LOCALIZATION OF THE NATURAL TRANSFORMATION MACHINERY AND MECHANISMS OF DNA SELECTION IN *CAMPYLOBACTER JEJUNI*

by

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DEDICATION

To my mother, Jan Beauchamp

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LIST OF ACRONYMS AND ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
Cas	CRISPR associated
cm	Chloramphenicol
CRISPR	Clustered, regularly interspaced, short palindromic repeats
CSP	Competence stimulating peptide
Cts	*Campylobacter* transformation system
dsDNA	Double-stranded DNA
DUS	DNA uptake sequence
eDNase	Extracellular Dnase
GBS	Guillain-Barré Syndrome
gDNA	Genomic DNA
HGT	Horizontal gene transfer
kan	Kanamycin
LacZ	Beta-galactosidase
LB	Luria Bertani
M	Modification
m4C	N^4-methylctyosine
m5C	N^5-methylcytosine
m6A	N^6-methyladenine

MCS	Multiple Cloning Site
me^+	Methylated DNA
me^0	Unmethylated DNA
MH	Mueller Hinton
MTases	DNA methyltransferases
NA	Naladixic Acid
NTPase	NTP cleaving enzyme
PhoA	Alkaline phosphatase
PT	Phosphorothioated
PCR	Polymerase Chain Reaction
QS	Quorum sensing
R	Restriction
REase	Restriction enzyme
RM	Restriction modification
S	Specificity
SAM	S-adenosylmethonine
SMRT	single-molecule real-time
ssDNA	Single-stranded DNA
T2SS	Type II secretion system
T4SS	Type IV secretion system
T6SS	Type VI secretion system
Tfp	Type IV pilus biogenesis system

ABSTRACT

The human pathogen *Campylobacter jejuni* is naturally competent for transformation with its own DNA. In this thesis work, we investigated key gene products of the transformation apparatus as well as the mechanism by which *C. jejuni* selects DNA for uptake and recombination. We demonstrate that two NTPases, CtsP and CtsE, are required for transformation. Their localization to the membrane is through a mechanism not generally observed with other competence ATPases. Further, CtsP interacts with another competence protein, CtsX, a single-pass transmembrane protein lacking significant homology to other proteins. Also investigated was the mechanism of DNA selection in *C. jejuni*. This bacterium is very selective in the DNA it uses during transformation, only using self or DNA derived from closely related strains. We demonstrate that this selection is based on adenine-methylation within a specific sequence motif, RAATTY. This site is broadly conserved in *Campylobacter* species and is over-represented in the *C. jejuni* genome. Methylation at RAATTY is conferred by CtsM, an orphan DNA methyltransferase also highly conserved in *Campylobacter*. CtsM is dispensable for transformation, but genomic DNA from a *ctsM* mutant serves as a transformation substrate with efficiency several orders of magnitude below DNA from a *ctsM*$^{+}$ strain. A single methylated site is sufficient to confer wild type transformability to otherwise untransformable DNA. We also demonstrate that DNA lacking RAATTY methylation is transported

inside the cell, implying that discrimination of methylated versus unmethylated DNA does not occur on the outer membrane. Finally, this work provides a potentially powerful tool to carry out rapid genome editing of *C. jejuni*, requiring only that an incoming homologous DNA fragment be RATTY-methylated for uptake and recombination. This is the first example of a methylation dependent mechanism of DNA selection during transformation.

CHAPTER I

INTRODUCTION

1.1 Introduction

Natural transformation, or competence, is the process by which bacteria encode a dedicated transport system to bring DNA from their environment into the cell and recombine it into their genome. This process uses protein machinery conserved in systems of protein secretion and pilus assembly; this machinery is similar in most bacteria that are naturally competent. Notwithstanding this similarity, competent bacteria have distinct mechanisms to regulate the recognition and uptake process, enabling them to optimize DNA uptake by enhancing the likelihood of taking up DNA from closely related bacteria. These mechanisms include activating competence under circumstances where the bacteria are likely to encounter DNA, either through quorum sensing or by promoting active DNA release.

DNA methyltransferases (MTases), and the modifications they provide, are even more widespread than natural transformation. These enzymes often work in concert with restriction endonucleases in restriction modification (RM) systems. These systems protect the bacteria from incoming DNA. MTases not

part of a restriction modification system regulate gene expression through epigenetic DNA modification.

C. jejuni is a naturally competent bacterium that causes bacterial gastroenteritis. Competence in *C. jejuni* appears to be a constitutive trait, although the efficiency can be affected by different conditions tested in the laboratory [1, 2]. *C. jejuni* is also competent during *in vivo* conditions such as in the chicken intestinal tracts and within biofilms [3, 4].

The predominant machinery for DNA uptake is encoded by the *cts* system, which includes gene products similar to those used in both secretion and pilus assembly systems in a range of bacteria [5]. At least in the laboratory, *C. jejuni* can efficiently select its own DNA and discriminate against DNA from other species; the mechanism by which this occurs has not been determined. The *C. jejuni* genome encodes a number of RM systems and orphan MTases, which may contribute in part to its ability to discriminate DNA.

1.2 *Campylobacter jejuni*

Campylobacter species are Gram-negative, microaerophilic, spiral-shaped, motile bacteria [6]. They can be found in the gastrointestinal tract of many animals and avian species where they are often considered commensal bacteria, particularly in avians. They have a small genome, approximately 1.6-1.7 Mbp in size [7-9]. They have flagella at each pole of the cell and are small in size ranging from 0.5-5 µm long and 0.2-0.8 µm wide. Unlike many other bacteria, they primarily rely on amino acids for energy. As they are colonizers of both

mammals and birds, they grow equally well at 37°C and 42°C, the respective body temperatures of those animals.

1.2.1 Campylobacteriosis

Campylobacter spp. are among the major cause of acute bacterial gastroenteritis worldwide. Most of these cases are from either *C. jejuni* or *C. coli.* In 2014, 88% of the speciated isolates causing campylobacteriosis in the United States were *C. jejuni* and 9% were *C. coli* [10]. The infectious dose is very small, ingenstion of fewer than 500 bacteria are required to cause illness [11]. Transmission generally occurs from contaminated food or water sources. A major source of infection by humans is poultry; in 2011, *Campylobacter* was found on 47% of raw chicken samples in grocery stores that were tested through the National Antimicrobial Resistance Monitoring System (NARMS) [10]. In humans, *C. jejuni* will colonize the intestinal epithelium causing a range of symptoms from mild, watery diarrhea to severe, bloody diarrhea. *C. jejuni* is estimated to infect over 1.3 million individuals annually in the United States [11]. Infection is also associated with the development of Guillain-Barré syndrome (GBS), a severe immune mediated neurological disease that results in paralysis [11]. According to the CDC, as many of 40% of the GBS cases in the country may be triggered by campylobacteriosis [11].

C. jejuni has also been declared to be an urgent threat by the CDC, due to the rise of antibiotic resistance. According to a 2013 report, there are 310,000 drug-resistant *Campylobacter* infections per year [12]. Resistance to ciprofloxacin, one of the main drugs for treating infections, has risen to almost

25% of cases [12]. There is also documented resistance to azithromycin, one of the other main drugs for treating *Campylobacter* infections [12].

1.2.2 Genetic diversity

Bacterial species can vary widely in the extent of genome conservation. *Borrelia burgdorferi* exists mainly as a clonal population with little genetic diversity between sequenced strains. In contrast, *Helicobacter pylori* has a non-clonal structure with such extensive diversity that it is common to find a high degree of intra-strain sequence variation even within a single gene [13-16]. As *Campylobacter* spp. are closely related to *Helicobacter,* it is not surprising that it has extensive genetic diversity as well. This was first recognized when multilocus sequence typing (MLST) was applied to *Campylobacter* isolates [17-19]. One such study looked at seven housekeeping genes from thirty-three *C. jejuni* isolates. Of the 33 isolates tested, only two had identical sequences for all seven genes, and a homoplasy ratio between 0.36 and 0.48 was determined [17]. The homoplasy test indicates the frequency of recombination within a population, with values ranging from 0 to 1. Zero indicates clonal descent and one indicates non-clonal descent where the population has been freely recombining [15]. Another study looking at more strains concluded that intra-and interspecies recombination frequently occurs in *Campylobacter* [19]. The amount of genetic diversity observed in *C. jejuni* is less that of *Streptococcus pneumoniae,* but similar to that of *Neisseria meningitides*. Whole genome sequencing of *Campylobacter* strains confirms these earlier studies postulating a high degree of genetic exchange [20].

The two major mechanisms for generating diversity within bacterial species are i) through replication errors or DNA damage and ii) through horizontal gene transfer (HGT). Accumulation of mutations leads to a slow and steady diversification over time. HGT results in transfer of large pieces of DNA and therefore quick, significant change. The three major mechanisms of HGT are conjugation, transduction, and natural transformation. Conjugation is the contact-dependent transfer of DNA between two strains. Transduction is the transfer of DNA between bacteria by bacteriophage. Natural transformation is the acquisition of DNA from the environment by a bacterium through a dedicated protein transport system. *C. jejuni* undergoes HGT from all of these processes, contributing to its genetic diversity.

Some of the genome diversity can also be attributed to its phase variation system. Phase variation occurs due to alteration within polyG:C tracts. Changes in the number of nucleotides within a region containing a polyG:C tract, most likely due to slipped-strand mispairing during chromosome replication, shifts the gene in and out of frame and thus cause phenotypic variation [21]. Production of lipo-oligosaccharide, a key molecule underlying GBS, is regulated by phase variation [22-24]. At least eight other genes are thought to be phase variable as well, including a MTase with potential regulatory roles that will be discussed in Section 1.5.5 [21, 25, 26]

1.3 Natural transformation

Natural transformation has been described in over 65 species [27]. While many species can be artificially induced to take up DNA by manipulating their

membranes, naturally competent bacteria encode dedicated machinery used to transport DNA from the extracellular space into the cytosol where it can recombine into the genome. Generally this machinery resembles that of Type II secretion systems (T2SS) or Type IV pilus biogenesis systems (Tfp) [28]. *H. pylori* is a major exemption, as it uses a Type IV secretion system (T4SS) typically associated with conjugation (T4SS) [29, 30]. *C. jejuni* carries a plasmid, pVir, that encodes a T4SS that has been implicated in competence, but it is not the major contributor to this trait as the Cts system noted above predominates for competence [5, 31]. Deletion of transformation genes encoded on pVir decreases transformation efficiency but the decrease is negligible as compared to deletion of cts genes where few to no transformants are ever detected.

1.3.1 Proposed benefits to natural transformation

There are three principal hypotheses for fitness advantages conferred by a dedicated DNA uptake system; they are not mutually exclusive. These are 1) DNA for food, 2) DNA for repair, and 3) DNA for evolution. These have been extensively reviewed but brief summaries of each, and the major evidence supporting them, are provided below [32, 33].

