Development of an in vitro model and fluorescent protein expression system for the study of highly abortigenic Campylobacter jejuni

by

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ABSTRACT

Campylobacter spp. are a leading cause of sheep abortions worldwide; *Campylobacter fetus* ssp. *fetus* has historically been the major culprit. Increasingly, however, *C. jejuni* has been isolated from cases of sheep abortion, and it has now replaced *C. fetus* ssp. *fetus* as the predominant cause of *Campylobacter*-related ovine abortion in the United States. Emergence of a single tetracycline resistant clone (clone SA) has been implicated as the primary reason for this shift. Virulence factors for clone SA have still not been completely elucidated, and it is not known how this bacterium reaches the uterine and placental tissue from the gut. Although an effective animal model has been described, development of an *in vitro* cell culture model would provide a cost-effective and reliable alternative.

In this study, we assessed the ability of IA3902, a clinical isolate of clone SA, to invade and survive within two different cell lines, AH-1 ovine placental trophoblasts and RAW264.7 murine macrophages. Our results indicate that IA3902 is actually less invasive and has lower survival within AH-1 trophoblast cells than the nonabortigenic isolate NCTC11168; in contrast, IA3902 displayed increased invasion and survival in RAW macrophages as compared to NCTC11168. Next, we tested the hypothesis that *C. jejuni*’s invasive abilities in AH-1 cells would be enhanced in the presence of progesterone, which is present in high levels in the serum of ewes at the time that *C. jejuni* abortion typically occurs. Contrary to our hypothesis, progesterone treatment of AH-1 cells actually decreased invasion of *C. jejuni* into trophoblasts. Finally, we developed a system of mCherry fluorescent protein expression in both IA3902 and NCTC11168 to facilitate noninvasive detection of bacteria in an *in vitro* system. mCherry was successfully expressed in *Campylobacter* as observed via...
fluorescent microscopy and measured using a fluorospectrometer; however, low background fluorescence values and an apparent fitness cost in mCherry-expressing *Campylobacter* may limit its utility for certain studies. These findings indicate that *C. jejuni* clone SA’s ability to survive within immune cells may provide the driving force for its high abortigenicity, rather than an increased tropism for placental tissues. They also provide a basis for future use of *in vitro* systems for the study of clone SA, and identify RAW264.7 murine macrophages as a suitable cell culture model for further pathogenesis studies.
CHAPTER I. GENERAL INTRODUCTION

Introduction

*Campylobacter* is a small, Gram negative bacteria that is among the leading causes of food-borne gastrointestinal disease worldwide. The most common culprit of gastrointestinal disease, *C. jejuni*, is estimated to affect nearly 1.3 million persons per year in the United States alone (1), and outbreaks of food-borne campylobacteriosis have steadily increased since 2009 (2). Worldwide, *C. jejuni* is responsible for 5-14% of all diarrheic disease (3). Despite the *Healthy People 2020* objective target of reducing incidence to 8.5 cases per 100,000 people in the United States by the year 2020 (4), the incidence of *Campylobacter* has remained unchanged or slightly increased in recent years, and preliminary 2013 estimates put the current incidence at 13.8 cases per 100,000 people (5). The cost of human campylobacteriosis in the United States each year is estimated to be between 1.5 and 6.8 billion dollars (6,7). It is evident that the burden of disease is substantial; however, due to its more fastidious culture characteristics, much less is known about this organism than many bacteria causing similar disease, e.g. *E. coli* or *Salmonella*.

*Campylobacter* infections occur at all ages, but are more prevalent in children under 5; other influencing factors include seasonality (increased incidence during summer months), infecting strain (variations in virulence exist among strains), and socioeconomic status, among others (7,8). Transmission to humans occurs via a variety of sources, including consumption of contaminated milk, food, or water, and contact with infected pets or livestock (9). By far the most frequent source, however, is contaminated undercooked poultry products (10-12). Chickens (and, to a lesser extent, turkeys and ducks) can be colonized with *C. jejuni*
from a relatively early age without showing any overt signs of disease (9,13). *Campylobacter*

is highly infectious; infective doses as low as 500 to 800 cfu have been reported in human

patients (14). After infection, *C. jejuni* colonizes the intestine and may result in clinical signs

ranging from a mild, watery diarrhea to severe, bloody diarrhea requiring hospitalization.

Rarely, post-infection sequelae such as reactive arthritis, Guillain-Barré syndrome (GBS), or

Miller-Fischer syndrome may result (7).

In addition to poultry, *Campylobacter* spp. are also widespread among livestock;

most often, they colonize the intestinal tract without causing any clinical signs, but they may

be associated with important illnesses, including infertility and abortion in ruminants

(10,15,16). Abortions due to *Campylobacter* are an important management problem in the

sheep industry, and can cause major economic losses to producers; reported abortion rates in

affected flocks range from 5-50% (17). Disease occurs following an initial oral exposure,

with subsequent bacteremia, placentitis, fetal infection, and abortion; typically, abortions due
to *C. jejuni* are seen during the last trimester of pregnancy (17).

In the past, *Campylobacter*-related abortions have been largely attributed to *C. fetus*
spp. *fetus*; however, ongoing work at Iowa State University has demonstrated that a single
tetracycline-resistant clone of *C. jejuni*, clone SA, has gained predominance in the United

States (18,19). The exact mechanisms of this strain’s high pathogenicity are as yet unknown.

Use of an established pregnant guinea pig model has allowed demonstration of this strain’s

high abortigenicity (20) as well as elucidation of a critical virulence factor, LuxS (21), and a

combination of genomic- and proteomic-based approaches has recently shed more light on

the relationship of this strain to non-abortifacient isolates (22). However, much still remains

unknown.
In order to study the pathogenesis of this strain in greater detail, an *in vitro* cell culture model would be of great value. The identification of a cell line in which clone SA shows high levels of invasion and/or survival as compared to non-abortigenic isolates would allow us to quickly and inexpensively identify potential virulence factors of this strain. Combination of an *in vitro* model with a simple bacterial tag, such expression of a fluorescent protein, would further simplify quantification and would allow for tracking of *C. jejuni* intracellularly without the need for cellular destruction. Here, we report recent progress in development of an *in vitro* model system for characterization of highly abortifacient *C. jejuni* clone SA, as well as the development of a fluorescent protein expression system in *C. jejuni*. The outcomes of this study further our understanding of clone SA’s pathogenesis, and identify a potential cell line for further development as an *in vitro* modeling system.

**Thesis Organization**

This thesis consists of two chapters. The first chapter is a literature review, which includes a general overview of *Campylobacter* pathogenesis and epidemiology (focusing on *C. jejuni*), as well as a review of cellular and animal model systems. The second chapter describes the development of a cellular model for characterization of invasion and intracellular survival of clone SA, as well as the development of a fluorescent protein expression system in that strain. References cited throughout the text can be found immediately following each chapter.
1.1 History

The first published account of *Campylobacter* is attributed to Theodore Escherich in 1886 (23); the bacteria were further described in 1913 by John McFadyean and Stewart Stockman, who were investigating a flock of sheep with an abortion rate approaching 33%. In smears of uterine mucus, they identified an organism which they accurately characterized as comma-shaped and actively motile, with some end-to-end joining producing long spiral forms (24,25). After an apparently identical organism was identified in aborting cattle just a few years later, the organism was named *Vibrio fetus* (25,26).

In the years following, *Vibrio* continued to be identified, primarily by veterinarians, and by 1944 two further species, *Vibrio jejuni* and *Vibrio coli*, had been identified from the feces of cattle and pigs, respectively (25,27). These organisms remained in the *Vibrio* genus until 1963, when Sebald and Véron placed *V. fetus* into a new genus, *Campylobacter*, based on its microaerophilic nature and low G+C content (28). Ten years later, Véron and Chatelain performed more comprehensive studies, and classified four distinct species (*C. fetus* ssp. *fetus*, *C. fetus* ssp. *venerealis*, *C. coli*, and *C. jejuni*) into the *Campylobacter* genus (23,29).

1.2 Classification

The family Campylobacteraceae now contains two genera, *Campylobacter* and *Arcobacter*. There are currently 25 species in the *Campylobacter* family (30), and at least 14 of these have been isolated from cases of human disease (23). However, *C. jejuni* and *C. coli* together account for >95% of human *Campylobacter* infections (31), and are the focus of most efforts to control campylobacteriosis.
As a genus, *Campylobacter* are small (0.2-0.8 µm x 0.5-5 µm), Gram-negative, slender, curved rods (23). They are sometimes referred to as "gull-shaped" organisms because of their tendency to join end-to-end and form "S" or "V" shapes. They have low G+C content, ranging from 29 to 47 percent of molar weight (23). These organisms are microaerophilic, and optimal growth is achieved in an environment containing 5% O₂, 10% CO₂, and 85% N₂ (27,32). A restricted temperature range for growth is also seen, with the optimal temperature for most species being 37˚C (*C. jejuni*, *C. coli*, and *C. lari* being the notable exceptions that grow optimally at 42˚C); no growth is seen at temperatures below 30˚C (32). These two characteristics, taken together, place severe restrictions on growth outside of an animal host, and preclude multiplication (though not survival) in food during storage and processing (27,32).

The majority of the species are motile via a single polar unsheathed flagellum at one or both ends of the cell. Notable exceptions are *C. gracilis*, which is nonmotile, and *C. showae*, which has multiple flagellae (27). Energy is obtained via amino acids or tricarboxylic acid cycle intermediates; *Campylobacter* species do not readily ferment or oxidize carbohydrates (23,27). Most strains are resistant to cephalothin - a fact which is often used in their laboratory identification (16) - and resistance to fluoroquinolones is emerging (33).

