Campylobacter rectus BACTERIAL-HOST INTERACTIONS IN PATHOGENESIS

A Thesis

by

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ABSTRACT

Campylobacter rectus is a Gram-negative oral anaerobe and a causative agent of periodontitis. Additionally, *C. rectus* strains have been isolated from patients with oral and extraoral abscesses, gingival crevices, and appendicitis. This organism has been implicated in bacteremia and is associated with pre-term births and low birth weight, indicating its importance as an emerging pathogen. The goal of this thesis was to establish genetic tools to begin clarifying mechanisms responsible for *C. rectus* pathogenesis. In particular, secretion systems identified as important for pathogenesis in related bacterial species were selected for further study. Bacterial-host interaction assays were used to identify host responses stimulated by the CiaB protein as well as the role of the Hcp protein in host cell adherence. Toward this goal, complete deletions of the *ciaB* and the *hcp* genes were generated in *C. rectus* and reference genes for RT-qPCR in *C. rectus* were validated to permit assessment and verification of the mutant strains. Host response was assessed by exposing a human placental epithelial cell line, BeWo, to both *C. rectus* wild-type and *ciaB* mutant strains for six-hours under anaerobic conditions and inflammatory mRNA expression was measured using RT-qPCR. A significant upregulation in 41 inflammatory genes was measured in response to Δ*ciaB* while only three were significantly upregulated in response to wild-type. THP-1 human monocyte cell viability was also assessed over a 96-hour time course after exposure to wild-type and Δ*ciaB*. Additionally, adherence efficiencies of wild-type and Δ*hcp* to BeWo cells

were calculated after a one-hour time point. Adherence rates between wild-type and Δ*hcp* were not significantly different. The studies in this thesis suggest *C. rectus* uses CiaB as a mechanism to evade the host cell immune response. Analysis of the Δ*hcp* mutant prepared this strain for further studies like those used for the CiaB mutant strain to fully characterize the importance of the type VI secretion system to *C. rectus* virulence. Results from this foundational thesis research will expedite future studies examining the underlying molecular mechanisms of *C. rectus* pathogenesis.

DEDICATION

To my parents for their unconditional love and support at every step of my journey. To Casey, who has helped me keep sight of my goals and provided endless support, patience, and love. And finally, to all my past teachers, professors, and mentors: I could not have made it this far without their guidance and advice.

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The reference gene analyses depicted in Chapter II were conducted in part by Dr. Dana Blackburn, formerly of the Department of Veterinary Pathobiology. The mutants in Chapter II were generated by Bridget E. Conley (*ciaB*) and Erin A. Harrell (*hcp)*, formerly of the Department of Microbiology at North Carolina State University. All other work conducted for the thesis was completed by the student independently.

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The discovery, features, and metabolic properties of *Campylobacter rectus*

Campylobacter rectus is a Gram-negative anaerobic bacterium and a causative agent of periodontal disease. *C. rectus* is a small, straight rod that is typically 0.5 μm wide and 4 μm long and possesses a single polar flagellum which provides motility [1, 2]. Originally named *Wolinella recta* in 1981 by Tanner *et al.* [2], it was later reclassified into the *Campylobacter* genus in 1991 by Vandamme *et al*. [3] following more extensive rRNA sequence analysis.

C. rectus grows optimally under anaerobic conditions with media containing mycoplasma broth supplemented with formate and fumarate and is unable to use carbohydrates for fermentative growth [4]. *C. rectus* has a formate-fumarate driven metabolism and uses formate or hydrogen from other oral species as electron donors while fumarate and nitrate act as an electron acceptor for metabolism [4, 5]. The use of formate and fumarate from other oral organisms signifies a symbiotic relationship between *C. rectus* and other oral bacteria, indicating the existence of a complex microbiota of the oral milieu is pertinent to optimal growth conditions and pathogenesis of *C. rectus.*

The connection between *Campylobacter rectus* and adverse health outcomes

Periodontal disease is caused by a plethora of oral pathogenic bacteria and is often characterized using the microbial complex [6]. *C. rectus* is classified as an intermediate

oral pathogen and is labeled in the "orange complex" [6]. The exact role of each bacterium in periodontal disease is relatively unknown, however the initial colonizers of the complex, such as *Streptococcus* and *Actinomyces* species, are able to colonize and provide the necessary environment for more intermediate pathogenic bacteria, including *C. rectus* and *Fusobacterium nucleatum,* to colonize. The presence of these intermediate oral pathogens creates the ideal environment for the most aggressive oral pathogens: *Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola* [6, 7].

Periodontitis affects 47% of adults in the United States and *C. rectus* being present in 90% of those cases [8]. *C. rectus* is most commonly isolated from periodontal locations [9], however strains have been isolated from other regions of the body such as extraoral abscesses [10], gingival crevices [11], and appendicitis cases [12]. Furthermore, *C. rectus* has been detected in peripheral blood from orally infected patients as well as in placental tissue from infected mice [13], and is known to be associated with adverse human pregnancy outcomes such as low birth weight and preterm labor [10, 14]. Estradiol and progesterone stimulate the growth of *C. rectus in vitro* and correlates with increased physiological levels of estradiol in pregnant women [14, 15]. Additionally, when compared to their healthy counterparts, pregnant women with increased levels of *C. rectus* present in their oral microbiome were more likely to have gum bleeding and deep periodontal pocket depth [15]. The association of periodontal disease and adverse pregnancy outcomes make *C. rectus* an increasingly important organism to study in human health.

Characteristics of *Campylobacter rectus*

Lipopolysaccharide

Although it is well-established that *C. rectus* is a causative agent of periodontal disease, the methods of pathogenesis associated with *C. rectus* are largely understudied and as a result, not well understood. However, previous studies have discovered *C. rectus* produces several virulence factors including lipopolysaccharide (LPS) [16]. LPS is a common virulence factor in Gram-negative bacteria and is known in *C. rectus* 33238 to stimulate the plasminogen activator-plasmin system to release prostaglandin E and interleukin (IL)-1 from human gingival fibroblast (HGF) cells [17, 18]. Although LPS isolated from *C. rectus* 33238 has been shown to stimulate inflammation, its presence is not necessary for toxic effects [16]. Furthermore, *C. rectus* 33238 LPS has activity similar to LPS isolated from *Escherichia coli* [19].

Surface layer

Another characterized virulence factor of *C. rectus* is the surface-layer protein (s-layer). The s-layer of *C. rectus* is a paracrystalline cell surface structure and seems to play a role in altering host-pathogen interactions [20, 21]. Studies have shown that *C. rectus* 33238 loses its s-layer after 15 to 17 *in vitro* passages and isolates lacking the s-layer are less virulent in a mouse abscess model [22, 23]. S-layer-positive isolates are more resistant to phagocytosis by leukocytes or to complement-mediated killers [24]. Finally, a mutation in the s-layer of *C. rectus* 314 and 33238 decreases binding to epithelial cells and the *C. rectus* 314 s-layer mutant stimulates increased expression of pro-inflammatory cytokines from epithelial cells [21]. These discoveries about the s-

layer function are essential in beginning to understand the mechanisms behind *C. rectus* pathogenesis.

Toxins

Cytotoxic activities have been reported in *C. rectus* and are suggested to be caused by pore-forming protein toxins in the RTX (repeats in the structural toxins) family [25]. RTX toxins are known to be major virulence factors of many Gram-negative bacteria and include the alpha-hemolysin HlyA in *E. coli,* the leukotoxins AaLtA in *Aggregatibacter actinomycetemcomintans,* and the adenylate cyclase CyaA of *Bordetella pertussis* [26-29]. *C. rectus* cytotoxic activity was first suggested in 1990, discovering that whole cells of *C. rectus* 33238 suppress the proliferation of undifferentiated monomyelocytic HL-60 cells. This study also found both undifferentiated and differentiated HL-60 cells are killed post-exposure to live and opsonized *C. rectus* 33238 [30]. Furthermore, heat-killed *C. rectus* 33238 has decreased killing effects when compared to live and opsonized bacteria [30]. Although media supernatants from *C. rectus* 33238 are toxic to HL-60 cells and human polymorphonuclear leukocytes (PMNs), whole cell suspensions of *C. rectus* do not appear to have a killing effect [31]. Consequently, Kuhnert et al. [32] found RTX protein-coding genes in the *C. rectus* 33238 genome, terming these genes *csxA* and *csxB* (*C. rectus* s-layer-RTX toxin). These toxins are hypothesized to play a role in host interactions in other campylobacters [33].

Host response and inflammation stimulation

The stimulation of a host response is significant in disease pathogenesis and is a vital step in the progression of periodontal disease. Previous studies have indicated whole cells and components of *C. rectus* are capable of stimulating a host response. Formalin-killed *C. rectus* 33238 cells induce the production of interleukin (IL)-6 and IL-8 from HGF cells, while *C. rectus* surface associated material (SAM) induces IL-6 production from HGF, peripheral blood mononuclear cells (PBMCs), and Mono-Mac-6, a myelomonocytic cell line [34, 35]. Additionally, LPS from *C. rectus* 33238 stimulates IL-1 secretion from murine macrophages [36]. The s-layer is proposed to play a major role in *C. rectus* pathogenesis as an s-layer mutant in *C. rectus* 314 and 33238 stimulates a significant increase of the pro-inflammatory cytokines IL-6, IL-8, and tumor necrosis factor–alpha (TNF-α) from HEp-2 epithelial cells [21]. The production of pro-inflammatory cytokines is understood to be responsible for the immune response and inflammation commonly seen in periodontal disease.

CiaB as a virulence factor in *Campylobacter rectus*

Another strong candidate for virulence of *C. rectus* is a protein deemed *Campylobacter* invasion antigen B (CiaB), which was first described in *C. rectus* 314 by LaGier and Threadgill in 2014 [37]. This study found *C. rectus* 314 encodes an 1,830-base pair *ciaB* gene, encoding a 69.8 kilodalton (k-Da) protein, which shares a 53% sequence identity and 70% similarity with the *ciaB* gene in *C. jejuni.* BLASTx analysis identified similarity to genes in other species annotated as *ciaB*, a hypothetical protein, and a lipoprotein signal peptidase [37]. Moreover, using as quantitative reverse

transcription PCR (RT-qPCR), *ciaB* was found to be expressed by *C. rectus* 314, a clinical isolate, as well as *C. rectus* 33238*,* a periodontal pocket isolate [37, 38]. *C. rectus ciaB* is predicted to be localized to the cytoplasm and does not contain membrane helices or a signal peptide [37]. *C. rectus ciaB* is necessary for maximal host cell invasion of human placental cells (BeWo), but not for maximal adherence [38]. These findings indicate *C. rectus* CiaB possesses a similar function as what is observed in *C. jejuni*, however, the role of CiaB in host responses is still unknown.

