CAMPYLOBACTER JEJUNI MOTILITY IS REGULATED BY CO-CULTURE WITH EPITHELIAL CELLS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of ALISON BRIANA LANE find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Campylobacter jejuni is a Gram-negative, motile, spiral-shaped bacterium, and a common cause of gastroenteritis in humans. The flagellum of *C. jejuni*, which confers motility and serves as a secretion system for the export of virulence proteins, is composed of a basal body, hook, and filament. The filament is comprised of two proteins, termed FlaA and FlaB. While these two proteins are 94% homologous, the *flaA* and *flaB* genes differ in that they are expressed from separate promoters controlled by different sigma factors (σ^{28} and σ^{54}, respectively). FlaA is required for *C. jejuni* motility. A *C. jejuni* mutant that only synthesizes FlaB is non-motile and has truncated flagella, but is still able to invade host cells better than mutants lacking both FlaA and FlaB. The purpose of this study was to determine the expression levels of the *C. jejuni flaA* and *flaB* flagellar genes in response to conditions that the organism encounters throughout its life cycle. We hypothesized that in a host, bacterial motility may be significantly reduced over time, but that the organism maintains a functional flagellar secretion apparatus. Promoter activity of these two genes was determined in response to a variety of culture conditions. Consistent with our hypothesis, we found that promoter activity of *flaA* (required for full motility) was

downregulated in the presence of epithelial cells, while activity of *flaB* (and other σ^{54}-controlled genes essential for formation of a functional secretion apparatus) remained unchanged. These findings create the opportunity for further investigation of the mechanism behind this phenomenon to gain better a greater understanding of the regulation of flagellar gene expression in *C. jejuni.*

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To the memory of Tara Valentine Clarke

Thank you for all of the laughter, good times, and country dancing we shared in Pullman!

Not a day goes by when I don't miss you with all my heart.

Chapter 1

INTRODUCTION

1.1 Background

Campylobacter jejuni is a common foodborne pathogen and one of the most prevalent causes of bacterial gastroenteritis both worldwide and in the United States, where over 2.4 million cases are estimated to occur annually (24). Although other *Campylobacter* species are able to cause human disease, a five-year surveillance study in the United States implicated *C. jejuni* in 99% of cases (37). The overall incidence of campylobacteriosis is likely under-reported, and recent estimates have suggested that one of 38 cases is actually accounted for (24). This Gram-negative, spiral-shaped bacterium is motile and microaerophilic, making it well-suited to both its natural habitat as a commensal organism in the digestive tract of chickens and other avian species, and to the human gastrointestinal tract where it can cause disease. *C. jejuni* can grow at both 37°C and 41°C, the internal temperatures of humans and most birds, respectively. It is typically acquired via consumption of undercooked poultry meats or cross-contamination from raw poultry products during food preparation, although contaminated milk and water have also been implicated as sources. The infective dose of *C. jejuni* can be quite low, with ingestion of as few as 500 organisms being sufficient to cause disease (33).

The majority of *C. jejuni* enteritis cases are sporadic, and the incidence of campylobacteriosis in developed countries follows a seasonal pattern, with most cases reported in summer and early fall (8). Age- and sex-specificity is also observed. In the United States, *C. jejuni* is more frequently isolated from males than females, and from infants and young adults (37). In contrast,

C. jejuni is endemic in much of the developing world, and infections show no discernable seasonal distribution. Campylobacteriosis in these areas primarily affects children under the age of two (after which immunity has typically developed), and can cause severe dehydration in this patient population (3, 9).

The primary clinical feature of campylobacteriosis is diarrhea, which can range from mild and watery to severe, accompanied by the presence of blood and leukocytes. Other symptoms may include fever, abdominal pain, nausea, and vomiting. These differences in disease presentation may be due to variation in genomic content or expression of the bacteria, host factors, or most likely a combination of both. The disease is typically self-limiting, ranging from 2 to 7 days. In severe cases, antibiotic treatment may be necessary. Erythromycin is currently the drug of choice due to increasing emergence of *C. jejuni* strains resistant to fluoroquinolones such as ciprofloxacin, previously the most common treatment for campylobacteriosis (3).

Rarely, post-infection complications associated with *C. jejuni* enteritis may occur. The most prevalent, Guillain-Barré syndrome (GBS), is a demyelinating polyneuropathy causing acute flaccid paralysis (28). It is estimated that one in a thousand cases of campylobacteriosis results in GBS (2). Although prior infection by a number of bacteria and viruses has been implicated as the antecedent to the development of GBS, *Campylobacter* is by far the most common, estimated to account for 30-40% of GBS cases (27). GBS is thought to be caused by an autoimmune cross-reaction of anti-*Campylobacter* antibodies with nervous tissue, as some *C. jejuni* lipooligosaccharides have structural similarity to gangliosides found on nerve cells (28). Miller-Fisher syndrome is a variant of GBS that demyelinates cranial nerves, leading to paralysis of eye

muscles (ophthalmoplegia), and loss of control and reflexes of ocular motor nerves (34). Prior infection with *C. jejuni* has also been linked to development of rheumatologic disorders such as reactive arthritis, however, the mechanism of this association is currently not well understood (28).

1.2 Virulence Factors

Several factors contributing to the virulence of *C. jejuni* have been identified, and previous work indicates that the flagellum of the organism plays an important role in colonization of a host and in pathogenesis. The *C. jejuni* flagellum is comprised of a basal body, hook, and filament, and confers motility in addition to serving as a type III secretion system (TTSS) for export of virulence proteins into host cells (21).

After ingestion by a human host and passage through the stomach, *C. jejuni* enters the lumen of the small intestine and reaches epithelial cells via chemotaxis toward the mucin layer (22). The bacterium then translocates from the apical to basolateral side of the epithelial cell barrier via a transcellular or paracellular route (22). Motility is clearly an essential part of these early steps in colonization, and has been demonstrated to be of importance in both invasion of cultured host cells and experimental infection of animals (26, 38).

Upon reaching the basolateral surface of an epithelial cell, *C. jejuni* binds to the surface of host cells via a variety of adhesins. These are likely to be constitutively synthesized proteins, due to the fact that metabolically inactive bacteria bind to epithelial cells (18). Several adhesins have been identified. The most well-characterized adhesin is CadF (*Campylobacter* adhesion to

fibronectin), which binds fibronectin in the host extracellular matrix (19). *C. jejuni* flagella may also play a role in adherence, although this phenomenon is not well understood and conflicting data has been found (22).

Following the adherence of *C. jejuni* to host epithelial cells, the flagellum continues to contribute to the pathogenic process by functioning as a TTSS for the export of the *Campylobacter* invasion antigens (Cia proteins). Without the presence of the flagellar structure, secretion of these virulence proteins will not occur (21). Synthesis and secretion of these proteins has been demonstrated to be essential for invasion of cultured epithelial cells by *C. jejuni* (20). Several studies using animals have demonstrated that *C. jejuni* invasion of epithelial cells contributes significantly to the ability of this organism to cause disease (22). There is a wide variation in invasive ability between strains, with strains isolated from clinical specimens tending to be more invasive *in vitro* than environmental isolates (29).

1.3 Flagellar Structure and Regulation

The *C. jejuni* flagellum consists of three main components: the basal body, hook, and filament. Each of these structures is in turn composed of several different proteins. The flagellar filament of *C. jejuni* is comprised of two flagellin proteins, FlaA and FlaB. Under standard growth conditions, the amount of FlaA in the filament is greater than that of FlaB (14). Mutational studies of the flagellar filament genes support this assertion, indicating that a $FlaA^+ FlaB^-$ mutant will produce a flagellum composed solely of FlaA that is equal in size to wild-type flagella, while a $FlaA^- FlaB^+$ filament comprised of only FlaB is severely truncated (13). Motility of these mutants is similarly affected, with $FlaA^+ FlaB^-$ being slightly less motile than wild-type

organisms and FlaA$^-$ FlaB$^+$ having significantly reduced motility in comparison. However, maximum motility is only achieved when both FlaA and FlaB are incorporated into the flagellar filament, and organisms expressing neither *flaA* nor *flaB* lack flagella and are non-motile (4, 39).

The *flaA* and *flaB* genes are each 1731 base pairs in length (separated by a 173 bp region) and have nucleotide sequences that are 95% identical (30). However, expression of the two flagellar filament genes is controlled by two different promoters, a situation that is uncommon amongst the Gram-negative enteropathogens. The *flaA* gene is expressed from a σ^{28} promoter, much like the flagellar genes of *Escherichia coli* and *Salmonella typhimurium* (13). A σ^{54} promoter controls expression of *flaB* in *C. jejuni*. *Caulobacter crescentus,* which lacks σ^{28}, has all of its flagellar filament genes are under control of a σ^{54} promoter (4, 25). In *C. jejuni*, several other genes essential for flagellar structure and function are also controlled by σ^{54}, including the flagellar basal body and hook genes, and many of the flagellar biosynthesis genes. The only other organisms sharing this dual-sigma factor reguation of flagellar genes are the Gram-negative pathogens *Helicobacter pylori*, *Vibrio cholerae* and *Vibrio parahemolyticus* (31, 35).

The *C. jejuni* flagellum, like that of many other Gram-negative bacteria, is assembled in a sequential fashion (23). Regulation of this process in *C. jejuni*, however, varies from that of other enteric pathogens. First, the Class I flagellar genes, controlled by the housekeeping sigma factor σ^{70}, are expressed. These include genes encoding some of the initial proteins required to assemble the basal body. Expression of the *flhA* gene in particular appears to be essential for activating expression of both Class II and Class III flagellar genes (11). The Class II genes are controlled by a σ^{54} promoter, and encode the majority of the hook and basal body genes in

addition to the minor flagellin *flaB*. Like all sigma factors in the σ^{54} family, an additional activator protein is needed for transcription of σ^{54}-controlled genes (17). In *C. jejuni*, expression of these genes is also dependent on the FlgS/FlgR two-component regulatory system, the signal for which is as yet unknown. After phosphorylation by the FlgS sensor kinase, the FlgR response regulator interacts with RpoN (σ^{54}), increasing its synthesis and transcriptional activity (16, 42). The interaction between FlgR and RpoN leads to the completion of the hook and basal body structure. As *flaB* is also controlled by σ^{54}, a functional flagellar secretion apparatus is present at this point. Until this stage in flagellar assembly, expression of the σ^{28}-controlled Class III genes, encoding the flagellar filament and cap proteins, is weakly repressed by the presence of the anti-sigma factor FlgM (16). When Class I and II genes have been expressed and their products assembled into a secretion apparatus, FlgM escapes the bacterium, derepressing FliA (σ^{28}) and leading to asubsequent increase in expression of the Class III genes (including *flaA*, the major flagellar filament protein), and completion of a full-length flagellum.

The role played by the *C. jejuni* flagellum in pathogenesis appears to be two-fold. First, for the organism to be fully motile, both *flaA* and *flaB*, in addition to the σ^{54}-controlled hook and basal body genes, must be expressed. However, for the flagellum to serve as a secretory apparatus for the components of the flagellum itself (e.g., FlaA and FlaB), regulatory proteins (e.g. an anti-sigma factor), and virulence proteins (e.g. Cia proteins), only the σ^{54} regulon must be expressed. Expression of the σ^{54}-controlled genes encoding the hook and basal body is absolutely necessary for the flagellum to contribute to both motility and to act as a secretion apparatus.

1.4 Luciferase Reporter Gene Systems

The hallmarks of a good reporter gene system are that its product can be easily detected, has no background activity (i.e., the activity is not naturally present in the host organism), and a high level of sensitivity. Several different reporter genes have been used to assess promoter activity in *Campylobacter* species. This includes the widely used *E. coli lacZ* gene, which encodes β-galactosidase (41). An exogenous substrate must be added to assay activity, and because the majority of β-galactosidase substrates cannot cross cell membranes, cell lysis is required prior to determination of enzyme activity. Additionally, β-galactosidase can remain active after cell death, and therefore may not be an accurate indicator of real-time promoter activity. Another reporter gene previously used in *C. jejuni* is *astA*, which encodes the enzyme arylsulphatase (16). Like β-galactosidase, activity of this enzyme is determined from a whole-cell lysate spectrophotometrically. One additional drawback is that *astA* must be deleted from the strain to be used as a host before transformation with the reporter vector to eliminate background activity. Promoter fusions with chloramphenicol transferase (CAT) genes have also been used as reporters, with the amount of enzyme produced assayed by ELISA to determine promoter activity (4).

Exogenous bacterial luciferases have previously been demonstrated to function successfully as reporters in *Campylobacter* species, in the form of both the *luxAB* genes that encode the actual luciferase and the *lux* operon (*luxCDABE*) in its entirety (1, 32). Luciferases in general have several properties that make them well-suited for use in this system. First, the luciferase aldehyde substrate can easily cross membranes, eliminating the need for either a permease or cell lysis to assay reporter enzyme activity. Additionally, measurable luciferase activity will only

occur in live cells (because $FMNH_2$, produced only in metabolically active cells, is required for the light-producing reaction) while other reporter enzymes such as β-galactosidase can retain their activity even in non-viable cells as long as there is substrate present.

