Mechanisms and roles of the LuxS system, methyl recycling, and DNA methylation on the physiology of Campylobacter jejuni

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Mechanisms and roles of the LuxS system, methyl recycling, and DNA methylation on the physiology of Campylobacter jejuni

by

Kathy T. Mou

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Microbiology

Program of Study Committee:
Paul J. Plummer, Major Professor
   Gwyn Beattie
   Lisa Nolan
   Michael Yaeger
   Qijing Zhang

Iowa State University
Ames, Iowa
2015

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TABLE OF CONTENTS

LIST OF FIGURES iv
LIST OF TABLES vi
NOMENCLATURE vii
ACKNOWLEDGEMENTS ix

ABSTRACT x

CHAPTER 1. LITERATURE REVIEW AND INTRODUCTION 1
General biology of Campylobacter jejuni 1
Broad host range of C. jejuni owing to its genetic diversity 2
Pathogenesis and associated virulence factors of C. jejuni 4
C. jejuni efflux pumps and multi-drug resistance 7
C. jejuni is a major threat to human and animal health 10
Control and prevention of C. jejuni infections 12
Quorum sensing 14
Role of LuxS in the pathogenesis of C. jejuni 16
DNA methylation 20
Restriction-Modification systems 21
Roles of DNA methylation in cell biology and virulence 22
DNA MTases in C. jejuni 23
Using SMRT sequencing to profile methylomes 26
Specific aims and significance of this research 28
Organization of this dissertation 30

CHAPTER 2. THE IMPACT OF THE LUXS MUTATION ON PHENOTYPIC EXPRESSION OF FACTORS CRITICAL FOR CAMPYLOBACTER JEJUNI COLONIZATION 31
Abstract 31
Introduction 33
Materials and Methods 39
Results 45
Discussion 51
Acknowledgements 58
Figures legends 59
Tables 68

CHAPTER 3. A COMPARATIVE ANALYSIS OF METHYLOME PROFILES OF CAMPYLOBACTER JEJUNI SHEEP ABORTION ISOLATE AND GASTROENTERIC STRAINS USING PACBIO DATA 73
Abstract 73
Introduction 74
### Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>78</td>
</tr>
<tr>
<td>Results</td>
<td>80</td>
</tr>
<tr>
<td>Discussion</td>
<td>98</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>105</td>
</tr>
<tr>
<td>Author Contributions</td>
<td>106</td>
</tr>
<tr>
<td>Figures legends</td>
<td>107</td>
</tr>
<tr>
<td>Tables</td>
<td>114</td>
</tr>
<tr>
<td><strong>CHAPTER 4. CHARACTERIZATION OF THE CJSA_RS00180 DNA METHYLTRANSFERASE IN CAMPYLOBACTER JEJUNI IA3902</strong></td>
<td>118</td>
</tr>
<tr>
<td>Abstract</td>
<td>118</td>
</tr>
<tr>
<td>Introduction</td>
<td>119</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>123</td>
</tr>
<tr>
<td>Results</td>
<td>128</td>
</tr>
<tr>
<td>Discussion</td>
<td>131</td>
</tr>
<tr>
<td>Figures legends</td>
<td>138</td>
</tr>
<tr>
<td>Tables</td>
<td>144</td>
</tr>
<tr>
<td><strong>CHAPTER 5. General Conclusions</strong></td>
<td>149</td>
</tr>
<tr>
<td>General Discussion</td>
<td>149</td>
</tr>
<tr>
<td>Recommendations for Future Research</td>
<td>154</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>156</td>
</tr>
<tr>
<td><strong>APPENDIX</strong></td>
<td>176</td>
</tr>
<tr>
<td>Chapter 3 Supplementary Data and Tables</td>
<td>176</td>
</tr>
</tbody>
</table>
CHAPTER 2

Figure 1. Expression of *cmeB* (A) and *cmeR* (B) in W7ΔluxS and W7ΔluxSc compared to W7 wildtype over a 48 hour growth period. 

Figure 2. Transmission electron micrographs reveal no morphological changes in cell shape or flagella in wildtype, *luxS* mutant (ΔluxS), and *luxS* complement (ΔluxSc) of both strain backgrounds A) W7 and B) IA3902.

Figure 3. Penetration of W7, W7ΔluxS, and W7ΔluxSc through mucus while incubating at 37°C (A, B) or 42°C (C, D).

Figure 4. Penetration of IA3902, IA3902ΔluxS, and IA3902ΔluxSc through mucus while incubating at 37°C (A, B) or 42°C (C, D).

CHAPTER 3

Figure 1. Distinct areas of hyper- and hypomethylation in the whole-genome methylome plot of IA3902.

Figure 2. Circos plot displaying the distributions of each motif across the genome of IA3902.

Figure 3. Distinct areas of hyper- and hypomethylation in the whole-genome methylome plot of 11168.

Figure 4. Distinct areas of hyper- and hypomethylation in the whole-genome methylome plot of 81-176.

Figure 5. Detection of N6-methyladenine (m6A) base modification in both IA3902 strains.

CHAPTER 4

Figure 1. Diagram of insert in pRS00180 plasmid construct.

Figure 2. Comparison of the 1130bp region of CISA_RS00180 gene with methyltransferase genes of other *Campylobacter* species.
Figure 3. Growth of IA3902ΔRS00180 broth culture at 42°C over a 48 hour period was like IA3902 wildtype.

Figure 4. Motility assay of IA3902 and IA3902ΔRS00180 incubated at 42°C.
LIST OF TABLES

CHAPTER 2

Table 1. List of all bacterial strains used in this study. 68

Table 2. Primers used in this study. 69

Table 3. Normalization scores of 17 morphological characteristics of W7 and IA3902 strains grown at 37°C (A) and 42°C (B). 70

Table 4. LC-ESI-MS/MS analysis shows expected AMC metabolite levels within luxS mutant compared to wildtype and luxS complement. 72

CHAPTER 3

Table 1. Methylome motifs detected within the C. jejuni IA3902 genome. 114

Table 2. Putative Restriction-Modification (RM) systems detected in IA3902. 115

Table 3. Methylome motifs detected within the C. jejuni IA3902ΔluxS genome. 117

CHAPTER 4

Table 1. Bacterial strains (A) and plasmids (B) used in this study. 144

Table 2. Primers used in this study. 145

Table 3. Comparison of methylome motifs detected within C. jejuni IA3902 and IA3902ΔRS00180 genome. 147

Table 4. Penetration of IA3902 and IA3902ΔCJSA0032 through mucus show no significant differences at each fraction. 148
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AHL</td>
<td>acylated-homoserine lactone</td>
</tr>
<tr>
<td>AI</td>
<td>autoinducer</td>
</tr>
<tr>
<td>AMC</td>
<td>activated methyl cycle</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>DPD</td>
<td>4,5-dihydroxy-2,3-pentanedione</td>
</tr>
<tr>
<td>FQ</td>
<td>fluoroquinolone</td>
</tr>
<tr>
<td>GBS</td>
<td>Guillain-Barré Syndrome</td>
</tr>
<tr>
<td>HGT</td>
<td>horizontal gene transfer</td>
</tr>
<tr>
<td>HSL</td>
<td>homoserine lactone</td>
</tr>
<tr>
<td>IPD</td>
<td>interpulse duration</td>
</tr>
<tr>
<td>m4C</td>
<td>N4-methylcytosine</td>
</tr>
<tr>
<td>m5C</td>
<td>C5-methylcytosine</td>
</tr>
<tr>
<td>m6A</td>
<td>N6-methyladenine</td>
</tr>
<tr>
<td>MATE</td>
<td>multidrug and toxic compound extrusion</td>
</tr>
<tr>
<td>Mb</td>
<td>Megabases</td>
</tr>
<tr>
<td>MDR</td>
<td>multi-drug resistant</td>
</tr>
<tr>
<td>MFS</td>
<td>major facilitator superfamily, Miller Fisher Syndrome</td>
</tr>
<tr>
<td>MTase(s)</td>
<td>methyltransferase(s)</td>
</tr>
<tr>
<td>NGS</td>
<td>next-generation sequencing</td>
</tr>
<tr>
<td>PACE</td>
<td>proteobacterial antimicrobial compound efflux</td>
</tr>
<tr>
<td>PW</td>
<td>pulse width</td>
</tr>
<tr>
<td>qPCR</td>
<td>real-time quantitative PCR</td>
</tr>
</tbody>
</table>
REase(s)  restriction enzyme(s) or restriction endonuclease(s)
REBASE  Restriction Enzyme dataBASE
R-M     restriction-modification
RND     resistance-nodulation-cell division
SA      sheep abortion
SAH     $S$-adenosylhomocysteine
SAM     $S$-adenosylmethionine
SRH     $S$-ribosylhomocysteine
SMR     small multidrug resistance
SMRT    single-molecule, real-time
ACKNOWLEDGEMENTS

This thesis was made possible by the collective efforts and support of colleagues, friends, and family. First, I would like to express my sincerest gratitude to my major professor, Dr. Paul Plummer, as he has been an invaluable mentor at every step of my graduate studies. I am thankful for his kindness, patience, expertise, and the time he has spent answering all of my questions, challenging me and instilling confidence in myself as a scientist, improving my writing, and helping me gain access to innumerable opportunities, all of which have helped me develop into a successful independent researcher. I am very grateful for the opportunity to work in his laboratory. I would also like to thank my program of study committee: Drs. Qijing Zhang, Michael Yaeger, Lisa Nolan, and Gwyn Beattie for their guidance, suggestions, and encouragement.

I thank all the members of the Plummer lab and Zhang lab, whom I have had the privilege to work with, and have invested countless number of hours to help assist me with experiments and training, sharing their wisdom and expertise in all things *Campylobacter*, and have always been available for me to exchange thoughts and ideas with. My research successes would not have been possible without your support and guidance. I also thank the countless number of former and current employees at the Iowa State University College of Veterinary Medicine and at the Interdepartmental Microbiology program for their support throughout my graduate career.

Lastly, I would like to sincerely thank my parents Dach and Sherry, my sister Connie, Alan, Marty, Brent, Anna, and all my friends for their unwavering love and invaluable support network. For without their continuous love and support, I would not be where I am today.
ABSTRACT

Campylobacter jejuni is one of the leading causes of human bacterial gastroenteritis, and campylobacteriosis in sheep. The genetic diversity of this organism, the potential for multiple sources to transmit C. jejuni to humans, and the possession of a variety of virulence factors and antimicrobial resistance mechanisms make C. jejuni a serious health problem worldwide. The autoinducer-2 (AI-2)/LuxS system has been the focus of several studies for its potential applications to attenuate C. jejuni virulence. A study from our group found that the LuxS enzyme plays a critical role in virulence and fitness of C. jejuni IA3902 and 11168 strains. Mutagenesis of the luxS gene negatively impacted C. jejuni colonization of the gastrointestinal tract of several host species. However, the physiologic basis for this colonization defect is unclear. In addition to AI-2 production, LuxS is also a key enzyme involved in the activated methyl cycle (AMC).

The AMC is an important source for the formation of S-adenosylmethionine, a methyl donor crucial to biological processes like DNA methylation. DNA methylation has also been linked with a diverse number of important physiological and pathogenic mechanisms in many bacteria, but is poorly understood in C. jejuni. The collective work from this thesis attempts to address some of the knowledge gaps on the role of LuxS, methyl recycling, and DNA methylation in C. jejuni physiology. Collectively, results from our study showed that luxS mutation interrupted the AMC resulting in significant changes to intracellular concentrations of several key metabolites. However, the colonization-associated factors tested on our luxS mutants in this thesis do not show evidence of being the primary mechanisms responsible for the luxS mutant’s decreased colonization ability. We proceeded to analyze the role of LuxS on DNA methylation and found that the luxS mutation had no appreciable effect on the methylome profile of the
mutant. We also compared the methylome profiles of three important C. jejuni strains and found significant strain variability in the methylomes, which suggest a potential role for DNA methylation in Campylobacter pathobiology. The methylome studies also revealed a novel putative methyltransferase which we later confirmed and definitively assigned to a specific methylation motif. While mutagenesis of the methyltransferase gene resulted in a loss of methylation of its cognate motif we were unable to show an effect on the growth or motility phenotypes tested in our study. In summary, the luxS mutation demonstrated physiological effects on the AMC, but the colonization mechanisms affected by the mutation are still unknown. However, DNA methylation studies revealed strain-specific methylation profiles, including a unique methyltransferase, which may serve a biological and/or pathogenic purpose specific to the strain.
CHAPTER 1. LITERATURE REVIEW AND INTRODUCTION

General biology of *Campylobacter jejuni*

*Campylobacter jejuni* is a gram-negative bacterium that is shaped like a spiral rod and possesses one or two single unsheathed polar flagella at one or both ends of the cell (Wagenaar and Jacobs-Reitsma, 2008). Its helical cell shape is what gives it its distinct corkscrew-like motility. During unfavorable growth conditions, it forms a viable non-culturable form in the shape of spheroid or coccoid bodies (Humphrey et al., 2007). It is non-spore forming and grows in microaerobic conditions (3-10% oxygen, 5-10% carbon dioxide) at temperatures ranging from 30°C up to the optimal growth temperature of 42°C, making it a thermotolerant organism (Kelly, 2008; Wagenaar and Jacobs-Reitsma, 2008; Wieczorek and Osek, 2013). It has also been found to survive better at 4°C than at room temperature, which makes contaminated refrigerated foods and liquids a potential source for *C. jejuni* infections (Hofreuter, 2014).

*C. jejuni* primarily obtains its energy and carbon sources from amino acids or intermediates of the tricarboxylic acid cycle (Debruyne et al., 2008). It was originally thought to be restricted in carbohydrate catabolism due to a lack of the 6-phosphofructokinase gene and its inability to metabolize exogenous glucose and most hexose sugars (Kelly, 2008; Hofreuter, 2014). However, recent studies have found that *C. jejuni* is not entirely asaccharolytic, as it is able to utilize L-fucose for growth. This ability provides a competitive advantage for the organism in host species that have more fucose available than other preferred substrates (Muraoka and Zhang, 2011; Stahl et al.,
2011). In addition, some *C. jejuni* isolates harbor enzymes capable of glucose catabolism and can use glucose for *de novo* synthesis of amino acids and cell surface carbohydrates (Szymanski, 2015).

Phylogenically *C. jejuni* is classified under the phylum *Proteobacteria*, class *Epsilonproteobacteria*, order *Campylobacterales*, family *Campylobacteraceae*, and genus *Campylobacter* (Kaakoush et al., 2015). The genus *Campylobacter* was established in 1963 and is grouped with three other genera under the family *Campylobacteraceae*. The three genera include *Arcobacter*, *Sulfurospirillum*, and *Bacteroides ureolyticus*, which are also gram-negative, asaccharolytic organisms that require microaerophilic growth and contain a low G+C content (Debruyne et al., 2008).

The genome of *C. jejuni* varies between 1.6-1.8 Mb long depending on the strain (Champion et al., 2008). In this dissertation, we will focus on two commonly studied laboratory strains (11168 (Parkhill et al., 2000) and 81-176 (Korlath et al., 1985; JCVI, 2007)) which originated from human diarrheal cases, and a clinical isolate (IA3902) of the clone SA (*Sheep Abortion*) (Sahin et al., 2008; Sahin and Zhang, 2010). The genomes of all three strains are approximately 1.6 Mb. In addition to its circular chromosome, 81-176 also possesses two plasmids, named *pTet* and *pVir*, which are 0.045 Mb and 0.037 Mb, respectively. IA3902 also has a *pVir* plasmid (0.037 Mb) while the 11168 strains that have been sequenced to this date do not possess any plasmids (NCBI, 2015).

**Broad host range of *C. jejuni* owing to its genetic diversity**

*C. jejuni* is found in a diverse range of hosts and environments. It is a commensal organism of the intestinal tract of poultry, food-producing animals, wild animals (with
birds being the most common wildlife host), companion animals, and insects (Humphrey et al., 2007; Wagenaar and Jacobs-Reitsma, 2008). Poultry are the most important vehicle for C. jejuni transmission to humans (Sahin et al., 2015). However, all animals that are natural hosts of C. jejuni in addition to contaminated food (poultry products, meats), unpasteurized milk, and water sources can serve as a source for C. jejuni transmission to humans (Acheson and Allos, 2001).

The fitness advantage for C. jejuni to survive in all these hosts and environments is due, in large part, to the significant amount of genetic variation found between strains and within strains of this organism (Gilbreath et al., 2011). Evidence of the non-clonal nature of this organism is provided by the fact that 20% of the genome varies between C. jejuni strains, and there are over 7,906 sequence types represented in the 34,408 isolates for C. jejuni/coli present in pubMLST database (van Putten et al., 2009; Jolley and Maiden, 2010). The genome of 11168 lacks many DNA repair genes that are found in other bacterial species like E. coli, including glycosylases and genes involved in direct DNA repair, mismatch repair, and the SOS response (Parkhill et al., 2000). The genome of 11168 also contains hypervariable sequences with homopolymeric tracts which are found in clusters of genes displaying high antigenic diversity such as flagellar modification, lipooligosaccharide and capsule biosynthesis (Parkhill et al., 2000). The hypervariable sequences are also found in genes responsible for DNA restriction and modification, which play a major role in horizontal gene transfer (HGT) and recombination events (van Putten et al., 2009; Vasu and Nagaraja, 2013). With the lack of repair genes, homopolymeric tracts can undergo phase variation via slipped-strand mispairing, and spontaneous mutations can develop in other portions of the genome—all
of which benefit and contribute to the survival of the organism in diverse niches and environments. Additional attributes that contribute to the genetic diversity of *C. jejuni* include its natural competency in the uptake of exogenous chromosomal or plasmid DNA from the environment, and the large repertoire of virulence and colonization factors it possesses to facilitate host-pathogen interactions (Wang and Taylor, 1990; Young et al., 2007; Gilbreath et al., 2011).

**Pathogenesis and associated virulence factors of *C. jejuni***

Current literature shows that *C. jejuni* possess a large variety of virulence factors that enable its survival within the host intestinal environment, though the mechanisms of many are poorly understood (Dasti et al., 2010). After ingestion by the host, the organism must survive passage through the stomach acid followed by the highly alkaline secretion from the bile duct as it moves into the small intestine. It initially colonizes the small intestine during early infection and subsequently moves to the target portion of the intestine, which is the colon (Guerry, 2007; Hu and Kopecko, 2008). Because the mucin of the mucus layer of the intestine is a strong chemoattractant, *C. jejuni* will translocate through the gut mucosal layer (van Putten et al., 2009). From there it will encounter the intestinal epithelial cells and through mechanisms that are under investigation, will adhere and invade the epithelial cells (Young et al., 2007). The interaction of *C. jejuni* translocation through the intestinal mucosa disrupts the normal secretory and absorptive capacity of the intestine, thus causing gastrointestinal disease (Hu and Kopecko, 2008).
One of the most crucial features for pathogenesis of *C. jejuni* is its flagella. They are vital for motility to resist the peristaltic forces and flushing from the small intestine, drive through viscous mucosal layers, chemotaxis, and adherence and invasion of the intestinal epithelial cells (Larson et al., 2008). Flagella also serve as export apparatuses secreting virulence proteins that have broad regulatory functions, are involved in small molecule and macromolecule metabolism, cell processes, or other miscellaneous functions (Larson et al., 2008). Flagella are heavily glycosylated to protect them from proteolytic cleavage (Larson et al., 2008). Furthermore, the glycan composition of the flagella can mediate autoagglutination, which is a preliminary step to the formation of microcolonies and biofilms (Guerry, 2007). Both glycosylation and flagella are controlled by phase variation, which provide antigenic variations that help *C. jejuni* evade detection by the immune system (Guerry, 2007; Lertsethtakarn et al., 2011).

In addition to flagella, the helical shape of *C. jejuni* is also important for pathogenesis. Combined with polar flagella, both work together to provide the corkscrew-like rotation that enable the bacteria to push through moderate to highly viscous substances such as the mucosal layer (Lertsethtakarn et al., 2011). The lipooligosaccharides and capsule on *C. jejuni* are both highly variable, which provide extensive variation in the structure and also help to evade the immune system (Young et al., 2007). Both features also provide adherence and invasion capabilities, along with numerous other proteins such as CadF (adhesion and invasion), CapA (adhesion), glycoproteins (adhesion and invasion), PEB1 (periplasmic binding protein), JlpA (surface-exposed lipoprotein crucial for cell binding), and *Campylobacter* invasive
antigens (secreted out of a flagellar export apparatus to assist in invasion of the host cell) (Young et al., 2007; Dasti et al., 2010).

The pathogenesis of the *C. jejuni* clone SA clinical isolate results in abortions, which is in contrast to other commonly studied *C. jejuni* laboratory strains like 11168 and 81-176 that are primarily associated with gastrointestinal disease (Wu et al., 2013). Although the route of exposure and pathogenesis involving intestinal colonization and invasion of the intestinal mucosa are presumed to be the same, IA3902 proceeds to cause bacteremia upon escaping the intestines and entering the bloodstream (Sahin et al., 2008). In a pregnant ewe, IA3902 has a specific tropism towards fetoplacental tissue and will colonize the placenta, infecting the fetus, and subsequently inducing an abortion (Burrough et al., 2009).

Comparative genomics showed that the genome of IA3902 is highly syntenic with that of 11168, with no known pathogenicity islands or virulence genes known to be associated with the induction of abortion. IA3902 contains a highly homologous *pVir* plasmid to 81-176 and a chromosomal insertion of the *tetO* tetracycline resistance gene, although *pVir* was not required for inducing abortions. The differences found between the genomes of 11168 and IA3902 were the presence of large numbers of single nucleotide polymorphisms and insertions/deletions throughout the chromosome. In addition, divergent transcriptomic and proteomic profiles between IA3902 and 11168 in genes involving energy generation, respiration, motility, nutrient utilization were identified (Wu et al., 2013). The divergent profiles suggest that small genomic changes can have significant influence on the gene/protein expression patterns and consequently the virulence phenotype of each strain.
**C. jejuni efflux pumps and multi-drug resistance**

Another major contributing factor to *C. jejuni* survival in the intestinal environment is the expression of one or more multi-drug efflux pumps. Efflux pumps transport a wide variety of molecules out of the bacterial cell, including bile salts, toxic substances found in the intestinal tract, organic solvents, and various antimicrobial drugs. This contributes to the innate antimicrobial resistance of the bacteria and enables it to adapt to and survive under high external concentrations of these substrates (Blair et al., 2014). Of growing importance is the identification and characterization of multi-drug resistant (MDR) pumps in numerous bacterial species of public health concern, such as the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (Boucher et al., 2009; Li et al., 2015). Efflux pumps are divided into two types: primary transporters and secondary transporters. Primary transporters, which consist of the ATP-binding cassette (ABC) superfamily, use ATP energy to efflux molecules out of the cell (Delmar et al., 2014). Secondary transporters use an electrochemical gradient to power efflux activity, and include the major facilitator superfamily (MFS), small multidrug resistance (SMR) superfamily, resistance-nodulation-cell division (RND) superfamily, and the multidrug and toxic compound extrusion (MATE) superfamily (Delmar et al., 2014). A sixth family of efflux pumps, the proteobacterial antimicrobial compound efflux (PACE) family, was recently found as the newest family of multidrug efflux pumps (Hassan et al., 2015). *C. jejuni* harbors 14 putative drug efflux pumps, with at least one from the ABC, RND, MATE, MFS, and SMR families (Zhang and Plummer, 2008). As of this date, the only well-characterized efflux systems of *C. jejuni* are
CmeABC and CmeDEF. CmeGH and ArsP have also been characterized to a lesser extent.

CmeABC is in the RND family and is organized into a 3-gene operon that codes for a tripartite efflux pump with three components: CmeA (periplasmic fusion protein), CmeB (inner membrane efflux transporter), and CmeC (outer membrane channel protein) (Lin et al., 2002). This efflux pump confers resistance to a diverse array of compounds (bile salts, ciprofloxacin, erythromycin, ethidium bromide and various detergents), making it an MDR pump. CmeABC works synergistically with other antimicrobial resistance mechanisms, such as mutations in target genes, to generate high-level resistance to important classes of drugs used to treat human and animal infections, including macrolides, fluoroquinolones (FQs), and tetracyclines (Luo et al., 2003; Ge et al., 2005; Cagliero et al., 2006). CmeR is the transcriptional repressor for CmeABC and binds to an inverted repeat within the promoter region of CmeABC to inhibit its expression (Guo et al., 2008). Bile salts induce the expression of CmeABC by binding to CmeR protein to prevent it from binding to CmeABC promoter, thus allowing CmeABC expression to be induced (Lin et al., 2005a). As such, bile resistance was found to be the natural function of CmeABC and that presence of bile salts may also decrease susceptibility of C. jejuni to antibiotics (Lin et al., 2005a). It was recently found that C. jejuni isolates from different host species possessed various mechanisms that contributed to differential over-expression of CmeABC, which, under selective pressure, would promote the emergence of antibiotic-resistant mutants (Grinnage-Pulley and Zhang, 2015).
CmeDEF is also an RND pump like CmeABC, with a 3-gene operon coding for an outer membrane channel protein (CmeD), periplasmic fusion protein (CmeE), and an inner membrane transporter (CmeF) (Akiba et al., 2006). The efflux mechanism of CmeDEF is secondary to CmeABC and its contribution to resistance is normally masked by the function of CmeABC. However, both are important in maintaining Campylobacter cell viability as a cmeF/cmeB double mutation was lethal to 11168 (Akiba et al., 2006). Another efflux system recently characterized in C. jejuni is the MFS transporter CmeGH, which consists of a multidrug efflux transporter (CmeG) and a periplasmic protein (CmeH) (Jeon et al., 2011). CmeG is an MDR pump, although it preferentially extrudes FQs and may have a role in oxidative stress defense. CmeH was not found in some C. jejuni strains, suggesting it is not essential for the antimicrobial resistance phenotype. The most recently identified efflux transporter in C. jejuni is ArsP, which is suggested to be an ABC transporter that mediates resistance to organic arsenic (Shen et al., 2014).

