections were boiled in buffer (Tris/EDTA pH 9.0, sodium citrate pH 6.0) in a pressure cooker for 20 minutes. The sections were then incubated with 1% Proteinase K in Pre-Buffered Saline (PBS) at 37°C for 15 minutes. This was followed with 3 washes in PBS, blocking with antibody buffer (PBS, 2% bovine serum albumin (BSA)). Antibodies against *Campylobacter* species (goat anti-Campylobacter, Kierkegaard and Perry Laboratories), (1:500 dilution) were incubated on the sections at 7°C overnight. The sections were washed three times with PBS and incubated with FITC-conjugated mouse anti-goat antibodies (Invitrogen, 1:1000 dilution), rhodamine phalloidin (Invitrogen, 1:1000 dilution), and DAPI (Invitrogen, 1:1000 dilution) were incubated on the sections at 37°C for 2 hours. The sections were washed with PBS three times, fixed using Pro-Long Gold Anti-fade, and visualized via immunofluroescent microscopy.

Isolation of Primary Chick Intestinal Cells

Isolation of intestinal cells from 2-week old chickens was performed as described in Byrne et al (Byrne}. The small intestines were removed, slit open, cut into small fragments and put in Hanks balanced salt solution (HBSS) with gentamicin (100μg ml^{-1}), streptomycin (100μg ml^{-1}), penicillin (100μg ml^{-1}), 0.1mM EDTA and 0.1mM DTT. The tissue was digested for 4 hours at 37°C in Dulbecco's modified Eagles medium F-12 (DMEM F-12) with 300 units ml^{-1} collagenase (Sigma-Aldrich). The suspension was

centrifuged at 800 xg for 5 minutes at 5°C, and the supernatant was washed and resuspended DMEM F-12 (Sigma-Aldrich) supplemented with 10% FCS, 100U ml^{-1} streptomycin, 100μg ml^{-1} penicillin and 8μg ml^{-1} insulin. Viability was assessed by trypan blue exclusion.

In vivo Gentamicin Protection Assay

Ceca from infected chicks were removed, opened, and washed three times with PBS. A cecum was divided, weighed and the tissue was manually homogenized, making a single-cell suspension. The homogenized tissue was resuspended in media alone (RPMI containing 5% Fetal Bovine Serum) or media containing 100μg ml^{-1} gentamicin to kill extracellular bacteria. The mixtures were incubated for 2 hours at 37°C in a 5% CO_2 incubator. The mixture was centrifuged at 10,000 x g and washed with PBS three times. The cells were lysed with 0.1% trypsin in PBS and plated for cfu of *C. jejuni* on MH agar containing 10μg ml^{-1} TMP and 30ug ml^{-1} cefeparazone.

Real-time quantitative reverse transcriptase-PCR

RNA expression was determined using real-time quantitative RT-PCR (qRT-PCR), using the Stratagene MxPro 3000. Primer sequences used in real-time quantitative RT-PCR were from a previously published study [10]. Ceca from infected chicks were washed with PBS and stored at 4°C in RNA Later (Sigma). Total cecal RNA was extracted using Qiagen RNeasy for Tissue Samples. Amplification and detection of transcripts were carried out using equivalent amounts of total RNA from total chick ceca. cDNA was pooled in equal amounts from each infection group. Quantification was

carried out by normalizing to amplicons generated from the gene *gapdh*. Each analysis was performed in triplicate. The normalized C_t values for each transcript were then compared to uninfected chick ceca C_t values for the corresponding day of infection by the $\Delta\Delta$ C_t method.

tRFLP

Terminal Restriction Fragment Length Polymorphism (tRFLP) analysis was performed on the cecal contents of seven day-old conventional chicks and chicks raised in germ-free facilities. Four chicks per group were analyzed. Cecal tissue were homogenized and DNA extracted using DNeasy Blood & Tissue Kit (Qiagen). DNA was amplified using the illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare). Primers for this reaction were FAM-labeled 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3'. PCR reactions were purified using QIAquick PCR purification kit (Qiagen) and digested with Msp1 (NEB) and Hha1 (NEB). Digests were purified using the QIAquick Nucleotide removal kit and sequenced (UM Sequencing Core). Although bacteria were not recovered on non-selective blood agar plates, the tRFLP results demonstrated that the chicks hatched in the germ-free facility contained bacterial DNA and were referred to as "limited-flora". Bacteria inside the shell is not unexpected, as bacterial transfer to eggs from hen cloacae and vaginal contamination has been reported [11].

454-Sequencing

DNA isolated and amplified from cecal tissue of infected chicks used in tRFLP analysis (see above) was used sequencing. Samples were sent to Research and Testing LLC, and the sequences were classified using RDP Classifier in collaboration with Dr. Gary Huffnagle.

Immunofluorescent Microscopy

Cecal cross sections were immersed in 10% neutral buffered formalin, embedded in paraffin and cut in 5 micron sections onto microscope slides. The tissue was deparaffinized in xylene, and washed 3 times in PBS, and incubated for 2 hours with DAPI (Invitrogen 1:10000 dilution). The sections were washed three times with PBS, fixed using Pro-Long Gold Anti-fade (Invitrogen), and visualized.

Inductively coupled plasma mass spectrometry

Cecal contents were diluted in 1% HNO_3 and run on a Thermo Scientific Finnigan Element inductively coupled plasma-high resolution mass spectrometer (ICP-HRMS) using instrument-standard conditions.

Isolation of Zinc-Binding Proteins

Zinc-binding proteins from the cecal contents were isolated as previously described by Choi *et al* [12]. Chelating sepharose fast flow gel was purchased from GE Healthcare and used for the matrix for immobilized metal. Sepharose gel was packed onto a column and washed with distilled water. A 0.1% $ZnSO_4$ solution was applied until

zinc appeared in the eluate, as detected by the PAR assay (see below). Solutions of 50mM Tris-HCl buffer (pH 8.0), 0.1M sodium phosphate (pH 6.5), and 0.1M sodium acetate (pH 6.5) were used as washing buffer, the first and second eluting buffers, respectively. EDTA-treated cecal contents or cecal content media were applied over the column.

Fractions were run on 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) separation gels according to standard procedures. SDS-PAGE gels were stained by Coomasie staining and proteins were isolated for sequencing and identification by mass spectrometry.

Statistical Analysis

Analysis of variance was used in experiments to test significance between varying test groups. For comparisons, unpaired *t* tests were used. $p<0.05$ was considered statistically significant (*) and it is noted where $p<0.005$ (**).

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CHAPTER III

Campylobacter jejuni Invasion of the Chick Cecum Stimulates an Inflammatory Response and Leads to Asymptomatic Colonization

Summary

Campylobacter jejuni is one of the leading causes of bacterial gastroenteritis worldwide. *C. jejuni* is a normal inhabitant of the chicken intestinal tract, and humans most commonly acquire the infection through the ingestion of contaminated poultry products, prompting efforts to discover the mechanisms of asymptomatic colonization of chickens. To identify factors influencing the establishment of colonization of chickens by *C. jejuni*, we analyzed the early events of colonization in a day-of-hatch chick infection model. We showed that *C. jejuni* invades the chick cecal epithelium *in vivo,* recruits heterophils to the site of colonization, and stimulates increased expression of pro-inflammatory cytokines, but colonization is not suppressed by the inflammatory response. Both invasion and heterophil recruitment, but not colonization, are dependent on *virB11*. In contrast, neither *virD4* nor CDT were necessary for invasion or heterophil recruitment, indicating that invasiveness but not colonization density is correlated with heterophil recruitment. We propose that early heterophil recruitment in chicks colonized with *C. jejuni* is due to invasion of the cecal epithelium.

Introduction

Campylobacter jejuni is a Gram-negative, thermophilic bacterium that is the leading cause of bacterial food-borne enteric diarrhea in humans. Common clinical signs of *C. jejuni* infection are fever, abdominal cramping, and diarrhea. The primary mode of human *Campylobacter* infection is through ingestion of contaminated chicken. In contrast to its infection of humans, *Campylobacter jejuni* colonizes the deep crypts of the chick ceca in high numbers (10^9 cfu g^{-1} cecal contents) without causing disease [1]. Determining mechanisms by which *C. jejuni* colonize chick ceca may eventually lead to new approaches towards limiting human exposure to this zoonotic pathogen.

The use of chicks as a model of infection is widely used to study bacterial factors that contribute to colonization of a natural host. To date there is very limited research on fundamental aspects of colonization, including attachment, invasion, host response and resolution. While attachment to and invasion of human epithelial cells by *C. jejuni* is well documented and is a crucial step of the infection process, [2], very little is known concerning interactions between *C. jejuni* and chick epithelium *in vivo.* Several reports have documented invasion of *C. jejuni* into various chicken cell lines *in vitro*, including primary intestinal cells [3-5]. Invasion of chicken intestinal epithelium has not been documented *in vivo*, although *C. jejuni* can at times be recovered from the spleen and liver, suggesting it is able to traverse the epithelium [6]. Understanding whether *C. jejuni* is able to invade the chick epithelium is fundamental to elucidating the mechanisms by which *C. jejuni* establishes its niche in the chicken gastrointestinal tract.

Hallmarks of human campylobacteriosis include high levels of IL-8 induction and neutrophil recruitment, which culminate in inflammation and diarrhea. Similarly, pro-inflammatory cytokines, including IL-6, IL-8 and IL-1β, are induced by *C. jejuni* in a number of chicken cell lines, including chicken kidney cells, avian macrophage cell line, chicken embryo intestinal cells, and primary chicken intestinal epithelial cells, as well as in cecal tissue during chick infection [3] [5, 7] [3, 5, 7]. In contrast to humans, however, the induction of pro-inflammatory cytokines by chicken epithelial cells both *in vivo* and *in vitro* does not result in disease. Taken together, the knowledge to date suggests that the lack of disease during chick colonization by *C. jejuni* is not due to a deficiency in one of the main aspects of virulence; attachment, invasion, or ability to induce host pro-inflammatory cytokines. Rather, it suggests that *C. jejuni* stimulates an inflammatory reaction within the chick during colonization, which is quickly resolved and does not result in disease manifestations.

Human campylobacteriosis represents a significant public health problem. Understanding the process by which *C. jejuni* colonizes chickens may suggest methods to disrupt chicken colonization, thereby decreasing transmission to humans. Here we describe experiments investigating the dynamics of colonization of *C. jejuni* 81-176, a human pathogenic isolate, in the cecum of young chickens. We demonstrate that *C. jejuni* invades the chick cecal epithelium, possibly triggering the recruitment of heterophils and up-regulation of genes encoding pro-inflammatory cytokines. This invasion and heterophil recruitment is dependent on the *C. jejuni* factor *virB11*, and eventually subsides resulting in asymptomatic colonization by *C. jejuni*.

Results

Chick Colonization Dynamics of Six Isolates of *C. jejuni*

In order to understand what factors contribute to *C. jejuni* colonization within the chick cecum, we established a chick colonization model, modified from that described earlier [11]. Using this model, day-of-hatch chicks were inoculated with 10^6 cfu of one of three human clinical isolates (81-176, 81116, and 11168-O) or one of three chicken isolates (CR1 1-1, CR1 2-1, and CR1 3-1) to test whether strain source influences colonization potential. Chicks inoculated with PBS served as a negative control.

Colonization of the chick cecum was monitored 1, 4, and 7 days post-inoculation (Fig. 7A). All three human isolates were recovered at approximately 10^9 cfu g^{-1} cecal contents on day one. Strain 81-176 was recovered at 10^8 cfu g^{-1} cecal contents on days four and seven. Colonization by strains 81116 and 11168-O decreased to 10^7 to 10^8 cfu g^{-1} cecal contents on days four and seven. The three chicken commensal strains were recovered from chicks at variable levels on day one, from 10^6 to 10^8 cfu g^{-1} cecal contents. On days four and seven, all three chick isolates colonized the chicks at 10^6-10^7 cfu g^{-1} cecal contents. These results show variable colonization dynamics of the six different strains tested and we asked whether various host factors, such as an innate inflammatory response to the different strains, or specific bacterial factors could be causing this variability

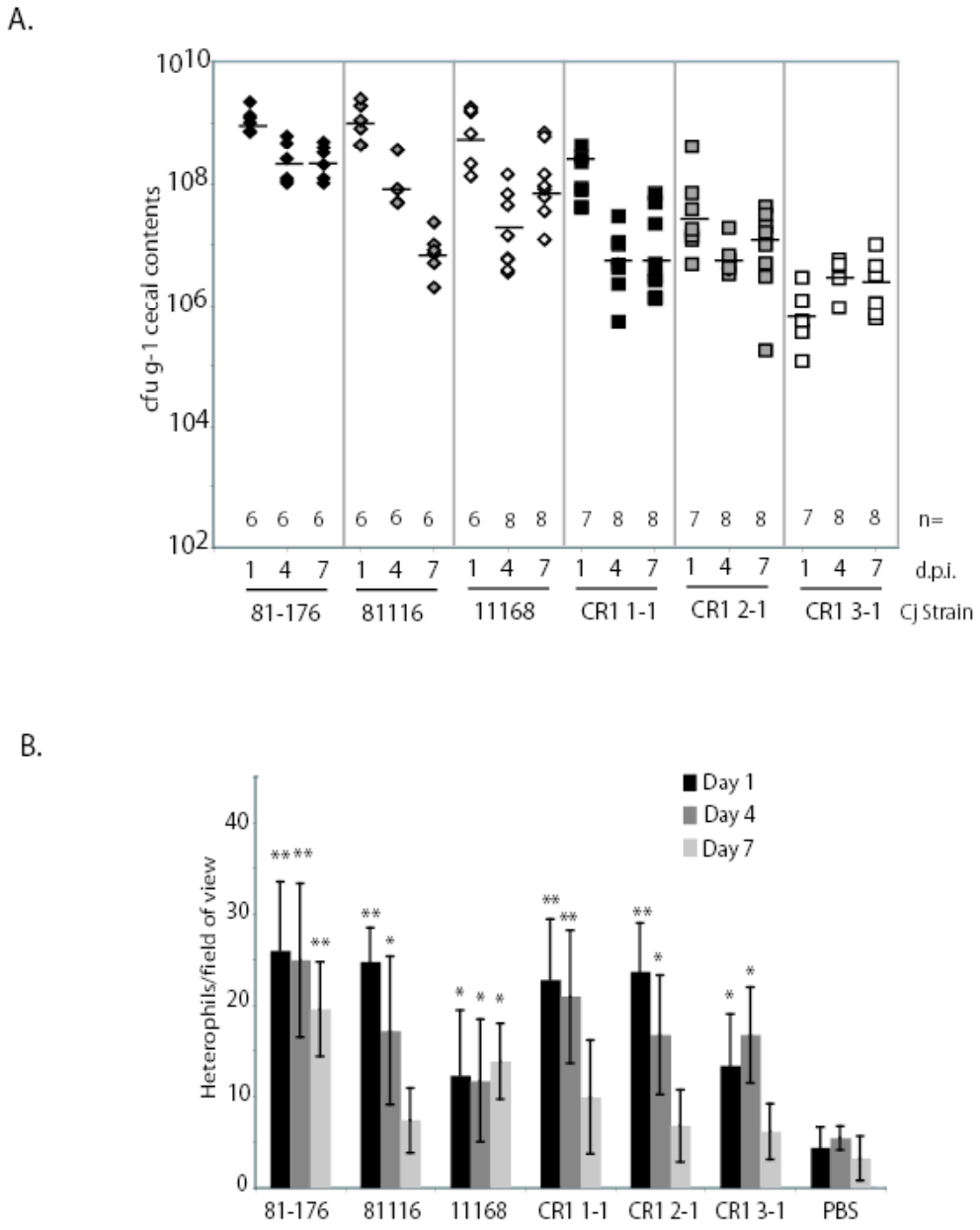


Figure 7. Colonization and Heterophil Response to 6 *C. jejuni* Strains (A). *C. jejuni* colonization in chick ceca inoculated with 8-1-176 (black diamonds), 81116 (gray diamonds), 11168 (white diamonds), CR1 1-1 (black squares), CR1 2-1 (gray squares), or CR1 3-1 (white squares) on days one, four, or seven post inoculation. Birds were orally inoculated with 10^6 cfu of the indicated strains. Limit of detection is 10^2 cfu g^{-1} cecal contents. The number of birds tested for each group is reported (n) Each point represents the level of colonization in an individual chick. (B). The average number of heterophils in the chick cecum on days one, four, and seven for the indicated six strains. Significance compared to age-matched PBS inoculated birds *p<0.05, **p<0.005.

Heterophil Recruitment in Response to *C. jejuni* Infection by Histopathology

Like mammalian neutrophils, avian heterophils are an early line of defense against invading pathogens. Heterophils can be recognized in H&E-stained sections of cecum (Fig 8), and quantification of heterophils in tissue sections was used as a marker of an inflammatory reaction in *C. jejuni* infected chicks.

We examined the chick heterophil response to the *C. jejuni* human isolates 81-176, 81116, and 11168-O, as well as the chicken commensal isolates CR1 1-1, CR1 2-1, and CR1 3-1 on days one, four, and seven post-inoculation. Histopathology of infected chick ceca compared to PBS-treated chicks revealed elevated heterophil recruitment on day one post-inoculation in chicks infected with all strains of *C. jejuni* (Fig. 7B). Heterophil recruitment remained elevated for both strains 81-176 and 11168-O on days four and seven, compared to the PBS-treated chicks. Significant heterophil recruitment was observed on day four in 81116 infected chicks, and dropped to background levels on day seven. The chicks infected with the three chicken isolates of *C. jejuni* had significant heterophil recruitment on day four, which decreased to background levels on day seven. The results suggest that the chick innate immune system responds to five of the six isolates of *C. jejuni*, but with variable kinetics, possibly due to variations in motility or inherent strain or source differences.

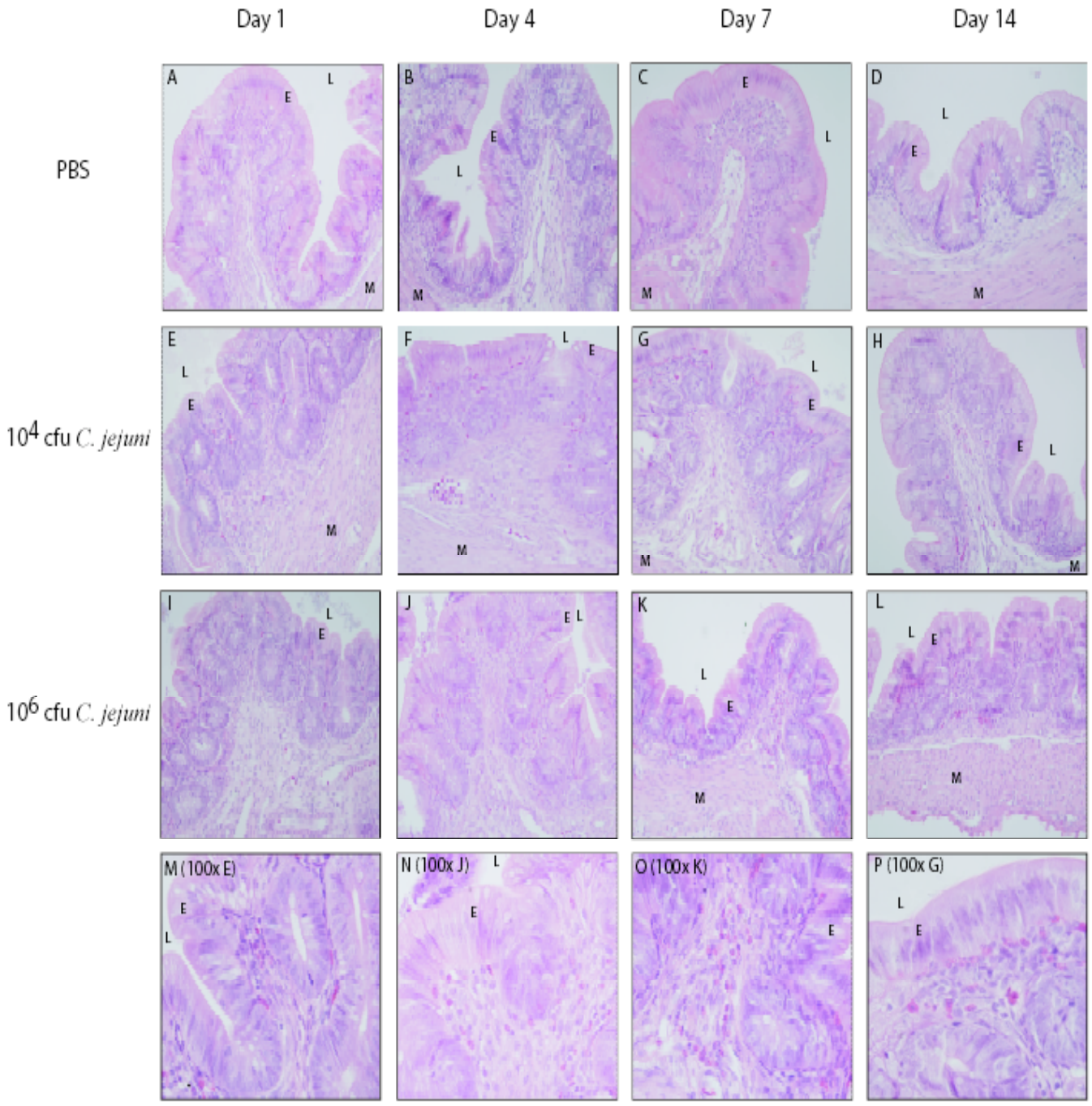


Figure 8. H&E Staining of Infected Tissue. Cecal tissue sections from chicks infected with PBS, 10^4 cfu *C. jejuni* 81-176 Str^R, or 10^6 cfu *C. jejuni* 81-176 Str^R on days one, four, seven, and fourteen post-inoculation stained with H&E. Heterophils (pink granular cells) can be observed in the *C. jejuni* infected tissue. Other signs of inflammations are not observed.

Heterophil Recruitment Requires *virB11*, but not *cdtB* or *virD4*

We hypothesized that bacterial invasion of the chick cecum led to the recruitment of heterophils observed during colonization. We asked whether *C. jejuni* motility and other putative virulence factors are required for the stimulation of heterophil recruitment.

Several *C. jejuni* strains, including 81-176 and 81116, in this study, carry the plasmid pVir, which is responsible for its high levels of invasion into intestinal epithelial cells and virulence in ferrets [12]. The pVir plasmid encodes two putative ATPases based on homology to *H. pylori vir* genes, *virB11* and *virD4*. They are both thought to be a part of a type IV secretion system [12]. The six strains used in this study were tested for the virB11 gene by PCR. Strains 81-176, 81116, CR1 1-1, CR1 2-2, and CR1 3-1 all tested positive for the presence of virB11 by PCR and subsequent sequencing (Fig. 9). To assess the contribution of *virB11* and *virD4* in heterophil recruitment, day-of-hatch chicks were inoculated at three doses, 10^2, 10^4, and 10^6 cfu with 81-176 mutants lacking *virD4* or *virB11*. Colonization was monitored after 7 days and was compared to the *C. jejuni* 81-176-parent strain. The Δ*virD4* strain was recovered from the chick ceca at similar levels at the wild-type strain for all doses examined, and heterophil levels resembled those from wild-type infected birds (Fig. 10). Conversely, Δ*virB11* colonization was attenuated at the 10^2 cfu dose only, and there was no significant heterophil recruitment in the Δ*virB11* infected chicks at all doses examined (Fig 10B). This suggests that *virB11*, while non-essential for colonization, is involved in heterophil recruitment to the infected tissue.

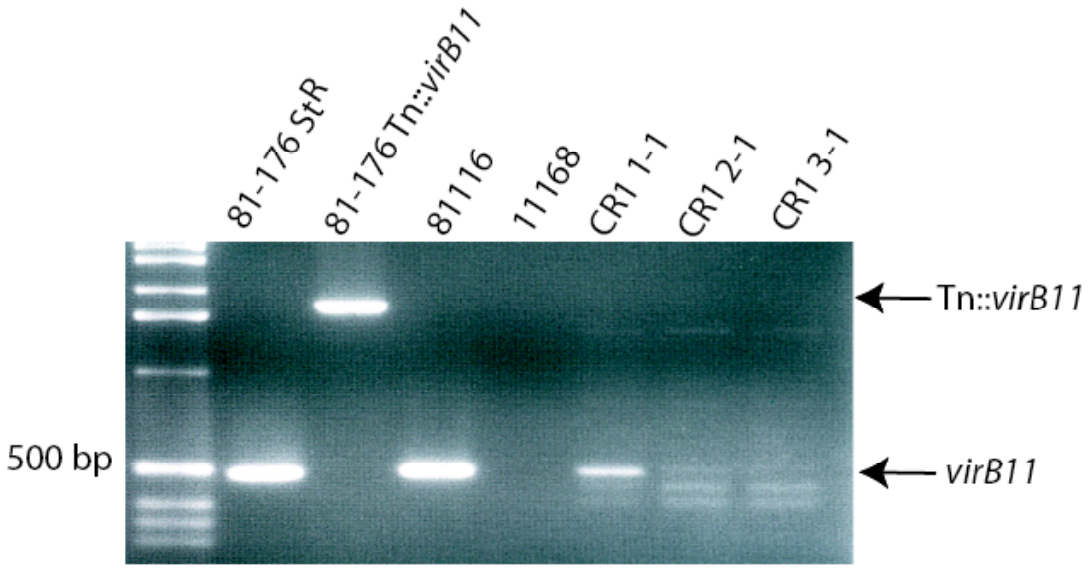


Figure 9. *virB11* Detection by PCR. Colony PCR on the indicated strain for *virB11*. Sequencing of indicated *virB11* band for all strains confirmed positive identification. Smaller bands in CR1 1-1, CR1 2-1, and CR1 3-1 were also *virB11* sequence. Reason for breakdown is unknown.

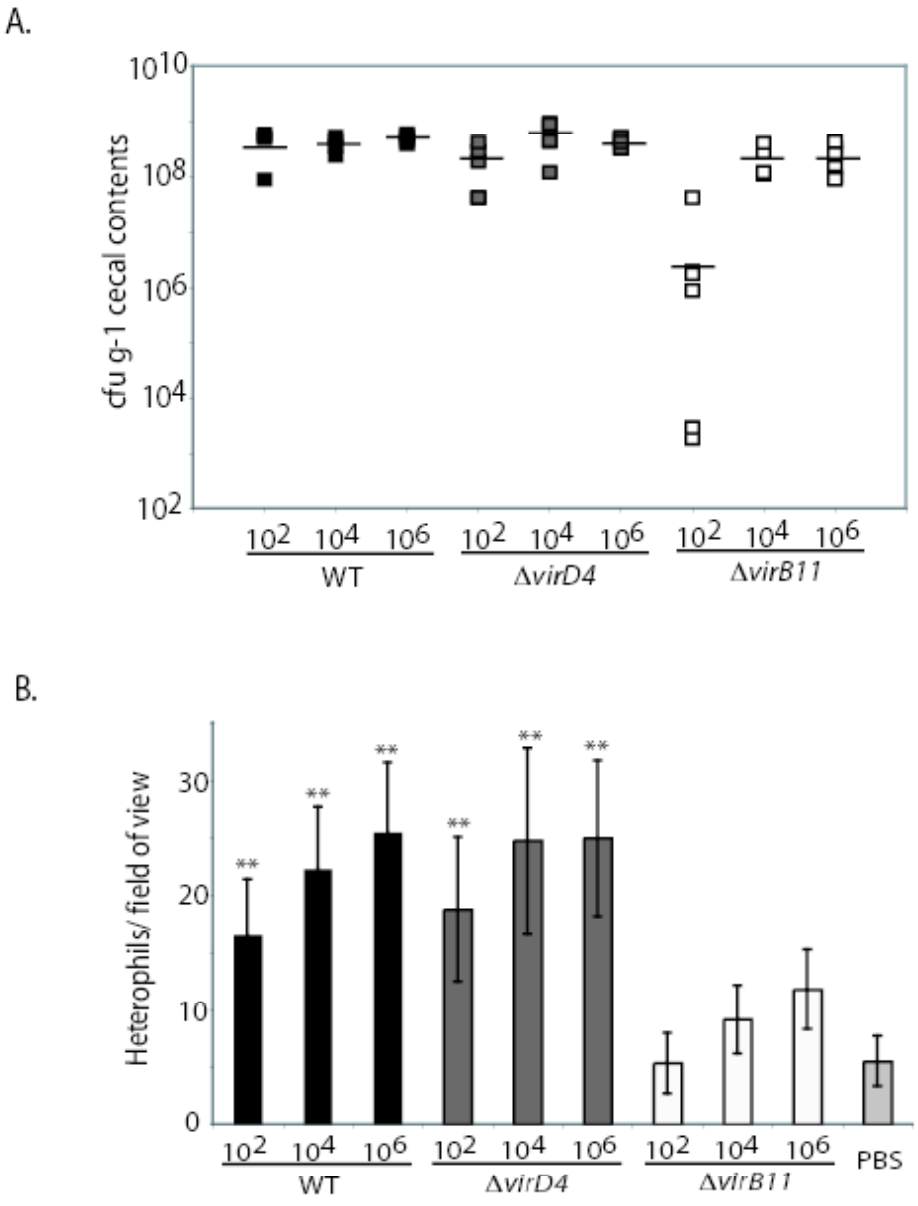


Figure 10. Colonization and Heterophil Response in Δ*virB11* and Δ*virD4* Infections
(A). Cecal colonization of 81-176 [13], Δ*virD4* [14] and Δ*virB11* (white) in chicks inoculated with 10^2, 10^4, 10^6 cfu of each strain after 7 days. Each point represents bacterial recovery from an individual chick. (B). Average heterophil levels in the ceca of chicks infected with WT [13], Δ*virD4* [14], Δ*virB11* (white), and PBS (light gray) at the indicated doses. Significance compared to age-matched PBS inoculated birds *p<0.05, **p<0.005

Cytolethal distending toxin (CDT) is a putative virulence factor, however its precise role in pathogenesis remains unclear. CDT is required for apoptosis in monocytic cell lines *in vitro* and is responsible for the secretion of interleukin (IL)-8 from intestinal epithelial cells [15]. We asked whether CDT is responsible forthe modest level of heterophil recruitment observed during *C. jejuni* infection. CdtB is known to be the toxic component of the toxin and is thought to act as a DNase; localizing to the nucleus of the host cells and causing DNA damage [16]. A Δ*cdtB* mutant was inoculated into day-of-hatch chicks at an inoculum of 10^6 cfu and was recovered at 10^8 cfu g^{-1} cecal contents on days one, four, seven and fourteen post-inoculation demonstrating that *cdtB* is not required for efficient chick colonization, as previously described (Fig. 11A) [17, 18]. Further, heterophil levels in Δ*cdtB* infected chicks were elevated compared to PBS infected chicks and resembled that of wild-type infected birds, demonstrating that *cdtB* is not required for heterophil recruitment during an infection (Fig. 11B).

C. jejuni Invasion of Human and Chicken Cells by an *in vitro* Gentamicin Protection Assay

C. jejuni motility and invasion has been shown to play a major role in establishing colonization in the chick as well as pathogenesis in infection models [19-21]. To ascertain whether invasion of the chick cecal epithelium was contributing to the heterophil

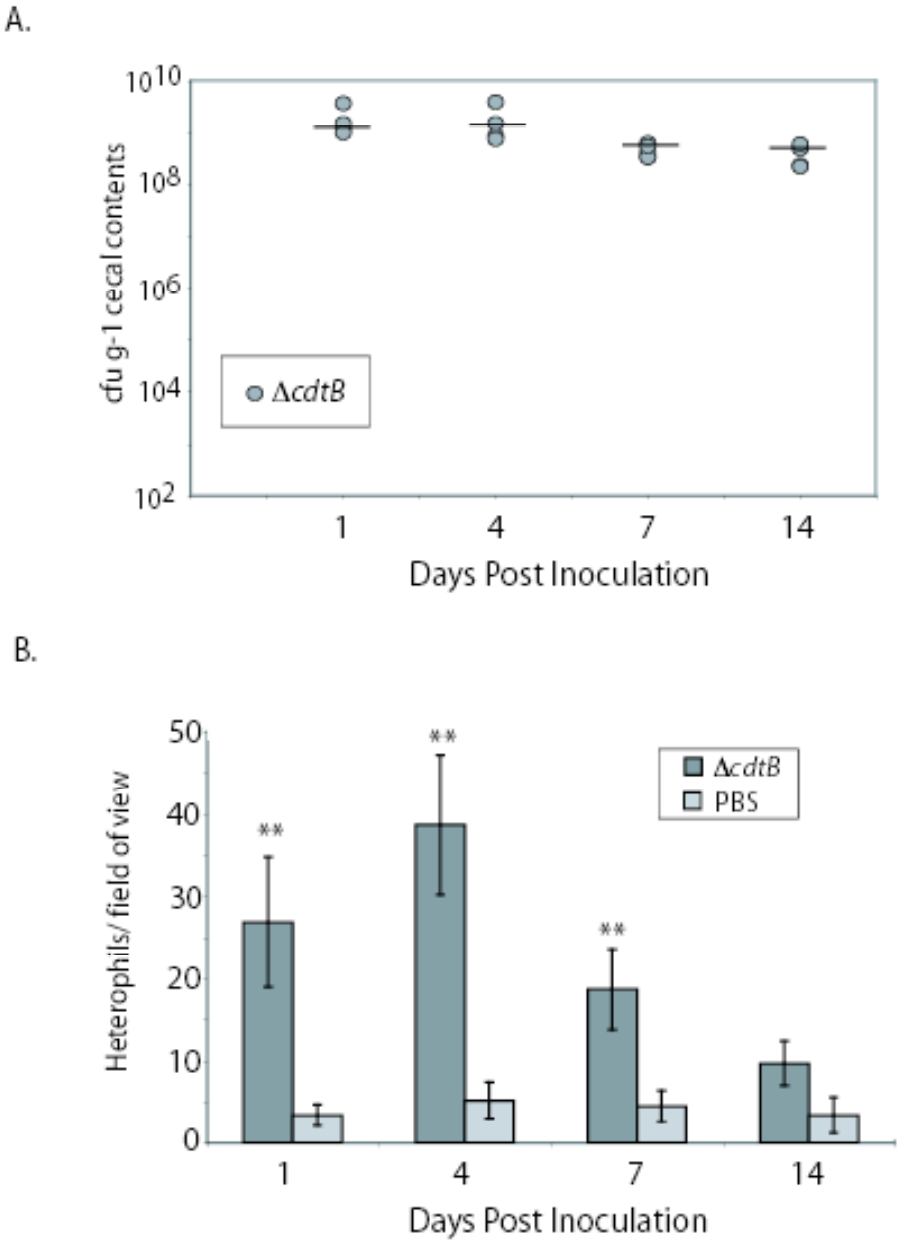


Figure 11. Colonization and Heterophil Response to Δc*dtB* Infection A). Cecal colonization of 81-176 Δ*cdtB* in chicks inoculated with 10^4 cfu on days 1, 4, 7, and 14 post inoculation. Each point represents bacterial recovery from an individual chick. (B) Average heterophil levels in the ceca of chicks inoculated with Δ*cdtB* mutant (dark grey) and PBS (light gray) on days 1, 4, 7, and 14 post-inoculation. Significance compared to age-matched PBS infected birds. *p<0.05, **p<0.005

recruitment observed in response to infection, invasion of INT407 cells and primary chicken intestinal cells was examined for several *C. jejuni* strains. It was previously reported that *virB11* is necessary for efficient invasion of INT407 [22]. We confirmed these results, demonstrating that *virB11*, and not *virD4* or *cdtB* are necessary for invasion of INT407 cells as well as primary chicken intestinal cells (Fig. 12). The chicken commensal strains, CR1 1-1, CR1 2-1, and CR1 3-1, were also tested for their ability to invade INT407 and primary chick intestinal cells and were able to invade both cell types albeit with slight, but significantly lower rate of invasion (Fig. 12).

C. jejuni Localization in the Chick Cecum by an *in vivo* Gentamicin Protection Assay

C. jejuni invades chicken epithelial cells in cell culture and is speculated to be able to invade chicken intestinal cells *in vivo* [5, 23]. As described in the methods, we used a gentamicin protection assay on cecal homogenates from infected chicks to quantify *C. jejuni* that were intracellular, associated with the cells, or in the luminal contents. In this assay, bacteria isolated from the tissue incubated with media alone represents those in close association with the tissue, either extracellular or intracellular. Bacteria isolated from tissue to which gentamicin was added represents bacteria that had become resistant to the antibiotic, presumably intracellular. *C. jejuni* was added to the third fraction containing gentamicin to demonstrate effective killing of extracellular bacteria during incubation with gentamicin.

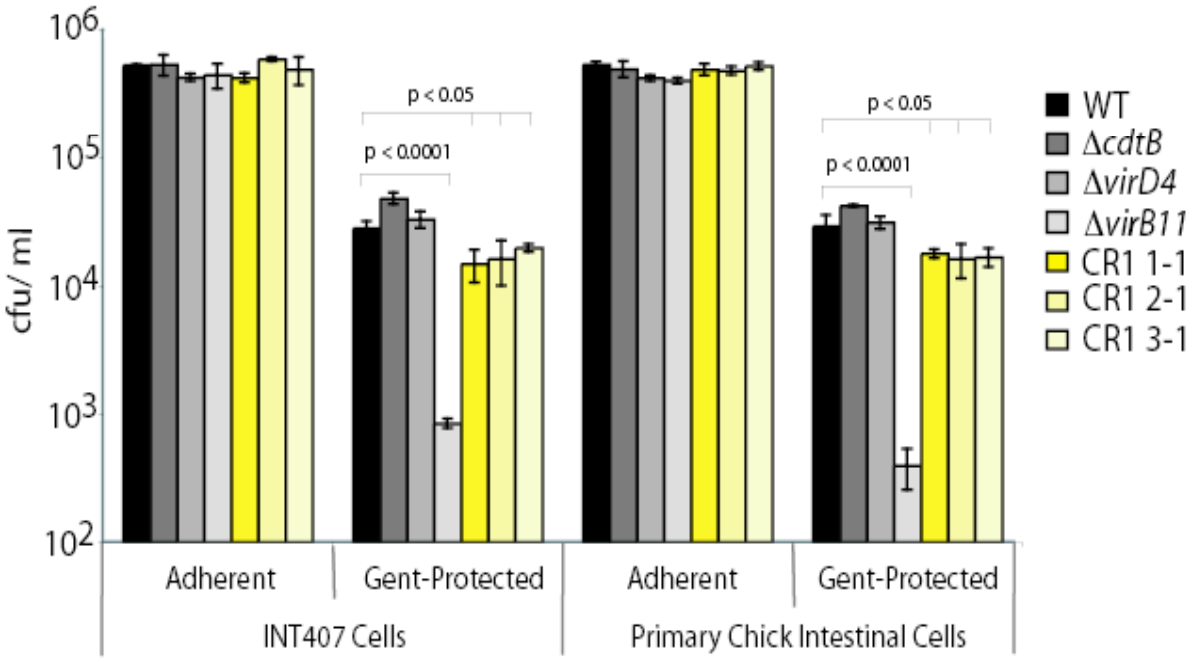


Figure 12. Cell Culture Invasion of INT407 and Primary Chicken Intestinal Cells. Cell culture invasion, as tested by the gentamicin protection assay, by *C. jejuni* strains 81-176Str^{R}, Δ*cdtB*, Δ*virD4*, Δ*virB11*, CR1 1-1, CR1 2-1, and CR1 3-1 of INT407 cells and primary chicken intestinal cells. Recovery of total cell associated, or adherent bacteria, as well as gentamicin-protected bacteria, or invasive bacteria, are reported in cfu/ml. Cells were individually inoculated with 2x10^{7} cfu of each strain. Significance to WT levels is indicated.

While consistently high numbers of bacteria (10^6 cfu g^{-1} cecal tissue) were recovered from media- treated tissue on each day sampled, bacteria protected from gentamicin killing (10^4 cfu g^{-1} cecal tissue) were recovered from infected ceca only on days four and seven post inoculation (Fig. 13A). The number of gentamicin-protected bacteria dropped considerably by day fourteen post-inoculation (to fewer than 10^2 cfu g^{-1} cecal tissue). The recovery of similar levels of bacteria in the tissue incubated with gentamicin and an additional 10^6 cfu *C. jejuni* gives us confidence that the gentamicin-protected bacteria did not simply survive inefficient killing by the antibiotic in the milieu of the tissue extracted from the infected animals. Similarly, *C. jejuni* added to tissue extracts from animals inoculated with PBS were killed to high levels when gentamicin was added. Tissue incubated with 10^6 cfu *C. jejuni* without gentamicin yielded 10^6 cfu *C. jejuni,* demonstrating that extracellular *C. jejuni* was not lost in the assay by washes or bactericidal factors in the tissue itself (Fig. 13C). Based on these results, we conclude that *C. jejuni* is primarily localized to the luminal contents, but a fraction of the population invades the intestinal epithelium between days four and seven post inoculation in this model of colonization.

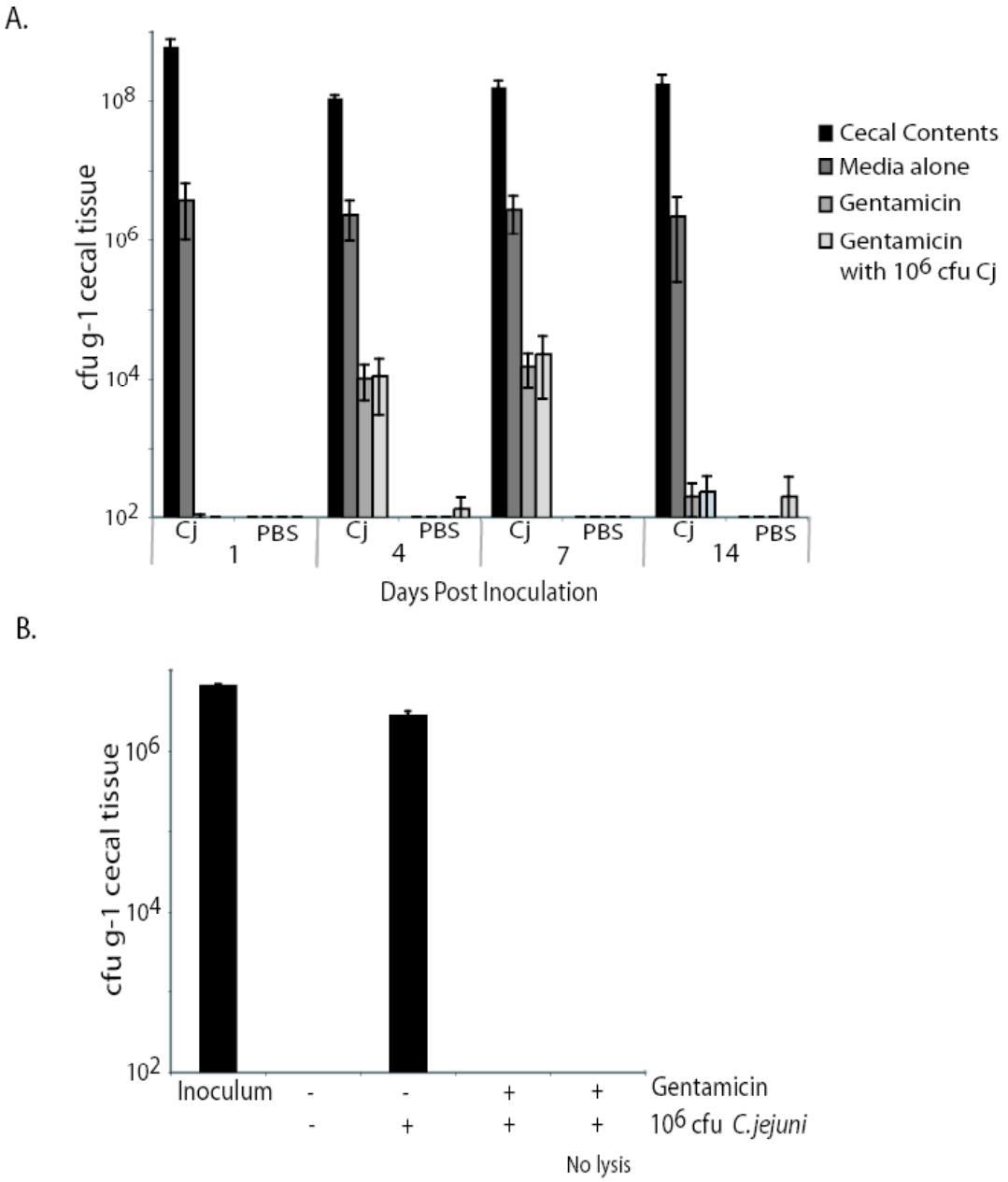


Figure 13. *In vivo* Gentamicin Protection Assay. Infected chick ceca were removed and their contents examined for *C. jejuni* recovery on days one, four, seven, and fourteen post inoculation [13]. Tissue incubated with media alone represents total cell associate bacteria (dark gray), gentamicin treated tissue samples represent intracellular bacteria (light gray), and tissue incubated with gentamicin with an additional 10^6 cfu *C. jejuni* demonstrates the efficiency of gentamicin killing of extracellular bacteria (lightest gray). (B). PBS infected chick ceca were removed and incubated with media alone, 10^6 cfu *C. jejuni, C. jejuni* and gentamicin, or *C. jejuni* and gentamicin with no cell lysis. Tissue treated with *C. jejuni* alone demonstrates that extracellular *C. jejuni* is not lost in the assay without the treatment of gentamicin.

C.

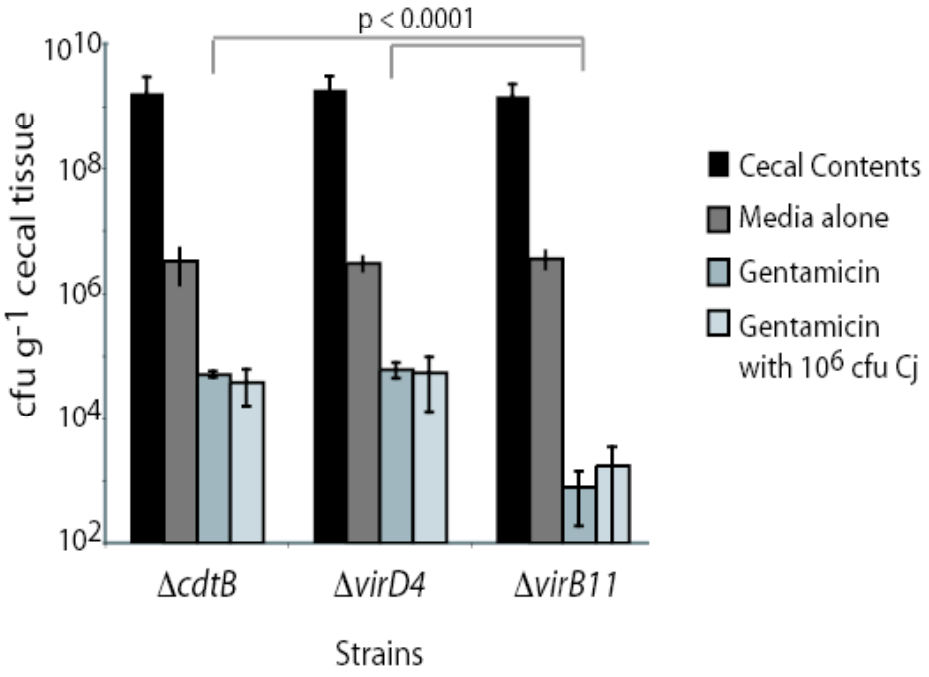


Figure 13 (continued) (C). In *vivo* gentamicin protection assay of Δ*cdtB,* Δ*virD4,* and Δ*virB11*. Tissue from birds infected with the indicated strains on day seven post inoculation were incubated with media alone, gentamicin, or gentamicin with an additional 10^6 cfu *C. jejuni*

Similar to the results in cell culture, the Δ*virB11* mutant was not attenuated for adherence to the chick cecal cells *in vivo*, but the number of gentamicin-protected bacteria recovered was significantly reduced compared to WT, Δ*cdtB*, and Δ*virD4* on day 7 post-inoculation (Fig. 13B). This suggests that *C. jejuni* invasion of tissue culture cells and the chicken epithelial cells *in vivo* is *virB11* dependent. Further, as the Δ*virB11* mutant colonization did not stimulate significant heterophil response in the chick ceca, we have demonstrated a correlation between the ability of a strain to invade epithelial cells and its ability to stimulate the innate immune response in the cecal tissue of infected chicks.

