SIGNALS AND SENSORY MECHANISMS THAT IMPACT *CAMPYLOBACTER JEJUNI*-HOST INTERACTIONS

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DEDICATION

To my wife, Lauren.
For never letting me lose sight of my dreams.

SIGNALS AND SENSORY MECHANISMS THAT IMPACT *CAMPYLOBACTER JEJUNI*-HOST INTERACTIONS

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center

Dallas, Texas

August 2015

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The University of Texas Southwestern Medical Center, 2015

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Campylobacter jejuni is a leading cause of bacterial diarrheal disease worldwide and a frequent commensal organism of the intestinal tract of poultry and other agriculturally-important animals. Upon infection of the avian host, *C. jejuni* likely responds to external stimuli present within the intestinal tract to establish commensalism. The sensing mechanisms and subsequent physiological responses by *C. jejuni* can be crucial for initial growth and colonization and long-term persistence within the infected host. However, how many of the signals and sensing mechanisms affecting *C. jejuni* biology are not fully understood. In this work, I explored signal transduction mechanisms and possible *in vivo* signals that may influence the colonization capacity of *C. jejuni*.

One method *C. jejuni* employs to monitor environmental stimuli are through two-component regulatory systems (TCSs). I analyzed the potential of *C. jejuni Cjj81176_1484* (*Cjj1484*) and *Cjj81176_1483* (*Cjj1483*) to encode a cognate TCS that influences expression of genes possibly important for *C. jejuni* growth and colonization. Through transcriptome analysis, I discovered that the

regulons of the Cjj1484 histidine kinase and the Cjj1483 response regulator contain many common genes, which suggests these proteins likely form a cognate TCS. I found that this TCS generally functions to repress expression of specific proteins with roles in metabolism, iron/heme acquisition, and respiration. Furthermore, the TCS repressed expression of *Cjj81176_0438* and *Cjj81176_0439*, which had previously been found to encode a gluconate dehydrogenase complex required for commensal colonization of the chick intestinal tract. However, the TCS and other specific genes whose expression is repressed by the TCS were not required for colonization of chicks. I observed that the Cjj1483 response regulator binds target promoters both in unphosphorylated and phosphorylated forms and influences expression of some specific genes independently of the Cjj1484 histidine kinase. I propose that this TCS may sense signals found in the host intestinal tract, wherein repression of genes may be relieved.

In addition to characterizing the Cjj1484/Cjj1483 TCS, I explored the role of metabolites that are commonly found in the intestines – organic acids and short-chain fatty acids (SCFAs) – in *C. jejuni* commensal colonization. *C. jejuni* has both acetate and lactate utilization pathways, as well as a pathway for acetate production. I observed that acetogenesis mutants incapable of producing acetate were deficient for colonization of the avian intestinal tract early during infection, but not at later points during infection. Furthermore, I found that an acetogenesis mutant was impaired during growth in a defined media containing solely amino acids and organic acids as carbon sources. Transcriptome analysis of the acetogenesis mutant identified the SCFA-induced regulon which contains metabolically important genes, many of which have been implicated in *C. jejuni* colonization and virulence. In addition, I found that *peb1c*, which was downregulated in the acetogenesis mutant, was important for colonization of the chick ceca. I further confirmed *in vitro* that physiological concentrations of the SCFAs acetate and butyrate activated expression of the SCFA-induced regulon whereas the organic acid lactate repressed these genes. I found that *in vivo* expression of the SCFA-induced regulon was highest in regions of the intestinal tract where SCFAs are present in the greatest concentration. Furthermore, butyrate counteracted the inhibitory effects of lactate when the two compounds were combined in culture *in vitro*. I propose that *C. jejuni* senses the concentration of SCFAs and organic acids to discriminate between different regions of the

intestinal tract and to coordinate expression of colonization genes in the preferred niche for colonization. In effect, SCFA sensing and signaling allows *C. jejuni* to home to appropriate sites of the host for colonization and long-term persistence.

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PREVIOUS PUBLICATIONS

Luethy PM, Huynh S, Parker CT, Hendrixson DR. 2015. Analysis of the Activity and Regulon of the Two-component Regulatory System Composed by Cjj1484 and Cjj1483 of *Campylobacter jejuni.* J Bacteriol. 197(9):1592-1605.

Zhang Y, **Luethy PM**, Zhou R, Kroos L. 2013. Residues in Conserved Loops of Intramembrane Metalloprotease SpoIVFB Interact with Residues near the Cleavage Site in Pro-σ^K. J Bacteriol. 195(21):4936-4946.

Lee JS, Son B, Viswanathan P**, Luethy PM**, Kroos L. 2011. Combinatorial regulation of fmgD by MrpC2 and FruA during *Myxococcus xanthus* development. J Bacteriol. 193(7):1681-1689.

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LIST OF ABBREVIATIONS

6xHis	hexahistidine tag
A	alanine
Ac	acetate
Ac-CoA	acetyl-CoA
ackA	acetate kinase A
AcP	acetyl-phosphate
Acs	acetyl-CoA synthetase
AMP	antimicrobial peptide(s)
bp	base pairs
But	butyrate
C. jejuni	*Campylobacter jejuni*
Caco-2	human epithelial colorectal adenocarcinoma cells
CCV	*Campylobacter* containing vacuole
CDM	*Campylobacter* defined media
cDNA	complementary DNA
CDS	coding sequence
CDT	cytolethal distending toxin
cfu	colony forming units
Cjj0682	*Cjj81176_0682*
Cjj0683	*Cjj81176_0683*
Cjj1483	*Cjj81176_1483*
Cjj1484	*Cjj81176_1484*
D	aspartic acid
D58	aspartic acid residue 58

DCIP	2,6-dichlorophenolindophenol
DFO	deferoxamine mesylate
E	glutamic acid
E. coli	*Escherichia coli*
EHEC	enterohemorrhagic *Escherichia coli*
EMSA	electrophoretic mobility shift assay(s)
FLAG	FLAG octapeptide tag
Fur	ferric uptake regulator
g	gram
GBS	Guillain-Barré syndrome
GEO	Gene Expression Omnibus
GGT	γ-glutamyltranspeptidase
GST	glutathione S-transferase
h	hour(s)
H	histidine
H195	histidine residue 195
HK	sensor histidine kinase
Hsp90α	heat shock protein 90α
INT 407	human intestinal epithelial cells
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase
L. monocytogenes	*Listeria monocytogenes*
LB	Lysogeny broth
Lc/Lac	lactate
LEE	locus for enterocyte effacement

Li-AcP	lithium acetyl-phosphate
LOS	lipo-oligosaccharide
Lrp	leucine-responsive regulatory protein
M	molar
Mb	megabases
MBP	maltose binding protein
mg	milligrams
MH	Mueller-Hinton
min	minute(s)
mL	milliliters
mM	millimolar
MOMP	major outer membrane protein
mRNA	messenger RNA
N	asparagine
ncRNA	non-coding RNA
ng	nanograms
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometers
OD	optical density
P	phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PerR	peroxide stress regulator

pmoles	picomoles
PMS	phenazine methosulfate
Pta	phosphotransacetylase
qRT-PCR	semi-quantitative real-time polymerase chain reaction
RNAseq	RNA sequencing
rpm	rotations per minute
RpoA	RNA polymerase α subunit
RR	response regulator
RT-PCR	reverse transcriptase polymerase chain reaction
S. enterica	*Salmonella enterica*
SCFA	short-chain fatty acid(s)
SDS	sodium dodecyl sulfate
Sm^R	streptomycin resistant
TCA	tricarboxylic acid
TCS	two-component regulatory system
TEV	Tobacco Etch Virus
UTR	untranslated region
vec	vector
WT	wild-type
µg	microgram
µL	microliter
µM	micromolar

CHAPTER ONE

Literature Review

Introduction to *Campylobacter jejuni*

C. jejuni is a Gram negative, spiral-shaped bacterium that belongs to the ϵ-proteobacteria family, and was first isolated from human diarrheal stools (1). In the laboratory, *C. jejuni* grows optimally in microaerobic conditions (10 % CO_2, 5 % 0_2, 85 % N_2). Furthermore, *C. jejuni* is a moderate thermophile that grows well at temperatures between 37 and 42 °C but will not grow at temperatures below 33 °C. The genome of *C. jejuni* reveals that it has a relatively small genome size containing 1.6 Mb that code for an estimated 1,700 proteins (2). In nature, *C. jejuni* is a natural commensal organism of the intestinal tract of many animals and avian species, including chickens, and the bacterium typically resides in these hosts without causing disease. However, *C. jejuni* is also one of the leading causes of foodborne diarrheal disease in the United States and worldwide, making this pathogen an important organism to study and understand (3, 4).

Pathogenesis of *C. jejuni* disease

Transmission of *C. jejuni* to humans

Due to the high percentage of contaminated poultry meats available at commercial outlets, one of the highest risks for *C. jejuni* infection includes the handling and/or consumption of raw and undercooked poultry meats (5). Although consumption of contaminated poultry is the most common route of infection, infection due to the consumption of contaminated water has increased. *C. jejuni* may reach water sources through fecal contamination by wild and domestic animals or from sewage discharge (6-9). Outbreaks of *C. jejuni* disease due to contaminated water, in many cases, occur in individuals without access to a treated water supply (10-14).

The consumption of raw, unpasteurized milk has also led to an increase in *Campylobacter* diarrheal disease (15-17). Curiously, there have even been outbreaks in which proper pasteurization occurred, but the process was inadequate to kill *C. jejuni* (9, 18). The direct contamination of milk occurs via fecal contamination from cows and goats (19, 20). Thus, proper safety precautions must be applied to prevent contamination as much as possible during the milking process.

Symptoms of *C. jejuni* disease in humans

Infection of humans with *C. jejuni* can occur by consuming as little as 500-800 organisms from a contaminated source (21, 22). Following migration to the gastrointestinal tract, *C. jejuni* cells adhere to and invade the epithelial lining of the lower intestinal tract and colon (23). This interaction leads to inflammation, which may culminate in watery to bloody diarrhea following a typical incubation time of three to seven days (24). Most infections caused by *C. jejuni* are self-limiting, although abdominal pain may remain for several days. Interestingly, excretion of infectious bacteria in feces continues on average 38 days following recovery (25). Normal treatment includes fluid replacement therapy, but in severe cases, antibiotics can be prescribed for *C. jejuni* diarrheal disease.

Although most cases of *C. jejuni* infection resolve without incident, late-onset complications can occur. One such complication is a reactive arthritis of the hands and feet (26, 27). Incidence of *Campylobacter*-associated reactive arthritis is 1-5 % of those infected, and while the symptoms may last several weeks to months, full recovery is normal (28). The cause of *Campylobacter*-associated reactive arthritis is unknown, although it is thought to be related to the production and modification of lipo-oligosaccharide (LOS; (29, 30)).

A second, more serious sequelae of *C. jejuni* infections is Guillain-Barré syndrome (GBS), which is an acute paralysis of the peripheral nervous system. Patients with GBS are more likely to have had a recent *C. jejuni* infection, with antibodies to *C. jejuni* likely to be found 1-2 weeks following infection (31, 32). Interestingly, *C. jejuni* isolates from GBS patients express LOS which mimics human

gangliosides. It is thought that this molecular mimicry may lead to an antibody-mediated autoimmune response towards the peripheral nervous system that results in paralysis (33).

Virulence factors of *C. jejuni* involved in human disease

Following infection of the intestinal tract, *C. jejuni* requires many virulence factors to elicit disease in humans (34). These factors include chemotactic motility via a flagellum, which promotes migration that aids in colonizing the mucus layer of the colon. Once *C. jejuni* has colonized the mucus layer, the bacterium utilizes an extensive repertoire of proteins to adhere to and invade intestinal epithelial cells, where *C. jejuni* undergoes a transient intracellular lifestyle. *C. jejuni* can also secrete toxins, such as the cytolethal distending toxin (CDT), during infection. As described above, *C. jejuni* LOS mimics human gangliosides, and combined with a capsular polysaccharide and glycosylation of proteins, helps lead to immune evasion. Due to limited human volunteer studies, the mechanisms for many of these factors are not well understood. However, as described below, the necessity of these virulence factors for adherence and invasion of intestinal epithelial cells *in vitro*, as well as for colonization and virulence in the murine and ferret models, has been studied.

Flagellar motility and chemotaxis

C. jejuni is an amphitrichously flagellated bacterium that requires flagella for locomotion. Human challenge studies revealed that only motile strains from a mixed inoculum of motile and non-motile strains are recovered following diarrheal symptoms (22).

In addition, *C. jejuni* has a chemotaxis system that influences the direction of flagellar rotation to promote movement towards or away from specific signals in the environment. The system, which includes the classical CheAY two-component regulatory system (TCS), as well as *C. jejuni* specific chemoreceptors Tlp1-Tlp10, displays chemotactic motility towards amino acids found in high concentration in the intestinal tract, organic acids, and mucus (35-37). *C. jejuni* mutants lacking *cheA* and *cheY* are attenuated for colonization and virulence in mouse and ferret models of infection (38, 39). This

finding suggests that chemotaxis for energy-related purposes are an important mechanism for disease and colonization.

Adherence and invasion of epithelial cells

Before *C. jejuni* can invade intestinal epithelial cells of humans, it must first adhere to the cellular surfaces. *C. jejuni* appears to rely on non-pilus adhesions such as CadF, JlpA, CapA, FlpA, Cj1496c, and Peb1. These proteins have been shown *in vitro* to specifically bind fibronectin, Hsp90α, and other unknown factors to promote adhesion to intestinal epithelial cells (40-52).

Evidence for invasion of intestinal epithelial cells by *C. jejuni* has been found through the examination of intestinal biopsy samples from infected patients, as well as in many *in vitro* tissue culture experiments utilizing human intestinal epithelial cells (23, 53-56). Aside from proteins that affect motility, which has been shown in numerous studies to be required for invasion, identification of proteins that play a direct role in *C. jejuni* invasion has been somewhat elusive. Recently, CiaI and FedA, proteins that are coexpressed with flagellar proteins but not required for motility, were found to impact invasion of intestinal epithelial cells (57-59). A secreted protein, FlaC, which shares homology to the FlaA and FlaB flagellins, binds to epithelial cells and is also important for cell invasion (60).

Once invasion is stimulated, cell membrane pseudopods containing polymerized microtubules engulf *C. jejuni*, and the *Campylobacter* containing vacuole (CCV) moves to the perinuclear region of the cell near the Golgi (61-64). Internalization has been shown to significantly alter *C. jejuni* physiology, including a complete reprograming of metabolic processes such as favoring fumarate respiration and the induction of the oxidative stress response (65, 66).These physiological and metabolic changes may assist *C. jejuni* in coping with the harsh environment of the CCV while the bacterium engages in a transient intracellular lifestyle.

Cytolethal distending toxin

CDT is encoded by the genes *cdtABC* and is a tripartite toxin similar to that of other bacterial species (67). Following secretion out of *C. jejuni* via outer membrane vesicles, CdtA and CdtC bind to eukaryotic cells and deliver the intracellular cytotoxic component, CdtB, which may have DNase activity

(68-71). Following localization to the nucleus, CdtB can cause cell cycle arrest at the G2/M phase, progressive cellular distension, chromatin fragmentation, and apoptotic cell death (67, 71-73). CDT is required for persistent colonization in the mouse model of infection, but not for commensal colonization of chickens, perhaps indicating a role in virulence and diarrheal disease (74-76).

Lipo-oligosaccharide and capsule

C. jejuni produces a LOS that varies greatly in its modification of the outer core between strains (77, 78). This high variability exhibited by LOS is due to modifications with different types of sialic acid structures that resemble ganglioside on human neurons. These modifications promote immune evasion against an antibody-mediated response and likely contribute to autoimmunity that leads to GBS (34). Recent evidence suggests that modification of LOS and other surface structures with phosphoethanolamine by the EptC enzyme is required for colonization during the mouse model of infection (79).

The capsule of *C. jejuni* is made of highly variable polysaccharide units which are components of the antigen used for strain serotyping (80). Variability in capsule structure in *C. jejuni* is due to phase variation of the structural genes and the ability of the organism to modify the capsule with *O*-methyl phosphoramadate (80-83). Capsule plays an important role in *C. jejuni* biology and pathogenesis. Without genes involved in capsule formation and *O*-methyl phosphoramadate modification, the bacterium becomes sensitive to serum and is attenuated for virulence in the ferret model of diarrheal disease and the mouse model of colonization (84, 85). Furthermore, capsule was found to be required for adhesion and invasion of INT 407 intestinal epithelial cells *in vitro* (84, 86).

O-linked and N-linked glycosylation of proteins

C. jejuni glycosylates serine and threonine resides on flagella through *O*-linked glycosylation (34, 87). Many *C. jejuni* strains encode pathways for *O*-linked glycosylation with legionaminic acid, although the 81-176 strain does not contain this pathway. This strain, which is the focus of our research program, contains pathways for the glycosylation of flagella with pseudoaminic acid (2, 88). *O*-linked glycosylation of flagellin is required for proper formation of the flagellar filament (89). Cells which

produce flagella but are defective for *O*-linked glycosylation are non-motile, defective for adherence to and invasion of INT 407 cells *in vitro*, and have decreased virulence in the ferret model of diarrheal disease (90). Recent work has found that the major outer membrane protein (MOMP) was found to be *O*-glycosylated at a threonine residue, which in turn promotes adhesion to intestinal epithelial cells *in vitro* (91).

Glycosylation of asparagine residues on many different proteins occurs due to a process called *N*-linked glycosylation, which was first discovered in *C. jejuni* (34, 92). Performed by the Pgl proteins, *C. jejuni* mutants for *N*-linked glycosylation were shown to be deficient in attachment and invasion of INT 407 and Caco-2 cells *in vitro* (93, 94). Furthermore, *C. jejuni N*-linked glycosylation mutants showed significant decreases in colonization in the mouse model of disease (93, 94).

C. jejuni commensal colonization in avian species

Colonization of chickens can begin as early one week after hatch and may persist as long as 42 weeks (95, 96). Upon positive detection of *C. jejuni* in a flock, bird-to-bird transmission occurs rapidly, resulting in near 100 % colonization of birds within the flock (97-99). Following infection, *C. jejuni* colonizes the avian intestinal tract, residing predominantly in the mucus layer of the ceca and large intestine (100). In these regions, *C. jejuni* can readily achieve colonization levels exceeding 10^7 colony forming units (cfu) and up to ~10^{10} cfu per gram of content (101). *C. jejuni* also colonizes the upper intestinal tract, although at levels much less than that of the ceca and large intestine (101). While the colonization of birds has been thought to be harmless, new evidence suggests that some modern breeds of chickens may actually suffer inflammatory and diarrheal disease upon infection (102).

Contamination of poultry meats occurs upon slaughter of the bird. (103-105). Following processing, *C. jejuni* on poultry meats may range from 10^2 to 10^6 cfu per carcass or 10^1 to 10^4 cfu per 100g of meat (103, 106-108). Indeed, contamination levels of commercially available poultry products continue to be a common source of infection, as a recent study has found that 43 % of tested commercially available poultry meat products were contaminated with *Campylobacter* (109).

Factors required for colonization of the avian host

Although animal models such as the mouse and ferret have been used to study *C. jejuni* virulence, these models lack consistent human disease phenotypes (34). In contrast, the commensal colonization of chickens by *C. jejuni* is an attractive model due to naturally high colonization levels of *C. jejuni* in the chicken intestinal tract. Furthermore, understanding the specific factors required to promote colonization of the avian intestinal tract is important for developing novel methods for limiting *C. jejuni* in agriculture and exposure of humans to contaminated meats (34).

Flagellar motility and chemotaxis

Signature-tagged transposon mutagenesis found flagellar motility and chemotaxis to be important factors required for commensal colonization of the chick (101). Indeed, without flagella, *C. jejuni* can still colonize the gastrointestinal tract of chicks, albeit at greatly reduced numbers compared to motile strains (110-113). Mutants lacking the chemoreceptors *docB* (*tlp10*) and *docC* (*tlp4*) are reduced for commensal colonization of chicks (114). Furthermore, *C. jejuni* mutants lacking *cheA* and *cheY*, which can no longer translate chemotactic signals to flagellar motility, are deficient for commensal colonization of the chick ceca (101).

LOS and capsule

As described above, the modification of LOS contributes to immune evasion and autoimmune disease in the human host (34). Recent evidence suggests that modification of LOS and other surface structures with phosphoethanolamine by the EptC enzyme is required for commensal colonization of the chick (79). Furthermore, a mutant in capsule polysaccharide synthesis was found to be required for colonization of the chicken (115). Recent evidence also suggests that capsular heptose modification is required for colonization and persistence in the chick intestinal tract (116).

Adherence and invasion

Although the adhesins CadF, CapA, FlpA, Cj1496c, and Peb1 are thought to mostly play roles in attachment and invasion of human intestinal epithelial cells, they have also been shown to be required for full levels of colonization in the chicken intestinal tract (43, 46, 47, 49). The proteins CiaI and FedA were

found to impact not only invasion of human intestinal epithelial cells, but also commensal colonization of the chicken (57-59). Interestingly, CiaI secretion is required for commensal colonization of the avian host, but not for invasion of human intestinal epithelial cells *in vitro* (59).

Glycosylation

As *O*-linked glycosylation primarily occurs on flagellin, and is required for formation of the flagellar filament, mutants lacking *O*-linked glycosylation are most likely deficient in colonization of the chick intestinal tract due to a lack of flagellar motility (87, 89). However, flagella are not the only proteins found to be *O*-glycosylated. *O*-glycosylation also occurs on a threonine residue of MOMP which in turn promotes commensal colonization of the chick gut (91). The other form of glycosylation in *C. jejuni*, *N*-linked glycosylation, has also been shown to be important for commensal colonization. Mutants in the *N*-glycosylase pathway showed significant decreases for commensal colonization in chickens (94, 101, 117). One identified protein that is *N*-glycosylated is Cj1496c, which is important for invasion of human intestinal epithelial cells during virulence and for colonization of the chick ceca (49). However, the primary function of the *N*-glycosylation pathway is to produce free oligosaccharides in response to osmotic changes and bacterial growth (118). The importance of free oligosaccharide in virulence and colonization is yet to be understood.

Acquisition of nutrients and metabolites by *C. jejuni*

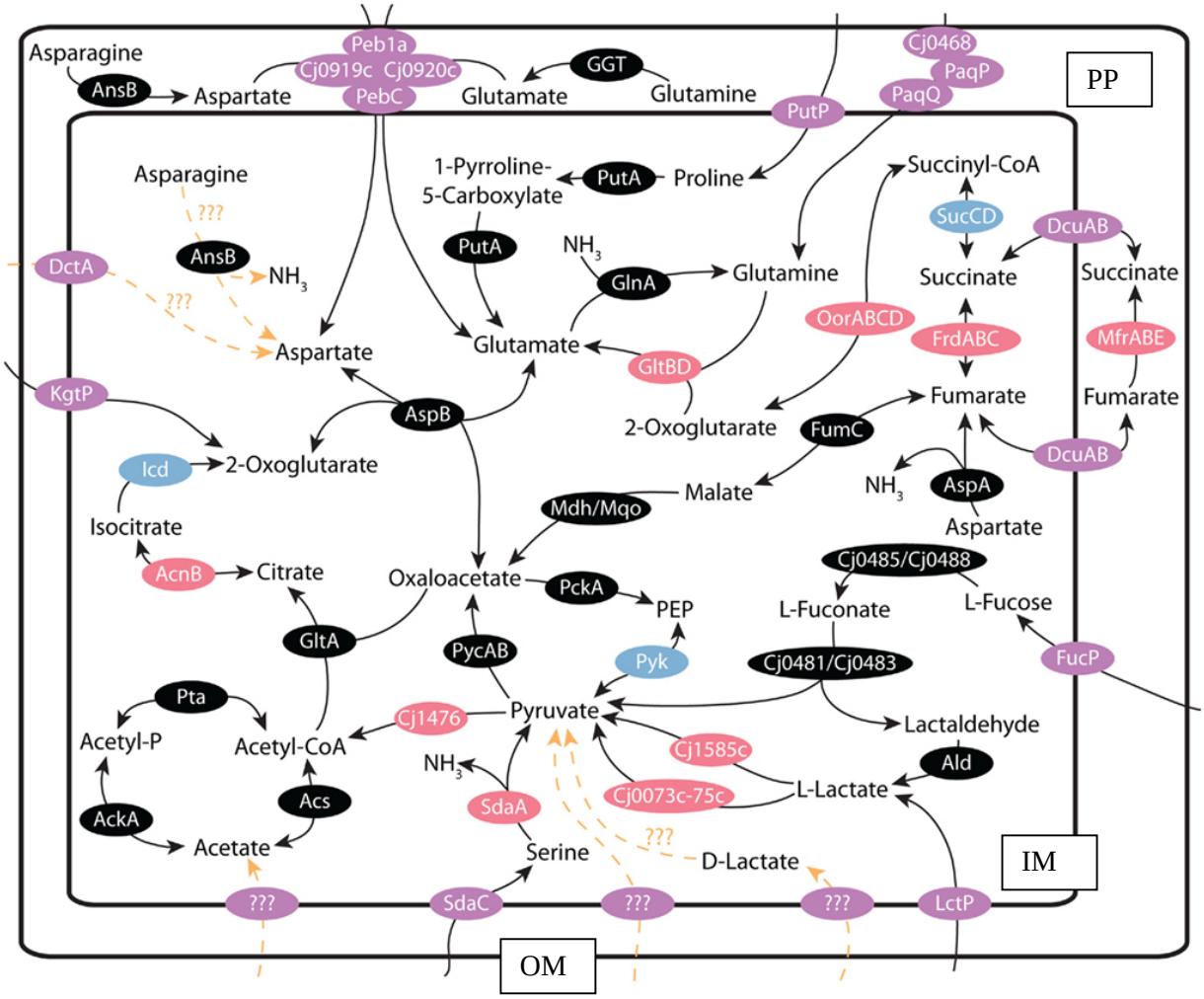
Transcriptional profiling of *C. jejuni* strains during colonization of the chick ceca found genes that were differentially expressed *in vivo* compared to expression *in vitro* (119). Furthermore, transcriptome profiling by RNA sequencing (RNAseq) was also utilized to identify transcripts expressed *in vivo* but not *in vitro* (120). These and other studies revealed the importance of metabolism in the commensal lifestyle of *C. jejuni* (101). Specifically, transcripts for the acquisition and metabolism of amino and organic acids as well as factors involved in the tricarboxylic acid (TCA) cycle and electron transport were found to be increased during colonization (119). Many of these metabolic pathways require iron to function, although excess iron can lead to oxygen-related damage. Not surprisingly,

transcripts for iron and heme acquisition, as well as the oxidative stress response, were found to be increased during colonization (119, 120).

Amino and organic acid acquisition and metabolism

Although *C. jejuni* effectively colonizes both avian and human hosts, the organism remains fastidious compared to other organisms in regards to nutritional requirements. Annotation of the *C. jejuni* genome confirmed the early observations indicating that the bacterium could not utilize glucose and other carbohydrates for metabolism (2). Further metabolic studies confirmed that *C. jejuni* could not utilize glucose and also could not utilize fructose, galactose, rhamnose, lactose, maltose, trehalose, and sucrose for respiration (121, 122). Although the genome lacks genes encoding glycolytic enzymes such as glucokinase and 6-phosphofructokinase, a complete gluconeogenesis pathway is encoded (2). This pathway, which is predicted to result in the synthesis of glucose from phosphoenolpyruvate (PEP), has not been studied *in vitro*. However, work analyzing an intermediary reaction which fuels gluconeogenesis has been performed. In contrast to other bacteria, *C. jejuni* lacks a PEP carboxylase and synthase, prohibiting the direct conversion of PEP to oxaloacetate (123). To counteract this deficiency, *C. jejuni* encodes enzymes that result in cyclic generation of pyruvate-to-oxaloacetate-to-PEP-to-pyruvate (Figure 1; (123)). Finally, to further complicate its carbohydrate diet, *C. jejuni* encodes the genes necessary for the non-oxidative portion of the pentose phosphate pathway as well as a glycerol-3-phosphate transporter (121, 123, 124). However, the genome contains absolutely no enzymes required for the oxidative portion of the pentose phosphate pathway, prohibiting the generation of pentose sugars.

To efficiently grow and synthesize carbohydrates, lipids, and proteins, *C. jejuni* acquires and catabolizes other carbon sources, specifically amino acids (Figure 1). Many *in vitro* growth assays have shown that there are five amino acids that promote growth in liquid culture in the following preferred order: serine, aspartate, asparagine, glutamate, and proline (125, 126). Furthermore, serine, aspartate, proline, and glutamate are the most prevalent amino acids found in the excreta of chickens (127).



Adapted from Stahl, M et al. (2012; (128))

Figure 1. Nutrient acquisition and metabolism in *C. jejuni*. Detailed in this diagram are the transporters of nutrients and the subsequent metabolic reactions that occur within *C. jejuni* to generate metabolites required for central metabolism. These pathways include transport and catabolism of amino acids as well as organic acids. Enzyme and enzyme complexes that contain iron-sulfur complexes (pink), enzymes containing magnesium (blue), transport complexes (purple), and hypothetical reactions (orange dashed lines) are shown.

Serine, the most preferred amino acid of *C. jejuni*, is first imported into the cell by active transport through the sole serine transport protein in *C. jejuni*, SdaC (129). Following uptake, serine is deaminated and converted to pyruvate by the SdaA serine dehydratase (Figure 1; (129)). This process of serine catabolism was shown to be absolutely necessary for chick and murine colonization (129, 130).

Aspartate transport in *C. jejuni* is primarily performed by the Peb1 ABC transport system, which contains two permeases, an ATP-binding protein, and one periplasmic binding protein (Figure 1; (131)). A mutation in the binding protein of the Peb1 system revealed that transport and/or adhesion mediated by Peb1 is required for commensal colonization of chicks (47). Although the majority of L-aspartate is transported by the Peb1 system, a C4-dicarboxylate transport system consisting of DctA, DcuA, and DcuB was also shown to allow for minor transport of the amino acid (Figure 1; (125, 131)). Once inside *C. jejuni*, aspartate can enter many pathways. Deamination by AspA results in production of fumarate, which can immediately feed into the TCA cycle (Figure 1; (125)). Aspartate can also be used to fuel the production of many other amino acids, including lysine, methionine, threonine, and isoleucine.

Although most *C. jejuni* strains encode a cytoplasmic form of the AnsB asparaginase, *C. jejuni* 81-176 (the strain studied by our research program) encodes a variant that can be secreted to the periplasm via a Sec-dependent secretion signal (Figure 1; (132)). Nevertheless, these strains do not contain a putative asparagine transporter. Thus, it is hypothesized that the cytoplasmic form of AnsB deaminates asparagine originating from peptides while the periplasmic form deaminates free asparagine from the environment (128). Deamination of asparagine in the periplasm results in aspartate, which can then be transported into the cytoplasm through the Peb1 system, as described above (Figure 1).

Glutamate is transported into *C. jejuni* by the Peb1 transport system. Glutamate can then be converted to aspartate or glutamine (Figure 1; (125, 131)). Formation of aspartate is achieved through transamination of glutamate by AspB, which also requires oxaloacetate as a substrate. This reaction not only generates aspartate but also α-ketoglutarate as well (Figure 1; (125)). Glutamate, on the other hand, can also be synthesized by the GltBD glutamate synthase using glutamine and α-ketoglutarate as substrates (133). Glutamine metabolism in *C. jejuni* was initially discovered during ^{14}C-labeled

respiration experiments, and a glutamine transporter consisting of the Paq system allowed for uptake (Figure 1; (134, 135)). Interestingly, despite containing this transport system, only a few strains (including 81-176) could grow using glutamine as a sole carbon source (132). These strains possess *ggt*, which encodes a secreted form of γ-glutamyltranspeptidase (GGT) capable of hydrolyzing glutamine to glutamate and ammonia (Figure 1; (132, 136)). Interestingly, the presence of *ggt* appears to increase the fitness of *C. jejuni* for persistent colonization of the avian host and colonization in MyD88$^{-/-}$ deficient mice (132, 137). GGT-activity also provides for resistance to isothiocyanantes (138).

Of the five preferred amino acids, proline is least preferred, but it is still an important nutrient source (126). Transport of proline into the cell is made possible by the products of *putA* and *putP* (130). The PutP transporter is a sodium/proline symporter, and is highly conserved in *C. jejuni* strains. Once L-proline is transported into the cell by PutP, PutA, a predicted dual-function proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase, converts proline to glutamate (Figure 1; (130)). The transport of proline into the bacterium has been found to be required for efficient colonization of the murine intestine (130).

Outside of these five amino acids, no others were found to promote growth in liquid culture when supplemented alone (125). However, *C. jejuni* still produces transport systems to acquire other amino acids. This includes the LIV system, which is an ABC transporter system for leucine, isoleucine, and valine (139). In addition to this system, there are other putative amino acid transport systems for alanine, threonine, cysteine, and histidine, although their function remains to be experimentally confirmed (2, 140, 141). Whereas direct investigation of *de novo* biosynthesis of amino acids has only been documented for leucine and cysteine in some *C. jejuni* strains, auxotrophies for methionine, proline, valine, isoleucine, and leucine have been found in other isolates (139, 142-145). Furthermore, in contrast to strains containing GGT, which have the ability to utilize glutathione as a carbon source, other enzymes with the ability to utilize peptides to promote growth in *C. jejuni* remain to be discovered (132).

In addition to the production of organic acids from amino acid catabolism, *C. jejuni* can also utilize these and other TCA cycle intermediates directly from the milieu for its carbon metabolism. α-

ketoglutarate is predicted to be transported into *C. jejuni* by the KgtP permease and may be used directly in the TCA cycle or in glutamate and glutamine metabolism (Figure 1; (2)). Succinate, fumarate, and malate are transported into the cell through DcuA and DcuB (Figure 1; (125)). Aside from direct use in the TCA cycle, fumarate and succinate may play additional roles in *C. jejuni.* Fumarate is an electron acceptor during oxygen-independent respiration, resulting in the generation of succinate (146). Furthermore, *C. jejuni* possesses enzymes which convert fumarate to succinate (FrdABC), and succinate to fumarate (MfrABE; Figure 1; (147, 148)). Pyruvate, which has been shown to be utilized as a carbon source in *C. jejuni*, however, has no transporter identified to date (123). In *C. jejuni*, pyruvate can be converted to acetyl-CoA by the essential enzyme pyruvate::acceptor oxidoreductase (POR; (149)). Acetyl-CoA can then enter the TCA cycle, the acetogenesis pathway, or be used to synthesize fatty acids (126, 133, 150, 151). Pyruvate may also play a role in PEP synthesis (123). *C. jejuni* can also generate pyruvate through fucose utilization (Figure 1; (152, 153)). However, fucose utilization genes are not universal to all *C. jejuni* strains (including 81-176, the strain studied in our research program; (124)). Curiously, strains that encode genes necessary for fucose metabolism lack *ggt* and *ansB*, suggesting selective pressure in some strains for use of specific metabolites (122, 154, 155).

Iron acquisition by *C. jejuni*

Analysis of the *C. jejuni* genome reveals that it encodes for five iron-acquisition systems, although only four are present within *C. jejuni* strain 81-176 (2, 124). Without iron, which is required for many metabolic processes, growth of *C. jejuni* is strongly inhibited (156). One of the important functions of iron in these metabolic processes is for iron-sulfur complexes that are essential for many metabolic enzymes. Importantly, it was shown that enzymes containing iron-sulfur complexes are sensitive to damage by molecular oxygen and reactive oxygen species, endangering important metabolic processes in *C. jejuni* under undesirable atmospheric conditions (157).

Unlike many bacteria, *C. jejuni* does not produce siderophores (2, 124). Rather, *C. jejuni* utilizes siderophores produced from host microbes present throughout the digestive tract, including *E. coli* (158,

159). The microbial-derived ferric-enterobactin is imported into the cell through the CfrA or CfrB protein and subsequently enters the cytosol through the CeuBCDE transport system, where it is then hydrolyzed by unknown enzymes (156, 160-162). Not unexpectedly, CfrA and CfrB have been shown to be required for colonization of chicks by *C. jejuni* (156, 162). Recent studies have demonstrated that TonB-ExbB-ExbD energy transduction systems are involved in ferric-enterobactin import (163).

C. jejuni can also utilize iron resources that it scavenges from the host. These include compounds that include heme, such as hemin, hemoglobin, hemin-hemopexin, and hemoglobin-haptoglobin (164). The ChuABC transporter system is the main pathway through which *C. jejuni* can acquire host-derived heme compounds. ChuA acts as the heme receptor, ChuB is predicted to be a permease, ChuC is predicted to be the ATPase, and ChuD is predicted to be the periplasmic binding protein (2, 165). Interestingly, a *chuA* mutant will grow poorly when heme is the only iron source, but mutants in *chuBCD* do not exhibit this phenotype (164, 165). This suggests that other ABC transport systems may serve a redundant function to that of the Chu system for heme uptake. Furthermore, a *chuA* mutant does not affect the cecal colonization of chicks, although it may be important in other hosts or environments.

A second method for acquisition of iron from host-derived sources is through the uptake of lactoferrin and transferrin. Through a yet undescribed mechanism, lactoferrin or transferrin uptake is performed by CtuA (166). The subsequently released iron is then transported into the cytosol through the CfbpABC transport system (166). Mutations in *ctuA* result in drastic decreases in colonization of *C. jejuni* in both the chick ceca and rabbit ileal loop model of colonization (156, 167).

Although all bacteria need iron, excess iron can combine with atmospheric oxygen to produce reactive oxygen species that are detrimental to bacteria (168, 169). Thus, tight regulation of iron transport genes at the transcriptional level is required to maintain an appropriate homeostasis. Primary regulation of genes involved in iron uptake is modulated by the ferric uptake regulator (Fur), while PerR is the peroxide stress regulator protein (170, 171). Fur functions as a sensor of intracellular Fe^{2+}. When Fe^{2+} concentrations in the cell are high, Fur binds Fe^{2+} as a cofactor and subsequently forms a dimer which can then bind to DNA at promoter sequences, acting as a repressor (172, 173). When cellular Fe^{2+}

concentrations decrease, Fur dissociates from promoters, and transcription of Fur-regulated genes can proceed. PerR, which is a Fur homolog and also influenced by iron concentrations, has a regulon of 109 genes, including *katA* and *ahpC*, which encode proteins responsive to oxidative stress (174). Indeed, a *perR* mutant strain is hyper-resistant to oxidative stress, demonstrating that PerR acts as a repressor of gene expression (174). In iron-limited conditions, genes that encode for iron uptake and oxidative stress response were upregulated (156, 175). Conversely, upon addition of iron, *C. jejuni* iron uptake systems are downregulated (156, 175). Both *fur* and *perR* mutant strains of *C. jejuni* colonize the chick ceca significantly less than that of the wild type, suggesting Fur and PerR regulation *in vivo* plays an important role in limiting iron and oxygen toxicity (156, 174).

Short-chain fatty acids (SCFAs) in the gastrointestinal tract

Production and spatial distribution of SCFAs in the gastrointestinal tract

Increasing evidence suggests that intestinal bacteria play an important role in diet and nutrition in hosts more than previously thought. Fermentation of sugars, peptides, and glycoprotein precursors by bacteria in the gut results in the production of short-chain fatty acids (SCFAs), which are a fatty acids containing 1 to 6 carbon atoms (176-178). Production of SCFAs begins when primary fermenters, particularly *Bacteriodetes* species, convert simple sugars to organic acids and hydrogen gas (179). The resulting organic acids (which include acetate, propionate, and succinate) will then be further fermented by secondary fermenters, including *Clostridium* species and other butyrate producers, into additional SCFAs (179). In addition to these classes of fermenters, acetogens can utilize sugars and the excess hydrogen gas in the intestine to create increasing amounts of acetate (180, 181). In combination, these reactions result in a human colonic concentration of SCFAs wherein acetate is present in the highest amount, followed by propionate, which is in a concentration greater than or equal to the concentrations of butyrate (182). Lactate, an organic acid that is produced during the fermentation process in the gut, is the result of converting glycolytic-produced pyruvate to lactate. Lactate is produced by many species in the

small intestines, including *Lactobacillus* species, and can be consumed by other intestinal bacteria as a way to form SCFAs (183, 184).

It has been shown that total SCFA concentration in the human and avian colon can reach 70 to 130 mM, although this concentration varies between species and diet composition (178, 182, 185-189). For example, one study of 64 week old laying hens found that concentrations of total SCFAs ranged from 114 to 160 mM depending on whether the chickens exhibited low or high feather pecking behavior, respectively (188). This study also found similar ratios of SCFAs in the ceca of the chicken to that of the colon of other animals (acetate:propionate:butyrate at 3:2:1), although butyrate levels were lower than reported in other species (188, 190). Lactate levels in these birds were highest in the ileum (roughly 20-30 mM) and lowest in the ceca (roughly 1-5 mM; (188)). Furthermore, a recent study found that animal age and diet composition can effect intestinal SCFA concentrations, specifically in broiler chickens (189). SCFA concentrations in the ceca of hens changed both in response to dietary composition and age (189). However, this group found that concentrations of butyrate were much higher than those of propionate (189). Lactate levels in the chick intestinal tract were also found to be influenced by animal diet (183). In addition to the influences by diet and age, SCFA concentration is influenced by microbiota composition, thus resulting in a distribution of SCFAs throughout the intestinal tract (183, 191, 192). For instance, the microbial composition of the small intestine differs from that of the large intestine, which results in differing concentrations of SCFAs than found in the large intestine (186, 193).