The active extrusion of drugs out of the bacterial cells through multiple efflux pumps, combined with synthesis of modification enzymes, alterations or protection of antibiotic targets, and reduced permeability to drugs are all mechanisms for antimicrobial resistance in C. jejuni (Zhang and Plummer, 2008). Research has shown that these resistance mechanisms develop and transmit easily among Campylobacter as they are naturally competent and lack many DNA repair genes. Together, Campylobacter can easily become drug resistant by acquiring drug resistant elements via HGT and developing spontaneous mutations that produce drug-resistance (Wieczorek and Osek, 2013).
C. jejuni is a major threat to human and animal health

As one of the leading causes of human bacterial gastroenteritis, C. jejuni is a serious public health problem worldwide. In the U.S. alone, Campylobacter species (with C. jejuni as the primary causative agent) are responsible for over 1.3 million infections, 13,000 hospitalizations, and 120 deaths each year (CDC, 2013a). In addition, Campylobacter infections cost the US an estimated 1.9 billion dollars annually (ERS, 2014). With an infectious dose as low as 360 bacteria and incubation period ranging from 18 hours to 8 days, C. jejuni infection can last up to 2 weeks (Young et al., 2007; Hara-Kudo and Takatori, 2011). Its classical symptoms are manifested as abdominal pain/cramping, diarrhea, fever, headache, dizziness and myalgia (Blaser and Engberg, 2008). Although not as common, intestinal complications (such as intestinal hemorrhage, appendicitis, colitis, toxic megacolon) and extraintestinal infections (including bacteremia, hepatitis, abortion, and renal and urinary tract disease) can also occur. A subset of individuals develop serious long term infections like postinfectious irritable bowel syndrome, and neurological and rheumatological sequelae such as Guillain-Barré Syndrome (GBS), Miller Fisher Syndrome (MFS), and reactive arthritis (Blaser and Engberg, 2008). GBS is characterized as the most common form of acute neuromuscular paralysis worldwide (where poliomyelitis has been eradicated) while MFS is a non-paralytic subform of GBS (Humphrey et al., 2007; Blaser and Engberg, 2008). The onset of GBS and MFS is attributed to the molecular mimicry between the lipooligosaccharide of C. jejuni and the surrounding nervous tissue, which induces cross-reactive antibodies and leads to autoimmune-mediated disease (Kaakoush et al., 2015).
Because of its short duration and clinically mild symptoms that are typically self-limiting, there is no specific treatment for campylobacteriosis in humans other than oral rehydration and electrolyte balance (Maćkiw et al., 2012). However, for systemic, severe or complicated *Campylobacter* infections, antibiotic therapy is needed. Macrolides have commonly been used to treat *Campylobacter* enteritis in addition to fluoroquinolones, although the rate of resistance to fluoroquinolones have essentially made this class of drugs ineffective in some geographic areas (Blaser and Engberg, 2008).

In animal health, a 2010 survey from the USDA showed that campylobacteriosis is one of the most commonly reported microbial causes of sheep abortions in the US (USDA, 2013). In addition, *C. jejuni* is now the most common cause of campylobacteriosis in sheep among all *Campylobacter* species (USDA, 2014). Historically, multiple species and strains of *Campylobacter* were responsible for *Campylobacter*-associated sheep abortions including (starting with the most frequent causative agent) *C. fetus* subsp. *fetus*, *C. jejuni*, and *C. coli* (Delong et al., 1996; Burrough, 2011). However, starting from the late 1980s, a major shift in the causative agent and epidemiology of *Campylobacter*-associated sheep abortions was observed in the U.S. A unique clone of *C. jejuni*, named clone SA, has replaced *C. fetus* as the predominant cause of *Campylobacter*-associated sheep abortions in the country (Sahin et al., 2008). In addition, this clone is resistant to tetracyclines, which is the only class of antibiotics approved to treat *Campylobacter*-associated abortions. Unlike the U.S., *Campylobacter* isolates from sheep abortions in other countries showed high genetic diversity and no presence of predominating clones among the isolates (Sahin et al., 2012; Wu et al., 2014). What is also troubling about clone SA is that it has been found in
multiple outbreak cases of human gastroenteritis, primarily due to raw-milk consumption (Sahin et al., 2012). In addition, clone SA was found as a commensal organism in healthy sheep and cows, as well as a pathogen in cow and goat abortion cases. The zoonotic potential of clone SA and finding ruminants as a significant reservoir for *Campylobacter* transmission from animals to humans has added to increasing efforts to control zoonotic transmission of *Campylobacter* originating from ruminant sources.

**Control and prevention of *C. jejuni* infections**

Numerous approaches to reduce the shedding of *C. jejuni* in animals at the production level (particularly poultry), at the processing level, and implementation of stricter biosecurity protocols and good hygiene measures have been utilized to control the spread of *C. jejuni* (Kaakoush et al., 2015; Sahin et al., 2015). Unfortunately, these methods could not control the spread of antimicrobial resistance in *C. jejuni*, which continues to grow as an emerging threat to public health worldwide (Chen et al., 2010; Maćkiw et al., 2012; CDC, 2014; 2015; Pham et al., 2015). In the U.S., the CDC has reported drug-resistant *Campylobacter* as a serious threat to public health, with 310,000 cases attributed to drug-resistant *Campylobacter* infections (CDC, 2013a). *C. jejuni* possess widespread intrinsic and acquired resistance to several important drug classes through a diverse set of resistance mechanisms, especially to FQ and macrolides (FDA, 2015), beta-lactams, tetracyclines, and aminoglycosides (Zhang and Plummer, 2008).

Antibiotic resistance is known to come at a fitness cost to the bacteria possessing these resistant traits. However, more studies are showing that antibiotic resistance is
providing a positive in vivo advantage as shown in several important pathogens: *P. aeruginosa*, *A. baumannii*, *Salmonella enterica* serovar Typhi, *E. coli*, and *V. cholerae* (Baker et al., 2013; de Lastours et al., 2014; Roux et al., 2015). FQ resistance was also found to enhance the fitness and survival of *C. jejuni* based on an analysis of isolates derived from chickens (Luo et al., 2005). However, the mechanism of how FQ resistance enhances *C. jejuni* fitness is at present unknown. In addition, the specific mutation in the DNA gyrase gene *gyrA* was found in a majority of FQ-resistant *Campylobacter* isolates in animal reservoirs and human patients and enables *C. jejuni* to persist in chickens in the absence of antibiotic selection pressure. The latter point goes along with studies that found FQ-resistant *Campylobacter* in poultry farms that have discontinued the use of this drug for up to four years (Luangtongkum et al., 2009).

While promising approaches have been developed to vaccinate poultry against *Campylobacter* colonization, attempts to create vaccines against human campylobacteriosis have been difficult, as the cross-protection conferred by the vaccine strain(s) is insufficient to protect against field *Campylobacter* isolates that come with enormous genetic and antigenic diversity (Sahin et al., 2008; Ghunaim and Desin, 2015). In addition, safety concerns, failure to confer protection, and the need for deeper understanding of *Campylobacter* pathogenesis and *Campylobacter*-immune system interaction have all made vaccine development particularly difficult (Kirkpatrick and Tribble, 2011; Maue et al., 2014). Thus attempts to combat the emergence of antibiotic resistance have pushed research efforts towards understanding the pathobiology of *C. jejuni*, how resistance mechanisms arise, and to identify alternative drug candidates and novel treatment schemes.
Quorum sensing

Quorum sensing is a key system that regulates the expression of virulence factors and holds promise as a target to attenuate bacterial virulence (Defoirdt et al., 2010). It has shown novel biotechnological applications and with a potential for broad spectrum effect on a multitude of species or diseases (Pereira et al., 2013; Popat et al., 2015). The first described quorum sensing system was in the bioluminescent marine bacterium *Vibrio fischeri*. This system was the first example of how gene expression and cell-population density are coupled and also laid the foundation for all subsequent quorum sensing studies of gram-negative bacteria (Engebrecht et al., 1983; Engebrecht and Silverman, 1984; 1987; Bassler, 1999). Quorum sensing is defined as the “cell-cell communication process used by bacteria to coordinate gene expression in response to changes in population density” (Ng and Bassler, 2009). This system enables bacteria to monitor population density and respond to environmental cues in unison through several generalized steps. Low-weight signal molecules called autoinducers (AIs) are synthesized within the cell and then released outside of the cell through passive or active mechanisms. As the population number increases, the extracellular concentration of AIs also increase. When the AIs accumulate to a minimum detection level, cognate receptors will bind to AIs and trigger a cascade of signal transduction events that eventually lead to a population-wide change in gene expression (Ng and Bassler, 2009).

There are three general groups of quorum sensing systems: acylated-homoserine lactone (AHL)/LuxIR-based systems in gram-negative bacteria, autoinducing peptide/Agr-systems in gram-positive bacteria, and the AI-2/LuxS system found in both gram-positive and gram-negative bacteria (Vendeville et al., 2005; Gospodarek et al.,
A fourth system, the AI-3 system, has also been discovered recently and characterized in *E. coli* and *Salmonella* thus far (Karavolos et al., 2013). The first study to show the production of quorum-sensing molecule in *C. jejuni* found this organism possessed the AI-2/LuxS system (Elvers and Park, 2002). The LuxS enzyme is a Fe$^{2+}$ metalloenzyme that has greater identity with *V. harveyi*. *E. coli* and *Pasteurella multocida* than *H. pylori*, the closest phylogenetic neighbor of *C. jejuni* (Elvers and Park, 2002; De Keersmaecker et al., 2006). A study also found naturally occurring mutations in LuxS of some *C. jejuni* strains that resulted in production of high levels or undetectable levels of AI-2 synthesis (Plummer et al., 2011). Because of this, it is important to fully characterize isolates used for quorum sensing research (Plummer, 2012). In addition, the natural mutation could represent an adaptive strategy to switch between high and low AI-2 production in response to changes in the environment (Pereira et al., 2013).

The primary function of LuxS is its participation in the activated methyl cycle (AMC), which is important for the recycling and maintaining cellular concentrations of $S$-adenosylmethionine (SAM), the primary methyl donor for a variety of metabolic and biosynthetic reactions (Schauder et al., 2001). LuxS is directly involved in the hydrolysis of $S$-ribosylhomocysteine to form two molecules: homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). Homocysteine is converted, through several additional steps, to form SAM. DPD is the precursor molecule for AI-2 (Gospodarek et al., 2009) and spontaneously cyclizes into many different types of AI-2 depending on the level of boron in the environment (Waters and Bassler, 2005; Ng and Bassler, 2009).

Several studies have looked into whether *C. jejuni* contain receptors or transport systems to sense quorum molecules. No AI-2 receptor homologues were identified in the
genome of \textit{C. jejuni} and AI-2 molecules do not import into the cell by an ABC transporter system (Rezzonico and Duffy, 2008; Adler et al., 2015). However, alternative AI-2 receptors and transport systems (such as a two-component signaling system) have been speculated. Another study also found that the virulence expression profile of certain strains of \textit{C. jejuni} is influenced in the presence of homoserine lactone (HSL)-type molecules, making it appear as though \textit{C. jejuni} can detect and respond to HSL-like quorum molecules (Moorhead and Griffiths, 2011).

Varying levels of AI-2 activity were also observed when \textit{C. jejuni} was grown in growth media and food environments ranging from Brucella broth, Mueller Hinton broth, Modified Eagles Medium, milk, and chicken juice (Cloak et al., 2002; Holmes et al., 2009; Gölz et al., 2012a). Temperature also showed an effect on AI-2 levels (Cloak et al., 2002; Gölz et al., 2012a) and luxS expression (Ligowska et al., 2011). The significance of these AI-2 levels and LuxS activity in the varying conditions remains to be determined, but these studies suggest that LuxS is important for the environmental adaptation of \textit{C. jejuni} under certain conditions.

\textbf{Role of LuxS in the pathogenesis of \textit{C. jejuni}}

The AI-2/LuxS system serves as an important regulator for the expression of many significant virulence factors such as biofilm formation, motility, toxin expression, antibiotic resistance, and persistence \textit{in vivo} in important bacterial pathogens like \textit{E. coli}, \textit{V. cholerae}, \textit{S. typhimurium}, \textit{H. pylori}, \textit{H. influenzae}, \textit{Streptococcus} species, etc. (Pereira et al., 2013). In \textit{C. jejuni}, the mutagenesis of LuxS in \textit{C. jejuni} has shown mixed
phenotypic changes in many studies. A key example is growth rate. Most studies for laboratory strains 11168 (Elvers and Park, 2002; Holmes et al., 2009; Plummer et al., 2012) and 81116 (Jeon et al., 2003), and clinical isolates IA3902 (Plummer et al., 2012) and M129 (Reeser et al., 2007) showed no differences in growth rates between wildtype and luxS mutant. However, Adler et al. has suggested that the type of mutation made on the luxS gene can impact the growth rate of the mutant (Adler et al., 2014). For instance, they found that 11168 with a deletion mutation in the luxS gene showed an obvious lag in growth compared to wildtype no matter what temperature or type of media the strains were grown in. In another study, the 11168 luxS mutant showed no growth differences from wildtype (Plummer et al., 2012). However, Adler et al. did not have a 11168 luxS complement. Thus it cannot be said whether the luxS mutation was responsible for the growth discrepancy observed by Adler et al., or if it could be attributed to downstream polar effects of the mutation. For 81-176, three studies showed differences in growth rates between luxS mutant and wildtype but because these studies did not include a luxS complement, it cannot be assumed that the luxS mutation is responsible for the changes in growth rates (He et al., 2008; Quiñones et al., 2009; Adler et al., 2014).

Oxidative stress response, in vitro adherence and/or invasion, and in vivo colonization phenotypes of luxS mutants were also tested and showed different responses depending on the strain background. For oxidative stress response, 11168 and its isogenic luxS strain did not show any difference in susceptibility when exposed to hydrogen peroxide (Elvers and Park, 2002), but 81-176 luxS mutant showed greater sensitivity to hydrogen peroxide than wild type (He et al., 2008). When testing in vitro adherence and invasion ability, Elvers and Park found no changes between luxS mutant and 11168
wildtype using the Caco-2 cell line (Elvers and Park, 2002) while Quiñones found that the 81-176 luxS mutant had significantly reduced adherence ability to the LMH chicken cell line (Quiñones et al., 2009). In an in vivo chicken colonization experiment also in the same study by Quiñones et al., the 81-176 luxS mutant showed reduced ability to colonize the lower gastrointestinal tract of the chicken (Quiñones et al., 2009). Plummer et al. showed evidence strongly suggesting that the IA3902 luxS mutant has a defect in colonization ability as the IA3902 luxS mutant could induce abortions in a pregnant guinea pig abortion model when inoculated into pregnant animals intraperitoneally, but could not induce abortions when inoculated orally (Plummer et al., 2012). Although it is unknown whether the mutant had a defect in colonizing the guinea pigs, when they inoculated the same mutant in a chicken colonization model, the mutant could not colonize in chickens. In addition, strain background had an apparent effect on the colonization ability of the luxS mutant as the 11168 luxS mutant could colonize the gastrointestinal tract of chickens up to wildtype levels when mono-inoculated in chickens. When co-inoculated with wildtype, it was outcompeted in colonizing the chicken gastrointestinal tract.

Unlike the growth rate, oxidative stress, and in vitro and in vivo colonization phenotypes, motility tests (including for swarming behavior) consistently showed reduced motility in the luxS mutant compared to wildtype for 11168 (Elvers and Park, 2002; Holmes et al., 2009), 81116 (Jeon et al., 2003; Plummer et al., 2011), and 81-176 (He et al., 2008; Quiñones et al., 2009). However, for 81-176, He et al. reported swarming behavior was no different between luxS mutant and wildtype at 42°C (He et al., 2008). Adler et al., on the other hand, argued that swarming behavior would depend on
how the luxS mutant was made: whether by insertional mutation or a deletion mutation (Adler et al., 2014). However, because they did not include a luxS complement of the appropriate strain background, it cannot be determined whether the mutation in the luxS gene is the cause for the observed swarming behaviors.

Additional phenotypes were tested on luxS mutants as well as effects of those phenotypes on the expression of luxS gene, but by fewer studies: effect of luxS mutation on C. jejuni response to probiotic cell extracts (Mundi et al., 2013), cytolethal distending toxin expression (Jeon et al., 2005; Ligowska et al., 2011), biofilm formation (Reeser et al., 2007), chemotaxis (Quiñones et al., 2009), loss of mRNA degradation function (Haddad et al., 2012), and capsular polysaccharide biosynthesis (Corcionivoschi et al., 2009). Three microarray studies showed that less than 2% of genes were differentially expressed between wildtype and its cognate luxS mutant in strains 11168 and 81-176 under in vitro conditions (He et al., 2008; Holmes et al., 2009; Plummer, 2012). This suggests that under in vitro basal growth conditions, LuxS is not a global regulator and that the observed gene expression changes are most likely linked to the lost metabolic functions of LuxS. However, the possibility that the quorum sensing role of LuxS would have major regulatory effects on gene expression under in vivo conditions cannot be ruled out.

One can deduce that studies testing the same phenotypes and showed varying results would require careful design to consider differences in the strain background, type of mutation made in the luxS gene, need for complementation of the luxS gene to ensure the luxS mutation is true, and experimental conditions tested on the mutant (Adler et al., 2014). To add to the complication is the uncertainty of whether the phenotypic changes
observed in a mutagenized *luxS* strain are due to disruption of the AMC, quorum sensing, or both (Vendeville et al., 2005; Plummer, 2012; Pereira et al., 2013). Regardless of which role(s) of LuxS are important for differing phenotypes, we can appreciate the deep effect a *luxS* mutation has on phenotypes that are crucial for the adaptation and survival of *C. jejuni* in the host.

**DNA methylation**

Participation of the LuxS enzyme in the AMC is vital for the formation of S-adenosylmethionine (SAM), a major methyl donor for methylation, synthesis of polyamines, AHLs, and various vitamins (Parveen and Cornell, 2011). SAM is a crucial source for methylation of macromolecules such as nucleic acids, proteins, carbohydrates, lipids and other molecules in prokaryotic and eukaryotic organisms. Of particular importance is the use of methyl groups from SAM to methylate DNA. DNA is methylated at specific bases, with the three most common base modifications in prokaryotes being N4-methylcytosine (m4C), C5-methylcytosine (m5C) and N6-methyladenine (m6A) (Korlach and Turner, 2012). *C. jejuni* is, as of now, known to possess both m6A and m5C base modifications (Murray et al., 2012; Mou et al., 2015; O’Loughlin et al., 2015). Enzymes that carry out base modifications, known as methyltransferases (MTases), recognize specific sequences motifs and transfer methyl groups from SAM to the specific base located at the motif (Vasu and Nagaraja, 2013). MTases can also be associated with restriction enzymes (REases), which cleave at the
phosphodiester bond of cognate sequence motifs, generating 5’ or 3’ blunt or “sticky” ends.

**Restriction-Modification systems**

Together, REases and MTases are classified as Restriction-Modification (R-M) systems that divide out to four major types based on enzyme composition, regulation of their expression, co-factor requirements, sequence specificities, and the symmetry of the motif on the double-stranded DNA (Bujnicki, 2001; Roberts et al., 2003). There are also solitary REases and MTases, which, as their name implies, have no associated MTase or REase, and don’t belong with a specific R-M class (Vasu and Nagaraja, 2013). According to the Restriction Enzyme dataBASE (REBASE), which is a comprehensive web-based database containing information about all genes, enzymes, and genomes involved in DNA restriction and modification, *C. jejuni* are predicted to have a combination of any of the four major R-M systems (Types I-IV) (Roberts et al., 2010). However, the only known characterized R-M systems in *C. jejuni* are Types I and II, which are described in more detail below (Murray et al., 2012; Mou et al., 2015; O’Loughlin et al., 2015).

The enzymes in Type I R-M systems are characterized as multi-subunit proteins made up of three types of subunits: R subunit catalyzes restriction, M subunit carries out methylation, and S subunit binds to cognate sequence motifs (Roberts et al., 2003). The subunits function as a single complex and cleave nonspecifically (up to tens of thousands of base pairs away from target sequence), making them useless for recombinant DNA
techniques (Bujnicki, 2001). On unmethylated DNA, the enzyme acts mainly as REases, but on hemimethylated DNA, the enzyme acts as MTases (Roberts et al., 2003).

Type II enzymes are the most diverse, abundant, and widely-studied R-M systems, and are composed of 2 separate enzymes (a MTase and REase) but some subdivisions can have R-M genes fused into a single gene (Bujnicki, 2001; Roberts et al., 2003). The REases cleave a defined fragmented pattern near or within the sequence motif and are thus heavily used for their application in genetic engineering (Roberts et al., 2003).

**Roles of DNA methylation in cell biology and virulence**

The function of the MTase in DNA methylation has an enormous impact on a diverse number of phenotypes involved in cell maintenance, defense, selfish behaviors to benefit the R-M system, phase variation, and host-pathogen interactions. Cell maintenance functions vary from controlling gene expression, cell cycle, DNA repair, DNA replication, posttranscriptional regulation, phase variation, growth and metabolism, and stress response (Jeltsch, 2002; Low and Casadesús, 2008; Chen et al., 2014). The most well-known function that MTase is involved in is in bacterial defense systems (Vasu and Nagaraja, 2013). The host genome is protected via methylation by cognate MTases. The ability to recognize self from non-self is a primitive immune system to protect the host bacterial cell from phages and phage DNA. MTases, and R-M systems in general, also exhibit selfish behaviors to ensure its survival in the bacterial host (Kobayashi, 2001). When cell division of the host yields daughter cells that are diluted in
R-M systems, there is a greater chance for unmethylated DNA. The unmethylated DNA is susceptible to double-strand breaks by REases, which result in REase-mediated cell death also known as postsegregational killing.

R-M systems not only affect the expression of phase variable genes, they can also undergo phase variation called ‘phasevarions’ (Srikhanta et al., 2010; Casadesús and Low, 2013). Unlike individual phase variation systems that only generate heterogeneity in a single phenotypic trait, a phasevarion has R-M activity that also acts as an epigenetic regulator, helping to generate heterogeneity in multiple phenotypic traits (Casadesús and Low, 2013). Phasevarions are advantageous to the survival of the bacteria as it helps the bacteria evade the host immune system, generate diverse antigenic surface structures, increase the fitness of the bacteria under certain environmental conditions, protect from diverse bacteriophage populations, and control when to uptake foreign DNA (Vasu and Nagaraja, 2013). R-M systems also have a necessary role in the dynamics of host-pathogen interactions and the survival of the pathogen, including the expression of virulence traits such as flagella biosynthesis and motility, cell envelope biosynthesis, toxin synthesis and export, stress responses, DNA repair systems, chemotaxis, and antibiotic resistance (Marinus and Casadesus, 2009; Parveen and Cornell, 2011).

DNA MTases in *C. jejuni*

In the last two decades, there has been increasing interest in studying the role of methylation in *C. jejuni* pathobiology. The first evidence of methylation in *C. jejuni* was identified by Edmonds et al. who analyzed the distribution of methylated adenines in
GATC motifs in the chromosomal DNA of several *Campylobacter* species, including *C. jejuni* (Edmonds et al., 1992). Later on, the phenotypes of three *C. jejuni* methyltransferases have been characterized through mutation of the MTase genes *cj1461*, *cj0588*, and *cj0031*. The gene *cj1461* codes for a solitary DNA MTase in 81-176 (no cognate REase) that performs m6A methylations (Kim et al., 2008). Mutation of this gene resulted in phenotypic changes, including decreased motility, defective flagellar morphology, hyperadherence to epithelial cells, and severe decrease in invasion efficiency. The gene for *cj0588* codes for an rRNA MTase in 81-176 that methylates ribonucleic acids, specifically the 23S rRNA in the 50S ribosomal subunit (Sałamaszyńska-Guz et al., 2014). Mutation of *cj0588* resulted in similar effects as *cj1461* mutation where motility was reduced, phenotypic changes to adherence and invasion (in this case, decreased ability to do both), increased quantities of free 50S subunits and decreased amounts of 70S ribosomes, and more resistance to the antimycobacterial drug capreomycin.

*Cj0031* (from 11168) is a unique Type IIG MTase that undergoes phase variation and is postulated to behave like a phasevarion (Anjum, 2013). This MTase is believed to be involved in the regulation of adhesion, invasion and biofilm formation but had no effect on motility. Analysis of its gene sequence showed that the gene possesses a polyG tract that fuses *cj0031* and *cj0032* together, under a single promoter. A repeat tract length of 9 guanine nucleotides or G9 results in the in-frame fusion of the two genes, thereby producing a functional product- a phase ON phenotype. However, a repeat tract of G10 or G8 separates the reading frames of the two genes, leading to a phase OFF genotype with production of a non-functional or truncated MTase. When *C. jejuni* colonies possessing
G10 repeat tract were passaged through a chicken, the resulting populations that were isolated from the chicken shifted to the phase ON G9 genotype, suggesting that the role of the phasevarion is necessary to facilitate rapid adaptation of C. jejuni to various environments within hosts. We have also discovered a homologue of cj0031 in IA3902 called RS00180 (formerly known as CJS_0032) but the polyG tract in RS00180 was replaced with a non-phase variable 1130bp sequence, resulting in a constitutive “phase ON” MTase (Mou et al., 2015). The significance of this phase ON MTase in IA3902 pathogenesis is currently under investigation and will be further discussed in chapter 4 of this thesis.

An example of a Campylobacter MTase that generates multi-drug resistance was discovered within the last year. The \textit{erm}(B) gene coding for an rRNA MTase was detected in \textit{C. jejuni} and \textit{C. coli} isolates from various human and animal sources in China (Wang et al., 2014). It is also an HGT element contributing to macrolide resistance, along with several other important drug classes. This is a huge concern as prevalence of macrolide resistance in Campylobacter has been historically very low in comparison to FQ resistance, thus making them an optimal drug choice for treating Campylobacter infections (Luangtongkum et al., 2009; FDA, 2015). However, the discovery of a MTase that creates highly drug-resistant \textit{Campylobacter} isolates and is disseminated by HGT can rapidly change the landscape of Campylobacter drug resistance. Emphasis on efforts to control and prevent the spread of this element is needed before it becomes even more difficult to provide effective clinical treatment options.
Using SMRT sequencing to profile methylomes

Until recently, the biggest hurdle in methylation studies has been the difficulty of detecting methylated bases with the technologies available at the time. These bulk methods lacked the sensitivity and accuracy to detect the exact location of the base modification (Korlach and Turner, 2012). When sequencing methods were invented, they greatly reduced the time and labor-intensive procedures for studying methylation, with the capacity to directly detect these base modifications. While second generation sequencing methods were at a disadvantage for detecting methylation (the sample preparation protocols required DNA amplification which lose base modifications and cannot be detected during actual sequencing), the automated dye terminator chemistry used in Sanger sequencing was able to identify the three base modifications (m4C, m5C and m6A), though it cannot detect every methylated base in the genome (Rao and Buckler-White, 1998).

As sequencing costs continue to decline, various single molecule sequencing technologies, and in particular, single-molecule real-time (SMRT) sequencing, have been developed and gone several steps further by combining DNA sequencing of native, unamplified DNA with the direct detection and location of methylated bases (Korlach and Turner, 2012). In this dissertation, we used SMRT sequencing technology to detect methylation patterns in C. jejuni. Briefly, SMRT sequencing directly measures the activity of a single DNA polymerase bound to a circular molecule of template DNA. When the nucleotide is bound to the polymerase, a pulse of fluorescence specific to the nucleotide is released (Flusberg et al., 2010; Davis et al., 2013). The length of time that the nucleotide is bound to the polymerase is recorded as the pulse width (PW) (Clark et
al., 2012). The fluorescence pulse ends when the polymerase cleaves the fluorophore before translocating to the next base in the template strand. The interpulse duration (IPD), which is the time between successive nucleotide-bound states of the enzyme, is also recorded to measure the kinetics of the polymerase. When the polymerase encounters a methylated base, its kinetics are changed whereby DNA synthesis slows down and the enzyme delays the incorporation of the complementary nucleotide (Sánchez-Romero et al., 2015). PW and IPD values vary from those of non-methylated DNA bases, with IPD ratios becoming statistically longer on methylated bases and the obvious identification of methylated bases on chromatograms. (Flusberg et al., 2010; Sánchez-Romero et al., 2015). SMRT sequencing is also able to distinguish between the different types of methylated bases as they each have their own kinetic signature.