Using the *lux* operon of *Photorhabdus (Xenorhabdus) luminescens* as a reporter system to determine gene expression in *C. jejuni* offers several advantages. The *luxCDABE* operon of *P. luminescens* encodes five proteins, two of which (LuxA and B) make up the luciferase itself. LuxC, D, and E are all involved in synthesis of the long-chain aldehyde substrate (RCOH) that is necessary for the light-producing oxidation reaction of luciferase to take place (43). When the entire *P. luminescens lux* operon is placed under the control of the promoter in question (as opposed to only the *luxAB* genes encoding luciferase itself), it is unnecessary to add additional aldehyde substrate. The other two substrates, $FMNH_2$ and oxygen, are typically present in the bacterial cell in sufficient quantities for the reaction to proceed. The *P. luminescens* luciferase in particular is also advantageous in comparison to other bacterial luciferases because it retains activity up to 45°C (40). In contrast, the commonly used lucifersases from *Vibrio fischeri* and *Vibrio harveyi* can function at temperatures of up to 30°C and 37°C. In our assays, it was advantageous to utilize the *P. luminescens* luciferase because *C. jejuni* is found in both humans and chickens, with internal temperatures of 37° and 41°C, respectively. For this study, luciferase reporter vectors were constructed by inserting the *lux* operon of *P. luminsecens* into the multiple cloning site of the *Campylobacter* shuttle vector pRY111, downstream of the promoter sequence of the gene in question.

Chapter 2

MATERIALS AND METHODS

Culture of Bacterial Strains

C. jejuni wild-type strain F38011 and sigma factor mutants derived from *C. jejuni* F38011 were cultured on Mueller-Hinton agar plates supplemented with 5% bovine blood (MH-blood agar) under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C. All strains were passed to fresh plates every 24 to 48 hr. *Escherichia coli* S17-1 λ *pir*, used for cloning, was grown aerobically at 37°C on Luria-Bertani (LB) agar plates or in LB broth. If necessary, media was supplemented with antibiotics at the following concentrations: streptomycin (Str), 20 μg/mL; nalidixic acid (Nal), 50 μg/mL; tetracycline (Tet), 12.5 μg/mL; and chloramphenicol (Chl), 20 μg/mL.

Construction of Sigma Factor Mutants

To create a mutation in the *rpoN* gene (encoding σ^{54}) of *C. jejuni* F38011, the 5' end of the F38011 *rpoN* gene was amplified (forward primer, 5'-GGG TTA ATT TCT TGG TCT TG; reverse primer, 5'-AAC CGC GGA AAG CTA GCG TGT TCA AAA TAT CCT TCC TCA TTT AG, contained *Nhe*I and *Sac*II restriction sites) and ligated into pCR2.1 (Invitrogen, Carlsbad, CA). The 3' end of the *rpoN* gene from *C. jejuni* F38011 was also amplified (forward primer, 5'-TTG CTA GCG CTA CTC TTT ATA AAA TAG GGC TTA TG, contained *Nhe*I restriction site; reverse primer, 5'-AAC CGC GGT AAA GCT ACT AAA GCA ATA GCT CCT AAG, contained *Sac*II restriction site). The 3' fragment of the *rpoN* gene was ligated to the 5' fragment in pCR2.1, followed by digestion with *Nhe*I and insertion of the *tetO* gene encoding

tetracycline resistance. This vector was then digested with *EcoR*I to liberate the fragment containing the 5' and 3' ends of *rpoN* flanking the *tetO* gene, which was subsequently ligated into the suicide vector pBSK (Stratagene, La Jolla, CA) that had been modified to include an *aphA-3* gene cassette encoding resistance to kanamycin. This vector was electroporated into the *C. jejuni* F38011 wild-type strain, and Tet resistant, Kan sensitive recombinant *rpoN* mutants were selected.

A *C. jejuni* F38011 *fliA* (σ^{28}) mutant was constructed in a similar fashion by amplifying the 5' and 3' ends of the *fliA* gene from the chromosome of *C. jejuni* NCTC 11168 (5' end: forward primer 5'-TTG GAA GAC ATT TTA ATA GAA G; reverse primer 5'-AAC CGC GGA AAG CTA GCC ACA AGC TCA TCT TGC TCT TTC, contained *Sac*II and *Nhe*I restriction sites. 3' end: forward primer 5'-TTG CTA GCC ACG AAG TGC TAG ATG ATC TTA AAG, contained *NheI* restriction site; reverse primer 5'- AAC CGC GGA TTT CTT TGA TTT CAT CTT TAT C, contained *Sac*II restriction site). Each fragment was digested with the appropriate restriction enzymes, and ligated with the *tetO* gene into pBSK-Kan, as described above. The resulting vector was electoporated into *C. jejuni* F38011, and recombinant *fliA* mutants were identified by sensitivity to Kan and resistance to Tet.

Luciferase Reporter Vectors

The previously described 6.45 kb shuttle vector pRY111 was modified to include the *luxCDABE* operon from *Photorhabdus luminescens*. The 4.774 kb *luxCDABE* cassette was obtained from pCS26 and inserted into the *Not*I site of pRY111, creating an 11.22 kb plasmid termed pRY111-lux. Promoter regions of *flaA* and *flaB* (flagellins) and *porA* (major outer membrane protein)

were PCR-amplified from *C. jejuni* NCTC 11168 and digested with *BamH*I, yielding fragments of 216 bp, 460 bp, and 331 bp respectively. The promoter fragments were ligated upstream of the *lux* operon into the *BamH*I site of pRY111-lux, creating pRY111-$flaA_{prom}$-lux, pRY111-$flaB_{prom}$-lux, and pRY111-$porA_{prom}$-lux. These three vectors, in addition to pRY111-lux (to be used as a promoterless control), were electroporated into *E. coli* S17-1 λ *pir* and selected with 20 μg/mL Chl. Each of the four constructs was subsequently transformed into Str and Nal resistant *C. jejuni* F38011 wild-type, *fliA* (σ^{28}) mutant (tet^R), and *rpoN* (σ^{54}) mutant (Tet^R) strains via conjugation. Transconjugants were identified on MH-blood plates containing Str, Nal, and Chl (with the addition of Tet for the sigma factor mutants) to select for only those *C. jejuni* harboring the pRY111-lux vectors. This process resulted in twelve combinations of the three *C. jejuni* strains containing the four different luciferase reporter vectors.

Luciferase Assays – Environmental Factors

To compare the effects of pH on activity of the flagellar filament gene promoters, bacteria were prepared as described above. After adjusting the OD_{540} to 0.1, 1 mL of each culture was centrifuged for 2 min at 6000 x *g* and resuspended in 1 mL of fresh MH broth adjusted using 2 N H_2SO_4 or 2 N NaOH to pH 5.5, 6.5, 7.3, or 8.0. Plates containing 200 μL of each bacterial suspension in quadruplicate were incubated on a shaker at 37°C in microaerobic conditions and measurements of luciferase activity and OD_{595} were taken each hr.

To determine if short chain fatty acids (SCFA) affected activity of the promoters, broth cultures of *C. jejuni* were grown overnight and OD_{540}-adjusted to 0.2. 100 μL of each was added in quadruplicate to 100 μL of MH broth containing 60 mM acetate, butyrate, propionate, or NaCl

(to control for osmolarity effects of the SCFAs). This resulted in a final OD_{540} of 0.1 and a final concentration of 30 mM of each SCFA. Plates were shaken in standard incubation conditions and luciferase activity and OD_{595} were determined every hr.

To assess the effects of temperature on *flaA* and *flaB* promoter activity, *C. jejuni* strains containing each of the four pRY111-lux luciferase reporter vectors were grown overnight in MH broth with 20 μg/mL chloramphenicol. The OD_{540} of each culture was adjusted to 0.1, and 200 μL was added in quadruplicate to 96-well white-walled, clear bottom tissue culture plates, in addition to wells containing MH broth alone as a media-only control. One plate was placed at 37°C and the other at 41°C, both shaking in microaerobic conditions. Luciferase activity and OD_{595} were determined hourly using a Wallac Victor^2 luminometer (Perkin Elmer, Waltham, MA).

When examining the effects of the bile salt deoxycholate (DOC), bacteria were prepared as described above for testing SCFA. 100 μL of bacteria at an OD_{540} of 0.2 were added 1:1 in 96-well tissue culture plates to deoxycholate prepared in MH broth at twice the desired final concentration. The resultant final concentrations of DOC were 0%, 0.2%, and 0.4%. Plates were incubated shaking at 37°C and measurements taken as above.

Culture of Epithelial Cells

INT 407 human intestinal epithelial cells (ATCC CCL6; American Type Culture Collection, Manassas, VA) were maintained in minimal essential media (MEM, Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and

5% L-glutamine (1.8mM). LMH chicken hepatocellular carcinoma cells (ATCC CRL-2117; American Type Culture Collection, Manassas, VA) were cultured in Waymouth MB 752/1 media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS. The cells were cultured at 37°C in a humidified, 5% CO_2 incubator and passed when they reached 80 to 90% confluency, approximately every 48 hr.

Luciferase Assays – Cell-Associated Bacteria

INT 407 or LMH epithelial cells were seeded in a 24-well tissue culture plate with approximately 2 x 10^5 cells per well in MEM or Waymouth media + 10% FBS and grown overnight to 80-90% confluency. After rinsing once with MEM + 1% FBS, a 1mL suspension of *C. jejuni* (harboring a luciferase reporter vector) at an OD_{540} of 0.18 (approximately 5 x 10^8 CFU) was added to each well of INT 407 cells. Plates were centrifuged for 5 min at 600 x *g* to promote bacteria-host cell contact and incubated at 37°C in a humidified, 5% CO_2 incubator. Luciferase and OD_{595} readings were taken hourly. Similar measurements were determined using *C. jejuni* exposed to fixed host cells. After being seeded and grown overnight as described above, INT 407 cells were fixed by the addition of 250 µL of 2% paraformaldehyde, followed by a 10 min incubation at 4°C. Each well was washed thoroughly 3 times with PBS, after which bacteria were added and luciferase measurements determined as above.

^{35}S-Metabolic Labeling

After overnight growth on MH-blood plates, *C. jejuni* F38011 wild-type bacteria were harvested in RPMI 1640 (CellGro, Herndon, VA) supplemented with 65 mg/L cysteine, 300 mg/L glutamine, and 1% dialyzed FBS. The OD_{540} of the suspension was adjusted to approximately

0.3, and 1 mL was added to wells of live and fixed INT 407 cells. Following 5 min of centrifugation at 600 x *g*, the plate was incubated in humidified conditions at 37°C. After one hour and 30 min, 2.3 µL of Expre^{35}S^{35}S Protein Labeling Mix (NEN, Boston, MA) containing ^{35}S -methionine was added to one well of bacteria plus live INT 407 and one well of bacteria plus paraformaldehyde-fixed INT 407. After 30 min of labeling, at the two-hour time point, 500 µL of supernatant was removed from a well, centrifuged, and resuspended in 250 µL of water. 100 µL of the suspension was combined 1:1 with 1% SDS and incubated for 5 min at ambient temperature to facilitate cell lysis. Subsequently, 800 µL of 1 mM HCl in acetone was added, and allowed to incubate 5 min to precipitate proteins. Following 5 min of centrifugation, the supernatant was removed and the proteins were resuspended in 50 µL water and stored at -20° C. This process was repeated at two-hour intervals for 10 hours. After all samples had been collected, they were thawed and 10 µL was added to a vial of scintillation fluid and counted on a liquid scintillation analyzer (Packard Instrument Co., Downer's Grove, IL) to determine the amount of ^{35}S methionine incorporated.

Transmission Electron Microscopy

C. jejuni F38011 wild-type bacteria were grown overnight on MH-blood plates and harvested in MEM supplemented with 1% FBS. After adjusting the OD_{540} of the suspension to approximately 0.3, 1 mL was added to each well of INT 407 cells as described above. The tissue culture plate was centrifuged for 5 min at 600 x *g*, and subsequently incubated in humidified conditions at 37°C. After 2, 12, and 24 hr of incubation, samples were prepared for TEM as follows. The supernatant from 1 well of bacteria and INT 407 cells was removed, and a droplet of approximately 5 µL was added to the surface of a formvar coated nickel grid and allowed to

sit for 1 min, after which absorbent blotting paper was used to remove the liquid. A 5 μL drop of 2% phosphotungstic acid was added to the grid to stain it for 1 min. After removal of the liquid, a drop of distilled H_2O was placed on the grid and immediately absorbed using blotting paper. The grid was allowed to dry for approximately 15 min at ambient temperature, and was subsequently analyzed using a JEOL transmission electron microscope (JEOL, Tokyo, Japan).