DNA for food

The idea of DNA as a source of nutrients proposes that competence exists primarily to transport a source of nucleotides into the bacterium instead of synthesizing them *de novo* [34, 35]. For this hypothesis, recombination that occurs (DNA for repair or evolution) is a bonus of acquiring DNA as a metabolic substrate. The main arguments underlying this hypothesis are 1) using DNA for

food nets an immediate gain whereas any benefits from recombination may only be selected for long term. 2) Nutritional stress can induce competence in some species, including *Haemophilus influenzae* and *Bacillus subtilis*, though this is not true of all bacteria. In fact, some bacteria, such as *Streptococcus pneumoniae,* induce competence in rich media at low cell density, conditions in which there is presumably no nutritional stress [35-38]. 3) DNA damage does not induce competence in all bacteria, notably *H. influenzae* and *B. subtilis.* If competence were used for repair, DNA damage would be predicted to induce competence. 4) While homologous DNA can recombine, non-homologous DNA may be degraded and used as a nutrient source. 5) DNA that is in the extracellular environment might be damaged and therefore would not be a suitable substrate for recombinational repair or evolution [39, 40]. However, many bacteria have evolved mechanisms to co-regulate competence with bacterial killing or DNA donation so that the DNA they acquire would be in good condition [41, 42]. This has been demonstrated in *S. pneumoniae* and *Neisseria gonorrhoeae* and will be discussed in more detail later. Work in *E. coli* and *Vibrio cholerae* support this hypothesis as each of these bacteria can grow on DNA as a sole carbon source so DNA can be used for nutritional purposes [43-46]. However, it is unclear if the mechanism for transport of the DNA is identical to that used in competence.

A number of findings do not support a hypothesis that DNA is a food source. For example, in addition to its competence system, *B. subtilis* has an extracellular nuclease and uptake systems for nucleotides and nucleosides. It is unclear why a separate DNA uptake system would be maintained if the cell were

able to take in nucleotides directly [28]. As will be described in detail below, both *V. cholerae* and *C. jejuni* possess extracellular DNases whose activity inhibits competence [47-50]. Furthermore, the advantage of using a complex, multiprotein competence machinery that requires ATP cleavage to bring in DNA, as opposed to simply synthesizing nucleotides, is not clear [5, 28, 51, 52]. DNA uptake motors have been described as some of the strongest linear motors characterized to date and are energetically costly to the cell [51, 53-55].

DNA for repair

The major argument that DNA is taken up primarily to repair errors in the chromosome is that many species of competent bacteria are either biased towards or selective for DNA from closely related individuals. This will be discussed later, but briefly these mechanisms include using DNA uptake sequences as in *Neisseria* and *Haemophilus*, using restriction modification systems as in *Helicobacter* and *Pseudamonas*, using a competence pheromone as in *V. cholerae* and *S. pnuemoniae*, or using CRISPR/Cas as in *N. gonorrhoeae*. By selecting the DNA brought into the cell during competence, the bacteria can maintain genetic integrity. It should be noted that studies in *H. pylori* or *Legionella pneumophila* have provided evidence against this hypothesis. When their DNA uptake systems were inactivated, there was no change in their susceptibility to DNA damage inducing agents [56, 57]. Also, as noted above, neither *B. subtilis* nor *H. influenzae* induce competence in response to DNA damage. However, *S. pneumoniae* does induce competence upon DNA damage. Normally the protease HtrA degrades CSP. When misfolded proteins accumulate

because of stress of genomic errors, they become the preferred substrate for HtrA which allows CSP to accumulate and induces competence.[58]**.**

DNA for evolution

The third hypothesis for natural transformation is that the ability to import DNA and recombine it onto the chromosome might allow for rapid evolution in times when diversity is be beneficial, such as during high population densities, nutrient stress or starvation, or DNA damage. As all of these are known to induce transformation in at least some bacteria, there is a great deal of evidence for this hypothesis. However, directly testing this hypothesis is challenging [59]. Most experiments have a bias towards seeing benefits from acquiring DNA. There have been a few studies, including one with long-term *in vitro* passage of *H. pylori* where competent bacteria increased their fitness faster than their non-competent counterparts [60]. Furthermore, because competence is a complex process that is often co-regulated with other processes such as fratricide or bacteriocin production, Type VI secretion, or quorum sensing, unlinking the evolutionary benefits that derive directly from competence as opposed to these other traits is challenging [42, 61-66].

As mentioned in the introduction to this section, none of these hypotheses are mutually exclusive. Considering the diversity in competent bacteria, in the regulation of the systems, and in the makeup of the systems themselves, it seems likely that there is no single hypothesis that completely describes the fitness advantage conferred by the ability to take up intact DNA. It is also

possible that different forces drove the development of competence and thus different hypotheses hold for different bacteria.

1.3.2 Regulation of transformation

The strategies for regulation of transformation are diverse and vary in different bacteria. Some bacteria, such as *H. pylori* and *N. gonorrhoeae,* are constitutively competent [56, 67]. Other bacteria, such as *S. pneumoniae* and *B. subtilis*, tightly regulate their competence machinery such that they are only competent for small windows of time. In other cases, such as *V. cholerae,* competence is co-regulated with other bacterial processes such as quorum sensing and type VI secretion. As the regulation of competence has been extensively reviewed, I will only go into brief detail for *S. pneumoniae* and *V. cholerae* [28, 68-71].

In *V. cholerae,* competence is regulated by TfoX, the master competence regulator [72]. This is a homolog of the Sxy regulator in *H. influenzae* [72]. The TfoX regulon has not been completely deciphered, but we know that it regulates the biosynthesis of the structural genes in the Tfp machinery used by *V. cholerae* to bring DNA into the cell [73-75]. Some of these genes also require input from HapR, the master regulator of quorum sensing (QS) as well as QstR (QS-dependent and TfoX-dependent regulator) [48, 76, 77]. TfoX is induced by growth on chitin and HapR is induced by high cell densities and accumulation of CAI-1, the species-specific autoinducer of competence [48, 72]. Both TfoX and HapR activate QstR which goes on to activate a subset of genes involved in transporting the DNA from the periplasm to the cytosol (*comEC* and *comEA*) as

well as the T6SS operon. [48, 76] [63]. QS also regulates production of an eDNase, Dns, that is expressed at low cell densities and prevents transformation [47]. Expression of *dns* is repressed by HapR [47]. Repression of *dns* allows the competence machinery to gain access to intact DNA, facilitating DNA uptake. This extensive co-regulation of competence, QS, and T6SS results in competence expression during high cell densities by cells actively producing machinery to kill other bacteria and release DNA.

In *S. pneumoniae,* competence is induced by environmental cues such as antibiotic treatment or by early exponential growth, both which require the competence pheromone, CSP [37, 78, 79]. CSP is produced as peptide inside the cell and transported out through the ABC transporter ComAB [80, 81]. ComAB also processes CSP to its active form [81, 82]. Extracellular CSP is recognized by the two component system ComDE. ComE, the response-regulator, is phosphorylated by ComD, the histidine kinase, and then activates the early competence genes as well as the alternative sigma factor, *comX* [80, 83]. ComX activates the rest of the genes including the structural genes required for DNA transport. These transcription factors also activate fratricide effectors and bacteriocins, along with their respective immunity proteins [42, 66, 78, 84-86]. As in *V. cholerae,* co-regulation of these systems results in the activation of competence in an environment where there is likely to be DNA available for uptake.

1.3.3 Transformation Machinery

Generally, the machinery used in transformation resembles T2SS and Tfp systems, with the major exception being *H. pylori,* which uses a T4SS to transport DNA. This is depicted in Figure 1 [29, 51, 87-90]. Despite differences between Gram-positive and Gram-negative bacteria, there are many conserved features between the systems. These mechanisms have best been described in *B. subtilis* for Gram-positive organisms and in *N. gonorrhoeae* and *V. cholerae* for Gram-negative organisms and I will focus on those in this discussion [87, 89-91]. I will also include *H. pylori* as although it has a different system, in some aspects it functions similarly [88]. Information from bacteria such as *S. pneumoniae* will be included to fill in gaps in knowledge.

The goal of transformation is to transport DNA into the cytosol where it can recombine with the chromosome. Because the cell wall and membranes differ between Gram-positive and Gram-negative bacteria, each class of bacteria must to overcome different challenges to transport DNA into the cytosol. For Gram-positive bacteria, DNA has to be transported through a thick cell wall and then through one membrane into the cytosol. Gram-negative bacteria have two membranes through which they must transport DNA. Therefore in these bacteria, transport into the periplasm is the first step and this renders the incoming DNA resistant to eDNases [87]. In order to become eDNase resistant in Gram-positive bacteria, the DNA must be transported into the cytosol [87].

For both types of bacteria, it appears dsDNA is bound on the outside of the cell with varying degrees of specificity depending on the requirement for an uptake sequence. Once the DNA is bound, the transformation machinery can

transport the DNA into either the cytosol in the case of Gram-positive bacteria or into the periplasm in Gram-negative bacteria [87]. In Gram-negative bacteria, dsDNA is transported into the periplasm where it is bound by proteins that facilitate its transport to another pore through which it is then translocated into the cytosol. During the translocation process, one strand is degraded.

Step one: DNA binding and transport towards the cytosol.

As briefly described above, the first step in transformation is binding of the DNA to the cell surface. In *B. subtilis,* a pseudopilus is made composed of the ComG proteins (ComGC, GD, GE, GG) [92]. In intact cells, the pseudopilus is required for the interaction of the transforming DNA with the DNA binding protein ComEA which localizes at the cytoplasmic membrane [92]. When protoplasts (cells lacking peptidoglycan) are made, the pseudopilus is not required for the ComEA-transforming DNA interaction [92]. This indicates that the pseudopilus functions to penetrate the peptidoglycan and create a pore for the DNA to enter. It is unclear if the DNA directly interacts with the pilus, though it seems unlikely as the ComG proteins do not bind DNA [92]. However, in *S, pneumoniae* the competence pilus has been shown to directly bind to DNA. [93].

