ANALYSIS OF BACTERIAL-HOST INTERACTIONS BETWEEN

CAMPYLOBACTER JEJUNI AND THE AVIAN HOST

DURING COMMENSALISM

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DEDICATION

To my parents, Paul and Linda Bingham.
For their unconditional love and support throughout my life.

To my wonderful husband, Lalo.
For the many adventures we have been through
and those that lie ahead.

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ANALYSIS OF BACTERIAL-HOST INTERACTIONS BETWEEN *CAMPYLOBACTER JEJUNI* AND THE AVIAN HOST DURING COMMENSALISM

by

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Campylobacter jejuni is a leading cause of bacterial enteritis in humans throughout the world. In contrast to the disease seen in humans upon infection, *C. jejuni* promotes an asymptomatic, intestinal colonization of many animals, especially avian species, to result in commensalism. The primary route of transmission to humans is through the consumption or handling of undercooked poultry meats, making *C. jejuni* of particular importance to the agricultural

industry. The direct interplay between *C. jejuni* and the natural avian host was examined to better understand the interactions that contribute to commensalism. We analyzed the colonization dynamics of *C. jejuni* over 28 days and identified a previously uncharacterized prolonged, robust colonization of the bursa of Fabricius, a major lymphoid organ. *C. jejuni* localized to the mucus layer lining the epithelium of the bursal lumen, with no invasion of or damage to host tissue apparent. However, *C. jejuni* was detected invading the cecal epithelium of chicks but only at day 1 post-infection, which may contribute to the observed transient, infection of the spleen and liver. Additionally, certain colonization factors of *C. jejuni* were shown to promote persistence in specific organs. Mutants lacking catalase and the cytolethal distending toxin demonstrated a reduction in levels in the bursa but not the ceca during prolonged colonization, whereas an unencapsulated mutant showed a global colonization defect of all organs. These findings suggest that persistent colonization of the bursa and the ceca, and the ability of the avian host to largely confine *C. jejuni* to mucosal surfaces may be specific for the development of commensalism.

Separate analyses of additional colonization factors of *C. jejuni* revealed the importance of two putative cytochrome *c* peroxidases (CCP), DocA and Cjj0382, in promoting efficient cecal colonization. Further analysis of DocA and Cjj0382 revealed that both proteins have typical characteristics of CCPs, as they are periplasmic proteins with heme-dependent peroxidase activity. Our data

suggest that although DocA and Cjj0382 have characteristics of CCPs, they likely perform different physiological functions for the bacterium during colonization. Overall, this study enhances our understanding of the interactions between *C. jejuni* and a natural host that contribute to the development of commensalism.

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PREVIOUS PUBLICATIONS

Bingham-Ramos, L. K., Ribardo, D. A., Richardson, J. A., and Hendrixson, D. R. (2009) Analysis of prolonged colonization of avian lymphoid tissue and transient spread to systemic sites by *Campylobacter jejuni* during commensalism. In submission.

Bingham-Ramos, L. K. and Hendrixson, D. R. (2008) Characterization of two putative cytochrome *c* peroxidases of *Campylobacter jejuni* involved in promoting commensal colonization of poultry. *Infect. Immun.* 76: 1105-1114.

Chapman, L.M., Roling J. A., **Bingham, L. K.**, Herald, M. R., and Baldwin, W. S. (2004) Construction of a subtractive library from hexavalent chromium-treated winter flounder (*Pseudopleuronectes americanus*) reveals alterations in non-selenium glutathione peroxidases. *Aquat. Toxicol.* 67: 181-194.

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A	adenine
Ala	alanine
C	cytosine
CCP	Cytochrome *c* peroxidase
Cdt	cytolethal distending toxin
cfu	colony forming units
Cys	cysteine
C. jejuni	*Campylobacter jejuni*
DC	dendritic cell
docA	determinant of chick colonization A
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
Fdh	formate dehydrogenase
Frd	fumarate reductase
G	guanine
GBS	Guillain-Barré Syndrome
h	hour(s)
HBS	heme-binding sites

HD11	avian monocyte cell line
His	histidine
Ig	Immunoglobulin
IL	interleukin
INF-γ	interferon gamma
INT407	human embryonic epithelial cell line
IPTG	isopropylthio-β-D-galactoside
kb	kilobases
LB	Luria-Beratani medium
LMH	chicken hepatocellular carcionma epithelial cell line
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAMP	microbial-associated molecular pattern
Mb	megabases
MH	Mueller-Hinton medium
min	minute(s)
MOMP	major outer membrane protein
NK cells	natural killer cells
NOD	nucleotide-binding oligomerization domain
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline

PBSG	PBS containing 0.1% gelatin
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonyl fluoride
PRR	pattern recognition receptors
Rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute media
SDS	sodium dodecyl sulfate
Sm^R	streptomycin resistant
S. pullorum	*Salmonella enterica* serovar Pullorum
T	thymine
THP-1	human monocyte cell line
TGF-β	transforming growth factor – beta
Th1	T helper 1
TLR	Toll-like receptor
TNF-α	tumor necrosis factor - alpha
TMP	trimethoprim
WCL	whole cell lysates

CHAPTER ONE

Introduction

Campylobacter jejuni, a Gram-negative bacterium, is a frequent cause of bacterial enteritis in humans in both industrialized and developing countries throughout the world (33, 36, 132). Epidemiological studies have estimated that every year two million people are infected with *C. jejuni* in the United States (147). Infection of humans can result in a mild to severe, inflammatory enteritis leading to a bloody diarrheal syndrome (19). Whereas infection of humans is usually self-limiting, secondary sequelae associated with having a previous *C. jejuni* infection can occur. One such complication is Guillain-Barré syndrome which is an autoimmune disorder that results in an acute paralysis of the peripheral nervous system and in rare cases can lead to death (65, 87).

Sporadic cases of *C. jejuni* enteritis in humans are most often associated with the consumption or handling of poultry meats (53). In contrast to infection of humans, *C. jejuni* asymptomatically colonizes the intestinal tracts of many wild and agriculturally important animals, including avian species, to result in a commensal relationship between the bacterium and the host. Whereas infection of these animals is harmless to the hosts, it creates large reservoirs of *C. jejuni* in the environment that can contaminate the human food or water supply.

The mechanisms by which *C. jejuni* causes disease in humans are not well understood due to the lack of a convenient animal model. Furthermore, little is known regarding the interactions between *C. jejuni* and poultry that result in a commensal relationship. This dissertation serves to provide an increased understanding of the unique interplay between *C. jejuni* and the natural avian host that contribute to commensalism.

The work performed in this study has provided insights into the colonization dynamics of *C. jejuni* and has led to a better understanding of the development of commensalism in poultry. The specific ability of *C. jejuni* to promote prolonged colonization of the intestinal tract and the gut-associated lymphoid tissue of the bursa, and the ability of the avian host to largely confine the bacterium to the mucosal surfaces of these organs may serve to prevent inflammation and damage to intestinal tissues. Further studies also revealed the importance of certain factors of *C. jejuni* in specifically promoting colonization and persistence in the ceca or bursa. Additionally, the characterization of two homologous factors of *C. jejuni*, DocA and Cjj0382, revealed that these similar proteins are both involved in promoting efficient cecal colonization, although it is likely that they each perform different functions *in vivo*.

Overall, this work has provided an increased understanding of the complex interactions between *C. jejuni* and poultry that lead to commensalism. Understanding this commensal relationship will hopefully provide insights into

the bacterial-host interactions that lead to different outcomes of infection in humans and avian species. Furthermore, the findings presented in this work suggest a possible conservation of commensal-host behaviors across the animal kingdom.

CHAPTER TWO

Literature Review

Historical Perspective and Classification of *C. jejuni*

Campylobacter was first described in 1886 by Theodor Escherich in which he observed spiral-shaped organisms in the colon of children who died of 'cholera infantum' and in stool samples from children suffering from enteric disease (26, 46). Unfortunately, Escherich was unable to culture the organism and the bacterium was not recognized as the etiological agent of the observed illnesses (26). Several years later in 1913, similar spiral-shaped bacteria were isolated from aborted sheep fetuses by McFadyean and Stockman, and in 1919 the same bacteria were isolated from aborted bovine fetuses by Smith and Taylor and termed, *Vibrio fetus* (153). For the next several decades, these *Vibrio*-like bacteria were identified predominantly in animals with or without symptoms of disease (43). Another *Vibrio*-like organism, originally termed *Vibrio jejuni* and later to be reclassified as *Campylobacter jejuni*, was isolated from the feces of cattle with diarrhea (157), from blood cultures of humans with gastroenteritis (102), and from aborted sheep fetuses (25). Presumably, this same species was also documented to be the causative agent of an outbreak of bacterial gastroenteritis in a group of inmates in 1938. This was the first documented

outbreak of *C. jejuni* and was likely due to the consumption of contaminated milk, resulting in 355 illnesses in two adjacent state prisons (26). Even though *C. jejuni* had been identified for nearly 100 years, the importance of this species as a pathogen of humans was largely disregarded until the 1970s.

Due to the spiral- or curved-shape of the bacterium, campylobacters were originally classified as members of the *Vibrio* genus. It was not until 1963 that the improper taxonomic classification was formally addressed by Sebald and Véron and a new genus, *Campylobacter*, was introduced reassigning two *Vibrio* species, *Vibrio fetus* and *Vibrio bubulus*, to the *Campylobacter* genus (148). These organisms were spiral-shaped but did not conform to other characteristics typical of *Vibrio* species such as their microaerophilic growth requirements, non-fermentative growth, and low DNA base composition. In 1973, Véron and Chatelain introduced two additional *Vibrio*-like species to the *Campylobacter* genus, *Campylobacter jejuni* and *Campylobacter sputum* (170). Today, there are 17 different species of *Campylobacter* with *C. jejuni* and *C. coli* being the most important to human public health (43).

C. jejuni is the most prevalent cause of bacterial gastroenteritis in humans (33). Infection is generally associated with the consumption or handling of contaminated poultry meats, and thus the bacterium is of significant importance to both human medicine and the agricultural industry (53). *C. jejuni* is a member of the ε-proteobacteria class which also include the *Wolinella* and *Helicobacter*

genera (45). *Campylobacter* species share the following characteristic features. They are Gram-negative bacteria with a characteristic curved, spiral shape as shown in the electron micrograph of *C. jejuni* in Figure 1. A single flagellum located at one or both poles of the bacterium confers a corkscrew-like motility required for colonization of multiple hosts (43). For optimal growth, these microaerophilic organisms require an oxygen-limiting environment. Additionally, the bacteria are asaccharolytic and must ferment amino acids and peptides to generate energy (154, 169). The *Campylobacter* genus has the lowest guanosine and cytosine (G + C) content of the ε-proteobacteria class, in which *C. jejuni* has a G + C content of about 30%. Furthermore, the genomes of this class of bacteria are small; the genome of *C. jejuni* is approximately 1.6 Mb encoding almost 1700 proteins (45, 134).

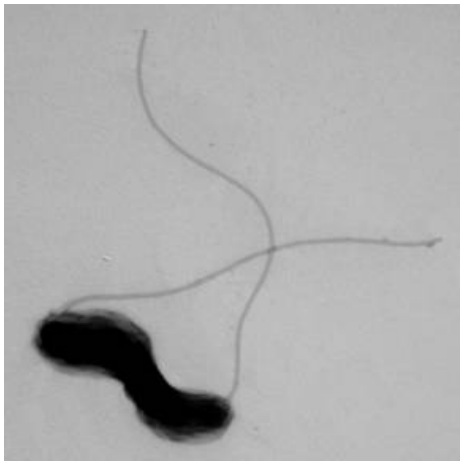

Figure 1. Transmission electron micrograph of *C. jejuni* 81-176.

Epidemiology

Since the addition of *C. jejuni* to the *Campylobacter* genus in 1973 and the development of laboratory techniques to isolate and identify *Campylobacter* species by Butzler and Skirrow (27, 152), *C. jejuni* has become recognized as a leading cause of bacterial gastroenteritis throughout the world in both industrialized and developing countries (33, 36, 147). *C. jejuni* is capable of interacting with multiple hosts to result in different biological relationships. Infection of humans often results in a pathogenic diarrheal disease as mentioned above, whereas infection of many animals, particularly avian species, results in an asymptomatic colonization. Sporadic cases of *C. jejuni* are most commonly associated with the consumption or handling of contaminated poultry meats (53). Thus, while *C. jejuni* develops a harmless commensal relationship with poultry, this interaction creates reservoirs of *C. jejuni* in the food supply and the environment that can greatly impact human health. In contrast, outbreak cases of *C. jejuni* are generally linked to the consumption of contaminated water or unpasteurized milk (54).

In industrialized countries, *C. jejuni* infection is most commonly due to consuming or handling contaminated meat, particularly poultry products. Infection in industrialized countries occurs in a wide age distribution but the incidence rate of infection is significantly higher in children, especially children under four years of age (26, 132). This high incidence rate may be due to the

increased likelihood of parents to seek medical attention when symptoms appear in their child. Higher rates of *C. jejuni* infection also occur during young adulthood and in the elderly (3, 132). In contrast, *C. jejuni* infection in developing countries usually results in a less severe form of disease and may even be asymptomatic (36). Disease rarely occurs in the adult population and is generally limited to young children under 5 years of age that have not been previously exposed to *C. jejuni*. Exposure at a young age may offer protection against disease upon subsequent exposure to the bacterium later in life (36).

The true incidence rate of *C. jejuni* infection is thought to be underreported since the majority of people who become ill usually do not seek medical treatment and a large portion of those that seek treatment are not cultured for *C. jejuni*. Thus, epidemiological studies have been performed to account for these factors and have estimated that the number of cases of *C. jejuni* each year in the United States may be as great as two million (147). The incidence of *C. jejuni* in other parts of the world can vary significantly from country to country, and frequency comparisons between countries can be difficult without a standardized system for reporting cases. In New Zealand, the incidence rate of *C. jejuni* has increased greatly in the past two decades to more than 400 cases per 100,000 persons, surpassing the rates of other countries around the globe (7, 88). In contrast, other countries such as Italy or Portugal did not report a single case of *C. jejuni*, making it difficult to appreciate the epidemiology of *C. jejuni* throughout

the world (88). Nevertheless, *C. jejuni* is a pathogen of significant importance to public health as it is prevalent throughout the world and can be contracted easily by consuming contaminated food products, contact with animals carrying the bacterium, or exposure to environmental reservoirs.

Clinical Presentation of Infection

Infection with *C. jejuni* results in an enteritis that develops into an acute diarrheal disease. The infective dose of *C. jejuni* has been observed to be quite low with as few as 500 to 800 organisms capable of establishing infection in humans (16, 143). Upon infection with *C. jejuni*, there is an incubation period lasting approximately three days before symptoms develop (19, 88). Initial symptoms may include fever and severe abdominal cramping followed by the development of diarrheal disease. Flu-like symptoms including nausea, vomiting, headache, and myalgia have also been observed in some patients. Depending on the severity of infection, enteritis can range from moderate, watery diarrhea to severe, bloody diarrhea lasting about three to four days (17, 20, 37). Clinical pathology studies have shown that patients infected with *C. jejuni* develop inflammation of the colon and rectum, which is likely due to an infiltration of neutrophils and lymphocytes throughout the lamina propria (20, 37, 121, 167).

Fortunately, *C. jejuni* infection is a self-limiting disease and patients usually make a full recovery without treatment, but fluids and electrolytes may be

administered to assist in recovery (19). In some cases of severe disease, antimicrobial therapy may be necessary to clear the infection and expedite recovery. The most common antibiotic regimens for *C. jejuni* infection include the macrolide, erythromycin, or fluoroquinolones. However, the development of increasing resistance to fluoroquinolones in recent years has resulted in more common treatment with erythromycin (3, 19). Without antibiotic treatment, *C. jejuni* can be shed in the feces for several weeks even though disease symptoms may have completely resolved (19).

Secondary Sequelae Associated with Infection

Even though *C. jejuni* infections are usually self-limiting with patients making a full recovery within about a week, secondary sequelae associated with *C. jejuni* enteritis have been observed. A reactive arthritis of the knees and joints known as Reiter syndrome is known to occur as a post-infectious complication (88). However, Guillain-Barré Syndrome (GBS) is the most common secondary sequelae associated with *C. jejuni*, occurring at a frequency of about one in every 1,000 infected individuals (2, 87). Studies have found that approximately 66% of patients with GBS had a previous gastrointestinal or respiratory infection and it is estimated that 20–50% of these patients had a previous infection with *C. jejuni* (65, 87). Thus, previous exposure to *C. jejuni* is one of the leading risk factors for GBS.

GBS is an autoimmune disorder that results in an acute paralysis of the peripheral nervous system (65). Symptoms of GBS usually begin to develop about one to three weeks after recovery from *C. jejuni* enteritis, in which a numbness or tingling sensation develop in the arms or legs followed by the loss of strength. This gradual weakness spreads throughout the body and can result in paralysis of the limbs, trunk, face, pharyngeal, and tongue musculature. The length of paralysis varies from patient to patient but can last for several weeks to months (87). Most patients make a complete recovery but in some cases symptoms may persist for several years. Complications involving the respiratory tract may occur in some cases, resulting in a 2–3% mortality rate (87, 88).

Molecular mimicry is likely the mechanism by which infection with *C. jejuni* leads to an autoimmune disorder (190). Studies have shown that the lipooligosaccharide (LOS) of *C. jejuni* contains modifications that mimic host nerve ganglioside structures. Yuki *et al*. demonstrated that when rabbits are immunized with the LOS of *C. jejuni* the subsequent antibody response is cross-reactive to rabbit gangliosides which leads to limb weakness and paralysis (191). Therefore, it has been suggested that infection of humans with *C. jejuni* can result in an antibody response against bacterial LOS which can cross-react with human gangliosides causing damage to neurons in the peripheral nervous system, to result in paralysis (87).

The development of GBS in *C. jejuni*-infected individuals has hindered studies using human volunteers to explore virulence mechanisms of the bacterium. These same issues are also hampering studies involving the development of an effective vaccine against *C. jejuni* for human use. However, a recent study has identified two strains of *C. jejuni* that lack the genes necessary for the synthesis of ganglioside mimics which could potentially be useful in human trials for vaccine candidates (138). Additional studies must be performed to determine if vaccination strategies using these strains would offer similar levels of protection against other virulent strains of *C. jejuni*.

Pathogenesis of *C. jejuni* Infection

C. jejuni must overcome several host barriers in order to initiate and maintain successful colonization. After a susceptible host is exposed to *C. jejuni* either through consumption of contaminated meats, milk, or water, the ingested bacteria must survive passage through the harsh, acidic environment of the stomach. Upon arriving in the intestinal tract, *C. jejuni* is dependent on the corkscrew-like motility conferred by the rapid rotation of the flagella to move through the viscous mucus lining the intestinal epithelium (48). Lastly, the bacteria may adhere to and penetrate through the intestinal epithelium to invade the underlying lamina propria.

The exact mechanism of *C. jejuni* pathogenesis has not yet been elucidated, however a potential mechanism based on the cumulative knowledge from pathogenesis studies has been postulated by Hu and Kopecko (84). Studies have shown that *C. jejuni* adheres to intestinal epithelial cells *in vitro*, facilitating penetration and invasion of the bacterium through the epithelium (89, 104, 106, 135, 145, 167). Furthermore, clinical pathology studies have observed that *Campylobacter* enteritis results in invasion and inflammation throughout the rectal mucosa (17, 37, 121). From these combined observations we can speculate that adherence and invasion may induce the production and secretion of toxins, such as the cytolethal distending toxin (Cdt), altering fluid absorption within the intestinal tract and resulting in inflammation and diarrheal disease (177). Further investigations are needed to fully understand the mechanisms of *C. jejuni* pathogenesis, however previous studies have made great strides in revealing many interesting and important factors involved in the infectious process.

Potential Virulence Factors

The virulence mechanisms of *C. jejuni* are poorly understood largely due to the fact that progress has been hindered by the lack of a convenient animal model to study pathogenesis. However, the completion of the genome sequence of *C. jejuni* in 2000, and the subsequent sequencing of additional strains of *C. jejuni* has greatly benefited studies of pathogenesis (51, 82, 134). Analysis of the

genome sequence and the use of *in vitro* model systems have led to the identification of some potential virulence factors and characterization of their putative role during pathogenesis.

Flagella and flagellar motility

Flagella and flagellar motility have been shown to be essential virulence factors in promoting the pathogenesis of *C. jejuni*. Motility of *C. jejuni* is conferred by a single flagellum located at one or both poles of the bacterium (43). Compared to other motile bacterial species, the flagella of *C. jejuni* facilitate increased movement through highly viscous intestinal mucus which is advantageous during colonization (48). Additionally, the ability of *C. jejuni* to invade intestinal epithelial cells *in vitro* is dependent on the presence of flagella (59, 172, 185). Studies using human volunteers have been performed to examine the role of the flagellum in promoting successful *C. jejuni* infection. Non-motile bacteria that lack flagella cannot efficiently colonize the gastrointestinal tract of humans, and thus are unable to initiate and maintain an association with the mucus layer of the intestinal epithelium (16).

Not only do flagella confer motility to *C. jejuni* but the flagellar export apparatus is also responsible for secreting non-flagellar proteins. CiaB and other related Cia proteins (CiaA-H) promote invasion of *C. jejuni* into intestinal epithelial cells, and it has been shown that a functional flagellar export apparatus

is required for secretion of these proteins (106, 107). More recently, FlaC which has some homology to the flagellin proteins, FlaA and FlaB, but is not required for flagellar biosynthesis was also found to promote invasion of *C. jejuni*. This protein is also dependent on the flagellar export apparatus for secretion (160).

Another important virulence mechanism associated with the flagellum of *C. jejuni* is that its production is susceptible to phase variation. Phase variation of the flagellum allows the bacterium to switch from a flagellated, motile phase to an aflagellated, non-motile phase (72). This phenomenon occurs by altering the length of homopolymeric adenine or thymine (A or T) tracts in *flgR*, which encodes the response regulator of a two-component system, FlgSR, in *C. jejuni* that is required for the transcription of σ^{54}-dependent flagellar genes (72, 76). Additionally, the gene for the cognate histidine kinase, *flgS*, undergoes random mutation events that either shorten the length of homopolymeric A tracts by one nucleotide or result in the loss of a heteropolymeric repeat in the coding region of *flgS* (73). Phase-variation and random mutation events that affect flagellar biosynthesis may be advantageous for the bacterium to adapt to the different conditions encountered during infection, to escape an immune response, or to facilitate transmission.

Capsule

Questions regarding the possible presence of a capsular polysaccharide in *C. jejuni* were stimulated during the genome sequencing project in which a locus with significant homology to the capsular polysaccharide biosynthesis genes of other bacterial species was identified (96, 134). This observation was initially met with uncertainty because a capsule had not been previously observed in *C. jejuni* (97). However, the independent disruption of three genes in the putative capsular polysaccharide biosynthesis locus resulted in the loss of the capsular polysaccharide which was originally thought to be the O-antigen of the lipopolysacchride (LPS) of *C. jejuni* (98). These studies also revealed that the majority of *C. jejuni* strains produce a LOS that lack the O-antigen repeats typically found in LPS (98). Since the discovery of the capsular polysaccharide of *C. jejuni*, *in vitro* and *in vivo* studies have revealed its importance as a likely virulence factor during infection.

Studies have demonstrated that capsule production is also subject to phase variation, a phenomenon which likely has a role in evasion of the host immune response (6). The capsule has also been shown to be important in adherence to and invasion of host cells *in vitro* and in promoting resistance to human sera. Studies examining the effect the capsule has on pathogenesis of *C. jejuni* in a ferret model revealed that ferrets infected with a capsular polysaccharide mutant were less likely to develop diarrhea as compared to ferrets infected with wild-type

C. jejuni (6). Currently, promising vaccines against capsular polysaccharide are being developed and have been shown to induce a robust immune response in a mouse challenge model as well as provide protection against disease development upon subsequent infection (125).

Cytolethal distending toxin

A major potential virulence factor produced by *C. jejuni* is the cytolethal distending toxin (Cdt). Several other pathogens have been found to produce Cdt, including other *Campylobacter* species, pathogenic *Escherichia coli*, *Salmonella enterica* serovar Typhi, *Shigella dysenteriae*, *Helicobacter hepaticus*, and *Haemophilus ducreyi*, however the role of this toxin during pathogenesis is not completely understood (55). *In vitro*, the Cdt of *C. jejuni* causes cell cycle arrest during the transition between the G_2 phase and M phase before mitosis is initiated (177). The functional toxin is comprised of three proteins CdtA, CdtB, and CdtC. All three proteins are needed in concert to interact and form an active holotoxin complex, with CdtB functioning as the active subunit, and CdtA and CdtC likely functioning as binding proteins to assist in delivery of CdtB into target cells (109).

In vitro studies using human and avian cell lines have revealed that the absence of Cdt does not have an effect on the ability of *C. jejuni* to adhere to or invade avian cells. Additionally, disruption of each of the Cdt subunits does not have an effect on colonization of the chick ceca at day 7 post-infection (15). In

contrast, *in vitro* studies using human cell lines have suggested that Cdt may play a role in the invasion and intracellular survival of *C. jejuni* (15). Cdt has also been found to mediate the secretion of the pro-inflammatory chemokine, IL-8, from human intestinal cell lines upon infection with *C. jejuni* (80) and induce apoptosis in a human monocyte cell line (79). Furthermore, specific anti-Cdt neutralizing antibodies are only elicited during human infection and not during chicken infection (1). Together these studies suggest that Cdt may be an important virulence determinant during infection of humans but does not play a significant role in maintaining commensal colonization.

O- and N-linked glycosylation

The genome of *C. jejuni* encodes two independent glycosylation systems allowing both *O*-linked and *N*-linked glycosylation. *O*-linked glycosylation modifies serine or threonine residues on flagellin, whereas *N*-linked glycosylation involves modification of asparagine residues on proteins (165). The role of flagellin glycosylation as a virulence determinant is still being investigated, however studies have shown that glycosylation is necessary for flagellar biosynthesis as disruption of components of the flagellin glycosylation locus results in non-motile and non-flagellated bacteria (58). Decreased adherence to and invasion of host epithelial cells *in vitro* and reduced virulence in ferrets has also been observed in flagellin glycosylation mutants (62, 99). However, it is

difficult to discern if these defects are a direct result of disrupting glycosylation or simply due to disruption of flagellar biosynthesis. In addition to the phase-variable mechanisms of flagellar biosynthesis, as described above, glycosylation of flagellin is also phase variable via slipped-strand mispairing mechanisms which cause reversible non-motile and motile phenotypes that may be beneficial in adaptation to different environmental conditions (91).

In contrast, the role of *N*-linked glycosylation of *C. jejuni* proteins is not very well understood. *N*-linked glycosylation is conserved in all strains of *C. jejuni* as well as other *Campylobacter* species and may have a role in promoting virulence (165). Disruption of protein glycosylation can reduce the adherence and invasion efficiency of *C. jejuni* to intestinal epithelial cells *in vitro*, and negatively affects colonization in chicks and mice (75, 91, 164). However, the majority of glycosylated proteins have been found to be periplasmic, and thus the importance of these modifications to the pathogenicity of *C. jejuni* still remains to be investigated (165, 189).

Animal Models of Pathogenesis

Identifying a convenient animal model of *C. jejuni* that mimics the enteritis observed in humans has been challenging as many animals can be colonized with the bacterium but do not develop disease. Initial human volunteer studies were useful in identifying flagellar motility as a vital factor for promoting

infection, however the discovery of *C. jejun*i-associated GBS abruptly halted progress of identifying virulence factors important in human infection (16, 65). The identification and characterization of virulence factors has predominantly been performed *in vitro* and thus, a model system to study disease progression and mechanisms of virulence *in vivo* would be beneficial. While several different animal models to study *C. jejuni* pathogenesis have been developed, there is currently not an ideal model prominently accepted by the *Campylobacter* field. Some animal models that have been investigated to date include mice, hamsters, ferrets, rabbits, dogs, pigs, and non-human primates (120). Progress has been made in understanding a few virulence determinants and some aspects of innate immunity that influence infection, however the majority of these model systems have been unsuccessful in consistently reproducing the same disease symptoms that occur in humans.

The model system that most closely mimics symptoms of human enteritis upon infection with *C. jejuni* is the ferret. This animal model initially appeared to have potential as ferrets can become colonized with *C. jejuni* for up to 12 days and develop diarrhea lasting 2-3 days (12, 52). However, in order for successful colonization to occur, ferrets must be challenged with a very high inoculum of *C. jejuni*, and sodium bicarbonate and opium must be administered to neutralize stomach acid and reduce gut peristalsis, respectively (6, 52). Furthermore, this model is expensive and results can be variable and difficult to reproduce. The

lack of suitable reagents and knockout strains lacking certain host factors for immunological studies also limit the analysis of the interaction between the host and pathogen during infection.