Motility Assays

Motility assays were performed using MH broth containing 0.4% Bacto agar (Becton Dickinson, Franklin Lakes, NJ). A suspension of bacteria was prepared at an OD_{540} of 1.0, and 2 μL was spotted on the center of the motility plate. After 48 hr of incubation in microaerophilic conditions, the zone of migration from the initial inoculation point was determined.

Western Blots with Anti-Flagellin Antibodies

Whole cell lysates of the *C. jejuni* F38011 wild-type strains were prepared at 0, 2, 6, and 10 hours of co-culture with live INT 407 epithelial cells and separated in duplicate using 12% SDS-PAGE. One gel was stained with Coomassie blue R-250, and the proteins from the other were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Bedford, MA). Immunoblots were performed by incubating the membrane overnight at 4°C with a 1:300 dilution of a rabbit-α-*C. jejuni* flagellin antibody (in PBS-Tween + 5% non-fat dry milk). After 3 washes with PBS-Tween, a HRP-conjugated goat-α–rabbit IgG (whole molecule) diluted 1:1000 in PBS-Tween + 5% non-fat dry milk was added as a secondary antibody and incubated at room temperature for 2 hours. Following 2 washes with PBS-Tween

and a final wash with PBS, blots were developed using 4-chloro-1-napthol tablets (Sigma, St. Louis, MO) to visualize reactive proteins.

Luciferase Assays – Conditioned Media

MEM alone and MEM supplemented with 1% FBS were added to flasks containing confluent viable INT 407 cells, and allowed to incubate at 37° overnight. The following morning, the supernatant media was collected and used to make suspensions of *C. jejuni* F38011 containing the pRY111-$flaA_{prom}$-lux or pRY111-$flaB_{prom}$-lux reporter vectors. In addition, bacterial suspensions were prepared using unconditioned MEM and MEM + 1% FBS. Luciferase assays were then performed as described above.

Chapter 3

RESULTS

Construction of luciferase reporter vectors

The *luxCDABE* operon from *Photorhabdus luminescens* was successfully cloned into the *C. jejuni* shuttle vector pRY111. Promoter regions of the flagellins and major outer membrane protein genes were cloned into this vector upstream of the *lux* operon. The resultant reporter vectors (pRY111-$flaA_{prom}$-lux, pRY111-$flaB_{prom}$-lux, and pRY111-$porA_{prom}$-lux), in addition to a promoterless control vector (pRY111-lux), were conjugated into *C. jejuni* F38011 wild-type bacteria and sigma factor mutant strains. The luciferase activity of each strain/vector combination was determined under standard growth condtions. As expected, the pRY111-lux promoterless control showed no activity in all three *C. jejuni* strains. Bacteria harboring the pRY111-$porA_{prom}$-lux vector showed the highest levels of activity, consistent with the fact that major outer membrane protein is expressed constitutively from a σ^{70} promoter. The activity of the flagellar gene promoters was reflective of previous reports regarding the protein composition of the flagellum, with the luciferase activity of *C. jejuni* F38011+pRY111-$flaA_{prom}$-lux being approximately 5 to 10-fold higher than F38011+pRY111-$flaB_{prom}$-lux. The sigma factor mutant strains had comparable relative luciferase activities to *C. jejuni* F38011, with the exception that the *fliA* mutant containing pRY111-$flaA_{prom}$-lux and the *rpoN* mutant containing pRY111-$flaB_{prom}$-lux had no detectable activity. This result was expected due to the fact that expression of a promoter should not occur in the absence of the appropriate sigma factor. Following determination of baseline activities, each bacterial strain and reporter vector combination was used in subsequent experiments.

Effects of media pH, short chain fatty acids, temperature, and deoxycholate on *flaA* and *flaB* promoter activity

Media pH altered measurable luciferase activity of the *flaA* and *flaB* promoters in a similar manner to one another. Promoter activity of both (standardized using both OD_{595} and levels of *porA* (major outer membrane protein, constitutively expressed)) had the highest luciferase measurements in Mueller-Hinton broth at pH 6.5, while bacteria cultured in media with pH 5.5, 7.3, and 8.0 all showed decreased activity (Figure 1A and 1B). Figure 1C illustrates that the changes in the level of *flaA* expression relative to *flaB* are minimal and are unaffected by media pH.

Activity of the *flaA* σ^{28} and *flaB* σ^{54} promoters was assessed in the presence of 30 mM of each of the short chain fatty acids (SCFAs) acetate, propionate, and butyrate. In comparison to *C. jejuni* to which 30 mM NaCl had been added (to control for osmolarity effects of the SCFAs), over the course of 8 hours, luciferase activity was reduced between approximately 1.5 to 20-fold by addition of SCFAs (Figure 2A and 2B). Propionate had the greatest effect in decreasing activity of the *flaA* and *flaB* promoters, specifically on *flaB*, which was reduced nearly to zero. This dramatic decrease in expression *flaB* in response to propionate caused the fold difference between *flaA* and *flaB* to increase sharply (Figure 2C).

When grown at 41°C, luciferase activity driven by both the *flaA* σ^{28} and *flaB* σ^{54} promoters was noticeably reduced compared to the standard temperature of 37°C (Figure 3A and 3B). This trend was observed both initially and over the course of several hours of growth. At both temperatures, however, *C. jejuni* F38011 containing each luciferase reporter vector had

comparable growth rates as determined by OD_{595}. The ratio of *flaA* and *flaB* promoter expression increased after 12 hours of culture at both 37°C and 41°C, (Figure 3C), indicating that temperature does not have a differential effect on flagellar gene expression.

The presence of the bile salt deoxycholate (DOC) at various physiologically relevant concentrations reduced both *flaA* and *flaB* promoter activity in similar fashions. Over time, luciferase activity of both *flaA* and *flaB* in *C. jejuni* treated with DOC gradually declined while promoter activity of both flagellin genes for bacteria in MH broth alone remained at steady levels (Figure 4). The levels of *flaA* expression as compared to *flaB* expression changed similarly over time, indicating that the presence or absence of DOC has little effect on the ratio of the two flagellin gene products.

Response of *flaA* and *flaB* promoter activity to co-culture with epithelial cells.

As shown in Figure 5A, *flaA* activity showed an initial upregulation during the first two hours that *C. jejuni* was cultured in the presence of viable INT 407 cells, followed by a gradual decrease in measurable luciferase activity. However, *flaB* activity (controlled by σ^{54}) remained relatively constant throughout the course of co-culture with INT 407 cells, decreasing after approximately 14 hr. Growth rates of the two *C. jejuni* F38011 strains containing the pRY111-$flaA_{prom}$-lux and pRY111-$flaB_{prom}$-lux reporter vectors, as monitored by OD_{595}, were comparable (Figure 5B). When the fold difference between *flaA* and *flaB* promoter expression levels was determined by taking the ratio of their luciferase activities, a steady decline was noted (Figure 5C). Activity of the *flaA* σ^{28} promoter was a maximum of 9 times higher than that of the *flaB* σ^{28} promoter at the 2 hr time point, but by 12 hr had decreased to only 1.5-fold higher. *C. jejuni*

co-cultured with LMH chicken epithelial cells displayed similar responses in *flaA* promoter activity as was seen with the INT 407 human epithelial cells, with an initial increase during the first 2 hr and a gradual decline after that point (Figure 6). As with INT 407 cells, *flaB* promoter activity was unaffected by the presence of the LMH cells, remaining constant throughout the course of the assay.

When *C. jejuni* containing the pRY111-$flaA_{prom}$-lux and pRY111-$flaB_{prom}$-lux reporter vectors was cultured in the presence of INT 407 cells fixed with paraformaldehyde, activity of the *flaB* promoter was comparable to what was observed with viable cells and remained relatively constant until approximately 12 hr. In contrast, as compared to co-culture with viable cells, the same initial increase in *flaA* promoter activity was observed during the first two hr, however after this point a sharp decline of about 10-fold occurred (Figure 7B). By subtracting luciferase activities obtained with fixed INT 407 cells from activities measured with viable INT 407 cells, the amount of *flaA* promoter activity attributable to viable cells was determined (Figure 7C). Although a steady decline in *flaA* expression was observed with live cells, in comparison to epithelial cells fixed with paraformaldehyde, it is clear the continued expression of *flaA* from 3 hr to 11 hr is specific to viable INT 407 cells.

***C. jejuni* co-cultured with viable and fixed INT 407 cells remain metabolically active**

The metabolic activity of *C. jejuni* in the presence of INT 407 epithelial cells was determined using a radiolabeled ^{35}S-methionine incorporation assay. During co-culture with INT 407 cells, both live and paraformaldehyde-fixed, metabolic activity increased over the course of 10 hours (Figure 9). Throughout the first 8 hours of the assay, the relative levels of novel protein

synthesis (as measured by amount of radiolabeled ^{35}S-methionine incorporated) were similar for bacteria cultured with both live and fixed epithelial cells.

Effects of co-culture with INT 407 epithelial cells on flagellar structure and bacterial motility

Visualization of *C. jejuni* during co-culture with INT 407 cells using transmission electron microscopy revealed a time-dependent variation in the number of flagellated versus non-flagellated bacteria (Figure 8A). After 2 hours in the presence of host cells, 32% of *C. jejuni* possessed full-length flagella. At 12 hours after inoculation, however, the percentage of flagellated bacteria had decreased to approximately 6%, with the remaining 94% completely lacking any visible flagellar structure (Figure 8B). In contrast, *C. jejuni* F38011 cultured in MH broth alone did not show the same decrease in percentage of flagellated bacteria. At 2 hr, 52% of organisms had a full-length flagellum, and at 12 hr, 46% remained flagellated. In accordance with these results, motility of the bacterial population decreased over time during co-culture with viable INT 407 epithelial cells (Figure 8C). Additionally, whole-cell lysates of *C. jejuni* co-cultured with viable INT 407 cells were immunoblotted with an α-*C. jejuni* flagellin antibody. Figure 10 reveals that at 0, 2, and 6 hours, flagellin is detectable, while after 10 hours of co-culture with INT 407 cells, no corresponding band was visible (Figure 10B).

Media conditioned with INT 407 cells maintains luciferase activity of *flaA* and *flaB*

Luciferase assays of *flaA* and *flaB* promoter activity were performed with media pre-conditioned overnight with and without INT 407 viable cells. The activity of *flaA* was barely above background in MEM alone, however, in the same media conditioned with INT 407 cells,

promoter activity gradually declined as seen in the presence of live cells (Figure 10A). Likewise, *flaB* activity was maintained fairly steadily over time by MEM conditioned with INT 407 cells, but was negligible in media alone (Figure 10B). The effects of FBS on the flagellar filament gene expression was also assessed. *C. jejuni* assayed for *flaA* and *flaB* activity in MEM alone had extremely low expression of both genes (data not shown). However, in MEM which had been supplemented by 1% FBS, measurable *flaA* activity was detected for the first hour of the assay, after which it dropped sharply and was minimally active. Promoter activity of *flaB* was also initially maintained by MEM + 1% FBS, but declined steeply after approximately 1.5 hr.

Chapter 4

DISCUSSION

Enteric organisms, whether they are normal flora or pathogens, must survive, colonize and grow in the harsh intestinal environment, and find a niche amongst competition from other bacteria. Many of the processes required for colonization of the intestine and invasion of host cells are very energetically costly, and bacteria often use environmental signals to allow efficient expression of appropriate genes at the correct time and in the correct location. Similarly, *Campylobacter jejuni* encounters a variety of conditions throughout its life cycle. This study sought to determine if the same phenomenon was observed in *C. jejuni*, with particular attention to the regulation of flagellar genes during co-culture with host epithelial cells. To assess changes in flagellar gene expression, we utilized a luciferase reporter vector system.

As *C. jejuni* passes through the gastrointestinal tract of both humans and avian species, it must make its way through environments of varying acidity. This may range from as low as pH 1-2 in an empty human stomach or the ventriculus (gizzard) of a chicken, to as high as pH 7-8 in the human colon. We observed maximal activity of the *flaA* and *flaB* promoters in media at a pH of 6.5. The pH in the human duodenum and jejunum, where *C. jejuni* colonizes and causes disease, ranges from 6-7. The flagellum of *C. jejuni* has been demonstrated to play a key role in the organism's ability to bind and invade epithelial cells of the small intestine. Thus, it makes sense that maximum activity levels of the flagellar filament gene promoters will occur a pH that reflects this environment. Previous reports have indicated highest levels of *flaA* promoter activity occur at a pH of 5, with the suggestion that acidic conditions cause the flagellum to dissociate. This could lead to an increase in FlaA synthesis to compensate for this loss in

flagellar protein subunits (1). However, the assays performed by Allen and Griffiths (2001) were done at a single time point, perhaps explaining the discrepancy with the results presented in the present study. We observed that the *flaA* and *flaB* promoter activities were only slightly repressed at the acidic pH of 5.5, while at increased pH of 7.3 and 8, expression of both flagellar filament genes was significantly decreased in comparison. While media pH clearly affects the levels of both the σ^{28}-controlled *flaA* promoter and the σ^{54}-controlled *flaB* promoter, this phenomenon was not found to be differential, and the relative amounts of the flagellar filament proteins remained the same. As the OD_{595} of the cultures did not vary in media of different pH values, this effect was not caused by differences in bacterial growth.