### 1.3 Epidemiology of Human Infections

The most common causes of campylobacteriosis, primarily *C. jejuni* and, to a lesser extent, *C. coli*, are ubiquitous in the environment as well as in the intestinal tracts of wild birds and many mammalian species. *C. jejuni* in particular is able to colonize the intestinal
tract of chickens without causing any overt disease in this species (9). Cattle are another common reservoir, though estimates vary widely; the prevalence of the pathogen in the digestive tract of healthy cattle has been shown to range from 0.8% - 80% (15,34-38).

Humans are infected most commonly when handling or ingesting raw or undercooked poultry (10-12); infection can also occur after contact with cattle or consumption of contaminated beef or raw milk (27). These two sources together are thought to account for more then 90% of human infections (10). Less common sources of infection include other food animal species (sheep, pigs, turkeys, ducks), domestic animals (cats, dogs, ferrets), contaminated water, and shellfish. Sheep are commonly colonized (39,40), with shedding rates often approaching 100% near the time of lambing (39). Most are colonized within 1-5 days of birth (39), and remain so throughout life; a recent study showed the majority of sheep and goat carcasses at slaughter were contaminated with *Campylobacter* (41).

Major limitations in data availability have thwarted attempts to measure the exact impact and prevalence of campylobacteriosis. Commonly, official estimates are derived from laboratory-based surveillance, but this probably represents only a very small fraction of those infected (42). Studies in the United States, England and Wales, and the Netherlands have estimated that for every reported *Campylobacter* infection, there are between 7 to 38 persons that actually become ill (43-45). In addition to mild unreported illnesses, seroconversion studies show a poor correlation between antibody detection and any gastrointestinal illness, and seroconversion rates far outstripped estimates of disease prevalence in several European Union countries in one study (46). Hence, serology is often of little value as a diagnostic tool.
1.4 Disease Syndromes

1.4.1 Human gastroenteritis

Despite its impact on foodborne and gastrointestinal disease worldwide, *Campylobacter*'s role and significance in this regard wasn't recognized until the 1970's (25,47). Prior to that time, it had been considered largely a reproductive pathogen of animals. This may seem surprising at first glance; however, its fastidious and slow-growing nature allows it to be easily overgrown in fecal cultures by the facultative anaerobes (e.g., *E. coli*) unless selective culture techniques are utilized. Advances in microbiological techniques have greatly improved our ability to detect and isolate *Campylobacter* from mixed populations, especially fecal and environmental samples.

Following ingestion, *C. jejuni* will colonize the lower intestinal tract. Infections may be asymptomatic; where clinical signs occur, they are typically self-limiting and include fever, vomiting, and watery or bloody diarrhea with abdominal pain. Although most infections are mild, severe dehydration can result and some patients may require hospitalization (9). In fact, *Campylobacter* are estimated to account for about 17% of hospitalizations resulting from food-borne infections, and for 5-6% of food-borne illness related deaths (44). Recent estimates by the CDC implicate *Campylobacter* in over 8,000 food-borne illness-related hospitalizations annually in the United States, a figure surpassed only by *Salmonella* spp. and Norovirus (1). In rare cases, bacteria entering the bloodstream may cause sepsis (48) and other systemic consequences, such as myocarditis (49).
1.4.2 Extraintestinal Disease in Humans

Rarely, long term complications may ensue after campylobacteriosis, the most serious being development of Guillain-Barré syndrome (GBS). This is an acute demyelinating polyneuropathy characterized by flaccid paralysis; disease onset is typically 1-2 weeks following an acute infectious illness (50). While other viruses and bacteria can initiate GBS, C. jejuni is by far the most frequent cause, with documented evidence of recent infection with this pathogen in 30-40% of GBS cases (51,52). The pathogenesis of GBS results when an immune-mediated response against the gangliosides is triggered by an immunologically similar antigen on the bacterium. Studies of O-side chain serotypes of the lipooligosaccharides have revealed that certain Campylobacter serotypes, in particular O:19, are linked to GBS (52,53). In addition to GBS and the GBS variant Miller Fisher Syndrome (MFS) (50), increased incidences of inflammatory bowel disease (54) and reactive arthritis (55) have been correlated with a prior history of campylobacteriosis.

1.4.3 Ovine abortion

Campylobacter species have long been associated with genitourinary disease in cattle, sheep, goats (16), and, rarely, humans (56). Sheep are the main species in which placental and fetal infection is common, and ovine campylobacteriosis typically manifests as late term abortions; in bovids, early embryonic death and/or infertility are more common (16).

There are two main species of Campylobacter associated with ovine abortion, C. fetus ssp. fetus and C. jejuni. Clinically, abortions caused by either species are identical, and it is not possible to distinguish the two without further testing (16). Abortions occur late in pregnancy (during the last 6 weeks, or approximately days 105-150); infected ewes may also
have diarrhea, depression, an elevated temperature, and/or vaginal discharge several days prior to parturition (16). High levels of *Campylobacter* are shed in aborted fetal and placental tissues, and when a naïve flock is exposed to these materials, an abortion storm can result in which up to 50% of ewes may abort (57). Frequently, aborted fetuses lack specific lesions due to *in utero* autolysis; where histology is possible, necrosis is seen in the chorionic villi with arteriolitis and leukocyte infiltration into the lamina propria (16,58). Bronchopneumonia and hepatitis are also common findings in the fetus (58). Diagnosis is by culture and/or observation of the organisms in placental smears, and is substantiated by histopathology; serology is not useful as a diagnostic tool as many sheep can carry the organism in the intestine and gall bladder without showing any clinical signs (39,40).

Historically, most ovine abortions were attributed to *Campylobacter fetus ssp. fetus*, while *C. jejuni* had been thought of as largely a gastrointestinal pathogen (16). Based on a report that *C. jejuni* could also be a cause of abortion (59), Hedstrom et al. inoculated pregnant ewes with intravenous *C. jejuni* at days 114 and 123 of gestation, and found that all ewes aborted at 7-12 days post-inoculation, with no other clinical signs beyond an elevation in rectal temperature prior to abortion (58). Since that time, abortions due to *C. jejuni* have increasingly been recognized in North America (18,60), and it has now replaced *C. fetus ssp. fetus* as the predominant cause of *Campylobacter*-related ovine abortion in the United States. In a study of ovine abortion isolates from Iowa, California, Idaho, Oregon, Nevada, and South Dakota during 2003-2007, it was found that 68 of 74 *Campylobacter* isolates were *C. jejuni*. Strikingly, 93% of those *C. jejuni* isolates belonged to a single genetic clone, named clone SA (for "sheep abortion"), and all members of that clone were resistant to tetracycline (18).
Further studies have confirmed the widespread nature of *C. jejuni* clone SA (which belongs to MLST sequence type [ST]-8) in sheep flocks United States (19); additionally, this clone has been associated with human gastrointestinal infections following consumption of raw milk (61). Tetracycline resistance would seem to be a major factor driving the predominance of clone SA; use of this drug is common in sheep flocks in the United States, especially as it is the only class of antibiotics approved for control and prevention of *Campylobacter* abortion storms in this country (18). In Great Britain, where use of tetracyclines in livestock is highly restricted, less than 5% of *C. jejuni* isolates from cases of ruminant abortions were resistant to this class of antibiotics (19). Supporting the importance of tetracycline resistance, the *tet*(O) gene-- the main gene providing tetracycline resistance to *Campylobacter* (62)-- is invariably found on the chromosome in clone SA, whereas it is more typically plasmid-borne (19,22).

While tetracycline use may provide a driving force for the spread of clone SA, its extreme predominance in U.S. flocks suggests that other factors are at play as well; this is especially true given that other genotypes of *C. jejuni* can carry tetracycline resistance, albeit much less commonly associated with abortions. Virulence factors of clone SA are not well understood, and it is not known exactly how this bacterium reaches the uterine and placental tissues from the gut. However, clone SA has been shown to have a distinct transcriptomic and proteomic profile from the human isolate NCTC11168 (which was shown to be non-abortifacient in a pregnant guinea pig model (20)), despite high genomic sequence homology between these two strains (22). In particular, transcription of genes for energy production and conversion, motility, and anaerobic respiration are upregulated in IA3902 (a representative isolate of clone SA) while transcription of genes encoding iron uptake systems and utilization
of proline, glutamate, aspartate, and lactate are downregulated (22). Additionally, there are notable genetic differences between the two strains in the regions encoding for synthesis and modification of capsule and flagellin (22).

1.5 Pathogenesis

A number of confirmed or putative virulence factors have been identified in *C. jejuni*, including capsular polysaccharides (CPS) and lipooligosaccharides (LOS), adhesins, protein glycosylation, flagella/motility, and the cytolethal distending toxin (CDT) (63). Here, we will focus on CPS/LOS, adhesins, and flagella, which are the factors most associated with cellular infection and invasion.

1.5.1 Capsular polysaccharides (CPS)/Lipooligosaccharides (LOS)

In contrast to many other enteric pathogens, *C. jejuni* express both a capsular polysaccharide (CPS) and a lipooligosaccharide (LOS) in lieu of a full-length lipopolysaccharide (LPS) (64). The CPS and LOS produced by different strains are highly variable and structurally complex, and many genes are further subject to phase variation (65); this inherent genetic and phenotypic diversity aids in thwarting immune system responses and adaptation to various environmental conditions.

Probably the best characterized role of LOS in *Campylobacter* pathogenesis is its relationship to Guillain-Barré syndrome (GBS), which is discussed in further detail above. The LOS in certain strains of *C. jejuni* mimic the human gangliosides on peripheral nerves, leading to autoimmune demyelination and severe neurologic deficits (52). While a number of studies have shown increased sensitivity to various antimicrobial compounds in LOS mutants
(66-69), no direct role in pathogenesis has to date been identified. Additionally, no alterations in cellular invasion have been seen in *C. jejuni* harboring mutations in several individual genes of the LOS cluster (70); however, large scale deletion of the LOS biosynthesis region did abolish cellular invasion (68). Thus, the definitive role of LOS in *Campylobacter* pathogenesis remains somewhat murky.