The mouse may be the best available animal model for analyzing virulence due to its convenience, availability, and well-characterized immune system, yet it is still not an ideal system. *C. jejuni* can colonize the intestinal tract of wild-type mice upon administration of a high inoculum of at least 10^8 cfu, but the resulting colonization is transient and does not result in intestinal pathology or disease development. Recently, several groups have investigated the use of mice with limited enteric flora or immunocompromised mice to study *C. jejuni* pathogenesis (35, 119, 175). Mice with limited enteric flora can be consistently and reproducibly colonized with *C. jejuni*, however this colonization occurs without the development of inflammation or symptoms of disease (35). Limited gut flora mice colonized with mutants of *C. jejuni* lacking factors known to play a role in virulence such as motility and chemotaxis were defective in establishing colonization of the intestinal tract, suggesting that this model system may be valuable in identifying colonization determinants of *C. jejuni* (35).

Other groups have investigated murine models that lack various components of the innate immune system to examine if *C. jejuni* can stably colonize these animals and if this colonization results in enteritis resembling human infection. Mice deficient in MyD88, an important signaling component

for most toll-like receptors (TLR), can be stably colonized with a high inoculum of *C. jejuni*, but no pathological signs of disease are apparent (175). In contrast, mice deficient in IL-10, a regulatory cytokine that suppresses the effector function of macrophages, T cells, and NK cells, develop colitis but not a diarrheal disease upon infection with *C. jejuni* (119, 126). While immunocompromised or limited gut flora murine model systems do offer insights into particular aspects of disease progression, ultimately there are still limitations to what can be gleaned from these studies. Furthermore, while manipulation of the immune system or the gut flora allows *C. jejuni* to stably colonize these mice, the symptoms and pathology do not mimic those observed during human infection. Further studies will need to be performed to examine the feasibility and reproducibility of these model systems and their usefulness in studying mechanisms of pathogenesis, and in identifying and characterizing important virulence determinants.

Immune Response to *C. jejuni* During Infection

Immune detection and evasion

The first line of defense against infection with *C. jejuni* involves the innate immune system, in which pattern recognition receptors (PRRs) recognize microbial-associated molecular patterns (MAMPs) to induce the proper immune response to clear the pathogen. A class of intracellular cytoplasmic PRRs,

nucleotide-binding oligomerization domain (NOD) proteins, recognize peptidoglycan components of both Gram-negative bacteria (NOD1 and NOD2) and Gram-positive bacteria (NOD2) and may have a role in recognizing *C. jejuni* (34, 86). There is evidence that NOD1 is important in promoting microbial defense against *C. jejuni* as reduced expression of NOD1 results in an increased survival of *C. jejuni* within human intestinal epithelial cells (195). NOD2 may also have a role during infection with *C. jejuni* however this role is minimal compared to the activity of NOD1 (195).

C. jejuni is also capable of avoiding immune recognition by some common PRRs. TLR5 recognizes the evolutionarily conserved sites of bacterial flagellin allowing recognition of the bacterium by the host innate immune system (69). However, the flagellin of *C. jejuni* lacks the TLR5 recognition sites that are conserved in the majority of flagellins of other bacterial species (4). Thus, not only can the flagella of *C. jejuni* promote infection but this structure can also evade recognition by the host immune system. Furthermore, *C. jejuni* is an A/T rich organism and thus TLR9, which recognizes CpG dinucleotides, may not be efficiently stimulated during infection with *C. jejuni* (39). However, MyD88, a signaling molecule involved in the majority of TLR pathways, has been shown to be important in clearance of *C. jejuni* during colonization in mice, and thus it is likely that other TLRs are important in the recognition of *C. jejuni* (175).

Phagocytosis

Upon invasion of the intestinal epithelium, *C. jejuni* likely encounters dendritic cells and macrophages, however conflicting results have been reported regarding the ability of *C. jejuni* to survive within human monocytes (101, 173). Kiehlbauch *et al*. observed that after phagocytosis, *C. jejuni* is able to survive within human monocytes for six to seven days (101). In contrast, a subsequent study using human peripheral blood monocytes from multiple donors determined that *C. jejuni* was efficiently killed within 24 to 48 hours post-infection, suggesting that *C. jejuni* are not capable of intracellular survival (173). The conflicting observations may simply be due to the difference in treatment of cells, as monocytes capable of killing intracellular *C. jejuni* were stimulated with cytokines prior to infection whereas monocytes incapable of killing *C. jejuni* were not. Additionally, several studies using intestinal epithelial cell lines have found that *C. jejuni* can be detected intracellularly for more than three days post-infection, however the bacteria begin to lose viability within the first 24 hours after infection (29, 40, 41, 105). Thus, there is still uncertainty regarding the ability of *C. jejuni* to survive and persist within human monocytes *in vitro*. Furthermore, the ability of *C. jejuni* to survive intracellularly during infection *in vivo* still remains to be examined.

Innate immune response

Several studies have found evidence of pro-inflammatory cytokine secretion during infection with *C. jejuni*. *In vitro* studies using human monocyte-derived dendritic cells or a THP-1 human monocyte cell line found that infection with *C. jejuni* results in the induction of several pro-inflammatory cytokines including the interleukins (IL), IL-1α, IL-1β, IL-6, and IL-8, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) (83, 92, 151). Additionally, secretion of IL-12 was observed during infection of human monocyte-derived dendritic cells suggesting a Th1-polarized adaptive immune response (83). Human intestinal epithelial cells have also been found to secrete the cytokines IL-8 and TNF-α in response to infection with *C. jejuni* (80, 193, 195). Cdt may play a role in this induction of IL-8 as all three components (CdtA, CdtB, and CdtC) are needed for the secretion of IL-8 in intestinal epithelial cells (80, 193).

The role of human β-defensins, a family of antimicrobial peptides, has also been explored revealing that infection of human intestinal epithelial cells with *C. jejuni* induced increased expression of genes encoding these factors (194, 195). Furthermore, recombinant β-defensins were found to be bactericidal to *C. jejuni* by disrupting the integrity of the bacterial membrane (194). Additional studies must be performed to examine the importance of other β-defensins as well as antimicrobial peptides such as cathelicidins during *C. jejuni* infection.

Humoral immune response

Detection of *C. jejuni* by the innate immune system and production of pro-inflammatory cytokines in response to infection leads to the generation of an adaptive immune response against *C. jejuni*. The humoral response is generated to a number of different antigens of *C. jejuni* including flagellin and the major outer membrane protein (MOMP) (31). Within six to seven days after the onset of illness, circulating antibodies to *C. jejuni* can be detected (88). During the first two to three weeks after exposure to *C. jejuni* serum IgM, IgG, and IgA levels peak (18). Serum IgM and IgG levels decline over the course of approximately two or three months, respectively. Alternatively, IgA levels decline more rapidly returning to normal less than one month after the onset of illness, suggesting the importance of IgA at early time points after the onset of illness (18, 77, 162).

Previous studies have shown that infection with *C. jejuni* may lead to protective immunity against subsequent infections (21, 90). Surveys and serological tests have been performed during outbreak cases of *C. jejuni* in which contaminated milk was likely the source of infection. Through these studies it was revealed that subjects who did not have any previous exposure to raw milk became ill upon infection with *C. jejuni*, whereas subjects that had previously consumed raw milk were asymptomatically infected. Furthermore, previous exposure to raw milk correlated with elevated antibody levels to *C. jejuni* upon

subsequent exposure to unpasteurized milk (21, 90). In separate studies, human volunteer experiments revealed that upon re-challenge with *C. jejuni* one month after initial exposure patients were protected against illness, however *C. jejuni* could still be recovered from the stool of the majority of patients (16). These studies suggest protective immunity develops, however the length of protective immunity or the level of cross-protection between different strains of *C. jejuni* was not addressed.

Commensal Colonization of Animals

In contrast to its relationship with human hosts, *C. jejuni* can colonize the intestinal tract of many animals of agricultural importance including bovine, swine, and avian species as well as household pets such as dogs or cats, without resulting in the development of disease symptoms (66, 68). The colonization of poultry is of particular importance as it is the primary means by which *C. jejuni* is transmitted to humans (53, 180). *C. jejuni* is found in poultry flocks at extremely high rates due to the ease of horizontal transmission throughout the flock, likely by coprophagy. Chicks initially hatch free of *C. jejuni* but become colonized within the first two to three weeks after hatch and can remain colonized until the time of slaughter which is usually around eight weeks of age (85, 111, 137). Surveillance studies have estimated that 67–83% of poultry meats in retail grocery stores are contaminated with *C. jejuni* (67, 93, 192). Thus, thoroughly

cooking meat and the proper handling of raw poultry meats are of extreme importance to reduce the risk of infection.

Chickens are the preferred model for studying commensal colonization as avian species are natural hosts of *C. jejuni* (11, 94, 161). The infectious dose of *C. jejuni* is quite low with an inoculum of less than 100 organisms sufficient to efficiently colonize the intestinal tract (14, 32, 44, 75, 94, 163, 188). *C. jejuni* primarily colonizes the intestinal tract of chickens with a particular affinity for the large intestine and the ceca. Chickens contain two cecal sacs that branch off of the large intestine, as depicted in Figure 2. Transmission electron microscopy revealed that *C. jejuni* mainly associates with the mucus layer lining the intestinal tract (11, 123). There is also some evidence that *C. jejuni* can spread to the spleen and liver, however this is not well characterized and ultimately does not affect the outcome of this commensal relationship as no symptoms of disease or changes in pathology are present during colonization (11, 38, 103, 122, 188). The interactions between *C. jejuni* and the avian host required for the development of a commensal relationship rather than disease are still not well understood and will be discussed in Chapter 4.

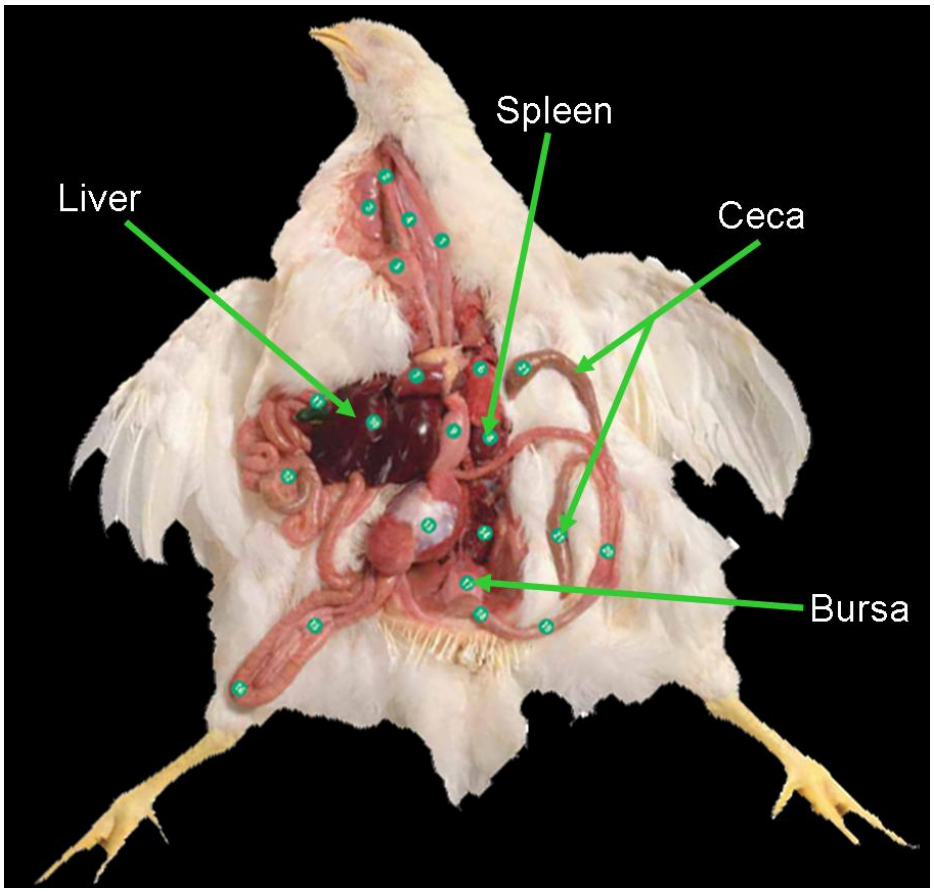

Figure 2. Diagram of the chicken anatomy. Organs of interest in this dissertation including liver, spleen, ceca, and bursa (indicated by arrows). Image is adapted from Lohmann Animal Health International and used with permission.

Potential Colonization Factors

To better understand how *C. jejuni* creates a commensal relationship with avian species, many studies have identified and characterized factors of *C. jejuni* involved in colonization of the intestinal tract. Genetic selections and direct mutagenesis approaches have been used to identify factors of *C. jejuni* involved in promoting efficient colonization of chickens. A study involving signature-tagged transposon mutagenesis identified 22 different genes of *C. jejuni* involved in colonization of the chick ceca including factors involved in motility, chemotaxis, protein glycosylation, amino acid transport, and factors of unknown function (75). This study also revealed the importance of a putative cytochrome *c* peroxidase (CCP), termed *docA* for determinant of chick colonization, in promoting efficient cecal colonization. When chicks are infected with a Δ*docA* mutant, a severe defect in cecal colonization is observed when compared to wild-type *C. jejuni* (75). The role of DocA and a paralogue, Cjj0382, during colonization has been characterized and will be discussed in Chapter 5.

Flagellar motility is likely one of the most studied factors that is known to be required to promote and maintain chick colonization. Disruption of the components of the flagellum or regulators of flagellar gene expression leads to significant defects in cecal colonization and persistence (72, 75, 130, 174, 182). The presence of capsular polysaccharide is also vital during chick colonization. A capsule-deficient mutant of *C. jejuni* has a severe defect in colonization being

unable to maintain colonization after 1 week post-infection in a 1-day old infection model (91).

Many response regulators of *C. jejuni* and presumably some genes of their regulons have also been found to have a role in commensal colonization using direct mutagenesis approaches (64, 113, 140, 181, 182, 186). Additionally, the fibronectin-binding protein (CadF) was found to be essential for cecal colonization as chicks infected with a *cadF* mutant were no longer colonized by day 7 post-infection (197). Factors involved in the invasion of human epithelial cells such as CiaB, have also been shown to be important during cecal colonization (196). Overall, many factors have been found to be important in promoting efficient colonization of the intestinal tract. However, the majority of studies mentioned above were limited to studying colonization at early time points after infection (less than 2 weeks post-infection) and predominantly focused on examining colonization of the intestinal tract. Our findings which will be discussed in Chapter 4 reveal an increased complexity of colonization factors, suggesting that heightened scrutiny needs to be applied when analyzing colonization factors as some may be required for long-term colonization or specific colonization of different organs. Collectively, the identification of different colonization factors of *C. jejuni* will hopefully provide valuable information regarding the interactions between *C. jejuni* and poultry that lead to a commensal relationship.

Immune Response to *C. jejuni* During Colonization of Poultry

Invasion and phagocytosis

The interplay between *C. jejuni* and the avian immune system during colonization that leads to the development of a commensal relationship is not understood. Studies have shown that during chick colonization, *C. jejuni* closely associates with the mucus layer overlying the intestinal epithelium (11, 110, 123). However, research is still being performed to understand the interaction between *C. jejuni* and avian epithelial cells during colonization and why this interaction results in a commensal relationship rather than the disease which is observed in humans. *In vitro* invasion assays revealed that *C. jejuni* can invade chicken intestinal cells as well as chicken kidney cells which are commonly used as a model for avian intestinal epithelial cells. However, these findings are surprising since *C. jejuni* colonization does not result in disease (28, 110, 156). One study has postulated that differences in mucus composition in chickens and humans may have a role in affecting the different outcomes of infection. Chicken intestinal mucus, but not mucus derived from the human intestine, has been shown to reduce the ability of *C. jejuni* to invade human intestinal cells suggesting that chicken mucus may provide protection against *C. jejuni* invasion *in vivo* (28).

Upon colonization of the intestinal tract, it is likely that *C. jejuni* may interact with innate immune cells such as dendritic cells or macrophages. Chicken peritoneal macrophages can readily phagocytose *C. jejuni* within 30 minutes with the majority of bacteria are no longer viable by 6 hours post-infection (129). Studies using an avian monocyte cell line revealed that *C. jejuni* can survive intracellularly for up to 8 hours but bacteria are no longer viable after 24 hours post-infection (156). While *C. jejuni* can invade chicken intestinal epithelial cells and survive within innate immune cells *in vitro*, this may not occur to the same extent *in vivo* as *C. jejuni* does not cause disease in chickens, and a noticeable damaging inflammatory response or significant systemic infection does not occur during colonization.

Innate immune response

In vitro studies measuring transcripts of chicken cytokines have shown that infection of chicken kidney cells (used as a model for chicken epithelial cells) or an avian monocyte cell line results in the induction of the pro-inflammatory cytokines, IL-1β, IL-6, and two IL-8 orthologues, CXCLi1, and CXCLi2 (156). Furthermore, a study examining the cytokine response in ileal and cecal tissue of chicks infected with *C. jejuni* revealed a strong induction of CXCLi1 and CXCLi2, and variable expression of IL-1β and IL-6, however this did not correlate with any changes in pathology (155). The findings of these studies are

unexpected and controversial because they suggest that upon recognition by the avian host an inflammatory response is stimulated. However, no significant changes in pathology during colonization with *C. jejuni* have been observed and disease does not develop. Additionally, the expression of the regulatory cytokines, IL-10 and TGF-β, have been investigated but their expression cannot be detected, suggesting commensal colonization is not due to down-modulation of the immune response by *C. jejuni* (155). These findings highlight the importance of increasing our understanding of the innate immune response to *C. jejuni* during avian colonization and how this interaction results in a commensal relationship.

Humoral immune response

Several studies have documented the development of antibody responses during colonization with *C. jejuni*. Chicks infected with *C. jejuni* 1-day post-hatch develop both mucosal and systemic immunoglobulin responses specific to *C. jejuni*. Systemic IgG levels peak 8 to 9 weeks post-infection whereas systemic IgA levels peak at 5 weeks post-infection (30, 128). Furthermore, mucosal IgA levels have been shown to increase significantly over the course of 4 weeks post-infection, and plateau at this level for the remainder of the 8-week experiment (128). The production of mucosal IgA may serve to prevent penetration of *C. jejuni* through the intestinal epithelium, limiting colonization to the mucus layer lining the epithelium (116). Even though specific systemic and mucosal

immunoglobulin responses can be generated to *C. jejuni*, this has no effect on the colonization levels in chicks (128).

Control and Prevention of Poultry Colonization

Methods of controlling *C. jejuni* colonization in poultry flocks are actively being addressed but due to the ubiquitous distribution of *C. jejuni*, effective control measures are not currently available. A recent study has concluded that 97% of *C. jejuni* cases are attributed to consumption of contaminated meats whereas wild animal and environmental sources of *C. jejuni* account for only 3% of cases (180). Thus, actively controlling the presence of *Campylobacter* in poultry could greatly reduce the incidence of human infection with *C. jejuni*. One method of controlling the transmission of *C. jejuni* within poultry flocks is to treat chicks orally with lytic bacteriophages. Administration of bacteriophages resulted in a three- to five-log reduction in cecal colonization as compared to *C. jejuni*-infected chicks that did not receive bacteriophage therapy (112, 171). This strategy could significantly reduce the transmission of *C. jejuni* throughout a poultry flock, however there is a risk of selecting bacteriophage-resistant *C. jejuni* in the environment.

Vaccinating poultry flocks against *C. jejuni* is another means of controlling poultry colonization. Several different vaccine strategies have been developed but have proven to be unsuccessful as only reduction in the levels of

colonization have been observed but not complete ablation of colonization (100, 141, 178, 183). Coordinating vaccine administration at an age that will confer the highest amount of protection with the time at which chicks become colonized on the farm is another factor that must be taken into account for the development of a successful vaccine. Thus, developing a vaccine for use in poultry may be challenging but has the potential to significantly reduce the transmission of *C. jejuni* from poultry to humans.

Conclusions

C. jejuni is a pathogen of significant importance to public health. *C. jejuni* is capable of promoting different biological relationships with multiple hosts, causing disease in humans but creating a harmless, asymptomatic colonization in many animals. Even though great strides have been made to understand the mechanisms by which *C. jejuni* causes diseases in humans, this relationship is still not very well understood. Additionally, even though many studies have examined colonization factors and the immune response to *C. jejuni* colonization in the natural avian host, how this commensal relationship develops is unknown. The work presented here serves to increase the understanding of how *C. jejuni* interacts with poultry to result in a commensal relationship. This was accomplished by directly examining the bacterial-host interactions between *C. jejuni* and poultry, which revealed previously uncharacterized and potentially

important niches for the bacterium in the avian host. Furthermore, the role of specific factors of *C. jejuni* during colonization were analyzed and indicated a role for peroxidases, capsule, Cdt, and catalase in various aspects of short- and long-term colonization. These findings may also provide insight into how these interactions may differ in humans to result in disease.

CHAPTER THREE

Materials & Methods[§]

Bacterial Strains and Growth Conditions

Campylobacter jejuni strain 81-176 is a clinical isolate that can cause disease in human volunteers and promote commensal colonization of the chick gastrointestinal tract (14, 16, 75, 108). *C. jejuni* strains were stored in 85% MH broth-15% glycerol solution at -80 °C. *C. jejuni* was typically grown on Mueller-Hinton (MH) agar containing 10 μg/ml trimethoprim (TMP) under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) at 37 °C. Antibiotics for *C. jejuni* were used at the following concentrations: chloramphenicol, 15 μg/ml; kanamycin 50 μg/ml; streptomycin, 0.1, 0.5, 1, 2, or 5 mg/ml; or cefoperazone, 30 μg/ml. *Salmonella enterica* serovar Pullorum (strain 8453, (144)) was grown in Luria-Bertani (LB) agar or broth or on Brilliant Green agar (Difco) at 37 °C. *S. pullorum* was stored in 80% LB broth-20% glycerol solution at -80 °C. *Escherichia coli* DH5α was grown in Luria-Bertani (LB) agar or broth at 37 °C. All stock *E. coli* strains were stored in 80% LB broth-20% glycerol solution at -80 °C. Antibiotics were used at the following concentrations as necessary:

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ampicillin, 100 μg/ml; chloramphenicol, 15 μg/ml; kanamycin, 50 μg/ml; or tetracycline, 12.5 μg/ml.

Biphasic Culture System

To examine the growth rates of different strains of *C. jejuni*, bacteria were grown from frozen stocks for 48 h on MH agar with TMP at 37 °C under microaerobic conditions. Strains were then streaked heavily on MH agar with TMP and grown for 16 h. Bacteria were resuspended from the plates and diluted to an OD_{600} 0.1 with MH broth. Ten milliliters of culture was added to T25 flasks containing 5 ml solidified MH agar and incubated under microaerobic conditions at 37 °C for 24 h. Growth rates were analyzed by monitoring the OD_{600} of each strain.

Construction of *C. jejuni* Mutants

Construction of deletion mutants

To delete *kpsM* from the chromosome of 81-176 Sm^R (DRH212; (74)), a 2.35-kb fragment including approximately 900 nucleotides upstream and 700 nucleotides downstream of the *kpsM* coding sequence was amplified from the chromosome of *C. jejuni* 81-176 by PCR using primers with 5' *Bam*HI restriction sites (82). This fragment was then ligated into *Bam*HI-digested pUC19 to create pDRH2457. In-frame chromosomal deletion mutants were constructed as

previously described (74). To interrupt *kpsM*, *kan-rpsL* (obtained from *Sma*I-digested pDRH437; (76)) was ligated into the *Spe*I site within *kpsM* in pDRH2457 to create pJMB103. PCR-mediated mutagenesis was used to create an in-frame deletion of *kpsM* (118). pDRH2457 was used as the template with primers designed to make an in-frame fusion of codon 1 to codon 239, deleting the intervening 237 codons to create pJMB113. DRH212 was transformed by electroporation with pJMB103 and transformants were recovered on MH agar with kanamycin to obtain JMB111 (81-176 Sm^R *kpsM*::*kan-rpsL*). This insertional mutant was then transformed by electroporation with pJMB113 and transformants that had replaced the *kpsM*::*kan-rpsL* with the in-frame deletion construct on the chromosome were selected on MH agar with streptomycin. One transformant was recovered and designated JMB206 (81-176 Sm^R Δ*kpsM*).

To delete the *cdt* locus (including *cdtA*, *cdtB*, and *cdtC*) from the chromosome of 81-176 Sm^R (DRH212; (74)), we initially cloned two DNA fragments containing portions of the *cdt* locus. A 2.1-kb DNA fragment extending from the start codon of *cdtA*, through the coding sequence of *cdtB,* and to the stop codon of *cdtC* was amplified from the chromosome of *C. jejuni* 81-176 by PCR using primers with 5' *Bam*HI restriction sites. This fragment was ligated into *Bam*HI-digested pUC19 to create pDRH577. A second fragment of 3.6-kb that includes approximately 750 nucleotides upstream and downstream of the *cdt* coding sequence was also amplified by PCR using primers with 5' *Bam*HI

restriction sites (82). This fragment was then ligated into *Bam*HI-digested pUC19 to create pLKB560. To interrupt *cdt*, *cat-rpsL* (obtained from *Sma*I-digested pDRH265; (74)) was ligated into the *Eco*RV site within the *cdt* locus in pDRH577 to create pDRH2646. PCR-mediated mutagenesis was used to create an in-frame deletion of *cdt*. pLKB560 was used as the template with primers designed to make an in-frame fusion of codon 72 of *cdtA* to codon 179 of *cdtC*, removing the intervening partial coding sequence for *cdtA* and *cdtC* and the entire coding sequence of *cdtB*. DRH212 was transformed by electroporation with pDRH2646 and transformants were recovered on MH agar with chloramphenicol to obtain LKB602 (81-176 Sm^R *cdt*::*cat-rpsL*). This mutant was then transformed by electroporation with pLKB561 and MH agar with streptomycin was used to select for transformants that had replaced *cdt*::*cat-rpsL* with the in-frame deletion construct on the chromosome of *C. jejuni* to create LKB624 (81-176 Sm^R Δ*cdt*).

DRH212 (81-176 Sm^R) and DRH1169 (81-176 Sm^R Δ*docA*) were constructed as described previously (74, 75). DRH1169 contains a deletion of codons 2 – 293 of *docA* from the chromosome of *C. jejuni*. To construct a deletion mutant of *cjj0382*, we first amplified a 2.8-kb fragment that includes approximately 800 nucleotides upstream and downstream of the *cjj0382* coding sequence from the chromosome of *C. jejuni* 81-176 by PCR using primers with 5' *Bam*HI restriction sites. This fragment was cloned into *Bam*HI-digested pUC19 to create pDRH308. In-frame, chromosomal deletion mutants were constructed as

previously described (74). To interrupt *cjj0382*, *cat-rpsL* (obtained from *Sma*I-digested pDRH265; (74)) was ligated into *Swa*I-digested pDRH308 to create pDRH1573. PCR-mediated mutagenesis was used to create an in-frame deletion of *cjj0382* (118). pDRH308 was used as the template with primers designed to delete the entire coding sequence of *cjj0382* to create pDRH1748. DRH212 was transformed by electroporation with pDRH1573 and transformants were recovered on MH agar with chloramphenicol to obtain LKB140 (81-176 Sm^R *cjj0382*::*cat-rpsL*). This insertional mutant was then transformed by electroporation with pDRH1748 and MH agar with streptomycin was used to select for transformants that had replaced *cjj0382*::*cat-rpsL* with the in-frame deletion construct on the chromosome of *C. jejuni.* This approach allowed the recovery of LKB151 (81-176 Sm^R Δ*cjj0382*).

An in-frame deletion mutant of *katA* was generated by first amplifying a 3.1-kb fragment with approximately 800 nucleotides upstream and downstream of the *katA* coding sequence from the chromosome of *C. jejuni* 81-176 using primers with 5' *Bam*HI restriction sites. This fragment was cloned into *Bam*HI-digested pUC19 to create pLKB106. An *Msc*I site was generated by PCR-mediated mutagenesis by making a C-to-G mutation at nucleotide 181 of the *katA* coding sequence to create pLKB141. This plasmid was then digested with *Msc*I for insertion of the *Sma*I-digested *cat-rpsL* cassette into the coding sequence of *katA* to create pLKB147. pLKB106 was also used for PCR-mediated mutagenesis to

make an in-frame fusion removing the entire coding sequence of *katA* to create pLKB131. DRH212 was transformed by electroporation with pLKB147 to create LKB236 in which *katA* has been interrupted with *cat-rpsL*. This insertional mutant was then transformed by electroporation with pLKB131 and transformants were recovered on MH agar with streptomycin to obtain LKB246 (81-176 Sm^R Δ*katA*).