SCFAs and organic acids play an important role in many human and animal tissues, including metabolism. Acetate, for example, is easily absorbed from the colon and subsequently transported to the liver (194). Following this event, acetate can be easily transported through the blood to other cells, including adipocytes, which use acetyl-CoA synthetase to promote lipogenesis. Furthermore, absorbed acetate may be used as a primary substrate during cholesterol synthesis and can be used by muscle cells for energy through oxidation. Once absorbed by the liver, propionate can be used as an energy source through gluconeogenesis in hepatic cells (195). In contrast, the presence of propionate in hepatic cells

also inhibits the production of cholesterol. In the colon, butyrate acts as the primary source for metabolism of colonocytes (196, 197).

The effect of SCFAs on the intestinal microbiota

As detailed above, SCFAs can act as a food source for other bacteria found within the gastrointestinal tract. However, production of SCFAs by gut bacteria can also have an adverse effect on the native flora. As the concentration of SCFAs increases, pH in the intestine decreases, which can alter the composition of the microbiota (194, 196). Following the decrease in pH, early *in vitro* studies found SCFA-induced toxicity to be caused by the nonionized form entering the bacterial cytoplasm (198-200). Due to their nature as small, uncharged molecules, the nonionized SCFAs are thought to diffuse across the bacterial membrane, leading to an accumulation of protons and SCFA anions (201-204). The resultant increased intracellular pH due to high proton concentration can disturb the proton motive force as well as interrupt metabolic processes such as methionine and homocysteine metabolism (205, 206). Furthermore, the increase in SCFA anions in the cytoplasm can influence osmotic balance through the depletion of amino acid pools (207). However, some organisms may exhibit different responses to fluctuating SCFA concentrations. For instance, exposure of *Bacillus subtilis* to propionate and acetate inhibited amino acid transport, while in *E. coli*, exposure to acetate exhibited an opposite effect, resulting in an increase in uptake of some amino acids (208, 209).

Short-chain fatty acids in the intestinal tract can modify host immunity indirectly as well. One method through which this can occur is by the induction of antimicrobial peptides (AMPs; (210)). Interestingly, colonic epithelial cells, when exposed to butyrate, upregulate expression of a gene encoding the AMP LL-37 (211). Further studies have found that SCFAs can modulate expression of AMPs from a variety of cells. These studies found that butyrate, as well as acetate and propionate, can influence expression of host defense peptides in chickens (212, 213). Furthermore, when the SCFAs were included in the food source of chickens, it was found that the host defense peptide expression not only increased, but that levels of *Salmonella enterica* in the host ceca also decreased (212, 213).

SCFAs influence behaviors of intestinal bacterial pathogens

The effect of SCFA exposure on intestinal pathogens is a burgeoning field. The bacteria in which effects have been studied include *Salmonella*, enterohemorrhagic *E. coli (*EHEC), *Listeria monocytogenes*, and *Shigella* species. Of the bacteria listed, *Salmonella* and its response to SCFAs has been the most intensely studied. Interestingly, at low levels, SCFAs can be used by *Salmonella* as a carbon source (214). However, in conditions in which the environmental pH is low and the SCFA concentrations are high, growth is strongly inhibited (215-217). There are many studies in which SCFAs have been shown to influence gene expression of virulence factors in several *Salmonella* species (218-224). Whereas high levels of acetate increased levels of virulence gene expression through the intermediary molecule acetyl-phosphate, high levels of propionate and butyrate strongly decrease expression of invasion genes in *S. enterica* serovar Typhimurium (225). More significantly, concentrations of SCFAs normally found in the distal ileum allowed for wild type levels of invasion gene expression, while concentrations of SCFAs found in the large intestine inhibited expression of these genes (225). Further *in vivo* data has shown that high levels of SCFAs in chickens protect the host from *Salmonella* infection and that antibiotic-treated mice with low levels of SCFAs succumb to *Salmonella* infection more often (226, 227). Together, this data suggests spatial monitoring for invasion as well as possible protective avenues from *Salmonella* infection.

In EHEC, expression of genes in the locus for enterocyte effacement (LEE) is strongly influenced by butyrate, but not by acetate or propionate (228). Interestingly, butyrate acted through the leucine-responsive regulatory protein Lrp (228, 229). In contrast to the promotion of adherence, SCFAs induce production of flagella in EHEC through both Lrp-dependent and Lrp-independent mechanisms (230). This raises the possibility that certain phenotypes are influenced by the microbial environment, which was supported by evidence that EHEC strains upregulated expression of adherence genes in concentrations of SCFAs found in the colon, but not in the ileum (231). While this suggests a spatial-recognition through sensing of SCFAs, a study found that EHEC incubated with bovine tissues and SCFAs exhibited decreased bacterial load, revealing that more work for this pathogen is required (232).

L. monocytogenes, when exposed to butyrate, exhibits alterations in physiology and gene expression. Specifically, butyrate alters the membrane fatty acid composition by increasing the amount of straight-chain fatty acids more so than from exposure to acetate or lactate (233-235). Furthermore, high levels of butyrate strongly inhibit the transcription of virulence factors in *L. monocytogenes* (234). Finally, although less work to examine the effect of SCFAs on *Shigella* species has been performed, one clinical trial has found that patients with *Shigella* infections who receive butyrate-containing enemas clear the infection faster due to an increase in cathelicidin (236).

SCFAs and *Campylobacter jejuni*

While other bacteria are capable of generating and/or utilizing many SCFAs, *C. jejuni* has only been shown to utilize lactate and acetate (126, 237). Although butyrate and propionate are also present within the gut lumen, no putative transport or utilization pathways within *C. jejuni* are known. Interestingly, it has been documented that the MfrABE complex can catabolize crotonate to butyrate in the periplasm, but the effects of this conversion are unknown (148).

Although no evidence exists for lactate excretion in *C. jejuni*, L-lactate and D-lactate catabolism has been observed (237). The majority of lactate is brought into *C. jejuni* through the LctP permease, with a yet unknown secondary permease thought to transport lactate in minor amounts (237). Following transport, the lactate is converted into pyruvate by a non-flavin iron-sulfur containing oxidoreducatase that is NAD-independent and specific to L-lactate (Figure 1; (237)). A second enzyme was found to have the same activity, but is not present in the 81-176 *C. jejuni* strain (237). Furthermore, while D-lactate metabolism has been shown, no D-lactate dehydrogenases have been identified in *C. jejuni* (237).

As detailed above, acetate is a product of the acetogenesis pathway which begins by the production of acetyl-CoA from pyruvate (149). The acetyl-CoA is converted to acetyl-phosphate (AcP) by phosphotransacetylase (Pta), and AcP is subsequently converted into acetate by acetate kinase (AckA; Figure 1; (126)). The produced acetate is excreted out of the cell through an unknown transporter during logarithmic growth phase (126). Upon entering late logarithmic or stationary phase growth, *C. jejuni* will

take up excreted acetate, and may use it as a carbon source following conversion of acetate to acetyl-CoA by acetyl-CoA synthetase (Acs; Figure 1; (126)). This change to acetate uptake is known as the 'acetate switch', and is common in many bacteria (181). The process of generating acetate appears to be important for *C. jejuni* biology, as a mutation in *pta* resulted in a modest decrease in colonization of the chick ceca (101).

The effect of SCFAs on the biology of *C. jejuni* has not been extensively explored. Many studies have explored the effect of feeding chickens with SCFAs and determining the level of *C. jejuni* in the intestinal tract (238-240). Results from these studies have not consistently shown an inhibitory effect by SCFAs (238-240). Curiously, when chicks are pre-colonized by lactic acid producing *Lactobacillus* species, the ability of *C. jejuni* to colonize the ceca is reduced (241). Following this trend, research to reduce *C. jejuni* load on processed poultry carcasses has shown that treatment with a dilute lactic acid solution aids in the reduction of *C. jejuni* organisms present (242, 243). It is unclear if either of these effects is due to the acidity of lactic acid or other means.

The effect of SCFAs on *C. jejuni* virulence gene expression is also poorly understood. While one study attempted to measure the effect of SCFA exposure on intestinal cell invasion, no effect was found in regards to *C. jejuni* physiology (244). In contrast, intestinal cells resisted *C. jejuni* infection if pretreated with SCFAs before exposure to *Campylobacter* (244). Regardless, the effect of acetate and other SCFA metabolism upon *C. jejuni* biology remains to be discovered.

Two-component regulatory systems in *C. jejuni*: A means to sense environmental signals

Environmental signals, such as metabolites and nutrients, can have broad effects on bacterial biology. To sense and respond to these signals, many bacteria utilize two-component regulatory systems (TCSs). Upon sensing of a specific signal, a TCS responds through the activation and/or repression of transcription of different sets of genes (245). Genomic analyses show that many bacteria contain TCSs, and on average contain 52 pairs of TCSs (246). However, some bacteria produce over a hundred while others produce none (246) .

The typical TCS pair consists of a sensor histidine kinase (HK) and a DNA-binding response regulator (RR; (246, 247)). Modulation of transcription by TCSs begins once the HK senses its specific signal. Signals can range from environmental cues, such as temperature, atmospheric gas levels, and color of light, to metabolic signals, such as presence of amino acids, TCA cycle intermediates, and quorum-sensing molecules (245). Following binding of the signal at the sensing domain, the HK undergoes autophosphorylation at a conserved histidine residue through the hydrolysis of ATP (245, 247). The resulting phosphohistidine serves as the phosphate source for an aspartic acid residue on the receiver domain of the RR (247). The subsequent phosphorylation of this residue results in a conformational change in the RR, which can then modulate transcription by binding DNA for activation or repression (245, 247). Some HK can also exhibit phosphatase activity (247). The ability to control the level of phosphorylated RR allows for the HK to fine tune the output of gene expression in response to minute changes in environmental signal.

C. jejuni encodes twelve putative two-component regulators and seven putative two-component histidine kinases, which results in seven possible cognate TCS pairs (2). To date, only five of these putative cognate pairs have been characterized extensively. These include RacRS, FlgSR, DccRS, CprRS, and PhosSR. Many of these have been found to play a role in *C. jejuni* biology, specifically in the avian host, and their functions are outlined below.

RacRS was first characterized as a system required for colonization of the chick intestinal tract, and the regulon included genes differentially expressed as a response to temperature (248). Furthermore, it was found that the RacR RR could function as both a repressor and activator of gene expression (248). Later investigation of the system confirmed the requirement for colonization and thermoregulatory properties of the RacRS TCS, and found that mutants exhibited a filamentous growth form (249). *C. jejuni racRS* mutants were altered for transcription of genes required for fumarate metabolism and uptake in O_2-limited conditions (250).

The FlgSR TCS is required for σ^{54}-dependent activation of *C. jejuni* flagellar genes (110, 251, 252). Further analysis has identified the formation of the flagellar type III secretion system, the MS ring,

and rotor structures of the flagellar motor as a signal dictated by the FlgS HK to result in activation of σ^{54}-dependent genes (253-255). Genes whose transcripts are eventually activated through the flagellar signaling are required for motility and/or commensal colonization (58).

The TCS CprRS was first identified through a microarray screen looking for genes that were differentially expressed upon infection of intestinal epithelial cells (256). Analysis of the CprRS regulon found that the TCS was important for transcription of genes that were involved in biofilm formation and stress tolerance (257). CprR was found to be essential for *C. jejuni* viability while CprS was not (257). Recent results also suggest that CprR must maintain a phosphorylated state for *C. jejuni* to remain viable despite the non-essentiality of CprS (258). Furthermore, the TCS is a required component for commensal colonization of the chick intestinal tract, and it also controls genes related to the cell envelope (257, 258).

The DccRS TCS is also important for commensal colonization of chicks as well as for the colonization of the mouse model of infection (259, 260). Genes originally identified within the DccRS regulon in *C. jejuni* encode for putative membrane and periplasmic proteins, which are also required for commensal colonization of the chick ceca (260). Further analysis also found a putative macrolide efflux pump which may be regulated by this TCS (259).

The final characterized TCS of *C. jejuni* consists of PhosSR. While this TCS is not required for commensal colonization of the avian host, the TCS influences transport of phosphate (261). As expected, this TCS was found to be activated by phosphate limitation (261).

CHAPTER TWO

Materials and Methods[†]

Bacterial strains and plasmids

C. jejuni strains were routinely grown from freezer stocks in microaerobic conditions (10 % CO_2, 5 % O_2 and 85 % N_2) on Mueller-Hinton (MH) agar containing 10 μg/mL trimethoprim at 37 °C for 48 h. Strains were then restreaked onto appropriate media and grown for an additional 16 h. When appropriate, antibiotics were added to media at the following concentrations: 20 μg/mL chloramphenicol, 100 μg/mL kanamycin, 30 μg/mL cefoperazone, or 0.1, 0.5, 1, 2, or 5 mg/mL streptomycin. *E. coli* DH5α and BL21 strains were grown on Luria-Bertani (LB) agar or LB broth containing 100 μg/mL ampicillin, 100 μg/mL kanamycin, 12.5 μg/mL tetracycline, or 20 μg/mL chloramphenicol when necessary. *C. jejuni* strains were stored at -80 °C in a mixture of 85 % MH broth and 15 % glycerol. *E. coli* strains were stored at -80 °C in a mixture of 80 % LB broth and 20 % glycerol.

Construction of *C. jejuni* mutants

Campylobacter jejuni mutants were constructed through electroporation using previously published methods (262). Briefly, the loci of genes to be deleted was amplified via PCR from the *C. jejuni* 81-176 chromosome using primers containing 5' BamHI sites located approximately 750 base pairs (bp) upstream and downstream of the locus. The PCR product was cloned into the BamHI site of pUC19 to create the following plasmids: pDAR812 (pUC19::*Cjj81176_1484-1483*), pLKB156 (pUC19::*chuCD*), pPML770 (pUC19::*Cjj81176_0063-0064*), pPML771 (pUC19::*Cjj81176_1386-1385*), pPML772 (pUC19::*Cjj81176_1619-1620*), pPML456 (pUC19::*ggt*), pPML706 (pUC19::*Cjj81176_0682-0683*), pPML725 (pUC19::*peb1c*), and pPML963 (pUC19::*acs*). If required, restriction sites were created

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within the coding sequence of some genes through PCR-mediated mutagenesis (263, 264). These reactions generated an HpaI site in *Cjj81176_1484* (pPML101) and *Cjj81176_1619* (pPML772) and an EcoRV site within *Cjj81176_0683* (pPML706). A *cat-rpsL* resistance cassette or *kan-rpsL* resistance cassette was removed from pDRH265 or pDRH436, respectively, by SmaI digestion and ligated into the appropriate resistance sites to interrupt each gene on the plasmids (252, 262).

Plasmids were subsequently electroporated into *C. jejuni* 81-176 Sm^R (DRH212) or *C. jejuni* 81-176 Sm^R Δ*astA* (DRH461) to interrupt the respective genes on the *C. jejuni* chromosome with either the -*cat-rpsL* or *kan-rpsL* cassette. Transformants were recovered on MH agar plates containing chloramphenicol or kanamycin, and following recovery, mutations were confirmed by colony PCR. Isogenic mutants of *C. jejuni* 81-176 Sm^R include: PML321 (*Cjj81176_1484*::*cat-rpsL*), PML322 (*Cjj81176_1483*::*cat-rpsL*), PML1262 (*Cjj81176_0064*::*cat-rpsL*), PML1274 (*chuC*::*cat-rpsL*), PML1280 (*Cjj81176_1386*::*cat-rpsL*), PML1305 (*Cjj81176_1619*::*cat-rpsL*), PML1049 (*peb1c*::*cat-rpsL*), PML1059 (*ggt*::*cat-rpsL*), and PML1065 (*Cjj81176_0683*::*cat-rpsL*). Isogenic mutants of *C. jejuni* 81-176 Sm^R Δ*astA* include: PML324 (Δ*astA Cjj81176_1483*::*cat-rpsL*), PML1006 (Δ*astA acs*::*kan-rpsL*), and PML1009 (Δ*astA pta ackA*::*cat-rpsL acs*::*kan-rpsL*) (252, 262). In addition, creation of *C. jejuni* 81-176 Sm^R Δ*astA* containing mutants in *pta*, *ackA*, and both *pta* and *ackA* were created by electroporation of 81-176 Sm^R Δ*astA* (DRH461) with previously constructed plasmids pJMB553, pJMB653, and pJMB955, respectively, to result in the strains JMB611 (Δ*astA pta*::*cat-rpsL*), PML1239 (Δ*astA ackA*::*cat-rpsL*), and JMB957 (Δ*astA pta ackA*::*cat-rpsL*) (254).

In-frame deletions were constructed by PCR-mediated mutagenesis (263). The mutations were verified by DNA sequencing, resulting in: pPML113 (pUC19::Δ*Cjj81176_1483*), pPML334 (pUC19::Δ*Cjj81176_1484*), and pPML1144 (pUC19::Δ*Cjj81176_0683*). These plasmids were then electroporated into *C. jejuni* strains containing *cat-rpsL* interruptions in the respective genes on the chromosome and mutants were recovered on MH agar containing 0.5-5 mg/mL streptomycin. Deletion of each gene was verified by colony PCR, resulting in the creation of the following *C. jejuni* 81-176 Sm^R mutant strains: PML335 (Δ*Cjj81176_1483*), PML337 (Δ*astA* Δ*Cjj81176_1483*), PML360

(Δ*Cjj81176_1484*), and PML1160 (Δ*Cjj81176_0683)*. In addition, creation of 81-176 Sm^R Δ*astA* Δ*pta* was performed by electroporation of 81-176 Sm^R Δ*astA pta*::*cat-rpsL* (JMB611) with the previously constructed plasmid pJMB627, to result in strain JMB638 (Δ*astA* Δ*pta*) (254).

To create point mutants in the predicted catalytic residues of Cjj1484 and Cjj1483, PCR-mediated mutagenesis was performed using pDAR812 as a template to create pPML239 (pUC19::$Cjj1483_{D58A}$), pPML340 (pUC19::$Cjj1483_{D58E}$), pPML732 (pUC19::$Cjj1483_{D58N}$), and pPML817 (pUC19::$Cjj1484_{H195A}$) (263). pPML239 was electroporated into PML322, and pPML340 and pPML732 were electroporated into PML324 to replace *Cjj1483*::*cat-rpsL* on the chromosome with the respective genes containing point mutants. Mutants were recovered on MH agar containing 0.5-5 mg/mL streptomycin and were verified by colony PCR and DNA sequencing to result in PML242 (81-176 Sm^R $Cjj1483_{D58A}$), PML739 (81-176 Sm^R Δ*astA* $Cjj1483_{D58E}$), and PML769 (81-176 Sm^R Δ*astA* $Cjj1483_{D58N}$).

Construction of plasmids for *in trans* complementation of *C. jejuni*

To create plasmids to be used for *in trans* complementation, a fragment beginning 400 bp upstream of the *Cjj1483* translational start site and ending at the *Cjj1483* stop codon was amplified by PCR from pDAR812 using primers with 5' BamHI sites. Following restriction digest, the fragment was ligated into the BamHI site of pRY112 to create pPML533 (pRY112::*Cjj1483*) (265). A fragment containing the 203 bp upstream of the start codon of *flaA* followed by an N-terminal FLAG-tag was amplified by PCR using primers with a 5' XbaI site and a 3' BamHI site. Following restriction digest, the fragment was inserted into the corresponding sites in pRY108 to create pDAR1604 (pRY108::P*flaA*-N FLAG). This plasmid allowed for creation of N-terminal FLAG-tagged proteins from the strong *flaA* promoter of *C. jejuni*. To create a *Cjj1484* complementation plasmid, codons 2 – stop codon of *Cjj1484* were amplified from pDAR812 with primers containing 5' BamHI sites. This fragment was digested with BamHI and then ligated into the BamHI site of pDAR1604 to create pPML968 (pDAR1604::*Cjj1484*). In addition, we amplified $Cjj1484_{H195A}$ from pPML817 in a similar manner described above and cloned the gene into the BamHI site of pDAR1604 to create pPML1119 (pDAR1604::$Cjj1484_{H195A}$).

To overexpress the *pta* and *ackA* genes *in trans* from a plasmid in *C. jejuni*, a fragment containing the 203 bp upstream of the start codon of *flaA* followed by the start codon and an NcoI site was amplified by PCR using primers with a 5' XbaI site and a 3' BamHI site. Following restriction digest, this fragment was inserted into the corresponding sites in pRY108 to create pDAR1423 (pRY108:: P*flaA*-NcoI). A 2.7 kb fragment including codon 2 of *pta* through codon 396 of *ackA* was amplified by PCR using primers with 5' and 3' NcoI sites. The fragment was digested with NcoI and inserted into the NcoI site in pDAR1423 to create pPML1071 (pDAR1423::*pta ackA*).

Plasmids pRY112, pPML533, pPML968, pPML1119, pDAR1604, pPML1071, and pDAR1423 were transformed into chemically competent *E. coli* DH5α / pRK212.1 which contains the conjugation transfer element (266). These plasmids were then conjugated into appropriate *C. jejuni* strains as previously described (267). Transconjugants containing pPML533 and pRY112 were recovered on MH agar containing trimethoprim, streptomycin, and chloramphenicol. Transconjugants containing pPML968, pPML1119, pDAR1604, pPML1071, and pDAR1423 were recovered on MH agar containing trimethoprim, streptomycin, and kanamycin. Presence of the plasmid was confirmed by colony PCR.

Primer Extension Analysis

Wild-type (WT) *C. jejuni* was grown from a freezer stock on MH agar containing trimethoprim for 48 h at 37 °C under microaerobic conditions, then restreaked on MH agar and grown for another 16 h. Total RNA was extracted with RiboZol (Amresco). To identify the transcriptional start site of *Cjj1483*, a primer (5'-ATCAATGATTCTCTAGCTT-3') that bound 50 bases downstream of the start codon was used. The primer was end-labeled with [γ^{32}P]-ATP using a polynucleotide kinase from the Excel Cycle-Sequencing Kit (Epicentre Tech) and then mixed with RNA and SuperscriptII reverse transcriptase (Invitrogen) to create labeled cDNA. A sequencing ladder was generated using the end-labeled primer and pDAR812 as a template. The cDNA and sequencing ladder were run on a 6 % acrylamide gel, dried, and analyzed using a Storm 820 Phosphorimager according to manufacturer's instructions (Amersham Biosciences).

Chick colonization assays

The ability of WT or isogenic mutant 81-176 Sm^R *C. jejuni* strains to colonize the ceca of chicks after oral inoculation was determined as previously described (101). Briefly, fertilized chicken eggs (SPAFAS) were incubated for 21 days at 37.8 °C with appropriate humidity and rotation in a Sportsman II model 1502 incubator (Georgia Quail Farms Manufacturing Company). One day after hatch, chicks were orally inoculated with 100 µL of MH broth containing approximately 10^2 cfu of either a wild-type or mutant strain. Strains were prepared for infection by suspending *C. jejuni* strains from MH agar plates after 16 h growth at 37 °C and microaerobic conditions in MH broth and diluting to achieve the appropriate inoculum for oral gavage of chicks. Dilutions of the inoculum were plated on MH agar to assess the number of bacteria in each inoculum. Seven or 14 days post-infection, chicks were sacrificed, the small intestinal, cecal, or large intestinal contents were removed and suspended in phosphate buffered saline (PBS), and serial dilutions were plated on MH agar containing trimethoprim and cefoperazone. Following 72 h of growth at 37 °C in microaerobic conditions, bacteria were counted to determine cfu per gram of cecal contents. Recovered colonies were analyzed by colony PCR to verify that WT and mutant strains were isolated from respectively infected chicks.

Collection of RNA for microarray analysis

WT *C. jejuni* 81-176 Sm^R (DRH212) and isogenic Δ*Cjj1484* (PML360), Δ*Cjj1483* (PML335), and Δ*pta* Δ*ackA* (JMB957) mutants were grown from freezer stocks on MH agar containing trimethoprim at 37 °C in microaerobic conditions for 48 h. Strains were then restreaked on MH agar and grown for an additional 16 h. *C. jejuni* growth was suspended from the plates in MH broth and diluted into 25 mL of MH broth to an OD_{600} of approximately 0.05. Strains were then grown statically at 37 °C in microaerobic conditions for 8 h to achieve mid-log phase growth. Following growth, strains were suspended in 10X stop solution (95 % ethanol plus 5 % phenol), incubated on ice for 20 min, pelleted by centrifugation, and stored at -80 °C (256). Pellets were suspended in 1 mL of RiboZol (Amresco) and RNA removed by chloroform extraction. Total RNA (60 µg) was treated with DNaseI (Invitrogen) and then purified

through an RNeasy Mini Column (Qiagen). RT-PCR was performed to confirm the absence of DNA from the RNA samples.

Transcriptome analysis with DNA microarrays

The DNA microarray-based transcriptome analysis was performed as previously described (268). Briefly, gene expression comparisons were performed indirectly by comparing the transcriptome profile of WT *C. jejuni* 81-176 Sm^R with the profile of *C. jejuni* 81-176 Sm^R Δ*Cjj1484* (PML360), 81-176 Sm^R Δ*Cjj1483* (PML335), or 81-176 Sm^R Δ*astA* Δ*pta* Δ*ackA* (JMB957). Total RNA samples (20 μg) for each strain were labeled with Cy3-dUTP during cDNA production by reverse transcriptase and mixed with Cy5-dUTP-labeled reference genomic DNA from WT *C. jejuni* 81-176 Sm^R before being hybridized separately to the custom cDNA microarrays (GEO accession: Platform GPL6315 on Corning UltraGAPS slides). Microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA). The microarray experiments were performed with two technical replicates per array with two replicate features for each coding sequence (CDS) per array. GenePix 4.0 software was used to process the spot and background intensity, and data normalization was performed to compensate for differences in the amount of template amount or unequal Cy3 or Cy5 dye incorporation. GeneSpring 7.3 software (Silicon Genetics, Palo Alto, CA) was used to analyze the normalized data, and a parametric statistical *t* test was used to determine the significance of the centered data at a *P* value of <0.05, adjusting the individual *P* value with the Benjamini-Hochberg false discovery rate multiple test correction in the GeneSpring analysis package. Microarray data were deposited in the Gene Expression Omnibus (GEO) database.

Semiquantitative real-time RT-PCR (qRT-PCR) analysis

C. jejuni strains were grown from freezer stocks on MH agar containing trimethoprim at 37 °C in microaerobic conditions for 48 h. Strains were then restreaked on MH agar and grown for an additional 16 h. *C. jejuni* growth was suspended from the plates in MH broth and diluted into 25 mL of MH broth to

an OD_{600} of approximately 0.1. Strains were then grown statically at 37 °C in microaerobic conditions for 8 h to achieve mid-log phase growth. Total RNA was extracted with RiboZol (Amresco) and RNA was treated with DNaseI (Invitrogen). RNA was diluted to a concentration of 50 ng/μL before analysis. qRT-PCR was performed using a 7500 real-time PCR system (Applied Biosystems) with *secD* mRNA detection as an endogenous control. mRNA transcript levels in strains DRH212 and DRH461 served as WT controls to determine relative gene expression in isogenic mutants.

To examine the effect of different short-chain fatty acids (SCFAs) on gene expression in *C. jejuni*, WT and isogeneic *pta* and *ackA* mutant strains were grown from freezer stocks on MH agar as detailed above. Following the additional 16 h growth, the *C. jejuni* strains were suspended from plates in *Campylobacter* Defined Media (CDM), which is a media containing nutrients at specific concentrations to support growth, and diluted into 25 mL of CDM broth to an OD_{600} of approximately 0.1 (150). Strains were grown in CDM alone, CDM containing 0, 10, 25, 50, or 100 mM potassium acetate (Sigma), CDM containing 0, 12.5, or 25 mM sodium butyrate (Acros Organics), CDM containing 0, 10, 25, 50, or 100 mM sodium L-lactate (Sigma), or CDM containing 12.5 mM sodium butyrate and 25 mM sodium L-lactate. Growth was performed statically at 37 °C in microaerobic conditions for 8 h to achieve mid-log phase growth. Total RNA was extracted with RiboZol (Amresco) and RNA was treated with DNaseI (Invitrogen). RNA was diluted to a concentration of 50 ng/μL before analysis. qRT-PCR was performed using a 7500 real-time PCR system (Applied Biosystems) with *secD* mRNA detection as an endogenous control. mRNA transcript levels in the strain DRH461 grown in CDM alone served as the WT control to determine relative gene expression in the wild type and isogenic mutant at the different concentrations of SCFAs.

To examine the effect of overexpression *in trans* of *pta* and *ackA* on gene expression in *C. jejuni*, PML1102 (Δ*pta* Δ*ackA* + *pta ackA*), PML1125 (Δ*pta* Δ*ackA* + vector), and PML1140 (WT + vector) were grown in MH and CDM broth containing kanamycin and trimethoprim. Total RNA was extracted and purified as detailed above. mRNA transcript levels in the strain PML1140 served as the WT control and *secD* mRNA detection as the endogenous control to determine relative gene expression.

To examine levels of expression of colonization and virulence genes throughout the chick intestinal tract, one day old chicks were orally inoculated with 100 μL of MH broth containing approximately 10^2 cfu of WT *C. jejuni*, as described above. At seven days post-infection, chicks were sacrificed, the small intestine, cecal, or large intestine contents were removed and total RNA was extracted with Ribozol (Amresco). RNA was then purified as described above. mRNA transcript levels in the ceca served as the organ load control and *secD* mRNA detection as the endogenous control to determine relative gene expression.

Analysis of growth of *C. jejuni* strains

WT *C. jejuni* 81-176 Sm^R (DRH212) and isogenic Δ*Cjj1484* (PML360) and Δ*Cjj1483* (PML335) mutants were grown from freezer stocks on MH agar containing trimethoprim at 37 °C in microaerobic conditions for 48 h. WT *C. jejuni* 81-176 Sm^R Δ*astA* with vector (PML1140), Δ*pta* Δ*ackA* with vector (PML1125), and Δ*pta* Δ*ackA* with plasmid containing *pta ackA* (PML1102) were grown from freezer stocks on MH agar containing kanamycin at 37 °C in microaerobic conditions for 48 h. Strains were then restreaked on MH agar and grown for an additional 16 h. *C. jejuni* growth was suspended from the plates in MH broth or CDM broth and diluted into 25 mL of MH broth or CDM broth containing trimethoprim or both trimethoprim and kanamycin to an OD_{600} of approximately 0.1. Iron-rich growth conditions were created by adding Fe_2SO_4 to MH broth to a final concentration of 40 μM. Iron-limited growth conditions were created by adding the iron chelator deferoxamine mesylate (DFO) to MH broth to a final concentration of 20 μM (175). Cultures were grown at 37 °C in microaerobic conditions without shaking, and growth was measured via spectrometry at an OD_{600}. All strains were analyzed in triplicate.

Gluconate dehydrogenase activity in *C. jejuni* strains

WT *C. jejuni* 81-176 Sm^R (DRH212) and the isogenic Δ*Cjj1484* mutant (PML360) were grown from freezer stocks on MH agar containing trimethoprim at 37 °C in microaerobic conditions for 48 h. Strains were then restreaked on MH agar and grown for an additional 16 h. *C. jejuni* growth was

suspended from the plates in MH broth and diluted into 25 mL of MH broth containing trimethoprim to an OD_{600} of approximately 0.1. Cultures were grown statically at 37 °C in microaerobic conditions until growth reached mid-log phase. Cells were pelleted and washed twice in 20 mM $NaPO_4$ (pH 7.0), disrupted by sonication, and centrifuged at 13,000 rpm for 5 min to remove cell debris. Gluconate dehydrogenase assays were performed as previously described (269). Briefly, each reaction contained 1 mL of 0.1 M KPO_4 buffer (pH 6.0), 0.1 mL of 1 mM 2,6-dichlorophenolindophenol (DCIP), 0.1 mL of 3 mM phenazine methosulfate (PMS), 0.1 mL of 1 M D-gluconate, and 0.2 mg of total protein from the *C. jejuni* cell lysates in a total volume of 3 mL. Enzyme activity was determined through continuous measurement of DCIP reduction at 600 nm at 25 °C. Three separate assays were performed with each strain analyzed in triplicate.

Determination of acetate concentrations *in vitro*

WT *C. jejuni* 81-176 Sm^R Δ*astA* (DRH461) or isogenic *pta* and *ackA* mutants were grown from freezer stocks on MH agar containing trimethoprim at 37 °C in microaerobic conditions for 48 h. Strains were then restreaked on MH agar and grown for an additional 16 h. *C. jejuni* growth was suspended from the plates in CDM broth and diluted into 25 mL of CDM broth containing trimethoprim to an OD_{600} of approximately 0.1. Cultures were grown at 37 °C in microaerobic conditions without shaking for 8 h to achieve mid-log phase. At this time, cultures were centrifuged for 5 minutes at 13,000 rpm to pellet cells and the supernatant was removed. The supernatant was then centrifuged again for 5 mins at 13,000 rpm to remove any remaining *C. jejuni* cells. Samples from the supernatants of DRH461 (WT), JMB638 (Δ*pta*), PML1239 (*ackA*::*cat-rpsL*), and JMB957 (*pta ackA*::*cat-rpsL*) were used as directed in a microplate assay for detection of acetic acid (Megazyme), with un-inoculated CDM broth serving as a control. Three separate assays were performed with each strain analyzed in triplicate.

Motility assays

WT *C. jejuni* 81-176 Sm^R (DRH212) and isogenic Δ*Cjj1484* (PML360) and Δ*Cjj1483* (PML335) mutants were grown from freezer stocks on MH agar containing trimethoprim at 37 °C in microaerobic conditions for 48 h. Strains were then restreaked on MH agar and grown for an additional 16 h. *C. jejuni* growth was suspended from the plates in MH broth and diluted into 10 mL of MH broth containing trimethoprim to an OD_{600} of approximately 0.8. Strains were stabbed in MH motility agar (MH broth + 0.4 % agar) with an inoculation needle and incubated for 30 h at 37 °C in microaerobic conditions. The size of the motility zone was measured for each strain.

Purification of proteins and generation of antisera

Primers containing in-frame 5' BamHI restriction sites to codon 2 and the stop codon were used to amplify *Cjj1483* from pDAR812, $Cjj1483_{D58A}$ from pPML239, $Cjj1483_{D58E}$ from pPML340, and $Cjj1483_{D58N}$ from pPML732. Following restriction digestion, the fragments were ligated into the BamHI site of pGEX-4T-2, generating pPML165, pPML278, pPML757, and pPML758, respectively. The plasmids were then transformed into BL21 (DE3) *E. coli*, and induced with 1 mM IPTG. The N-terminal glutathione S-transferase (GST) tagged proteins were purified from the soluble fraction with glutathione Sepharose beads (GE Healthcare). Following GST tag removal by thrombin-mediated cleavage, the recombinant proteins were eluted from benzamidine Sepharose following manufacturer's instructions (GE Healthcare). Glycerol was added to a final concentration of 10 % and proteins were stored at -80°C. Recombinant WT Cjj1483 was then used to immunize five mice to generate polyclonal antisera (Cocalico Biologicals).

Primers containing in-frame 5' BamHI restriction sites to codon 2 and the stop codon were used to amplify *Cjj1484* from pDAR812. Following restriction digestion, the fragments were ligated into the BamHI site of pET28-6xHis-MBP to generate pPML848 (270). The plasmid encoding the N-terminal 6xHis-maltose binding protein (MBP) fusion was transformed into BL21 (DE3) *E. coli* and induced with 300 μM IPTG at 16 °C. Recombinant protein was purified from the soluble fraction with Ni-

nitrilotriacetic acid (NTA) agarose (Qiagen) as previously described (271). The 6xHis-MBP tag was removed following cleavage with Tobacco Etch Virus (TEV) protease, and the recombinant protein was purified from the tag with amylose agarose, as per manufacturer's instructions (New England Biolabs). Glycerol was added to a final concentration of 10 % and proteins were stored at -80 °C. Recombinant Cjj1484 was then used to immunize five mice to generate polyclonal antisera (Cocalico Biologicals).

In vitro phosphorylation of Cjj1483 proteins with acetyl-phosphate

Radiolabeled Ac[^{32}P] was created as described previously (272). Fifty pmoles of Cjj1483 proteins were incubated with 10 μL of Ac[^{32}P]-generating reaction for 20 min at 37 °C. Proteins were separated on a 12.5 % SDS/PAGE without boiling and the gel was analyzed using a Storm 820 Phosphorimager according to manufacturer's instructions (Amersham Biosciences).

Electrophoretic mobility shift assays (EMSAs)

Recombinant Cjj1483 proteins were purified as described above. A 320-bp DNA fragment spanning -297 to +20 relative to the transcriptional start site were amplified for the *Cjj0438* and *Cjj1386* promoters. EMSAs were performed based on a modified protocol (273). Briefly, 0.5 - 2.0 μM of WT or Cjj1483 mutant proteins in the presence or absence of 50 mM lithium acetyl-phosphate (Li-AcP) was incubated with ^{32}P-labelled DNA at 4 °C for 20 min. Competition experiments were performed through the addition of unlabeled DNA for the genes mentioned above and *aphA-3*$_P$ as a non-specific control. Unlabeled DNA was added at 1:1, 2:1, and 5:1 ratios relative to ^{32}P-labeled DNA, and 2 μM of Cjj1483 with or without Li-AcP was used. After electrophoresis, analysis was performed with a Storm 820 Phosphorimager according to manufacturer's instructions (Amersham Biosciences).

Statistical analyses

Tests of statistical significance in gene expression, gluconate dehydrogenase activity, *in vitro* growth, and acetate production were performed by using the Student's *t* test (two-tailed distribution with

two-sample, equal variance calculations). Statistically-significant differences between strains possessing *P*-values < 0.05 or 0.01 are indicated in the figures, tables, figure legends, and/or table legends. To determine statistical significance of chick colonization assays, Mann-Whitney *U* analyses was performed, with statistically-significant differences between wild-type and mutant strains indicated with *P*-values < 0.05 in the figures and/or figure legends.

CHAPTER THREE

Analysis of the Activity and Regulon of the Two-component Regulatory System Composed by Cjj1484 and Cjj1483 of *Campylobacter jejuni* [‡]

Introduction

Despite increasing surveillance and food safety measures, the number of cases of foodborne diarrheal disease caused by *Campylobacter jejuni* has increased over the last few decades. As such, *C. jejuni* remains one of the leading causes of diarrheal disease in the United States and many other developed countries throughout the world (3, 4). *C. jejuni* is a natural commensal bacterium of many wild and agriculturally-important animals, particularly poultry (274, 275). In chickens, *C. jejuni* promotes a persistent colonization of the mucus layer lining the ceca and large intestine without causing disease (100). These zoonotic infections lead to contamination of poultry meats for human consumption (5). In humans, *C. jejuni* adheres to and invades epithelial cells of the lower intestinal tract and colon, which stimulates inflammation that often leads to watery or bloody diarrhea (23). Infection of humans is typically self-limiting. However, Guillain-Barré syndrome, an autoimmune disorder that results in an acute paralysis of the peripheral nervous system, may develop after diarrheal disease (32, 33).

Many pathogenic bacteria utilize two-component regulatory systems (TCSs) to link environmental cues to transcription of specific genes. TCSs may function by activating and/or repressing transcription of different sets of genes. Typical TCSs are composed of a sensor histidine kinase (HK) and a DNA-binding response regulator (RR; (246, 247)). Upon sensing a specific signal, the HK autophosphorylates at a conserved histidine residue (245). This phosphohistidine then serves as the substrate for the RR, which catalyzes the transfer of the phosphate to an aspartic acid on its receiver domain. Once phosphorylated, the RR can influence gene expression by binding to promoter regions of target genes to mediate either transcriptional activation or repression. In some instances, the HK also

exhibits phosphatase activity that can dephosphorylate the response regulator (247). This latter mechanism is a means to provide finite control of the response regulator in the absence of a stimulus.

Relative to many bacterial species, *C. jejuni* contains few TCSs, with only up to seven cognate TCSs predicted to be encoded in the genome (276, 277). Through studies by many groups, five cognate TCSs of *C. jejuni* have been characterized, including FlgSR, DccRS, PhosSR, CprRS, and RacRS. Many of these have been implicated in controlling expression of genes required for host interactions. For instance, the FlgSR TCS monitors formation of the flagellar type III secretion system, the MS ring, and rotor structures to activate expression of σ^{54}-dependent flagellar genes required for motility and commensal colonization (58, 110, 251-255). Furthermore, DccRS, CprRS, and RacRS are required for WT levels of commensal colonization of the chick ceca (248, 249, 257, 259, 260). Whereas DccRS directly influences expression of genes encoding colonization determinants (260), CprRS is required for expression of genes for biofilm formation (257). RacRS is required for expression of genes encoding proteins for the heat shock response and fumarate metabolism depending on the strain and growth conditions used for analysis (248-250). Additionally, the PhosSR TCS is required for expression of 12 genes, five of which encode proteins required for phosphate acquisition (261). However, this TCS is not required for *in vivo* colonization under the parameters tested.