Polysaccharide capsules are, in general, very important virulence structures in the bacteria that express them (64); this also seems to be the case in *C. jejuni*, although the role of CPS in disease is incompletely understood. In *C. jejuni*, the capsule genes are arranged into three regions, where region 2 contains genes encoding the synthesis of specific capsular polysaccharides, and regions 1 and 3 contain transport and assembly genes such as *kpsM, kpsE, kpsS*, and *kpsC* (64). A mutant deficient in the CPS transporter gene *kpsM* has been shown to have a modestly reduced ability to invade INT-407 cells *in vitro* and reduced virulence in a ferret-based disease model (71). Similarly, a study by Bachtiar et al. showed that a *kpsE* mutant had reduced invasiveness *in vitro* and reduced colonization of chickens compared to the wild-type strains (72). Paradoxically, *C. jejuni* seems to reduce its expression of surface capsular polysaccharides when co-cultured with intestinal epithelial cells (73); however, in that study, there was correspondingly reduced cellular invasion when capsular synthesis was decreased. Similar to the situation in many other pathogens, one of the roles of CPS in *C. jejuni* seems to be in mediating resistance to complement-mediated killing (69,71); whether this is required to initiate colonization or after invasion has not yet been elucidated (64), but it is likely to play an important role where systemic disease is concerned.
1.5.2 Adhesins

A variety of human and non-human origin cell lines have been utilized to study the interaction of *C. jejuni* with host cells, and these have led to the identification of a number of putative adhesion or binding factors. These include the fibronectin-binding outer membrane protein CadF (74), the surface lipoprotein JlpA (75), the periplasmic binding protein PEB1 (76), and the major outer membrane protein (MOMP) (77), among others. CadF is expressed in all strains of *C. jejuni* and *C. coli*, although the gene varies slightly between the two species. CadF's importance as a virulence factor was demonstrated by experiments showing that wild-type *C. jejuni* bound to and invaded INT-407 epithelial cells much more efficiently than wild-type *C. coli*; when isogenic *cadF* mutant strains were created, this difference in cellular invasion was significantly reduced (78). In addition to its role as a binding protein, CadF seems to be important in triggering bacterial internalization via a Rho GTPase signaling pathway (79). JlpA is a 42.3-kDa protein that has previously been reported as mediating *C. jejuni* adherence by binding to Hsp-90 on the surface of HEp-2 epithelial cells (80). A more recent investigation utilizing targeted mutagenesis has disputed this finding and contends that PEB3, and not JlpA, is required for host cell binding (81). Flanagan et al. also reported mutagenesis of *JlpA* did not significantly alter binding to epithelial cells, while CadF and FlpA seem to play important roles in colonization (82).

The major outer membrane protein (MOMP), also called PorA, is one of two porins identified to date in *C. jejuni* (77,83). It is important in the envelope structure of the bacteria (83), and was also investigated as a vaccine candidate (84,85). Mutational analysis of MOMP has been difficult to achieve, as mutations in *porA* are probably lethal to the bacterium (82). However, its importance in binding to intestinal epithelial cells has been demonstrated via
immunoblotting (77). It has also recently been identified, along with the flagellar protein FlaA, as a blood group antigen binding agent that is required for chicken colonization (86). Although immunity to C. jejuni is largely strain-specific, the sera of human patients recovering from C. jejuni has been repeatedly shown to contain antibodies against MOMP (84,85,87,88), and vaccination utilizing a recombinant MOMP has been shown to provide some protection against heterologous strains in a mouse intestinal colonization model (89), further reinforcing the idea that it is of key importance in establishing bacterial pathogenicity.

1.5.3 Flagella/Motility

Campylobacter jejuni possesses a single, polar flagellum that is essential in its ability to colonize mammalian hosts and invade eukaryotic cells (90). The first evidence of its importance in virulence came from studies by Black et al., who fed human volunteers a mixture of motile and non-motile phase variants and thereafter recovered only motile forms from stool samples (14). Subsequent studies have confirmed the requirement for motility in intestinal colonization in a variety of animal models, including mice (91), chickens (92), rabbits (93), and ferrets (94).

The flagellum is composed of two flagellin monomers, FlaA and FlaB, which are encoded by flaA and flaB, respectively. Both monomers are required for expression of a normal flagella and motility; however, loss of flaA seems to be more detrimental, as this causes a severely truncated flagella that is greatly reduced in motility (95). Mutants of flaA are unable to efficiently colonize intestinal cells in vitro (96,97); similarly, other mutations affecting motility completely or near completely eliminate internalization into intestinal epithelial cells (98-101). However, in the case of flaA, this effect may not entirely be due to
the decrease in motility; Wassenaar et al. showed that \textit{flaA} mutants showed a severe deficit in their ability to colonize chickens regardless of motility conferred by FlaB flagellae (102).

Another important function of the flagellar apparatus in \textit{Campylobacter} is protein secretion. The flagellum serves as a type III secretion system (T3SS) and secretes both flagellar and non-flagellar proteins (103-105). A set of proteins termed the "\textit{Campylobacter} invasion antigens", or Cia, require intact flagella for their successful secretion (103) and are integral in cellular infection. In particular, CiaB is required for the invasion of INT-407 cells (103,106,107) and contributes to induction of apoptosis in a THP-1 macrophage model (108). Mutants defective in CiaB also show severe deficits in their ability to colonize chicks (109). CiaC is not required for host cell invasion but mutants do show a reduction in cellular internalization in INT-407 cells (110). CiaI mutants show a defect in intracellular survival as well as increased colocalization of the lysosomal marker cathepsin D with the \textit{Campylobacter}-containing vacuole (CCV; see below) (111). Mutation of a secreted flagellar protein, FlaC, results in no morphological changes in the flagella but causes a severe deficit in invasion of HEp-2 cells (105). Lastly, apart from their roles in motility and protein secretion, flagella are associated with other important functions, including biofilm formation (112,113) and autoagglutination (114). Taken together, the data provide compelling evidence that flagella and their associated components are crucial for the interaction of \textit{C. jejuni} and host cells.

1.6 Interaction with Eukaryotic Cells

By virtue of its microaerophilic, temperature-specific nature, \textit{C. jejuni} has necessarily developed a close relationship with the vertebrate gastrointestinal tract. However, details of
its interactions with host cells are as yet poorly understood, especially in comparison to other intestinal pathogens such as \textit{E. coli} and \textit{Salmonella}. There do appear to be three critical factors for a successful interaction between \textit{C. jejuni} and host cells: (1) the ability to facilitate its own uptake into host cells, (2) the ability to elude or modulate delivery to host lysosomes, and (3) the ability to alter host cell gene expression and thereby stimulate production of proinflammatory cytokines (115).

It has been clearly demonstrated via both examination of biopsy samples from human patients naturally infected with \textit{C. jejuni} (116) as well as experimental infection of primates (117) and \textit{in vitro} infection of human intestinal epithelial cells (118) that \textit{C. jejuni} is able to gain intracellular access to these nonphagocytic cells. It is not completely understood how this occurs or what bacterial factors may be important, and many of the forces at work are likely to be multifactorial and complex. To complicate the issue, \textit{C. jejuni} displays marked differences in colonization ability among strains, and genomic instability during intestinal colonization of humans, chickens, and mice has been shown (119-122).

Several mechanisms for \textit{C. jejuni}'s} uptake into intestinal epithelial cells have been proposed, though none have yet been proven. What is known is that \textit{C. jejuni}'s uptake into cells seems to markedly differ from that of many common pathogens. Many pathogens, including \textit{Salmonella enterica} serovar Typhimurium (123-125), \textit{Listeria monocytogenes} (126-128), and \textit{Shigella flexneri} (129-132) rely heavily upon actin-mediated mechanisms for their successful uptake into host cells. In contrast, Oelschlaeger et al. demonstrated that uptake of \textit{C. jejuni} is actin-independent, and disruption of the actin cytoskeleton does not prevent its invasion (133). In fact, disruption of the actin cytoskeleton has been shown to actually increase the efficiency of \textit{C. jejuni} cellular uptake (134). The internalization process
for *C. jejuni* appears instead to be dependent upon microtubules, and administration of drugs that cause microtubule depolymerization will prevent its uptake (133). Cholesterol-, glycolipid-, and sphingolipid-enriched domains in the cell membrane called lipid rafts or caveolae may also play an important role in *C. jejuni*’s internalization process, and it has been shown that sequestration of host cholesterol inhibits *C. jejuni* entry into cells (135). One study utilizing a dominant interfering mutation of the caveolae-associated protein caveolin-1 in the T84 human intestinal epithelium cell line demonstrated that this protein is required for efficient uptake of *C. jejuni* into intestinal epithelial cells (134). It has also been pointed out, however, that Caco-2 cells lacking caveolin-1 (and thus, caveolae) are still efficiently colonized, and Caco-2 cells engineered to produce caveolin-1 do not show increased bacterial internalization (136). Subsequent siRNA knockdown studies in HeLa cells have shown that non-caveolae cholesterol-rich cell membrane domains, rather than caveolae themselves, are integral in internalization, while caveolin-1 plays a role in the ensuing signal cascade (136).