For construction of 81-176 Sm^R Δ*docA* Δ*cjj0382*, LKB151 (81-176 Sm^R Δ*cjj0382*) was transformed by electroporation with pDRH1161 (pUC19 containing *docA*::*cat-rpsL*; (75)) to create LKB165 (81-176 Sm^R Δ*cjj0382 docA*::*cat-rpsL*). This strain was then transformed by electroporation with pDRH1147 (pUC19 containing Δ*docA*; (75)) resulting in LKB177 (81-176 Sm^R Δ*docA* Δ*cjj0382*). To create 81-176 Sm^R Δ*docA* Δ*katA*, DRH1169 (81-176 Sm^R Δ*docA*) was transformed by electroporation with pLKB147 to create LKB170 (81-176 Sm^R Δ*docA katA*::*cat-rpsL*). This strain was then transformed by electroporation with pLKB131 resulting in LKB181 (81-176 Sm^R Δ*docA* Δ*katA*). To create 81-176 Sm^R Δ*cjj0382* Δ*katA*, LKB151 was transformed by electroporation with pLKB147 to create LKB164 (81-176 Sm^R Δ*cjj0382 katA*::*cat-rpsL*). This strain was then transformed by electroporation with pLKB131 resulting in LKB231 (81-176 Sm^R Δ*cjj0382* Δ*katA*). Lastly, to generate an 81-176 Sm^R Δ*docA* Δ*cjj0382* Δ*katA* mutant, LKB181 (81-176 Sm^R

Δ*docA* Δ*katA*) was transformed by electroporation with pDRH1573 to create LKB213 (81-176 Sm^R Δ*docA* Δ*katA cjj0382*::*cat-rpsL*). This strain was then transformed by electroporation with pDRH1748 resulting in LKB226 (81-176 Sm^R Δ*docA* Δ*cjj0382* Δ*katA*).

Complementation of deletion mutants

The 81-176 Sm^R Δ*docA* and Δ*cjj0382* mutants were complemented *in trans* with pRY112 derivatives containing *docA* or *cjj0382* (184). Primers were designed with 5' *Bam*HI restriction sites to amplify DNA fragments containing *docA* and *cjj0382* with their putative promoter elements. Each fragment was digested with *Bam*HI and ligated into *Bam*HI-digested pRY112 to create pLKB260 and pLKB268 (containing *docA* or *cjj0382*, respectively). These plasmids or pRY112 (vector-only control) were then transferred to DH5α/pRK212.1 for subsequent conjugation into the 81-176 Sm^R Δ*docA* or Δ*cjj0382* mutants (50, 63).

Construction of heme-binding site point mutants

Heme-binding site (HBS) mutants were constructed by creating point mutations in the first cysteine and last histidine residue of the CXXCH motif of each binding site in *docA* and *cjj0382*, generating Cys-to-Ser and His-to-Ala mutants. All point mutants were generated by PCR-mediated mutagenesis using

pDRH765 (75) for *docA* HBS mutants or pLKB366 (pUC19 containing *cjj0382* with myc epitope at C' terminus) for *cjj0382* HBS mutants. For construction of pLKB366, pUC19::*cjj0382* was used for PCR-mediated mutagenesis to add the myc epitope to the C' terminal end of *cjj0382*. For construction of *docAC56S*, a G-to-C point mutation at nucleotide 167 in the *docA* coding sequence was generated, creating pLKB477. For construction of *docAH60A*, C-to-G and A-to-C point mutations at nucleotides 178 and 179 in the *docA* coding sequence were generated, creating pSMS176. For construction of *docAH203A*, C-to-G and A-to-C point mutations at nucleotides 607 and 608 in the *docA* coding sequence were generated, creating pSMS201. DRH1168 (81-176 Sm^R *docA*::*cat-rpsL*, (75)) was transformed by electroporation with pLKB477, pSMS176, and pSMS201 to create LKB506 (81-176 Sm^R *docAC56S*), LKB122 (81-176 Sm^R *docAH60A*), and LKB101 (81-176 Sm^R *docAH203A*), respectively. For construction of *cjj0382C80S*, a G-to-C point mutation at nucleotide 239 in the *cjj0382* coding sequence was generated, creating pLKB428 (pUC19 containing *cjj0382C80S*-myc). For construction of *cjj0382H84A*, C-to-G and A-to-C point mutations at nucleotides 250 and 251 of the *cjj0382* coding sequence were generated, creating pLKB429. For construction of *cjj0382C225S*, a G-to-C point mutation at nucleotide 674 in the *cjj0382* coding sequence was generated, creating pLKB430. For construction of *cjj0382H229A*, C-to-G and A-to-C point mutations at nucleotides 685 and 686 in the *cjj0382* coding sequence were generated, creating

pLKB431. LKB140 (81-176 Sm^R *cjj0382*::*cat-rpsL*) was transformed by electroporation with pLKB428, pLKB429, pLKB430, and pLKB431 to create LKB469 (81-176 Sm^R *cjj0382C80S*), LKB455 (81-176 Sm^R *cjj0382H84A*), LKB447 (81-176 Sm^R *cjj0382C225S*), and LKB450 (81-176 Sm^R *cjj0382H229A*), respectively.

Table 1. Bacterial strains used in this study.

Strain	Description	Reference
81-176	wild-type *C. jejuni*	(108)
DRH212	81-176 Sm^R	(74)
81-176 Sm^R derivatives		
DRH1168	*docA*::*cat-rpsL*	(14, 75)
DRH1169	Δ*docA*	(14, 75)
JMB111	*kpsM*::*cat-rpsL*	This study
JMB206	Δ*kpsM*	This study
LKB101	*docA H203A*	(14)
LKB122	*docA H60A*	(14)
LKB140	*cjj0382*::*cat-rpsL*	(14)
LKB151	Δ*cjj0382*	(14)
LKB164	Δ*cjj0382 katA*::*cat-rpsL*	(14)
LKB165	Δ*cjj0382 docA*::*cat-rpsL*	(14)
LKB170	Δ*docA katA*::*cat-rpsL*	(14)
LKB177	Δ*cjj0382* Δ*docA*	(14)
LKB181	Δ*docA* Δ*katA*	(14)
LKB213	Δ*docA* Δ*katA cjj0382*::*cat-rpsL*	(14)
LKB226	Δ*docA* Δ*cjj0382* Δ*katA*	(14)

Table 1. (continued)

Strain	Description	Reference
LKB231	Δ*cjj0382* Δ*katA*	(14)
LKB277	Δ*cjj0382* / pRY112::*cjj0382*	(14)
LKB307	Δ*docA* / pRY112	(14)
LKB310	Δ*cjj0382* / pRY112	(14)
LKB313	Δ*docA* / pRY112::*docA*	(14)
LKB455	*cjj0382 H84A*	(14)
LKB447	*cjj0382 C225S*	(14)
LKB450	*cjj0382 H229A*	(14)
LKB469	*cjj0382 C80S*	(14)
LKB506	*docA C56S*	(14)
LKB602	*cdt*::*cat-rpsL*	This study
LKB624	Δ*cdt*	This study

Table 2. Plasmids used in this study.

Plasmid	Description	Reference
pDRH265	pUC19::*cat-rpsL*	(74)
pDRH308	pUC19::*cjj0382*	(14)
pDRH437	pUC19::*kan-rpsL*	(76)
pDRH577	pUC19::*cdt* CDS	This study
pDRH765	pUC19::*docA*	(14, 75)
pDRH1147	pUC19::Δ*docA*	(14, 75)
pDRH1161	pUC19::*docA*::*cat-rpsL*	(14, 75)
pDRH1573	pUC19::*cjj0382*::*cat-rpsL*	(14)
pDRH1748	pUC19::Δ*cjj0382*	(14)
pDRH2457	pUC19::*kpsM*	This study
pDRH2646	pUC19::*cdt*::*cat-rpsL*	This study
pJMB103	pUC19::*kpsM*::*cat-rpsL*	This study
pJMB113	pUC19::Δ*kpsM*	This study
pLKB106	pUC19::*katA*	(14)
pLKB131	pUC19::Δ*katA*	(14)
pLKB141	pUC19::*katA*-M*scI*	(14)
pLKB147	pUC19::*katA*::*cat-rpsL*	(14)

Table 2. (continued)

Plasmid	Description	Reference
pLKB260	pRY112::*docA*	(14)
pLKB268	pRY112::*cjj0382*	(14)
pLKB366	pUC19::*cjj0382-myc*	(14)
pLKB428	pUC19::*cjj0382 C80S*	(14)
pLKB429	pUC19::*cjj0382 H84A*	(14)
pLKB430	pUC19::*cjj0382 C225S*	(14)
pLKB431	pUC19::*cjj0382 H229A*	(14)
pLKB477	pUC19::*docA C56S*	(14)
pLKB560	pUC19::*cdt*	This study
pSMS176	pUC19::*docA H60A*	(14)
pSMS201	pUC19::*docA H203A*	(14)

Chick Infections

Fertilized white leghorn eggs (SPAFAS) were incubated for 21 days at 37.8 °C under appropriate humidity and rotation of the eggs in a Sportsman II model 1502 incubator (Georgia Quail Farms Manufacturing Company). Chicks were housed in brooders and given food and water *ad libitum*. For infection of 1-day old chicks, birds were orally gavaged using a feeding needle with 100 µl of

wild-type *C. jejuni* 81-176, mutants of 81-176 Sm^R (DRH212; (74)), or *S. pullorum* approximately 12 to 36 hours after hatch. Infection of 14-day old chicks was performed identically to 1-day old chicks, except chicks were left uninfected until day 14 post-hatch. Some 14-day old chicks were sacrificed prior to infection to ensure that these chicks were not colonized by *C. jejuni*. Chicks were infected orally with 100 µl of MH broth. Colonization levels were assessed as described below.

Prolonged chick colonization assays

To prepare *C. jejuni* strains for inoculation, the bacteria were grown from frozen stocks on MH agar with TMP for 48 h at 37 °C under microaerobic conditions. The bacteria were restreaked on MH agar containing TMP and grown for 16 hours under the same conditions. Bacteria were resuspended from plates and diluted in MH broth to an OD_{600} of 0.4 to approximate 10^9 cfu per ml to create a high inoculum. For low inocula, bacteria were further diluted in PBS to approximate 10^3 cfu per ml. To prepare *S. pullorum* for inoculation, bacteria were grown from frozen stocks on LB agar at 37 °C overnight. Bacteria were then grown in LB broth overnight at 37 °C, diluted to an OD_{600} 0.48 in LB broth to approximate 10^9 cfu per ml. One hundred microliters (10^8 cfu) of these preparations (containing 10^8 or 10^2 cfu) were used to orally infect chicks.

One-day old chicks or 14-day old chicks were orally gavaged with *C. jejuni* or *S. pullorum* as described above. At 1, 4, 7, 14, 21, or 28 days post-infection, five to six chicks were sacrificed and the ceca, bursa, spleen, and liver were removed. Cecal contents were extracted and resuspended in PBS at a final concentration of 0.1 g per ml. The bursa, spleen, and liver from each chick was homogenized separately and resuspended in PBS to achieve a final concentration of tissue at 0.1 g per ml. Ten-fold serial dilutions were spread on MH agar with 10 μg/ml TMP and 30 μg/ml cefoperazone for *C. jejuni* or Brilliant Green agar (Difco) for *S. pullorum* to determine the levels of the bacteria in the organs. Blood samples were obtained by cardiac puncture and 100 μl of blood were spread on appropriate agar to evaluate the presence of *C. jejuni* or *S. pullorum* in the blood.

Cecal colonization assays

To assess the cecal colonization capacity of wild-type and mutant strains of *C. jejuni* 81-176 Sm^R, 1-day old chicks were orally infected with 100 μl of PBS containing approximately 10^2, 10^4, and 10^6 cfu of wild-type or mutant strains of *C. jejuni*. To prepare strains for infection, bacteria were grown from frozen stocks for 48 h on MH agar with TMP at 37 °C under microaerobic conditions. Strains were then streaked heavily on MH agar with TMP and grown for 16 h. Bacteria were resuspended from the plates and diluted to the appropriate

inoculum in PBS. Dilutions of the inocula were plated to determine the number of bacteria in the inoculation dose. At seven days post-infection, chicks were sacrificed and the cecal contents were recovered, resuspended in PBS, and dilutions were plated on MH agar containing 10 μg/ml TMP and 30 μg/ml cefoperazone to determine the number of *C. jejuni* per gram of cecal content.

Creation of Antisera

Polyclonal antiserum against C. jejuni

C. jejuni 81-176 was grown from freezer stocks onto MH agar containing TMP for 48 h at 37 °C under microaerobic conditions. Bacteria were restreaked onto two MH agar plates and grown for 16 h under the same conditions. Cells were resuspended from the plates in 5 ml of PBS, washed twice with PBS, and resuspended in 10 ml of 0.4% formalin in water. After overnight agitation at 4 °C, cells were collected by centrifugation, washed twice in PBS and resuspended in 1.25 ml of PBS. A 100 μl aliquot of the suspension was spread on MH plates with TMP to confirm that the preparation contained non-viable bacteria. Rabbits were immunized with this preparation to generate polyclonal antiserum against bacterial components by a commercial vendor (Cocalico Biologicals). The derived antiserum was designated α-*C. jejuni* 81-176 Rab527.

Polyclonal antisera against DocA and Cjj0382

Primers were designed to amplify the coding sequences of *docA* from codon 33 to the stop codon, *cjj0382* from codon 50 to the stop codon, and *atpF*, encoding the b subunit of ATP synthase, from codon 54 to the stop codon. In-frame *Bam*HI restriction sites were added to the 5' ends of the primers. After amplification from the chromosome of *C. jejuni* strain 81-176, the gene fragments were digested with *Bam*HI and ligated into *Bam*HI-digested pGEX-4T-2 (GE Healthcare). Transformation into *E. coli* BL21(DE3) allowed for recovery of plasmids designated pDRH2302 (containing a portion of *atpF*), pDRH2556 (containing a portion of *docA*), and pDRH2557 (containing a portion of *cjj0382*). For purification of GST-fusion proteins, 500 ml cultures were grown in LB at 37 ºC and induced with 1 mM IPTG for 4 hours. Bacteria were lysed with an EmusliFlex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in^2. Proteins were purified from the soluble fraction with Glutathione Sepharose 4B according to manufacturer's instructions (GE Healthcare). Each protein was then injected into five different mice for production of polyclonal antisera (Cocalico Biologicals).

Preparation of Tissue Samples for Histology and Immunofluorescent Confocal Microscopy

One-day old chicks were orally gavaged with approximately 10^8 cfu of *C. jejuni* or *S. pullorum* in 100 µl volume of MH or LB broth as described above. A

control group of chicks remained uninfected. At days 1, 4, 7, and 14 post-infection, three chicks from each infection group were sacrificed. The bursa and ceca from each chick were removed immediately. The ends of the ceca were removed and the cecal lumen was flushed with PBS followed by 10% formalin. Bursal and cecal tissues were immersed in 10% formalin for 48 h at room temperature with agitation. The formalin was then removed and the tissue was immersed in PBS until processing. All samples were processed in paraffin and sectioned at 5 μm. For histological examination, the tissue specimens were stained with hematoxylin and eosin by standard procedures. To examine heterophil infiltration, three chicks from each infection group at each time point were examined. For each chick, twelve separate fields were examined at 40X magnification to visually quantify the number of heterophils present. Data are reported as the average number of heterophils in a field for three chicks in each group.

Immunofluorescent Confocal Microscopy

Sectioned tissues in paraffin were heated at 58 °C for 30 min, deparafinized through washes of xylene, and then graded alcohols to water. Antigenic epitopes were retrieved by microwave twice in 1X antigen retrieval Citra solution (BioGenex) at 95 °C for 5 min. Citra was removed and slides were cooled to room temperature via several partial changes in distilled water over 20

min. Slides were then equilibrated in PBS for 5 min and tissue was permeabilized with 0.3% Triton X-100 in PBS for 5 min followed by three washes for 5 min in PBS to remove the detergent. Tissue autofluorescence was quenched with 10 mM glycine in PBS for 5 min followed by three washes in PBS for 5 min each. Excess PBS was then removed from the slides and non-specific binding was blocked with 3% normal goat serum in PBS for 30 min. After the incubation, the serum was decanted. α-*C. jejuni* Rab527 antiserum or α-*Salmonella* antiserum (Salmonella core antigen (5D12A); Santa Cruz Biotechnology) was diluted 1:100 in PBS and applied to tissues. Antiserum was incubated with tissue overnight at 4 °C in a humid chamber. The next day, slides were washed three times in PBS with excess PBS removed after the last wash. Goat α-rabbit FITC-conjugated secondary antibody (Santa Cruz Biotechnology) was diluted 1:100 in PBS for detection of *C. jejuni* and goat α-mouse FITC-conjugated secondary (Jackson Laboratories) was diluted 1:25 in PBS for detection of *S. pullorum*. These antibody solutions were applied to slides at room temperature for 30 min in the dark. Subsequently, slides were washed three times in PBS for 5 min in the dark. To counterstain specimens, slides were incubated in Hoescht diluted 1:5,000 in PBS for 2 min. Slides were then washed four times for 5 min in PBS, excess PBS was removed, and coverslips were mounted with Vecta Shield (Vector Laboratories, Inc.). Slides were stored at -20 °C until examined. Images of

specimens were obtained using a Zeiss Pascal laser scanning confocal microscope with Zeiss LSM5 software.

Intracellular Survival Assays After Phagocytosis by Avian HD11 Cells

Avian HD11 monocytes were routinely cultivated in T75 tissue culture flasks in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS) in a 5% CO_2 incubator (13). For infection studies, monocytes were removed from flasks by mild trypsinization (0.25% trypsin/EDTA solution) and seeded at 10^6 cells per well of a 48-well tissue culture plate in the presence of phorbol 12-myristate 13-acetate (PMA) at a final concentration of 50 ng/ml to activate the monocytes. After 48 h of incubation at 37 °C in 5% CO_2, the cells were centrifuged at 1300 rpm for 5 min and the media was replaced with fresh media without PMA. The cells were then allowed to rest for 24 h at 37 °C.

C. jejuni was prepared for intracellular survival studies by growing the bacteria from frozen stocks on MH agar containing TMP under microaerobic conditions at 37 °C for 48 h. The bacteria were then streaked on fresh MH agar containing TMP and grown for an additional 16 h at 37 °C under microaerobic conditions. The bacteria were suspended from the plates and diluted to OD_{600} of 0.4. For preparation of *S. pullorum*, the bacterium was grown from frozen stocks on LB at 37 °C overnight. The bacteria was then grown in LB broth at 37 °C overnight and then diluted to OD_{600} of 0.46. Both the diluted *C. jejuni* and *S.*

pullorum cultures were centrifuged and the bacteria were resuspended in RPMI 1640 with 10% FCS to achieve a bacterial concentration of approximately 2 x 10^8 cfu per ml.

For infections, resting activated monocytes were centrifuged for 5 min at 1300 rpm. The media was replaced with 0.5 ml of the bacterial preparations resuspended in RPMI 1640 with 10% FCS to achieve a final concentration of 10^8 cfu per well. This time point was designated -2 h of infection. Two hours after infection, the monolayer was washed three times by replacing media with 0.5 ml of fresh RPMI 1640 containing 10% FCS and centrifuging at 1300 rpm for 5 min. After the final wash, 0.5 ml of RPMI 1640 containing 10% FCS and 250 μg/ml gentamicin was added to each well to kill extracellular bacteria (time point designated 0 h of infection). The tissue culture plate was incubated in 5% CO_2 at 37 °C for the remainder of the assay. The number of viable bacteria associated with (-2 and 0 h of infection; before gentamicin treatment) or surviving in (2, 4, 6, 8, 24, 48, 72, and 96 h of infection; after gentamicin treatment) the activated monocytes was determined by harvesting the cells by mild trypsinization and plating dilutions on appropriate media. Each assay was performed in triplicate and the assay was repeated at least three times. The data from a representative assay are reported.

Real-Time RT-PCR Analysis

One-day old chicks were infected with 10^4 cfu wild-type *C. jejuni* 81-176 Sm^R (DRH212) as described above and total RNA was extracted with Trizol reagent (Invitrogen) from the cecal contents of chicks seven days post-infection and then treated with DNase prior to analysis. A final concentration of 50 ng/µl of RNA was used in a SYBR green PCR master mix. Real-time RT-PCR was performed using a 7500 Real Time PCR System (Applied Biosystems). Detection of mRNA for *gyrA*, encoding DNA gyrase, served as an endogenous control and the *in vivo* transcript levels of *docA* and *cjj0382* were compared to each other.

Table 3. Primers used for real-time RT-PCR analysis of *docA* and *cjj0382*.

Primer	Primer Sequence 5' – 3'
docA F	AACTTTATGGAGAAGTTACGGTAGAAAAC
docA R	ATAGCGATCAAAAGGAGAATTTGG
cjj0382 F	TTGCTGAAACTGCTCCATATTTTC
cjj0382 R	GCCAAGTTGCACACTACCCATT
gyrA F	CGACTTACACGGCCGATTTC
gyrA R	ATGCTCTTTGCAGTAACCAAAAAA

Fractionation of *C. jejuni* Strains

Fractionation of *C. jejuni* into subcellular compartments for analysis of localization of proteins was performed as previously described with slight modifications (159). Briefly, *C. jejuni* strains were grown from frozen stocks on MH agar with either TMP or chloramphenicol at 37 ºC under microaerobic conditions for 48 h. Strains were then streaked heavily on MH agar and grown for 16 h. After growth, each strain was resuspended in MH broth, and diluted to an OD_{600} 0.8. This method of preparing each strain was used for all fractionation procedures described below. For whole-cell lysates (WCL), 1 ml of bacterial culture was pelleted, washed once in PBS, and resuspended in 50 µl 1X SDS-PAGE loading buffer. To generate periplasmic and cytoplasmic fractions, 20 ml of bacterial culture were washed twice with 2 ml of PBS containing 0.1% gelatin (PBSG) and then resuspended in 2 ml of PBSG containing 20 mg/ml polymixin B sulfate (Sigma) to compromise the outer membrane and release the periplasmic contents. After centrifugation with a Sorvall Biofuge *pico* microcentrifuge at 16,000 × *g* for 30 min, the supernatant was saved and the recovered pellets consisted of whole spheroplasts. The supernatants were centrifuged again for 30 min at 16,000 × *g* to ensure removal of contaminating spheroplasts from the periplasmic fraction. The pellets consisting of whole spheroplasts were resuspended in 1 ml PBSG and sonicated with a Branson Sonifier 450 set at an amplitude of 4.5 with a constant duty cycle. Samples were kept on ice between

bursts and each sample was sonicated four times. Bursts were approximately eight seconds long with a 2 min resting period between each burst. After centrifugation at 16,000 × *g* for 30 min to remove insoluble membranes, the supernatants represented soluble cytoplasmic proteins. To obtain inner and outer membrane fractions, 5 ml aliquots of bacteria were pelleted and washed once with 1 ml of 10 mM HEPES (pH 7.4). Bacteria were then resuspended in 1 ml of 10 mM HEPES for sonication as described above and total membranes were recovered after centrifugation for 30 min at 16,000 × *g*. Membranes were resuspended in 10mM HEPES containing 1% *N*-lauroylsarcosine sodium salt to solubilize the inner membrane fraction. The soluble inner membrane proteins were separated from the insoluble outer membrane proteins by centrifugation for 30 min at 16,000 × *g*.

Protein Analyses

Protein homology and domain analyses

Homology searches were performed with BLASTP and PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Analyses of N-terminal signal sequences were performed with the SignalP 3.0 server (http://cbs.dtu.dk/services/SignalP/).

Detection of humoral immune response to infection

One-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* or *S. pullorum* as described above. For detection of the systemic IgG response, blood was collected via cardiac puncture from four *C. jejuni*- and *S. pullorum*-infected chicks at 1, 7, 14, and 28 days post-infection. Serum was recovered from each sample and stored at -20 °C. For detection of a mucosal IgA response, total IgA was extracted from contents of the large intestines of *C. jejuni*- and *S. pullorum*-infected chicks at 1, 7, 14, and 28 days post-infection as previously described for isolation of murine mucosal IgA from feces with slight modifications (8). Briefly, intestinal contents were weighed and resuspended in IgA extraction buffer (PBS with 0.05% Tween 20, 0.5% fetal calf serum (FCS), 2.7 mM EDTA, 0.6 mM PMSF and 0.2 mg per ml trypsin soybean inhibitor) to a final concentration of 0.1 g of contents per ml. Samples were incubated on ice for 20 min, and then centrifuged at 20,000 x g for 15 min at 4 °C. The supernatant was recovered and stored at -20 °C.

For preparation of whole cell lysates, *C. jejuni* or *S. pullorum* was grown as described previously for chick infections and diluted to an OD_{600} of 0.8. One milliliter of bacterial culture was pelleted, washed once in PBS, and resuspended in 100 µl of 1X SDS-PAGE loading buffer. Proteins from these entire WCL preparations were separated by SDS-PAGE using 10% polyacrylamide gels with a preparative comb. Immunoblot analysis was performed using a Miniblot System

C-Shell apparatus (Immunetics) which allowed multiple serum IgG or mucosal IgA samples to be analyzed on one membrane. To detect serum IgG, a 1:50 dilution of chick serum was used as the primary antibody and peroxidase-conjugated rabbit anti-chicken IgY (equivalent of mammalian IgG) at a dilution of 1:30,000 was used as the secondary antibody (Sigma). To detect the mucosal IgA, a 1:1 dilution of supernatant recovered from the extraction procedure with intestinal contents was used as the primary antibody and horseradish peroxidase-conjugated goat anti-chicken IgA at a dilution of 1:3,000 was used as the secondary antibody (AbD Serotec).

Localization of DocA and Cjj0382

Proteins were separated by SDS-PAGE using 10% acrylamide gels. For WCL, protein samples were loaded to represent the proteins recovered from 200 µl of bacterial cultures which had been equilibrated to the same density as described above. For the fractions representing outer membrane, periplasmic, inner membrane, and cytoplasmic proteins, the amounts loaded represented protein samples obtained from 400 µl of bacterial culture. To detect DocA or Cjj0382, a 1:1,500 dilution of murine α-DocA M10 antiserum or a 1:2,000 dilution of murine α-Cjj0382 M17 antiserum was used, respectively. For control immunoblots to detect known proteins contained within specific fractions, α-RpoA M1 at a dilution of 1:1,500 was used to detect RpoA, a cytoplasmic protein

(159); α-AtpF M3 at a dilution of 1:1,000 was used to detect AtpF, an inner membrane protein; and α-FlgP M1 at a dilution of 1:1,500 was used to detect FlgP, an outer membrane protein (159). Horseradish peroxidase-conjugated goat α-mouse immunoglobulin G antiserum was used as the secondary antibody (BioRad).

Arylsulfatase Assays

To determine the purity of the protein preparation derived from the periplasmic fractionation procedure, the amount of sample derived from an equivalent of 400 μl of bacterial culture described above was tested for arylsulfatase activity in assays similar to ones previously described (70, 76, 187). For comparison, we also analyzed the cytoplasmic fractions from an equivalent amount of bacterial culture. Samples were incubated in a final volume of 400 μl of 10 mM nitrophenylsulfate and 1 mM tyramine for 1 hour at 37 ºC. Reactions were stopped and the amount of nitrophenol released in each sample was determined by a spectrophotometric reading at OD_{410}. The obtained readings were compared to a standard curve of OD_{410} readings from known concentrations of nitrophenol to determine the number of arylsulfatase units produced by each sample. Each sample was tested in triplicate.

Heme Stain Assay

The method of Feissner *et al.* was used to detect heme bound to DocA and Cjj0382 in soluble periplasmic fractions (47). Approximately 25 μg of proteins isolated from the periplasmic fraction as described above, were mixed with SDS-PAGE loading buffer that lacked β-mercaptoethanol. Samples were then loaded onto 10% SDS-polyacrylamide gels without boiling the samples. Preparing the samples in this manner is considered a non-denaturing condition that is required for this assay so that heme compounds remain covalently bound to proteins. After the proteins were transferred to a nitrocellulose membrane, the membrane was washed three times with PBS and exposed to SuperSignal West Femto chemiluminescent substrate (Pierce Biotechnology) for 5 min. The peroxidase activity of the bound heme reacted with the chemiluminescent substrate to generate light which was detected upon exposure to film.

Peroxide Resistance Assays

Wild-type and mutants strains of *C. jejuni* were grown from frozen stocks on MH agar with TMP at 37 ºC under microaerobic conditions. Bacteria then were streaked heavily on MH agar and grown for 16 h, resuspended in MH broth and diluted to an OD_{600} 0.4. One ml samples of bacteria were treated with 0.5 mM hydrogen peroxide, cumene hydroperoxide, or *tert*-butyl hydroperoxide for 30 min at 37 ºC under microaerobic conditions. After incubation, serial dilutions

were plated on MH agar with TMP and the number of surviving bacteria relative to the number of viable bacteria at the beginning of the assay was determined. Each assay was performed three times in triplicate.

Statistical Analyses

Statistical analysis on results from chick colonization experiments was performed by the Mann-Whitney U test with a *p*-value of < 0.01 for analyses in Chapter 4 and < 0.05 for analyses in Chapter 5. A two-tailed, unpaired student's t-test ($p < 0.05$) was performed to determine statistically significant differences between heterophil infiltration of uninfected chicks and *C. jejuni*- or *S. pullorum*-infected chicks.