Another cognate TCS may be encoded by *Cjj81176_1484 (Cjj1484*; *Cj1492c* as annotated in the *C. jejuni* NCTC11168 genome; (277)) and the immediately downstream gene, *Cjj81176_1483* (*Cjj1483*; *Cj1491c* as annotated in the *C. jejuni* NCTC11169 genome; (277)). *Cjj1484* is predicted to encode a putative sensor HK, whereas *Cjj1483* appears to encode a putative DNA-binding RR. This TCS appears to be conserved in a wide variety of *C. jejuni* strains. In this study, we investigated the potential for these genes to encode a cognate TCS that may influence transcription of genes required for host interactions. Our findings indicate that Cjj1484 and Cjj1483 appear to function as a cognate TCS mainly involved in transcriptional repression of genes encoding proteins involved in commensal colonization of chicks and metabolic processes related to redox potential and iron or heme acquisition when grown *in vitro*. In addition, the TCS is modestly required for transcription of an invasion gene. Furthermore, we provide

evidence that the Cjj1483 RR may function independently of the Cjj1484 HK to modulate transcription of some genes. Finally, we show that Cjj1483 can be phosphorylated and that specific DNA binding to target promoters occurs with and without phosphorylation. We propose that the previously uncharacterized *Cjj1484-1483* TCS of *C. jejuni* represses expression of some colonization factors outside the host and likely functions in combination with other transcription factors to fine tune expression of specific genes involved in various processes that are important for the biology of *C. jejuni*.

Results

Genomic and transcriptional organization of the *Cjj1484-Cjj1483* locus

We analyzed the potential for *Cjj1484* and *Cjj1483* to encode a TCS that influences expression of specific genes in *C. jejuni* 81-176. On the *C. jejuni* 81-176 chromosome, these genes are predicted to be in an operon with *Cjj1484* (encoding a putative HK) at the 5' end and *Cjj1483* (encoding a putative RR) immediately downstream and in the same orientation as *Cjj1484* (Figure 2A). In order to assess the transcriptional organization of this locus, we performed primer extension analysis to identify potential transcriptional start sites and promoters for these genes. We detected a strong primer extension product that begins 180 bases upstream of the start codon of *Cjj1483*, but we were not able to detect a product representing a transcriptional start site for *Cjj1484* (Figure 2B and data not shown). However, RNAseq analyses of *C. jejuni* 81-176 performed by another group identified transcriptional start sites immediately upstream of both genes (278). We attempted to determine if *Cjj1484* and *Cjj1483* were co-transcribed from the promoter upstream of *Cjj1484*, but we were unable to detect a product from reverse-transcriptase PCR for a bicistronic mRNA (data not shown). To discern whether Cjj1483 can be translated from transcripts originating upstream of *Cjj1484* or *Cjj1483*, we constructed an 81-176 Δ*Cjj1484* mutant, which removed the promoter for *Cjj1483*. In this mutant, we detected production of Cjj1483 at approximately half the level of the WT strain (Figure 2C). These findings combined indicate that *Cjj1483* is mostly like co-transcribed with *Cjj1484* from the promoter upstream of *Cjj1484* and also independently transcribed from its own promoter.

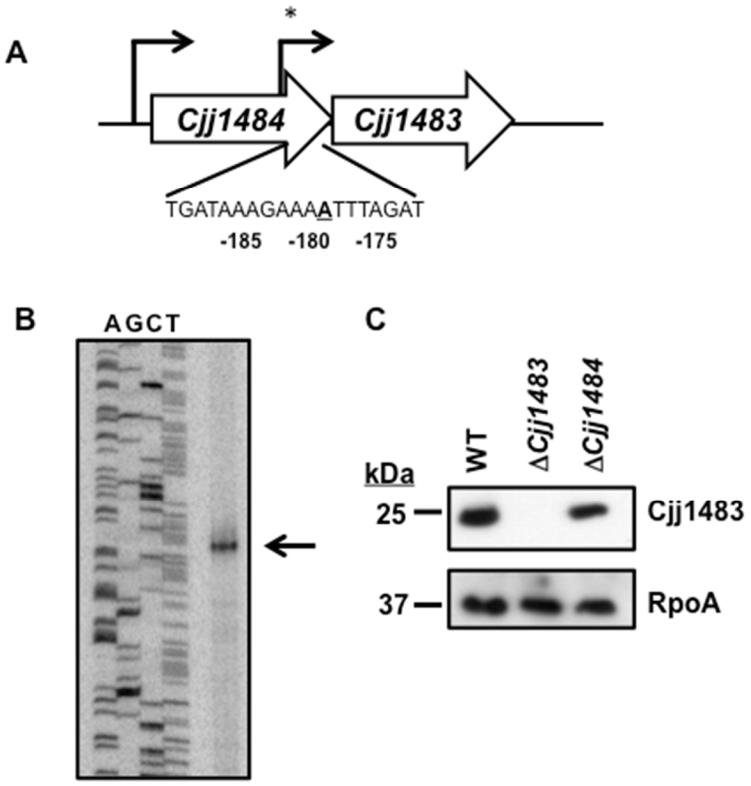

Figure 2. Genetic and transcriptional organization of the Cjj1484-Cjj1483 TCS. (A) Organization of *Cjj1484* and *Cjj1483* on the *C. jejuni* 81-176 chromosome. Arrows indicate transcriptional start sites for each gene as determined by RNAseq analysis (278) or by primer extension (*) in this work as shown in (B). The DNA sequence below indicates the transcriptional start site for *Cjj1483* that occurs at the adenosine residue 180 bases upstream of the translational start site for the gene as determined by primer extension. (B) Primer extension analysis for *Cjj1483*. The product of primer extension analysis was run alongside a sequencing ladder using the same primer for the primer extension reaction. Arrow indicates the product of primer extension. (C) Immunoblot of Cjj1483 production in whole-cell lysates of WT *C. jejuni* 81-176 Sm^R and isogenic mutants lacking *Cjj1483* or *Cjj1484*. Cjj1483 was detected with specific antiserum generated against recombinant Cjj1483. The Δ*Cjj1484* mutant lacks the region of the gene containing the promoter and transcriptional start site for a monocistronic *Cjj1483* transcript depicted in (A). Detection of RNA polymerase α subunit (RpoA) served as a loading control for the whole-cell lysates.

Analysis of *C. jejuni* Δ*Cjj1484* and Δ*Cjj1483* mutants in commensal colonization of chicks

We assessed whether the putative Cjj1484-Cjj1483 TCS was necessary for colonization of a natural host by infecting 1-day old chicks with an inoculum of 10^2 cfu of WT *C. jejuni* 81-176 Sm^R or isogenic Δ*Cjj1484* and Δ*Cjj1483* mutants. Seven days post-infection, the levels of *C. jejuni* in the chick ceca were determined. WT *C. jejuni* colonized on average at 1.55×10^9 cfu per gram of cecal content (Figure 3). We did not detect any statistically significant colonization defects due to deletion of *Cjj1484* or *Cjj1483* as these mutants colonized at similar levels as the WT strain. These data suggest that this putative TCS is likely not required for interaction with the natural avian host.

Transcriptome analysis of *C. jejuni* Δ*Cjj1484* and Δ*Cjj1483* mutants

In order to identify a biological role for the putative Cjj1484-Cjj1483 TCS in *C. jejuni*, we performed transcriptome analysis using DNA microarrays to identify a potential regulon for Cjj1484 and Cjj1483. WT and mutant *C. jejuni* strains were grown to mid-log phase at 37 °C in microaerobic conditions for isolation of mRNA to be used in transcriptome analysis. Genes in which expression was increased or decreased by at least two-fold were classified as putative members of the regulon controlled by Cjj1484 or Cjj1483 (Table 1 includes an abbreviated list of genes analyzed in this study; Tables 2 and 3 include all genes identified). Genes selected for additional analysis met one or more of the following criteria: 1) highest degree of expression differences in mutants relative to the WT strain; 2) expression of the gene was dysregulated in both the Δ*Cjj1484* and Δ*Cjj1483* mutants; 3) genes identified in the analysis that were likely grouped in operons and co-transcribed; and/or 4) genes encoding *C. jejuni* proteins whose function has previously been characterized. Select genes were analyzed by semi-quantitative qRT-PCR to validate the results from DNA microarrays analysis (Figure 4).

In combining the results from the DNA microarray and qRT-PCR analyses, we discovered genes could be grouped into four classes based on how mutation of the Cjj1484 HK and the Cjj1483 RR affected transcription of the genes. Generally, the Cjj1483 RR had a repressive effect on transcription of target genes, but the Cjj1484 HK demonstrated both positive and negative effects on transcription. We

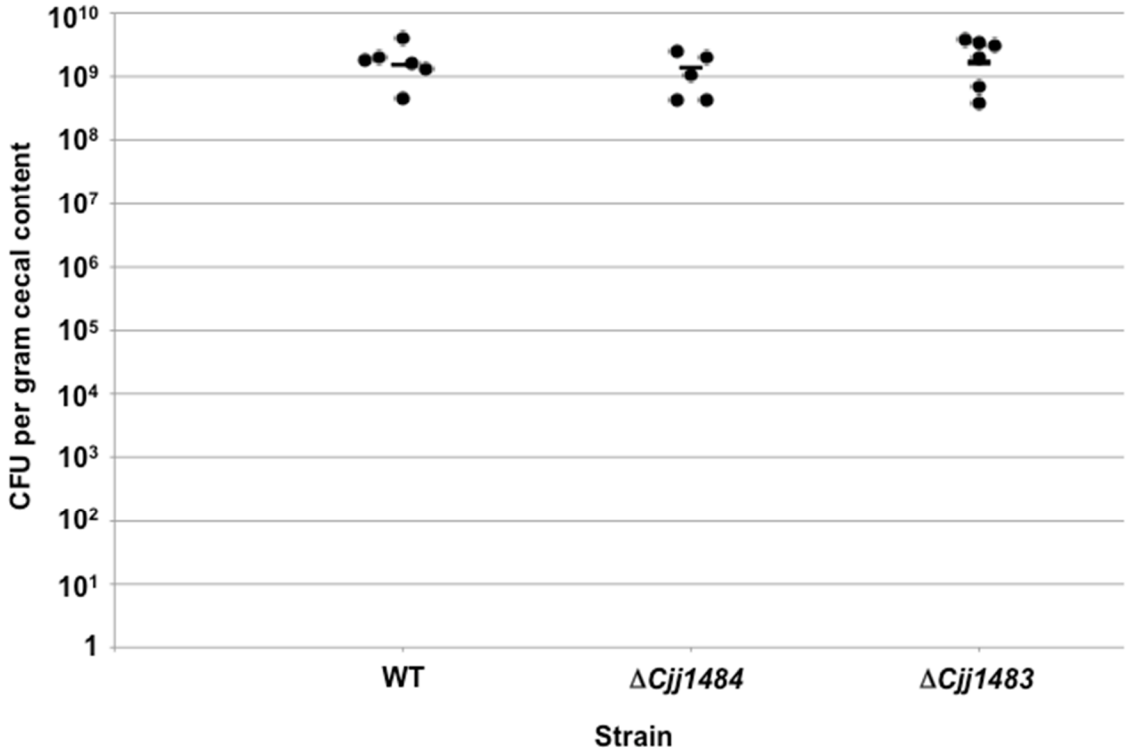

Figure 3. Commensal colonization capacity of wild-type *C. jejuni* and isogenic mutants lacking *Cjj1484 or Cjj1483*. One-day-old chicks were orally inoculated with approximately 10^2 cfu of wild-type *C. jejuni* 81-176 Sm^R or isogenic mutant strains. Each dot represents the amount of *C. jejuni* recovered from the ceca of each chick at day 7 post-infection. The geometric mean for each group is depicted by the horizontal bar. Statistical analysis was performed using the Mann-Whitney *U* test. Both mutants colonize at levels equal to the WT strain and showed no statistically significant differences.

Locus Tag	Gene Name	Putative Function	Ratio WT/ Δ*Cjj1484*[b]	Ratio WT/ Δ*Cjj1483*[c]
Class I[d]				
Cjj81176_0438		gluconate dehydrogenase, subunit III	0.07	0.14
Cjj81176_0439		gluconate dehydrogenase, subunit I	0.08	0.13
Cjj81176_1603[e]	*chuC*	hemin-transport ATP-binding protein		0.09
Cjj81176_0064[c]		putative cytochrome c protein		0.32
Class II				
Cjj81176_0210		possible transferrin transport protein	2.51	0.47
Cjj81176_0211		possible transferrin transport protein	2.70	0.45
Cjj81176_1619	*exbB2*	ferric enterobactin transport protein	3.44	0.33
Cjj81176_1620	*exbD2*	ferric enterobactin transport protein	2.62	0.39
Cjj81176_0063[e]		hypothetical protein		0.27
Cjj81176_0315[f]	*peb3*	glycoprotein; putative adhesion or transport protein	3.52	
Class III				
Cjj81176_1385[e]		hypothetical protein		0.19
*Cjj81176*_1386[e]		hypothetical protein		0.11
Class IV				
Cjj81176_1257[e]	*ciaC*	invasion protein		2.23

Table 1. Condensed list of genes differentially expressed in *C. jejuni* 81-176 Sm^R Δ*Cjj1484* or Δ*Cjj1483* compared to WT *C. jejuni* 81-176 Sm^R by microarray analysis. Shown are a subset of genes identified to be differentially expressed in mutants that were further analyzed in this work. A complete list of genes that were differentially expressed in mutants are shown in Tables 2 and 3. [b] Expression of genes was increased or decreased by two-fold in the *C. jejuni* Δ*Cjj1484* mutant. [c] Expression of genes was increased or decreased by two-fold in the *C. jejuni* Δ*Cjj1483* mutant. [d] Genes were divided into one of four classes as described in the text based on their expression in *C. jejuni* Δ*Cjj1484* and Δ*Cjj1483* mutants. [e] Expression of these genes was not affected in *C. jejuni* Δ*Cjj1484* as determined by microarray analysis. [f] Expression of this gene was not affected in *C. jejuni* Δ*Cjj1483* as determined by microarray analysis.

Locus Tag	Gene Name	Putative Function	Ratio WT/ mutant	Reference
81176_0315	*peb3*	glycoprotein; putative adhesin or transport protein	3.52	(279-281)
81176_1619	*exbB2*	ferric enterobactin transport protein	3.44	(163)
81176_1339	*flaA*	major flagellin	2.87	(282)
81176_0211		possible transferrin transport protein	2.70	(156, 166)
81176_1620	*exbD2*	ferric enterobactin transport protein	2.62	(163)
81176_0210		possible transferrin transport protein	2.51	(156, 166)
81176_0722	*glnA*	glutamine synthetase	2.35	
81176_0580		hypothetical inner membrane protein	2.06	
81176_0438		gluconate dehydrogenase, subunit III	0.07	(269)
81176_0439		gluconate dehydrogenase, subunit I	0.08	(269)
81176_0885		putative cytochrome c	0.33	

Table 2. Complete list of genes differentially expressed in *C. jejuni* 81-176 Sm^R Δ*Cjj1484* compared to WT *C. jejuni* 81-176 Sm^R. Expression of genes was increased or decreased by two-fold in the *C. jejuni* Δ*Cjj1484* mutant.

Locus Tag	Gene Name	Putative Function	Ratio WT/ mutant	Reference
81176_0204		putative periplasmic glycoprotein	5.87	(283)
81176_0075		putative cytochrome c subfamily protein	3.95	
81176_0206		phase-variable motility protein	3.77	(284, 285)
81176_1312		phase-variable flagellin glycosylation protein	3.73	(286)
81176_1208		putative periplasmic protein	3.17	
81176_1435		capsular polysaccharide modification protein	2.93	(287)
81176_0283	*eptC*	lipid A phosphoethanolamine transferase	2.51	(79, 288)
81176_0722	*glnA*	putative glutamine synthetase	2.37	
81176_1257	*ciaC*	invasion protein	2.23	(289)
81176_1422	*hddC*	capsular polysaccharide biosynthesis	2.13	(94)
81176_1603	*chuC*	hemin transport ATP-binding protein	0.09	(165)
81176_1386		hypothetical protein	0.11	
81176_0439		gluconate dehydrogenase, subunit I	0.13	(269)
81176_0438		gluconate dehydrogenase, subunit III	0.14	(269)
81176_0463	*mfrA*	methylmenaquinol:fumurate reductase protein A	0.16	(148)
81176_1385		hypothetical protein	0.19	
81176_1604	*chuD*	hemin transport substrate-binding protein	0.22	(165)
81176_0063		hypothetical protein	0.27	
81176_0464	*mfrB*	methylmenaquinol:fumurate reductase protein B	0.28	(148)
81176_0433	*frdA*	fumarate reductase/succinate dehydrogenase flavoprotein subunit	0.32	(147)
81176_0064		cytochrome c family protein	0.32	
81176_1619	*exbB2*	ferric enterobactin transport protein	0.33	(163)
81176_1602	*chuB*	hemin transport permease protein	0.34	(165)
81176_1389		hypothetical protein	0.36	
81176_1392	*metC*	hypothetical protein	0.38	
81176_1620	*exbD2*	ferric enterobactin transport protein	0.39	(163)
81176_1353	*ceuD*	enterochelin transport, ATP-binding protein	0.39	(290)
81176_1699	*rplV*	50S ribosomal protein L22	0.39	
81176_0123	*dcuA*	C4-dicarboxylate transporter	0.40	(125)
81176_0122	*aspA*	aspartate ammonia-lyase	0.41	(125)
81176_0561	*oorA*	2-oxoglutarate:acceptor oxidoreductase	0.41	
81176_1693	*rplX*	50S ribosomal protein L24	0.42	
81176_0471	*cfrB*	enterobactin receptor	0.42	(162)
81176_0432	*frdC*	fumarate reductase, cytochrome b subunit	0.42	(147)
81176_0291		putative endoribonuclease L-PSP	0.43	

81176_0478	*thiC*	thiamine biosynthesis protein	0.45	
81176_1600	*chuZ*	heme oxygenase	0.45	(165)
81176_1580	*rplsK*	30S ribosomal protein S11	0.45	
81176_0211		possible transferrin transport protein	0.45	(156)
81176_1160		LOS biosynthesis	0.45	
81176_1162		LOS biosynthesis	0.46	
81176_0128		putative periplasmic protein	0.46	
81176_0465	*mfrE*	methylmenaquinol:fumurate reductase protein E	0.47	(148)
81176_0210		possible transferrin transport protein	0.47	
81176_0758		putative periplasmic protein	0.47	
81176_0066		putative cytochrome c	0.48	
81176_0655	*hypE*	hydrogenase isoenzyme formation protein	0.50	

Table 3. Complete list of genes differentially expressed in *C. jejuni* 81-176 Sm^R Δ*Cjj1483* compared to WT *C. jejuni* 81-176 Sm^R. Expression of genes was increased or decreased by two-fold in the *C. jejuni* Δ*Cjj1483* mutant.

found that Class I genes include *chuC* (encoding a transport protein for hemin), *Cjj81176_0438* and *Cjj81176_0439* (both of which encode subunits of the gluconate dehydrogenase complex that is required for colonization of chicks (269)), and *Cjj81176_0064* (encoding a putative cytochrome c protein). Mutation of Cjj1484 and Cjj1483 resulted in a 2- to 7-fold increase in expression of these genes as shown by qRT-PCR (Figure 4).

Class II genes included *Cjj81176_0210* and *Cjj81176_0211* (encoding putative transport proteins for transferrin), *exbB2* and *exbD2* (encoding putative transport proteins for enterobactin; (163)), *peb3* (encoding a glycoprotein that may function as an adhesin or transport protein; (279-281)), and *Cjj81176_0063* (encoding a protein of unknown function). Compared to the WT strain, mutation of the Cjj1484 HK resulted in 2.5- to 5-fold decreases in expression of most of these genes, but mutation of the Cjj1483 RR resulted in 2- to 7-fold increases in expression by qRT-PCR (Figure 4). These results suggest that the RR has a repressive effect on transcription, whereas the HK may function to remove repression, likely by acting on the Cjj1483 RR.

Class III genes included *Cjj81176_1385* and *Cjj81176_1386*, which encode two hypothetical proteins of unknown function. For these genes, mutation of the Cjj1483 RR resulted in a 2- to 5-fold

increase in expression, suggesting that the RR functions as a repressor for these genes (Figure 4). However, mutation of the Cjj1484 HK did not affect transcription of these genes. This finding may indicate that the RR functions independently of the HK to affect expression of these genes.

Class IV genes are exemplified by *ciaC*, encoding a protein required for invasion (289). For *ciaC*, transcription was reduced 2- to 2.5-fold by mutation of both the Cjj1484 HK and the Cjj1483 RR, indicating that the TCS had a positive effect on transcription of this gene.

Phenotypic analysis of the *C. jejuni* Δ*Cjj1484* and Δ*Cjj1483* mutants

In our transcriptome analysis of the *C. jejuni* Δ*Cjj1484* or Δ*Cjj1483* TCS mutants, genes encoding various iron-binding and transport systems were found to be dysregulated in the mutants relative to the WT strain. For example, *chuC* was expressed at higher levels than WT in both TCS mutants, whereas expression of *Cjj0210*, *Cjj0211*, *exbB2*, and *exbD2* was reduced in the mutant lacking the Cjj1484 HK, but increased in the mutant lacking the Cjj1483 RR. Due to these findings, we investigated whether the TCS mutants demonstrated altered growth in Mueller-Hinton (MH) broth with varying levels of iron. In normal MH broth at 37 °C in microaerobic conditions, both the Δ*Cjj1484* and Δ*Cjj1483* mutants grew equally well as the WT *C. jejuni* strain over the course of 32 h (Figure 5A). In MH broth supplemented with 40 μM Fe_2SO_4, we noted a slight enhancement of growth of the Cjj1483 RR mutant relative to the WT strain over the course of the assay that was statistically significant (Figure 5B). In contrast, the Cjj1484 HK mutant did not consistently demonstrate increased growth relative to the WT strain in this high iron condition. In MH broth containing 20 μM of the iron chelator deferoxamine mesylate (DFO) that stimulated iron-limiting conditions, neither the Δ*Cjj1484* mutant nor the Δ*Cjj1483* mutant consistently demonstrated a growth difference compared to the WT strain (Figure 5C). Other than a small enhancement of growth in the Cjj1483 RR mutant in high iron conditions, dysregulation of expression of iron transport systems does not significantly impact the ability of the Cjj1484-Cjj1483 TCS mutants to grow *in vitro*.

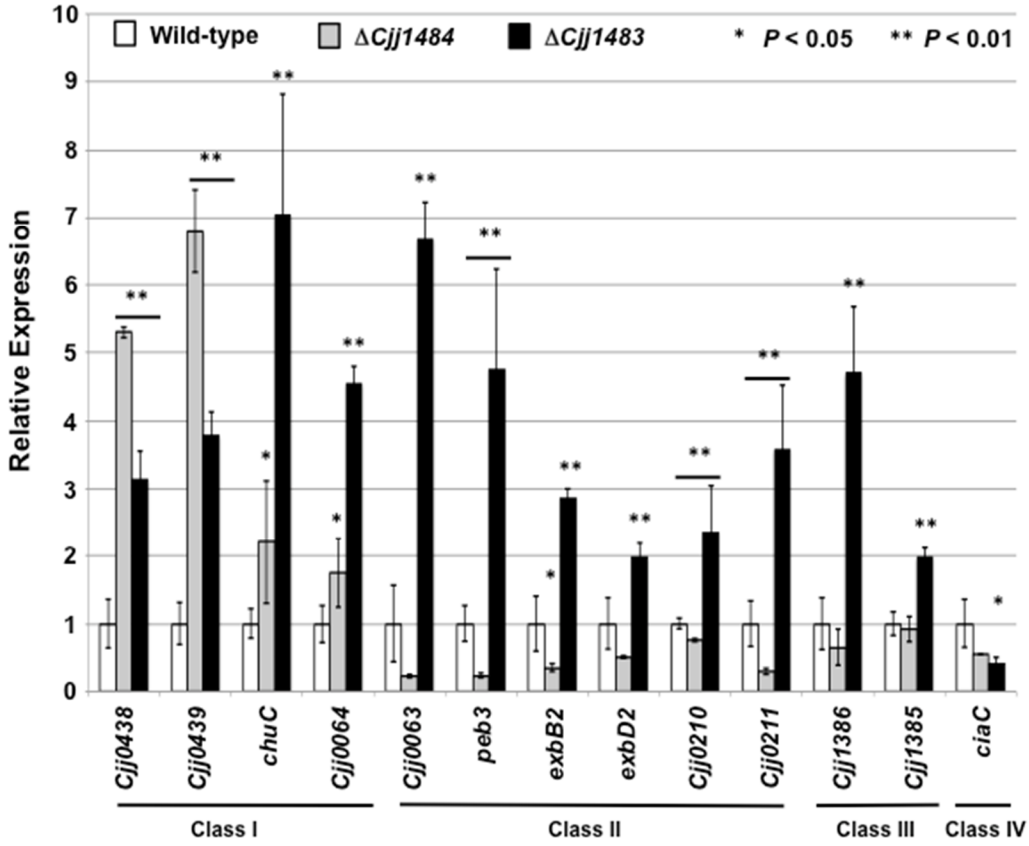

Figure 4. Semi-quantitative real-time PCR analysis of transcription of a subset of genes initially identified by microarray analysis of WT and mutant *C. jejuni* strains. The expression of each gene in the WT strain (white) as measured by qRT-PCR was set to 1. Expression of each gene in the Δ*Cjj1484* (grey) and Δ*Cjj1483* (black) mutants is shown relative to WT *C. jejuni*. All strains were examined in triplicate and the error bars indicate the standard deviation. Statistically significant differences in gene expression between WT *C. jejuni* and mutant strains is indicated (* *P*-value < 0.05; ** *P*-value < 0.01) and was determined by the Student's *t*-test. Bars indicate instances where gene expression in both Δ*Cjj1483* and Δ*Cjj1484* were significantly different than WT *C. jejuni*.

Due to increased *in vitro* expression of *Cjj0438* and *Cjj0439*, which both encode components of a *C. jejuni* gluconate dehydrogenase complex (269), in each of our TCS mutants, we analyzed whether this increased transcription translated to increased gluconate dehydrogenase activity in cellular lysates. For this analysis, we compared gluconate dehydrogenase activity in WT *C. jejuni* and the Cjj1484 HK mutant, which was observed to have an approximately 5- to 7-fold increased transcription of *Cjj0438* and *Cjj0439*. In our assays, we found that the Δ*Cjj1484* mutant did indeed demonstrate an 80 % increase in

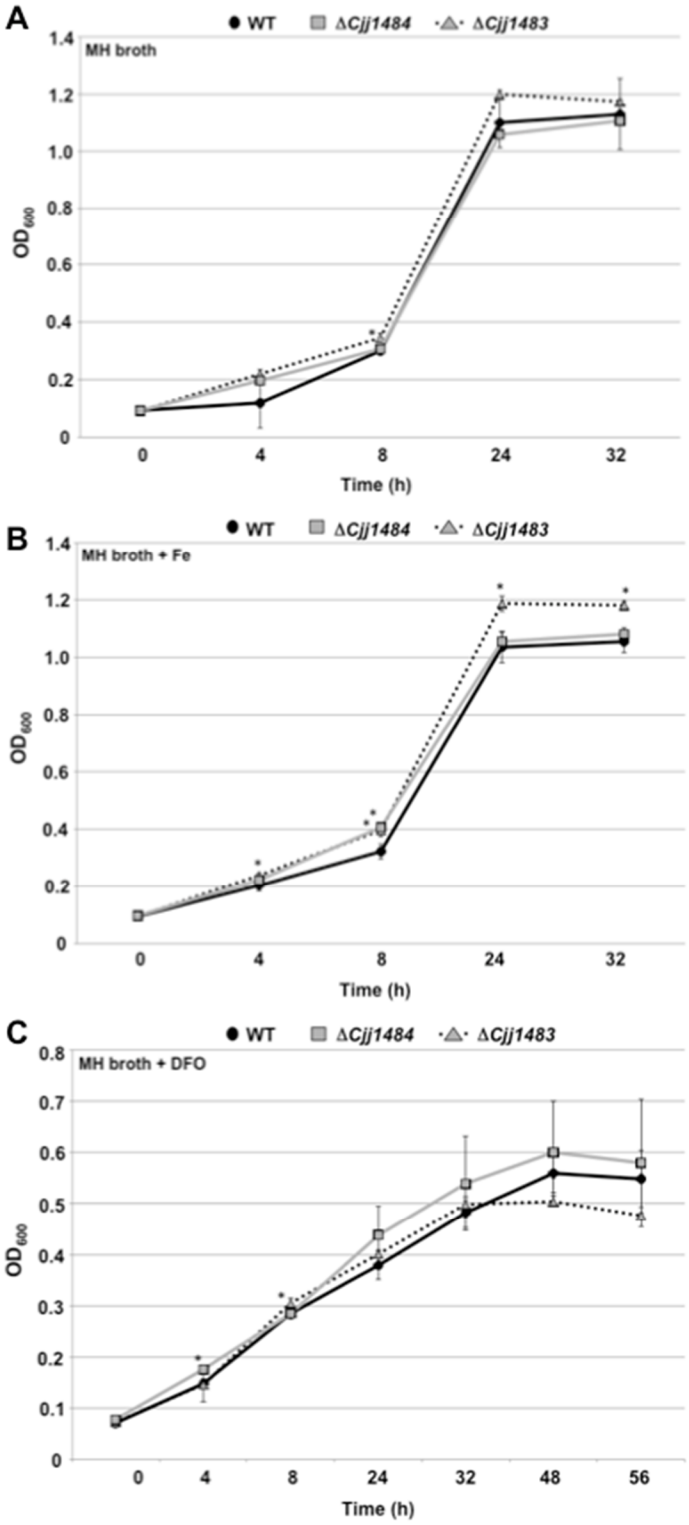
A
WT
ΔCjj1484
ΔCjj1483
MH broth
OD600
Time (h)
B
MH broth + Fe
C
MH broth + DFO

Figure 5. Analysis of growth of WT and mutant *C. jejuni* strains. *C. jejuni* strains were grown in (A) Mueller-Hinton (MH) alone, or MH broth containing (B) 40 μM Fe_2SO_4 to simulate high iron levels or (C) 20 μM of deferoxamine mesylate (DFO), an iron chelator, to simulate iron-depleted conditions. Strains were grown in microaerobic conditions at 37 °C for 32-56 h. All strains were analyzed in triplicate and the data are presented as an average of OD_{600} readings for strains at each timepoint. Error bars indicate standard deviations. Asterisks (*) indicate data points of mutants that are statistically significant ($P < 0.05$) than the WT strain.

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gluconate dehydrogenase activity (Table 4). Thus, disruption of the Cjj1484-Cjj1483 TCS caused aberrant gluconate dehydrogenase activity in *C. jejuni*.

We also observed that expression of *flaA*, encoding the major flagellin required for flagellar motility of *C. jejuni* was reduced approximately 3-fold in the Δ*Cjj1484* mutant (Table 2). However, we did not detect any motility defects in strains lacking the Cjj1484 HK or the Cjj1483 RR in motility agar (data not shown), indicating that disruption of the TCS does not significantly impact the motility of *C. jejuni*.

Analysis of phosphotransfer through the Cjj1484-Cjj1483 TCS and effects on gene expression

Bioinformatic analysis indicates that Cjj1484 contains domains typical of many bacterial HKs (Figure 6A). A PAS9 domain that may function as a specific sensor domain for Cjj1484 is located within the N-terminal 120 residues. Bioinformatic analysis of this domain reveals some conservation of residues for a heme-binding pocket. However, due to an inability to purify sufficient levels of Cjj1484, it is unclear if the PAS domain senses heme or a related compound. This domain is followed by a region that would be expected to form the dimerization and histidine phosphotransfer (DHp) domain in most bacterial HKs. This domain usually contains the conserved histidine residue that is autophosphorylated upon sensing a signal (247). While this domain in Cjj1484 is only weakly predicted, H195 is the best candidate for a histidine residue to be modified by autophosphorylation. An HATPase domain that typically binds ATP for the autophosphorylation reaction of HKs is present at the C-terminus (247). No transmembrane

Strain	Activity[a]
81-176 WT	5.61 +/- 1.55
81-176 Δ*Cjj1484*	10.05 +/- 3.35[b]

Table 4. Gluconate dehydrogenase activity in *C. jejuni* 81-176 Sm^R and *C. jejuni* Sm^R Δ*Cjj1484*. [a] Lysates of strains after microaerobic growth at 37 °C were analyzed by a coupled enzyme assay. The amount of gluconate dehydrogenase is reported as nmol of DCIP reduced per minute per mg of protein in the lysates. Data represent the average of three assays with each strain analyzed in triplicate. [b] Indicates statistical significance compared to the WT strain ($P < 0.05$).

domains are apparent within Cjj1484 to suggest that it is linked to the inner membrane of *C. jejuni*. Thus, Cjj1484 is most likely a cytoplasmic HK.

In a prototypical TCS, mutation of the phosphoaccepting histidine of the HK disrupts autophosphorylation and prevents subsequent phosphotransfer to the cognate RR. Considering this mechanism, we mutated H195 to monitor whether this residue is autophosphorylated to serve as the phosphodonor for Cjj1483. However, WT Cjj1484 is largely undetectable in whole-cell lysates, making it difficult to assess whether $Cjj1484_{H195A}$ is stable in *C. jejuni* (Figure 6B). Therefore, we analyzed production of WT Cjj1484 and $Cjj1484_{H195A}$ overexpressed *in trans* from the *flaA* promoter in *C. jejuni* Δ*Cjj1484*. Although we were able to detect overexpressed WT Cjj1484, we were unable to detect $Cjj1484_{H195A}$ upon expression from the same *flaA* promoter (Figure 6B). We conclude that mutation of H195 likely creates an unstable Cjj1484 protein and this point mutant was not further analyzed.

Cjj1483 displays features common to many bacterial RRs. The first 120 residues compose a receiver domain with D58 as the most likely aspartate residue that is phosphorylated by phosphotransfer from the Cjj1484 HK or other potential phosphodonors (Figure 6A). At the C-terminus of Cjj1483 is a predicted wing-helix DNA-binding domain that likely facilitates interactions between the RR with target promoters. For many RRs, mutation of the conserved phosphoaccepting aspartic acid to an alanine or asparagine prevents phosphotransfer and alters function. In some RRs, alteration of the aspartate to a glutamate mimics a phosphoaspartate, resulting in RR activities associated when the RR is naturally phosphorylated by its cognate HK (291). However, in other response regulators, this alteration

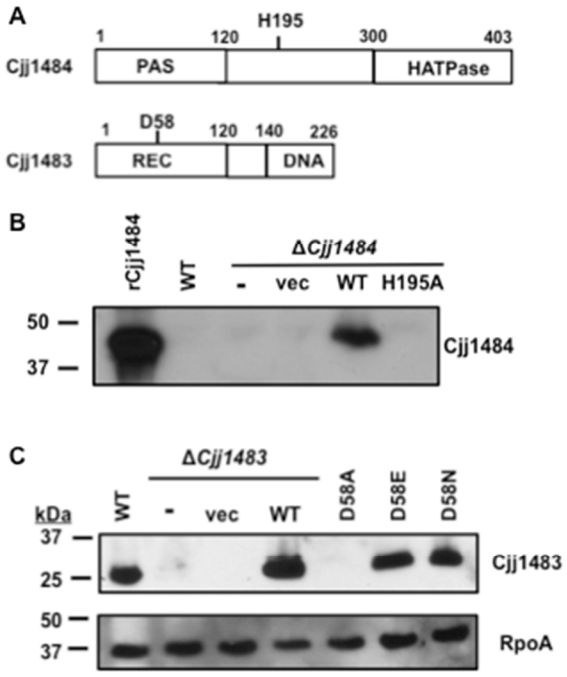


Figure 6. Construction and stability of Cjj1484-Cjj1483 TCS point mutants. (A) Putative domain organization of the Cjj1484 HK and the Cjj1483 RR. Predicted domains were identified by BLAST analysis. Conserved residues that are predicted to participate in phosphotransfer for each protein are indicated. The Cjj1484 HK is predicted to contain a PAS sensory domain and a C-terminal HATPase domain for binding and hydrolysis of ATP. Cjj1484 does not have a strongly predicted dimerization and histidine phosphotransfer (DHp) domain, but H195 is the most likely histidine that is modified by autophosphorylation. The Cjj1483 RR is predicted to contain an N-terminal REC domain common to many response regulators and a C-terminal winged-helix DNA-binding domain. (B) Immunoblot analysis of WT Cjj1484 and Cjj1484$_{H195A}$ in *C. jejuni*. Specific antiserum to recombinant Cjj1484 (rCjj1484) was generated and then used to analyze production of WT and mutant Cjj1484 proteins in *C. jejuni* whole-cell lysates. *C. jejuni* Δ*Cjj1484* was complemented *in trans* with a plasmid expressing WT *Cjj1484* or *Cjj1484*$_{H195A}$ (H195A) from the promoter for *flaA* (encoding the major flagellin of *C. jejuni*) to overexpress the proteins. In addition, Δ*Cjj1484* was not complemented (-) or complemented with vector alone (vec). (C) Immunoblot analysis of WT Cjj1483 or Cjj1483 with various mutations at D58 in *C. jejuni* whole-cell lysates. *C. jejuni* Δ*Cjj1483* was complemented *in trans* with a plasmid expressing WT *Cjj1483* from its native promoter as shown in Figure 1A. In addition, Δ*Cjj1483* was not complemented (-) or complemented with vector alone (vec). For the Cjj1483$_{D58A}$, Cjj1483$_{D58E,}$ and Cjj1483$_{D58N}$ mutants, genes encoding each mutation replaced the WT *Cjj1483* gene on the chromosome of *C. jejuni* so that the genes were expressed from native promoters. Detection of RpoA served as a loading control for the whole-cell lysates.

inactivates the protein (similar to an alanine mutation) causing the RR to constitutively function as it does in the unphosphorylated state.

Similar to our observations above, WT Cjj1483 was easily detected in whole-cell lysates and its production was restored in a Δ*Cjj1483* mutant upon expression of the gene *in trans* from its native promoter (Figure 6C). However, we were unable to detect Cjj1483$_{D58A}$ when the respective allele replaced WT *Cjj1483* at the native locus. In contrast, Cjj1483$_{D58E}$ and Cjj1483$_{D58N}$ were stable and produced at WT levels in *C. jejuni* (Figure 6C). As such, these latter mutant Cjj1483 proteins were used in all subsequent studies.

We first attempted to biochemically analyze the potential phosphotransfer mechanism through the Cjj1484-Cjj1483 TCS. We were able to abundantly purify WT Cjj1483, Cjj1483$_{D58E}$, and Cjj1483$_{D58N}$ as soluble proteins from *E. coli*. However, we were unable to consistently express and purify a soluble form of the WT Cjj1484 HK. Therefore, we were unable to perform autokinase assays with Cjj1484 and subsequent phosphotransfer assays to Cjj1483. To determine whether D58 was the potential phosphoaccepting residue of Cjj1483, we performed autophosphorylation assays with radiolabelled acetyl-phosphate (AcP). WT Cjj1483 was modified by autophosphorylation with AcP, but both Cjj1483$_{D58E}$ and Cjj1483$_{D58N}$ were not (Figure 7A). These findings strongly suggest that D58 is the phosphoaccepting aspartate of the Cjj1483 RR.

With this in mind, we analyzed gene expression in *C. jejuni* 81-176 *Cjj1483*$_{D58E}$ and *Cjj1483*$_{D58N}$ mutants by qRT-PCR. Compared to the Δ*Cjj1483* mutant, Cjj1483$_{D58E}$ and Cjj1483$_{D58N}$ repressed transcription of *chuC*, *Cjj1386*, and *exbB2* similar to WT Cjj1483 (Figure 7B). Since both mutant proteins demonstrated the same activity, we assume that these proteins are both mimicking the unphosphorylated state of Cjj1483 in an activity to repress expression of these genes. Thus, these results together suggest that Cjj1483 likely binds these promoters to repress transcription in *C. jejuni* in the absence of phosphorylation.

In contrast, Cjj1483$_{D58E}$ and Cjj1483$_{D58N}$ had different effects on expression of *Cjj0438*. We found that *Cjj0438* expression was increased 5-fold in the D58E mutant, similar to the Δ*Cjj1483* mutant

(Figure 7B). However, the level of expression of *Cjj0438* in *C. jejuni* 81-176 *Cjj1483*$_{D58N}$ was similar to that of the WT strain. We envision two possible interpretations of these results. In one case, Cjj1483$_{D58E}$ might be mimicking a phosphorylated version of the protein in *C. jejuni* that cannot specifically repress transcription from the *Cjj0438* promoter. Alternatively, mutation of D58 to glutamate may disrupt the binding activity of Cjj1483 RR to the *Cjj0438* promoter, but not to other target promoters.

Colonization ability of mutants lacking genes within the Cjj1484-Cjj1483 TCS regulon

As we showed above, the *Cjj1484-Cjj1483* TCS is required to repress expression of the *Cjj0438-0439* operon, which encodes a gluconate dehydrogenase complex required for WT levels of commensal colonization of the chick ceca (269). Having identified more members of the Cjj1483 regulon, we assessed if any other genes repressed by the RR are required for colonization of chicks. We generated mutants in *chuC, exbB2, Cjj0064,* and *Cjj1386* in *C. jejuni* 81-176 Sm^R. Chicks were infected with WT *C. jejuni* 81-176 Sm^R or the isogenic mutants with an inoculum of 10^2 cfu. Chicks were then sacrificed at day 7 post-infection and the levels of *C. jejuni* were enumerated in the ceca of chicks. In this assay, WT *C. jejuni* colonized between 2.7 x 10^7 to 1.17 x 10^9 cfu per g cecal content with a geometric mean of 2.95 x 10^8 cfu per g cecal content (Figure 8). Mutants lacking *exbB2, Cjj0064* or *chuC* colonized chicks at levels similar to WT *C. jejuni*. Although the colonization levels did not reach statistical significance, we did notice a modest 3.5-fold decrease in colonization for the *Cjj1386* mutant.