On the bacterial side, motility seems to be an essential factor in efficient intestinal cell colonization, as was previously discussed. However, nonmotile mutants are still recovered from models of *in vitro* infection, albeit at low levels, suggesting that this is not an absolute requirement (101). Adhesins are integral to the process of binding and internalization, and have also been discussed above. CadF may play a particular role, in that it seems to both bind to cells and trigger the internalization process (79). Proteins secreted through the flagellar apparatus such as CiaB and CiaC also seem to be integral to the binding and internalization process; however, their exact roles in this process remain undefined, and as Watson and Galán point out, evidence to support a direct role of any
flagellar component or secreted protein in directly triggering bacterial entry is scant at best (115). Mutations affecting protein glycosylation or capsular synthesis also affect invasion into host cells. However, the role of protein glycosylation may be related to motility, in that glycosylation is essential for flagellar assembly (137); a direct role has yet to be established. Stronger evidence exists for the role of the polysaccharide capsule in cellular invasion, and knockout mutants of a number of capsular transport and assembly genes have consistently borne this out (69,71,72,97). In contrast, mutants in which the LOS core structure is severely truncated show no alteration in their ability to invade Caco-2 cells, despite increased susceptibility to complement-mediated killing and decreased intracellular survival time (65).

Once actually inside the cell, Campylobacter resides within a specialized compartment that has been termed the Campylobacter-containing vacuole, or CCV (134,138,139). In macrophages, the CCV efficiently fuses with the phagolysosomal compartment and rapid killing is typically accomplished (134). However, in epithelial cells, the CCV avoids fusion with lysosomes, and the bacteria are able to survive within the CCV (134); the mechanism causing this alteration in the canonical endocytic pathway is unclear. Most studies have reported a loss in viability after about 24 hours within intestinal epithelial cells (139-141); one study, utilizing a 48 hour "preculture" period under strictly anaerobic conditions to recover C. jejuni following the invasion of T84 cells and live/dead staining coupled with FACS analysis, reported that there was no loss in viability at 24 hours and suggested that there may be a shift in the physiologic state of the bacterium within epithelial cells such that it becomes extremely oxygen-sensitive (134).

Intestinal epithelial cells are capable of mounting a robust innate immune response (142), and induction of proinflammatory cytokines by these cells, such as IL-8, is a major
step in the pathogenesis of campylobacteriosis. The proinflammatory state induced by *Campylobacter* is a consequence of the activation of NF-κB and the MAP kinase signaling pathways (143-145); however, the mechanism of activation appears to be unusual, as it is independent of TLR2, TLR4, NOD1, or NOD2 activation (146). It has also been proposed that cytolethal distending toxin, the only toxin produced by *C. jejuni* that has thus far been characterized (115), may directly stimulate cytokine production via DNA damage (147).

Simultaneously, an anti-inflammatory pathway is also activated via the phosphotidylinositol-3 kinase/Atk pathway (145), which may represent an attempt by the bacterium to overcome the host inflammatory response and thus prolong intracellular survival.

### 1.7 Model systems for the study of campylobacteriosis

Despite its worldwide prevalence and economic importance, many questions clearly still remain regarding the pathogenesis of genitourinary disease caused by *Campylobacter* spp. The lack of a reliable, inexpensive, and reproducible animal model for *in vivo* study has been a major roadblock to more extensive study. A model utilizing pregnant Guinea pigs is currently the most well-established and reliable laboratory model of abortion, in that they are susceptible to infection and clinical signs are similar to those seen in sheep (148-150). This model has been successfully utilized for the study of clone SA (20,21,151); however, studies have been hampered by expense and handling limitations, and large-scale experiments are impractical. Mouse models have been used with some success to demonstrate bacteremia (152,153), but not reproductive disease. Chickens are a utile and commonly employed model for colonization studies (97,154,155); the usefulness of this model in the study of mammalian reproductive processes is nevertheless, by definition, limited. Use of an ovine model would
clearly be prescient, and has been employed in past studies (58,156); again, their use is limited by expense, as well as housing and animal welfare considerations. Additionally, many sheep can be silent carriers with no overt clinical symptoms of campylobacteriosis, thus necessitating extensive screening and/or strict sourcing requirements prior to undertaking experimental infections.

In light of these difficulties, development of a cellular model for the study of abortifacient *C. jejuni* is clearly of pressing concern. A number of *in vitro* models have been used successfully to study the interaction of *C. jejuni* with intestinal epithelial cells and immune cells. Epithelial cell lines which have been utilized include the polarized human colorectal cell line Caco-2 (118,157,158), the nonpolarized intestinal line INT-407 (98,133,159,160), and cell lines of human cervical provenance including HEP-2 (139,161,162) and HeLa (136,143), among others. A variety of immune cells have also been utilized for the study of *Campylobacter*, include the murine macrophage lines RAW264.7 (163) and J774 (164,165), bone marrow derived macrophages (166,167) and dendritic cells (166,168).

Despite the wealth of information these studies have yielded regarding intestinal and immune cell pathogenesis, to date no study (to the author's knowledge) has attempted to study the pathogenesis of highly abortigenic *C. jejuni* *in vitro*. The multifactorial nature of the abortion syndrome makes identifying a single paramount cell line a daunting task; however, several cell types seem to be good candidates to elucidate at least portions of disease pathology. Histopathology of aborted placental and fetal tissues in animals infected with *C. jejuni* has consistently shown a strong tropism for placental trophoblasts and inflammatory cells (20,58). The AH-1 ovine placental trophoblast cell line was derived by
Haldorson et al. in 2006 to study the pathogenesis of *Neospora caninum* (169), but has not yet been utilized in the study of *Campylobacter*.

In order to cause abortion, *C. jejuni* must first translocate to the uterus and placenta from its normal location in the gastrointestinal tract, and evidence exists that this strain is able to cause septicemia at a high rate after oral inoculation (20). It is not known, however, whether *Campylobacter* travels free in the bloodstream or is ferried within inflammatory cells; one study utilizing a gfp-expressing strain of *C. jejuni* showed high association with neutrophils and (to a lesser extent) macrophages in the bloodstream of experimentally inoculated mice (122). Due to their high numbers in the intestinal tract and robust accumulation in placental tissues, macrophages seem a likely candidate for *C. jejuni* translocation. Although a variety of macrophage lines have been utilized in the study of *Campylobacter*, the vast majority of these studies have focused on relatively well-characterized strains such as 81-176 and NCTC11168; it is not known whether clone SA invades or survives in these cells at a different rate than these strains. If survival were greatly improved, abortion might be less a function of increased tropism for placental tissue than of increased systemic bacterial load. Regardless of the cell culture system utilized, the identification of a cell line allowing robust and differential growth of clone SA as compared to non-abortigenic strains of *C. jejuni* would afford a method of quickly identifying and characterizing key virulence factors without the expense and difficulty of animal models.

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CHAPTER 2: DEVELOPMENT OF AN IN VITRO MODEL AND FLUORESCENT PROTEIN EXPRESSION SYSTEM FOR THE STUDY OF HIGHLY ABORTIGENIC CAMPYLOBACTER JEJUNI

A paper to be submitted for publication

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Abstract

Campylobacter spp. are a leading cause of sheep abortions worldwide; Campylobacter fetus ssp. fetus has historically been the major culprit. Increasingly, however, C. jejuni has been isolated from cases of sheep abortion, and it has now replaced C. fetus ssp. fetus as the predominant cause of Campylobacter-related ovine abortion in the United States. Emergence of a single tetracycline resistant clone (clone SA) has been implicated as the primary reason for this shift. Virulence factors for clone SA have still not been completely elucidated, and it is not known how this bacterium reaches the uterine and placental tissue from the gut. Although an effective animal model has been described, development of an in vitro cell culture model would provide a cost-effective and reliable alternative.

In this study, we assessed the ability of IA3902, a clinical isolate of clone SA, to invade and survive within two different cell lines, AH-1 ovine placental trophoblasts and RAW264.7 murine macrophages. Our results indicate that IA3902 is actually less invasive and has lower survival within AH-1 trophoblast cells than the nonabortigenic isolate
NCTC11168; in contrast, IA3902 displayed increased invasion and survival in RAW macrophages as compared to NCTC11168. Next, we tested the hypothesis that *C. jejuni*’s invasive abilities in AH-1 cells would be enhanced in the presence of progesterone, which is present in high levels in the serum of ewes at the time that *C. jejuni* abortion typically occurs. Contrary to our hypothesis, progesterone treatment of AH-1 cells actually decreased invasion of *C. jejuni* into trophoblasts. Finally, we developed a system of mCherry fluorescent protein expression in both IA3902 and NCTC11168 to facilitate noninvasive detection of bacteria in an *in vitro* system. mCherry was successfully expressed in *Campylobacter* as observed via fluorescent microscopy and measured using a fluorospectrometer; however, low background fluorescence values and an apparent fitness cost in mCherry-expressing *Campylobacter* may limit its utility for certain studies. These findings indicate that *C. jejuni* clone SA’s ability to survive within immune cells may provide the driving force for its high abortigenicity, rather than an increased tropism for placental tissues. They also provide a basis for future use of *in vitro* systems for the study of clone SA, and identify RAW264.7 murine macrophages as a suitable cell culture model for further pathogenesis studies.

**Introduction**

*Campylobacter* is a small, Gram-negative, comma-shaped bacterium that is a common cause of food-borne gastrointestinal disease worldwide. One of the most common culprits of gastrointestinal disease, *Campylobacter jejuni*, is estimated to affect nearly 1.3 million persons per year in the United States alone (1). In addition, *Campylobacter* spp. consistently rank among the most prominent causes of infectious sheep abortions worldwide. Outbreaks within a flock can be devastating, with abortion rates in naïve flocks reaching up
to 50% (2,3). Historically, *C. fetus* ssp. *fetus* has been the major culprit identified in ruminant genitourinary disease. In the late 1980's, it began to be recognized that *C. jejuni* was also capable of causing abortion (4,5); since that time, there has been a remarkable shift in disease epidemiology in the United States. In the last 25 years, *C. jejuni* has replaced *C. fetus* ssp. *fetus* as the predominant cause of *Campylobacter*-related ovine abortion in this country (6), and there has been a shift from a variety of circulating strains (7) towards a single dominant clone. This clone is named "clone SA" (for "sheep abortion). All recent isolates of clone SA are tetracycline resistant (6), and this clone has also been associated with human illness after consumption of raw milk (8).