Microscopy of *C. jejuni*-infected Chick Ceca

Cecal tissue samples from infected animals were fixed and visualized by electron microscopy (Fig. 14). *C. jejuni*, notable for its characteristic spiral shape, was easily visible in the infected tissues. C. *jejuni* primarily inhabits the mucous layer of cecal crypts[24, 25] and *C. jejuni* was found in the cecal crypts, both within the mucosal layer and lumen, as well as adherent to the epithelium (Fig. 14).

To clarify the interaction of *C. jejuni* with the chick epithelium, we used immunofluorescent microscopy as described in Materials and Methods (Fig. 15). *C. jejuni* was seen in the lumen, with high concentrations in the mucosal layer early in infection. By day four, *C. jejuni* was found close to the intestinal epithelium layer, in contrast to other intestinal normal flora detectable in the mucosal layer and lumen by DAPI staining (Fig. 15). In 12 of 131 fields of view containing bacteria, in chicks at days four and seven post-inoculation, we detected *C. jejuni* in the sub-mucosa, indicative of an ability to invade the epithelia (Fig. 14).

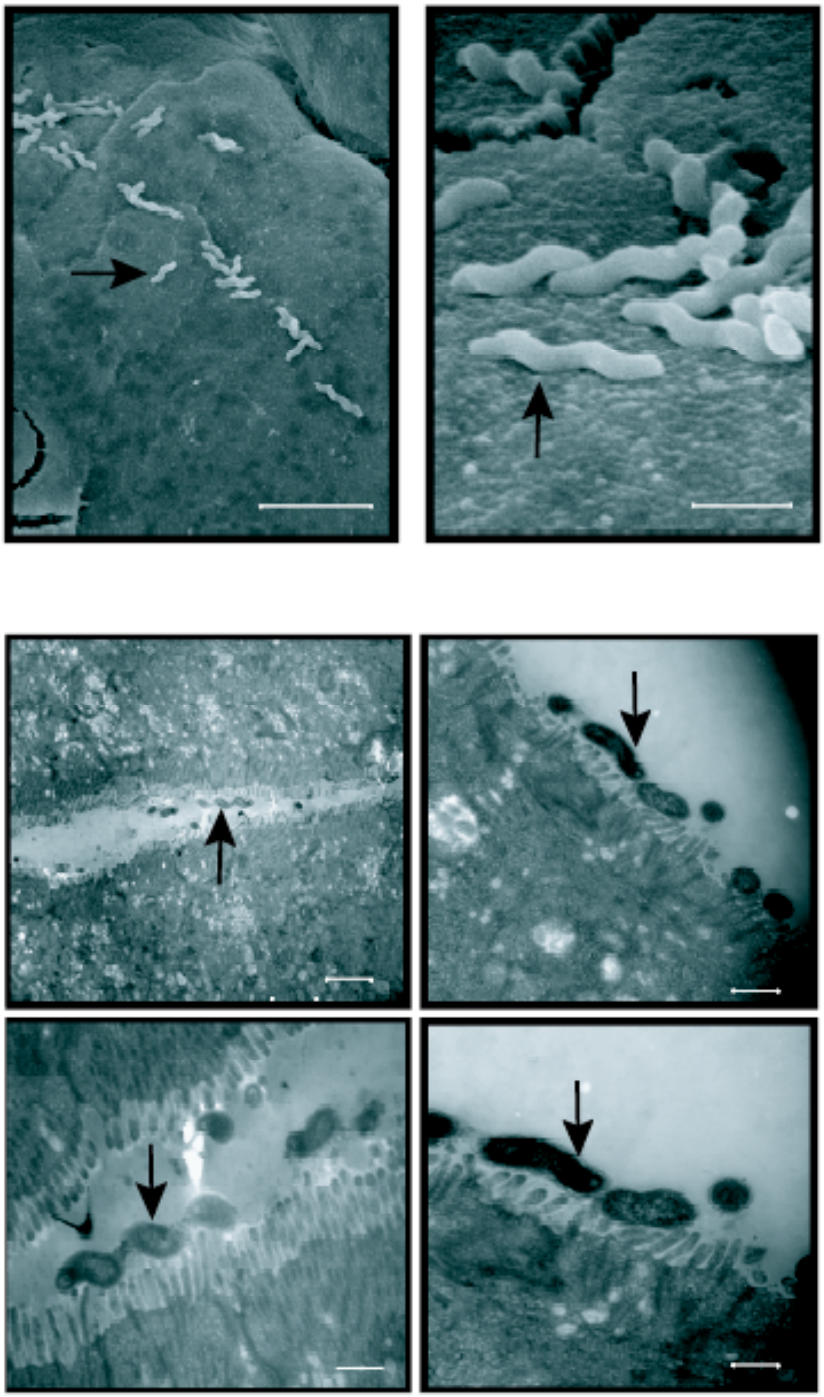


Figure 14. Electron Microscopy. (A) Scanning electron micrographs of *C. jejuni* infected chick ceca day 4 post inoculation. (B) Transmission electron micrographs depicting C. *jejuni* in close association with the epithelial layer in the chick ceca day 4 post inoculation. White lines denote 5 microns (top left panel) and 1 micron (top right panel), 2 microns (middle left), 500nm (middle right), 500nm (bottom left) and 50nm (bottom right). Arrows denote *C. jejuni.*

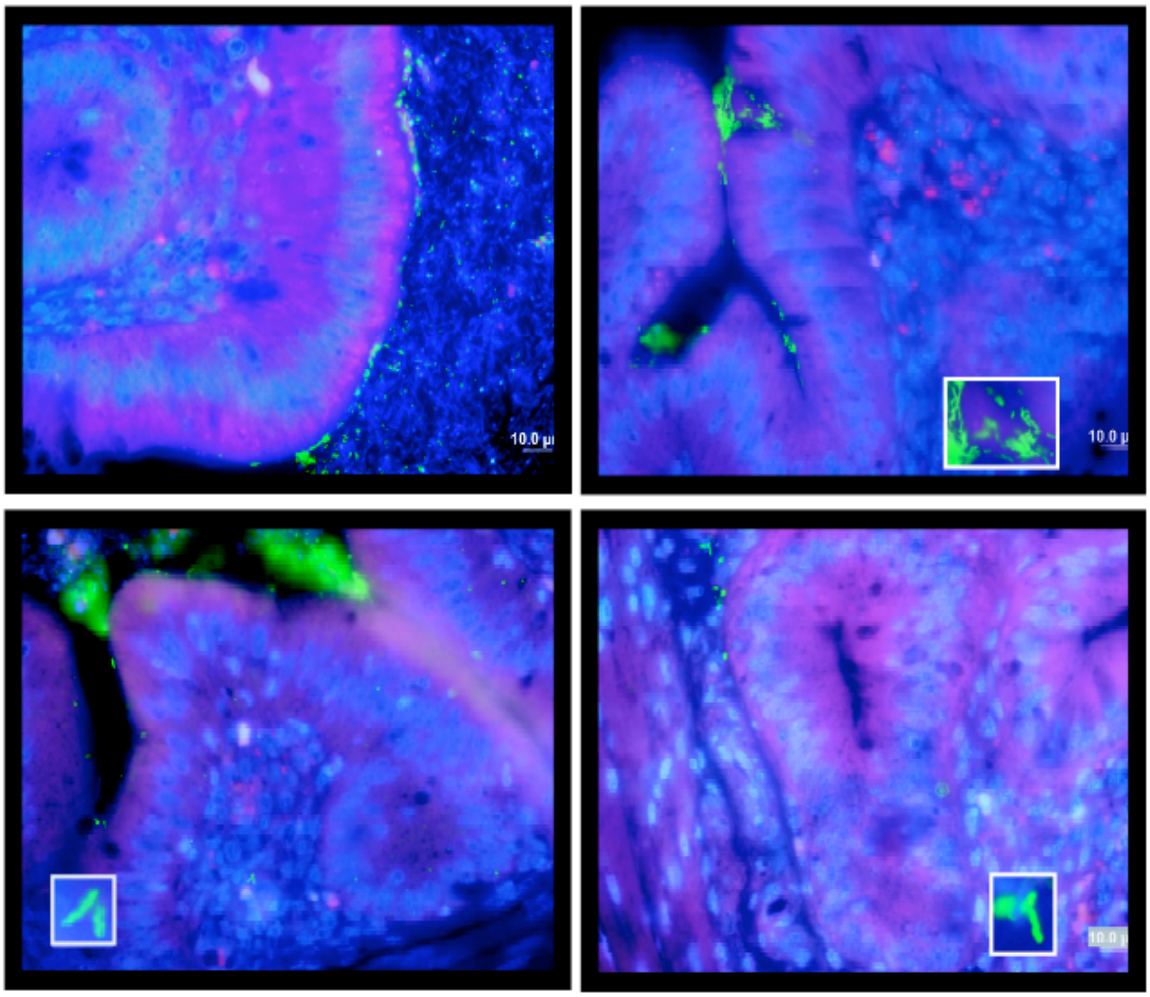


Figure 15. Immunofluorescent Microscopy. Immunofluorescent micrographs of infected chick ceca at four days post inoculation. Tissues were incubated with DAPI (blue) rhodamine-phalloidin (red), and anti-*Campylobacter* antibodies (green). Insets are enlarged images of *C. jejuni* in the tissue.

Kinetics of *C. jejuni* Colonization

Day-of hatch chicks were inoculated with *C. jejuni* 81-176(Str^r), at doses of 10^4 cfu or 10^6 cfu. Two doses were used to assess whether the quantity of bacteria introduced into the chick plays a role in how *C. jejuni* establishes colonization and stimulates the innate immune response.

Infection over two weeks with wild-type *C. jejuni* 81-176(Str^r) at inocula of 10^4 cfu and 10^6 cfu demonstrated that kinetics of cecal colonization reflects the inoculum to some degree (Fig. 16). When inoculated with10^4 cfu, *C. jejuni* colonized to levels of 10^4 cfu g^{-1} cecal contents up to 4 days post inoculation. By day seven, the amount of *C. jejuni* colonization increased and remained high until fourteen days post inoculation, ranging between 10^7-10^9 cfu g^{-1} cecal contents. When inoculated with 10^6 cfu, *C. jejuni* colonized at 10^8-10^9 cfu g^{-1} of cecal contents very early after inoculation and remained at that level throughout the course of the experiment (Fig. 16). These findings are consistent with what we have previously demonstrated with *C. jejuni* colonization dynamics, with slight variability in levels of colonization of chicks inoculated with 10^4 cfu on day four post-inoculation [11].

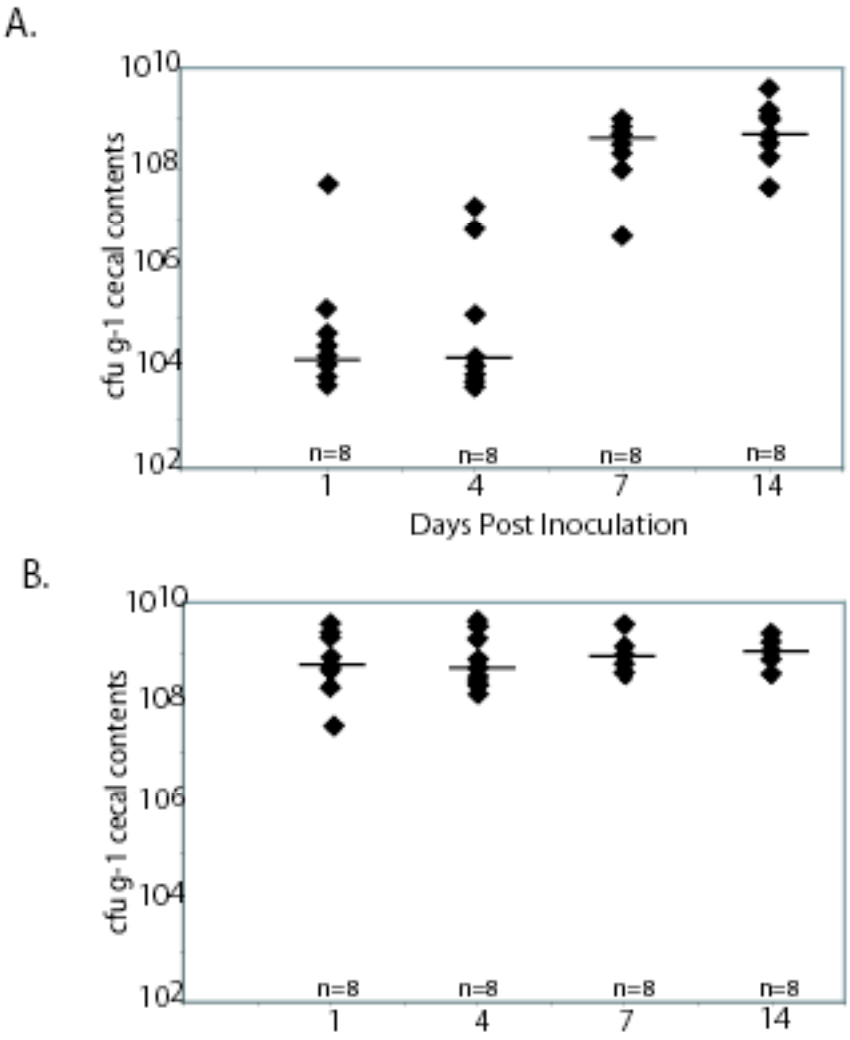


Figure 16. Colonization and Heterophil Response to *C. jejuni* Strain 81-176Str^{R}. Each dot represents bacterial recovery from an individual chick. (A). Colonization of chick ceca inoculated with 10^4 and (B) 10^6 cfu. Limit of detection is 10^2 cfu g^{-1} cecal contents.

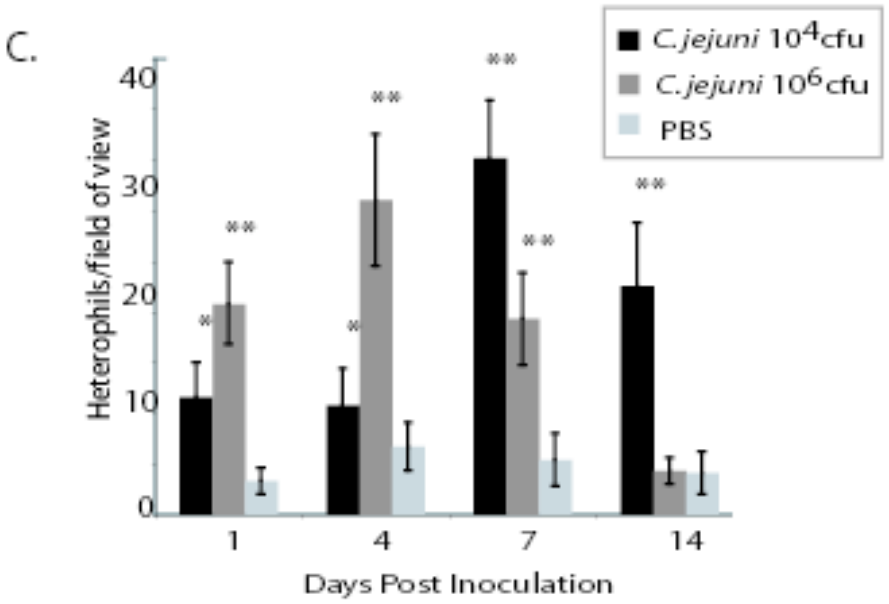


Figure 16. (continued) (C). Average number of heterophils in the chick cecum on days one, four, seven, and fourteen in chicks inoculated with 81-176Str^{R}. Significance compared to age-matched PBS inoculated birds *$p<0.05$, **$p<0.005$.

By histopathology, heterophil levels in the cecal tissue of chicks inoculated with 10^4 cfu 81-176(Str^r) were slightly elevated on days one and four post inoculation, compared to PBS inoculated chicks. Heterophil levels increased on days seven and fourteen inoculated with 10^4 cfu 81-176(Str^r) (Fig. 16C). This increase in heterophil recruitment coincides with the increase of *C. jejuni* colonization in the chick cecum on days seven and fourteen post inoculation (Fig. 16A). In chicks inoculated with 10^6 cfu C. jejuni, the number of heterophils observed was significantly greater than those in the chicks inoculated with 10^4 cfu. The number of heterophils correlates with the level of colonization by *C. jejuni*. Although no other visible signs of epithelial damage were observed in the cecal samples (Fig. 8), significant heterophil recruitment could signify the ability of the chicks to recognize *C. jejuni* as an invading microbe as polymorphonuclear cell recruitment is not typically seen during colonization by normal flora [26].

Systemic Spread of *C. jejuni*

C. jejuni has been recovered from systemic sites in broiler chicks asymptomatically colonized with *C. jejuni* [6]. As shown, local invasion of cecal tissue by *C. jejuni* is evident, and we asked whether local invasion of the tissue could lead to systemic spread of *C. jejuni.* To test this, recovery of *C. jejuni* was examined from the cecal contents, spleen, and liver from chicks inoculated with 10^6 cfu wild-type *C. jejuni* over two weeks. *C. jejuni* was recovered from both the spleen and liver on days 7 and 14 post-inoculation, in agreement with previous reports (Fig. 17). Whether this systemic spread is a result of local invasion of the cecum is unknown.

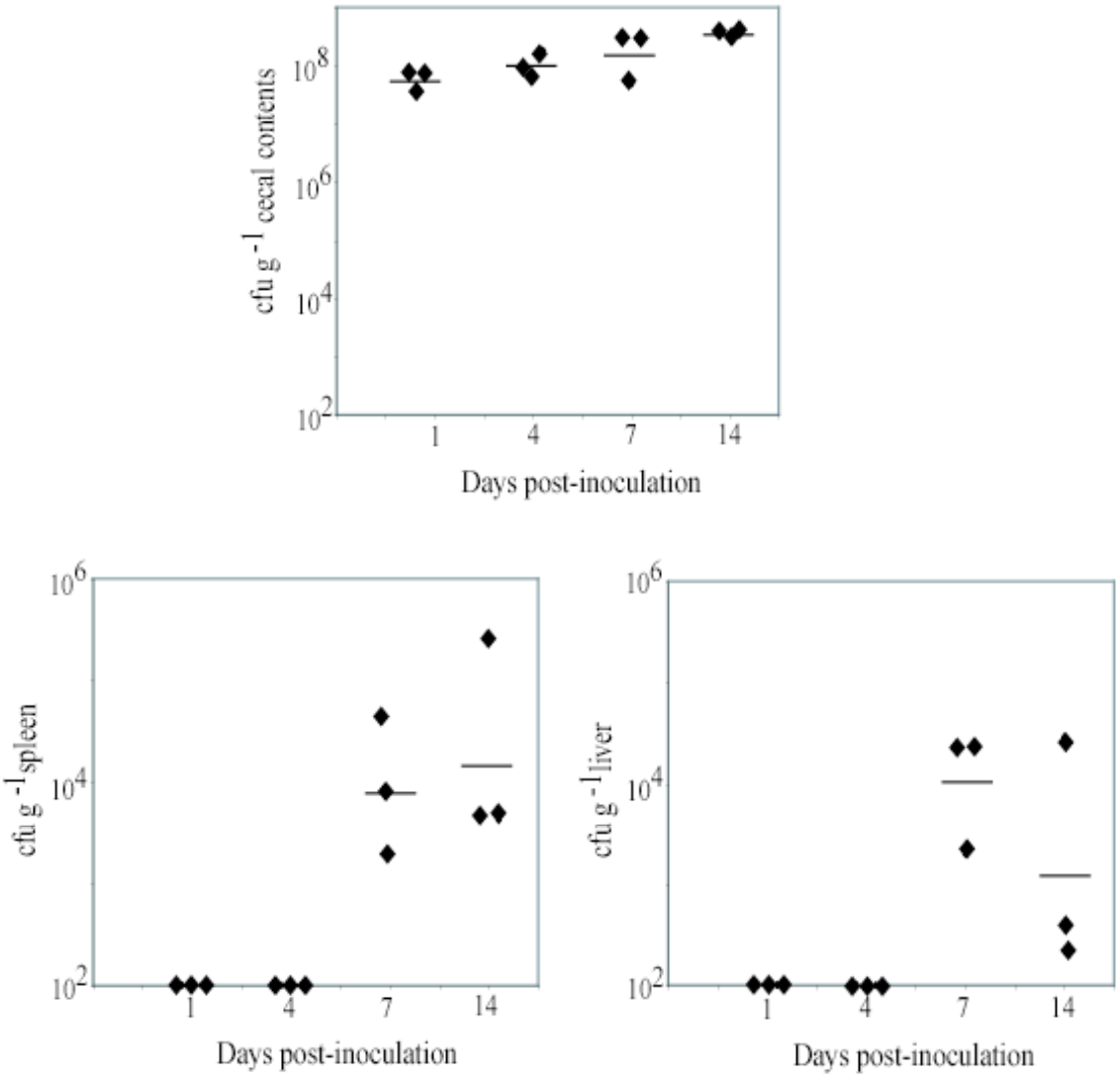
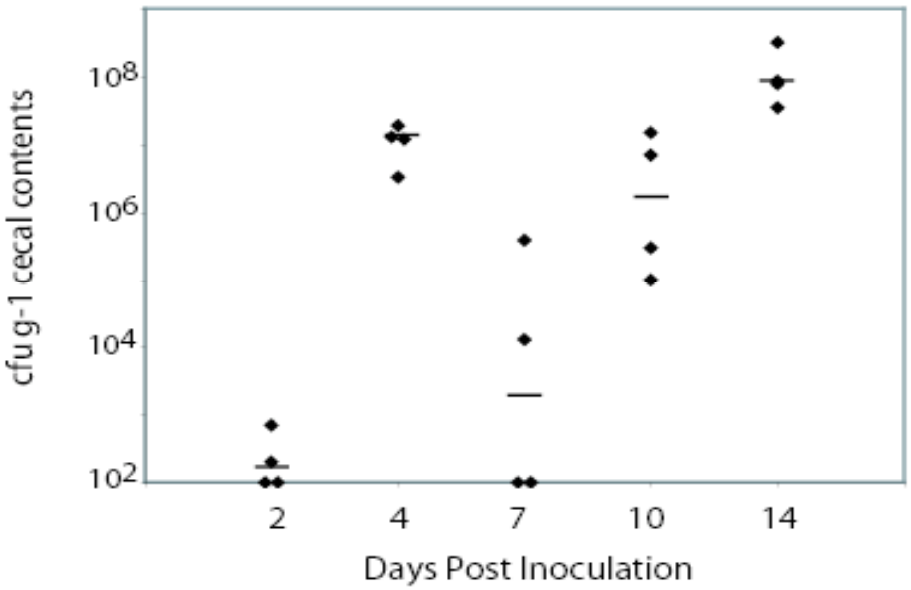


Figure 17. *C. jejuni* Recovery From Systemic Sites. Bacterial recovery from cecal contents (top), spleen (bottom left), and liver (bottom right) of chicks over two weeks, inoculated with 10^6 cfu wild-type *C. jejuni*.

Low-Dose Infection *C. jejuni* Colonization Dynamics

Chicks in broiler flocks most likely are exposed to *C. jejuni* upon hatch at low levels at a consistent rate, unlike the day-of-hatch model which uses relatively large doses of the bacteria at once (10^4-10^6 cfu). In order to mimic a more natural infection, day-of-hatch chicks were orally inoculated with 100 cfu of wild-type *C. jejuni* daily for 5 consecutive days. Recovery from the cecal contents was monitored for two weeks. *C. jejuni* was recovered at low levels 2 days post-inoculation, which increased on day 4 post-inoculation to 10^7 cfu g^{-1} cecal contents. Heterophil levels were significantly higher on day 4 post-hatch, which corresponds with an increase in *C. jejuni* recovery from the chick cecum.

C. jejuni recovery dropped to 10^3 cfu g^{-1} cecal contents on day 7 post hatch, which corresponds with a low level of heterophil recruitment. Why *C. jejuni* recovery fell on day 7 is unknown. Although *C. jejuni* colonization significantly increases to 14 days post-inoculation, heterophil recruitment does not (Fig. 18). These results suggest that after the initial inflammatory response, *C. jejuni* did not elicit a second response, independent of the level of colonization. The lack of a second immune response could be due to *C. jejuni* phase variation, introduction of microbiota, alterations in the epithelial layer or mucus layer, or changes in the host response to *C. jejuni*, due to maturation of the immune system, production of sIgA antibodies against *C. jejuni,* or development of tolerance against *C. jejuni*.



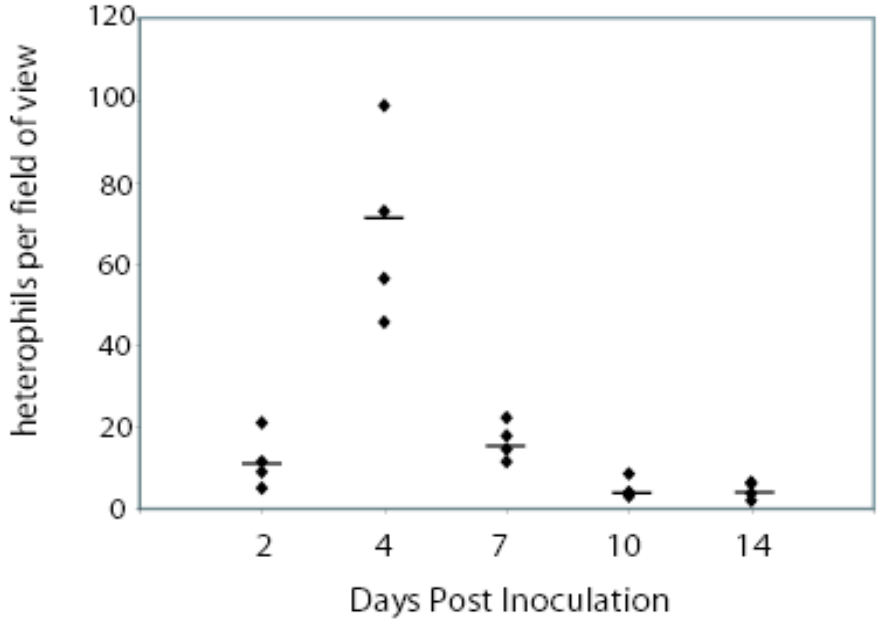
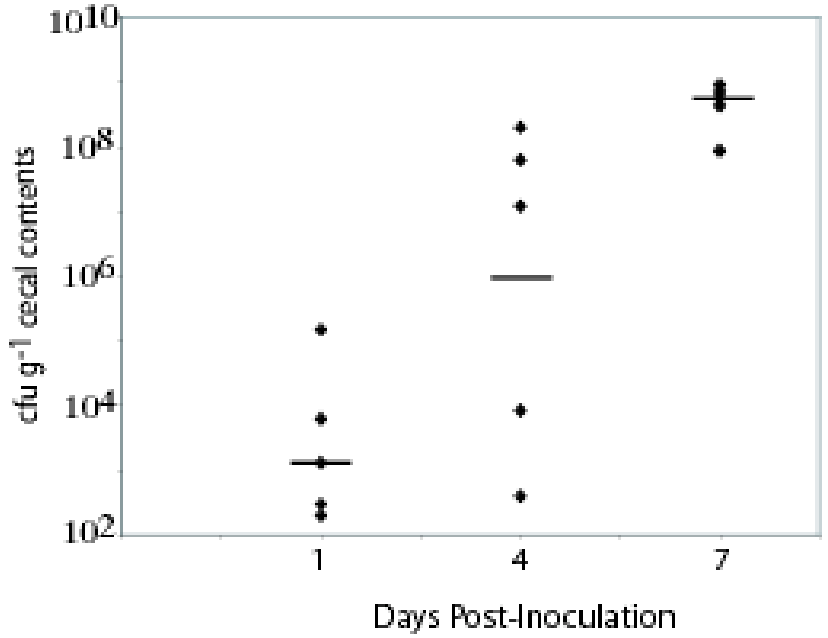


Figure 18. Colonization and Heterophil Response During a Low-Dose Infection. (A). *C. jejuni* recovery from cecal contents from birds inoculated with 100 cfu *C. jejuni* for 5 consecutive days. (B) Heterophil levels in the cecal tissue of infected chicks.

Day-7 Infection *C. jejuni* Colonization Dynamics

In a further effort to recapitulate natural conditions of *C. jejuni* colonization of chicks, 7-day-old chicks were orally inoculated with 10^4 cfu of wild-type *C. jejuni,* and colonization and heterophil recruitment were monitored for one week. Broiler chicks are not colonized with *C. jejuni* until 10-14 days post-hatch, presumably due to the presence of anti-*C. jejuni* maternal antibodies. *C. jejuni* was recovered at low levels one day post-inoculation, which increased to 10^9 cf u g^{-1} cecal contents on day 7 post-inoculation. This is similar to *C. jejuni* recovery from orally-inoculated day-of-hatch chicks described above (Fig. 19 and Fig. 16). However, there was no significant heterophil recruitment in response to *C. jejuni* colonization when chicks were inoculated on day 7, compared to day-of-hatch inoculation (Fig. 19 and Fig.16).

These results suggest that other factors independent of the level of *C. jejuni* colonization could be influencing the innate immune response to *C. jejuni* colonization. Factors that could be influencing *C. jejuni*-host interactions could include the presence of a more established microbiota, age-dependent development of the epithelial layer and mucus layer in the cecum, or maturation of the immune system.



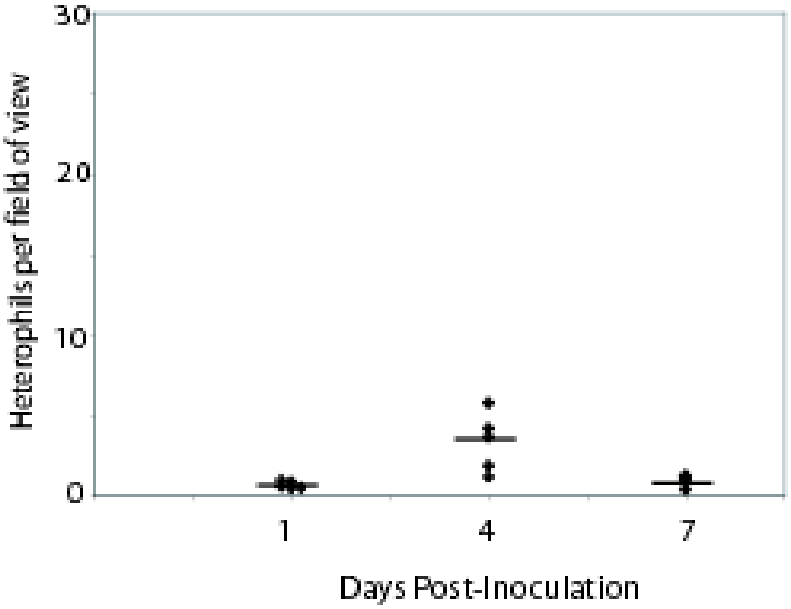


Figure 19. Colonization and Heterophil Response During Day-7 Inoculation. (A) Recovery of C. jejuni from the ceca of chicks inoculated with 10^4 cfu of wild-type *C. jejuni* on day 7 post-hatch. Colonization was monitored on day 1, 4, and 7 post-inoculation. (B). Heterophil levels per field of view in chicks as determined by histopathology.

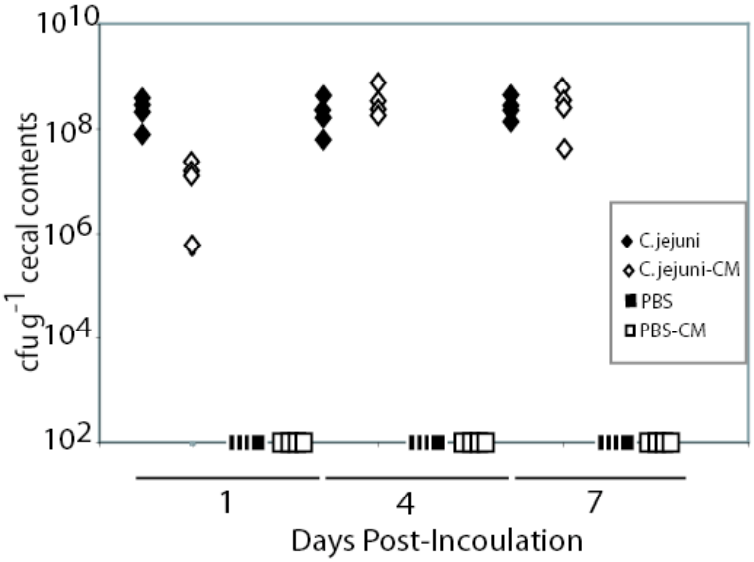
Chick Cecal Response to Cecal Extracts from Week Old Chicks

In the day-of-hatch colonization model, chicks are infected with large doses of bacteria (*C. jejuni*), with clear evidence of a significant innate immune response to the infection in terms of a heterophil response. To further investigate the nature of the response to *C. jejuni* infection specifically, the non-specific response to a large dose of bacteria in these animals was examined.

To examine this, we asked whether the constituents of the chicken microbiota would elicit a similar response, or whether the response observed was specific to *C. jejuni*. Four groups of day-of-hatch chicks were analyzed in this experiment (Fig. 20). In two groups, the chicks were infected with *C. jejuni* or treated with PBS alone, as previously described. In two other groups, the chicks were infected with cecal material [27] from older (seven-day-old) chicks that had originally been treated with PBS alone (PBS-CM) or *C. jejuni* (*C. jejuni*-CM). *C. jejuni* colonized the chick cecum whether inoculated alone, or along with other flora from older animals on days one, four and seven post-inoculation (Fig. 20A). The lower initial colonization of *C. jejuni* from cecal material is most likely due to a lower inoculation dose (10^5 cfu), harvested from the cecal material.

Heterophil levels were analyzed by histopathology in the infected groups. Although PBS-CM infected chicks had significant heterophil levels on day one post inoculation, this resolved by day four post inoculation (Fig. 20B). Also, the levels of heterophils observed in PBS-CM chicks were significantly lower than those seen in *C. jejuni* inoculated chicks (Fig. 20B). Chicks inoculated with *C. jejuni*-CM had elevated heterophils levels, similar to those inoculated with *C. jejuni* alone. These levels were

A.



B.

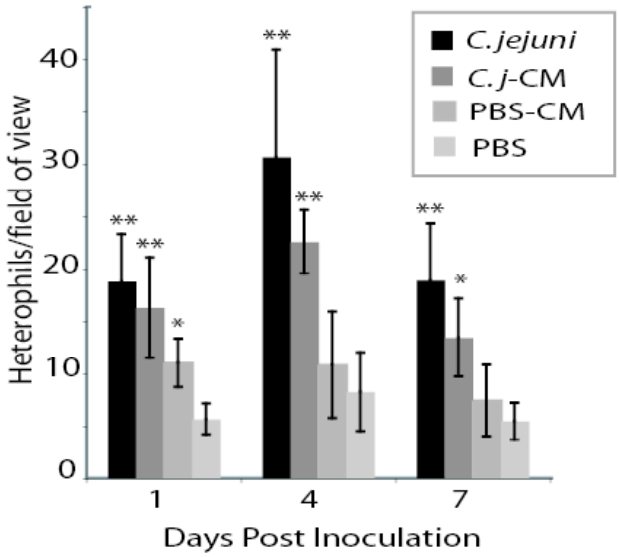


Figure 20. Colonization and Heterophil Response to Cecal Material (A). Cecal colonization of birds inoculated with *C. jejuni*, PBS, or Cecal Material [27] from 7-day birds infected with *C. jejuni* (Cj-CM, open diamond) or PBS (PBS-CM, open square). Samples were taken on one, four, and seven days post-inoculation. Each dot represents an individual chick. Limit of detection is 10^2 cfu g^{-1} cecal contents. (B). Average number of heterophils in the chick cecum on days one, four, and seven in chicks inoculated with *C. jejuni*, Cj-CM, PBS, or PBS-CM. Significance compared to age-matched PBS inoculated birds *$p<0.05$, **$p<0.005$.

elevated on days one, four and seven compared to chicks inoculated with PBS alone (Fig. 20B). We conclude that although chicks may respond to their own microbiota through recruitment of heterophils in the day-of-hatch chick colonization model, the majority of the response observed is dependent on the presence of *C. jejuni.*

Chick Cecal Response to *S. typhimurium*

As polymorphonuclear cell recruitment is indicative of an invasive pathogen, we asked how the chick response to *C. jejuni* compared to the chick response to an invasive pathogen, such as *Salmonella typhimurium*. Colonization and heterophil recruitment were examined over two weeks in day-of-hatch chicks infected with 10^6 cfu of *C. jejuni* or S. *typhimurium*. *C. jejuni* colonized the chick ceca within one day at levels up to 10^8 cfu g^{-1} cecal contents and remained at high levels, while PBS inoculated chicks remained un-colonized with either *C. jejuni* or *S. typhimurium* through the two week infection period. In contrast, *S. typhimurium* was recovered at 10^8 cfu g^{-1} cecal contents for only one-day post inoculation. *S. typhimurium* colonization were reduced on day four post inoculation, and were recoverable above our level of detection on days seven and fourteen post inoculation in only two chicks (Fig. 21A).

Histopathology revealed increased levels of heterophil recruitment (308.8 heterophils/ field of view) compared to PBS inoculated chicks (7.2 heterophils/ field of view) and *C. jejuni* inoculated chicks (19.5 heterophils/field of view) on day one post-inoculation (Fig. 21B). Heterophil levels remained higher in the *S. typhimurium* infected animals, compared to the *C. jejuni* infected chicks over the two-week infection, despite the rapid decline of colonization by *S. typhimurium*. These results demonstrate that

although *C. jejuni* induces heterophil recruitment in infected chick ceca, infection with *S. typhimurium* leads to a more aggressive host response. This is most likely due to the more invasive nature of *S. typhimurium* compared to *C. jejuni*.

We asked whether *C. jejuni* would be sensitive to an aggressive immune response, such as the one seen in response to *S. typhimurium* infection. To test this, we infected chicks with either *C. jejuni* or *S. typhimurium* alone, or with a 1:1 mixture of the two bacteria. Colonization and histopathology were examined on days three, seven and ten post-inoculation. In the co-infection, both *S. typhimurium* and *C. jejuni* were detected at high levels on day three post-inoculation (Fig. 21C). By day seven, S. *typhimirium* colonization decreased, and bacteria were recoverable above our level of detection in two birds, similar to the results of the single infection of *S. typhimurium*. *C. jejuni* was detected at 10^8 to 10^9 cfu g^{-1} cecal contents on days three, seven and ten post-inoculation , despite an aggressive inflammatory response similar to what was observed in the *S. typhimurium* infected chicks. These results demonstrate that the robust inflammatory response stimulated in response to an *S. typhimurium* infection is ineffective at clearing *C. jejuni.*

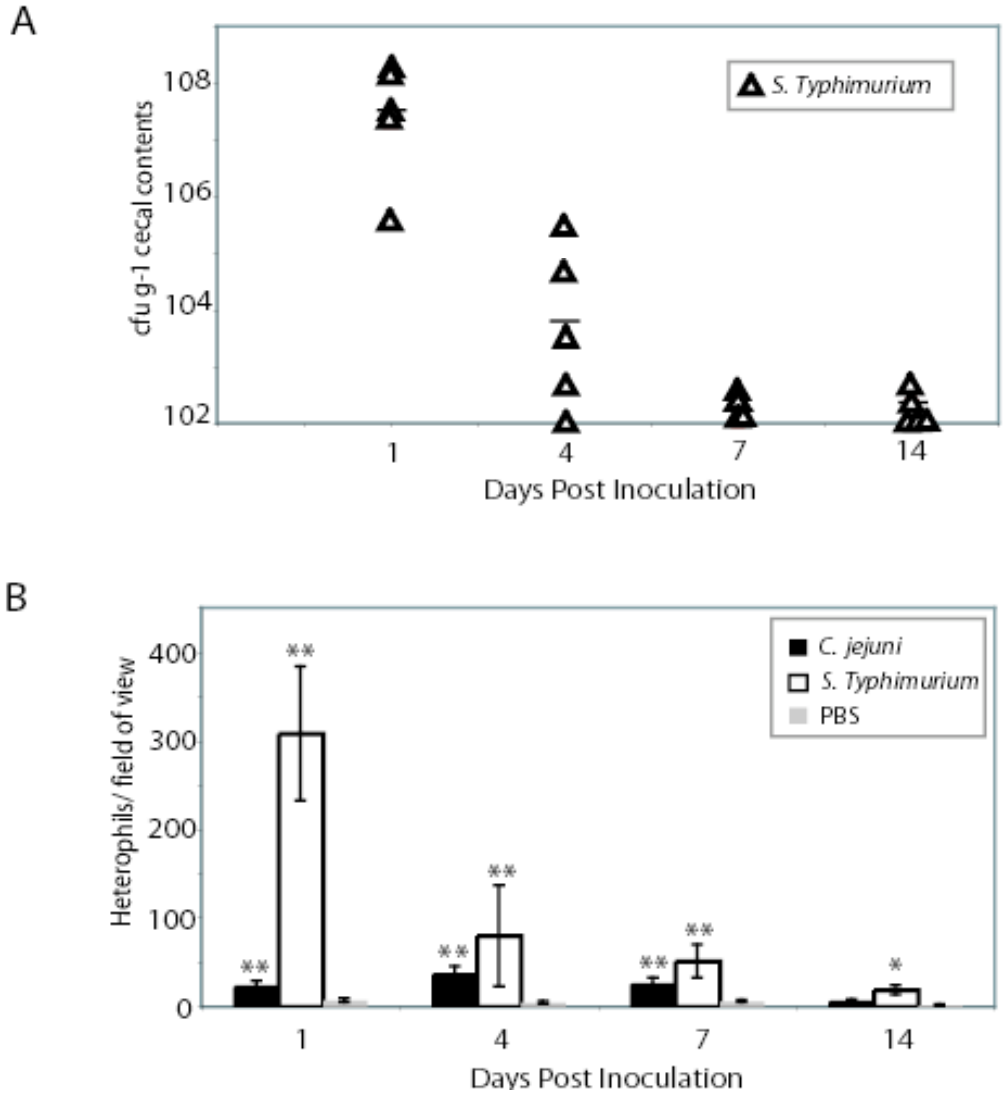


Figure 21. Colonization and Heterophil Response to *S. typhimurium* (A). Cecal colonization of birds inoculated with 10^6 cfu *S. typhimurium* (triangle) on days one, four, seven and fourteen post-inoculation. (B). Average number of heterophils in the chick cecum on days one, four, seven, and fourteen in birds inoculated with *C. jejuni* [13], *S. typhimurium* (white), or PBS [14]. Significance compared to age-matched PBS inoculated birds *p<0.05, **p<0.005.

C

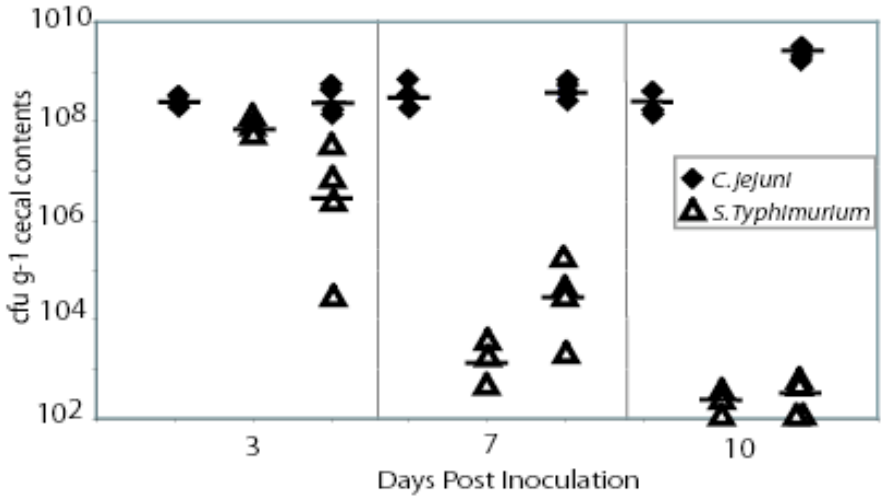


Figure 21. (continued) (C). Colonization in the chick cecum during a co-infection of *C. jejuni* (black diamond) and *S. typhimurium* (open triangle). Colonization was monitored on days three, seven and ten for chicks inoculated with 10^6 cfu *C. jejuni* alone, 10^6 cfu *S. typhimurium* alone, or 1:1 mixture of 10^6 cfu each of *C. jejuni* and *S. typhimurium*.

***C. jejuni* stimulates pro-inflammatory cytokines.**

Based on histopathology observations, *C. jejuni* appears to stimulate a more limited inflammatory response than does a more virulent pathogen like *S. typhimurium.* To examine the cytokine response due to infection of the two pathogens, we employed qRT-PCR on RNA extracted from infected chick ceca and examined the quantity of transcripts of various pro-inflammatory cytokines and chemokines, as well as anti-inflammatory cytokines. We examined chicks infected with 10^6 cfu *C. jejuni* 81-176 (Str^r), PBS-CM, *S. typhimurium,* and *C. jejuni* Δ*virB11* over two weeks.

Several transcripts indicative of a pro-inflammatory response were significantly up-regulated in chicks inoculated either *C. jejuni* or *S. typhimurium*, including *IL-6, IL-8, IFN-α, myD88,* and *iNOS* (Fig 22). Generally, pro-inflammatory transcripts were up-regulated from days one to four post inoculation, and decreased by day seven. These data correspond with the influx of heterophils upon *C. jejuni* infection. Chicks inoculated with PBS-CM had lower transcripts of pro-inflammatory cytokines compared to chicks inoculated with either *C. jejuni* or *S. typhimurium*. This demonstrates that the activation of pro-inflammatory cytokines is dependent on the presence of *C. jejuni*. Response to *S. typhimurium* showed similar trends compared to *C. jejuni* with respect to the pro-inflammatory cytokines. However, the transcripts for *IL-6, IL-8, myD88*, and *iNOS* were at least 10 fold higher in chicks inoculated with *S. typhimurium*, compared to chicks infected with *C. jejuni*. Transcripts of *tgf-β,* a potent anti-inflammatory cytokine, was also up-regulated in the presence of *C. jejuni* over the two week infection. Again, transcript levels of *tgf-β* were more robust in *S. typhimurium* infected chicks compared to *C. jejuni* infected chick ceca, and remained high at fourteen days post-inoculation.

Chicks colonized with a Δ*virB11* mutant *C. jejuni* strain had similar expression of pro-inflammatory cytokines as PBS infected birds (Fig 22). This further supports the suggestion that *virB11*-dependent invasion of the chicken cecal tissue is responsible for the up-regulation of pro-inflammatory cytokines and recruitment of heterophils during the initial stages of colonization.