CiaB in other campylobacters

C. jejuni, a bacterium closely related to *C. rectus*, utilizes its flagellum as a type III secretion system (T3SS) to secrete proteins essential for virulence processes such as host cell invasion [39]. *C. jejuni* proteins that are required for high rates of invasion have been termed *Campylobacter* invasion antigens (Cia). Although approximately 18 Cia proteins have been discovered in *C. jejuni*, only four have been characterized [40-43]. The most extensively characterized Cia protein in *C. jejuni*, CiaB, is a 73 k-Da secreted protein and is required for the secretion of all other Cia proteins [42]. Additionally, CiaB is vital to host cell invasion, apoptosis, and overall virulence in animal models [42, 44, 45], however, the underlying molecular mechanisms of *C. jejuni* CiaB are still unclear. Furthermore, the *ciaB* gene has been identified in other campylobacters such as *Campylobacter fetus, Campylobacter coli*, *Campylobacter lari, Campylobacter curvus,* and *Campylobacter concisus* [37, 46-48]. The discovery of the *ciaB* gene in multiple campylobacters indicates the gene may play an important role in overall campylobacter pathogenesis.

Secretion systems as virulence factors

Secretion systems play a vital role in bacterial pathogenesis by secreting proteins or DNA into host cells. Such secreted proteins are vital for numerous things including evasion of the immune system, adherence and invasion of host cells, ruffling of the host extracellular matrix, and the stimulation of an immune response [45, 49]. Six classes of secretion systems have been identified in Gram-negative bacteria: T1SS (type I secretion system), T2SS, T3SS, T4SS, T5SS and T6SS [50]. Each secretion system class is noted to perform different functions. T1SS, T2SS, and T5SS are classified as delivering proteins to the bacterial surface or into the environment while T3SS, T4SS, and T6SS are more commonly associated with pathogenesis mechanisms [49, 51].

A secretion system often related with bacterial pathogens is the T3SS, which distributes proteins directly into eukaryotic cells to stimulate a host response [52]. For example, in *Campylobacter jejuni,* the flagellar T3SS is found to secrete proteins vital to host cell interactions, known as the Cia proteins [53]. Similar to *C. jejuni*, genomic research has revealed the presence of a T3SS in *C. rectus*, however T3SS functionality has yet to be verified.

The function of the most recently discovered secretion system, T6SS, is dependent on the bacterial species and has been found to perform many roles, such as toxin secretion, stimulation of immune responses from the host cell, anti-virulence to evade the immune system, and bacterial competition [54]. The T6SS in some bacteria is critical to the adherence and invasion of host cells. For example, in *Campylobacter jejuni*, a 50% reduction in both adherence and invasion to colon epithelial and

macrophage cells was observed in T6SS mutants with deletions in *hcp*, encoding a T6SS secreted hemolysin protein, and the T6SS structural *icmF* gene [55]. Conversely, the *Helicobacter hepaticus* T6SS hinders adherence and invasion. However, previous studies observed an increase in adherence and invasion when the T6SS function was inactivated, suggesting that *H. hepaticus* may utilize its T6SS as a survival function [56].

Current genomic research has identified the presence of a T3SS and T6SS in the *C. rectus* genome, however it is still unknown how these systems contribute to *C. rectus* pathogenesis. In the following chapters, research aimed at investigating CiaB and the T3SS as well as Hcp and the T6SS will be discussed to address some important features of these two systems in *C. rectus.* In particular, the importance of these two systems to host interactions will be explored.

Campylobacter rectus 33238 and 314 characteristics

C. rectus 33238 is the only publically sequenced strain. This strain was isolated from a periodontal pocket and is currently a well-established laboratory strain of *C. rectus* [57]. *C. rectus* 33238 was sequenced in 2008 by the J. Craig Venter Institute as a reference genome for the species. Genomic sequence revealed that this strain has a genome size of approximately 2.5 megabases (Mb) and GC content of 44.8% [58].

Another useful strain, *Campylobacter rectus* 314, is a clinical strain isolated from a periodontitis patient [22]. *C. rectus* 314 was sequenced by our lab in 2016 and found to also have a genome size of 2.5 Mb, with a comparable GC content of 44.9%. Studies using 33238 and 314 are described in this thesis.

Inflammation and periodontal disease

Periodontal disease (PD) affects millions of Americans and is an inflammatory disease affecting the supporting tissues and structures surrounding the teeth [59]. PD results from oral bacteria localizing in periodontal pockets and stimulating a response from the host, such as inflammation [60, 61]. This chronic inflammation can lead to the destruction of gum tissues, bone, and tooth-supporting structures [62]. First evidence of host response participation in PD was demonstrated when treatment with a prostaglandin inhibitor reduced the levels of bone loss [63]. Furthermore, studies involving a non-human primate model suggested cytokines play a significant role in PD, finding inhibition of IL-1 and tumor necrosis factor (TNF) slows the progression of periodontal bone loss and detachment [64, 65]. More evidence supporting the role of cytokines in PD include a study demonstrating significant levels of IL-1β in gingival tissue from periodontitis patients while no IL-1β was detectable in patients with normal gingival tissue [66].

Pro-inflammatory cytokines, like interferon-gamma (IFN-γ), IL-6 and TNF, are thought to play a destructive role in the host, as mice deficient for these receptors exhibited reduced bone loss in oral gavage models infected with *P. gingivalis* or *A. actinomycetemcomitans* [67, 68]. IL-6 has been found to be produced from gingival mononuclear cells only in adults with PD [69]. Chemokines also affect PD bone loss by recruiting neutrophils, which aid in protection against bacterial invasion. A study investigating the chemokine receptor CXCR2 found an increase in periodontal bone loss when CXCR-2 deficient mice were given an oral gavage of *P. gingivalis* when compared

to wild-type mice [70]. This study suggests chemokines are a major contributor in the protection of the host from bacterial-induced bone loss.

In summary, previous studies suggest the nature of cytokines and chemokines produced by the host response is an important factor in determining if a bacterial infection is able to localize in the oral cavity and the extent of gum tissue and bone damage caused in response to the infection.

Rationale and summary of experiments

Campylobacter rectus has been recognized as a major contributor in periodontal disease and other adverse human health outcomes, however the molecular methods of pathogenesis are not well-understood. The goal of this thesis was to begin to elucidate and characterize virulence mechanisms underlying *C. rectus* pathogenesis through bacterial-host interaction assays to identify host responses related to two secretion systems (T3SS and T6SS). In particular the importance of the CiaB protein to host response, as well as the role of the Hcp protein in host cell adherence was investigated. Toward this goal, complete deletions of the *ciaB* and *hcp* genes have been generated in *C. rectus.* These mutant strains were used to complete the following specific aims:

Aim 1: Establish stable reference genes for RT-qPCR in Campylobacter rectus

One important aspect of studying the involvement of a gene in pathogenesis is the ability to measure the expression levels under a variety of conditions through the use of reverse transcription quantitative PCR (RT-qPCR). However, it is imperative to normalize gene expression to obtain accurate RT-qPCR results. In order to establish normalized expression, reference gene (such as housekeeping genes) are commonly used

to determine relative expression of a stably expressed target gene [71]. The stability of many reference genes used in bacterial gene expression studies have been validated prior to analysis, however many are used without verification [72]. Additionally, it is important to establish more than one reference gene that will reduce strain-to-strain expression variation. Once stable reference genes are established in a bacterium, RT-qPCR can be used as a helpful tool to provide insight into the many aspects of bacterial pathogenesis. Prior to the studies described in this thesis, no published studies had investigated global gene expression in *C. rectus*, meaning stable reference genes were not available for this emerging pathogen. Determining the best reference genes is helpful in understanding expression patterns and regulation of virulence-associated genes in *C. rectus.* Additionally, the validation of reference genes permitted the assessment of transcriptional polarity for mutated regions and assist with confirmation of mutant status.

Aim 2: Determine the role of CiaB in host cell immune expression

Previous research has shown bacterial-host interactions with *C. rectus* induce various host responses such as the *in vitro* secretion of pro-inflammatory cytokines [73, 74]. In *C. jejuni*, CiaB induces secretion of IL-1β and is responsible for the secretion of other Cia proteins, such as CiaD, which is found to stimulate the secretion of IL-8 [42, 43, 75]. In this project, we focused on the role of CiaB in host cell immune gene expression in response to interactions with *C. rectus* CiaB. Assessment of the *ciaB* function was based on measuring mRNA expression of human genes associated with Gram-negative bacterial infections using RT-qPCR.

Aim 3: Assess the role of CiaB in host cell viability

Pathogens induce host cell death as a mechanism to exploit host cells in order to establish an infection. Earlier studies have shown the loss of function of the CiaB protein in *C. jejuni* decreases apoptosis by 50 percent in differentiated human THP-1 cells (human monocyte, ATCC TIB-202) [75]. Previous research has found CiaB in *C. rectus* has a similar function to CiaB in *C. jejuni [38]*, therefore, it was anticipated that CiaB would cause a decrease host cell viability. Host cell viability was measured using a cell culture-based assay utilizing trypan blue [30, 31].

Aim 4: Evaluate the impact of the type VI secretion system (T6SS) Hcp protein in adherence efficiencies in Campylobacter rectus

Adherence to host cells is common among pathogenic bacteria. The T6SS in several related bacteria has been found to be critical to the adherence and invasion of host cells. In *C. jejuni*, a 50% reduction in both adherence and invasion to colon epithelial and macrophage cells was observed in T6SS mutants, including deletions in genes responsible for the Hcp secreted protein and a T6 structural protein [55]. However, the T6SS has been found in *H. hepaticus* to decrease host cell adherence and invasion [56]. It was hypothesized that T6SS is critical to the pathogenesis of *C. rectus*, in particular, host cell adherence rates of a *C. rectus* Δ*hcp* mutant was examined.

CHAPTER II

MATERIALS AND METHODS

Bacterial isolates and routine growth conditions

Two strains of *C. rectus* were used in the experiments in this thesis: 33238, a strain isolated from a periodontal pocket [57] and 314, a clinical strain isolated from a periodontitis patient [22]. Both strains were routinely grown on Mycoplasma formate fumarate liquid and agar media (MFF) (Mycoplasma Broth Base 20 g/L, 0.2% sodium formate, 0.2% ammonium fumarate) at 37°C in anaerobic conditions (5% CO_2, 10% H_2, 85% N_2) [4]. *C. rectus* mutant strains were routinely grown on MFF agar media containing 100 μg/mL spectinomycin.