DNA-binding activity of the Cjj1483 RR to the *Cjj0438* promoter

In order to better understand the ability of Cjj1483 to influence gene expression in *C. jejuni*, we analyzed the potential DNA-binding activity of the RR for target promoters of genes within its regulon. Electrophoretic mobility shift assays (EMSAs) with recombinant WT Cjj1483, Cjj1483$_{D58E}$, and Cjj1483$_{D58N}$ and a DNA fragment encompassing the *Cjj0438* promoter were employed. As shown in Figure 9A, unphosphorylated WT Cjj1483 bound to the *Cjj0438* promoter. The amount of the DNA bound by Cjj1483 increased as the concentration of the protein increased. We also analyzed the effect of

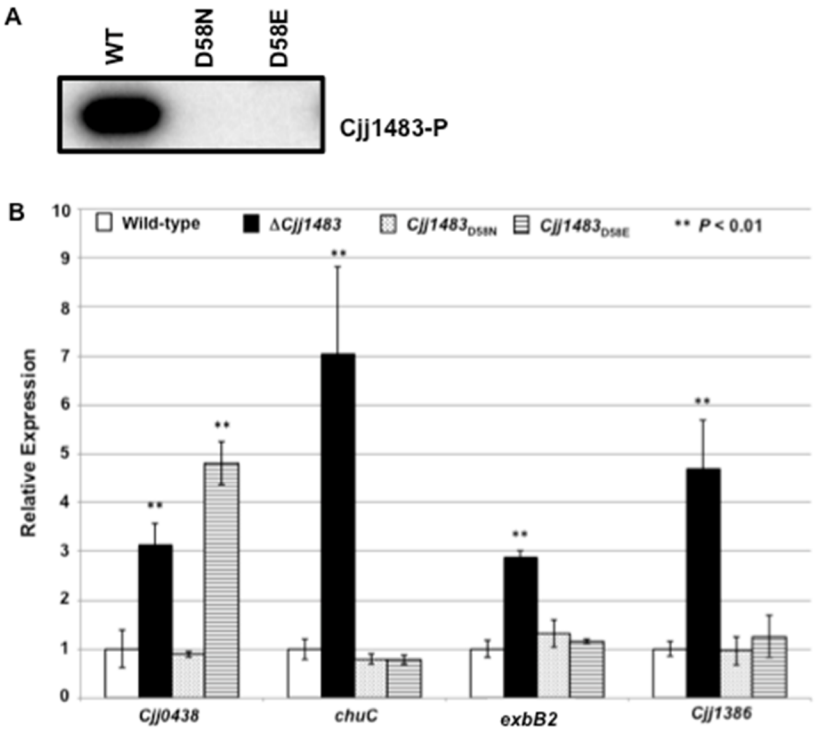


Figure 7. Analysis of Cjj1483 D58 mutants on expression of target genes. (A) *In vitro* phosphorylation assay of WT and mutant Cjj1483 RR proteins. Recombinant WT Cjj1483, Cjj1483$_{D58E}$, and Cjj1483$_{D58N}$ proteins were incubated with Ac[^{32}P] for 20 minutes, separated by SDS-PAGE, and then exposed to a PhosphorImager. Signal indicates the ability of the WT protein, but not the Cjj1483 D58 mutants to autophosphorylate. (B) Analysis of expression of select genes within the Cjj1483 regulon in WT *C. jejuni* or isogenic mutants producing Cjj1483$_{D58E}$ or Cjj1483$_{D58N}$ mutant proteins. The expression of each gene in the WT strain (white) as measured by qRT-PCR was set to 1. Expression of each gene in Δ*Cjj1483* (black), *Cjj1483*$_{D58N}$ (dotted bar), and *Cjj1483*$_{D58E}$ (striped bar) mutants is shown relative to WT *C. jejuni*. All strains were examined in triplicate and the error bars indicate the standard deviation. Statistically significant differences in gene expression between WT *C. jejuni* and mutant strains is indicated (** *P*-value < 0.01) and was determined by the Student's *t*-test.

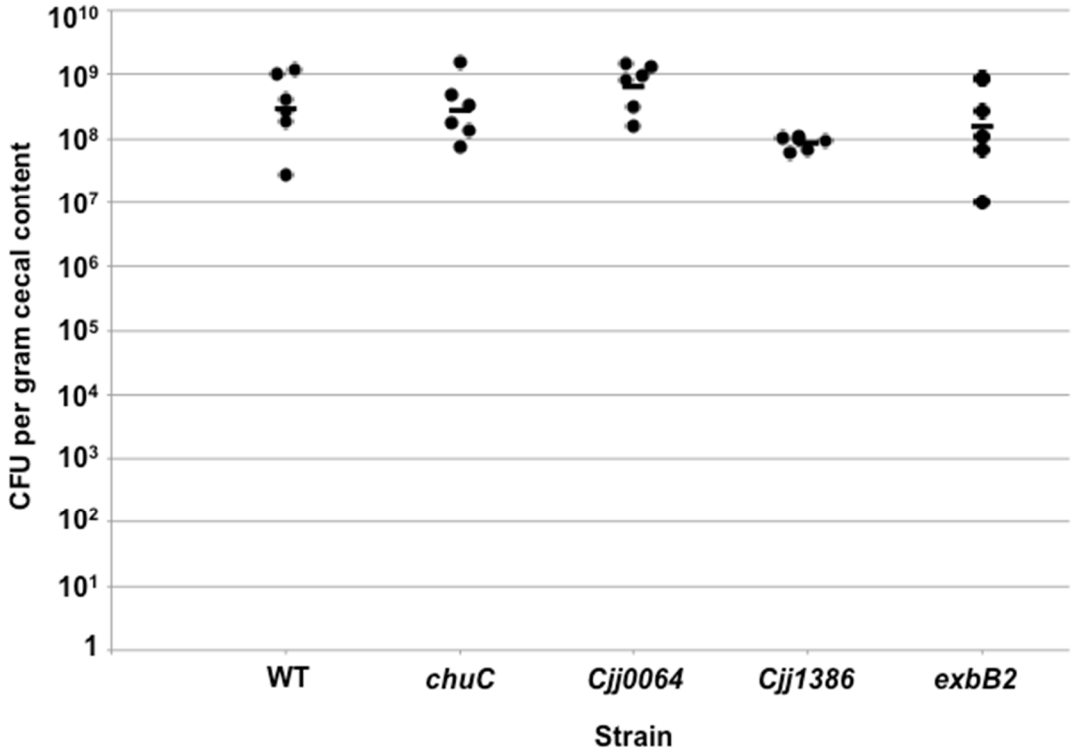


Figure 8. Commensal colonization capacity of wild-type *C. jejuni* and mutant strains lacking specific genes within the Cjj1483 regulon. One-day-old chicks were orally inoculated with approximately 10^2 cfu of WT *C. jejuni* 81-176 Sm^R or isogenic mutant strains. Each dot represents the amount of *C. jejuni* recovered from the ceca of each chick at day 7 post-infection. The geometric mean for each group is depicted by the horizontal bar. Statistical analysis was performed using the Mann-Whitney *U* test. All mutants showed no statistically significant differences.

phosphorylation of Cjj1483 on DNA binding. For this analysis, Cjj1483 was pretreated with Li-AcP for 20 minutes and used in DNA-binding assays. We did notice a slight enhancement in the DNA-binding activity of Cjj1483 relative to the unphosphorylated form, but it is unclear whether this enhanced binding is significant (Figure 9A, left). Unphosphorylated Cjj1483 did not bind to a promoter for *aphA*-3, encoding kanamycin resistance, but the phosphorylated form did demonstrate a non-specific, weak-binding ability to this promoter (Figure 9A, right). To determine whether binding to the *Cjj0438* promoter was specific, we performed DNA-binding assays with an excess of a specific competitor (unlabeled *Cjj0438* promoter) or a non-specific competitor (unlabeled *aphA-3* promoter). When Cjj1483 was not phosphorylated, DNA-binding could still be observed with a 5-fold excess of unlabeled *Cjj0438* promoter (Figure 9B, left). Furthermore, the *aphA3* promoter did not compete with *Cjj0438* for binding by unphosphorylated Cjj1483 (Figure 9B, right). When Cjj1483 was pretreated with Li-AcP to stimulate phosphorylation prior to EMSAs, Cjj1483 binding to the *Cjj0438* promoter was reduced by a two-fold excess of unlabeled DNA and completely reduced at a five-fold excess. Again, the *aphA3* promoter did not effectively compete for binding by phosphorylated Cjj1483 (Figure 9B, right). Results from these competitive binding assays may indicate that the Cjj1483 RR can bind in an unphosphorylated and phosphorylated form, but the phosphorylated form may have a specific, but reduced binding affinity to target promoters. As a note, binding by the phosphorylated Cjj1483 RR occurred with other non-specific DNA that we analyzed (data not shown). This observation indicates that analysis of *in vitro* DNA binding by the Cjj1483 RR may be problematic in terms of assessing or interpreting with confidence the degree of binding specificity.

We next examined the DNA-binding ability of $Cjj1483_{D58E}$ and $Cjj1483_{D58N}$ to *Cjj0438*. Compared to WT Cjj1483, $Cjj1483_{D58E}$ was unable to bind the *Cjj0438* promoter, but $Cjj1483_{D58N}$ did appear to bind this DNA (Figure 9C). Considering that $Cjj1483_{D58E}$ was unable to repress expression of *Cjj0438* in *C. jejuni* (Figure 7B), we propose that this mutation may be mimicking a constitutive phosphorylated state and that WT Cjj1483 likely has full DNA-binding ability to repress expression of *Cjj0438* in an unphosphorylated form. In support of this hypothesis, $Cjj1483_{D58N}$ bound to the *Cjj0438*

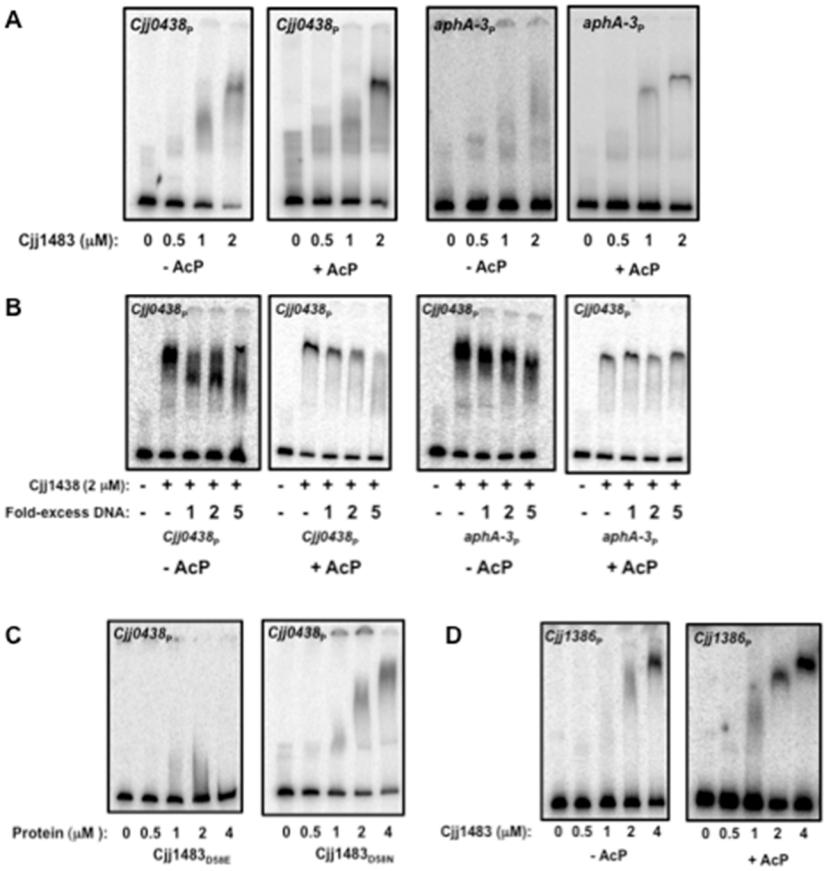


Figure 9. Electrophoretic mobility shift assays for analysis of DNA-binding activity of the Cjj1483 RR. For (A and B), purified WT Cjj1483 protein was added to various promoter DNAs at concentrations ranging from 0-2 μM. For (C and D), WT Cjj1483 or Cjj1483$_{D58E}$ and Cjj1483$_{D58N}$ mutant proteins were added at concentrations ranging from 0-4 μM. For (A, B, and D), '-AcP' and '+AcP' indicates whether or not Cjj1483 was pretreated with Li-AcP to autophosphorylate the protein prior to addition to DNA-binding assays. (A) Binding of Cjj1483 to the radiolabeled promoters for *Cjj0438* or *aphA-3* in the absence of competition. (B). Binding of Cjj1483 to the radiolabeled promoter for *Cjj0438* in the presence of unlabeled competitor DNA (*Cjj0438* promoter; left panels) or an unlabeled non-competitor DNA (*aphA-3* promoter; right panels). (C) Binding of Cjj1483$_{D58E}$ and Cjj1483$_{D58N}$ mutant proteins to the radiolabeled promoter for *Cjj0438* in the absence of competition. (D). Binding of Cjj1483 to the radiolabeled promoter for *Cjj1386* in the absence of competition.

promoter and repressed expression of the gene (Figure 7B and 9C), suggesting that this protein mimics a constitutively unphosphorylated form that binds DNA specifically to repress gene expression.

Analysis of Cjj1483 binding to the *Cjj1386* promoter

In order to further analyze the biological activity of the *Cjj1484-Cjj1483* TCS in *C. jejuni*, we assayed the binding ability of the Cjj1483 RR to the promoter region for the previously uncharacterized *Cjj1386-Cjj1385* operon. As shown in Figure 4, deletion of *Cjj1484* did not have a strong effect on expression of either *Cjj1386* or *Cjj1385*. However, deletion of *Cjj1483* resulted in a 2- to 5-fold increase in expression of *Cjj1385* and *Cjj1386*, respectively. These data suggested that Cjj1483 may function as a repressor for this promoter independently of the putative cognate Cjj1484 HK.

We performed EMSAs with the promoter for *Cjj1386-Cjj1385* and purified Cjj1483 alone or after phosphorylation by Li-AcP. We observed that both the unphosphorylated and phosphorylated RR could bind to the promoter of *Cjj1386*, with perhaps phosphorylation promoting Cjj1483 binding to the DNA at a lower protein concentration (Figure 9D). We attempted to analyze if this binding was specific for the promoter of *Cjj1386* by analyzing the binding ability of Cjj1483 in the presence of excess unlabeled *Cjj1386* promoter DNA and non-specific *aphA3* promoter DNA. However, neither DNAs in excess efficiently competed for binding (data not shown). Regardless, our collective data are consistent with the Cjj1483 RR repressing transcription from the *Cjj1386-1385* promoter in *C. jejuni* in either an unphosphorylated or phosphorylated state that is independent of the Cjj1484 HK. It is likely that Cjj1483, which can be expressed independent of Cjj1484 from a monocistronic transcript (Figure 2B), is periodically produced without the Cjj1483 HK and can influence gene expression in an unphosphorylated state or with phosphorylation originating from a non-cognate HK or phosphodonor.

Discussion

In this work, we investigated one of the remaining uncharacterized putative TCS of *C. jejuni* encoded by *Cjj1484* and *Cjj1483* on the *C. jejuni* 81-176 genome. Our findings include evidence that the

Cjj1484 HK and the Cjj1483 RR likely function as a cognate TCS to influence expression of a common set of genes. In addition, we provide evidence that Cjj1483 influences expression of genes independently of its cognate Cjj1484 HK. In either case, the TCS or Cjj1483 alone appear to largely repress transcription of genes under *in vitro* growth conditions. Most genes whose expression is controlled by the Cjj1484-Cjj1483 TCS encode proteins that function in various metabolic processes including heme and/or iron uptake and respiration. In addition, this TCS represses transcription of one known colonization factor, the gluconate dehydrogenase complex (269). Biochemcial analysis allowed us to make predictions about the DNA-binding activity of Cjj1483 to target promoters in relation to its phosphorylation state. Although the Cjj1484-Cjj1483 TCS was not required for commensal colonization of the natural avian host, we suspect that the system could play a role with other transcriptional regulators in finely controlling transcription of metabolic genes that may be important *ex vivo* and during transmission from one host to another.

The regulons for Cjj1484 and Cjj1483 were found to contain many overlapping genes. Our findings strongly suggests that the Cjj1484 HK and the Cjj1483 RR function as a cognate TCS. Further support for these factors forming a cognate TCS includes their apparent operonic organization on the *C. jejuni* chromosome. Complete verification would require biochemical analysis to reveal specific phosphotransfer from Cjj1484 to Cjj1483. We attempted to perform such an analysis, but we were unable to purify the Cjj1484 HK in a soluble state in sufficient quantities. We also acquired evidence that transcription of certain genes, such as *Cjj1386* and *Cjj1385*, are specifically influenced by the Cjj1483 RR alone, but were unaffected by mutation of the Cjj1484 HK. Additionally, we identified a promoter within the *Cjj1484* coding sequence that expresses *Cjj1483* without the HK. This finding gives credence that the Cjj1483 RR may be produced without the HK in certain situations to influence expression of specific genes independently of the Cjj1484 HK. Therefore, while Cjj1483 appears to have a classical function as a RR in a cognate TCS with influence from its cognate HK for regulation of specific genes, the RR appears to be expressed and function independently of the HK in controlling expression of other genes.

We experienced several difficulties in performing a complete biochemical and genetic analysis of the Cjj1484-Cjj1483 TCS, which may indicate some unusual features of this TCS compared to other

systems. First, the WT Cjj1484 HK could only be detected in *C. jejuni* upon overexpression of the gene *in trans*, indicating that the HK is either produced at extremely low levels or is rapidly turned over. Second, we could not absolutely prove that H195 is the histidine residue that is modified by autophosphorylation upon sensing a stimulus. Mutation of this residue resulted in a protein that was undetectable in *C. jejuni* even upon overexpression. For the Cjj1483 RR, we showed that mutation of D58 to an asparagine or glutamate prevented the protein from autophosphorylating by using radiolabelled AcP as a phosphodonor. Curiously, mutation of D58 of Cjj1483 to an alanine resulted in an unstable protein that could not be detected. In many RRs, this mutation does not cause instability. Currently, it is unclear whether this mutational analysis and resultant stability issues for the Cjj1484-Cjj1483 TCS implies a significantly altered biochemistry of signal perception, phosphotransfer, and transduction compared to other TCSs.

By combining results from expression analysis and DNA-binding assays using WT and mutant TCS proteins, we are able to propose four putative models for how the Cjj1484-Cjj1483 TCS influences expression of different classes of genes depending on the phosphorylation state and activities of the Cjj1484 HK and Cjj1483 RR (Figure 10). We propose that for the class I genes, which include the *Cjj0438* operon, the *Cjj0063* operon, and *chuC*, the Cjj1484 HK and the Cjj1483 RR function together to repress expression of these genes. For these genes, we propose that the Cjj1483 RR in an unphosphorylated state binds promoter DNA to repress gene expression and the Cjj1484 HK may have a phosphatase activity to maintain Cjj1483 in an unphosphorylated state. This model would explain why mutation of *Cjj1484* causes derepression of expression of these genes similar to a mutant lacking the Cjj1483 RR. Specifically for *Cjj0438*, we noticed that mutation of the phosphorylated aspartate in Cjj1483 caused different effects on DNA binding to the *Cjj0438* promoter and expression of the gene. $Cjj1483_{D58N}$ bound the *Cjj0438* promoter DNA effectively, but $Cjj1483_{D58E}$ did not. In addition, $Cjj1483_{D58N}$ repressed expression of *Cjj0438,* but $Cjj1483_{D58E}$ did not. We interpret these data as suggesting that $Cjj1483_{D58N}$ likely mimics an unphosphorylated state that can still bind DNA to repress gene expression, but $Cjj1483_{D58E}$ may function as a constitutively-phosphorylated protein that is unable to bind the *Cjj0438* promoter and repress expression. Curiously, we did observe that $Cjj1483_{D58E}$ was still

able to repress expression of *chuC*, which may indicate that alteration of the aspartate residue by certain mutations ultimately affects the ability of the RR to recognize and bind different promoters. Also, as further discussed below, other regulators affect expression of *chuC*, which may have an influence on the ability of Cjj1483$_{D58E}$ to repress expression of this gene.

For the class II genes, which include *Cjj0063*, *peb3*, and the *exbB2* and *Cjj0210* operons, we found that the mutation of the Cjj1484 HK resulted in repression of gene expression, but expression of the genes in the Cjj1483 RR mutant was derepressed. We propose that like *Cjj0438* and *chuC*, Cjj1483 in the unphosphorylated form may mediate repression. This repression may be relieved by Cjj1484 promoting phosphorylation of the RR (Figure 10). This model would explain how the lack of Cjj1483 phosphorylation in the HK mutant may lead to increased repression of these genes.

For *Cjj1386* operon, which composes class III genes, we did not find any evidence that the Cjj1484 HK was involved in expression of this operon. However, in our DNA-binding assays, we noted that the both unphosphorylated and phosphorylated Cjj1483 RR bound *Cjj1386* promoter DNA, with perhaps the phosphorylated form of Cjj1483 binding DNA at a lower protein concentration. Regardless, we propose that the Cjj1483 RR can repress gene expression independently of the HK, with a potential non-cognate phosphodonor possibly being used to autophosphorylate the Cjj1483 RR and enhance its DNA binding (Figure 10). Lastly, we provided evidence that both Cjj1484 and Cjj1483 were required for WT levels of expression of the class IV gene *ciaC*, suggesting that phosphotransfer through the TCS resulting in phosphorylation of Cjj1483 is likely a transcriptional activator for *ciaC* (Figure 10). In all, our data suggest various modalities for how the Cjj1484-Cjj1483 can influence expression of different sets of genes.

In this work, we identified genes within the Cjj1484-Cjj1483 TCS regulon and provided some evidence for direct regulation by the TCS. Genes within this regulon encode proteins involved in different metabolic activities such as gluconate respiration (Cjj0438 and Cjj0439), heme uptake (ChuC), ferric enterobactin transport (ExbB2 and ExbD2), and a possible transferrin transport system (Cjj0210 and Cjj0211). Although we did not observe any large differences in the ability of *C. jejuni* mutants lacking the

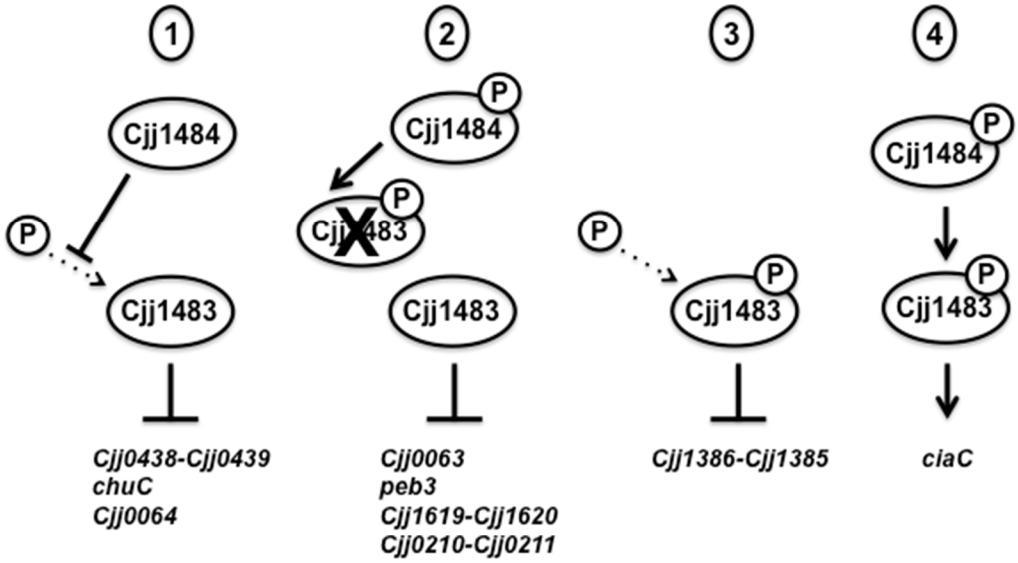


Figure 10. Putative models for how the Cjj1484-Cjj1483 TCS may mediate regulation of different classes of *C. jejuni* genes. For models 1-3, Cjj1483 is proposed to function as a transcriptional repressor whereas for model 4, Cjj1483 likely functions as a transcriptional activator. In each model, it is indicated whether Cjj1483 must be phosphorylated to mediate transcriptional regulation and how phosphorylation or prevention of phosphorylation may occur. Dotted arrows indicate phosphorylation that may occur through non-cognate phosphodonors. In model 1, Cjj1484 is predicted to serve as a phosphatase to maintain Cjj1483 in an unphosphorylated state, whereas in model 2, Cjj1484 is predicted to phosphorylate Cjj1483 to inhibit its repressive activity. In model 3, the Cjj1484 HK plays no role in gene expression. In this model, phosphorylation of Cjj1483 via non-cognate phosphodonors may mediate repression by the response regulator. In model 4, phosphorylation of the Cjj1483 RR via Cjj1484 appears to be required for Cjj1483 to function as a transcriptional activator. See Discussion for more details.

TCS to grow in iron-rich or iron-depleted conditions, we did observe increased gluconate dehydrogenase activity in a mutant lacking the Cjj1484 HK, suggesting at least one physiological consequence of an impaired Cjj1484-Cjj1483 TCS. Currently the functions of two other members, Cjj1385 and Cjj1386, are unknown. In bacteria, it is common for expression of heme and iron acquisition systems or respiration to be moderately influenced by diverse regulatory systems. We propose that regulation of these genes is likely complex in *C. jejuni*. Indeed, in searching the *C. jejuni* literature, we found that expression of these genes is influenced by multiple regulatory factors or growth conditions. For instance, previous studies have shown that expression of many genes within the Cjj1484-Cjj1483 regulon is reduced in high iron conditions and hyperosmotic shock, but increased in acidic pH (292-294). In addition, the ferric uptake regulator (Fur), which monitors the iron status of the bacterium, largely represses expression of these genes (295). For *Cjj0210-Cjj0211*, *chuC*, *exbB2*, and *exbD2*, evidence exists that the peroxide regulator (PerR) also influences expression of these genes (174). A recent study of the RacRS TCS of *C. jejuni* revealed that *chuC*, *Cjj0210-Cjj0211*, and *Cjj1385-Cjj1386* expression were all decreased in a *racR* mutant (250). However, expression of the Cjj1484-1483 TCS, which is the subject of this work, was significantly higher in the *racR* mutant (250). Further investigation was unable to demonstrate that RacR interacted specifically with the promoters for *chuC*, *Cjj0210-Cjj0211*, or *Cjj1385-Cjj1386*, creating some speculation whether the RacRS TCS may only indirectly regulate expression of these genes. In our current work, we demonstrated that the Cjj1483 RR directly bound to the promoter of *Cjj1386*, indicating a strong possibility that the RacRS TCS may mediate regulation of expression of this gene and possibly others through the Cjj1484-Cjj1483 TCS. Considering these other global transcriptome analyses in *C. jejuni*, it is likely that expression of genes within the Cjj1484-Cjj1483 regulon is multifactorial with multiple regulators finely controlling expression of these genes to an appropriate level that benefits the biology of *C. jejuni* in different situations.

We were unable to demonstrate that Cjj1484-Cjj1483 TCS was required for commensal colonization of the natural avian host. However, this TCS *in vitro* largely represses expression of genes, such as *Cjj0438* and *Cjj0439* that are required to be expressed *in vivo* for WT levels of colonization of

chicks. Thus, mutations of Cjj1484 or Cjj1483 cause enhanced transcription of *Cjj0438, Cjj0439* and other possible colonization factors that are within the Cjj1484-Cjj1483 regulon. Results from our work suggest that an aberrant increase in expression of genes caused by mutation of the Cjj1484-Cjj1483 TCS does not appear to negatively affect the ability of *C. jejuni* to promote colonization of chicks. Although the data did not meet the statistical significance, we did note that *Cjj1386* had a 3.5-fold decrease in colonization. This finding may indicate that the *Cjj1386* operon encodes proteins that modestly impact colonization. Although the Cjj1484-Cjj1483 TCS does not appear to be required for colonization, it is possible that this TCS could play an important role in repressing expression of colonization factors when the bacterium is outside a host. This model would suggest that *in vivo*, the repressive activity of the TCS is likely decreased so that expression of *Cjj0438* and *Cjj0439* is sufficient to promote colonization. Further studies will be required to determine if the TCS has an important role in regulating expression of genes *ex vivo* that may assist the bacterium in surviving outside a host or prime the bacterium for transmission to a new host. Furthermore, it is possible that this TCS could play an important role in regulating expression of genes required for infection of humans to promote diarrheal disease.

CHAPTER FOUR

Short-chain Fatty Acids Influence Transcription of *Campylobacter jejuni* Determinants Required for Commensal Colonization and Virulence

Introduction

Dietary nutrients fuel metabolic reactions of gut microbiota in hosts. One common reaction performed by gut bacteria is the fermentation of carbohydrates into organic and short-chain fatty acids (SCFAs). Due to the residence of bacterial species in different regions of the intestinal tract that produce or utilize these metabolites, spatial gradients of these organic acids and SCFAs are created (185). For example, lactate-producing bacteria are often found in the upper regions of the intestinal tract whereas bacterial species that produce acetate and butyrate as end products are often in greater abundance in the lower regions of the intestinal tract and colon (179, 183). The production of these organic acids and SCFAs by the microbiota can benefit the host. For instance, the SCFA butyrate produced by the microbiota is the preferred energy source of colonocytes (196, 197). Other SCFAs such as acetate and propionate are absorbed from the intestines and serve as energy sources or substrates for lipid biosynthesis by other organs (185).

Production of the organic acid lactate or SCFAs by the host microbiota can also benefit bacterial pathogens, not only by serving as carbon and energy sources in metabolism, but also by influencing the behavior of pathogens to facilitate virulence and colonization mechanisms (179). Furthermore, the spatial distribution of lactate and SCFAs can assist pathogens in homing to appropriate niches in the gut and facilitate processes important for colonization of these areas (179). For instance, *Salmonella* species increase expression of adhesins at physiological levels of SCFAs found in the small intestines (225), whereas opposite SCFA-dependent spatial regulation was observed in enterohemorrhagic *E. coli* (EHEC). Rather, EHEC, when in the present of colonic (in contrast to small intestinal) SCFA concentrations, activates production of flagella, motility, adherence, and virulence factors (230).

Campylobacter jejuni is a commensal organism of the intestinal tract of poultry and many other animals in the wild and in agriculture. As a consequence of these zoonotic infections, a large amount of meats in the food supply are contaminated with *C. jejuni*, resulting in the bacterium being a leading cause of diarrheal disease in humans. In chickens, a natural host for *C. jejuni*, the bacterium must sense and differentiate between various regions of the avian gut and compete with other commensal organisms to find preferred niches to establish a persistent colonization in the intestinal tract. As a result, *C. jejuni* predominantly colonizes the lower regions of the chick intestinal tract including the ceca and large intestine (101). Significant colonization does occur in the upper intestinal tract (i.e., the small intestines), but at magnitudes lower than the ceca and large intestines (101). Similarly, *C. jejuni* preferentially infects the lower intestinal tract of humans including the colon and rectum, with adherence to and invasion of the epithelium resulting in an inflammatory diarrheal disease. *C. jejuni* can persist in the human intestinal tract and be shed for many weeks after resolution of disease.

Unlike many other enteric pathogens, *C. jejuni* displays a severely limited carbohydrate catabolism due to lack of one or more enzymes to utilize glucose as a carbon source. Fucose utilization has been reported *in vitro* for a subset of *C. jejuni* strains that contain a genomic island encoding genes for this process (153). Instead, *C. jejuni* predominantly relies on amino acids and peptides for carbon to fuel various metabolic pathways, including the TCA cycle and gluconeogenesis for LOS biosynthesis and capsular polysaccharide production (128, 296). *C. jejuni* has a preference for consumption and utilization of specific amino acids over others (126, 132). These include serine, aspartate, glutamate, and proline, as shown *in vitro*. As such, *C. jejuni* strains also produce specific transporters for these amino acids (50, 130, 131). However, a subset of *C. jejuni* strains has an expanded metabolic repertoire and can also use asparagine and glutamine during *in vitro* growth (132). Curiously, strains that are able to catabolize fucose generally lack the ability to use asparagine and glutamine and *vice versa* (296).

In addition to amino acids, *C. jejuni* catabolizes organic acids and SCFAs such as pyruvate, lactate, and acetate. Exogenously-acquired lactate is oxidized to pyruvate in the *C. jejuni* cytoplasm, with some strains producing two different enzymes for the conversion to pyruvate (237). In addition, pyruvate

can be generated from serine (via SdaA) or PEP (via Pyk; (123)). Pyruvate can also be catabolized via the acetogenesis pathway of *C. jejuni*, which converts pyruvate to acetyl-CoA ((Ac-CoA) via pyruvate oxidoreductase (POR)), Ac-CoA to acetyl phosphate ((AcP); via phosphotransacetylase; Pta), and AcP to acetate (via acetate kinase; AckA). This pathway in *C. jejuni* results in the secretion of acetate by an unknown transporter during logarithmic growth (126). Like other bacteria, *C. jejuni* undergoes the 'acetate switch' in stationary phase after depletion of favored carbon sources, in which excreted acetate is acquired and converted to Ac-CoA by Ac-CoA synthase (Acs), which can be used to fuel the TCA cycle (126, 181).

C. jejuni must compete with the gut microbiota for colonization of niches in the avian host to promote commensalism or in the human host to promote disease. In addition, *C. jejuni* behavior is likely influenced by metabolites and other products of the microbiota. For example, lactic acid bacteria present in the avian gut have been reported to have inhibitory activities that reduce *C. jejuni* commensal colonization (297). Currently, it is unclear the extent by which lactate, bacteriocins, or other factors produced by the bacteria that alter the environment contribute to the bactericidal and bacteriostatic properties against *C. jejuni* (241, 297). On the other hand, lactate itself can be catabolized by *C. jejuni* and support growth of the bacterium (237). Furthermore, it is unclear if the potential negative effect of lactic acid bacteria and derived metabolites on *C. jejuni* replication or survival may correlate with the reduced ability of *C. jejuni* to colonize the small intestines relative to lower intestinal tract other types of bacteria and metabolites are more prevalent.

In a previous study, we identified a transposon insertion in *C. jejuni pta* caused a 10-fold defect in commensal colonization of the chick ceca at day 7 post-infection (101). Since this mutation disrupts the acetogenesis pathway, it is possible that the lack of production of AcP or ultimately acetate may affect the ability of *C. jejuni* to initiate colonization of its natural host. As such, we explored the reason why an acetogenesis mutant is reduced for commensalism in the avian host. From these studies, we discovered that transcription of specific *C. jejuni* genes necessary for commensal colonization are differentially influenced by organic acids and SCFAs that are produced in the avian intestinal tract by gut microbiota or

by *C. jejuni* itself. We discovered that lactate, which is prevalent in the upper regions of the intestinal tract suppressed expression of colonization determinants. However, SCFAs such as acetate and butyrate, which are present in greater concentrations in the lower intestinal tract greatly stimulated expression of these factors. We postulate that the different spatial distribution of these metabolites in the chick gut allow *C. jejuni* to discriminate between different regions of the gut and coordinate expression of colonization factors in preferred niches for growth. This mechanism effectively allows *C. jejuni* to home to ideal niches for colonization and persistence. Our work suggest new findings for the types of signals that *C. jejuni* monitors *in vivo* to establish commensalism in an avian host and are likely relevant for initiating infection in the human host to promote diarrheal disease.

Results

The acetogenesis pathway is required for initial commensal colonization of the intestinal tract of chicks

In a previous study, we identified a mutant with a transposon insertion in *pta* with a reduced commensal colonization capacity for the chick ceca at day 7 post-infection (101). On the *C. jejuni* chromosome, *pta* (encoding phosphotransacetylase) is located immediately upstream of *ackA* (encoding acetate kinase). Both Pta and AckA are components of the acetogenesis pathway in many bacteria that converts Ac-CoA to AcP (via Pta) and AcP to acetate and ATP (via AckA) during the exponential phase of growth, which leads to excretion of acetate (Figure 11; (181)). As carbon sources are depleted into late log and stationary phase, many bacteria experience the 'acetate switch', in which acetate is transported into the cell and converted to Ac-CoA by Acs (Ac-CoA synthase; (181)). Additional analysis of this original *pta*::Tn mutant revealed a polar defect on transcription of *ackA* (data not shown). Thus, we were uncertain whether the colonization defect of the original mutant was due specifically to lack of Pta, AckA, or both enzymes.

Therefore, we assessed the commensal colonization capacity of non-polar *C. jejuni* 81-176 Sm^R Δ*pta* or Δ*ackA* mutants and also analyzed *C. jejuni* 81-176 Sm^R Δ*pta* Δ*ackA*, which contained a deletion of both genes. After oral inoculation of 1-day old chicks with approximately 10^2 cfu, WT *C. jejuni* 81-176

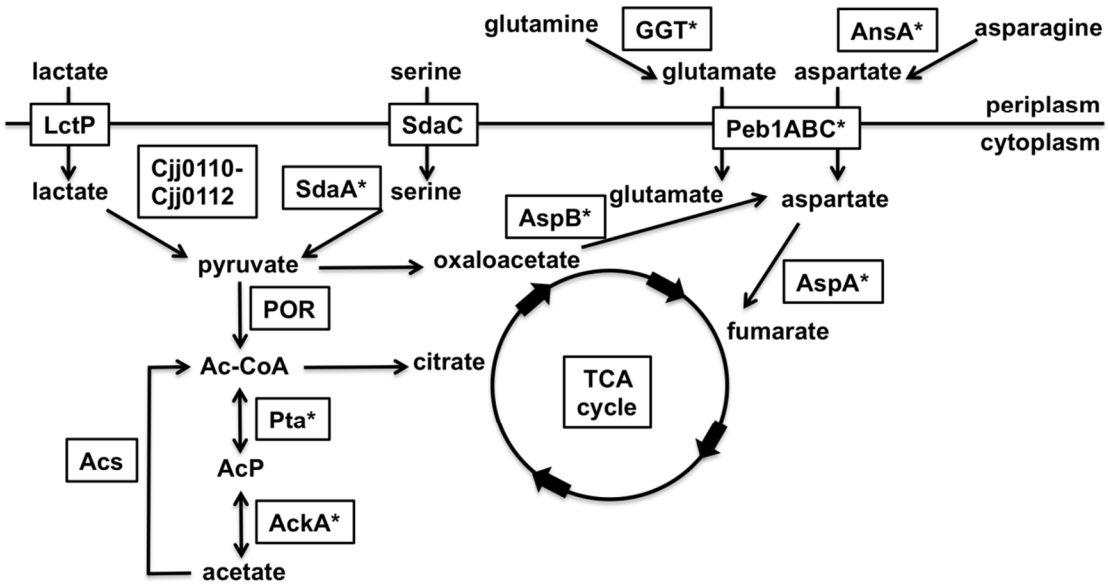


Figure 11. Metabolic reactions in *C. jejuni* affected by select amino acid transport and catabolic pathways. A simplified schematic of the *C. jejuni* transport pathways and the catabolic reactions for amino acids whose expression are affected by exposure to lactate or SCFAs as presented in this work. Boxes indicate factors required for the associated processes. Asterisks indicate those factors that are shown in this work or have been previously shown to be required for commensal colonization of the avian intestinal tract or infection of the murine intestinal tract.

Sm^R efficiently colonized throughout the avian intestinal tract at days 7 and 14 post-infection, with highest levels in the ceca and large intestines ($10^8 – 10^9$ cfu per gram content) and lower levels in the proximal and distal small intestines (10^4 - 10^6 cfu per gram content; (Figure 12A and 12B)). For Δ*pta* and Δ*ackA*, levels of colonization throughout the intestinal tract were comparable to WT at days 7 and 14 post-infection. The only exception was a 4-fold reduction in colonization of the ceca at day 7 post-infection by *C. jejuni* Δ*ackA*, which was statistically significant. We also noted a 17-fold reduction in colonization of the proximal small intestines by Δ*pta* at day 14 post-infection, but this reduction did not meet statistical significance.

In contrast, *C. jejuni* Δ*pta* Δ*ackA* displayed a large colonization defect throughout the intestinal tract at day 7 post-infection (Figure 12A and 12B). We observed 29- and 229-fold reductions in colonization of the proximal and distal small intestines, respectively, with no *C. jejuni* detected in both regions in two of the chicks. In the lower intestinal tract, we observed 98- and 294-fold decreases in colonization of the ceca and large intestines, respectively, by Δ*pta* Δ*ackA* relative to WT *C. jejuni* at day 7 post-infection (Figure 12A). For one chick, the level of *C. jejuni* Δ*pta* Δ*ackA* in the large intestines was below the limit of detection. By day 14 post-infection, the Δ*pta* Δ*ackA* mutant colonized all intestinal regions at close to WT levels (Figure 12B).