In recent years, SMRT sequencing has become increasingly popular for its use in profiling methylomes of important pathogens, including E. coli (Fang et al., 2012), Helicobacter pylori (Krebes et al., 2014), Salmonella enterica (Pirone-Davies et al., 2015), Klebsiella pneumoniae (Doi et al., 2014), Enterococcus faecalis (Huo et al., 2015), and Campylobacter jejuni (Murray et al., 2012; Mou et al., 2015; O’Loughlin et al., 2015). In C. jejuni, SMRT sequencing has revealed several new findings on the methylome of this pathogen, including sequence motifs, base modification types, associated methyltransferases, and methylome characterization (including hypo- and hypermethylated regions of the genome, the genes present in these areas) and comparison of methylomes between different strains of C. jejuni (Murray et al., 2012; Mou et al., 2015; O’Loughlin et al., 2015). In particular, our group found that the genome-wide methylation patterns and the types of genes that were hypo- and hypermethylated were
very similar between 11168 and 81-176. However, IA3902 had completely different set of methylation patterns as well as the genes found in the hypo- and hypermethylated regions. These findings will be discussed at length in chapter 3 of this thesis.

Although more investigation is needed to understand the significance of these methylated areas and the expression of the genes, we can speculate that methylation may play a role in the unique pathogenicity of the gastroenteric 11168 and 81-176, and the abortifacient IA3902. However, much remains to be understood on the mechanisms of methylation in the pathogenesis of *Campylobacter*.

**Specific aims and significance of this research**

The overriding goal of our research group is to identify novel targets for controlling *C. jejuni* disease by elucidating the molecular mechanisms of potential targets and their role in *C. jejuni* pathogenesis. Multiple studies showed phenotypic effects of important *C. jejuni* disease traits when mutations were made in AI-2/LuxS system and methylation-associated genes. The research presented in this dissertation aims to close gaps in our knowledge on quorum sensing, LuxS, and methylation in the adaptation and survival of *C. jejuni*.

Our main objective was to define the mechanisms of LuxS on methyl recycling, DNA methylation, and *C. jejuni* physiology. Our central hypothesis is that the AI-2/LuxS system, its role in methyl recycling, and DNA methylation are vital for *C. jejuni* physiology and phenotypic expression of virulence factors important for host colonization and general pathogenesis mechanisms. We accomplished our central hypotheses with the following specific aims: 1) Investigate the effects of the *luxS* mutation on the expression and function of key colonization factors of *C. jejuni* to
delineate the molecular mechanisms of LuxS (including its roles in AI-2 production and AMC methyl recycling) in these factors (Chapter 2).  2) Compare DNA methylation patterns between a luxS mutant and wildtype to assess whether mutation of luxS would disrupt DNA methylation processes; secondly, we will compare methylome profiles between gastroenteric and abortifacient \textit{C. jejuni} strains to establish whether the differences in disease presentations of these strains could be associated with their methylome profiles (Chapter 3).  3) Characterize the CJSA_RS00180 putative methyltransferase gene of \textit{C. jejuni} clone SA clinical isolate IA3902 and evaluate its role in growth, motility and mucin penetration (Chapter 4).

These studies and future studies will provide insight into the role of AI-2/LuxS quorum sensing system and methylation in \textit{C. jejuni}’s ability to cause enteric and systemic disease. In addition, if the LuxS system and/or methylation are required for pathogenesis to occur, they can serve as a novel measure to control \textit{C. jejuni} transmission and colonization in gastrointestinal tract. The potential to identify significance of quorum sensing and/or methylation systems in \textit{C. jejuni} disease also provides attractive cell-signaling disruption techniques and methyltransferase vaccine targets. This is an especially timely project contributing efforts to identify alternative novel targets to combat the rise of antibiotic resistance as currently available drug options to treat \textit{C. jejuni} infections are limited.
Organization of this dissertation

This dissertation is organized in the journal format with 5 chapters. Chapter 1 contains the literature review of *Campylobacter jejuni*. Each of chapters 2-4 address the following topics: 1) the molecular mechanisms for how AI-2/LuxS system affects the expression of colonization factors that are pivotal to *C. jejuni* pathogenesis, 2) thorough analysis of the methylome profile of a *luxS* mutant as well as methylome comparisons between three *C. jejuni* strains, and 3) the characterization of a methyltransferase mutant and how the methyltransferase impacts the *C. jejuni* physiology. Each chapter contains a manuscript that has been published or will be submitted for publication. Chapter 5 is a general conclusion summarizing the studies presented in this dissertation and future research directions. Following the end of chapter 5 are references cited, tables, and figures.
CHAPTER 2. THE IMPACT OF THE LUXS MUTATION ON PHENOTYPIC EXPRESSION OF FACTORS CRITICAL FOR CAMPYLOBACTER JEJUNI COLONIZATION

A paper to be submitted to Veterinary Microbiology

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Abstract

Studies have collectively shown the wide impact that luxS mutation has on the expression and function of various aspects of Campylobacter jejuni virulence. However, many important traits critical to each stage of C. jejuni pathogenesis remain unexplored. Previous work by our laboratory has demonstrated that LuxS mutagenesis negatively impacts colonization of the gastrointestinal tract of several host species, however the specific physiologic basis for this colonization defect is unclear. In this study, we use a mechanistic approach to understand how the luxS mutation affects the expression of key physiologic factors important to the colonization ability of C. jejuni, including the CmeABC efflux system, cell morphology, and motility through mucin substrate. Efflux pump expression was compared between C. jejuni 11168, its isogenic luxS mutant, and a chromosomally encoded luxS complement during a growth study (under basal conditions) using qPCR. A wildtype, luxS mutant and genetic complement from two C. jejuni strains (11168 and IA3902) were also evaluated for differences in cell morphology and motility.
through mucin using an ImageStream® imaging flow cytometer and a mucin penetration assay, respectively. In addition, we measured and compared the activated methyl cycle (AMC) metabolite levels of the IA3902 luxS mutant to wildtype to assess how a luxS mutation would disrupt the AMC, a cycle in which LuxS plays a crucial role for recycling the methyl donor S-adenosylmethionine. Results showed that mutagenesis of the luxS gene completely disrupted the activated methyl cycle with altered concentrations of AMC metabolites both upstream and downstream of LuxS. Multidrug efflux pump genes cmeABC showed no significant changes in expression levels within the luxS mutant. Similarly, while the cmeR response regulator had consistently lower expression levels, this failed to reach a level of statistical significance. Though motility through mucin was not completely unaffected by the luxS mutation, the lack of differences in cell morphology between wildtype and luxS mutant suggest that morphology is not responsible for the slight changes in mucin penetration observed in one of our luxS mutants. Though additional studies are warranted, these findings suggest that the CmeABC multi-drug efflux pump, cell morphology and mucin penetration are not major mechanisms responsible for the luxS mutant’s decreased ability to colonize and/or translocate out of the intestine in its host.

Key Words: Campylobacter jejuni, AI-2/LuxS, efflux pump, cell morphology, mucin, motility
Introduction

The gram-negative microaerophilic bacterium *Campylobacter jejuni* is the most common cause of bacterial-associated food borne diarrhea in the United States, with reports of over 2 million cases each year (CDC, 2013b). The latest report from the USDA Economic Research Service calculated that the annual cost of foodborne illnesses attributable to *Campylobacter* in the U.S. amounted to over $1.9 billion dollars (ERS, 2014). *Campylobacter jejuni* infections in various animal species, especially in sheep and cattle, can manifest as enteritis, abortions and infertility, which can lead to severe economic losses (Sanad, 2011; Leedom Larson and Spickler, 2013). Of even greater concern to both human and animal health is the increasing prevalence of antibiotic-resistant *C. jejuni* worldwide, especially to drugs normally used for treating *Campylobacter* enteritis such as macrolides, fluoroquinolones, aminoglycosides, and beta-lactams (Alfredson and Korolik, 2007; Wieczorek and Osek, 2013). For example, a highly virulent *C. jejuni* clone (clone SA or Sheep Abortion), which emerged in the last few decades, has now become the primary cause of *Campylobacter*-associated sheep abortions in the U.S. and carries a tetracycline-resistance plasmid (Sahin et al., 2008). Tetracycline is the only class of antibiotics approved for treating sheep abortions in the U.S., making control of infectious abortions by this clone even more difficult (Sahin et al., 2008). Recent evidence has also linked clone SA with a number of human gastroenteritis cases, bringing attention to this clone not only for its tetracycline resistance, but also for its zoonotic potential (Sahin et al., 2012). The emergence of this clone and other antibiotic resistant *C. jejuni* strains has led to a dire need for novel
approaches to control *C. jejuni* infections while minimizing the potential for development of additional antibiotic resistance phenotypes.

One area of research that has gained a foothold as a promising alternative drug target for controlling bacterial infections is quorum sensing. This cell density-dependent mechanism relies on bacterial monitoring for the accumulation of a sufficient number of bacterial cells in a population (quorum) to initiate the production of signaling molecules (autoinducers) that would in turn elicit a coordinated behavioral response to external stimuli (Miller and Bassler, 2001). Quorum sensing has been demonstrated to play a central regulatory role in bacterial communities including regulation of virulence, antibiotic production, survival in various environmental stresses, and cell growth and metabolism (Bhardwaj et al., 2013). Most *Campylobacter* species possess the autoinducer-2 (AI-2)/LuxS system, which consists of the LuxS enzyme and its product, the signaling molecule AI-2 (Gölz et al., 2012a). LuxS is part of the activated methyl cycle (AMC), which functions to recycle S-adenosylmethionine (SAM), a primary methyl donor for various biological processes including DNA methylation (Winzer et al., 2003). In addition to its contribution in the AMC, LuxS also produces AI-2 as a result of the enzymatic cleavage of S-ribosylhomocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (De Keersmaecker et al., 2006). DPD is unstable and spontaneously cyclizes to form varying structures of autoinducer molecules collectively known as AI-2 (Waters and Bassler, 2005).

In *C. jejuni*, LuxS has been associated with a wide range of virulence traits, including biofilm formation, autoagglutination, motility, oxidative stress, and cytolethal distending toxin regulation, as reviewed by Plummer (Plummer, 2012). Moreover,
Plummer et al. showed clear evidence for the critical role of LuxS in the virulence and fitness of two different *C. jejuni* strains (also used in this paper) in two types of *Campylobacter* pathogenesis models (Plummer et al., 2012). In the pregnant guinea pig abortion model, the *luxS* mutant of the clinical isolate of clone SA, IA3902, was unable to induce abortions following oral inoculation, but was able to induce abortions following intraperitoneal inoculation. These results suggest that the LuxS mutant has a defect in its ability to colonize and/or translocate out of the intestinal environment. In addition, the IA3902 *luxS* mutant failed to colonize the chicken colonization model. The *luxS* mutant of W7, a highly motile variant of the commonly studied laboratory strain 11168, could colonize the chicken colonization model when monoinoculated, but was outcompeted when co-inoculated with wildtype. In summary, these results highlight the importance of LuxS in the fitness and/or colonization ability of *C. jejuni* in the host gut environment.

While our long-term goal is to determine if the LuxS system could serve as a potential target for developing therapeutic strategies against *C. jejuni*, much remains to be determined regarding the mechanisms by which the LuxS system contributes to the pathogenesis of *C. jejuni*. For example, the exact mechanism underlying the benefit of a functional LuxS system in the critical stage of *C. jejuni* colonization of the intestinal tract has yet to be defined. One well described element of *C. jejuni* pathogenesis is the CmeABC efflux pump, which is required for successful *C. jejuni* colonization of the host intestinal tract (Koolman et al., 2015). The *cmeB* gene is part of the *cmeABC* three-gene operon that forms the CmeABC multidrug efflux pump (Lin et al., 2002). Along with its natural function to resist bile salts, CmeABC is also a major contributor to resistance to a wide variety of antimicrobial drugs (Lin et al., 2002; Lin et al., 2005b; Guo, 2010). This
multi-substrate efflux pump is crucial for *C. jejuni* to survive and colonize the intestinal tract, which hosts a diverse array of potentially lethal substances (Lin et al., 2003). CmeABC expression is regulated, in part, by CmeR, which functions to transcriptionally repress the efflux system in the absence of bile salts (Guo et al., 2008). CmeR also functions as a pleiotropic regulator of a number of other characterized and uncharacterized membrane transporters, proteins involved in C₄-dicarboxylate transport and utilization, enzymes for capsular polysaccharide biosynthesis, and hypothetical proteins of unknown functions.

Only one prior study has shown evidence of an interaction between LuxS and an efflux system in *C. jejuni* (He et al., 2008). This study screened the differential gene expression profiles between a wildtype *C. jejuni* 81-176 strain and a *luxS* mutant under untreated and hydrogen peroxide-treated conditions. Microarray analysis identified one operon of efflux pump genes (*cmeDEF*) which had higher expression levels in the *luxS* mutant compared to wildtype. However, no further studies have been conducted to explain what relationship the efflux system has with AI-2/LuxS regulation under stress conditions.

Two other important features of *C. jejuni* that are crucial for colonization of the intestinal tract are motility and cell shape. Studies have consistently shown that *luxS* mutagenesis reduces motility in 11168 (Elvers and Park, 2002; Holmes et al., 2009), 81116 (Jeon et al., 2003; Plummer et al., 2011), 81-176 (He et al., 2008; Quiñones et al., 2009), and the *luxS* mutants used in this study (Plummer et al., 2012). Motility is required for colonization of the gastrointestinal tract and is important for the movement of the organism out of the intestinal lumen and into the mucosal lining of the intestine. The
mucosal lining of the gut serves as an important first line of defense to protect the gastrointestinal epithelia from luminal bacteria (Young et al., 2007). Bacterial interaction with the mucins of the mucosal lining is also the first step in colonization of the gut mucosal surfaces (Naughton et al., 2013a). Mucin is a primary component of the intestinal mucosal layer, acting as an adherence target for intestinal pathogens and also as a strong chemoattractant for C. jejuni (Usui et al., 1999; Stahl et al., 2011). Motility of C. jejuni increases in the presence of highly viscous mucus, which is also correlated with increased virulence in vitro (Alemka et al., 2012). This was evident as mucin-producing cell lines promoted C. jejuni infection in contrast to non-mucin producing cell lines (Naughton et al., 2013b). Analysis of the protein expression profiles of C. jejuni incubated in porcine mucin (Hong et al., 2014) demonstrated a diverse range of proteins that were up- or downregulated, including motility, adherence, energy metabolism, respiration, and even CmeA protein. Thus mucin is a strong promoter for C. jejuni motility and expression of proteins important to the disease process. However, it remains to be determined how LuxS affects motility and interaction of C. jejuni through substances like mucus.

In conjunction with the motility function of the flagella, the helical shape of C. jejuni is also necessary to provide the “corkscrew-like” rotation needed to push through the thick mucus layer (Lertsethtakarn et al., 2011). In addition, its cell shape and associated short O-side chains of its lipooligosaccharide are purported to protect C. jejuni from non-specific binding to mucin that could retard its ability to access the intestinal epithelial cells (Alemka et al., 2012). Few studies have defined the genetic components responsible for cell shape in C. jejuni (Frirdich et al., 2012; Ikeda and Karlyshev, 2012).
However, of the studies recently published, morphology has been shown to be critical for virulence traits including motility, biofilm formation, colonization, and host cell interactions (Frirdich et al., 2012; Frirdich et al., 2014). Whether LuxS helps to dictate cell shape remains to be determined.

In an attempt to better delineate the role of LuxS in maintaining normal intracellular levels of AMC metabolites and host colonization, we set out to characterize the impact of the LuxS system on several colonization factors important to *C. jejuni*. The colonization factors evaluated included the expression of the *cmeABC* efflux system operon, expression of the *cmeR* global regulator, mucin penetration, and cell shape. Because previous studies demonstrated a decreased ability of *luxS* mutants to colonize the animal host and induce systemic infection, we hypothesized that mutagenesis of the LuxS system would disrupt the AMC cycle resulting in altered intracellular concentrations of AMC metabolites. Additionally, we hypothesized that the *luxS* mutation would decrease *cmeABC* efflux pump expression and, simultaneously, cause significant morphological changes to the mutant (cell shape and flagella) that would be associated with altered motility through the mucin layer.

Our experimental design to test these hypotheses used a mechanistic approach to characterize the phenotypic effects of the *luxS* mutation on the expression or functions of these colonization factors. These effects included analysis of gene expression of the *cmeABC* system in the presence of a *luxS* mutation, cell shape, motility through mucin, as well as the intracellular effect of the *luxS* mutation on the AMC via measuring AMC metabolite levels.
Materials and Methods

Bacterial strains and culture conditions

*Campylobacter jejuni* strains were routinely cultured in Mueller-Hinton (MH) broth or agar plates (Difco) at 42°C in gas jars with a microaerobic atmosphere (5% O₂, 10% CO₂, 85% N₂). A list of all *C. jejuni* strains used in this study is described in Table 1. W7 is a highly motile spontaneous variant of the commonly studied *C. jejuni* NCTC 11168 (ATCC 700819) (Plummer et al., 2012). IA3902 is a clinical isolate of *C. jejuni* clone SA (Sheep Abortion) obtained from a sheep abortion outbreak in Iowa in 2006 (Sahin et al., 2008) and has been sequenced (Wu et al., 2013). The only strain created in this study was the chromosomally encoded luxS complement of W7 (W7ΔluxSc). This strain was created by natural transformation of W7ΔluxS with genomic DNA from IA3902ΔluxSc as performed previously (Jeon and Zhang, 2007). Transformants were selected on MH agar plates supplemented with chloramphenicol (4µg/ml) and kanamycin (30µg/ml) and confirmed by PCR. Strains containing an antimicrobial resistance marker were grown in media containing 4µg/ml chloramphenicol or 30µg/ml kanamycin.

Expression of cmeB and cmeR genes in *Campylobacter jejuni* luxS mutant grown under basal conditions

Growth studies

*C. jejuni* strains W7, W7ΔluxS, and W7ΔluxSc were each prepared for growth study inoculum using confluent lawns grown to 24 hours, harvested in a small volume of MH broth media and adjusted to an optical density (OD₆₀₀) of 0.5. Inocula were then further diluted 1:100 by adding 600µl of normalized cultures to 60ml MH broth
(approximate cell densities of $10^5$-$10^7$ colony forming unit (CFU)/ml) in 250ml flasks. Suspensions were grown in gas jars (Mitsubishi Gas Chemical Co., Inc.) under microaerobic conditions at 42°C for 48 hours. Sample aliquots from each strain were harvested at the time of inoculation and at time points 3, 8, 16, 24, 32, and 48 hours for bacterial enumeration. Aliquots from time points 8, 16, 24, and 32 hours were also used for quantitative real-time PCR (qPCR) studies. Three independent growth studies were performed for each strain. Growth of each strain was assessed by using the drop plate method (Chen et al., 2003) to plate serial dilutions (1:10) of bacterial suspensions on MH agar. Log 10 transformation of CFU/ml was performed and assessed for any significant growth differences between strains using two-way analysis of variance (ANOVA) with repeated measures in GraphPad Prism (La Jolla, CA, USA). A $p$-value of 0.05 or less was considered significant. Results are the average of three independent experiments.

Quantitative real-time PCR analysis of cmeB and cmeR transcription under basal conditions

*C. jejuni* cultures were collected at time points 8, 16, 24 and 32 hours from the growth studies and mixed with 2 volumes of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA). RNAprotect cultures were stored in -80°C for no more than a week before total RNA isolation was performed using an RNeasy Protect Bacteria Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was further treated with the TURBO DNA-free kit (Life Technologies, Carlsbad, CA) to remove any residual DNA contamination. RNA concentration was then measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Quality and size distribution of RNA was determined using gel electrophoresis with ethidium bromide staining. RNA
samples were stored in -80°C until ready for quantitative real-time PCR (qPCR) analysis. Primers used in the qPCR assay for amplifying expression levels of cmeB, cmeR, and 16S rRNA are listed in Table 2. The primers made in this study were designed using Primer3 online interface (http://bioinfo.ut.ee/primer3/). qPCR assays were run using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) and the MyiQ Single-Color Real-Time PCR detection system (Bio-Rad) as done previously with some modifications described here (Lin et al., 2005b). Each dilution of the RNA template was run in triplicate with reaction volumes of 15µl. Thermal cycling conditions consisted of cDNA synthesis at 50°C for 10 min and 60°C for 5 min followed by reverse transcriptase inactivation at 95°C for 3 min. Amplification then proceeded with 40 cycles of denaturation at 95°C for 10 s followed with annealing for each primer pair (Table 2) at 58°C for 30 s. Relative changes in cmeB and cmeR expression between W7 wildtype, W7ΔluxS, W7ΔluxSc were calculated using the “ISU Equation” described in previous methods (Gallup and Ackermann, 2006) and modified for this project by the Iowa State University Office of Biotechnology’s DNA Facility ISU qPCR Consultation Service (Ames, IA). Each gene was normalized using 16S rRNA expression levels as a control. Statistical analyses were performed using two-way ANOVA with repeated measures (GraphPad Prism) to assess significance of changes in gene expression levels. A p-value of 0.05 or less was considered significant. Results are the average of three independent experiments.

**Cell image capture and morphology analysis using ImageStream**

Cultures of bacterial strains were grown at 37°C or 42°C for 24 hours. Cultures were then adjusted to an approximate OD$_{600}$ of 0.1, fixed with 3.6% formalin, washed
with DPBS (1X concentration), and stained with Baclight Green stain (Life Technologies, Carlsbad, CA) to a final concentration of 100nM following the manufacturer’s protocol. Images of cells for each strain were captured with a 60x objective using the ImageStream® multispectral imaging flow cytometer (Amnis Corp., Seattle, WA). Gating was applied on area and intensity to select for images containing single cells positive for fluorescence under channel 2, which contains the blue (488nm) excitation field. Morphological features (area, aspect ratio intensity, aspect ratio, diameter, elongatedness, height, length, major axis intensity, major axis, minor axis intensity, minor axis, perimeter, shape ratio, thickness min/max, width) were calculated for at least 1500 cells of each strain using channel 2 filter images by IDEAS® image analysis software (version 4.0, Amnis Corp., Seattle, WA). Further calculations were made using the Fisher’s Discriminant ratio or RD value (difference of means/sum of standard deviation) to compare features between wildtype and luxS mutant or wildtype and luxS complement in W7 or IA3902 grown at the same temperature. A value of over 1 represented significant difference between the compared samples. At least three independent experiments containing three technical replicates of each strain were performed.

**Transmission electron microscopy**

Aliquots of bacterial strains and their respective isogenic luxS mutants were analyzed by electron microscopy on at least three separate occasions. Strains were grown to log or stationary phase in MH broth under basal conditions. 3µl of each strain were added to Formvar/carbon-coated copper grids and air-dried for 1-3 minutes. Remaining liquid was removed using a filter paper and the grids were washed with deionized
distilled water. Grids were air-dried again for the same amount of time and then negatively stained with 1% uranyl acetate for one minute. Excess stain was removed using filter paper and grids were air dried for at least a minute. Samples were then observed in a JEOL JM-2100 scanning/transmission electron microscope at an accelerating voltage of 200kV at the Microscopy and NanoImaging Facility at Iowa State University in Ames, IA.

*Mucin penetration assay*

Mucin penetration was simulated for bacterial strains using methods adapted from previous studies (Liu et al., 2008; Naresh and Hampson, 2010). One-ml syringes containing 1ml porcine gastric mucin (Sigma-Aldrich) dissolved in PBS buffer to a concentration of 30mg/ml were used as mucin columns. The concentration 30mg/ml was selected as it was the highest concentration of mucin that could be used without the mucin viscosity interfering with the syringe function of the assay. One hundred microliters of log-phase bacterial cultures were added to the top of mucin columns and allowed to settle for 30 min at either 37°C or 42°C. Fractions of 0.1ml (the first 0.1ml collected is fraction 1, with subsequent fractions in chronological order until the last 0.1ml-fraction or fraction 10) were collected from the bottom of mucin columns, plated on MH agar containing Oxoid™ Preston *Campylobacter* selective supplement (Thermo Scientific, Wilmington, DE), incubated for at least 24 hours in microaerobic conditions, and then enumerated for bacterial quantification. Three independent experiments were performed for each strain at each temperature and the averages of three experiments were statistically analyzed using two-way ANOVA with repeated measures (GraphPad Prism). Data were assessed for significant differences in number of bacteria (CFU or colony
forming units) compared to wildtype at each fraction and total CFU from fractions 1-9. Fraction 10 was not included in any of the analysis as this fraction included the very top of the mucin layer mixed with culture inoculum, thus making it impossible to determine if the bacteria in this sample had truly penetrated the mucin. A p-value of 0.05 or less was considered significant.

**Analysis of activated methyl cycle metabolites levels in C. jejuni cell pellets using LC-ESI-MS/MS.**

The relative levels of AMC metabolites in IA3902, luxS mutant (IA3902ΔluxS), and luxS complement (IA3902ΔluxSc) were profiled using a previously described LC-ESI-MS/MS (liquid chromatography-electrospray ionization-mass spectrometry) method (Halliday et al., 2010). To extract metabolites, C. jejuni strains were grown from freezer stocks at 42°C with microaerophilic gas and passaged to MH broth. After overnight growth, the optical densities of broth cultures were diluted to an OD$_{600}$ of 0.1-0.15 in MH broth. Cell pellets were collected as described by Halliday et al. (Halliday et al., 2010) and were stored at -80°C until time of submission to the Centre for Analytical Bioscience at the University of Nottingham (Nottingham, England) for metabolite analysis. Chemicals, preparation of stock solutions and standards, metabolite extractions, chemical derivation, instrumentation, and calibration and validation of methods used in the LC-ESI-MS/MS analysis were performed exactly as done by Halliday et al. (Halliday et al., 2010).
Results

*Correlation in gene expression of luxS and efflux pump genes cmeB and cmeR*

Only one prior study has published findings correlating the functions of LuxS and *C. jejuni* efflux systems. The authors found that under stress-induced conditions, a non-functional LuxS in *C. jejuni* strain 81-176 altered the expression of several key virulence genes, including efflux pump genes (*cmeDEF*) (He et al., 2008). The observations from He et al. and our preliminary work merited further study into deciphering the mechanisms of the role of LuxS in efflux pump expression. We hypothesized that mutation of the *luxS* gene in *C. jejuni* would decrease the expression of efflux pump genes from the CmeABC efflux system, thus making the organism more sensitive to the bile salts in the intestine and limiting colonization. This hypothesis was tested by investigating how the expressions of the *cmeABC* efflux genes changed when the *luxS* gene was mutated. To do this, *C. jejuni* W7 wildtype, W7 mutant containing a non-functioning *luxS* gene (W7ΔluxS), and a chromosomally encoded genetic complement of the W7 *luxS* mutant (W7ΔluxSc) were grown at 42°C in broth culture under basal conditions for 48 hours. No growth differences were found between the strains, which were consistent with growth results from previous studies (Plummer et al., 2012).

No statistically significant differences in *cmeB* and *cmeR* transcriptional levels were found between wildtype and W7ΔluxS over the average of three independent growth experiments (Figure 1). *cmeB* gene expression in W7ΔluxS fluctuated slightly from 16 to 32 hours with fold-changes (compared to wildtype) of 1.328 (16h) down to 0.8941 (24h) and then returning to 1.044 (32h). However, none of these differences were
statistically significant. We observed steadily decreasing levels of cmeR transcription from 8 to 32 hours with fold changes (compared to wildtype) starting at 1.51 down to 0.529 from an average of three independent experiments; however, these changes did not reach the level of statistical significance.