CHAPTER FOUR

Analysis of Prolonged Colonization of Avian Lymphoid Tissue and Transient Spread to Systemic Sites

Introduction

A current topic in *C. jejuni* biology has focused on analyzing the interactions occurring between the bacterium and the avian host that allow *C. jejuni* to reach high levels and persist in the intestinal tract over time without symptoms of disease. Extensive epidemiological studies have been performed to understand transmission and persistence of *C. jejuni* in chickens on the farm and how this natural infection contributes to contamination of meats intended for the human food supply (as reviewed in (150)). From these studies, it has been revealed that chicks hatch free of *C. jejuni* and usually become colonized with the bacterium during the first two weeks of life, likely due to coprophagy or consumption of contaminated environmental reservoirs. Laboratory studies have provided some characterization of the intestinal niches colonized by the bacterium and the innate and adaptive immune responses resulting from infection (11, 122, 128, 155). These studies have revealed that *C. jejuni* can colonize throughout the intestinal tract of the avian host, with the highest concentration of the bacterium located in the ceca (frequently above 10^9 cfu per gram of cecal content) (11, 75). Microscopic analyses of cecal specimens from chicks infected with *C. jejuni*

suggest that the bacterium is mainly located within the cecal lumen and in the mucus layer covering the cecal epithelium (11, 110, 123). Evidence for *C. jejuni* invading the avian intestinal tract *in vivo* is lacking. However, a few studies have found that *C. jejuni* can invade primary avian intestinal or kidney cells *in vitro* (28, 110, 156). Infection of these cells *in vitro* leads to increased expression of some pro-inflammatory cytokines (23, 110, 156). One study has observed an induction of pro-inflammatory cytokines and an increase in heterophil migration into cecal and ileal tissue in chicks infected with *C. jejuni* (155), but the significance of these *in vitro* and *in vivo* findings is puzzling since the bacterium does not cause apparent disease in chickens.

The ability of *C. jejuni* to colonize outside the intestinal tract and the levels of colonization and persistence at these sites has not been adequately addressed. A few studies have reported *C. jejuni* at extraintestinal sites such as the bursa of Fabricius, liver, spleen, gall bladder, and blood of infected birds, suggesting that *C. jejuni* can breach the intestinal epithelial barrier *in vivo* to some extent (11, 38, 94, 103, 122, 188). However, these studies were largely superficial, primarily indicating the presence or absence of bacteria with little, if any, extensive detail regarding the degree of colonization or the length of persistence at these extraintestinal sites. Furthermore, no studies have addressed where *C. jejuni* might invade the avian gut epithelium *in vivo* and how *C. jejuni* could potentially reach these systemic sites.

To obtain a better understanding of bacterial-host interactions that promote colonization and the development of commensalism we studied the global colonization dynamics of *C. jejuni* in chickens and the host response to infection during prolonged colonization (up to 4 weeks). We also performed a comparative analysis of the bacterial-host interactions that lead to commensalism by *C. jejuni* or progress to disease by *Salmonella enterica* serovar Pullorum (*S. pullorum*). *S. pullorum* is a strict avian pathogen that infects the intestinal tract and promotes both a diarrheal disease and a lethal systemic infection (158). Therefore, experiments with this bacterium serve as a positive control for determining attributes of infection associated with a pathogen. By comparing the infectious processes mediated by *S. pullorum* and *C. jejuni*, we could determine which activities of *C. jejuni* or the host response to *C. jejuni* infection are likely specific for the development of commensalism.

Other research efforts to enhance our knowledge of this commensal relationship have focused on identifying colonization factors of *C. jejuni* required for *in vivo* growth in poultry. By using a negative selection procedure with signature-tagged transposon mutants, we identified over 20 colonization factors of *C. jejuni* required for optimal growth in the chick ceca (75). Additional studies have analyzed defined mutants lacking various activities to identify other colonization factors. From these studies, flagellar motility (72, 73, 75, 130, 174, 182), certain metabolic pathways (10, 14, 61, 133, 168, 176), and transcriptional

regulatory systems (24, 64, 113, 140, 163) have been shown to be required for efficient colonization of the intestinal tract, more specifically the ceca. Although a few of these studies have analyzed persistence in the ceca for up to 5 weeks, most analyses of *C. jejuni* colonization in poultry have been limited to short periods of time (usually from one to 14 days). Thus, information regarding the dynamics of colonization within and potentially outside the intestinal tract over time and the colonization factors of *C. jejuni* required for persistence in various organs is lacking.

Through this study we have identified lymphoid tissue as a major reservoir for *C. jejuni* during persistent colonization. In addition to initiating and maintaining colonization of the ceca of 1-day old birds over time, we found that *C. jejuni* promotes a robust colonization of the mucus layer lining the bursa of Fabricius, a major gut-associated lymphoid organ, which can be maintained for at least 4 weeks of infection. In addition to bursal colonization, we noticed a transient systemic infection of the spleen and liver that occurred at early time points after oral infection of 1-day old chicks but did not persist. *C. jejuni* was also found invading the cecal epithelium only at day 1 post-infection, a finding that, to the best of our knowledge, is the first report of the ability of *C. jejuni* to invade cecal tissue *in vivo*. These colonization dynamics were specific for *C. jejuni* as colonization with *S. pullorum* resulted in an initial high level of

colonization and systemic spread coordinated with invasion of cecal and bursal tissue resulting in inflammation.

We also extended the studies to determine if certain factors of *C. jejuni* were specifically required for cecal or bursal colonization. Analyses of defined mutants of *C. jejuni* revealed that an unencapsulated mutant has a global defect for colonization of all organs examined, whereas mutants lacking the cytolethal distending toxin or catalase have a more specific colonization defect for the bursa compared to other organs. These studies revealed that, in addition to intestinal surfaces, *C. jejuni* can initiate and maintain colonization of bursal lymphoid tissue, an organ dense with cellular effectors of both innate and adaptive immunity, and this colonization may be important for contributing to the development of commensalism between the bacterium and the avian host.

Results

C. jejuni promotes a robust, prolonged colonization of the bursa during commensalism

Most laboratory studies analyzing growth of *C. jejuni* in chicks have primarily focused on intestinal colonization for a short period of time (usually under 1 to 2 weeks after infection) with only a few studies having extended colonization studies for up to 5 weeks. Some studies have provided some evidence that *C. jejuni* may not be strictly confined to intestinal lumen surfaces,

but may also reach extraintestinal and systemic sites such as the spleen, liver, and bursa of Fabricius (11, 38, 94, 103, 122, 188). These studies were limited as most did not report the level of colonization at these sites (only that specific organs contained viable *C. jejuni*), nor did they examine the presence of *C. jejuni* over an extended period of time.

For an increased depth of understanding the global colonization dynamics of *C. jejuni* in multiple organs of chicks during commensalism, we monitored the levels of the bacterium in intestinal contents and lymphoid tissue as well as at systemic sites such as the spleen and liver over four weeks following oral infection. In this study, intestinal colonization was analyzed by measuring the loads of the bacterium in cecal contents as the ceca contains the highest viable numbers of *C. jejuni* compared to other regions of the gut (11, 75). Because birds do not have mesenteric lymph nodes associated with their gastrointestinal tract, we analyzed the level of colonization of the bursa. The bursa contains gut-associated lymphoid tissue, appearing as a diverticulum between the large intestine and cloaca. The bursa also has an epithelium containing M cells which are specialized in sampling intestinal antigens and transporting them to underlying follicles that contain B cells undergoing maturation (22). For systemic colonization, we monitored the occurrence and levels of *C. jejuni* in the blood, spleen, and liver.

Oral gavage of 1-day old chicks with 10^8 cfu of *C. jejuni* strain 81-176 resulted in a prolonged, high-level colonization of the ceca at all time points studied during infection. Between days 1 and 14 post-infection, *C. jejuni* reached approximately 10^9 cfu per gram of cecal content (Figure 3A). After this time, the levels of colonization decreased approximately 50-fold and were maintained around 4 x 10^7 cfu per gram of cecal content for the remainder of the experiment (28 days post-infection). These findings of high colonization levels and persistence in the ceca were expected based on previous short-term colonization studies performed with this strain of *C. jejuni* (14, 75).

When we explored potential extraintestinal sites for colonization by *C. jejuni*, we found that the organ with the second highest viable numbers of *C. jejuni* outside the intestinal tract was the bursa. Colonization of the bursa was robust and persisted until the end of the assay (28 days post-infection) (Figure 3A). After an initial level of colonization around 10^5 cfu per gram of tissue, the number of viable *C. jejuni* in the bursa increased 100-fold in the first seven days of infection. A decrease in the load of *C. jejuni* in the bursa was observed between days 7 and 28 post-infection with levels maintaining between 10^4 to 10^5 cfu per gram of tissue towards the end of the assay. Thus, *C. jejuni* has the ability to persist and increase in viable numbers in a major avian lymphoid organ rich in cellular effectors of innate and adaptive immunity.

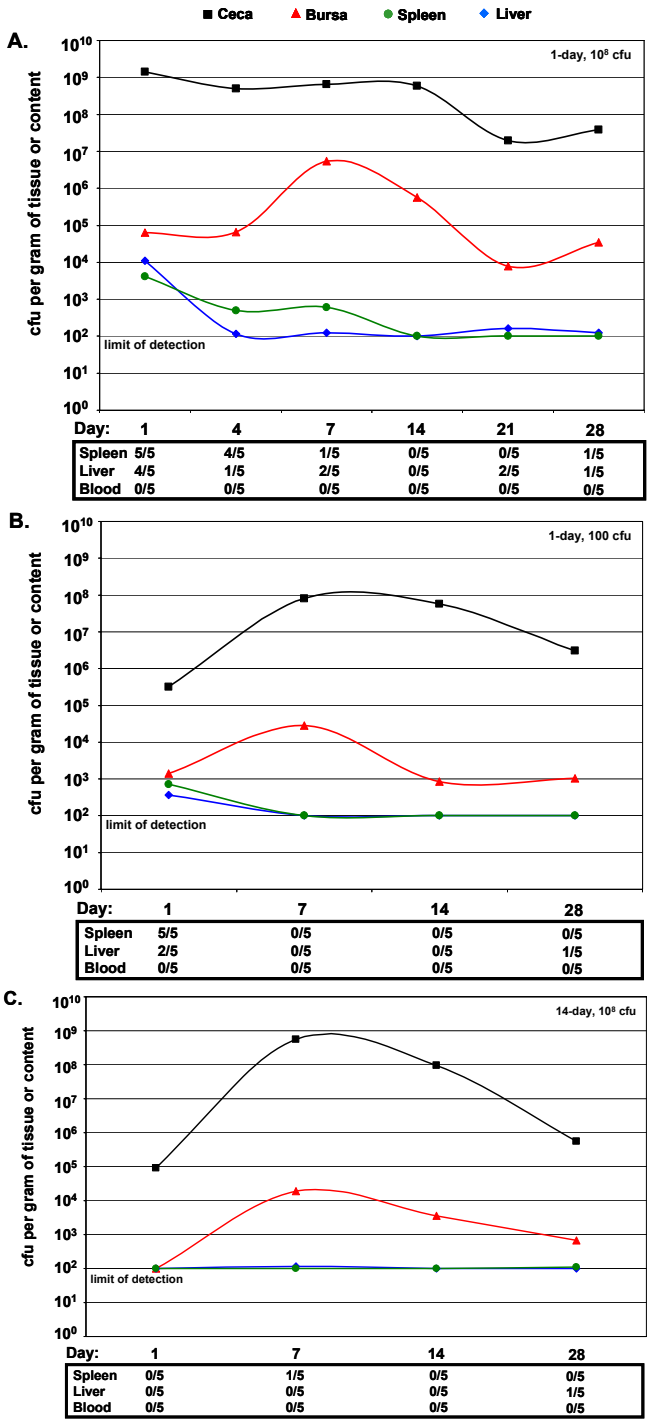

Figure 3. Distribution and levels of *C. jejuni* in various organs during prolonged colonization of chickens. One-day old chicks were orally gavaged

with (A) 10^8 cfu or (B) 100 cfu of *C. jejuni* 81-176. In (C), 14-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* 81-176. At day 1, 4, 7, 14, 21, or 28 post-infection, five chicks were sacrificed. Cecal contents were recovered and suspended in PBS. Bursa, spleen, and liver tissue were homogenized in PBS. Dilutions of the homogenized samples were spread on agar to select for *C. jejuni*. For blood, 100 µl of a blood sample from cardiac puncture was directly spread on agar and each bird was judged positive or negative for the presence of bacteria. The levels of *C. jejuni* in various samples are reported as cfu per gram of content or tissue. Symbols indicate the geometric means of the levels of colonization for ceca (■), bursa (▲), spleen (●) and liver (♦) for 5 chicks. The limit of detection in the assay is 100 cfu per gram of content or tissue. Box below graph indicates number of chicks out of five that were positive for *C. jejuni* in the spleen, liver, and blood at each time point.

In contrast to the colonization dynamics of *C. jejuni* in the ceca and bursa, we found that the bacterium could initially colonize the spleen and liver at low levels but this infection did not persist. At 1-day post-infection, the bacterium was present in these organs at approximately 10^4 cfu per gram of tissue (Figure 3A). *C. jejuni* was found in the spleen of all five chicks and the liver of four out of five chicks at day 1. However, after day 7 post-infection, *C. jejuni* was only found in the spleen and liver of a few chicks and the levels of bacteria at these sites were near or below the level of detection in the assay (100 cfu per gram of tissue). *C. jejuni* was not isolated from the blood of any chick at any time point. Therefore, we observed that *C. jejuni* is not strictly confined to gastrointestinal tract during commensalism, but can also promote a persistent infection of lymphoid tissue and a transient infection of systemic sites.

We and others have reported that *C. jejuni* strain 81-176 can colonize the ceca of chicks when given orally at a dose of 100 cfu or less (14, 75, 163). Because we initially used an inoculum at a higher order of magnitude (10^8 cfu) in the experiment described above, we considered that the large inoculum may have overwhelmed the birds, resulting in an abnormal infection of the bursa or spread to systemic sites. Therefore, we performed a similar prolonged colonization study by orally infecting 1-day old chicks with 100 cfu of *C. jejuni* and analyzing colonization out to 28 days post-infection. In this experiment, we again observed prolonged cecal and bursal colonization for the entire course of the experiment, but the levels of *C. jejuni* in this organ were 10- to 1,000-fold lower than chicks dosed at 10^8 cfu (compare Figures 3A and 3B). We were also able to isolate *C. jejuni* from the spleen of all chicks and the liver of two out of five chicks at day 1 post-infection. After this time, *C. jejuni* was absent in the spleen of all birds and was present in the liver of only one bird at day 28 post-infection. Again, *C. jejuni* was not isolated from the blood of any chicks at any time point. These results demonstrate that *C. jejuni* promotes a prolonged colonization of the ceca and the bursa upon oral gavage and a transient systemic infection, regardless of the size of the inocula.

We considered if the ability of *C. jejuni* to promote persistent bursal colonization and systemic infection was also influenced by the maturity of the immune system of the avian host. In the 1-day old chick infection model used in

experiments described above, the young chicks are known to have less developed innate and adaptive immune systems. For instance, the adaptive immune system is not thought to be fully functional in chicks until at least eight to ten days after hatch (9). To determine if *C. jejuni* could persist in the ceca and bursa and promote a systemic infection in birds with a more mature immune system, we performed a similar colonization assay by orally gavaging 14-day old birds with 10^8 cfu of *C. jejuni* 81-176. A subset of 14-day old chicks was sacrificed prior to oral gavage and found to be negative for *C. jejuni* in the ceca, ensuring that these birds had not been infected with the bacterium post-hatch. At day 1 post-infection, *C. jejuni* colonized the ceca at levels 15,000-fold lower than birds infected 1-day post-hatch (compare figures 3C and 3A). However, the levels of colonization seen in these groups of birds were similar by day 7. After day 7, the levels of *C. jejuni* in the 14-day old infected birds declined at a faster rate than 1-day old infected birds but did remain around 5×10^5 cfu per gram of cecal content at the end of the assay. *C. jejuni* was not observed in the bursa above the limits of detection for the assay at 1 day post-infection of 14-day old birds. However, a similar dynamic of bursal colonization was seen after this time point in these birds as compared to that of infected 1-day old birds with levels of *C. jejuni* increasing in the bursa at day 7 and then slightly decreasing with time (compare Figures 3C and 3A). The transient systemic infection of the liver and spleen seen in infected 1-day old birds was almost absent at all time points in infected 14-day old birds,

with only one bird being positive for *C. jejuni* in the spleen at day 7 post-infection and in the liver at day 28 post-infection. In summary, *C. jejuni* can specifically colonize the ceca and bursa of chicks and spread to systemic sites regardless of inoculation level, but systemic infection was largely ablated in more immunologically mature birds.

Robust, prolonged colonization of the ceca and bursa is specific for C. jejuni rather than S. pullorum

We next performed a similar analysis with *S. pullorum* to compare the global colonization dynamics of this bacterium in young chicks to *C. jejuni*. *S. pullorum* is a strict avian pathogen that colonizes the same organs of young chicks as *C. jejuni* (e.g., ceca, bursa, spleen, and liver) (71, 144, 179). In contrast to the outcome of *C. jejuni* infections in chicks, *S. pullorum* causes a diarrheal disease and a systemic infection known as pullorum disease resulting in high mortality (up to 50%) in young birds (158). Studies using *S. pullorum* allowed us to compare its colonization dynamics in the ceca, bursa, spleen, and liver over a prolonged period with those of *C. jejuni* so we could determine what events during colonization may be specific for *C. jejuni* to result in the development of commensalism between *C. jejuni* and the avian host.

To this end, we orally gavaged 1-day old birds with approximately 10^8 cfu of *S. pullorum* and determined the levels of the bacterium in intestinal and

lymphoid tissue (the ceca and bursa, respectively) and at systemic sites (the spleen, liver, and blood) for 28 days after infection. As shown in Figure 4, the initial levels of *S. pullorum* in the ceca and bursa were comparable to that of *C. jejuni* at day 1 post-infection (compare Figures 4 and 3A). However, *S. pullorum* did not maintain the same level of colonization of the ceca and bursa as *C. jejuni* over the course of the infection. After day 1 post-infection, a gradual decline in the number of *S. pullorum* in the ceca was observed over time. By day 28 post-infection, only around 8 x 10^4 cfu of *S. pullorum* per gram of cecal content was present, a level of colonization that was 500-fold lower than that of *C. jejuni* in birds at day 28 post-infection (compare Figures 4 and 3A). *S. pullorum* also initially colonized the bursa at levels 5- to 200-fold higher than *C. jejuni* at days 1 and 4 post-infection. However, the bacterium could not maintain this level of infection in the bursa. By day 28 post-infection, *S. pullorum* was only found in the bursa of three of five chicks and the loads of *S. pullorum* in the bursa of these chicks was under 500 cfu per gram of tissue, a level 100-fold lower than *C. jejuni* in the bursa of birds at day 28 post-infection (compare Figures 4 and 3A).

In contrast to *C. jejuni*, *S. pullorum* promoted a systemic infection in chicks that persisted for a longer period of time than the systemic infection promoted by *C. jejuni*. *S. pullorum* was found in the spleen of all chicks and in the liver of 90% of chicks out to day 14 post-infection (Figure 4). In

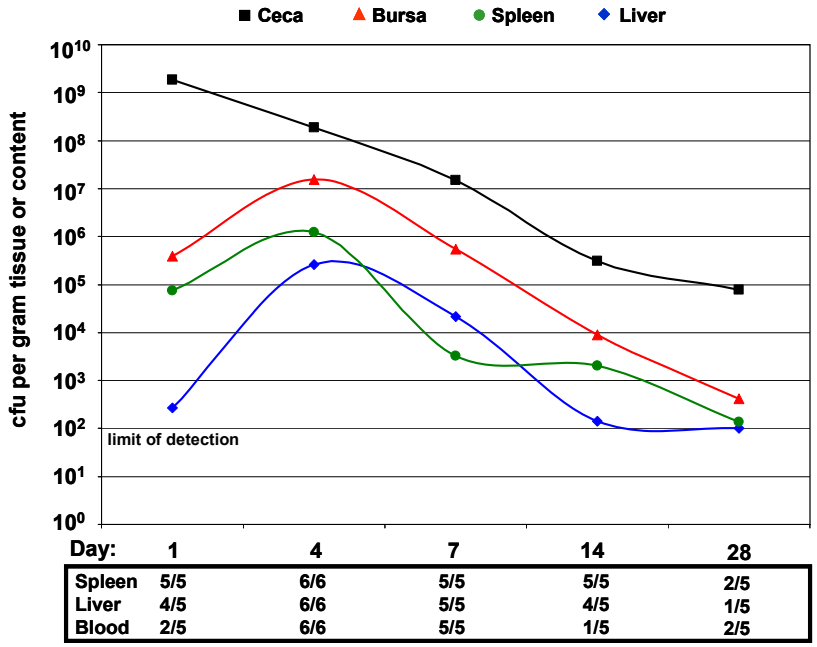

Figure 4. Distribution and levels of *S. pullorum* in various organs during prolonged colonization of chickens. One-day old chicks were orally gavaged with 10^8 cfu of *S. pullorum*. At day 1, 4, 7, 14, and 28 post-infection, five to six chicks were sacrificed. Cecal contents were recovered and suspended in PBS. Bursa, spleen, and liver tissue were homogenized in PBS. Dilutions of the homogenized samples were spread on Brilliant green agar to select for *S. pullorum*. For blood, 100 µl of a blood sample from cardiac puncture was directly spread on agar and each bird was judged positive or negative for the presence of bacteria. The levels of *S. pullorum* in various samples are reported as cfu per gram of content or tissue. Symbols indicate the geometric means of the levels of colonization for ceca (■), bursa (▲), spleen (●) and liver (♦). The limit of detection in the assay is 100 cfu per gram of content or tissue. Box below graph indicates number of chicks out of five to six that were positive for *S. pullorum* in the spleen, liver, and blood at each time point.

addition, the levels of *S. pullorum* in the spleen were higher than *C. jejuni* during the first 14 days after infection (compare Figures 4 and 3A). We also observed a higher level of *S. pullorum* in the liver of chicks between days 4 and 14 post-infection than *C. jejuni* at similar time points during infection. Whereas no *C. jejuni* was found in the blood of chicks at any time point, *S. pullorum* was isolated from the blood of at least one chick at every time point. At days 4 and 7 post-infection, *S. pullorum* was present in the blood of all chicks (Figure 4). Approximately 50% of the chicks died between days 3 and 7 post-infection which is generally when infections of the spleen, liver, and blood peaked. By comparing the colonization dynamics of these two bacteria in the avian host, we found that: 1) *C. jejuni* specifically maintains a prolonged, robust, commensal colonization of intestinal and lymphoid tissue; and 2) colonization of systemic sites such as the liver and spleen by *C. jejuni* is transient and at low levels relative to *S. pullorum*. Thus, the ability of *S. pullorum* to promote a systemic infection while *C. jejuni* is primarily confined to intestinal or lymphoid tissue may influence the different outcomes observed during infection of the avian host.

C. jejuni invades cecal tissue in vivo at day 1 post-infection

Although not sufficiently explored, *C. jejuni* is generally believed to localize to the mucus layer lining the cecal epithelium during colonization of poultry. Only *in vitro* studies have suggested an ability of *C. jejuni* to invade

avian intestinal or kidney cells (23, 28, 110, 156). Since we observed a measureable level of *C. jejuni* at systemic sites such as the spleen and liver during early time points of infection, we hypothesized that *C. jejuni* may cross the intestinal epithelium at some location to reach these sites. As most *C. jejuni* resides in the ceca, we performed an extensive analysis of this organ to determine if *C. jejuni* can breach the cecal epithelium. To this end, we searched for *C. jejuni* that had invaded the cecal epithelium in specimens from infected chicks by confocal microscopy using an antiserum developed in rabbits against formalin-fixed *C. jejuni* 81-176.

One day-old chicks were orally gavaged with 10^8 cfu of *C. jejuni*, and cecal specimens were obtained at days 1, 4, and 7 post-infection. As shown in Figure 5A, we found *C. jejuni* associated with the luminal surface of the cecal crypt epithelium at all time points, confirming other studies that used non-specific methods such as transmission electron microscopy or histological analysis to observe bacteria appearing to be *C. jejuni* colonizing the ceca (11, 110, 123). However, further analysis of cecal tissue from chicks infected with *C. jejuni* at day 1 post-infection did reveal some *C. jejuni* penetrating the cecal epithelium and invading underneath the basolateral surface of the epithelium of all infected chicks examined (Figure 5A and data not shown). By days 4 and 7 post-infection this invasion was no longer evident and *C. jejuni* was only found associated with

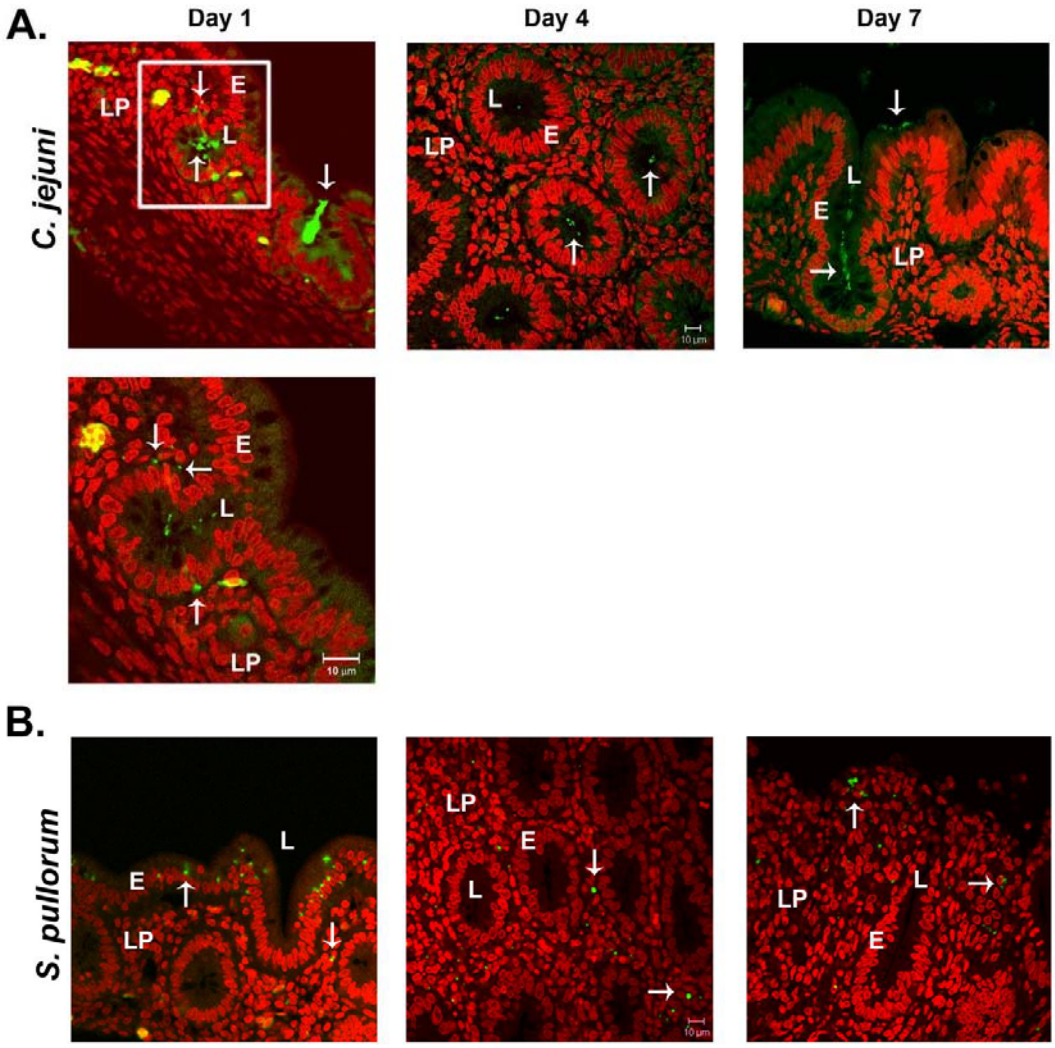

Figure 5. Localization of *C. jejuni* and *S. pullorum* in cecal specimens by confocal microscopy. One-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* 81-176 or *S. pullorum*. Chicks were sacrificed at days 1, 4 and 7 post-infection. Cecal tissues were removed, fixed, and processed. *C. jejuni* (A) and *S. pullorum* (B) were detected with specific antibodies to each bacterium followed by a FITC-labeled secondary antibody and appear green. Nuclei of eukaryotic cells were counterstained with Hoescht and appear red. Specimens from left to right are from infected chicks at days 1, 4, and 7 post-infection. Specimens from chicks at day 1 (left) and 7 (right) post-infection are longitudinal sections of cecal crypts and the lamina propria. The cecal specimens from chicks at day 4 (middle) post-infection show transverse sections viewing into cecal crypts and the

surrounding lamina propria. Images are at 63X magnification. Bar indicates 10 μm. (A) *C. jejuni*-infected chicks. Single image in second row is a 100X magnification of the area outlined in the square in the day 1 *C. jejuni* specimen above. Bar indicates 10 μm. Arrows indicate individual *C. jejuni* that have invaded or many *C. jejuni* localized to the luminal surface of the cecal epithelium. (B) *S. pullorum*-infected chicks. Arrows indicate *S. pullorum* that have invaded the epithelium or lamina propria. Structural areas of the tissue are indicated as follows: LP, lamina propria; E, epithelium; and L, cecal lumen. Yellow areas in images are autofluorescent red blood cells.