A representative isolate of *C. jejuni* clone SA, IA3902, has now been sequenced (9), and efforts are underway to more extensively characterize the factors driving its evolution, virulence, and abortifacient nature. Towards this end, a pregnant Guinea pig model has been successfully utilized for the study of clone SA pathogenesis (10,11), and has led to the identification of several critical virulence factors, such as LuxS (12). However, major limitations to this model exist, including expense, the impracticality of large-scale studies, and the confounding effects of handling stress in these delicate subjects. Given these concerns, the establishment of a reliable *in vitro* model to study the reproductive pathogenesis of *Campylobacter* would clearly be of great advantage. Histopathologic studies of abortifacient *C. jejuni* have shown a predilection for infection of the placental trophoblasts and inflammatory cells (5,10), especially the macrophages that are recruited *en masse* to the uterus and placenta during pregnancy (36). A stable line of ovine placental trophoblasts (AH-1) has previously been derived (13) and has been used to study both *Neospora* (13) and *Chlamydophila* (14) but not, to the authors knowledge, in studies of *Campylobacter*
pathogenesis. Macrophages have been utilized in a number of studies to determine *C. jejuni*'s ability to colonize and survive within inflammatory cells; however, no studies to date have elucidated clone SA's abilities in this regard.

During pregnancy, a number of endocrinological changes take place in the ewe. Serum levels of the sex hormone progesterone are high in sheep throughout pregnancy and increase further during the third trimester (15,16). This increase coincides with the usual onset of *C. jejuni*-induced abortion, which can occur from approximately day 108 through day 150 (17). In contrast, circulating estrogen levels in the ewe are typically quite low (5-20 pg/ml) until immediately prior to parturition, at which time there is a sudden and intense spike in estrogen (16,18). Due to the high circulating serum hormone levels occurring during the peak time of *Campylobacter* abortion in sheep, we hypothesized that the sex hormones, and progesterone in particular, would enhance *C. jejuni* infection of AH-1 ovine placental trophoblasts.

As an additional tool towards development of an *in vitro* cell culture system, it was desirable to develop a method of quickly identifying and quantifying bacterial growth without the necessity of destroying cells or of recovering *C. jejuni* in culture, where difficulties can abound. Fluorescent proteins provide an ideal means of tracking bacteria, in that they are intrinsic, resistant to photo-bleaching, and do not require extra processing steps or cellular destruction for visualization. Several systems have been developed in *Campylobacter* to date, including organisms expressing green fluorescent protein (*gfp*) (19) and its derivatives cyan fluorescent protein (*cfp*) and yellow fluorescent protein (*yfp*) (20). However, experience with these systems in our laboratory has been mixed (O. Sahin, personal communication) and none have gained wide acceptance. Additionally, if use in *in
vivo as well as in vitro studies is desired (e.g., monitoring subjects for the development of C. jejuni septicemia), a protein with fluorescence in the far-red spectrum would achieve better tissue penetration, and thus better detection, than gfp-based proteins. The "mFruit" family of fluorescent proteins includes a number of monomeric fluorescent proteins in a range of colors that were derived from the tetrameric protein DsRed via a combination of directed evolution and random mutagenesis (21). Of these, the fluorescent protein mCherry offers a very short maturation time (t_{0.5} = 15 minutes at 37°C), excellent pH resistance (pKa < 4.5), and high photostability (t_{0.5} = 68 seconds) (21,22). Additionally, it has one of the longest emission wavelengths of the mFruit proteins (610 nm), facilitating deeper tissue penetration and making it an excellent candidate for noninvasive in vivo imaging. Creation of a strain of C. jejuni able to stably express this protein would allow for high resolution in vivo tracking of bacterial infection in susceptible animals.

The aims of this study were thus threefold: 1) to characterize two likely cellular models (AH-1 ovine placental trophoblasts and RAW264.7 murine macrophages) for in vitro study of the pathogenesis of infection with the highly abortifacient C. jejuni clone SA; 2) to determine whether the sex hormones progesterone and β-estradiol would enhance C. jejuni's invasion of or persistence in AH-1 ovine placental trophoblasts; and 3) to develop and characterize a system of mCherry fluorescent protein expression in C. jejuni and determine its suitability for further use in in vitro and in vivo models.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. All isolates of C. jejuni were routinely
cultured from frozen stocks (-80°C) onto Mueller-Hinton (MH) agar or broth (Difco, Franklin Lakes, NJ) at 42°C in a microaerobic atmosphere (85% N₂, 10% CO₂, and 5% O₂) and passaged every 24 to 48 hours. *C. jejuni* IA3902 is a representative clinical isolate of clone SA from a case of sheep abortion that has been described previously (6). NCTC11168 is a nonabortifacient *C. jejuni* isolate from a case of human enteritis (23) that was used as a control strain in this study. *E. coli* was grown in Luria-Bertani (LB) broth or agar (Difco) at 37°C. Where necessary, media was supplemented with chloramphenicol (Cm; 15 µg/ml).

**Cell culture conditions.** RAW264.7 murine macrophages (ATCC #TIB-71) were routinely cultured in DMEM (Corning Cellgro, Tewksbury, MA) supplemented with 10 mM HEPES, 2 mM glutamax, and 10% fetal bovine serum (Thermo Scientific, Logan, UT). AH-1 ovine placental trophoblasts (obtained from Dr. Tim Baszler at Washington State University) were routinely cultured in Opti-MEM without phenol red (Life Technologies, Grand Island, NY) supplemented with 2 mM glutamax, 10 mM HEPES, and 10% fetal bovine serum. Where noted, this media was also supplemented with progesterone or β-estradiol (Sigma-Aldrich, St. Louis, MO) at a concentration of 0.1 µg/ml or 1.0 µg/ml. Cells were seeded onto 24 and 96 well plates at a density of 1 x 10⁶ cells/ml approximately 3-4 hours prior to infection. Live cells were counted in a hemacytometer, and cell culture viability was monitored via the use of trypan blue dye exclusion.

**Gentamicin Protection Assays.** *C. jejuni* internalization and survival assays were carried out in a similar manner as described previously (24). Fresh cultures of *C. jejuni* were grown for 16-20 hours in MH broth on a shaker in microaerobic conditions, followed by
centrifugation at 8,000 rpm for 10 minutes and removal of supernatant. After resuspension in sterile phosphate-buffered saline (PBS; Thermo Scientific), an aliquot was taken for measurement of $\text{OD}_{600\text{nm}}$ via spectrophotometer (Bio-Rad smartspec$^{\text{tm}}$3000, Hercules, CA) and cultures were diluted in sterile PBS to a final concentration of $5 \times 10^9$ cfu/ml. Cells were then infected with either IA3902 or NCTC11168 at a multiplicity of infection (MOI) of 100. Infection was allowed to proceed for 2 hours, at which time the cell culture media was removed and cells were washed 3x with sterile PBS. Fresh cell culture media supplemented with 100 $\mu$g/ml gentamicin was then added and cells were incubated for a further 2 hours in order to destroy any remaining extracellular bacteria. Media was again removed, cells were washed 1x with sterile PBS, and complete culture media (without antibiotics) was added; this was considered $T=0$. Cells were lysed at 0, 12, 24, or 48 hours after infection using 0.1% Triton-X 100 and aliquots plated onto MH agar to determine bacterial survival. All plates were incubated at 42˚C for 48 hours in microaerobic conditions, after which time colony-forming units (cfu) were counted.

**Preincubation of *C. jejuni* with sex hormones.** Fresh cultures of *C. jejuni* were grown for 16-20 hours in MH broth containing either no hormones or 1.0 $\mu$g/ml progesterone or $\beta$-estradiol on a shaker in microaerobic conditions. Bacterial cultures were then centrifuged at 8,000 rpm for 10 minutes and the supernatant poured off. After resuspension in sterile PBS, an aliquot was taken for $\text{OD}_{600\text{nm}}$ measurement via spectrophotometer and cultures were diluted in sterile PBS to a concentration of approximately $5 \times 10^9$ cfu/ml. Infection of cell cultures proceeded as above, with no hormones added to the cell culture media at any time.
**Generation of Campylobacter shuttle plasmid harboring mCherry.** The *mCherry* gene was amplified using PCR from the PRSET-mCherry plasmid (a generous gift of the laboratory of Dr. Bryan Bellaire of Iowa State University). Primers utilized were mCherry-F3 (CGCTTCCGCAGGAGATTTAATGGTGAGCAAGGCGAGGAGG) and mCherry-R3 (TTTCGAGCTTTACTTGTACGCTCGTCCATGCCG); underlined sequences represent restriction sites for *SacII* and *SacI*, respectively. The pRY112 shuttle plasmid (25) contains the selectable marker gene CmR (conferring chloramphenicol resistance), and our lab has previously cloned the promoter region of the *cmeABC* gene (pABC) (26) into this vector (27). This plasmid and the *mCherry* PCR product were serially digested with *SacI* and *SacII*, and ligated together. DH5α *E. coli* was then naturally transformed with the ligation product and streaked onto LB agar containing chloramphenicol (LB-Cm). After 24 hours of growth, colonies were harvested into LB-Cm broth, and grown for a further 24 hours. Plasmid isolation was performed using the Qiaprep Spin Miniprep Kit (Qiagen, USA) and the presence of the *pABC-mCherry* construct was confirmed via PCR using primers pABC-F (AAAAGGATCCTAAATGGAATCAATAG) and mCherry-R3. The resulting plasmid was designated pRY112-pABC-mCh.