Bacterial growth conditions to find reference genes

Prior to RNA extraction, *C. rectus* 33238 and 314 were inoculated into 5 mL of either MFF broth or tryptic soy broth containing formate fumarate (TSBFF) (tryptic soy broth 30 g/L, 0.2% sodium formate, 0.2% ammonium fumarate) and grown anaerobically for 8 hr at 37°C. Following the incubation period, these cultures were used to inoculate 50 mL of MFF or TSBFF, respectively, to an optical density at 600 nm (OD_{600}) of 0.02. Cultures were grown overnight at 37°C to an OD_{600} of 0.25. Once the desired OD was achieved, 3 mL was removed from each culture and centrifuged at 14,800 rpm for 1 min. The supernatant was removed and cultures were resuspended in 750 μl of RNALater (Thermo Fisher Scientific, Waltham, MA, USA). Four biological replicates were analyzed per media type.

Bacterial RNA isolation

After at least a 10 min incubation in RNALater (Thermo Fisher Scientific, Waltham, MA, USA), RNA was extracted using the RNeasy Mini Kit (Qiagen, Valenica, CA, USA) following the manufacturer's instructions (RNAprotect Bacteria Reagent Handbook) with the following modifications: samples suspended in RNALater (Thermo Fisher Scientific, Waltham, MA, USA) were centrifuged at 14,800 rpm for 1 min. The supernatant was removed and samples were resuspended in 200 µl of ice-cold lysis buffer (20 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 150 mM β-mercaptoethanol, 0.5% SDS, 150 µg/mL proteinase K). Samples were then incubated for 5 min on ice and then for 5 min at 65°C. Following incubation, steps 9-10 of protocol 4 from Qiagen's RNAprotect Bacteria reagent handbook (Enyzmatic Lysis and Proteinase K Digestion of Bacteria; Qiagen) were performed, followed by step 1 of the manufacturer's protocol 7 (Purification of Total RNA from Bacterial Lysate Using the RNeasy Mini Kit), including the optional treatment using RNase-Free DNase Set (Qiagen, Valenica, CA, USA) for on-column DNase digestion. To eliminate any DNA still present, another DNase digest was performed with the DNA-free DNA Removal Kit (Thermo Fisher Scientific, Waltham, MA, USA), adding 3 µL each of DNase and 10X DNase I Buffer to 24 µL of RNA from each sample, incubating for 90-min at 37°C. Post-incubation, 6 µL of DNase inactivation reagent was added to inactivate the DNase. Samples were incubated at room temperature with the inactivation reagent for 2 min before being centrifuged at 10,000 rpm for 90 sec. Supernatants were removed and stored at -20ºC.

Bacterial RNA quality and quantification

RNA samples were tested for any remaining DNA presence using PCR with primers for *glyA* and *rpoA* targets (Table 1). PCR was performed using the Qiagen Taq PCR Core kit (Qiagen, Valencia, CA, USA) with final concentrations of 1X coral load buffer, 0.2 mM dNTPs, 0.4 μM forward and reverse primers, and 0.625 units of Taq polymerase, using the following parameters on a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA): 94°C for 3 min, then 35 cycles of: 94°C 30 sec, 55°C 30 sec, 72°C 1 min, with a final elongation of 72°C for 5 min. Once RNA samples were determined to be DNA-free, samples were quantified using a Qubit Fluorometer 2.0 (ThermoFisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. RNA quality was visualized following manufacturer's protocol on the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA).

Quantitative reverse transcription PCR for bacterial reference genes

The following commonly used bacterial reference genes were validated in *C. rectus* 33238 and 314 using RT-qPCR: 16S ribosomal RNA (*16S*), DNA gyrase (*gyrA*), recombinase A (*recA*), serine hydroxymethyltransferase (*glyA*), RNA polymerase (*rpoA*), and RNA polymerase sigma factor (*rpoD*) [72]. RT-qPCR was performed on a Bio-Rad C1000 Touch Thermal Cycler with the CFX96 Real-Time System with the iTaq Universal SYBR Green One-Step Kit according to manufacturer's instructions for 10 μL reactions using 250 ng RNA from each sample. The thermocycler was set to the following parameters: 10 min at 50°C, 1 min at 95°C, followed by 40 cycles of 95°C 15

sec, 55°C 15 sec, 72°C 30 sec, with a 65-95°C melt curve analysis with 0.5°C increase and 5 sec per cycle. Reactions were performed with primers for the six different reference genes (Table 1) with no template controls included for each primer set. Additionally, no reverse transcriptase controls for all samples were included using the *glyA* and *rpoA* primer sets. Results were analyzed using BestKeeper and NormFinder programs [76, 77]. Statistical differences between quantification cycle (Cq) values in each media condition were calculated using a Student t-test using JMP 12 Pro software (SAS, Cary, NC, USA).

Bacterial mutant generation

A complete gene replacement of the *ciaB* gene was constructed in *C. rectus* 33238 as previously described by Blackburn, *et al.* [38]. A complete deletion of the *hcp* gene was generated in *C. rectus* 33238 by the DS Threadgill lab using the Gibson Assembly Kit (New England Biolabs, Ipswich, MA, USA) following manufacturer's instructions. Primers were designed for the genes immediately up and downstream of the *hcp* gene and the spectinomycin cassette was inserted into the pUC19 suicide plasmid digested with BamH1. All plasmids were sequence verified (Eton Biosciences, Research Triangle Park, NC, USA). The suicide plasmid was electroporated into *C. rectus* 33238 as previously described [38]. The *hcp* mutant was verified using PCR screens for the presence of *spec*^{r} and the presence of the *hcp* gene. PCR reactions contained 1X coral load buffer, 0.2 mM dNTPs, 0.4 μM of either *spec*^{r} forward and reverse primers or pre-hcp forward and post-*hcp* reverse primers (Table 1), 0.625 units of Taq polymerase, and nuclease-free water to a final volume of 25 μL. The following parameters were used on a

Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA): 94°C for 3 min, then 35 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 1 min, with a final elongation of 72°C for 5 min.

Mutant polar effects and gene expression

C. rectus wild-type (WT) 33238 and mutant bacterial RNA was extracted following the methods previously described [38]. RT-qPCR reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad C1000 Thermocycler; Bio-Rad) under the following parameters: 10 min at 50°C, 1 min at 95°C, followed by 40 cycles of 95°C 15 sec, 55°C 15 sec, 72°C 30 sec, with a 65-95°C melt curve analysis with 0.5°C increase and 5 sec per cycle. Assessment of transcriptional activity included genes located upstream and downstream of *ciaB* and *hcp,* as well as of the *ciaB* gene. Reactions were performed with primers for the nearest up (pyridine nucleotide-disulfide) and downstream acyl-CoA thioester hydrolase genes of *ciaB*, genes upstream (diadenosine tetraphosphate hydrolase) and downstream (Rhs element VgrG protein) of *hcp,* as well as primers designed for the *ciaB* gene (Table 1) with no template controls included for each primer set. Additionally, no reverse transcriptase controls for all samples were included using the *rpoA* primer set. Data were normalized using the three recommended *C. rectus* 33238 reference genes (*rpoA, gyrA,* and *recA*). Expression analysis was performed using the Bio-Rad's CFX Manager software and statistical analysis of significant differences between WT and mutant gene expression was calculated using a Student t-test in JMP 12 Pro software.

Growth curves

A 36 hr growth curve of *C. rectus* 33238 WT, Δ*hcp,* and Δ*ciaB* in MFF broth was conducted to analyze growth characteristics of each strain. *C. rectus* 33238 WT, Δ*ciaB*, and *vhcp* strains were inoculated into 5 mL of MFF broth and grown anaerobically overnight at 37°C. Following the incubation period, these cultures were used to inoculate 5 mL of MFF broth to an OD_{600} of 0.01. The OD_{600} was read every 4 hr for 36 hr, with the exception of readings at 16 and 20 hr. Differences in growth were analyzed using an ANOVA statistical test in JMP Pro 12 statistical software. Three biological replicates were analyzed per bacterial strain.

Identification tests

Identification tests were performed on *C. rectus* 33238 WT and Δ*ciaB* strains to ensure no biochemical or motility differences existed between the two strains. Tests were conducted as previously described [78]. Tests included alpha-hemolysis on Brucella blood agar plates (Anaerobe Systems, Morgan Hill, CA, USA), motility on MFF plates containing 0.4% agar [79], oxidase test, Lysine Iron Agar (LIA), Ornithine broth, and resistance to the antibiotic Cephalothin (Table 2) [80, 81].

Human cell culture

BeWo cells were generously provided by Dr. Sakhila K. Banu (Texas A&M University, College Station, TX, USA) and are derived from a human placenta choriocarcinoma (ATCC CCL-98). BeWo cells were routinely cultured in F-12K medium (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (VWR International, Radnor, PA,

USA) and Penicillin-Streptomycin (100 U/mL) (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2. Cells were split 1:4 every 48-72 hr. THP-1 cells, a human leukemic monocyte cell line (ATCC TIB-202), were routinely cultured in RPMI-1640 medium (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (VWR International, Radnor, PA, USA) and penicillin-streptomycin (100 U/mL) (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2.

Bacterial growth conditions for assays

C. rectus WT 33238, Δ*ciaB,* and Δ*hcp* strains were inoculated into 5 mL of MFF broth approximately 20 hr before all host response assays for pre-growth and incubated anaerobically for 8 hr. After pre-growth, 5 mL of MFF was inoculated to reach an OD_{600} of 0.01 and then diluted by half. Cultures were then incubated overnight to reach an OD_{600} of 0.15. Bacterial cultures were centrifuged for 2 min at 14,800 rpm and resuspended in F-12K or RPMI 1640 cell culture medium supplemented with 1% FBS.