These data support the conclusion that the absence of a functional LuxS enzyme (W7ΔluxS) had no effect on the expression of efflux genes cmeB and cmeR. Despite the lack of significant differences in efflux gene expression, the consistency of the decreasing expression levels of cmeR suggest a potential trend that could have biological significance in different environments to those tested in this study and would warrant additional work in the future.

**Effect of luxS mutation on cell morphology**

Cell morphology is critical for motility and overall colonization of *C. jejuni* in the host intestinal environment. To date, only one study has looked at cell morphology of a luxS mutant in *C. jejuni* 81116 using transmission electron microscopy and found no differences in cell shape or flagellar morphology compared to wildtype (Jeon et al., 2003). We utilized two approaches to measure cell morphology of luxS mutants at the population level and individual cell level. To compare morphology within a population, we used the ImageStream® multispectral imaging flow cytometer to measure 17 different morphological features for W7 and IA3902 wildtype, luxS mutant and luxS complement strains. The RD value, which is the difference of means between two strains divided by the sum of standard deviation, was calculated to compare features between W7 or IA3902 strains grown in the same temperature. As shown in Table 3, none of the
comparisons for any of the morphological features had an RD value over 1, which suggests that the luxS mutation had no impact on the cell morphology features measured in this study.

Transmission electron microscopy was also used for closer inspection of individual cell morphology. In agreement with the ImageStreamX® results, we also found no subjective evidence of changes in cell and flagellar morphology between wildtype, luxS mutant, and complement of both strain backgrounds in three separate replicates (Figure 2).

**Effect of luxS mutation on motility through mucin**

Motility is vital for *C. jejuni* to interact with mucins and establish colonization of the intestinal mucosal lining. Though numerous studies have found differences in motility between a *C. jejuni* wildtype and luxS mutants on motility agar (Elvers and Park, 2002; He et al., 2008; Holmes et al., 2009), none have tested the motility of a luxS mutant in mucin in vitro. Given the biological importance and relevance of mucin penetration in the physiology of *C. jejuni*, we simulated motility through mucin, comparing the wildtype, luxS mutant and luxS complement strains of both W7 and IA3902 using a mucin column incubated at 37°C or 42°C. When we compared the bacterial counts at each fraction of the mucin column, we found no significant differences in most of the fractions regardless of the incubation temperature (Figures 3A, 4A and 4C). The only fraction that showed statistically significant differences between wildtype and luxS mutant mucin penetration was that of W7ΔluxS and the wildtype in fraction 9 when incubated at 42°C (Figure 3C). In this fraction the W7ΔluxS strain showed a decreased CFU count compared to the
wildtype. Given this result it seemed that there were two plausible possibilities that would explain this finding. Either less of the mutant cells were able to penetrate the mucin at all, or the mutant cells had readily penetrated the mucin and were now located in the deeper fractions of the mucin column. We therefore calculated the total CFU (colony forming units) of W7ΔluxS from fractions 1 through 9 (i.e. the sum of the CFU counts in all fractions) and compared to W7 and W7ΔluxSc. While we did not find any statistically significant differences in the mean total CFU of W7ΔluxS compared to wildtype (Figure 3D), we did see that the mean CFU of W7ΔluxS in fractions 1-9 was noticeably lower than W7 and W7ΔluxSc. Taken together we believe that this may contribute to the significantly lower number of W7ΔluxS observed at fraction 9 of the mucin column and suggest that the W7ΔluxS mutant does have a mucin penetration defect at 42°C. The mucin penetration data of W7ΔluxSc at 42°C show that complementing the luxS gene partially restored the mucin penetration ability of the strain suggesting that the mucin penetration defect observed in W7ΔluxS is indeed LuxS-mediated. We also compared the total CFU count from fractions 1-9 for W7ΔluxS at 37°C, and IA3902ΔluxS incubated at 37°C or 42°C to wildtype to see if were any differences in total number of luxS mutants that were present in these fractions. When we compared with their respective wildtype, we found no significant differences in total CFU counts (Figures 3B, 4B and 4D), consistent with the findings of the individual fraction analysis for each of these samples.

Though the number of W7ΔluxS cells in fraction 9 (42°C) was statistically lower than W7, the biological significance of this observation regarding the physiology of W7ΔluxS remains unclear. Regardless, evidence strongly suggests that the motility of the
luxS mutant through mucin is not affected by morphology based on the lack of morphological differences found between wildtype and luxS mutants.

**Effect of luxS mutation on the AMC function**

Lastly, to assess whether the luxS mutation affected the function of the AMC, and to what extent, we utilized the LC-ECI-MS/MS method to analyze intracellular levels of AMC metabolites in IA3902 wildtype, luxS mutant (IA3902ΔluxS), and luxS complement (IA3902ΔluxSc). Results of the metabolite levels, shown as a ratio of metabolite peak areas to internal standards, support our predicted results for our strains (Table 4).

We expected the luxS mutation to cause a decrease in homocysteine levels, as this metabolite requires a functional LuxS enzyme for its synthesis. Indeed, this metabolite was almost undetectable in IA3902ΔluxS while IA3902 and IA3902ΔluxSc showed normal levels (Table 4). Homocysteine is the product of LuxS catalysis of S-ribosylhomocysteine (SRH) to homocysteine and DPD (Winzer et al., 2002). In addition, because LuxS is non-functional in the mutant, SRH should accumulate at higher levels in the cell. This is clearly evident in IA3902ΔluxS with SRH levels being three times that of wildtype (Table 4).

Cystathionine levels were at least 5 times lower in IA3902ΔluxS (0.0030) compared to wildtype (0.0159) and IA3902ΔluxSc (0.0162), which is expected as homocysteine can be a precursor for the formation of cystathionine (Selhub, 1999). Methionine levels were also reduced in IA3902ΔluxS (1.7195) compared to wildtype (2.8010), although far from complete depletion. This would make sense as homocysteine
levels are already low in IA3902ΔluxS and any remaining homocysteine would be converted to methionine, which will also be comparably lower than wildtype.

We found the S-adenosylmethionine (SAM) levels reduced by roughly 25% in IA3902ΔluxS compared to wildtype and IA3902ΔluxSc (Table 4). SAM is a critical methyl donor for a variety of metabolic and biosynthetic reactions, including methylation of macromolecules such as DNA (Parveen and Cornell, 2011). The slightly lower levels of SAM suggest that the luxS mutation could disrupt the primary function of the AMC in recycling SAM.

Slightly higher levels of the toxic metabolite S-adenosylhomocysteine (SAH) were observed in IA3902ΔluxS in contrast to wildtype and IA3902ΔluxSc (Table 4). This is assumed to be due to the rapid depletion of SAM levels while simultaneously accumulating more SAH. The lack of a functional LuxS enzyme may then slow down or halt the conversion of SAH to SRH. Accumulation of SAH can also inhibit various methylation reactions and polyamine synthesis, thereby altering growth and potentially killing the organism (Cornell et al., 1996). However, because we did not observe any growth differences between our wildtype and mutant, we can only assume that the levels of SAH accumulating in IA3902ΔluxS are not high enough to prohibit growth. However, the SAH levels may have enough impact to negatively affect the functions of one or more cellular processes including methylation and polyamine synthesis.
Discussion

We set out to investigate the impact of the luxS mutation on colonization factors that have not been examined previously and are critical to C. jejuni pathogenesis. The factors focused on in this study included multi-drug efflux pump systems, global regulators, cell morphology, and motility. Based on preliminary work and evidence from previous C. jejuni publications that have looked at similar phenotypes in the luxS mutant, we hypothesized that the luxS mutation would affect each of these factors, as well as the function of the AMC in the cell. The results of this study partially support our hypothesis that luxS mutation has a phenotypic effect on some of the traits we analyzed. For one, the luxS mutation does cause a phenotypic change in the AMC intermediate levels observed in the mutant. This was confirmed by analysis of AMC metabolite levels in IA3902ΔluxS, which contained lower levels of homocysteine and higher levels of SRH than wildtype and complement. This is expected as LuxS is responsible for the conversion of SRH to homocysteine and DPD (De Keersmaecker et al., 2006). The absence of a functional LuxS would lead to accumulation of SRH and simultaneous decreased levels of available homocysteine as it is continually used to form methionine and subsequently the SAM methyl donor.

Recently we profiled the methylome of our IA3902 and IA3902ΔluxS strains (Mou et al., 2015). We hypothesized that the luxS mutation would severely reduce the level of methylation in the luxS mutant compared to wildtype. The logic behind the hypothesis was that the luxS mutation would disrupt the AMC cycle and, as a result, reduce the amount of available SAM to donate methyl groups for DNA methylation, which relies on SAM as its primary methyl donor (Parveen and Cornell, 2011). However,
we found the luxS mutant methylome profile was unchanged compared to wildtype, suggesting the luxS mutation had no effect on DNA methylation. Our AMC metabolite results further support those results as there was only a 25% reduction in SAM metabolite levels in our luxS mutant compared to other metabolites, which had much higher percentages of reduction in comparison to wildtype (99.3% reduction of homocysteine, 81% reduction of cystathionine, and 38.6% reduction of methionine levels in luxS mutant compared to wildtype). The smaller reduction level of SAM in the luxS mutant suggests that the luxS mutant still had a sufficient amount of SAM to result in no significant changes to DNA methylation. Although we do not know the minimum level of SAM required for biological processes like DNA methylation, the fact that there were measurable levels of methionine in the luxS mutant also confirms that there is still some source providing the methionine other than the AMC cycle, thus enabling processes like DNA methylation to function relatively normally. These findings have additional significance when one considers the dual role of LuxS and phenotypic changes associated with its mutagenesis. If these phenotypes are associated with alterations in metabolic function of the AMC, these data would argue that it is not likely to be associated with significant changes in methionine or SAM.

The wildtype and IA3902ΔluxSc showed a complete AMC with comparable levels of almost all major AMC metabolites. The only exception was that no SRH was detected in the complement. This is not a major surprise as the complement strain is believed to have an inherently higher level of LuxS activity than wildtype, due to being expressed in the complement strain by a constitutive promoter instead of its native
promoter. This would, in turn, yield a higher rate of conversion of SRH to homocysteine and DPD than wildtype, and thus quickly depleting the supply of available SRH.

As for the efflux gene expression levels in our luxS mutant, though cmeB and cmeR efflux gene expression levels were not statistically significant, there was a general trend of decreased expression of cmeR efflux gene in the luxS mutant. While any potential biological significance of this trend is unclear, it does provide a rationale for evaluating these changes in different environmental conditions, especially in vivo. One could speculate that the decreased expression of cmeR gene in W7ΔluxS may be triggered by accumulating levels of SAH, as evidenced from the AMC metabolite levels analysis. The lower levels of cmeR gene expression would result in less CmeR protein and thus less repression of CmeABC along with other uncharacterized efflux systems regulated by this repressor. This in turn may lead to increased efflux expression and function that would then assist in extruding the toxic SAH metabolite out of the cell. This type of response is evident in P. aeruginosa where efflux systems were shown to be important in removing toxic metabolites linked to the quorum sensing system function of the organism (Aendekerk et al., 2005).

The unchanged levels of cmeB expression is puzzling as the decreasing cmeR expression would also be expected to lead to fewer CmeR proteins to repress the CmeABC operon, and therefore increased opportunity for more cmeB expression. This lack of change in cmeB expression would argue that the trend of decreased cmeR expression is not likely biologically significant in the conditions tested. Preliminary work from our group (data not shown) found no changes in cmeB expression when we exposed broth cultures of W7ΔluxS to lethal or sublethal doses of extracellular SAH. This, along
with our *cmeB* expression data of W7ΔluxS grown under basal conditions, suggest that CmeABC expression is not induced by SAH and that the cell may not recruit CmeABC efflux activities in response to high extracellular SAH levels that are accumulating inside W7ΔluxS. Despite the lack of change in *cmeB* expression, there is potential for the *luxS* mutation to trigger expression of other efflux systems associated with the CmeR regulator such as putative efflux transporters Cj0035c, Cj0561c, and Cj1619 (Guo et al., 2008). These efflux transporters, rather than CmeABC, may help extrude toxic SAH metabolite out of the cell.

The expression of CmeDEF, an efflux operon that is not regulated by CmeR, was tested in a *luxS* mutant in *C. jejuni* 81-176 under treated and untreated stress conditions (He et al., 2008). The authors found a greater increase in *cmeDEF* expression in the *luxS* mutant compared to wildtype when treated with hydrogen-peroxide. However, the mutant also showed slight increases in *cmeDEF* expression under untreated conditions. Although we did not test *cmeDEF* expression in our *luxS* mutant in this study, this efflux transporter, along with the efflux transporters described above, warrant further evaluation.

Numerous studies have already demonstrated potential roles of multidrug efflux pumps in quorum sensing for *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Bacteroides fragilis*, *Escherichia coli*, *Enterbacteriaceae*, and *Vibrio cholerae* (Martinez et al., 2009). However, in those studies, quorum molecules were found to act as substrates for the efflux pumps or that quorum sensing regulated virulence phenotypes that involve multidrug efflux pumps, such as antibiotic resistance. These published data show strong evidence of quorum sensing in the organisms and the involvement of efflux
pumps in the functions of quorum sensing and vice versa. Our data do not support this mechanism in *C. jejuni*.

In regards to motility through mucin and cellular morphology, we found no evidence of changes in morphology (using real-time imaging flow cytometry and transmission electron microscopy) in our *luxS* mutants. Motility through mucin was also mostly unaffected by the *luxS* mutation, especially for IA3902. However, motility through mucin was slightly affected in one of our *luxS* mutants (W7ΔluxS) when incubated at 42°C, but showed no significant defects in mucin penetration when incubated at 37°C. Plummer et al. previously found that the IA3902 and W7 luxS mutants showed decreased motility compared to wildtype (but with no statistical difference) on a motility agar assay (Plummer et al., 2012). Follow-up studies of those same mutants in our experiments suggest that the decreased motility is not attributed to cell and flagellar morphology based on transmission electron microscope and flow cytometry analyses. We did observe significantly lower number of W7ΔluxS at fraction 9 of the mucin column compared to wildtype, as well low, but not statistically significant, average total CFU of W7ΔluxS in fractions 1 through 9 at 42°C. Complementation of the *luxS* gene partially restored mucin penetration in W7ΔluxSc, indicating that the mucin motility defect is correlated with the *luxS* mutation. The decreased mucin motility of W7ΔluxS may be associated or caused by the motility decrease seen in W7ΔluxS (also incubated at 42°C) by Plummer et al. (Plummer et al., 2012). However, it is unknown what mechanism of the *luxS* mutation would lead to the observed mucin penetration results for W7ΔluxS. IA3902ΔluxS showed no defects in mucin penetration but showed decreased motility on
the motility agar assay. These observations may be specific to the assay as motility is assessed through the use of plates rather than through a homogenous liquid medium.

*V. cholerae* nonmotile strains were found to penetrate mucin much slower than wildtype (Liu et al., 2008). However, when the nonmotile strains were pre-mixed with mucin, they penetrated mucin at the same rate as wildtype. We did not premix our *luxS* mutant with mucin and even with the reduced motility compared to wildtype, the *luxS* mutation and reduced motility had no effect or was not enough to affect its mucin penetration ability. Jeon et al. found decreased motility on the motility agar test in the *luxS* mutant of *C. jejuni* 81116 (though not significantly different from wildtype), but no changes in cell shape or flagellar morphology under transmission electron microscope analysis (Jeon et al., 2003). Though 81116 is not genetically identical to 11168, the fact that our IA3902 and W7 *luxS* mutant strains exhibited the same motility and cell morphology phenotypes as the 81116 *luxS* mutant indicates that what we observed in our *luxS* mutants is not unique and may share similar LuxS-mediated mechanisms as 81116 *luxS* mutants. However, no further studies looking at the phenotypic effects and mechanisms of the LuxS system on other colonization factors in 81116 have been examined.

Other studies have also witnessed similar motility and morphology phenotypes in a *luxS* mutant of a close relative of *Campylobacter, Helicobacter pylori* (Osaki et al., 2006). The mutant exhibited reduced motility on a semi-solid agar and poor colonization in a Mongolian gerbil model, but found no changes in flagellar and cell morphology. Subsequent studies of the LuxS system in *H. pylori* reveal the role of AI-2 mediated
quorum sensing mechanisms in important colonization traits such as biofilm formation, flagellar gene expression, and chemotactic response (Götz et al., 2012b).

The findings from this study reveal how limited our understanding is of the underlying mechanisms of LuxS on the colonization step of *C. jejuni* pathogenesis. The effects of the *luxS* mutation on other aspects of *C. jejuni* pathogenesis steps are also needed to provide a more comprehensive look of the potential regulatory role of LuxS in *C. jejuni*. Potential *C. jejuni* virulence attributes that need to be tested include *in vitro* adhesion and invasion abilities, taxis towards chemoattractants like chicken mucin, L-asparagine, formate, and D-lactate (Vegge et al., 2009); and how stress conditions, like oxidative stress (He et al., 2008), would change the efflux expression in a *luxS* mutant.

In conclusion, we did not find any significant differences in *cmeABC* and *cmeR* expression and cell morphology between our *luxS* mutant and wildtype, which suggest that these phenotypes are not affected by the *luxS* mutation. However, mucin penetration ability was not completely unaffected by the *luxS* mutation. The *luxS* mutation also significantly disrupted the AMC cycle, especially by reducing the available levels of metabolites like homocysteine and cystathionine, which require the immediate enzymatic function of LuxS to catalyze the reactions. The results presented here, collectively with previous LuxS studies in *C. jejuni*, call attention to the complexity and our limited understanding of the LuxS system’s role in *C. jejuni* colonization and general pathobiology. The questions postulated above will help the field move towards complete characterization of the LuxS mechanisms in *C. jejuni* disease and enable utilization of that knowledge towards development of novel therapeutics against *C. jejuni* diseases.
Acknowledgements

We thank Dr. Qijing Zhang and members of the Zhang lab for their *Campylobacter* expertise and occasional use of their lab facilities for part of this project, Dr. Dave Barrett’s services for measuring AMC metabolite levels in our samples, and Dr. Bonnie Bassler for the *Vibrio harveyi* strains used in the AI-2 assays. This work was supported by the Agriculture and Food Research Initiative Fellowships Grant Program of the USDA National Institute of Food and Agriculture Grant number 2013-67011-21155, the National Institute for Allergy and Infectious Diseases grant number K08AI07052303, and Iowa State University startup funds.
Figure legends.

Figure 1.

Expression of *cmeB* (A) and *cmeR* (B) in W7ΔluxS and W7ΔluxSc compared to W7 wildtype over a 48 hour growth period. Samples of broth culture grown at 42°C were taken at time points 8, 16, 24, and 32 hours to measure transcript levels of *cmeB* and *cmeR* using qPCR. Bars represent the mean transcript fold-change ± SD of three independent experiments, relative to W7, with 16s rRNA used as an internal control. No significant differences were found.

Figure 2.

Transmission electron micrographs reveal no morphological changes in cell shape or flagella in wildtype, *luxS* mutant (ΔluxS), and *luxS* complement (ΔluxSc) of both strain backgrounds A) W7 and B) IA3902.

Figure 3.

Penetration of W7, W7ΔluxS, and W7ΔluxSc through mucus while incubating at 37°C (A, B) or 42°C (C, D). Columns of 30mg/ml porcine gastric mucin in PBS were prepared in 1-ml tuberculin syringes, with bacteria inocula applied to the top of the column. The columns were incubated at 37°C (A, B) or 42°C (C, D) for 30 min. After the incubation period, each fraction consisting of 0.1ml mucin was collected from the bottom of the column and plated for bacterial quantification. Bars represent the mean CFU/ml ± SD of three independent experiments. Fraction 1 represents the bottom of the column, fraction 9 at the top. Graphs for Figures 3A and 3C depict mean CFU/ml ± SD at each fraction. Figures 3B and 3D depict mean CFU/ml ± SD of total bacteria from fractions 1 through 9. Asterisks indicate significantly lower number of W7ΔluxS at the specific fraction of mucin column compared to wildtype.

Figure 4.

Penetration of IA3902, IA3902ΔluxS, and IA3902ΔluxSc through mucus while incubating at 37°C (A, B) or 42°C (C, D). Assay was the same as described in Figure 3. Bars represent the mean CFU/ml ± SD of three independent experiments. Fraction 1 represents the bottom of the column, fraction 9 at the top. Graphs for Figures 4A and 4C depict mean CFU/ml ± SD at each fraction. Figures 4B and 4D depict mean CFU/ml ± SD of total bacteria from fractions 1 through 9. No significant differences were found.
Figure 1.

A.

B.
Figure 2.

A.

W7

W7ΔluxS
Figure 2 (continued).

**W7ΔluxSc**

**B.**

**IA3902**
Figure 2 (continued).

IA3902ΔluxS

IA3902ΔluxSc
Figure 3.

A.

B.
Figure 3 (continued).

C.

D.
Figure 4.

A.

B.
Figure 4 (continued).

C.

D.
Tables.

Table 1. List of all bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Description</th>
<th>Source</th>
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</thead>
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<tr>
<td>W7</td>
<td>Highly motile variant of NCTC 11168</td>
<td>(Plummer et al., 2012)</td>
</tr>
<tr>
<td>W7ΔluxS</td>
<td>W7 mutant with $kan^R$ inserted into luxS</td>
<td>(Plummer et al., 2012)</td>
</tr>
<tr>
<td>W7ΔluxSc</td>
<td>W7ΔluxS with a chromosomal complement of LuxS, contains $cm^R$ and $kan^R$ genes (acquired from IA3902ΔluxSc)</td>
<td>This study</td>
</tr>
<tr>
<td>IA3902</td>
<td>C. jejuni isolate of clone SA</td>
<td>(Sahin et al., 2008)</td>
</tr>
<tr>
<td>IA3902ΔluxS</td>
<td>IA3902 mutant with $kan^R$ inserted into luxS</td>
<td>(Plummer et al., 2012)</td>
</tr>
<tr>
<td>IA3902ΔluxSc</td>
<td>IA3902ΔluxS with a chromosomal complement of LuxS, contains $cm^R$ and $kan^R$ genes</td>
<td>(Plummer et al., 2012)</td>
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Table 2. Primers used in this study.

<table>
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<td>CACAAAAAGTTTTTAGCCAGACAAG</td>
<td>This study</td>
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<td>cmeR-R</td>
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<td>(Han et al., 2008)</td>
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<td>(Han et al., 2008)</td>
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Table 3. Normalization scores of 17 morphological characteristics of W7 and IA3902 strains grown at 37°C (A) and 42°C (B). At least 1500 cell images for each strain were captured and calculated for morphology differences using 17 different morphological features for comparison. The resulting normalization scores or RD values of luxS mutant and luxS complement were normalized to their respective wildtype. RD values of over 1 were considered significant differences, although no significant differences were detected in any of the morphology characteristics compared here.

A.

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<th>Strain</th>
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<th>Aspect Ratio</th>
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<th>Diameter</th>
<th>Elongatedness</th>
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<th>Length</th>
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Table 3 (continued).

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Table 4. LC-ESI-MS/MS analysis shows expected AMC metabolite levels within *luxS* mutant compared to wildtype and *luxS* complement. Values are presented as ratios of metabolite peak areas to internal standards corrected to the endogenous concentration of the metabolites in a standard cell pellet. Metabolite abbreviations: SAM (S-adenosylmethionine), SAH (S-adenosylhomocysteine), SRH (S-ribosylhomocysteine), Hcyst (Homocysteine), Met (Methionine), Cysta (Cystathionine)

<table>
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<tr>
<th>Strain</th>
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<th>SAH</th>
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CHAPTER 3. A COMPARATIVE ANALYSIS OF METHYLOME PROFILES OF CAMPYLOBACTER JEUNI SHEEP ABORTION ISOLATE AND GASTROENTERIC STRAINS USING PACBIO DATA


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Abstract

Campylobacter jejuni is a leading cause of human gastrointestinal disease and small ruminant abortions in the United States. The recent emergence of a highly virulent, tetracycline-resistant C. jejuni subsp. jejuni sheep abortion clone (clone SA) in the United States, and that strain’s association with human disease, has resulted in a heightened awareness of the zoonotic potential of this organism. Pacific Biosciences’ Single Molecule, Real-Time sequencing technology was used to explore the variation in the genome-wide methylation patterns of the abortifacient clone SA (IA3902) and
phenotypically distinct gastrointestinal-specific *C. jejuni* strains (NCTC 11168 and 81-176). Several notable differences were discovered that distinguished the methylome of IA3902 from that of 11168 and 81-176: identification of motifs novel to IA3902, genome-specific hypo- and hypermethylated regions, strain level variability in genes methylated, and differences in the types of methylation motifs present in each strain. These observations suggest a possible role of methylation in the contrasting disease presentations of these three *C. jejuni* strains. In addition, the methylation profiles between IA3902 and a *luxS* mutant were explored to determine if variations in methylation patterns could be identified that might explain the role of LuxS-dependent methyl recycling in IA3902 abortifacient potential.

**Introduction**

As a major cause of human gastroenteritis worldwide, *Campylobacter jejuni* is responsible for over 400 million cases of diarrhea each year (Ruiz-Palacios, 2007) and is among the leading causes of foodborne disease related hospitalizations in the United States (Scallan et al., 2011). In the past two decades, a highly virulent *C. jejuni* clone, named clone SA, has emerged to become the predominant cause of *Campylobacter*-associated sheep abortions in the United States (Sahin et al., 2008). This clone is tetracycline-resistant, leaving drug treatment options limited as tetracycline is the only approved class of antibiotics for treating sheep abortions (Sahin et al., 2008). In addition, recent findings suggest a zoonotic potential for transmission of clone SA from animals to humans (Sahin et al., 2012). Though there is only evidence linking clone SA with human
gastroenteritis cases, its ability to cause more severe disease in humans cannot be ruled out.

Concurrently, the widespread emergence of antibiotic resistant *Campylobacter* in both human and animal medicine, combined with limited alternative treatment options has shifted research efforts towards identifying alternative therapeutic and preventative strategies against *Campylobacter* (Luangtongkum et al., 2009). This underscores the importance of improving our understanding of *Campylobacter* pathogenesis to develop appropriate and effective novel treatment interventions.

A recent study by Wu et al. (Wu et al., 2013) found that the genome of IA3902, a clinical isolate of clone SA, is remarkably syntenic with that of *C. jejuni* subsp. *jejuni* gastroenteric strains NCTC 11168 (Parkhill et al., 2000) and to a lesser extent that of 81-176 (Russell et al., 1989). The pVir plasmids of IA3902 and 81-176 are also syntenic (Wu et al., 2013). However, the disease presentations between the gastroenteric strains 11168 and 81-176, and abortigenic IA3902 are very different. More specifically, our research group demonstrated that 11168 would not induce abortion following oral inoculation in the pregnant guinea pig model (Burrough et al., 2009). The differences in disease presentation were found not to be due to the presence of major pathogenicity islands or virulence genes associated with an abortion phenotype (Wu et al., 2013). However, comparative genomic analysis by Wu et al. (Wu et al., 2013) identified several differences in global gene expression profiles between IA3902 and 11168, which were attributed to small genomic changes within the chromosomes, including a large number of single-nucleotide polymorphisms and indels. We expanded on this hypothesis and
propose that DNA methylation may explain the differences in disease presentation in *C. jejuni*.