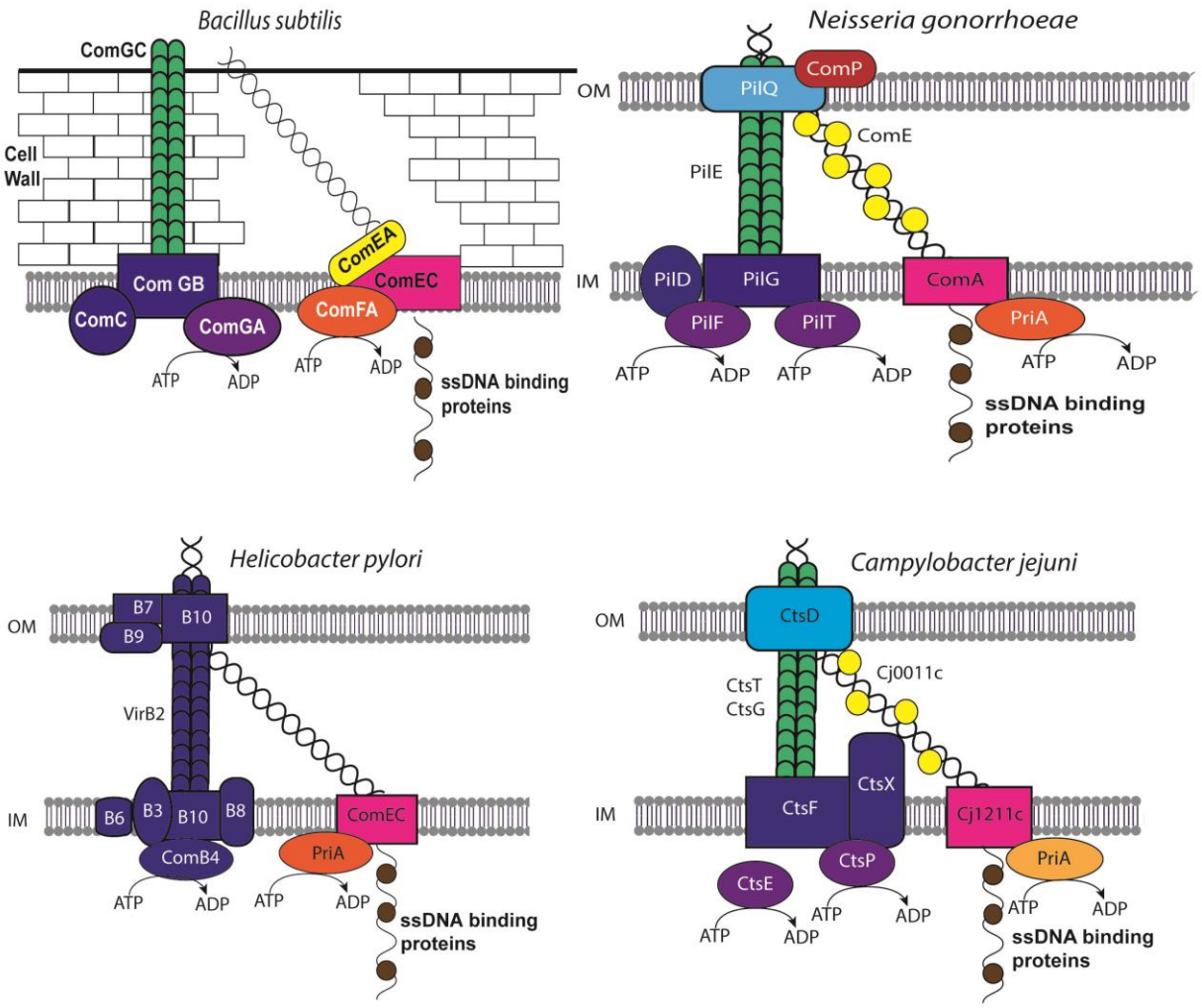

Fig 1.1. Model transformation systems. The transformation systems of *B. subtilis, N. gonorrhoeae, H. pylori* and *C. jejuni* are modeled above. Homologous or conserved proteins are color coordinated. Green denotes pilins or pseudopilins. Yellow denotes ComE-like DNA binding proteins. Pink denotes ComA or ComEC DNA transporters. Orange denotes either PriA or ComFA. Light orange denotes protein is found in the genome of *C. jejuni* but has not yet been implicated in competence. Purple denotes ATPases that power the pilus and transformation. ssDNA binding proteins are denoted in brown. Proteins that are not conserved are denoted in navy blue.

In Gram-negative bacteria, the DNA has to be transported sequentially through both membranes before it enters the cytosol. In *Neisseria*, the secretin PilQ in the outer membrane can bind to both dsDNA and ssDNA in a sequence-independent manner [94]. The minor pilin, ComP, binds specifically to the DNA uptake sequence (DUS) and is thought to be responsible for the import of DUS-containing DNA [95]. In *Neisseria,* functional type IV pili are required for transformation; this is not true of all Gram-negative bacteria [96]. Functional type IV pili require two ATPases, PilF and PilT for assembly and disassembly of the pilus respectively. The pore of PilQ is at least 6.5 nm at its narrowest point which is sufficiently large to allow for pili to translocate through [97]. The pili lumen are rather hydrophobic and measure about 1.2 nm in diameter [96]. This is not sufficiently large to allow dsDNA, approximately 2.4 nm, to pass through. These data lead to two main hypotheses for how the DNA enters the periplasm. The first is it binds directly to the pilus and as the pilus is disassembled by the action of PilT, the DNA is pulled into the periplasm. The second hypothesis is that retraction of the pilus opens a gap in the secretin and that gap is filled by DNA. Once the DNA is brought into the periplasm, it is bound by ComE, a homolog of ComEA in *B. subtilis* [88]. As a corollary to the second hypothesis proposed above, it is hypothesized that the binding of ComE to the incoming DNA might work as a ratchet mechanism that facilitates movement of the DNA across the membrane, though currently there are no experimental data to support that hypothesis [88].

Step two: transport across the cytoplasmic membrane

In both Gram-positive and Gram-negative bacteria, before the DNA is transported into the cytosol, it is double-stranded. At some point in the transport process, the DNA is processed into ssDNA; this happens in a strand-independent manner [98, 99]. The mechanism for this degradation has yet to be elucidated. Transport across the cytoplasmic membrane relies on the homologous membrane proteins ComEC and ComA of *B. subtilis* and *N. gonorrhoeae* respectively [100]. These proteins are thought to function as an oligomer with multiple transmembrane passes. The transport process is highly specific for DNA as DNA that has been modified such as covalently labelled fluorescent DNA is unable to be transported across the cytoplasmic membrane in both *H. pylori* and *B. subtilis* [101]. The motor that powers this process is not clear, though a strong pulling force has been recorded when uptake was measured with laser tweezers in *B. subtilis* [54]. This indicates that there is some molecular motor involved. ComFA, a DEAD-box helicase, is important for translocating DNA in *B. subtilis* [102]. However, genes encoding for ComFA homologs are not found in the genomes of many competent bacteria indicating that this helicase is not a universal component of transformation systems. PriA, a related helicase, is found ubiquitously in bacteria because it is involved in the restart of chromosomal replication after the replication fork has stalled. It is important in transformation in *Neisseria,* though it is unclear if it is involved in DNA import or later in recombination [103].

Single stranded DNA binding proteins are thought to bind to the DNA once it enters the cytoplasm to prevent reverse translocation [104]. These proteins

include single-strand DNA-binding protein (Ssb) and DprA [77, 105]. This binding also helps prevent DNA degradation. DprA is also required for loading RecA onto incoming DNA, and this step is required for homologous recombination [105].

Helicobacter pylori competence machinery

As noted earlier, *H. pylori* uses a T4SS to transport DNA across the membranes. There is also a similar system in *C. jejuni* but it is not the major transformation system, at least in laboratory settings [5, 31]. These systems are highly similar to the machinery used in conjugation. The crystal structure of the outer membrane components of the *H. pylori* system, VirB, has been determined [106]. This revealed an unexpectedly large structure of 17.2 nm with a 3.2 nm pore [106]. *In vivo*, it is likely that this pore is smaller because the VirB2 protein, thought to coat the inner portion of the channel, was not included in this structure. As might be expected with such a large pore as well as a completely different transport structure, the initial steps of DNA transport appear to be different in *H. pylori.* Loose contact of the DNA with the outer pore appears to take place and pilus retraction could play a role in uptake [101]. What happens to the DNA once it enters the periplasm is unclear as well, because *H. pylori* lacks a *comE* homolog. However, the pilin VirB2 binds to DNA in *Agrobacterium* and might functionally replace ComE [107]. After transport across the outer membrane, transport across the inner membrane in *H. pylori* appears similar to that of other competent bacteria. It uses a ComEC homolog to translocate the

DNA into the cytosol [101]. Once in the cytosol, the DNA is bound by single-stranded DNA binding proteins such as Ssb, DprA, and RecA [56].

1.3.4 Recombination

Transforming DNA enters the cytosol single-stranded. From this state, it must recombine in order to complete the transformation process. This process has been most well studied in *S. pneumoniae* so I will use that model for this discussion. When the ssDNA enters the cytoplasm, it is incorporated into a nucleoprotein complex [108, 109]. In this state, termed eclipse, the DNA is not capable of recombining onto the chromosome but it is protected from DNases [110]. In *S. pneumoniae,* eclipse lasts about 10 minutes at 37°C after which the DNA has integrated onto the chromosome [105]. The recombination process requires RecA, SsbB and DprA [105]. SsbB binds most of the incoming ssDNA, protecting it from DNases [105]. DprA binds either to the SsbB bound DNA and replaces it or to the free ssDNA[105]. DprA can then facilitate the loading of RecA [105]. RecA catalyzes heteroduplex formation and subsequent recombination [105]. This step requires a high degree of homology between the transforming DNA and the existing gDNA [111].

1.4 DNA selection during natural transformation

While natural transformation can be beneficial to the recipient cell, it can also result in the acquisition of deleterious mutations. Therefore, bacteria have evolved a number of mechanisms to select DNA during natural transformation, preferring to use DNA from closely related bacteria. Some of these methods are specific for transformation, whereas others work on the other forms of HGT,

conjugation and transduction. The methods I will discuss are restriction-modification (RM) systems, DNA uptake sequences (DUS), co-regulation of competence with quorum sensing, active DNA release, and CRISPR-Cas.

1.4.1 Restriction Modification systems

Restriction modification systems (RM systems) enable bacteria to combat phage infections. All endogenous DNA is modified using a DNA methyltransferase (MTase) which protects the DNA from degradation by a cognate restriction enzyme (REase). When a phage injects its DNA into the cell, it will lack the appropriate modifications and therefore be degraded by the REases. There are many types of these systems which will be discussed in depth in Section 1.5. In this section, I will examine how bacteria use these systems to select the DNA they use as a substrate for natural transformation.

Helicobacter pylori encodes a large number of RM systems. The J99 and 26695 strains are predicted to encode up to 26 putative RM systems [112]. One of those systems, M.HpyAXII/R.HpyAXII, targets GTAC sites and restricts unmethylated plasmid and chromosomal DNA during natural transformation [112]. Other RM systems, RM. Hpy188III and RM.HpyCH4V, act similarly. These systems also decrease the length of the DNA that recombines onto the chromosome [113]. This was determined by assessing the endpoints of DNA that recombined onto the chromosome by genome sequencing in strains containing or lacking the RM systems [114]. Similarly, *Pseudomonas stutzeri* JM300 also uses an RM system to select DNA during natural transformation, though the exact system was not described [115]. The NlaIV RM system is

found in 19/20 *Neisseria meningitidis* strains as well as in other *Neisseria* strains. This system restricts plasmid transformation but does not restrict the transfer of DNA that is homologous except for a point mutation encoding antibiotic resistance [116]. DpnA, a MTase that targets ssDNA, is also important during transformation in *Streptococcus pneumoniae* [117]. This MTase is induced during competence and is important for acquisition of large, unmethylated pieces of DNA during transformation [117]. However, this MTase is not required for transformation of point mutations, even if the incoming DNA is not methylated [117].