Host response assays

Approximately 20 hr prior to host response assays, BeWo cells were washed with Dulbecco's Phosphate-Buffered Saline (DBPS) (Thermo Fisher Scientific, Waltham, MA, USA), detached from culture flasks with 0.5% Trypsin EDTA (Thermo Fisher Scientific, Waltham, MA, USA), and cell concentrations were determined using a Countess Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA) using manufacturer's instructions. BeWo cells were then seeded in 6-well cell culture plates (Greiner Bio-One, Monroe, NC, USA) at a density of $5x10^5$ cells in triplicate for each

variable. Cells were added in a 2-mL volume of F-12K media supplemented with 10% FBS and Penicillin-Streptomycin (100 U/mL) and incubated for 20 hr in 5% CO_2 at 37°C. Approximately 30 min prior to adding bacteria to cells, BeWo cells were washed again 3 times with DPBS in order to remove any remaining antibiotic and 2 mL F-12K containing 1% FBS was added to each well. *C. rectus* 33238 WT and mutant strains were added to each well to reach a multiplicity of infection (MOI) of 200. *E. coli* LPS (0111:B4 strain; Invivogen, San Diego, CA, USA) was added at a concentration of 2.5 μg per well as a positive control [73]. Three wells of uninfected cells were included as a negative control. Plates were centrifuged at 1000 rpm for 5 min for maximal bacteria-host contact and incubated anaerobically at 37°C for 6 hr, as previous research testing a variety of time points for maximal host cell invasion has indicated maximal *C. rectus* invasion occurs at 5 hr [38]. Additionally, Wang *et al.* [21] found that maximal host mRNA expression and cytokine secretion levels in response to *C. rectus* occurs after 6 hr of bacterial-host exposure. Following the 6 hr incubation period, the supernatant was removed from the wells, centrifuged for 5 min at 1500 rpm, removed from the pellet, and stored at -20°C for later use [73]. Cells were washed 3 times with DPBS, overlaid with 800 μl RNAlater (Thermo Fisher Scientific, Waltham, MA, USA), sealed with ParaFilm, and stored at 4ºC. Three biological replicates were conducted per variable.

BeWo RNA isolation

After at least a 48 hr incubation in RNALater (Thermo Fisher Scientific, Waltham, MA, USA), RNALater was gently removed from cells by pipetting and total eukaryotic RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA)

per the manufacturer's instructions (RNeasy Mini Kit Quick-Start Protocol) with the following modifications: after homogenization using the QiaShredder (Qiagen, Valencia, CA, USA), samples were incubated at room temperature for 5 min prior to the addition of fresh 70% ethanol. Samples were also incubated at 55ºC for 5 min after 40 µL RNase-free water was added to the column, prior to elution. Methods also included the optional on-column DNAse digestion treatment. All RNA samples were treated with one additional DNase digestion, following methods previously described above using the DNA-free DNA Removal Kit (Thermo Fisher Scientific, Waltham, MA, USA) [38]. To ensure no DNA was still present, PCR was performed with primers designed for the glyceraldehyde-3-phophate dehydrogenase (GAPDH) gene of BeWo cells (Table 1).

PCR was performed using the Qiagen Taq PCR Core kit (Qiagen, Valenica, CA, USA) with final concentrations of 1X coral load buffer, 0.2 mM dNTPs, 0.4 µM forward and reverse primers, and 0.625 units of Taq polymerase, using the following parameters on a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA): 94°C for 3 min, then 35 cycles of: 94°C 30 sec, 55°C 30 sec, 72°C 1 min, with a final elongation of 72°C for 5 min. RNA samples were quantified and quality was evaluated using a Take3 Micro-Volume plate with the Cytation 5 microplate spectrophotometer (BioTek, Winooski, VT, USA).

cDNA synthesis

One reaction of 15 µL of total RNA from each sample was converted to cDNA using the iScript Advance Reverse Transcriptase Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. cDNA synthesis was initiated by

reverse transcription for 20 m at 46ºC followed by RT inactivation for 1 m at 95ºC using a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). After conversion, cDNA samples were stored at -20ºC.

Quantitative reverse transcription PCR for human inflammatory gene expression

Expression profiling of a predesigned 96-well gene panel containing genes specific to human inflammatory response to Gram-negative infection (Bio-Rad, Hercules, CA) was performed using 22 ng cDNA per well. Each qPCR contained 1 µl diluted cDNA sample, 1x SsoAdvanced universal SYBR supermix (Bio-Rad, Hercules, CA, USA), and 1x PrimePCR assays for the designated target. Reactions were performed for three biological replicates per variable at 20 µL final volume per well. RT-qPCR was performed using a Bio-Rad C1000 Touch Thermal Cycler with the CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA), starting with activation at 95ºC for 2 min followed by 40 cycles of denaturation at 95 ºC for 5 sec and annealing/extension at 60 ºC for 30 sec, and ending with a melt curve in 0.5 ºC increments from 65-95ºC at 5 sec/step. For each sample, the level of a given qPCR product was normalized to the qPCR levels of three housekeeping genes (GAPDH, TBP, and HPRT1). Analysis of data was conducted using Bio-Rad CFX manager.

Viability assays

Approximately 20 hr prior to viability assays, THP-1 cells were centrifuged at 1,000 rpm for 5 min, resuspended in 50 mL DPBS, and centrifuged again at 1,000 rpm for 5 min. Following three washes, cells were resuspended in RPMI-1640 supplemented with 1% FBS and seeded in a 24-well tissue culture plate (Greiner Bio-One, Monroe,

NC, USA) at a density of $5x10^5$ cells per well and incubated at 37°C with 5% CO_2 [30]. Upon reaching an OD_{600} of 0.15, 1 mL of bacterial cells were harvested via centrifugation for 2 min at 14,800 rpm, resuspended in 1 mL RPMI-1640 with 1% FBS, and added to each respective well at an MOI of 100 [30]. Plates were incubated at 37°C in anaerobic conditions for 24, 36, 72, and 96 hr [21, 30, 38]. At each time point, viability of THP-1 cells was calculated using a Countess Cell Counter (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) per manufacturer's instructions. A positive control of 10 μg/mL of *E. coli* LPS (0111:B4 strain; Invivogen, San Diego, CA, USA) was included [82]. Uninfected cells were used as a negative control and each variable was conducted in biological triplicates. At every time point, cell viability for each well was calculated in duplicate. Significant differences in viability were analyzed using an ANOVA statistical test by JMP 12 software.

Adherence assays

BeWo cells were washed with DPBS, detached from culture flasks with 0.5% trypsin, and cell concentrations were determined with a Countess Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's protocol. Cells were then seeded in 24-well tissue culture plates (Greiner Bio-One, Monroe, NC, USA) with $1x10^5$ BeWo cells and incubated at 37°C with 5% CO_2 for 20 hr [38]. About 30 min prior to adding bacteria, BeWo cells were washed 3 times with DPBS and covered with 1 mL of F12-K medium containing 1% FBS. When bacterial cultures reached an OD_{600} of 0.15, BeWo cells were washed 3 times with DPBS to remove any remaining antibiotic presence before 250 μL fresh media was added to each well. Bacterial cells were added

at an MOI of 100 to each well after preparation as described above and plates were centrifuged at 1000 rpm for 5 min. Plates were incubated at 37°C in anaerobic conditions for 1 hr [21, 38]. After incubation, wells were washed three times with DPBS and 250 μL of 0.25% trypsin was added to each well for 5 min. Cells were collected into sterile microcentrifuge tubes containing cell medium with 1% FBS to inhibit the trypsin. Samples were serially diluted and plated on MFF agar in anaerobic conditions. Following 48-72 h incubation, CFUs were counted and the percent adherence rate was determined based on the total number of bacteria recovered divided by initial bacterial input.

CHAPTER III

RESULTS

Analysis of reference genes

All of the potential reference genes, except the 16S rRNA gene, were used for gene stability analysis as the 16S Cq values were too low for analysis. There was no significant difference in Cq values for each reference gene between samples grown in MFF and TSBFF with the exception of *C. rectus* 33238 *gyrA* Cq levels ($P<0.05$) (Figure 1). This gene was still included in analysis as the difference in Cq values was minor. According to the BestKeeper program analyses, there was not one shared reference gene that was the most stable for both strains of *C. rectus* (Table 3), however the top three most stable genes were the same for both strains. The order of the top three reference genes using the BestKeeper software for 33238 were *rpoA, recA,* and *gyrA*, while the order of the top three reference genes for 314 were *recA, rpoD,* and *gyrA* or *rpoA*. BestKeeper stability recommendations are based on pair-wise correlations between each sample's geometric mean, arithmetic mean, minimal and maximal values, standard deviations, and coefficient of variances [77]. Analysis using the NormFinder program found the top three stable reference genes for strain 33238 to be *recA, gyrA*, followed by *rpoA* (Table 4). The NormFinder program recommends *gyrA, recA,* and *glyA* as the best combination of reference genes for strain 314 (Table 4). NormFinder recommendations are based on each sample's coefficient of variation and signal intensity to find estimation of expression variation [76].

Mutant gene expression and polar effects

The *ciaB* gene was successfully deleted and potential polarity effects were tested. PCR and RT-qPCR analysis showed the lack of the *ciaB* gene and mRNA expression in the *ciaB* mutant (Figure 2). Moreover, no polarity effects were found in mRNA expression between WT and the *ciaB* mutant strains of up and downstream genes (Figure 2). PCR analysis confirmed the absence of the *hcp* gene in the *hcp* mutant (data not shown).

Growth curves

In order to determine whether wild-type and mutant strains of *C. rectus* exhibit different rates of growth, *C. rectus* 33238 WT, Δ*ciaB*, and Δ*hcp* strains were grown in MFF broth without antibiotics for 48 hr (Figure 3). Growth of *C. rectus* 33238 WT and both mutant strains significantly increased over 24 hr ($P<0.05$). All three strains reached their peak OD_{600} at 24 h (Figure3). Numbers of viable *C. rectus* 33238 WT and mutant strains decreased significantly after 32 hrs ($P<0.05$). The only significant difference in growth rates was seen at 12 hr, where Δ*ciaB* growth was significantly less than the other two strains ($P<0.01$).

Identification tests

To determine if any unexpected phenotypic differences (i.e., those not expected to result from deletion of *ciaB*) between *C. rectus* 33238 WT and Δ*ciaB* strains existed, several biochemical tests were performed. Both strains had similar amounts of motility in 0.4% agar, tested positive for alpha-hemolysis, and oxidase activity and negative for lysine-iron agar. No growth was visualized in the ornithine decarboyxylase test. Both

strains were susceptible to the antibiotic Cephalothin (Table 2). Results indicated no unexpected phenotypic differences between WT and Δ*ciaB* strains.