Enzymes that carry out DNA methylation activities are part of restriction-modification (R-M) systems, which are best known for their role in prokaryotic defense mechanisms (Vasu and Nagaraja, 2013). R-M systems are grouped into four classes (Types I, II, III, and IV) and are classified based on the enzyme composition and associated cofactors, specific base position methylated in the recognized sequence motif, and the symmetry of the motif on the double-stranded DNA (Roberts et al., 2003). In addition to prokaryotic defenses, methylation serves numerous other important roles associated with gene expression and regulation, cell maintenance, and virulence (Marinus and Casadesus, 2009).

We first explored whether the methylation patterns between IA3902 and *C. jejuni* strains 11168 (Parkhill et al., 2000) and 81-176 (Russell et al., 1989) were different. This was determined by using Pacific Biosciences’ Single-Molecule, Real-Time (SMRT) sequencing technology (Flusberg et al., 2010) to characterize the genome methylation patterns for IA3902. Previous methods for detecting DNA methylation and other common epigenetic markers at the genomic level have been difficult due to the lack of quick and simple methods sensitive enough to detect such markers (Korlach and Turner, 2012). However, the advent of Pacific Biosciences’ SMRT sequencing has made it possible to detect such markers quickly and directly map genome-wide methylation patterns of bacteria (Davis et al., 2013). We utilized the methylation data of 11168 and 81-176 from a recent publication (Murray et al., 2012) to compare the methylation profiles with IA3902.
Previously, we reported that a *luxS* mutation in IA3902 significantly lowers its virulence (Plummer et al., 2012). Therefore, the mutation in the LuxS enzyme and its effect on the methylation of IA3902 was also investigated. *C. jejuni* possess the Autoinducer-2/LuxS system, which is well-known in other bacterial species for its quorum sensing role as well as a promising drug target candidate (Sintim et al., 2010). A study from our group found the *luxS* mutation compromised the abortion phenotype of IA3902, when administered orally in a pregnant guinea pig model, but not intraperitoneally (Plummer et al., 2012). The fact that the *luxS* mutant was unable to cause abortions when inoculated orally in the guinea pig model suggests that the mutant was compromised in its ability to colonize, invade the enteric epithelium, and enter systemic circulation (Plummer et al., 2012).

Interestingly, LuxS is a dual-purpose enzyme that is also an important component of the activated methyl cycle (AMC), which is a primary source of methyl groups for DNA methylation (Parveen and Cornell, 2011). In *C. jejuni*, studies have already shown the importance of methylation for virulence expression, such as adhesion and invasion of host intestinal epithelia (Kim et al., 2008; Anjum, 2013). These two traits coincide with those hypothesized to be affected by the *luxS* mutation in the pregnant guinea pig sheep abortion model (Plummer et al., 2012). Based on these observations, we hypothesize that the *luxS* mutation could impact methylation and ultimately attenuate the virulence expression and abortion phenotype of IA3902.

The work in this study provides a comprehensive analysis of the methylome of IA3902. In addition, the in-depth analysis of the methylation motif distributions and
genes located within these hyper- and hypomethylated regions of interest together provide extensive insights into the pathobiology of these C. jejuni strains.

Materials and Methods

Bacterial strains and growth conditions

C. jejuni subsp. jejuni IA3902 is a clinical isolate of clone SA and its full genome sequence has been determined (Wu et al., 2013). IA3902 and its LuxS mutant (IA3902ΔluxS) (Plummer et al., 2012) were routinely grown in Mueller-Hinton broth or agar incubated in gas jars at 42°C in a microaerobic environment (5% O₂, 10% CO₂, 85% N₂) until 24 h (stationary growth phase). C. jejuni subsp. jejuni NCTC 11168 and 81-176 both originated from human gastroenteritis, have been genome-sequenced, and are commonly studied by researchers around the world (Parkhill et al., 2000; Hofreuter et al., 2006).

Genomic DNA isolation and preparation for sequencing

DNA extraction from IA3902 and IA3902ΔluxS was performed using Wizard Genomic DNA purification kit (Promega, Madison, WI). The NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and Qubit fluorometer (Life Technologies, Grand Island, NY) were used to measure DNA quantity and quality. Approximately 10 μg of genomic DNA per strain were sent to Pacific Biosciences for library preparation and SMRT sequencing.
**Sequencing library preparation and SMRT sequencing**

Preparation of IA3902 and IA3902ΔluxS DNA samples for Single-Molecule, Real-Time (SMRT) sequencing was performed as previously described (Travers et al., 2010; Clark et al., 2012). Genomic DNA was randomly sheared to approximately 15 kb using gTUBEs (Covaris, Inc., Woburn, MA). Fragmented DNA was damage repaired, end repaired, and ligated to hairpin adapters using standard SMRTbell template preparation protocols (Pacific Biosciences, Menlo Park, CA). SMRTbell templates with sizes greater than 5-10kb were size-selected with Blue Pippin (Sage Science, Beverly, MA). SMRT sequencing was carried out using P4/C2 chemistry with 4 SMRT Cells per sample. Genomes were assembled into single contigs using the HGAP algorithm as part of the SMRT analysis suite v2.0 (Chin et al., 2013).

**Bioinformatic analysis of methylation motifs**

Identification of methylated motifs from SMRT sequencing data on IA3902, IA3902ΔluxS, 11168, and 81-176 was performed using the SMRT analysis suite v2.0 as previously described (Murray et al., 2012). Methylated motif information from other *Campylobacter* strains was obtained from the Restriction Enzyme database (REBASE) (Roberts et al., 2010). The output from SMRT sequencing analysis also included each motif’s methylation site, methylation score and the extent of genome methylation for each sequence motif (Supplementary Datasheets 2-5).

Based on the methylation data, and genome annotations available for IA3902 and *C. jejuni* strains in NCBI, genes that were methylated in each strain were identified (Quinlan and Hall, 2010). The methylation motifs associated with each gene were also analyzed in this paper. For every gene, we assigned a functional group or role category as
described elsewhere (Wu et al., 2013). Then, for every methylation motif, we obtained the total number of methylated genes in each role category.

**Bioinformatic analysis of whole-genome methylation motif distribution plots**

The statistical software package R was used to generate methylation motif distribution plots for the whole genome of each strain using a bin size of 1000bp. Methylation sites on both strands of the DNA were considered. Mean and standard deviations are reported in the results. In this study, bins that have more than 41-45 methylation sites (the actual number mean + three times standard deviation varies for each strain) per bin were considered as hypermethylated regions. Bins that have 4 or less methylation sites were considered as hypomethylated regions.

**Results**

**SMRT sequencing and methylome analysis of IA3902**

SMRT sequencing of IA3902 yielded one circular chromosome (1.64Mb) and one circular plasmid (0.037Mb). Its genome encoded seven total recognition sites for methylation, also known as sequence motifs (Table 1). At least 98.8% of all seven motifs present in the genomes were detected by SMRT sequencing. We found two pairs of bipartite motifs (TAAYN$_5$TGC/GCAN$_5$RTTA, and GAGN$_5$RTG/CAYN$_5$CTC), a palindromic motif (RAATTY/YTTAAR), and two non-paired motifs (CAAAYG and GAAGAA) (methylated adenines are underlined). Motifs were compared to predicted IA3902 data in the Restriction Enzyme Database REBASE (http://rebase.neb.com/rebase/rebase.html), a web-based database containing comprehensive information about all genes, enzymes, and genomes involved in DNA
restriction and modification (Roberts et al., 2010). Each bipartite motif is recognized and modified by the same enzyme (e.g. TAAYN₅TGC is recognized by the R-M enzyme CjeIAORF994P, and GAGN₅RTG is recognized by M.CjeIAII).

The motifs CAAAYG and GAAGAA were novel discoveries as they have not been previously predicted for IA3902 in REBASE. Both motifs have been submitted to REBASE for inclusion in the database. These were also the only motifs methylated on just one DNA strand while all other motifs were methylated on both strands. The methylome of IA3902 revealed that more than 98% of the identified motifs were modified. More specifically, the adenine base was the only base methylated in the sequence motifs (Table 1). This type of modification, N6-methyladenine (m6A), was also the only form of base methylation found in two commonly studied C. jejuni subsp. jejuni gastroenteric strains NCTC 11168 and 81-176 in an earlier study (Murray et al., 2012).

Five enzymes were identified that are responsible for the m6A methylation on the seven motifs (Table 2). The enzymes detected in the IA3902 strains are part of Types I or II R-M systems. Type I systems are enzyme complexes made up of subunits with restriction (R), methylation (M), and specificity (S) activities (Vasu and Nagaraja, 2013). In this study, M.CjeIAII was the only known Type I methyltransferase (MTase). On the other hand, Type II systems are simpler and ubiquitous (Bujnicki, 2001). They encode two proteins with separate activities: one as an endonuclease and the other as a MTase. In this case, all other MTases identified in this study were Type II, including CjeIAORF32P, M.CjeIAI, CjeIAORF654P, and CjeIAOF994P.

As of recently, three of the detected motifs (TAAYN₅TGC, RAATTY, and GAGN₅RTG) have already been reported in REBASE. These three motifs were also
predicted to be recognized by three IA3902 R-M enzymes (M.CjeIAl, M.CjeIAI, and CjeIAOF994P, respectively) (Table 1). There was, however, no mention of any IA3902 R-M enzyme that recognized GAAGAA and CAAAYG. We predicted that the R-M enzymes CjeIAORF32P and CjeIAORF654P would recognize GAAGAA and CAAAYG, respectively (Table 2). This prediction was based on CjeIAORF654P having 97% and 96% DNA sequence identity with previously characterized R-M enzymes of other Campylobacter jejuni strains, CjeFIII (C. jejuni 81-176) and CjeNIII (C. jejuni NCTC 11168) (Supplementary Datasheet 1, Table 3). Since the closest IA3902 motif to these two motifs was CAAAYG, we thus predicted that CjeIAORF654P would recognize CAAAYG. As the remaining R-M enzyme without a candidate motif assigned to it, CjeIAORF32P was predicted to recognize GAAGAA by default (Supplementary Datasheet 1, Table 5). However, these were only predictions and the function of the MTases will need to be tested for confirmation. Thus far, the results have revealed novel information about the forms of methylation and associated motifs found in IA3902.

**Comparative analysis of IA3902 motifs with C. jejuni gastroenteric strains 11168 and 81-176**

Evidence for differing methylation patterns between closely-related C. jejuni strains NCTC 11168 and 81-176 (Murray et al., 2012) prompted our investigation for comparisons with IA3902. The results from Wu et al. (Wu et al., 2013) suggested that small genomic changes are cause for differences in global gene expression profiles and thus disease presentations. One such small genomic change is DNA methylation, which lead to the hypothesis that IA3902 methylome profile will be different from the gastroenteric strains and possibly explain for the unique hyper virulence of each strain.
Supplementary Datasheet 1, Tables 1-5 show a list of all motifs known and detected in this study for *C. jejuni* strains IA3902, 11168, and 81-176. In addition, the associated MTases of the motifs are listed.

Of the motifs identified, the following were identical or homologous between the 3 strains (Supplementary Datasheet 1, Tables 1-3): TAAYN₅TGC/GC₅N₅RTTA, RAATYPE, and the set CAAAYG (IA3902), GKAAYG (11168), and GCAAGG (81-176). The motifs GAGN₅RTG/CAYN₅CTC (IA3902) and GAGN₅GT/AC₅N₅CTC (11168) were homologous, but only between IA3902 and 11168 (Supplementary Datasheet 1, Table 4). Motifs homologous between only IA3902 and 81-176 included GAAGAA (IA3902) and GGRCA (81-176) (Supplementary Datasheet 1, Table 5). The only motifs that did not have a homologue with IA3902 were GAGAN₅GMT motif in 11168, and CAAYN₅ACT/GTN₅RTTG motif in 81-176.

When we analyzed each strain’s MTases and their associated motifs, we discovered that M.CjeIAII (IA3902) showed 100% identity with M.CjeNIV (11168) and M.CjeFIV (81-176), were all Type I restriction enzymes, and recognized the motif TAAYN₅TGC (Supplementary Datasheet 1, Table 1). In Supplementary Datasheet 1, Table 2, M.CjeIAI (IA3902) showed 100% identity with M.CjeNI (11168) and M.CjeFI (81-176), and all three enzymes were Type II DNA MTases that recognized the RAATYPE motif. CjeIAORF654P (IA3902), as described earlier, showed 96% homology with CjeNIII (11168), and 97% homology with CjeFIII (81-176) (Supplementary Datasheet 1, Table 3). The motifs recognized by these Type II enzymes, GKAAYG (11168) and GCAAGG (CjeFIII of 81-176), were homologous with IA3902 motif CAAAYG.
In Supplementary Datasheet 1, Table 4, the Type II enzyme CjeIAORF994P (IA3902) and CjeNII (11168) were 86% homologous, and both recognized similar motifs: $G\text{AGN}_3\text{RTG}$ (IA3902) and $G\text{AGN}_3\text{GT}$ (11168). Lastly, CjeIAORF32P (IA3902) had 65% homology with CjeFV (81-176). Since CjeFV recognized the motif GGRCA, we thus grouped it with CjeIAORF32P and its recognized motif GAAGAA (Supplementary Datasheet 1, Table 5). Altogether, each of the seven motifs of IA3902 was homologous with at least one other motif in 11168 and/or 81-176.

**Comparative analysis of methylome distributions of IA3902, C. jejuni 11168 and 81-176**

The methylomes of IA3902, 11168 and 81-176 contained a total number of methylated bases ranging from 13,835 (81-176), 14,632 (11168), and 15,748 (IA3902). The oriC regions of all three strains were realigned to the exact same region (dnaA gene from nucleotide positions 1-1323, and the origin of replication, or oriC, from nucleotide positions 1324-1482). Although the motifs and MTases were similar between the three strains, distinguishing aspects of each strain’s methylome density plot revealed otherwise.

The density distributions of all motifs were evaluated across the genomes of each of the three C. jejuni strains, shown in Figures 1-4. The density distributions of each motif across the genome of IA3902 are shown in Figure 2. The plots showed a general even distribution of all methylation motifs across the genome except for several distinct regions of hyper- and hypo-frequency of methylation motifs. For ease of description, these regions are called hyper- and hypomethylated regions. Genes in a bin with methylation motif frequency values greater than the mean frequency plus three times the standard deviation were deemed hypermethylated genes. The mean frequency values for
IA3902, 11168, and 81-176 were 20.15, 18.62, and 18.12, respectively. The standard deviation values were 8.39, 7.55, and 7.92, respectively. Thus, genes would be considered hypermethylated if the number of methylation motifs within these genes (motif frequencies) were greater than 45 (IA3902) or 41 (11168 and 81-176). Genes in a bin with methylation motif frequency values of four or less were considered hypomethylated genes.

**Major hypomethylated regions**

Observation of the plots in Figures 1, 3, and 4 revealed the presence of three defined hypomethylated regions within the genome of each strain. These regions were considered major hypomethylated areas since they contained multiple adjacent bins that were hypomethylated. The locations of these major hypomethylated areas were clearly different between IA3902 and its gastroenteric counterparts 11168 and 81-176, which are indicated in bold in Supplementary Datasheet 1, Table 6. In addition, the genes at these hypomethylated areas in 11168 and 81-176 were extraordinarily similar while IA3902 had a very different and much wider set of genes (Supplementary Datasheet 1, Table 7).

More specifically, 11168 and 81-176 both had 16S and 23S ribosomal RNA genes in these major hypomethylated areas as shown in Supplementary Datasheet 1, Table 7. 81-176 also had genes coding for hypothetical protein and putative outer-membrane proteins. Genes in the major hypomethylated regions of IA3902, however, varied considerably from the other two strains, including: cell division protein FtsA, flagellar basal-body rod protein, glutamine synthetase type I, DNA gyrase subunit A, putative multidrug efflux transporter, putative ferredoxin, selenocysteine synthase, and a hypothetical protein (Supplementary Datasheet 1, Table 7). Flagellar basal-body rod
protein (Konkel et al., 2004), DNA gyrase subunit A and putative multidrug efflux transporter (Iovine, 2013), and putative ferredoxin (van Vliet et al., 2001) are known virulence genes in *Campylobacter*. These and all other genes in the major hypomethylated regions of IA3902 (with the exception of selenocysteine synthase and hypothetical protein) are also associated with virulence in other bacterial species (Klose and Mekalanos, 1997; Ran Kim and Haeng Rhee, 2003; Sjöblom et al., 2008; Kawai et al., 2011; Schweizer, 2012; Swick et al., 2013). The hypomethylation of a large number of genes in a concentrated area of the genome (major hypomethylated region) may suggest a role in gene or virulence expression. However, it remains to be determined whether this is the case and is an indication of gene regulation.

**Minor hypomethylated regions**

In addition to the major hypomethylated areas, there were numerous single hypomethylated bins scattered across the genome, which, for ease of description, will be called minor hypomethylated regions. The locations of these regions are described in Supplementary Datasheet 1, Table 6. Genes unique to IA3902 in these areas and listed in Supplementary Datasheet 1, Table 7 included: tetrapyrrole methylase family protein, 16S ribosomal RNA methyltransferase RsmE, phosphoribosylformylglycinamidine synthase I, olyopolyglutamate synthase/dihydrofolate synthase, uracil phosphoribosyltransferase, putative peptide ABC-transport system periplasmic peptide-binding protein, DNA topoisomerase I, putative metallo-beta-lactamase family protein, dimethyladenosine transferase, and several hypothetical proteins. None of these genes are well-known *Campylobacter* virulence factors. However, the genes such as uracil phosphoribosyltransferase (Koyama et al., 2012), putative peptide ABC-transport system
periplasmic peptide-binding protein (Garmory and Titball, 2004), DNA topoisomerase I (Galán and Curtiss, 1990), putative metallo-beta-lactamase family protein (Maltezou, 2009), and dimethyladenosine transferase (Chiok et al., 2013) are important for the virulence of other organisms and also serve as potential drug targets. For example, the putative metallo-beta-lactamase family protein found in IA3902 is among a growing list of contributing sources to antimicrobial resistances in ESKAPE pathogens (Bassetti et al., 2013). These antibiotic-resistant pathogens are the cause of a majority of US hospital infections and consist of Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter species (Boucher et al., 2009).

Of the genes unique to 11168 in the minor hypomethylated areas (Supplementary Datasheet 1, Table 7), only fumarate reductase subunits (Kassem et al., 2012), ATP-dependent protease La (Cohn et al., 2007), and molecular chaperone GroEL (Klančnik et al., 2006) were known for their roles in C. jejuni virulence. These genes, along with other minor hypomethylated genes (glutamine transporter permease and cystathionine beta-lyase), have virulence homologues in other bacterial organisms (Ejim et al., 2004; Buettner et al., 2008; Zhu et al., 2009; Breidenstein and Hancock, 2012; Kupper et al., 2014). In 81-176, the only genes that were unique to this strain, found in the minor hypomethylated regions, and have a role in C. jejuni virulence included RelA/SpoT family protein (Gaynor et al., 2005) and flagellar hook-associated protein FlgK (Fernando et al., 2007; Neal-McKinney and Konkel, 2012) (Supplementary Datasheet 1, Table 7). These two genes and other genes in the minor hypomethylated regions of 81-176 (succinyl-CoA synthetase (subunits alpha and beta), cell division protein FtsZ,
flagellar basal-body rod protein, putative cytochrome oxidase maturation protein (cbb3-type), heavy metal translocating P-type ATPase, uridylate kinase, and 1-deoxy-D-xylulose 5-phosphate reductoisomerase) are also important virulence determinants in other bacterial species (Ji and Silver, 1995; Ran Kim and Haeng Rhee, 2003; Henry et al., 2005; Dozot et al., 2006; Tchawa Yimga et al., 2006; Wu et al., 2006; Jiménez de Bagüés et al., 2007; Lee et al., 2007; Brown and Parish, 2008).

**Hypermethylation**

Even fewer similarities were found in the types of genes that were hypermethylated in all three strains. Genes were considered hypermethylated in a strain if motif frequencies were greater than three standard deviations above the mean: 45 (IA3902) or 41 (11168 and 81-176). IA3902 had two hypermethylated regions, 11168 had one, and 81-176 had four regions. The locations of these hypermethylated regions and the genes present in these regions are shown in SupplementaryDatasheet 1, Tables 8 and 9, respectively.

The first hypermethylated region in IA3902 was located near the oriC and consisted of the following genes (Supplementary Datasheet 1, Table 8): chromosomal replication initiator protein DnaA, glutamate synthase subunit beta, ribonuclease HII, ExsB protein, DsbB family disulfide bond formation protein, methyl-accepting chemotaxis protein, cytochrome c551 peroxidase, FAD-dependent thymidylate synthase, CTP synthetase, and hypothetical proteins. The genes present in the second hypermethylated region towards the end of the genome included putative periplasmic toluene tolerance protein and putative integral membrane protein (Supplementary Datasheet 1, Table 8). Of these genes, only DsbB family disulfide bond formation protein
(Łasica et al., 2010), methyl-accepting chemotaxis protein (Vegge et al., 2009; Li et al., 2014) and cytochrome c551 peroxidase (Hendrixson and DiRita, 2004) are recognized virulence genes in C. jejuni. The Dsb protein (Heras et al., 2009), methyl-accepting chemotaxis protein (Dons et al., 2004; Terry et al., 2005; Nishiyama et al., 2012), FAD-dependent thymidylate synthase (Ulmer et al., 2008), and putative periplasmic toluene tolerance protein (Sardessai and Bhosle, 2002) had virulence homologues in other bacterial organisms too. All motifs were found in the hypermethylated genes of IA3902, and RA\_ATTY was detected in every hypermethylated chromosomal gene (Supplementary Datasheet 1, Table 8).

As for the pVir plasmid in IA3902, a number of hypermethylated genes were also found (Supplementary Datasheet 1, Table 9). Such genes included VirB4, VirB8, and VirB9 virulence proteins; phage protein, TrbM-like protein, and numerous hypothetical proteins. Of these genes, VirB9 (Kienesberger et al., 2011) is the only known gene in Campylobacter with virulence capabilities. VirB4 (Juhas et al., 2008; Gokulan et al., 2013; Sánchez-Zauco et al., 2013) and VirB8 (Baron, 2006) are well-known for their involvement in Type IV secretion systems, but their virulence roles are unknown in Campylobacter. All motifs except for TA\_YN\_TGC were found in the plasmid genes. Only RA\_ATTY was found in most genes, except for CJSA\_pVir0050 and CJSA\_pVir0051.

The C. jejuni strain 11168 had only one hypermethylated gene located in the middle of the genome, with the motifs RA\_ATTY and ACN\_CTC (Supplementary Datasheet 1, Table 8). This gene, para-aminobenzoate synthase component I, unlike other bacterial organisms (Shinohara et al., 2005), has no known role in C. jejuni virulence. 81-
176 had four hypermethylated regions: three at the beginning of the genome and one near the end of the genome (Supplementary Datasheet 1, Table 8). The genes present in these regions have no known association with *Campylobacter* virulence (Supplementary Datasheet 1, Table 9). However, RarA has an antimicrobial resistance role in other bacterial species (De Majumdar et al., 2013; Veleba et al., 2013), which, like the other genes not yet found to be involved in *Campylobacter* virulence, could have a potential role for virulence in *C. jejuni*. In addition, all motifs were found in the hypermethylated genes of 81-176 except for TA\_YN\_TGC.

**General trends in *C. jejuni* strain methylation motifs of hypo- and hypermethylated genes**

A common theme among all three strains was that RA\_TTY was the only motif found in almost every hyper- and hypomethylated gene. The only genes of the genome that did not contain a RA\_TTY motif were: Flagellar basal-body rod protein (CJSA\_0661), putative ferredoxin (CJSA\_1311), 16S ribosomal RNAs (Cjr01, Cjr04, CJJ81176\_1711, CJJ81176\_1724), 23S ribosomal RNAs (Cjr02, Cjr05, Cjr08, CJJ81176\_1707, CJJ81176\_1727), and putative periplasmic protein (CJJ81176\_0993).

Another similarity linking IA3902 with one of the gastroenteric strains was the hypomethylation of the flagellar basal-body rod protein. This was the only hypomethylated gene in common between IA3902 and 81-176, although they were located in different types of hypomethylated regions (major in IA3902, minor in 81-176) (Supplementary Datasheet 1, Table 6). In addition, both strains had the TA\_YN\_TGC motif in the flagellar basal-body rod protein gene.
While there were some similarities among all three strains, we also noticed several differences distinguishing IA3902 from 11168 and 81-176. For instance, IA3902 had a far greater number of genes that were hypo- and hypermethylated in contrast to 11168 and 81-176. This was especially evident with the number of hypermethylated genes in IA3902 chromosome and plasmid surpassing the number of hypermethylated genes in 11168 and 81-176 combined. Eight well-characterized C. jejuni virulence genes were found in both the hyper- and hypomethylated regions of IA3902. In contrast, only three or two known C. jejuni virulence genes were detected in the hypomethylated regions of 11168 and 81-176, respectively. IA3902 was the only strain to have all its motifs present in the hypermethylated genes. 11168 and 81-176 had at least one motif that was not found in a hypermethylated gene. IA3902 was also the only strain that had hypermethylation at the oriC site, specifically in the dnaA gene. The significance for hypermethylation at this site in only IA3902 is presently unknown and will require further investigation.

Another difference was the tendency for one motif to dominate over the others in a specific hypomethylated region (major or minor), and only observed in 11168 and 81-176 (Supplementary Datasheet 1, Table 6). For example, in 11168, RAATTY was found most often in the minor hypomethylated areas and GKAAYG was found most often in the major hypomethylated areas. This same pattern was observed in 81-176. Specifically, RAATTY was more prevalent in the minor hypomethylated genes while GGRCA was more prevalent in the major hypomethylated genes. However, for IA3902, the homologue to GKAAYG and GGRCA, GAAGAA, was not the most common motif in the major
hypomethylated regions. Instead, it was RAATTY, which is prevalent in the minor as well as the major hypomethylated areas.

The genes in the minor hypomethylated regions (Supplementary Datasheet 1, Table 7) shared only between 11168 and 81-176 included cmeB efflux pump, elongation factor Tu, and Type I restriction enzyme (R subunit). CmeB efflux pump was the only known virulence gene in *C. jejuni* (Lin et al., 2002) among these set of genes while elongation factor Tu has a virulence role in other bacterial species (Kunert et al., 2007; Wang et al., 2013; Al-Maleki et al., 2014; Mohan et al., 2014). The motifs seem to be of the same type when comparing the same gene between 11168 and 81-176 (Supplementary Datasheet 1, Table 6). For example, 11168 had RAATTY and ACN<sub>5</sub>CTC in all three genes while 81-176 had RAATTY and GGRCA in all three of its genes. A trend was observed among this example and the flagellar basal-body rod protein gene present in both IA3902 and 81-176. The pattern observed was that a gene present in two different strains had the same type of motif and was recognized by Type II R-M enzymes. This may indicate conservation in the sequence motif and its associated R-M enzyme for the expression of the particular gene. This conservation may be part of what defines the species or important for the survival of the organism. However, the significance for the conservation of a sequence motif will require further epigenetic studies in other *Campylobacter* species to understand its importance in the evolution of the species.
**Comparative analysis of IA3902 methylated genes by role category with C. jejuni gastroenteric strains 11168 and 81-176**

The functional roles of the genes were examined based on methylation pattern (hyper- and hypomethylation). Genes were classified into role categories (functional role of the genes) for each motif and all motifs combined. The percent total was then calculated for methylated genes enriched with a specific motif for each role category and made side-by-side comparisons between all three strains (Supplementary Datasheet 1, Table 10).