In all of these examples, it is unclear how a REase, which always targets dsDNA could target the ssDNA that is transported into the cell during natural transformation. Claverys *et al* propose an elegant model to address this question which also explains the discrepancy that has been demonstrated between how these systems succeed in targeting the transfer of antibiotic cassettes but not of point mutations [117]. In this model, incoming unmethylated ssDNA with sufficient homology successfully recombines onto the chromosome creating DNA that is me^0/me^+ (gDNA is fully methylated (me^+) whereas the incoming DNA lacks the proper methylation (me^0)). The transfer of a cassette results in a bubble of single stranded, unmethylated DNA. The transfer of a point mutation does not create this bubble as there is only one base pair difference. Any DNA in the me^0/me^+ form can be methylated by endogenous DNA MTases and this results in two fully methylated strands of DNA (me^+/me^+). The DNA in the cassette, as it is still single stranded, cannot be methylated. DNA replication occurs. The newly

synthesized strand is unmethylated (me^0). In the case of the cassette transfer, this results in a double-stranded region lacking methylation (me^0/me^0) that can be degraded by REases. This is in contrast to the point mutant, which had been methylated by the endogenous MTases and now is me^0/me^+ and thus is resistant to REase activity [117].

1.4.2 DNA Uptake Sequences

Some bacteria recognize a specific sequence in order to select the DNA they utilize in transformation. Termed DNA uptake sequences (DUS), these are 10-12 base pair sequences that are overrepresented in the genomes of the bacteria that carry them. They tend to occur approximately once per kilobase and tend to be enriched at genes associated with genome maintenance [118]. They have been identified in *Neisseria* species as well as *H. influenza* [119-121]. In *Neisseria*, this DUS (5'-ATGCCGTCTGAA-3') is recognized by a component of the Tfp. ComP is a minor pilin that is highly conserved throughout the *Neisseria* and was demonstrated to bind to the DUS in a sequence-dependent manner [95]. Although this sequence was thought to be absolutely required for transformation in *Neisseria*, more recent work demonstrated that different isolates do not require the transforming DNA to contain a DUS, though it does increase the transformation efficiency [122]. The DUS has also been shown to increase the transformation efficiency of ssDNA, which has an overall lower efficiency than otherwise identical dsDNA [123].

1.4.3 Quorum sensing/competence peptide

Linking competence to QS allows the bacterium to induce competence only in environments enriched for DNA from highly related individuals. Competence has been extensively studied in the Gram positive bacteria *Bacillus subtilis* and *Streptococcus pneumoniae* which have similar, though independently evolved mechanisms for regulating competence [124]. In both of these systems, competence genes are activated by small competence pheromones or peptides [81, 125]. These peptides are secreted and then recognized by two component systems that transmit the signal to activate the competence specific transcription factor that then turns on the competence regulon [80, 82, 126-128]. In *B. subtilis,* this peptide is called ComX and in *S. pneumoniae,* it is called CSP [81, 125]. Both of these peptides activate competence in a cell density dependent manner. In *B. subtilis,* competence is induced during stationary phase but only in about 10% of cells [129, 130]. In *S. pneumoniae,* CSP induces competence during early exponential phase and most cells become competent, but they only stay competent for approximately 15 minutes [78, 131]. While this mechanism is reminiscent of QS, these microbes do not strictly depend on cell density to induce signaling. Instead, CSP accumulation (and therefore sensing) can be induced by external cues as well as cell density and can be better described as cell-cell signaling [37, 79]. These systems allow for competence to be tightly regulated and induced by contact (through peptides) with other, closely related cells. This increases the likelihood that the DNA these cells take up is similar to their own.

In Gram negative bacteria, the link between QS and competence has primarily been studied in *Vibrio cholerae.* This bacterium expresses competence genes using *tfoX* which is induced by growth on chitin [72, 132]. As *Vibrio* species normally inhabit aquatic environments where they colonize the chitinous surfaces of zooplankton, this allows for the induction of competence in the natural environment. Transformation is more efficient at high cell density, indicating a role for QS [47, 72]. HapR, the master regular of QS, represses the eDNase Dns [47]. When Dns is present, it degrades DNA before it can be used by the competence system [47, 133]. HapR is also a positive regulator of *comEA* and *pilA* [48, 72, 134]. *V. cholerae* also produces two autoinducers, CAI-1 and AI-2 [135, 136]. CAI-1 functions as a *Vibrio* specific signal where AI-2 is a more general autoinducer and can be made and sensed by many different types of bacteria [135-137]. Correspondingly, strains deficient in CAI-1 are almost completely deficient in natural transformation whereas the defect for strains lacking AI-2 is much smaller [48, 77, 134]. This indicates that *Vibrio* has optimized its expression of competence genes in order to preferentially take up DNA from closely related bacteria. However, as competence is still initiated by AI-2, it can also take up DNA from more distantly related species, enabling greater genetic diversity. Because of its species specificity and its role in natural transformation, CAI-1 is termed a competence pheromone, similar to CSP in *S. pneumoniae* [48, 77].

1.4.4 Active DNA release

Similar to QS, some bacteria secrete bacteriocins or autolysins that result in the release of DNA. When these systems are coregulated, competence is turned on in an optimal situation to acquire DNA. In many cases, the bacteria are closely related and this increases the odds of taking up similar DNA. This has been best characterized in *S. pneumoniae* which produces fratricide effectors. These are bacteriolytic cell wall hydrolases that work on bacteria closely related to the producer strain. They are also co-regulated with the competence genes and are only produced after the bacteria have upregulated competence [66, 138]. Fracticide immunitiy is induced upon induction of competence, but sibling cells that have not become competent are susceptible to these effectors and are killed, providing a source for DNA [65, 66, 138]. In addition to fracticide effectors, *S. pneumoniae* produces bacteriocins to compete with other bacteria, including within mammalian hosts. Different strains express unique bacteriocins with corresponding immunity proteins. Bacteriocin production is co-regulated with the competence system [42, 78]. The bacteriocin locus is induced by the competence pheromone, CSP [42]. Bacteriocins kill neighboring cells lacking the proper immunity, leading to increased availability of DNA that the competence system can use [42]. Furthermore, the bacteriocin pheromone can be secreted by the competence system, leading to increased synergy between these two systems [42]. Unlike fracticide effectors that will only work on non-competent cells, bacteriocins have the potential to work on many other closely related cells because even if the cell has become competent and upregulated its bacteriocin locus, it may lack the correct immunity factors. This

can increase the likelihood the induction of competence will result in the uptake of DNA.

T6SS enable bacteria to directly inject effector proteins into other bacterial cells in order to kill them. As previously discussed, in *V. cholerae,* competence is co-regulated with QS. It is also co-regulated with the *V. cholerae* T6SS. The master regulator of competence, TfoX, also induces the T6SS. Likewise, the T6SS is turned on when *V. cholerae* grows on a chitin surface. Killing as a result of T6SS activity also triggers uptake of DNA in the predator cells, indicating a functional link between the activation of T6SS and competence [63].

1.4.5 CRISPR Cas Systems

CRISPR (Clustered, regularly interspaced, short palindromic repeat) loci are essentially sequence specific bacterial immune systems and primarily defend against phage attack, though they work against conjugative plasmids as well. The CRISPR loci consist of arrays of short, approximately 40bp, repetitive sequences separated by short spacer sequences. The spacers are derived from (and are therefore specific to) mobile genetic elements such as phage DNA. When a phage injects its DNA into a cell containing a CRISPR that contains spacers specific to that phage derived from an earlier encounter, CRISPR RNAs (crRNAs) are transcribed and are used as guides by the CRISPR associated (Cas) genes to bind to and degrade the incoming DNA in a sequence specific manner. It has been assumed that this system could not work against transforming DNA as half of the time the incoming DNA would not match the spacer because that strand was degraded. However, in both *S. pneumoniae* and

N. meningitidis CRISPR can target DNA brought into the cell through natural transformation [139, 140].

S. pneumoniae does not normally encode a CRISPR/Cas system; Bikard *et al* moved the CRISPR loci from *Streptococcus pyogenes* SF370 into *S. pneumoniae* [139]. They also engineered spacers that would target incoming chromosomal DNA during natural transformation. When the transforming DNA did not match the spacer, equal transformation efficiencies were observed for both CRISPR containing and WT strains [139]. However, when the transforming DNA matched the spacer, no transformants were observed for the CRISPR containing strain whereas the WT strain had no change in transformation efficiency [139]. They further demonstrated that this system worked inside an animal model [139]. Although this demonstrated that CRISPR/Cas could work against transforming DNA, it was in an artificial system and did not explain how the incoming single-stranded transforming DNA could be targeted by a system that is extremely sequence specific.

Zhang *et. al.,* identified a naturally occuring CRISPR/Cas system in *N. meningitidis* that encoded spacers to other *Neisseria* genomes and demonstrated that this CRISPR/Cas system could prevent natural transformation [140]. Where targets could be putatively identified, all the spacers targeted other *Neisseria* strains, ranging from other *N. meningitidis* genomes to *N. gonorrhoeae* and *N. lactamica* [140]. When a *N. meningitidis* strain containing the CRISPR/Cas system was transformed with DNA matching the spacers, no transformants were obtained [140]. However, when the transforming DNA did not match the spacers,

this strain was readily transformed [140]. This effect was independent of the vector used, the integration locus, or the selection marker and worked for many spacers, as long as they shared homology with the transforming DNA [140]. *Neisseria* primarily uses natural transformation, as opposed to conjugation or transduction, to acquire genetic diversity. The fact that the spacer arrays primarily target other related *Neisseria* strains indicate this CRISPR/Cas might be a mechanism to select DNA. This is in contrast to other mechanisms described thus far where systems are optimized to take up closely related DNA. In this case *Neisseria* selects for DNA that is not closely related. This could help explain the extensive genetic diversity found in these species.

Although neither study offered an explanation for how the CRISPR system could efficiently target incoming ssDNA without a strand bias, Johnston *et. al.* provide a hypothesis similar to the mechanism for ssDNA targeting by RM systems [141]. Essentially, they propose that CRISPR/Cas targeting does not occur until after the first round of replication. At this point, the DNA is double stranded again, so targeting can occur independent of which strand was brought into the cell. Secondly, because CRISPR/Cas is efficient and there is no mechanism to avoid targeting, unlike RM systems, this also explains how no transformants are ever identified. For RM systems, occasional transformants are found presumably because the MTase can methylate the me^0/me^0 DNA before the REase degrades it [141].