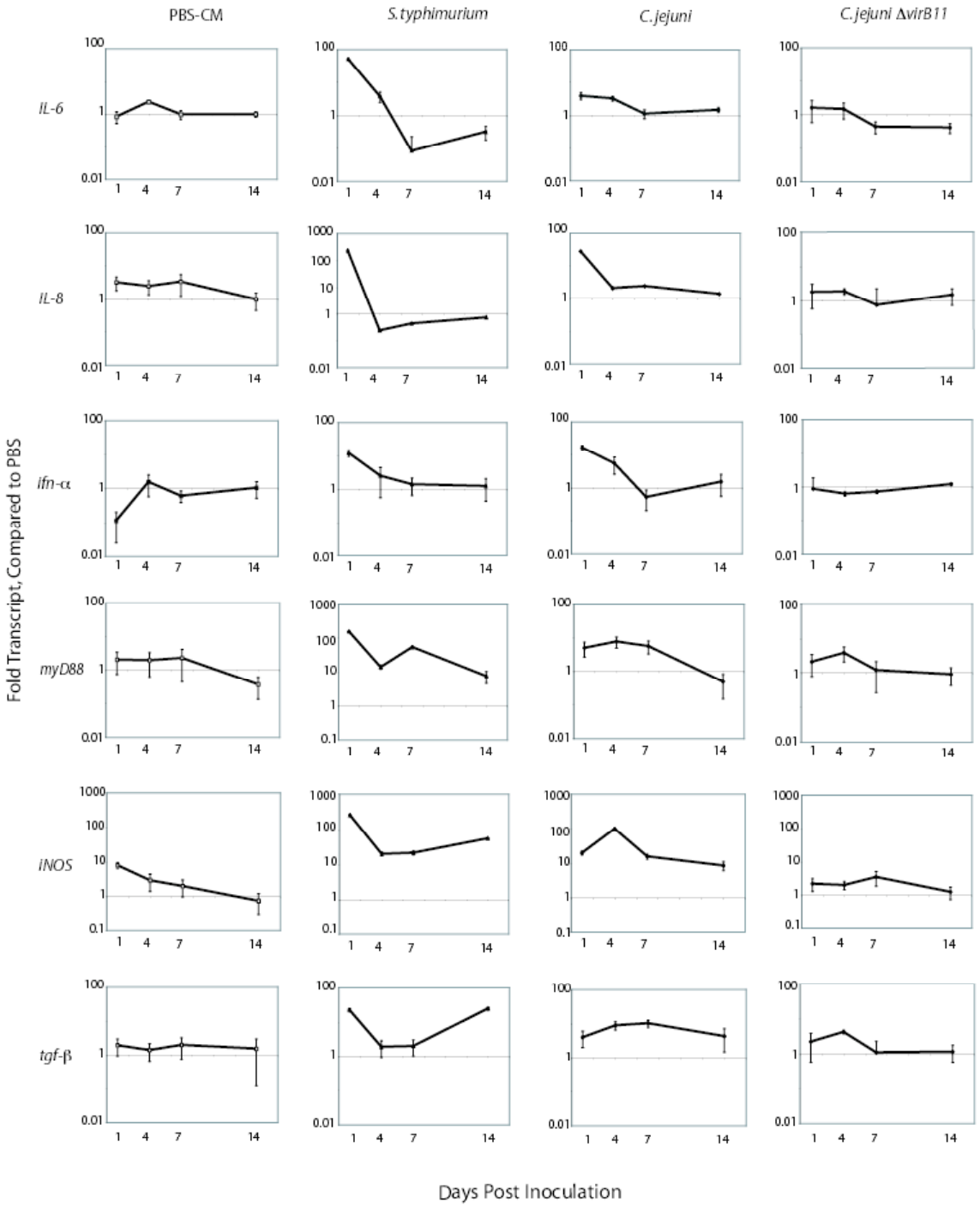


Figure 22. Quantitative RT-PCR of Cytokine Transcripts from Infected Chicks
Quantitative RT-PCR on cecal tissue from birds infected with 10^6 cfu *C. jejuni*, PBS-CM, *S. typhimurium*, or *C. jejuni* Δ*virB11* on days one, four, seven and fourteen post-inoculation. Amplification of the immune modulating transcripts *IL-6, IL-8, ifn-α, myD88, iNOS* and *tgf-β* were compared to PBS infected birds over the time-course and standardized to *gapdh* amplicons

Discussion

C. jejuni induces inflammation during human infection by invading the intestinal mucosa, leading to severe watery to bloody diarrhea [28] Profuse diarrhea has been demonstrated in several animal models, including ferrets, rabbits and piglets [19, 20, 29]. However, *C. jejuni* is able to colonize a number of animal species asymptomatically, including cows, pigs, sheep, chickens and germ-free mice [1, 30-32] Although contamination by chicken feces is the main route of infection to humans, due to the high prevalence of *C. jejuni* colonization in chicken broiler flocks, little is known about the interaction between *C. jejuni* and its avian host.

Previous work demonstrated the ability of *C. jejuni* to invade various cell lines of human and chicken origin, including INT407 cells and primary chicken embryo intestinal cells, chicken kidney cells, and primary chicken intestinal cells [3, 7]. In the current study, we demonstrated that *C. jejuni* invades the chicken cecal epithelium *in vivo*, as well as primary chick intestinal cells in cell culture. Such interactions of *C. jejuni* with the chicken cecal epithelium *in vivo* have not previously been documented. This observation is in contrast to those of Beery *et al* and Meinersmann *et al* who were unable to demonstrate *C. jejuni* invasion of the chick intestinal epithelium *in vivo* [24, 25].

Bacterial invasion of host tissue often leads to the up-regulation of pro-inflammatory cytokines, causing inflammatory cell recruitment. In the current study we observed significant up-regulation of inflammatory cytokines such as IL-6, IL-8 and IFN-α in *C. jejuni* infected cecal tissue, but not in response to *C. jejuni* Δ*virB11* infected chicks or chicks fed cecal contents from older animals. This is similar to previously

published reports of *C. jejuni* activation of pro-inflammatory cytokines in chicken cells and tissue [3, 7, 23, 33]. Heterophils, similar to mammalian neutrophils, are the first line of defense against invading pathogens and are recruited with the up-regulation of IL-8. This is compatible with our results demonstrating elevated numbers of heterophils in the chick cecal tissue, directly corresponding with the level of *C. jejuni* colonization. Heterophil recruitment peaked at an average of 34 heterophils per field of view occurring on day four post-inoculation in birds inoculated with 10^6 cfu *C. jejuni*. Previously published studies vary in the heterophilic responses of chickens to *C. jejuni*. In one study, no granulocyte infiltration was noted in response to *C. jejuni* infection [4]. However, heterophil recruitment has been observed in response to *C. jejuni* infections in a previous study, and was associated with an increased expression of pro-inflammatory cytokines in a 2-week infection model [23], but not in day-old chicks in the same study. As we have demonstrated here, there are significant differences in the ability of strains to elicit heterophil recruitment and differences between our study and others could be due to variations of the *C. jejuni* strains used in the different studies. Alternatively, differences in chicken strains could account for the differences seen in previous studies compared to ours. However, based on these previous studies and the work presented here, *C. jejuni* colonization of the chick cecum can induce a pro-inflammatory response with variations in heterophil association.

In this study, the heterophil recruitment in response to *C. jejuni* infection was less robust than the response to the invasive pathogen *S. typhimurium*, which resulted in ten times more heterophil recruitment on day one post –inoculation. These results suggest that *C. jejuni* may not cause as much tissue damage in the chick cecum as *S.*

typhimurium, possibly due to a lower invasive potential. An *in vivo* gentamicin protection assay which demonstrated only 0.1% of the total bacteria in the cecal contents were gentamicin protected on days 4 and 7 post-inoculation. We suggest that this low level of invasion observed by *C. jejuni* stimulates the modest level of heterophil recruitment in the chick cecum. In addition to the observation that *C. jejuni* does not stimulate the same level of inflammation as *S. typhimurium*, we also demonstrate that *C. jejuni* is resistant to killing in the midst of a large inflammatory response to S. *typhimurium* infection. We suggest that this resistance is due to the localization of *C. jejuni* in the mucus layer and luminal contents during colonization, away from the robust inflammatory response occurring in the tissue.

A small percentage of *C. jejuni* strains carry the putative virulence plasmid, pVir, including strains from human, bovine, and chicken sources [34, 35]. The pVir plasmid contains several genes homologous to a type IV secretion system in *H. pylori*, including the gene *virB11*. Here we report that *virB11* is necessary for efficient colonization of chicks when infected at a low dose of 10^2 cfu, but not at higher doses of 10^4 and 10^6 cfu. However, chicks colonized with the Δv*irB11* mutant showed a significant attenuation of heterophil influx detected in the infected ceca on day seven post-inoculation, in spite of colonization levels similar to wild-type. This phenotype is most likely due to an invasion defect reported for Δv*irB11*, as previously shown and recapitulated here in INT407 cells and primary chick intestinal cells. The Δv*irB11* strain also had a defect in invasion of the tissue as determined by the *in vivo* gentamicin protection assay on day seven post-inoculation. This finding furthers supports the hypothesis that *C. jejuni* invasion of the chick cecum is responsible for the heterophil response observed in the

tissue, as the invasive-deficient Δv*irB11* mutant is able to colonize but not stimulate heterophil recruitment. This finding also validates the observation of heterophil recruitment in response to an active bacterial process, and not a general response to *C. jejuni* or bacteria alone. Indeed, heterophil recruitment was not observed in response to normal cecal microbiota from older animals, as well as in response to colonization by the *C. jejuni* Δv*irB11* mutant.

Intriguingly, birds infected with 10^6 cfu of wild-type *C. jejuni* had insignificant heterophil recruitment on day fourteen post-inoculation. One explanation for this could be the production of antibodies against *C. jejuni*. Chickens commonly produce both systemic and mucosal antibodies against *C. jejuni* antigens [36]. Although the protective nature of maternal antibodies has been explored in *C. jejuni* infected chicks, the role and mechanisms behind systemic antibody production against a colonizing strain of *C. jejuni* in chicks has never been investigated. One reason for the drop in heterophil recruitment on day fourteen post-inoculation would be due to the formation and secretion of sIgA against *C. jejuni*. Secretory IgA at mucosal membranes is known to protect the host against invasive bacteria, by binding to antigens and "trapping" the bacteria in the mucus. This hypothesis is supported by the fact that the chick adaptive immune system is not developed upon hatching, and is only functional after 10-14 days post hatch. Further, the chick mucus layer has previously been implicated in inhibiting mobility of *C. jejuni*, as invasion of human cells was inhibited with the addition of chicken mucus, contrary to the increased invasion of cells with the application of human mucus in a cell culture model [5]. The reason for this phenotype will be the focus of future work.

In summary, we demonstrate that *C. jejuni* invades the chick cecum and also stimulates a modest level of heterophil recruitment and up-regulation of pro-inflammatory cytokines early during infection in a day-of-hatch chick colonization model. A *virB11*-deficient strain had attenuated invasiveness and also induced fewer heterophils, suggesting that invasion of the chick cecum, and not just colonization, is necessary to induce the pro-inflammatory response in the chick. Although the rate of invasion of *C. jejuni* in the chick cecum is low (0.1%), we argue that this level of invasion is sufficient to induce the modest level of heterophil recruitment seen in response to infection. Although the Δ*virB11* mutant was able to colonize the chick cecum at a high level, insufficient invasion of the cecal cells most likely lead to the attenuation in heterophil recruitment. Future work will focus on such factors that influence colonization, including production of antibodies, composition of mucus, and the role of the microbiota in the ability of *C. jejuni* to establish colonization in the chick cecum

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CHATPER IV

A *Campylobacter jejuni znuA* Orthologue is Essential for Growth in Low Zinc Environments and Chick Colonization

Summary

Campylobacter jejuni is a leading cause of bacterial gastroenteritis in the U.S. and is primarily acquired through the ingestion of contaminated poultry products. Here we describe the *C. jejuni* orthologue of ZnuA in other Gram negative bacteria. ZnuA (Cj0143c) is the periplasmic component of a putative zinc ABC transport system and is encoded on a zinc-dependent operon with *cj0142c* and *cj0141c*, which encode the other two likely components of the transport system of *C. jejuni*. Transcription of these genes is zinc dependent. A mutant lacking *cj0143c* is growth-deficient in zing-limiting media as well as in the chick gastrointestinal tract. The protein is glycosylated at asparagine 28, but this modification is dispensable for zinc-limited growth and chick colonization. Affinity purified FLAG-tagged Cj0143c binds zinc *in vitro*. Based on our findings and on homology to *E. coli* ZnuA, we conclude that Cj0143c encodes the *C. jejuni* orthologue of ZnuA.

Introduction

Campylobacter jejuni is a leading cause of bacterial gastroenteritis worldwide. Symptoms of acute campylobacteriosis include self-limiting watery to bloody diarrhea, along with nausea, fever, and abdominal cramping. *C. jejuni* is a microaerophilic gram-negative bacterium that commonly colonizes the gastrointestinal tract of broiler chickens. Passage of *C. jejuni* to humans occurs primarily through consumption of contaminated poultry products.

An important trait for bacterial pathogens is their ability to take up trace elements. Iron is commonly considered the most important trace element necessary for bacterial growth inside of mammalian hosts. However, other trace divalent metals play critical roles during infection, including zinc and manganese [1, 2]. Zinc plays both structural and catalytic roles in over 300 proteins within *E. coli*, being incorporated into regulatory proteins, ribosomal proteins, and many other enzymes. Although zinc is essential, excess zinc is toxic to cells by competing with other metals for enzymes binding sites, and internal zinc homeostasis is tightly regulated in both eukaryotic and bacterial cells. The intracellular concentration of zinc within *E. coli* is 0.2mM, however little to no free zinc exists within the cytoplasm [3].

Several transport systems have been identified that are involved in intracellular zinc homeostasis. Under conditions of limited metal availability, such as those within the host, a high affinity zinc transport system is responsible for the uptake of zinc, as initially reported in *E. coli* [3]. This system is encoded by the *znuABC* genes and is part of the

family of ATP-binding cassette (ABC) transporters. ZnuA is a periplasmic meta4llochaperone, ZnuB is a membrane permease, and ZnuC is an ATPase component [3, 4].

The *znuABC* genes in *E. coli* are regulated by Zur (zinc-uptake regulator), a member of the Fur (ferric-uptake regulator) family of metalloregulatory proteins. In the presence of zinc, Zur forms a dimer and binds a palindromic sequence, termed the Zur box, thereby repressing expression of the *znuABC* genes. Zur is sensitive to femtomolar concentrations of zinc within *E. coli*, once again demonstrating the stringent regulation of zinc within cells [5].

The amount of zinc available within a host is assumed to be limited, similar to that of iron, and ZnuABC transporters are required for virulence of several pathogens, including *E. coli, Salmonella enterica*, *Brucella abortus*, *Neisseria gonorrhoeae, Pastuerella multocida*, and *Haemophilus* species [6, 7] [3, 4]. *C. jejuni* must survive in zinc-deprived environments during its infectious cycle, hence it is likely that zinc homeostasis plays a major role in its ability to survive within hosts as well.

To assess the role zinc plays in *C. jejuni* growth and within a host, we identified and characterized ZnuA in *C. jejuni*. Our results demonstrate that *cj0143c* encodes for the metallochaperone ZnuA. ZnuA is essential for *C. jejuni* growth in zinc-limiting media, as well as within the chick gastrointestinal tract. ZnuA is *N*-glycosylated by the Pgl system, but we demonstrate that glycosylation of the protein does not affect its function [8].

Results

Characterization of *cj0143c*.

As part of an effort to characterize glycoproteins of *C. jejuni* [6] the putative glycoprotein Cj0143c was identified in *C. jejuni* as having extensive homology to ZnuA in *E. coli*, a high affinity zinc binding protein for an ABC-transporter [3]. A CLustalW alignment of *cj0143c* from *C. jejuni* 81-176, other *C. jejuni* strains, *C. coli, H. hepaticus, Synechocystis*, and *E.coli* was performed (Figure 23A). *C. jejuni* 81-176 *znuA* had high homology with the *znuA* gene from other *C. jejuni* strains (96-99%), but limited homology to *znuA* from *C. coli* RM228 (61%), *E. coli* (21.5%), *Synechosystis* (32.8%) and *H. hepaticus* (26%). Three conserved histidine residues, which in *E. coli* ZnuA comprise a high-affinity binding site, are designated in boxes. Beginning at residue 162 in the *C. jejuni* 81-176 sequence is a 14 amino acid stretch of which 13 residues are His (H) or Glu (E). This H/E rich domain *per se* is in the other *C. jejuni* strains and orthologues from other species have a His/Asp-rich region (green line in Figure 23A). *Synechocystis* ZnuA contains a 20 amino acid stretch rich in histidine and acidic residues that forms a loop by crystal structure [17]. This loop in the *Synechocystis* ZnuA is not essential for zinc binding to the high-affinity binding site of ZnuA, but may play a regulatory role in transport [18].

The gene *cj0143c* was first identified as encoding a glycoprotein, modified by the *N*-glycosylation system in *C. jejuni* [19]. The Pgl system assembles a conserved heptasaccharide and transfers it onto periplasmic proteins by the transferase, PglB [8]. PglB recognizes a conserved sequence, termed the sequon, (D/E-X-N-Y-S/T, X,Y ≠ P) [20]. A sequence matching this consensus, DQNTS, is denoted by the blue bar in Figure

1a, and is conserved in all *Campylobacter jejuni* strains. Based on homology, we predict that *cj0143c (C. jejuni* 81-176 gene number CJJ81176_0179*)* and its two adjacent ORFS, *cj0141c (*CJJ81176_0177*)* and *cj0142c* (CJJ81176_0178) together form a zinc ABC transporter, similar to ZnuABC. (See Fig 23C).

***cj0141c, cj0142c*, and *cj0143c* are co-transcribed and expression is elevated in low zinc media.**

To test whether *cj0141c, cj0142c*, and *cj0143c* form an operon, RT-PCR was performed on RNA extracted from wild-type *C. jejuni* grown in Mueller Hinton (MH) broth containing 10 μM EDTA, using three primer sets spanning across the putative operon (Fig. 24A). A transcript encoding all three ORFs would be predicted to be amplified by primer pair P3. A 650bp product was detected for P1, a 1400bp product for P2, and a 1850bp product for P3 in the RT reactions (Fig. 24B). These species are indicative of transcription across all three ORFs. Based on these results, we conclude that *cj0143c, cj0142c*, and *cj0141c* are co-transcribed.

To assess whether expression of the operon is influenced by zinc concentrations, quantitative RT-PCR was performed for the *cj0143c* transcript, on cells grown in the presence of varying concentrations of EDTA, a common chelator of divalent cations. Wild-type *C. jejuni* was cultured for 24 hours in MH broth, MH broth with 1μM EDTA, 2μM EDTA, 10μM EDTA, and 10μM EDTA with 10 μM Zn. The fold increase of *cj0143c* transcript in the different conditions was compared to transcript levels in bacteria grown in MH (Fig. 25A). Expression of *cj0143c* was increased 2-3 fold in the presence of EDTA (Fig. 25A). When zinc was added with EDTA, transcription resembled that of

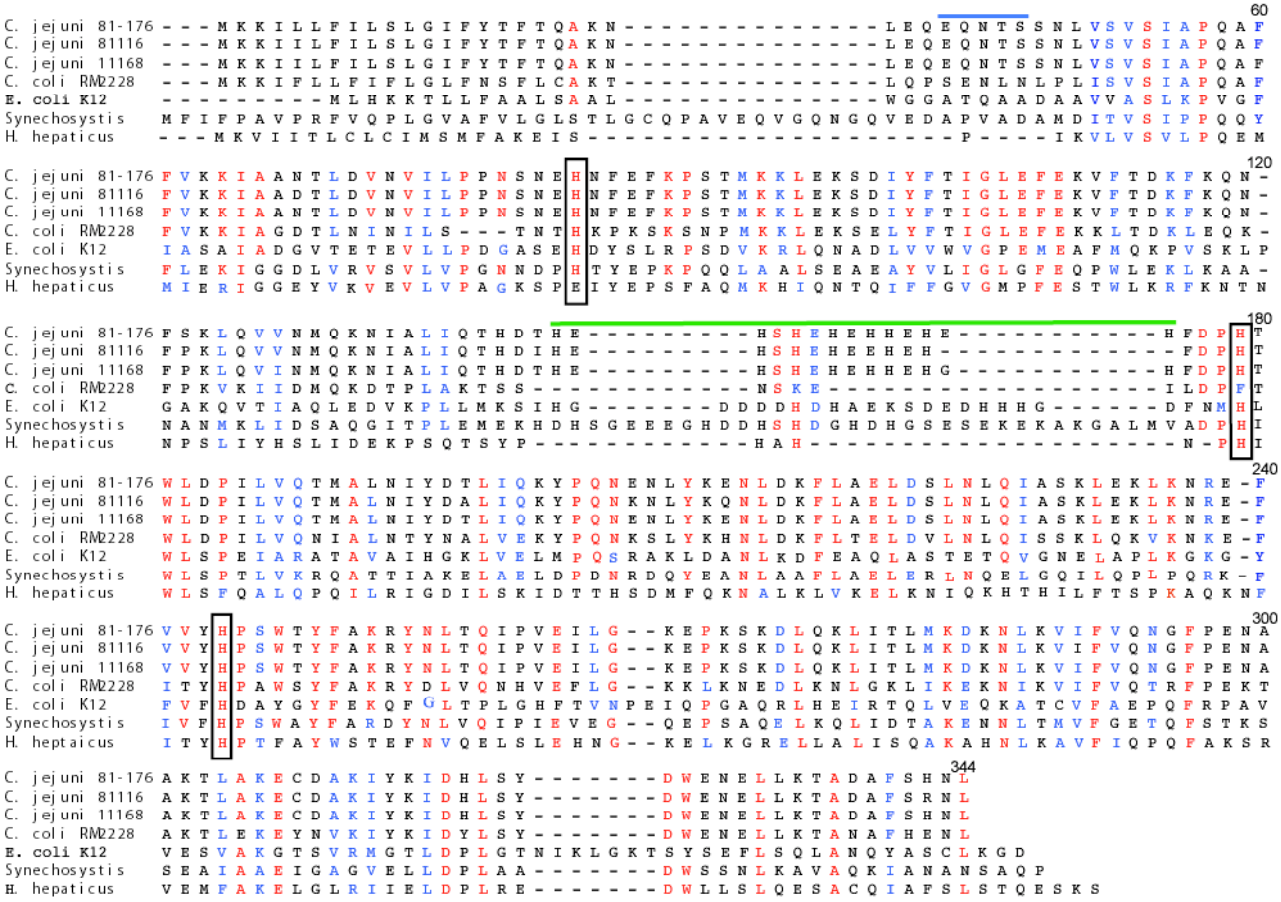

Figure 23. Alignment of *znuA* and Opeon Structure (A)ClustalW alignment of ZnuA from *Campylobacter jejuni* 81-176, *C. jejuni* 11168, *C. jejuni* 81116, *Campylobacter coli* RM2228, *E. coli* K12, *Synechosystis* 6803, and *Helicobacter heptaicus* 51449. Conserved residues (red) and similar charged residues (blue) demonstrate a high degree of homology, especially around the zinc binding pocket. The conserved zinc-binding histidines are denoted by black boxes. A hyper-variable region which contains a predicted zinc-binding arm (green line) in *Synechosystis* is conserved between the *C. jejuni* strains. The sequon, or the site recognized for *N*-glycosylation by the Pgl system in *C. jejuni* is designated by the blue line.

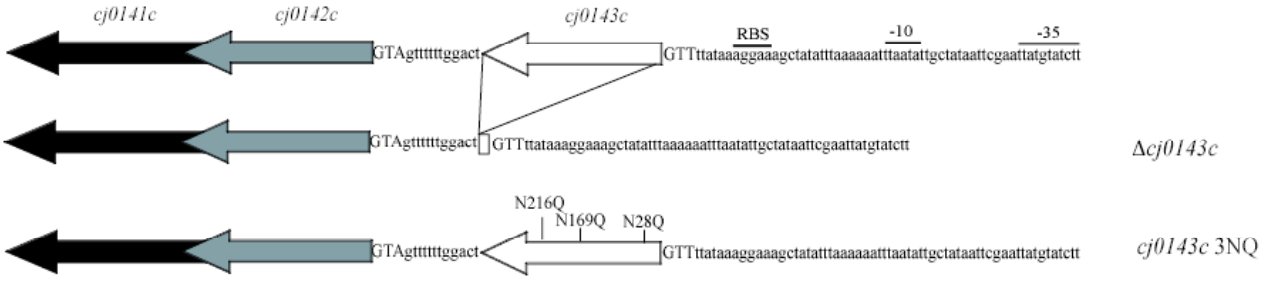

Figure 23 (continued) (B) Genetic organization of *cj0141c, cj0142c*, and *cj0143c* with consensus promoter sequence (black lines) and ribosome binding site (RBS) upstream of *cj0143c* . An in-frame deletion of *cj0143c* was constructed, as well as an allele containing point-mutations in three putative *N*-glycosylation sites.

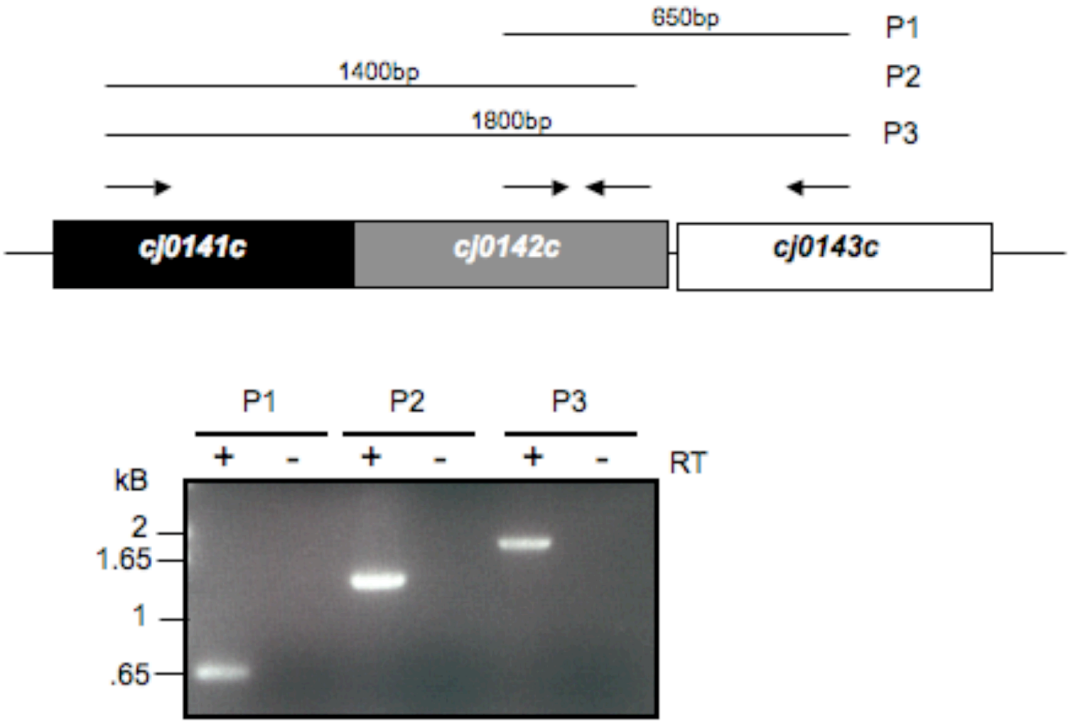

Figure. 24. RT-PCR of *znuABC* operon (A) RT-PCR was performed on the locus to determine whether the *cj0141c, cj0142c,* and *cj0143c* are co-transcribed. The three primer sets (P1, P2, P3) were designed to span the three genes. Their predicted product sizes are reported. (B) RT-PCR on RNA extracted from wild-type *C. jejuni* DRH2.12 using the primer sets figured in (A). The RT (+) and no-RT control (-) are represented for each primer set.

MH, indicating that the expression of *cj0143c* in the presence of EDTA is zinc-dependent.

Deletion of *cj0143c* Results in a Growth Defect in Zinc Limiting Media.

We hypothesized that *cj0143c* is required for *C. jejuni* growth in zinc-limiting environments. We constructed a strain with an in-frame chromosomal deletion of *cj0143c* (Δc*j0143c)* as well one expressing an allele with altered glycosylation sites (*cj0143c*3NQ), as described in Materials and Methods. Quantitative RT-PCR with primers for *cj0141c*, *cj0142c*, and *cj0143c* transcripts was performed on WT, Δc*j0143c*, and *cj0143c*3NQ grown in CDM containing or lacking zinc (Figure 26B). *cj0143c* transcript was not detected in Δc*j0143c*, while both *cj0141c* and *cj0142c* were detected, confirming that the in-frame deletion mutation of *cj0143c* is not polar on downstream transcription. Further, in comparing expression during growth without added zinc to growth with added zinc, *cj0141c* and *cj0142c* were expressed 10-fold higher in the Δ*cj0143c* mutant than in WT and *cj0143c*3NQ (Figure 26B). We think this occurs because the Δ*cj0143c* mutation creates a zinc-starved condition within the bacteria. Although the media does not contain added zinc, contaminating trace amounts of zinc may be present.

Δc*j0143c* and *cj0143c*3NQ grew similarly to wild-type in rich MH broth cultures. However, growth of *Δ*c*j0143c* was severely attenuated after 24 hours when cultured in Campylobacter Defined Media (CDM) lacking added zinc (Fig.27). Growth of Δc*j0143c* was restored when $ZnSO_4$ was added to CDM (Fig. 27). Culturing these strains under conditions lacking other individual trace elements, including Fe^{+2}, Ca^{+2}, Mg^{+2}, Cu^{+2},

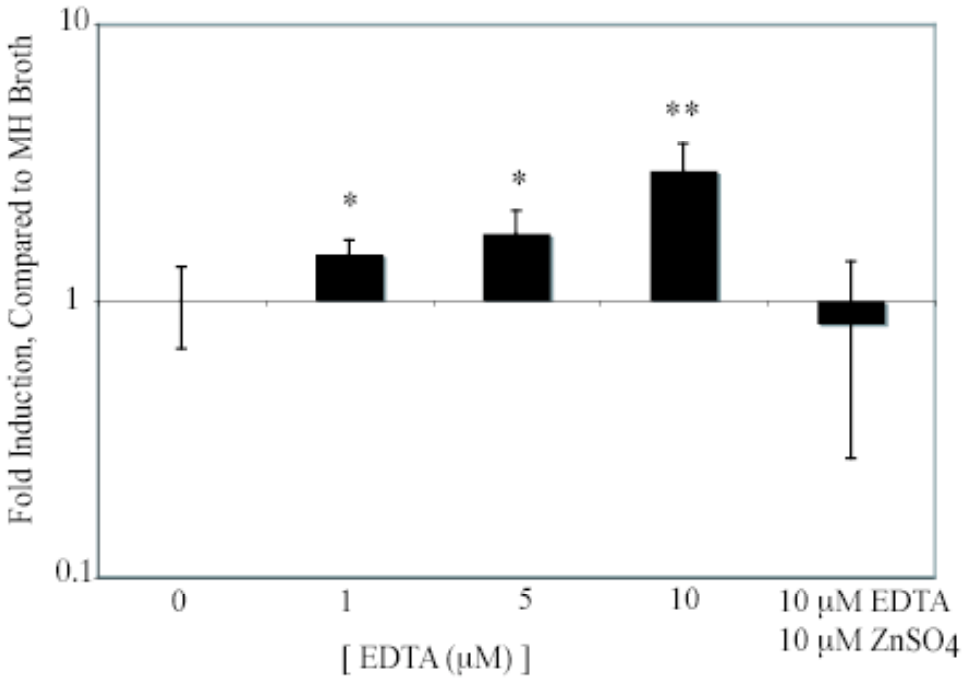

Figure 25. Quantitative RT-PCR of *znuA*. Quantitative RT-PCR of *cj0143c* transcript levels in wild-type *C. jejuni* grown in the indicated conditions; MH broth, 1µM EDTA, 2µM EDTA, 10µM EDTA, and 10µM EDTA + 10µM $ZnS0_4$. Compared to MH broth transcript, *cj0143c* is expressed more in the presence of EDTA. * $p<0.05$, ** $p< 0.005$

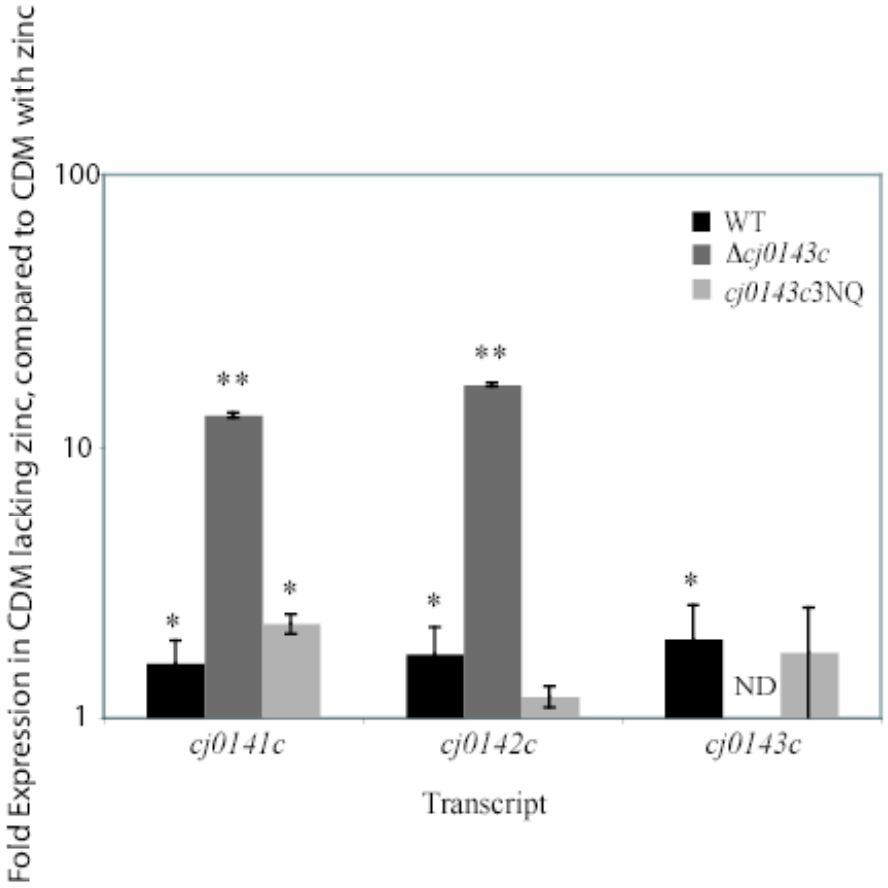

Figure 26. Quantitative RT-PCR of znuA in Defined Media. Quantitative RT-PCR of *cj0141c*, *cj0142c*, and *cj0143c* transcripts in wild-type *C. jejuni* [21], Δ*cj0143c* (dark grey), and cj0143c 3NQ (light grey) grown in CDM lacking zinc compared to bacteria grown in MH. Transcript of *cj0143c* in Δ*cj0143c* was not detected, while *cj0141c* and *cj0142c* transcript was detected, demonstrating an in-frame, non-polar Δ*cj0143c* mutation. ** $p < 0.005$

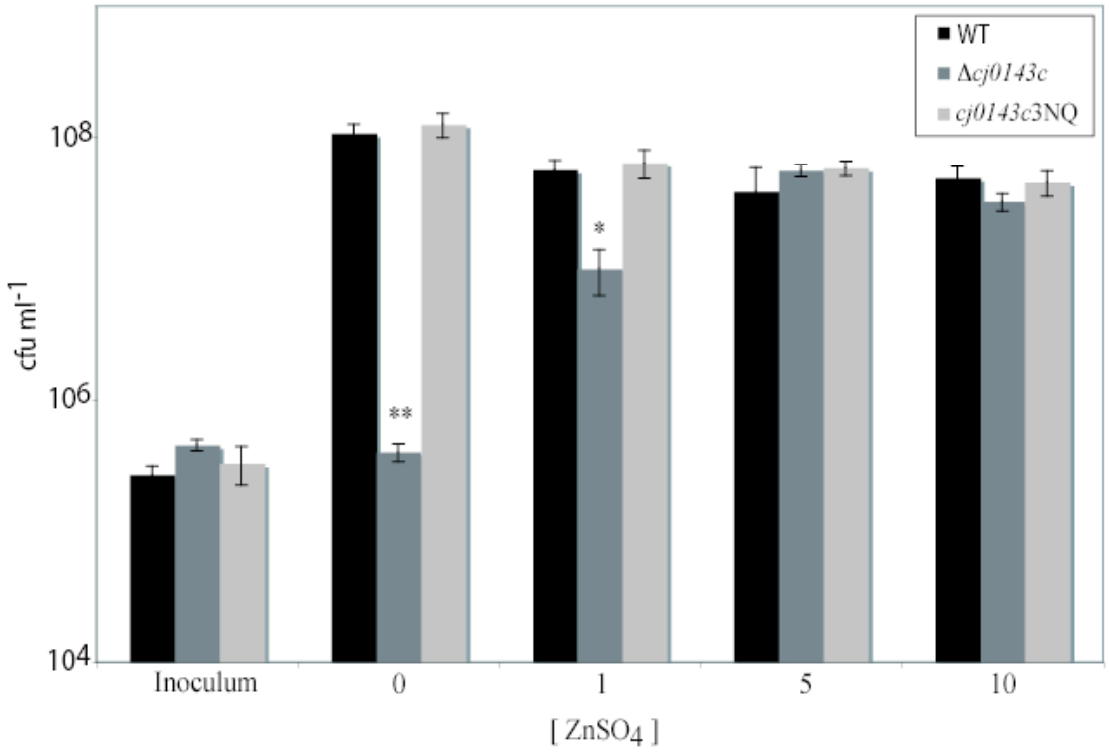

Figure 27. Growth of *ΔznuA* in Defined Media. Growth of wild-type [21], *Δcj0143c (*grey*)*, and *cj0143*c3NQ (dots) at 24 hours in defined media without the addition of zinc. *Δcj0143c* is unable to grow in the defined media lacking zinc. The media is supplemented with increasing concentrations of $ZnS0_4$ and growth of *Δcj0143c* is restored to wild-type levels. * $p< 0.05$, ** $p< 0.005$

Mn^{+2} was also tested, and Δc*j0143c* was not significantly attenuated in any other condition, suggesting that the requirement for *cj0143c* is specific for zinc-limited growth (data not shown).

We also tested zinc-limited growth using EDTA, hypothesizing that *Δcj0143c* would be challenged under this zinc-depleted condition. We grew wild-type, *Δcj0143c*, and *cj0143c*3NQ in MH broth with increasing concentrations of EDTA for 24 hours with an inoculum of 10^5 cfu ml^{-1}. At 10 and 25μM EDTA, wild-type and the *cj0143c*3NQ mutant grew well, but the *Δcj0143c* mutant did not (Figure 28A). All three strains were unrecoverable in the presence of 100μM EDTA (Figure 28A). This coincides with the toxicity of EDTA for *Helicobacter pylori,* with a minimal inhibitory concentration as low as 0.1mM EDTA [22]. Addition of equimolar concentrations of $ZnSO_4$ to MH broth containing EDTA rescued the growth of *Δcj0143c* (Figure 28B); in contrast, $FeSO_4$ at concentrations up to 20μM was unable to rescue growth of Δ*cj0143c* in 10μM EDTA (Figure 28E).

Taken together, these data suggest that *cj0143c* is necessary for *C. jejuni* growth in low zinc environments, and that it is specific for zinc.

Cj0143c is glycosylated at a single site. To assess the glycosylation state of *cj0143c*, we constructed plasmids expressing *cj0143c* and *cj0143c*3NQ with C-terminal FLAG epitopes (pCj0143c-FLAG and pCj0143c3NQ-FLAG) from plasmid pBW210. These constructs were introduced into wild-type, *Δcj0143c*, and *ΔpglB C. jejuni* backgrounds. A *C. jejuni ΔpglB* mutants lacks the glycosyl transferase *pglB*, which is necessary for the final step in *N*-linked protein glycosylation [6, 8]. All strains grew similarly to wild-type

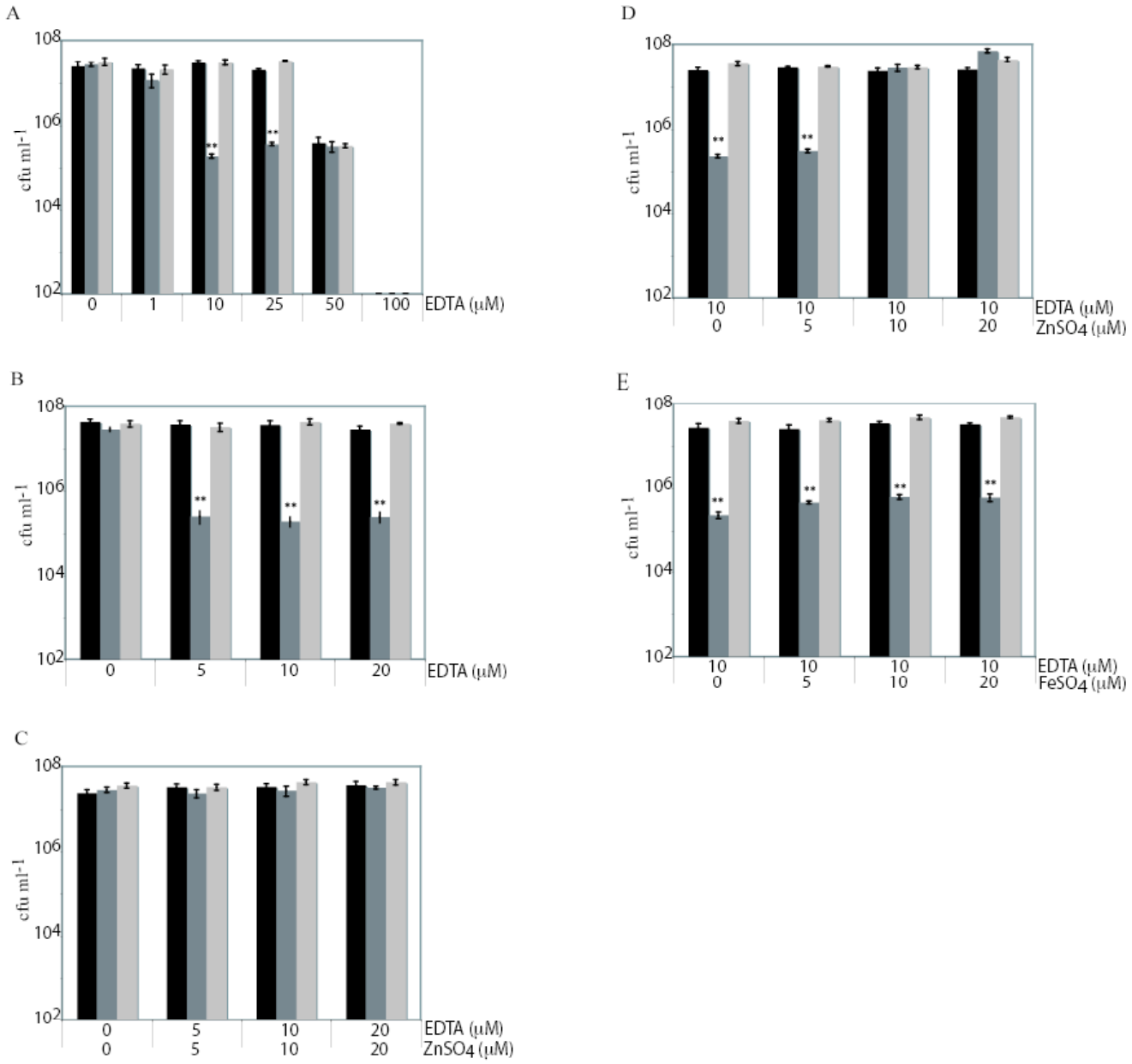

Figure 28. Growth of *ΔznuA* in EDTA (A-C) Wild-type [21], *Δcj0143c* (dark grey*)*, and *cj0143c*3NQ (light grey) grown for 24 hours in MH broth containing increasing levels of EDTA. (C) Strains were grown in MH broth containing increasing levels of EDTA and supplemented with increasing amounts of $ZnSO_4$, demonstrating $ZnSO_4$ is able to rescue the growth defect of *Δcj0143c* in MH broth containing high levels of EDTA. (D-E) Increasing amounts of either $ZnSO_4$ (D) or $FeSO_4$ (E) were added in MH broth containing 10μM EDTA. Growth of Δ*cj0143c* is more restricted in the presence of EDTA, and was rescued with $ZnSO_4$ only.

in rich and low-zinc media, suggesting that the FLAG-tagged proteins remain functional and complement the *Δcj0143c* mutant (data not shown).

Immunoblots of whole cell lysates of the indicated strains grown on MH agar for 24 hours were performed, probing with anti-FLAG antibody. WT and Δ*cj0143c* carrying pCj0143c-FLAG expressed two species recognized by the anti-FLAG antibody (Fig.29). A single species was observed in WT and Δ*cj0143c* carrying pCj0143c3NQ-FLAG. When pCj0143c-FLAG was expressed in a Δ*pglB* mutant of *C. jejuni*, which is unable to carry out *N*-linked protein glycosylation, only a single species was detected with anti-FLAG antibody. This species has a mobility similar to that of Cj0143c3NQ and to that of the lower of the two species observed in cells expressing pCj0143c-FLAG. Given these results and the updated sequon consensus sequence, we conclude that Cj0143c is glycosylated at a single site, most likely at residue N28. We conclude the upper band in figure 6a is the glycosylated species and the lower band is unglycosylated.

Cj0143c Localization. Based on homology to ZnuA, we predicted Cj0143c is localized to the periplasm. To test this, sub-cellular fractions of cells expressing pCj0143c-FLAG were analyzed with anti-FLAG antibody. Bands corresponding to the glycosylated and un-glycosylated forms of Cj0143c in wild-type, *Δcj0143c*, and *ΔpglB* backgrounds were located in the periplasmic fraction only (Fig. 30). The activity of isocitrate dehydrogenase, a cytoplasmic enzyme, was use to demonstrate purity of each fraction. Isocitrate dehydrogenase activity was highest in the cytoplasmic fraction, and activity in the periplasmic fraction may reflect contamination. However, little to no signal was

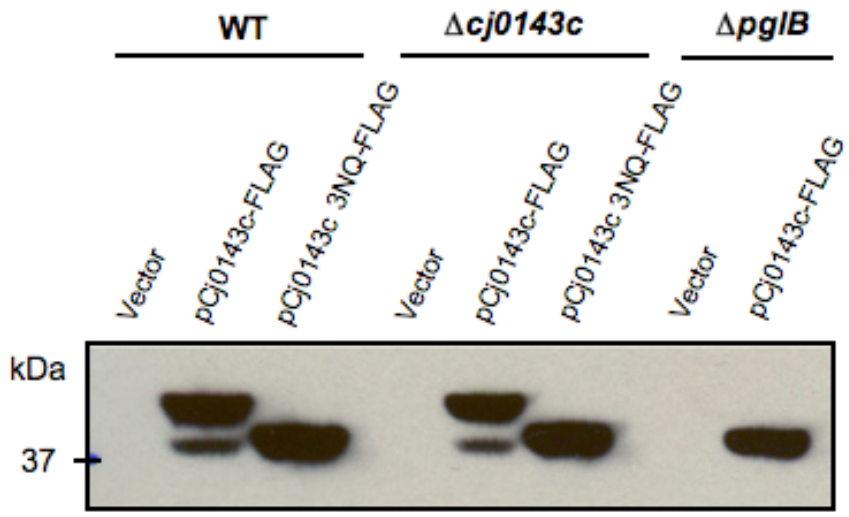

Figure 29. Glycosylation of ZnuA . Glycosylation States of Cj0143c. Whole cell lysates were collected from wild-type DRH2.12*, Δcj0143c* and *ΔpglB* containing vector only, pCj0143c-FLAG or p*Cj0143c 3*NQ-FLAG. Samples were normalized based on OD_{600}, run on a SDS –Page Gel and blotted with anti-FLAG antibody.