RAATTY was found in 90-100% of the genes in all role categories for each strain. This corresponded with its prevalence in the hypo- and hypermethylated genes. In addition, RAATTY contained the greatest number of high percentage methylated genes (ranging from 90-100% methylation) in all role categories except for two. These two exceptions were “Mobile and extrachromosomal element functions” and “rRNA and stable RNAs,” which not only had no detectable RAATTY motifs, but also no other motif. The potential reason for not finding motifs in the “rRNA and stable RNAs” category is because modifications on RNA are post-transcriptional (Helm, 2006; Grosjean, 2009). The genes in this category may have motifs on the RNAs recognized by DNA or RNA MTases that may not be detectable by DNA sequencing. Instead, the motifs require other methods for detection such as m6A immunoblotting, m6A-sensitive ligase reaction, and m6A-sensitive reverse transcription or using SMRT technology to reverse transcribe cDNA from RNA templates (Meyer and Jaffrey, 2014).

We also compared role categories between each strain based on motif homology and listed the role categories from highest to lowest percentage in total methylated genes.
(Supplementary Datasheet 1, Table 11). A few notable differences were found that both distinguished IA3902 from the gastroenteric strains, and from each individual strain. The only categories with genes that were 100% methylated, without regard to a specific motif, and in all three strains were: central intermediary metabolism; DNA metabolism, replication, and repair; regulatory functions, signal transduction, small molecule degradation, and transport and binding proteins. Categories with 100% methylation unique to IA3902 were amino acid biosynthesis and protein fate.

We also found two role categories that either had the highest percent number of genes with a particular motif, or had the lowest or zero percent number of genes with the motif when we compared the three strains. The role category “small molecule degradation” had the highest percent number of genes with the motif GKA\_AYG (11168) at 100%. On the other hand, the same category for CAA\_AYG (IA3902) was at 50%. In 81-176, motif GCA\_AGG was even lower at 20%. This same category also had the highest percent number of genes with the motif GA\_GN\_5\_RTG in 11168 (66.67%). However, GA\_GN\_5\_RTG was not found in any of the genes in the “small molecule degradation” category for IA3902. Another role category, “pseudogenes/degenerate CDS”, had the highest percentage of genes with the methylated motif GA_GA\_A at 61.11% for IA3902. The homologous motif in 81-176, GG\_RCA, was not detected in any of the genes in this category.

**Identification and analyses of MTases not detected in this study**

We searched through REBASE to identify what bacterial species had enzymes with the RAATTY recognition sequence. We discovered that 62.5% of the enzymes
belonged to *C. jejuni*, which may suggest a particular association between RAATTY and the identity of *C. jejuni*.

This study identified a number of different MTases in IA3902. However, there were several *C. jejuni* MTases with IA3902 homologues not detected in our study. This may have happened because none of the sequence motifs we identified in IA3902 were predicted targets for the MTase homologues described below based on data available on REBASE.

Based on one of the few publications on *C. jejuni* MTases, we found that IA3902 possessed a homologue to the 11168 MTase Cj1461. The function of this DNA MTase was found linked to virulence-related phenotypes including motility, adhesion, and invasion (Kim et al., 2008). The homologue in IA3902 is a putative DNA methylase (CJSA_1385), but no record of CJSA_1385 was found in REBASE. When we ran the sequence in REBASE to find MTase homologues, the closest identity was M.CjeR14ORF8290P. This Type II, site-specific DNA MTase had the highest DNA sequence identity (98%) with CJSA_1385 among other MTases in the *Campylobacter* group, and is an enzyme isolated from *Campylobacter jejuni* subsp. *jejuni* R14. The closest neighbors to M.CjeR14ORF8290P have motif specificities for 5'-GA\(\text{ATC}\)-3', which suggests the motif recognized by CJSA_1385 is or is similar to G\(\text{ATC}\). The 81-176 homologue of Cj1461 was CJJ81176_1454, also a Type II site-specific DNA MTase with 98% identity to Cj1461 and CJSA_1385.

Another 11168 MTase that IA3902 had a homologue for was CjeNORF31P (locus tag Cj0031), which is a Type IIS R-M enzyme with 5'-CC\(\text{CGA}\)-3’/5’-CC\(\text{CGAA}\)-3’ as its predicted recognition site or motif (Anjum, 2013). It is a phase variable adenine
MTase known for its role in coordinated switching of gene expression, in particular adhesion, invasion, biofilm formation capability, and motility (Anjum, 2013). The IA3902 homologue for Cj0031 is CJSA_0032, which is also a Type II R-M enzyme. Though Cj0031 was thought to contain a frameshift in a previous methylome study (Murray et al., 2012), it has subsequently been demonstrated that Cj0031 is a phase variable gene (Anjum, 2013). The phase variable region of Cj0031 contains a polyG tract of 8-10 guanine nucleotides. Slipped strand mispairing during DNA replication allows for variability in the length of the polyG tract and thus inducing a frameshift that results in premature termination of translation. In the case of 11168, phase variable ON isolates have a 9 nucleotide polyG tract that allows for translation of Cj0031 to continue. In contrast, a polyG tract length of either 8 or 10 will result in termination of the translation and the loss of function of this MTase. The 11168 isolate that was sequenced in the original methylome paper was evidently a phase OFF variant that resulted in a non-active MTase (Anjum, 2013).

When we used BLAST to compare Cj0031 with the corresponding CJSA_0032 gene, we found the polyG tract was gone and the 3’ end of the gene was replaced with a non-phase variable 1130bp sequence in IA3902. This replacement resulted in a constitutive “phase ON” MTase in contrast to 11168. Furthermore, by using BLAST to compare the non-phase variable region of CJSA_0032 with other Campylobacter strains we found four strains with the exact same or similar sequences that were also identified as Type II R-M enzymes. C. jejuni subsp. jejuni ICDCCJ07001 and C. coli RM1875 had the highest percent match with CJSA_0032 at a value of 97% (sequences from both strains were 1130bp long). C. jejuni subsp. doylei 269.97 strain had 89% homology with
a sequence length of 1119bp. Lastly, C. lari RM2100 had 85% homology with a sequence length of 1126bp.

Two genes coding for RNA MTases in 11168 (Cj0588 and Cj0693c) were also identified. BLAST analysis revealed that at least 99% of these two genes’ sequences were homologous with genes in IA3902 (CJSA_0556 and CJSA_0657, respectively). A potential reason for why our study could not detect these two RNA MTases in IA3902 may be due to the inability for the DNA sequencing aspect of SMRT technology to detect RNA-specific MTases. However, other methods could be performed to detect the RNA MTases as described earlier in the results (Meyer and Jaffrey, 2014).

**Comparative methylome analysis of IA3902 wildtype and luxS mutant**

SMRT sequencing was also carried out on the luxS mutant of IA3902 and it also yielded one circular chromosome and plasmid. Mutagenesis of the luxS gene was also confirmed in the luxS mutant strain when we compared its genome sequence with that of the wildtype strain. Sequencing analyses of the methylation sites (Table 3) and base modification type (Figure 5) revealed very little differences between the two genomes. The luxS mutant possessed the same seven total sequence motifs as the wildtype (Table 3) and more than 99% of the motifs had the m6A base modification (Table 3, Figure 5). Since results showed the methylation types and patterns were the same between both the wild type and the luxS mutant strains, this demonstrates that LuxS did not have any effect on the IA3902 methylation phenotype when grown in Mueller-Hinton broth.
**Discussion**

When we compared the methylation profile of IA3902 with 11168 and 81-176, there were both similarities and differences. A majority of their motif sequences and MTases were homologous (Supplementary Datasheet 1, Tables 1-5), with one or two motifs and MTases without an IA3902 homologue in 11168 and 81-176. The methylation motif distribution plot (Figures 1-4) and the number and types of genes within the hypo- and hypermethylated regions (Supplementary Datasheet 1, Tables 7 and 9) were vastly different between IA3902 and the other two *C. jejuni* strains. For example, a higher number of hyper- and hypomethylated genes were found in IA3902 in contrast to the other two *C. jejuni* strains. IA3902 also had a higher proportion of well-characterized *C. jejuni* virulence genes than 11168 and 81-176 individually and combined. This suggests that restriction and modification activities may play a stronger role in the expression of IA3902 genes (including virulence genes) more so than the gastroenteric strains. The high proportion of hyper- and hypomethylated virulence genes in IA3902 may thus be correlated with the strain’s hyper virulence and abortion-causing phenotype.

Most of the genes in the major hypomethylated region of 11168 and 81-176 were 16S and 23S rRNA genes (Supplementary Datasheet 1, Table 7). The reason for this is unclear, and to our knowledge similar findings of hypomethylation of ribosomal genes in other bacteria has not been described. Interestingly, MTases can also modify ribosomal RNA genes by post-transcriptional methylation and are most well-characterized in *Escherichia coli* (Baldridge and Contreras, 2014). Several studies have found that knocking out the MTase gene conferred an increased level of antibiotic resistance (LaMarre et al., 2011; Mikheil et al., 2012; Monshupanee et al., 2012; Sałamaszyńska-
Guz et al., 2014). These findings, combined with our identification of these genome regions as hypomethylated, suggest that further studies are warranted to determine the physiologic rationale for the hypomethylation of these rRNA genes.

It is interesting that all three *C. jejuni* strains had such a high number of and multiple types of R-M systems for its genome size compared to other organisms in the 1.5 to 2Mb genome size class (Vasu and Nagaraja, 2013). RAATTY was also the most prevalent motif of almost every hyper- and hypomethylated gene in all three strains. The prevalence of RAATTY and presence of so many R-M systems may be attributed to one or several reasons. First, they may serve to help stabilize the host chromosome. R-M systems parallel toxin-antitoxin systems, which are abundant and help to stabilize neighboring chromosomal regions of the genome (Mruk and Kobayashi, 2013). Especially with the small genome size of *C. jejuni* and natural transformative ability of the organism, R-M systems (along with the high prevalence of RAATTY motifs) can help to maintain genomic islands acquired during horizontal gene transfer events.

Second, R-M systems and the RAATTY motif may help to genetically isolate IA3902 from 11168 and 81-176, particularly as the strains have different tissue tropism and disease presentations. Enforced methylation is a common phenomenon in Type I, II and IV R-M systems (Ishikawa et al., 2010). Enzymes in these systems cause cell death when the host bacteria exhibits altered methylation patterns, thus ensuring the epigenetic status of the population. With the small genome size of *C. jejuni*, perhaps the Type I and II R-M systems help to protect the methylation pattern of the host genome and prevent any harmful changes that would threaten its survival. It is also interesting that all three *C. jejuni* strains possess the m6A form of base modification. The numerous MTases and
associated R-M systems that specifically form m6A modifications have diverse roles in cell maintenance and virulence (Wion and Casadesus, 2006; Low and Casadesús, 2008). It is thus logical that *C. jejuni* possess so many MTases and R-M systems to carry out as many functions for its small genome and limited number of genes. RAATTY may also help to maintain the identity of *C. jejuni*. We searched through REBASE to identify what bacterial species had enzymes with the RAATTY recognition sequence. We discovered that 62.5% of the enzymes belonged to *C. jejuni*, which may suggest a particular association between RAATTY and the identity of *C. jejuni*.

As for the polyG tract in Cj0031 of 11168, it is predicted to be replaced with an 1130bp sequence in CJSA_0032 of IA3902 through a horizontal gene transfer event, while the beginning and end regions of the two genes remained the same. In addition, based on sequence homology, we believe the 1130bp sequence was likely acquired from one of several *Campylobacter* strains. It is a well-known phenomenon for R-M systems to undergo horizontal gene transfer events (Sharp et al., 1992; Nobusato et al., 2000; Bujnicki, 2001). In addition, such events can occur with DNA fragments as large as the 1130bp fragment found in this study (Aras et al., 2002). R-M systems are even referred to as “selfish mobile elements” with the sole purpose of promoting their survival (Kobayashi, 2001), which may explain the phase ON state of CJSA_0032.

One possible explanation for the selection of the phase ON state is environmental pressures favoring this state. The possession of a phase variable MTase can generate multiple cell types with distinct virulence expression profiles via global changes in methylation (Srikhanta et al., 2005; Krebes et al., 2014). This was evident in the study (Anjum, 2013) when phase ON variants of Cj0031 in 11168 were selected over phase
OFF after passage \textit{in vivo} in a chicken host. It was predicted that phase ON Cj0031 allowed the MTase to regulate expression of other genes required for host adaptation. Thus it can be predicted that the phase ON state may enable IA3902 to thrive in its specific niche and generate its abortifacient virulence profile.

The unique methylation patterns as a result of the phase ON state of CJS0_0032 may have caused the formation of IA3902 in becoming a new “biotype” (Vasu and Nagaraja, 2013) or a different variant of 11168, with a distinct identity from 11168 and other \textit{C. jejuni} strains. Phase variable R-M systems can control the acquisition of foreign DNA, thus serving as both a defense mechanism as well as regulating the influx of DNA. It may be that IA3902 lost the phase variable function in CJS0_0032 as a way to prevent suicidal restriction of its DNA, or that the function was redundant (Fox et al., 2007).

The results from this study have provided new insights for understanding the impact of methylation on \textit{C. jejuni} virulence and evolution. Despite the emergence of sequencing technologies to detect methylation in prokaryotic genomes, very few studies have looked at the significance of methylation in genes such as the ones identified in this study. Though aspects of this study have revealed some clues, there were also major limitations that added challenges and complexities to this study. One limitation to this study is that the methylome analysis is based on strains grown in basal conditions. Additional work is needed to develop a more complete appreciation of the role of culture conditions in the methylomes of these strains. Using different culture conditions that would enable changes in gene expression would also provide a better understanding of what genes under what conditions have their expression regulated by methylation. Another limitation of this study is that the number of passages and differences in time of
isolation between the strains could result in changes to the methylome. This assertion is, to our knowledge, true of most comparative genomic studies that contain strains from varying sources, period of disease, and differing number of passages. However, findings from this study still hold value for developing new hypotheses and provide the first information regarding the role of the methylome in these strains. The inability to include a large number of replicate strains in this study was a third limitation. Methylation-calling in SMRT sequencing requires greater sequencing depth than that for base-calling alone. The inclusion of the IA3902 luxS mutant strain provided a replicate and demonstrated the repeatability of the process while also providing validity to the results presented. Since both strains demonstrated identical methylomes, additional replicates were out of the scope of this study.

In addition to the study limitations, much remains to be explored in terms of the significance of methylation (hypermethylation vs. hypomethylation) on the genes examined in this study. While work is ongoing to gain insight into the functional roles of methylation in *C. jejuni* virulence expression, several studies have already found potential roles for methylation in regulating the expression of genes, including those associated with virulence. In *E. coli*, MTases were associated with increased expression of cation transport as well as decreased expression in cell projection, flagellar motility, and flagellum (Fang et al., 2012). Similar to the hypermethylation observed in IA3902 at the oriC region, hypermethylation at the oriC site was discovered in *Shewanella oneidensis* (Bendall et al., 2013). The authors from the *S. oneidensis* study paralleled this phenomenon with what has been observed in *E. coli* and suggest this hypermethylation may have a role in regulating genome replication. In *Mycoplasma pneumoniae*,...
hypermethylation was observed in two functional gene groups (defense mechanisms and genes coding for membrane proteins or lipoproteins) and one of its main virulence factors (Lluch-Senar et al., 2013). Hypermethylation was also discovered in regions containing putative DnaA boxes, suggesting that DNA methylation may play a role in DNA replication in *M. pneumoniae*.

Several studies have also explored the impact of phase variable MTases on gene expression in other bacterial species. In *Neisseria* sp., it was found that changing methylation patterns and altered gene expression by its phase variable MTases gives the organism opportunities to adapt in the host (Srikhanta et al., 2009). The phase variable MTase activity and random switching of virulence factors expression were also tested in *Haemophilus influenzae* (Srikhanta et al., 2005). MTases were found to have enhanced expression of several types of genes that enabled improved fitness of *H. influenzae* to environmental and physiological stresses. The findings from these methylation studies give indication that methylation (hyper- and hypomethylation) of genes observed in our *C. jejuni* strains, including virulence genes, and the MTases identified in this study could be regulating the expression of these genes.

Earlier, it was discussed how R-M systems serve to maintain the species of a bacteria (Vasu and Nagaraja, 2013). We are also greatly interested in determining whether a species and its virulence are defined by the R-M systems present in its genome. Moreover, how would the MTase specific to one species behave in another species? This also brings to question if methylation is a key evolutionary event for other *Campylobacter* species as it may be for IA3902, 11168, and 81-176. In practical applications, uncovering the mechanisms for methylation on *Campylobacter* gene
expression would make DNA methylation a promising drug target against diseases of *Campylobacter* and related organisms, as has been exploited in other bacteria (Mashhoon et al., 2004; Feder et al., 2008; McKelvie et al., 2013).

In terms of the impact of the *luxS* mutation on the methylation patterns of IA3902, we found no significant differences between the *luxS* mutant and wildtype. Both strains’ genomes only displayed the m6A base modification (Figure 5). In addition, the seven motifs, along with the MTases recognizing the motifs, were also the same for both strains (Tables 1 and 3). We conclude that the mutation in the *luxS* gene had no appreciable effect on the genome-wide methylation on IA3902. This suggests that a non-functioning *luxS* gene had no effect on the methylation profile of IA3902, and thus cannot explain for the attenuated virulence of IA3902ΔluxS (Plummer et al., 2012).

There may be several reasons why no differences were observed in the methylation patterns between IA390 and IA3902ΔluxS. One hypothesis is that methylation may have been affected but because an exogenous source of methionine was provided (e.g. the Mueller-Hinton media it was grown in), this allowed the organism to preserve its methylation status. Or, the *luxS* mutant employed an alternative mechanism to bypass the step requiring LuxS enzyme to complete the AMC cycle. The mutant could have also substituted the AMC cycle with another pathway to generate methyl groups and complete methylation. Although there is no proof for these hypotheses, they warrant further study. Despite the lack of differences found between IA3902 and its *luxS* mutant, we obtained novel information about the motifs and MTases of IA3902. For example, this study revealed two novel motifs, CAAAYG and GAAGAA, and their putative assignments to R-M enzymes CjeIAORF654P and CjeIAORF32P, respectively.
In conclusion, the methylation patterns of IA3902 were not affected by a non-functional \textit{luxS} gene. In addition, though the motif sequences and MTases between IA3902 and the gastroenteric 11168 and 81-176 strains were similar, their methylation profiles (including types of genes methylated and the hypo- and hypermethylation regions in the methylation motif distribution plots) were very different and enriched with known virulence genes. The results from this study have raised many questions regarding the impacts of methylation in \textit{C. jejuni} virulence. However, these findings also revealed the immense potential methylation plays in \textit{Campylobacter} pathobiology. The impact from this will not only help determine the disease pathogenicity of this and other organisms, but also serve as a strong candidate for developing novel therapeutics against \textit{C. jejuni} diseases.

\textbf{Acknowledgements}

We thank Jonas Korlach of Pacific Biosciences for graciously providing SMRT sequences on IA3902. Many thanks to Bill Miller for his assistance with providing the role category tables to classify the genes in IA3902, 11168, and 81-176. We thank Orhan Sahin and Qijing Zhang for editorial assistance of this manuscript. Finally we also thank Stuart Thompson for providing us detailed information regarding the growth conditions of the \textit{C. jejuni} isolates used in the original methylome paper. This work was supported by the Agriculture and Food Research Initiative Fellowships Grant Program of the USDA National Institute of Food and Agriculture grant number 2013-67011-21155, grant number K08AI07052303 from the National Institute for Allergy and Infectious Diseases, and Iowa State University startup funds.
Author Contributions

Figure legends.

Figure 1.

**Distinct areas of hyper- and hypomethylation in the whole-genome methylome plot of IA3902.** Distribution plots of all motifs are combined per 1kb across the genome. Gray arrows indicate hypermethylated areas, which have methylation frequencies of at least 45 and higher. Asterisks indicate major hypomethylated regions, which contain several adjacent bins with methylation frequencies of 4 or less. Black arrows indicate origin of replication (oriC) sites.

Figure 2.

**Circos plot displaying the distributions of each motif across the genome of IA3902.**

**Key**

<table>
<thead>
<tr>
<th>Color</th>
<th>Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>5’-GCANNNNNRTTA-3’</td>
</tr>
<tr>
<td>Blue</td>
<td>5’-GAGNNNNNRTG-3’</td>
</tr>
<tr>
<td>Green</td>
<td>5’-CAAAAYG-3’</td>
</tr>
<tr>
<td>Orange</td>
<td>5’-GAAGAA-3’</td>
</tr>
<tr>
<td>Purple</td>
<td>5’-RAATTY-3’</td>
</tr>
</tbody>
</table>

Figure 3.

**Distinct areas of hyper- and hypomethylation in the whole-genome methylome plot of 11168.** Distribution plots of all motifs are combined per 1kb across the genome. Gray arrows indicate hypermethylated areas, which have methylation frequencies of at least 41 and higher. Asterisks indicate major hypomethylated regions, which contain several adjacent bins with methylation frequencies of 4 or less. Black arrows indicate origin of replication (oriC) sites.

Figure 4.

**Distinct areas of hyper- and hypomethylation in the whole-genome methylome plot of 81-176.** Distribution plots of all motifs are combined per 1kb across the genome. Gray arrows indicate hypermethylated areas, which have methylation frequencies of at least 41 and higher. Asterisks indicate major hypomethylated regions, which contain several adjacent bins with methylation frequencies of 4 or less. Black arrows indicate origin of replication (oriC) sites.

Figure 5.

**Detection of N6-methyladenine (m6A) base modification in both IA3902 strains.** The following kinetic score distributions show significantly higher numbers of adenine
residues above the background in IA3902 wildtype (A) and luxS mutant (B). This indicates that the only type of base modification in the wildtype and luxS mutant is m6A.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Tables.

**Table 1. Methylose motifs detected within the *C. jejuni* IA3902 genome.**

*a* Sequence motifs are listed in the 5′ to 3′ direction. Underlined bases indicate the methylated base on the sequence. Motifs containing Y have either T or C nucleotide, while motifs with R have an A or G nucleotide.

*b,c* Complementary motif sequences

d Last column lists assigned restriction-modification (R-M) enzymes predicted to recognize respective motif(s).

c The motifs and associated methyltransferases (MTases) are excellent candidates for the respective R-M system types. However, because the designations are not definitive, further tests are required to confirm the functional statuses of the MTases.

<table>
<thead>
<tr>
<th>Motif(^a)</th>
<th>Modification Type</th>
<th># of Motifs Detected</th>
<th># of Motifs in Genome</th>
<th>% Motifs Detected</th>
<th>Partner Motif</th>
<th>R-M enzymes(^d,e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAGNNNNNNRTG(^b)</td>
<td>m6A</td>
<td>717</td>
<td>717</td>
<td>100</td>
<td>CAYNNNNNCTC</td>
<td>CjeIAORF9 94P</td>
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<tr>
<td>CAYNNNNNCTC(^b)</td>
<td>m6A</td>
<td>716</td>
<td>717</td>
<td>99.86</td>
<td>GAGNNNNNRTG</td>
<td>CjeIAORF9 94P</td>
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<tr>
<td>CAAAAYG</td>
<td>m6A</td>
<td>1760</td>
<td>1760</td>
<td>100</td>
<td></td>
<td>CjeIAORF6 54P</td>
</tr>
<tr>
<td>TAYNNNNNTGC(^c)</td>
<td>m6A</td>
<td>499</td>
<td>499</td>
<td>100</td>
<td>GCANNNNNRTTA</td>
<td>M.CjeIAII</td>
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<td>GCANNNNNRTTA(^c)</td>
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<td>27514</td>
<td>99.29</td>
<td>RAATTY</td>
<td>M.CjeIAI</td>
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</table>
Table 2. Putative Restriction-Modification (RM) systems detected in IA3902.

a The Gene and Names of the systems are given as assigned by REBASE following submission of the data.
b The motifs and associated MTases are excellent candidates for the respective R-M system types. However, because the designations are not definitive, further tests are required to confirm the functional statuses of the MTases.

<table>
<thead>
<tr>
<th>R-M system type</th>
<th>Locus</th>
<th>Gene a</th>
<th>Name a,b</th>
<th>Gene description</th>
<th>Associated sequence motif (5’ to 3’ direction)</th>
<th>Partner Motif</th>
<th>Sequence motif identified in this study?</th>
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</thead>
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<td>I</td>
<td>CJSA_1465</td>
<td>R</td>
<td>CjeIAIIP</td>
<td>Putative restriction type I enzyme R protein</td>
<td>TAAYNNNNNTGC</td>
<td>GC_NNNNNRTTA</td>
<td>Yes</td>
</tr>
<tr>
<td>I</td>
<td>CJSA_1467</td>
<td>S</td>
<td>CjeIAII</td>
<td>Putative restriction type I enzyme S protein</td>
<td>TAAYNNNNNTGC</td>
<td>GC_NNNNNRTTA</td>
<td>Yes</td>
</tr>
<tr>
<td>I</td>
<td>CJSA_1469</td>
<td>M</td>
<td>CjeIAII</td>
<td>Putative restriction type I enzyme M protein</td>
<td>TAAYNNNNNTGC</td>
<td>GC_NNNNNRTTA</td>
<td>Yes</td>
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<tr>
<td>II</td>
<td>CJSA_0032</td>
<td>RM</td>
<td>CjeIAORF32P</td>
<td>Type II restriction modification enzyme</td>
<td>Unknown (putatively GAAGAA)</td>
<td></td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 2 (continued).

<table>
<thead>
<tr>
<th>R-M system type</th>
<th>Locus</th>
<th>Gene</th>
<th>Name \textsuperscript{a,b}</th>
<th>Gene description</th>
<th>Associated sequence motif (5' to 3' direction)</th>
<th>Partner Motif</th>
<th>Sequence motif identified in this study?</th>
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<tr>
<td>II</td>
<td>CJSA_0199</td>
<td>M</td>
<td>CjeIAI</td>
<td>D12 N6 adenine-specific DNA methyltransferase</td>
<td>RAATTY</td>
<td>RAATTY</td>
<td>Yes</td>
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<tr>
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<td>CJSA_0654</td>
<td>RM</td>
<td>CjeIAORF654P</td>
<td>Putative restriction modification enzyme</td>
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<td></td>
<td>Yes</td>
</tr>
<tr>
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<td>RM</td>
<td>CjeIAORF994P</td>
<td>Restriction modification enzyme</td>
<td>G\textsubscript{AGNNNNNRTG}</td>
<td>CYNNNNNNTCTC</td>
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Table 3. Methylome motifs detected within the *C. jejuni* IA3902ΔluxS genome.

*a* Sequence motifs are listed in the 5’ to 3’ direction. Underlined bases indicate the methylated base on the sequence. Motifs containing Y have either T or C nucleotide, while motifs with R have an A or G nucleotide. 

*b,c* Complementary motif sequences

*d* Last column lists assigned restriction-modification (R-M) enzymes predicted to recognize respective motif(s).

*e* The motifs and associated MTases are excellent candidates for the respective R-M system types. However, because the designations are not definitive, further tests are required to confirm the functional statuses of the MTases.