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the luminal surfaces of the ceca. Further analysis of Z scans of cecal tissue from *C. jejuni*-infected birds confirmed invasion of the cecal epithelium at day 1 post-infection (data not shown). Specificity of the antiserum for detection of *C. jejuni* was ensured by the lack of detection of any bacteria in cecal specimens from uninfected chicks (data not shown). Thus, only at the earliest time points of infection analyzed were we able to detect *C. jejuni* invading the cecal epithelium.

To determine if this association of *C. jejuni* with host tissue was specific for commensalism, we also examined the interaction between *S. pullorum* and host tissue. In contrast to *C. jejuni*, *S. pullorum* was rarely found associated with the luminal surfaces of the cecal epithelium (Figure 5B). Rather, we detected abundant *S. pullorum* invading the epithelium or in the lamina propria of cecal specimens, demonstrating that invasion had occurred. This invasion into the lamina propria was evident in multiple Z scans of cecal specimens (data not

shown). Therefore, minimal cecal invasion at the earliest time points of infection followed by confinement of *C. jejuni* to the lumen of the ceca appears to be specific for the development of commensalism.

C. jejuni is confined to the lumen of bursal tissue

To increase our understanding of how *C. jejuni* initiates and maintains a persistent colonization of the bursal lymphoid tissue, we identified the niches colonized by *C. jejuni* within the bursa by using confocal microscopy to determine if the bacterium is localized strictly to luminal mucosal surface or if *C. jejuni* can invade the bursal epithelium to reside in the intrafollicular tissue.

To this end, 1-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* or *S. pullorum* and bursal specimens were removed from multiple chicks at days 1, 4, and 7 post-infection. Throughout the period of colonization analyzed (days 1 to 7 post-infection), we consistently found *C. jejuni* associated with the mucus layer lining the epithelium of the bursa of infected chicks (Figure 6A), suggesting only a luminal association of *C. jejuni* with the bursa. We do not believe that the lack of *C. jejuni* in the bursal intrafollicular tissue is due to a technical inability since this antiserum could detect *C. jejuni* invading cecal epithelium (Figure 5A). In contrast, *S. pullorum* was consistently found within bursal tissue

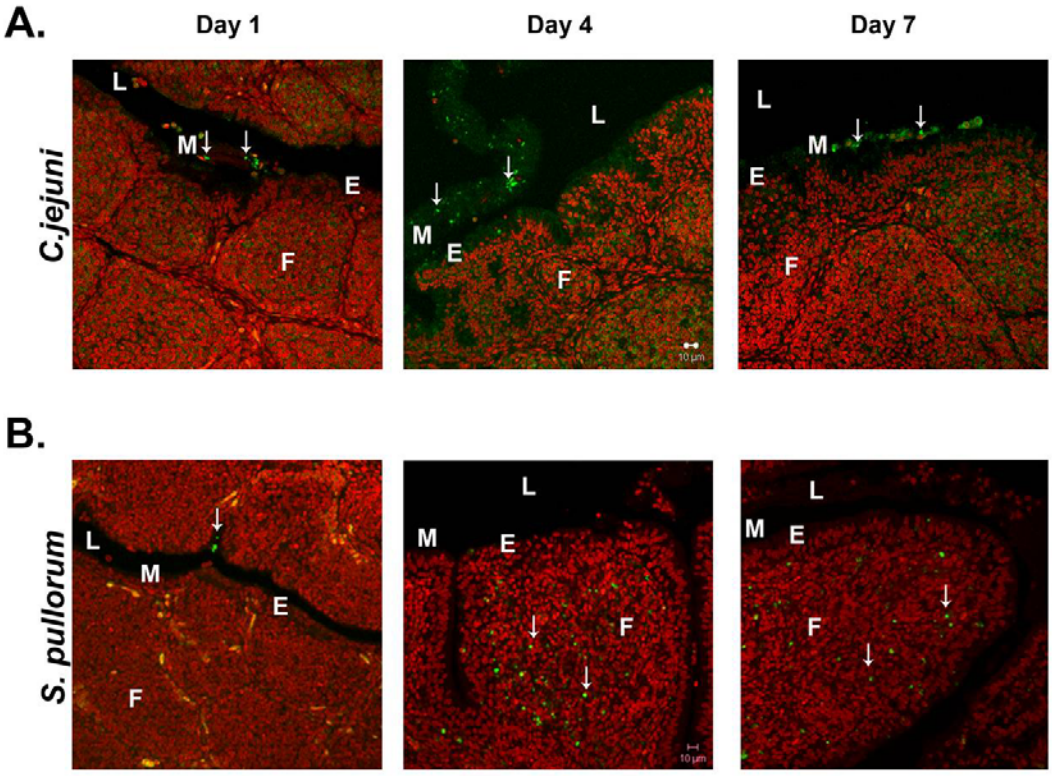

Figure 6. Localization of *C. jejuni* and *S. pullorum* in bursal specimens by confocal microscopy. One-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* 81-176 or *S. pullorum*. Chicks were sacrificed at days 1, 4 and 7 post-infection. Bursal tissues were removed, fixed, and processed. *C. jejuni* (A) and *S. pullorum* (B) were detected with specific antibodies to each bacterium followed by a FITC-labeled secondary antibody and appear green. Nuclei of eukaryotic cells were counterstained with Hoescht and appear red. Specimens from left to right are infected chicks at days 1, 4, and 7 post-infection. Bars indicate 10 μm. (A) *C. jejuni*-infected chicks. Arrows indicate *C. jejuni* confined to the mucus layer overlying the bursal epithelium. (B) *S. pullorum*-infected chicks. Arrows indicate *S. pullorum* in the mucus layer (day 1) or invading within bursal follicles (day 4 and 7). Structural areas of the specimens are indicated as follows: M, mucus layer; E, epithelium; F, follicular tissue; and L, bursal lumen. All images are at 40X magnification.

and underneath the bursal epithelium of infected chicks at days 4 and 7, but was only found associated with the luminal surface of the bursal epithelium at day 1 (Figure 6B). Unlike *C. jejuni*, multiple Z scans of bursal specimens of *S. pullorum*-infected chicks revealed extensive invasion of bursal tissue (data not shown). Thus, we conclude that *C. jejuni* is predominantly and perhaps exclusively limited to the mucus layer lining the bursal epithelium.

The inflammatory response elicited by C. jejuni infection is minimal compared to that provoked by S. pullorum

Previous studies have determined that the inflammatory response to *C. jejuni* after colonization of the ceca is fairly mild and transient, if existent at all (155). To more closely examine the host response to *C. jejuni* during cecal and bursal colonization over the course of 2 weeks, we analyzed tissues from *C. jejuni*- or *S. pullorum*-infected chicks. Although the host response to *S. pullorum* infection has been previously characterized, we wanted to use the resulting dramatic inflammatory response as a positive control to directly compare to any type of host response observed during commensal *C. jejuni* infection.

One-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* or *S. pullorum*. A control group of chicks remained uninfected with either bacterium. At days 1, 4, 7, and 14 post-infection, cecal and bursal tissues were processed and stained with hematoxylin and eosin for observation of histological changes or

lesions associated with bacterial infection. Over the course of the experiment, uninfected birds displayed normal cecal morphology (Figure 7). The epithelium remained intact and the depth of crypts increased with time. Very few heterophils, macrophages, or apoptotic bodies were observed in the tissue of uninfected chicks.

The cecal sections of *C. jejuni*-infected birds had no lesions or histopathologic differences from uninfected birds (Figure 7) despite high loads of *C. jejuni* present in cecal tissue over the course of the experiment (~10^9 cfu per gram of cecal content, Figure 3A). At day 4 post-infection, we observed a slight increase in the number of heterophils present in the cecal lamina propria and the epithelium compared to uninfected birds (Figure 7). However, at days 7 and 14 post-infection, the levels of heterophils decreased to levels observed in uninfected chicks. This observation was confirmed by enumerating heterophils in cecal sections of multiple *C. jejuni*-infected chicks. We observed a statistically significant four-fold increase ($p < 0.01$) in the number of heterophils per microscopic field at day 4 post-infection of *C. jejuni*-infected birds relative to uninfected birds (Figure 8). However, by days 7 and 14 post-infection, the number of heterophils decreased and was equivalent to the numbers found in uninfected birds. There was no ulceration or significant infiltration of macrophages in these chicks.

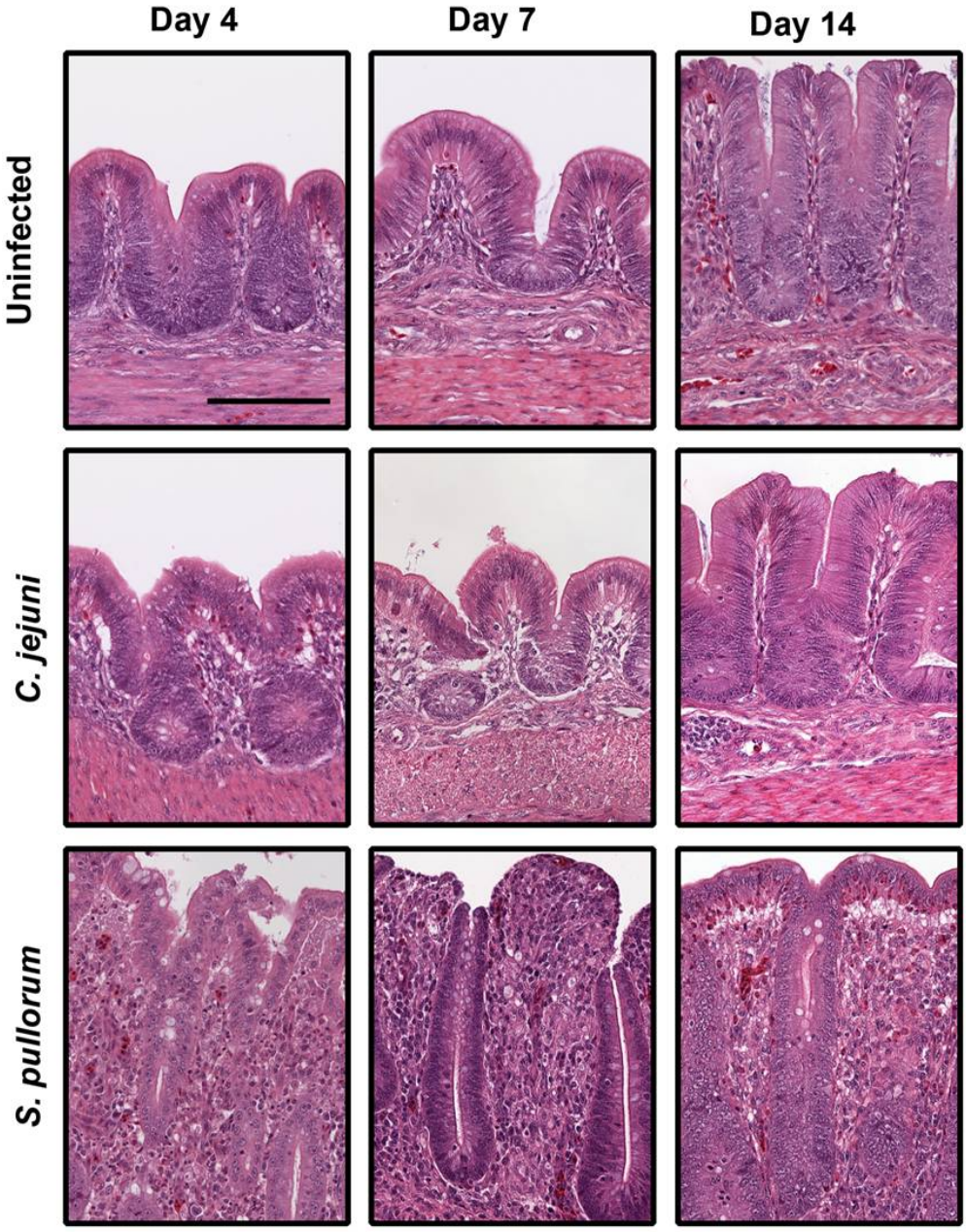

Figure 7. Histology of cecal tissue during colonization by *C. jejuni* or *S. pullorum*. One-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* 81-176 or *S. pullorum*, or remained uninfected. Chicks were sacrificed at day 1, 4, 7, and 14 post-infection. Day 1 data not shown. Cecal tissues were removed, fixed, processed, and stained with hematoxylin and eosin. Histology of cecal tissue visualized at 40X magnification. Bar indicates 100 μm.

In contrast, cecal tissue from *S. pullorum*-infected birds displayed dramatic differences compared to uninfected and *C. jejuni*-infected birds at all time points except for day 1 post-infection. No differences in cecal tissue from uninfected, *C. jejuni*-infected, or *S. pullorum*-infected chicks at day 1 post-infection were observed (data not shown). At day 4 post-infection, the cecal mucosa of *S. pullorum*-infected chicks was thickened with inflammation extending from the epithelium, through the lamina propria, and into the submucosa (Figure 7 and data not shown). The inflammatory cell population within the cecal mucosa consisted primarily of macrophages. In addition, the lamina propria was hyperemic in these *S. pullorum*-infected chicks. Multifocal necrosis of the overlying epithelium and hyperplasia of the crypt epithelial cells were also noted. At day 7 post-infection, the macrophage infiltrate persisted in the cecal mucosa (Figure 7). The epithelium of the crypt was necrotic, producing effacement of cecal crypts and the surviving crypt epithelium was hyperplastic. By day 14 post-infection, the inflammatory infiltrate in the lamina propria now included large numbers of lymphocytes and an increased number of heterophils. Quantification of the numbers of heterophils that had infiltrated the cecal tissue of *S. pullorum*-infected chicks revealed relatively similar numbers of heterophils as uninfected chicks at days 4 and 7 post-infection (Figure 8). However, the number of heterophils in cecal tissue was approximately 50-fold higher than uninfected or

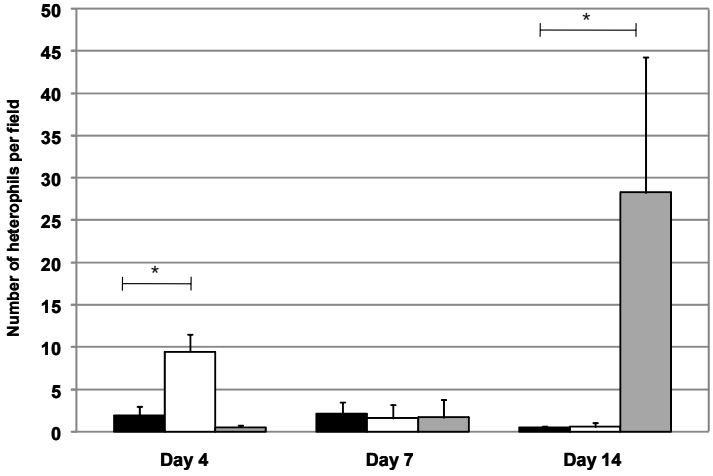

Figure 8. Quantification of heterophil infiltration in cecal tissue during colonization by *C. jejuni* or *S. pullorum*. One-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* 81-176 or *S. pullorum*, or remained uninfected. Chicks were sacrificed at day 4, 7, and 14 post-infection. Cecal tissue was removed, fixed, processed, and stained with hematoxylin and eosin. For an individual group at each time point, cecal specimens of three different chicks were examined. For each chick, twelve different fields were examined to visually quantify the number of heterophils present. Data are reported as the average number of heterophils in a field. Columns represent data from uninfected (black), *C. jejuni*-infected (white), or *S. pullorum*-infected (grey) chickens. Error bars represent the standard deviation. Analysis using the two-tailed, unpaired student's t-test was performed to determine statistically significant differences between heterophil infiltration of uninfected chicks with infected chicks. Asterisks and bars indicate pairs with statistically significant differences ($p < 0.05$).

C. jejuni-infected birds at day 14 post-infection (Figure 8). This difference in heterophil infiltration of *S. pullorum*-infected birds was statistically significant compared to uninfected and *C. jejuni*-infected birds. The crypt epithelium of *S. pullorum*-infected birds at 14-days post-infection remained hyperplastic and the majority of crypts were reepithelialized. These changes in the cecal tissue of *S.*

pullorum-infected birds compared to *C. jejuni*-infected birds were dramatic when considering that the levels of *S. pullorum* were equivalent to or 1,000-fold lower than that of *C. jejuni* in the cecal tissue of infected birds (Figures 3A and 4).

We next analyzed changes in bursal tissue between uninfected, *C. jejuni*-infected, and *S. pullorum*-infected birds over similar time points of infection. Over the course of the experiment, bursal tissue from uninfected birds displayed normal architecture with an intact, prominent follicle-associated epithelium and defined lymphoid follicles with normal apoptotic activity at all time points (Figure 9 and data not shown). The bursal tissue from *C. jejuni*-infected birds only showed minor differences from uninfected birds at day 1 and 4 post-infection (Figure 9 and data not shown). A slight increase in hyperemia and a minor decrease in apoptic activity of questionable significance were visually observed at day 4 post-infection in *C. jejuni*-infected birds in comparison to uninfected birds. However, by day 7 post-infection, these minor differences were not apparent. Thus, despite bacterial loads reaching 10^5 to 10^6 cfu per gram of tissue (Figure 1A), no dramatic changes in bursal tissue of *C. jejuni*-infected chicks were detected.

In contrast, many changes to bursal tissue were observed in the *S. pullorum*-infected birds. At day 4 post-infection, dramatic atrophy and ablation of lymphoid follicles were observed (Figure 9). Atrophic follicles contained large numbers of apoptotic cells and the interfollicular spaces were infiltrated with

macrophages. The overlying epithelium was ulcerated and necrotic cellular debris accumulated on the epithelial surface and within the bursal lumen. At day 7 post-infection, the majority of the lymphoid follicles of the bursa of *S. pullorum*-infected chicks were ablated with very few intact lymphoid follicles remaining and the perifollicular space was filled with macrophages (Figure 9). The overlying epithelium had small focal ulcerations and necrotic cellular debris was found in the lumen. At day 14 post-infection, the appearance of the bursa of *S. pullorum*-infected birds began returning to normal (Figure 9). The lymphoid follicles were repopulated with lymphocytes and the intrafollicular tissue contained smaller numbers of mononuclear cells than the bursa of *S. pullorum*-infected birds at earlier points of infection. The epithelium was hyperplastic with an increase in mucin-secreting cells. Thus, despite both bacteria being able to colonize the bursa, the destruction of tissue in the bursa of *S. pullorum*-infected birds was more severe compared to *C. jejuni*-infected birds.

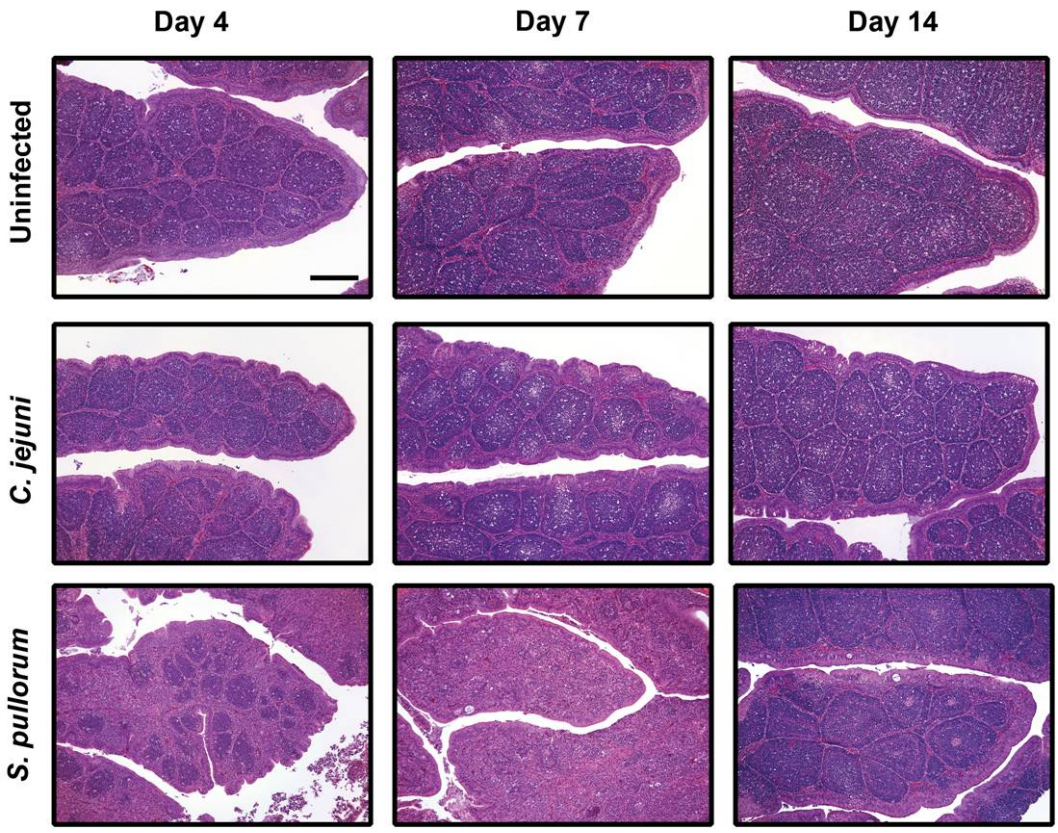

Figure 9. Histology of bursal tissue during colonization by *C. jejuni* or *S. pullorum*. One-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* 81-176, or *S. pullorum*, or remained uninfected. Chicks were sacrificed at days 1, 4, 7, and 14 post-infection. Day 1 data not shown. Bursal tissues were removed, fixed, processed, and stained with hematoxylin and eosin. Histology of bursal tissue was visualized at 20X magnification. Bar indicates 200 μm.

C. jejuni does not survive intracellularly after phagocytosis by activated avian monocytes

The minimal inflammatory response observed during infection with *C. jejuni*, even at time points in which the bacterium was found to invade the cecal epithelium, and the lack of persistence of *C. jejuni* at systemic sites compared to *S. pullorum* may be influenced by the ability of the bacterium to survive after phagocytosis by cells such as macrophages. *Salmonella* species are known to survive after phagocytosis by macrophages, which contribute to their ability to promote invasion and systemic disease in a susceptible host (49). We compared any potential differences in the ability of *C. jejuni* or *S. pullorum* to survive after phagocytosis by avian macrophages. For this approach, we used avian HD11 monocytes which can be activated by PMA to differentiate into macrophage-like cells. After infection of activated HD11 monocytes with *C. jejuni* or *S. pullorum* followed by a two-hour incubation period, we added gentamicin to kill extracellular bacteria and then monitored the ability of the bacteria to survive intracellularly over time. As shown in Figure 10, *C. jejuni* had a reduced ability to survive inside avian monocytes over time compared to *S. pullorum*. Within 4 to 8 hours after gentamicin treatment, the number of viable intracellular *C. jejuni* was 10- to 100-fold lower than *S. pullorum*. Whereas viable *S. pullorum* was found inside cells out to 96 hours, the levels of viable *C. jejuni* fell below the limit

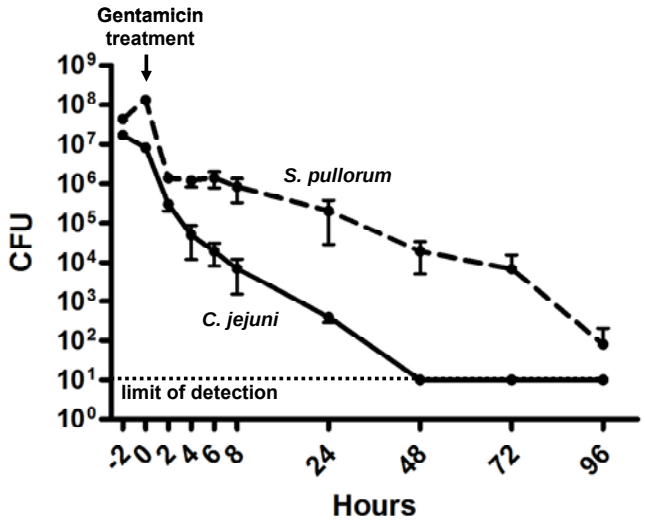

Figure 10. Survival of *C. jejuni* and *S. pullorum* after phagocytosis by activated avian monocytes. HD11 avian monocytes were activated by PMA and then infected at a MOI of 100 (~ 10^8 cfu per well). After two hours of infection (time 0 h), cells were washed and gentamicin was added to kill extracellular bacteria. Intracellular survival of *C. jejuni* (solid line) or *S. pullorum* (dashed line) was monitored over 96 hours. Each assay was performed in triplicate and the surviving bacteria for each time point is presented as the average cfu. Bars indicate standard deviation. The assay was repeated at least three times and a representative assay is shown.

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of detection between 24 and 48 hours post-gentamicin treatment. These results suggest that *C. jejuni* may have a reduced ability to survive after phagocytosis by avian macrophages *in vivo* which may reduce its ability to survive at systemic sites.

The adaptive immune response to C. jejuni consists of production of serum IgG and mucosal IgA

Considering that we observed differences in some innate immune responses in chicks due to infection with *C. jejuni* or *S. pullorum*, we next examined potential differences in the humoral immune responses of the avian host upon infection with these bacteria. The type of humoral immune responses generated against bacteria upon infection is often influenced by whether the bacteria primarily remain at luminal sites in intestinal or lymphoid tissue (generating a mucosal IgA response), or traverse the intestinal barrier to reach more systemic sites (generating a systemic IgG response). Since *S. pullorum* was able to promote infection of the intestinal mucosa and a systemic infection, we predicted that both a mucosal IgA response and a systemic IgG response could be detected. Because *C. jejuni* was predominantly localized to the intestinal mucosa, we predicted that infected chicks would generate a mucosal IgA response. Due to the transient systemic infection, we suspected that a systemic IgG response against *C. jejuni* may possibly be detected.

For this approach, we examined the systemic IgG and mucosal IgA responses to *C. jejuni*- or *S. pullorum*-infected birds over time. To analyze the systemic IgG responses, blood was collected at days 1, 7, 14, and 28 post-infection from four chicks at each time point and the sera were recovered. Detection of serum IgG specific for *C. jejuni* or *S. pullorum* was accomplished by

immunoblotting analyses with proteins from lysates of the bacteria. As shown in Figure 11A, some cross-reactive maternal serum IgG against each bacterium were present at day 1 post-infection. Over time, these maternal antibodies waned and the chicks began generating serum IgG against *C. jejuni* or *S. pullorum* by day 14 post-infection, correlating with the maturation of the adaptive immune system (9, 30, 128, 146). In *C. jejuni*-infected birds, the systemic IgG response does not appear to intensify by day 28 post-infection. However, the level of the IgG response against *S. pullorum* was more robust and extensive by day 28 post-infection. Thus, the systemic humoral immune response was less severe against *C. jejuni* than *S. pullorum* in infected chicks.

We then examined if *C. jejuni* and *S. pullorum* elicited a mucosal IgA response from the host during prolonged colonization. To this end, we recovered proteins from the contents of the large intestines and used this preparation in immunoblotting analyses to detect IgA specifically reactive to *C. jejuni* or *S. pullorum*. Isolation of IgA was difficult, but we were able to detect an IgA response against the bacteria at day 28 post-infection (Figure 11B). Unlike the serum IgG response, comparable mucosal IgA responses against each bacterium were detected. In conclusion, the avian host responds to each bacterium after infection by generating an adaptive immune response, with a more robust systemic IgG response observed in *S. pullorum*-infected birds.