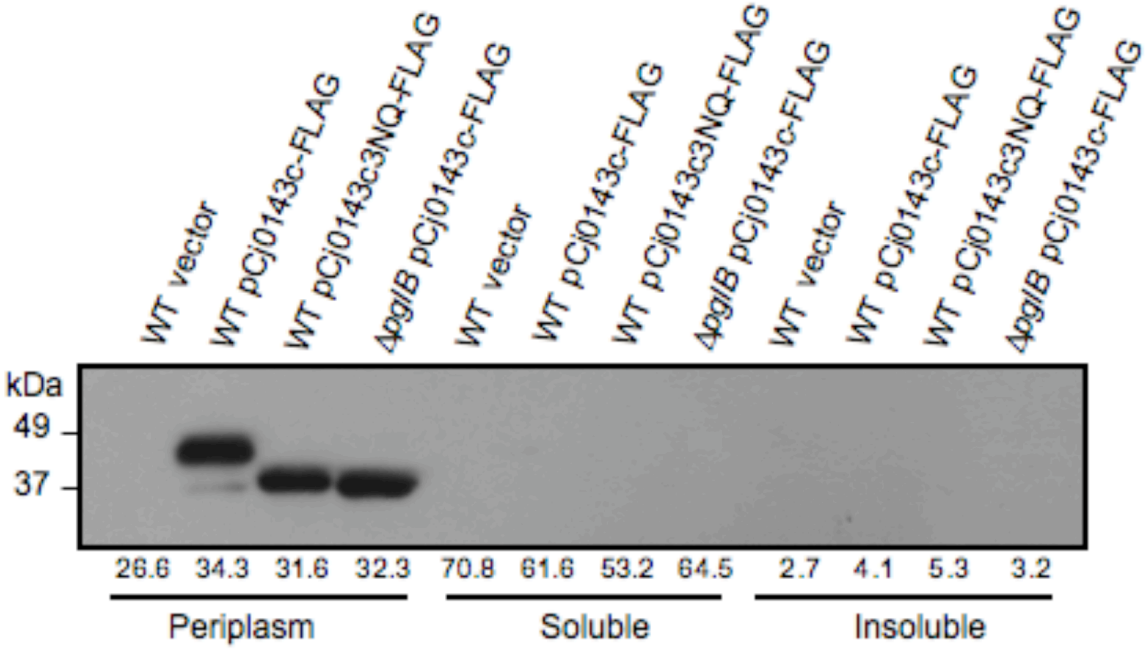

Figure 30. Localization of ZnuA. Wild-type DRH2.12 expressing vector alone, pCj0143c-FLAG, or pCj0143c3NQ-FLAG were fractionated into periplasmic, soluble, insoluble fractions. The samples were run on SDS-Page gel and blotted with anti-FLAG antibody. Cj0143c-FLAG and Cj0143c3NQ-FLAG are localized to the periplasmic fraction in WT and *ΔpglB* backgrounds. The pictured blot is a representative of three separate localizations. Isocitrate dehydrogenase activities for the samples are below the lanes.

detected for Cj0143c-FLAG in the cytoplasmic or membrane fractions, suggesting that Cj0143c is localized to the periplasm.

Cj0143c is more stable in the presence of EDTA.

Due to the fact that intracellular concentrations must be strictly controlled, zinc must be maintained at strict levels. External environmental zinc levels may change rapidly in the various hosts and conditions *C. jejuni* encounters. *C. jejuni* must response quickly to these changes, and therefore it is reasonable that this system could be regulated both transcriptionally as well as at the protein level.

We asked whether either the protein levels or glycosylation of Cj0143c was affected based on varying growth conditions such as temperature (37°C versus 42° C), growth phase, or varying levels of zinc. We grew the strain *Δcj0143c* carrying pCj0143c-FLAG in a variety of conditions, including increasing levels of EDTA (Fig 31). The quantity of Cj0143c-FLAG and Cj0143c3NQ-FLAG in Δ*cj0143c* increased with the addition of EDTA (Fig. 31). In this experiment, Cj0143c-FLAG was expressed from the cat^R promoter on plasmid pEco101, so any increase in the amount of Cj0143c-FLAG under zinc-limiting conditions is not due to transcriptional regulation.

Cj1496c is a previously characterized periplasmic glycoprotein in *C. jejuni* [12]. Cj1496c-FLAG was used as a control to demonstrate that the EDTA effect was specific for Cj0143c-FLAG and not due to a global effect EDTA could have on *C. jejuni*, such as through the activity of a metal-dependent protease or plasmid-specific stabilization.

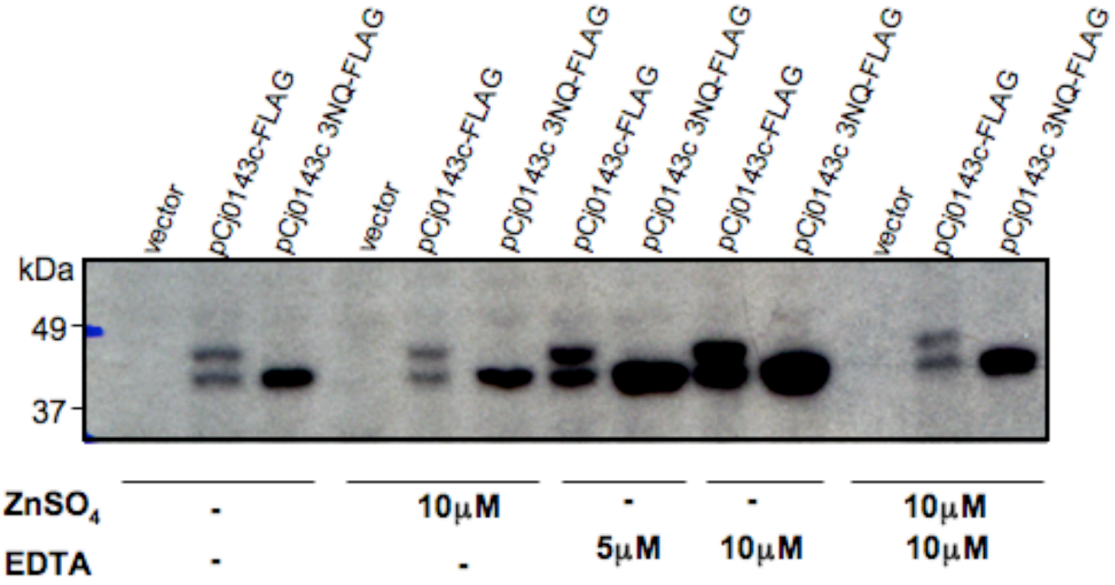

Figure 31. **ZnuA Stabilizes with Increasing Amounts of EDTA.** Δ*cj0143c* carrying pCj0143c-FLAG or pCj0143c3NQ-FLAG, and Δ*cj1496c* carrying pCj1496c-FLAG were grown for 24 hours in MH broth, MH + 5uM EDTA, MH + 10uM EDTA, and MH +10uM EDTA and 10uM ZnSO4. Cells were normalized by OD600. Cj0143c-FLAG and Cj0143c 3NQ-FLAG were more abundant in the presence of EDTA.

Cj0143c Binds Zinc *In vitro*.

To test the zinc-binding capacity of Cj0143c, 4-(2-pyrimidylazo)resorcinol (PAR) was used in an *in vitro* zinc binding assay. We took advantage of the fact that a PAR:Zn^{+2} complex forms a color that can be measured at 490nm [23]. The proteins Cj0143c-FLAG, Cj0143c3NQ-FLAG, and Cj1496c-FLAG were purified using anti-FLAG affinity beads as described in Materials and Methods. Cj1496c-FLAG was used as a control in this assay, as it does not contain a zinc-binding site.

The purified proteins were incubated with EDTA or $ZnSO_4$ for one hour. Proteins were eluted from the beads, and zinc content of each was measured by adding 0.2mM PAR. The amount of zinc (μM) was determined by comparing the absorbance to that of a standard curve of PAR/$ZnSO_4$. The samples were standardized to the quantity of purified protein in each sample.

We determined the concentration of zinc per μg purified protein for vector alone, Cj0143c-FLAG, Cj0143c3NQ -FLAG, and Cj1496c-FLAG incubated with buffer, 1mM EDTA, or 1mM $ZnSO_4$.(Fig. 32). Zinc was detected in the samples incubated with buffer alone for Cj0143c-FLAG and Cj0143c3NQ-FLAG at concentrations of 50 μM Zn^{+2} per μg protein. This most likely represents zinc that remained bound to the proteins during purification. Addition of 1mM EDTA reduced the detection of zinc in the protein complex considerably. With the addition of 1mM $ZnSO_4$, both Cj0143c-FLAG and

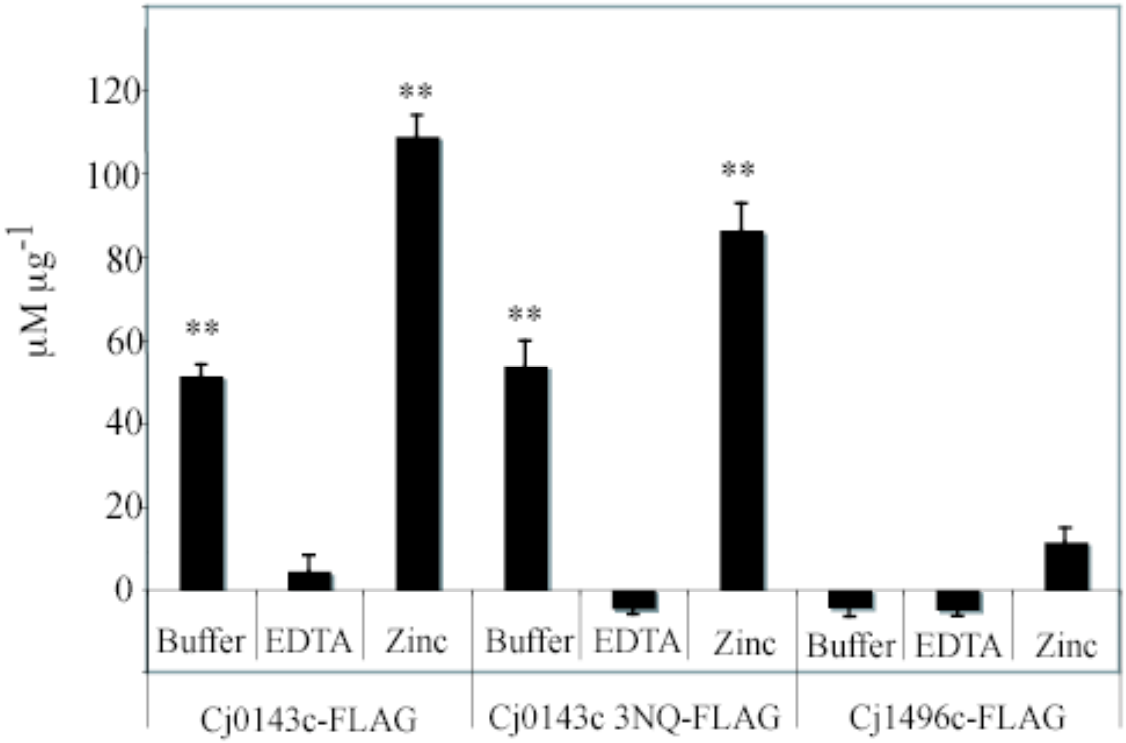

Figure 32. ZnuA Binds Zinc. Cj0143c and 3NQ Cj0143c bind zinc *in vitro*. Cj0143c-FLAG and Cj0143c 3NQ-FLAG were immobilized on sepharose beads conjugated to anti-FLAG antibody. The protein:bead complex was incubated with buffer, EDTA, or $ZnSO_4$. Protein quantity (ug) was determined by BioRad assay. The amount of zinc was quantified by measuring the absorbance of the PAR::zinc complex in each sample and calculating the total μM concentration of zinc. The values are represented by μM of Zinc per μg of purified protein. Cj0143c and Cj0143c3NQ-FLAG are able to bind zinc *in vitro* and at similar levels. Cj1496c-FLAG, a periplasmic-localized glycoprotein is not able to bind zinc *in vitro*. ** $p < 0.005$

Cj0143c3NQ-FLAG contained approximately 100 μM Zn^{+2} per μg protein. The samples of Cj1496c-FLAG bound zinc poorly or below detection levels.

These results demonstrate that Cj0143c-FLAG and Cj0143c3NQ-FLAG are able to bind zinc *in vitro.* The increase in zinc content with the addition of $ZnSO_4$ most likely represents the saturation of binding sites, including the predicted high-affinity zinc-binding site (histidines boxed in Figure 23a), as well as the zinc binding arm that we speculate exists in the protein (green line, Figure 23a).

Cj0143c is required for Chick Colonization.

Campylobacter jejuni colonizes the chick gastrointestinal tract to high levels. Factors that influence this natural colonization are beginning to be uncovered [1] and may serve as targets for developing approaches to limit chick colonization and thereby reduce human exposure. To assess whether *cj0143c* is necessary for *C. jejuni* interactions with the host, wild-type *C. jejuni*, *Δcj0143c, Δcj0143c* containing pCj0143c-FLAG and *cj0143c*3NQ were orally inoculated into day old white leghorn chicks at a dose of 10^4 cfu. After 7 days, the cecal contents of the infected chicks were plated for cfu per gram of cecal contents. Both the *cj0143c*3NQ mutant and *Δcj0143c* containing pCj0143c-FLAG colonized at wild-type levels, however Δ*cj0143c* was unrecoverable at our limit of detection, signifying that *cj0143c* is necessary for colonization (Fig 33).

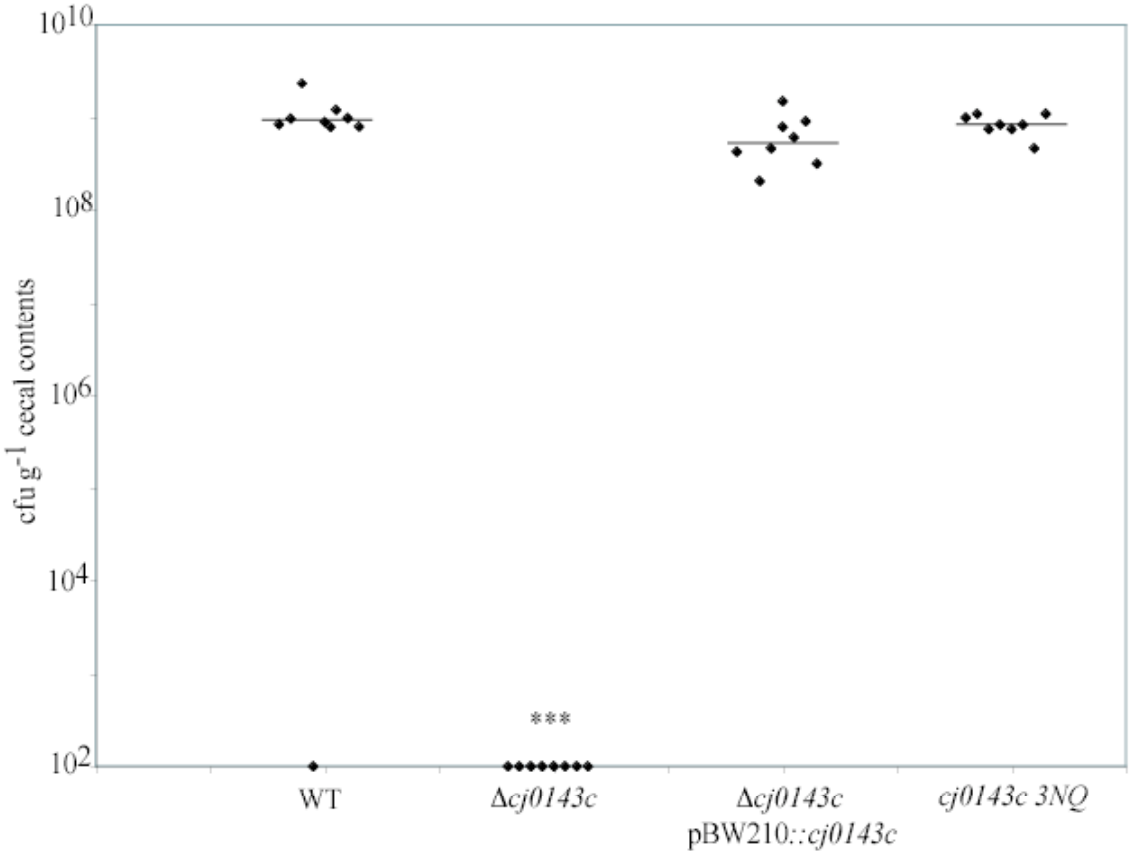

Figure 33. Chick Colonization by Δ*znuA*. Day old chicks were inoculated with 10^4 cfu of WT, Δ*cj0143c,* Δ*cj0143c* containing pCj0143c-FLAG, or *cj0143c3*NQ. After 7 days, the chicks were euthanized and their cecal contents were plated for *C. jejuni* cfu/g of cecal matter, with a limit of detection of 10^2 cfu/ml. Each point represents an individual chick. *** $p < 0.001$

Discussion

Campylobacter jejuni likely encounters varying levels of trace elements, including zinc, during its life cycle. Zinc is an essential trace element but can also be toxic to bacteria and therefore intracellular concentrations must be tightly regulated [3]. Bacteria regulate zinc homeostasis through zinc uptake and efflux systems.

A number of bacterial zinc transport systems have been characterized, including ZntA, ZupT, and ZnuABC. The ZntA system is a conserved bacterial zinc efflux system. ZupT is involved in uptake of zinc in *E. coli*, and is a member of the ZIP family of zinc transporters [24]. Based on sequence homology and previous characterization, *C. jejuni* contains ZntA (*cj1155c*) and ZupT *(cj0263*) systems. Here, we propose that *cj0143c, cj0141c*, and *cj0142c* encode the proteins of the ZnuABC zinc uptake system, with Cj0143c as the zinc binding protein (ZnuA), Cj0142c as the ATPase component (ZnuC) and Cj0141c as the integral membrane protein (ZnuB).

The ZnuABC system is essential for a number of bacterial pathogens in colonization and virulence of hosts [15, 22, 25, 26]. This work shows that *znuABC* in *C. jejuni* is essential for growth in low zinc environments, as well as for colonization of the chick ceca. The necessity of *C. jejuni* ZnuA in the colonization of the chick ceca, along with previous literature showing *znuA* as an important factor for survival within hosts for *E. coli, Brucella abortus*, *Haemophilus* spp, and *Neisseria gonorrhoeae*, and *Pastuerella multocida* suggest that available zinc is scarce within mammalian hosts [27] [3, 4, 6, 7]. This is similar to the low availability of iron, for which there are specific host binding proteins, transferrin and lactoferrin [28] The intestines are the major site of zinc

absorption, where intestinal metallothionein is thought to sequester zinc in the intestinal walls [29]. Metallothionein is an intracellular, cysteine-rich protein with a high binding affinity for heavy-metal ions, especially zinc. It is involved in zinc homeostasis as well as providing zinc for metalloenzymes involved in defense against pathogens [29, 30]. Whether metallothionein plays a direct role in the defense against microbial pathogens is currently unknown. However, mice deficient in metallothionein are more sensitive to *Helicobacter pylori* gastritis, as compared to wild-type mice [30]. Further, serum and plasma zinc concentrations are depressed when chickens are infected with *Salmonella gallinarum* or *E. coli,* suggesting a potential antimicrobial strategy of limiting the availability of zinc [31-33]. Competition for trace elements such as iron and zinc between a host and pathogen is critical for establishing infection. As chicken is one of the main sources for acquiring *C. jejuni*- related illness, factors that significantly decrease the ability of *C. jejuni* to colonize this food source could be a target for drug development.

The ZnuABC system is highly regulated in *E. coli,* being repressed by the zinc uptake regulator (Zur). Zur is part of the family of Fur regulators. Fur family proteins regulate metal homeostasis and act as global regulators for growth and in many cases, virulence [26, 34]. Zur represses zinc-responsive genes in the presence of zinc and is known to be sensitive to femtomolar quantities of zinc in *E. coli* [5]. Based on BLAST analysis, *C. jejuni* does not contain a *zur* homolog.

Fur regulates zinc-responsive genes within *Pasteurella multocida* [27], in the absence of Zur. The sequence upstream of *cj0143c* does not contain a Zur or Fur-box, as previously defined (Figure 23b). Previous microarray analysis demonstrated *cj0142c* was

up-regulated 2.2 fold in response to iron-limited conditions, but not in a *fur* mutant [35]. In a different microarray screen, *cj0141c-cj0143c* were not regulated in response to iron availability [36]. Based on these data and sequence analysis, we predict that the *C. jejuni znuABC* genes are not regulated by Fur. We demonstrated by RT-PCR that the *znuABC* genes in *C. jejuni* 81-176 are co-expressed under zinc-limiting conditions. With the absence of Zur and Fur regulation, we suggest that another form of regulation may be occurring for this system in *C. jejuni*.

PerR (peroxide stress regulator) was previously identified as a Fur homolog in *C. jejuni* that is responsive to iron limitation as well as to peroxide stress [37]. In *B. subtilis,* PerR is a peroxide-sensitive regulator that binds Fe^{+2} or Mn^{+2} as a cofactor and represses genes including *katA* (catalase*), aphPCF* (alkyl hydroperoxide), *mrgA* (Dps-like DNA binding protein), *hemAXCDBL* (heme biosynthesis operon), *zosA* (zinc uptake system) *fur*, and *perR* {Fuangthong, 2002 #207}. Gaballa *et al* further demonstrated that in *B. subtilis*, zinc uptake by ZosA is regulated by PerR and contributes to oxidative stress resistance [38]. Along with data showing Fur regulation of zinc-responsive genes in *Pasturella multocida*, these data demonstrate the ability for cross-talk of cation-responsive gene regulation within bacterial systems. In the absence of a *zur* homolog in *C. jejuni,* cross- regulation by PerR, or an uncharacterized system, are attractive alternatives for the transcriptional regulation of the *znuABC* system.

ZnuA from *C. jejuni* binds zinc *in vitro* irrespective of glycosylation, as we demonstrated using the PAR assay. Approximately 50μM zinc per μg of protein was detected for Cj0143c-FLAG when incubated with buffer alone, and 100μM zinc per μg protein with the addition of 1mM $ZnSO_4$. The quantity 50 μM of zinc per μg of protein

equates to 1.78 atoms of zinc for every molecule of ZnuA. This is approximately what was reported for *E. coli* ZnuA, which contains a high-affinity binding site, and a lower affinity site [39]. The addition of 1mM $ZnSO_4$ presumably saturated the protein with zinc with approximately 100 μM zinc per μg protein. This equates to 3.56 atoms of zinc per molecule of ZnuA, demonstrating the ability of *C. jejuni* ZnuA to bind to multiple zinc molecules.

ZnuA in *Synechocystis* contains a flexible loop of histidine and acidic residues, which has been hypothesized to perform a chaperone or regulatory function [1, 19]. At very low concentrations of zinc, only the high-affinity site binds zinc. When zinc concentrations are high, zinc association with the flexible loop may block zinc transport through structural changes [1, 30]. *C. jejuni* ZnuA has a region containing histidine and glutamates (Fig 23A) and similar to *Synechocystis* ZnuA, can bind multiple zinc molecules in high zinc concentrations (Fig 32). Further, our data suggests Cj0143c-FLAG is more stable in zinc-limiting conditions. We propose that zinc binding to the histidine rich-arm of Cj0143c results in a less stable conformation of the protein, leading to a second level of regulation. This may represent a previously uncharacterized system of cation regulation. Future work in the flexible loop region of ZnuA, possible regulators, such as PerR, and protein stability will provide insight into how this system is tightly regulated in *C. jejuni*.

Cj0143c was identified as a glycoprotein modified by the Pgl system, [12] and was identified to have a single glycosylation site in this study. However, glycosylation of Cj0143c is not necessary for the function of the protein in the assays examined, including chick cecal colonization. The level of glycosylation remained unchanged in

different conditions, including temperature, zinc content, growth phase or atmosphere (data not shown). The *N*-glycosylation system is necessary for a number of phenotypes, including colonization of both chicks and mice(Young, 2007). Although these studies demonstrate the importance of the *N*-glycosylation system in *C. jejuni* pathogenesis, the precise function is still under question. The glycosylation consensus sequence has been identified in several proteins, including Cj1496c and Cj0143c from proteomic analysis [16, 19]. Both Cj1496c and Cj0143c are required for colonization of chicks, however, the glycosylation of both proteins is not (this study) [6]. Conversely, the glycosylation of VirB10, a putative structural component of the pVir type IV secretion system, was demonstrated to be necessary for competence [20]. The exact mechanism of why the *N*-glycosylation system is necessary in chicks, along with why the proteins Cj1496c and Cj0143c are glycosylated is still unclear.

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CHAPTER V

Microbiota Influences on Intestinal Zinc in the Chick Cecum

Summary

Campylobacter jejuni commonly colonizes the gastrointestinal tract of chickens asymptomatically. ZnuA is a periplasmic binding protein for a high-affinity zinc ABC transporter. *C. jejuni znuA* is not essential for colonization of limited-flora chicks, but is necessary for colonization in conventional chicks. Conventionalization of *C. jejun*i Δ*znuA*-colonizing limited-flora chicks with cecal microbiota excludes the *C. jejuni* Δ*znuA* mutant. Measurable zinc in the ceca of conventional chicks is lower than that of chicks raised in germ-free conditions. We hypothesize that the microbiota use and store zinc, and modify host metabolic processes, thereby limiting the amount of available zinc for *C. jejuni* to compete for. Without its high affinity zinc transporter, *C. jejuni* is unable to compete with the microbiota in conventional chicks.

Introduction

Zinc is an essential trace element required for multiple cellular functions such as enzymatic reactions, DNA synthesis, and gene expression [1]. Over 300 enzymes and thousands of transcription factors contain one or more zinc atoms [2]. The availability and localization of intracellular zinc control the activities of metabolic enzymes and apoptosis. Mild zinc deficiencies in mammals can lead to an imbalance of the Th1 and Th2 functions, leading to a defect in the Th1 pathway [3]. The positive effects of zinc supplementation on boosting the Th1 immune pathway make it a common method for treating diarrhea in children and in developing countries [4].

Levels of zinc must be tightly regulated, as too little zinc does not support cellular growth while too much zinc is toxic [5]. Mammalian cells maintain zinc homeostasis though transport and export proteins (such as hZip proteins or ZnT-1), sequestration in vesicular compartments, or the zinc-binding protein metallothionein [6-8]. Metallothioneins are a family of proteins rich in cysteines that bind multiple atoms of metals, including zinc. The function of metallothioneins is unclear, but it is speculated that they protect against metal toxicity and participate in uptake, transport, and regulation of zinc in biological systems [9-11]. Unlike iron sequestration molecules such as lactoferrin, zinc sequestration molecules for scavenging purpose has never been described [12].

Similar to mammalian cells, zinc homeostasis must be tightly regulated in bacterial cells, however much less is known about these systems. In *E. coli*, excess zinc is exported from the cell via the zinc transporter ZntA, whose expression is under the

control of a response regulator ZntR [13]. Under low zinc conditions, zinc is brought into the bacteria through the ZnuABC transporter, which in many bacteria, is under negative control by the response regulator Zur [14]. The ZnuABC transport system is necessary for virulence and host colonization in a several bacterial species including *E. coli*, *Haemophilus spp*, *Salmonella* Typhimurium and *Campylobacter jejuni* [15-18].

Campylobacter jejuni is the causative agent of campylobacteriosis in humans, a self-limiting gastroenteritis. It is primarily acquired through the ingestion of contaminated poultry products, as *C. jejuni* asymptomatically colonizes the gastrointestinal tract of chickens. We previously characterized the high affinity zinc binding protein (ZnuA) for the ABC transporter ZnuABC in *C. jejuni.* ZnuA is essential for *C. jejuni* cecal colonization in a day-of-hatch chick colonization model, presumably due to a limiting amount of available zinc in the intestinal tract of the chicks [18].

That available zinc may be limiting in animals by the action of the gut microbiota was suggested by earlier work showing that conventional animals require more dietary zinc than germ-free animals [19]. The work presented here demonstrates that the microbiota of chicks affects the amount of available zinc in the chick intestines, presumably using and storing the available zinc, making the environment zinc-limiting. Without its high affinity zinc uptake system, *C. jejuni* is unable to compete for the available zinc. This is the first demonstration of zinc competition between microbiota in the gastrointestinal tract of a host.

Results

znuA is Required for Replication in the Chick Cecum

C. jejuni requires *znuA* for colonization in the chick cecum after seven days in a day-of-hatch chick colonization model [18]. Inability to colonize could be due to such factors as an inability to reach the cecum or replicate. To understand the mechanism behind this phenotype, day-of-hatch chicks were orally inoculated with 10^5 cfu of either wild-type (81-176 Str^R) or Δ*znuA* mutant of *C. jejuni*. Colonization of the cecum was monitored at 6, 12, 24, and 48 hours post inoculation. Both wild-type and the Δ*znuA* mutant were recoverable from the cecum at 10^3 cfu g^{-1} cecal contents by 6 hours post-inoculation (Fig 34). However, after 12 hours, there was a significant reduction in recovery of the Δ*znuA* mutant compared to wild-type. After 48 hours, recovery of the Δ*znuA* mutant was barely above the level of detection (Fig 34). Thus, the Δ*znuA* mutant is able to transiently colonize the cecum.

znuA is Not Required for *C. jejuni* Colonization of Germ-Free Mice and Limited-Flora Chicks

We hypothesized that the presence of a microbiota in the ceca could influence the ability of the Δ*znuA* mutant to colonize. Non-*Campylobacter* bacterial species are recovered from the chick cecum by 24 hours post-hatch on non-selective media, correlating with the decline in the Δ*znuA* mutant recovery. To test the contribution of the cecal microbiota in inhibiting colonization by the Δ*znuA* mutant, chicks were hatched and housed under germ-free conditions. Cecal contents of chicks housed under germ-free

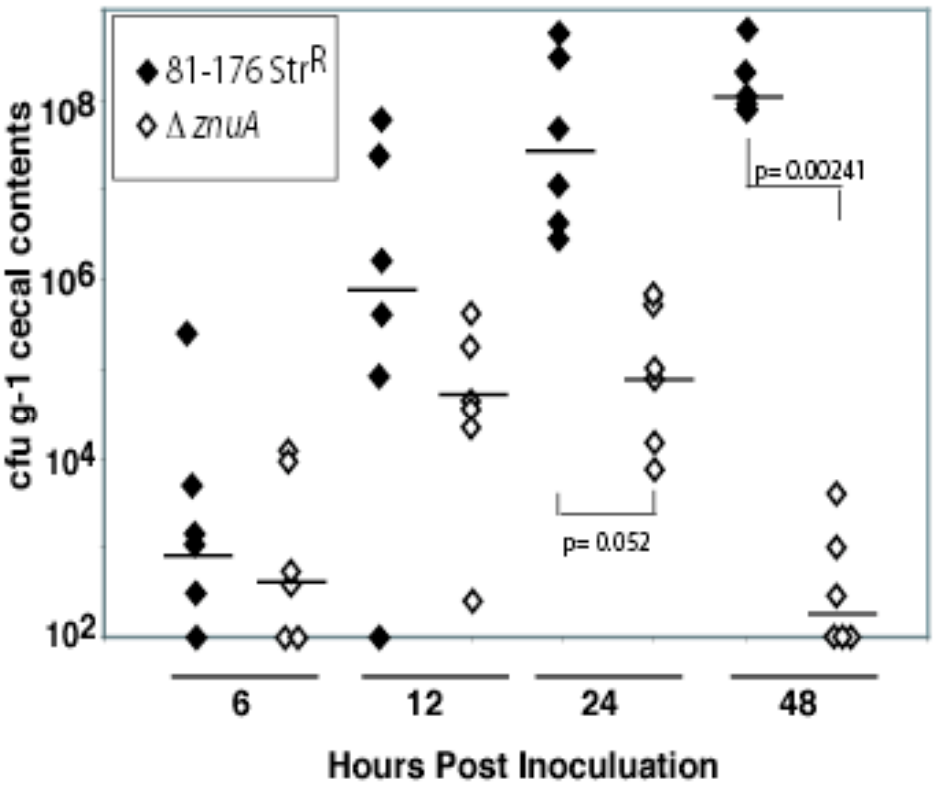

Figure 34. *ΔznuA* Mutant Early Colonization Events. *C. jejuni* cecal colonization of conventional day-of-hatch chicks inoculated with 10^5 cfu of either 81-176Str^R (black diamonds) or the Δ*znuA* mutant (open diamonds) at 6,12,24, and 48 hours post-inoculation. Each point represents the bacterial recovery in a single chick.

conditions were analyzed by tRFLP, demonstrating a reduction in the quantity and diversity of intestinal flora (Fig. 35). Chicks housed and hatched under germ-free conditions will be termed "limited-flora" due to the reduced number of bacteria in the ceca compared to conventionally raised chicks as analyzed by DAPI-stained immunofluorescent microscopy (Fig. 36) and tRFLP analysis (Fig. 35). The cecal microbiota of 7-day old conventional and limited-flora chicks were analyzed by 454-sequencing and are composed of similar bacteria phyla. Both populations are comprised primarily of Firmicutes; mores specifically of the Clostridiaceae family (Fig 37, Tables 3 and 4). These data are consistent with previous reports of cecal microbiota composition and horizontal transfer of microbiota in the egg, and demonstrate that the limited-flora chicks have a reduced number of total bacteria, but of similar composition compared to the conventional chick microbiota. [21, 23]

Limited-flora chicks were inoculated with either wild-type *C. jejuni* or the *C. jejuni* Δ*znuA* mutant. After seven days, both wild-type and the Δ*znuA* mutant were able to colonize the chick ceca at similar levels in limited-flora chicks (Fig 38). This was not specific to chicks, as both wild-type and the Δ*znuA* mutant were able to colonize germ-free mice at similar efficiencies (Fig. 38). These results demonstrate that the microbiota influences whether the *C. jejuni* Δ*znuA* mutant colonizes in the chick cecum and suggests that intestinal zinc levels differ between conventional and limited-flora chicks.

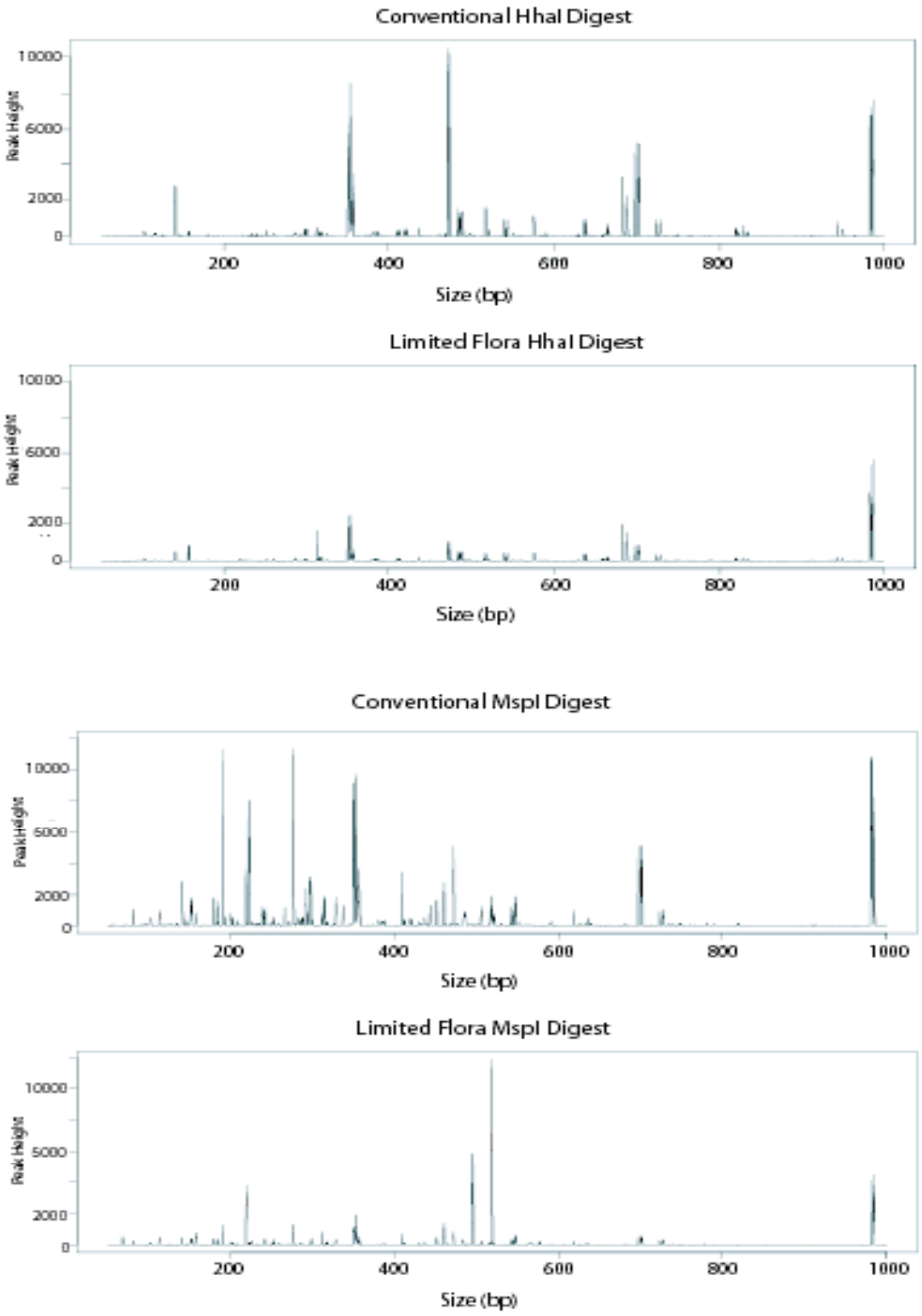

Figure 35. tRFLP Chromatogram of Conventional and Limited-flora Chicks. Results from cecal tissue from 7-day old conventional and limited-flora chicks digested with either Hha1 or Msp1. Chromatograms represent an average of four chicks per groups

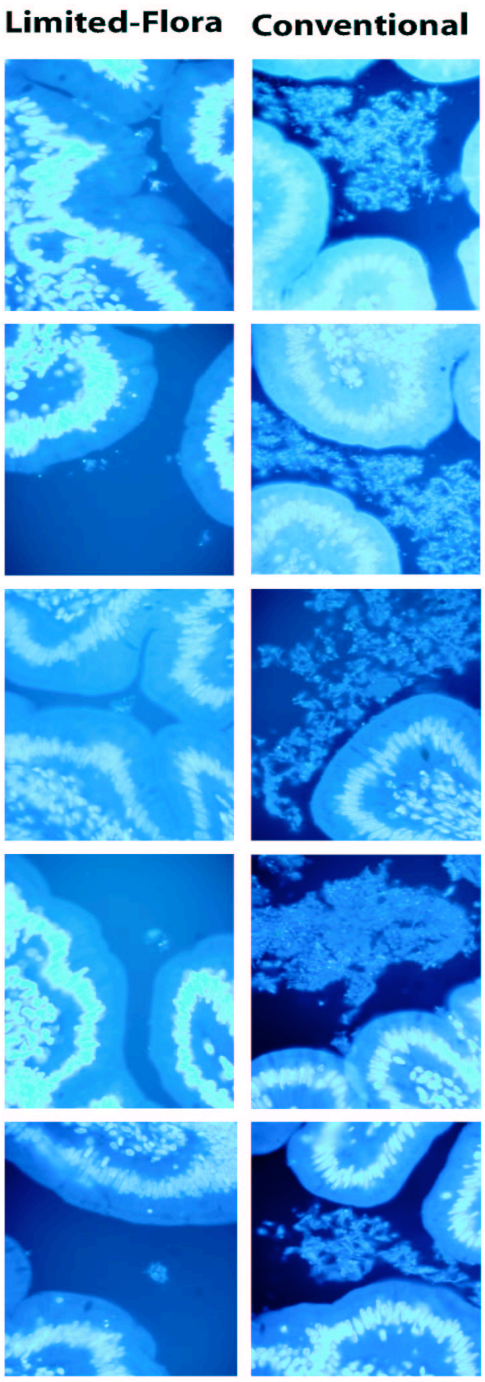

Figure 36. DAPI Stained Cecal Tissue from Conventional and Limited-flora Chicks. The lack of bacterial abundance and diversity of flora in limited-flora chicks is evident compared to conventional chicks after 7 days.

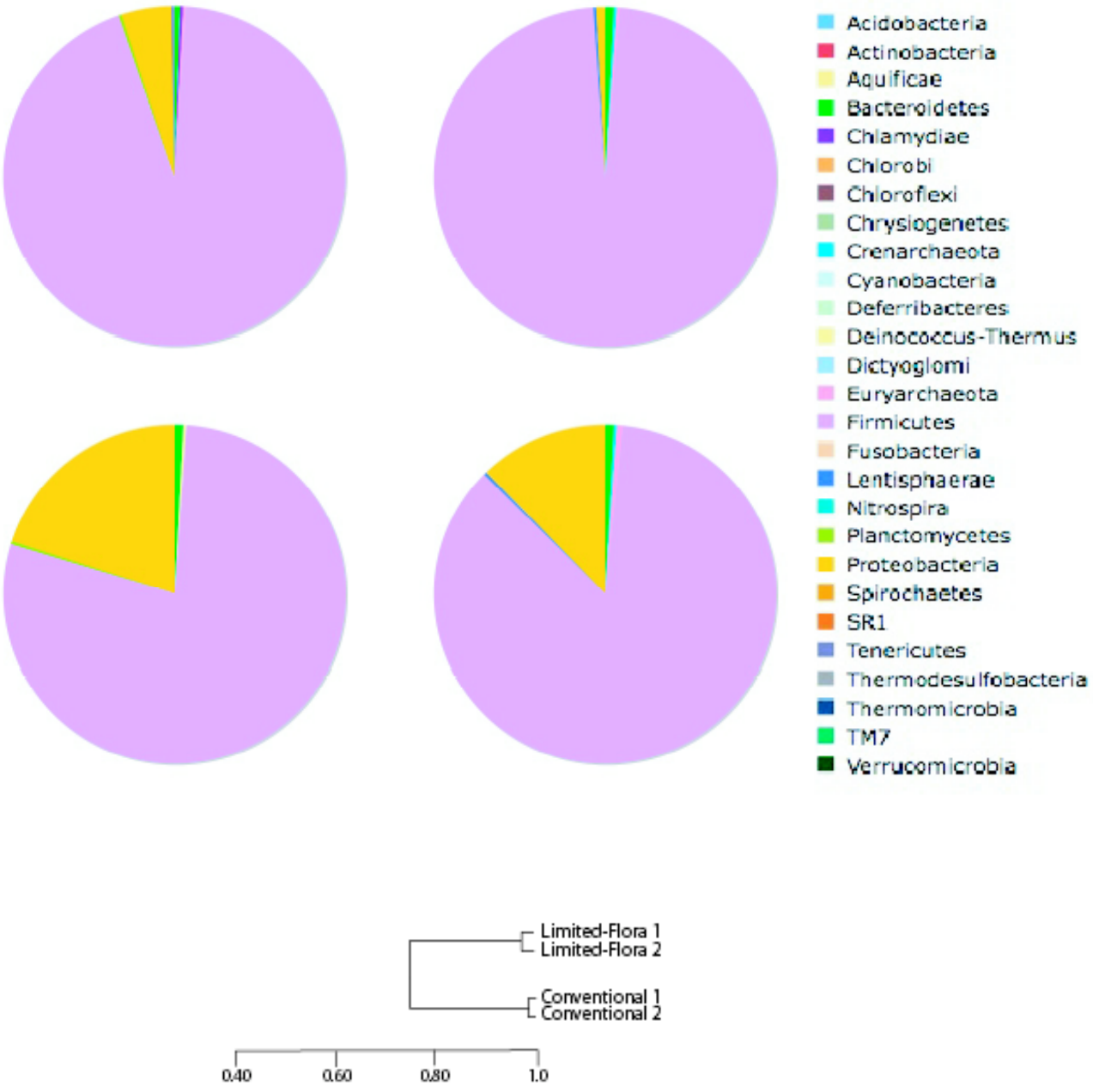

Figure 37. Phyla Composition from 454-sequencing of Conventional and Limited-flora Chicks. Analysis of Phyla composition demonstrates a high abundance of Firmicutes in both groups, with a higher abundance of Proteobacteria in limited-flora chicks. . UPGMA-mapping based on Morista-Horn statistics of relatedness show distinct groups between conventional and limited-flora chicks based on composition of the microbiota.

Table 2. Phyla Identities from 454 Sequencing of Conventional and Limited-flora Chicks. Percent of sequences of each Phyla per total sequences in two conventional and two limited-flora chicks after 7 days.

	Conventional	Conventional 2	Limited Flora 1	Limited Flora 2
Acidobacteria	0.0000	0.0000	0.0000	0.0000
Actinobacteria	0.0440	0.0368	0.0293	0.1243
Aquificae	0.0527	0.0368	0.0293	0.0000
Bacteroidetes	0.5626	0.6804	0.7847	0.5777
Chlamydiae	0.0352	0.0368	0.0147	0.0073
Chlorobi	0.0088	0.0184	0.0000	0.0146
Chloroflexi	0.0088	0.0000	0.0073	0.0073
Chrysiogenetes	0.0000	0.0184	0.0000	0.0000
Crenarchaeota	0.1494	0.1839	0.0953	0.2779
Cyanobacteria	0.0264	0.0184	0.0000	0.0000
Deferribacteres	0.0000	0.0000	0.0000	0.0512
Deinococcus-Thermus	0.0352	0.1287	0.0587	0.0804
Dictyoglomi	0.0176	0.0000	0.0000	0.0000
Euryarchaeota	0.2989	0.0919	0.0513	0.5119
Firmicutes	93.4951	97.6830	78.7768	85.9588
Fusobacteria	0.0527	0.0184	0.0000	0.0073
Lentisphaerae	0.0615	0.1103	0.0000	0.0293
Nitrospira	0.0000	0.0000	0.0000	0.0000
Planctomycetes	0.0967	0.0552	0.0513	0.0731
Proteobacteria	4.8875	0.8275	20.0645	12.1837
Spirochaetes	0.0088	0.0000	0.0073	0.0000
SR1	0.0000	0.0000	0.0000	0.0000
Tenericutes	0.0791	0.0368	0.0000	0.0512
Thermodesulfobacteria	0.0000	0.0000	0.0000	0.0000
Thermomicrobia	0.0000	0.0000	0.0073	0.0146
TM7	0.0000	0.0000	0.0073	0.0000
Verrucomicrobia	0.0791	0.0184	0.0147	0.0293

Table 3. Genus Identities from 454-Sequeincing of Conventional and Limited-Flora Chicks.