Growth and transcriptome analysis of the *C. jejuni* acetogenesis mutant

We developed a few hypotheses for why the *C. jejuni* acetogenesis mutant lacking both Pta and AckA has a commensal colonization defect in chicks. Elimination of Pta and AckA may result in accumulation of Ac-CoA and reduce the amount of free CoA. Reduction of CoA may impair growth as it is an essential co-factor for enzymes in many metabolic and physiological pathways. However, *C. jejuni* Δ*pta* Δ*ackA* grew modestly better than WT in late log and into early stationary phase during growth in complex Mueller-Hinton (MH) broth (Figure 13A). We also analyzed growth in *Campylobacter* defined media (CDM), in which organic and amino acids are the primary carbon sources (150). WT *C. jejuni*

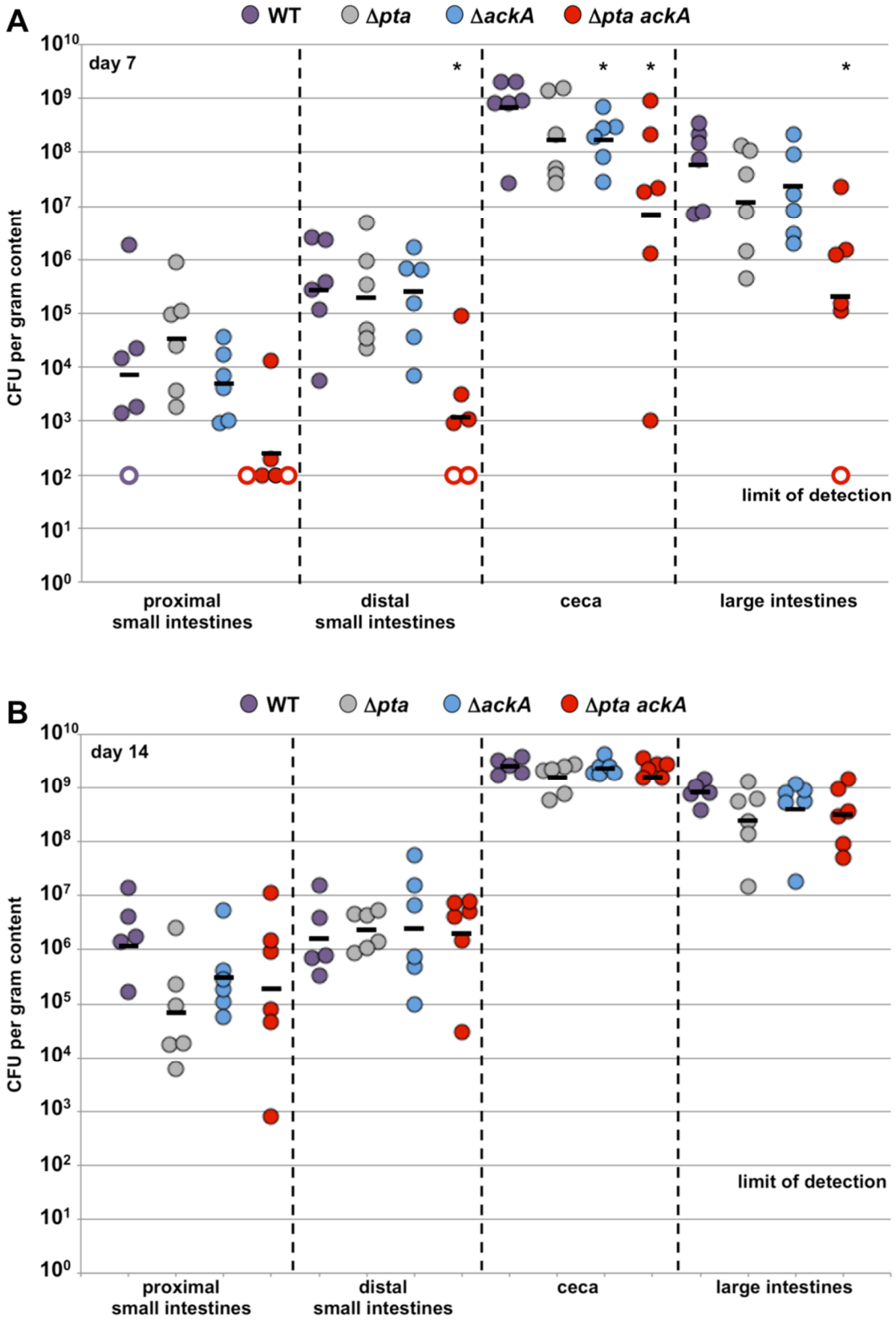

A
WT
Δpta
ΔackA
Δpta ackA
day 7
CFU per gram content
limit of detection
proximal small intestines
distal small intestines
ceca
large intestines
B
day 14

Figure 12. Colonization dynamics of WT *C. jejuni* and isogenic acetogenesis mutants over time in the avian intestinal tract. One-day old chicks were orally infected with approximately 100 cfu of WT *C. jejuni* 81-176 Sm^R (purple) or isogenic Δ*pta* (grey), Δ*ackA* (blue), or Δ*pta ackA* (red) mutants, with different defects in the *C. jejuni* acetogenesis pathway. Chicks were sacrificed at (A) day 7 or (B) day 14 and the levels of each *C. jejuni* strain in the proximal small intestines, distal small intestines, ceca, and large intestines was determined (reported as CFU per gram of content). Each closed circle represents the level of *C. jejuni* in a single chick. Open circles represent chicks with *C. jejuni* levels below the limit of detection (<100 cfu per gram of content). Horizontal bars represent geometric mean for each group. Statistical analysis was performed using the Mann-Whitney *U* test (* $P < 0.05$).

grew significantly slower in CDM than in MH broth (compare Figure 13A and 13B). In comparison, Δ*pta* Δ*ackA* showed a significant reduction in growth relative to WT *C. jejuni* over the course of the assay (Figure 13B). Complementation of *C. jejuni* Δ*pta* Δ*ackA* with a plasmid to express both *pta* and *ackA in trans* largely restored growth of the mutant to WT levels (Figure 13B). Thus, the acetogenesis mutant appears to have a growth defect when amino acids and organic acids are the main available carbon sources.

Another possible reason for the colonization defect of *C. jejuni* Δ*pta* Δ*ackA* could be due to lack of generation of acetate or AcP, which is a metabolite that can serve as a phosphodonor to activate response regulators and influence gene transcription (298, 299). In addition, acetate can serve as a signal for some signal transduction pathways and impact transcription of specific genes. Indeed, in CDM broth, the level of acetate produced by *C. jejuni* Δ*pta* Δ*ackA* was 10-fold lower than WT (Table 5). Note, quantitation of AcP is difficult due to the volatility of the metabolite.

Considering that acetogenesis was impaired in *C. jejuni* Δ*pta* Δ*ackA*, we tested whether the mutant displayed a significant alteration in transcription of genes that may be important for colonization relative to WT *C. jejuni*. For this analysis, we grew WT *C. jejuni* and the Δ*pta* Δ*ackA* mutant in MH broth in microaerobic conditions at 37 °C to mid-log phase and then isolated mRNA for transcriptome analysis using DNA microarrays. A select list of genes whose expression was increased or decreased by at least two-fold in Δ*pta* Δ*ackA* are reported in Table 6 (for a full list of genes whose expression was changed, see Table 7 and Table 8). We selected a subset of genes for validation by semi-quantitative real-time PCR

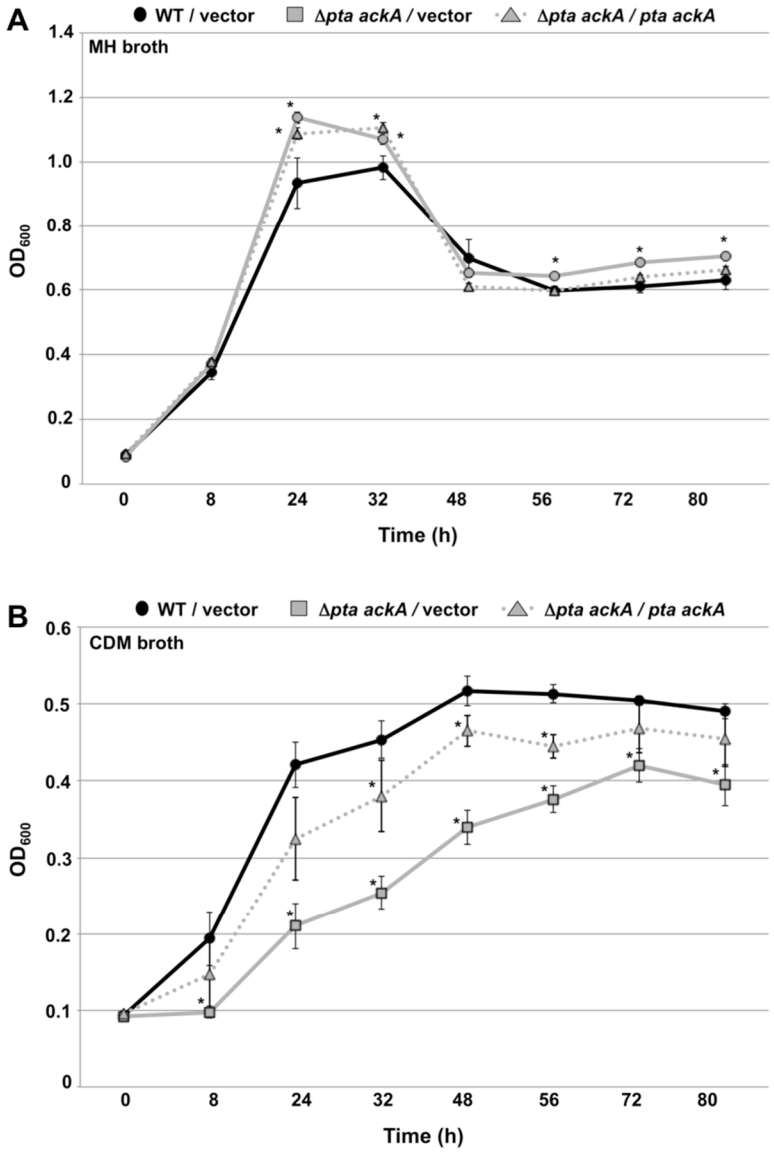


Figure 13. Analysis of growth of *C. jejuni* strains in rich and defined media. *C. jejuni* strains were grown in (A) rich Mueller-Hinton (MH) broth or (B) *Campylobacter* defined media (CDM) broth in microaerobic conditions at 37 °C for 80 h. All strains were analyzed in triplicate and the data are presented as an average of OD_{600} readings for strains at each time point. Error bars indicate standard deviations. Asterisks (*) indicate data points of mutants that are statistically significant ($P < 0.05$) than the WT strain.

Strain	Acetate concentration (mM)[a]
Wild-type	3.43 +/- 0.52
Δ*pta*	0.29 +/- 0.07*
Δ*ackA*	0.31 +/- 0.02*
Δ*pta* Δ*ackA*	0.30 +/- 0.01*

Table 5. Acetate production by WT *C. jejuni* and isogenic acetogenesis mutants. [a] *C. jejuni* strains were grown in CDM broth for 8 h to mid-log phase. The concentration of acetate was measured in culture-free supernatants in triplicate. * $P < 0.05$ as calculated by Student's two-tailed t-test.

(qRT-PCR) based on those encoding proteins previously found to be involved in colonization of chicks, virulence mechanisms, or important biological and metabolic functions for *C. jejuni*.

Many genes whose expression was reduced in Δ*pta* Δ*ackA* encode amino acid transport systems or enzymes for the utilization of amino acids in catabolism (Figure 11). For reasons explained below, we term this collection of genes the 'SCFA-induced regulon' of *C. jejuni*. Many of these factors had previously been found to be required for optimal interactions of *C. jejuni* with hosts. Thus, these transcriptional defects may contribute to the colonization defect in chicks and the growth defect in CDM broth we observed. For example, expression of *ggt*, *ansA* (also referred to as *ansB*), *peb1c*, *sdaA*, and *sdaC*, which encode factors for acquisition or utilization of glutamine, glutamate, serine, asparagine, and aspartate were 2.4- to 11.6-fold lower in the acetogenesis mutant (Figure 14A). GGT is a γ-glutamyltransferase that converts glutamine or glutathione to glutamate (132). This activity is required by *C. jejuni* for long-term colonization of the chick ceca (137). AnsA is an L-asparaginase to convert asparagine to aspartate (132). Whereas GGT is required for WT levels of colonization of the intestines of *myd88*$^{-/-}$ mice, AnsA is required for growth in the liver of these mice upon intraperitoneal infection (124, 132). Peb1c is the ATPase component of an amino acid transport system specific for the acquisition of aspartate, glutamate, and glutamine into *C. jejuni* (131). *peb1c* is the first gene of an operon that also includes *peb1a* and *peb1b*, which encode the binding component and permease of the Peb1 system. SdaC is required for serine transport and SdaA is a L-serine dehydratase that converts serine to pyruvate, which

Locus Tag	Gene Name	Putative Function	Ratio WT/ Δ*pta* Δ*ackA*[b]
Cjj81176_0038	*rrc*	Rbo/Rbr-like protein of *C. jejuni*, rubrerythrin-like protein	9.50
Cjj81176_0056	*ansA*	L-asparginase	7.28
Cjj81176_0067	*ggt*	γ-glutamyl transferase	8.11
Cjj81176_0122	*aspA*	aspartate ammonia-lyase	7.51
Cjj81176_0123	*dcuA*	anaerobic C4-dicarboxylate transporter	5.22
Cjj81176_0124	*dcuB*	anaerobic C4-dicarboxylate transporter	
Cjj81176_0682		possible di-/tri-peptide transporter	2.61
Cjj81176_0683		possible di-/tri-peptide transporter	5.90
Cjj81176_0927	*peb1a*	aspartate/glutamate transporter, permease component	2.74
Cjj81176_0928	*peb1b*	aspartate/glutamate transporter, solute-binding component	5.48
Cjj81176_0929	*peb1c*	amino-acid transporter, ATP-binding component	5.11
Cjj81176_1615	*sdaA*	L-serine dehydratase	2.66
Cjj81176_1616	*sdaC*	L-serine transporter	2.75
Cjj81176_0315	*peb3*	glycoprotein; putative adhesion or transport protein	0.22

Table 6. Condensed list of genes differentially expressed in *C. jejuni* 81-176 Sm^R Δ*pta* Δ*ackA* compared to WT *C. jejuni* 81-176 Sm^R by microarray analysis. Shown are a subset of genes identified to be differentially expressed in mutants that were further analyzed in this work. A complete list of genes that were differentially expressed in mutant are shown in Tables 7 and 8. [b] Expression of genes was increased or decreased by two-fold in the *C. jejuni* Δ*pta* Δ*ackA* mutant.

C. jejuni 81-176 gene tag	NCTC11168 gene tag	Fold Change (WT/Δ*pta* Δ*ackA*)	Gene Annotation	Verified or predicted function
Cjj0711	*Cj0688*	82.74	*pta*	Phosphate acetyltransferase
Cjj0712	*Cj0689*	10.02	*ackA*	Acetate kinase A
Cjj0038	*Cj0012c*	9.499	*rrc*	Rbo/Rbr-like protein of *C. jejuni*, rubrerythrin-like protein
Cjj0067	--	8.108	*ggt*	gamma-glutamyltransferase
Cjj0204	*Cj0168c*	7.99		Hypothetical protein
Cjj0122	*Cj0087*	7.51	*aspA*	Aspartate ammonia-lyase
Cjj0056	*Cj0029*	7.278	*ansA*	L-asparaginase
Cjj0292	*Cj0265c*	6.567		putative cytochrome C-type haem-binding periplasmic protein
Cjj0697	*Cj0671*	6.188	*dcuB*	C4-dicarboxylate transporter, anaerobic
Cjj0683	--	5.896		putative di-/tripeptide transporter
Cjj0928	*Cj0921c*	5.475	*peb1a*	Amino-acid ABC transporter, periplasmic solute-binding protein
Cjj0123	*Cj0088*	5.215	*dcuA*	C4-dicarboxylate transporter, anaerobic
Cjj0929	*Cj0922c*	5.112	*peb1c*	Amino-acid ABC transporter, ATP-binding protein
Cjj0474	*Cj0449c*	5.054		Conserved hypothetical protein
Cjj0291	*Cj0264c*	5.017		trimethylamine N-oxide(TMAO) reductase
Cjj1731	*Cj0913c*	4.994	*hup*	DNA-binding protein HU homolog
Cjj0440	*Cj0416*	4.989		hypothetical protein
Cjj1358	*Cj1356c*	4.904		Conserved hypothetical integral membrane protein
Cjj1198	*Cj1183c*	4.653	*cfa*	Cyclopropane fatty acyl phospholipid synthase
Cjj0382	*Cj0358*	4.486		Cytochrome c551 peroxidase
Cjj0743	*Cj0720c*	4.379	*flaC*	Flagellin
Cjj1170	*Cj1153*	4.187		Cytochrome-related conserved hypothetical protein
Cjj0443	*Cj0420*	4.075		Conserved hypothetical protein
Cjj0107	*Cj0069*	4.068		Conserved hypothetical protein
Cjj0393	*Cj0370*	4.056	*rpsU*	30S ribosomal protein S21
Cjj0880	*Cj0864*	3.924	*dsbA*	Thiol:disulfide interchange protein DsbA, putative
Cjj0464	*Cj0438*	3.89	*mfrB*	methylmenaquinol:fumurate reductase protein B
Cjj0465	*Cj0439*	3.731	*mfrC*	methylmenaquinol:fumurate reductase protein C
Cjj0037	*Cj0011c*	3.726		Conserved hypothetical protein, putative ComEA-related protein
Cjj0473	*Cj0448c*	3.522		MCP-domain signal transduction

				protein (Tlp6)
Cjj0641	*Cj0612c*	3.465	*cft*	Ferritin
Cjj0881	*Cj0865*	3.421	*dsbB*	Disulfide bond formation protein B
Cjj0974	*Cj0950c*	3.389		Conserved hypothetical lipoprotein
Cjj0682	*Cj0654c*	3.38		pseudogene (putative transmembrane transport protein)
Cjj1519	*Cj1534c*	3.37		Conserved hypothetical protein
Cjj0917	*Cj0909*	3.315		Conserved hypothetical protein
Cjj1242	*Cj1228c*	3.205	*htrA*	Periplasmic serine protease DO; heat shock protein HtrA
Cjj0463	*Cj0437*	3.203	*mfrA*	methylmenaquinol:fumurate reductase protein A
Cjj0448	*Cj0428*	3.173		Conserved hypothetical protein
Cjj1675	*Cj1682c*	3.118	*gltA*	Citrate synthase
Cjj0624	*Cj0596*	3.029	*peb4*	Cell binding factor 2 precursor, major antigenic peptide Peb4
Cjj1382	*Cj1380*	3.026		Conserved hypothetical protein, probable periplasmic protein
Cjj0415	*Cj0392c*	2.959	*pyk*	Pyruvate kinase
Cjj1016	*Cj0998c*	2.891		Conserved hypothetical protein
Cjj1457	*Cj1464*	2.883	*flgM*	anti-sigma factor for sigma28 involved in regualtion of flagellar gene expression
Cjj0758	*Cj0735*	2.881		Hypothetical protein, similar to C. jejuni Cj0735
Cjj1525	*Cj1540*	2.811	*tupA*	tungstate ABC transporter component, substrate-binding protein
Cjj1443	*Cj1450*	2.792	*ciaI*	invasion protein, probable ATP/GTP-binding protein
Cjj1338	*Cj1338c*	2.773	*flaA*	Flagellin
Cjj0432	*Cj0408*	2.76	*frdC*	Fumarate reductase, cytochrome b subunit
Cjj1616	*Cj1625c*	2.746	*sdaC*	L-serine transporter
Cjj0927	*Cj0920c*	2.738	*peb1b*	Amino acid ABC transporter, permease protein
Cjj1179	*Cj1164c*	2.721		Conserved hypothetical protein
Cjj0591	--	2.67		conserved hypothetical protein
Cjj0416	*Cj0393c*	2.666	*mqo*	predicted malate:quinone oxidoreductase
Cjj1615	*Cj1624c*	2.659	*sdaA*	L-serine dehydratase
Cjj0682	--	2.613		putative di-/tripeptide transporter
Cjj1257	*Cj1242*	2.601	*ciaC*	invasion protein
Cjj0577	*Cj0552*	2.579		Conserved hypothetical integral membrane protein
Cjj0950	--	2.52		conserved hypothetical protein
Cjj0434	*Cj0410*	2.434	*frdB*	Fumarate reductase, iron-sulfur protein
Cjj0113	*Cj0076c*	2.428	*lctP*	L-lactate permease

Cjj0764	*Cj0742*	2.427		outer membrane protein, putative
Cjj1139	*Cj1121c*	2.42	*wlaK*	Putative aminotransferase
Cjj0446	*Cj0426*	2.418		Conserved hypothetical protein, possible ABC transporter (ATP-binding protein)
Cjj0383	*Cj0360*	2.392		Phosphoglucomutase/phosphomannomutase family protein
Cjj0722	*Cj0699c*	2.365	*glnA*	Glutamine synthetase
Cjj1444	*Cj1451*	2.353	*dut*	Deoxyuridinetriphosphatase, putative
Cjj0048	*Cj0021c*	2.348		Conserved hypothetical protein, putative fumarylacetoacetate hydrolase family protein
Cjj0907	*Cj0898*	2.331		HIT family hydrolase
Cjj0783	*Cj0762c*	2.324	*aspB*	Aspartate aminotransferase
Cjj0869	*Cj0853c*	2.321	*hemL*	Glutamate-1-semialdehyde 2,1-aminomutase
Cjj0392	*Cj0369c*	2.307		Ferredoxin domain-containing integral membrane protein
Cjj1191	*Cj1176c*	2.286	*tatA1*	\ putative twin-arginine translocation protein, TatA/E family
Cjj1752	*Cj1360c*	2.27	*10SaRNA*	putative proteolysis tag for 10Sa_RNA
Cjj0063	--	2.269		conserved hypothetical protein
Cjj1522	*Cj1537c*	2.265	*acs*	Acetyl-CoA synthetase
Cjj0414	*Cj0391c*	2.248	*fedB*	secreted flagellar co-expressed determinant required for colonization
Cjj1485	*Cj1493c*	2.233		Conserved hypothetical membrane protein
Cjj0553	*Cj0528c*	2.215	*flgB*	Flagellar basal body rod protein FlgB
Cjj1213	*Cj1198*	2.204	*luxS*	Autoinducer-2 production protein LuxS
Cjj1471	*Cj1478c*	2.194	*cadF*	Outer membrane fibronectin-binding protein
Cjj0852	*Cj0835c*	2.175	*acnB2*	Aconitate hydratase 2
Cjj1093	*Cj1075*	2.149	*fliW*	flagellar chaperone protein
Cjj1526	*Cj1541*	2.146		Conserved hypothetical protein, LamB/YcsF family protein
Cjj0397	*Cj0373*	2.142		2-hydroxyacid dehydrogenase family protein
Cjj0620	*Cj0592c*	2.141		Conserved hypothetical protein
Cjj0572	*Cj0547*	2.129	*flaG*	Possible flagellar protein
Cjj1494	*Cj1502c*	2.129	*putP*	Sodium/proline symporter
Cjj0556	*Cj0531*	2.112	*icd*	Isocitrate dehydrogenase
Cjj1207	*Cj1192*	2.103	*dctA*	C4-dicarboxylate transport protein
Cjj0231	*Cj0200c*	2.067		Conserved hypothetical protein
Cjj1339	--	2.063	*flaB81176*	flagellin B
Cjj0720	*Cj0697*	2.06	*flgG2*	Flagellar distal rod protein FlgG
Cjj1402	*Cj1403c*	2.056	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase

Cjj1339	*Cj1339c*	2.036	*flaB11168*	Flagellin
Cjj0953	--	2.013		conserved hypothetical protein

Table 7. Complete list of genes with increased expression in the *C. jejuni* 81-176 Sm^R WT strain compared to the *C. jejuni* 81-176 Sm^R Δ*pta* Δ*ackA* mutant. Expression of genes was increased by at least two-fold in the *C. jejuni* WT strain over the Δ*pta* Δ*ackA* strain.

C. jejuni 81-176 gene tag	NCTC11168 gene tag	Fold Change (Δ*pta* Δ*ackA*/WT)	Gene Annotation	Verified or predicted function
Cjj0315	*Cj0289c*	4.631	*peb3*	glycoprotein; putative adhesin or transport protein
Cjj1088	*Cj1170c*	3.934		Conserved hypothetical protein
Cjj0643	*Cj0614*	3.738	*pstC*	Phosphate ABC transporter, permease protein
Cjj1087	*Cj1169c*	3.077		Conserved hypothetical protein, probable periplasmic protein
Cjj1387	*Cj1385*	3.066	*katA*	Catalase
Cjj1435	--	2.836	--	putative sugar transferase
Cjj0515	*Cj0494*	2.796		hypothetical protein
Cjj0001	*Cj1707c*	2.581	*rplC*	50S ribosomal protein L3
Cjj0644	*Cj0615*	2.554	*pstA*	Phosphate ABC transporter, permease protein
Cjj0752	*Cj0729*	2.543		Conserved hypothetical protein
Cjj1301	*Cj1284*	2.499	*ktrA*	TRK system potassium uptake protein TrkA, putative
Cjj1699	*Cj1702c*	2.45	*rplV*	50S ribosomal protein L22
Cjj1569	*Cj1584c*	2.408	*dppA*	Peptide ABC transporter, periplasmic substrate-binding protein
Cjj0642	*Cj0613*	2.394	*pstS*	Phosphate ABC transporter, periplasmic phosphate-binding protein
Cjj0585	*Cj0560*	2.385		putative integral membrane protein
Cjj0076	*Cj0038c*	2.379		Conserved hypothetical protein
Cjj1388	*Cj1386*	2.369		Conserved hypothetical protein, ankyrin repeat family protein
Cjj1620	*Cj1629*	2.328	*exbD2*	Biopolymer transport protein, TolR/ExbD family
Cjj0549	*Cj0524*	2.29		Conserved hypothetical membrane protein, possible Na-dependent transporter
Cjj0616	*Cj0588*	2.244	*tlyA*	Hemolysin A
Cjj0878	*Cj0862c*	2.198	*pabB*	Para-aminobenzoate synthase, glutamine amidotransferase component I
Cjj0005	*Cj1711c*	2.187	*ksgA*	Dimethyladenosine transferase
Cjj1697	*Cj1700c*	2.184	*rplP*	50S ribosomal protein L16
Cjj1246	*Cj1232*	2.182		Conserved hypothetical protein
Cjj1512	--	2.162	--	conserved hypothetical protein
Cjj1702	*Cj1705c*	2.148	*rplW*	50S ribosomal protein L23
Cjj0977	*Cj0954c*	2.125		Conserved hypothetical protein, DnaJ homolog
Cjj1694	*Cj1697c*	2.114	*rplN*	50S ribosomal protein L14

Cjj0119	*Cj0082*	2.112	*cydB*	Cytochrome bd oxidase, subunit II
Cjj1029	*Cj1011*	2.109		Conserved hypothetical protein
Cjj0715	*Cj0692c*	2.102		Conserved hypothetical membrane protein
Cjj0118	*Cj0081*	2.043	*cydA*	Cytochrome bd oxidase, subunit I
Cjj0004	*Cj1710c*	2.035		Conserved hypothetical protein, putative beta-lactamase family protein
Cjj1703	*Cj1706c*	2.02	*rplD*	50S ribosomal protein L4
Cjj1655	*Cj1664*	2.018		Conserved hypothetical protein, possible periplasmic thiredoxin
Cjj1059	*Cj1040c*	2.017		Conserved hypothetical protein, probable transmembrane transport protein
Cjj1619	*Cj1628*	2.017	*exbB2*	Biopolymer transport protein, MotA/TolQ/ExbB family

Table 8. Complete list of genes with increased expression in the *C. jejuni* 81-176 Sm^R Δ*pta* Δ*ackA* strain compared to the *C. jejuni* 81-176 Sm^R WT strain. Expression of genes was increased by at least two-fold in the Δ*pta* Δ*ackA* strain over the *C. jejuni* WT strain.

not only feeds the acetogenesis pathway but can also be converted to oxaloacetate (through PycAB) to feed the TCA cycle (129). A previous study found that Peb1a of the Peb1 system and SdaA are required for WT levels of commensal colonization of chicks and for growth in the intestines and liver of *myd88*$^{-/-}$ mice (47, 51, 129, 130).

We also noted that expression of *Cjj81176_0683* (*Cjj0683*) and *Cjj81176_0682* (*Cjj0682)* was reduced 112.4- and 9.7-fold, respectively (Figure 14A). Not all strains of *C. jejuni* have a locus containing these genes. In most strains that do, the locus is composed by only one gene that encodes a large protein that is predicted to function as a di- or tri-peptide permease of a transport system (142). In *C. jejuni* 81-176, it appears that a mutation has occurred in this gene to give rise to two genes (*Cjj0683* and *Cjj0682*), which together may not produce a functional pair of proteins for a similar putative permease. We also noted that *rrc* transcription was reduced 2.6-fold in the acetogenesis mutant, which might have implications for oxidative stress or iron responses (300).

Although the microarray analysis suggested that transcription of *aspA*, *dcuA*, and *dcuB*, was significantly reduced, we were unable to verify a decrease in transcription by qRT-PCR (Figure 14A). In a previous study, these genes are responsible for the uptake of aspartate and conversion of aspartate to fumarate. AspA had previously been shown to be required for intracellular survival and commensal colonization of chicks (65, 125).

Other commensal colonization determinants whose expression was reduced according to microarray analysis include, *Cjj81176_0382*, *fedB*, *ciaI*, and *cadF* (Table 7). Each of these genes are required for WT levels of commensal colonization of the chick ceca (57-59, 301). In addition, *C. jejuni* Δ*ciaI* has an approximately two-fold reduction in invasion in human colonic cells (57, 59). Microarray analysis also revealed a reduction in expression of *frdB* and *frdC*, which with *frdA*, encode a succinate dehydrogenase complex to convert succinate to fumarate (147). FrdA is required for WT levels of cecal colonization of chicks (147). For other *C. jejuni* determinants required for invasion or intracellular survival in human colonic cells, we noted a reduction in expression of *aspB* and *ciaC* by microarray analysis (Table 7). AspB is required for invasion of epithelial cells and for colonization of the murine

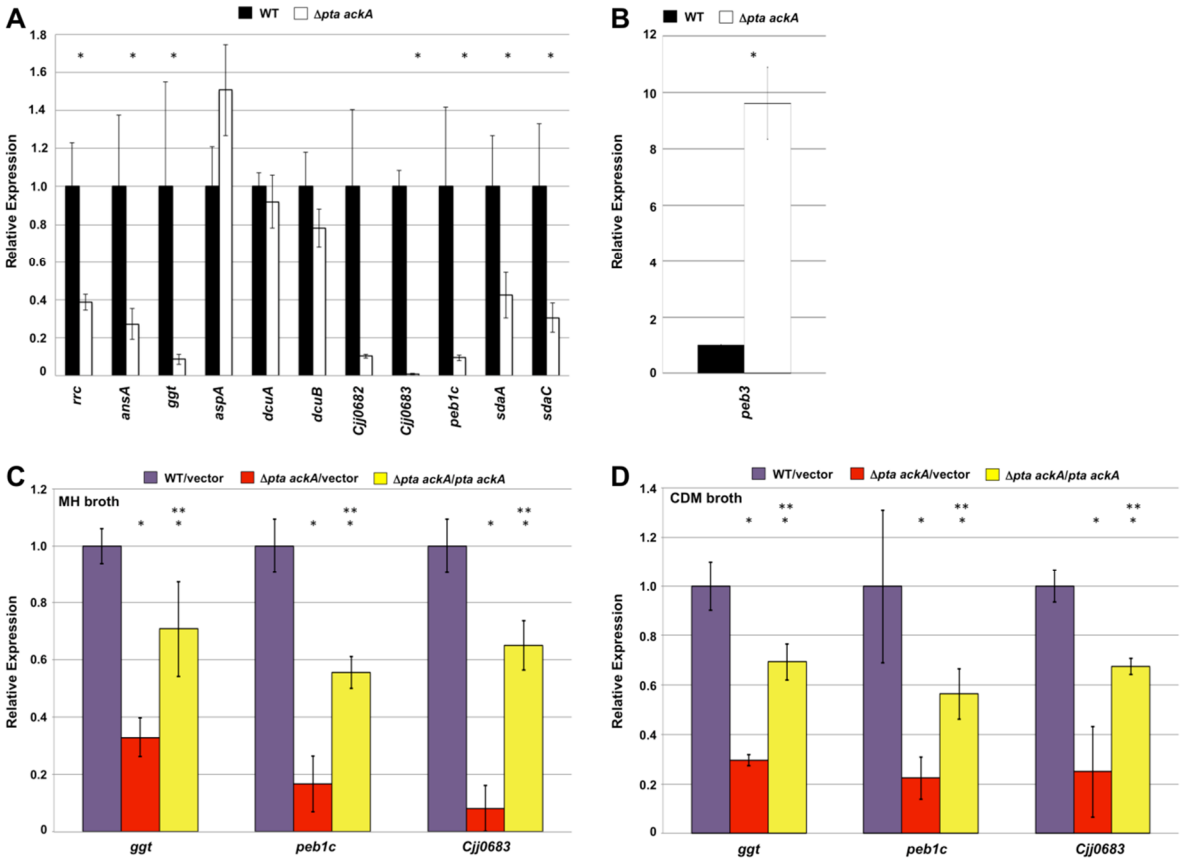


Figure 14. Transcriptional analysis of the SCFA-induced regulon of *C. jejuni*. (A and B) Semi-quantitative real-time PCR analysis of transcription of a subset of genes initially identified by microarray analysis of WT *C. jejuni* and *C. jejuni* Δ*pta* Δ*ackA*. The expression of each gene in the WT *C. jejuni* 81-176 Sm^R (black) as measured by qRT-PCR was set to 1. Expression of each gene in the Δ*pta ackA* mutant (white) is shown relative to the WT strain. (A) Genes whose expression was reduced in Δ*pta ackA* relative to WT *C. jejuni* in the microarray analysis. (B) Gene whose expression was increased in Δ*pta ackA* relative to WT *C. jejuni* in the microarray analysis. (C and D) Semi-quantitative real-time PCR analysis of transcription of select members of the SCFA-induced regulon of *C. jejuni* in different media in WT *C. jejuni* and Δ*pta* Δ*ackA* with or without *in trans* complementation. The expression of *ggt*, *peb1c*, and *Cjj0683* in WT *C. jejuni* 81-176 Sm^R with empty vector (purple) as measured by qRT-PCR was set to 1. Expression of each gene in Δ*pta* Δ*ackA* with empty vector (red) or Δ*pta* Δ*ackA* with vector containing WT *pta ackA* (yellow) is shown relative to the WT strain. Strains were examined in triplicate after growth in MH broth (A-C) or in CDM broth (D). Error bars indicate standard deviations. Statistically significant differences in gene expression between WT *C. jejuni* and mutant strains (*, $P < 0.05$) or between Δ*pta* Δ*ackA* with vector alone or Δ*pta* Δ*ackA* complemented with WT *pta ackA* (**, $P < 0.05$) as performed by the Student's *t*-test are indicated.

liver, spleen, and intestine (65). Metabolically, AspB is required to synthesize aspartate from oxaloacetate and glutamate (Figure 11). The exact function of CiaC in invasion is unclear. Other notable genes whose expression was reduced include *mfrABE* (previously annotated as sdhABC) and *Cjj0291-Cjj0292*, which are required for fumarate reduction and TMAO/DMSO reduction, respectively, which may be important for energy production during host colonization (146, 148).

We did find by microarray analysis that expression of a smaller number of genes appeared to be increased in *C. jejuni* Δ*pta* Δ*ackA* relative to WT *C. jejuni*. We verified that expression of one gene, *peb3*, was increased 10-fold (Figure 14B). Peb3 is a surface-localized glycoprotein that has potential adhesive properties for eukaryotic cells (279, 281, 302).

For all analysis reported in the remainder of this work, we analyze transcription of *ggt*, *peb1c*, and *Cjj0683* as representatives of the SCFA-induced regulon. Upon complementation of Δ*pta* Δ*ackA* with the vector to express *pta* and *ackA in trans*, we noted that expression of the SCFA-induced regulon after growth in MH broth were significantly higher than the mutant harboring vector alone although levels of expression were not completely to WT levels (Figure 14C).

We also assessed whether transcription of the SCFA-induced regulon was reduced in CDM broth, which may contribute to the growth defect of Δ*pta* Δ*ackA* mutant in this media (Figure 13B). Relative to WT *C. jejuni*, the Δ*pta* Δ*ackA* mutant showed 4- to 5-fold reductions in expression of *ggt*, *peb1c*, and *Cjj0683* (Figure 14D). Upon complementation of Δ*pta* Δ*ackA* with *pta* and *ackA*, expression of these genes was partially restored to WT levels and was significantly higher than in Δ*pta* Δ*ackA* with vector alone. Thus, the acetogenesis pathway of *C. jejuni* influences expression of the SCFA-induced regulon.

Colonization capacity of Δ*ggt*, Δ*peb1c*, and Δ*Cjj0683* mutants

We next investigated whether genes within the SCFA-induced regulon are required for initial commensal colonization of the chick ceca. We constructed *C. jejuni* mutants lacking *ggt*, *peb1c*, or *Cjj0683* and infected 1-day old chicks with approximately 10^2 cfu. At day 7 post-infection, WT *C. jejuni*

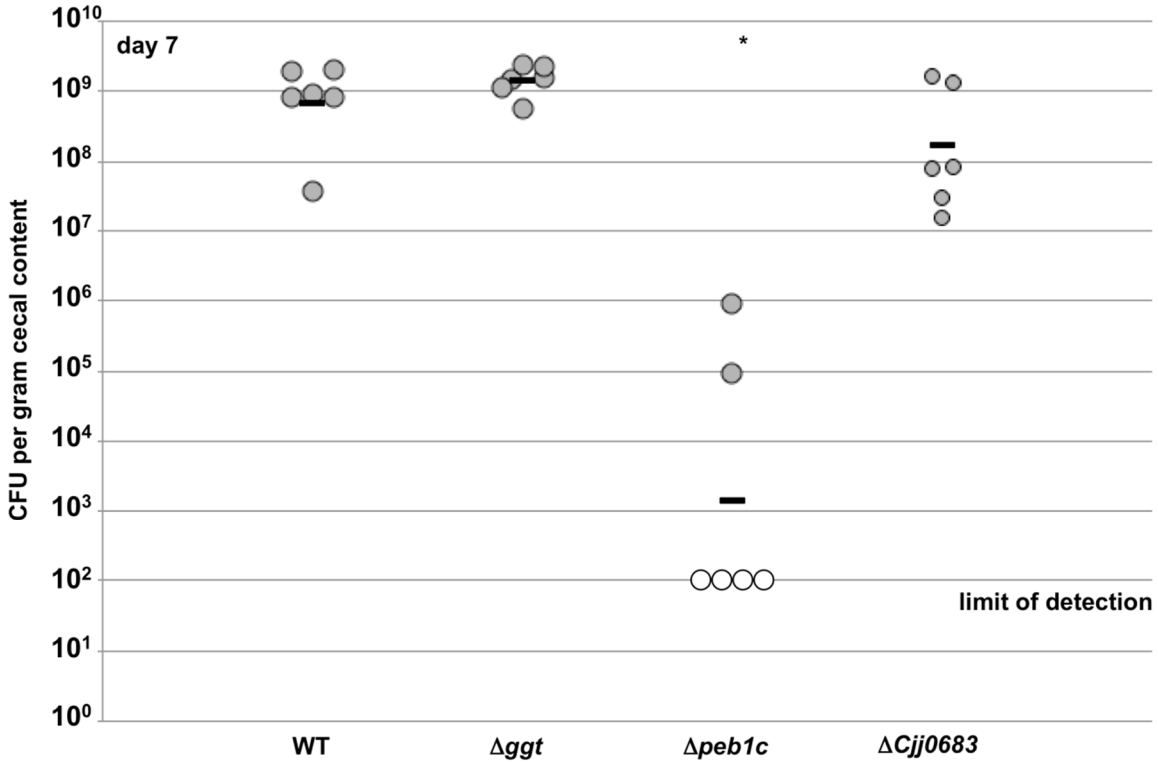


Figure 15. Cecal colonization capacities of WT *C. jejuni* and isogenic mutants lacking a single select gene of the SCFA-induced regulon for the avian intestinal tract. One-day old chicks were orally infected with approximately 100 cfu of WT *C. jejuni* 81-176 Sm^R or isogenic Δ*ggt*, Δ*peb1c*, or Δ*Cjj0683* mutants. Chicks were sacrificed at day 7 post-infection and the levels of each *C. jejuni* strain in ceca were determined (reported as CFU per gram of cecal content). Each closed circle represents the level of *C. jejuni* in a single chick. Open circles represent chicks with *C. jejuni* levels below the limit of detection (<100 cfu per gram of content). Horizontal bars represent geometric mean for each group. Statistical analysis was performed using the Mann-Whitney *U* test (* $P < 0.05$).

colonized at around 10^9 cfu per gram of cecal content (Figure 15). We observed a similar level of colonization by the Δ*ggt* and Δ*Cjj0683* mutants. As a note, GGT has previously been shown to be required for persistent colonization of chicks at day 21 post-infection, but not at an earlier timepoint of infection (137). In contrast, *C. jejuni* Δ*peb1c* demonstrated a drastic reduction in cecal colonization of over 100,000-fold with the bacterium undetectable in four of the six chicks. This colonization defect supports a previously study where a *peb1a* mutant in another *C. jejuni* strain demonstrated a cecal colonization (and dissemination in mice) defect (47, 51).