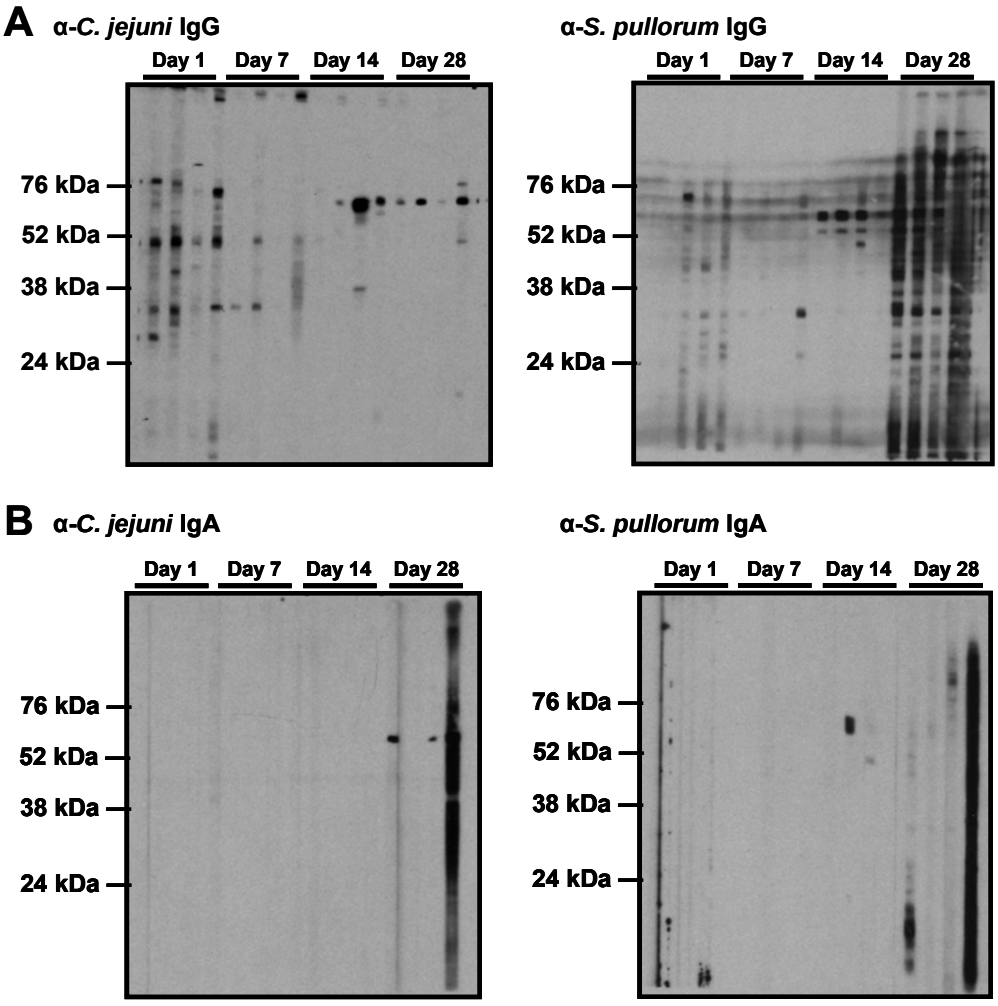

Figure 11. Systemic IgG and mucosal IgA responses to *C. jejuni* and *S. pullorum* during prolonged colonization. One-day old chicks were orally gavaged with 10^8 cfu of *C. jejuni* 81-176 or *S. pullorum*. Chicks were sacrificed at days 1, 7, 14, and 28 post-infection. Blood was collected by cardiac puncture to obtain serum IgG samples and contents of the large intestines were collected to obtain mucosal IgA samples. Samples were collected from four different chicks at each time point. SDS-PAGE was performed with whole-cell lysates of *C. jejuni* and *S. pullorum* followed by immunoblotting analyses with serum IgG or mucosal IgA samples. Each lane was probed with the respective immunoglobulin preparation from an individual chick. (A) Systemic IgG responses from *C. jejuni*- or *S. pullorum*-infected chicks. (B) Mucosal IgA responses from *C. jejuni*- or *S. pullorum*-infected chicks.

C. jejuni relies on certain factors for specifically promoting wild-type levels of colonization of the bursa

Comparison of the colonization dynamics and avian host response to infection with *C. jejuni* or *S. pullorum* suggested that the robust, prolonged bursal colonization observed during infection with *C. jejuni* may be a significant event that contributes to the establishment of commensalism. Considering that the environments of the ceca and the bursa are likely different due to nutrient availability and cellular composition of the organs, we hypothesized that certain factors of *C. jejuni* may be required for specifically initiating and maintaining colonization in either the ceca or the bursa, or both. To determine if certain colonization factors exist, we analyzed specific mutants of *C. jejuni* for prolonged colonization of multiple organs. We chose to analyze three mutants, lacking *kpsM*, *cdt*, or *katA*, that have previously been reported to have different colonization abilities for various portions of the chick intestinal tract (1, 14, 15, 91). None of these mutants have been analyzed for prolonged colonization of the ceca or for the ability to spread to the bursa or systemic sites and persist in these organs.

A *kpsM* mutant of *C. jejuni* has been shown to lack production of the polysaccharide capsule due to disruption of the export system necessary to secrete the capsule (6, 98). A previous study has shown that a *kpsM* mutant could be isolated from clocal swabs up to one week post-infection of 14-day old chicks but

not at later time points (91). We deleted *kpsM* from the genome of 81-176 Sm^R (creating JMB206) and used this mutant to orally gavage 1-day old chicks with 10^8 cfu. At day 1 post-infection, the Δ*kpsM* mutant was initially found at wild-type levels in the ceca and bursa (compare Figures 12A and 3A). However, from day 7 to day 28 post-infection, severe decreases in colonization of the bursa and ceca were observed, with statistically significant differences in levels between wild-type and Δ*kpsM* mutant bacteria. By day 28 post-infection, the levels of the Δ*kpsM* in the ceca and bursa were below the limit of detection for the assay (< 100 cfu per gram of tissue or content). In addition, the Δ*kspM* mutant was largely incapable of promoting systemic infection of the spleen and liver, with only one chick positive for *C. jejuni* in the spleen at day 1 post-infection. From this analysis, we conclude that capsular polysaccharide production is required for prolonged colonization of both the ceca and bursa and for transient spread to systemic sites.

Whereas production of the cytolethal distending toxin (Cdt) is believed to be important for pathogenesis during infection of humans, others have shown that *cdt* mutants have no appreciable colonization defect for the ceca at five to seven days post-infection (1, 15). We generated a mutant of *C. jejuni* that lacks the entire *cdt* locus (genes *cdtA*, *cdtB*, and *cdtC*) to determine if Cdt is required for colonization or persistence in cecal or bursal tissue. We observed comparable levels of the Δ*cdt* mutant and the wild-type strain in the ceca throughout 28 days

post-infection, with only a slight, statistically insignificant reduction at days 1 and 7 post-infection (compare Figures 12B and 3A). Bursal colonization by the Δ*cdt* mutant was similar to the wild-type strain at day 1 post-infection, but the levels of the mutant did not increase in the bursa at day 7 post-infection as observed with the wild-type strain. At days 7 and 14 post-infection, the levels of the Δ*cdt* mutant were significantly lower than the wild-type strain but by day 28 post-infection, the levels of the Δ*cdt* mutant were only 10-fold lower than the wild-type levels in the bursa of infected birds (compare Figures 12B and 3A). Analysis of systemic infection by the Δ*cdt* mutant revealed that the mutant was generally able to spread to and persist in the spleen and liver but the levels were near the limit of detection. Thus, we conclude that the Δ*cdt* mutant appears to have a specific defect for increasing and maintaining levels of *C. jejuni* in the bursa during prolonged colonization.

Similarly, a Δ*katA* mutant appeared to also have a specific colonization defect for the bursa. Previous studies from our group (discussed in Chapter 5) have revealed that this mutant has a severe defect for peroxide resistance *in vitro*, but only a moderate 10-fold decreased colonization ability for the ceca after seven days of colonization when given to 1-day old chicks at 10^6 cfu (14). As expected, we again noticed this modest cecal colonization defect by this mutant out to day 7

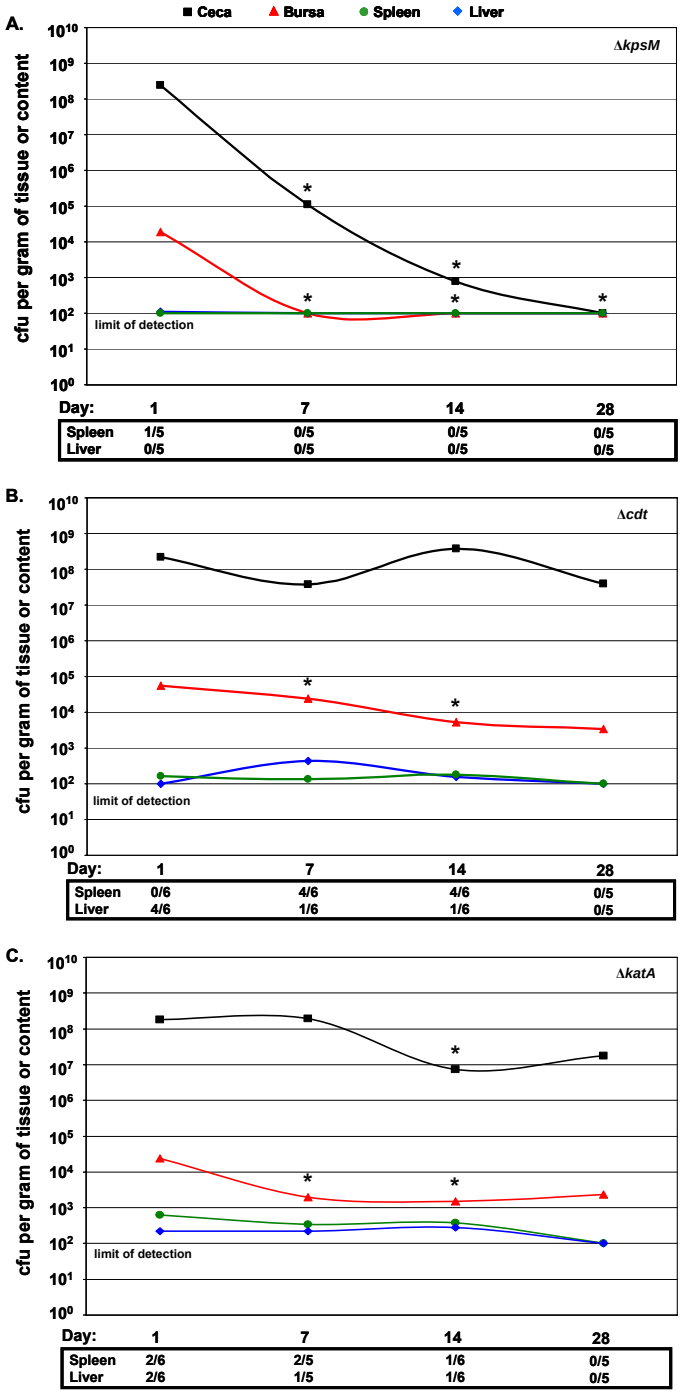

Figure 12. Analysis of prolonged colonization defects of *C. jejuni* 81-176 mutants in chickens. One-day old chicks were orally gavaged with 10^8 cfu of

(A) 81-176 Sm^R Δ*kpsM* (JMB206), (B) 81-176 Sm^R Δ*cdt* (LKB624), or (C) 81-176 Sm^R Δ*katA* (LKB246; (14)). At day 1, 7, 14, and 28 post-infection, five chicks were sacrificed. Cecal contents were recovered and suspended in PBS. Bursa, spleen, and liver tissue were homogenized in PBS. Dilutions of the homogenized samples were spread on agar to select for *C. jejuni*. The levels of *C. jejuni* in various samples are reported as cfu per gram of content or tissue. Symbols indicate the geometric means of the levels of colonization for ceca (■), bursa (▲), spleen (●) and liver (♦) for five chicks. The limit of detection in the assay is 100 cfu per gram of content or tissue. Box below graph indicates number of chicks out of five that were positive for *C. jejuni* in the spleen and liver at each time point. Statistical analysis was performed using the Mann-Whitney U test using the colonization data presented from the wild-type strain in Figure 1A and the colonization data presented in this figure for the mutants. Asterisks (*) indicates a significant difference between the colonization levels of the wild-type strain and the mutant strain ($p < 0.01$). In (A), the single asterisk at day 28 represents statistically significant differences for both cecal and bursal colonization as compared to the wild-type strain.

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post-infection. A significant decrease in *C. jejuni* levels in the ceca at day 14 was observed, but by day 28 post-infection there was no difference between the Δ*katA* mutant and the wild-type strain (compare Figures 12C and 3A). Initial colonization levels in the bursa at day 1 post-infection were reduced by deleting *katA* although this difference was not statistically significant. However, over the course of the experiment, the levels of the Δ*katA* mutant dropped dramatically at days 7 and 14 post-infection (2700-fold and 400-fold, respectively). By the end of the assay, the Δ*katA* mutant was at about 15-fold lower levels than the wild-type strain in the bursa at day 28 post-infection. Despite being present at lower numbers in the spleen and liver at day 1 post-infection, the levels of the Δ*katA*

mutant were approximately the same as the wild-type strain in similarly infected chicks at days 7 and 14 post-infection. In conclusion, we have determined that capsule production is required for colonization of multiple organs while Cdt and catalase are more specifically required for colonization and persistence in the bursa of infected chicks. Thus *C. jejuni* has colonization factors that are likely specifically utilized for prolonged colonization and growth in different organs.

Discussion

C. jejuni is able to promote infection of humans and poultry, but the outcomes of infection are vastly different. Thus, the bacterium and the hosts are likely to interact with each other differently to result in disease in humans or commensalism in birds. Previous studies that have analyzed colonization of the natural avian host by *C. jejuni* have primarily focused on the ability of the bacterium to colonize the ceca, the identification of colonization determinants required for growth in this organ, or the avian host response after infection. While these studies are relevant and have provided important insights into the biology of *C. jejuni* in the natural host, they have not addressed a potentially broader perspective in analyzing if *C. jejuni* can exist and thrive outside the intestines *in vivo* and what the consequences are for the bacterium and the host for spreading to more systemic sites. In this study, we have extended our understanding of the activities of *C. jejuni* in the natural host by analyzing the

global colonization dynamics of *C. jejuni* during commensal colonization of chickens. We have provided more insight into the organs *C. jejuni* can colonize from 24 hours after oral gavage through four weeks post-infection. We have found that *C. jejuni* can invade cecal epithelium and that this invasion may lead to a transient but non-persistent systemic infection. Even more intriguing, we found that *C. jejuni* can promote a robust, persistent colonization of lymphoid tissue within the bursa of Fabricius. Colonization of the bursa may have consequences for promoting interactions with the host innate and adaptive immune systems that may contribute to proper host responses for the development of commensalism.

Moreover, we have also performed a comparative analysis of infection with the invasive pathogen, *S. pullorum*, to directly compare the interactions of *C. jejuni* and poultry to those of an avian pathogen. This analysis assisted in determining which interactions between *C. jejuni* and poultry are specific for the development of commensalism. In contrast to the colonization dynamics of *C. jejuni, S. pullorum* was not capable of maintaining colonization in these organs at comparable levels. *S. pullorum* invaded the cecal and bursal epithelium to provoke extensive inflammation and damage, and promoted high levels of systemic infection which likely contributed to a more robust systemic IgG response than that of *C. jejuni*. Additionally, potential differences in the survival of the bacteria after phagocytosis by activated avian monocytes were also noted. These observations highlight potential activities of *C. jejuni* and the avian host

that likely influences the development of commensalism rather than disease upon infection.

Even though the appearance of *C. jejuni* in the bursa has been previously noted (38), the levels and persistence of the bacterium in this organ during commensalism had never been fully explored. The bursa functions as gut-associated lymphoid tissue in poultry with an epithelium specialized for transport and underlying follicles with maturing B cells (22). Unlike mammalian species, birds lack mesenteric lymph nodes associated with their gastrointestinal tract. We observed that *C. jejuni* could initially colonize the bursa after oral gavage and then increase 100-fold in viable numbers in this organ over the course of the experiment. This increase is striking considering that the bursa is the organ associated with the intestinal tract that is the most concentrated in cellular components of the immune system. We suspect that colonization of this organ may allow the bacterium to interact with components of the innate and adaptive immune system to drive proper host responses, such as generation of mucosal IgA, which likely contribute to the development of commensalism. Alternatively, colonization of the bursa may simply be due to its anatomical location in relation to the intestinal tract. The bursa is derived as a diverticulum between the large intestine and cloaca, and has an epithelium that is contiguous with the intestinal tract. Colonization of this organ may simply be an extension of colonization of the intestines and an extraintestinal reservoir of the bacterium for possible

reseeding of the intestinal tract. Future studies will focus on understanding how colonization of this lymphoid organ by *C. jejuni* contributes to the development of commensalism. These types of studies may involve infecting normal and bursectomized chicks with *C. jejuni* and monitoring the colonization dynamics of the bacterium and certain innate and adaptive immune responses over time, such as development of IgA. Additionally, it will be interesting to investigate if the increase in viable numbers of *C. jejuni* in the bursa during the first week of infection is due to increased growth of the bacterium in the bursa or increased trafficking of the bacterium to bursal tissue.

In vitro studies with primary intestinal cells and avian kidney cell lines have suggested that *C. jejuni* can invade chick epithelial cells (28, 110, 156), but no supportive evidence for invasion has been obtained from *in vivo* studies. We have potentially provided the *in vivo* findings to support that *C. jejuni* is able to invade the cecal epithelium at least up to 24 hours after oral gavage of 1-day old chicks. Coincidentally, this time point after gavage is when we observed the most viable numbers of *C. jejuni* at systemic sites such as the spleen and liver. Therefore, this invasion of the cecal epithelium may potentially lead to the brief transient infection we observed in splenic and hepatic tissue. Whether or not this cecal invasion specifically leads to systemic spread remains to be investigated more thoroughly. Ultimately, the observed invasion and systemic infection does not contribute to a pathogenic outcome in poultry because the avian host appears

to be able to remove *C. jejuni* from these sites without any obvious inflammation or signs of disease progression.

Pathogenic enteric bacteria such as *S. pullorum* are known to invade the intestinal epithelium, initiating a cascade of inflammatory events such as the production of pro-inflammatory cytokines, migration of innate immune cells, and damage to host tissues. However, the reason for the lack of inflammation in *C. jejuni*-infected chicks remains unknown but has been the subject of a few hypotheses. It has been postulated that only a very localized pro-inflammatory response may be generated by the avian host in intestinal tissue that may control *C. jejuni* to limit disease. In *in vitro* analyses with primary chicken epithelial cells and *in vivo* studies with cecal and ileal tissue from *C. jejuni*-infected birds, increased mRNA transcripts for pro-inflammatory cytokines such as IL-1β, IL-6, and chemokines CXCLi1 and CXCLi2 (both putative orthologues to human IL-8) were observed (23, 155, 156). However, this induction has not yet been correlated with increased production of the respective cytokines in birds due to limited reagents to detect these avian proteins. In short, despite some evidence supporting a pro-inflammatory response initiated by *C. jejuni*, this type of response does not ultimately result in great changes to tissue or progression to disease.

Another hypothesis for the lack of inflammation during commensal colonization is that *C. jejuni* may modulate the immune system of the avian host

by inducing expression of certain anti-inflammatory cytokines such as IL-10 or TGF-β that may temper any pro-inflammatory responses (155). This latter hypothesis has been explored but expression of these cytokines was not observed in cecal or ileal tissue of *C. jejuni*-infected birds (155). Thus, *C. jejuni* may not be actively suppressing the local immune response in infected avian tissue. Alternatively, the lack of inflammation in the intestinal tract of *C. jejuni*-infected birds may be related to the levels of *C. jejuni* invading the tissue. Smith *et al* have suggested that the level of invasion across the intestinal epithelium by *C. jejuni* may not be significant to initiate obvious inflammation or damage to avian intestinal tissue (155). We favor this hypothesis based on our observations in this study. We observed an extensive invasion of *S. pullorum* across cecal and bursal epithelia that correlated with inflammation and tissue destruction. However, epithelial invasion of *C. jejuni* was only observed at 1 day post-infection. Thus, insignificant levels of invasion of the avian epithelium *in vivo* and the eventual confinement of *C. jejuni* to the luminal surfaces may limit insults to avian intestinal tissue to prevent any damaging pro-inflammatory response.

Prolonged colonization of the ceca and bursa by *C. jejuni* may be specific for the development of commensalism. Because the nutrient availability and cellular composition of the ceca and bursa are likely very different, we hypothesized that factors of *C. jejuni* may be differentially required to initiate or maintain colonization of the ceca or bursa. While our study was not extensive in

analyzing numerous colonization factors, we did find that certain factors of *C. jejuni* appear to promote optimal colonization and persistence in different organs. For instance, we found that production of the capsular polysaccharide is required by the bacterium to initiate infection of the spleen and liver and to maintain infection of the ceca and bursa. Thus, this colonization factor is required for infection of and persistence in multiple organs over time. However, the cytolethal distending toxin, which was previously found to be dispensable for short-term colonization of the ceca, was required to maintain wild-type levels of *C. jejuni* in the bursa. This finding was also very similar for a mutant of *C. jejuni* lacking catalase, which is sensitive to peroxide stress. Considering we have previously performed a negative selection procedure using signature-tagged transposon mutants to identify genes of *C. jejuni* required for short-term colonization of the ceca (75), it will be interesting to explore if this technology can be adapted to identify genes of the bacterium required for bursal colonization. Furthermore, future studies analyzing the colonization factors of the bacterium may need to explore not only short-term colonization but also persistent colonization of multiple organs to fully delineate the importance of the factors for *in vivo* growth of *C. jejuni*.

The observations from this study have revealed increased dimensions and complexities in understanding the interactions between *C. jejuni* and its natural host. How *C. jejuni* and the avian host interact with each other is reminiscent of

commensals in the mammalian intestinal tract (discussed in further detail in Chapter 6) and suggests interactions between gut commensals and hosts are evolutionary conserved amongst animals. The knowledge gleaned from this study may help to determine what aspects of the infectious process or the host response to *C. jejuni* infection may be different in humans that contribute to the development of disease.

CHAPTER FIVE

Characterization of Two Putative Cytochrome *c* Peroxidases of *C. jejuni*[§]

Introduction

To understand the requirements of *C. jejuni* for colonization of poultry that result in commensalism, we previously employed a genetic selection procedure using signature-tagged transposon mutants to identify genes of the bacterium involved in growth within the avian ceca (75). Among the 22 different genes we identified were ones required for flagellar motility, protein glycosylation, and a putative cytochrome c peroxidase (CCP) that we annotated as *docA* (for *d*eterminant *o*f chick *c*olonization). Deletion of *docA* from *C. jejuni* strain 81-176 results in 10- to 10^5-fold lower bacterial loads in the ceca of chicks at seven days post-infection compared to those of chicks infected with wild-type bacteria (75).

Bacterial CCPs are periplasmic proteins that reduce potentially toxic hydrogen peroxide compounds to water (5). Two heme molecules that are bound by each CCP protein receive electrons from cytochrome c to reduce hydrogen peroxide to water without generating other reactive oxygen intermediates (Figure

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13). Despite having a peroxidase activity *in vitro*, the physiological roles of this activity and, more globally, the contribution of the CCPs to biological functions in bacteria are not well understood (5). The CCP of *Neisseria gonorrhoeae* may have a part in providing resistance to hydrogen peroxide since a mutant lacking the CCP is slightly more sensitive to the compound than wild-type bacteria (166). However, the level of resistance to hydrogen peroxide provided by the cytoplasmic catalase of *N. gonorrhoeae* is much greater, suggesting that the primary role of the CCP in this bacterium may not be protection from oxidative stress. Considering that many Gram-negative bacteria do not have a CCP and these proteins are absent from Gram-positive organisms, CCPs are not believed to have a universal role in bacteria in promoting resistance to oxidative stress (5).

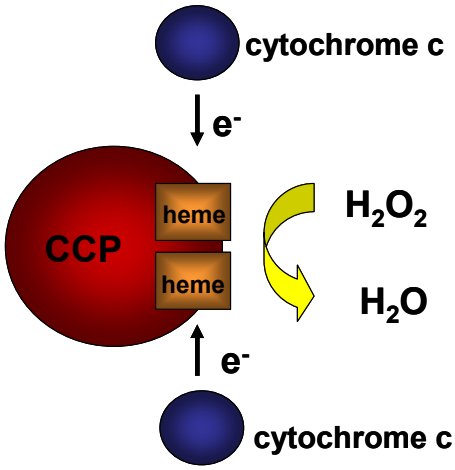

Figure 13. Function of cytochrome c peroxidases (CCPs). CCPs are heme-binding proteins that reduce toxic peroxide compounds through the activity of two heme compounds. This reaction occurs via a two-step process in which each heme receives electrons from cytochrome c to reduce hydrogen peroxide to water and oxygen.

Bioinformatic analysis of the *C. jejuni* 81-176 genome has predicted that the gene *cjj0382* encodes another putative CCP. In order to understand the importance of *docA* and *cjj0382* in colonization, we compared the ability of mutants lacking each gene to promote commensal colonization of 1-day old chicks. We characterized the biochemical properties of DocA and Cjj0382 to determine if they had characteristics of typical CCPs. We also explored their role, along with KatA, a cytoplasmic catalase, in promoting peroxide resistance. This study provides insight into the colonization determinants that are required for optimal *in vivo* growth of *C. jejuni* in poultry.

Results

*Δ*docA and *Δ*cjj0382 mutants display different colonization capacities*

Through a genetic selection procedure using signature-tagged transposon mutants that identified genes of *C. jejuni* 81-176 involved in commensal colonization of the chick ceca, we discovered that a *docA* mutant of *C. jejuni* is severely attenuated for colonization (75). Bioinformatic analysis suggests that DocA, which is predicted to function as a CCP, is similar to another putative CCP encoded by *cjj0382* in *C. jejuni* 81-176 (51). DocA and Cjj0382 share 62% similarity and 44% identity which include two different putative heme-binding motifs in each protein. These heme-binding sites are predicted to be essential for

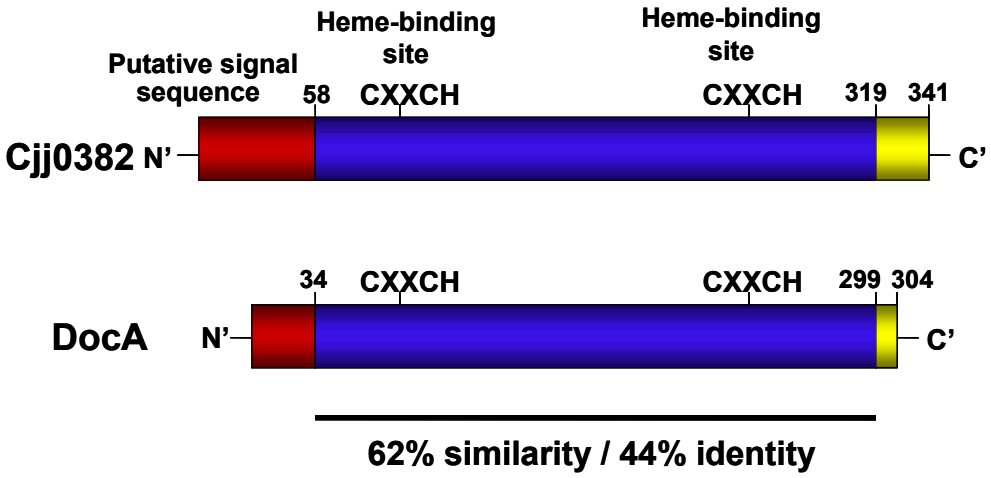

Figure 14. Putative functional domains of Cjj0382 and DocA. Cjj0382 and DocA have predicted N-terminal signal sequences and are most divergent in the first 58 and 33 amino acids of each protein, respectively. Both proteins contain two CXXCH heme binding site motifs. DocA and Cjj0382 are 62% similar and 44% identical in the central 266 amino acid region.

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binding heme for the peroxidase activity that reduces hydrogen peroxide to water (5, 42). The largest region of non-homology occurs in the N-terminal regions of the proteins which contain predicted N-terminal signal sequences for transport out of the bacterial cytoplasm. The domain organization of DocA and Cjj0382 is shown in Figure 14. The presence of two CCPs in an individual bacterium appears to be uncommon (5). Phylogenetic analyses have predicted that Cjj0382 clusters closely to other 'classical' CCPs that are typically found in various Gram-negative bacteria (5). However, DocA is not closely related phylogenetically to any predicted bacterial CCP and is currently the sole member of a separate clade (Figure 15).