During the breakdown of food by digestion and intestinal flora, high concentrations of short-chain fatty acids (SCFA) are produced as byproducts throughout the gastrointestinal tract. In the human small intestine, SCFA are present at 20-40 mM, while in the colon a concentration of greater than 100 mM is found. The amount of specific SCFA varies by anatomical location as well, with acetate being the major SCFA found in the small intestine, compared to the cecum and colon where propionate and butyrate predominate (6). SCFA have been shown to regulate invasion genes in *Salmonella* species (C. Altier, personal communication). The presence of SCFA at varying concentrations may serve as an environmental signal to enteric bacteria such as *C. jejuni*, indicating the appropriate segment of the intestinal tract for colonization and to regulate gene expression accordingly. We found that in comparison to MH broth, the presence of 30 mM acetate, propionate, or butyrate at reduced promoter activity of both *flaA* and *flaB*. Propionate had the greatest effect on both promoters, and completely abolished *flaB* expression during the initial stages of the assay. The effect on *flaA* was not nearly as drastic, leading to

changes in the fold difference and ratio of *flaA* to *flaB*, suggesting a differential effect. While acetate and butyrate did affect flagellar filament gene promoter activity, the effect appeared to be non-differential, downregulating expression of both *flaA* and *flaB* in the same manner.

During passage through the gastrointestinal tract of humans and chickens, *C. jejuni* also encounters varying concentrations of bile salts. While concentration, composition, and toxicity of bile salt components vary between species, they have the same detergent and antimicrobial effects. In the human host, this ranges from approximately 0.2-2% overall (15), with deoxycholate (DOC) accounting for 15% of the total. Bile salts are known to regulate virulence-associated genes in a number of Gram-negative enteric pathogens, including *Salmonella* and *Vibrio* species (15). We found that at three different physiologically relevant concentrations of DOC, both *flaA* and *flaB* promoter activities were downregulated over time. Regardless of the concentration of DOC used, no differential effect on the two promoters was observed, suggesting that the levels of FlaA to FlaB produced relative to each other remain the same. This is in contrast to previous results examining *flaA* promoter activity from Allen, which found that bile and DOC upregulate its expression (1). However, their data was gathered at a single time point after 12 hours of culture, and changes in *flaA* promoter activity over time were not determined. Using a proteomics approach, Fox *et al.* observed an upregulation of both *flaA* and *flaB* in response to bile (12). However, at the 18 hour time point, the bacteria may have entered stationary phase, thus it is difficult to compare their results to those obtained in the present study.

As *C. jejuni* colonize, survive and multiply in both avian species and humans, the role of temperature in regulation of flagellar genes was investigated. In contrast to humans, the core

temperature of birds is 41°C. While promoter expression of both the *flaA* and *flaB* genes was decreased when bacteria were cultured at 41°C relative to 37°C, when the fold difference between the two was determined, the ratio of *flaA* to *flaB* expression increased over time. This effect was independent of bacterial growth, as the OD_{595} of the cultures remained equivalent throughout the course of the assay. It has been previously that *flaA* in *C. coli* is upregulated at 42°C, with a resulting increase in motility of the organisms (13). In accordance, we observed greatly increased motility when *C. jejuni* was cultured at 41°C when compared to 37°C (data not shown).

While each individual environmental factor tested had an effect on flagellar filament gene expression, with the exception of growth at 41°C and the addition of 30 mM propionate, they affected *flaA* and *flaB* promoter activity in the same manner. In other words, there was little differential regulation of the σ^{54} and σ^{28} operons observed. Although it would follow that the overall amounts of FlaA and FlaB proteins were correspondingly decreased, the ratio (relative levels of the two proteins) remained the same in most cases. This implies little differential regulation occurs between the fully motile state of *C. jejuni* and the presence of a functional secretion apparatus in response to each environmental signal. Perhaps in combination with one another or with some other signal found in the intestinal milieu, each of these factors could contribute to differential regulation of flagellar filament gene expression, but alone they do not appear to have an effect.

After passage through the upper gastrointestinal tract of a chicken or human host, *C. jejuni* colonizes the cecum and jejunum, respectively. We hypothesized that when *C. jejuni* is

associated with host cells, motility is shut down but a functional flagellar secretion apparatus is still present. While a full-length flagellum is essential in the initial stages of colonization, its production and maintenance is a very energetically costly process for the organism. Regulation of this process in response to host cells has been observed in other enteropathogens such as *Salmonella,* which is known to turn off flagellin expression after entering phagocytes (7). Other organisms downregulate production of flagellin as a strategy to evade detection by the immune system, however, this is not an issue for *C. jejuni* as its flagellin is not recognizable by the TLR5 receptors on host cells because it is lacking the residues required for recognition by TLR5 (5).

Luciferase assays of flagellar filament gene promoter activity revealed differential regulation of *flaA* and *flaB* in response to co-culture with INT 407 epithelial cells. An initial surge in *flaA* activity occurred in the first 2 hours, followed by a gradual decrease until the 10 hour time point. At this stage, *flaA* expression levels off. In contrast, *flaB* promoter activity remained constant throughout co-culture with INT 407 cells, declining only at stationary phase. This was observed with both human INT 407 and chicken LMH epithelial cells, indicating that this phenomenon likely occurs whether *C. jejuni* is existing as a commensal or as a pathogen. This differential regulation could correspond to an initial upregulation in motility during the beginning stages of colonization, as bacteria make their way from the intestinal lumen towards the intestinal epithelium and translocate across it. After this point, a full-length flagellum might not be necessary, and *C. jejuni* could downregulate *flaA* while maintaining *flaB* and the other σ^{54}-controlled flagellar structural genes that are required for a functional export apparatus. This would allow for continued secretion of the Cia proteins and invasion of host cells, which will not occur without a flagellar secretion apparatus (21). INT 407 cells fixed with paraformaldehyde

are also sufficient to maintain constant *flaB* activity over time, while *flaA* expression levels show the same initial increase until 2 hours, then drop precipitously. This indicates that some unidentified factor from viable INT 407 cells supports the gradual decline in *flaA* activity.

When *C. jejuni* were co-cultured with viable INT 407 cells and examined by transmission electron microscopy, it was observed that in the presence of live INT 407 cells, the percentage of flagellated bacteria significantly decreased between 2 and 12 hours of co-culture. This change corresponds to the time points with the greatest and least *flaA* activity as determined by luciferase assays. This implies that the decrease in luciferase activity is due to an overall population change in an "all or nothing" fashion rather than each individual bacterium making less FlaA. *C. jejuni* is known to regulate motility in a biphasic manner (flagellated versus aflagellate) through variable expression of flagella (10). We have demonstrated that this phenomenon occurs in response to co-culture with host cells. If the amount of measureable *flaA* promoter activity is decreasing over time, and the population of bacteria is becoming progressively less flagellated, we would expect the motility of a *C. jejuni* to decrease over the course of co-culture with INT 407 cells. Results of motility assays support this assertion.

Chapter 5

FUTURE DIRECTIONS

The present study has revealed a novel and interesting regulation of the *C. jejuni* flagellar structure and motility phenotype when the organism is co-cultured with host epithelial cells. A gradual decline in promoter activity of *flaA* was observed, with a corresponding decrease in percentage of flagellated organisms and bacterial motility. This finding suggests that in a host, bacterial motility may be significantly reduced over time, but that the organism maintains a functional flagellar secretion apparatus. Further investigation into the molecular mechanism of this phenomenon is certainly warranted. An important step will be identification of the effector molecule present during co-culture with viable epithelial cells and in media conditioned with these cells that induces the gradual downregulation of expression of *flaA* and other σ^{28}-controlled genes without affecting *flaB* and the σ^{54} operon. Additionally, it may be possible to gain greater insight into the regulatory hierarchy of *C. jejuni* flagellar assembly and function and how resultant phenotypes affect colonization of hosts and pathogenic potential.

The first step in the regulation of flagellar genes has been identified as the FlgS/FlgR two-component regulatory system (42). The initial signal for this system that leads to subsequent expression of flagellar genes is unknown, but it seems likely that this pathway is involved in the regulation of *flaA* expression demonstrated in response to intestinal epithelial cells. A possibility is that some effector molecule(s) secreted from host cells is acting as either an agonist, antagonist, or combination of both. Some possible candidates include cytokines released from epithelial cells as part of the inflammatory response, soluble fibronectin from host cells that has

dissolved into the media, a small-molecule byproduct of intestinal metabolism, or another previously unidentified factor.

The interplay that occurs between both commensal and pathogenic organisms and their human hosts is complex, including not only responses of eukaryotic cells to the presence of bacteria, but also responses of bacteria to host cells. In addition to the previously mentioned possibilities, an example recently discovered in *Salmonella* that relates specifically to flagellar gene expression is the upregulation of flagellin expression in response to lysophospholipids secreted from intestinal epithelial cells (36). These molecules are typically involved in signaling between epithelial cells, but recent evidence reveals that it induces *de novo* synthesis of flagellin by *Salmonella.* In this particular instance, this primarily benefits the host, as it makes the invading pathogen more detectable by the TLR5 receptors on the intestinal epithelial cells, leading to a greater immune reponse against *Salmonella.* While a parallel mechanism of flagellar regulation could be occurring in *C. jejuni*, the consequence for the organism would not be exactly the same. While the human body does mount an antibody response to *C. jejuni* flagellin, it does not occur via the TLR5 pathway, as the flagellin of *C. jejuni* does not contain the typical amino acid sequence recognizable by TLR5 receptors.

It is clear from the work presented in this study that media conditioned with epithelial cells (and not co-culture with live host cells themselves) can cause the gradual downregulation of *flaA,* indicating that a factor secreted into the media by the INT 407 cells can maintain *flaA* expression. Initial steps to identify the biological properties of this molecule will include treatment of conditioned MEM with dialysis, boiling, and heating, and use of the resultant

treated conditioned media in luciferase and motility assays. Depending on the response (or lack of response) from the *flaA* reporter vector, it will be possible to narrow down the identity of the effector molecule based on size and whether or not it is a protein. From that point, the next steps in the pathways leading to the gradual downregulation of *flaA* expression in response to co-culture with epithelial cells can be elucidated.

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41. **Wosten, M. M., M. Boeve, M. G. Koot, A. C. van Nuene, and B. A. van der Zeijst.** 1998. Identification of *Campylobacter jejuni* promoter sequences. J Bacteriol **180:**594-599.

42. **Wosten, M. M., J. A. Wagenaar, and J. P. van Putten.** 2004. The FlgS/FlgR two-component signal transduction system regulates the fla regulon in *Campylobacter jejuni.* J Biol Chem **279:**16214-16222.

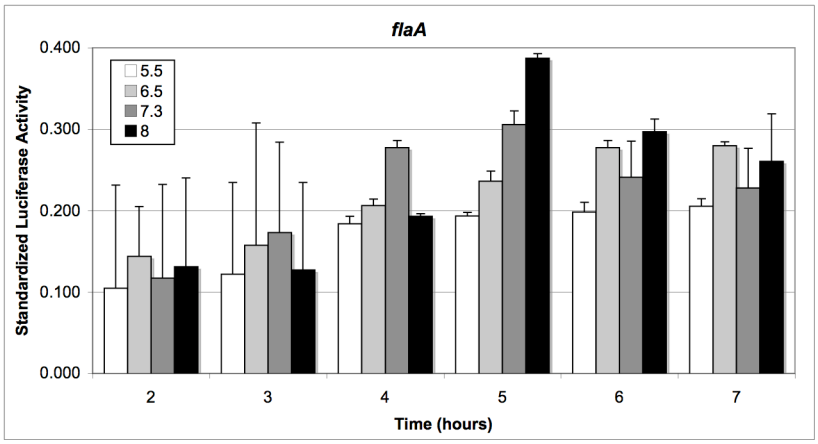
43. **Xi, L., K. W. Cho, and S. C. Tu.** 1991. Cloning and nucleotide sequences of lux genes and characterization of luciferase of *Xenorhabdus luminescens* from a human wound. J Bacteriol **173:**1399-1405.

Figure 1. Media pH affects *flaA* and *flaB* promoter activity in a non-differential manner.