Host response assays

In order to assess the role of the *ciaB* gene in host cell response, *C. rectus* 33238 WT and Δ*ciaB* strains were exposed to BeWo cells for 6 hr. BeWo mRNA expression levels were assessed using the Bio-Rad predesigned assay specific for 88 genes common in human inflammatory response to Gram-negative bacteria. Overall, a trend in upregulation of BeWo inflammatory mRNA expression was seen in response to *C. rectus* 33238 compared to expression from uninfected BeWo cells, however the pro-inflammatory response to Δ*ciaB* was greater than the response to WT. Results show 41 genes were significantly upregulated in response to Δ*ciaB* (Table 5). Some of the most significant genes include IL-8, TGFBR2, and TNF (P<0.001), CASP7, CCR7, CD14, EGR1, IL-6, LGALS3BP, PTGS2, RAF1, TLR4, TNFRSF1A, TNFRSF1B, and TRIM 25 (P<0.01), and CXCL2, MGMT, and SOCS3 (P<0.05) (Figure 4). In comparison, 3 genes were significantly upregulated in response to WT, including PTGS2, TNF and TRIM25 (P<0.05). Additionally, 6 genes had a statistical trend in upregulation in response to WT: IL-8, CD14, MGMT, CCR7, CXCL2, and TNFRSF1B (P<0.1) (Figure 4). BeWo cell responses to the positive control, *E. coli* LPS, were upregulated for several genes, including CD14 (2.04±0.50) and IL-8 (37.94±22.35) (Table 5).

Viability assays

Viability of undifferentiated THP-1 monocytes was measured after exposure to *C. rectus* 33238 WT and Δ*ciaB* strains over a 96 hr time period. The effects of *C. rectus*

33238 WT and Δ*ciaB* strains on cell viability are seen in Figure 5. There is no significant difference between the viability of THP-1 monocytes when challenged with *C. rectus* 33238 WT or Δ*ciaB* strains. However, there was a significant decrease in the viability of the uninfected cells versus those challenged with WT or Δ*ciaB* at the 24 and 96 hr time points ($P<0.01$). Cells infected with 10 μg/mL *E. coli* LPS (0111:B4 strain) were significantly less viable than uninfected cells at every time point ($P<0.01$). Cell viability when infected with *E. coli* LPS-infected cells was significantly lower than uninfected cells at every time point ($P<0.01$). The viability of unchallenged cells only significantly decreased after 96 hr ($P<0.05$).

Adherence assays

The role of the T6SS Hcp protein was assessed in the ability of *C. rectus* to adhere to host cells. After a 1 hr incubation time with BeWo cells, the adherence efficiency of *C. rectus* Δ*hcp* was not significantly different than that of the *C. rectus* 33238 WT strain (Figure 6).

CHAPTER IV

DISCUSSION

This study was performed to further elucidate important virulence mechanisms of *Campylobacter rectus*. More specifically, the role of two proteins, CiaB and Hcp, in host cell interactions was investigated. Both proteins are associated with secretion systems and contribute to pathogenesis in closely related species [38, 42, 55, 56].

To date, little has been discovered about the molecular methods behind *C. rectus* pathogenesis. Earlier research has identified several virulence factors, such as lipopolysaccharide, a surface layer protein, cytotoxic activity, chemotaxis, and other effector proteins like *Campylobacter* invasion antigen proteins (Cia) [20, 25, 31, 37, 83]. Furthermore, *C. rectus* s-layer protein is crucial for host cell adherence and immune evasion while CiaB is important in the invasion of human placental cells [38]. This is important in the ability of *C. rectus* to colonize in locations other than in the oral cavity.

The goals of this study were to: a) establish stable reference genes for RT-qPCR in *C. rectus*, b) determine the role of CiaB in host cell immune expression and secretion, c) assess the role of CiaB in host cell viability, and d) evaluate the impact of Hcp in host cell adherence efficiencies.

Reference genes

RT-qPCR has become an important tool in molecular biology for measuring gene expression. In order to obtain accurate and reliable results, it is essential to normalize gene expression by using internal controls such as reference genes that are stably

expressed under a variety of conditions. In this study, six commonly used reference genes were tested. The 16S samples were excluded from the study because the Cq values were too low for analysis, likely due to the abundance of rRNA compared to mRNA present in the 16S total RNA samples. The sample could have been diluted, however doing so could introduce new potential errors [71]. Because the top most stable reference genes slightly varied between the BestKeeper and NormFinder, finding stable reference genes may not be as straight forward as picking the three most stable reference genes in one strain and applying it to all strains. Ideal reference genes have the possibility of varying from strain to strain due to variations between strains, meaning each strain used in RT-qPCR would need to have reference genes validated prior to expression analyses. Using at least *recA* and *gyrA* as reference genes in *C. rectus* RT-qPCR studies could be a reasonable alternative as these two genes were selected as the most stable in both *C. rectus* 3328 and 314 by two different statistical programs. Establishing stable reference genes for RT-qPCR for *C. rectus* was fundamental in this thesis in allowing a) the assessment of transcriptional polarity effects due to the deletion mutations, and b) the sampling of gene expression for mutant strains versus wild-type strains to help characterize mutant strains.

Mutant characterization

This research utilized the identified reference genes for *C. rectus* to assess transcriptional polarity effects caused by the *ciaB* and *hcp* mutations and to ensure the *ciaB* deletion mutant had no measurable *ciaB* mRNA expression (Figure 2). The investigation of transcriptional polarity effects and loss of expression for deleted genes is

an effective process to confirm observed phenotypic differences are likely due to the mutated gene alone. This is especially important as no molecular tools currently exist to complement mutations in *C. rectus*. No significant changes in up or downstream gene expression was observed in either of the mutant strains, implying the phenotypic characterization observed was due solely to the single gene deleted. Additionally, expression of the *ciaB* gene was absent in the *ciaB* deletion mutant, confirming, as expected, the loss of function of the *ciaB* gene. Additional experiments are necessary in order to verify the *hcp* gene is no longer being expressed in the *hcp* mutant strain.

Other methods utilized in this research to determine if the deletions of the *ciaB* and *hcp* genes caused any unexpected phenotypic differences from the WT included growth curves (Figure 3) and previously established biochemical tests commonly used for *Campylobacter* identification (Table 2) [81]. No significant changes in growth were observed between the WT and mutant strains, indicating the deletion of the *ciaB* and *hcp* genes had no unexpected effects on growth. Additionally, no difference was seen between the mutant and WT strains in any of the biochemical tests, further indicating the unique phenotypes seen for the mutant are likely due to the engineered mutation alone and not any other underlying effects such as rearrangements due to mutagenesis [78, 81].

Host response

In the present study, the host response of BeWo cells to *C. rectus* 33238 WT and *ciaB* mutant was measured, using RT-qPCR to assess mRNA expression of 88 genes commonly associated with human Gram-negative bacterial infections. Although *C. rectus* is an oral pathogen, initial studies on *C. rectus* host interactions include a variety

of human cells lines, likely because *C. rectus* has been isolated from other areas of the body such as the placenta, brain, and lungs [13, 21, 38, 73]. Human placental cells were chosen for this study as previous research has revealed *C. rectus* is capable of invading BeWo placental cells and also creates a higher risk of adverse pregnancy outcomes for the affected individual [38, 73].

Pro-inflammatory activity was observed in the BeWo cell line in response to *C. rectus* 33238 WT and Δ*ciaB*. However, an increased pro-inflammatory response was seen in response to the mutant strain (Figure 4), indicating CiaB may contribute to evasion of the host immune response. LPS is a potent activator of the host cell response involving the CD14 and mammalian Toll-like receptor (TLR) 4 system, which were both upregulated in the presence of *C. rectus* 33238 WT and Δ*ciaB*. This response to both strains was anticipated to stimulate CD14 and TLR4, as *C. rectus* produces LPS [16]. In placental cells, activation of the TLR4 signaling pathway stimulates the NF-κB signaling cascade [84]. Many of the genes seen upregulated in this study are involved in one or both of these pathways [85-87]. Genes in these pathways, including the ones upregulated in this study, are responsible for pro-inflammatory responses such as cytokine release, DNA repair, cell signaling, leukocyte recruitment and activation, and host apoptosis [85-88].

Of the 41 genes upregulated in response to *C. rectus* Δ*ciaB* or WT, most were involved in inflammation and cell proliferation. Because more pro-inflammatory genes were upregulated in the presence of Δ*ciaB,* it is suggested the presence of CiaB predominately suppresses the immune system rather than stimulates it. CD14 and TLR4

recognize the LPS of Gram-negative bacteria and activate the response system that induces pro-inflammatory genes such as TNF, IL-6, IL-8, and PTGS2 [89]. Other genes significantly upregulated are responsible for cell signaling, such as LGALS3BP, TNFRSF1A, TNFRSF1B, TGFB2, TRIM25, and SOCS3 [90]. Interestingly, TRIM25 plays a role in inducing cytokines in the RIG-I-like receptor signaling pathway, which is known for viral RNA recognition in the NF-κB pathway [91]. However, TRIM25 has also been found to regulate ubiquitination of the protein K63, which is involved in signal transduction pathways as well as trafficking of targeted proteins [91].

Further support of the increased inflammatory response triggered by Δ*ciaB* includes the increased mRNA expression from genes involved in recruitment and activation of leukocytes and T-cells, like CCR7, CXCL2, and CSF2 [90]. EGR1 mRNA expression was also upregulated in response to the *ciaB* mutant strain. EGR1 is involved in cell proliferation and is found to regulate pro-inflammatory gene expression in human placental cells [92, 93]. The upregulation o a cell proliferation involved gene in this study further supports the increased inflammatory response triggered by Δ*ciaB*.

Overall, the increase in pro-inflammatory expression when CiaB is not present suggests CiaB plays an anti-inflammatory role in *C. rectus* and aids the bacterium in the evasion of the immune system. Although the specific host response to CiaB has not been investigated in *C. jejuni*, results from this study are somewhat contradictory to what was anticipated. While there have been no other Cia proteins detected in *C. rectus,* CiaB in *C. jejuni* is responsible for the secretion of other Cia proteins, such as CiaD, which is found to be responsible for stimulating the secretion of IL-8 [43]. However, the CiaI

protein in *C. jejuni* enhances bacterial survival within human epithelial cells by preventing host lysosomes from eliminating internalized *C. jejuni* [40]. Because *C. rectus* CiaB appears to play a role in the invasion of host cells, it would be expected that inflammation would decrease because fewer bacteria were able to invade. Similar to *C. jejuni* WT and *ciaB* mutant strains [42], *C. rectus* WT and *ciaB* mutant host cell invasion rates are less than 5% [38]. CiaB could also possess functions other than host invasion, such as assisting in intracellular survival. Further studies are necessary to fully understand the mechanism by which CiaB impacts inflammation, such as examining mRNA expression of more genes associated with the innate immune system, measuring secreted cytokine levels, and assessing different time points throughout the bacterial-host interaction.