1.4.6 Production of extracellular DNases

Campylobacter strains have not been described to have any of the previously described mechanisms of limiting or selecting DNA during transformation. However, certain *C. jejuni* strains produce extracellular DNases (eDNases) that can degrade DNA non-specifically, preventing it from being used for transformation. Three different eDNases have been identified, *dns,* Cje0556 and Cje1441 [49, 50]. The presence of a single eDNase is sufficient to eliminate transformation and strains encode between zero and three eDNases [49, 50, 142]. 42% of sequenced *Campylobacter* strains do not encode any of the nucleases [142]. Only 13% of genomes contained more than a single eDNase and only 0.9% of strains contained all three [142]. These nucleases are also encoded on integrated elements indicating they might be transferred among strains [49, 50]. Thus far, regulation of these nucleases and of the competence genes has not been described. Like the case with *V. cholerae* and its eDNase *dns,* there may be a condition(s) where the strains encoding these nucleases become competent by turning them off and upregulating the competence genes.

1.5 DNA Methyltransferases and Restriction Modification Systems

MTases are enzymes that attach a methyl group to a DNA base. There are three different groups based on which carbon on which base the methyl group is attached to: N^6-methyladenine (m6A), N^5-methylcytosine (m5C), or N^4-methylcytosine (m4C) [143]. m6A enzymes specifically methylate the amino group at the C-6 position of adenines. m4C MTases specifically methylate the amino group at the C-4 position of cytosines while m5C methylate the C-5 carbon of cytosines [143]. Work in bacteria has primarily focused on m6A and to a lesser

degree m4C MTases. This has been due both to the prevalence of m6A MTases in bacterial genomes as well as the lack of straightforward method for detecting methylated bases. Single-molecule real-time (SMRT) sequencing has allowed for the easy detection of modified bases during genome sequencing, especially of modified adenines [144]. SMRT sequencing uses unamplified DNA as the sequencing template and then measures the amount of time it takes to add a new base to the growing strand. Bases that have already been modified cause the DNA polymerase to add the next base more slowly [144]. From this kinetic data and the sequence it is possible to infer which base is modified [144]. Modified cytosines are not detected with as high degree of accuracy so these motifs, and the enzymes that provide them, have been less studied [144].

In bacteria, DNA MTases are often part of RM systems [145]. As previously discussed, these systems function to protect the bacterial genome from incoming DNA, whether that is bacteriophage DNA or DNA from other bacteria brought in through conjugation or natural transformation. The endogenous DNA contains a certain methylation pattern based on the MTases encoded in its genome. These methyl marks prevent the cognate REases from degrading the chromosome because methylation inhibits cleavage, except for Type IV RM systems. Incoming DNA will lack the proper methylation and so becomes a target for the REases and can then be degraded, protecting the host chromosome. As of this writing, over 12,000 bacterial and archaeal genomes have been sequenced. Of those, only 4% do not contain a single RM system (http://rebase.neb.com). As of 2010, nearly 4000 enzymes had been identified

with about 300 different specificities. This was from only 2,450 bacterial and archaeal genomes [146]. As the number of sequenced genomes has increased dramatically, there are likely to be many more identified enzymes and specificities. In a recent study of 230 newly SMRT sequenced genomes, 215/230 genomes had DNA modification [147]. Generally speaking, the number of RM systems increases with the size of the genome with outliers in *Helicobacter*, *Campylobacter* and *Neisseria* which contain proportionally more RM systems for the size of their genomes [147, 148]. The presence of more RM systems also correlates with naturally competent bacteria [148]. Bacteria that are not competent tend to have fewer systems compared to those of similar genome size that are competent. Notably, those bacteria previously indicated to be outliers above are all naturally competent. As discussed earlier, this indicates a role for RM systems in protecting the genome, especially among bacteria that specifically bring in DNA. There are four major different types of these systems: Type I, Type II, Type III and Type IV.

1.5.1 Type I RM systems

Type I systems are not well characterized and are complex. There are three polypeptides that function together in a complex. They are named after their function: R for restriction, M for modification, and S for specificity. The complex formed by these polypeptides is $R_2M_2S_1$. This complex contains both restriction and modification capabilities. The S subunit recognizes the target domain as well as binds to the DNA. The recognition sequence for Type I RM systems is complex and asymmetrical. There are typically two halves separated

by a series (between six and nine) of nonspecific bases. The methyltransferase domain generally modifies adenines in each half of the recognition sequence. This polypeptide contains both a SAM (S-adenosylmethonine) binding motif and methyltransferase activity. The R subunit is the largest and cleaves DNA at a site distant, between ~100 bp to tens of thousands of base pairs from the recognition sequence. [148] This complex is large—the R subunit is approximately one thousand amino acids while the M and S subunits are between 450 and 500 amino acids.

Ten total strains of *Campylobacter*, ranging from *C. jejuni* to *C. subantarcticus,* have had their methylomes been determined by SMRT sequencing [149-153]. However, all the genes responsible for these motifs have not been experimentally determined. For the sake of this review, I will focus on identified RM systems in the *C. jejuni* strains 81-176 and 11168. They have been SMRT sequenced and all motifs have been matched to RM system genes. They share one Type I system in common which recognizes the motif TA<u>A</u>YN_5TGC/ATTRN_5<u>A</u>CG [149]. Here, methylated bases are indicated by underlining. This system is made up of Cj81176_0776 (M), 0777 (S), and 0780 (R) in 81-176 and Cj1549c (R), 1551c (S), and 1553c (M) in 11168. In addition, 81-176 has an additional type I system that recognizes the motif CA<u>A</u>YN_6ACT/GTTRN_6TG<u>A</u>, made up of Cj81176_1534 (R), 1536 (S), and 1539 (M) [149]. Both of these motifs have been confirmed to be methylated by SMRT sequencing, indicating these systems are active. Most other SMRT sequenced

Campylobacter strains contain at least one active Type I system [150-153] . The exception is *C. subantarticus* LMB24377 which has none [152].

1.5.2 Type II RM systems

Type II systems are the most well studied of the RM systems and are generally the simplest. The REases require divalent cations, usually Mg^{2+}. The REases generally act as homodimers (except Type IIT); each cuts one strand of the DNA resulting in a double stranded break. Most recognize short DNA sequences, usually four to eight base pairs long [154]. These are highly utilized in genetic engineering. This family is also very heterogenous because this grouping is based on enzymatic activity as opposed to phylogeny. [155] There are over 350 prototype systems in this family. Because of this diversity, these systems have been further divided into subgroups [155]. These families overlap so each unique RM system can also belong to multiple families.

Type IIP enzymes recognize palindromes and cleave at fixed symmetrical locations. These can be within the sequence or immediately adjacent [156]. EcoRI is an example of this type. It recognizes the palindrome GAATTC and cleaves within the sequence. As EcoRI was one of the first REase identified, it is the prototype for this family [156].

The Type IIC family includes all enzymes that have a hybrid structure in which all enzymatic activity is encoded on one polypeptide [156]. All members of Type IIB, IIG, and some Type IIH are also included in this family [156]. Type IIB systems have REases that cleave on both sides of the recognition sequence [156]. Type IIG enzymes are a subtype of the of Type IIC family. The

distinguishing characteristic of these enzymes is that SAM is critical—it can stimulate or inhibit activity [156]. Type IIA and IIP enzymes are sometimes members of the IIG family. Type IIH enzymes have a gene structure similar to Type I, with different polypeptides encoding each function, but enzymatically function as Type II enzymes [156].

Type IIA RM systems recognize asymmetric recognition sequences [156]. Members of this family have REases that cleave either in the sequence or away from it [156]. Generally, they have one REase and two MTase genes [156]. This is because each MTase must modify a different sequence on each strand of DNA. For these systems, the recognition sequences are similar to Type IIB. Type IIS enzymes are a subset of Type IIA enzymes [156]. They cleave asymmetrically and at least one cleavage occurs outside of the recognition sequence [156].

Type IIE systems have enzymes that require two copies of the recognition sequence to be functional [156]. One of the sites is cleaved and the other is the allosteric effector [156]. EcoRII is an example of this type of enzyme. Similar to Type IIE, IIF enzymes also require two copies of the recognition sequence but in this case, both copies are cleaved [156]. BspMI and NgoMIV are examples of this type of enzyme. Type IIT systems have REases that are composed of heterodimers.

All of the identified Type II systems in *C. jejuni* are Type IIG systems [26, 149]. This means that SAM is critical for these systems and that all enzyme activity is encoded in one polypeptide. Both 81-176 and 11168 share one active

Type II system in common and this system recognizes GCAAGG [149]. It is encoded by CJJ81176_0713 in 81-176 and Cj0690c in 11168. 81-176 has an additional system, encoded by CJJ81176_0068 that recognizes GGRCA [149]. 11168 has two more systems that are active. The first is encoded by Cj1051c, and recognizes GAGN$_5$GT [26, 149]. The second system is encoded by Cj0031c [26]. This gene has a polyG tract of eight to ten base pairs two-thirds of the way through the gene [26]. These leads to phase variation in which the system becomes inactivated. This enzyme recognizes the sequence CCYGA [26]. All SMRT sequenced strains of *Campylobacter* have been found to have at least one active Type II system and many have more [149-153].

1.5.3 Type III

Type III systems have features of both Type I and Type II systems. Like Type II, the R and M subunits are separate proteins, but the M subunit confers the specificity for both functions. The REase requires ATP in order to cleave the DNA and the MTase requires SAM in order to methylate the DNA. Like Type I systems, Type III systems recognize asymmetric sequences. Type III systems function as heterotrimers (M_2R_1) or heterotetramers (M_2R_2) [148]. These enzymes compete among themselves for modification or restriction within the same catalytic cycle [148]. They recognize short, asymmetric sequences of five or six base pairs [148]. Once a sequence is recognized, the complex translocates approximately twenty-five basepairs down the DNA and cleaves the 3' side of the target [148]. The DNA is only cleaved when two recognition sequences are in an inverse orientation with respect to each other [148]. There have not been any

active Type III systems identified in 81-176 or 11168. One is predicted for RM1221 which has not been SMRT sequenced so it is not known if this system is active.