**Transformation of C. jejuni.** Strain IA3902 was naturally transformed with the pRY112-pABC-mCh plasmid using an agar-based method as described previously (28). Several attempts at natural transformation of strain NCTC11168 were unsuccessful; thus, electroporation was utilized for transformation of this strain as described previously (29). Briefly, bacteria were grown overnight and then washed 3-4 times in ice-cold wash buffer (272 mM sucrose, 15% glycerol). A 50 µl aliquot was then mixed with 4.0 µg of the
pRY112-pABC-mCh plasmid and electroporated (2.5 kV, 200 Ohms, 25 μF; Gene Pulser, Bio-Rad, USA). The cuvette was flushed with SOC medium (Life Technologies) and plated onto MH agar with chloramphenicol (MH-Cm). Colonies growing on MH-Cm agar were transferred to MH-Cm broth and grown overnight, after which time expression of the plasmid was confirmed in all strains via immunofluorescence microscopy (Olympus IX-61 inverted microscope; Olympus America, Center Valley, PA). PCR was also performed to confirm the presence of the pABC-mCherry construct using primers pABC-F and mCherry-R3. The resulting strains were designated 3902-mCh and 11168-mCh, respectively.

**Motility assay.** All *C. jejuni* strains used in this experiment were tested for motility using semisolid MH agar. Strains were grown for 16-20 hours in MH broth and optical density was measured via spectrophotometer. After adjustment to an OD$_{600nm}$ value of 0.5, strains were inoculated into 0.4% Mueller-Hinton agar via a single stab into the center of the plate. After incubation under microaerobic conditions for 30h at 42°C, the distance of swarming motility was measured at the widest portion of the swarm halo.

**Growth kinetics and fluorescence determination.** In order to determine the effects of various substances on the growth of wild-type strains of *C. jejuni* as well as mCherry-harboring strains, growth curves were performed. Fresh cultures of *C. jejuni* were grown for 16-20 hours in MH broth on an orbital shaker at 160 rpm. Aliquots were then taken for measurement via spectrophotometer and cultures were diluted in fresh MH broth to a starting OD$_{600nm}$ of 0.05. After dilution, taurodeoxycholic acid (25 mg/ml) or acetylsalicylic acid (200 μg/ml) was added to the culture media. All cultures were incubated in microaerobic
conditions with shaking (160 rpm) at 42˚C. Aliquots were removed and \(\text{OD}_{600\text{nm}}\) values were measured at the time points indicated (0, 4, 8, 12, and 24 hours); fluorescence values were also measured in triplicate at each time point using a fluorometer (FLUOstar Omega, BMG Labtech, Offenburg, Germany).

**Plasmid Retention Assay.** To determine the retention of the pRY112-pABC-mCh plasmid in the absence of selective pressure, a plasmid retention assay was performed. Strains were inoculated from frozen stocks onto MH (wt strains) or MH-Cm (mCherry-harboring strains) agar and grown for 24 hours under standard culture conditions. Ten colonies of each strain were then selected at random and inoculated into 5 ml of MH broth without antibiotic supplementation. Broths were incubated microaerobically on a shaker (160 rpm) at 42˚C for 24 hours, at which time aliquots from each strain were diluted 1000-fold (to approximately \(10^6\ \text{cfu/ml}\)) and 100 µl was reinoculated into 5 ml of fresh MH broth. Passaging of strains continued in a similar manner every 24 hours for 20 days. On days 1, 3, 5, 10, 15, and 20, 100 µl aliquots of 1000- and 10,000-fold dilutions of each strain were spread onto MH agar. Wild-type strains NCTC11168 and IA3902 were also spread onto MH-Cm agar. Plates were incubated under standard culture conditions for 48h. At that time, growth of wt strains on MH agar was verified and absence of spontaneous development of antibiotic resistance was confirmed via the absence of wt growth on MH-Cm plates. For strains 11168-mCh and 3902-mCh, 100 colonies were selected at random at each time point from the MH agar plates, and plated in tandem onto fresh MH and MH-Cm agar plates. After incubation for 48 hours under standard culture conditions, growth of each colony on the two plates was compared and recorded. Colonies not growing on MH-Cm were restreaked from the MH agar onto a new
MH-Cm plate for confirmation, and PCR was utilized to test for the absence of the mCherry gene construct in those colonies.

**Immunofluorescence microscopy.** Infected slides were prepared for immunofluorescence microscopy in a manner previously described (30). RAW cells were added to EtOH prepared coverslips and grown to approximately 80% confluency, at which time cells were infected with *C. jejuni* at an MOI of 100. At 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 24 hours, and 48 hours post-infection, coverslips were harvested and fixed in 4% paraformaldehyde in PBS for at least 24 hours at 4°C. Monolayers were permeabilized with a bovine serum albumin-PBS solution containing a final concentration of 0.1% saponin (BSP buffer) prior to incubation with primary antibody (LAMP 1-Alexa Fluor 488; sc-19992 AF488) for 2 hours at room temperature. Antibody was purchased from Santa Cruz Biotechnology (Dallas, TX) and used at a concentration of 1:200. Coverslips were then washed 3x with cold BSP prior to being mounted on glass slides using 1 drop of ProLong® Gold antifade reagent with DAPI (Life Technologies). Immunofluorescence microscopy was performed using an Olympus IX-61 inverted microscope equipped with blue (DAPI), red (mCherry), and green (Alexa Fluor 488) filter sets and a cooled CCD camera. Final images were prepared using ImageJ v1.48b image analysis software (Rasband, 1997).

**Statistical Analysis.** Statistical analysis was performed using commercially available statistical software (JMP Pro v.10.0, SAS Institute, 2012). Differences among groups were analyzed using Tukey’s HSD test and were considered statistically significant at values of \( p < 0.05 \).
Results

NCTC11168 efficiently invades AH-1 ovine placental trophoblasts.

To determine whether AH-1 ovine placental trophoblasts might serve as a suitable in vitro model for infection, it was first desirable to determine whether C. jejuni is capable of invading and/or surviving in these cells. The human fecal isolate NCTC11168 and a representative isolate of the SA clone, IA3902, were used for this purpose.

We hypothesized that since IA3902 is highly abortigenic, it would be able to infect the placental trophoblasts more efficiently than the non-abortifacient isolate NCTC11168. In fact, our data showed precisely the opposite (Fig. 1); 11168 was able to invade (T=0) and persist (T=12 and later time points) within the cells more efficiently than IA3902. This difference was most pronounced at earlier time points (T=0 and T=12), but persisted through 24 hours. After that time, few viable bacteria of either strain were recovered. These results indicate the suitability of the AH-1 cell line for the study of the pathogenic traits of C. jejuni, since both strains were able to invade at a relatively high rate (approximately $10^4$ cfu/ml).

Treatment of AH-1 cells with sex hormones is protective against C. jejuni invasion

Next, we desired to determine whether the sex hormones progesterone and β-estradiol would have any effect on the receptiveness of AH-1 cells to infection with C. jejuni. Abortions due to C. jejuni almost invariably occur late in pregnancy, when progesterone levels in the serum are quite high (0.02 µg/ml) and the uterine venous concentration can reach 0.05 µg/ml (31,32). In contrast, levels of β-estradiol and other estrogens are quite low
in the serum (5-20 pg/ml) until just prior to parturition, when a large spike occurs (with levels reaching up to 900 pg/ml), concurrent with a decrease in serum progesterone levels (16,18). It was our hypothesis that the high level of circulating progesterone might cause immunosuppression in the AH-1 cells and allow more efficient colonization by *C. jejuni*.

As seen in Fig. 2A, when AH-1 cells were treated for 48 hours prior to infection with a high dose of progesterone (1 µg/ml; PH), there is a moderate, though not statistically significant, decrease in invasion of 11168, as well as a significant decrease in survival of 11168 at 12 hours. More surprisingly, there is a significant decrease in invasion of AH-1 cells by IA3902 (Fig. 2B); no significant differences were noted in survival at 12 hours. No difference in survival was noted at 24 or 48 hours for either strain. Despite this, it should be noted that for 11168, there were several outlier wells in which abundant growth was unexpectedly seen after 24 or 48 hours of incubation, and these outliers were all within the progesterone treatment groups; no such outliers were noted for IA3902. Treatment of AH-1 cells with a lower dose of progesterone (0.1 µg/ml; PL) had no effect on the invasion or intracellular survival of either strain (Fig. 2). These results are somewhat contrary to expectations, and are surprising given the high circulating levels of progesterone at the time of peak *C. jejuni* infection of the fetoplacental unit.

When AH-1 cells were treated with β-estradiol at a low dose of 0.1 µg/ml (EL) or a high dose of 1.0 µg/ml (EH) for 48 hours prior to infection (Fig. 3), the only significant difference noted was a decrease in invasion by IA3902 (at T=0) when cells were treated with low-dose β-estradiol. The significance of that difference is unclear; however, the rest of the data are consistent with our hypotheses. Estrogen values do not drastically alter during pregnancy in the ewe until immediately prior to parturition; thus, we would not expect
estrogens to play an important role in the pathogenesis of *C. jejuni*.