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<tr>
<th>Motif a</th>
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<th># of Motifs in Genome</th>
<th>% Motifs Detected</th>
<th>Partner Motif</th>
<th>R-M enzymes d,e</th>
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<td>718</td>
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<tr>
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CHAPTER 4. CHARACTERIZATION OF THE CJSA_RS00180 DNA METHYLTRANSFERASE IN CAMPYLOBACTER JEJUNI IA3902

A paper to be submitted to PLOS ONE

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Abstract

The number of studies characterizing restriction-modification systems in Campylobacter jejuni have grown in the past decade, thanks to continuous developments in next generation sequencing technologies, like Pacific Biosciences’ Single-Molecule, Real-Time (SMRT) sequencing. A recent study from our group conducted comparative methylome profile analysis between three C. jejuni strains, including two human gastroenteric strains 11168 and 81-176 and an abortifacient clonal isolate IA3902, in an effort to determine whether methylation patterns play a role in their disparate disease phenotypes. Methylome analysis revealed a unique putative methyltransferase (MTase) in IA3902 called CJSA_RS00180 (formerly known as CJSA0032), which was further characterized in this study. We created a CJSA_RS00180 mutant and used SMRT sequencing to confirm that the mutation eliminated its MTase function and confirm that the putative cognate methylation motif of CJSA_RS00180 MTase was GAAGAA. Additional colonization-related phenotypic studies characterizing the MTase’s role in vitro growth and motility revealed that mutation of this gene did not affect its growth or
motility on a semi-solid agar assay or in a mucin column. Though no observable
differences in growth and motility were observed here, future studies will continue to
characterize the effects of the CJSA_RS00180 mutation on expression and function of
genes that contain the GAAGAA methylation motif, and to evaluate the colonization and
virulence potential of IA3902ΔRS00180 in animal studies.

Key Words: Campylobacter jejuni, CJSA_RS00180, IA3902, methyltransferase,
methylation, motif

Introduction

Next generation sequencing (NGS) technologies, such as Pacific Biosciences’
Single-Molecule, Real-Time (SMRT) sequencing, have greatly accelerated the study of
bacterial DNA methylation at the genomic scale. Methylation is one type of DNA
modification present in all bacteria and carried out by DNA methyltransferases (MTases)
that are part of restriction modification (R-M) systems (Roberts et al., 2003). Aside from
their most well-known function in prokaryotic defenses, R-M systems also play important
roles in gene expression and regulation, cell maintenance, and virulence (Marinus and
Casadesus, 2009).

Numerous studies have utilized SMRT sequencing to characterize methylomes of
several bacterial species (Lee et al., 2015; Pirone-Davies et al., 2015; Zhang et al., 2015)
and some have found a correlation between R-M systems and bacterial virulence (Fang et
al., 2012; Lluch-Senar et al., 2013; Huo et al., 2015; Kyuma et al., 2015). In
Campylobacter jejuni, the use of SMRT sequencing has led to rapid methylome
characterization of several _C. jejuni_ strains, including commonly studied laboratory
strains 11168 and 81-176 (Murray et al., 2012), a clinical isolate of the clone SA (Sheep Abortion) IA3902 (Mou et al., 2015), and an isolate from a human gastroenteritis case (O’Loughlin et al., 2015).

_C. jejuni_ is a zoonotic pathogen that was recently estimated as not only one of the primary causes of food-borne illnesses in humans, but also a leading cause of hospitalizations in the United States (Scallan et al., 2011). In humans, _C. jejuni_ associated disease is usually manifested in the form of acute, self-limiting gastroenteritis (Acheson and Allos, 2001). In domestic and food production animals, _C. jejuni_ is typically a commensal organism (Wagenaar and Jacobs-Reitsma, 2008). However, small ruminants, and other less common livestock and domestic species, can have significant infectious abortion outbreaks associated with some strains of _C. jejuni_ (Van Donkersgoed et al., 1990; Wu et al., 2014). Over the last few decades, a highly virulent clone of _C. jejuni_, clone SA (Sheep Abortion), has gained widespread attention for becoming the predominant cause of _Campylobacter_-associated sheep abortions in the U.S. (Sahin et al., 2008). Major concerns over antibiotic resistance found in this clone, and _Campylobacter_ in general, have led to a significant need to identify alternative therapy and prevention measures that are effective against this organism (Kaakoush et al., 2015). Such research efforts greatly benefit from an improved understanding of the epidemiology and biology of these _Campylobacter_ organisms.

Clone SA is particularly intriguing because of its emergence as the predominant cause for _Campylobacter_-associated sheep abortions (Sahin et al., 2008), the hypervirulence this clone demonstrates in abortions (Burrough et al., 2009), and its
recent ties to human zoonotic disease (Sahin et al., 2012). Interest in this clone has driven research to determine what elements are responsible for the hypervirulence and emergence of this clone. One study did comparative genomic, transcriptomic and proteomic analysis to identify potential genetic factors that could explain the unique disease phenotypes of 11168 versus IA3902 (Wu et al., 2013). Though global gene expression profiles were different between the two, they did not find any specific abortion-associated genetic factors in IA3902.

To begin to understand whether methylation could play a role in their gene expression profiles, we used SMRT sequencing and compared the methylome profiles of IA3902, 11168, and 81-176 (Mou et al., 2015). Indeed, the methylation patterns and types of genes methylated in IA3902 were different from 11168 and 81-176. This suggests that methylation could contribute to their unique gene expression profiles and possibly even the unique hypervirulence of IA3902 in comparison with 11168 and 81-176 strains.

In that same study, we also identified a novel methylation motif in IA3902 (GAAGAA) that was putatively assigned to the CjeIAORF32P R-M system. The predicted MTase for this system, CJSAR00180 (formerly known as CJSV_0032), also has a homologue in 11168 (Anjum, 2013). The 11168 homologue is the Cj0031 MTase which contains a polyG tract of 8-10 guanine nucleotides that is subject to slipped-strand mispairing during DNA replication (Anjum, 2013). Chicken colonization studies showed that wildtype variants with the phase ON state of cj0031 (and therefore a functional Cj0031 MTase) were positively selected for over wildtype variants containing the phase OFF state of cj0031 (non-functional Cj0031 MTase). This suggests that a functional
Cj0031 MTase contributes to *C. jejuni* adaptation and colonization of the host. BLAST analysis showed that the polyG tract in *cj0031* is located within a 1130bp non-homologous region of the CJSA_RS00180 gene. Unlike *cj0031*, CJSA_RS00180 gene does not contain a polyG tract or any homopolymeric tract in that region, and as a result, is believed to be in a constitutive phase ON state. Whether the phase ON state of CJSA_RS00180 gene functions similarly to *cj0031* and is an important colonization factor remains to be determined.

Several other studies have also linked the function of MTases with specific factors important for *C. jejuni* virulence such as motility, adherence, invasion, and colonization of the host (Kim et al., 2008; Anjum, 2013; Sałamaszyńska-Guz et al., 2014). However, these studies give a limited view of the functional impact these MTases have. The genome-wide impact that these MTases possess and how their methylation activities link with the phenotypic expression of these and other factors remains to be understood. Fully characterizing the genotypic and phenotypic facets of these methyltransferases will further our understanding of the various roles MTases have in the biology and virulence of *Campylobacter jejuni*, including the potential to affect the organism’s disease phenotype.

In order to further explore how methylation regulates the expression and function of factors important to *C. jejuni* pathogenesis, it is critical that we first confirm the putative role of MTases such as CJSA_RS00180 from IA3902, which was identified in our previous study, as a methyltransferase (Mou et al., 2015). Based on identification of methylated GAAGAA motifs and predicted MTases for this motif in REBASE, we hypothesized that CJSA_RS00180 is a MTase that methylates GAAGAA motifs and that
mutation of the MTase gene would completely eliminate methylation of this motif. In addition, the function of this MTase is predicted to be crucial for normal cell growth and motility in IA3902. This hypothesis is based on the results of our previous study, which identified several genes associated with growth and colonization phenotypes that were hyper- and hypomethylated at the GAAGAA motif, compared to the remainder of the genome. To characterize the CJSA_RS00180 MTase, we created a CJSA_RS00180 mutant and performed several genotypic and phenotypic assays on the mutant. These assays included profiling the mutant's methylome, examining its growth, as well as comparing its motility with wildtype on both semi-solid agar and through a mucin substrate.

**Materials and Methods**

**Bacterial strains and culture conditions**

Routine cultures of *Campylobacter jejuni* strains were grown in Mueller-Hinton (MH) broth or agar plates (Difco) at 42°C in a microaerobic atmosphere generated by compressed gas (5% O₂, 10% CO₂, 85% N₂). *C. jejuni* strains with chloramphenicol resistance were grown on broth or agar plates containing 10μg/ml concentration of chloramphenicol when appropriate. *Escherichia coli* competent cells used for cloning studies were grown at 37°C in Luria-Bertani broth or agar plates. When needed, chloramphenicol (15μg/ml) and ampicillin (100μg/ml) were added to broth or agar plates. All strains used in this study are described in **Table 1**.
**Insertional mutation of the methyltransferase gene CJSA_RS00180 of C. jejuni**

The IA3902ΔRS00180 was constructed by inserting a chloramphenicol resistance cassette (*cat*) into the middle of the gene (Figure 1). To construct the mutant, primers were designed using the New England Biolabs NEBuilder online tool for High-Fidelity DNA Assembly (2013) for the following DNA sequences: two separate fragments of CJSA_RS00180 coding and flanking sequences (45931F/45931R and 49804F/49804R), and *cat* gene (*cat*F and *cat*R) (Table 2). Cloning fragments were generated by PCR using 2X Phusion High-Fidelity Master Mix (Finnzymes, Thermo Scientific, Waltham, MA), 200ng of *Campylobacter* genomic DNA, and 500uM primers. pUC19 vector was restriction digested with XbaI (New England Biolabs, Ipswich, MA) and dephosphorylated using Antarctic Phosphatase (New England Biolabs, Ipswich, MA). All DNA fragments and digested pUC19 were purified with a PCR clean-up kit (Qiagen, Venlo, Netherlands). 0.2pmol DNA fragments were cloned with 0.2pmol digested pUC19 plasmid using NEB HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA), resulting in the pRS00180 construct containing *cat* cassette flanked by the CJSA_RS00180 fragments in pUC19 plasmid (Figure 1). pRS00180 construct was transformed into NEB 5-alpha competent *E. coli* (New England Biolabs, Ipswich, MA) and transformants were selected on LB agar plates containing chloramphenicol (15µg/ml), ampicillin (100 µg/ml), and ChromoMax IPTG/X-Gal Solution (Fisher Scientific, Pittsburgh, PA). pRS00180 was purified from *E. coli* and verified by PCR and sequencing using universal primers U and R-1 that target the multiple cloning site in pUC19, provided by the Iowa State University DNA Facility (Ames, IA) (Table 2). pRS00180 was introduced as a suicide vector into IA3902 by electroporation and
Campylobacter transformants were grown on MH agar plates containing chloramphenicol (10µg/ml). 10 colonies were screened and confirmed for insertional mutation via PCR and sequencing with 45931F-a and 49804R primers (data not shown) (Table 2). This mutation resulted in the deletion of 3.374kb of CJSA_RS00180 coding sequence and simultaneous insertion of the cat gene into the same location.

**DNA Sequencing Preparation, SMRT Sequencing of C. jejuni IA3902ΔRS00180, and Bioinformatics Analysis of IA3902ΔRS00180 Methylation Motifs**

DNA was extracted from IA3902ΔRS00180 grown for 24 h in a microaerobic environment under the same growth conditions described previously, and assessed for DNA quality and quantity (Mou et al., 2015). Approximately 15µg of genomic DNA was submitted to Arizona Genomics Institute at University of Arizona (Tucson, AZ) for library preparation and SMRT sequencing. Library preparation and SMRT sequencing were performed similarly to previous methods with a few modifications (Mou et al., 2015). SMRTbell templates with sizes between 7-50kb were size-selected and two SMRT Cells were used to sequence the DNA sample.

SMRT sequencing data of IA3902ΔRS00180 were analyzed for methylated motifs using the SMRT analysis suite v2.0 as previously described (Murray et al., 2012). The output from IA3902ΔRS00180 SMRT sequencing analysis included each motif’s methylation site, methylation score and extent of genome methylation for each sequence motif. IA3902ΔRS00180 methylation data were compared with methylation data of IA3902 from our previous methylome profile study to identify any differences including
whether GAAGAA motif methylation was completely eliminated in the MTase mutant (Mou et al., 2015).

**Growth studies**

*C. jejuni* strains IA3902 and IA3902ΔRS00180 were prepared for growth study inoculum using confluent lawns grown to 24 hours, harvested in a small volume of MH broth media, and adjusted to an optical density (OD$_{600}$) of 0.5. Inocula were then further diluted 1:100 by adding 700µl of OD-adjusted cultures to 70ml MH broth (approximate cell densities of 10$^6$-10$^7$ colony forming unit (CFU)/ml) in 250ml flasks. Suspensions were grown in gas jars (Mitsubishi Gas Chemical Co., Inc.) under microaerobic conditions at 42°C for 48 hours. Sample aliquots from each strain were harvested at time of inoculation and at time points 3, 8, 14, 24, 32, and 48 hours for bacterial enumeration. Growth of each strain was assessed by using the drop plate method (Chen et al., 2003) to plate serial dilutions (1:10) of bacterial suspensions on MH agar. Log 10 transformation of CFU/ml was performed and assessed for any significant growth differences between strains using two-way analysis of variance (ANOVA) with repeated measures in GraphPad Prism (La Jolla, CA, USA). A $p$-value of 0.05 or less indicated significant growth differences. Results are the average of three independent experiments.

**Motility assay using semi-solid MH agar**

The motility assay was performed similar to previous methods using a semisolid MH agar medium to test the motility phenotype (Plummer et al., 2012). Strains were harvested from confluent lawns grown overnight and suspended in MH broth. Cultures were adjusted to an OD$_{600}$ of 0.3 (approximate cell densities of 10$^8$ CFU/ml). A single
stab inoculation was made into the center of the plate, and plates were incubated at 42°C in a microaerobic environment for 48 h. Distance of swarming motility was measured from the center of the stab to the widest portion of the swarm halo. The experiment was conducted three separate times with three technical replicates each time for each strain. Combined results from all three experiments were analyzed with the paired Student’s t test and were considered significantly different if the p-value was 0.05 or less.

**Mucin penetration assay**

Mucin penetration was simulated for IA3902 and IA3902ΔRS00180 using methods adapted from previous studies (Liu et al., 2008; Naresh and Hampson, 2010). One-ml tuberculin syringes containing 1ml porcine gastric mucin (Sigma-Aldrich) dissolved in PBS buffer to a concentration of 30mg/ml were used as mucin columns. The concentration 30mg/ml was selected as it was the highest concentration of mucin that could be used without the mucin viscosity interfering with the syringe function of the assay. One hundred microliters of log-phase bacterial cultures adjusted to an OD$_{600}$ of 0.01 (cell densities of approximately $10^7$ CFU/ml) were added to the top of mucin columns and incubated for 30 min at 42°C. Fractions of 0.1ml (the first 0.1ml collected is fraction 1, with subsequent fractions in chronological order until the last 0.1ml-fraction or fraction 10) were collected from the bottom of mucin columns, plated on MH agar containing Oxoid™ Preston *Campylobacter* selective supplement (Thermo Scientific, Wilmington, DE), incubated for at least 24 hours in microaerobic conditions, and then enumerated for bacterial quantification. Fraction 10 included both the very top portion of the mucin and the inoculum and was thus excluded from the data calculations, as it was impossible to determine what portion of those organisms in this fraction had truly
penetrated the agar. Three independent experiments were performed and were statistically analyzed with two-way ANOVA with repeated measures (GraphPad Prism) to assess significant differences in the number of mutant vs. wildtype bacteria in each fraction. A $p$-value of 0.05 or less was considered significant.

**Results**

*Sequence features of CJSA_RS00180 in C. jejuni IA3902*

CJSA_RS00180 was previously predicted to be a MTase that recognizes the sequence motif GAAGAA (Mou et al., 2015). Bioinformatic analysis revealed that the 3774 bp gene encoding the MTase (nucleotides 46231 to 50004 in IA3902 genome) translates into a 1257aa sequence. The majority of the protein aligns with the Type II R-M system domain located from 2-1250aa. Type II R-M systems contain separate restriction endonucleases (REases) and MTase enzymes that usually act independently of each other, but can also be fused into a single composite gene (Roberts et al., 2003). In the case of CJSA_RS00180, it did not contain any REase domain within the gene and, instead, contained a conserved SAM MTase domain at the 808-913aa region of CJSA_RS00180. No endonuclease-encoding genes were found adjacent to the CJSA_RS00180 gene, suggesting that this MTase functions alone and does not have a cognate REase.
Sequence homologies of CJSA_RS00180 with other Campylobacter species

BLAST results showed that gene sequence homology with CJSA_RS00180 ranged from 46-99% in Campylobacter species including C. lari, C. jejuni subsp. jejuni, C. jejuni subsp. doylei, and C. coli. Campylobacter lari subsp. concheus LMG 11760 showed the closest sequence homology with 99% coverage to CJSA_RS00180 gene and 81% identity within the coverage. The MTase gene cj0031 in 11168, another homologue of CJSA_RS00180, showed gene homology with 65% coverage to CJSA_RS00180 and 98% identity within the coverage. The sequence length of cj0031 is 3733bp and translates to a 1243aa-long protein, which is only 14aa shorter than CJSA_RS00180.

The region of the genes encoding the nonhomologous domains of cj0031 and CJSA_RS00180 gene spanned 1130bp and was found towards the 3’ end of both genes (Figure 2). The previously described polyG tract in cj0031 was located within this 1130bp non-homologous region, while no polyG tract or similar homopolymeric tract was found in this same region within CJSA_RS00180 gene. As described earlier, cj0031 is a MTase gene prone to phase variation specifically at the polyG tract. During DNA replication, slipped-strand mispairing enables variability of the tract’s length and dictates whether the gene will result in an active (phase ON) or truncated (phase OFF) protein. However, the absence of a polyG or similar homopolymeric tract in the 1130bp region of CJSA_RS00180 indicated that this portion is non-phase variable and that the MTase gene is in a constitutive phase ON state. BLAST analysis further found exact or similar matches of the non-phase variable portion of CJSA_RS00180 to Type II R-M enzyme sequences found in C. jejuni subsp. jejuni ICDCCJ07001, C. coli RM1875, C. jejuni subsp. doylei 269.97, C. lari subsp. concheus LMG 11760, and C. lari RM2100. The
finding of this non-phase variable region in other *Campylobacter* species suggest that the non-phase variable sequence in CJSA_RS00180 may have originated from a horizontal gene transfer event from other *Campylobacter* species (Figure 2).

**Insertional mutagenesis of CJSA_RS00180 eliminates MTase function to methylate GAAGAA motif**

To confirm whether CJSA_RS00180 is a MTase and specifically methylates GAAGAA motifs, we disrupted CJSA_RS00180 by insertional mutagenesis (see Materials and Methods section). The construction of the mutation is shown in Figure 1. We conducted SMRT sequencing on IA3902ΔRS00180, which quickly and simultaneously sequences the genome and detects methylated bases within the sequences. The methylation results showed that the only methylated motif not detected in our mutant was GAAGAA (Table 3). The lack of detection for methylated GAAGAA motifs lead to the conclusions that CJSA_RS00180 is indeed a MTase, and that GAAGAA is its cognate motif. All six other methylated motifs that were previously reported for IA3902 were identified in our mutant’s methylation data (Mou et al., 2015).

**Effects of the mutation of CJSA_RS00180 on growth, motility and mucin penetration**

Several studies have found that mutating the MTase genes in *C. jejuni* have functional consequences on a variety of phenotypes including motility, *in vivo* colonization, antibiotic resistance, and *in vitro* adhesion and invasion ability (Kim et al., 2008; Anjum, 2013; Salamaszyńska-Guz et al., 2014). To simulate some of those phenotypic studies in our mutant, we first tested the growth ability of IA3902ΔRS00180. When we compared growth between wildtype and IA3902ΔRS00180 under basal
conditions over a 48 hour period, we found no significant changes in growth and growth rate for our mutant in all three independent experiments (Figure 3).

We next investigated the effect of the mutation on *C. jejuni* motility, a trait that is vital to the pathogenesis of *C. jejuni*. We first used a motility assay on semi-solid MH agar (Plummer et al., 2012) and found no significant motility differences between wildtype and mutant in all three biological replicates (Figure 4). We then tested the ability of IA3902ΔRS00180 to penetrate through mucin. Interaction with mucin is a key step to establishing colonization of the intestinal mucosal lining. As mucin is a strong promoter for *C. jejuni* motility, we wanted to determine the effect of the mutation on mucin penetration. We assessed penetration ability of IA3902ΔRS00180 clone 5 through porcine mucin and found no differences between wildtype and mutant (Table 4).

**Discussion**

We recently identified a putative CJSA_RS00180 MTase (Mou et al., 2015) and in this study, hypothesized that CJSA_RS00180 is indeed a MTase that methylates GAAGAA motifs. Thus mutation of this MTase gene would eliminate methylation of this motif. The collective evidence from this study proved all of those statements to be true. First, bioinformatic analysis identified CJSA_RS00180 as a MTase belonging to the Type II R-M system, possessing domains characteristic of Type II R-M systems and SAM MTase, and shared homology with a large number of other *Campylobacter* Type II R-M enzymes. The identification of the 1130bp non-phase variable region of CJSA_RS00180 in Type II MTase genes of other *Campylobacter* species suggests that IA3902 may have
acquired the non-phase variable region through a horizontal gene transfer event. This is likely as R-M systems are often acquired through horizontal gene transfer events (Vasu and Nagaraja, 2013). The lack of identification of REase genes adjacent to and within this MTase gene indicated that this MTase works independent of a REase, which is characteristic of Type II MTases (Roberts et al., 2003). In addition, we did not detect any methylated GAAGAA motifs in the IA3902ΔRS00180 SMRT sequencing results, demonstrating that the mutation in CJSA_RS00180 gene eliminated the MTase function to specifically methylate GAAGAA motifs. This evidence confirms that CJSA_RS00180 is a MTase and that GAAGAA is its cognate motif.

In a recent study by O’Loughlin et al., the authors discovered a MTase in the C. jejuni F38011 strain that is a putative homologue of Cj0031 (O’Loughlin et al., 2015). This homologue was identified as CJH00185, which is a Type II R-M MTase like CJSA_RS00180. The 3491bp MTase gene contained a frameshift mutation that split the sequence into two ORFs, and encoded two separate proteins with lengths of 662aa and 511aa. In addition, the three or four guanine bases in the polyG tract region of Cj0031 (depending on the 11168 genome used for comparison) were not found in CJH00185 in the same location. When we conducted BLAST analysis comparing this MTase gene with CJSA_RS00180, the sequence homology between CJH00185 and CJSA_RS00180 was even less than the homology of Cj0031 with CJSA_RS00180, with 57% coverage and 99% identity within the coverage. Interestingly, the range of homology excludes the non-phase variable sequence of CJSA_RS00180, just like with Cj0031.

The drastic differences in gene structure of the MTase genes cj0031, CJSA_RS00180, and CJH00185 is striking. The 1130bp region in cj0031 is phase
variable as it contains a polyG tract. In CJSA_RS00180, the same region is not phase variable as it contains no homopolymeric tract. Instead, this gene is in a constitutive phase ON state. In CJH00185, the same region is also non-phase variable region, but the MTase gene sequence contains a frameshift mutation that splits the sequence into two potential protein-encoding ORFs. We speculate these unique changes are a function of the physiology of the organism and may have ecological benefits for adaptation and survival in the host intestinal environment. For example, the phase ON state of \textit{cj0031} is vital for successful colonization and adaptation to the chicken gut (Anjum, 2013). \textit{cj0031} is also a phasevarion, which acts like an epigenetic regulator and regulates the expression of multiple genes through its methylation function (Casadesús and Low, 2013). Its role as a phasevarion may help to regulate the expression of genes necessary for \textit{C. jejuni} strain 11168’s adaptation within the chicken gut. On the other hand, the phasevarion may incur a fitness cost to the organism and was therefore replaced with a non-phase variable region via horizontal gene transfer (Vasu and Nagaraja, 2013). The absence of a phasevarion like \textit{cj0031} in CJSA_RS00180 and CJH00185 may serve a functional role specific to the strain’s adaptation to its particular niche. For example, the constitutive expression of CJSA_RS00180 may help increase the fitness and/or hypervirulence of IA3902 by regulating genes required for physiology and adaptation.

We also hypothesized that the function of this MTase is critical to cell growth and factors important for colonization. Previous analysis from our group found the following chromosomal genes that contained the GAAGAA motif and were either hypo- or hypermethylated at this motif (Mou et al., 2015): hypomethylated genes included tetrapyrole methylase family protein (CJSA_0145), glutamine synthetase, type I
(CJSA_0663), DNA gyrase subunit A (CJSA_0970), putative ferredoxin (CJSA_1311), putative peptide ABC-transport system periplasmic peptide-binding protein (CJSA_1496), DNA topoisomerase I (CJSA_1595), and a hypothetical protein (CJSA_1621); hypermethylated genes included chromosomal replication initiator protein DnaA (CJSA_0001), hypothetical protein (CJSA_0008), glutamate synthase subunit beta (CJSA_0009), ExsB protein (CJSA_0016), methyl-accepting chemotaxis protein (CJSA_0019), and putative periplasmic toluene tolerance protein (CJSA_1306).

When we observed the growth of IA3902ΔRS00180, the lack of growth rate differences in comparison to wildtype was rather surprising. Multiple genes involved in growth and cellular maintenance possess the GAAGAA motif in their sequence. Some of these genes were also found hypermethylated in IA3902 such as DnaA chromosomal replication initiator protein. The lack of methylation of genes like DnaA would be expected to have drastic effects on expression and full-functioning of these genes, which in turn, would affect replication and growth of the mutant. Though no obvious growth differences were detected in our mutant, the conditions we tested the mutant under were limited to in vitro growth in a rich media. Thus, we cannot rule out the possibility that growth of the mutant would be altered in other more stressful environmental conditions, such as those encountered in vivo. As such, further analysis of the mutant using different growth conditions, profiling gene expression, or use of additional in vitro or in vivo models is warranted.

Similar to our results, and contrary to our hypothesis, other studies have also observed no growth rate changes in their MTase mutants. For example, the CJH00185 deletion mutant of the C. jejuni F38011 strain showed similar growth rates with wildtype
(O’Loughlin et al., 2015). The same observation was made in a *cj0588* RNA methyltransferase mutant in *C. jejuni* 81-176 strain (Sałamaszyńska-Guz et al., 2014). In contrast, another DNA MTase mutant in 81-176, *cj1461* mutant, showed growth defects in comparison to wildtype (Kim et al., 2008).

As for motility and mucin penetration, we detected no significant defects in these phenotypes for IA3902ΔRS00180. This was also surprising as we expected the function of one of the genes requiring CJSA_RS00180 methylation, a methyl-accepting chemotaxis protein (CJSA_0019), would be affected in a MTase mutation background. Mucin is a strong chemoattractant that promotes *C. jejuni* infection (Vegge et al., 2009; Stahl et al., 2011; Naughton et al., 2013a). Although the exact role of CJSA_0019 in motility and chemotaxis is unknown, we postulated that the lack of methylation would impact the function of this gene and would cause a defect in motility or its ability to interact and penetrate the mucin column. However, based on the results obtained, the methylation status of this gene is not required for the motility function and interaction with mucin in the conditions evaluated.