1.5.4 Type IV (Mcr-like) systems

Type IV systems cut modified DNA as opposed to unmodified DNA. They most commonly cut methylated DNA but they have also been demonstrate to cut phosphorothioated (PT) DNA, hydroxymethylated or glucosyl-hydroxymethylated DNA [148, 157]. PT is a modification in which a sulfur atom replaces the non-bridging oxygen in the phosphate backbone. This modification creates linked dinucleotides: d(A_{PS}A), d(C_{PS}A), d(C_{PS}C), d(G_{PS}A), G_{PS}G, d(T_{PS}A), and d(T_{PS}C) [158]. Very little sequence specificity is required for these enzymes. In many of the identified systems, cleavage requires NTP hydrolysis; various members have been found to require diverse NTPs including UTP, CTP, GTP, dATP, and ATP [159]. These enzymes have been hard to identify because in order to identify them, a functioning genetic system is required [159]. As of 2010, of the 1303 putative Type IV REases in REBASE, only three had been biochemically characterized [157]. The rest were bioinformatic predictions.

McrBC-like RM systems have not been identified in either 11168 or 81-176. However, both strains do encode McrBC-like genes [160]. In 11168 these genes are encoded by Cj0139 and Cj0140 and in 81-176 by CJJ81176_0174, 0175, and 0176 [160].

1.5.5 Orphan MTases

Finally, there is a group of DNA MTases that do not work with a cognate REase. This family is termed orphan MTases. Like the MTase polypeptides or proteins of the other families, these methylate specific sequences. These enzymes have not been demonstrated to play a role in RM systems. The well characterized ones, Dam in *E. coli* and CcrM in *Cauliobacter crescensus,* have a number of roles from gene regulation to cell cycle progression. These orphan MTases tend to have higher conservation as compared to MTases that are involved in restriction modification systems. There is also a link between hypomethylation and gene regulation at sites specific to orphan MTases.

Dam MTase is highly conserved throughout the γ-Proteobacteria. It is a SAM-dependent MTase that methylates the adenine in the motif G<u>A</u>TC and plays a role in regulating the cell cycle. In many species, such as *V. cholerae* and *Yersina enterocolitica* Dam is required [161, 162]. In other species, such as *Salmonella enterica and Klebsiella pneumoniae,* it is required for virulence [161, 163]. In *E. coli* it plays a role in mismatch repair, the SOS response and interactions with host cells [164-167]. This MTase regulates many genes although the mechanism is not clear in all cases. In the cases where the mechanism has been determined, there is a competition between other DNA binding proteins and Dam. These proteins bind to promoter regions in a methylation dependent manner. The DNA binding proteins Lrp and OxyR prefer un-methylated or hemi-methylated DNA respectively. This has been demonstrated for the regulation of the *pap* operon in *E. coli* in which Lrp and PapI compete with Dam for the promoter. This is also true for *agn43,* involved in the

synthesis of non-fimbrial adhesions, in which OxyR and SeqA compete with Dam [168, 169]. This mechanism seems consistent with the distribution of GATC sites, which accumulate more in the promoter regions as opposed to coding regions [164-166].

CcrM was first identified in *C. crescensus* and methylates adenines in GANTC motifs with a preference for hemi-methylated DNA [170, 171]. This MTase is tightly regulated during the cell cycle and only accumulates in pre-divisional cells, coinciding with the end of replication [170]. It is required for viability in *C. crescensus, Rhizobium meliloti, Agrobacterium tumefaciens* and *Brucella abortus* [170-173]. Furthermore, overexpression of this MTase results in cells that have more than one chromosome as well as other cell cycle defects. For *B. abortus*, CcrM overexpression results in attenuated replication in mouse macrophages [173]. Like Dam, CcrM regulates many other genes and the mechanism is similar. This can be demonstrated for the regulation of *dnaA*. This gene, located near the origin, has two GANTC sites in the promoter and functions best when fully methylated. Upon chromosomal replication, this site becomes hemi-methylated, decreasing the efficiency of transcription from the promoter. It remains in this state until CcrM accumulates and can methylate the promoter [174, 175]. CcrM has also been demonstrated to regulate *ctrA* P1 which plays a role in the initiation of DNA replication and is a master transcriptional regulatorv [176]. Finally, CcrM regulates its own transcription because transcription from *ccrM* is repressed by CcrM [177]. This promoter is also activated by CtrA[178].

Orphan MTases are widespread. A recent paper reported at least one active orphan MTase in 111 sequenced species spread among 15/20 phyla analyzed [147]. They also made an observation that these MTases tend to be more evolutionarily conserved than MTases that are part of RM system. They found 57% of orphan MTases are conserved at the genus level compared to 9% of those that are part of RM systems [147]. While the conservation decreases somewhat when comparing family and class, orphan MTases are still significantly more conserved at these levels [147]. This has also been demonstrated bioinformatically where there is a much higher probability that an orphan MTase will be conserved than one in a RM system [179].

When orphan MTases are grouped into families based on recognition motif and the taxonomic relationship between host organisms, Dam-like and CcrM-like MTases were most common [147]. The third most common family methylates the motif 5'-RAATTY-3' and was found in six Spirochaetaceae as well as ten sequenced *Campylobacter* strains [147, 149-153]. Orphan MTases also seemed to operate distinctly from those in RM systems. Generally, all the sites in a genome are methylated when the methylation was associated with a MTase from functional RM system [147]. In contrast, there are distinct patterns of hypomethylation associated with orphan MTases [147]. This had previously been demonstrated for Dam MTase in *E. coli.* This is the result of competing activities of Dam and regulatory proteins and can lead to epigenetic modifications of gene expression [180-182]. Considering RAATTY methylation, both Spirochaetaceae and *Campylobacter* were enriched in hypomethylated sites

relating to transcription [147, 153]. However, whether this plays a functional role in gene regulation has not been demonstrated.

Based on SMRT sequencing, only one active orphan MTase has been identified in all SMRT sequenced *Campylobacter* strains [149-153]. As described above, this methylates the motif RAATTY and is identified as EcoRI-like because this recognition sequence is a more general version of the EcoRI recognition sequence, GAATTC. This is the only active orphan MTase that has been found in either 81-176 or 11168 [149]. However, both are predicted to encode at least nine more though they were not found to be active (Table 1.1) [160]. The role of this conserved orphan MTase will be discussed in more detail in Chapter III.

11168	81-176
Cj0495	CJJ81176_0516
Cj0722c	CJJ81176_0745
Cj0590	CJJ81176_0618
Cj0976	CJJ81176_0995
Cj0979c	CJJ81176_0998
Cj1300	CJJ81176_1314
Cj1325	CJJ81176_0207
Cj1419c	CJJ81176_1418
Cj1420c	CJJ81176_1419
Cj1426c	

Table 1.1: Predicted orphan adenine methyltransferases. Gene designations are given for each predicted MTase. Genes on the same line share homology.

1.6 Transformation in *C. jejuni*

1.6.1 Genes required for transformation

As previously noted, *C. jejuni* can encode up to two distinct competence machineries [5, 31]. One of those, encoded on a plasmid (Vir), resembles the *H. pylori* T4SS machinery while the other encoded on the chromosome (Cts), resembles T2SS or Tfp, similar to other competent bacteria. Loss of essential

components of the Vir system led to at best one half-log decrease in transformation efficiency whereas loss of any of Cts components resulted in the inability to detect transformants [5]. Because of this, I have focused my work on the Cts system and that is the system I will detail here. For a model of the *C. jejuni* Cts competence system, see Figure 1.

By performing a transposon screen, Wiesner *et. al.,* identified the major components of a Tfp/T2SS system. These genes were named based on homology to previously characterized competence genes and termed them *Campylobacter* transformation system proteins (*cts*). They include those encoding a secretin, *ctsD*; pseudopilins, *ctsG* and *ctsT*; two ATPases, *ctsP* and *ctsE*; and as a structural component of these systems, *ctsF*. Also identified was a gene encoding a transmembrane protein that has no overall homology to other proteins and termed *ctsX* [5]. While potential pseuopilins have been identified, *C. jejuni* has never been demonstrated to produce a pilus. This leads us to hypothesize that retraction of a pseudopilus, as opposed to a pilus, opens up a gap in the secretin CtsD, which then allows DNA to enter the cell. Interestingly, *C. jejuni* does express two transformation-associated ATPases, CtsE and CtsP. CtsE is most similar to more typical production ATPases whereas CtsP is not similar to retraction ATPases [183]. However, retraction of pseudopili—as opposed to bona fide pili—may require an ATPase of a different specificity. The roles of these two ATPases as well as CtsX, the required membrane protein of unknown function, will be discussed more in Chapter II.

recA was the first gene identified to be required for transformation in *C. jejuni* [184]. DprA is also required for both natural transformation as well as electroporation with plasmid DNA [185]. This phenotype is similar to that in *H. pylori,* indicating these proteins behave similarly in both systems [185].

Cj0011c is a ComE/ComEA homolog required for transformation [186]. It is a non-specific DNA binding protein capable of binding to ssDNA and dsDNA [186]. Like ComE in *Neisseria,* it localizes to the periplasm of *C. jejuni* and is not surface exposed [186]. The transformation defect of cells lacking Cj0011c is less severe than that of *B. subtilis* cells lacking ComEA [28, 186]. This indicates that *C. jejuni* might have additional DNA binding proteins that function during transformation.

Cj1211c is also required for competence in *C. jejuni* [187]. This protein had a strong degree of homology to *H. pylori* ComE3 but not to other functional ComE3 homologs such as ComEC in *B. subtilis* and ComA in *N. gonorrhoeae* [187]. These proteins function as the inner membrane translocator. Although it is required for transformation in *C. jejuni*, a Cj1211c mutant has no DNA binding and uptake defect [187]. As the binding and uptake assay measures if the DNA becomes resistant to eDNases (i.e. the DNA is transported inside the outer membrane of the cell), this phenotype indicates a role downstream of initial import. This includes transporting the DNA to the cytosol once it has been brought into the periplasm and so Cj1211c could be the inner membrane translocator in *C. jejuni.*

N-linked protein glycosylation (*pgl*) has also been shown to have an effect on transformation. When insertional mutations were made in two key genes in this process, *pglB* and *pglE*, there was a significant defect in transformation [188]. The authors initially investigated this phenotype because of VirB10, a component of the Vir competence system with a glycosylation motif [188]. However, the glycosylation of VirB10, which does affect its function, is not sufficient to explain the defect [188]. The transformation defect in *pgl* insertional mutants is greater than in a strain lacking a functional Vir system. Thus far, no other competence proteins have been found to be targets of glycosylation.

Polyphosphate (poly-P) kinases have also been demonstrated to be important for transformation in *C. jejuni* [189]. These enzymes play roles in bacterial survival and stress tolerance [190, 191]. A Δ*ppk1* strain had an approximately 1.5 log defect in natural transformation [189]. In other bacteria, PPK1 is important for the production of cell membrane channels that allow for DNA uptake [190, 191]. Whether this is the role of PPK1 in *C. jejuni* was not determined.

As I previously described the role of eDNases on transformation in Section 1.4.6, I will just summarize the previous section. *C. jejuni* can produce up to three eDNases and all are capable of inhibiting natural transformation.