Exogenous acetate restores expression of the SCFA-induced regulon to a *C. jejuni* acetogenesis mutant

Because deletion of both *pta* and *ackA* caused a reduction in expression of the SCFA-induced regulon *in vitro*, we analyzed whether this defect was due to mutation of *pta*, *ackA*, or both genes. In MH and CDM broth, we observed a consistent reduction of expression of the SCFA-induced regulon in *C. jejuni* lacking either *pta* or *ackA* to the levels observed in *C. jejuni* Δ*pta* Δ*ackA* (Figure 16A and 16B). These data suggest that disruption of any component of the acetogenesis pathway affects expression of the SCFA-induced regulon.

Like *C. jejuni* Δ*pta* Δ*ackA*, the single Δ*pta* or Δ*ackA* mutants also produced much less acetate than WT *C. jejuni* during *in vitro* growth in CDM (Table 5). We considered whether expression of the SCFA-induced regulon required the *in vitro* production of acetate by *C. jejuni*. To this end, we monitored expression of *ggt*, *peb1c*, and *Cjj0683* in WT and Δ*pta* Δ*ackA* after growth in CDM supplemented with physiological concentrations of acetate found in the chick ceca, which can reach up to 100 mM (189, 303). We grew *C. jejuni* strains in CDM broth rather than MH broth to allow for accurate control of concentrations of media components. For WT *C. jejuni*, addition of 100 mM acetate did not influence expression of *ggt* or *peb1c* and only modestly increased expression of *Cjj0683* (Figure 17A). However, we observed a dose-dependent increase in expression of the SCFA-induced regulon by acetate in *C. jejuni* Δ*pta* Δ*ackA*. We began observing increases in expression of these genes with 50 mM acetate, and at 100

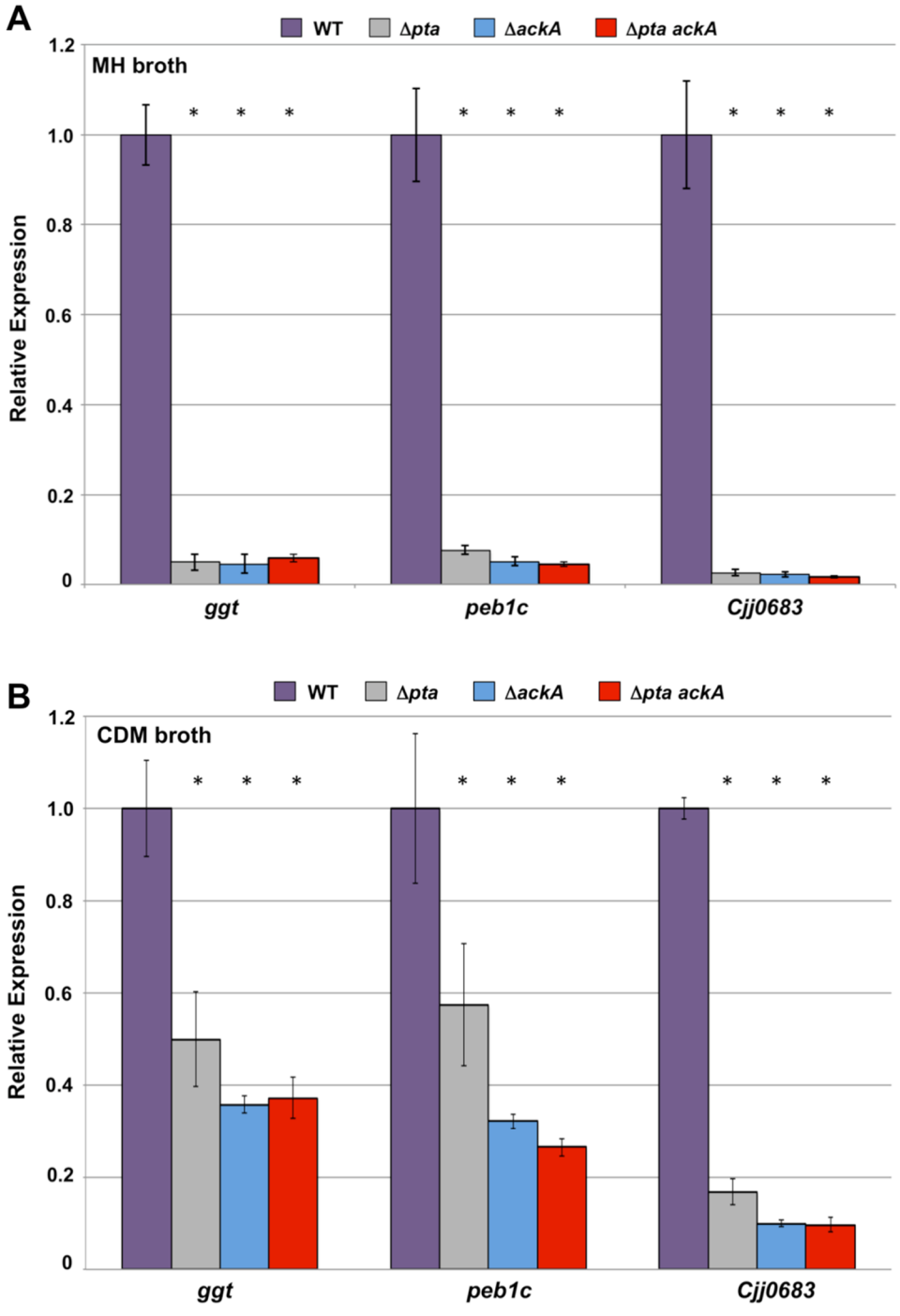

A
WT
Δpta
ΔackA
Δpta ackA
MH broth
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0
ggt
peb1c
Cjj0683
B
WT
Δpta
ΔackA
Δpta ackA
CDM broth
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0
ggt
peb1c
Cjj0683

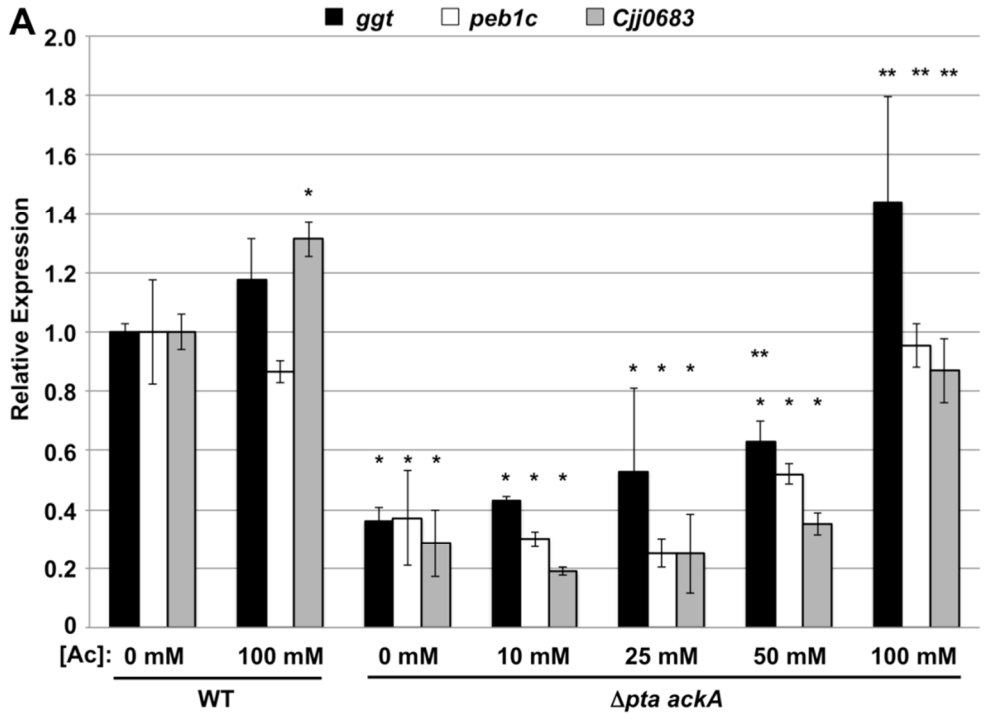
Figure 16. Transcriptional analysis of the SCFA-induced regulon in isogenic *C. jejuni* acetogenesis mutants. (A and B) Semi-quantitative real-time PCR analysis of transcription of a subset of *ggt*, *peb1c*, and *Cjj0683* in WT *C. jejuni* (purple bars) and isogenic acetogenesis mutants lacking Δ*pta* (grey bars), Δ*ackA* (blue bars), or Δ*pta* Δ*ackA* (red bars). The expression of each gene in the WT *C. jejuni* 81-176 Sm^R as measured by qRT-PCR was set to 1. Expression of each gene in the mutants is shown relative to the WT strain. Strains were grown in (A) rich MH broth or (B) CDM in microaerobic conditions to mid-log phase and were examined in triplicate. Error bars indicate the standard deviation. Statistically significant differences in gene expression between WT *C. jejuni* and mutants is indicated (* *P*-value < 0.05) and was determined by the Student's *t*-test.

mM acetate, the levels of expression of the genes in Δ*pta* Δ*ackA* were fully restored to WT levels (Figure 17A).

We postulated that acetate may be used in a catabolic pathway or as a signaling molecule itself in *C. jejuni* to positively affect expression of the SCFA-induced regulon. Currently, the only known catabolic pathway for acetate in *C. jejuni* is the conversion of acetate to Ac-CoA via Acs that occurs during the 'acetate switch' that many bacteria experience in late phases of growth (181). We tested the potential for Acs to convert acetate to Ac-CoA for the acetate-dependent effects on expression of *ggt*, *peb1c*, and *Cjj0683*. Compared to the WT strain, *C. jejuni* Δ*acs* did not show a reduction in expression of these genes (Figure 17B). In addition, expression of the genes in *C. jejuni* Δ*pta* Δ*ackA* Δ*acs* was similarly low as Δ*pta* Δ*ackA* (Figure 17B). Upon addition of 100 mM acetate to Δ*pta* Δ*ackA* Δ*acs*, we found a 3.7- to 9.2-fold increase in expression that was just 25 % lower than those of the WT strain without acetate or Δ*pta* Δ*ackA* with 100 mM acetate (Figure 17B). We interpret these data as suggesting that acetate conversion by Acs only accounts for a small amount restoration of gene expression upon addition of acetate to the acetogenesis mutant. Instead, we propose that acetate is either used by unknown catabolic pathway or as a signaling molecule to stimulate expression of the SCFA-induced regulon in *C. jejuni*.

SCFAs and lactate opposingly influence expression of the SCFA-induced regulon

We examined if other SCFAs present in the avian lower intestinal tract could affect expression of the SCFA-induced regulon. Butyrate is another SCFA that is present in the lower intestinal tract of chicks, normally between 12.5 and 25 mM (189, 303). Indeed, addition of butyrate to WT *C. jejuni*


A
ggt
peb1c
Cjj0683
Relative Expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
[Ac]: 0 mM 100 mM 0 mM 10 mM 25 mM 50 mM 100 mM
WT
Δpta ackA

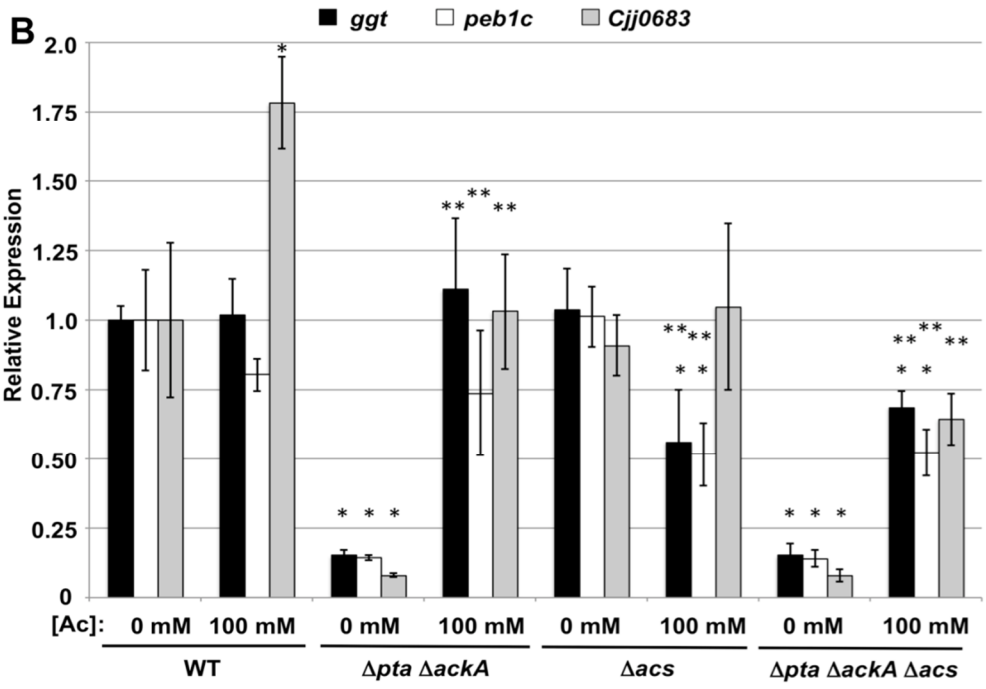

B
ggt
peb1c
Cjj0683
Relative Expression
2.0
1.75
1.50
1.25
1.0
0.75
0.50
0.25
0
[Ac]: 0 mM 100 mM 0 mM 100 mM 0 mM 100 mM 0 mM 100 mM
WT
Δpta ΔackA
Δacs
Δpta ΔackA Δacs

Figure 17. Expression of the SCFA-induced regulon in WT and isogenic acetogenesis mutants upon exogenous acetate supplementation. (A and B) Semi-quantitative real-time PCR analysis of transcription of *ggt*, *peb1c*, and *Cjj0683* in WT *C. jejuni* and isogenic mutants grown in CDM or CDM with different concentration of exogenous acetate (Ac). All media was equilibrated to pH 7.0 prior to growth of bacteria to eliminate effects due to acidification by exogenous acetate. The expression of *ggt* (black bars), *peb1c* (white bars), and *Cjj0683* (grey bars) in the WT *C. jejuni* 81-176 Sm^R grown without acetate as measured by qRT-PCR was set to 1. Expression of each gene in the mutants grown with or without acetate is shown relative to the WT strain. Error bars indicate standard deviations. Statistically significant differences in gene expression between WT *C. jejuni* without acetate and strains with or without acetate (*, $P < 0.05$) or between each individual strain without acetate or with acetate (**, $P < 0.05$) as performed by the Student's *t*-test are indicated.

caused a 4-fold increase in expression of two genes of the SCFA-induced regulon, *ggt* and *Cjj0683*, but *peb1c* expression remained unchanged (Figure 18A). With *C. jejuni* Δ*pta* Δ*ackA*, we observed a striking restoration of gene expression with butyrate supplementation. With 12.5 mM butyrate, expression of the SCFA-induced regulon was restored to levels of WT *C. jejuni* grown in CDM alone (Figure 18A). At 25 mM butyrate, we observed further stimulation that exceeded WT *C. jejuni* without butyrate by 2- to 7-fold. In comparison to *C. jejuni* Δ*pta* Δ*ackA* grown with acetate supplementation, butyrate promoted a greater level of gene expression on a lower molar concentration, suggesting that *C. jejuni* is more sensitive to butyrate for stimulation of the SCFA-induced regulon (compare Figure 17A to 18A).

Typically, the concentrations of organic acids such as lactate and SCFAs are inversely correlated in differing regions of the intestinal tract in avian species and humans. For example, lactate is usually present in higher concentrations of the upper intestinal tract while SCFAs are more limiting. However, in the lower intestinal tract SCFAs are more abundant relative to lactate (178, 303-305). Therefore, we tested whether lactate may impact expression of the SCFA-induced regulon. After growth of WT *C. jejuni* in CDM with different concentrations of lactate, we observed a dose-dependent reduction of expression of the SCFA-induced regulon (Figure 18B). At 25 mM, which is reported to best represent the physiological concentrations of lactate in the upper regions of the intestinal tract, we observed 2- to 5-fold reduction in gene expression (Figure 18B; (188)). In *C. jejuni* Δ*pta* Δ*ackA*, we found very little effects of lactate on gene expression. We did observe up to a 3.5- to 6- fold increase in expression in *peb1c* and *Cjj0683*, but these increases were still 5-fold lower than WT *C. jejuni* without supplementation (Figure 18B). These

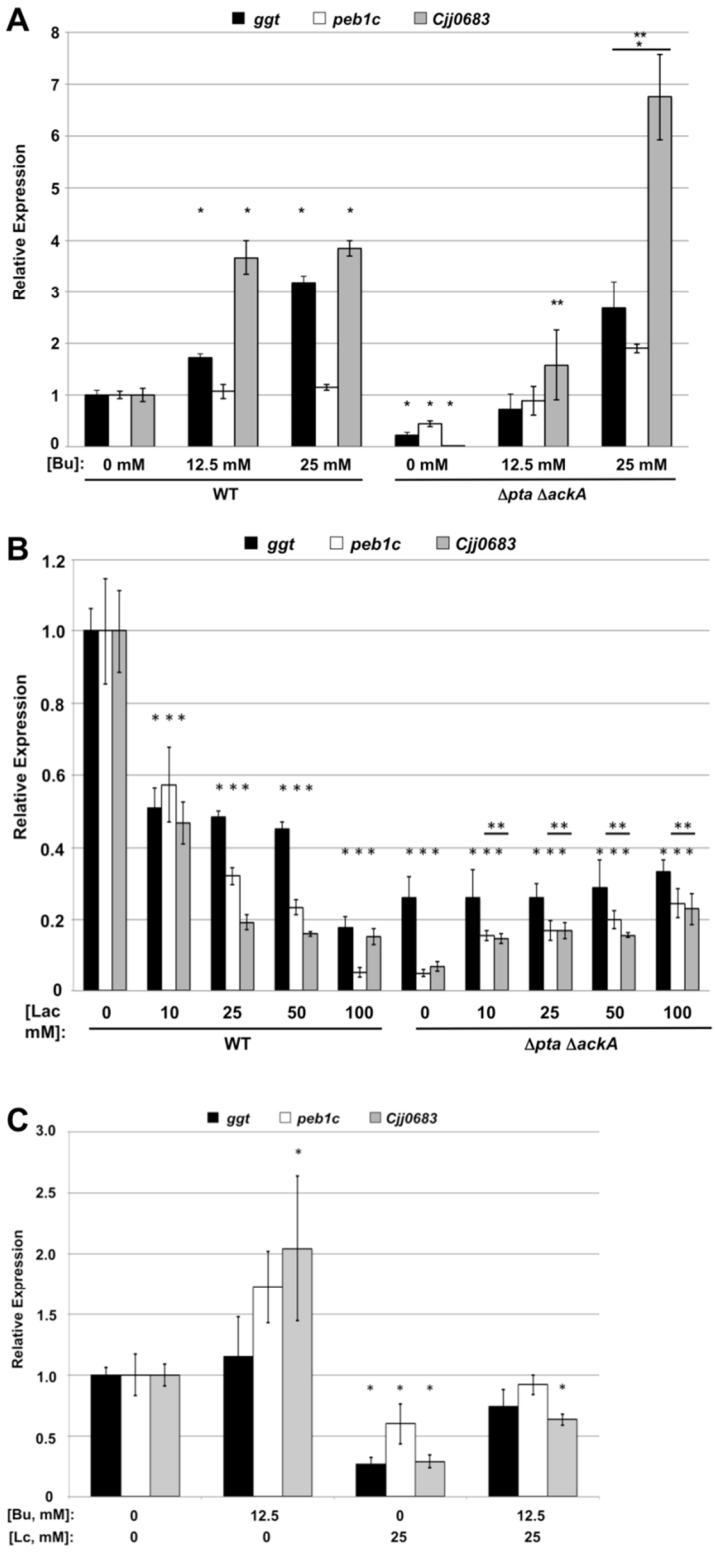

A
ggt
peb1c
Cjj0683
Relative Expression
[Bu]:
0 mM
12.5 mM
25 mM
0 mM
12.5 mM
25 mM
WT
Δpta ΔackA
B
ggt
peb1c
Cjj0683
Relative Expression
[Lac mM]:
0
10
25
50
100
0
10
25
50
100
WT
Δpta ΔackA
C
ggt
peb1c
Cjj0683
Relative Expression
[Bu, mM]:
[Lc, mM]:
0
0
12.5
0
0
25
12.5
25

Figure 18. Effect of butyrate and lactate on expression of the SCFA-induced regulon in WT and *C. jejuni* Δ*pta* Δ*ackA*. (A-C) Semi-quantitative real-time PCR analysis of transcription of *ggt*, *peb1c*, and *Cjj0683* in WT *C. jejuni* and isogenic Δ*pta* Δ*ackA* mutants grown in CDM or CDM with different concentrations of butyrate (Bu) (A), lactate (Lc) (B), or a mixture of butyrate and lactate (C). For (C), only the WT strain was analyzed with the indicated media supplementations. All media was equilibrated to pH 7.0 prior to growth of bacteria to eliminate effects due to acidification by exogenous butyrate or lactate. The expression of *ggt* (black bars), *peb1c* (white bars), and *Cjj0683* (grey bars) in the WT *C. jejuni* 81-176 Sm^R grown without any supplementation was measured by qRT-PCR was set to 1. Expression of each gene in the mutants grown with or without supplementation is shown relative to the WT strain. Error bars indicate standard deviations. Statistically significant differences in gene expression between WT *C. jejuni* without supplementation and strains with or without supplementation (*, $P < 0.05$) or between each individual strain without with or without supplementation (**, $P < 0.05$) as performed by the Student's *t*-test are indicated.

data indicate that lactate has an opposite effect on transcription of the SCFA-induced regulon in WT *C. jejuni*, with lactate possibly functioning *in vivo* to dampen expression of these genes in the upper regions of the intestinal tract where lactate is a predominant metabolite over SCFAs.

Considering the inverse correlation of concentrations of lactate and SCFAs in different regions of the chick gut and the differing effects of lactate and SCFAs on expression of the SCFA-induced regulon, we analyzed the net effect of a mixture of lactate and butyrate on gene expression in WT *C. jejuni*. For this assay, we supplemented CDM with physiological concentrations of butyrate in the ceca (12.5 mM) and lactate in the small intestines (25 mM; (188, 189)). Consistent with our data described above, we noticed stimulation of gene expression with butyrate alone and a repression of gene expression with lactate alone (Figure 18C). However, in the presence of butyrate and lactate, the level of expression of the SCFA-induced regulon was close to WT *C. jejuni* grown without either metabolite. These data suggest that lactate and SCFAs opposingly affect expression of the SCFA-induced regulon. Furthermore, the data suggest that butyrate can counteract the repressive effects of lactate on expression of these genes and may function *in vivo* to stimulate expression of factors necessary for colonization, especially in the lower intestinal tract.

We then investigated whether we could find evidence for spatial-dependent differences in *in vivo* expression of the SCFA-induced regulon by *C. jejuni* during colonization of the chick gut. We reasoned that if lactate and SCFAs were impacting the *in vivo* expression of the SCFA-induced regulon, we may

observe higher concentrations of expression of these genes in the lower intestinal tract than the upper intestinal tract. Thus, we monitored expression of the SCFA-induced regulon in WT *C. jejuni* colonizing different regions of the chick intestinal tract at day 7 post-infection. The highest level of expression of the SCFA-induced regulon occurred in the ceca, which contains the highest loads of *C. jejuni* during colonization (Figure 19 and Figure 12A). Note that expression of genes in each region of the intestinal tract was relative to the expression of a control housekeeping gene, not relative to the absolute numbers of *C. jejuni* present at the site of colonization. In the large intestines, which contain the second highest levels of *C. jejuni*, we observed a 2.5-fold lower decrease in expression of *ggt* and *Cjj0683*; the level of expression of *peb1c* on average was similar to *C. jejuni* in the ceca (Figure 19). The levels of expression of the SCFA-induced regulon in the upper regions of the intestinal tract including both regions of the small intestines, however, were 5- to 20-fold lower that *C. jejuni* in the ceca (Figure 19). These data correlate with the highest level of expression of the SCFA-induced regulon occurring in regions of the avian intestinal tract with highest levels of activating SCFAs and lower levels of inhibitory lactate (189, 303). Thus, we propose that *C. jejuni* monitors SCFAs in the environment to initiate production of catabolic pathways and colonization factors necessary for colonization and persistence in favored niches in the natural avian host.

Discussion

Bacteria sense and respond to their environments to locate niches with appropriate nutrients for growth, to evade detrimental conditions, and to alter their surroundings to persist. For bacterial commensals and pathogens, the ability to monitor host surroundings allow bacteria to discriminate between different sites in the host, microniches in a tissue, and even different compartments inside a host cell. In this work, we discovered that *C. jejuni* responds to organic acids and SCFAs by altering transcription of genes required for colonization, which we have termed the 'SCFA-induced regulon' of *C. jejuni*. Members of the SCFA-induced regulon include many genes for transport and utilization of amino acids used in *C. jejuni* catabolism. Furthermore, we provided *in vivo* correlative evidence that a similar

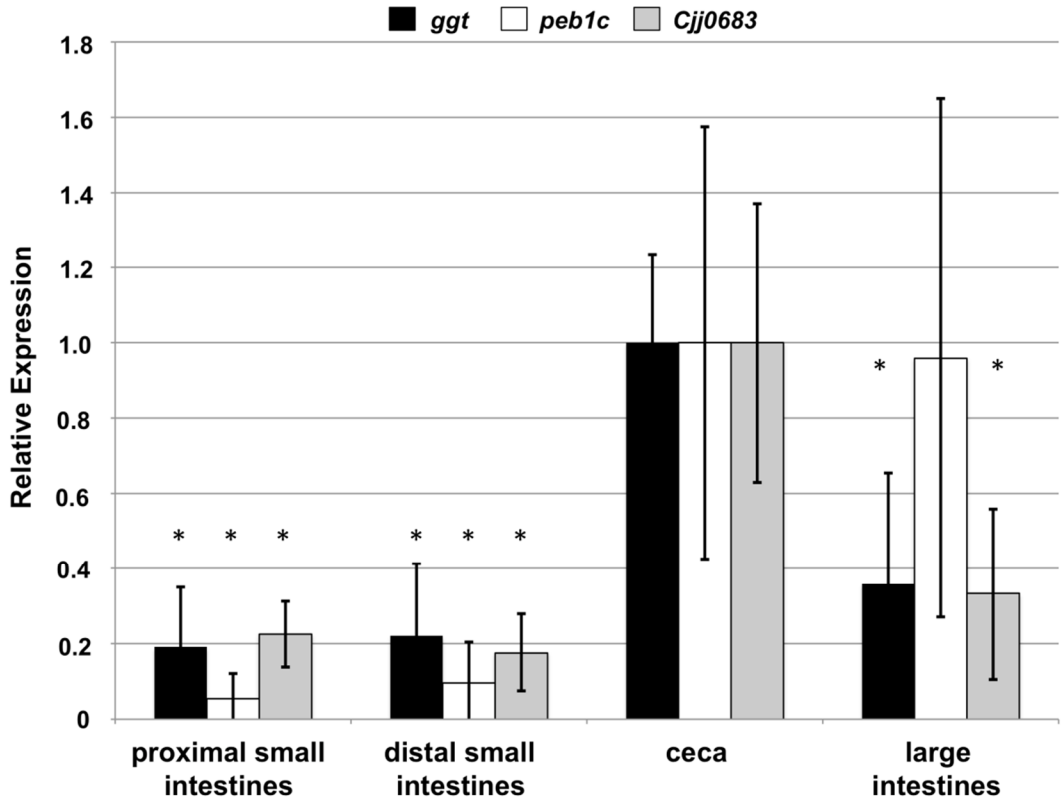


Figure 19. *In vivo* expression of the SCFA-induced regulon in *C. jejuni* colonizing different regions of the avian intestinal tract. Semi-quantitative real-time PCR analysis of transcription of *ggt*, *peb1c*, and *Cjj0683* in WT *C. jejuni* isolated from different regions of the avian intestinal tract at day 7 post-infection. The expression of *ggt* (black bars), *peb1c* (white bars), and *Cjj0683* (grey bars) in the WT *C. jejuni* 81-176 Sm^R isolated from the ceca as measured by qRT-PCR was set to 1. Expression of each gene in *C. jejuni* from different regions of the intestinal tract is shown relative to the WT strain isolated from the ceca. In total, gene expression was analyzed from six different chicks and combined. Error bars indicate standard deviations. Statistically significant differences in gene expression between WT *C. jejuni* isolated from the ceca and other regions of the intestinal tract (*, $P < 0.05$) as performed by the Student's *t*-test are indicated.

response occurs throughout the intestinal tract of the natural avian host during commensalism. In the lower intestinal tract where SCFAs are most prevalent and *C. jejuni* most abundant, *C. jejuni* expressed the highest levels of the SCFA-induced regulon. Thus, a SCFA-rich environment in the ceca and large intestines induced the catabolic genes and pathways *C. jejuni* needs for optimal growth and colonization in these preferred sites in the natural avian host. In contrast, the lowest levels of expression of the SCFA-induced regulon occurred in the small intestines, which contain high concentrations of lactate, and where *C. jejuni* colonizes to a much lower extent. To the best of our knowledge, our work provides one of the first examples and strongest correlations for metabolites that are sensed *in vivo* by *C. jejuni* that impact expression of colonization factors necessary for commensalism in the natural avian host. We suspect that a similar spatial arrangement of lactate and SCFAs may be sensed by *C. jejuni* in the human host, which may lead to the production and colonization and virulence determinants to promote infection and disease.

We propose that *C. jejuni* may discriminate between different regions of the avian intestinal tract by monitoring and responding to the differing concentrations of lactate and SCFAs in the avian host. These metabolites are largely generated by the intestinal microbiota. Thus, we suspect that the microbiota is spatially influencing the ability of *C. jejuni* to colonize the avian host. However, we also believe that we found evidence for the microbiota temporally influencing the colonization dynamics of *C. jejuni*. During an early time point of infection (day 7 post-infection), we observed *C. jejuni* being dependent on its own acetogenesis pathway for optimal colonization of different regions of the intestinal tract. In our model system, the microbiota is relatively immature in chicks directly after hatch when infection is initiated with lactate-producing bacteria dominating the intestinal tract, especially in the small intestines (183). SCFA-producing bacteria gradually emerge as more prevalent members of the lower intestinal tract days later. Therefore, 1-day old chicks that were infected with *C. jejuni* in our assays would be predicted to have an immature microbiota that produces more lactate than SCFAs. The levels of SCFAs produced by the microbiota are likely not sufficient at this early time point of infection to be sensed by *C. jejuni* and activate expression of the SCFA-induced regulon. Thus, *C. jejuni* may need to generate its own acetate *in vivo* in young chicks to stimulate expression of these genes necessary for optimal colonization. However,

as the chicks age, SCFA-producing bacteria become major members of the cecal and large intestinal microbiota, and contribute to significant SCFA concentrations that can be sensed by *C. jejuni* (183). This surge of SCFAs likely negates the need for *C. jejuni* to produce its own acetate via the acetogenesis pathway to keep transcription of the SCFA-induced regulon sufficient for colonization (such as what we observed at day 14 post infection; Figure 12B). We propose that *C. jejuni* employs its acetogenesis system as a compensatory mechanism to ensure the supply of a SCFA to induce an optimal level of expression of the SCFA-induced regulon if it is in an intestinal region that lacks sufficient levels of these metabolites.

It is possible that this different effect that lactate and SCFAs have on the expression of the SCFA-induced regulon may facilitate a homing mechanism for *C. jejuni* to identify the optimal sites of colonization, which are the lower regions of the intestinal tract. The higher level of lactate in the upper small intestines is likely one factor to reduce the expression of the SCFA-induced regulon and the colonization capacity of *C. jejuni.* We suspect that the acetogenesis pathway of *C. jejuni* does not generate sufficient acetate to counteract the suppressive effects of lactate. However, in the lower intestinal tract SCFAs are more abundant and lead to activation of the SCFA-induced regulon. Even though some lactate is present in the lower intestinal tract, we suspect that SCFAs produced by the microbiota and perhaps even the acetate generated by *C. jejuni* is sufficient to overcome the suppressive effects of lactate. Indeed, we observed that when butyrate and lactate were supplemented together at physiological concentrations, butyrate could counteract the effects of lactate on WT *C. jejuni* for expression of the SCFA-induced regulon. Undoubtedly, we suspect that *C. jejuni* is monitoring other factors, conditions, and metabolites in the host gut to discern appropriate niches for colonization in addition to SCFAs.

We have provided clear evidence that *C. jejuni* responds to lactate and SCFAs by altering the transcription of specific factors required for growth and colonization in the natural host. Currently, it is unclear if lactate and SCFAs enter a catabolic pathway to produce byproducts that ultimate impact transcription of the SCFA-induced regulon or if there is a sensing and signal transduction mechanism to detect these metabolites. At least for acetate, we analyzed the only known catabolic pathway in *C. jejuni.*

However, we observed that acetate supplementation largely restored expression of the SCFA-induced regulon in an *acs* mutant that was unable to convert acetate to Ac-CoA. Considering that we observed effects of acetate, butyrate, and lactate on expression of the SCFA-induced regulon, we propose that the simplest explanation is that there is a system to sense these metabolites and lead to different effects on transcription of the SCFA-induced regulon.

In other bacteria such as *E. coli* and *Salmonella*, SCFAs are sensed by homologous BarA-UvrY two-component regulatory systems (306). SCFAs are detected by the BarA sensor kinase, which results in phosphorylation of the UvrY response regulator and transcription of the non-coding RNAs (ncRNAs) CsrB and CsrC. These ncRNAs sequester the mRNA-binding protein CsrA. Without sequestration, CsrA represses translation of mRNAs encoding proteins that function in various aspects of physiology such as carbon metabolism, motility, peptide uptake, and virulence. Although *C. jejuni* produces CsrA, it does not encode homologs of the BarA-UvrY system, CsrB, or CsrC (307). Thus, it is currently unclear how *C. jejuni* may sense lactate and SCFAs to lead to specific transcriptional responses. It is also unclear whether these metabolites are sensed by the same or different signal transduction mechanisms.

We also noticed that on occasion not all members of the SCFA-induced regulon were collectively influenced to the same degrees by lactate or SCFAs. Currently, without a transcriptional mechanism to explain how these metabolites influence expression of these genes we are unable to comment on these differences in expression. We suspect while a SCFA-dependent mechanism exist to influence expression of these genes, each of the genes may be influenced by other systems and regulators, creating complex mechanisms for their expression in different environments.

The repressive effects that we observed for lactate on the transcription of the SCFA-induced regulon sheds new insights into how this metabolite may negatively impact *C. jejuni* growth *in vivo*. It has been reported that lactate has antimicrobial properties for *C. jejuni* and lactate-producing bacteria are potential probiotics to reduce *C. jejuni* in poultry flocks in agriculture (241). Infection of chicks with these bacteria has been shown to reduce the level of *C. jejuni* in the ceca, although the mechanism through which these probiotics work remains to be elucidated (241). Curiously, lactate itself has been

administered to chicken carcasses after slaughter and is effective in reducing the levels of *C. jejuni* on the surfaces of commercial poultry meats (242, 243). The potential antimicrobial properties of lactate *in vivo* remain to be determined. In one report, the acidification of the environment by lactate reduced the viability of *C. jejuni*, but there was evidence for lactate having pH-independent effects on *C. jejuni*. In our assays where CDM was supplemented with lactate or any SCFAs, pH was not a factor as all media was balanced to neutral pH. We observed that lactate functioned in a mechanism to repress transcription of the SCFA-induced regulon, which contains genes for colonization determinants. Therefore, it is possible that a probiotic effect of lactate and lactate-producing bacteria functions to reduce levels of *C. jejuni* colonization by lowering transcription of genes required for *in vivo* growth and catabolism.

Our work provides strong evidence for metabolites, specifically lactate and SCFAs produced by gut microbiota, to greatly impact the behavior of *C. jejuni* and its ability to colonize the natural avian host. We suspect that similar mechanisms may occur in the human host to impact the ability of *C. jejuni* to express virulence factors for pathogenesis of diarrheal disease. Much future work will be required to unravel how lactate and SCFAs are sensed and how the response mechanisms lead to opposing effects on transcription of colonization and virulence determinants. Furthermore, our work may have implications for how lactate, and possibly other unidentified metabolites, may negatively impact *C. jejuni* growth and transcription *in vivo*, providing an opportunity to explore different antimicrobial strategies to reduce *C. jejuni* not only in the human host, but also in agriculture and the human food supply.

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CHAPTER FIVE

Discussion

Overview

C. jejuni is one of the world's leading causes of food-borne diarrheal diseases in humans. In addition, the bacterium has the ability to infect the intestinal tract of a multitude of hosts, including agriculturally-important animals such as chickens to promote commensalism. Adaptation for life inside the infected host requires that *C. jejuni* senses and responds to a multitude of environmental signals. The work presented herein describes a previously uncharacterized two-component regulatory system (TCS) that *C. jejuni* may utilize to aid in responding to conditions inside and outside the intestinal environment. We showed that although this system itself was not required for commensal colonization of the avian host, it acted through multiple possible mechanisms to repress transcription of many genes involved in metabolite acquisition, including a known colonization factor. In addition, we explored the role of organic acids and short-chain fatty acids (SCFAs), which are metabolites commonly found throughout the intestinal mucosa, in *C. jejuni* commensal colonization of the natural avian host. Through this work, we discovered that production of the SCFA acetate by *C. jejuni* acts as a signal for transcriptional activation of the SCFA-induced regulon, which contains genes responsible for important metabolic processes required for commensal colonization or virulence. In addition, we discovered that concentrations of the SCFAs acetate and butyrate normally found in the lower intestinal tract stimulated expression of the SCFA-induced regulon. We also were able to identify the organic acid lactate as a signal that inhibits transcription of this regulon. Furthermore, we found a correlation between the spatial distribution of differential concentrations of lactate and SCFAs within the avian intestinal tract and where *C. jejuni* colonizes and expresses the SCFA-induced regulon. The results from this work suggest that lactate and SCFAs act as signals to allow *C. jejuni* to discriminate between different regions of the avian (and

possibly human) intestinal tract to coordinate expression of genes required for catabolism, colonization, and possibly virulence.

The *C. jejuni* Cjj1484-Cjj1483 TCS is a Cognate TCS

In many bacteria, histidine kinases (HKs) and response regulators (RRs) participate in coordinated phosphotransfer events, linking the two regulatory proteins together as a cognate TCS that controls transcription of genes encoding a regulon. Often, components of cognate TCSs are present within an operon on a bacterial genome. Upon examination of the *C. jejuni* genome, we observed this arrangement for the Cjj1484-Cjj1483 TCS, with *Cjj1484* encoded directly upstream of *Cjj1483.* Furthermore, a separate study had found an independent promoter upstream of *Cjj1484* that may be sufficient for transcription of both genes (120). Indeed, upon immunoblotting analysis, we found that this promoter allowed for *Cjj1483* transcription at a high level. In addition, transcriptome analysis of *Cjj1484* and *Cjj1483* mutants revealed many overlapping genes between the two regulons, providing further evidence that these proteins form a cognate TCS. Direct phosphotransfer and/or dephosphorylation of Cjj1483 by the HK Cjj1484 would allow us to definitively verify Cjj1484-Cjj1483 as a cognate TCS. However, due to difficulties in purifying sufficient soluble Cjj1484, we can only speculate the direct role of Cjj1484 for Cjj1483-mediated repression or activation of gene expression.

The Cjj1484-Cjj1483 TCS Mainly Functions as a Repressor of Transcription

Following sensing of a specific signal, TCSs can activate or repress transcription to modulate gene expression. Upon analyzing the transcriptome data, we found that many genes in the Cjj1484-Cjj1483 TCS regulon were repressed by the Cjj1483 RR. In contrast, only a subset of the genes was repressed by the Cjj1484 HK, whereas others required the HK for activation. Furthermore, there was one gene which required the Cjj1484-Cjj1483 TCS for full WT levels of expression. By combining expression and DNA promoter-binding analysis of WT and mutant Cjj1483 proteins, we were able to propose four different models by which the cognate Cjj1484-Cjj1483 TCS may function to repress or activate gene

expression: 1) Cjj1484 maintains an unphosphorylated Cjj1483, which binds DNA to repress transcription; 2) Cjj1483 binds DNA to repress transcription until Cjj1484 serves as a phosphodonor, which subsequently relieves repression; 3) Cjj1483 may use a non-cognate source for phosphorylation, inducing repression; 4) phosphotransfer from Cjj1484 to Cjj1483 leads to activation of transcription. Further biochemical, expression, and DNA-binding analyses are required before these models can be finalized.