Viability

Functional monocyte and macrophage cells are essential components of the immune system and response to infection in numerous ways, such as inflammation and cell proliferation [94]. Previous research has found *C. rectus* 33238 to suppress proliferation of uninduced promyelocytic cells (HL-60) and is lethal to induced HL-60 cells [30]. The present study expanded upon prior knowledge and incorporated the THP-1 human monoyctic cell line, testing the effects of CiaB on cell viability. No significant change in viability was seen between *C. rectus* WT or Δ*ciaB,* implying CiaB does not have an effect on undifferentiated THP-1 viability. However, THP-1 cell viability of cells exposed to WT or Δ*ciaB* was significantly less than unchallenged cells at 24 hr and 96 hr, indicating the presence of *C. rectus* may have an impact. The viability of

uninfected cells was only significantly less after 96 hr, indicating THP-1 cells could survive in anaerobic conditions, however the assay may benefit from different conditions, such as exposing the cells to bacteria under anaerobic conditions for 5 hr, then transferring to normal cell culture conditions (37°C in 5% CO_2). This would provide enough time for the bacteria to potentially invade the THP-1 cells before being transferred to the optimal environment for the THP-1 cells. Additionally, further experiments are necessary to determine if CiaB has an impact on the viability of differentiated THP-1 cells, as differentiation induces THP-1 cells into a macrophage-like phenotype and thus enhances cellular responses [95].

Adherence

Secretion systems and effector proteins are important contributors to bacterial pathogenesis. In *C. rectus,* the CiaB protein is partly responsible for impacting host cell invasion [38], and was determined by this study to play a role in pro-inflammatory activity in the host. The research in this thesis also provides some preliminary insight into the involvement of the Hcp protein from the type VI secretion system in *C. rectus* pathogenesis, specifically focusing on adherence to host cells. This was done to establish whether a difference would be seen in adherence when the *hcp* gene was deleted, as earlier research has indicated the T6SS is involved in adhering to host cells [55, 56]. Although no difference was observed in adherence efficiencies between the WT and *hcp* mutant, adherence could be observed after longer cell exposure, or, Hcp could play an important role in host cell invasion. If future experiments elucidate no role in host

invasion, Hcp could potentially contribute to other methods of virulence such as biofilm formation or bacterial competition [96].

Research in *C. jejuni* involving T6SS found a significant decrease in both adherence and invasion of host cells when *hcp* is deleted [55]. Subsequently, in studies involving *H. hepaticus,* a significant change in host adherence was not observed between WT and an *hcp* mutant until after a 6 hr exposure [56]. In the present study, adherence was only observed after a 1 hr bacterial exposure. Previous studies involving *C. rectus* CiaB have shown the deletion of *ciaB* does not impact adherence efficiencies but reduces host cell invasion [38], indicating additional studies will be needed to determine whether there is a difference in invasion frequency of the *hcp* mutant.

CHAPTER V

CONCLUSIONS

This study has identified optimal reference genes for use in RT-qPCR analysis of the importance of CiaB and Hcp in *Campylobacter rectus* pathogenesis as assessed by the stimulation of a response from host cells for CiaB, and adherence (prelude to invasion) to host cells for Hcp. The ability of CiaB to stimulate a host response from human placental cells likely contributes to the virulence of *C. rectus* and its ability to cause adverse health effects especially during pregnancy. Based on results found in this research, future studies can more efficiently and thoroughly investigate mechanisms of pathogenesis in this bacterium. For example, CiaB is secreted via the flagellar type III secretion system in *Campylobacter jejuni* and requires a stimulatory signal for secretion [42, 53, 97], however, the secretion mechanism of CiaB has yet to be investigated in *C. rectus*. Additionally, the type VI secretion system (T6SS) and one of the secreted T6 proteins, Hcp, has been found in both the related species *Campylobacter jejuni* and *Helicobacter hepaticus* to play an important role in pathogenic events such as host cell adherence, invasion, and immune response stimulation or evasion [55, 56]. In *C. rectus*, the role of Hcp is still unclear, as host adherence levels were not affected in this research.

The work in this thesis has provided further insight into virulence mechanisms in *C. rectus* and opened avenues for future research involving the role of secretion systems and effector proteins in *C. rectus* pathogenesis. The studies described in this thesis are

foundational to future studies related to host response to *C. rectus* mutants as measured by gene expression. Future investigations should be directed toward: a) determining if CiaB is a secreted protein; b) measuring *ciaB* mRNA expression in response to various stimulants such as hormones and the presence of host cells; and c) assessing the role Hcp plays in *C. rectus* host cell invasion efficiency and host response stimulation.

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APPENDIX A

FIGURES

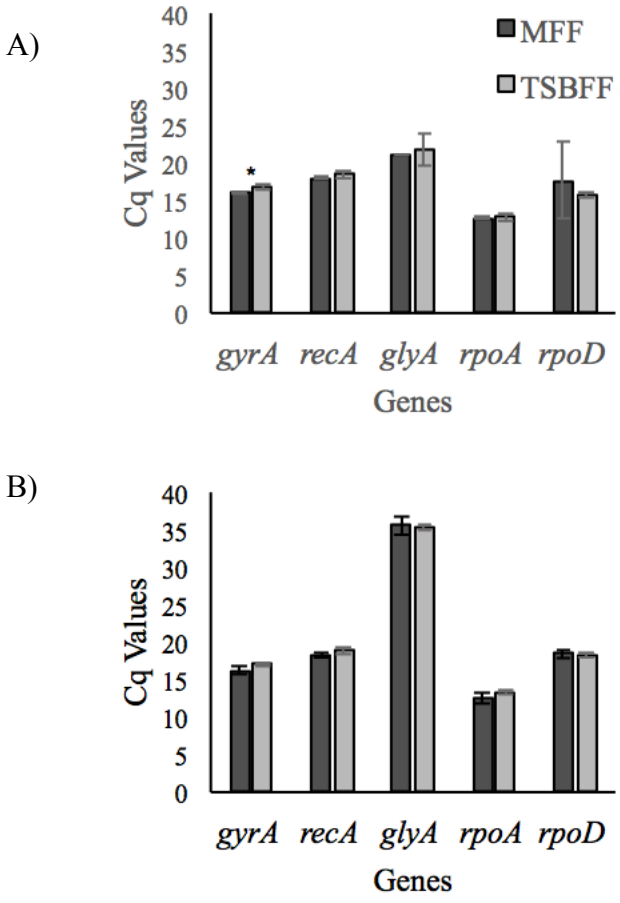
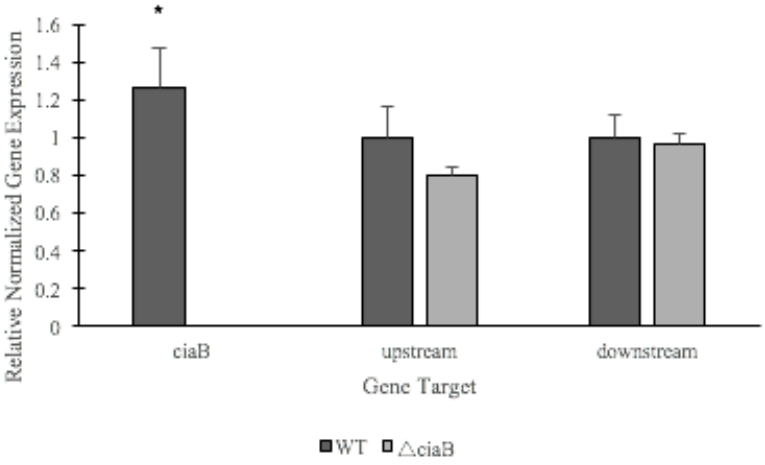


Figure 1. Observed Cq values of 5 reference genes from RT-qPCR. The Cq values of the potential reference genes in *C. rectus* strains 33238 (A) and 314 (B), were used in the determination of reference genes by BestKeeper and NormFinder. Dark gray bars indicate Cq values after growth in MFF broth and light gray bars indicate Cq values after growth in TSBFF broth. Statistical differences between culture conditions are denoted by a single asterisk ($P<0.05$). Error bars represent standard deviation.

A)



B)

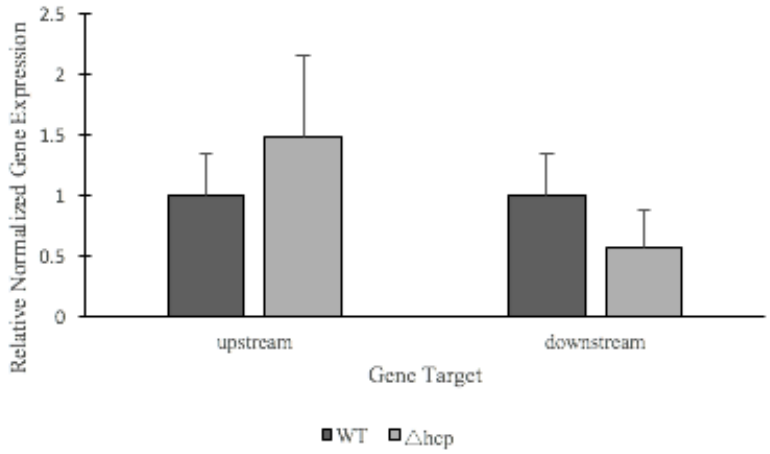


Figure 2. *C. rectus* gene mRNA expression. The expression levels of *C. rectus* 33238 wild-type and Δ*ciaB* (A) and Δ*hcp* (B). mRNA expression was normalized to the amount of reference genes including *recA, gyrA,* and *rpoA* RT-qPCR product from the same samples. Statistical differences between relative normalized gene expression are denoted by a triple asterisk ($P<0.001$). Error bars represent standard deviation.