1.6.2 Regulation

As described previously, bacteria regulate competence to different degrees. Some such as *H. pylori* and *N. gonorrhoeae* are constitutively competent whereas others such as *S. pneumoniae* and *B. subtilis* carefully

regulate this process. *C. jejuni* is competent in all growth phases, though there is some variation in the efficiency. The highest transformation efficiency observed (10^{-4}) occurred in exponential growth and the efficiency decreased during stationary phase (10^{-6}) [2]. There is also an inverse relationship between CO_2 concentrations and transformation efficiency. *C. jejuni* grows best at higher CO_2 concentrations (~10%). At 10% CO_2, the average transformation efficiency was 10^{-4} but as the CO_2 concentration was decreased to 5% and 0.7% the transformation efficiency rose to about 10^{-3} and 10^{-2} respectively [2]. This might indicate that *C. jejuni* is more competent in times of stress, such as low CO_2 concentrations. The loss of transformation efficiency in stationary phase was not as dramatic at low CO_2 concentrations [2].

The effect of temperature and oxygen levels on transformation has also been measured. Temperature in the normal growth range of *C. jejuni,* 37°C and 42°C, had minimal effect on transformation, though there were more transformants at 42°C [1]. As the temperature decreased to 30°C or 20°C degrees, transformation dropped significantly and essentially no transformants were detected at 20°C [1]. Oxygen levels also affected transformation. Anaerobic conditions were inhibitory to transformation at all temperatures [1]. Microaerobic and aerobic conditions were much more favorable, with the best transformation efficiency seen in microaerobic conditions at 42°C [1].

pH also has an effect on transformation. At pH levels from 7 to 11, the transformation efficiency remained stable at 10^{-3} [1]. There was a precipitous drop in transformation efficiency outside of this window [192]. This is contrast to

the vitality of the cells. Cells grew best at pH 7 but at higher pHs, approximately 60% or fewer cells were able to survive [1].

As has been previously described for other systems and concurrent with our data concerning the Cts ATPases in Chapter II, transformation requires energy. When overall cellular energy was depleted either with HQNO or sodium azide, transformation was inhibited [1]. This was true either if the cells were treated prior to transformation or during transformation [1].

Finally, transformation requires active translation but not active transcription. In the presence of transcription inhibitors, transformation is not affected but when translation inhibitors such as erythromycin and tetracycline are added, transformation is inhibited [1]. The mechanism that might require translation but not transcription for natural transformation has yet to be determined.

1.6.3 DNA selection

It has been described that *C. jejuni* and *C. coli* are selective for the DNA that they use in transformation [2, 193]. In *C. coli,* uptake of radiolabeled *C. coli* gDNA was able to be outcompeted by unlabeled *C. jejuni* or *C. coli* DNA but not by *E. coli* derived DNA [193]. *Campylobacter* does not seem to contain a conventional DNA uptake sequence. Furthermore, DNA purified from *E. coli* or generated via PCR, does not transform WT cells. However when identical DNA is purified from *C. jejuni,* the DNA transforms as well as *C. jejuni* gDNA, the preferred substrate. This indicates that selection is not based on sequence per se. Cj1051c has been reported to play a role in DNA selection in *C. jejuni* strain

11168 [194]. However, this gene is not conserved among all *Campylobacter* strains and is therefore not sufficient to explain the overall specificity of *Campylobacter* [194]. The mechanism of DNA selection will be discussed in Chapter III.

1.6.4 *In vivo* transformation

Most work studying *C. jejuni* transformation has used *in vitro* conditions. However, transformation has been examined in the *in vivo* conditions of both the chicken intestinal tract and biofilms. *C. jejuni* is well adapted to colonize chicken intestine and different strains colonize the intestine simultaneously [195]. It was hypothesized that because multiple strains could be present, transformation could occur in the chicken. This was tested experimentally by coinfecting chickens with strains containing two different antibiotic markers and looking for double resistant strains [3]. These were detected in every group tested as early as two days post infection and as late as 29 days post infection. This work was flawed in some respects because the rate of transformation was not calculated. Thus it was not determined if the double-resistant colonies that appeared to be transformants were the result of independent genetic exchanges or the expansion of one successful transformation [3].

Similar to other competent species such as *S. pneumoniae*, *C.jejuni* makes biofilms. This is thought to be one way it survives in water in the environment. Genetic exchange has been demonstrated in biofilms *in vitro* [4]. This transformation occurs at a higher rate in the biofilms than for planktonic cells and transformation is abolished in the presence of added DNases [4].

Furthermore, the biofilm itself is thought to contain DNA as biofilm formation is reduced in the presence of DNases [4, 196].

1.7 Conclusions

Natural transformation is a trait of many diverse bacteria. Despite the diversity of bacteria, the machinery used to transport the DNA into the cell is highly homologous in many species, resembling a T2SS or Tfp. Despite the similarity in the machinery used, these systems have diverse regulatory mechanisms ranging from constitutive expression in *N. gonorrhoeae* of the competence genes to brief, synchronized expression in *S. pneumoniae.* Many different mechanisms exist to optimize DNA uptake, especially DNA from similar bacteria. Some use uptake sequences to select for similar DNA. Others co-regulate competence with the expression of bacteriocin genes to promote active DNA release. Another uses CRISPR Cas and many uses RM systems.

DNA MTases are also widespread across the bacterial phyla. Most bacteria encode at least one and it is often a part of a RM system. These systems protect the bacteria from incoming DNA and are more common in naturally competent bacteria. The four types of RM systems differ in how they recognize DNA, how the enzymes are encoded on the genome, and how they modify the DNA. Orphan MTases are more widely conserved than MTases that are in RM systems. Two of these, Dam and CcrM, have been well studied and are involved in gene regulation. Many more orphan MTases have recently been identified and bioinformatic and sequencing data support the hypothesis that these newly identified orphan MTases play a role in gene regulation as well.

C. jejuni is a Gram negative, commensal of avian species and pathogen of some mammalian species including humans. The species exhibits a great deal of genomic diversity. This is at least partially attributable to the fact that it is capable of all three mechanisms of HGT. Natural transformation has been studied in this bacteria and genes important for this process have been identified. Like most other bacteria, these genes look like a T2SS or Tfp system. *C. jejuni* is highly selective in the DNA it uses for transformation though it does not seem to encode a conventional uptake sequence. The degree of competence varies in different environmental conditions, but in all conditions tested, the bacteria are competent unless eDNases are also expressed. Finally, competence is even active in *in vivo* conditions such as chick intestinal tracts and biofilms.

1.8 Thesis outline

The central goal of this thesis was to examine to aspects of *C. jejuni* natural transformation. In Chapter II, I examine individual components of the transformation machinery. I focus on the two identified ATPases, CtsP and CtsE, and the protein that has no overall homology to those in other systems, CtsX. I look at the requirement of these proteins in transformation as well as how they localize. With a former student, I determine that these proteins are required for transformation and that while the two ATPases are similar to other competence ATPases, these have unique characteristics regarding their membrane localization. We also determine that CtsX is a transmembrane protein that can interact with CtsP, though we still do not understand its role in competence. In Chapter III, I investigate at the process of DNA selection in *C. jejuni*. I explore a

methylation pattern that is required for transformation and demonstrate that a single, methylated motif on the DNA is sufficient for transformation by that DNA, independent of its origin. I demonstrate that selection must occur inside of the cell, though the exact mechanism is yet to be determined. In Chapter IV, I summarize the findings of this thesis and identify questions that have yet to be answered.

CHAPTER II

CHARACTERIZATION AND LOCALIZATION OF THE *CAMPYLOBACTER JEJUNI* TRANSFORMATION SYSTEM PROTEINS CTSE, CTSP AND CTSX

2.1 Abstract

The human pathogen *Campylobacter jejuni* is naturally competent for transformation with its own DNA. Genes required for efficient transformation in *C. jejuni* include those similar to components of type II secretion systems found in many Gram negative bacteria [5]. Two of these, *ctsE* and *ctsP*, encode proteins annotated as putative nucleotide binding NTPases or NTP binding proteins. Here we demonstrate that the nucleotide binding motifs of both proteins are essential for their function in transformation of *C. jejuni*. Localization experiments demonstrate that CtsE is a soluble protein while CtsP is membrane-associated in *C. jejuni*. A bacterial twohybrid screen identified interaction between CtsP and CtsX, an integral membrane protein also required for transformation. Topological analysis of CtsX using LacZ and PhoA fusions demonstrate it to be a bitopic, integral membrane protein with a cytoplasmic amino terminus and a periplasmic carboxyl terminus. Notwithstanding its interaction with membrane-localized CtsX, CtsP inherently associates with the membrane, requiring neither CtsX or several other Cts proteins for this association.

2.2 Introduction

The Gram-negative bacterium *C. jejuni* is a leading cause of bacterial gastroenteritis worldwide [197]. *C. jejuni* often colonizes the avian intestinal tract and, consequently, a common route of infection is through consumption of contaminated poultry [198].

A number of Campylobacters are naturally competent for transformation, meaning that they can take up macromolecular DNA from the environment and incorporate it heritably into their genomes [2, 193]. The ability to acquire DNA from the environment may contribute to the extensive genetic diversity observed among strains of *C. jejuni* [17, 18]. Horizontal gene transfer *in vivo* has been demonstrated during experimental infection of chicks, a natural host for this pathogen [3].

Multiple genes whose products are involved in natural transformation of *C. jejuni* have been identified [5, 31, 185, 188, 199]. Using transposon mutagenesis and a genetic screen for loss of competence, we isolated mutations that mapped to 11 genes encoded in *C. jejuni* strain 81-176 [5]. Mutations in these result in reduction in transformability to levels four orders of magnitude below wild type [5]. Among these are six genes arranged in a likely operon, some of which encode proteins similar to components of type II secretion and type IV pilus biogenesis systems and are homologous to proteins important for natural transformation in other organisms [200]. Two of these, *ctsE* and *ctsP*, encode putative NTPases or NTP binding proteins, according to the annotated genome of *C. jejuni* strain 11168 [5, 21].

CtsE is a member of the type II secretion/type IV secretion system superfamily of NTPases collectively referred to as the PulE - VirB11 family [183, 201]. Members of this family are involved in diverse processes including secretion, pilus biogenesis, competence for natural transformation, and conjugation [183]. PulE – VirB11 family members share several elements, including the nucleotide-binding motifs – Walker boxes A and B – an Asp box, and a His box [202-204]. CtsE also has a tetra-cysteine motif conserved among the GspE and PilB/HofB subfamilies [205, 206].