**Length of exposure of AH-1 cells to sex hormones alters magnitude of effect**

Since a difference was seen in the ability of *C. jejuni* to invade AH-1 cells after exposure to progesterone as described above, we next asked how long cells would need to be exposed to this hormone in order to see that difference. Cells were exposed to a high dose of progesterone (1.0 µg/ml) for 48 hours, 24 hours, 3 hours, or 15 minutes prior to infection with either NCTC11168 or IA3902. As can be seen (Fig. 4A), cells that were unexposed to hormones were invaded by 11168 (T=0) at a significantly higher rate than those that were exposed to progesterone for either 3 hours, 24 hours, or 48 hours. There was not a significant difference between untreated cells and those exposed to progesterone for 15 minutes. At T=12, untreated cells had much higher bacterial loads than those treated for 24 or 48 hours, and cells treated for 15 minutes were also significantly more infected than the cells treated for 48 hours. By 24 hours post-infection, cells treated for 48 hours were significantly less infected than untreated cells or cells treated for 3 hours. At 48 hours post-infection, there was no difference between untreated cells and treated cells; however, significantly more 11168 persisted in cells treated for 15 minutes than in cells treated for 24 or 48 hours prior to infection. Thus, treatment of AH-1 cells with progesterone for at least 24 hours prior to infection seems to be necessary to cause a significant decrease in *C. jejuni* invasion.

For IA3902, the differences were less pronounced (Fig. 4B). At T=0, untreated cells and those treated for 15 minutes or 3 hours were all significantly more invaded by *C. jejuni* than cells treated for longer periods of time (24 or 48 hours). However, by 12 hours, there were no significant differences in bacterial persistence. Interestingly, there were again
several wells with unexpected abundant growth seen at either 24 or 48 hours; these outliers all occurred in cells treated with progesterone for short periods (either 15 minutes or 3 hours).

**Treatment of *C. jejuni* with sex hormones does not alter intracellular invasion or survival**

To more accurately determine whether the effects of progesterone and ß-estradiol were due to changes in cellular protein expression or were due to effects upon the bacteria themselves, *C. jejuni* were grown in MH broth containing 1.0 µg/ml of progesterone (Pretx PH) or ß-estradiol (Pretx EH) for approximately 20 hours prior to infection. Bacteria were then pelleted via centrifugation and resuspended in sterile PBS such that any remaining extracellular hormone was eliminated. AH-1 cells were then infected as described above, with no hormones used in the cell culture media. As seen in Fig. 5, treatment of *C. jejuni* strains NCTC11168 (Fig. 5A) or IA3902 (Fig. 5B) with either progesterone or ß-estradiol did not significantly alter cellular invasion or intracellular survival at any of the time points tested.

**IA3902 efficiently invades RAW264.7 Murine Macrophages**

Invasion of AH-1 ovine placental trophoblasts by IA3902 was relatively low, especially in comparison to the non-abortive strain NCTC11168. Thus, it was desirable to determine whether this invasion defect was a general property of IA3902, or was specific to the AH-1 cell line. To determine this, infections of RAW264.7 murine macrophages were undertaken.
RAW264.7 murine macrophages were infected in the same manner as AH-1 placental trophoblasts, described above. As can be seen (Fig. 6), IA3902 both invaded and survived within macrophages more efficiently than NCTC11168 at 0, 12, and 24 hours post-infection. In comparison to AH-1 cells, in which survival was occasionally vigorous at 48 hours (Fig. 1), no viable bacteria of either strain were recovered at 48 hours from the RAW264.7 murine macrophages.

Expression of mCherry fluorescent protein in two *C. jejuni* strains

In addition to developing a viable *in vitro* cell culture model, it was also desirable to develop a method of quickly identifying and quantifying bacteria both *in vitro* and *in vivo*. To this end, the mCherry fluorescent protein was cloned and inserted into the pRY112 plasmid along with the promoter for the *cmeABC* gene cassette; the resulting plasmid was referred to as pRY112-pABC-mCh. The plasmid was successfully inserted into IA3902 via natural transformation and into NCTC11168 via electroporation, and its expression was confirmed via use of immunofluorescence microscopy (see Fig. 9b). These strains were referred to as 3902-mCh and 11168-mCh, respectively.

Characterization of *C. jejuni* strains expressing the pRY112-pABC-mCh plasmid

**Motility**

Following successful transformation of both IA3902 and 11168 with the pRY112-pABC-mCh plasmid, it was first desirous to assess the motility of the 11168-mCh and 3902-mCh strains in comparison to their wt counterparts. As motility is one of the major virulence
factors identified in both cellular invasion and intestinal colonization models of *C. jejuni* infection, any reduction would greatly impair the utility of these strains as a model for infection. As seen in Fig. 7, no significant alteration in motility was noted in mCh-harboring strains as compared to their wt counterparts.

**Growth Curve and Baseline Fluorescence values**

To characterize expression of the pRY112-pABC-mCh plasmid and to determine whether its expression had any measurable effect on cell viability, a growth curve experiment was undertaken (Fig. 8). Both the 3902-mCh and 11168-mCh strains were significantly hampered in their growth as compared to the wt strains at both 12 and 24 hours; the expression of this plasmid thus seems to cause a considerable fitness cost in these strains.

Additionally, measures of fluorescence were taken at each time point to determine the amount of baseline expression of the mCherry protein (Fig. 9a). As the mCherry-harboring strains did not grow as well as wt strains, fluorescence values were normalized to OD$_{600nm}$ values to adjust for differences in bacterial numbers. Baseline fluorescence values were not significantly increased over wt strains at time points prior to 12 hours. At T=12h, both the 3902-mCh and 11168-mCh strains were significantly more fluorescent than their wt counterparts. At T=24h, the fluorescence of 3902-mCh was again significantly greater than its wt counterpart; 11168-mCh was also more fluorescent than 11168, although the difference was not quite significant ($p = 0.0567$).

**Induction of fluorescence using inducers of the cmeABC promoter**

Next, we attempted to determine whether substances that are known inducers of the
cmeABC promoter could increase expression of the pRY112-pABC-mCh construct and thus increase the fluorescence of these strains. Two such inducers are acetylsalicylic acid (33) and bile salts (34). Fresh cultures of 3902-mCh, 11168-mCh, and wt strains were diluted to an OD$_{600nm}$ of 0.05 and acetylsalicylic acid or a representative bile salt, sodium taurodeoxycholic acid, were added at a concentration of 200 µg/ml or 25 mg/ml, respectively.

Surprisingly, acetylsalicylic acid did not increase the fluorescence of the mCherry-harboring strains (Fig. 10), and in fact actually significantly decreased the fluorescence of the 3902-mCh strain by 24 hours. The fluorescence of the 11168-mCh strain was also decreased at 24 hours; however, this decrease was not quite significant (p = 0.07).

In contrast, taurodeoxycholic acid did significantly increase the fluorescence of both mCherry-harboring strains (Fig. 11). Fluorescence intensity was significantly enhanced for both 3902-mCh and 11168-mCh at 4 and 24 hours. At 8 and 12 hours, only 3902-mCh was significantly more fluorescent than its wt strain; however, the difference between 11168-mCh and its wt approached significance (p = 0.07). This increase in fluorescence was seen despite a concurrent decrease in OD$_{600nm}$ values and dismal growth through 24 hours; however, samples plated at 12 and 24 hours confirmed viability of the cells, and wt growth in the presence of taurodeoxycholic acid was similarly thwarted (data not shown).

**Stability of the pRY112-pABC-mCh construct in C. jejuni**

As a major application of mCherry-harboring strains is to assess *Campylobacter*-cell interactions using both *in vitro* and *in vivo* models, it is important to determine the stability of
the plasmid to ensure that there is a correlation between loss of fluorescence and absence of bacteria. To this end, a plasmid retention assay was performed by serially passaging 11168-mCh and 3902-mCh in MH broth (Fig. 12). Samples were taken at days indicated (days 1, 3, 5, 10, 15, and 20), and 100 colonies were randomly passaged onto MH-Cm agar to determine the percent retaining the pRY112-pABC-mCh plasmid. Although the vast majority of bacteria of both strains retained the plasmid through day 5, both strains show significant loss of the plasmid at day 10, and have nearly completely lost the plasmid by day 20. This exponential loss is probably due in large part to the fitness cost the plasmid appears to impose (see Fig. 8), and would severely limit the usefulness of the construct for long-term experiments.

Use of strains harboring mCherry in microscopy applications

Lastly, it was important to determine whether the mCherry harboring strains could be useful in tracking infection in vitro. RAW cells were grown to approximately 80% confluency on coverslips and infected with either 3902-mCh or 11168-mCh at an MOI of 100. Coverslips were harvested and fixed in 4% paraformaldehyde at 30 minutes, 2 hours, 4 hours, 8 hours, 12 hours, 24 hours, and 48 hours after infection. After at least 24 hours of fixation, coverslips were stained with an antibody to LAMP-1 and fixed with DAPI (Fig. 13). Immunofluorescence microscopy revealed mCherry expression within endosomal vesicles of RAW murine macrophages. Results are in general consistent with previous studies (35) and show that mCherry fluorescence appears to co-localize at a high rate with LAMP-1, an early endosomal marker, by 1-2 hours post infection.
Discussion

Here, we have attempted to develop two different cell culture models for use in *in vitro* study of *Campylobacter* genitourinary pathogenesis, with an aim towards identifying a suitable system of study for *C. jejuni* clone SA. Surprisingly, clone SA was much less efficient than the nonabortifacient strain NCTC11168 at infecting and surviving within AH-1 ovine placental trophoblasts. This seems to indicate that the high virulence of this strain in pregnant animals is probably due more to its ability to actually gain access to the bloodstream and placenta, rather than a specific tropism for the placental tissue. This assumption is supported by the results of infections of RAW264.7 murine macrophages, in which IA3902 was significantly more invasive and persistent than NCTC11168. It has previously been shown that both IA3902 and NCTC11168 are able to produce evidence of bacteremia after oral inoculation at a relatively high rate (92% and 67%, respectively); however, only IA3902 causes uterine inflammation and abortion in pregnant Guinea pigs (10). Immune cells are recruited in large number to the fetus and placenta during pregnancy (36,37), and transport within these cells may represent a major mechanism via which *C. jejuni* localize there. The difference in survival and persistence in macrophages may thus present a deciding factor in pathogenesis of abortion. Previous studies utilizing a green fluorescent protein-expressing strain of *C. jejuni* showed a strong affiliation with granulocytes (neutrophils and, to a lesser extent, macrophages) in the bloodstream (19). Thus, if *C. jejuni* are "ferried" within immune cells in the bloodstream, macrophages are one likely candidate to serve as hosts. Indeed, they have been known to play a role in aiding the pathogenesis of a variety of exploitative bacteria, including providing a cue for expression of
virulence genes in *Salmonella* (38) and harboring and spreading *Mycobacterium tuberculosis* (39).