	Conventional 1	Conventional 2	Limited-Flora 1	Limited-Flora 2
Abiotrophia	0.0000	0.0000	0.0000	0.0000
Acetanaerobacterium	0.1959	0.1783	0.0142	0.0000
Acetitomaculum	0.0170	0.0000	0.0142	0.0000
Achromobacter	0.0256	0.0000	0.0000	0.0000
Acidaminobacter	0.0000	0.0000	0.0000	0.0000
Acidaminococcus	0.0000	0.0000	0.0000	0.0070
Acidicaldus	0.0085	0.0000	0.0000	0.0000
Acidilobus	0.0085	0.0000	0.0000	0.0070
Acidimicrobium	0.0000	0.0000	0.0000	0.0000
Acidovorax	0.1107	0.0000	0.0000	0.0000
Acinetobacter	0.0000	0.0000	0.0071	0.0349
Actinobacillus	0.0000	0.0000	0.0000	0.0000
Actinomyces	0.0000	0.0000	0.0000	0.0000
Adhaeribacter	0.0000	0.0000	0.0000	0.0000
Aeromonas	0.0170	0.0178	0.0000	0.0000
Afipia	0.0000	0.0000	0.0000	0.0000
Aggregatibacter	0.0000	0.0000	0.0000	0.0000
Agrobacterium	0.0000	0.0000	0.0000	0.0000
Akkermansia	0.0767	0.0178	0.0000	0.0140
Alistipes	0.0256	0.0000	0.0142	0.0140
Alkaliflexus	0.0000	0.0000	0.0000	0.0000
Alkalispirillum	0.0000	0.0000	0.0000	0.0000
Alkanindiges	0.0085	0.0000	0.0000	0.0000
Allobaculum	0.0000	0.0000	0.0142	0.0000
Alloiococcus	0.0085	0.0000	0.0000	0.0000
Aminobacterium	0.0000	0.0000	0.0000	0.0000
Aminomonas	0.0000	0.0000	0.0000	0.0070
Anaerobacter	0.2641	0.2675	0.8525	0.9642
Anaerobaculum	0.0000	0.0000	0.0000	0.0000
Anaerobiospirillum	0.0000	0.0000	0.0071	0.0000
Anaerococcus	0.0000	0.0000	0.0000	0.0000
Anaerofilum	0.0000	0.0000	0.0000	0.0000
Anaerofustis	0.0000	0.0000	0.0000	0.0070
Anaeroglobus	0.0085	0.0000	0.0000	0.0070
Anaeromusa	0.0000	0.0000	0.0000	0.0000
Anaeromyxobacter	0.0085	0.0000	0.0000	0.0000
Anaerophaga	0.0511	0.0000	0.0000	0.0000
Anaerostipes	0.0000	0.0000	0.0000	0.0489
Anaerotruncus	6.9676	8.0614	0.4049	0.0070
Anaerovorax	0.0000	0.0000	0.0000	0.0140
Aquifex	0.0426	0.0000	0.0284	0.0000
Arcobacter	0.0000	0.0000	0.0000	0.0000
Asteroleplasma	0.0767	0.0178	0.0000	0.0489
Asticcacaulis	0.0681	0.0000	0.0000	0.0000

Atopobacter	0.0085	0.0000	0.0071	0.0210
Atopobium	0.0000	0.0000	0.0000	0.0000
Atopostipes	0.0170	0.0000	0.0000	0.0000
Azospirillum	0.1022	0.0000	0.0213	0.0140
Azovibrio	0.0000	0.0178	0.0000	0.0000
Bacillus	0.0000	0.0000	0.0142	0.0279
Bacteroides	0.1448	0.3924	0.5541	0.2306
Beijerinckia	0.0000	0.0000	0.0213	0.0000
Belnapia	0.0000	0.0535	0.0000	0.0000
Bergeriella	0.0000	0.0000	0.0000	0.0000
Beutenbergia	0.0000	0.0000	0.0000	0.0000
Blastopirellula	0.0000	0.0000	0.0000	0.0000
Bradyrhizobium	0.0000	0.0000	0.0000	0.0000
Brevinema	0.0000	0.0000	0.0071	0.0000
Bryantella	0.1618	0.1248	0.0000	0.0000
Buchnera	0.0681	0.0000	0.0142	0.0699
Bulleidia	0.5792	0.0178	0.0000	0.0000
Burkholderia	0.0000	0.0357	0.0000	0.0000
Butyrivibrio	0.0085	0.0000	0.0000	0.0070
Byssovorax	0.0000	0.0000	0.0000	0.0279
Caldilinea	0.0085	0.0000	0.0000	0.0000
Caldisphaera	0.0341	0.0535	0.0000	0.0000
Caldivirga	0.0000	0.0000	0.0142	0.0559
Caminicella	0.0085	0.0000	0.0071	0.0210
Campylobacter	0.0085	0.0000	0.0071	0.0000
Capnocytophaga	0.0000	0.0000	0.0000	0.0000
Carboxydothermus	0.0170	0.0000	0.0000	0.0070
Cardinium	0.0085	0.0000	0.0000	0.0000
Catenibacterium	0.0085	0.0000	0.0000	0.0000
Catonella	0.0085	0.0178	0.0000	0.0000
Centipeda	0.0000	0.0000	0.0000	0.0000
Cerasibacillus	0.0000	0.0000	0.0000	0.0000
Cetobacterium	0.0170	0.0000	0.0000	0.0000
Chloroherpeton	0.0085	0.0178	0.0000	0.0140
Chloronema	0.0000	0.0000	0.0071	0.0070
Chromohalobacter	0.0000	0.0000	0.0000	0.0000
Chryseomonas	0.0341	0.0000	0.0000	0.0489
Chrysiogenes	0.0000	0.0178	0.0000	0.0000
Citrobacter	0.1533	0.0357	0.8880	1.4673
Cloacibacterium	0.0000	0.0000	0.1208	0.1397
Clostridium	11.7206	11.9493	47.9150	55.1146
Coenonia	0.0000	0.0178	0.0000	0.0070
Collinsella	0.0085	0.0000	0.0000	0.0000
Coprobacillus	0.0170	0.0000	0.0000	0.0000
Coprococcus	0.0170	0.0178	0.0071	0.0000
Corynebacterium	0.0000	0.0000	0.0000	0.0000
Craurococcus	0.0000	0.0000	0.0000	0.0000
Croceibacter	0.0000	0.0357	0.0000	0.0070
Cryobacterium	0.0000	0.0000	0.0071	0.0210
Cryomorpha	0.0085	0.0000	0.0000	0.0000
Cryptanaerobacter	0.0085	0.0000	0.0000	0.0140

Cupriavidus	0.0000	0.0000	0.0071	0.0000
Delftia	0.0341	0.0000	0.0071	0.0000
Dendrosporobacter	0.0000	0.0000	0.0000	0.0000
Dermabacter	0.0000	0.0000	0.0000	0.0000
Desemzia	0.0085	0.0000	0.0142	0.0210
Desulfitibacter	0.0000	0.0357	0.0000	0.0000
Desulfonauticus	0.0085	0.0000	0.0000	0.0140
Desulfonispora	0.0170	0.0000	0.0000	0.0000
Desulforegula	0.0000	0.0000	0.0000	0.0070
Desulfospira	0.0085	0.0000	0.0000	0.0140
Desulfurococcus	0.0000	0.0000	0.0000	0.0070
Dialister	0.0000	0.0000	0.0000	0.0000
Dichelobacter	0.0000	0.0000	0.0000	0.0000
Dictyoglomus	0.0085	0.0000	0.0000	0.0000
Dokdonia	0.0000	0.0000	0.0000	0.0000
Dolosigranulum	0.0000	0.0000	0.0071	0.0070
Dorea	3.6371	2.8536	7.9420	4.9469
Eggerthella	0.0000	0.0000	0.0000	0.0000
Empedobacter	0.0000	0.0000	0.0000	0.0000
Emticicia	0.0000	0.0178	0.0000	0.0000
Enhydrobacter	0.0000	0.0000	0.0000	0.0000
Enterobacter	0.0000	0.0000	0.0000	0.0000
Enterococcus	1.9165	0.8204	1.6552	2.9486
Escherichia	0.0341	0.0178	0.5967	0.2096
Ethanoligenens	0.0681	0.0000	0.0071	0.0000
Faecalibacterium	0.4940	0.3567	0.1137	0.1467
Fastidiosipila	0.6729	0.4637	0.0355	0.0070
Ferroplasma	0.0256	0.0535	0.0213	0.1048
Filifactor	0.0000	0.0000	0.0000	0.0000
Finegoldia	0.0000	0.0000	0.0000	0.0000
Flavimonas	0.0085	0.0178	0.0000	0.0140
Flavobacterium	0.0000	0.0000	0.0000	0.0000
Flexithrix	0.0000	0.0000	0.0000	0.0070
Fluviicola	0.0000	0.0000	0.0000	0.0070
Fulvimarina	0.0000	0.0000	0.0000	0.0070
Fusibacter	0.0000	0.0178	0.0000	0.0000
Fusobacterium	0.0000	0.0000	0.0000	0.0000
Gallibacterium	0.0085	0.0000	0.0000	0.0000
Gelria	0.0170	0.0000	0.0000	0.0070
Gemella	0.0000	0.0000	0.0000	0.0000
Gemmata	0.0000	0.0178	0.0000	0.0070
Geodermatophilus	0.0000	0.0000	0.0000	0.0000
Geoglobus	0.0170	0.0178	0.0000	0.3354
Geopsychrobacter	0.0000	0.0000	0.0000	0.0000
Gp4	0.0000	0.0000	0.0000	0.0000
Granulicatella	0.1278	0.0000	0.0000	0.0000
Haemophilus	0.0000	0.0000	0.0000	0.0000
Hafnia	0.0000	0.0000	0.0000	0.0000
Haliscomenobacter	0.0085	0.0000	0.0000	0.0070
Hallella	0.0000	0.0000	0.0000	0.0000
Halobacterium	0.0000	0.0000	0.0000	0.0000

Halochromatium	0.0000	0.0000	0.0000	0.0000
Halogeometricum	0.0000	0.0000	0.0000	0.0070
Halomonas	0.0000	0.0000	0.0000	0.0000
Halorhabdus	0.0170	0.0000	0.0000	0.0000
Halostagnicola	0.0000	0.0000	0.0071	0.0000
Haloterrigena	0.0000	0.0000	0.0000	0.0000
Halothermothrix	0.0000	0.0000	0.0000	0.0000
Halovivax	0.0000	0.0000	0.0071	0.0000
Helicobacter	0.0000	0.0000	0.1350	0.0000
Herbaspirillum	0.0000	0.0178	0.0000	0.0000
Hespellia	1.5673	1.4268	0.1634	0.0000
Hippea	0.0000	0.0000	0.0000	0.0000
Holdemania	0.0341	0.0000	0.0000	0.0000
Holospora	0.0000	0.0000	0.0000	0.0070
Hydrogenivirga	0.0085	0.0357	0.0000	0.0000
Ignisphaera	0.0085	0.0178	0.0213	0.0070
Isobaculum	0.0170	0.0000	0.0000	0.0210
Johnsonella	0.0000	0.0000	0.0000	0.0000
Kaistella	0.0000	0.0000	0.0000	0.0000
Kingella	0.0000	0.0000	0.0000	0.0000
Klebsiella	0.0000	0.0000	0.0000	0.0000
Kordia	0.0000	0.0000	0.0000	0.0000
Lachnobacterium	0.1022	0.0357	0.0142	0.0140
Lachnospira	33.0920	37.0965	11.4868	12.7935
Lactobacillus	0.1618	0.0000	0.0639	0.0000
Lactococcus	0.0000	0.0000	0.0000	0.0000
Lactovum	0.0000	0.0000	0.0000	0.0000
Laribacter	0.0000	0.0000	0.0000	0.0000
Larkinella	0.0341	0.0178	0.0000	0.0000
Leclercia	0.0000	0.0000	0.0071	0.0070
Lentisphaera	0.0511	0.0892	0.0000	0.0279
Leptolinea	0.0000	0.0000	0.0000	0.0000
Leptonema	0.0085	0.0000	0.0000	0.0000
Leptotrichia	0.0000	0.0000	0.0000	0.0000
Leuconostoc	0.0170	0.0000	0.0000	0.0140
Levilinea	0.0000	0.0000	0.0000	0.0000
Limnobacter	0.0000	0.0000	0.0000	0.0000
Listonella	0.0000	0.0000	0.0000	0.0140
Longispora	0.0000	0.0000	0.0000	0.0000
Luteimonas	0.0085	0.0000	0.0000	0.0000
Macrococcus	0.0000	0.0000	0.0000	0.0000
Magnetobacterium	0.0000	0.0000	0.0000	0.0000
Mahella	0.0000	0.0178	0.0071	0.0210
Marinilabilia	0.0000	0.0000	0.0071	0.0000
Marinithermus	0.0000	0.0000	0.0000	0.0000
Megasphaera	0.0000	0.0000	0.0000	0.0000
Melissococcus	0.0000	0.0000	0.0071	0.0210
Mesonia	0.0000	0.0000	0.0071	0.0000
Methanocaldococcus	0.0085	0.0000	0.0071	0.0000
Methanohalobium	0.1107	0.0000	0.0000	0.0000
Methanomethylovorans	0.0085	0.0000	0.0000	0.0210

Methanopyrus	0.0000	0.0178	0.0000	0.0000
Methanosphaera	0.0000	0.0000	0.0000	0.0000
Methanothermobacter	0.0000	0.0000	0.0000	0.0000
Methanothermococcus	0.0000	0.0000	0.0000	0.0000
Methanothrix	0.0000	0.0000	0.0000	0.0000
Methanotorris	0.0085	0.0000	0.0000	0.0000
Methylarcula	0.0000	0.0000	0.0000	0.0070
Methylibium	0.0000	0.0000	0.0000	0.0000
Methylobacterium	0.0000	0.0000	0.0071	0.0070
Methylothermus	0.0000	0.0000	0.0000	0.0000
Microbacterium	0.0000	0.0000	0.0000	0.0070
Microcella	0.0000	0.0000	0.0000	0.0769
Micrococcus	0.0000	0.0000	0.0000	0.0000
Microscilla	0.0085	0.0178	0.0000	0.0000
Microvirga	0.0000	0.0000	0.0000	0.0000
Mitsuaria	0.0000	0.0000	0.0000	0.0000
Moellerella	0.0000	0.0000	0.0000	0.0070
Mogibacterium	0.0000	0.0000	0.0000	0.0000
Moryella	0.0000	0.0000	0.0000	0.0000
Mucispirillum	0.0000	0.0000	0.0000	0.0419
Muricoccus	0.0085	0.0000	0.0000	0.0140
Mycobacterium	0.0000	0.0000	0.0000	0.0000
Mycoplasma	0.0000	0.0000	0.0000	0.0000
Natronobacterium	0.0000	0.0000	0.0000	0.0000
Natronolimnobius	0.0000	0.0000	0.0000	0.0000
Naxibacter	0.0000	0.0000	0.0000	0.0000
Neisseria	0.0000	0.0000	0.0000	0.0000
Neochlamydia	0.0000	0.0178	0.0000	0.0000
Neoehrlichia	0.0085	0.0000	0.0000	0.0000
Nicoletella	0.0000	0.0000	0.0000	0.0000
Nitratifractor	0.0170	0.0178	0.0000	0.0070
Nitratiruptor	0.0000	0.0000	0.0071	0.0000
Odyssella	0.0000	0.0000	0.0000	0.0000
Oligotropha	0.0000	0.0000	0.0000	0.0000
Olleya	0.0085	0.0178	0.0000	0.0000
Oribacterium	0.0000	0.0000	0.0000	0.0000
Orientia	0.0000	0.0000	0.0000	0.0000
Oscillochloris	0.0000	0.0000	0.0000	0.0000
Owenweeksia	0.0000	0.0000	0.0000	0.0070
Oxobacter	0.0000	0.0000	0.0071	0.0070
Palaeococcus	0.0000	0.0000	0.0000	0.0070
Paludibacter	0.0341	0.0000	0.0000	0.0000
Papillibacter	1.3969	1.7835	0.1776	0.0140
Parabacteroides	0.0000	0.0178	0.0000	0.0000
Parachlamydia	0.0085	0.0000	0.0000	0.0000
Paracoccus	0.0000	0.0000	0.0000	0.0000
Parasporobacterium	0.4685	0.4637	0.0213	0.0000
Parvibaculum	0.0000	0.0000	0.0000	0.0000
Parvimonas	0.0000	0.0000	0.0000	0.0000
Parvularcula	0.0000	0.0000	0.0000	0.0140
Pasteurella	0.0000	0.0000	0.0000	0.0000

Pectobacterium	0.0000	0.0000	0.0000	0.0070
Pelomonas	0.0511	0.0535	0.0000	0.0000
Pelospora	0.0000	0.0000	0.0000	0.0000
Peptococcus	0.0085	0.0000	0.0000	0.0000
Peptoniphilus	0.0000	0.0000	0.0000	0.0000
Peptostreptococcus	0.0000	0.0000	0.0000	0.0000
Peredibacter	0.0000	0.0000	0.0071	0.0000
Petrimonas	0.0000	0.0000	0.0000	0.0000
Phenylobacterium	0.0000	0.0000	0.0000	0.0000
Phlomobacter	0.0000	0.0000	0.0000	0.0000
Phocoenobacter	0.0000	0.0000	0.0000	0.0000
Picrophilus	0.0511	0.0000	0.0000	0.0140
Pirellula	0.0937	0.0357	0.0426	0.0629
Piscirickettsia	0.0000	0.0000	0.0000	0.0000
Plantibacter	0.0000	0.0000	0.0000	0.0000
Polynucleobacter	0.0000	0.0000	0.0000	0.0000
Pontibacter	0.0085	0.0000	0.0000	0.0000
Porphyromonas	0.0085	0.0000	0.0000	0.0000
Prevotella	0.0170	0.0000	0.0071	0.0140
Procabacter	0.0085	0.0000	0.0000	0.0000
Propionibacterium	0.0341	0.0178	0.0000	0.0000
Proteiniphilum	0.0426	0.0535	0.0000	0.0210
Pseudobutyrivibrio	0.0085	0.0178	0.0000	0.0000
Pseudomonas	2.0102	0.2854	0.0710	0.3913
Pseudoramibacter	0.0085	0.0000	0.0000	0.0140
Pseudospirillum	0.0170	0.0000	0.0000	0.0000
Pseudoxanthomonas	0.0000	0.0000	0.0071	0.0000
Pullulanibacillus	0.0000	0.0000	0.0000	0.0000
Pyrolobus	0.0000	0.0000	0.0000	0.0070
Quadrisphaera	0.0000	0.0000	0.0000	0.0000
Quatrionicoccus	0.0085	0.0178	0.0000	0.0000
Ralstonia	0.0256	0.0000	0.0000	0.2026
Raoultella	0.0000	0.0000	0.0000	0.0210
Rhizobium	0.0085	0.0000	0.0000	0.0000
Rhodobacter	0.0000	0.0000	0.0000	0.0000
Rhodocista	0.0000	0.0000	0.0000	0.0000
Rhodomicrobium	0.0085	0.0000	0.0000	0.0000
Rhodopseudomonas	0.0000	0.0000	0.0000	0.0000
Rhodothalassium	0.0000	0.0178	0.0000	0.0000
Rhodothermus	0.0256	0.0000	0.0071	0.0349
Riemerella	0.0000	0.0000	0.0000	0.0070
Rikenella	0.0000	0.0000	0.0000	0.0000
Roseateles	0.1022	0.0000	0.0000	0.0000
Roseburia	0.0170	0.0000	0.0071	0.0489
Roseiflexus	0.0000	0.0000	0.0000	0.0000
Rothia	0.0000	0.0000	0.0000	0.0000
Rubritalea	0.0000	0.0000	0.0071	0.0140
Ruminobacter	0.0085	0.0178	0.0000	0.0000
Ruminococcus	0.0085	0.0357	0.0071	0.0000
Saccharomonospora	0.0000	0.0000	0.0142	0.0070
Salicola	0.0085	0.0000	0.0000	0.0070

Salinibacter	0.0000	0.0000	0.0000	0.0000
Salmonella	0.1022	0.0178	3.2891	1.2367
Samsonia	0.0000	0.0000	0.0071	0.0000
Sandarakinotalea	0.0000	0.0000	0.0000	0.0000
Sarcina	0.0170	0.0535	0.0071	0.0210
Scardovia	0.0000	0.0000	0.0000	0.0000
Schlegelella	0.0000	0.0000	0.0000	0.0000
Sebaldella	0.0000	0.0000	0.0000	0.0000
Selenihalanaerobacter	0.0000	0.0000	0.0000	0.0070
Shewanella	0.0000	0.0000	0.0000	0.0000
Shigella	0.5196	0.1070	13.2557	6.9312
Shimazuella	0.0000	0.0000	0.0071	0.0000
Shuttleworthia	0.0000	0.0000	0.0000	0.0000
Simkania	0.0170	0.0000	0.0071	0.0070
Simonsiella	0.0000	0.0000	0.0000	0.0000
Sinococcus	0.0000	0.0000	0.0000	0.0000
Slackia	0.0000	0.0000	0.0000	0.0000
Sneathia	0.0000	0.0000	0.0000	0.0000
Sodalis	0.0341	0.0000	0.0071	0.0000
Sorangium	0.0000	0.0000	0.0213	0.0070
Sphingobium	0.0000	0.0000	0.0000	0.0838
Sphingomonas	0.3237	0.0000	0.0071	0.0000
Sphingopyxis	0.0000	0.0000	0.0000	0.0000
Spirillum	0.0000	0.0000	0.0071	0.0000
Spirosoma	0.0000	0.0000	0.0000	0.0000
Sporacetigenium	0.0000	0.0000	0.0000	0.0000
Sporanaerobacter	0.0085	0.0000	0.0000	0.0000
Sporobacter	0.1278	0.0357	0.0000	0.0140
Sporobacterium	0.0341	0.0000	0.0000	0.0000
Sporohalobacter	0.0085	0.0178	0.0000	0.0000
Stackebrandtia	0.0000	0.0000	0.0000	0.0000
Staphylococcus	0.0000	0.0000	0.0000	0.1607
Stenothermobacter	0.0085	0.0000	0.0000	0.0000
Stenotrophomonas	0.1448	0.0000	0.0000	0.0000
Streptobacillus	0.0341	0.0178	0.0000	0.0070
Streptococcus	0.0170	0.0000	0.0000	0.0489
Stygiolobus	0.0085	0.0000	0.0000	0.0349
Subdoligranulum	0.1022	0.0000	0.0142	0.0140
Succiniclasticum	0.0256	0.0000	0.0000	0.0000
Succinimonas	0.0000	0.0000	0.0000	0.0000
Succinispira	0.0000	0.0178	0.0142	0.0070
Sulfophobococcus	0.0000	0.0000	0.0213	0.0070
Sulfurisphaera	0.0596	0.0535	0.0142	0.1048
Sulfurivirga	0.0000	0.0000	0.0000	0.0000
Sutterella	0.0000	0.0000	0.0000	0.0000
Swaminathania	0.0000	0.0000	0.0000	0.0000
Symbiobacterium	0.0000	0.0000	0.0000	0.0000
Syntrophococcus	1.1499	0.9096	0.1634	0.2236
Syntrophothermus	0.0000	0.0178	0.0071	0.0000
Tannerella	0.0426	0.0000	0.0000	0.0000
Tepidicella	0.0000	0.0000	0.0000	0.0000

Terasakiella	0.0000	0.0000	0.0000	0.0000
Terrimonas	0.0085	0.0000	0.0071	0.0140
Thalassobacillus	0.0000	0.0000	0.0000	0.0000
Thermacetogenium	0.0000	0.0000	0.0071	0.0000
Thermanaeromonas	0.0000	0.0000	0.0000	0.0000
Thermicanus	0.0000	0.0000	0.0000	0.0000
Thermoanaerobacterium	0.0000	0.0000	0.0142	0.0000
Thermobacillus	0.0596	0.0000	0.0000	0.0000
Thermobispora	0.0000	0.0000	0.0071	0.0000
Thermochromatium	0.0000	0.0000	0.0000	0.0000
Thermocladium	0.0256	0.0357	0.0071	0.0210
Thermodesulfatator	0.0000	0.0000	0.0000	0.0000
Thermodiscus	0.0000	0.0000	0.0071	0.0000
Thermofilum	0.0000	0.0000	0.0000	0.0070
Thermohalobacter	0.0085	0.0000	0.0071	0.0000
Thermomicrobium	0.0000	0.0000	0.0071	0.0140
Thermoproteus	0.0000	0.0000	0.0000	0.0000
Thermosphaera	0.0000	0.0000	0.0000	0.0000
Thermothrix	0.0681	0.0000	0.0142	0.0838
Thermovenabulum	0.0000	0.0000	0.0000	0.0000
Thiobacter	0.0000	0.0000	0.0000	0.0070
Thioreductor	0.0000	0.0000	0.0000	0.0000
Thorsellia	0.0000	0.0000	0.0000	0.0000
Trabulsiella	0.0000	0.0000	0.0071	0.0000
Treponema	0.0000	0.0000	0.0000	0.0000
Trichlorobacter	0.0000	0.0000	0.0000	0.0000
Turicella	0.0000	0.0000	0.0000	0.0000
Turicibacter	0.0000	0.0000	0.0497	0.0070
Veillonella	0.0000	0.0000	0.0000	0.0000
Verrucomicrobium	0.0000	0.0000	0.0000	0.0000
Victivallis	0.0085	0.0000	0.0000	0.0000
Vitreoscilla	0.0000	0.0000	0.0000	0.0000
Vulcanithermus	0.0341	0.1070	0.0568	0.0769
Waddlia	0.0085	0.0178	0.0071	0.0000
Weissella	0.0000	0.0000	0.0071	0.0070
Wolinella	0.0000	0.0000	0.0071	0.0000
Xenohaliotis	0.0426	0.0357	0.0213	0.0559
Xylanibacter	0.0000	0.0000	0.0000	0.0000
Xylella	0.0000	0.0000	0.0000	0.0070
Xylophilus	0.0000	0.0000	0.0000	0.0000
Yeosuana	0.0000	0.0178	0.0000	0.0000
Zymophilus	0.0085	0.0000	0.0000	0.0000

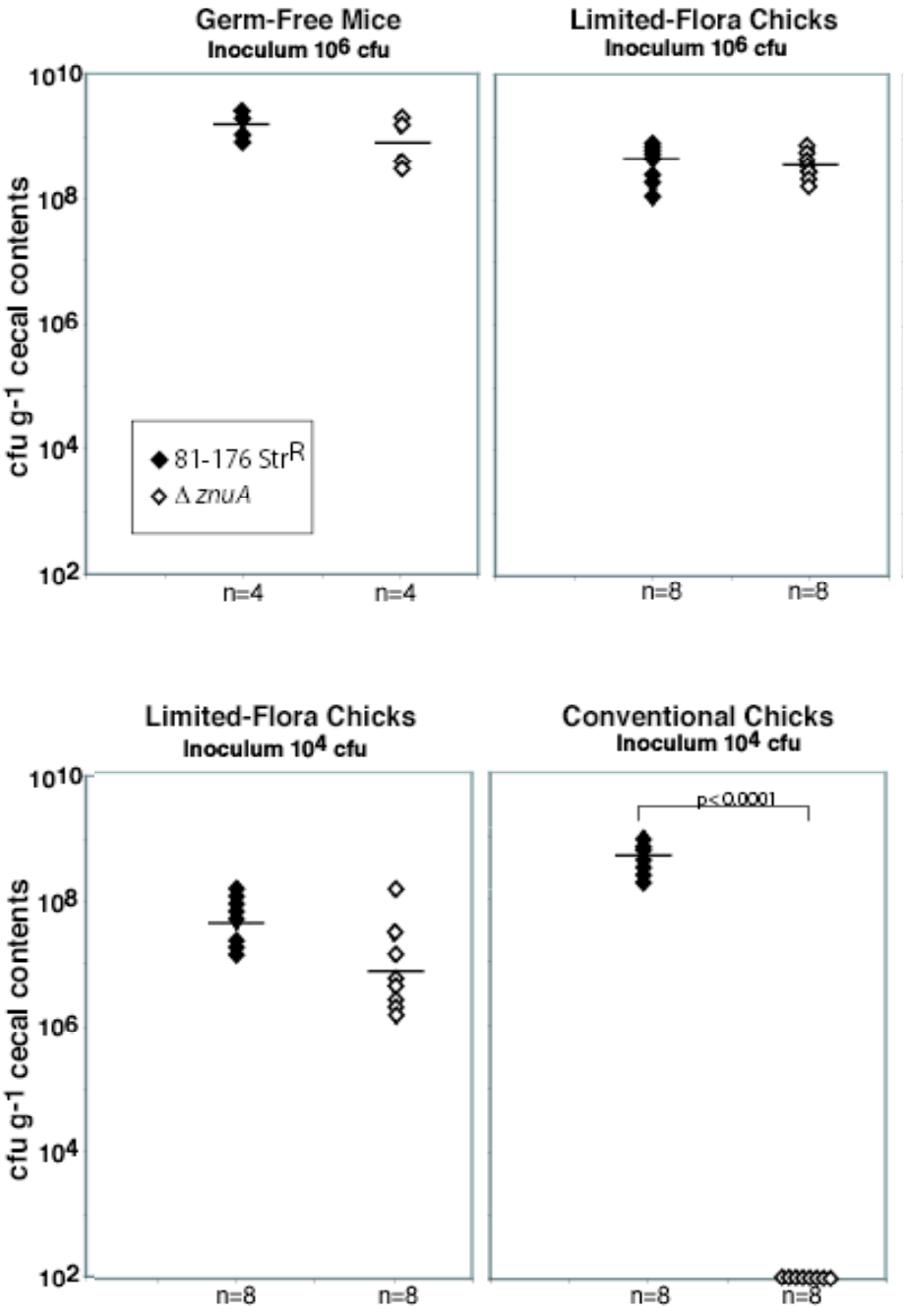

Figure 38. *C. jejuni* Colonization in Germ-Free Mice and Limited-Flora Chicks. *C. jejuni* cecal colonization of germ-free mice, germ-free chicks, and conventional-chicks. The animals were inoculated with the indicated doses of either 81-176 Str^R (black diamond) or the Δ*znuA* mutant (open diamond) and the cecal contents were plated for *C. jejuni* recovery after 7 days. Chicks were inoculated day-of-hatch and germ-free mice were inoculated post-weaning (one month-old).

C. jejuni ΔznuA Mutant Colonization of Limited-Flora Chicks Elicits an Inflammatory Response

As demonstrated in Chapter II, *C. jejuni* elicits a modest inflammatory response in the ceca of conventional chicks, marked by increased pro-inflammatory cytokine expression and heterophil recruitment. Chicks inoculated with the Δ*znuA* mutant did not have marked heterophil infiltrate, most likely due to an inability to colonize and invade the tissue.

Limited-flora chicks colonized with the Δ*znuA* mutant displayed marked heterophil infiltration after 7 days in the ceca. The levels of heterophil recruitment were significantly higher than PBS inoculated limited-flora chicks, and were similar to wild-type infected limited-flora chicks (Fig 39). These results demonstrate that the Δ*znuA* mutant is able to stimulate a similar inflammatory response as wild-type in chicks when able to colonize.

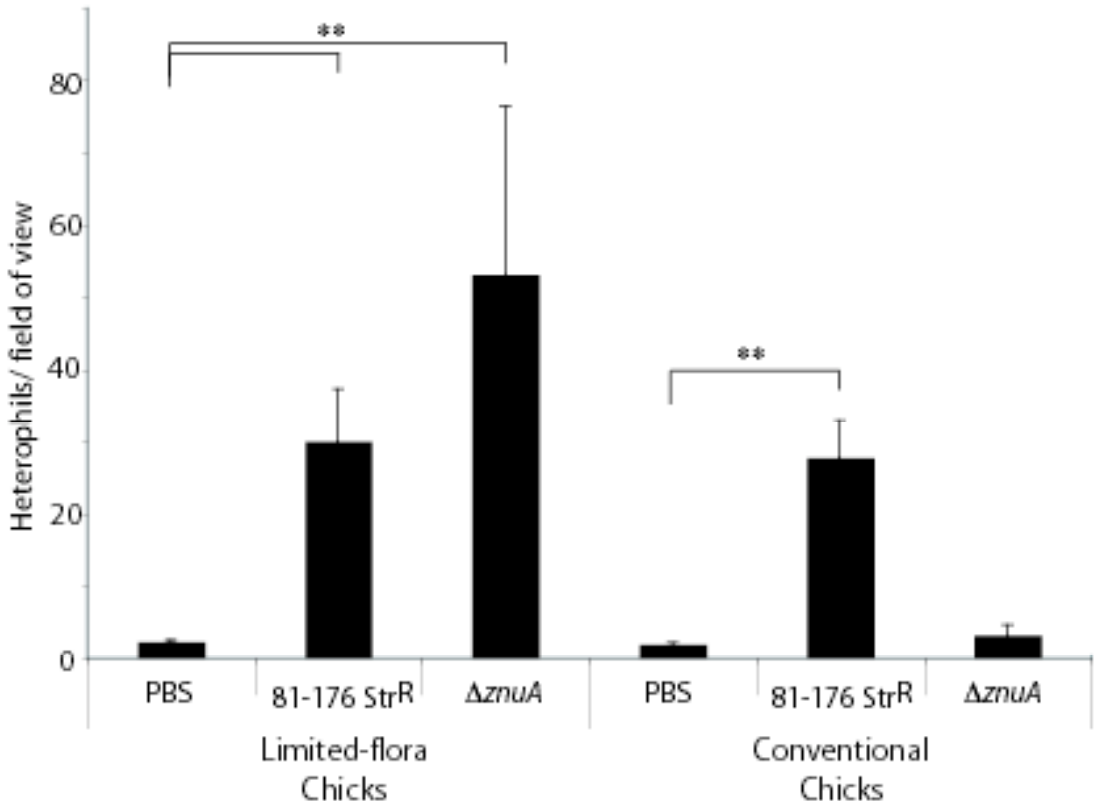

Figure 39. Enumeration of Heterophils in Limited-flora Chick Ceca. Limited-flora chicks and conventionally raised chicks were orally inoculated with either PBS, wild-type *C. jejuni*, or *C. jejuni* znuA. After 7 days, the number of heterophils per field of view was examined.

Conventionalization of *C. jejuni ΔznuA* Mutant-Colonizing Limited-Flora Chicks Excludes *C. jejuni* Colonization

To ascertain whether the microbiota are competing with the Δ*znuA* mutant, limited-flora chicks previously colonized with the Δ*znuA* mutant were conventionalized with age-matched conventional chick cecal contents. The Δ*znuA* mutant strain was introduced by oral inoculation into limited-flora day-of-hatch birds. On day three post-inoculation, birds were colonized with the Δ*znuA* mutant at 10^8 cfu g^{-1} cecal contents (Fig. 40). Half of the birds on day three post-inoculation were conventionalized with cecal contents from age-matched conventional chicks, while half were not. On day seven post-inoculation, the birds that were not conventionalized were colonized with Δ*znuA* at 10^8 cfu g^{-1} cecal contents. There was a significant reduction in recovery of the Δ*znuA* mutant in the conventionalized chicks compared to the un-conventionalized on day seven (median 1.7 x10^7 cfu g^{-1} cecal contents) and thirteen (median 6.1 x10^6 cfu g^{-1} cecal contents) post-inoculation (Fig. 40). This exclusion was specific for the Δ*znuA* mutant and not wild-type *C. jejuni* (Fig. 40). This suggests that the microbiota in the conventional chick ceca have a direct antagonistic effect on the Δ*znuA* mutant colonization, presumably due to zinc competition.

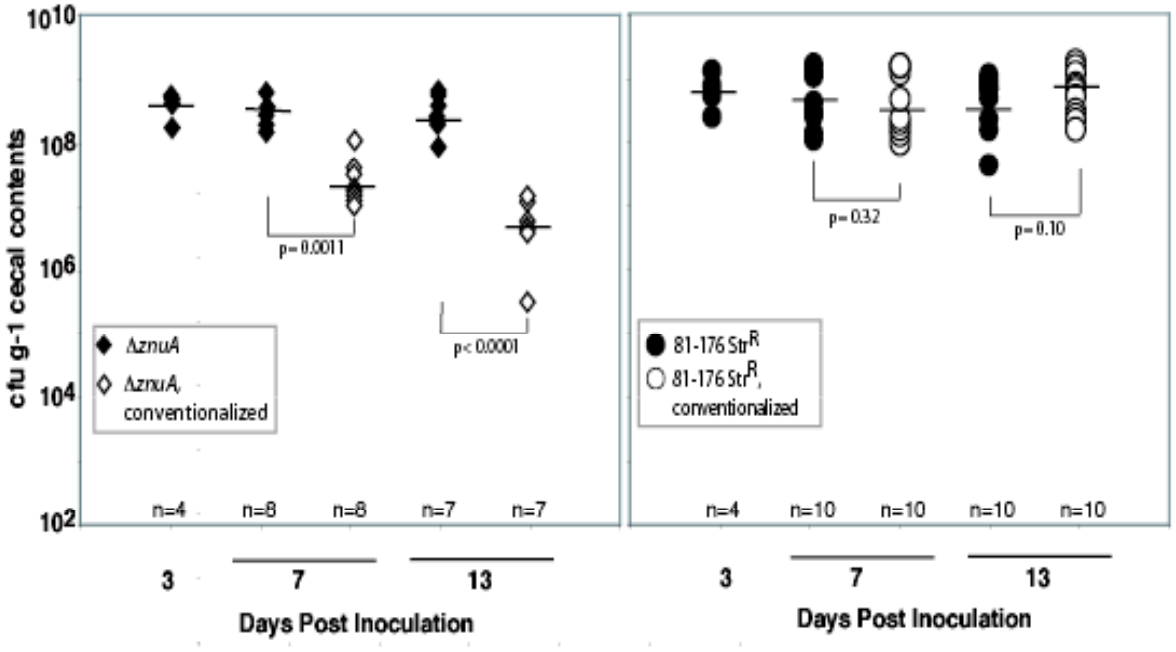

Figure 40. Conventionalization of *C. jejuni* –Colonizing Limited-Flora Chicks. Conventionalization of Δ*znuA*-colonizing or wild-type-colonizing limited-flora chicks. Day-of-hatch limited-flora chicks were inoculated with 10^6 cfu of either wild-type *C. jejuni* 81-176 Str^R or the Δ*znuA* mutant. Cecal contents were plated on day 3 post-inoculated. On day 3, birds colonized with wild-type *C. jejuni* or the Δ*znuA* mutant were conventionalized with cecal contents of age-matched conventional chicks (open circles or open diamonds), or not (black circles or black diamonds). Recovery of either wild-type or the Δ*znuA* mutant from the cecal contents was monitored on days 7 and 13 post-inoculation.

The Cecal Microbiota Influence Available Zinc in Cecal Contents and Subsequent Growth of *C. jejuni ΔznuA* Mutant

To test if the effect of *ΔznuA* exclusion in conventional chicks correlated to zinc levels, we analyzed the amount of total zinc in the cecal contents of conventional and limited-flora chicks by inductively coupled-plasma high resolution mass spectrometry (ICP-HRMS). There was significantly less zinc in the conventional cecal contents of seven day-old chicks compared to age-matched limited-flora chicks (Fig 41). There was also less iron and magnesium in the conventional chick cecal contents compared to the limited-flora chicks. However, the opposite trend was observed for copper and manganese ions (Fig 41).

We hypothesized that the *ΔznuA* mutant was unable to colonize conventional birds due to an inability to replicate in the zinc-limiting cecal contents. Chicken cecal contents from both limited-flora and conventional birds were isolated, diluted to 10% weight/ volume in PBS, and filter-sterilized to make a liquid medium termed chicken cecal media (CCM). Chicken cecal media prepared from both conventional and limited-flora birds were inoculated with either wild-type, the *ΔznuA* mutant, or *ΔznuA* complemented with a plasmid-encoded *znuA* gene (p*znuA*) and incubated for 24-hours. The *ΔznuA* mutant was attenuated for growth in the conventional CCM, but not in the limited-flora CCM. Attenuation was alleviated by adding 20μM $ZnSO_4$ to the conventional CCM (Fig 42). These results suggest that the *ΔznuA* mutant was unable to replicate in the conventional chick cecal contents due to a lack of available zinc, which is dependent on the presence of cecal microbiota.

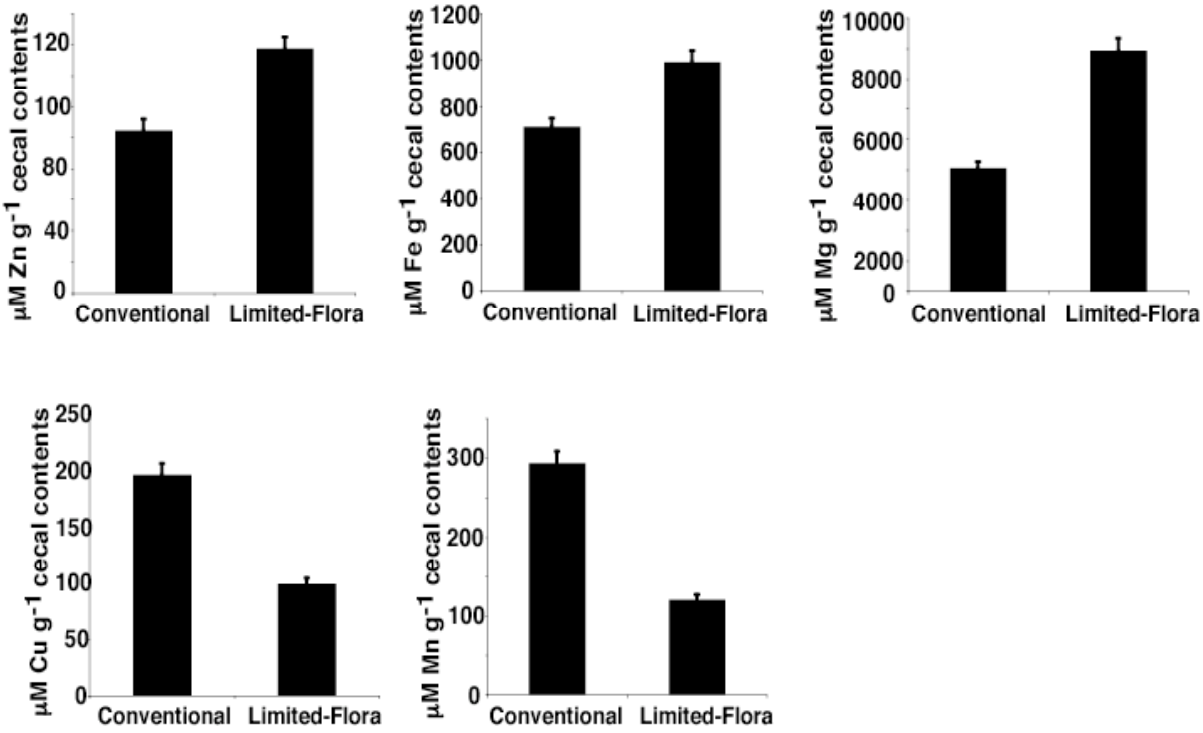

Figure 41. ICP-HRMS of Chick Cecal Contents. ICP-HRMS analysis of zinc, iron, magnesium, copper, and manganese atoms in conventional and germ-free cecal contents.

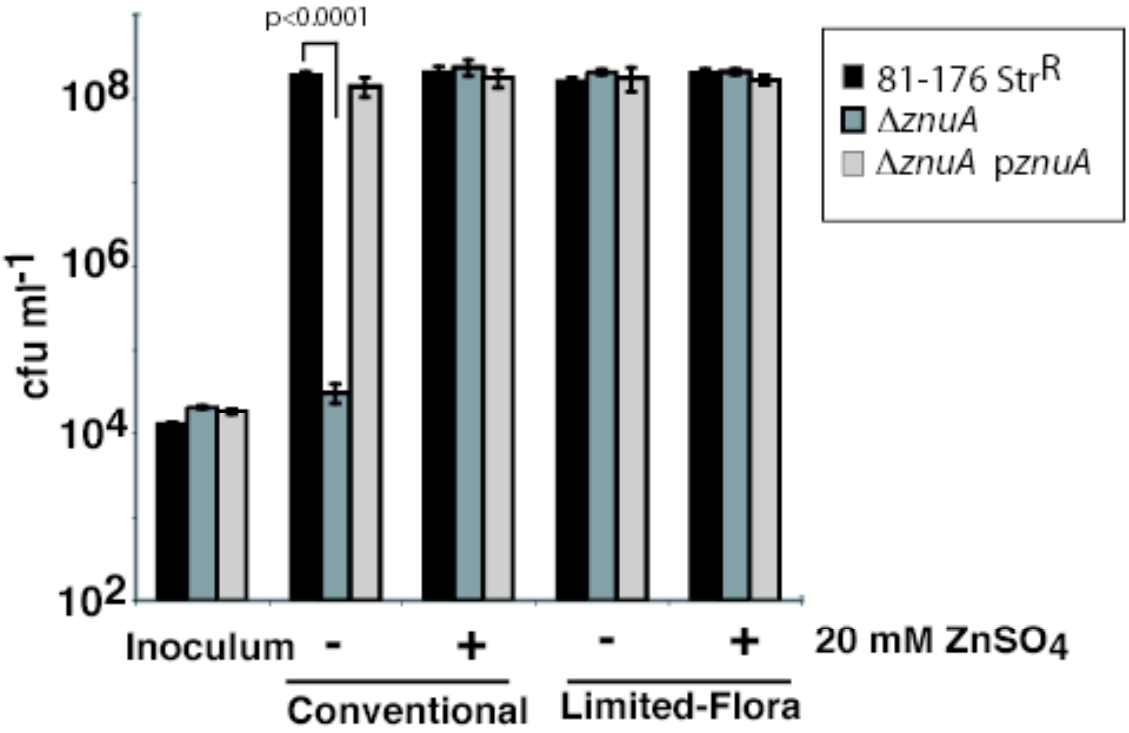

Figure 42. Growth of *C. jejuni* in Chicken Cecal Media. 81-176Str^R [24], the Δ*znuA* mutant (grey), and Δ*znuA* p*znuA* (light grey) were grown in conventional CCM or limited-flora CCM statically with and without 20μM $ZnSO_4$ for 24 hours. The cultures were diluted and plated for *C. jejuni* recovery and growth was reported as cfu ml^{-1}. Each culture was done in triplicate and shown is a representative of three separate trials.

Zinc-Binding Proteins in the Chick Cecal Contents Inhibit Growth of the *ΔznuA* Mutant

Zinc binding proteins in the cecal contents of conventional birds were isolated by immobilized metal chromatography in order to discern whether a specific component of the cecal milieu was responsible for the Δ*znuA* growth inhibition.

Growth of wild-type *C. jejuni* and the Δ*znuA* mutant was examined in the presence of the zinc-binding protein eluate in MH broth. After 24 hours, the growth of Δ*znuA* was attenuated in the presence of 5% of the cecal content zinc binding protein eluate (E1), but not in the presence of the eluate buffer (BE1). These results are similar to the attenuation observed in conventional cecal media (CCM) (Fig 43). This suggests that the zinc binding proteins in the cecal contents of chicks have an inhibitory effect on the Δ*znuA* mutant growth, even in rich media containing zinc. Zinc binding proteins isolated from the cecal contents of conventional chicks were identified by mass spectrometry (Table 5). The majority of the proteins isolated from the chick cecal contents identified in this screen were chick digestive proteins and are known to bind zinc.

Samples of the conventional CCM and limited-flora CCM were applied to SDS-PAGE gel electrophoresis for separation of proteins from the two media. Proteins present in the conventional CCM and not in the limited-flora CCM were isolated and sequenced by mass spectrometry (Table 5). Several of the proteins identified as zinc-binding proteins by immobilized metal chromatography described above in the chick cecum were also identified in this experiment. This demonstrates that the conventional CCM contains several zinc-binding proteins that the limited-flora CCM does not. Presumably, the

Table 4. Zinc Binding Protein Identification. Identification of proteins present in conventional CCM and not germ-free CCM (first column) and of zinc-binding proteins (from the first elution fraction) of the chick cecal contents isolated by immobilized metal chromatography. Proteins were separated by SDS-Page gel electrophoresis and sequenced by mass spectrometry.