The Cjj1484-Cjj1483 Regulon May Undergo Complex Regulation *in vivo*

In this work, we presented evidence that the Cjj1484-Cjj1483 TCS directly influences transcription of some genes. However, work by other groups has shown that transcription of many genes included in the Cjj1484-Cjj1483 regulon we discovered are also controlled by other factors. These factors include the regulatory proteins Fur and PerR (in response to variable iron conditions), hyperosmotic stress, pH levels, and the TCS RacRS (174, 250, 292-295). We also found evidence that in some situations, the Cjj1483 RR may be transcribed independently of Cjj1484. Considering these factors, it is likely that transcriptional regulation of the Cjj1484-Cjj1483 regulon is complex and possibly multifactorial, with some regulatory factors mediating action through the Cjj1484-Cjj1483 TCS. Furthermore, we propose that these regulatory systems may work in unity to promote finely controlled expression of the Cjj1484-Cjj1483 regulon in response to specific environments. As such, the signals that are sensed by the Cjj1484-Cjj1483 TCS may include factors that contribute to the complex regulation described above. Previous analysis of the gluconate dehydrogenase complex encoded by *Cjj0438-Cjj0439* found that expression levels increased in response to body temperature corresponding to that of the avian host (269), perhaps indicating temperature as a possible inducer of expression. However, we failed to observe temperature dependent effects on gluconate dehydrogenase activity and expression (data not shown). Regardless, while the specific signals are yet to be identified, delineating whether multiple regulatory factors affect expression of the Cjj1484-Cjj1483 regulon can be accomplished by generating multiple mutations in both the TCS and the possible other regulators. For example, this could include

generating a Δ*Cjj1483* Δ*fur* mutant, followed by expression analysis after exposure to high and low iron levels. By comparing expression in this strain to expression of Δ*Cjj1483* and Δ*fur* single mutants, we can determine whether removal of multiple regulatory factors results in an additive effect on transcription. Mutating the other known HKs of *C. jejuni* by using this method could also help in discerning whether non-cognate phosphodonors can influence Cjj1483-mediated repression.

The Cjj1484-Cjj1483 TCS may Assist *C. jejuni* in Adapting to *in vivo* and/or *ex vivo* Environments

Although the Cjj1484-Cjj1483 TCS and select genes within its regulon that we tested were not required for commensal colonization of the chick, the mode of regulation and the importance of these genes to *C. jejuni* biology must be considered. Most of the genes we found in this regulon have been shown to be involved in respiration and iron/heme acquisition. Furthermore, the majority of these factors were repressed by the Cjj1484-Cjj1483 TCS in the *in vitro* conditions we tested. I believe it is possible that upon infection of a host, *C. jejuni* may sense specific signals that abrogate repression of transcription through this TCS, thus promoting growth through nutrient acquisition. This could explain why *Cjj1484* and *Cjj1483* isogenic mutants were not deficient for chick colonization or *in vitro* growth, as these mutations resulted in derepression of transcription. To test this hypothesis, the RNA from the cecal contents from chicks infected with WT, Δ*Cjj1484*, and Δ*Cjj1483* mutants could be acquired, and expression of genes of the Cjj1484-Cjj1483 regulon examined. If the Cjj1484-Cjj1483 TCS is not actively repressing expression *in vivo*, we would expect to see equal levels of expression between the WT and mutant strains.

Although the Cjj1484-Cjj1483 TCS may relieve repression once inside of the chick gut, this may not be the main role of this regulatory system. A second possibility includes priming the bacterium once secreted from the host in the feces. Perhaps genes in the regulon are repressed *ex vivo*, which assists *C. jejuni* in using alternative pathways for survival or acquisition of nutrients immediately upon excretion. This may prevent *C. jejuni* from overexerting energy in an attempt to acquire nutrients that are scarce *ex vivo*, increasing the likelihood of survival. To test this, we could collect feces from chicks that have been

infected with WT or mutant strains of *C. jejuni* and analyze levels of expression of the Cjj1484-Cjj1483 regulon. We could also compare the survival in feces between the WT and mutant strains. Furthermore, expression from *C. jejuni* in the feces could be compared against expression from *C. jejuni* in the ceca, allowing for direct comparison between these two scenarios.

Finally, it is possible that this TCS may be involved in bird-to-bird transmission of *C. jejuni*, which occurs through the consumption of expelled feces. Following infection of chicks with the WT and mutant strains, we could remove the infected chicks from their soiled cages, insert uninfected chicks, and monitor transmission by recovery of *C. jejuni* in the ceca of the infected chicks. This experiment would also allow us to monitor a potential requirement for the Cjj1484-Cjj1483 TCS in survival following passage through the intestinal tract.

SCFAs and Lactate Opposingly Influence Expression of the SCFA-induced Regulon

During infection, pathogenic bacteria utilize signals from the environment to modulate transcription of important growth, colonization, and virulence factors. As a bacterium that colonizes the intestinal tract, *C. jejuni* encounters metabolites produced by the endogenous gut microbiota as it migrates from the upper intestinal tract to its natural site of colonization, the ceca. These metabolites include fermentation products such as the organic acid lactate, and the short-chain fatty acids (SCFAs) acetate, butyrate, and propionate. It is currently unknown if *C. jejuni* utilizes SCFAs for metabolism as transport and catabolic pathways are not obviously encoded in the genome. Furthermore, how lactate and SCFAs may affect *C. jejuni* biology *in vivo* had not been explored until this study.

<u>*C. jejuni* self-production of acetate is required for growth and colonization</u>

To begin to understand how SCFAs impacted *C. jejuni* biology, we first explored the only known pathway *C. jejuni* has to produce a SCFA. Acetate is generated through the acetogenesis pathway of *C. jejuni*, and by creating a Δ*pta* Δ*ackA* mutant, we found that self-production of acetate is required for full commensal colonization of *C. jejuni* at day 7 post-infection. In addition, we demonstrated that an

acetogenesis mutant was deficient for growth in a media that contains only organic and amino acids as carbon sources. As such, we postulated that acetate production by *C. jejuni* influenced metabolic pathways, leading to the growth and colonization defects observed in the acetogenesis mutant strain. To that end, we performed transcriptome analysis of the *C. jejuni* acetogenesis mutant. Indeed, we found that the acetogenesis *in vitro* positively influenced transcription of many genes involved in catabolic pathways for amino acids, including genes known to be colonization or virulence determinants. We termed the genes found in this analysis as the 'SCFA-induced regulon'. In addition, this data also led to the discovery of a new colonization determinant, Peb1c, which is part of a system for the transport of glutamate and aspartate (131).

The SCFAs acetate and butyrate positively influence transcription of the SCFA-induced regulon

In our attempts to understand why acetogenesis by *C. jejuni* influenced transcription of the SCFA-induced regulon, we postulated whether concentrations of acetate produced by the gut microbiota and found commonly in the chick ceca could influence gene expression. Therefore, we tested whether exogenous acetate could restore expression of the SCFA-induced regulon to the acetogenesis mutant. Indeed, we observed that the presence of acetate at physiological concentrations found in the chick ceca could restore expression of SCFA-induced regulon genes in the acetogenesis mutant strain.

Following this result, we were curious as to whether SFCAs other than acetate could stimulate expression of the SCFA-induced regulon. Thus, we performed a similar assay analyzing expression with physiological concentrations of butyrate commonly produced by the gut microbiota in the ceca. Impressively, we found that low levels of butyrate restored expression of SCFA-induced regulon genes to WT levels in the Δ*pta* Δ*ackA* mutant. Furthermore, we saw additional increases in expression when the amount of butyrate was doubled, including increases of expression in both the WT and mutant strains. Thus, butyrate, which influences expression at a lower molar concentration than acetate, may act as a more potent stimulator *in vivo* of the SCFA-induced regulon of *C. jejuni*.

The organic acid lactate represses expression of the SCFA-induced regulon

The physiological levels of SCFAs we analyzed were concentrations normally found in the ceca, where *C. jejuni* colonizes at the highest levels (101). In contrast, SCFA concentrations are reduced in the small intestines where *C. jejuni* colonizes at the lowest level. However, organic acids such as lactate predominate in these regions. We analyzed whether expression of the SCFA-induced regulon would be reduced in the presence of lactate and possibly impair the colonization capacity of *C. jejuni* in the small intestines. Expression of the SCFA-induced regulon in the WT strain decreased in response to increasing amounts of lactate. This result, in combination with the activation of expression by SCFAs, suggests that *C. jejuni* monitors the metabolites present in the intestinal milieu to regulate expression of genes important for growth and colonization.

C. jejuni Monitors SCFA Concentrations in the Intestines Through an Unknown Mechanism

Monitoring of SCFAs in *E. coli* and *Salmonella* species occurs by the BarA-SirA/UvrY TCS (306). Detection of SCFAs by the BarA HK results in phosphorylation of the UvrY RR and subsequent transcription of the ncRNAs CsrB and CsrC. The mRNA binding protein, CsrA, is sequestered by these ncRNAs, allowing translation of mRNAs encoding proteins involved in bacterial physiology and growth. *C. jejuni* produces CsrA which has been shown to have similar functions to that of *E. coli* CsrA (307). However, *C. jejuni* does not encode putative homologs of the BarA-UvrY TCS, CsrB, or CsrC, making it unclear whether *C. jejuni* senses SCFAs and other organic acids in a manner similar to other bacteria. Furthermore, it is also unclear whether *C. jejuni* senses SCFAs and lactate through the same or different signal transduction mechanisms. It is possible that these metabolites are catabolized by *C. jejuni*, leading to a metabolic flux that stimulates or inhibits expression of the SCFA-induced regulon. However, although transport of lactate has been shown, no putative transport systems are known for SCFAs in *C. jejuni*. In addition, *C. jejuni* only encodes catabolic pathways for acetate and lactate utilization. We analyzed whether the conversion of acetate to Ac-CoA by Acs was important for the acetate-dependent activation of the SCFA-induced regulon, and observed that the transcriptional effect was largely Acs-

independent. Thus, we propose that the most likely scenario for SCFA- and lactate-influenced expression of this regulon occurs through an as yet unidentified signal transduction system.

Some work has been performed in an attempt to identify a system to sense lactate and SCFAs in *C. jejuni*, but we have encountered difficulties in identifying such factors for multiple reasons. We attempted to create transcriptional reporters to genes in the SCFA-induced regulon to monitor gene expression in WT *C. jejuni* and acetogenesis mutants. However, the SCFA- or lactate-dependent regulation was not maintained in contrast to when we measured RNA levels directly by microarray analysis or qRT-PCR. We surmise that these data may indicate that the SCFA-induced regulon is under post-transcriptional control. This allows for the possibility of the CsrA-encoded by *C. jejuni* to function in a manner similar to other bacteria, albeit with a different TCS and ncRNAs that must still be discovered.

In many bacteria, concentrations of nutrients in the environment are sensed by structures in the UTR of transcripts that form a riboswitch. Typically, these riboswitches control translation of proteins needed for metabolic processes relating to the sensed nutrients. In some instances, however, riboswitches have been shown to modulate transcription, as in the case of Rho-independent termination. Furthermore, there have been cases where riboswitches act *in trans* to interrupt transcription of certain genes (308). It is possible that butyrate and other SCFAs, when at high enough concentrations, could serve as a ligand that allows for transcription through either of these mechanisms. In contrast, lactate may occlude SCFA binding, or may induce conformational changes that result in transcriptional termination or prevention altogether. By analyzing the 5' UTR of members of the SCFA-induced regulon, riboswitches that sense SCFAs and lactate may be revealed.

SCFAs and Lactate Spatially and Temporally Influence the Expression of *C. jejuni* SCFA-induced Regulon

C. jejuni utilizes SCFAs and lactate to discriminate between different regions of the intestinal tract

Previous work by other groups has investigated the spatial distribution of lactate and SCFAs produced by the microbiota throughout the avian and human intestinal tracts, finding that lactate is

present in the highest concentration in the upper intestinal tract, whereas SCFAs are at highest concentrations in the lower intestinal tract (178, 189, 303-305). Considering that physiological concentrations of SCFAs in the ceca activated transcription and physiological concentrations of lactate in the small intestines repressed transcription of the SCFA-induced regulon, we examined whether a similar transcriptional response occurred during *C. jejuni* colonization of the avian intestinal tract. Indeed, expression of the SCFA-induced regulon was highest in the ceca (where there are high levels of SCFAs and *C. jejuni* colonization) and lowest in the small intestines (where lactate levels are high and *C. jejuni* colonization is low). Thus, this strong correlative evidence provides credence that the SCFA-rich environments of the lower intestinal tract (ceca and large intestine) induce expression of catabolic genes *C. jejuni* requires for optimal growth and colonization in the avian host, whereas the opposite occurs in the upper intestinal tract where SCFA levels are low. Although most abundant in the upper intestinal tract, low concentrations of lactate can also be found in the lower intestinal tract. However, we found that when *C. jejuni* was subjected to both butyrate and lactate, butyrate negated the inhibitory effect of lactate, allowing for full WT expression of the SCFA-induced regulon. This suggests that butyrate, with its strong stimulatory influence, acts as an excellent beacon to definitively signal to *C. jejuni* that it is in the lower intestinal tract.

We suspect *C. jejuni* senses the similar spatial arrangement of SCFAs and lactate in the human intestinal tract to promote infection and diarrheal disease. Examination of the SCFA-induced regulon revealed many genes that influence virulence factors in the murine model of infection. These include GGT, AnsA, Peb1a, SdaA, and AspB, which have been shown to influence *C. jejuni* colonization of the mouse liver and intestines. Furthermore, the Peb1 system, AspA, AspB, CiaC, and CadF encode proteins that have been previously shown to influence adherence, invasion, and survival within human intestinal epithelial cells *in vitro*. Altogether, this suggests a role for SCFAs and acetogenesis in *C. jejuni*-mediated human diarrheal disease. Murine colonization and *in vitro* invasion experiments where the *C. jejuni* acetogenesis pathway, SCFA, and lactate concentrations are manipulated will need to be performed to test this hypothesis.

C. jejuni requires self-produced acetate early during infection before sufficient production of SCFAs by the host microbiota

To establish the spatial distribution of SCFAs and lactate observed throughout the intestinal tract, the gut microbiota that produce high levels of these metabolites are similarly distributed. However, for this to occur, a shift in the intestinal microbiota as the chick ages must also take place (183). Thus, we believe we have found evidence of SCFAs produced by the intestinal microbiota exerting temporal influence over colonization genes included in the SCFA-induced regulon. As described above, a *C. jejuni* mutant incapable of making its own acetate was deficient for colonization of all regions of the chick intestinal tract. We believe that this is due to an immature intestinal microbiota present in the newly-hatched chick on the day of infection. This immature microbiota contains primarily lactate-producing bacteria and low amounts of SCFA-producing bacteria (183). Although we showed that butyrate can counteract the inhibitory effects of lactate, we suspect that this immature microbiota does not produce sufficient levels of SCFAs to stimulate this effect. Instead, we propose that *C. jejuni* utilizes its acetogenesis pathway to generate levels of acetate necessary to stimulate the SCFA-induced regulon to initiate colonization and growth in the intestinal tract of young chicks. As the chick ages, SCFA-producing bacteria become primary members of the ceca and the large intestines, producing high levels of SCFAs (183, 189). We suspect that this increase in SCFA concentration strongly stimulates the SCFA-induced regulon, supplanting the necessity of the acetogenesis pathway in *C. jejuni* (as seen 14 days post-infection). 16s rRNA sequencing of the chick intestinal bacteria both over the chick's lifetime as well as in different intestinal organs could help in confirming our hypothesis.

Closing remarks

Analysis of the signal-transduction systems of *C. jejuni* has revealed possible environmental stimuli that may influence *C. jejuni* growth and persistence during commensal colonization of the natural avian host and pathogenic infection of the human host. In this work, we have demonstrated the utility of a previously uncharacterized TCS for fine-tuned control of genes important for *C. jejuni* colonization and

iron acquisition. More importantly, we have demonstrated how metabolites produced by the native microbiota aid *C. jejuni* in discriminating between regions of the intestinal tract during colonization, allowing *C. jejuni* to home to appropriate sites of the host for colonization and long-term persistence.

ACKNOWLEDGEMENTS

First, I would like to thank my parents for engaging me in science at a young age. They continue to expose me to interesting and thought-provoking experiences, all the while never losing faith in my direction even when I moved 1,000 miles from home.

I would also like to thank my thesis mentor, David Hendrixson. Through his teaching, advice, and criticisms, I have grown to become the scientist that I am today, and will continue to grow due to his tutelage. I am also fortunate to have had the opportunity to work with a brilliant group of current and upcoming scientists, each one of them exhibiting the depth of training our mentor is capable of. Thank you Deb, CJ, Angelica, Joe, and Mickey for making each day at the lab a light-hearted one, and I look forward to following your future successes.

Furthermore, I would like to thank the friends I have made while living in Dallas, including those from school and those from my hockey team. I would have surely gone crazy without the breaks from science you provided.

Finally, I would like to thank the most important person in my life, my wife and best friend, Lauren. Throughout this journey you have supported me not only as a scientist, but also as a person. I am eternally lucky to know someone like you.

REFERENCES

1. **Dekeyser P, Gossuin-Detrain M, Butzler JP, Sternon J.** 1972. Acute enteritis due to related vibrio: first positive stool cultures. J Infect Dis **125:**390-392.

2. **Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AH, Whitehead S, Barrell BG.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665-668.

3. **Crim SM, Iwamoto M, Huang JY, Griffin PM, Gilliss D, Cronquist AB, Cartter M, Tobin-D'Angelo M, Blythe D, Smith K, Lathrop S, Zansky S, Cieslak PR, Dunn J, Holt KG, Lance S, Tauxe R, Henao OL, Centers for Disease C, Prevention.** 2014. Incidence and trends of infection with pathogens transmitted commonly through food--Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2013. MMWR Morb Mortal Wkly Rep **63:**328-332.

4. **Olson CK, Ethelberg S, van Pelt W, Tauxe RV.** 2008. Epidemiology of *Campylobacter jejuni* infections in industralized nations, p 163-189. *In* Nachamkin I, Szymanski CM, Blaser MJ (ed), *Campylobacter*, 3rd ed. ASM Press, Washington, DC.

5. **Friedman CR, Hoekstra RM, Samuel M, Marcus R, Bender J, Shiferaw B, Reddy S, Ahuja SD, Helfrick DL, Hardnett F, Carter M, Anderson B, Tauxe RV.** 2004. Risk factors for sporadic *Campylobacter* infection in the United States: A case-control study in FoodNet sites. Clin Infect Dis **38 Suppl 3:**S285-296.

6. **EFSA.** 2005. Scientific report of the scientific panel on biological hazards on the request from the commission related to *Campylobacter* in animals and foodstuffs. Annex EFSA J **173:**1-105.

7. **Koenraad P, Rombouts F, Notermans S.** 1997. Epidemiological aspects of thermophilic *Campylobacter* in water related environments: a review. Water Environm Res **69:**52-63.

8. **NACMCF.** 1994. *Campylobacter jejuni* / *coli.* J Food Prot **57:**1101-1121.

9. **Blaser MJ, Taylor DN, Feldman RA.** 1983. Epidemiology of *Campylobacter jejuni* infections. Epidemiol Rev **5:**157-176.

10. **Moore JE, Caldwell PS, Millar BC, Murphy PG.** 2001. Occurrence of *Campylobacter* spp. in water in Northern Ireland: implications for public health. Ulster Med J **70:**102-107.

11. **Gallay A, De Valk H, Cournot M, Ladeuil B, Hemery C, Castor C, Bon F, Megraud F, Le Cann P, Desenclos JC.** 2006. A large multi-pathogen waterborne community outbreak linked to faecal contamination of a groundwater system, France, 2000. Clin Microbiol Infect **12:**561-570.

12. **Kuusi M, Nuorti JP, Hanninen ML, Koskela M, Jussila V, Kela E, Miettinen I, Ruutu P.** 2005. A large outbreak of campylobacteriosis associated with a municipal water supply in Finland. Epidemiol Infect **133:**593-601.

13. **Hanninen ML, Haajanen H, Pummi T, Wermundsen K, Katila ML, Sarkkinen H, Miettinen I, Rautelin H.** 2003. Detection and typing of *Campylobacter jejuni* and *Campylobacter coli* and analysis of indicator organisms in three waterborne outbreaks in Finland. Appl Environ Microbiol **69:**1391-1396.

14. **DeFraites RF, Sanchez JL, Brandt CA, Kadlec RP, Haberberger RL, Lin JJ, Taylor DN.** 2014. An outbreak of *Campylobacter* enteritis associated with a community water supply on a U.S. military installation. Msmr **21:**10-15.

15. **Jones PH, Willis AT, Robinson DA, Skirrow MB, Josephs DS.** 1981. *Campylobacter* enteritis associated with the consumption of free school milk. J Hyg (Lond) **87:**155-162.

16. **Peterson MC.** 2003. *Campylobacter jejuni* enteritis associated with consumption of raw milk. J Environ Health **65:**20-21, 24, 26.

17. **Mungai EA, Behravesh CB, Gould LH.** 2015. Increased outbreaks associated with nonpasteurized milk, United States, 2007-2012. Emerg Infect Dis **21:**119-122.

18. **Fahey T, Morgan D, Gunneburg C, Adak GK, Majid F, Kaczmarski E.** 1995. An outbreak of *Campylobacter jejuni* enteritis associated with failed milk pasteurisation. J Infect **31:**137-143.

19. **Schildt M, Savolainen S, Hanninen ML.** 2006. Long-lasting *Campylobacter jejuni* contamination of milk associated with gastrointestinal illness in a farming family. Epidemiol Infect **134:**401-405.

20. **Harris NV, Kimball TJ, Bennett P, Johnson Y, Wakely D, Nolan CM.** 1987. *Campylobacter jejuni* enteritis associated with raw goat's milk. Am J Epidemiol **126:**179-186.

21. **Robinson DA.** 1981. Infective dose of *Campylobacter jejuni* in milk. Br Med J (Clin Res Ed) **282:**1584.

22. **Black RE, Levine MM, Clements ML, Hughes TP, Blaser MJ.** 1988. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis **157:**472-479.

23. **van Spreeuwel JP, Duursma GC, Meijer CJ, Bax R, Rosekrans PC, Lindeman J.** 1985. *Campylobacter* colitis: histological immunohistochemical and ultrastructural findings. Gut **26:**945-951.

24. **Blaser MJ, Engberg J.** 2008. Clinical aspects of *Campylobacter jejuni* and *Campylobacter coli* infections, p 99-121. *In* Nachamkin I, Szymanski CM, Blaser MJ (ed), *Campylobacter*, 3rd ed. ASM Press, Washington, DC.

25. **Kapperud G, Lassen J, Ostroff SM, Aasen S.** 1992. Clinical features of sporadic *Campylobacter* infections in Norway. Scand J Infect Dis **24:**741-749.

26. **Peterson MC.** 1994. Rheumatic manifestations of *Campylobacter jejuni* and *C. fetus* infections in adults. Scand J Rheumatol **23:**167-170.

27. **Schaad UB.** 1982. Reactive arthritis associated with *Campylobacter* enteritis. Pediatr Infect Dis **1:**328-332.

28. **Pope JE, Krizova A, Garg AX, Thiessen-Philbrook H, Ouimet JM.** 2007. *Campylobacter* reactive arthritis: a systematic review. Semin Arthritis Rheum **37:**48-55.

29. **Bergman M, Heikema A, Kujif M, al. e.** 2007. Development of joint disease is associated with *Campylobacter jejuni* strains harboring lipo-oligosaccharide biosynthese gene cluster A. Zoonoses Public Health **54:**19-155.

30. **Mortensen NP, Kuijf ML, Ang CW, Schiellerup P, Krogfelt KA, Jacobs BC, van Belkum A, Endtz HP, Bergman MP.** 2009. Sialylation of *Campylobacter jejuni* lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. Microbes Infect **11:**988-994.

31. **Blaser MJ, Duncan DJ.** 1984. Human serum antibody response to *Campylobacter jejuni* infection as measured in an enzyme-linked immunosorbent assay. Infect Immun **44:**292-298.

32. **Hadden RD, Karch H, Hartung HP, Zielasek J, Weissbrich B, Schubert J, Weishaupt A, Cornblath DR, Swan AV, Hughes RA, Toyka KV.** 2001. Preceding infections, immune factors, and outcome in Guillain-Barre syndrome. Neurology **56:**758-765.

33. **Jacobs BC, Van Belkum A, Endtz HP.** 2008. Guillain-Barre Syndrome and *Campylobacter* Infection, p 245-262. *In* Nachamkin I, Szymanski CM, Blaser MJ (ed), *Campylobacter*, 3rd ed. ASM Press, Washington D. C.

34. **Young KT, Davis LM, Dirita VJ.** 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. Nat Rev Microbiol **5:**665-679.

35. **Zautner AE, Tareen AM, Gross U, Lugert R.** 2012. Chemotaxis in *Campylobacter jejuni*. Eur J Microbiol Immunol (Bp) **2:**24-31.

36. **Hugdahl MB, Beery JT, Doyle MP.** 1988. Chemotactic behavior of *Campylobacter jejuni*. Infect Immun **56:**1560-1566.

37. **Korolik V, Ketley J.** 2008. Chemosensory signal transduction pathway of *Campylobacter jejuni*, p 351-366. *In* Nachamkin I, Szymanski CM, Blaser MJ (ed), *Campylobacter*, 3rd ed. ASM Press, Washington, DC.

38. **Chang C, Miller JF.** 2006. *Campylobacter jejuni* colonization of mice with limited enteric flora. Infect Immun **74:**5261-5271.

39. **Yao R, Burr DH, Guerry P.** 1997. CheY-mediated modulation of *Campylobacter jejuni* virulence. Mol Microbiol **23:**1021-1031.

40. **Konkel ME, Garvis SG, Tipton SL, Anderson DE, Jr., Cieplak W, Jr.** 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol Microbiol **24:**953-963.

41. **Monteville MR, Konkel ME.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect Immun **70:**6665-6671.

42. **Monteville MR, Yoon JE, Konkel ME.** 2003. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. Microbiology **149:**153-165.

43. **Ziprin RL, Young CR, Stanker LH, Hume ME, Konkel ME.** 1999. The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. Avian Dis **43:**586-589.

44. **Jin S, Joe A, Lynett J, Hani EK, Sherman P, Chan VL.** 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol Microbiol **39:**1225-1236.

45. **Jin S, Song YC, Emili A, Sherman PM, Chan VL.** 2003. JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90alpha and triggers signalling pathways leading to the activation of NF-kappaB and p38 MAP kinase in epithelial cells. Cell Microbiol **5:**165-174.

46. **Ashgar SS, Oldfield NJ, Wooldridge KG, Jones MA, Irving GJ, Turner DP, Ala'Aldeen DA.** 2007. CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. J Bacteriol **189:**1856-1865.

47. **Flanagan RC, Neal-McKinney JM, Dhillon AS, Miller WG, Konkel ME.** 2009. Examination of *Campylobacter jejuni* putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization. Infect Immun **77:**2399-2407.

48. **Konkel ME, Larson CL, Flanagan RC.** 2010. *Campylobacter jejuni* FlpA binds fibronectin and is required for maximal host cell adherence. J Bacteriol **192:**68-76.

49. **Kakuda T, DiRita VJ.** 2006. Cj1496c encodes a *Campylobacter jejuni* glycoprotein that influences invasion of human epithelial cells and colonization of the chick gastrointestinal tract. Infect Immun **74:**4715-4723.

50. **Pei Z, Blaser MJ.** 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. J Biol Chem **268:**18717-18725.

51. **Pei Z, Burucoa C, Grignon B, Baqar S, Huang XZ, Kopecko DJ, Bourgeois AL, Fauchere JL, Blaser MJ.** 1998. Mutation in the peb1A locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect Immun **66:**938-943.

52. **Kervella M, Pages JM, Pei Z, Grollier G, Blaser MJ, Fauchere JL.** 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect Immun **61:**3440-3448.

53. **De Melo MA, Gabbiani G, Pechere JC.** 1989. Cellular events and intracellular survival of *Campylobacter jejuni* during infection of HEp-2 cells. Infect Immun **57:**2214-2222.

54. **Ketley JM.** 1997. Pathogenesis of enteric infection by *Campylobacter*. Microbiology **143 (Pt 1):**5-21.

55. **Konkel ME, Joens LA.** 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter spp*. Infect Immun **57:**2984-2990.

56. **Oelschlaeger TA, Guerry P, Kopecko DJ.** 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc Natl Acad Sci U S A **90:**6884-6888.

57. **Buelow DR, Christensen JE, Neal-McKinney JM, Konkel ME.** 2011. *Campylobacter jejuni* survival within human epithelial cells is enhanced by the secreted protein CiaI. Mol Microbiol **80:**1296-1312.

58. **Barrero-Tobon AM, Hendrixson DR.** 2012. Identification and analysis of flagellar coexpressed determinants (Feds) of *Campylobacter jejuni* involved in colonization. Mol Microbiol **84:**352-369.

59. **Barrero-Tobon AM, Hendrixson DR.** 2014. Flagellar biosynthesis exerts temporal regulation of secretion of specific *Campylobacter jejuni* colonization and virulence determinants. Mol Microbiol **93:**957-974.

60. **Song YC, Jin S, Louie H, Ng D, Lau R, Zhang Y, Weerasekera R, Al Rashid S, Ward LA, Der SD, Chan VL.** 2004. FlaC, a protein of *Campylobacter jejuni* TGH9011 (ATCC43431) secreted through the flagellar apparatus, binds epithelial cells and influences cell invasion. Mol Microbiol **53:**541-553.

61. **Hu L, Kopecko DJ.** 1999. *Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells. Infect Immun **67:**4171-4182.

62. **Biswas D, Itoh K, Sasakawa C.** 2000. Uptake pathways of clinical and healthy animal isolates of *Campylobacter jejuni* into INT-407 cells. FEMS Immunol Med Microbiol **29:**203-211.

63. **Biswas D, Itoh K, Sasakawa C.** 2003. Role of microfilaments and microtubules in the invasion of INT-407 cells by *Campylobacter jejuni*. Microbiol Immunol **47:**469-473.

64. **Watson RO, Galan JE.** 2008. *Campylobacter jejuni* survives within epithelial cells by avoiding delivery to lysosomes. PLoS Pathog **4:**e14.

65. **Novik V, Hofreuter D, Galan JE.** 2010. Identification of *Campylobacter jejuni* genes involved in its interaction with epithelial cells. Infect Immun **78:**3540-3553.

66. **Liu X, Gao B, Novik V, Galan JE.** 2012. Quantitative Proteomics of Intracellular *Campylobacter jejuni* Reveals Metabolic Reprogramming. PLoS Pathog **8:**e1002562.

67. **Lara-Tejero M, Galan JE.** 2001. CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. Infect Immun **69:**4358-4365.

68. **Elmi A, Watson E, Sandu P, Gundogdu O, Mills DC, Inglis NF, Manson E, Imrie L, Bajaj-Elliott M, Wren BW, Smith DG, Dorrell N.** 2012. *Campylobacter jejuni* outer membrane

vesicles play an important role in bacterial interactions with human intestinal epithelial cells. Infect Immun **80:**4089-4098.

69. **Lindmark B, Rompikuntal PK, Vaitkevicius K, Song T, Mizunoe Y, Uhlin BE, Guerry P, Wai SN.** 2009. Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from *Campylobacter jejuni*. BMC Microbiol **9:**220.

70. **Lee RB, Hassane DC, Cottle DL, Pickett CL.** 2003. Interactions of *Campylobacter jejuni* cytolethal distending toxin subunits CdtA and CdtC with HeLa cells. Infect Immun **71:**4883-4890.

71. **Lara-Tejero M, Galan JE.** 2000. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science **290:**354-357.

72. **Frisan T, Cortes-Bratti X, Thelestam M.** 2002. Cytolethal distending toxins and activation of DNA damage-dependent checkpoint responses. Int J Med Microbiol **291:**495-499.

73. **Whitehouse CA, Balbo PB, Pesci EC, Cottle DL, Mirabito PM, Pickett CL.** 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. Infect Immun **66:**1934-1940.

74. **Fox JG, Rogers AB, Whary MT, Ge Z, Taylor NS, Xu S, Horwitz BH, Erdman SE.** 2004. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Camplyobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. Infect Immun **72:**1116-1125.

75. **Abuoun M, Manning G, Cawthraw SA, Ridley A, Ahmed IH, Wassenaar TM, Newell DG.** 2005. Cytolethal distending toxin (CDT)-negative *Campylobacter jejuni* strains and anti-CDT neutralizing antibodies are induced during human infection but not during colonization in chickens. Infect Immun **73:**3053-3062.

76. **Biswas D, Fernando U, Reiman C, Willson P, Potter A, Allan B.** 2006. Effect of cytolethal distending toxin of *Campylobacter jejuni* on adhesion and internalization in cultured cells and in colonization of the chicken gut. Avian Dis **50:**586-593.

77. **Moran AP, Penner JL.** 1999. Serotyping of *Campylobacter jejuni* based on heat-stable antigens: relevance, molecular basis and implications in pathogenesis. J Appl Microbiol **86:**361-377.

78. **Prendergast MM, Moran AP.** 2000. Lipopolysaccharides in the development of the Guillain-Barre syndrome and Miller Fisher syndrome forms of acute inflammatory peripheral neuropathies. J Endotoxin Res **6:**341-359.

79. **Cullen TW, O'Brien JP, Hendrixson DR, Giles DK, Hobb RI, Thompson SA, Brodbelt JS, Trent MS.** 2013. EptC of *Campylobacter jejuni* mediates phenotypes involved in host interactions and virulence. Infect Immun **81:**430-440.

80. **Karlyshev AV, Linton D, Gregson NA, Lastovica AJ, Wren BW.** 2000. Genetic and biochemical evidence of a *Campylobacter jejuni* capsular polysaccharide that accounts for Penner serotype specificity. Mol Microbiol **35:**529-541.

81. **St Michael F, Szymanski CM, Li J, Chan KH, Khieu NH, Larocque S, Wakarchuk WW, Brisson JR, Monteiro MA.** 2002. The structures of the lipooligosaccharide and capsule polysaccharide of *Campylobacter jejuni* genome sequenced strain NCTC 11168. Eur J Biochem **269:**5119-5136.

82. **Szymanski CM, Michael FS, Jarrell HC, Li J, Gilbert M, Larocque S, Vinogradov E, Brisson JR.** 2003. Detection of conserved N-linked glycans and phase-variable lipooligosaccharides and capsules from *campylobacter* cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. J Biol Chem **278:**24509-24520.

83. **Karlyshev AV, Champion OL, Churcher C, Brisson JR, Jarrell HC, Gilbert M, Brochu D, St Michael F, Li J, Wakarchuk WW, Goodhead I, Sanders M, Stevens K, White B, Parkhill J, Wren BW, Szymanski CM.** 2005. Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. Mol Microbiol **55:**90-103.

84. **Bacon DJ, Szymanski CM, Burr DH, Silver RP, Alm RA, Guerry P.** 2001. A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. Mol Microbiol **40:**769-777.

85. **Maue AC, Mohawk KL, Giles DK, Poly F, Ewing CP, Jiao Y, Lee G, Ma Z, Monteiro MA, Hill CL, Ferderber JS, Porter CK, Trent MS, Guerry P.** 2013. The polysaccharide capsule of *Campylobacter jejuni* modulates the host immune response. Infect Immun **81:**665-672.

86. **Bachtiar BM, Coloe PJ, Fry BN.** 2007. Knockout mutagenesis of the kpsE gene of *Campylobacter jejuni* 81116 and its involvement in bacterium-host interactions. FEMS Immunol Med Microbiol **49:**149-154.

87. **Szymanski CM, Yao R, Ewing CP, Trust TJ, Guerry P.** 1999. Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. Mol Microbiol **32:**1022-1030.

88. **Thibault P, Logan SM, Kelly JF, Brisson JR, Ewing CP, Trust TJ, Guerry P.** 2001. Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. J Biol Chem **276:**34862-34870.

89. **Goon S, Kelly JF, Logan SM, Ewing CP, Guerry P.** 2003. Pseudaminic acid, the major modification on *Campylobacter* flagellin, is synthesized via the Cj1293 gene. Mol Microbiol **50:**659-671.

90. **Guerry P, Ewing CP, Schirm M, Lorenzo M, Kelly J, Pattarini D, Majam G, Thibault P, Logan S.** 2006. Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence. Mol Microbiol **60:**299-311.

91. **Mahdavi J, Pirinccioglu N, Oldfield NJ, Carlsohn E, Stoof J, Aslam A, Self T, Cawthraw SA, Petrovska L, Colborne N, Sihlbom C, Boren T, Wooldridge KG, Ala'Aldeen DA.** 2014. A novel O-linked glycan modulates *Campylobacter jejuni* major outer membrane protein-mediated adhesion to human histo-blood group antigens and chicken colonization. Open Biol **4:**130202.

92. **Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW, Aebi M.** 2002. N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. Science **298:**1790-1793.

93. **Szymanski CM, Burr DH, Guerry P.** 2002. *Campylobacter* protein glycosylation affects host cell interactions. Infect Immun **70:**2242-2244.

94. **Karlyshev AV, Everest P, Linton D, Cawthraw S, Newell DG, Wren BW.** 2004. The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks. Microbiology **150:**1957-1964.

95. **Lindblom GB, Sjorgren E, Kaijser B.** 1986. Natural *campylobacter* colonization in chickens raised under different environmental conditions. J Hyg (Lond) **96:**385-391.

96. **Pokamunski S, Kass N, Borochovich E, Marantz B, Rogol M.** 1986. Incidence of *Campylobacter spp.* in broiler flocks monitored from hatching to slaughter. Avian Pathol **15:**83-92.

97. **Humphrey TJ, Henley A, Lanning DG.** 1993. The colonization of broiler chickens with *Campylobacter jejuni*: some epidemiological investigations. Epidemiol Infect **110:**601-607.

98. **Shreeve JE, Toszeghy M, Pattison M, Newell DG.** 2000. Sequential spread of *Campylobacter* infection in a multipen broiler house. Avian Dis **44:**983-988.

99. **Colles FM, Jones TA, McCarthy ND, Sheppard SK, Cody AJ, Dingle KE, Dawkins MS, Maiden MC.** 2008. *Campylobacter* infection of broiler chickens in a free-range environment. Environ Microbiol **10:**2042-2050.

100. **Beery JT, Hugdahl MB, Doyle MP.** 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl Environ Microbiol **54:**2365-2370.

101. **Hendrixson DR, DiRita VJ.** 2004. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. Mol Microbiol **52:**471-484.

102. **Humphrey S, Chaloner G, Kemmett K, Davidson N, Williams N, Kipar A, Humphrey T, Wigley P.** 2014. *Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affects bird welfare. MBio **5:**e01364-01314.

103. **Berndtson E, Tivemo M, Engvall A.** 1992. Distribution and numbers of *Campylobacter* in newly slaughtered broiler chickens and hens. Int J Food Microbiol **15:**45-50.

104. **Mead GC, Hudson WR, Hinton MH.** 1995. Effect of changes in processing to improve hygiene control on contamination of poultry carcasses with *campylobacter*. Epidemiol Infect **115:**495-500.

105. **Rosenquist H, Sommer HM, Nielsen NL, Christensen BB.** 2006. The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. Int J Food Microbiol **108:**226-232.

106. **Atanassova V, Reich F, Beckmann L, Klein G.** 2007. Prevalence of *Campylobacter* spp. in turkey meat from a slaughterhouse and in turkey meat retail products. FEMS Immunol Med Microbiol **49:**141-145.

107. **Bryan F, Doyle M.** 1995. Health risks and consequences of *Salmonella* and *Campylobacter jejuni* in raw poultry. J Food Prot **58:**326-344.

108. **Jorgensen F, Bailey R, Williams S, Henderson P, Wareing DR, Bolton FJ, Frost JA, Ward L, Humphrey TJ.** 2002. Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. Int J Food Microbiol **76:**151-164.

109. **Authors.** 2014. The High Cost of Cheap Chicken, vol February 2014 Consumer Reports.

110. **Wosten MMSM, Wagenaar JA, van Putten JPM.** 2004. The FlgS/FlgR two-component signal transduction system regulates the *fla* regulon in *Campylobacter jejuni*. J Biol Chem **279:**16214-16222.

111. **Hendrixson DR.** 2006. A phase-variable mechanism controlling the *Campylobacter jejuni* FlgR response regulator influences commensalism. Mol Microbiol **61:**1646-1659.

112. **Nachamkin I, Yang XH, Stern NJ.** 1993. Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. Appl Environ Microbiol **59:**1269-1273.

113. **Wassenaar TM, van der Zeijst BA, Ayling R, Newell DG.** 1993. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. J Gen Microbiol **139 Pt 6:**1171-1175.

114. **Vegge CS, Brondsted L, Li YP, Bang DD, Ingmer H.** 2009. Energy taxis drives *Campylobacter jejuni* toward the most favorable conditions for growth. Appl Environ Microbiol **75:**5308-5314.

115. **Grant AJ, Coward C, Jones MA, Woodall CA, Barrow PA, Maskell DJ.** 2005. Signature-tagged transposon mutagenesis studies demonstrate the dynamic nature of cecal colonization of 2-week-old chickens by *Campylobacter jejuni*. Appl Environ Microbiol **71:**8031-8041.

116. **Wong A, Lange D, Houle S, Arbatsky NP, Valvano MA, Knirel YA, Dozois CM, Creuzenet C.** 2015. Role of capsular modified heptose in the virulence of *Campylobacter jejuni*. Mol Microbiol doi:10.1111/mmi.12995.

117. **Kelly J, Jarrell H, Millar L, Tessier L, Fiori LM, Lau PC, Allan B, Szymanski CM.** 2006. Biosynthesis of the N-linked glycan in *Campylobacter jejuni* and addition onto protein through block transfer. J Bacteriol **188:**2427-2434.

118. **Nothaft H, Liu X, McNally DJ, Li J, Szymanski CM.** 2009. Study of free oligosaccharides derived from the bacterial N-glycosylation pathway. Proc Natl Acad Sci U S A **106:**15019-15024.

119. **Woodall CA, Jones MA, Barrow PA, Hinds J, Marsden GL, Kelly DJ, Dorrell N, Wren BW, Maskell DJ.** 2005. *Campylobacter jejuni* gene expression in the chick cecum: evidence for adaptation to a low-oxygen environment. Infect Immun **73:**5278-5285.