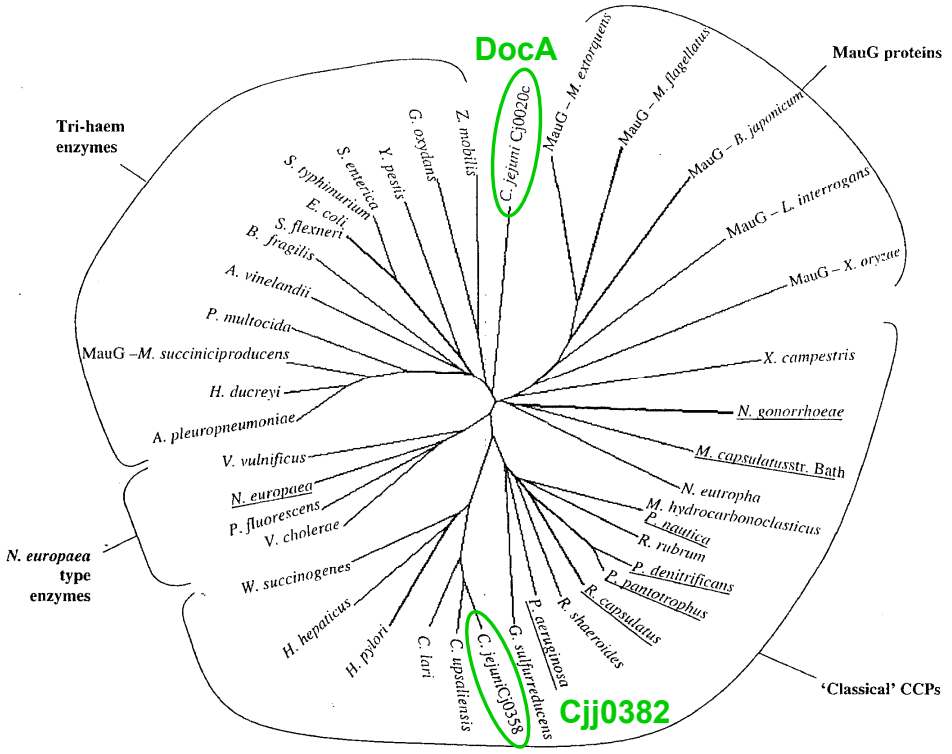

Figure 15. Phylogenetic tree illustrating relationships between bacterial CCPs. Cjj0382 is closely related to 'Classical' CCPs. DocA is not phylogenetically related to any CCP and is the only member of its clade. Image is adapted from Adv. Microb. Physiol. 2007; 52:73-106 and used with permission.

Since previous analysis by our laboratory revealed that a Δ*docA* mutant (which was constructed by fusing the start codon to the last 12 codons, deleting the intervening 292 codons) is attenuated for colonization of the chick ceca (75), we analyzed if Cjj0382 is required by *C. jejuni* for efficient colonization of chicks. We created a mutant of *cjj0382* in 81-176 Sm^R (DRH212; (74)) by deleting the entire coding sequence of the gene from the chromosome.

The ability of the Δ*cjj0382* mutant to colonize the chick ceca was compared to wild-type 81-176 Sm^R and 81-176 Sm^R Δ*docA* by orally infecting 1-day old chicks with inocula of approximately 10^6, 10^4, or 10^2 organisms. Wild-type bacteria consistently colonize the chick ceca at approximately 10^9 cfu per gram of cecal content at day seven post-infection, regardless of the inocula size (Figures 16A-C). At inocula of approximately 10^6 and 10^4, the Δ*docA* mutant colonizes at levels 10- to 1,000-fold lower than wild-type bacteria with a further reduction up to 10,000-fold at an inoculum of 10^2 bacteria (Figures 16A-1C). When we analyzed the Δ*cjj0382* mutant, we discovered that this strain displays a less severe colonization defect than the Δ*docA* mutant. At the higher inocula (10^6 and 10^4 bacteria), the Δ*cjj0382* mutant demonstrates levels of colonization similar to wild-type with only a very slight colonization defect of approximately two-fold at the 10^4 inoculum (Figures 16A and 16B). However, at the lowest inoculum (approximately 10^2 bacteria), we did observe a statistically significant 10- to 50-fold reduction in colonization for this mutant (Figure 16C). The defects in colonization of the mutants are specific for *in vivo* growth as we could not detect any differences in growth between the wild-type and mutant strains during *in vitro* growth in laboratory media (data not shown).

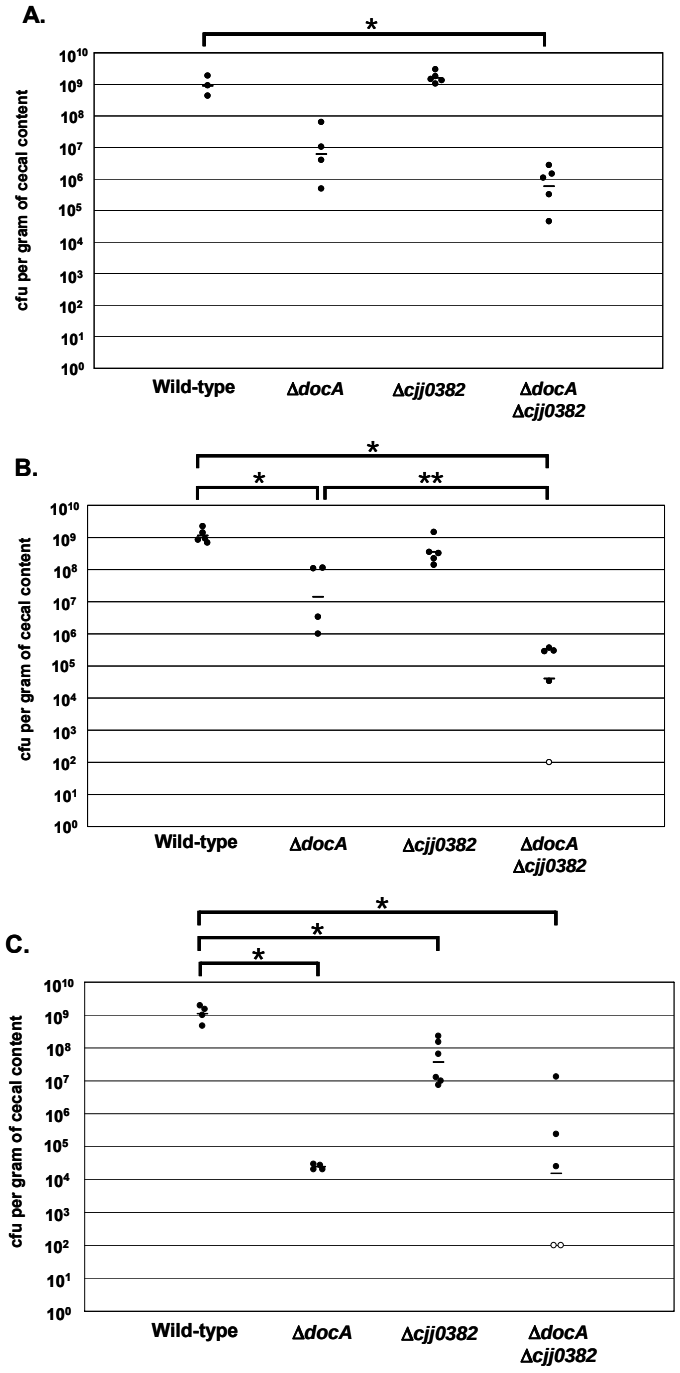

Figure 16. The Δ*cjj0382* mutant of *C. jejuni* displays a less severe colonization defect than the Δ*docA* mutant. One-day old chicks were orally infected with 100 µl of *C. jejuni* 81-176 Sm^R (DRH212), 81-176 Sm^R Δ*docA*

(DRH1169), 81-176 Sm^R Δ*cjj0382* (LKB151), or 81-176 Sm^R Δ*docA* Δ*cjj0382* (LKB177) at inocula of approximately (A) 10^6, (B) 10^4 or (C)10^2 bacteria. Chicks were sacrificed seven days post-infection to determine the cecal colonization capacity of each *C. jejuni* strain. Each filled symbol represents the amount of *C. jejuni* recovered from the ceca of a single chick which is reported as the number of cfu per gram of cecal content. Each open symbol represents chicks in which the level of *C. jejuni* colonization was below the limit of detection (<100 cfu per gram of cecal content). The geometric mean of the bacterial loads from each set of chicks is denoted by a line (—). Statistical analysis was performed using the Mann-Whitney U test ($P < 0.05$). *, indicates a significant difference between wild-type and mutant strains. **, indicates a significant difference between the 81-176 Sm^R Δ*docA* mutant and the 81-176 Sm^R Δ*docA* Δ*cjj0382* mutant. The actual inoculum doses ranged from: (A) 5 x 10^5 to 1.7 x 10^6 cfu, (B) 4.4 x 10^3 to 2.19 x 10^4 cfu, and (C) 58 to 208 cfu.

To more thoroughly characterize the requirements for DocA and Cjj0382 for colonization of the chick ceca, we made a mutant that lacks both *docA* and *cjj0382* (81-176 Sm^R Δ*docA* Δ*cjj0382*). At inocula of approximately 10^6 and 10^4, the colonization defect of the Δ*docA* Δ*cjj0382* mutant is greater than mutants lacking only *docA* or *cjj0382*, with the differences in colonization with the latter inoculum being statistically significant (Figures 16A-16B). In addition at the 10^4 inoculum, one chick contained levels of *C. jejuni* that were below the limit of detection. Even though two chicks contained *C. jejuni* at levels below the limit of detection with an inoculum of 10^2, we could not detect a significant difference between the overall colonization capacities of the Δ*docA* and the Δ*docA* Δ*cjj0382* mutant even though both mutants colonized at significantly lower levels than the wild-type strain (Figure 16C). Together these data suggest that both proteins are

involved in promoting efficient commensal colonization of the chick ceca yet a greater dependency on DocA for colonization compared to Cjj0382 is evident.

Either a lack of expression of *cjj0382* or an increased expression of *docA* relative to *cjj0382* during *in vivo* growth could explain why the Δ*cjj0382* mutant is only slightly attenuated for *in vivo* growth in chicks compared to the Δ*docA* mutant. To determine if either of these hypotheses were valid, we compared the relative levels of *docA* and *cjj0382* transcripts by real-time RT-PCR from wild-type *C. jejuni* directly isolated from the chick ceca seven days post-infection. Contrary to our hypotheses, we discovered that both genes are expressed *in vivo* and that the levels of expression of *cjj0382* were approximately 15-fold greater than *docA* (data not shown). Therefore, expression of *cjj0382* is not a mitigating factor for the difference in colonization capacities of the Δ*docA* and Δ*cjj0382* mutants. However, we can not rule out translational control mechanisms that may affect the different levels of production of DocA or Cjj0382 proteins *in vivo* to explain the less attenuated colonization phenotype of the Δ*cjj0382* mutant.

DocA and Cjj0382 are localized to the periplasm of C. jejuni

Since *C. jejuni* mutants lacking either DocA or Cjj0382 display dramatically different colonization phenotypes, we hypothesized that these two proteins may perform different physiological roles in the bacterium necessary for optimal colonization of poultry. Because both proteins are predicted to function

as bacterial CCPs, we investigated whether these proteins have features common to this class of proteins.

Since bacterial CCPs are periplasmic proteins, we first determined the location of DocA and Cjj0382 in *C. jejuni* by separating wild-type and mutant strains into outer membrane, inner membrane, periplasmic, and cytoplasmic fractions and performing immunoblot analysis on each fraction with antisera against DocA or Cjj0382. In whole-cell lysates, DocA and Cjj0382 appear as 34-kDa and 37-kDa proteins, respectively, in wild-type *C. jejuni* (Figures 17A and 17B). Analysis of wild-type and Δ*docA* mutants revealed DocA to be a soluble protein in the periplasmic and cytoplasmic fractions (Figure 17A). Similarly, Cjj0382 was found in the periplasm and cytoplasm (Figure 17B). Whereas localization to the periplasm is characteristic of a typical bacterial CCP, we suspect that the cytoplasmic forms of these proteins are likely nascently translated proteins that have yet to be secreted to the periplasm. As a control for a periplasmic protein, we performed arylsulfatase assays to detect AstA, an enzymatic protein with a predicted signal sequence for localization to the periplasm. In fractionation samples representing periplasmic and cytoplasmic proteins, arylsulfatase activity was present in both fractions (data not shown). Thus, proteins localized to the periplasm in *C. jejuni* appear in the cytoplasm as well. However, we do not believe that the presence of DocA or Cjj0382 in the periplasm is due to contamination of this fraction with cytoplasmic proteins as

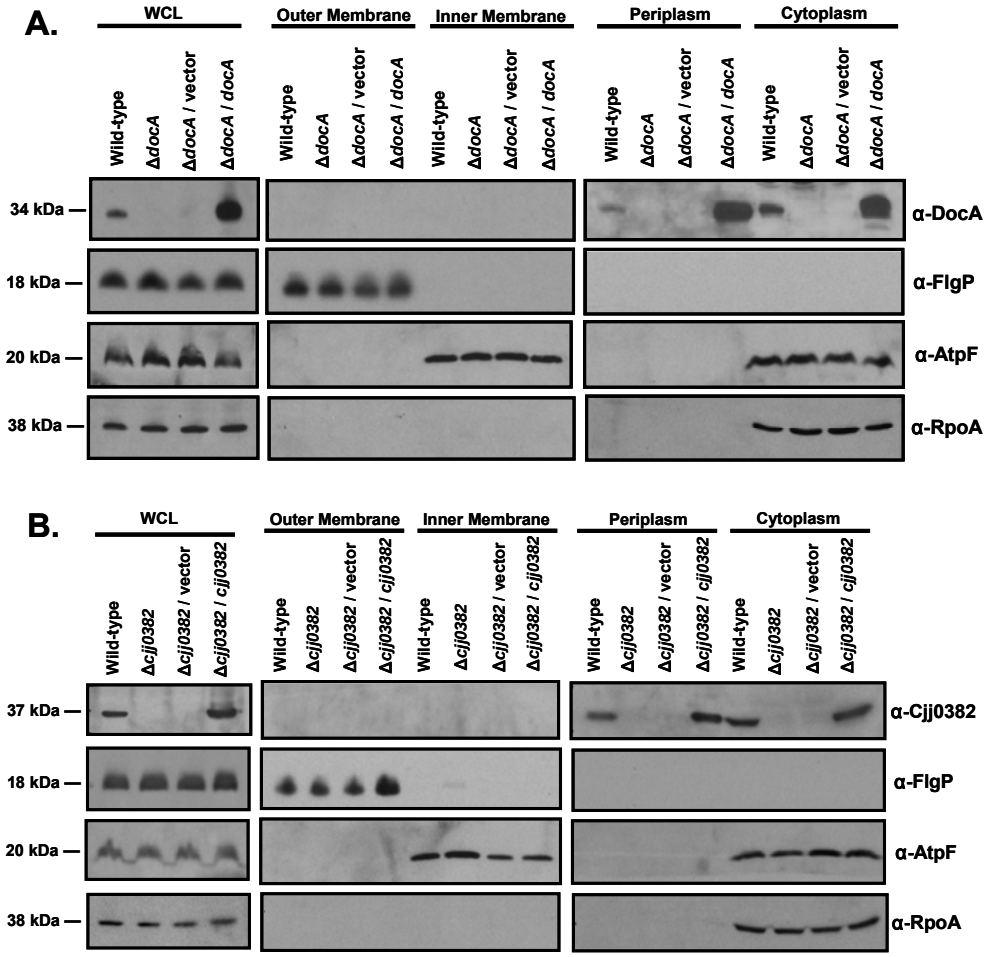

Figure 17. DocA and Cjj0382 are periplasmic proteins in *C. jejuni*. Proteins were separated by 10% SDS-PAGE. α-DocA and α-Cjj0382 antisera were used for detection of the respective proteins. (A) Analysis of DocA localization. Strains used include *C. jejuni* 81-176 Sm^R (DRH212), Δ*docA* (DRH1169), Δ*docA*/pRY112 (LKB307), and Δ*docA*/pRY112::*docA* (LKB313). (B) Analysis of Cjj0382 localization. Strains used include *C. jejuni* 81-176 Sm^R (DRH212), Δ*cjj0382* (LKB151), Δ*cjj0382*/pRY112 (LKB310) and Δ*cjj0382*/pRY112::*cjj0382* (LKB277). For both (A) and (B), fractions analyzed included those of the whole-cell lysates (WCL), outer membrane, inner membrane, periplasm, and cytoplasm. α-FlgP, α-AtpF, and α-RpoA were used to verify fractionation procedures for the outer membrane, inner membrane and cytoplasm, respectively. Proteins from WCL representing 200 μl of bacterial culture and proteins from the other fractions representing 400 μl of bacterial culture were used for immunoblotting.

RpoA, a component of RNA polymerase, was only detected in cytoplasmic fractions (Figures 17A and 17B). Neither DocA nor Cjj0382 were found in the outer or inner membrane fractions. Verification of the purity of outer and inner membrane fractions was confirmed by detecting the FlgP flagellar protein in the outer membrane (159) and AtpF, a component of ATP synthase, in the inner membrane (Figures 17A and 17B). In addition, the levels of the control proteins (RpoA, FlgP, and AtpF) are similar amongst the wild-type and mutant strains analyzed, ensuring that equal amounts of proteins were analyzed for each fraction from the various strains.

Complementation of the Δ*docA* or Δ*cjj0382* mutants *in trans* with a plasmid expressing each respective gene from their predicted native promoters restored the presence of the proteins in the periplasm and the cytoplasm, indicating that our antisera is specific for only DocA or Cjj0382. Our analysis suggests that like typical bacterial CCPs, both proteins are localized to the periplasm. However, these findings do not provide insight into the differing physiological roles that the proteins may have during commensal colonization.

DocA and Cjj0382 are heme-binding proteins

Most CCPs bind two heme compounds via different CXXCH heme-binding site motifs. DocA and Cjj0382 each contain two CXXCH motifs at residues 56-60 and 199-203 in DocA, and residues 80-84 and 225-229 in Cjj0382.

To determine if DocA and Cjj0382 bind heme, we performed a technique described previously by Feissner *et al.* that specifically detects heme-bound proteins (47). A very similar, but less sensitive method has been used to detect bound heme in the CCP of *N. gonorrhoeae* (166). As shown in Figures 18A and 18B, we could detect three separate heme-bound proteins with peroxidase activity from the periplasmic fraction of *C. jejuni* that migrate closely to each other. The lowest protein band appears to represent DocA while the uppermost band appears to be Cjj0382. The faint band representing DocA is absent in Δ*docA* mutants and appears as an abundant protein band when *docA* is over expressed *in trans* from a plasmid (Figure 18A). Similarly, the uppermost band representing Cjj0382 is absent in Δ*cjj0382* mutants but restored upon expression of the gene *in trans* (Figure 18B). The middle band in all blots likely represents another heme-bound periplasmic protein of *C. jejuni* whose identity currently remains unknown. This assay not only provides evidence that these proteins are likely to bind heme but also reveals that the hemes, and consequently DocA and Cjj0382, have the characteristic peroxidase activity associated with CCPs.

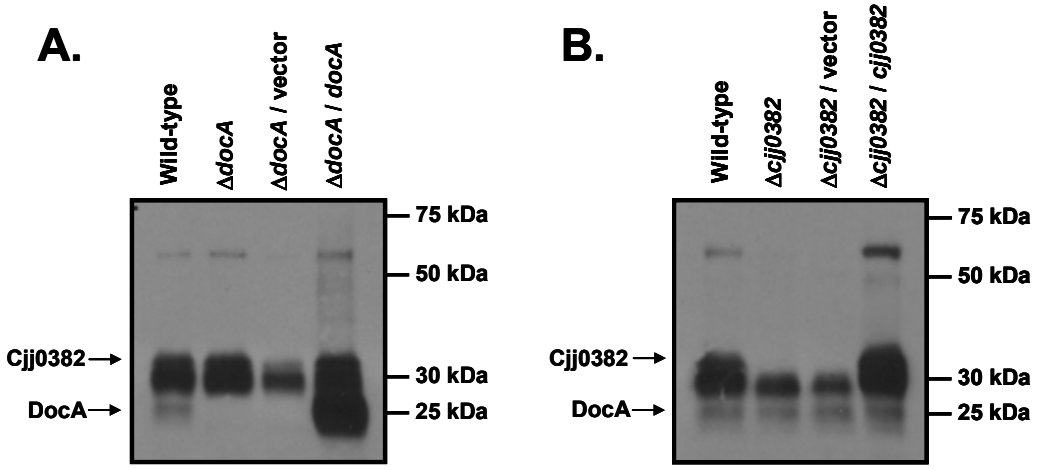

Figure 18. DocA and Cjj0382 have heme-associated peroxidase activity. A heme stain assay was performed on proteins isolated from the periplasm. (A) Analysis of DocA heme-binding ability. Strains used include *C. jejuni* 81-176 Sm^R (DRH212), Δ*docA* (DRH1169), Δ*docA*/pRY112 (LKB307), Δ*docA*/pRY112::*docA* (LKB313). (B) Analysis of Cjj0382 heme-binding ability. Strains used include *C. jejuni* 81-176 Sm^R (DRH212), Δ*cjj0382* (LKB151), Δ*cjj0382*/pRY112 (LKB310), and Δ*cjj0382*/pRY112::*cjj0382* (LKB277). The upper band represents Cjj0382, the faint lower band is DocA, and the middle band is an uncharacterized heme-bound protein of *C. jejuni*.

To further explore the ability of DocA and Cjj0382 to bind heme, we created point mutations in the CXXCH heme-binding site motifs of each protein by mutating cysteine residues to serine or histidine residues to alanine. However, mutation of the heme-binding sites in *docA* resulted in unstable mutant proteins that could not be detected in whole-cell lysates via immunoblotting (data not shown). Similar mutations in *cjj0382* resulted in unstable mutant proteins or proteins that were detected at lower levels in whole-cell lysates but none of these

proteins could be detected in the periplasm (data not shown). Thus, we are unable to confirm that the heme-binding motifs of DocA and Cjj0382 are required for attachment of heme.

Neither DocA nor Cjj0382 contributes substantially to resistance to hydrogen peroxide in vitro

The physiological role of many previously studied bacterial CCPs remains uncertain (5). A role in hydrogen peroxide resistance has been demonstrated for the CCP of *N. gonorrhoeae* in which a mutant lacking its CCP is slightly more sensitive to hydrogen peroxide (166). Considering that the heme-binding detection assay suggested that DocA and Cjj0382 have peroxidase activity, we tested the ability of DocA and Cjj0382 to promote survival of *C. jejuni* upon exposure to hydrogen peroxide. Wild-type and mutants of *C. jejuni* lacking *docA* or *cjj0382* were exposed to 0.5 mM hydrogen peroxide for 30 min and the number of bacteria surviving treatment was determined. By performing this analysis, we found that the Δ*docA*, Δ*cjj0382*, and Δ*docA* Δ*cjj0382* mutants demonstrate a wild-type level of resistance to hydrogen peroxide (Figure 19A).

C. jejuni produces catalase, encoded by *katA*, that has been shown to promote resistance to hydrogen peroxide (40, 60). We reasoned that the presence of cytoplasmic catalase in the Δ*docA* or Δ*cjj0382* mutants may be masking any resistance to hydrogen peroxide promoted by DocA or Cjj0382. We generated a

Δ*katA* mutant in *C. jejuni* 81-176 Sm^R and observed that this mutant is up to 10,000-fold more sensitive to hydrogen peroxide than wild-type bacteria (Figure 19A). We then constructed mutants lacking *docA*, *cjj0382*, or both genes in the Δ*katA* background and determined if we could observe any increased sensitivity to hydrogen peroxide that could be attributed to the lack of DocA or Cjj0382. In this assay, the level of survival of the Δ*katA* mutant to 0.5 mM hydrogen peroxide is set at 100%; the percent survival of all other mutant strains are calculated relative to that of the Δ*katA* mutant. Removal of *cjj0382* in the Δ*katA* mutant did not affect the survival of *C. jejuni*. Deletion of *docA* from the Δ*katA* or Δ*katA* Δ*cjj0382* resulted in no more than a 2-fold increase in sensitivity to hydrogen peroxide. These results combined largely indicate that neither DocA nor Cjj0382 contribute significantly to survival upon exposure to hydrogen peroxide *in vitro*. Rather, this phenotype is largely attributed to catalase. We also analyzed the sensitivity of *C. jejuni* to other peroxides such as cumene hydroperoxide and *tert*-butyl hydroperoxide, but we found no evidence that Cjj0382 or DocA contribute to survival upon exposure to these compounds either (data not shown). Taken together, these data suggest that DocA and Cjj0382 have peroxidase activity, but this activity does not contribute to an overall resistance to peroxide stress.

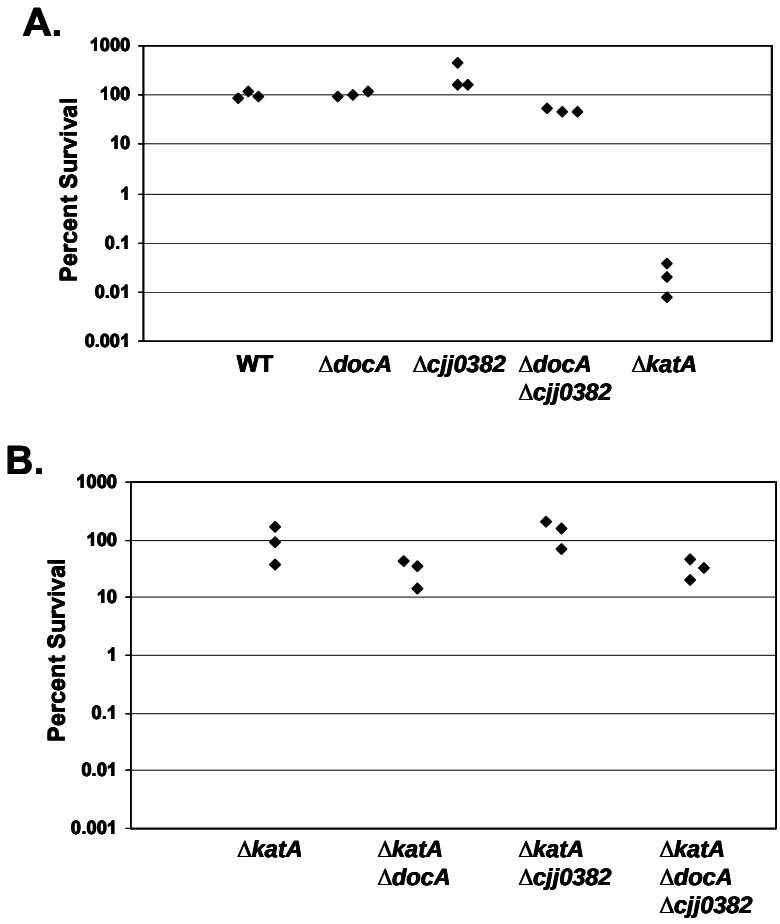

Figure 19. DocA and Cjj0382 do not promote significant resistance to hydrogen peroxide *in vitro*. *C. jejuni* strains were treated with 0.5 mM hydrogen peroxide for 30 minutes at 37 ºC under microaerophilic conditions. (A) The percent survival of wild-type bacteria was set at 100% and all other strains were normalized to this value. Strains used include *C. jejuni* 81-176 Sm^R (DRH212), Δ*docA* (DRH1169), Δ*cjj0382* (LKB151), Δ*docA* Δ*cjj0382* (LKB177), Δ*katA* (LKB246). (B) The percent survival of the Δ*katA* mutant was set at 100% and all other strains were normalized to this value. Strains used include *C. jejuni* Sm^R Δ*katA* (LKB246), Δ*katA* Δ*docA* (LKB181), Δ*katA* Δ*cjj0382* (LKB231), and Δ*katA* Δ*docA* Δ*cjj0382* (LKB226). For (A) and (B), each diamond represents the percent survival of a bacterial sample after exposure to hydrogen peroxide.

We explored if resistance to peroxide stress is a major determinant for *C. jejuni* in promoting commensal colonization of chicks by analyzing the ability of the Δ*katA* mutant, which is hypersensitive to hydrogen peroxide, to colonize the ceca of chicks. Previous studies in *Campylobacter coli* found that a catalase-deficient mutant has a colonization capacity similar to wild-type (139). In our colonization experiments, a Δ*katA* mutant shows 10- to 50-fold reduced bacterial levels in the ceca of chicks seven days post-infection regardless of the inocula size (Figure 20). In comparison to the Δ*docA* mutant, the Δ*katA* mutant is much less attenuated for colonization (Compare Figures 16A-C to Figure 20). Considering that the *katA* mutant is hypersensitive to hydrogen peroxide yet does not demonstrate a large defect in colonization, we suspect that peroxide stress is not a large factor for *C. jejuni* to overcome during cecal colonization.