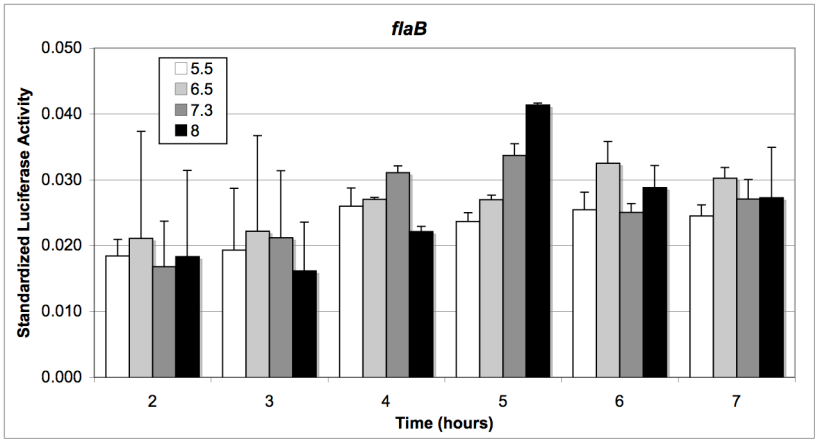
Panel A illustrates the effects of pH on *flaA* promoter activity, while Panel B shows these same effects on the *flaB* promoter. Over the course of 7 hours, both promoters responded in a similar fashion. The fold difference between the *flaA* and *flaB* promoters is depicted in Panel C, and indicates that while media pH does affect flagellar filament gene promoter activity, the ratio is independent of this factor and there is no differential effect observed.

Figure 1

A.


flaA
Standardized Luciferase Activity
0.400
0.300
0.200
0.100
0.000
5.5
6.5
7.3
8
2
3
4
5
6
7
Time (hours)

B.


flaB
Standardized Luciferase Activity
0.050
0.040
0.030
0.020
0.010
0.000
5.5
6.5
7.3
8
2
3
4
5
6
7
Time (hours)

C.

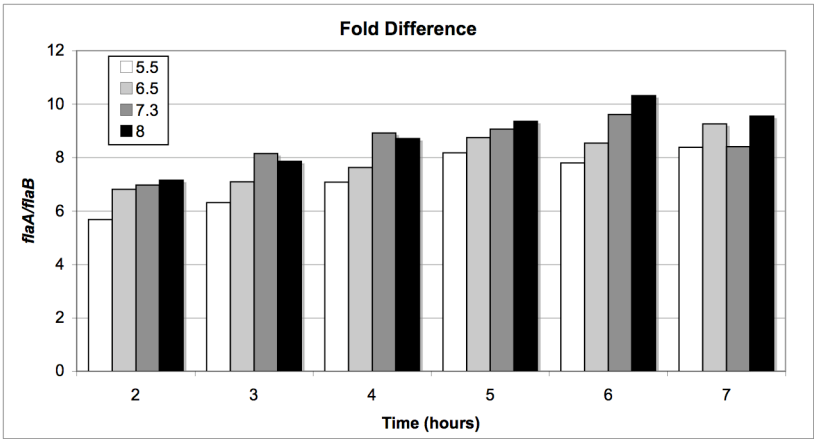
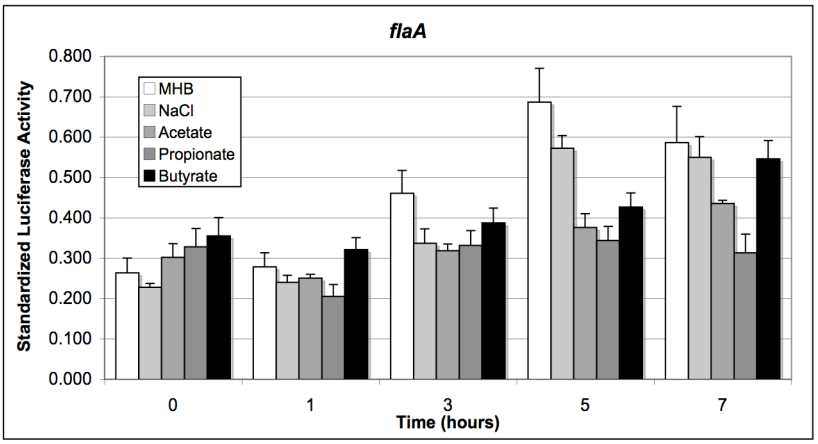

Fold Difference
flaA/flaB
12
10
8
6
4
2
0
5.5
6.5
7.3
8
2
3
4
5
6
7
Time (hours)

Figure 2. Propionate significantly reduces *flaB* promoter activity.

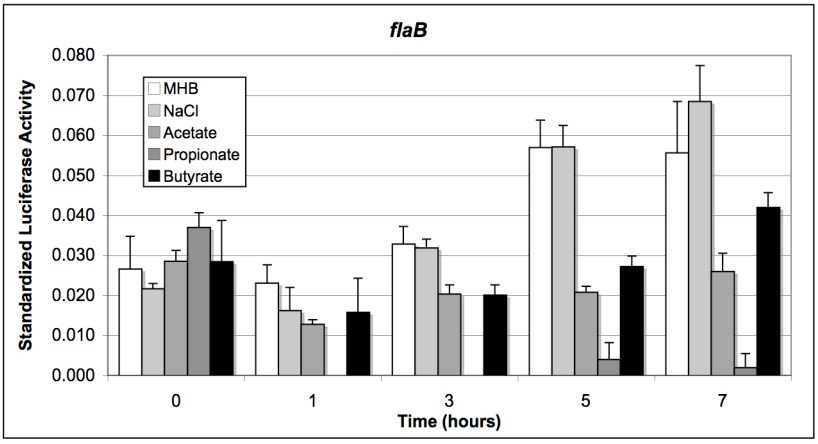
Effects of short chain fatty acids on the *flaA* promoter are seen in Panel A, and effects on *flaB* are shown in Panel B. In comparison to Mueller-Hinton broth alone or 30mM NaCl (added as an osmolarity control), over time, propionate had the greatest effect in decreasing activity of both promoters, impacting *flaB* significantly. Panel C illustrates the fold difference between *flaA* and *flaB*, and that while NaCl, acetate, and butyrate do not have a differential effect on the flagellar filament gene promoters, after 5 hours the presence of propionate drastically reduces *flaB* expression.

Figure 2

A.


flaA
Standardized Luciferase Activity
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
MHB
NaCl
Acetate
Propionate
Butyrate
0
1
3
5
7
Time (hours)

B.


flaB
Standardized Luciferase Activity
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
MHB
NaCl
Acetate
Propionate
Butyrate
0
1
3
5
7
Time (hours)

C.

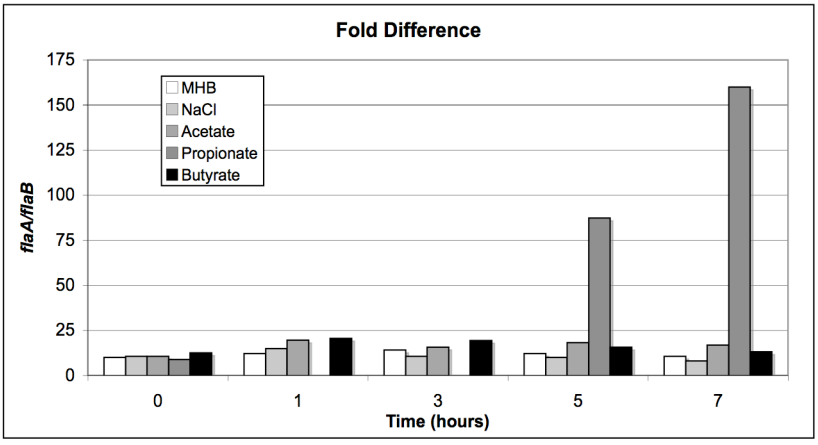
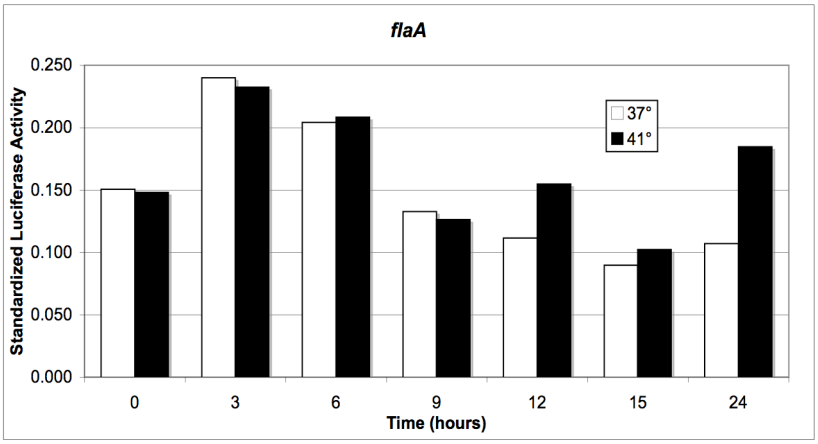

Fold Difference
flaA/flaB
175
150
125
100
75
50
25
0
MHB
NaCl
Acetate
Propionate
Butyrate
0
1
3
5
7
Time (hours)

Figure 3. Increased temperature reduces *flaA* and *flaB* promoter activity in a differential manner.

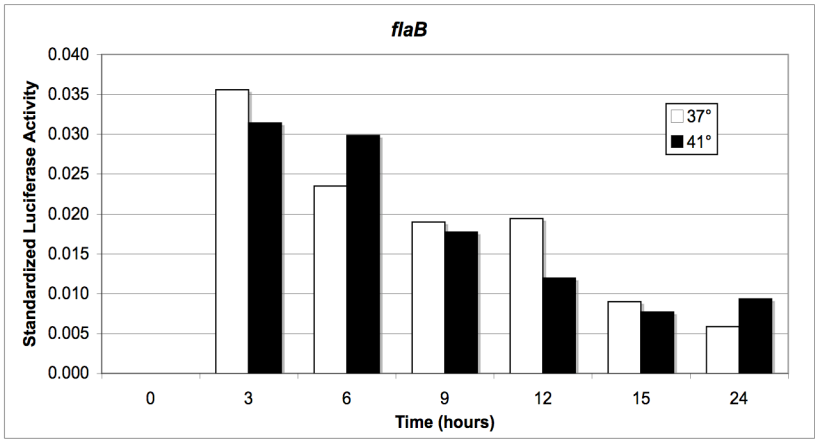
For both *flaA* (Panel A) and *flaB* (Panel B), when cultured at 41°C, luciferase activity was reduced compared to the standard temperature of 37°C. However, Panel C (depicting the fold difference) illustrates that the ratio of *flaA* and *flaB* promoter expression over time actually increased after the 12 hour time point.

Figure 3

A.


flaA
Standardized Luciferase Activity
0.250
0.200
0.150
0.100
0.050
0.000
37°
41°
0
3
6
9
12
15
24
Time (hours)

B.


flaB
Standardized Luciferase Activity
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
37°
41°
0
3
6
9
12
15
24
Time (hours)

C.

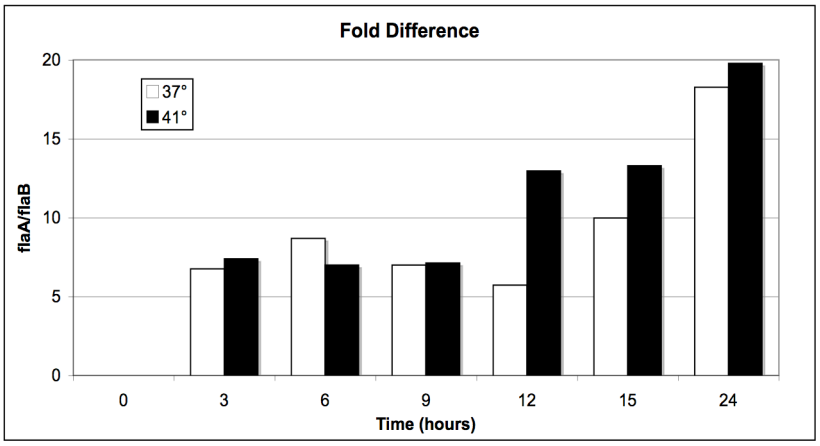
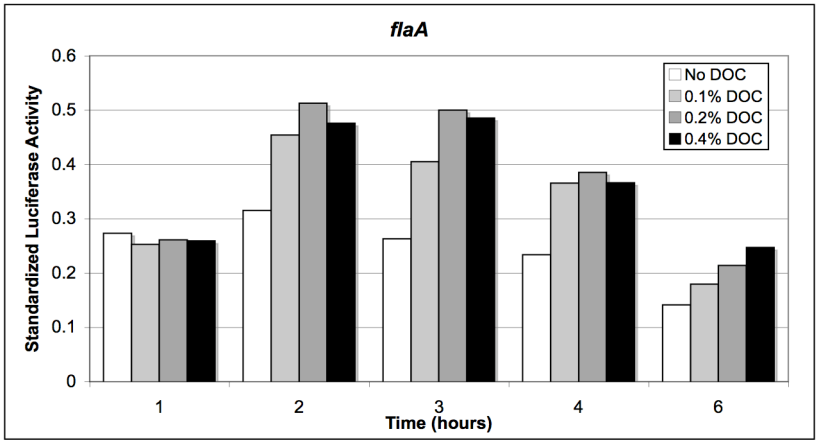

Fold Difference
flaA/flaB
20
15
10
5
0
37°
41°
0
3
6
9
12
15
24
Time (hours)

Figure 4. Deoxycholate decreases *flaA* and *flaB* promoter activity.