The apparently protective nature of progesterone against invasion by *Campylobacter* spp. is intriguing. We had hypothesized that a high level of progesterone would make the trophoblasts more susceptible to infection, since at the time of typical infection (the third trimester of pregnancy), serum and local uterine levels are relatively high in the ewe. Additionally, in driving fetal tolerance, progesterone's effects are largely anti-inflammatory; it promotes IL-10 synthesis in dendritic cells (40) and drives T cells towards a Th2 phenotype (41). Thus, we would expect its presence to dampen immune response to *C. jejuni*; paradoxically, the data indicate that high levels of progesterone actually decrease infection of AH-1 ovine placental trophoblasts by *C. jejuni*. A drop in progesterone level may actually be required, then, prior to maximal *C. jejuni* invasion and replication; interestingly, it was noted in one of the first studies of *C. jejuni* abortion that progesterone levels were normal 48 hours prior to abortion but dropped precipitously immediately prior to the abortive event (5). Possibly, this could be attributed to gastrointestinal inflammation causing increased blood flow to the gut and liver; 96% of progesterone is metabolised during a single passage through these organs (42), which could be expected to significantly lower the peripheral concentration if blood flow were diverted there. As progesterone is essential in the maintenance of pregnancy (41,43), a large enough decrease could directly cause abortion to occur. However, while subclinical hepatitis and gastrointestinal inflammation is a possibility, most ewes show few clinical signs prior to the abortive event (5). It should also be noted that the effects of progesterone during pregnancy are far-reaching and complex, especially with regards to the immune system, and thus far understood to a rather scarce degree (44); hence,
at least some of its effects on the trophoblasts are likely to be multifactorial and rather difficult to untangle. That it did not have an effect on the bacteria themselves is not surprising (as *C. jejuni* do not possess a known progesterone receptor), and serves as further confirmation that its effect is likely to be in altering cellular pathways in the trophoblasts themselves. More extensive studies of gene and protein expression would be necessary to pinpoint the extent of progesterone’s influence upon these cells and its possible mechanisms for inducing a protective state against *C. jejuni* infection.

These studies have also demonstrated successful cloning of the *mCherry* gene into *C. jejuni*. The promoter of *cmeABC* was utilized for *mCherry* expression as it is moderately constitutively active in *Campylobacter* (26,45). However, its activity does not appear to be high enough to warrant its use as a promoter in this instance. While fluorescence in *mCherry*-expressing strains was higher than that of the wt strains at times greater than 12 hours (Fig. 9a), the difference was not impressively large; the lack of difference at earlier time points also suggests that expression of the *mCherry* protein was too low to be readily detectable unless bacteria are in high concentration. Addition of taurodeoxycholic acid to increase expression through the *cmeABC* promoter was highly successful in increasing fluorescence, but also caused severe growth restriction at the concentration used. Acetylsalicylic acid, which also would be expected to increase expression through the *cmeABC* promoter, did not cause a growth defect but also did not improve and actually somewhat decreased fluorescence values. Selection of a promoter with higher innate activity, such as the MOMP promoter (46,47), may provide at least a partial solution. However, there also appears to be a large fitness cost to strains of *C. jejuni* expressing the plasmid, with consequently low retention after 5 passages. It should be noted that our results in the first 5 days are similar to
Miller et al., who showed excellent retention of plasmids expressing gfp, yfp, and cfp at day 5, but did not passage their cultures beyond that time (20). This still leaves open the question of whether the observed plasmid loss is specific to this plasmid construct or represents a general fitness cost to expression of other fluorescent proteins in C. jejuni.

Despite the obvious drawbacks of the mCherry strains created herein as a system for in vivo study of C. jejuni infection, the utility of the mCherry-expressing strains for in vitro microscopic studies of C. jejuni infection is evident. Visualization of bacteria within RAW murine macrophages facilitated determination of intracellular localization. The mCherry fluorescent protein has excellent proven tissue penetration (48,49) and would likely make an excellent candidate for in vivo tracking of Campylobacter infection if the problems outlined above could be overcome, e.g. via the use of a more active promoter or through chromosomal insertion of the mCherry gene.

In sum, we have identified a potential system for in vitro study of the pathogenesis of C. jejuni clone SA, as RAW264.7 murine macrophages seem to be permissive to infection and persistence of this strain. We have also determined that clone SA does not seem to have high pathogenicity for AH-1 ovine placental trophoblasts in an in vitro model, despite high tropism for these cells in vivo, and that progesterone seems to be protective for trophoblasts in spite of its anti-inflammatory effects during pregnancy.

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Figure 1. Invasion and intracellular survival of two strains of *Campylobacter jejuni* in AH-1 ovine placental trophoblasts. Data are expressed in log (cfu/ml) (mean ± SEM). * indicates $p < 0.05$, ** indicates $p < 0.01$. 
**Figure 2.** Effects of progesterone on 11168 (A) and 3902 (B) invasion and intracellular survival in AH-1 ovine placental trophoblasts. Time = -4 represents inoculum values. Data are representative of three independent experiments, each performed in triplicate. Box represents second and third quartiles with line at mean, and min/max range is indicated. * = $p < 0.05$. 
Figure 3. Effects of β-estradiol on 11168 (A) and 3902 (B) invasion and intracellular survival in AH-1 ovine placental trophoblasts. Time = -4 represents inoculum values. Data are representative of three independent experiments, each performed in triplicate. Box represents second and third quartiles with line at mean, and min/max range is indicated. * = p < 0.05.
Figure 4. Invasion and intracellular survival of NCTC11168 (A) and IA3902 (B) in AH-1 Ovine placental trophoblast cells treated with 1 µg/ml progesterone for 15 minutes, 3 hours, 24 hours, or 48 hours prior to infection. Data are representative of two independent experiments, each performed in triplicate. Box represents second and third quartiles with line at mean, and min/max range is indicated. *, p < 0.05; **, p < 0.01.
Figure 5. Pretreatment of NCTC11168 (A) or IA3902 (B) with 1.0 µg/ml progesterone (PH) or β-estradiol (EH). *C. jejuni* were cultured in the presence of hormones indicated for approximately 20 hours prior to use in infection assays of AH-1 ovine placental trophoblasts. Data are representative of three independent experiments, each performed in triplicate. Box represents second and third quartiles with line at mean, and min/max range is indicated.
Figure 6. Invasion and intracellular survival of two strains of *Campylobacter jejuni* in RAW264.7 murine macrophages. Data are expressed in log (cfu/ml) (mean ± SEM). Data are representative of three independent experiments, each performed in triplicate. **, *p* < 0.001.
**Figure 7.** Motility of mCh-expressing *C. jejuni* strains as compared to wt strains. Mean is indicated for each strain. Differences between wt and mCh-harboring strains were not significant. Data are representative of three independent experiments, each performed in triplicate.
Figure 8. Growth of mCherry-harboring strains of NCTC11168 and IA3902 as compared to their wt counterparts. Data are expressed as mean±SEM of OD$_{600nm}$ values. Data are representative of three independent experiments. *, 3902 > 3902-mCh and 11168 > 11168-mCh, $p<0.05$. 
Figure 9. Normalized fluorescence values for 11168-mCh, 3902-mCh, and their wt counterparts (A). Normalized values represent average blank corrected relative fluorescence units (RFU)/average OD$_{600nm}$ (mean±SEM). Data are representative of three independent experiments. *, 3902-mCh > 3902 and 11168-mCh > 11168, p < 0.05; †, 3902-mCh > 3902, p < 0.001. Figure 9B shows a representative image of *C. jejuni* 3902-mCh bacteria confirming expression of fluorescent mCherry protein; the 11168-mCh strain is similar in appearance.
Figure 10. Fluorescence values (mean ± SEM) of 11168-mCh and 3902-mCh grown in plain MH broth or MH broth containing 200 µg/ml acetylsalicylic acid (ASA). Data are representative of three independent experiments. †, 3902-mCh > 3902-mCh+ASA ($p = 0.002$) and 11168-mCh > 11168-mCh+ASA ($p = 0.07$).
Figure 11. Fluorescence values (mean±SEM) of 11168-mCh and 3902-mCh grown in plain MH broth or MH broth containing 25 mg/ml taurodeoxycholic acid (TCA). Data are representative of three independent experiments. **, 3902-mCh+TCA > 3902-mCh and 11168-mCh+TCA > 11168-mCh, p < 0.01. †, 3902-mCh+TCA > 3902-mCh (p < 0.05) and 11168-mCh+TCA > 11168-mCh (p = 0.07).
Figure 12. Percent retention of the pRY112-pABC-mCh plasmid within C. jejuni strains NCTC11168 and IA3902 after serial passage in MH broth.
Figure 13. Representative image showing expression of mCherry fluorescent protein (red) by C. jejuni strain 3902-mCh within endosomal vesicles of RAW264.7 murine macrophages at 2 hours post infection. Blue, DAPI (nuclear) stain; green, anti-LAMP-1 conjugated to Alexa Fluor488.
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