Protein ID	Source	Conventional vs. Germ Free	Zinc Binding Proteins E1	Zinc Binding Protein?
Aminopeptidase Ey	*Gallus gallus*	+	+	Y
Ovalbumin	*Gallus gallus*		+	Y
Ovotransferrin	*Gallus gallus*		+	Y
Procarboxypeptidase A	*Gallus gallus*	+	+	Y
Superoxide Dismutase	*Gallus gallus*	+		Y
Transthyretin chain	*Gallus gallus*	+	+	Y
Trypsin	*Gallus gallus*	+		
Glutamate dehydrogenase NADP	*Clostridium phytofermentans*	+		

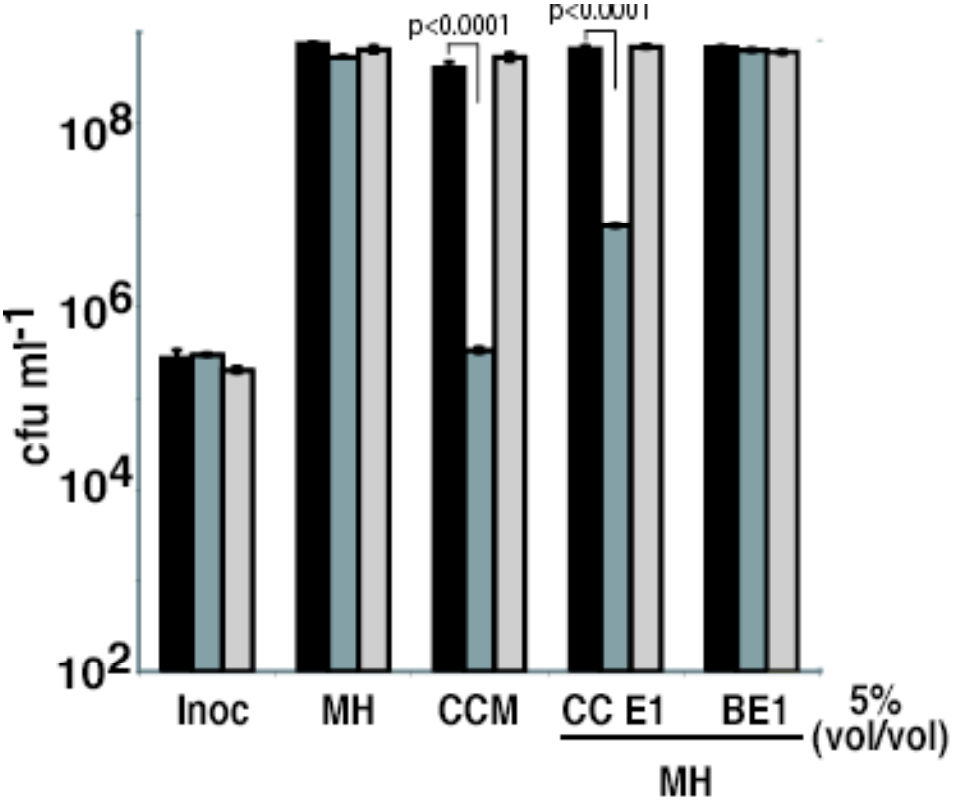

Figure 43. *C. jejuni* Growth with Zinc-Binding Proteins from Conventional Chick Cecal Contents. Growth of 81-176Str^R [24], the Δ*znuA* mutant (grey) and Δ*znuA* p*znuA* (light grey) after 24 hours in MH broth containing 5% of the first elution fraction (E1) of conventional cecal contents run on a zinc-binding column or 5% of the elution buffer (BE1). The cultures were diluted and plated for *C. jejuni* recovery and growth was reported as cfu ml^{-1}. Each culture was done in triplicate and shown is a representative of three separate trials.

presence of these zinc-binding proteins inhibits growth of the Δ*znuA* mutant in the conventional CCM and not the limited-flora CCM.

We hypothesized that the ΔznuA mutant growth inhibition in the presence of zinc-binding proteins was dependent on zinc concentrations. To test this, zinc was added to the cultures. Growth of the Δ*znuA* mutant in the presence of 5% zinc-binding proteins was restored to wild-type levels with the addition of 1mM $ZnSO_4$ (Fig 44). These results suggest that the presence of the zinc binding proteins inhibits the growth of the Δ*znuA* mutant by sequestering the available zinc away, which can be overcome with the addition of zinc to the cultures.

Heat-treatment Alleviates Growth Inhibition

To further demonstrate the inhibitory effects of the zinc-binding proteins in the chick ceca on the Δ*znuA* mutant growth, the conventional CCM and the zinc-binding protein E1 fraction were boiled for 30 minutes to denature the proteins. Growth of the Δ*znuA* mutant in the heat-treated conventional CCM and in MH containing 5% of the heat-treated E1 fraction was restored to wild-type levels, in opposition to the non-heat-treated media (Fig 45). The heat treatment presumably denatures the proteins in these fractions, releasing zinc into the media and alleviating the Δ*znuA* mutant growth attenuation. Growth of all three strains (wild-type, Δ*znuA*, and Δ*znuA* p*znuA)* was reduced in the heat-treated conventional CCM compared to non-treated, probably due to denaturation of key nutrients (Fig 45).

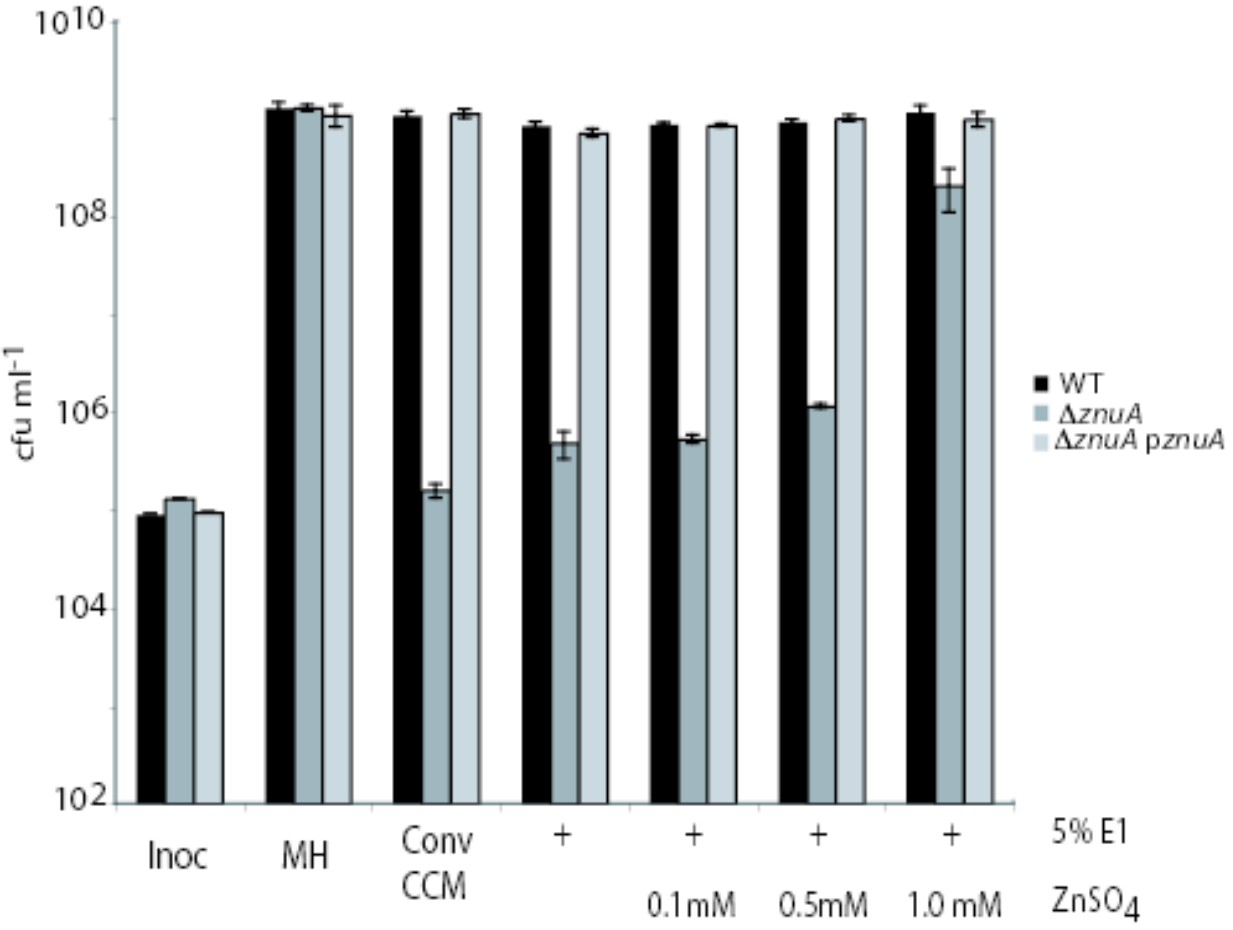

Figure 44. Growth of *C. jejuni* with Zinc-Binding Proteins and $ZnSO_4$. Growth of 81-176Str^R [24], the Δ*znuA* mutant (grey) and Δ*znuA* p*znuA* (light grey) in MH containing 5% E1 and increasing amounts of $ZnSO_4$. The cultures were diluted and plated for *C. jejuni* recovery and growth was reported as cfu ml^{-1}.

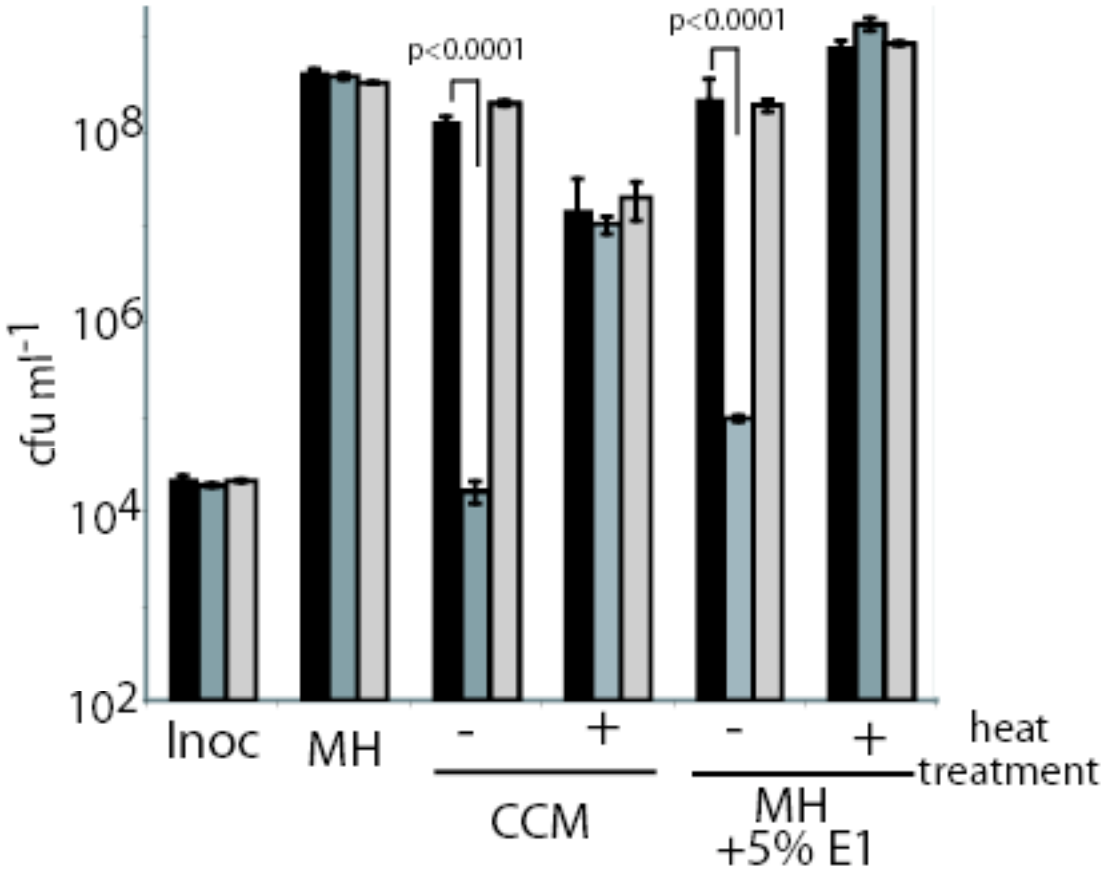

Figure 45. Heat Treatment of Zinc-Binding Proteins . Growth of 81-176Str^{R} [24], the Δ*znuA* mutant (grey) and Δ*znuA* p*znuA* (light grey) after 24 hours in conventional CCM that has been heat treated or in MH containing heat-treated E1-zinc binding proteins. The cultures were diluted and plated for *C. jejuni* recovery and growth was reported as cfu ml^{-1}.

Growth Inhibition of the Δ*znuA* Mutant in the Presence of Ovotransferrin

Ovotransferrin, identified as a zinc-binding protein in the conventional chick cecal contents, could be an important factor inhibiting the growth of the znuA mutant in the cecal contents. Ovotransferrin is known to bind divalent cations such as iron and zinc, and has been seen to inhibit the growth of several bacterial species through metal sequestration [30, 31]. We tested the ability of ovotransferrin to inhibit growth of *C. jejuni.* Growth of wild-type *C. jejuni* was significantly inhibited with the addition of 5mg ovotransferrin to MH broth, while growth of the Δ*znuA* mutant was inhibited with the addition of 10μg ovotransferrin (Fig 46). Whether ovotransferrin is a specific factor inhibiting growth of the Δ*znuA* mutant in the chicken cecal contents is unknown. It would be interesting to test whether zinc rescues the growth phenotype of the *znuA* mutant in the presence of ovotransferrin. Further, depletion of ovotransferrin from the conventional chicken cecal contents would elucidate whether it is the single factor inhibiting the growth of the Δ*znuA* mutant or whether it is combination of multiple zinc-binding proteins.

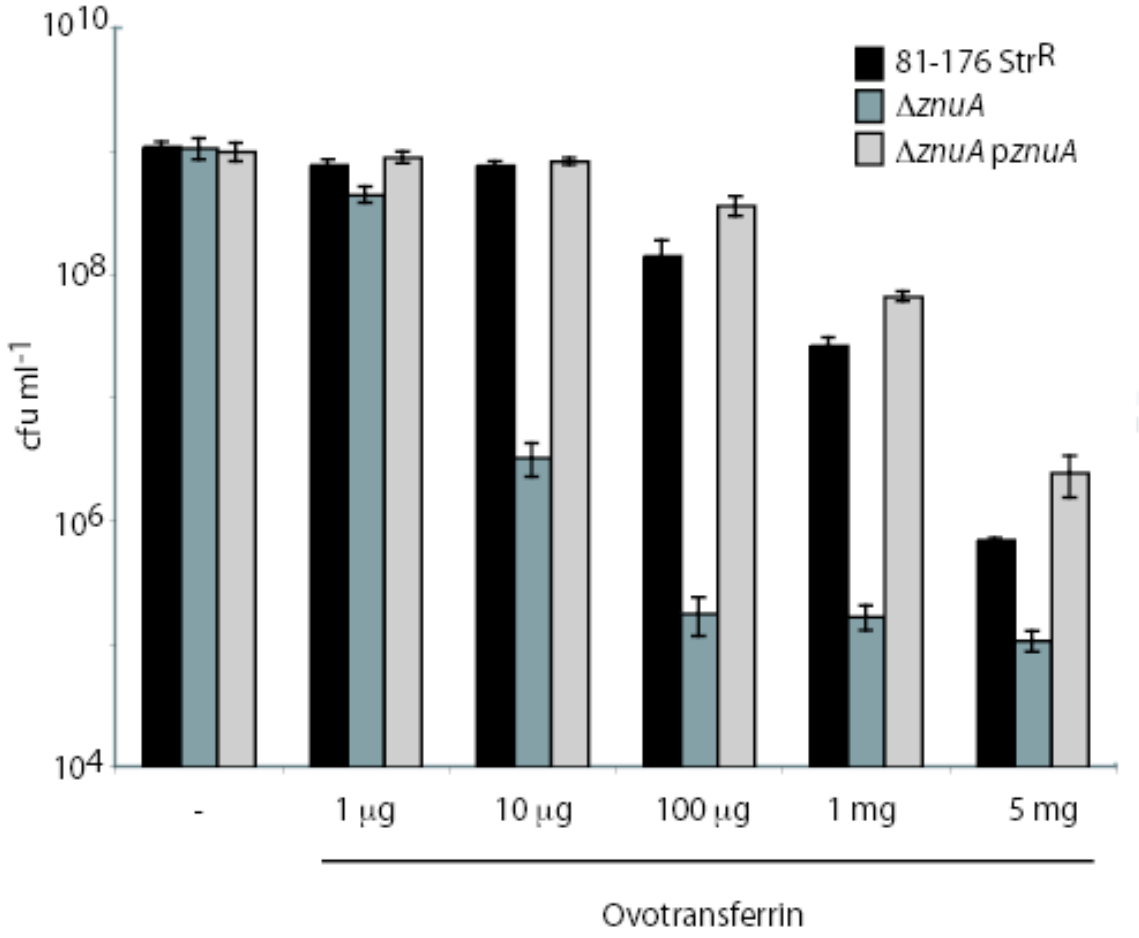

Figure 46. Growth in the Presence of Ovotransferrin. Growth of 81-176Str^{R} [24], the Δ*znuA* mutant (grey) and Δ*znuA* p*znuA* (light grey) after 24 hours in MH broth containing various quantities of ovotransferrin. The cultures were diluted and plated for *C. jejuni* recovery and growth was reported as cfu ml^{-1}.

Discussion

Indigenous microbiota compete with introduced bacteria in the gastrointestinal tract for space and nutrients. Antagonistic effects can be direct or indirect. Direct antagonism occurs by production of inhibitory metabolites or through competition for nutrients or adhesion sites. Indirect antagonism is a result of alterations of the physiological response of the host by the indigenous microbiota that in turn influence such aspects of gastrointestinal functions as immune-modulation or stimulation of peristalsis.

Conventional animals require more dietary zinc compared to their germ-free counterparts, presumably due to the indigenous microbiota using and storing the zinc atoms {Smith 1971}. However, experiments to test this phenomenon have never been carried out. Further, research into the competition between host and microbiota, microbiota and pathogen, and microbe versus microbe for the available dietary zinc has not been reported. Our work demonstrates zinc competition between the microbiota and *C. jejuni* in the chick cecum. A mutant lacking the high affinity zinc ABC transporter zinc-binding protein (Δ*znuA*) in *C. jejuni* is unable to compete for available zinc, does not replicate, and is cleared from the chick cecum. In the absence of the microbiota, the Δ*znuA* mutant is able to replicate and colonize the chick cecum. It is uncertain whether the effect of zinc competition in the chick cecum is a direct or indirect antagonism.

There were significant differences between the composition of the chick cecal contents in terms of host digestive proteins recovered, suggesting an indirect effect. Lower quantities of trypsin, aminopeptidase Ey, and precarboxypeptidase A in germ free

chicks suggests that there are metabolic and/or digestive changes in the limited flora chicks compared to the conventional chicks (Table 5). Indeed, lower levels of transthyretin (also known as pre-albumin) in germ-free chicks indicate an altered metabolic function compared to conventional animals, a common occurrence in germ-free animals. This alteration in host metabolism could be responsible for the zinc-limitation, as several of these host digestive proteins bind zinc. The decrease in zinc-binding digestive proteins, or a decreased rate of metabolism or cell proliferation in the intestines (functions that require the use of zinc), may explain the increased level of zinc in the limited-flora chicks. The role of the microbiota in influencing host immunity is well recognized. [25, 26] However the effects of the microbiota on digestive functions of the host are poorly understood.

Intestinal bacteria have been implicated in the degradation of secreted digestive enzymes including trypsin, chymotrypsin, glycoside hydrolases and aminopeptidase N [27, 28] [29]. In a recent study, conventionalization of neonatal gnotobiotic pigs resulted in higher aminopeptidase N expression compared to germ-free pigs [29]. However, enzymatic activity was reduced in conventionalized and *E. coli* infected pigs, suggesting protein destabilization by the resident bacteria, which was supported by *E. coli* degradation of purified aminopeptidase *in vitro* [29]. Our work demonstrated an abundance of aminopeptidase Ey protein in conventional chicks compared to limited-flora chicks, correlating with the increase of aminopeptidase N expression in the aforementioned study [29]. Although deactivation of the protein was not observed in conventionalized chicks, differences in the microbiota composition between chicks and pigs could explain observation, as *Lactobacillus fermentum* and *Klebsiella pneumoniae*

do not deactivate aminopeptidase N activity as well as *E. coli* [29]. These studies demonstrate that host metabolism may be altered based on bacterial enzymatic activities and composition of the residing microbiota.

Lower quantities of trypsin, aminopeptidase Ey, and precarboxypeptidase A in limited-flora chicks suggest that there are metabolic changes dependent on the presence of a microbiota (Table 5). This alteration in host metabolism could explain the higher level of zinc in limited-flora cecal contents, as several of the digestive proteins identified in the conventional cecal contents bind zinc. The decrease in zinc-binding proteins, or a decreased rate of metabolism or cell proliferation in the intestines (functions that require the use of zinc), would cause an increase in available zinc. However, the effects of the microbiota on digestive functions of the host are poorly understood. The presence of ovotransferrin in the chicken cecal contents, a protein found in high abundance in chicken egg whites capable of binding ions such as iron and zinc, could be depriving *C. jejuni* Δ*znuA* of zinc necessary for growth. Ovotransferrin has been shown to have antibacterial effects against *Psuedomonas, E. coli, Streptococcus mutans*, *Staphylococcus aureus, Bacillus cereus*, and *Salmonella enteritids* [30, 31]. Research into whether growth inhibition of the Δ*znuA C. jejuni* mutant in the chick cecal contents is due to the presence of a specific microbiota-dependent zinc-binding protein, competition with the dense microbiota population, or due to alterations in the host immune or metabolic functions, is currently underway.

Observations have been made on the effects of the microbiota on calcium and phosphorous metabolism. Germ-free rats have significantly higher excretion of calcium and phosphorous and greater absorption in the gut compared to conventional rats [32].

Calcium, phosphorous, and magnesium concentrations of the femur in germfree rats are higher than conventional rats [33]. These data demonstrate that the microbiota of rats decrease the amount of available calcium, phosphorous, and magnesium for the host, either through bacterial use and sequestration or changes in host metabolism due to bacterial activities. Similarly, we have demonstrated here that the magnesium content of the cecal contents is higher in germfree chicks compared to conventional chicks. Similar results were seen for zinc and iron, while opposing outcomes were observed for manganese and copper. In a study on germ-free rats, there was significantly more absorption of iron and copper compared to conventional rats, while manganese levels were the same between the two groups [34]. The differences observed in copper and manganese levels between the limited-flora chicks in our study and germ-free rats are unknown. However it could simply reflect differences in the species or diets.

Although research has characterized the many ways in which different organisms, from mammals to bacteria, use and require zinc, there is limited research on how these different organisms compete for the available zinc. Zinc homeostasis in all cells must be tightly regulated and several zinc-responsive transcription factors and transporters mediate homeostasis [6-8, 16]. The metallothionein family of proteins binds and sequesters zinc, primarily in higher eukaryotic systems. The metallothionein SmtA has been well studied in *Synechococcus* and has also been identified in a number of bacteria including *Synechococcus* spp, *Pseudomonas* spp, *Anabaena* spp, and *E. coli* [35][36]. SmtA binds zinc in elevated zinc environments, sequestering the zinc ions. Deletion of *smtA* in *Synechococcus* leads to decreased zinc tolerance [37].

A metallothionein that binds and sequesters zinc in low zinc environments, similar to the way that iron siderophores may function, has not been identified. However, bacterial species have evolved to survive within zinc-depleted environments, through the use of zinc-specific regulators and transporters, such as Zur and ZnuABC respectively. Additionally, ribosomal subunits in *Bacillus, Streptococcus*, *and E. coli* spp. are regulated based on the availability of zinc [38]. Under zinc-replete conditions, ribosomal proteins contain functional zinc binding sites. Under zinc-limiting conditions, transcription of ribosomal proteins that do not bind zinc proceeds as a result of direct Zur regulation. These non-zinc-binding proteins replace the zinc-binding ribosomal proteins. The zinc from the original zinc-binding ribosomal proteins is released into the cytoplasm and is incorporated into other zinc-requiring proteins [38]. In this manner, the ribosomal proteins serve as a storage mechanism for zinc under normal conditions, until the zinc is needed for other proteins under zinc-limiting conditions.

The presence of these intracellular zinc-storage and regulatory mechanisms in bacteria may explain the need for a functional high affinity zinc uptake system in *C. jejuni* for colonization of the chick cecum. The intestinal tract of higher eukaryotes is thought to contain 10^{12} bacteria per gram of contents. The amount of zinc readily available must be affected by the sheer number of bacteria using and storing available zinc, which we have shown to be in lower concentrations than other elements examined in the chick cecal contents regardless of the presence of a microbiota. This may explain why several bacterial species have been shown to require a functional high affinity ZnuABC transport system for colonization and/or virulence, including *C. jejuni, E. coli,*

Salmonella, *Neisseria, Pasteurella*, *Haemophilus*, and *Bacillus* species [15, 16, 18, 39, 40].

Not only does zinc sequestration and storage occur by the microbiota, but by the host as well. Zinc is an essential trace element, and higher eukaryotic cells have similar mechanisms for intracellular zinc storage as bacteria. Further, the presence of the microbiota affects metabolic functions and subsequent zinc utilization. Future research is needed to understand how the metabolic changes of the host dependent, on the presence of a microbiota, affect the amount of zinc available for not only the microbiota, but incoming pathogens as well.

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CHAPTER VI

Conclusions and Future Directions

Conclusions

The work presented here is focused on factors that contribute to *C. jejuni* colonization of the chick cecum. Due to the lack of a good small animal virulence model for *C. jejuni*, chicken colonization is routinely used to study *C. jejuni* interactions with a natural host. Although many factors are known to contribute to *C. jejuni* colonization of the chick cecum, very little is known about the model itself, including what host factors contribute to establishment of *C. jejuni* colonization. The broad aim of this dissertation was to discern how *C. jejuni* establishes a benign colonization and persists in chickens. To answer such a broad question, the specific aims of the project were focused on three aspects of bacterial-host interactions; (1) characterization of the host innate immune response to early events of *C. jejuni* colonization, (2) characterization of a *Campylobacter jejuni* colonization determinant, a high-affinity zinc ABC transporter, and (3) the role of the microbiota in *C. jejuni* colonization, specifically in competition for zinc in the chick cecum.

Although *C. jejuni* is commonly found colonizing the gastrointestinal tract of broiler chickens asymptomatically, previous studies detected the presence of *C. jejuni*-specific immunoglobulins in the sera of *C. jejuni*-colonizing chickens. The production of

protective systemic antibodies against *C. jejuni* is not indicative of a commensal relationship, where the systemic immune system is normally kept ignorant of its colonizing microbiota. Further, *C. jejuni* can invade a number of chicken cell types *in vitro*, and has been recovered from systemic sites of the chicken, indicating that *C. jejuni* may invade the chicken gut epithelium *in vivo*. Based on these previous studies, we hypothesized that *C. jejuni* may interact with the chicken epithelium and locally invade the tissue.

In Chapter III, we examined the early colonization events of *C. jejuni* in the chick cecum. Wild-type *C. jejuni* 81-176 Str^R induced a modest inflammatory response early in infection (days 4 to 7 post-inoculation), with marked heterophil recruitment and pro-inflammatory cytokine expression as determined by qRT-PCR (Fig 47). Different strains *of C. jejuni*, both human and chicken isolates, induced variable levels of inflammation. The modest level of inflammation was thought to be induced by *C. jejuni* invasion of the tissue. A *C. jejuni* mutant lacking *virB11*, while able to colonize the chick ceca, did not invade the tissue and did not stimulate heterophil recruitment or significant expression of pro-inflammatory cytokines compared to wild-type *C. jejuni* (Fig 47). Invasion of the tissue was confirmed by microscopy techniques. Not only was local invasion of the tissue was observed, systemic spread of *C. jejuni* to the spleen and liver was confirmed in our model, as well as production of systemic antibodies against specific *C. jejuni* antigens (Appendix D).

As described in Chapter III, we investigated *C. jejuni* factors required for chick colonization and found that *virB11* contributes to colonization and interaction with the host. The gene *cj0143c* was previously found to be essential for chick colonization

(Chapter IV). We characterized *cj0143c* as encoding a high-affinity zinc substrate binding protein for an ABC transporter, with homology to *znuA* in other bacterial systems. In *Campylobacter*, ZnuA is localized to the periplasm and is modified by the *N*-glycosylation system. However, the function of the glycosylation of *C. jejuni* ZnuA is unknown, as abolishing ZnuA glycosylation by mutation of the conserved glycan binding site does not affect the protein's localization, binding to zinc, function in low-zinc media, or colonization of the chick.

Similar to iron, the intestinal milieu is thought to be zinc-limiting, and the inability of a *C. jejuni ΔznuA* mutant to colonize the chick cecum supported this hypothesis. Zinc is an essential trace element, required as a cofactor for hundreds of metalloproteins and enzymes in both eukaryotic and prokaryotic cells. The concentration of intracellular zinc must be strictly controlled, as too little or too much zinc is toxic to cells. The quantity of zinc in the intestinal tract is zinc limiting, and as described in Chapter V, is dependent on the microbiota.

Intestinal microbiota are known to influence bacterial-host interactions in many ways, including priming the host immune system and digestive functions and competing with introduced bacteria for space and nutrients. Using chicks raised in germ-free conditions , we showed that the presence of a microbiota reduces the quantity of a number of host digestive proteins, many of which bind zinc. These proteins include ovotransferrin, ovalbumin, trypsin and transthyretin. The presence of these zinc-binding proteins inhibits the growth of the *ΔznuA* mutant *in vitro*. We suggest that the presence of these zinc-binding proteins in conventionally raised chicks reduce the amount of free zinc in the cecal contents, thereby inhibiting the growth of the *C. jejuni ΔznuA* mutant.

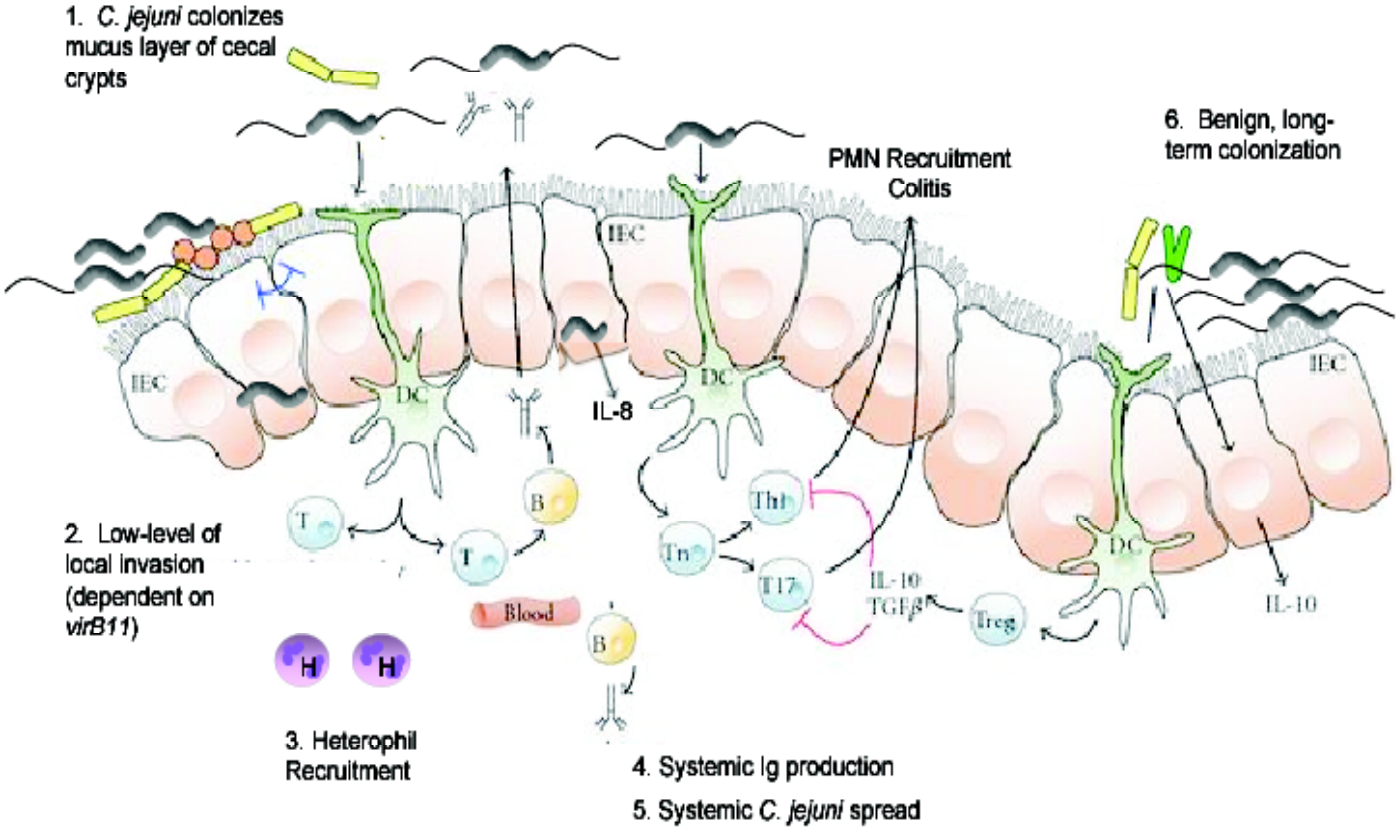

Figure 47. Model of *C. jejuni* Colonization of the Chick Cecum. A model of the events leading up to *C. jejuni* (grey bacteria) asymptomatic colonization in the chick cecum (epithelial layer shown) was constructed based on the work presented in this thesis. Upon entry to the chick cecum, *C. jejuni* locally invades the tissue (*virB11* dependent), which causes the recruitment of heterophils. The chick develops systemic antibodies against *C. jejuni* antigens, and it is unknown whether it is against the asymptomatic colonizing population of *C. jejuni* or against *C. jejuni* found in systemic sites. The role of sIgA production and systemic Ig production in *C. jejuni* colonization is currently unknown and the focus of future studies.

Alternatively, the sheer number of bacteria in conventionally raised chicks could be using and storing zinc, making the chick cecal milieu zinc-limiting.

The work presented here highlights a number of unique traits of *Campylobacter* colonization. First, we have demonstrated that a metabolic factor, zinc, is a colonization trait and putative virulence trait. An inability to regulate the intracellular concentration of zinc in *C. jejuni* results in an inability to replicate and colonize chickens. Bacterial survival and transmission in hosts depends on its ability to adapt to the changing environments. For pathogenic bacteria, adaptation to the new host environment often includes regulation of virulence genes, and therefore studies of virulence traits often focuses on these factors, such as motility, adhesions, or toxins, and regulation thereof. However, it is arguable that the ability to replicate and acquire nutrients necessary for survival within a new host environment could be considered virulence traits. Similar to the necessity of iron transporters for *Yersinia pestis* [1] and *Staphylococcus aureus* [2, 3] to cause disease, we have demonstrated that the acquisition of zinc within a host can be construed as a colonization trait and putative virulence factor in *Campylobacter jejuni.*

In addition, the work presented here reveals several novel characteristics of the microbiota-host relationship, opening the door for future studies aimed at understanding symbiotic relationships. While the chick provides an environment and nutrients for the bacterium, the role of *C. jejuni* on host has only begun to be explored. *C. jejuni* is found readily in the gastrointestinal tracts of chickens in broiler flocks. However, there are no observable effects in chicks that lack recoverable *C. jejuni*, leading the scientific community to classify *C. jejuni* as a commensal organism of the chicken gastrointestinal tract.

It can be argued that *C. jejuni* colonization has several beneficial effects for the chick based on the work presented here. In Chapter V, we demonstrated that the microbiota of the chick stimulates the production of digestive enzymes and while *C. jejuni* was not present in the flora of the gastrointestinal tract in these experiments, as part of the normal flora of chickens we can assume that *C. jejuni* may have a similar effect on the host.

Additionally, we demonstrated that *C. jejuni* stimulates a modest inflammatory response in the cecal tissue in Chapter III. While the host response does not result in symptomatic inflammation or *C. jejuni* clearance, this modest inflammatory response could aid in the development of the host immune response. Stimulation of the innate immune system by *C. jejuni*, which does not seem to affect the health of the chick, could aide in the response to pathogens. The role of the microbiota on the development of the immune system is widely studied. The indigenous intestinal microbiota intimately interact with the host defense machinery and is necessary for the development of the immune system balance; stimulation in the presence of a pathogen and tolerant immune readiness in the face of the microbiota (as reviewed in Chapter I) [4, 5]. While the indigenous microbiota typically stimulate an anti-inflammatory response of the host, mediated primarily by the cytokine IL-10 (Fig. 47), *C. jejuni* stimulated what seemed to be a controlled inflammatory response. While the mechanism behind this controlled response is still poorly understood, including mechanisms of recognition and events leading to a non-inflammatory state, future experiments will aim at understanding this dynamic relationship and resulting effects on the host.

Future Directions

This work has provided a comprehensive view of the initial stages of *C. jejuni* colonization of the chick cecum, but unanswered questions remain. One broad question is the applicability of these studies to natural *C. jejuni* chick colonization conditions. While several of the experiments in Chapter III were aimed at recapitulating natural conditions, we cannot be certain that the data presented here occur in natural broiler flocks. However, future directions of the specific aims of this work are discussed below.

A number of host zinc-binding proteins were identified that are present in higher quantities in conventional chicks compared to limited-flora chicks that could be affecting the level of available zinc in the cecal contents. We would like to explore whether a specific protein is lowering the amount of available zinc, much like a siderophore. To date, there is no known specific zinc-sequestration protein. One possible candidate for this action would be ovotransferrin, which was identified as a zinc-binding protein in chicken cecal contents. Ovotransferrin is known to bind and sequester iron in developing eggs, and inhibits growth of several bacterial species through this sequestration action. Ovotransferrin binds other divalent cations, including zinc. Does ovotransferrin inhibit *znuA* mutant zinc-dependent growth *in vitro*? Ovotransferrin is present in high quantities in eggs and in the gastrointestinal tract of young chicks, but is it present in older chicks? Could the *znuA* mutant colonize older chicks in the absence of ovotransferrin?

Although it is tempting to speculate that zinc is limiting because there exists a single factor that is binding and storing zinc, it is most likely a combination of several proteins, or even a consequence of a large number of organisms using zinc. Are there bacterial or host zinc- binding proteins that could be sequestering and storing zinc? Does

the size of the microbiota community affect the amount of zinc available? Do specific bacterial species of the microbiota require more zinc than others?

The necessity of zinc for *C. jejuni* replication and colonization is clear. Knowing the necessity for zinc for bacterial survival, we could design a small molecule screen to identify factors that inhibit ZnuA function in the chick gastrointestinal tract. Inhibition of ZnuA would lead to attenuation of *C. jejuni* growth and colonization similar to the conventionalization experiment in Chapter V where the microbiota out-competed a *znuA* mutant in limited flora chicks. Further, research into the composition of the microbiota could lead to identifying key populations that could out-compete *C. jejuni* colonization thru competition for key nutrients.

Further questions that arise from these studies are what roles the microbiota play on *C. jejuni* colonization. It would be interesting to investigate whether the composition of the microbiota is altered in the presence or absence of *C. jejuni*. How the microbiota changes over time in chicks, and whether *C. jejuni* colonization changes as well, over the life of chicken, would give insight into possible measures for *C. jejuni* control. We identified one specific factor (*znuA*) that is necessary for *C. jejuni* to compete with the developing microbiota in chicks. However, countless other *C. jejuni* factors, including adhesions or nutrient transporters, may be necessary for this competition with other microbiota in chick cecum.

The role the microbiota play on host metabolic processes is relatively unknown. The microbiota is known to influence host immune development, the host response to bacterial pathogens, and nutrient acquisition, as the microbiota supplies essential nutrients such as vitamin K, and breakdown food sources for efficient use by the host.

However, very little is known about whether the microbiota influence host metabolism; including the secretion of key digestive proteins, breakdown of food, and acquisition of nutrients. In Chapter V, several digestive proteins in the chick were in lower quantities in limited-flora chicks, suggesting a large impact on host metabolism, which opens up many questions. How does the composition or quantity of the microbiota influence host metabolism? Do members of the microbiota share or steal key nutrients from each other? Does the stimulation of host metabolic processes help or hinder other bacterial species colonization through alterations of the environment? Does *C. jejuni* stimulate the expression of similar digestive enzymes? Infection of limited-flora chicks by specific members of the microbiota of the ceca can be used to answer these questions.

ZnuA is necessary for colonization of the chick gastrointestinal tract, but there are many more basic questions about *znuA* in *C. jejuni.* While we demonstrated in Chapter IV that ZnuA is glycosylated by the *N*-glycosylation system, the purpose of this glycosylation is still unknown. Does glycosylation of proteins play more of a role in different environments, such as in water or soil? Does glycosylation of ZnuA affect its function in these environments or conditions? A broad question that remains unanswered is, why have specific proteins conserved the glycosylation moiety recognized by the pgl system?

The ZnuABC system is conserved in a number of bacterial species including *E. coli* and *B. subtilis*, and is regulated by the transcriptional regulator Zur in these systems. *C. jejuni* does not contain a Zur homologue. The upstream sequence of *znuA* does not contain a conserved Fur or Zur binding sequence. We demonstrated both transcriptional and translational regulation of *znuA* in Chapter IV. Future work on this system requires

characterization of the regulation of this system. We hypothesize that a flexible arm on *C. jejuni* ZnuA with a number of exposed histidines could bind zinc and regulate the stability of the protein. Regulation of protein stability based on available zinc would be advantageous for a bacterial system as it is more responsive than transcriptional regulation. Mutation of key residues and deletion of the flexible arm on *C. jejuni* ZnuA would highlight what role this region plays in the function of the protein. Further, *C. jejuni* PerR is a candidate for a transcriptional regulator of *znuA*, as it responds to peroxide stress and regulates iron-transport genes[6].

It is unquestionable that multiple factors influence the outcome of an infection in the gastrointestinal tract, including bacterial factors, the presence and composition of the microbiota and the host response. In Chapter III, we demonstrated that *C. jejuni* invades the chick cecal epithelium in a *virB11*-dependent manner, and stimulates a pro-inflammatory response characterized by heterophil infiltration of the cecal tissue and up-regulation of pro-inflammatory cytokines. However, these studies were performed in an un-natural environment and *C. jejuni* was given at relatively high doses. In efforts to recapitulate natural conditions, chicks were inoculated with a series of low-dose inoculations of *C. jejuni* for five consecutive days, as broiler chicks are constantly exposed and ingest *C. jejuni*. A more comprehensive study of the short and long term-effects of low-dose colonization, including *C. jejuni* invasion, host immune response and persistence, are needed to understand a more natural *C. jejuni* infection of chickens.

Continuing, the chicken cloaca undergoes reverse peristalsis, and it is speculated that *C. jejuni* may enter the chick gastrointestinal tract not by oral ingestion but rectally. It would be interesting to examine colonization dynamics of *C. jejuni* as well as a

transposon mutagenesis screen to identify bacterial factors that are necessary for chick colonization when infected rectally, and not orally. Bacterial stress factors and motility may not be as important for establishing colonization when fed rectally, as *C. jejuni* is not exposed to harsh stresses of the gastrointestinal tract when inoculated rectally. The day-of-hatch chick colonization model has a few drawbacks, including a bottleneck effect. Co-colonization experiments of two *C. jejuni* strains have not been successful due to this bottleneck, where only one strain readily colonizes the gastrointestinal tract and the second strain is lost. Rectal inoculation may remove this bottleneck.

Broiler chickens colonized by *C. jejuni* have been shown to have recoverable *C. jejuni* from systemic sites as well as systemic antibodies against *C. jejuni*. In Appendix C, chicks inoculated with *C. jejuni* in the day-of-hatch chick colonization model develop systemic antibodies against *C. jejuni.* We also recovered *C. jejuni* from systemic sites in Chapter III (Fig. 47). The production of systemic antibodies against *C. jejuni* colonization raises many questions. Are the antibodies produced against the colonizing *C. jejuni* population or the systemic- *C. jejuni* population (Fig. 47)? Are these antibodies protective against further *C. jejuni* colonization? Over time, would these antibodies clear *C. jejuni* colonization? It would be interesting to do a clearance infection, where after two or more weeks post-inoculation, *C. jejuni* is cleared by antibiotic treatment and re-challenged with the same strain and a different *C. jejuni* strain, to test whether antibodies produced during the first infection are protective against re-infection.

While we demonstrated that *C. jejuni* stimulates a modest inflammatory response in the chick cecal tissue, we know very little about how this response affects the host and incoming bacterial species, or the mechanism behind the resolution of the inflammatory

state. One hypothesis is that this stimulation helps to protect the chick against pathogens by priming the immune system to a readiness state. Research into the role of the microbiota and its influences on the development of the immune system suggests that the microbiota stimulates anti-inflammatory factors. However, in the chick, which does not have a developed adaptive immune system until two weeks post-hatch, stimulation and recruitment of innate immune effectors by friendly bacteria could aide in protection of the chicken against pathogenic bacteria. While we demonstrated the effects of *C. jejuni* and *S. typhimurium* co-infection in Chapter III, it would be interesting to perform serial infections of limited-flora chicks. For this, chicks initially infected with *C. jejuni* would be challenged with a pathogen or non-pathogen to test whether the immune response induced by *C. jejuni* aides in the clearance of the incoming bacteria. The use of probiotic bacteria in exclusion of pathogens is common, including in chicken flocks with commercially available products such as Broilact [7]. However, it is possible that the mechanism behind exclusion of pathogens by members of the microbiota may include stimulation of the host immune system.

In summary, the work presented here not only describes bacterial and host factors that contribute to *C. jejuni* colonization of the chick cecum, but it reveals dynamic relationships between bacteria, the host and the microbiota. The experiments described lay the foundation for future studies on the role of the microbiota on host digestive properties, competition for key nutrients, relationships between commensal and mutualistic bacteria and their host, as well as mechanisms of *C. jejuni* growth, replication and colonization of an important natural host, the chicken.

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Appendix A

Disruption of the *N*-glycosylation system inhibits colonization of Chick Ceca and Increases Flagellar Phase-Variation

Summary

The *N*-glycosylation system is required for chick colonization. A mutation in the *pglB* gene, a glycosyl-transferase, inhibits the transfer of assembled heptasaccharide to glycoproteins in *C. jejuni*. The Δ*pglB* mutant is unable to colonize chicks after seven days in the day-of-hatch chick colonization model. The work presented here demonstrates that the Δ*pglB* mutant is has a high rate of flagellar phase-variation, resulting in the down-regulation of flagellar genes. This decrease in flagellar assembly could explain the inability of the Δ*pglB* mutant to colonize chick ceca.

pglB is necessary for Persistent Colonization

The oligosaccaryltransferase, PglB, as well as other proteins in the *N*-linked protein glycosylation (pgl) pathway are essential for chick colonization as well as colonization of other hosts [1-3]. To examine this phenotype in more depth, chicks were inoculated with PBS, 10^4cfu wild-type *C. jejuni*, or 10^4 cfu of the Δ*pglB* mutant and colonization was examined on days one, four, seven and fourteen post-inoculation (Fig 48). The Δ*pglB* mutant was recovered from the ceca from 100% of the chicks on day one, but only two out of five chicks on day four. On days seven and fourteen, the Δ*pglB* mutant was not recoverable from the chick cecum, while wild-type was recovered at 10^8 cfu g^{-1} cecal contents. These results demonstrate that *pglB* is necessary for persistent colonization of the chick cecum.