The lack of motility differences in our mutant was similar to what was observed in the *cj0031* mutant (Anjum, 2013). In contrast, the *cj1461* MTase mutant showed significant reduction in motility both on soft-agar assay and under wet-mount microscopy (Kim et al., 2008). The authors attributed this motility defect to a defect in flagellar appearance when analyzed under transmission electron microscope. A similar reduction in motility was also observed in another 81-176 MTase mutant (*cj0588*) and exhibited 70% loss of motility in the mutant compared to wildtype (Sałamaszyńska-Guz et al., 2014).
The results from this study represent the groundwork to understanding the role of this MTase in the cell biology and virulence of IA3902. While we have no evidence so far for the functional requirement of this MTase in \textit{in vitro} growth in rich media and motility, we do not know what role CJSA_RS00180 plays during \textit{in vivo} motility or general colonization of the host. Anjum discovered that when a population of primarily phase OFF variants of Cj0031 was inoculated into a chicken colonization model, the output samples detected after the \textit{in vivo} passage were all phase ON variants (Anjum, 2013). This suggests that the phase ON variants of \textit{cj}0031 were positively selected for among the colonizing \textit{C. jejuni} population and the phase OFF variants were selected against. Moreover, \textit{cj}0031 expression \textit{in vivo} was required for coordinating and controlling the expression of other genes in the genome that enabled adaptation and colonization of the organism within the host intestinal tract. Since CJSA_RS00180 is not phase variable, we hypothesize that CJSA_RS00180 will function similarly to the phase ON state of Cj0031. Additionally, the constitutive expression of CJSA_RS00180 is predicted to be necessary for IA3902 to colonize and adapt to the host intestinal tract. In contrast, a mutation of this gene is expected to cause a severe defect in the mutant’s colonization ability. Future studies will test the colonization and virulence potential of this mutant using animal models such as chicken colonization model and the pregnant guinea pig abortion model.

If colonization is affected by the mutation in CJSA_RS00180, adhesion and invasion assays may provide additional insights. These assays can provide a closer look at examining which stages of colonization are affected by the mutagenesis. These assays have been tested in other \textit{C. jejuni} MTase mutants and while the results have been mixed
depending on the strain, MTase mutants were observed to either have no differences in adhesion and invasion (O’Loughlin et al., 2015), display hyper-adherence but decreased invasion ability (Kim et al., 2008), or severely reduced adhesion and/or invasion abilities compared to wildtype (Anjum, 2013; Sałamaszyńska-Guz et al., 2014).

Another potential area for future characterization of the MTase mutant is testing the effects of the mutation on the expression of genes containing the GAAGAA that are normally hypermethylated in IA3902 (Mou et al., 2015). Examples of phenotypes that can be tested include chemotaxis (Khanna et al., 2006; Vegge et al., 2009), glutamate synthase and DnaA expression and activity (qPCR, protein expression studies), and toluene tolerance (growth studies in the presence of toluene (Segura et al., 2008) or antimicrobial susceptibility testing).

Collectively, the work here provides the foundation for future studies that are greatly needed to fill in our limited understanding of the multi-functional roles and impact of putative restriction-modification enzymes such as CJSA_RS00180 in the biology, pathogenesis, and disease presentations of C. jejuni strains. These questions and other postulated above will help move the field towards complete characterization of MTase mechanisms, and ultimately gain valuable knowledge that may lead towards novel drug development against C. jejuni disease.
Figure legends.

Figure 1.

**Diagram of insert in pRS00180 plasmid construct.** Large white arrows represent the two fragments of CJSA_RS00180 coding sequence and its flanking genes. Large dark gray arrow labeled \( Cm^r \) represents the chloramphenicol resistance cassette. DNA fragments were amplified with primer pairs indicated by small arrows. The three DNA fragments were ligated with pUC19 vector using the NEB HiFi DNA assembly kit. pRS00180 was introduced as a suicide vector and replaced the functional chromosomal CJSA_RS00180 gene to form an insertional mutation. Figure not drawn to scale.

Figure 2.

**Comparison of the 1130bp region of CJSA_RS00180 gene with methyltransferase genes of other Campylobacter species.** ORFs are indicated by the arrows with the respective gene or locus tag designations shown to the right of the arrows. Percent homology of the entire MTase gene from other *Campylobacter* species with *C. jejuni* IA3902 CJSA_RS00180 MTase gene is indicated in black if 65% or less, or indicated in white if at least 82% or higher. The patterned regions within the arrows indicate 1130bp regions that either are homologous (vertical stripes) or not homologous (horizontal stripes) between CJSA_RS00180 gene with regions in the MTase genes of other *Campylobacter* species. The non-phase variable region of CJSA_RS00180 is found in Type II MTase genes of other *Campylobacter* species which suggest IA3902 may have acquired the non-phase variable region through a horizontal gene transfer event. Percent homology of the vertically striped regions from *C. lari* subsp. *concheus* LMG 11760, *C. jejuni* subsp. *doylei* 269.97, and *C. coli* RM1875 with CJSA_RS00180 are 85%, 89%, and 97%, respectively. Figure not drawn to scale.

Figure 3.

**Growth of IA3902ΔRS00180 broth culture at 42°C over a 48 hour period was like IA3902 wildtype.** Overnight cultures of IA3902 and IA3902ΔRS00180 were diluted in MH broth and incubated under microaerobic conditions at 42°C. Sample aliquots of cultures were taken at designated time points and enumerated by drop plating on MH agar and incubating for 24h at 42°C. Each data point represents the mean CFU/ml of three independent growth experiments ± SD. No significant differences in growth were found.
Figure 4.

**Motility assay of IA3902 and IA3902ΔRS00180 incubated at 42°C.** Motility was assayed on MH + 0.4% agar incubated microaerobically for 48h. Bars represent the mean radius (widest portion from the center of the stab to outermost edge of motility ring) of three independent experiments, and the standard deviations are represented by error bars.
Figure 2.

C. jejuni subsp. jejuni 11168
--- GGGGGGGGGGGG -- cj0031

C. jejuni subsp. jejuni S3
--- GGGGGGGGGG -- CJS3_0031

C. jejuni subsp. jejuni strain 00-0949
--- GGGGGGGGG -- PJ16_00180

C. jejuni subsp. jejuni F38011
--- -- CJH00185

C. jejuni subsp. jejuni IA3902
--- -- CJS_A500180

C. jejuni subsp. doylei 269.97
--- -- JJD26997_0043

C. coli RM1875
--- -- JJD26997_0043

C. lari subsp. concheus LMG 11760
--- -- CONCH_1400
Figure 3.
Figure 4.
Tables.

**Table 1. Bacterial strains (A) and plasmids (B) used in this study.**

A.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Description</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni IA3902</td>
<td>Wildtype</td>
<td>(Plummer et al., 2012)</td>
</tr>
<tr>
<td>C. jejuni IA3902ΔRS00180</td>
<td>IA3902 CJSA_RS00180 insertion mutation</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 ihi-1 hsdR17</td>
<td>New England Biolabs, Ipswich, MA</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19</td>
<td>pUC19 cloning and suicide vector with ampicillin resistance, and α-peptide of β-galactosidase</td>
<td>Invitrogen, Thermo Scientific, Waltham, MA</td>
</tr>
<tr>
<td>pRS00180</td>
<td>pUC19 plasmid containing chloramphenicol resistance cassette, and 500bp upstream and downstream sequences of CJSA_RS00180</td>
<td>This study</td>
</tr>
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</table>
Table 2. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’—3’)</th>
<th>Target gene</th>
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</thead>
<tbody>
<tr>
<td>45931F</td>
<td>GCTCGGTACCCGGGGATCCTAAAGAAT GTTTAAAAATTATTTAAAGATGTAAG</td>
<td>First fragment of CJSA_RS00180: 45931-46431bp (300bp upstream CJSA_RS00180 and first 200bp of CJSA_RS00180 gene) and overlap with pUC19</td>
</tr>
<tr>
<td>45931R</td>
<td>GTCCGCTCCAAGTTTTGAAATTTAACAT GGTTAAAAATG</td>
<td>First fragment of CJSA_RS00180: 45931-46431bp (300bp upstream CJSA_RS00180 and first 200bp of CJSA_RS00180 gene) and overlap with cat cassette</td>
</tr>
<tr>
<td>catF</td>
<td>TTTCAAAACTGGAGCGGACAACGAGTA</td>
<td>cat cassette and overlap with 45931-46431bp region</td>
</tr>
<tr>
<td>catR</td>
<td>TTACTATAGGTATCAGTGCGACAAACT GG</td>
<td>cat cassette and overlap with 49804-50304 region</td>
</tr>
<tr>
<td>49804F</td>
<td>CGCACTGATACCTATAGTAACCTAAA TTCCAAAAATC</td>
<td>Second fragment of CJSA_RS00180: 49804-50304bp (last 200bp of CJSA_RS00180 gene and 300bp downstream of CJSA_RS00180) and overlap with cat cassette</td>
</tr>
<tr>
<td>49804R</td>
<td>TGCATGCCTGAGGTCGACTTAGCCAT AAAAGTAGTTACACTTTAG</td>
<td>Second fragment of CJSA_RS00180: 49804-50304bp (last 200bp of CJSA_RS00180 gene and 300bp downstream of CJSA_RS00180) and overlap with pUC19</td>
</tr>
<tr>
<td>U</td>
<td>TGTTAAAACGACGGCCAGT</td>
<td>Universal (21M13) primer, targets pUC19 multi-cloning site</td>
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Table 2 (continued).

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<tr>
<th></th>
<th>CAGGAAACAGCTATGACC</th>
<th>Reverse (M13) Primer, targets pUC19 multi-cloning site</th>
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<tr>
<td>R-1</td>
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<tr>
<td>45931F-a</td>
<td>TCAACCCATACTACCGCAAAA</td>
<td>Overlap of 45931-46431bp and \textit{cat} cassette</td>
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Table 3. Comparison of methylome motifs detected within *C. jejuni* 1A3902 and 1A3902ΔRS00180 genome.

<table>
<thead>
<tr>
<th>Motif&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Modification Type</th>
<th>IA3902 % Motifs Detected</th>
<th>IA3902ΔRS00180 % Motifs Detected</th>
<th>Partner Motif</th>
<th>R-M enzymes&lt;sup&gt;d,e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAAGAA</td>
<td>m6A</td>
<td>99.8</td>
<td>0</td>
<td>CjeIAORF32P&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>GAGNNNNNRTG&lt;sup&gt;b&lt;/sup&gt;</td>
<td>m6A</td>
<td>99.86</td>
<td>98.9</td>
<td>CAYNNNNNCTC</td>
<td>CjeIAORF994P</td>
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<tr>
<td>CAYNNNNNCTC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>m6A</td>
<td>99.72</td>
<td>99.2</td>
<td>GAGNNNNNRTG</td>
<td>CjeIAORF994P</td>
</tr>
<tr>
<td>CAAAAYG</td>
<td>m6A</td>
<td>100</td>
<td>98.6</td>
<td></td>
<td>CjeIAORF654P</td>
</tr>
<tr>
<td>TAAAYNNNNTGC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>m6A</td>
<td>100</td>
<td>99.2</td>
<td>GCANNNNNRTTA</td>
<td>M.CjeIAlII</td>
</tr>
<tr>
<td>GCANNNNNRTTA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>m6A</td>
<td>99</td>
<td>98.8</td>
<td>TAAAYNNNNTGC</td>
<td>M.CjeIAlII</td>
</tr>
<tr>
<td>RAATTY</td>
<td>m6A</td>
<td>99.2</td>
<td>99.1</td>
<td>RAATTY</td>
<td>M.CjeIAI</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sequence motifs are listed in the 5' to 3' direction. Underlined bases indicate the methylated base on the sequence. Motifs containing Y have either T or C nucleotide, while motifs with R have an A or G nucleotide.

<sup>b,c</sup> Complementary motif sequences

<sup>d</sup> Last column lists assigned restriction-modification (R-M) enzymes predicted to recognize respective motif(s).

<sup>e</sup> The motifs and associated MTases are excellent candidates for the respective R-M system types. However, because the designations are not definitive, further tests are required to confirm the functional statuses of the MTases

<sup>f</sup> The putative role of CJSA_RS00180, part of the CjeIAORF32P R-M system, as a MTase is the subject of this study.
Table 4. Penetration of IA3902 and IA3902ΔRS00180 through mucus show no significant differences at each fraction. Columns of 30mg/ml porcine gastric mucin in PBS were prepared in 1-ml tuberculin syringes, with bacteria inocula applied to the top of the column. The columns were incubated at 42°C for 30 min. After the incubation period, each fraction consisting of 0.1ml mucin was collected from the bottom of the column and plated for bacterial quantification. Values represent the mean CFU/ml three independent experiments. Fraction 1 represents the bottom of the column, fraction 10 at the top. It was not possible to count the CFUs for fractions 7-10 as there were too many to count (TNTC = Too Numerous To Count).

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<th>Strain</th>
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<td>IA3902</td>
<td>1</td>
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<td>17.6667</td>
<td>498.3333</td>
<td>525.6667</td>
<td>680</td>
<td>692</td>
<td>TNTC</td>
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<tr>
<td>IA3902ΔRS00180</td>
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<td>72</td>
<td>437.3333</td>
<td>426.6667</td>
<td>1072.667</td>
<td>105.3333</td>
<td>1226.667</td>
<td>TNTC</td>
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CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

As a leading cause of human bacterial gastroenteritis, *C. jejuni* is a highly adaptable organism able to survive in a wide range of host environments, varying from the intestinal tracts of animal sources to contaminated food and water sources (Acheson and Allos, 2001; Humphrey et al., 2007; Wagenaar and Jacobs-Reitsma, 2008). Its adaptability and survival in diverse niches and environments is largely due to the high genetic diversity present within and between strains (Gilbreath et al., 2011). This genetic diversity includes a large variety of virulence factors that enable *C. jejuni* to survive in these environments (Dasti et al., 2010). In addition, it possesses widespread intrinsic and acquired resistance mechanisms to several important antimicrobial drug classes, making it a serious public health problem worldwide. As a result, research efforts have pushed towards understanding the molecular and cellular mechanisms responsible for its adaptability to such diverse environments. Such insights into its disease mechanisms may thus help the development of potential targets for intervention.

Quorum sensing is a key regulatory system for environmental adaptation. The identification of the AI-2/LuxS system in *C. jejuni* (Elvers and Park, 2002) and association with *C. jejuni* pathogenesis mechanisms (Plummer, 2012) makes the LuxS system a promising target to attenuate *C. jejuni* virulence. In addition to its role in AI-2 production, LuxS is also a key enzyme involved in the AMC. The AMC is vital for the formation of SAM, an important methyl donor for significant biological processes such
as DNA methylation. DNA methylation is a key process in bacteria with enormous impact on a diverse number of phenotypes in addition to cell maintenance and bacterial defense (Jeltsch, 2002; Low and Casadesús, 2008; Vasu and Nagaraja, 2013; Chen et al., 2014). Though few studies have looked into the role of methylation in C. jejuni pathobiology, the evidence so far show the potential impact of DNA MTases and/or methylation on important pathogenic and physiologic aspects of C. jejuni. However, there are significant gaps in our knowledge on AI-2/LuxS system, methyl recycling, and DNA methylation in the adaptation and survival of C. jejuni.

Our main objective was to define the mechanisms of LuxS on methyl recycling, DNA methylation, and C. jejuni physiology. Our central hypothesis is that the AI-2/LuxS system, its role in methyl recycling, and DNA methylation are vital for C. jejuni physiology and phenotypic expression of virulence factors important for host colonization and general pathogenesis mechanisms. Research from this thesis partially confirmed our central hypothesis and provides new insights into the key roles and mechanisms of LuxS, methyl recycling and DNA methylation in C. jejuni physiology based on data generated from these studies.

Plummer et al. previously showed clear evidence of the critical role LuxS plays in virulence and fitness of IA3902 and 11168 W7 strains, as LuxS mutagenesis negatively impacts colonization of the gastrointestinal tract of several host species by these strains (Plummer et al., 2012). However, the physiologic basis for this colonization defect is unclear. In Chapter 2, we utilized a mechanistic approach to understand how luxS mutation affects the expression and function of physiologic factors important to the colonization ability of C. jejuni, including the CmeABC efflux system, cell morphology,
and motility through mucin substrate. We also measured and compared AMC metabolite levels of our \textit{luxS} mutant to wildtype to assess how the \textit{luxS} mutation disrupted the AMC.

Results showed that the \textit{luxS} mutation completely disrupted the AMC by altering concentrations of AMC metabolites that were upstream and downstream of LuxS in the metabolic pathway. When we conducted a growth study, under basal conditions, to analyze the effects of the \textit{luxS} mutation on expression levels of multidrug efflux pump genes \textit{cmeABC}, we found no significant changes. Similarly, the \textit{cmeR} response regulator showed consistently lower expression levels but failed to reach a level of statistical significance. When we tested the \textit{luxS} mutant’s ability to penetrate through a mucin column assay, motility was somewhat affected by the \textit{luxS} mutation in specific strain-temperature environments. However, this effect was not universal and thus likely not the primary driver for the colonization defect of \textit{luxS} mutant observed previously (Plummer et al., 2012). We found no significant differences in cell and flagellar morphology at both the population level and at the individual cell level, suggesting that morphology is not responsible for the slight changes in mucin penetration observed in one of our \textit{luxS} mutants. All in all, these findings reveal that the CmeABC multidrug efflux pump, cell morphology and mucin penetration are not the major mechanisms underlying the \textit{luxS} mutant’s decreased ability to colonize and/or translocate out of the host’s intestine.

In recent years, the tetracycline-resistant hypervirulent \textit{C. jejuni} clone SA has become the predominant cause of \textit{Campylobacter}-associated sheep abortions in the US (Sahin et al., 2008). This clone also showed zoonotic potential (Sahin et al., 2012), which has heightened research efforts to improve our understanding of this organism’s biology and pathogenesis. Comparative genomic analysis of IA3902 and 11168 by Wu et al.
identified several differences between the two strains, including global gene expression profiles attributed to small genomic changes within the chromosome (Wu et al., 2013).

In Chapter 3, we used SMRT sequencing to profile the methylome of *C. jejuni* IA3902 and compared it with the methylome profiles of *C. jejuni* 11168 and 81-176. We found several features including methylation motifs, hypo- and hypermethylated regions, and specific genes methylated that distinguished IA3902 from 11168 and 81-176. In essence, their methylation profiles were very different and enriched with known virulence genes. These findings revealed the immense potential that methylation plays in *Campylobacter* pathobiology and will serve as the foundation for future *Campylobacter* methylome studies.

We also compared methylome profiles between IA3902 and its *luxS* mutant to assess whether mutation of *luxS* and disruption of the AMC would affect DNA methylation, which rely on AMC for the SAM methyl donor. Results showed no differences in methylome profiles, indicating that the *luxS* mutation had no effect on DNA methylation. The AMC metabolite results from Chapter 2 further support the methylome results, as measurable levels of SAM and methionine were found in the *luxS* mutant. These data suggest that the intracellular concentrations of these two metabolites were sufficient to not affect DNA methylation function.

The methylome profile of IA3902 revealed a unique putative MTase called CJSA_RS00180, which was predicted to methylate a motif specific to IA3902. CJSA_RS00180 also shares sequence homology with the 11168 Cj0031 MTase. Cj0031 possesses an 1130bp region that contains a phase variable polyG tract. The phase ON
state and expression of *cj0031* was found to play a beneficial colonization role in *C. jejuni*. CJSA_RS10080 does not contain a homopolymeric tract in the same 1130bp region which renders the gene in a constitutive phase ON state. To determine whether CJSA_RS00180 shares the same critical colonization role as Cj0031, we first confirmed the MTase function of CJSA_RS00180 in Chapter 4. In this study, we created a CJSA_RS00180 mutant and used SMRT sequencing to confirm that the mutation eliminated its MTase function and confirmed that its putative cognate methylation motif was GAAGAA. Additional colonization-related phenotypic studies characterized the MTase’s role in *in vitro* growth and motility and revealed that mutation of this gene did not affect its growth or motility on both a semi-solid agar assay and through a mucin column. Though no observable differences in growth and motility were found, future studies will continue to characterize the effects of the CJSA_RS00180 mutation on IA3902 colonization and virulence potential.

In summary, our central hypothesis was partially confirmed, whereby we showed the potential impact of DNA methylation in *Campylobacter* pathobiology when we compared the methylation profiles of IA3902, 11168, and 81-176 (Chapter 3). In addition, we confirmed that CJSA_RS00180 encodes a functional MTase that specifically methylates GAAGAA motifs in IA3902 (Chapter 4). However, our hypothesis regarding the role of LuxS on the physiologic factors tested in this thesis were not supported by the data with the exception of the disruption of the AMC (Chapter 2). The factors we tested, including CmeABC multidrug efflux pump, cell morphology, and mucin penetration showed that these mechanisms are not the main causes for the luxS mutant’s decreased colonization and/or translocation ability in the host intestine. Since we did not see any
significant changes in our studies, additional factors that are also crucial to the colonization step would be considered for future studies, including *in vitro* adhesion and invasion, chemotaxis, expression of other efflux systems in *C. jejuni*, and *in vivo* colonization.

**Recommendations for future research**

The conclusions from Chapter 2 bring to our attention the complexity and our limited understanding of the phenotypic impact of the *luxS* mutation on traits critical to *C. jejuni* pathobiology. We expected the *luxS* mutation to decrease the expression of *cmeABC* operon and *cmeR* regulator as we hypothesized the functions of the CmeABC efflux pump and CmeR regulator would be attenuated in the *luxS* mutant. However, despite the lack of significant expression changes observed in these two genes, there is the potential for the *luxS* mutation to induce the expression of other efflux systems associated with CmeR regulator like Cj0035c, Cj0561c, and Cj1619 (Guo et al., 2008), and those that function independently of CmeR such as CmeDEF.

In addition, future studies will examine the effects of the *luxS* mutation on other colonization-related phenotypes including *in vitro* adhesion and invasion, taxis towards chemoattractants (Vegge et al., 2009), and also how stress conditions would change the efflux expression in the *luxS* mutant (He et al., 2008).

Chapter 3 conclusions revealed many questions regarding the impact of methylation in *C. jejuni* virulence. In order to explore how methylation regulates the expression and function of factors important to *C. jejuni* virulence, we first confirmed the
putative role of CJSA_RS00180 from IA3902 in Chapter 4. While we did not find any observable differences in the growth and motility of our CJSA_RS00180 mutant, a potential area for further characterization of CJSA_RS00180 MTase mutant is testing the effects of the MTase mutation on the expression of genes containing the GAAGAA that are normally hypermethylated in IA3902. In addition, the conditions we used to test our mutant were limited to *in vitro* growth in rich media. Thus, we cannot rule out the possibility that the growth of the mutant would not be altered in other more stressful environmental conditions. Further analysis of the mutant warrant the use of different growth conditions, profile gene expression, or use additional *in vitro* or *in vivo* models.

CJSA_RS00180 shares sequence homology with 11168 MTase gene *cj0031*. The only difference between the two sequences is the presence of a phase variable region in *cj0031* that is non-phase variable in the same location of CJSA_RS00180. Phase ON variants of *cj0031* were found to be positively selected for among the colonizing *C. jejuni* population. This indicates that *cj0031* expression *in vivo* was required for coordinating the expression of genes in the genome of 11168, which then enable adaptation and colonization of the organism within the host intestinal tract. Since CJSA_RS00180 is not phase variable, we hypothesize that CJSA_RS00180 will function similarly to the phase ON state of Cj0031 and that a fully-functional CJSA_RS00180 MTase is required for successful colonization and adaptation to the host intestinal tract. On the other hand, mutation of the MTase gene is expected to cause a colonization defect in the mutant. Future studies will conduct animal studies to analyze the colonization ability of the MTase mutant.
REFERENCES


Evolution in an oncogenic bacterial species with extreme genome plasticity: *Helicobacter pylori* East Asian genomes. *BMC Microbiology* 11, 104.


Mruk, I., and Kobayashi, I. (2013). To be or not to be: regulation of restriction–
modification systems and other toxin–antitoxin systems. *Nucleic Acids Research*
42, 70-86. doi: 10.1093/nar/gkt711.

Cell-free preparations of *Lactobacillus acidophilus* strain La-5 and
*Bifidobacterium longum* strain NCC2705 affect virulence gene expression in
*Campylobacter jejuni*. *Journal of Food Protection* 76, 1740-1746. doi:
10.4315/0362-028x.jfp-13-084.

Muraoka, W.T., and Zhang, Q. (2011). Phenotypic and genotypic evidence for l-Fucose
utilization by *Campylobacter jejuni*. *Journal of Bacteriology* 193, 1065-1075. doi:

Murray, I.A., Clark, T.A., Morgan, R.D., Boitano, M., Anton, B.P., Luong, K.,
Fomenkov, A., Turner, S.W., Korfach, J., and Roberts, R.J. (2012). The
methyloomes of six bacteria. *Nucleic Acids Research* 40, 11450-11462. doi:
10.1093/nar/gks891.

Naresh, R., and Hampson, D.J. (2010). Attraction of *Brachyspira pilosicoli* to mucin.
*Microbiology* 156, 191-197. doi: 10.1099/mic.0.030262-0.


Naughton, J.A., Mariño, K., Dolan, B., Reid, C., Gough, R., Gallagher, M.E., Kilcoyne,
M., Gerlach, J.Q., Joshi, L., Rudd, P., Carrington, S., Bourke, B., and Clyne, M.
(2013b). Divergent mechanisms of interaction of *Helicobacter pylori* and
*Campylobacter jejuni* with mucus and mucins. *Infection and Immunity* 81, 2838-

Nebi (2015). "*Campylobacter jejuni* Genome Assembly and Annotation report".)

virulence protein is secreted from the flagellum and delivered to the cytosol of
host cells. *Front Cell Infect Microbiol* 2.

Ng, W.L., and Bassler, B.L. (2009). Bacterial quorum-sensing network architectures.
*Annu Rev Genet* 43, 197-222.

Nishiyama, S. - I., Suzuki, D., Itoh, Y., Suzuki, K., Tajima, H., Hyakutake, A., Homma,
*Vibrio cholerae* implicated in pathogenicity functions as a chemoreceptor for
multiple amino acids. *Infection and Immunity* 80, 3170-3178. doi:
10.1128/iai.00039-12.

modification gene homologues in *Helicobacter pylori*. *Gene* 259, 89-98. doi:
10.1016/S0378-1119(00)00455-8.

O’loughlin, J.L., Eucker, T.P., Chavez, J.D., Samuelson, D.R., Neal-McKinney, J.,
*Campylobacter jejuni* genome by SMRT DNA sequencing identifies restriction-
modification motifs. *PLoS ONE* 10, e0118533. doi:
10.1371/journal.pone.0118533.

Osaki, T., Hanawa, T., Manzoku, T., Fukuda, M., Kawakami, H., Suzuki, H.,
Mutation of luxS affects motility and infectivity of *Helicobacter pylori* in gastric
mucosa of a Mongolian gerbil model. *Journal of Medical Microbiology* 55, 1477-1485. doi: 10.1099/jmm.0.46660-0.


Sahin, O., and Zhang, Q. (2010). "Complete genome sequence of a highly virulent *Campylobacter jejuni* strain associated with sheep abortion in the United States.".


CHAPTER 3

All supplementary data and tables are accessible by the following web address: http://journal.frontiersin.org/article/10.3389/fmicb.2014.00782/abstract