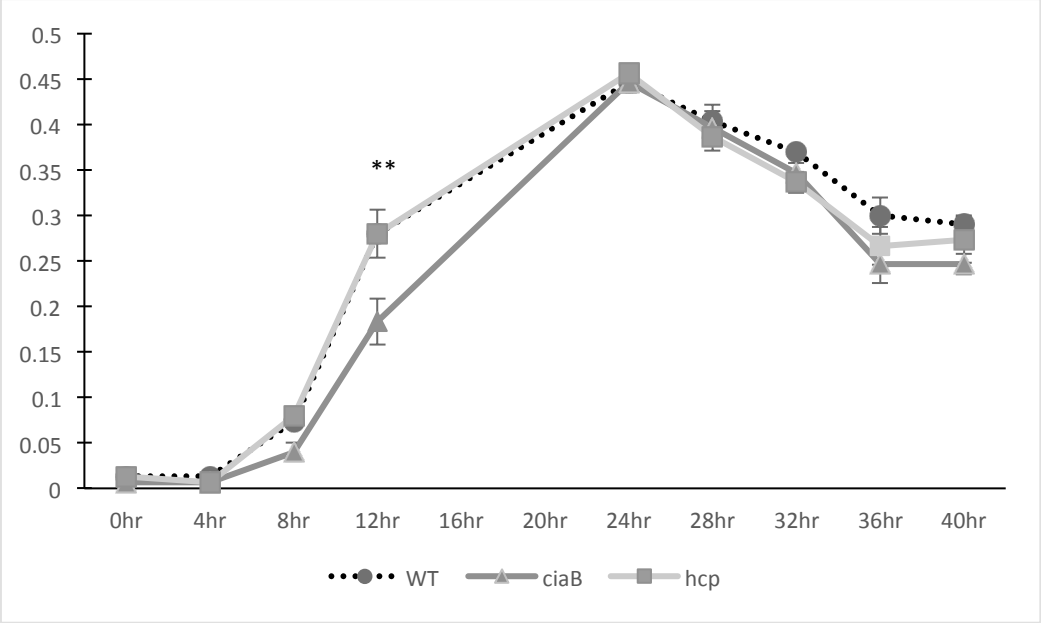


Figure 3. Growth curve of *C. rectus* 33238 wild-type, Δ*ciaB*, and Δ*hcp*. Spectrophotometric analysis (OD 600 nm) was recorded at 0, 4, 8, 12, 24, 28, 32, 36, and 40 hr. The growth of Δ*ciaB* was significantly less than that of WT of Δ*hcp* at 12 hr and is denoted by a double asterisk ($P<0.01$). Error bars represent standard deviation.

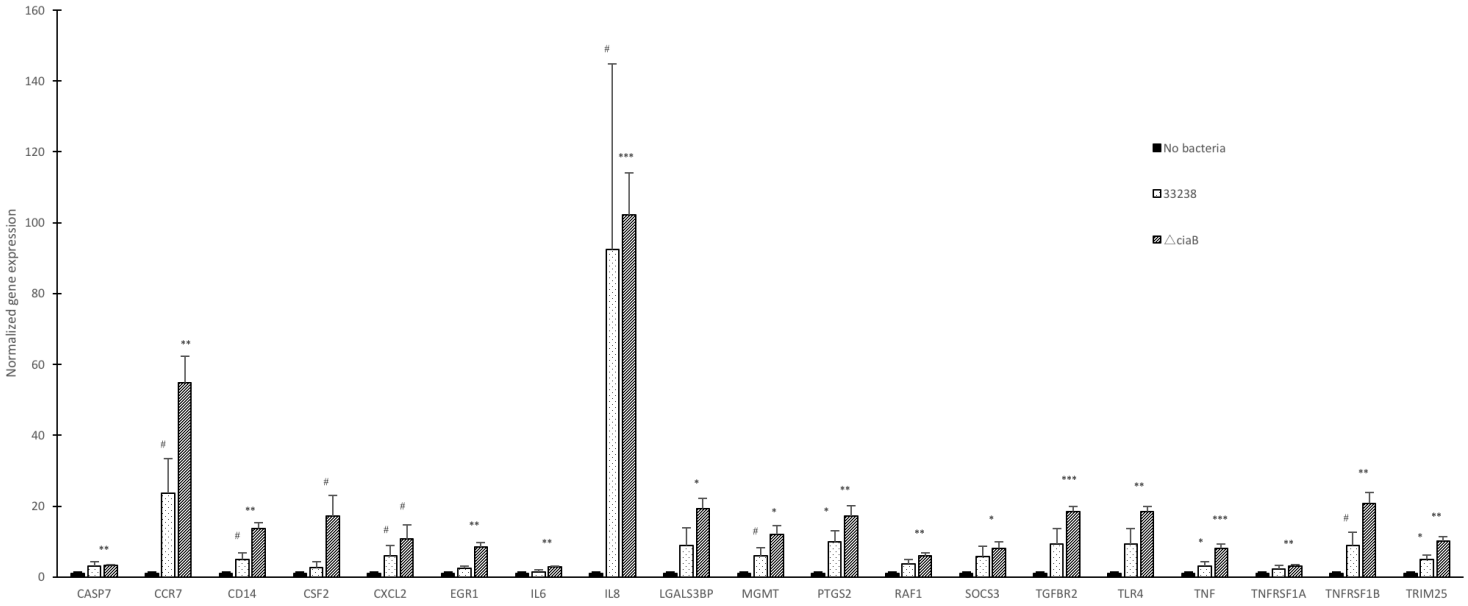


Figure 4. mRNA expression levels of BeWo cells when challenged with *C. rectus* 33238 wild-type or Δ*ciaB*. The mRNA expression levels of uninfected BeWo cells, or BeWo cells infected with *C. rectus* 33238 wild-type or Δ*ciaB*. mRNA expression was normalized to the amount of reference genes including GAPDH, HPRT1, and TBP RT-qPCR product from the same samples. Statistical differences between gene expression are denoted as follows: ***= $P<0.001$, ** = $P<0.01$, * = $P<0.05$, # = $P<0.1$. Error bars represent standard deviation.

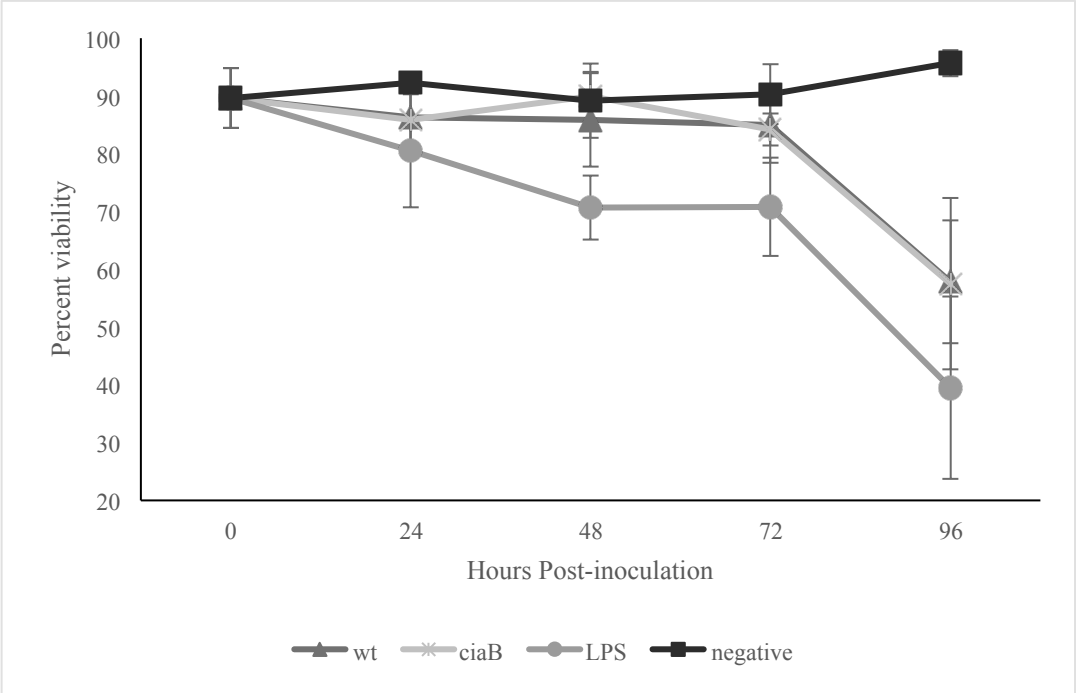


Figure 5. Viability assay of undifferentiated THP-1 monocytes. Viability of THP-1 monocytes was recorded at 0, 24, 48, 72, and 96 hr. As compared with the uninfected THP-1 cells, the viability of *C. rectus* WT infected cells was significantly different at 24 hr ($P<0.1$) and 96 hr ($P<0.01$); viability of Δ*ciaB* infected cells were significantly lower than uninfected cells at 24 hr ($P<0.05$), 72 hr ($P<0.1$) and 96 hr ($P<0.01$); viability of LPS infected cells was significantly decreased compared to uninfected THP-1 cells at 24 hr ($P<0.01$), 48 hr ($P<0.01$), 72 hr ($P<0.01$), and 96 hr ($P<0.01$). Error bars represent standard deviation.

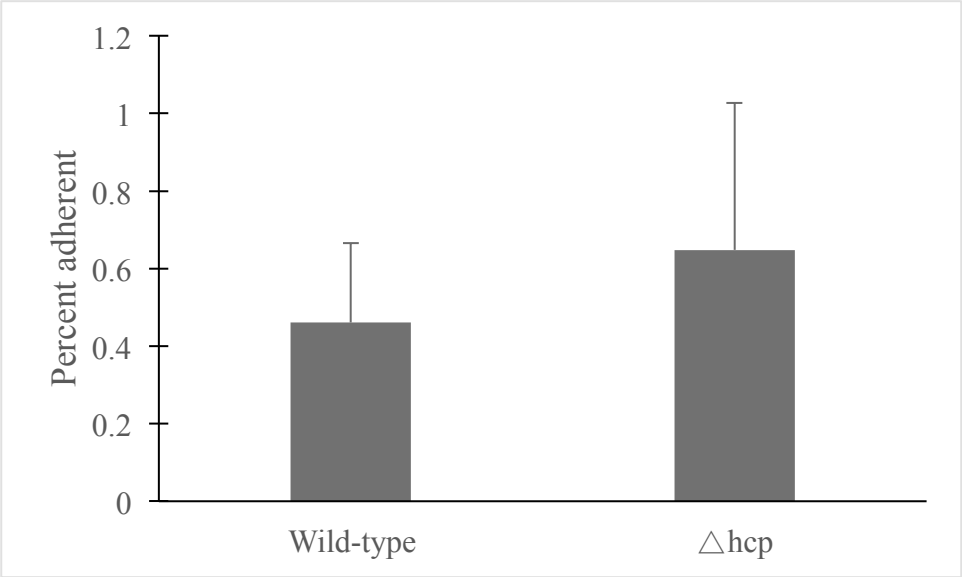


Figure 6. Adherence efficiency of *C. rectus* 33238 wild-type and Δ*hcp.* Adherence efficiencies to BeWo cells were calculated after a 1 hr exposure. Serial dilutions of input bacteria and output bacteria were plated and used to calculate adherence efficiencies, dividing total number of output bacteria recovered by initial bacterial input. Adherence efficiencies between WT and Δ*hcp* are not significantly different. Error bars represent standard deviation.

APPENDIX B

TABLES

Table 1. Primers used in this study.