Flagellar Genes are Down Regulated in a Δ*pglB* Mutant as Determined by Microarray Analysis

As the *N*-glycosylation system is thought to modify over forty proteins in *C. jejuni,* and a deletion in *pglB* may have pleiotropic effects on the cell, a microarray was performed on RNA extracted from wild-type *C. jejuni* and the Δ*pglB* mutant cells grown overnight on MH agar plates to understand what general effects a Δ*pglB* mutation has on the cell. A number of genes were down-regulated more than two-fold in the Δ*pglB* mutant compared to wild-type *C. jejuni*, no ORFS were up-regulated more than two-fold in the mutant. (Table 5 and 6). The majority of the down-regulated genes are responsible for flagellar regulation or construction of flagella. This was striking, as

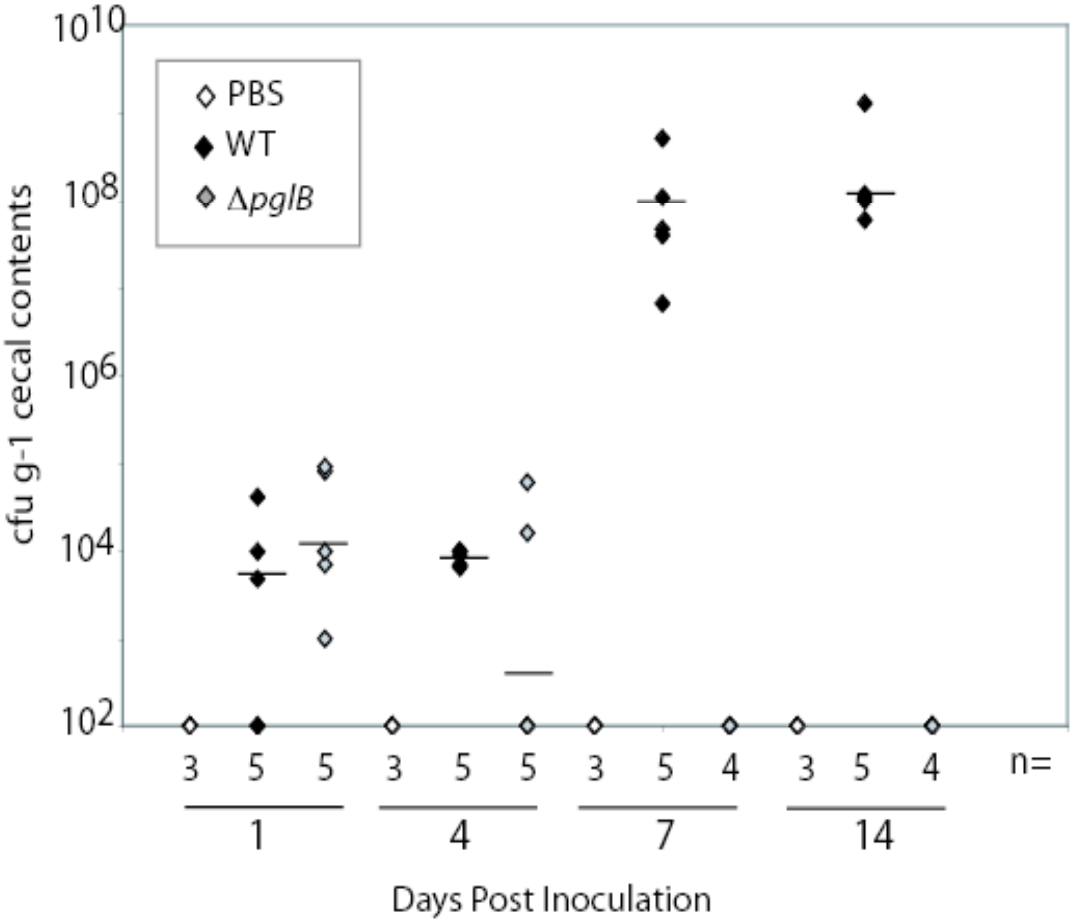

Figure 48. Chick Cecal Colonization of Δ*pglB* Mutant. Day-of-hatch chicks orally inoculated with PBS, wild-type *C. jejuni*, or Δ*pglB* mutant were examined for cecal recovery of the indicated strain on days 1, 4, 7, and 14 post inoculation. The Δ*pglB* mutant was not recovered after day 4 post-inoculation from the chick ceca.

Table 5. Genes Down-regulated in *ΔpglB* Mutant Compared to Wild-type *C. jejuni*

Gene Name	Common	Fold Change	Description
81-176fla3	flaB-81-176	2.444	Flagellin B
Cj0037c	Cj0037c	1.683	Cytochrome c family protein
Cj0040	Cj0040	26.43	Conserved hypothetical protein
Cj0041	Cj0041	6.896	Conserved hypothetical protein
Cj0042	flgD	19.79	Flagellar hook assembly protein FlgD
Cj0043	flgE	10.79	Flagellar hook protein FlgE
Cj0044c	Cj0044c	1.902	Conserved hypothetical protein
Cj0045c	Cj0045c	3.004	Putative iron-binding protein
Cj0056c	Cj0056c	1.85	hypothetical protein Cj0056c
Cj0061c	fliA	1.661	RNA polymerase sigma factor, sigma-F
Cj0062c	Cj0062c	2.763	Conserved hypothetical membrane protein
Cj0121	Cj0121	1.631	Conserved hypothetical protein
Cj0127c	accD	1.773	Acetyl-CoA carboxylase, carboxyltransferase, beta subunit
Cj0260c	Cj0260c	1.679	small hydrophobic protein
Cj0322	perR	1.722	Transcriptional regulator, Fur family
Cj0364	Cj0364	1.81	Conserved hypothetical protein
Cj0370	rpsU	2.007	30S ribosomal protein S21
Cj0391c	Cj0391c	2.501	Conserved hypothetical protein
Cj0416	Cj0416	2.715	hypothetical protein Cj0416
Cj0417	Cj0417	2.267	hypothetical protein Cj0417
Cj0428	Cj0428	1.809	Conserved hypothetical protein
Cj0456c	Cj0456c	1.74	Conserved hypothetical protein
Cj0457c	Cj0457c	2.319	Conserved hypothetical protein
Cj0525c	pbpB	1.682	Penicillin-binding protein
Cj0526c	fliE	1.76	Flagellar hook-basal body protein FliE
Cj0527c	flgC	2.492	Flagellar basal body rod protein FlgC
Cj0528c	flgB	10.46	Flagellar basal body rod protein FlgB
Cj0610c	Cj0610c	1.5	Conserved hypothetical periplasmic protein
Cj0612c	cft	1.56	Ferritin
Cj0687c	flgH	10.47	Flagellar basal body L-ring protein FlgH
Cj0697	flgG2	12.85	Flagellar distal rod protein FlgG
Cj0698	flgG	6.353	Flagellar distal rod protein FlgG
Cj0699c	glnA	2.414	Glutamine synthetase
Cj0724	Cj0724	1.74	Hypothetical protein
Cj0761	Cj0761	1.547	Conserved hypothetical protein
Cj0792	Cj0792	1.614	Conserved hypothetical protein
Cj0793	flgS	2.183	Two component system sensor kinase protein
Cj0794	Cj0794	2.122	Conserved hypothetical protein,
Cj0814	Cj0814	1.576	hypothetical protein Cj0814
Cj0816	Cj0816	1.911	hypothetical protein Cj0816

Cj0850c	Cj0850c	1.527	Transmembrane transport protein
Cj0859c	Cj0859c	2.815	Conserved hypothetical protein
Cj0876c	Cj0876c	1.567	Conserved hypothetical protein
Cj0887c	flaD	3.915	Flagellin family protein
Cj0975	Cj0975	1.657	putative outer-membrane protein
Cj0977	Cj0977	1.855	Conserved hypothetical protein
Cj0987c	Cj0987c	1.522	putative integral membrane protein
Cj0989	Cj0989	2.855	Hypothetical protein
Cj1002c	Cj1002c	1.542	Conserved hypothetical protein
Cj1026c	Cj1026c	3.455	Conserved hypothetical lipoprotein
Cj1034c	Cj1034c	2.395	DnaJ homolog
Cj1075	Cj1075	1.538	Conserved hypothetical protein
Cj1102	truB	1.617	tRNA pseudouridine synthase B
Cj1103	csrA	1.557	Carbon storage regulator
Cj1125c	wlaG	1.562	Polysaccharide biosynthesis protein (WlaG)
Cj1126c	wlaF	2.52	Oligosaccharyl transferase STT3 subunit superfamily
Cj1127c	wlaE	5.519	Polysaccharide biosynthesis protein (WlaE)
Cj1129c	wlaC	3.356	Polysaccharide biosynthesis protein (WlaC)
Cj1130c	wlaB	1.781	ABC transporter, ATP-binding/permease protein
Cj1153	Cj1153	1.648	Cytochrome-related conserved hypothetical protein
Cj1225	Cj1225	1.539	Conserved hypothetical protein
Cj1241	Cj1241	1.8	Putative transporter
Cj1242	Cj1242	53.71	Conserved hypothetical protein
Cj1293	flmA	2.894	UDP-GlcNAc C6-dehydratase/C4-reductase
Cj1294	Cj1294	2.065	Putative aminotransferase (DegT family)
Cj1295	Cj1295	1.535	Conserved hypothetical protein
Cj1315c	Cj1315c	1.508	Amidotransferase HisH (hisH)
Cj1316c	Cj1316c	2.221	Flagellin modification protein, PseA
Cj1318	Cj1318	1.913	hypothetical protein Cj1318 (1318 family)
Cj1338c	flaB	2.374	Flagellin
Cj1339c	flaA	2.291	Flagellin
Cj1357c	Cj1357c	1.562	Cytochrome c552 nitrite reductase catalytic subunit NrfA
Cj1383c	Cj1383c	1.514	Conserved hypothetical protein
Cj1384c	Cj1384c	1.761	Conserved hypothetical protein
Cj1386	Cj1386	1.603	Conserved hypothetical protein, ankyrin repeat family protein
Cj1443c	kpsF	1.778	Arabinose-5-phosphate isomerase, KpsF
Cj1450	Cj1450	2.299	Probable ATP/GTP-binding protein
Cj1456c	Cj1456c	1.912	putative periplasmic protein
Cj1461	Cj1461	2.165	Predicted DNA methyltransferase
Cj1462	flgI	21.43	Flagellar P-ring protein FlgI
Cj1463	Cj1463	3.137	Conserved hypothetical protein
Cj1464	Cj1464	2.53	Conserved hypothetical protein
Cj1465	Cj1465	2.406	Conserved hypothetical protein,

Cj1466	flgK	3.079	Flagellar hook-associated protein FlgK
Cj1467	Cj1467	1.59	Conserved hypothetical protein
Cj1531	dapF	1.668	Diaminopimelate epimerase
Cj1563c	Cj1563c	1.902	putative transcriptional regulator
Cj1616	chuC	1.538	Hemin ABC transporter, ATP-binding protein
Cj1617	chuD	1.764	Hemin ABC transporter, substrate-binding protein
Cj1631c	Cj1631c	2.138	Conserved hypothetical protein
Cj1632c	Cj1632c	2.256	putative periplasmic protein
Cj1647	iamA	2.2	ABC transporter, ATP-binding protein
Cj1648	Cj1648	2.115	Possible ABC transporter, periplasmic substrate-binding protein
Cj1649	Cj1649	1.602	Conserved hypothetical lipoprotein,
Cj1656c	Cj1656c	2.237	hypothetical protein Cj1656c
Cj1728c	Cj1728c	1.644	small hydrophobic protein
Cj1729c	flgE2	3.199	Flagellar hook protein FlgE
Cj1731c	ruvC	1.889	Crossover junction endodeoxyribonuclease RuvC
CJJ81176_0025	flgE2	10.44	flagellar hook protein FlgE
CJJ81176_0157		3.363	conserved hypothetical protein
CJJ81176_1311		1.91	aminotransferase, DegT/DnrJ/EryC1/StrS family
CJJ81176_1337	pseE	1.784	Involved in flagellin glycosylation
CJJ81176_1338	flaB	2.938	Flagellin B
CJJ81176_1339	flaB	2.779	flagellin A
CJJ81176_1340		1.562	motility accessory factor
Cjp27		1.567	Hypothetical protein
Cjp47		1.559	Hypothetical protein
Cjp48	Cjp48	2.085	Hypothetical protein
Cjp49	Cjp49	1.778	Hypothetical protein
Cjp52		1.638	Hypothetical protein
HS23,36CJ1333	pseE	1.606	Involved in flagellin glycosylation
HS23,36CJ1337	pseD	1.582	Involved in flagellin glycosylation

Table 6. Genes Up-regulated in Δ*pglB* Mutant Compared to Wild-type *C. jejuni*

Gene Name	Common	Fold Change	Description
Cj1366c	glmS	1.822	Glucosamine-fructose-6-phosphate aminotransferase
Cj0087	aspA	1.774	Aspartate ammonia-lyase
Cj1401c	tpiA	1.771	Triosephosphate isomerase
Cj1279c	Cj1279c	1.729	Fibronectin domain-containing lipoprotein
Cj1717c	leuC	1.726	3-isopropylmalate dehydratase, large subunit
Cj1096c	metK	1.721	S-adenosylmethionine synthetase
Cj0778	peb2	1.707	Peb2 accessory colonization factor AcfC (acfC)
Cj1115c	Cj1115c	1.705	Conserved hypothetical protein
Cj1719c	leuA	1.698	2-isopropylmalate synthase
Cj0361	lspA	1.682	Lipoprotein signal peptidase
Cj1573c	nuoG	1.626	NADH-ubiquinone oxidoreductase, subunit G
HS23,36ORF6		1.622	Orthologue of Cj1420c
Cj1092c	secF	1.614	Protein-export membrane protein SecF
Cj0576	lpxD	1.606	UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine N-acyltransferase
Cj0914c	ciaB	1.604	CiaB protein
Cj0088	dcuA	1.601	C4-dicarboxylate transporter, anaerobic
Cj1455	prfB	1.599	Peptide chain release factor 2
Cj0807	Cj0807	1.599	Putative short chain dehydrogenase
Cj1367c	Cj1367c	1.596	Possible nucleotidyltransferase
Cj0536	oorA	1.589	2-oxoglutarate:acceptor oxidoreductase, OorA subunit
Cj1707c	rplC	1.544	50S ribosomal protein L3
Cj1265c	hydC	1.535	Ni/Fe hydrogenase, cytochrome b subunit
Cj0362	Cj0362	1.531	Conserved hypothetical integral membrane protein
Cj1248	guaA	1.53	GMP synthase (glutamine-hydrolyzing)
Cj0133	Cj0133	1.523	Conserved hypothetical protein
Cj1497c	Cj1497c	1.523	Conserved hypothetical protein
Cj0274	lpxA	1.517	UDP-N-acetylglucosamine acyltransferase
Cj0845c	gltX	1.513	Glutamyl-tRNA synthetase
Cj1196c	gpsA	1.51	Glycerol 3-phosphate dehydrogenase
Cj1020c	Cj1020c	1.502	Putative cytochrome c family protein

several research groups have demonstrated that disruption of the *pglB* gene does not alter *C. jejuni* motility.

Isolation of motile variant of the Δ*pglB* mutant

Upon re-examination, the original frozen stock of the Δ*pglB* mutant had a reduced motile-phenotype in 0.4% MH motility agar. Single colonies were isolated from this Δ*pglB* stock and tested for motility. Motile-variants of the Δ*pglB* mutant were isolated and inoculated onto MH agar plates for 24 hours. Individual suspensions of these variants were stabbed into 0.4% MH motility and left for 24 hours at 37°C in the tri-gas incubator. Diameters of colonies in the soft agar were measured as an indicator of motility. The three isolated motile variants of the Δ*pglB* mutant had similar motility as wild-type, while the original stock was non-motile (Fig 49).

Microarray analysis of wild-type and Δ*pglB* mutant motile variant grown for 24 hours on MH plates was performed. The results of this microarray were similar to the first non-motile variant, with significant down-regulation of flagellar genes in the Δ*pglB* motile-variant strain compared to wild-type (Tables 7 and 8).

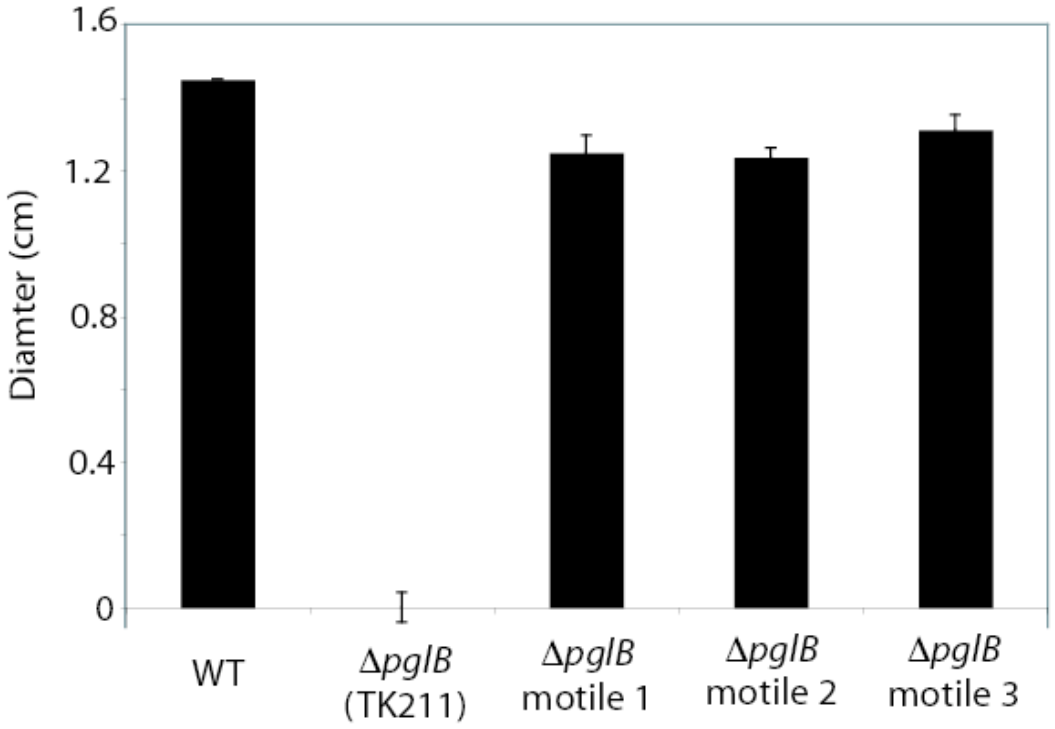

Figure 49. Motility of the Δ*pglB* Mutant and Variants. Suspensions of each indicated strain were stabbed into 0.4% MH motility agar . The diameter of the motile bacteria is reported.

Table 7. Genes Down-regulated in Δ*pglB*- motile Variant Mutant Compared to Wild-type *C. jejuni*

Gene Name	Common	Fold Change	Description
Cj0006	*Cj0006*	1.782	Probable integral membrane protein, putative Na+/H+ transporter/permease
Cj0007	*gltB*	3.688	Glutamate synthase, large subunit
Cj0009	*gltD*	3.47	Ribonuclease HII
Cj0010c	*rnhB*	2.192	ribonuclease HII
Cj0011c	*Cj0011c*	1.545	Conserved hypothetical protein, putative ComEA-related protein
Cj0037c	*Cj0037c*	1.6	Cytochrome c family protein
Cj0041	*Cj0041*	1.574	Conserved hypothetical protein
Cj0042	*flgD*	1.844	Flagellar hook assembly protein FlgD
Cj0043	*flgE*	1.996	Flagellar hook protein FlgE
Cj0345	*trpE*	1.922	Anthranilate synthase, component I
Cj0347	*trpF*	1.663	Phosphoribosylanthranilate isomerase
Cj0348	*trpB*	1.631	Tryptophan synthase, beta chain
Cj0528c	*flgB*	1.554	Flagellar basal body rod protein FlgB
Cj0697	*flgG2*	2.095	Flagellar distal rod protein FlgG
Cj0698	*flgG*	2.056	Flagellar distal rod protein FlgG
Cj0699c	*glnA*	3.235	Glutamine synthetase
Cj0887c	*flaD*	1.638	Flagellin family protein
Cj0982c	*Cj0982c*	2.283	Amino acid transporter, periplasmic solute-binding protein CjaA
Cj1126c	*wlaF*	1.508	Oligosaccharyl transferase STT3 subunit superfamily, putative (WlaF)
Cj1127c	*wlaE*	1.504	Polysaccharide biosynthesis protein (WlaE)
Cj1129c	*wlaC*	1.715	Polysaccharide biosynthesis protein (WlaC)
Cj1130c	*wlaB*	1.751	ABC transporter, ATP-binding/permease protein
Cj1199	*Cj1199*	1.76	Putative iron/ascorbate-dependent oxidoreductase
Cj1200	*Cj1200*	2.496	Conserved hypothetical periplasmic lipoprotein (NLPA family),
Cj1201	*metE*	2.419	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase
Cj1202	*metF*	2.658	5,10-methylenetetrahydrofolate reductase
Cj1203c	*Cj1203c*	1.704	Hypothetical membrane protein
Cj1242	*Cj1242*	1.924	Conserved hypothetical protein
Cj1338c	*flaA*	1.777	Flagellin
Cj1339c	*flaB*	1.784	Flagellin
Cj1462	*flgI*	1.758	Flagellar P-ring protein FlgI
Cj1728c		1.537	small hydrophobic protein
Cj1729c	*flgE2*	1.721	Flagellar hook protein FlgE
CJJ81176_0025	*flgE-81-176*	1.972	flagellar hook protein FlgE
CJJ81176_1338		1.892	flagellin B
CJJ81176_1339		2.016	flagellin B

Table 8. Genes Up-regulated in Δ*pglB*- motile Variant Mutant Compared to Wild-type *C. jejuni*

Gene Name	Common	Fold Change	Description
Cj0027	*pyrG*	1.585	CTP synthase (UTP-ammonia lyase)
Cj0028	*recJ*	1.823	Single-stranded DNA-specific exonuclease
Cj0029	*ansA*	1.703	L-asparaginase
Cj0086c	*ung*	1.614	Uracil-DNA glycosylase
Cj0087	*aspA*	2.288	Aspartate ammonia-lyase
Cj0088	*dcuA*	1.953	C4-dicarboxylate transporter, anaerobic
Cj0146c	*trxB*	1.766	Thioredoxin reductase
Cj0168c	*Cj0168c*	1.548	Hypothetical protein
Cj0175c	*Cj0175c*	1.585	Iron ABC transporter, periplasmic iron-binding protein
Cj0176c	*Cj0176c*	1.716	Hypothetical membrane protein
Cj0245	*rplT*	1.621	50S ribosomal protein L20
Cj0334	*ahpC*	1.708	Alkyl hydroperoxide reductase AhpC/TsaA family
Cj0379c	*Cj0379c*	1.52	Conserved hypothetical protein, putative reductase
Cj0420	*Cj0420*	1.527	Conserved hypothetical protein
Cj0429c	*Cj0429c*	1.673	Conserved hypothetical protein
Cj0430	*Cj0430*	1.795	Conserved hypothetical membrane protein
Cj0431	*Cj0431*	1.91	Conserved hypothetical protein, putative ATP/GTP-binding protein
Cj0432c	*murD*	1.656	UDP-N-acetylmuramoylalanine--D-glutamate ligase
Cj0433c	*mraY*	1.588	Phospho-N-acetylmuramoyl-pentapeptide transferase
Cj0559	*Cj0559*	1.509	Conserved hypothetical protein, putative pyridine nucleotide-disulphide oxidoreductase
Cj0715	*Cj0715*	1.876	Conserved hypothetical protein, putative transthyretin-like periplasmic protein
Cj0716	*Cj0716*	1.723	3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (DAHP synthetase)
Cj0736		1.753	hypothetical protein Cj0736
Cj0737		1.638	putative periplasmic protein
Cj0741		2.093	hypothetical protein Cj0741
Cj0742		1.935	
Cj0920c	*peb1a*	1.919	Amino acid ABC transporter, permease protein
Cj0921c	*peb1b*	1.834	Amino-acid ABC transporter, periplasmic solute-binding protein
Cj0998c	*Cj0998c*	1.687	Conserved hypothetical protein
Cj1183c	*cfa*	1.53	Cyclopropane fatty acyl phospholipid synthase
Cj1381	*Cj1381*	1.538	Conserved hypothetical protein, probable lipoprotein
Cj1382c	*fldA*	1.889	Flavodoxin
Cj1383c	*Cj1383c*	2.126	Conserved hypothetical protein
Cj1384c	*Cj1384c*	2.029	Conserved hypothetical protein
Cj1385	*katA*	1.849	Catalase

Cj1534c	*Cj1534c*	1.906	Putative bacterioferritin
Cj1537c	*acs*	1.781	Acetyl-CoA synthetase
Cj1545c	*Cj1545c*	2.12	Conserved hypothetical protein, drug resistance protein MdaB homolog, flavodoxin-like protein
Cj1613c	*Cj1613c*	1.733	Conserved hypothetical protein
Cj1615	*chuB*	1.809	Hemin ABC transporter, permease protein
Cj1616	*chuC*	1.519	Hemin ABC transporter, ATP-binding protein
Cj1617	*chuD*	1.513	Hemin ABC transporter, substrate-binding protein
Cj1659	*Cj1659*	1.556	periplasmic protein p19
CJJ81176_0063		1.755	conserved hypothetical protein
CJJ81176_0064		1.593	cytochrome c family protein
CJJ81176_0066		1.674	cytochrome c biogenesis protein
CJJ81176_0067		1.937	gamma-glutamyltransferase
CjPVIRORF7		1.522	

The Δ*pglB* mutant has higher flagellar phase variation

Based on the microarray results, we hypothesized that the Δ*pglB* mutant had lower expression of flagellar genes. To test this, an arysulfatase (*astA*) fusion was made on the chromosome in the *flaA* locus, the main subunit of flagella, in wild-type and Δ*pglB* backgrounds. White colonies on MH agar containing X-S denotes low *astA* expression, and therefore low *flaA* expression (phase OFF). Over 4,000 colonies were counted for each construct and done in triplicate. There was a significantly higher rate of white colonies in the Δ*pglB flaA::astA* construct, suggesting that the *pglB* mutant population had a higher number of phase-OFF cells. To test whether this phase variation is stable, a generational test was performed. For this, over 4,000 colonies were counted for each strain, for each passage or generation. For the second generation, individual colonies, either white or blue were resuspended and plated on X-S indicator plates. The results demonstrate that the second generation of white colonies in a Δ*pglB flaA::astA* construct, is relatively stable, with 70% of the cells retaining the white colony phenotype. There were no white colonies detected for WT *flaA::astA* strain for this experiment.

Taken together, a mutation in the *pglB* gene in *C. jejuni* causes a decrease in flagellar gene expression as determined by microarray. An *astA* gene fusion to the *flaA* gene in WT and Δ*pglB* backgrounds confirmed the microarray results, demonstrating an increase in phase variability at the f*laA* locus (Fig. 50).

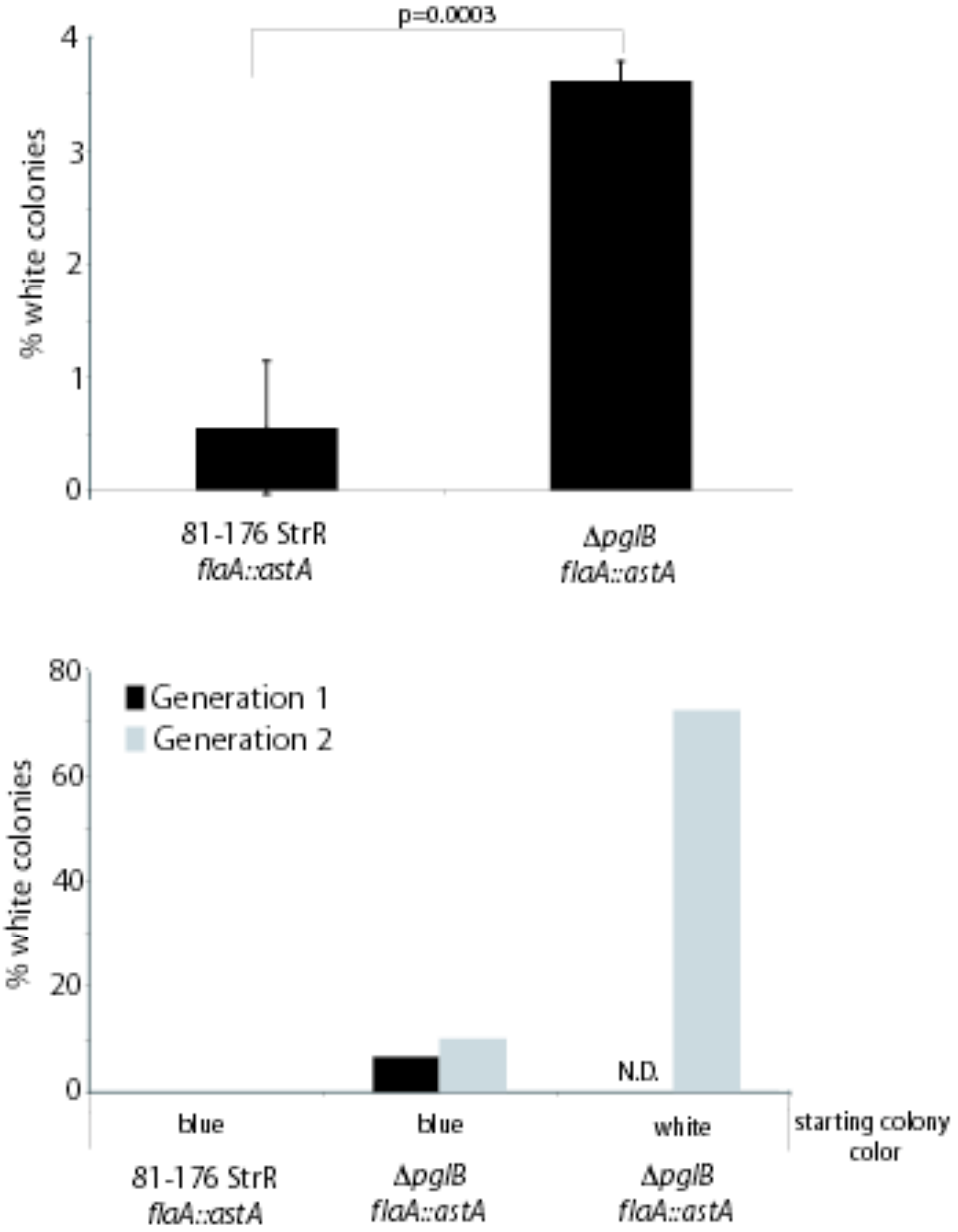

Figure 50. Arylsulfatase Assay of *flaA* Expression in Wild-type and Δ*pglB* Backgrounds. (A). Percent of white colonies in wild-type and Δ*pglB* background expressing *flaA:astA* fusions. White colonies signify low *flaA* expression. (B). Percent white colonies of the indicated strains after generational passage to test the stability of the phase variability.

Discussion

Recently, free oligosaccharides were identified in *C. jejuni* periplasmic fractions and were hypothesized to be the sugars assembled by the *N*-linked protein glycosylation pathway. The oligoosaccharyltransferase, PglB, transfers the assembled hetpassachride formed by the Pgl pathway from a carrier in the inner membrane onto specific proteins with the conserved glycosylation motif [4, 5]. Mutations in the Pgl pathway, including mutations in the *pglB* gene, reduce the level of colonization in chicks, reduced DNA uptake, and invasion in intestinal epithelial cells [2, 6-8]. The amount of free oligosaccharide found in *C. jejuni* was proportional to solute concentration in the media, as free oligosaccharide decreased with high salt and sucrose concentrations [9]. This suggests that the free oligosaccharide from of the *N*-glycosylation pathway could play a role in osmoregulation [9]. However, we were unable to reproduce these results.

O-glycosylation of flagellar proteins are known to affect flagellar synthesis. Here, a defect in the transfer of the *N*-glycosylation system heptassacharide to glycoproteins by the deletion of the oligotransferase, *pglB*, results in the decrease of motility through higher phase variation. This response to perturbations in periplasmic glycan concentrations is not wholly unexpected. As stated, the free periplasmic glycan in *C. jejuni* could be playing a role in osmoregulation for the cell. Alterations in this balance could result in the activation of stress pathways, leading to the down-regulation of flagellar genes. Indeed, mutations in periplasmic glycan pathways in *Rhizobium* have resulted in defects in flagellar assembly [10]. Therefore, taken together, we predict that the increase of free glycan in the periplasmic space due to the deletion of the *pglB* gene,

results in a stress response that causes an increase in phase variation at the flagellar gene loci.

A *C. jejuni* strain with a *pglB* deletion has a colonization defect after seven days in a day-of-hatch chick colonization model. C. *jejuni* strains lacking functional flagella also have a defect for colonization [11]. One explanation for the colonization defect for the Δ*pglB* mutant could be thee increased phase variation of the strain, resulting in a high population of non-motile Δ*pglB* mutants. We cannot disregard that the Δ*pglB* mutation could have pleiotropic effects on the bacteria, as over forty proteins were identified to be glycosylated by lectin binding studies and it is thought that many more glycoproteins exist [12]. Although *N*-glycosylation is understood to be important for *C. jejuni* biology, no obvious hypothesis has arisen for the role of glycosylation on individual proteins from the knowledge of which proteins are glycosylated [12-16].

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Appendix B

The Two-Component Regulatory System CprRS Controls Aspects of *Campylobacter jejuni* Pathogenesis by Mediating the Switch Between Planktonic and Biofilm Lifestyles

Summary

CprRS is a two-component response system in *C. jejuni* that is up-regulated during cellular infections. CprR is essential for *C. jejuni* growth. A strain with a mutation in the sensor kinase gene, *cprS*, displayed a growth defect as well as an increase in biofilm formation. Work presented here shows a *cprS* mutant is attenuated for colonization in a day-of-hatch chick cecal colonization model.

The work presented here was published in collaboration with Dr. Erin Gaynor:

Svensson SL *et al.* The Two-Component Regulatory System CprRS Controls Aspects of *Campylobacter jejuni* Pathogenesis by Mediating the Switch Between Planktonic and Biofilm Lifestyles. Mol Micro 2009, 71(1), 253-272

Introduction

CprRS is a two-component response system in *C. jejuni* that is up-regulated during cellular infections. CprR is essential for *C. jejuni* growth. A strain with a mutation in the sensor kinase, *cprS*, displayed a growth defect as well as an increase in biofilm formation, suggesting that the CprRS system may control aspects of *C. jejuni* physiology and metabolism involved in biofilm dynamics [1].

Biofilm formation has been implicated in transmission of a number of pathogens, including *C. jejuni*, *V. cholerae* and *Psuedomonas* [2-4]. *C. jejuni* biofilms can form on a variety of abiotic surfaces including those used in watering systems, and *C. jejuni* biofilms have been found in animal production watering systems [2] [5]. Biofilm formation could lead to the colonization of these animals, and is proposed to be a mechanism by which *C. jejuni* survives in the environment and transmits to animal species.

ΔcprS shows increased survival within INT 407 epithelial cells and colonizes the gastrointestinal tract of chicks better than the WT strain

To assess the involvement of CprRS in *in vivo* fitness, we tested the ability of Δ*cprS* to colonize chicks. WT and Δ*cprS* strains were used to infect one-day-old chicks at increasing inoculation doses. After one week, birds were sacrificed, and cecal contents were assayed for viable *C. jejuni* (Fig 501. While both strains colonized all birds, the average colonization level for WT across all inocula was 3.56×10^8 CFU, whereas birds infected with Δ*cprS* had an average colonization level of 1.22×10^9 CFU. This generally correlates with an approximately 1-log higher level of colonization for Δ*cprS* compared to wild-type at each dose; however, the differences did not reach statistical significance (p=0.32). No defect was observed for Δ*cprS* colonization of limited flora mice.

***cprR* OE does not affect chick colonization**

Mutagenesis of *cprR* has been attempted, with inactivation only possible when a second copy was present at a heterologous location [1, 6]. This suggests that the activity of the response regulator CprR is essential for *C. jejuni* growth under laboratory conditions. A strain was constructed that constitutively over expressed *cprR* (*cprR* OE). The *cprR* OE strain displayed altered motility phenotype over the growth curve, with decreased motility occurring at later time points (Gaynor, unpublished data). We tested the *cprR* OE strain in the day-of-hatch chick colonization model and found that it did not have a colonization defect when compared to wild-type (Fig. 52).

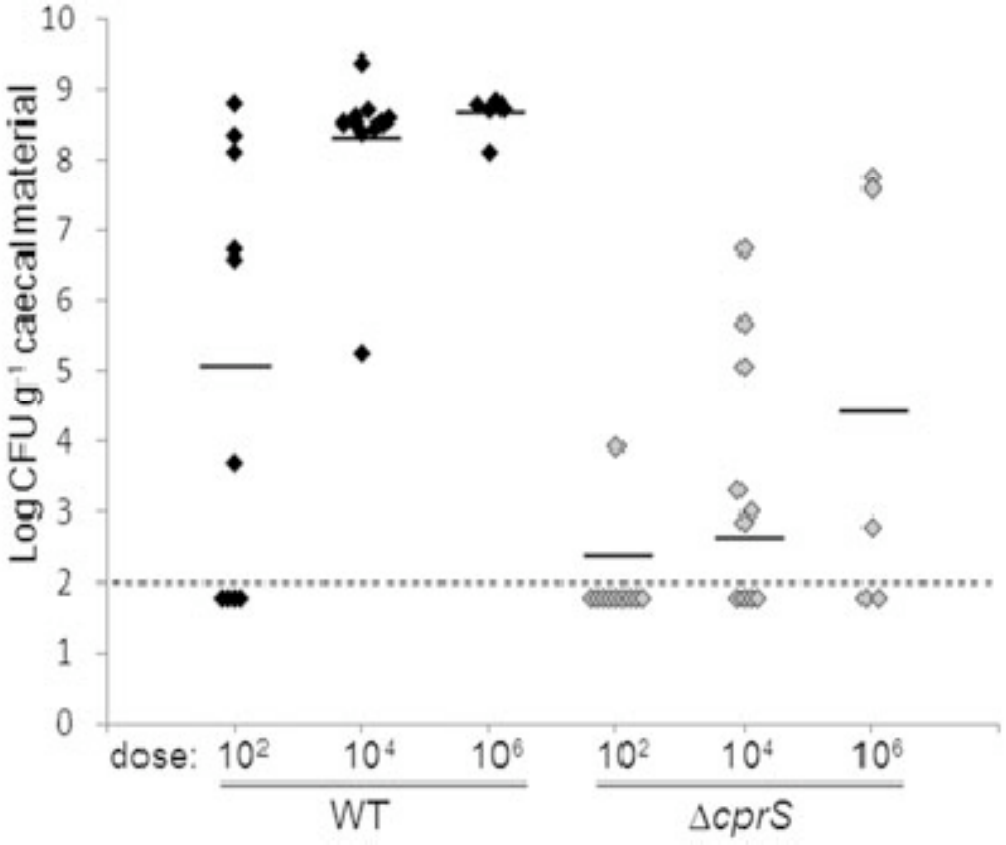

Figure 51. ***C. jejuni ΔcprS*** **Mutant Chick Colonization.** Day-of-hatch chicks were orally inoculated with wither WT or the Δ*cprS* mutant at the indicated doses. Recovery of each strain from the chick ceca was monitored on day 7 post-inoculation and reported in cfu g^{-1} cecal contents.

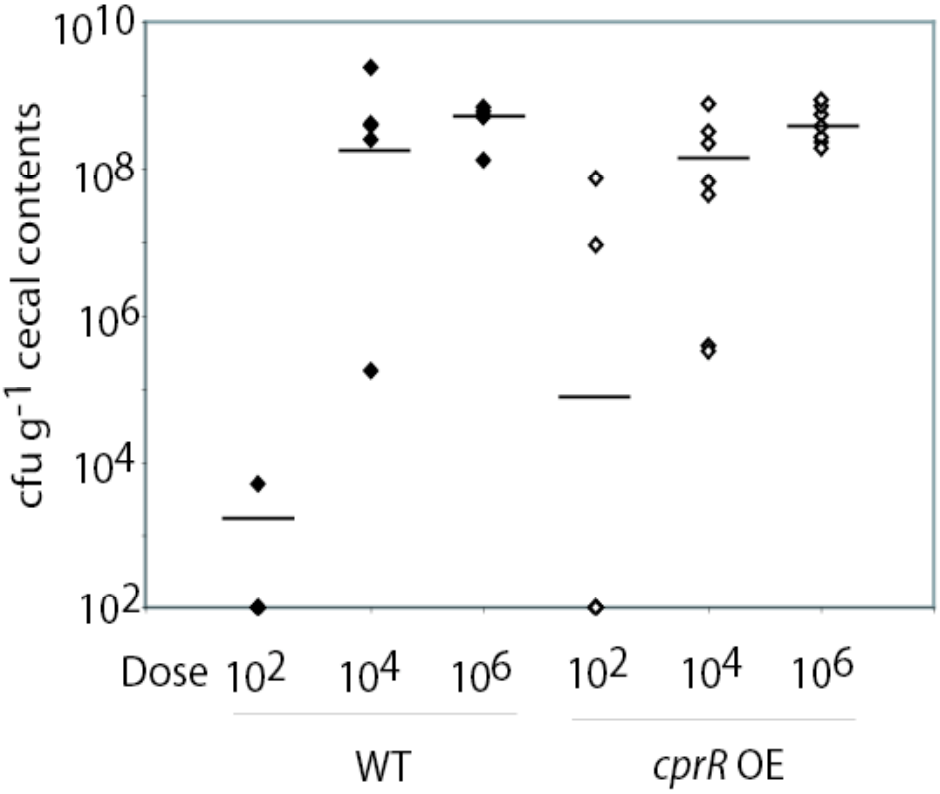

Figure 52. *C. jejuni cprR* OE Chick Colonization. Day-of-hatch chicks were orally inoculated with either wild-type or *cprR* OE *C. jejuni* at the indicated doses. Recovery of each strain was monitored on day 7 post-inoculation.

Discussion

The work presented here demonstrates that *cprS* is essential for efficient colonization of the chick cecum. The ability of the *cprS* mutant strain to produce more biofilm may explain the chick colonization defect. *V. cholera* mutants that produce more biofilm have lower colonization capacities than those of wild-type strains [7]. It has been proposed that *in vivo* signals cause the bacterial shift from biofilm to planktonic growth and the regulation of this switch may be important for *C. jejuni* transmission between the environment and various hosts, including chicks and humans.

Protein expression profiles of wild-type and the *cprS* mutant revealed differences involved in stress tolerance, cell surface structures, regulation, and metabolic pathways. Several oxidative stress proteins were up-regulated in the *cprS* mutant compared to wild-type including catalase (KatA), trioredoxin reductase (TrxZB) and alkyl hydropersoxide reducase (AhpC) [1]. Also, the major outer membrane protein (MOMP) and the flagellar filament protein FlaA were up-regulated in the *cprS* mutant compared to wild-type. Consistent with the proteomics analysis, the *cprS* mutant displayed increased motility as well as an increased sensitivity to osmotic and oxidative stress [1].

The significant attenuation observed for the *cprS* mutant in the day-of-hatch chick cecal colonization may be explained by the increased sensitivity to oxidative stress. In the characterization of the day-of-hatch chick colonization model, an increase in pro-inflammatory cytokines and recruitment of heterophils was observed in response to *C. jejuni*. Of note, this response was reliant on *virB11*-dependent invasion of the chick cecal epithelium (Chapter II). The increased motility of the *cprS* mutant may have stimulated

the host response during chick colonization. Further, the increased levels of MOMP observed in the proteomics analysis may increase outer membrane permeability to oxidative agents, resulting in the increase oxidative stress sensitivity and up-regulation of stress response proteins. This increase in oxidative and osmotic stress susceptibility could account for the chick colonization defect.

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Appendix C

Antibody Production Against *C. jejuni* in the Day-of-hatch Chick Colonization Model

Summary

C. jejuni invades the chick cecal epithelium during early stages of colonization and stimulates an innate immune response. We demonstrate here that the chicks mount an adaptive immune response to *C. jejuni* colonization, with the production of systemic immunoglobulins against *C. jejuni* specific antigens, including CjaA.

Results

Chicks produce systemic antibodies against *C. jejuni* antigens

Broiler chicks colonized with *C. jejuni* form maternal antibodies and systemic antibodies against *C. jejuni* antigens. We asked whether antibodies are produced against *C. jejuni* in the day-of-hatch chick colonization model. Chicks were inoculated with either PBS, wild-type *C. jejuni*, or *C. jejuni* lacking *pglB*, a strain that has been shown to be unable to stably colonize (Appendix A). The adaptive immune system of chicks does not develop until 10-14 days post-hatch. Therefore, serum from infected birds was collected on day 14 post-inoculation by cardiac puncture.

Proteins from whole cell lysate from wild-type and the Δ*pglB* mutant grown on MH agar plates for 24-hours were separated by SDS-Page gel electrophoresis and transferred onto nitrocellulose membranes. Western blot analysis was performed on these samples using the serum obtained from infected birds and anti-chicken Ig antibody conjugated to alkaline phosphatase as the secondary antibody (1:100).

A number of proteins from both wild-type and the Δ*pglB* mutant were recognized by the sera from wild-type infected birds, but not from uninfected or Δ*pglB* mutant infected birds (Fig. 52). An immuno-reactive band present in wild-type-lysate and not in the Δ*pglB* mutant lysate was identified as CjaA by mass spectrometry.

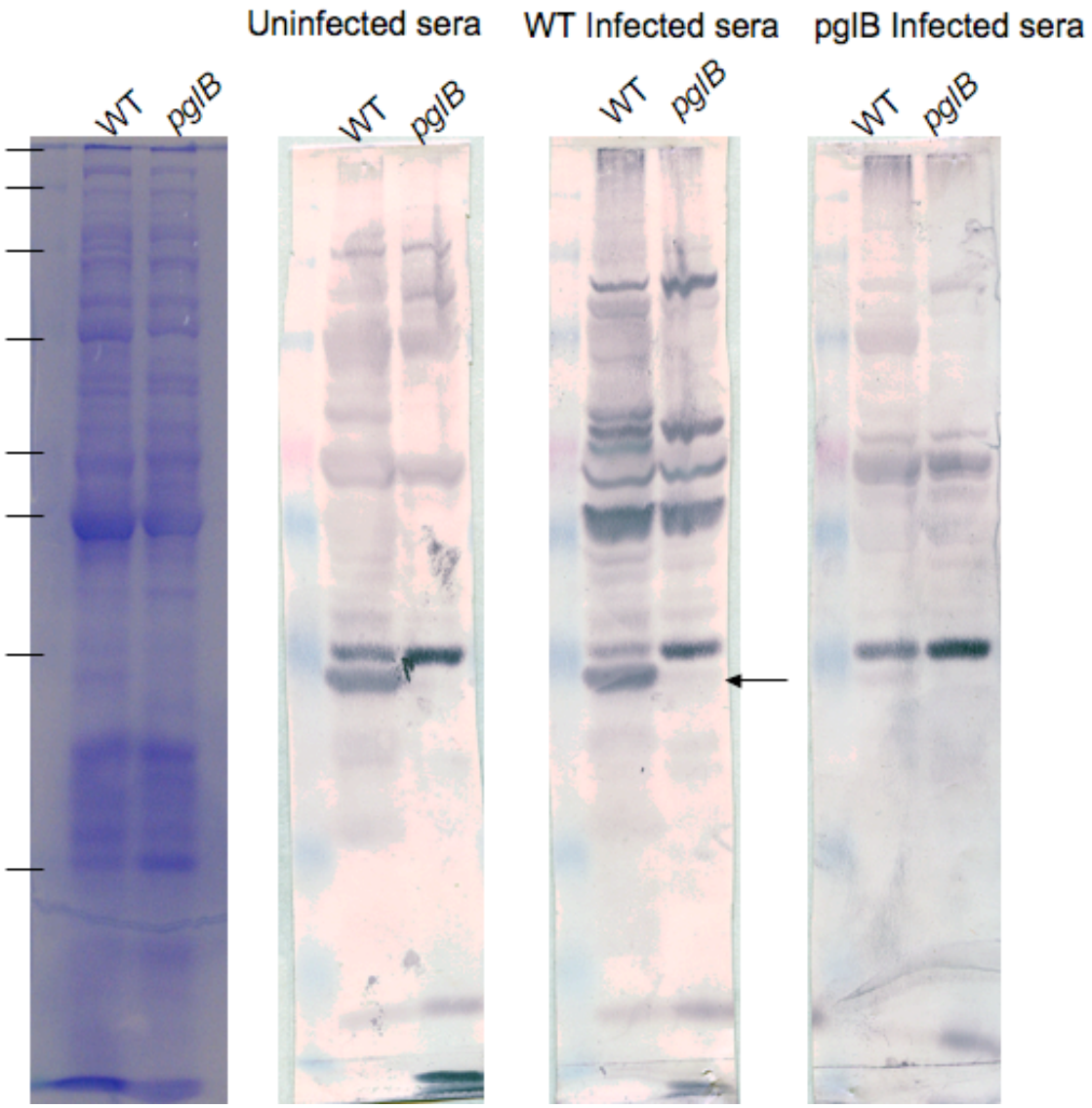

Figure 53. Western-Blot of *C. jejuni*-Infected Chick Sera. Whole cell lysates of wild-type and Δ*pglB* were run on an SDS-Page gel. (Left) Gel stained with Coomasie. Gels were transferred and exposed to sera from uninfected, wild-type infected, or Δ*pglB* infected chick sera. Arrow denotes position of CjaA in wild-type lysates.