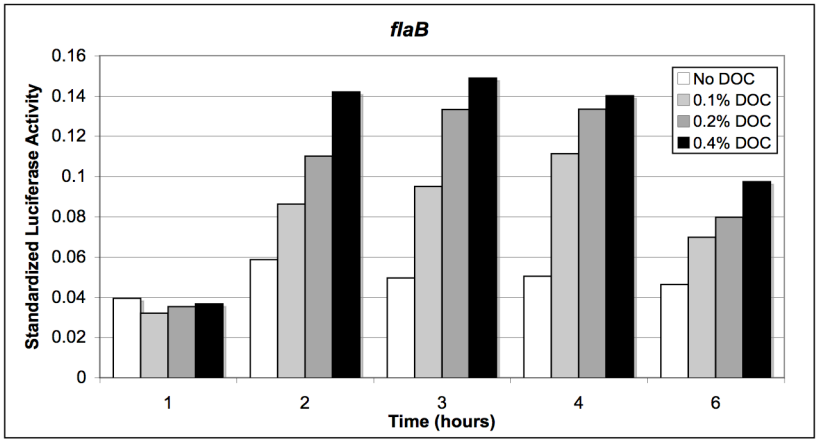
The presence of the bile salt deoxycholate (DOC) at various physiologically relevant concentrations reduced both *flaA* and *flaB* promoter activity in similar fashions, as seen in Panels A and B, respectively. The fold difference between *flaA* expression as compared to *flaB* expression changed similarly over time regardless of the concentration of DOC (Panel C), indicating that its presence or absence has little effect on the relative levels of the two flagellin gene products.

Figure 4

A.


flaA
Standardized Luciferase Activity
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
6
Time (hours)
No DOC
0.1% DOC
0.2% DOC
0.4% DOC

B.


flaB
Standardized Luciferase Activity
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
6
Time (hours)
No DOC
0.1% DOC
0.2% DOC
0.4% DOC

C.

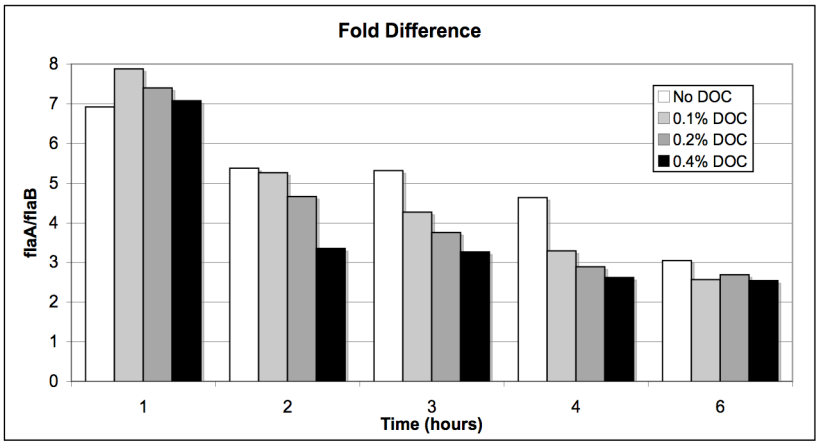
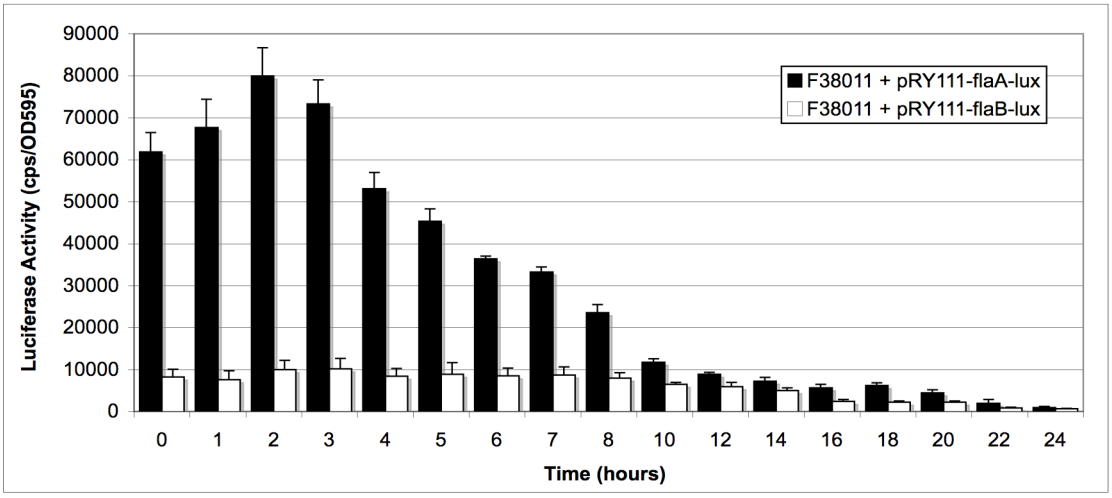

Fold Difference
flaA/flaB
8
7
6
5
4
3
2
1
0
1
2
3
4
6
Time (hours)
No DOC
0.1% DOC
0.2% DOC
0.4% DOC

Figure 5. Co-culture with INT 407 human epithelial cells decreases *flaA* promoter activity but does not affect *flaB*.

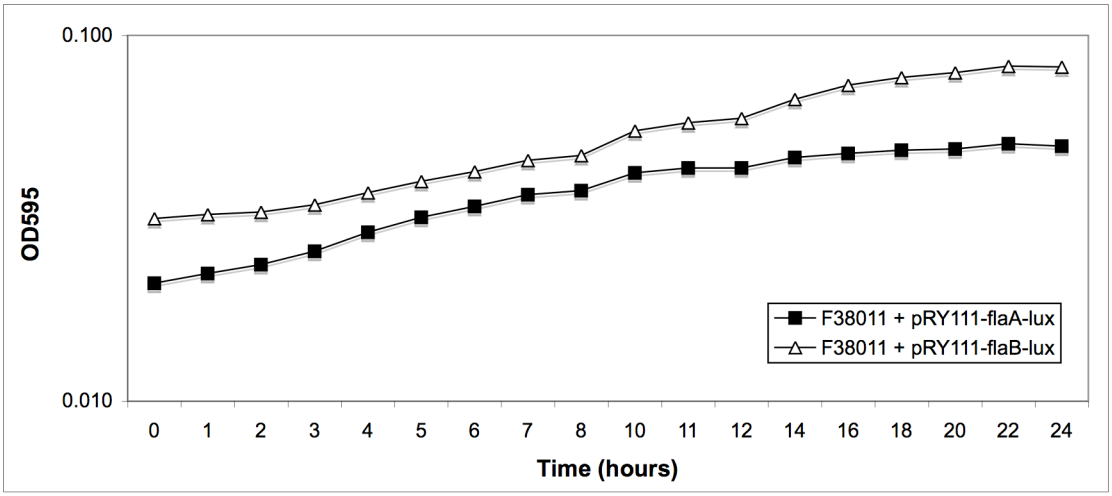
As shown in Panel A, *flaA* activity showed an initial upregulation during the first two hours that *C. jejuni* was cultured in the presence of viable INT 407 cells, followed by a gradual decrease in measurable luciferase activity. However, *flaB* activity remained relatively constant throughout the course of co-culture with INT 407 cells, decreasing after approximately 14 hr. Panel B illustrates that growth rates of the strains containing the pRY111-$flaA_{prom}$-lux and pRY111-$flaB_{prom}$-lux reporter vectors, as monitored by OD_{595}, were comparable. When the fold difference between *flaA* and *flaB* promoter expression levels was determined by taking the ratio of their luciferase activities (Panel C), a steady decline was noted, suggesting changes in the relative levels of *flaA* and *flaB* gene products.

Figure 5

A.


Luciferase Activity (cps/OD595)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0 1 2 3 4 5 6 7 8 10 12 14 16 18 20 22 24
Time (hours)
F38011 + pRY111-flaA-lux
F38011 + pRY111-flaB-lux

B.


OD595
0.100
0.010
0 1 2 3 4 5 6 7 8 10 11 12 14 16 18 20 22 24
Time (hours)
F38011 + pRY111-flaA-lux
F38011 + pRY111-flaB-lux

C.

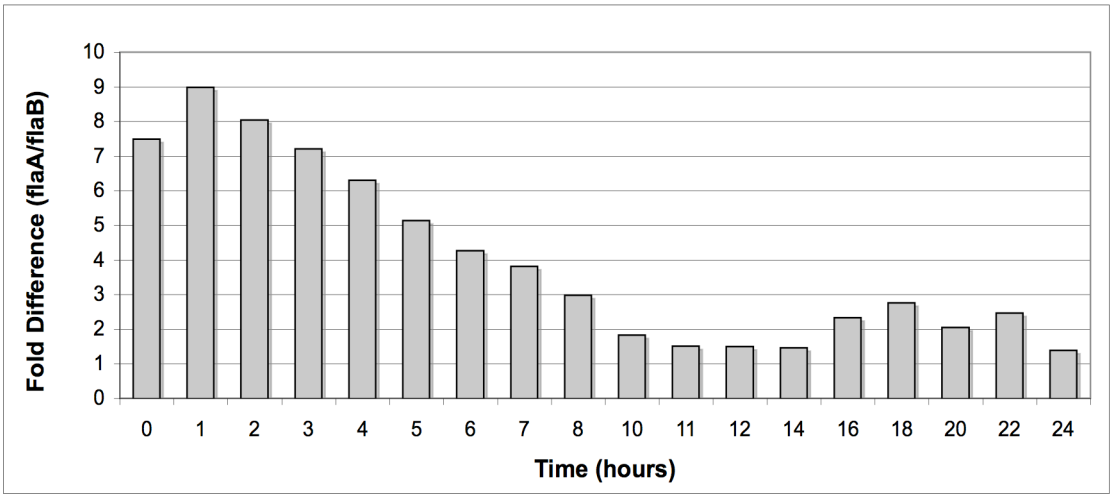

Fold Difference (flaA/flaB)
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 10 11 12 14 16 18 20 22 24
Time (hours)

Figure 6. Co-culture with LMH chicken epithelial cells decreases *flaA* promoter activity but does not affect *flaB*.

C. jejuni co-cultured with LMH chicken epithelial cells displayed similar responses in *flaA* promoter activity as was seen with the INT 407 human epithelial cells, with an initial increase during the first 2 hr and a gradual decline after that point. As with INT 407 cells, *flaB* promoter activity was unaffected by the presence of the LMH cells, remaining constant throughout the course of the assay.

Figure 6

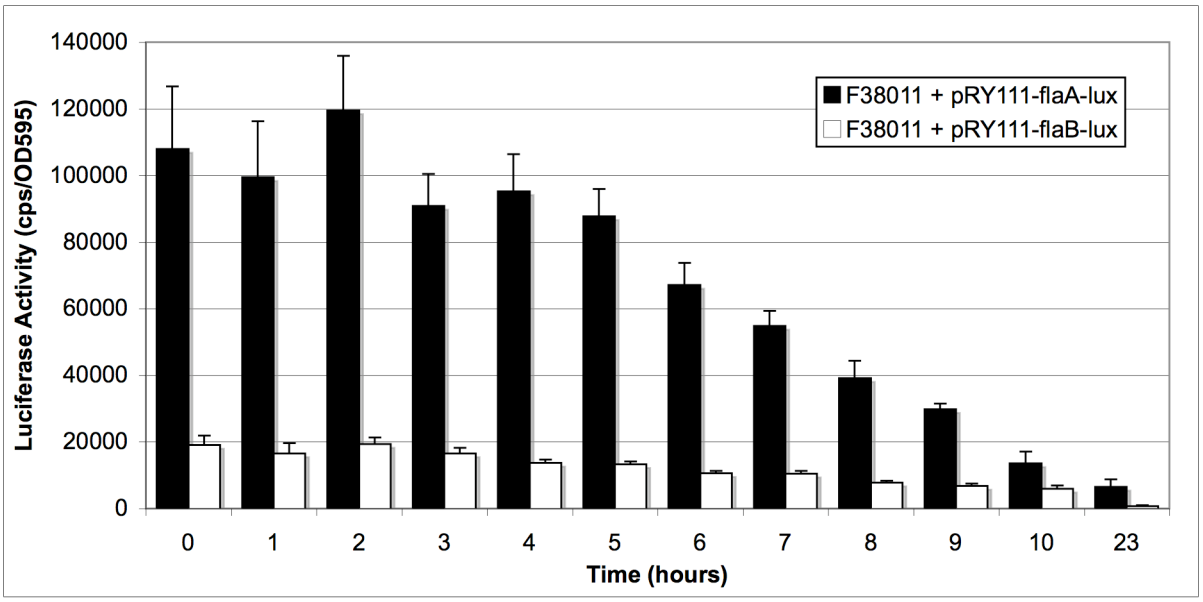
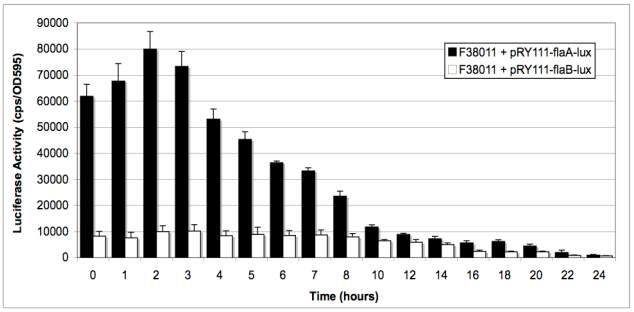

140000
120000
100000
80000
60000
40000
20000
0
Luciferase Activity (cps/OD595)
F38011 + pRY111-flaA-lux
F38011 + pRY111-flaB-lux
0
1
2
3
4
5
6
7
8
9
10
23
Time (hours)

Figure 7. The gradual decrease in *flaA* promoter activity is due to the presence of viable INT 407 cells.