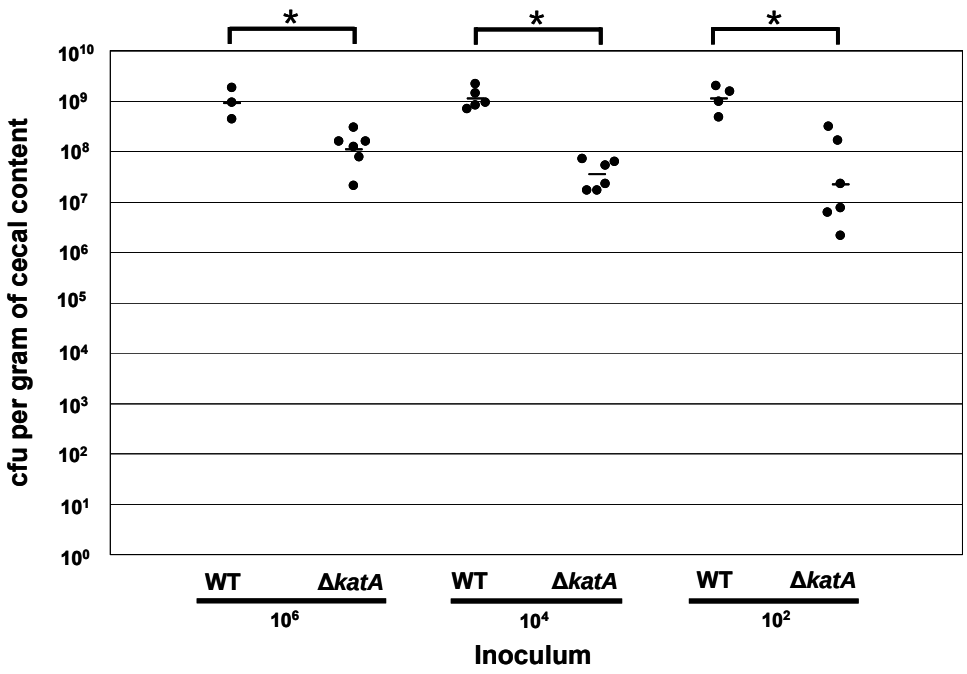

Figure 20. A Δ*katA* mutant of *C. jejuni* has a moderate defect for cecal colonization in chicks. One-day old chicks were orally infected with 100 µl of *C. jejuni* 81-176 Sm^R (DRH212) or 81-176 Sm^R Δ*katA* (LKB246) at inocula of approximately 10^6, 10^4, or 10^2 bacteria. Chicks were sacrificed seven days post-infection to determine the cecal colonization capacity of each *C. jejuni* strain. Each filled symbol represents the amount of *C. jejuni* recovered from the ceca of a single chick which is reported as the number of cfu per gram of cecal content. The geometric mean of the bacterial loads from each set of chicks is denoted by a line (—). Statistical analysis was performed using the Mann-Whitney U test ($P < 0.05$). *, indicates a significant difference between wild-type and mutant strains. The actual inoculum doses ranged from: 1.36 x 10^6 to 2.38 x 10^6 cfu (left), 5.9 x 10^3 to 8.2 x 10^3 cfu (middle), and 58 to 59 cfu (right). The colonization assay of wild-type *C. jejuni* shown in this figure is from the same experiments shown in Figures 16A-C.

Discussion

In this study, we found that *cjj0382* of *C. jejuni* encodes a protein with significant homology to DocA, a known determinant important for the bacterium to promote commensal colonization of poultry (75). A Δ*docA* mutant displays up to a 10,000-fold colonization defect, whereas the Δ*cjj0382* mutant shows a maximal 50-fold defect only at a low inoculum. These data suggest that DocA and Cjj0382 may not have redundant functions during *in vivo* growth even though both proteins are similar to each other and are predicted to serve as CCPs. Additional analysis confirmed that these two proteins have certain characteristics common to CCPs: 1) they are located in the periplasm; and 2) they bind heme for an inherent peroxidase activity that can be detected *in vitro*. However, neither DocA nor Cjj0382 provide any significant level of resistance to hydrogen peroxide *in vitro*. Further analysis suggested that the major resistance to hydrogen peroxide stress in *C. jejuni* is mediated by the cytoplasmic catalase. Since the *docA* mutant is 10- to 100-fold more attenuated for colonization than the catalase mutant, which is hypersensitive to hydrogen peroxide, we suspect that DocA may perform a physiological function *in vivo* other than promoting resistance to oxidative stress.

The observation that DocA and Cjj0382 do not play a major role in survival to exogenous hydrogen peroxide may not be surprising. In fact, only the CCP of *N. gonorrhoeae* has been shown to aid in survival to exposure to

hydrogen peroxide (166). However, catalase provides the majority of resistance to hydrogen peroxide in this bacterium. Instead of promoting resistance to hydrogen peroxide, the CCP in *Bacteroides fragilis* functions in survival of the bacterium to exposure to organic peroxides such as cumene hydroperoxide and *tert*-butyl hydroperoxide (78). However, we did not find that DocA or Cjj0382 protected *C. jejuni* from either of these organic peroxides. It is possible that DocA and Cjj0382 do provide resistance to hydrogen peroxide *in vivo*, but our *in vitro* assays are not sensitive enough to detect a difference in viability of the bacterium upon exposure to reactive oxygen species. Secondly, there may be additional factors or environmental conditions during *in vivo* growth that contribute to DocA and Cjj0382 mediating resistance to peroxides that we are unable to reproduce in our *in vitro* assays. Most likely, the majority of hydrogen peroxide protection in *C. jejuni* is due to catalase which we could observe in our *in vitro* assays and others have shown previously for *Campylobacter* species (40, 60).

One of the most common hypotheses for the physiological function of CCPs in various bacteria is that they may play a role during respiration or metabolism. For instance, when formate is used as an electron donor in *Campylobacter mucosalis*, hydrogen peroxide is generated as a by-product which may be toxic to the bacterium or harmful to other physiological activities (57). Evidence suggests that *C. jejuni* produces a formate dehydrogenase, but it is

unknown if this complex is required by the bacterium for colonization of chickens or if it generates periplasmic hydrogen peroxide (81). In *Thiosphaera pantotropha* and *Rhodobacter capsulatus*, hydrogen peroxide inhibits reduction of nitrate, an important activity for respiration (142). Thus, limiting hydrogen peroxide concentrations in the periplasm is essential for nitrate respiration. *C. jejuni* has nitrate reductase activity but this activity has not been tested for a critical function during *C. jejuni* colonization of chickens (136, 149). Multiple electron transport chains have been proposed for *C. jejuni* and some of these pathways result in transfer of electrons to cytochrome c (127, 149). Since DocA and Cjj0382 may be like most other CCPs in receiving electrons from cytochrome c, any metabolic pathways that pass down electrons to cytochrome c could be affected in mutants lacking DocA or Cjj0382. Further experimentation is necessary to determine if these metabolic pathways are important for *C. jejuni* in promoting colonization of poultry and if DocA or Cjj0382 influence these aspects of metabolism.

Infection of poultry with *C. jejuni* results in a commensal colonization of the ceca characterized by the lack of a significant inflammatory response from the host. Therefore, it may be hypothesized that the bacterium is not exposed to large amounts of hydrogen peroxide generated by an inflammatory response. Very few inflammatory cells such as heterophils (the avian equivalent to human neutrophils) or macrophages migrate to the cecal epithelium or lamina propria

during colonization, limiting the interactions of *C. jejuni* with these cellular components of innate immunity and their associated damaging oxidative bursts (11). Thus, surviving oxidative stress produced by an inflammatory response would be predicted to not be a major hurdle for *C. jejuni* to overcome during colonization of poultry. This hypothesis is supported by the cecal colonization phenotype of the Δ*katA* mutant which is hypersensitive to hydrogen peroxide but is only moderately attenuated for *in vivo* growth.

DocA and Cjj0382 have created interesting questions regarding the composition and biological activities of *C. jejuni*. First, possessing two different proteins that may function in some form as CCPs is somewhat unusual for a single bacterium. Most Gram-negative bacteria have only one CCP, if they have one at all, and these proteins are absent from Gram-positive organisms (5). Secondly, our results indicate that DocA and Cjj0382 likely perform non-redundant biological activities for *C. jejuni* during *in vivo* growth. Despite their homology, phylogenetic analysis of various bacterial CCPs suggest that Cjj0382 is grouped in a clade of 'classical' CCPs. However, DocA – despite its similarity to Cjj0382 – is the sole member of its own clade (5). This observation suggests that DocA may be truly unique amongst CCPs with a potentially uncommon or unique function. Further exploration is required to uncover the specific physiological functions of DocA and Cjj0382 *in vivo* that are critical for the ability of the bacterium to promote commensalism in poultry.

CHAPTER SIX

Discussion

Overview

C. jejuni is a major food-borne pathogen prevalent throughout the world (33, 36). The high prevalence of *C. jejuni* is likely due to the ability of the bacterium to develop unique relationships with different hosts, causing disease in humans but a harmless, asymptomatic colonization in many animals. Therefore, understanding the mechanisms by which *C. jejuni* infection leads to disease in humans, yet promotes a commensal relationship with avian species will greatly benefit the design of potential antimicrobial strategies to reduce and control the incidence of disease. The objective of this study was to enhance our understanding of the interactions between *C. jejuni* and poultry, and to identify and characterize factors that contribute to the formation of this commensal relationship.

Dynamic Interactions of *C. jejuni* with Poultry

In this study we provided extended insights into the colonization dynamics of *C. jejuni* that lead to a better understanding of the development of commensalism in the natural avian host. By performing a comparative analysis between an avian commensal, *C. jejuni*, and an avian pathogen, *S. pullorum*, we

were able to directly compare the bacterial-host interactions that contribute to different outcomes of infection.

We identified several interactions between *C. jejuni* and poultry that are specific for the development of commensalism. While some of these interactions were previously speculated to occur, they had not been thoroughly investigated. The ability of *C. jejuni* to promote colonization of the intestines has been well studied. However, prior to the work presented in this dissertation, little was known about either prolonged colonization or the ability of *C. jejuni* to colonize extraintestinal sites. The presence of *C. jejuni* in the liver, spleen, bursa, and blood has previously been noted, but these studies failed to report the degree of colonization and persistence at these sites (11, 38, 103, 122, 188). Through the studies presented here, we have observed that *C. jejuni* promotes a robust, prolonged colonization of the bursa of Fabricius, a major lymphoid organ of chickens. We also observed invasion of the cecal epithelium by *C. jejuni*, but only at the earliest time point of infection studied (day 1 post-infection), which correlates with the observed transient, systemic infection of the spleen and liver. Futhermore, by comparing the colonization dynamics of *C. jejuni* and *S. pullorum* we discovered that these dynamics of *C. jejuni* are specific for commensalism. Infection with *S. pullorum* resulted in initial high levels of colonization of the ceca and bursa, and systemic spread to the spleen and liver. This correlated with invasion of cecal and bursal tissue and massive inflammation. We believe that the

specific ability of *C. jejuni* to promote a prolonged colonization of intestines and gut-associated lymphoid tissue of the bursa, and the ability of the avian host to largely confine the bacterium to the luminal mucosal surfaces of these organs limits inflammation and damage that could occur in avian intestinal tissue.

Mechanism and Role of Bursal Colonization

We believe the robust, prolonged colonization of *C. jejuni* in the bursa may be of significant importance in promoting the development of a commensal relationship with poultry. A significant finding from our work that has not been previously reported is that *C. jejuni* promotes a prolonged bursal colonization during commensalism that is confined to the bursal epithelium. In analyzing bursal colonization, we noticed a few characteristics that require further investigation. Furthermore, additional studies will need to be performed to understand the role of bursal colonization in contributing to commensalism.

The first aspect of bursal colonization that needs to be further investigated is the means by which *C. jejuni* gets to the bursa and maintains colonization for a prolonged period. We hypothesize that *C. jejuni* is able to reach the bursa by two possible mechanisms: 1) bursal colonization may simply be due to the anatomical location of the bursa, and thus high intestinal colonization leads to bursal colonization; or 2) *C. jejuni* trafficks to the bursa via an unknown mechanism for antigen processing. Second, our studies revealed that between 4 and 7 days post-

infection, there is a 100-fold increase in the levels of *C. jejuni* in the bursa. However, it is currently unknown if this increase is either due to increased trafficking of *C. jejuni* to the bursa or increased proliferation of *C. jejuni* within the bursa.

How bursal colonization may benefit *C. jejuni* or the avian host during commensalism also needs to be investigated. From the perspective of the bacterium, colonization of the bursa may serve as a reservoir allowing reseeding of the intestinal tract with *C. jejuni* which may contribute to persistence at this site. However, evidence from colonization studies examining specific colonization factors of *C. jejuni* do not fully support this hypothesis as they have revealed that mutants with defects in bursal colonization can still colonize the ceca at wild-type levels. If bursal colonization directly and linearly influences cecal colonization, one may expect that defects in bursal colonization may result in decreased prolonged cecal colonization. From the perspective of the avian host, we hypothesize that colonization of the bursa serves to generate a protective mucosal IgA response that ultimately allows the host to confine the bacterium to mucosal surfaces for the development of a commensal relationship. Bursectomizing chicks either surgically or hormonally and then examining the interactions between *C. jejuni* and the avian host may reveal how bursal colonization impacts commensalism. This type of experimental approach would allow us examine how the absence of the bursa affects prolonged colonization of

different organs. Removing the bursa significantly reduces antibody-mediated responses, however it does not completely eliminate antibody synthesis (56). Thus, the differential antibody-mediated immune response generated during colonization and how it affects levels of *C. jejuni* in various tissues will be examined.

Importance of Invasion of the Cecal Epithelium by *C. jejuni*

We also found evidence that *C. jejuni* penetrates and invades the cecal epithelium at day 1 post-infection. This corresponds with the time in which we observed spread of *C. jejuni* to the spleen and liver. In future studies, we hope to elucidate the mechanism of systemic spread by investigating if invasion through the cecal epithelium enables *C. jejuni* to reach the spleen and liver. This can be accomplished by analyzing what cell types *C. jejuni* associates with after invasion of the cecal epithelium (such as dendritic cells or macrophages), and determining if *C. jejuni* can spread to the spleen and liver within these cells. Additionally, these studies would focus on determining how invasion of the cecal epithelium occurs at day 1 post-infection. *C. jejuni* were not present in the spleen or liver (within the limits of detection for our assay) in chicks infected 14 days post-hatch, suggesting invasion at day 1 post-infection is due to the fragility of the host tissue after hatch (allowing bacteria to easily penetrate through the epithelium), or is the result of an innate immune system that is not fully developed. Regardless, this

invasion is rapidly eliminated by the avian host as it is not observed at later time points of infection.

Studies of the interactions between *C. jejuni* and poultry have been largely limited by the lack of available reagents to examine the immunological response during colonization. However, with the continuing development of better reagents for studying the avian immune system, we hope in the future to provide a better understanding of the fate of *C. jejuni* and what cell types the bacterium may associate with after invasion.

Differential Survival of *C. jejuni* After Phagocytosis by Avian or Human Macrophages

We suspect that there may be major differences in the invasion capacity of *C. jejuni* for human and avian intestinal tissue which may contribute to the different outcomes of infection in these hosts. *C. jejuni* may have an increased ability to adhere to or invade human intestinal epithelium or survive within the lamina propria of the intestinal tissue, thereby overwhelming the local innate immune system to result in increased inflammation and damage. In considering possible differences in interactions of *C. jejuni* with components of the human or avian immune system, we and others have observed that activated avian monocytes are efficient at killing *C. jejuni* after phagocytosis (156). Conflicting studies are available regarding the efficiency of human monocytes to kill *C. jejuni*

after phagocytosis (79, 92, 101). If *C. jejuni* has a specific ability to survive after phagocytosis by human macrophages or if human macrophages have a specific defect in killing *C. jejuni*, the bacterium may not be easily removed in infected human tissue by these phagocytic cells. Future research studies will focus on some of these issues including a comparative analysis of the ability of primary human and avian macrophages to eliminate *C. jejuni* after phagocytosis by examining differences in engulfment, vacuole acidification, and phagosomal maturation.

Avian Host Factors Contributing to Commensalism

Several studies using primary chicken epithelial cells or various avian cell lines have observed that infection with *C. jejuni* results in the increased expression of pro-inflammatory cytokines (23, 110, 156). Additionally, a study examining cytokine secretion in ileal and cecal tissues during *C. jejuni* infection also resulted in an induction of pro-inflammatory cytokines (155). These findings suggest that the avian host recognizes *C. jejuni* during colonization, however why this recognition does not lead to any obvious signs of inflammation or result in disease development is not well understood.

There are a few different hypotheses as to how the avian host recognizes *C. jejuni* but does not generate a major inflammatory response during colonization. One possibility is that the pro-inflammatory response described

above is dampened by the secretion of regulatory cytokines. Studies investigating this hypothesis revealed that expression of regulatory cytokines (IL-10 and TGF-β) could not be detected during chick colonization with *C. jejuni* (155). However, while there are some similarities between the immune systems of chickens and mammals, functional differences have been found to exist (95). Thus, it is possible that regulatory cytokines responsible for dampening the pro-inflammatory response during *C. jejuni* colonization are present but different than in mammalian systems and are currently uncharacterized. A second hypothesis is that *C. jejuni* is recognized by the avian host but this response is controlled to avoid the initiation of a damaging inflammatory response. Studies performed by Larson *et al*. observed that infection of chicken hepatic cells (LMH) or human intestinal epithelial cells (INT407) with *C. jejuni* resulted in differential cytokine expression between the two cell types (110). Infection of human INT407 cells resulted in significantly higher expression of cytokines than avian LMH cells. These findings suggest that although pro-inflammatory cytokine secretion is induced during chick colonization, the level of this response is limited and therefore is not great enough to result in inflammation of host tissues. However, future studies need to be performed to determine if the pro-inflammatory cytokine response observed is relevant and if so, how this inflammatory response is controlled by the avian host.

C. jejuni Colonization Parallels Interactions of Mammalian Commensals

Whereas little is understood regarding the development of commensalism in the avian host, a few hypotheses have been proposed to explain how interactions between commensals or pathogens differ in the mammalian intestinal tract, and *in vivo* and *in vitro* analyses have provided support for these hypotheses (115, 131). One major hypothesis proposed by Macpherson and Uhr is that interactions between commensals and the mammalian host result in compartmentalization of the commensals to the intestinal lumen (114, 117). Generally, commensals of mammals promote very little adherence to or invasion of the intestinal epithelium. Commensals that reach the intestinal lamina propria or mesenteric lymph nodes are likely eliminated by macrophages or processed by dendritic cells for the generation of a mucosal IgA response (Figure 21) (114, 117). This IgA lessens the association of the bacteria with the intestinal epithelium to limit penetration of commensals across the epithelium (117). The net effect of these interactions between the commensal and a natural, mammalian host is that the bacteria are confined to the intestinal lumen, and any invading commensal is removed by a very limited and localized inflammatory response without generating damage or disease.

In contrast, pathogenic bacteria usually have a specific ability to adhere to and invade the intestinal epithelium of mammalian hosts. After invasion, the bacteria may resist clearance by cellular effectors of innate immunity such as

macrophages. Evasion of innate immunity may lead to systemic infection at sites in the blood, spleen, or liver. These infection processes lead to high levels of pro-inflammatory cytokines, damage to host tissues, and characteristic disease.

We observed many parallels between this model proposed by Macpherson and Uhr for how commensals are contained in the mammalian host with how *C. jejuni* is confined in the avian host. For instance, we found *C. jejuni* primarily localized to the lumen of intestinal and lymphoid tissue. We did note invasion of cecal tissue at the earliest time point examined after oral gavage as well as a transient and limited systemic infection of the spleen and liver. This systemic infection was apparently cleared by the host, and thus damage to host tissue or disease did not occur. Ultimately, over the course of the experiments, we observed that *C. jejuni* is primarily confined to the lumen of intestinal and lymphoid tissue and this localization is likely ideal for the bacterium to result in a prolonged colonization at mucosal surfaces (Figure 21). These findings are in stark contrast to the invasive pathogenic relationship we observed between *S. pullorum* and poultry. During *S. pullorum* infection, the bacteria are highly invasive resulting in extensive inflammation. Our finding of an extensive, prolonged bursal colonization may be opportune for the generation of an effective IgA response that could contribute to the further confinement of *C. jejuni* to mucosal intestinal surfaces. Thus, the behavior of this commensal in the avian intestinal tract is similar to those of gut commensals in mammalian hosts (114,

117). Despite divergent anatomy of the gastrointestinal tract and lymphoid organs between avian and mammalian species, our study suggests that there is conservation of commensal-host behaviors across the animal kingdom.

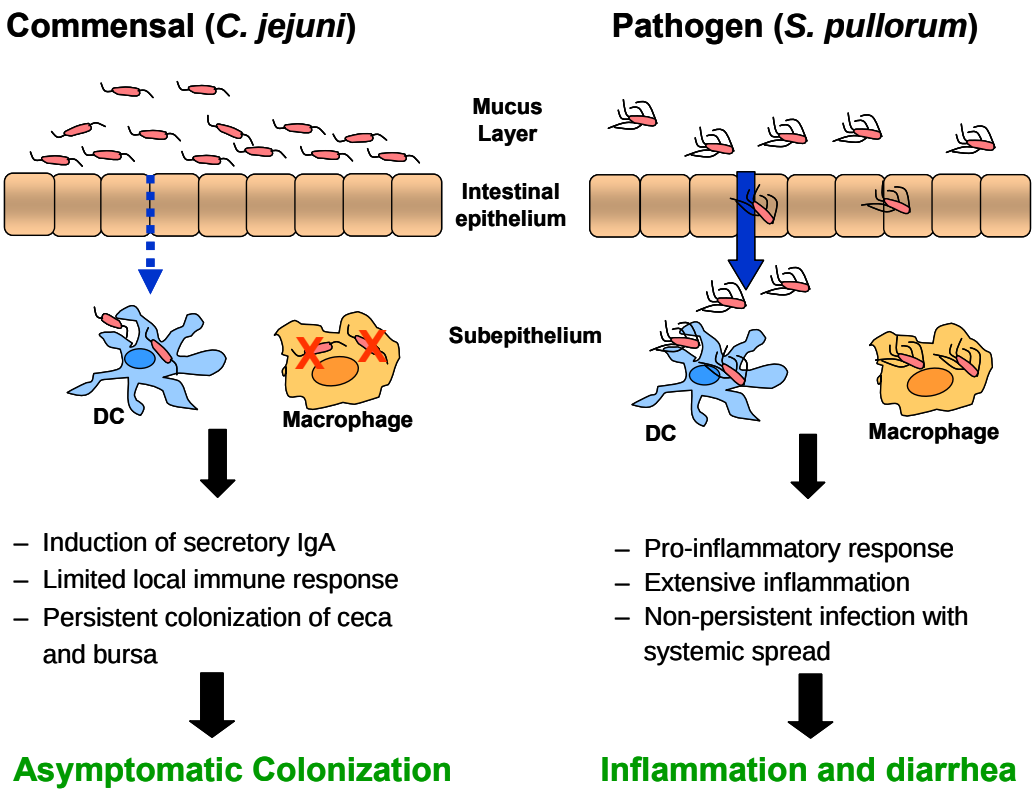

Figure 21. Model of the different interactions of commensals and pathogens with poultry. *C. jejuni* and *S. pullorum* interact with poultry promote different outcomes of infection. Confinement of *C. jejuni* to intestinal and lymphoid tissues and penetration of *S. pullorum* across the epithelium to reach systemic sites are likely determinants influencing the development of commensalism and disease in the avian host.

Analysis of Specific Factors Required for Colonization

Prolonged colonization factors

Through our studies, we revealed that *C. jejuni* can promote prolonged cecal and bursal colonization. The differences in the cellular composition and nutrient availability of these organs led us to examine if there are factors of *C. jejuni* that are specifically required for initiating or maintaining cecal or bursal colonization. We indeed found evidence for such colonization factors as we identified factors involved in promoting efficient bursal colonization as well as factors important in maintaining both cecal and bursal colonization. These findings suggest that commensal colonization may have more layers of complexity than initially presumed.

Previous studies examining factors of *C. jejuni* involved in promoting efficient colonization of chicks have failed to analyze prolonged colonization dynamics as most of these studies were limited to examining colonization levels at less than 2 weeks post-infection. Additionally, intestinal colonization has been the major focus of many of these works, ignoring other potentially important sites outside of the intestinal tract that *C. jejuni* may inhabit during colonization. Therefore, future studies examining colonization factors of *C. jejuni* should not be limited to intestinal colonization but should also explore persistent colonization of

multiple organs. This will create a more cohesive understanding of the global colonization dynamics of specific factors involved in colonization of poultry.

The prolonged colonization studies reported in this dissertation only examined a few different factors of *C. jejuni* that have been previously reported to have differential colonization abilities. We intentionally selected these factors based on past colonization analyses, and thus our studies were skewed toward identifying bursal-specific colonization factors. Therefore, future studies will analyze a wide range of factors including those that may have wild-type bursal colonization levels or a cecal-specific colonization defect. These studies should also aid in elucidating the interactions between *C. jejuni* and poultry that result in a commensal relationship.

DocA and Cjj0382 as Colonization Factors

Studies examining the importance of DocA or Cjj0382 revealed that both factors are involved in promoting efficient cecal colonization. However, it appears that these proteins do not perform redundant functions during colonization of poultry because mutants of *C. jejuni* lacking either *docA* or *cjj0382* have different colonization capacities. We also found that even though these proteins are located in the periplasm and have heme-dependent peroxidase activity (characteristics of typical CCPs), they do not appear to have a physiological role in promoting resistance to hydrogen peroxide. Instead, we

observed that hydrogen peroxide resistance is largely attributed to KatA, the cytoplasmic peroxidase. Further analysis revealed that a Δ*katA* mutant of *C. jejuni* demonstrates a less severe colonization deficiency than a mutant lacking *docA*.

To enhance our understanding of the functions of DocA and Cjj0382, collaborative studies were performed to examine if these proteins contribute to optimal growth upon utilization of substrates that generate periplasmic peroxides and superoxides as a by-product of metabolism. The oxidation of formate by the formate dehydrogenase (Fdh) complex leads to the production of peroxide in the periplasm (57). Therefore, the growth rates of different *C. jejuni* mutants were examined in the presence or absence of formate to determine if strains deficient in *docA* or *cjj0382* had a growth defect due to the inability of these strains to detoxify periplasmic peroxides produced during formate oxidation. All strains grew at rates similar to wild-type during exponential growth, however the Δ*cjj0382* mutant grown in the presence of formate had a lower final cell density than the same mutant grown without formate (Atack and Kelly, unpublished data). Fumarate reductase (Frd) activity results in the formation of superoxides in *E. coli* (124). *C. jejuni* produces Frd but studies have not been conducted to demonstrate superoxide formation by this protein. For these analyses, wild-type and mutant strains of *C. jejuni* were grown in oxygen-limiting conditions with fumarate as an electron acceptor. Both Δ*katA* and Δ*cjj0382* mutants had a severe

growth defect whereas a Δ*docA* mutant behaved similar to wild-type (Atack and Kelly, unpublished data). These studies suggest that Cjj0382 may have a role in detoxifying peroxides in the periplasm. However, the role of DocA remains elusive. Further studies will be required to elucidate the specific physiological roles of DocA and Cjj0382 in *C. jejuni*. However, based on the phylogenetic analyses of DocA and Cjj0382 (Figure 15), it appears that the function of DocA may be quite different than other classical bacterial CCPs since it is the sole member of its own clade. Additionally, prolonged colonization studies of DocA and Cjj0382 may reveal the importance these factors in colonization and persistence in multiple organs to further understand the function of these proteins *in vivo*.

Complementation of Colonization Factors of C. jejuni

Another important avenue of research that needs to be explored is to complement colonization factors and examine their colonization phenotypes to ensure the observed colonization defects are not due to secondary mutations in the chromosome of *C. jejuni*. The current complementation technology in *C. jejuni* involves *trans*-complementation with a plasmid. However, previous efforts to complement mutants with this plasmid *in vivo* have been unsuccessful. Reasons for this inefficiency may be due to the plasmid being unstable *in vivo* or the requirement of antibiotic selection to maintain the plasmid *in vivo*. Current

research efforts in our laboratory involve designing a complementation strategy that will allow complementation of genes on the chromosome of *C. jejuni*. A possible strategy that is currently being investigated involves using a gene of *C. jejuni*, *cjj1083* (encoding a nitroreductase), as a chromosomal complementation site. Wild-type *C. jejuni* producing Cjj1083 is sensitive to metranidazole, however disruption of this gene results in a metranidazole-resistant phenotype. Preliminary studies have shown that a non-motile mutant lacking *flhB* can be successfully complemented by inserting *flhB*, with its native promoter, into *cjj1083* to fully restore the motile phenotype (Ribardo and Hendrixson, personal communication). This chromosomal complementation strategy removes issues of plasmid instability and may be a suitable approach for *in vivo* complementation studies. Future research will examine the efficacy of this complementation strategy during *in vivo* colonization of chicks.

Concluding Remarks

The studies presented in this dissertation have led to an increased understanding of the interactions between *C. jejuni* and poultry as well as revealed the significance of different colonization factors that contribute to the formation of this commensal relationship. We have discovered interactions between *C. jejuni* and poultry that are specific to the development of a commensal relationship including a robust, prolonged colonization of *C. jejuni* in the ceca and

bursa. We have also revealed that specific factors of *C. jejuni* are required to promote colonization and persistence in the ceca and bursa. Additionally, we have characterized two paralogous proteins of *C. jejuni* that are both involved in promoting cecal colonization yet perform different functions *in vivo*. We hypothesize that the interactions between *C. jejuni* and poultry are quite similar to that of commensals of mammalian hosts suggesting conservation of commensal-host behaviors across the animal kingdom. Furthermore, we hope this study of the relationship between *C. jejuni* and the avian host will provide insights into the different possible bacterial-host interactions that occur between *C. jejuni* and humans to result in disease.

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