Detection of sIgA in the Cecal Mucus of *C. jejuni* Infected Chicks

In Chapter II, we characterized the chick innate immune response to *C. jejuni* colonization. After a modest inflammatory response, inflammation subsides and *C. jejuni* colonizes the cecal contents asymptomatically. We hypothesized that production of antibodies, specifically sIgA in the mucus secretions in the cecum, could be involved in the resolution of the inflammatory response. Specifically, sIgA could be "holding" *C. jejuni* in the mucus, inhibiting further local invasion of the tissue.

Chicks orally inoculated with 10^6 cfu *C. jejuni* or left uninfected were euthanized on days 7 and 14 post-inoculation. The ceca were opened and washed with PBS to remove debris, and mucus was collected by gentile scrapping. *C. jejuni* from MH agar plates, diluted in MH broth, was added to 96-well plates. Serial dilutions of mucus was added to the bacteria and incubated under microaerobic conditions for 2 hours. This allows specific anti-*C. jejuni* sIgA in the mucus to bind to the bacteria. The bacteria were then centrifuged and washed three times. Anti-chicken IgA with conjugated alkaline phosphates (1:1000) was added to the wells and incubated for an hour under microaerobic conditions. The bacteria were centrifuged and washed three times. The quantity of bacteria in each well was measured by OD600, followed by detection of alkaline phosphatase (OD405).

The results show a significant increase in the quantity of *C. jejuni* specific sIgA in the mucus of *C. jejuni* infected chicks after 2 weeks (Fig 54). This production corresponds with the decrease in C. jejuni invasion and inflammation (Chapter II). However, it is unknown whether the sIgA production is directly responsible for inhibiting *C. jejuni* invasion.

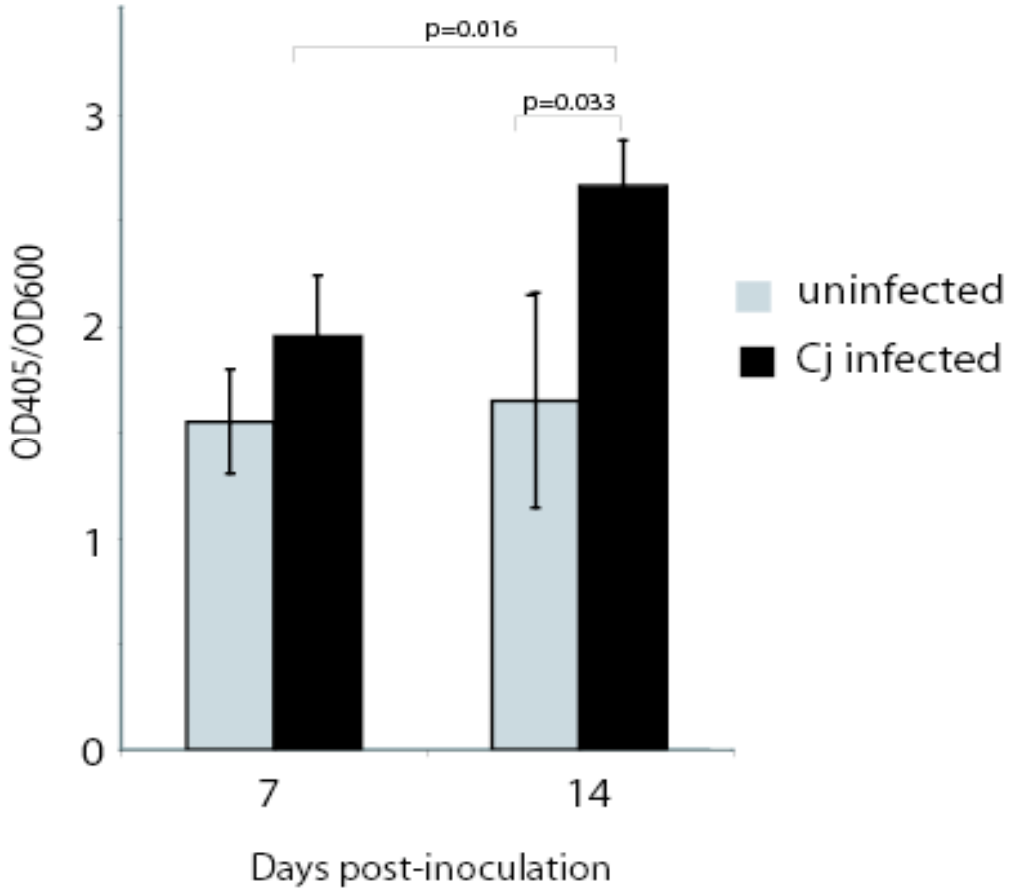

Figure 54. **Detection of sIgA in the Cecal Mucus of *C. jejuni* Infected Chicks.** sIgA, as detected by measurement of alkaline phosphatase (OD405) in mucus from uninfected (grey) and *C. jejuni* infected (black) chicks treated with anti-IgA antibodies conjugated to alkaline phosphatase. Samples were standardized based on amount of C. jejuni (OD600) in each well.

Discussion

Chickens colonized with *C. jejuni* produce systemic antibodies against specific *C. jejuni* antigens [1-4]. IgG, IgA and IgM circulating antibodies against flagellin and outer membrane proteins have been detected [1, 2]. Further, maternal antibodies against *C. jejuni* antigens are protective against the maternal colonizing *C. jejuni* strain in developing chicks out to 2 weeks of age [2, 4, 5]. After the maternal antibodies wane, the chicks are colonized with *C. jejuni* and, produce systemic immunoglobulins against their own colonizing strain of *C. jejuni*. The protective nature and mechanism of *C. jejuni* recognition by the host immune response have never been explored.

The *C. jejuni* CjaA protein, an amino acid-binding protein, was previously identified as immunogenic and contains a putative *N*-linked glyosylation site [6]. CjaA was characterized as an inner-membrane localized lipoprotein and a putative amino acid transporter in *C. jejuni* [6]. An avirulent *Salmonella* strain expressing CjaA protein was found to be protective in an oral immunization study, inhibiting the colonization of *C. jejuni* after the immunization [7]. It is interesting to note that the CjaA protein is not recognized in the Δ*pglB* mutant whole cell lysate. This suggests that the localization or function of CjaA may be impaired in the Δ*pglB* mutant.

Here we demonstrate specific *C. jejuni* sIgA production in the mucus of *C. jejuni* infected ceca. Whether invasion of *C. jejuni* is inhibited by this production is unknown, and it would be interesting to test whether addition of anti-*C. jejuni* antibodies to *C. jejuni* cultures or injected into the cecum of the chicks prior to inoculation would inhibit *C. jejuni* local invasion of the cecal tissue and the subsequent inflammatory response.

Further, the role of systemic and mucosal antibodies on *C. jejuni* colonization and persistence is unknown. It would be interesting to inhibit antibody production and examine how *C. jejuni* colonization and persistence is altered. The adaptive immune system of chicks is not fully developed at hatch, and addition of colchicine to the anal lips of chicks has been shown to inhibit antibody production by affecting bursal development [8]. *C. jejuni* infection of colchicine-treated birds would elucidate the roles both systemic and mucosal antibodies play in *C. jejuni* colonization of the chick.

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Appendix D

Murine Models of *C. jejuni* Colonization and Pathogenesis

Summary

In recent years, utilization of germ-free and genetically-engineered mice have led the way for studying *C. jejuni* virulence factors in a small-animal model. Here, we utilize germ-free mice and germ-free IL-10 -/- mice to study several *C. jejuni* factors in pathogenesis models, including *znuA, rpoN*, and *cdtB*. Germ-free mice are efficiently colonized by *C. jejuni* and germ-free IL-10 -/- mice are efficiently colonized and produce signs of disease when infected with *C. jejuni*. Colonization of germ-free mice does not require *znuA* or *rpoN*. Intestinal inflammation and death in germ-free IL-10 -/- mice is delayed when infected with *C. jejuni ΔcdtB* compared to wild-type *C. jejuni*.

C. jejuni Recovery from Germ-Free Mice Gastrointestinal Tract

While *C. jejuni* is unable to colonize conventional mice, previous studies demonstrated consistent colonization of germ-free mice. In an effort to study bacterial virulence factors, wild-type *C. jejuni* was orally inoculated into post-weaned germ-free mice. After 14-days, mice were euthanized and their gastrointestinal tract contents plated for *C. jejuni* recovery. *C. jejuni* was recovered in the small and large intestines, with highest colonization in the cecum and colon (Fig. 55).

C. jejuni Mutant Recovery from Germ-Free Mice

To test bacterial virulence factors in *C. jejuni* colonization and histopathology, germ-free mice were inoculated with 10^6 cfu of either wild-type, Δ*rpoN,* Δ*znuA* or un-inoculated. ZnuA is a high-affinity zinc binding periplasmic glycoprotein characterized in Chapter III, and a strain lacking *znuA* is unable to colonize conventional chicks [1]. RpoN is a sigma factor that regulates a number of flagellar genes and a strain lacking *rpoN* has a reduced motile-phenotype and is attenuated for chick colonization [2]. Wild-type, *rpoN*, and *znuA* were recovered from the gastrointestinal tract and spleen from infected mice at similar levels (Fig. 56).

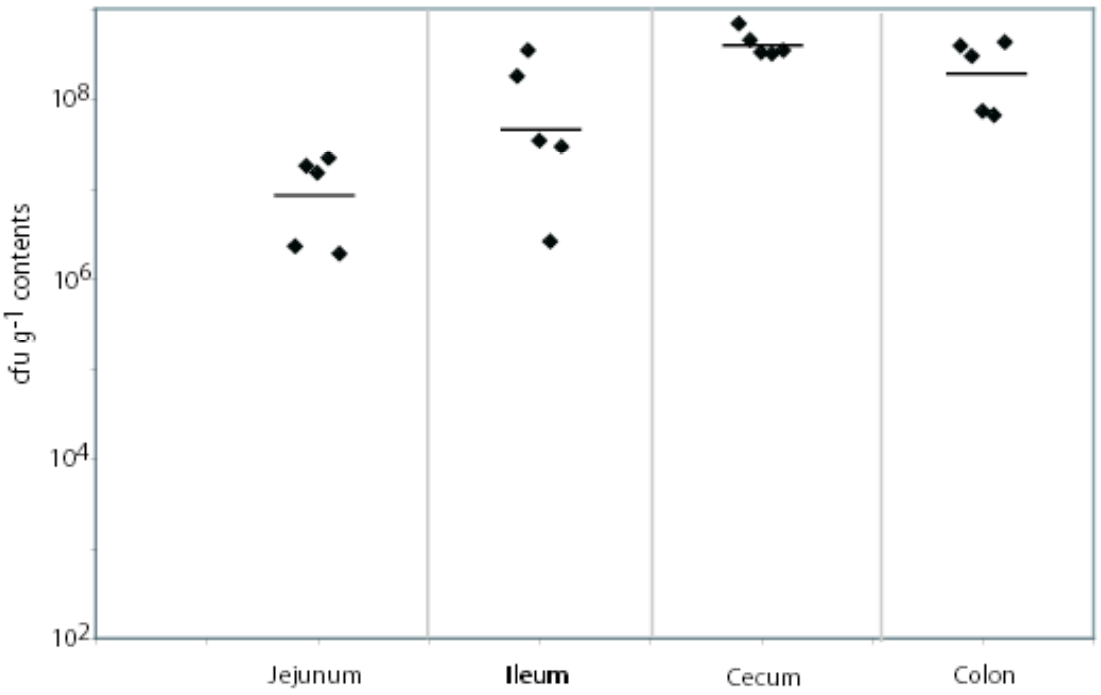

Figure 55. Wild-type Colonization of Germ-Free Mice. Recovery of wild-type *C. jejuni* from the indicated sections of germ-free mice intestinal tract after 14 days. Each dot represents an individual mouse.

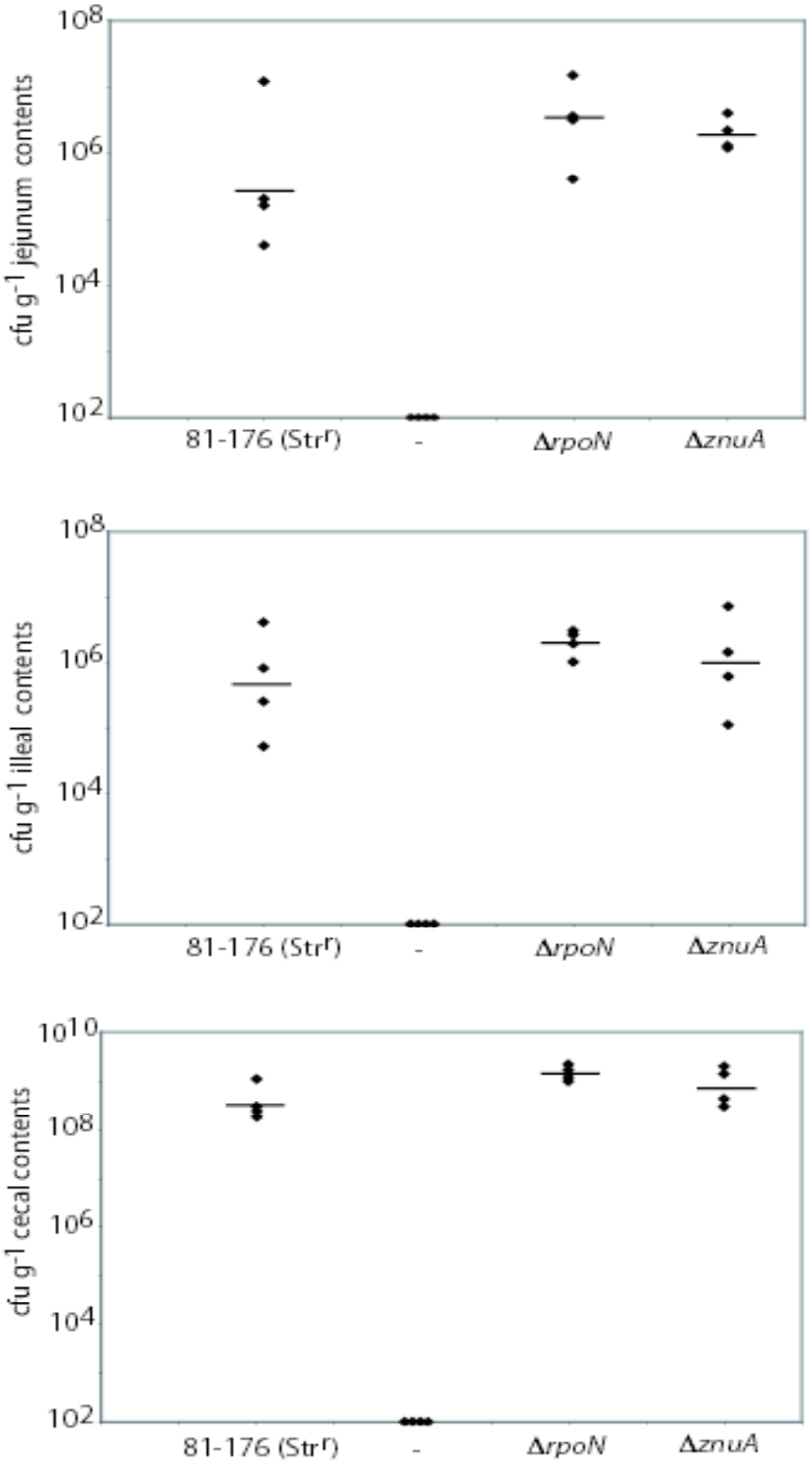

Figure 56. *C. jejuni* Mutant Colonization of Germ-Free Mice. Recovery of wild-type, Δ*rpoN*, and Δ*znuA* from the jejunum, illeul, cecal, colon, and spleen of germ-free mice after 2 weeks.

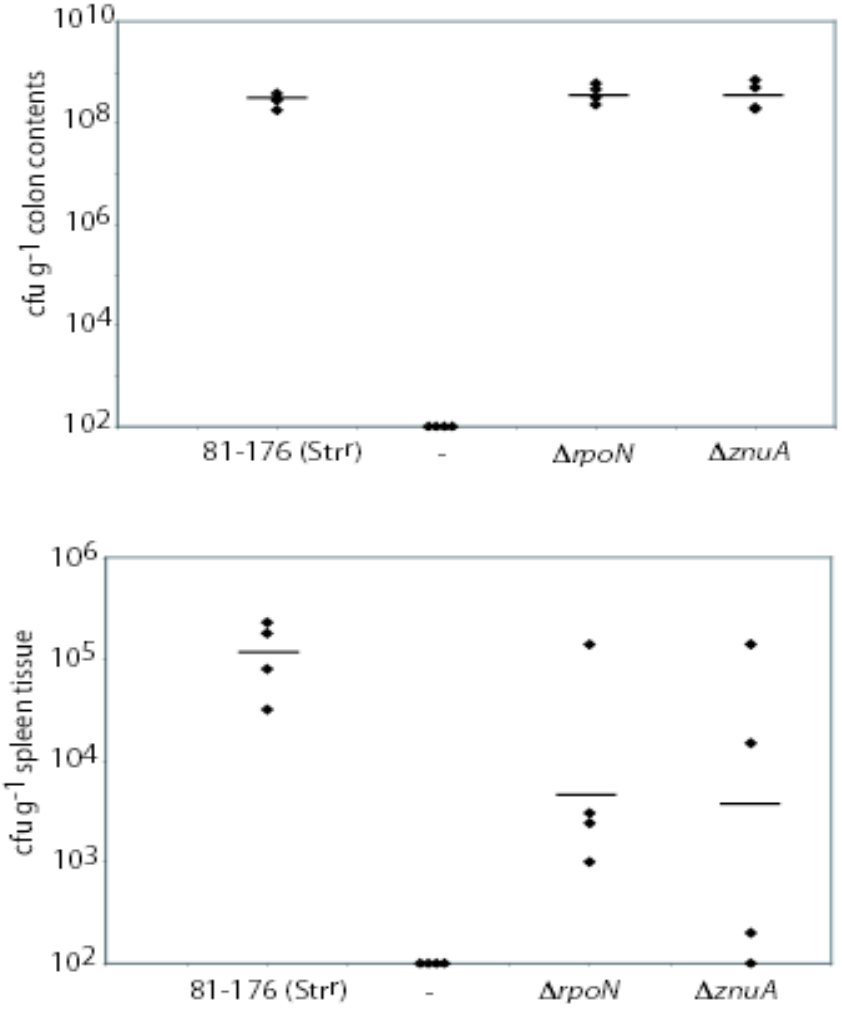

Figure 51 (continued). *C. jejuni* Mutant Colonization of Germ-Free Mice. Recovery of wild-type, Δ*rpoN* and Δ*znuA* from the jejunum, illeal, cecal, colon, and spleen of germ-free mice after 2 weeks.

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***C. jejuni* Pathogenesis in IL-10 -/- Germ-Free Mice**.

IL-10 is an anti-inflammatory cytokine that signals through the JAK/Stat pathway to down-regulated inflammatory genes as well as though inhibiting NF-KB signaling. Wild-type *C. jejuni* colonizes IL-10 -/- mice and causes histopathological lesions [3]. While cytolethal distending toxin (CDT) is necessary for inflammation in NF-KB-deficient (3X) mice, its effects in IL-10 -/- mice has never been examined [4].

We attempted to examine the effects of *C. jejuni* pathogenesis in IL-10 -/- germ-free mice. Mice were orally inoculated with 10^6 cfu of either wild-type *C. jejuni* or a mutant lacing *cdtB*. Mice inoculated with wild-type *C. jejuni* showed signs of distress after 3 days, were euthanized and their cecal contents plated for *C. jejuni* recovery. Mice inoculated with Δ*cdtB* did not have signs of distress, but were euthanized on day 3 as well. Both wild-type and Δ*cdtB* were recovered from the cecal contents of mice (Fig. 57). After 10 days, the remaining mice inoculated with Δ*cdtB* began to show signs of distress, were euthanized and their cecal contents plated for *C. jejuni* recovery. The Δ*cdtB* mutant was recovered at a high level from the cecal contents on day 10 post-inoculation (Fig. 57).

Tissue sections from the intestines of infected mice were examined by H&E staining for pathology. Mice inoculated with wild-type *C. jejuni* had severe pathology, including inflammatory cell infiltration and lesions in the cecum and colon after 3 days. Mice inoculated with *cdtB* did not have inflammation until 10 days post-inoculation (Fig. 57).

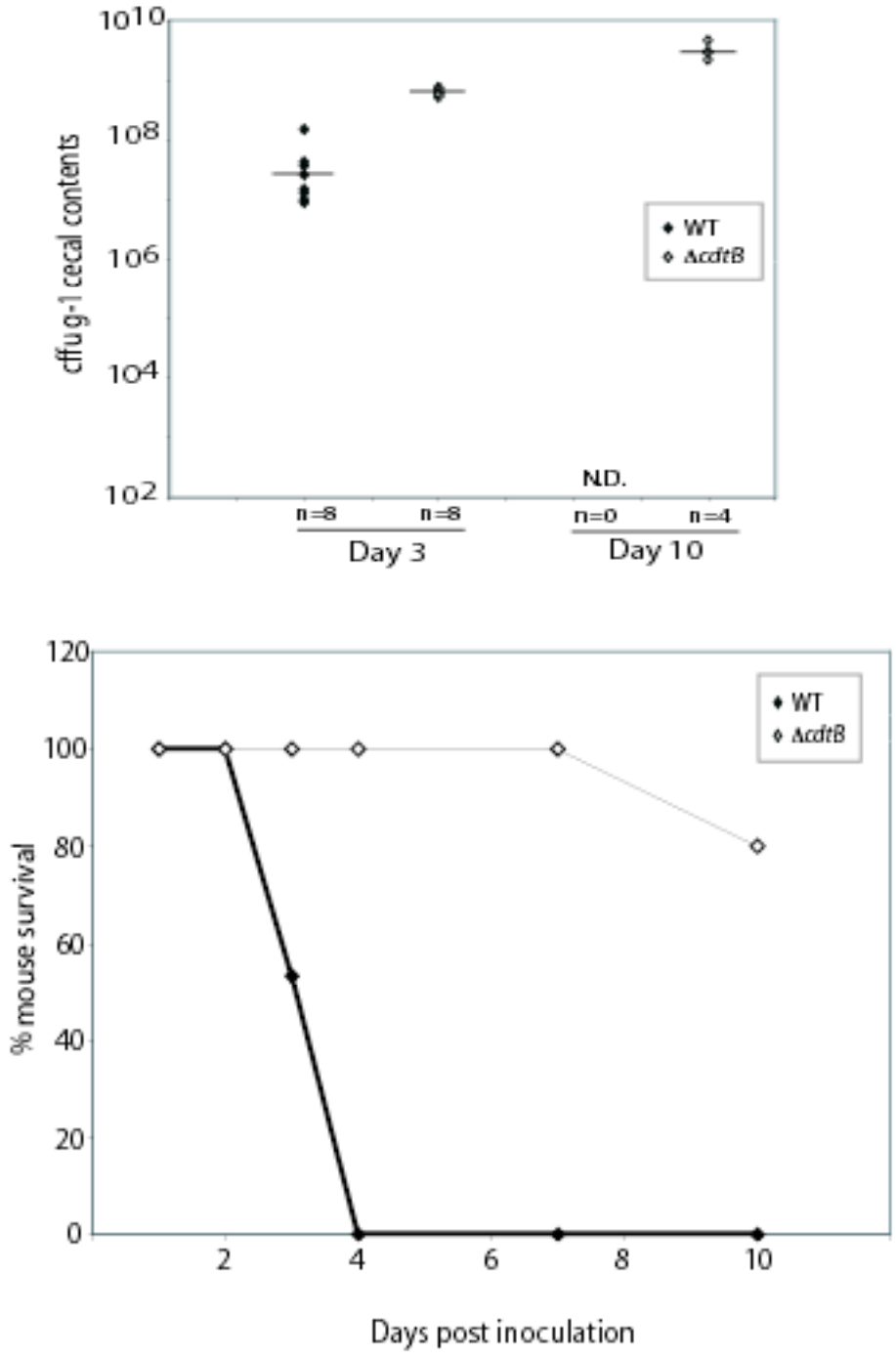

Figure 57. Cecal Colonization and Mouse Survival of *C. jejuni* infected IL-10-/- Germ-Free Mice. Mice were inoculated with either wild-type or Δ*cdtB C. jejuni* were examined for cecal recovery. Survival of mice inoculated with either wild-type or Δ*cdtB*.

Δ*znuA* Colonization of Germ-Free IL-10 -/- Mice.

We have shown that the *znuA* is necessary for *C. jejuni* colonization of conventional chicks and is not essential for colonization of limited-flora chicks or germ-free mice (Chapter IV). We asked whether Δ*znuA* is necessary for colonization of IL-10 -/- mice. Germ-free IL-10 -/- mice were inoculated with 10^6 cfu of either wild-type or *znuA* and euthanized after 2 days. The Δ*znuA* mutant was recovered at significantly higher levels than wild-type, demonstrating that Δ*znuA* is not essential for colonization of germ-free IL-10-/- mice and actually does better after 2 days (Fig. 58). The reason for this is unknown and is the subject of future studies.

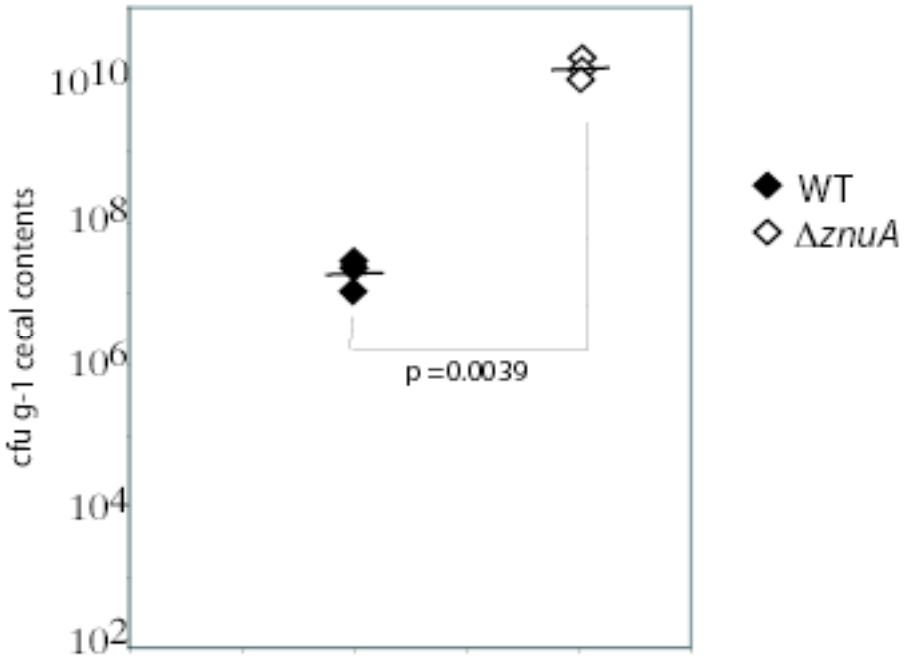

Figure 58. Δ*znuA* Mutant Colonization of Germ-Free IL-10-/- Mice. Recovery of wild-type and Δ*znuA* mutant from cecal contents of germ-free IL-10 -/- mice after 2 days.

Discussion

C. jejuni colonization of conventional mice is not stable and does not produce signs of disease. Germ-free and limited-flora mice are colonized well with *C. jejuni*, and when combined with gene-deleted or immuno-compromised mouse backgrounds, produce clinical signs of campylobacteriosis (reviewed in Chapter I).

We used germ-free mice to assess the contributions of *rpoN* and *znuA* in *C. jejuni* colonization. While these genes are necessary for colonization of conventional chicks, they are not necessary for colonization of germ-free mice and *znuA* is not necessary for colonization of germ-free chicks (Chapter V). Further, *znuA* is not essential for colonization of germ-free IL-10 -/- mice after two days, and even colonizes better than wild-type. Wild-type *C. jejuni* causes a severe inflammatory response after 3 days in germ-free IL-10 -/- mice. Histopathology examination of Δ*znuA*-inoculated mice was not performed. Future experiments will examine the immune response over time in Δ*znuA* infected germ-free IL-10 -/- mice compared to wild-type to ascertain whether a lack of immune stimulation is why the Δ*znuA* mutant is able to colonize better than wild-type. These results were recapitulated by another researcher in the DiRita Lab, showing Δ*znuA* colonizes germ-free IL-10-/- mice better than wild-type.

There are many un-answered questions. While we demonstrate the CDT is not necessary for colonization of germ-free IL-10-/- mice, we demonstrate that CDT is necessary for wild-type levels of inflammation. It is interesting that a Δ*cdtB* mutant colonization induces inflammation after 10 days, compared to 3 days during wild-type

infection. This suggests that other bacterial factors independent of Δ*cdtB* causes inflammation in this model. Mutations in flagellar genes, and a transposon mutagenesis screen in a *ΔcdtB* background could shed light on what factors cause inflammation in this model.

While Δ*znuA* and Δ*rpoN* mutants colonized germ-free mice at a similar efficiency as wild-type in the gastrointestinal tract, bacterial recovery of these mutants from the spleen was significantly lower than wild-type. This suggests these factors are necessary for systemic bacterial spread. Future research is necessary for confirm these results.

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Appendix E

5-Fluorouracil Depletion of Heterophils and Its Effects on *C. jejuni* Colonization

Summary

C. jejuni invasion of the cecal epithelium stimulates the recruitment of heterophils during early colonization events. Treatment of day-of-hatch chicks with 5-fluorouracil causes the depletion of circulating heterophils by 7 days post-hatch. We found that chicks treated with 5-fluorouracil and infected with *C. jejuni* resulted in a rapid death of the chicks, and systemic spread of *C. jejuni*. These results suggest that the innate immune response in the chicks is necessary for controlling *C, jejuni* systemic spread.

5-Fluorouracil (5-FU) is an anti-metabolic chemotherapeutic agent that incorporates into RNA and inhibits synthesis of thymidine nucleotides. Although used as a chemotherapeutic agent, 5-FU is commonly used to decrease neutrophil or heterophil production [1-3]. One side effect of 5-FU use is damage to mucosal layers in the body. The drug affects normal epithelial cells and hematopoeitic cells of the gastrointestinal tract, and mucosal epithelium of the gastrointestinal tract affected [4]. We asked whether disruption of the mucosal layer and innate defenses by administration of 5-FU to day-old chicks would affect *C. jejuni* colonization.

Chicks were injected with 5-FU at 200mg kg^{-1} body weight on day of hatch, and half were orally infected with *C. jejuni* at 10^6 cfu, while the other half were given PBS. *C. jejuni* colonization of the cecum and systemic spread to the spleen and liver were monitored for 7 seven days. PBS and PBS + 5-FU treated birds remained *C. jejuni*-free and healthy throughout the experiment. One day post-inoculation, *C. jejuni* colonization was reduced by statistically significant levels in the 5-FU treated animals, suggesting that a healthy mucosal layer is necessary for establishing a high level of infection (Fig. 59). Four days post-inoculation, *C. jejuni* colonization in both the non-treated and 5-FU treated birds were between 10^8cfu and 10^9 cfu g^{-1} cecal contents, similar to our previous observation (Chapter III). The 5-FU treated + *C. jejuni* inoculated birds were notably smaller and weaker. Between days five and seven, the majority of the chicks died. By day seven, the remaining chicks were moribund (Fig. 59).

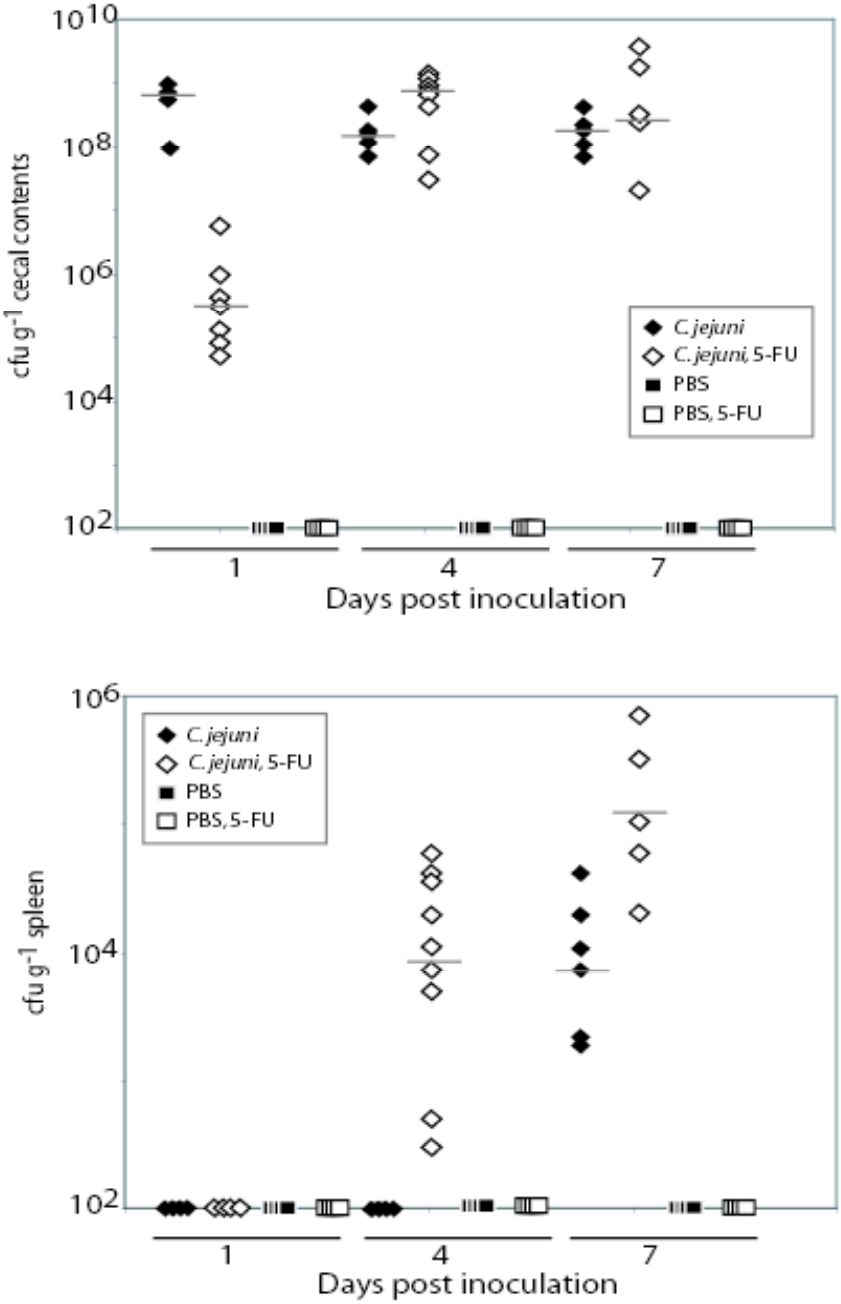

Figure 59. Recovery of *C. jejuni* from 5-FU Treated Chicks. Recovery of *C. jejuni* from the cecal contents (top) and spleen (bottom) of chicks treated with 5-FU and infected with *C. jejuni*.

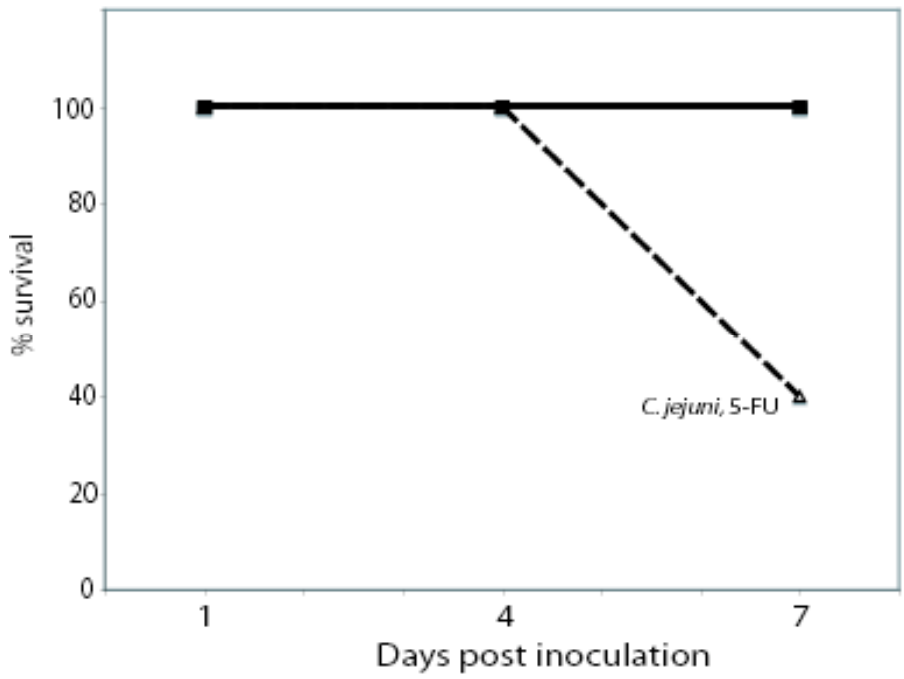

Figure 59 (continued). Recovery of *C. jejuni* from 5-FU Treated Chicks. Survival of chicks inoculated with C. *jejuni* or PBS, treated with 5-FU or left untreated. All the chicks in these studies remained healthy until day 7, chicks infected with *C. jejuni* and treated with 5-FU died and/or became moribund by day 7.

Heterophil levels for the chicks treated with PBS+ 5-FU were slightly elevated, but not significantly on days one and four post-inoculation (Fig. 60). However, heterophil levels were significantly reduced on day seven, less than those chicks inoculated with PBS alone (Fig. 60). This is consistent with published reports that 5-FU treatment significantly reduces heterophil levels in chicks between days five to ten [2]. Chicks inoculated with C. *jejuni* + 5-FU also had reduced heterophil levels on day seven, compared to chicks inoculated with PBS (Fig 60).

Strikingly, *C. jejuni* was recovered from the spleen of 5-FU + *C. jejuni* infected birds on day four post-inoculation at 10^4-10^5 cfu g^{-1} spleen, while *C. jejuni* was not recovered from spleen until day seven in un-treated animals (Fig. 59). Similar results were observed for liver (data not shown). Bacteria were not recovered from systemic sites in PBS or PBS+5-FU treated chicks under our culturing conditions. Epithelial damage in the cecum was not observed in the 5-FU treated animals, suggesting that epithelial integrity did not play a role in C. *jejuni's* systemic spread. Alternatively, the data suggests that the innate immune system, including heterophils, is necessary for controlling the systemic spread and bacterial load of *C. jejuni*. Additionally, these results confirm that that *C. jejuni* is able to invade the intestinal epithelium as the microbe was recovered from systemic sites in healthy (untreated) and compromised (5-FU) treated chicks between days four and seven, notwithstanding that the severity of systemic spread is dramatically increased in 5-FU treated chicks.

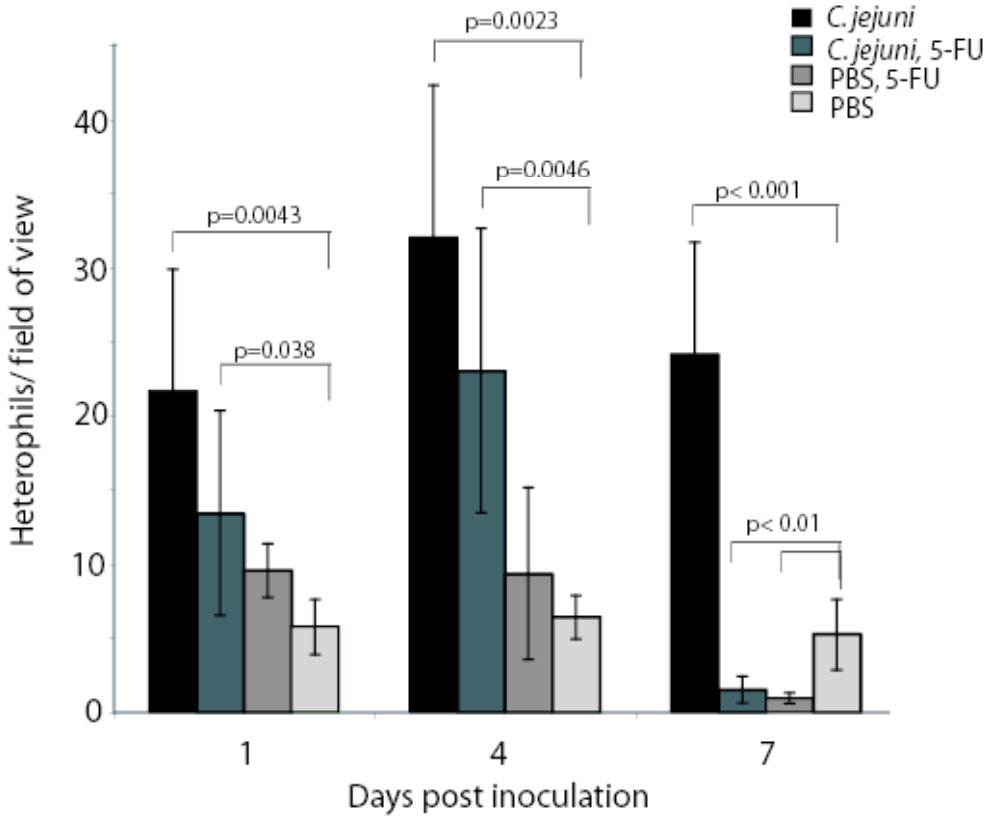

Figure 60. Heterophil Recruitment in 5-FU Treated Chicks. Quantification of heterophils in the cecal tissue of chicks infected with C. *jejuni* and left untreated or treated with 5-FU. There was significantly less heterophils in the tissue treated with 5-FU compared to PBS infected chicks.

Discussion

5-Flurouracil is a potent chemotherapy drug, and when injected into chickens, can cause up to a 10-fold reduction in circulating heterophils [5]. Previous research has shown that 5-FU treated chickens are more susceptible to systemic infections of *Salmonella* infections than non-treated birds [5]. 5-FU also alters the mucosal barrier of the gastrointestinal tract by affecting mucin production and epithelial layer integrity [4]. This can lead to systemic infection and death by normal flora in mouse animal models [6]. In our studies, chicks treated with 5-FU and infected with *C. jejuni* had a lower incidence of colonization at the earliest time-point, one-day post inoculation. *C. jejuni* was also recovered in systemic sites such as liver and spleen earlier in the 5-FU animals than in the untreated animals, perhaps consistent with the observation that 50% of the chickens in the 5-FU+*C. jejuni* treated cohort died by day 7. Although the 5-FU treatment may compromise the epithelial barrier, allowing *C. jejuni* access to systemic sites, death of the chicks suggests that an intact immune system, including heterophils, may indeed control *C. jejuni* systemic spread. *C. jejuni* is able to spread to systemic sites in un-treated animals, without causing apparent morbidity or mortality. However, the bacterial load at the systemic sites is less in the untreated animals than in the 5-FU treated animals. Further, PBS+ 5-FU treated chicks did not show signs of disease or distress, indicating *C. jejuni*, and not solely the effects of the 5-FU treatment, contributed to the death of the chicks treated with *C. jejuni* + 5-U. Also, 5-FU has toxic effects on some bacterial species due to incorporation into ribonucleic acid, but it does not have a

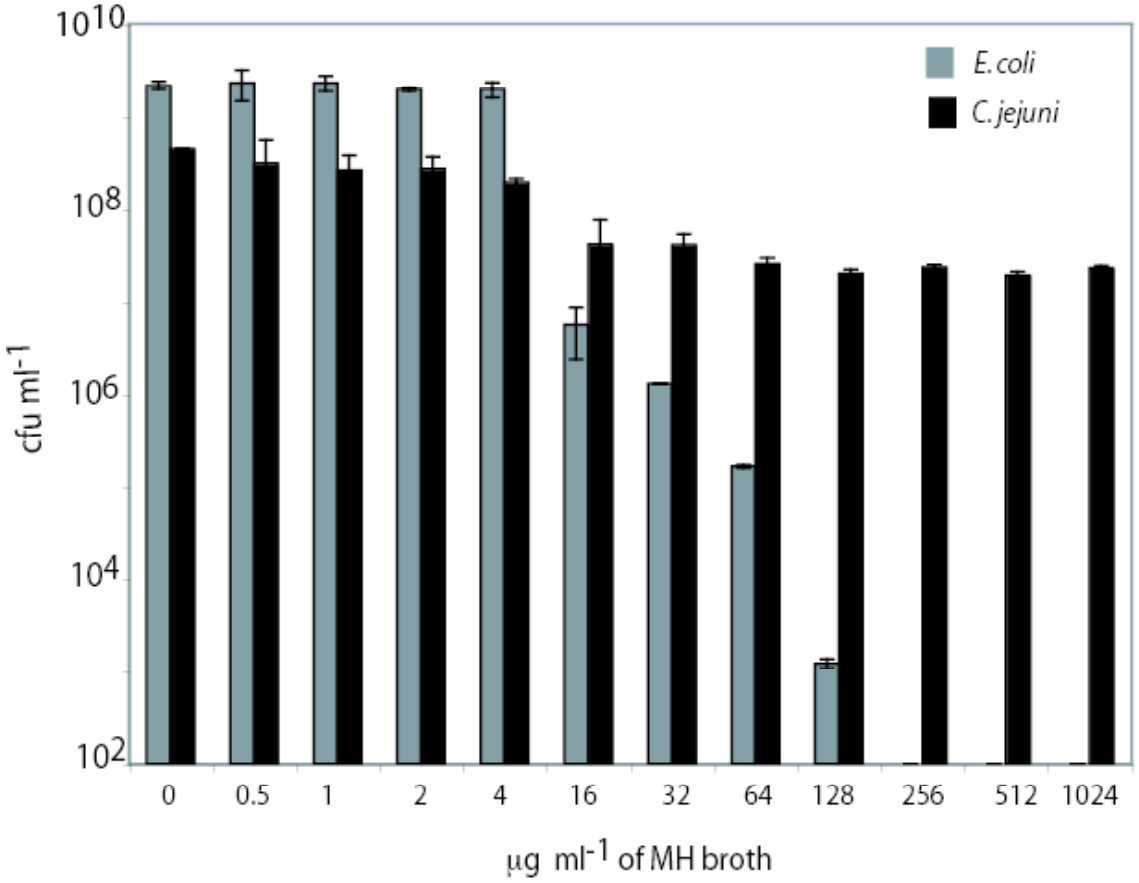

Figure 61. Growth of *C. jejuni* in the Presence of 5-FU. Growth of *C. jejuni* and *E. coli* DH5α after 24 hours in MH broth containing increasing amounts of 5-FU (μg/ml).

dramatic effect on *C. jejuni* growth in MH broth compared to *E. coli* [7](Fig. 61). Taken together, we conclude that an innate immune system is necessary for controlling *C. jejuni* within the chick, perhaps by restricting it to the intestinal mucosal epithelium at early time points after infection.

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