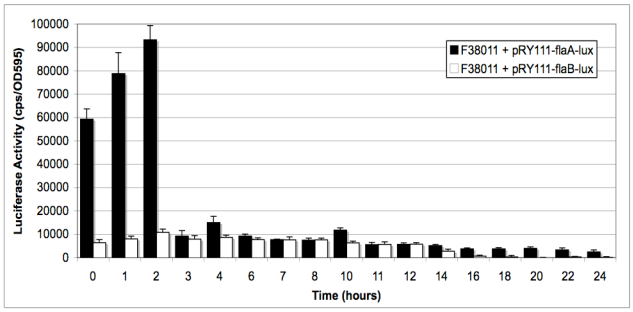
Activity of the *flaA* and *flaB* promoters in bacteria cultured in the presence of INT 407 cells fixed with paraformaldehyde is depicted in Panel B. Activity of the *flaB* promoter was comparable to what was observed with viable cells and remained relatively constant until approximately 12 hr. In contrast, as compared to co-culture with viable cells (Panel A), the same initial increase in *flaA* promoter activity was observed during the first two hr, however after this point a sharp decline of about 10-fold occurred. By subtracting luciferase activities obtained with fixed INT 407 cells from activities measured with viable INT 407 cells, the amount of *flaA* promoter activity attributable to viable cells was determined (Panel C).

Figure 7

A.


Luciferase Activity (cps/OD595)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0 1 2 3 4 5 6 7 8 10 12 14 16 18 20 22 24
Time (hours)
F38011 + pRY111-flaA-lux
F38011 + pRY111-flaB-lux

B.


Luciferase Activity (cps/OD595)
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0 1 2 3 4 6 7 8 10 11 12 14 16 18 20 22 24
Time (hours)
F38011 + pRY111-flaA-lux
F38011 + pRY111-flaB-lux

C.

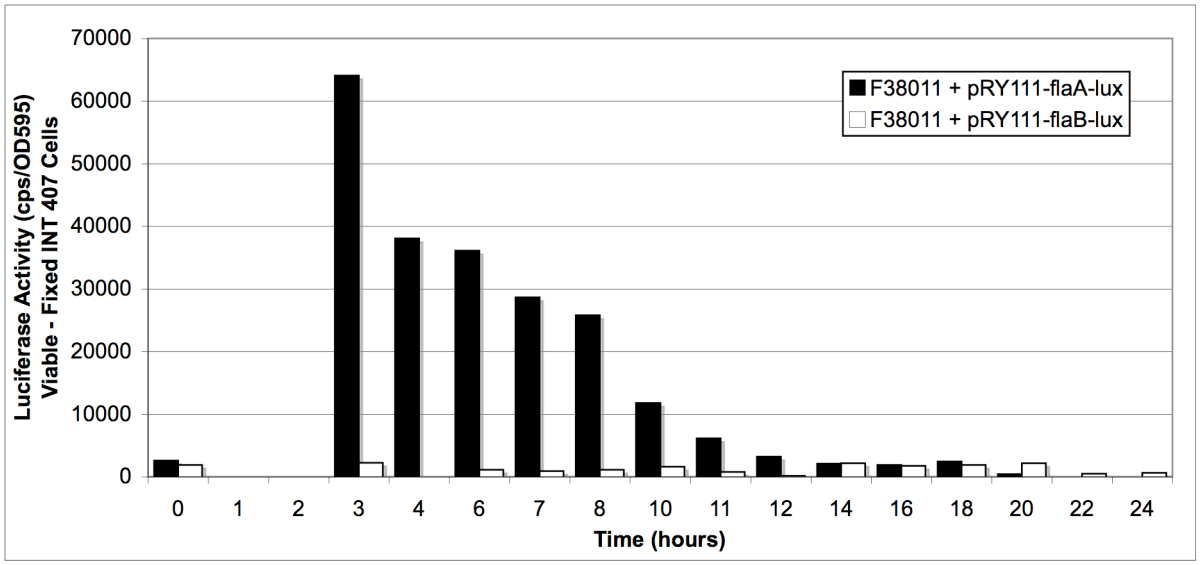

Luciferase Activity (cps/OD595)
Viable - Fixed INT 407 Cells
70000
60000
50000
40000
30000
20000
10000
0
0 1 2 3 4 6 7 8 10 11 12 14 16 18 20 22 24
Time (hours)
F38011 + pRY111-flaA-lux
F38011 + pRY111-flaB-lux

Figure 8. *porA* (Major outer membrane protein) expression levels remain steady during co-culture with INT 407 cells.

Expression levels of *porA* (Major outer membrane protein), a σ^{70}-controlled, constitutively expressed gene, were monitored during 12 hr of co-culture with INT 407 cells (both viable and fixed). Values were standardized to the OD_{595} of the bacterial suspension. The average value of all *porA* activities, with both viable and fixed INT 407 cells, was calculated and plotted as a line, demonstrating that variation in *porA* expression levels over time is negligible.

Figure 8

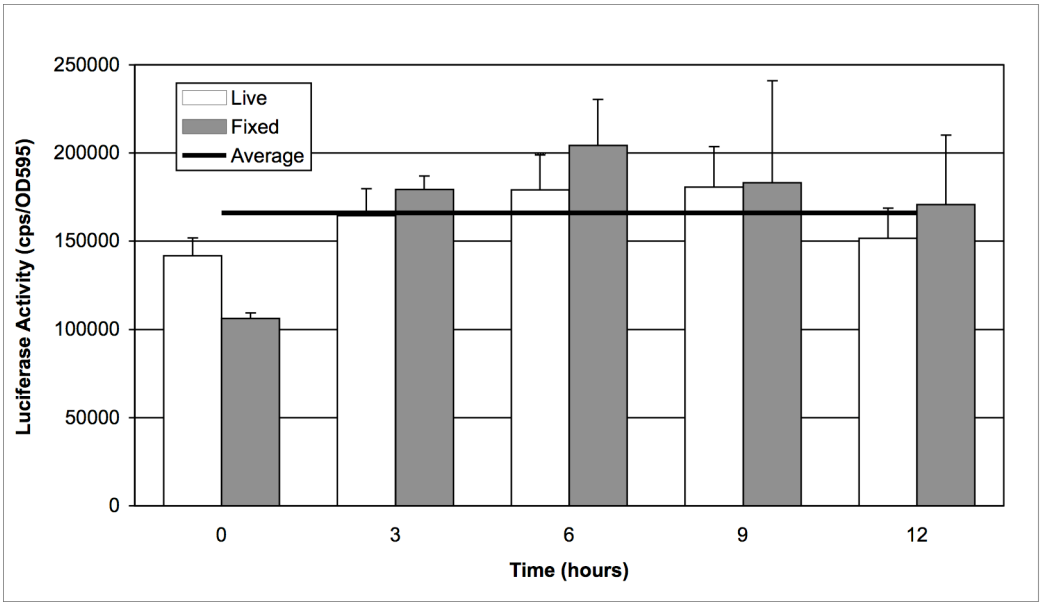

250000
200000
150000
100000
50000
0
Live
Fixed
Average
Luciferase Activity (cps/OD595)
0
3
6
9
12
Time (hours)

Figure 9. *C. jejuni* co-cultured with INT 407 cells is metabolically active.

A radiolabeled ^{35}S-methionine incorporation assay was used to assess the metabolic activity of *C. jejuni* in the presence of INT 407 epithelial cells. During co-culture with both live and paraformaldehyde-fixed INT 407 cells, metabolic activity increased over the course of 10 hours.

Figure 9

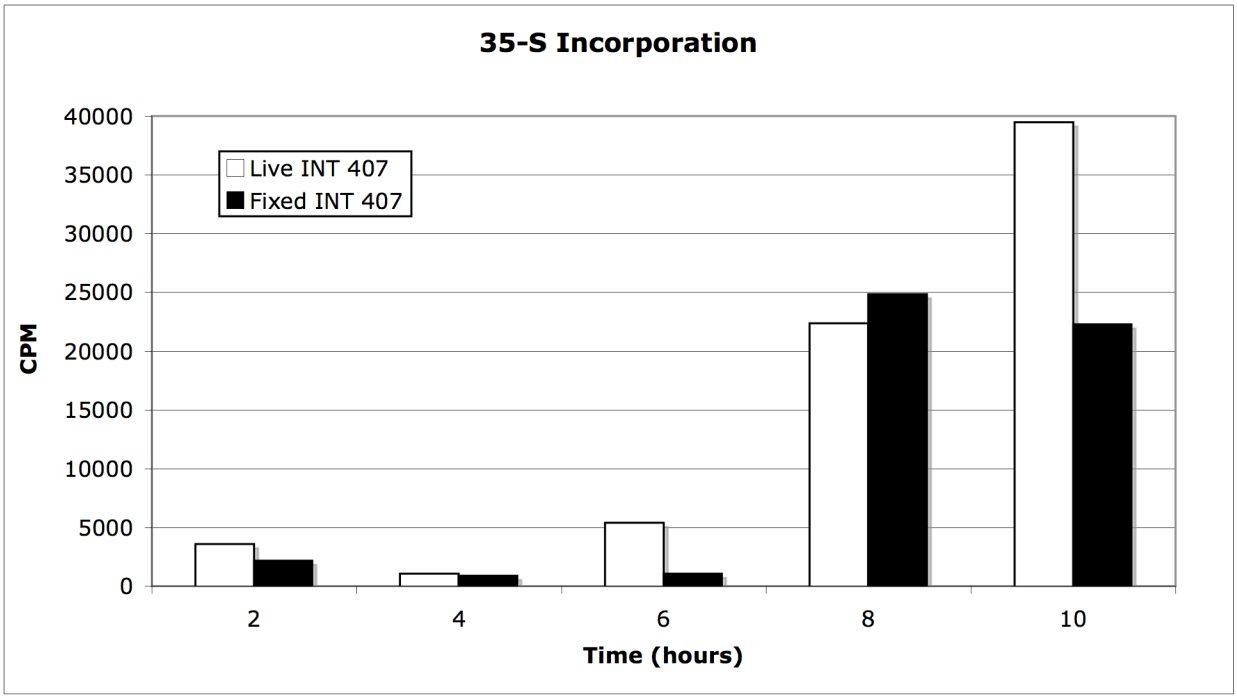

35-S Incorporation
CPM
40000
35000
30000
25000
20000
15000
10000
5000
0
Live INT 407
Fixed INT 407
2
4
6
8
10
Time (hours)

Figure 10. Co-culture with INT 407 cells reduces both the percentage of flagellated organisms and bacterial motility.