Target Gene	Forward Primer	Reverse Primer
16S	CGGTACCCAAGGAATAAGCA	TCCTTTACGCCCAGTGATTC
gyrA	AAAAACGGCATCGTAAAACG	GCTCGTCGTTCTCGTCTAGG
recA	TCGGACTTGACTTGGCTCTT	GCGATGATATGAAGCGTGAG
glyA	AGCGCATATACGCGAGAGAT	TACGACTAGACCGGCGATGT
rpoA	CACGCGAAGTTGCTACAAAG	CATCCATCAAAGCAAGCTCA
rpoD	CCAGGATGGCAAAGTCAAAT	CCGATGTTGCCTTCTTGAAT
	GGGGACAACTTTGTATAGAAAA GTTGCGCTGGCGGCCGGCA TGAGTT	GGGGACTGCTTTTTTGTACAAACTTG GATGGCGATAGCGCGCGAGAC
spec^r	GGGGACAAGTTTGTACAAAAAA GCAGGCTGCCGTAACGTG ACTGGCAAGAG	GGGGACCACTTTGTACAAGAAAGCT GGGTGCAAAAGATTT TAGACTAATGGTTAA
post-ciaB	GGGGACAGCTTTCTTGTACAAA GTGGCGCGCCGGTTCTTTG GACGGCT	GGGGACAACTTTGTATAATAAAGTT GCCGCGCAAATTTCCC AAACCGC
ciaB	AGCTAAACGGGCTTTTCTCC	GCCTTGGCACTCTCGTAAAC
ciaB upstream	CAGCTTGTCGAGGTGGATAAA	CATACGAGGCACGAGATGTAAA
ciaB downstream	TTTAGAAGGGATGGGCGAAC	CTAGGTCGATCTGGCTCATTATC
pre-hcp	ATTCGAGCTCGGTACCCGGGTC TTCGTTTTCATTTACCCAATG	TCACGTTACGTTTTACTCCTTTGAAA AAAATATTCATTATTG
post-hcp	TTTAGAAAACGCTTATAAATTT AGGAGACTTGTTTTTTG	CCTGCAGGTCGACTCTAGAGTATCCC GTTATAAACGGATTC
hcp upstream	GAGATGAAGCTTGGAGGTGTT	AAGAGACGCCAATCAGGTATTT
hcp downstream	GACGTAATCGAAGGGAGCTATG	GTATGCTGAGAGTCGGTGTTT
GAPDH	AGTCAGCCGCATCTTCTTTTGC	CTCCTGGAAGATGGTGATGGGA

Table 2. Biochemical tests used in this study.

Test	Function	WT	*ciaB* mutant
Alpha-hemolysis	Detection of the enzyme production capable of breaking apart blood cells	+	+
Motility	To detect presence of bacterial flagella	+	+
Oxidase	To identify bacteria containing cytochrome oxidase enzyme	+	+
Lysine Iron Agar (LIA)	Detects bacterial capability to produce Lysine Decarboxylase (LDC), Lysine Deaminase (LDA) enzymes, and/or Hydrogen Sulfide	LDA – LDC +	LDA – LDC +
Ornithine Broth	Detect bacterial ability to decarboxylate ornithine uing the enzyme Ornithine Decarboxylase (OCD)	-	-
Cephalothin resistance	Detection of Campylobacter species	-	-

Table 3. BestKeeper analysis for *C. rectus* strains 33238 and 314.

Strain	Gene	GM [Cq]	(min, max) [Cq]	SD [± Cq]	(min, max) [x-fold]	SD [± x-fold]
33238	gyrA	16.41	15.88,17.10	0.37	-1.4,1.57	1.27
	recA	18.29	17.87,19.23	0.33	-1.28,1.77	1.24
	glyA	21.43	20.04,24.69	0.87	-2.13,6.34	1.76
	rpoA	12.76	12.37,13.36	0.23	-1.26,1.45	1.16
	rpoD	16.66	14.93,25.22	2.14	-2.11,82.27	3.99
314	gyrA	16.67	15.44,17.44	0.46	-2.2,1.67	1.35
	recA	18.60	17.86,19.31	0.41	-1.56,1.54	1.30
	glyA	35.38	34.14,37.09	0.59	-1.99,2.62	1.46
	rpoA	13.00	11.46,13.80	0.46	-2.53,1.65	1.35
	rpoD	18.37	17.77,19.11	0.42	-1.34,1.45	1.31

Table 4. NormFinder Analysis for *C. rectus* strains 33238 and 314.

	Stability Value	
Gene	**33238**	**314**
gyrA	0.008	0.016
recA	0.003	0.016
glyA	0.027	0.029
rpoA	0.012	0.032
rpoD	0.065	0.031

Table 5. Genes and normalized relative expression levels with significant changes in mRNA expression compared to uninfected BeWo cells.
(*** = $P<0.001$, ** = $P<0.01$, * = $P<0.05$, # = $P<0.1$)

Target	Function	WT	Δ*ciaB*	*E. coli* LPS
AC013461.1	Cell cycle, apoptosis	1.70 ± 0.63 *	1.79 ± 0.2 *	1.81 ± 0.15 *
AIF1	Apoptosis, anti-inflammatory	0.46 ± 0.23	0.54 ± 0.07 #	0.92 ± 0.09
APOC2	Lipid biding	1.71 ± 0.92	6.87 ± 1.61 *	2.29 ± 0.45 #
APOL3	Lipid binding	0.28 ± 0.16 #	0.77 ± 0.10	1.19 ± 0.29
BAX	Apoptosis	1.72 ± 0.64	1.87 ± 0.22 *	1.69 ± 0.09 *
CASP7	Apoptosis	3.13 ± 1.27	3.24 ± 0.24 **	2.58 ± 0.49 *
CCR7	Chemokine recognition	23.63 ± 9.85 *	54.74 ± 7.52 *	2.17 ± 0.12 ***
CD14	Surface antigen	5.01 ± 1.7 *	13.67 ± 0.17 **	2.04 ± 0.50
CD55	Immune cell activation	1.18 ± 0.45	2.31 ± 0.21 #	0.76 ± 0.04
CSF2	Immune cell activation	2.68 ± 1.69	17.15 ± 5.86 #	2.25 ± 0.22
CXCL2	Inflammation	6.01 ± 2.97 *	10.83 ± 2.84 #	4.80 ± 1.65 #
CYP1A1	Lipid synthesis	2.21 ± 0.90	7.61 ± 1.27 **	0.31 ± 0.02
EGR1	Cell proliferation	2.36 ± 0.79	8.54 ± 1.14 **	3.79 ± 0.53 *
EZH2	Cell differentiation	2.24 ± 0.84	2.70 ± 0.36 **	1.82 ± 0.16
FKBP5	Protein transport	2.11 ± 0.76	1.83 ± 0.40 #	1.83 ± 0.44
FSTL1	Inflammation	1.07 ± 0.65	4.04 ± 0.61 **	2.67 ± 0.21 *
GDF15	Cell differentiation	3.44 ± 1.47	7.34 ± 1.07 **	0.94 ± 0.06
HLA-DMA	Protein transport	2.67 ± 1.06	4.69 ± 0.94 *	2.16 ± 0.17 *
IL6	Cytokine	1.46 ± 0.52	2.83 ± 0.22 **	0.34 ± 0.05 *
IL8	Chemokine	92.54 ± 52.22 #	102.27 ± 11.85 ***	37.94 ± 22.34 #
IRS2	Cell signaling	1.45 ± 0.48	2.21 ± 0.29 *	1.38 ± 0.13
JUP	Protein binding	11.43 ± 5.83	15.47 ± 2.55 *	3.58 ± 0.20 *
LGALS3BP	Cell signaling	8.89 ± 4.95	19.30 ± 2.88 *	3.12 ± 0.42 *
MBP	Apoptosis	2.27 ± 0.99	3.70 ± 0.85 *	0.64 ± 0.11 #
MCM3	DNA replication	1.63 ± 0.60	1.77 ± 0.16 *	1.27 ± 0.15
MGMT	DNA repair	5.92 ± 2.37 #	12.01 ± 2.45 *	4.22 ± 0.41 **
MIF	Immune cell recruitment	2.30 ± 0.83	2.21 ± 0.24 **	2.12 ± 0.23 **
MUC1	Cell signaling	1.76 ± 0.69	3.78 ± 0.36 **	1.42 ± 0.08
OAS3	Protein synthesis	1.94 ± 0.90	2.94 ± 0.24 **	1.16 ± 0.09
PSMA5	Proteasome	0.50 ± 0.16 #	0.54 ± 0.05 *	0.54 ± 0.07 #

Table 5 continued

Target	Function	WT	Δ*ciaB*	*E. coli* LPS
PSMB8	Proteasome	1.98 ± 0.77	1.84 ± 0.15**	1.32 ± 0.10 *
PTGS2	Inflammation	9.85 ± 3.16 *	17.14 ± 3.02 **	0.66 ± 0.05
RAF1	Apoptosis	3.62 ± 1.39	6.01 ± 0.87 **	2.60 ± 0.19 *
SLC6A4	Cell proliferation	1.23 ± 0.55	2.40 ± 0.27 **	1.29 ± 0.09
SOCS3	Anti-inflammatory	5.78 ± 2.93	7.95 ± 1.90 *	2.24 ± 0.11 *
TGFBR1	Cell proliferation	32.55 ± 1.03	3.90 ± 0.39 **	1.54 ± 0.09
TGFBR2	Cell proliferation	9.31 ± 4.40	18.36 ± 1.61 ***	2.85 ± 0.26 **
TLR4	Inflammation	3.14 ± 1.18	8.04 ± 1.32 **	0.06 ± 0.00
TMSB10	Cytoskeleton organization	0.83 ± 0.27	0.74 ± 0.07 #	1.00 ± 0.08
TNF	Inflammation	18.54 ± 7.46 *	54.84 ± 4.09 ***	0.82 ± 0.61
TNFRSF1A	Cell signaling	2.30 ± 0.93	4.02 ± 0.39 **	1.73 ± 0.16 #
TNFRSF1B	Cell signaling	8.85 ± 3.81 #	20.71 ± 3.13 **	2.80 ± 0.54 *
TRIM25	Transcription factor	4.99 ± 1.36 *	10.12 ± 1.14 **	13.17 ± 2.74 *
UBE2L3	Protein binding	0.78 ± 0.24	0.69 ± 0.06 #	0.72 ± 0.10
VEGFA	Cell proliferation	1.31 ± 0.45	2.68 ± 0.34 **	0.89 ± 0.11
YWHAZ	Cell survival	1.93 ± 0.74	1.97 ± 0.25 *	1.36 ± 0.13