120. **Taveirne ME, Theriot CM, Livny J, DiRita VJ.** 2013. The complete *Campylobacter jejuni* transcriptome during colonization of a natural host determined by RNAseq. PLoS One **8:**e73586.

121. **Line J, Hiett K, Guard J, Seal B.** 2011. Temperature affects sole carbon utilization patterns of *Campylobacter coli* 49941. Curr Microbiol **62:**821-825.

122. **Gripp E, Hlahla D, Didelot X, Kops F, Maurischat S, Tedin K, Alter T, Ellerbroek L, Schreiber K, Schomburg D, Janssen T, Bartholomaus P, Hofreuter D, Woltemate S, Uhr M, Brenneke B, Gruning P, Gerlach G, Wieler L, Suerbaum S, Josenhans C.** 2011. Closely

related *Campylobacter jejuni* strains from different sources reveal a generalist rather than a specialist lifestyle. BMC Genomics **12:**584.

123. **Velayudhan J, Kelly DJ.** 2002. Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. Microbiology **148:**685-694.

124. **Hofreuter D, Tsai J, Watson RO, Novik V, Altman B, Benitez M, Clark C, Perbost C, Jarvie T, Du L, Galan JE.** 2006. Unique features of a highly pathogenic *Campylobacter jejuni* strain. Infect Immun **74:**4694-4707.

125. **Guccione E, Leon-Kempis Mdel R, Pearson BM, Hitchin E, Mulholland F, van Diemen PM, Stevens MP, Kelly DJ.** 2008. Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. Mol Microbiol **69:**77-93.

126. **Wright JA, Grant AJ, Hurd D, Harrison M, Guccione EJ, Kelly DJ, Maskell DJ.** 2009. Metabolite and transcriptome analysis of *Campylobacter jejuni* in vitro growth reveals a stationary-phase physiological switch. Microbiology **155:**80-94.

127. **Parsons CM.** 1984. Influence of caecectomy and source of dietary fibre or starch on excretion of endogenous amino acids by laying hens. Br J Nutr **51:**541-548.

128. **Stahl M, Butcher J, Stintzi A.** 2012. Nutrient acquisition and metabolism by *Campylobacter jejuni*. Front Cell Infect Microbiol **2:**5.

129. **Velayudhan J, Jones MA, Barrow PA, Kelly DJ.** 2004. L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni*. Infect Immun **72:**260-268.

130. **Hofreuter D, Mohr J, Wensel O, Rademacher S, Schreiber K, Schomburg D, Gao B, Galan JE.** 2012. Contribution of amino acid catabolism to the tissue specific persistence of *Campylobacter jejuni* in a murine colonization model. PLoS One **7:**e50699.

131. **Leon-Kempis Mdel R, Guccione E, Mulholland F, Williamson MP, Kelly DJ.** 2006. The *Campylobacter jejuni* PEB1a adhesin is an aspartate/glutamate-binding protein of an ABC transporter essential for microaerobic growth on dicarboxylic amino acids. Mol Microbiol **60:**1262-1275.

132. **Hofreuter D, Novik V, Galan JE.** 2008. Metabolic diversity in *Campylobacter jejuni* enhances specific tissue colonization. Cell Host Microbe **4:**425-433.

133. **Gundogdu O, Bentley SD, Holden MT, Parkhill J, Dorrell N, Wren BW.** 2007. Re-annotation and re-analysis of the *Campylobacter jejuni* NCTC11168 genome sequence. BMC Genomics **8:**162.

134. **Westfall HN, Rollins DM, Weiss E.** 1986. Substrate utilization by *Campylobacter jejuni* and *Campylobacter coli*. Appl Environ Microbiol **52:**700-705.

135. **Lin AE, Krastel K, Hobb RI, Thompson SA, Cvitkovitch DG, Gaynor EC.** 2009. Atypical roles for *Campylobacter jejuni* amino acid ATP binding cassette transporter components PaqP and PaqQ in bacterial stress tolerance and pathogen-host cell dynamics. Infect Immun **77:**4912-4924.

136. **Shibayama K, Wachino J, Arakawa Y, Saidijam M, Rutherford NG, Henderson PJ.** 2007. Metabolism of glutamine and glutathione via gamma-glutamyltranspeptidase and glutamate transport in *Helicobacter pylori*: possible significance in the pathophysiology of the organism. Mol Microbiol **64:**396-406.

137. **Barnes IH, Bagnall MC, Browning DD, Thompson SA, Manning G, Newell DG.** 2007. Gamma-glutamyl transpeptidase has a role in the persistent colonization of the avian gut by *Campylobacter jejuni*. Microb Pathog **43:**198-207.

138. **Dufour V, Alazzam B, Ermel G, Thepaut M, Rossero A, Tresse O, Baysse C.** 2012. Antimicrobial activities of isothiocyanates against *Campylobacter jejuni* isolates. Front Cell Infect Microbiol **2:**53.

139. **Ribardo DA, Hendrixson DR.** 2011. Analysis of the LIV system of *Campylobacter jejuni* reveals alternative roles for LivJ and LivK in commensalism beyond branched-chain amino acid transport. J Bacteriol **193:**6233-6243.

140. **Muller A, Thomas GH, Horler R, Brannigan JA, Blagova E, Levdikov VM, Fogg MJ, Wilson KS, Wilkinson AJ.** 2005. An ATP-binding cassette-type cysteine transporter in *Campylobacter jejuni* inferred from the structure of an extracytoplasmic solute receptor protein. Mol Microbiol **57:**143-155.

141. **Garvis SG, Puzon GJ, Konkel ME.** 1996. Molecular characterization of a *Campylobacter jejuni* 29-kilodalton periplasmic binding protein. Infect Immun **64:**3537-3543.

142. **Vorwerk H, Mohr J, Huber C, Wensel O, Schmidt-Hohagen K, Gripp E, Josenhans C, Schomburg D, Eisenreich W, Hofreuter D.** 2014. Utilization of host-derived cysteine-containing peptides overcomes the restricted sulphur metabolism of *Campylobacter jejuni*. Mol Microbiol **93:**1224-1245.

143. **Tenover FC, Knapp JS, Patton C, Plorde JJ.** 1985. Use of auxotyping for epidemiological studies of *Campylobacter jejuni* and *Campylobacter coli* infections. Infect Immun **48:**384-388.

144. **Tenover FC, Patton CM.** 1987. Naturally occurring auxotrophs of *Campylobacter jejuni* and *Campylobacter coli*. J Clin Microbiol **25:**1659-1661.

145. **Blaser MJ, Perez GP, Smith PF, Patton C, Tenover FC, Lastovica AJ, Wang WI.** 1986. Extraintestinal *Campylobacter jejuni* and *Campylobacter coli* infections: host factors and strain characteristics. J Infect Dis **153:**552-559.

146. **Sellars MJ, Hall SJ, Kelly DJ.** 2002. Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen. J Bacteriol **184:**4187-4196.

147. **Weingarten RA, Taveirne ME, Olson JW.** 2009. The dual-functioning fumarate reductase is the sole succinate:quinone reductase in *Campylobacter jejuni* and is required for full host colonization. J Bacteriol **191:**5293-5300.

148. **Guccione E, Hitchcock A, Hall SJ, Mulholland F, Shearer N, van Vliet AH, Kelly DJ.** 2010. Reduction of fumarate, mesaconate and crotonate by Mfr, a novel oxygen-regulated periplasmic reductase in *Campylobacter jejuni*. Environ Microbiol **12:**576-591.

149. **St Maurice M, Cremades N, Croxen MA, Sisson G, Sancho J, Hoffman PS.** 2007. Flavodoxin:quinone reductase (FqrB): a redox partner of pyruvate:ferredoxin oxidoreductase that reversibly couples pyruvate oxidation to NADPH production in *Helicobacter pylori* and *Campylobacter jejuni*. J Bacteriol **189:**4764-4773.

150. **Leach S, Harvey P, Wali R.** 1997. Changes with growth rate in the membrane lipid composition of and amino acid utilization by continuous cultures of *Campylobacter jejuni*. J Appl Microbiol **82:**631-640.

151. **Kirkpatrick AS, Yokoyama T, Choi KJ, Yeo HJ.** 2009. *Campylobacter jejuni* fatty acid synthase II: structural and functional analysis of beta-hydroxyacyl-ACP dehydratase (FabZ). Biochem Biophys Res Commun **380:**407-412.

152. **Muraoka WT, Zhang Q.** 2011. Phenotypic and genotypic evidence for L-fucose utilization by *Campylobacter jejuni*. J Bacteriol **193:**1065-1075.

153. **Stahl M, Friis LM, Nothaft H, Liu X, Li J, Szymanski CM, Stintzi A.** 2011. L-fucose utilization provides *Campylobacter jejuni* with a competitive advantage. Proc Natl Acad Sci U S A **108:**7194-7199.

154. **Zautner AE, Herrmann S, Corso J, Tareen AM, Alter T, Gross U.** 2011. Epidemiological association of different *Campylobacter jejuni* groups with metabolism-associated genetic markers. Appl Environ Microbiol **77:**2359-2365.

155. **de Haan CP, Llarena AK, Revez J, Hanninen ML.** 2012. Association of *Campylobacter jejuni* metabolic traits with multilocus sequence types. Appl Environ Microbiol **78:**5550-5554.

156. **Palyada K, Threadgill D, Stintzi A.** 2004. Iron acquisition and regulation in *Campylobacter jejuni*. J Bacteriol **186:**4714-4729.

157. **Flint DH, Smyk-Randall E, Tuminello JF, Draczynska-Lusiak B, Brown OR.** 1993. The inactivation of dihydroxy-acid dehydratase in *Escherichia coli* treated with hyperbaric oxygen occurs because of the destruction of its Fe-S cluster, but the enzyme remains in the cell in a form that can be reactivated. J Biol Chem **268:**25547-25552.

158. **Miller CE, Williams PH, Ketley JM.** 2009. Pumping iron: mechanisms for iron uptake by *Campylobacter*. Microbiology **155:**3157-3165.

159. **Field LH, Headley VL, Payne SM, Berry LJ.** 1986. Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. Infect Immun **54:**126-132.

160. **Carswell CL, Rigden MD, Baenziger JE.** 2008. Expression, purification, and structural characterization of CfrA, a putative iron transporter from *Campylobacter jejuni*. J Bacteriol **190:**5650-5662.

161. **Zeng X, Xu F, Lin J.** 2009. Molecular, antigenic, and functional characteristics of ferric enterobactin receptor CfrA in *Campylobacter jejuni*. Infect Immun **77:**5437-5448.

162. **Xu F, Zeng X, Haigh RD, Ketley JM, Lin J.** 2010. Identification and characterization of a new ferric enterobactin receptor, CfrB, in *Campylobacter*. J Bacteriol **192:**4425-4435.

163. **Zeng X, Xu F, Lin J.** 2013. Specific TonB-ExbB-ExbD energy transduction systems required for ferric enterobactin acquisition in *Campylobacter*. FEMS Microbiol Lett **347:**83-91.

164. **Pickett CL, Auffenberg T, Pesci EC, Sheen VL, Jusuf SS.** 1992. Iron acquisition and hemolysin production by *Campylobacter jejuni*. Infect Immun **60:**3872-3877.

165. **Ridley KA, Rock JD, Li Y, Ketley JM.** 2006. Heme utilization in *Campylobacter jejuni*. J Bacteriol **188:**7862-7875.

166. **Miller CE, Rock JD, Ridley KA, Williams PH, Ketley JM.** 2008. Utilization of lactoferrin-bound and transferrin-bound iron by *Campylobacter jejuni*. J Bacteriol **190:**1900-1911.

167. **Stintzi A, Marlow D, Palyada K, Naikare H, Panciera R, Whitworth L, Clarke C.** 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. Infect Immun **73:**1797-1810.

168. **Andrews SC, Robinson AK, Rodriguez-Quinones F.** 2003. Bacterial iron homeostasis. FEMS Microbiol Rev **27:**215-237.

169. **Masse E, Arguin M.** 2005. Ironing out the problem: new mechanisms of iron homeostasis. Trends Biochem Sci **30:**462-468.

170. **van Vliet AH, Baillon ML, Penn CW, Ketley JM.** 1999. *Campylobacter jejuni* contains two fur homologs: characterization of iron-responsive regulation of peroxide stress defense genes by the PerR repressor. J Bacteriol **181:**6371-6376.

171. **van Vliet AH, Wooldridge KG, Ketley JM.** 1998. Iron-responsive gene regulation in a *campylobacter jejuni* fur mutant. J Bacteriol **180:**5291-5298.

172. **Coy M, Neilands JB.** 1991. Structural dynamics and functional domains of the fur protein. Biochemistry **30:**8201-8210.

173. **Stojiljkovic I, Hantke K.** 1995. Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). Mol Gen Genet **247:**199-205.

174. **Palyada K, Sun YQ, Flint A, Butcher J, Naikare H, Stintzi A.** 2009. Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. BMC Genomics **10:**481.

175. **Holmes K, Mulholland F, Pearson BM, Pin C, McNicholl-Kennedy J, Ketley JM, Wells JM.** 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology **151:**243-257.

176. **Miller TL, Wolin MJ.** 1979. Fermentations by saccharolytic intestinal bacteria. Am J Clin Nutr **32:**164-172.

177. **Cummings JH.** 1983. Fermentation in the human large intestine: evidence and implications for health. Lancet **1:**1206-1209.

178. **Cummings JH, Macfarlane GT.** 1991. The control and consequences of bacterial fermentation in the human colon. J Appl Bacteriol **70:**443-459.

179. **Sun Y, O'Riordan MX.** 2013. Regulation of bacterial pathogenesis by intestinal short-chain Fatty acids. Adv Appl Microbiol **85:**93-118.

180. **Rey FE, Faith JJ, Bain J, Muehlbauer MJ, Stevens RD, Newgard CB, Gordon JI.** 2010. Dissecting the *in vivo* metabolic potential of two human gut acetogens. J Biol Chem **285:**22082-22090.

181. **Wolfe AJ.** 2005. The acetate switch. Microbiol Mol Biol Rev **69:**12-50.

182. **Topping DL, Clifton PM.** 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev **81:**1031-1064.

183. **Rehman HU, Vahjen W, Awad WA, Zentek J.** 2007. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. Arch Anim Nutr **61:**319-335.

184. **Macfarlane S, Macfarlane GT.** 2003. Regulation of short-chain fatty acid production. Proc Nutr Soc **62:**67-72.

185. **Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ.** 2006. Colonic health: fermentation and short chain fatty acids. J Clin Gastroenterol **40:**235-243.

186. **Cummings JH.** 1981. Short chain fatty acids in the human colon. Gut **22:**763-779.

187. **Rechkemmer G, Ronnau K, von Engelhardt W.** 1988. Fermentation of polysaccharides and absorption of short chain fatty acids in the mammalian hindgut. Comp Biochem Physiol A Comp Physiol **90:**563-568.

188. **Meyer B, Zentek J, Harlander-Matauschek A.** 2013. Differences in intestinal microbial metabolites in laying hens with high and low levels of repetitive feather-pecking behavior. Physiol Behav **110-111:**96-101.

189. **Molnar A, Hess C, Pal L, Wagner L, Awad WA, Husveth F, Hess M, Dublecz K.** 2015. Composition of diet modifies colonization dynamics of *Campylobacter jejuni* in broiler chickens. J Appl Microbiol **118:**245-254.

190. **Cummings JH, Hill MJ, Bone ES, Branch WJ, Jenkins DJ.** 1979. The effect of meat protein and dietary fiber on colonic function and metabolism. II. Bacterial metabolites in feces and urine. Am J Clin Nutr **32:**2094-2101.

191. **Nava GM, Friedrichsen HJ, Stappenbeck TS.** 2011. Spatial organization of intestinal microbiota in the mouse ascending colon. Isme j **5:**627-638.

192. **Pedron T, Mulet C, Dauga C, Frangeul L, Chervaux C, Grompone G, Sansonetti PJ.** 2012. A crypt-specific core microbiota resides in the mouse colon. MBio **3**.

193. **Walter J, Ley R.** 2011. The human gut microbiome: ecology and recent evolutionary changes. Annu Rev Microbiol **65:**411-429.

194. **Roberfroid MB.** 2005. Introducing inulin-type fructans. Br J Nutr **93 Suppl 1:**S13-25.

195. **Venter CS, Vorster HH, Cummings JH.** 1990. Effects of dietary propionate on carbohydrate and lipid metabolism in healthy volunteers. Am J Gastroenterol **85:**549-553.

196. **Cook SI, Sellin JH.** 1998. Review article: short chain fatty acids in health and disease. Aliment Pharmacol Ther **12:**499-507.

197. **Fleming LL, Floch MH.** 1986. Digestion and absorption of fiber carbohydrate in the colon. Am J Gastroenterol **81:**507-511.

198. **Baskett RC, Hentges DJ.** 1973. *Shigella flexneri* inhibition by acetic acid. Infect Immun **8:**91-97.

199. **Hentges DJ.** 1967. Influence of pH on the inhibitory activity of formic and acetic acids for *Shigella*. J Bacteriol **93:**2029-2030.

200. **Weiner N, Draskoczy P.** 1961. The effects of organic acids on the oxidative metabolism of intact and disrupted *E. coli*. J Pharmacol Exp Ther **132:**299-305.

201. **Lambert RJ, Stratford M.** 1999. Weak-acid preservatives: modelling microbial inhibition and response. J Appl Microbiol **86:**157-164.

202. **Repaske DR, Adler J.** 1981. Change in intracellular pH of *Escherichia coli* mediates the chemotactic response to certain attractants and repellents. J Bacteriol **145:**1196-1208.

203. **Russell JB, Diez-Gonzalez F.** 1998. The effects of fermentation acids on bacterial growth. Adv Microb Physiol **39:**205-234.

204. **Salmond CV, Kroll RG, Booth IR.** 1984. The effect of food preservatives on pH homeostasis in *Escherichia coli*. J Gen Microbiol **130:**2845-2850.

205. **Axe DD, Bailey JE.** 1995. Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. Biotechnol Bioeng **47:**8-19.

206. **Roe AJ, O'Byrne C, McLaggan D, Booth IR.** 2002. Inhibition of *Escherichia coli* growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity. Microbiology **148:**2215-2222.

207. **Roe AJ, McLaggan D, Davidson I, O'Byrne C, Booth IR.** 1998. Perturbation of anion balance during inhibition of growth of *Escherichia coli* by weak acids. J Bacteriol **180:**767-772.

208. **Freese E, Sheu CW, Galliers E.** 1973. Function of lipophilic acids as antimicrobial food additives. Nature **241:**321-325.

209. **Kirkpatrick C, Maurer LM, Oyelakin NE, Yoncheva YN, Maurer R, Slonczewski JL.** 2001. Acetate and formate stress: opposite responses in the proteome of *Escherichia coli*. J Bacteriol **183:**6466-6477.

210. **Gallo RL, Hooper LV.** 2012. Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol **12:**503-516.

211. **Termen S, Tollin M, Rodriguez E, Sveinsdottir SH, Johannesson B, Cederlund A, Sjovall J, Agerberth B, Gudmundsson GH.** 2008. PU.1 and bacterial metabolites regulate the human gene CAMP encoding antimicrobial peptide LL-37 in colon epithelial cells. Mol Immunol **45:**3947-3955.

212. **Sunkara LT, Achanta M, Schreiber NB, Bommineni YR, Dai G, Jiang W, Lamont S, Lillehoj HS, Beker A, Teeter RG, Zhang G.** 2011. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. PLoS One **6:**e27225.

213. **Sunkara LT, Jiang W, Zhang G.** 2012. Modulation of antimicrobial host defense peptide gene expression by free fatty acids. PLoS One **7:**e49558.

214. **Horswill AR, Escalante-Semerena JC.** 1999. *Salmonella typhimurium* LT2 catabolizes propionate via the 2-methylcitric acid cycle. J Bacteriol **181:**5615-5623.

215. **Goepfert JM, Hicks R.** 1969. Effect of volatile fatty acids on *Salmonella typhimurium*. J Bacteriol **97:**956-958.

216. **McHan F, Shotts EB.** 1993. Effect of short-chain fatty acids on the growth of *Salmonella typhimurium* in an in vitro system. Avian Dis **37:**396-398.

217. **Van Immerseel F, De Buck J, Pasmans F, Velge P, Bottreau E, Fievez V, Haesebrouck F, Ducatelle R.** 2003. Invasion of *Salmonella enteritidis* in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. Int J Food Microbiol **85:**237-248.

218. **Boyen F, Haesebrouck F, Vanparys A, Volf J, Mahu M, Van Immerseel F, Rychlik I, Dewulf J, Ducatelle R, Pasmans F.** 2008. Coated fatty acids alter virulence properties of *Salmonella Typhimurium* and decrease intestinal colonization of pigs. Vet Microbiol **132:**319-327.

219. **Cardenal-Munoz E, Ramos-Morales F.** 2011. Analysis of the expression, secretion and translocation of the *Salmonella enterica* type III secretion system effector SteA. PLoS One **6:**e26930.

220. **Durant JA, Corrier DE, Ricke SC.** 2000. Short-chain volatile fatty acids modulate the expression of the hilA and invF genes of *Salmonella typhimurium*. J Food Prot **63:**573-578.

221. **Gantois I, Ducatelle R, Pasmans F, Haesebrouck F, Hautefort I, Thompson A, Hinton JC, Van Immerseel F.** 2006. Butyrate specifically down-regulates *salmonella* pathogenicity island 1 gene expression. Appl Environ Microbiol **72:**946-949.

222. **Gong H, Su J, Bai Y, Miao L, Kim K, Yang Y, Liu F, Lu S.** 2009. Characterization of the expression of *Salmonella* Type III secretion system factor PrgI, SipA, SipB, SopE2, SpaO, and SptP in cultures and in mice. BMC Microbiol **9:**73.

223. **Huang Y, Suyemoto M, Garner CD, Cicconi KM, Altier C.** 2008. Formate acts as a diffusible signal to induce *Salmonella* invasion. J Bacteriol **190:**4233-4241.

224. **Zabala Diaz IB, Ricke SC.** 2004. Influence of short chain fatty acids and lysine on *Salmonella typhimurium* cadA expression. Antonie Van Leeuwenhoek **85:**45-51.

225. **Lawhon SD, Maurer R, Suyemoto M, Altier C.** 2002. Intestinal short-chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA. Mol Microbiol **46:**1451-1464.

226. **McHan F, Shotts EB.** 1992. Effect of feeding selected short-chain fatty acids on the in vivo attachment of *Salmonella typhimurium* in chick ceca. Avian Dis **36:**139-142.

227. **Garner CD, Antonopoulos DA, Wagner B, Duhamel GE, Keresztes I, Ross DA, Young VB, Altier C.** 2009. Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a *Salmonella enterica* serovar typhimurium murine model of infection. Infect Immun **77:**2691-2702.

228. **Nakanishi N, Tashiro K, Kuhara S, Hayashi T, Sugimoto N, Tobe T.** 2009. Regulation of virulence by butyrate sensing in enterohaemorrhagic *Escherichia coli*. Microbiology **155:**521-530.

229. **Brinkman AB, Ettema TJ, de Vos WM, van der Oost J.** 2003. The Lrp family of transcriptional regulators. Mol Microbiol **48:**287-294.

230. **Tobe T, Nakanishi N, Sugimoto N.** 2011. Activation of motility by sensing short-chain fatty acids via two steps in a flagellar gene regulatory cascade in enterohemorrhagic *Escherichia coli*. Infect Immun **79:**1016-1024.

231. **Herold S, Paton JC, Srimanote P, Paton AW.** 2009. Differential effects of short-chain fatty acids and iron on expression of iha in Shiga-toxigenic *Escherichia coli*. Microbiology **155:**3554-3563.

232. **Cobbold RN, Desmarchelier PM.** 2004. In vitro studies on the colonization of bovine colonic mucosa by Shiga-toxigenic *Escherichia coli* (STEC). Epidemiol Infect **132:**87-94.

233. **Julotok M, Singh AK, Gatto C, Wilkinson BJ.** 2010. Influence of fatty acid precursors, including food preservatives, on the growth and fatty acid composition of *Listeria monocytogenes* at 37 and 10degreesC. Appl Environ Microbiol **76:**1423-1432.

234. **Sun Y, Wilkinson BJ, Standiford TJ, Akinbi HT, O'Riordan MX.** 2012. Fatty acids regulate stress resistance and virulence factor production for *Listeria monocytogenes*. J Bacteriol **194:**5274-5284.

235. **Mastronicolis SK, Berberi A, Diakogiannis I, Petrova E, Kiaki I, Baltzi T, Xenikakis P.** 2010. Alteration of the phospho- or neutral lipid content and fatty acid composition in *Listeria monocytogenes* due to acid adaptation mechanisms for hydrochloric, acetic and lactic acids at pH 5.5 or benzoic acid at neutral pH. Antonie Van Leeuwenhoek **98:**307-316.

236. **Raqib R, Sarker P, Mily A, Alam NH, Arifuzzaman AS, Rekha RS, Andersson J, Gudmundsson GH, Cravioto A, Agerberth B.** 2012. Efficacy of sodium butyrate adjunct

therapy in shigellosis: a randomized, double-blind, placebo-controlled clinical trial. BMC Infect Dis **12:**111.

237. **Thomas MT, Shepherd M, Poole RK, van Vliet AH, Kelly DJ, Pearson BM.** 2011. Two respiratory enzyme systems in *Campylobacter jejuni* NCTC 11168 contribute to growth on L-lactate. Environ Microbiol **13:**48-61.

238. **Heres L, Engel B, Urlings HA, Wagenaar JA, van Knapen F.** 2004. Effect of acidified feed on susceptibility of broiler chickens to intestinal infection by *Campylobacter* and *Salmonella*. Vet Microbiol **99:**259-267.

239. **Heres L, Engel B, Van Knapen F, Wagenaar JA, Urlings BA.** 2003. Effect of fermented feed on the susceptibility for *Campylobacter jejuni* colonisation in broiler chickens with and without concurrent inoculation of *Salmonella enteritidis*. Int J Food Microbiol **87:**75-86.

240. **Van Deun K, Haesebrouck F, Van Immerseel F, Ducatelle R, Pasmans F.** 2008. Short-chain fatty acids and L-lactate as feed additives to control *Campylobacter jejuni* infections in broilers. Avian Pathol **37:**379-383.

241. **Neal-McKinney JM, Lu X, Duong T, Larson CL, Call DR, Shah DH, Konkel ME.** 2012. Production of organic acids by probiotic lactobacilli can be used to reduce pathogen load in poultry. PLoS One **7:**e43928.

242. **Rasschaert G, Piessens V, Scheldeman P, Leleu S, Stals A, Herman L, Heyndrickx M, Messens W.** 2013. Efficacy of electrolyzed oxidizing water and lactic acid on the reduction of *Campylobacter* on naturally contaminated broiler carcasses during processing. Poult Sci **92:**1077-1084.

243. **Chaine A, Arnaud E, Kondjoyan A, Collignan A, Sarter S.** 2013. Effect of steam and lactic acid treatments on the survival of *Salmonella Enteritidis* and *Campylobacter jejuni* inoculated on chicken skin. Int J Food Microbiol **162:**276-282.

244. **Van Deun K, Pasmans F, Van Immerseel F, Ducatelle R, Haesebrouck F.** 2008. Butyrate protects Caco-2 cells from *Campylobacter jejuni* invasion and translocation. Br J Nutr **100:**480-484.

245. **Krell T, Lacal J, Busch A, Silva-Jimenez H, Guazzaroni ME, Ramos JL.** 2010. Bacterial sensor kinases: diversity in the recognition of environmental signals. Annu Rev Microbiol **64:**539-559.

246. **Stock AM, Robinson VL, Goudreau PN.** 2000. Two-component signal transduction. Annu Rev Biochem **69:**183-215.

247. **Gao R, Stock AM.** 2009. Biological insights from structures of two-component proteins. Annu Rev Microbiol **63:**133-154.

248. **Bras AM, Chatterjee S, Wren BW, Newell DG, Ketley JM.** 1999. A novel *Campylobacter jejuni* Two-component regulatory system important for temperature-dependent growth and colonization. J Bacteriol **181:**3298-3302.

249. **Apel D, Ellermeier J, Pryjma M, Dirita VJ, Gaynor EC.** 2012. Characterization of *Campylobacter jejuni* RacRS reveals roles in the heat shock response, motility, and maintenance of cell length homogeneity. J Bacteriol **194:**2342-2354.

250. **van der Stel AX, van Mourik A, Heijmen-van Dijk L, Parker CT, Kelly DJ, van de Lest CH, van Putten JP, Wosten MM.** 2014. The *Campylobacter jejuni* RacRS system regulates fumarate utilization in a low oxygen environment. Environ Microbiol doi:10.1111/1462-2920.12476.

251. **Joslin SN, Hendrixson DR.** 2008. Analysis of the *Campylobacter jejuni* FlgR response regulator suggests integration of diverse mechanisms to activate an NtrC-like protein. J Bacteriol **190:**2422-2433.

252. **Hendrixson DR, DiRita VJ.** 2003. Transcription of σ^{54}-dependent but not σ^{28}-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. Mol Microbiol **50:**687-702.

253. **Joslin SN, Hendrixson DR.** 2009. Activation of the *Campylobacter jejuni* FlgSR two-component system is linked to the flagellar export apparatus. J Bacteriol **191:**2656-2667.

254. **Boll JM, Hendrixson DR.** 2011. A specificity determinant for phosphorylation in a response regulator prevents in vivo cross-talk and modification by acetyl phosphate. Proc Natl Acad Sci U S A **108:**20160-20165.

255. **Boll JM, Hendrixson DR.** 2013. A regulatory checkpoint during flagellar biogenesis in *Campylobacter jejuni* initiates signal transduction to activate transcription of flagellar genes. MBio **4:**e00432-00413.

256. **Gaynor EC, Wells DH, MacKichan JK, Falkow S.** 2005. The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. Mol Microbiol **56:**8-27.

257. **Svensson SL, Davis LM, MacKichan JK, Allan BJ, Pajaniappan M, Thompson SA, Gaynor EC.** 2009. The CprS sensor kinase of the zoonotic pathogen *Campylobacter jejuni* influences biofilm formation and is required for optimal chick colonization. Mol Microbiol **71:**253-272.

258. **Svensson SL, Hyunh S, Parker CT, Gaynor EC.** 2015. The *Campylobacter jejuni* CprRS two-component regulatory system regulates aspects of the cell envelope. Mol Microbiol doi:10.1111/mmi.12927.

259. **Wosten MM, van Dijk L, Parker CT, Guilhabert MR, van der Meer-Janssen YP, Wagenaar JA, van Putten JP.** 2010. Growth phase-dependent activation of the DccRS regulon of *Campylobacter jejuni*. J Bacteriol **192:**2729-2736.

260. **MacKichan JK, Gaynor EC, Chang C, Cawthraw S, Newell DG, Miller JF, Falkow S.** 2004. The *Campylobacter jejuni dccRS* two-component system is required for optimal *in vivo* colonization but is dispensable for *in vitro* growth. Mol Microbiol **54:**1269-1286.

261. **Wosten MM, Parker CT, van Mourik A, Guilhabert MR, van Dijk L, van Putten JP.** 2006. The *Campylobacter jejuni* PhosS/PhosR operon represents a non-classical phosphate-sensitive two-component system. Mol Microbiol **62:**278-291.

262. **Hendrixson DR, Akerley BJ, DiRita VJ.** 2001. Transposon mutagenesis of *Campylobacter jejuni* identifies a bipartite energy taxis system required for motility. Mol Microbiol **40:**214-224.

263. **Makarova O, Kamberov E, Margolis B.** 2000. Generation of deletion and point mutations with one primer in a single cloning step. Biotechniques **29:**970-972.

264. **Higuchi R.** 1990. Recombinant PCR, p 77-183. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ (ed), PCR Protocols: a Guide to Methods and Applications. Academic Press, London.

265. **Yao R, Alm RA, Trust TJ, Guerry P.** 1993. Construction of new *Campylobacter* cloning vectors and a new mutational *cat* cassette. Gene **130:**127-130.

266. **Figurski DH, Helinski DR.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A **76:**1648-1652.

267. **Guerry P, Yao R, Alm RA, Burr DH, Trust TJ.** 1994. Systems of experimental genetics for *Campylobacter* species. Methods Enzymol **235:**474-481.

268. **Pryjma M, Apel D, Huynh S, Parker CT, Gaynor EC.** 2012. FdhTU-modulated formate dehydrogenase expression and electron donor availability enhance recovery of *Campylobacter jejuni* following host cell infection. J Bacteriol **194:**3803-3813.

269. **Pajaniappan M, Hall JE, Cawthraw SA, Newell DG, Gaynor EC, Fields JA, Rathbun KM, Agee WA, Burns CM, Hall SJ, Kelly DJ, Thompson SA.** 2008. A temperature-regulated

Campylobacter jejuni gluconate dehydrogenase is involved in respiration-dependent energy conservation and chicken colonization. Mol Microbiol **68:**474-491.

270. **Orchard RC, Kittisopikul M, Altschuler SJ, Wu LF, Suel GM, Alto NM.** 2012. Identification of F-actin as the dynamic hub in a microbial-induced GTPase polarity circuit. Cell **148:**803-815.

271. **Skerker JM, Prasol MS, Perchuk BS, Biondi EG, Laub MT.** 2005. Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis. PLoS Biol **3:**e334.

272. **Quon KC, Marczynski GT, Shapiro L.** 1996. Cell cycle control by an essential bacterial two-component signal transduction protein. Cell **84:**83-93.

273. **McIver KS, Myles RL.** 2002. Two DNA-binding domains of Mga are required for virulence gene activation in the group A *streptococcus*. Mol Microbiol **43:**1591-1601.

274. **Lindblom G-B, Sjorgren E, Kaijser B.** 1986. Natural campylobacter colonization in chickens raised under different environmental conditions. J Hyg (Lond) **96:**385-391.

275. **Pokamunski S, Kass N, Borochovich E, Marantz B, Rogol M.** 1986. Incidence of *Campylobacter* spp. in broiler flocks monitored from hatching to slaughter. Avian Pathology **15:**83-92.

276. **Fouts DE, Mongodin EF, Mandrell RE, Miller WG, Rasko DA, Ravel J, Brinkac LM, DeBoy RT, Parker CT, Daugherty SC, Dodson RJ, Durkin AS, Madupu R, Sullivan SA, Shetty JU, Ayodeji MA, Shvartsbeyn A, Schatz MC, Badger JH, Fraser CM, Nelson KE.** 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter species*. PLoS Biol **3:**e15.

277. **Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream M-A, Rutherford KM, van Vliet AHM, Whitehead S, Barrell BG.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665-668.

278. **Dugar G, Herbig A, Forstner KU, Heidrich N, Reinhardt R, Nieselt K, Sharma CM.** 2013. High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. PLoS Genet **9:**e1003495.

279. **Linton D, Allan E, Karlyshev AV, Cronshaw AD, Wren BW.** 2002. Identification of *N*-acetylgalactosamine-containing glycoproteins PEB3 and CgpA in *Campylobacter jejuni*. Mol Microbiol **43:**497-508.

280. **Rangarajan ES, Bhatia S, Watson DC, Munger C, Cygler M, Matte A, Young NM.** 2007. Structural context for protein N-glycosylation in bacteria: The structure of PEB3, an adhesin from *Campylobacter jejuni*. Protein Sci **16:**990-995.

281. **Min T, Vedadi M, Watson DC, Wasney GA, Munger C, Cygler M, Matte A, Young NM.** 2009. Specificity of *Campylobacter jejuni* adhesin PEB3 for phosphates and structural differences among its ligand complexes. Biochemistry **48:**3057-3067.

282. **Guerry P, Logan SM, Thornton S, Trust TJ.** 1990. Genomic organization and expression of *Campylobacter* flagellin genes. J Bacteriol **172:**1853-1860.

283. **Ding W, Nothaft H, Szymanski CM, Kelly J.** 2009. Identification and quantification of glycoproteins using ion-pairing normal-phase liquid chromatography and mass spectrometry. Mol Cell Proteomics **8:**2170-2185.

284. **Kim KS, Kim MJ, Kononen E, Lounatmaa K, Summanen P, Finegold SM.** 2012. Single nucleotide polymorphisms are randomly dispersed and mostly synonymous in partial rpoB and cpn60 genes of *Campylobacter showae* human isolates. Anaerobe **18:**626-629.

285. **Artymovich K, Kim JS, Linz JE, Hall DF, Kelley LE, Kalbach HL, Kathariou S, Gaymer J, Paschke B.** 2013. A "successful allele" at *Campylobacter jejuni* contingency locus Cj0170 regulates motility; "successful alleles" at locus Cj0045 are strongly associated with mouse colonization. Food Microbiol **34:**425-430.

286. **Hitchen P, Brzostek J, Panico M, Butler JA, Morris HR, Dell A, Linton D.** 2010. Modification of the *Campylobacter jejuni* flagellin glycan by the product of the Cj1295 homopolymeric-tract-containing gene. Microbiology **156:**1953-1962.

287. **van Alphen LB, Wenzel CQ, Richards MR, Fodor C, Ashmus RA, Stahl M, Karlyshev AV, Wren BW, Stintzi A, Miller WG, Lowary TL, Szymanski CM.** 2014. Biological roles of the O-methyl phosphoramidate capsule modification in *Campylobacter jejuni*. PLoS One **9:**e87051.

288. **Cullen TW, Trent MS.** 2010. A link between the assembly of flagella and lipooligosaccharide of the Gram-negative bacterium *Campylobacter jejuni*. Proc Natl Acad Sci U S A **107:**5160-5165.

289. **Christensen JE, Pacheco SA, Konkel ME.** 2009. Identification of a *Campylobacter jejuni*-secreted protein required for maximal invasion of host cells. Mol Microbiol **73:**650-662.

290. **Richardson PT, Park SF.** 1995. Enterochelin acquisition in *Campylobacter coli*: characterization of components of a binding-protein-dependent transport system. Microbiology **141 (Pt 12):**3181-3191.

291. **Klose KE, Weiss DS, Kustu S.** 1993. Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein. J Mol Biol **232:**67-78.

292. **Cameron A, Frirdich E, Huynh S, Parker CT, Gaynor EC.** 2012. Hyperosmotic stress response of *Campylobacter jejuni*. J Bacteriol **194:**6116-6130.

293. **Reid AN, Pandey R, Palyada K, Naikare H, Stintzi A.** 2008. Identification of *Campylobacter jejuni* genes involved in the response to acidic pH and stomach transit. Appl Environ Microbiol **74:**1583-1597.

294. **Butcher J, Stintzi A.** 2013. The transcriptional landscape of *Campylobacter jejuni* under iron replete and iron limited growth conditions. PLoS One **8:**e79475.

295. **Butcher J, Sarvan S, Brunzelle JS, Couture JF, Stintzi A.** 2012. Structure and regulon of *Campylobacter jejuni* ferric uptake regulator Fur define apo-Fur regulation. Proc Natl Acad Sci U S A **109:**10047-10052.

296. **Hofreuter D.** 2014. Defining the metabolic requirements for the growth and colonization capacity of *Campylobacter jejuni*. Front Cell Infect Microbiol **4:**137.

297. **Nazef L, Belguesmia Y, Tani A, Prevost H, Drider D.** 2008. Identification of lactic acid bacteria from poultry feces: evidence on anti-*campylobacter* and anti-*listeria* activities. Poult Sci **87:**329-334.

298. **Wolfe AJ.** 2010. Physiologically relevant small phosphodonors link metabolism to signal transduction. Curr Opin Microbiol **13:**204-209.

299. **Lukat GS, McCleary WR, Stock AM, Stock JB.** 1992. Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. Proc Natl Acad Sci U S A **89:**718-722.

300. **Yamasaki M, Igimi S, Katayama Y, Yamamoto S, Amano F.** 2004. Identification of an oxidative stress-sensitive protein from *Campylobacter jejuni*, homologous to rubredoxin oxidoreductase/rubrerythrin. FEMS Microbiol Lett **235:**57-63.

301. **Bingham-Ramos LK, Hendrixson DR.** 2008. Characterization of two putative cytochrome c peroxidases of *Campylobacter jejuni* involved in promoting commensal colonization of poultry. Infect Immun **76:**1105-1114.

302. **Rubinchik S, Seddon AM, Karlyshev AV.** 2014. A negative effect of *Campylobacter* capsule on bacterial interaction with an analogue of a host cell receptor. BMC Microbiol **14:**141.

303. **Annison EF, Hill KJ, Kenworthy R.** 1968. Volatile fatty acids in the digestive tract of the fowl. Br J Nutr **22:**207-216.

304. **Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT.** 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut **28:**1221-1227.

305. **Macfarlane GT, Gibson GR, Cummings JH.** 1992. Comparison of fermentation reactions in different regions of the human colon. J Appl Bacteriol **72:**57-64.

306. **Chavez RG, Alvarez AF, Romeo T, Georgellis D.** 2010. The physiological stimulus for the BarA sensor kinase. J Bacteriol **192:**2009-2012.

307. **Fields JA, Thompson SA.** 2008. *Campylobacter jejuni* CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. J Bacteriol **190:**3411-3416.

308. **Loh E, Dussurget O, Gripenland J, Vaitkevicius K, Tiensuu T, Mandin P, Repoila F, Buchrieser C, Cossart P, Johansson J.** 2009. A trans-acting riboswitch controls expression of the virulence regulator PrfA in *Listeria monocytogenes*. Cell **139:**770-779.