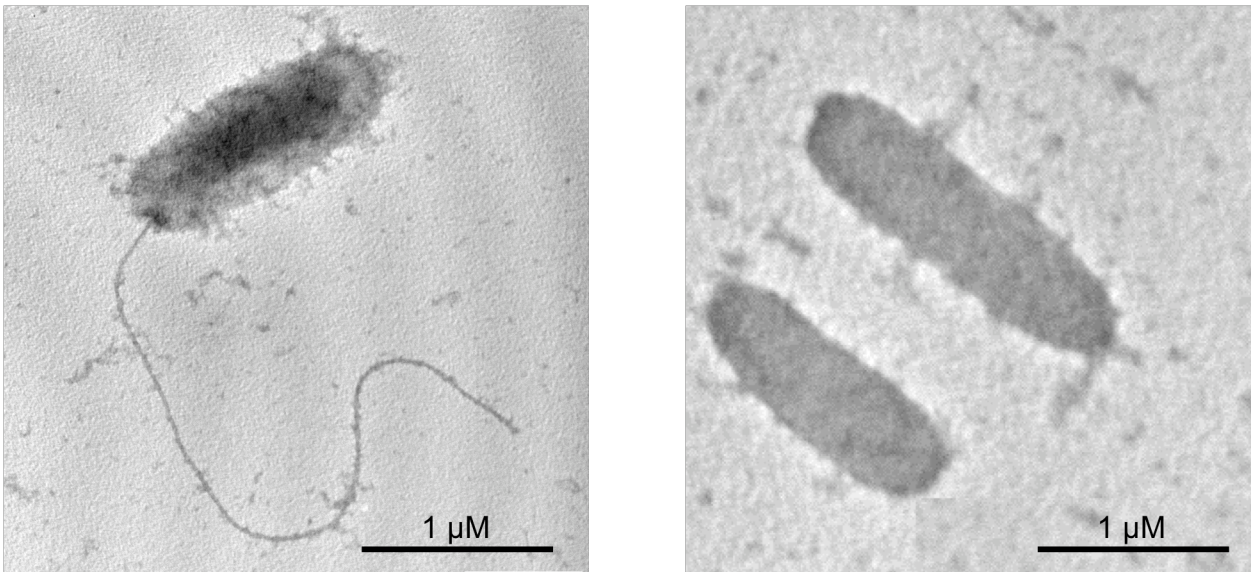
Tramsmission electron microscopy results to determine the percentage of flagellated bacteria are seen in Panel A. After 2 hours in the presence of host cells, 32% of *C. jejuni* possessed full-length flagella. At 12 hours after inoculation, however, the percentage of flagellated bacteria had decreased to approximately 6%, with the remaining 94% completely lacking any visible flagellar structure. In contrast, *C. jejuni* F38011 cultured in MH broth alone did not show the same decrease in percentage of flagellated bacteria. At 2 hr, 52% of organisms had a full-length flagellum, and at 12 hr, 46% remained flagellated. Panel A shows representative micrographs of bacteria with full-length flagella and completely lacking flagella. In accordance with these results, motility of the bacterial population decreased over time during co-culture with viable INT 407 epithelial cells (Panel C).

Figure 10

A.

	MH Broth	INT 407
2 hr	27/50 = 52%	16/50 = 32%
12 hr	23/50 = 46%	3/50 = 6%

B.



C.

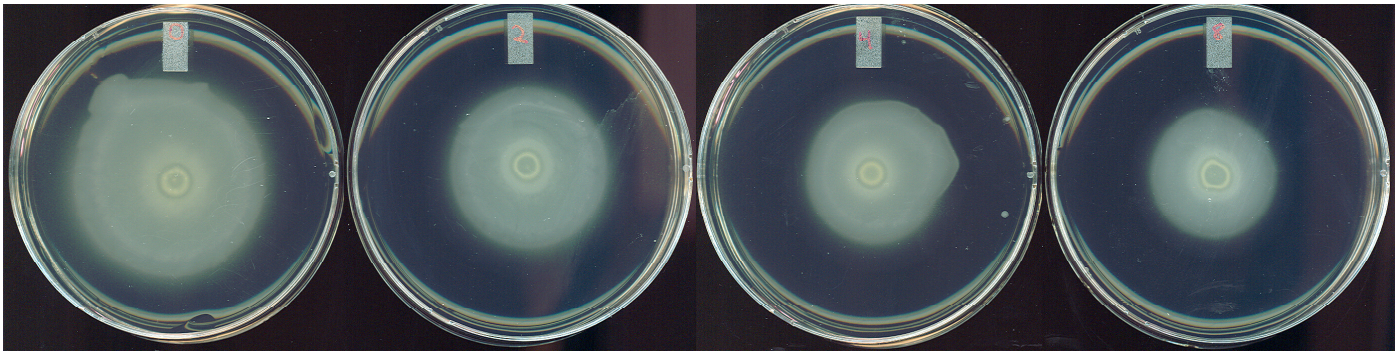
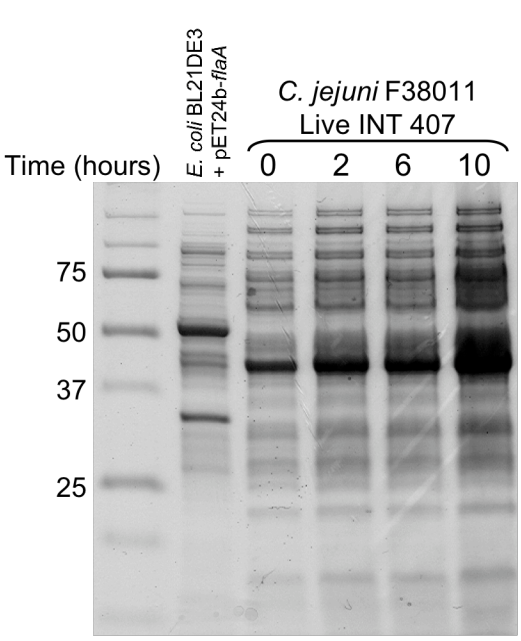


Figure 11. *C. jejuni* flagellin levels decrease over time throughout co-culture with viable INT 407 cells.

Whole-cell lysates of *C. jejuni* co-cultured for 0, 2, 6, or 10 hours with viable INT 407 cells were prepared and separated in duplicate with SDS-PAGE. One gel, shown in Panel A, was Coomassie stained to determine relative protein concentrations of the lysates. The second was transferred to a PVDF membrane and immunoblotted with an α-*C. jejuni* flagellin antibody, and revealed that at 0, 2, and 6 hours, flagellin is detectable, while after 10 hours of co-culture with INT 407 cells, no corresponding band was visible (Panel B).

Figure 11

A.


Time (hours)
E. coli BL21DE3 + pET24b-flaA
C. jejuni F38011 Live INT 407
0
2
6
10
75
50
37
25

B.

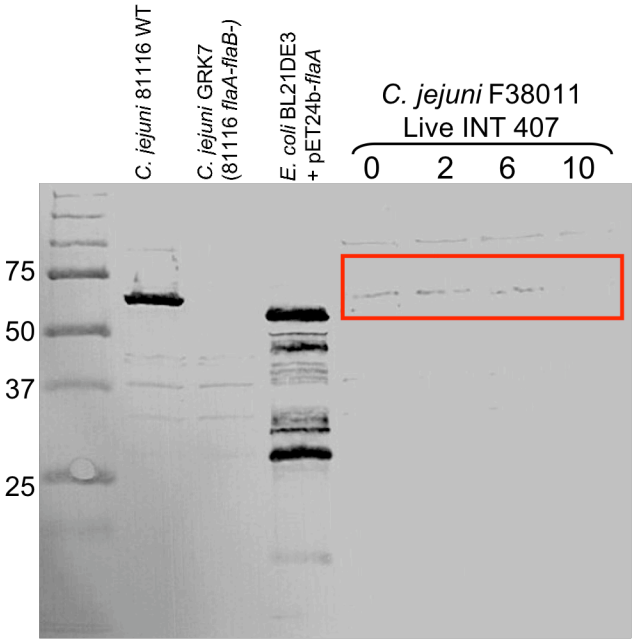
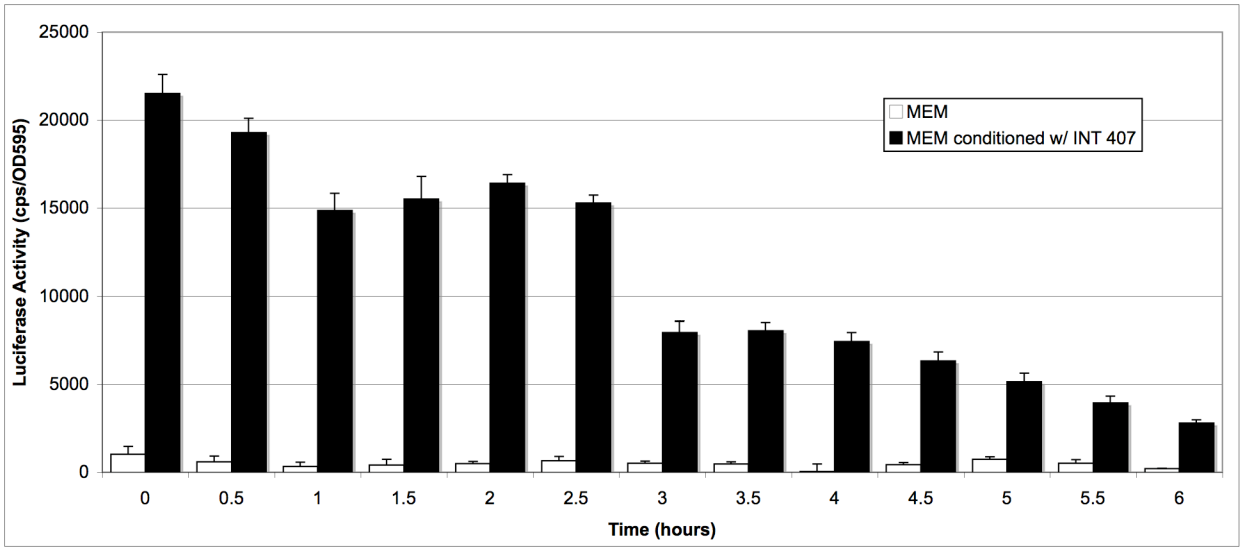

C. jejuni 81116 WT
C. jejuni GRK7 (81116 flaA-flaB-)
E. coli BL21DE3 + pET24b-flaA
C. jejuni F38011 Live INT 407
0
2
6
10
75
50
37
25

Figure 12. Media conditioned with viable INT 407 epithelial cells maintains activity of the *flaA* and *flaB* promoters.

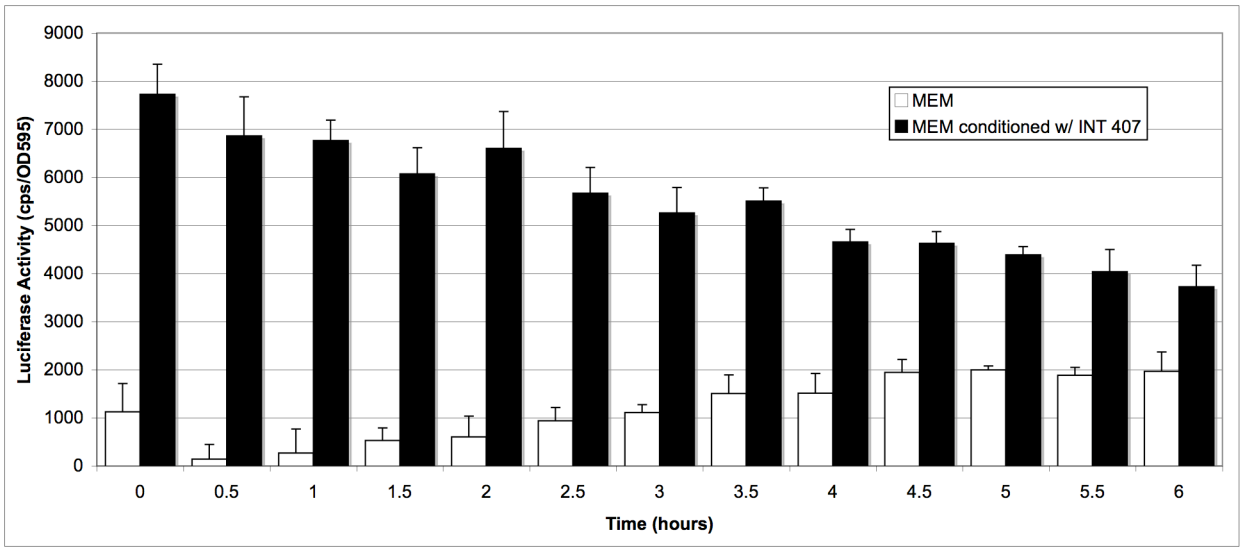
Results of luciferase assays of *flaA* and *flaB* promoter activity performed with media pre-conditioned overnight with and without viable INT 407 cells appear in Panels A and B. The activity of *flaA* was barely above background in MEM alone, however, in the same media conditioned with INT 407 cells, promoter activity gradually declined as seen in the presence of live cells (Panel A). Likewise, *flaB* activity was maintained fairly steadily over time by MEM conditioned with INT 407 cells, but was negligible in media alone (Panel B).

Figure 12

A.


25000
20000
15000
10000
5000
0
Luciferase Activity (cps/OD595)
MEM
MEM conditioned w/ INT 407
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Time (hours)

B.


9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Luciferase Activity (cps/OD595)
MEM
MEM conditioned w/ INT 407
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Time (hours)