ATPase activity has been demonstrated *in vitro* for several members of the PulE - VirB11 family [207-211]. It is hypothesized that this activity powers the transformation process, though this has yet to be conclusively shown. Phylogenetic analyses placed CtsE in the type II secretion subfamily of NTPases; in one such analysis CtsE fell between the ComG1 subfamily of Gram-positive NTPases involved in competence and the PilT and PilU subfamily involved in retraction of type IV pili [183, 201]. Another analysis placed CtsE in the Gram-positive competence subfamily, closely related to the GspE subfamily of type II secretion machinery [201]. Although ATPase activity has not been demonstrated for most of the type II secretion machinery, it has been hypothesized.

CtsE is one of two putative identified NTP binding proteins important for natural transformation of *C. jejuni*. The other is CtsP [5], which has Walker box A and B nucleotide binding motifs, but lacks other characteristics of the PulE – VirB11 superfamily including the His box, the Asp box and the tetra-cysteine motif. By BLAST analysis CtsP exhibits weak homology to several ATPases

including PilT homologues, ClpX ATP binding subunits, and members of the AAA family of ATPases. The Walker boxes of CtsP resemble those of the AAA^+ superfamily, but CtsP does not appear to have the minimal AAA consensus sequence [203, 212].

In this study we characterized CtsE and CtsP, the two putative NTPases/NTP binding proteins required for natural transformation of C. jejuni. We also carried out analysis of a third gene product encoded in the cts gene cluster, CtsX, unique to the C. jejuni transformation system. CtsX lacks significant sequence homology to other proteins, and shares no clearly conserved domains beyond a transmembrane domain. We determined subcellular localization of each of these proteins, and we investigated the roles of the nucleotide binding motifs in CtsE and CtsP. Further analysis investigated protein-protein interactions among constituents of the type II-like Cts system in C. jejuni [5], revealing association between CtsP and CtsX.

2.3 Results

2.3.1 The Walker Boxes of CtsE and CtsP are required for function *in vivo*

The Walker boxes A and B are conserved nucleotide binding motifs important for NTPase function [204], and both motifs are present in CtsP and CtsE. To determine the contribution of CtsP and CtsE for natural transformation of *C. jejuni* we tested whether Walker box motifs are important *in vivo*. Carboxyl-terminal FLAG fusions to each protein were expressed *C. jejuni* Δ*ctsP* and Δ*ctsE* mutants; the fusion proteins complemented the respective mutant alleles to near

wild-type levels of transformation, similar to complementation observed with untagged alleles of *ctsP* or *ctsE* (Figure 2.1). [5]

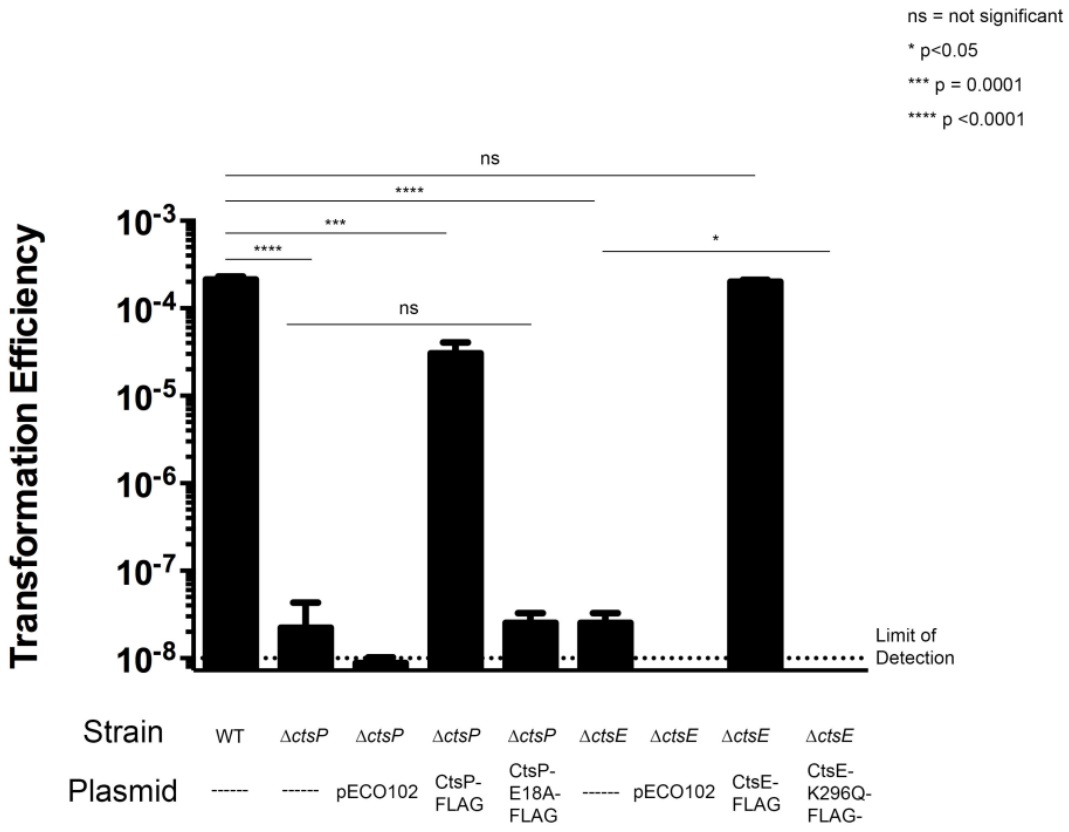

Figure 2.1. Transformation efficiency of C. jejuni CtsE and CtsP mutants. Strains are complemented with pECO102, pECO102 expressing the wild-type coding sequence with a C-terminal FLAG tag, or pECO102 expressing the coding sequence with a Walker Box mutation and a C-terminal FLAG tag. The data represent the average of three samples per strain from one experiment. Experiments were repeated at least three times with similar results. Error bars indicate standard deviation. The limit of detection is indicated with a dashed line. Stars indicate p values calculated using one-way ANOVA with Dunnett's multiple comparison test.

Walker box A (GxxxxGK(S/T), where x is any amino acid) forms a loop structure (the P-loop) in which the lysine can directly contact the phosphoryl group of the bound nucleotide [213, 214]. This Walker box motif is important for the function of many PulE – VirB11 superfamily members and mutation of

invariant residues abolishes ATP binding in a number of proteins [202, 215-217]. The conserved lysine codon (K296) of the Walker box A in *ctsE* was changed by site directed mutagenesis to encode a glutamine in CtsE-FLAG (CtsE-FLAG K296Q), which was expressed in a Δ*ctsE* strain to determine whether it could restore transformation. While the FLAG-tagged version of CtsE could complement a CtsE deletion mutant, the Walker Box A point mutant in CtsE (CtsE-FLAG K296Q) could not (Figure 2.1). Immunoblotting confirmed that CtsE-FLAG K296Q was expressed at levels similar to CtsE-FLAG (data not shown).

Several alleles of *ctsP-FLAG* with alterations to the same Walker box A lysine proved unstable (data not shown), preventing functional assessment of Walker box A in CtsP; we thus targeted Walker box B in CtsP for mutagenesis. The conserved sequence of Walker box B is hhhhDExx, where h is a hydrophobic amino acid and x is any amino acid. The coding sequence for the conserved glutamic acid residue (E81) was changed by site-directed mutagenesis to encode an alanine residue in CtsP-FLAG, which was stable (by immunoblotting, data not shown). CtsP-FLAG E81A was unable to complement a Δ*ctsP* mutant (Figure 2.1). Through these site-directed mutagenesis studies we have determined that intact nucleotide binding motifs are required for the function of both CtsE and CtsP in transformation.

2.3.2 Localization of CtsE and CtsP

Members of the PulE – VirB11 superfamily of NTPases are hypothesized to act at the inner membrane of Gram-negative bacteria through association with

membrane-localized proteins within their respective transport systems. To assess localization of CtsP-FLAG and CtsE-FLAG, *C. jejuni* strains expressing either fusion protein were fractionated into soluble and insoluble fractions as described previously [218]. The soluble fraction contains the cytoplasmic and periplasmic contents and the insoluble fraction contains the inner and outer membranes. Activity of isocitrate dehydrogenase, a cytoplasmic enzyme, was monitored to determine the purity of the fractions [218]. Immunoblot analysis of these fractions using anti-FLAG monoclonal antibody revealed different subcellular locations for these two putative NTPases. CtsP-FLAG was detected primarily in the membrane fraction, while CtsE-FLAG was located primarily in the soluble fraction (Figures 2.2A and 2.2B). To determine whether the cytoplasmic localization of CtsE-FLAG resulted from competition for membrane binding partners with the chromosomally encoded version of CtsE, we assessed localization of CtsE-FLAG in a Δ*ctsE* background. The tagged protein was still observed primarily in the cytoplasmic fraction in the absence of native CtsE (Figure 2.2B).

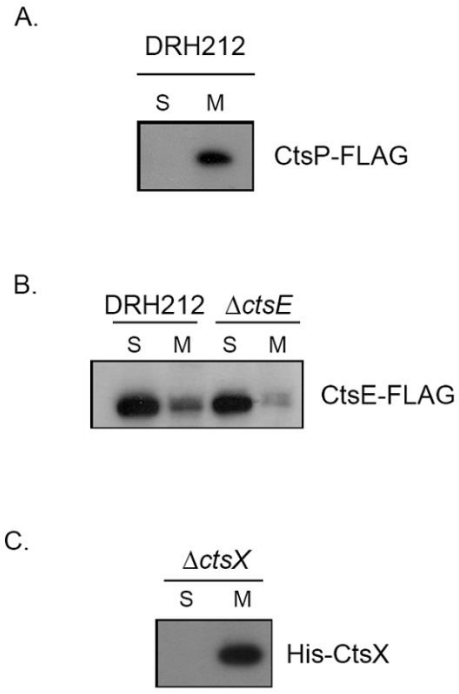

Figure 2.2. Localization of CtsE, CtsP, and CtsX fusion proteins. Cellular fractions of DRH212 expressing CtsP-FLAG (A), CtsE-FLAG (B), or His-CtsX (C) from pECO102 or Δ*ctsE* expressing CtsE-FLAG (B) were separated by 12% or 15% SDS-PAGE. The presence of the CtsP-FLAG and CtsE-FLAG fusion proteins was detected by immunoblotting using a monoclonal antibody against FLAG. His-CtsX was detected using a monoclonal antibody against His. S, soluble fraction, M, membrane fraction.

2.3.3 Bacterial two-hybrid screen for identification of Cts interaction partners

CtsP localizes to the membrane fraction of *C. jejuni*, but does not have a predicted hydrophobic region that could serve as a transmembrane domain. We hypothesized that interaction with another protein might be responsible for the membrane localization of CtsP. To explore this, we used a bacterial two-hybrid system based on functional reconstitution of adenylate cyclase activity [219]. For two-hybrid analysis, we tested the competence genes *ctsE*, *ctsX*, *ctsF*, *ctsD*,

ctsR, all of which are encoded contiguously with *ctsP*, and *ctsG*, *ctsW*, and *dprA*, which are unlinked to *ctsP*. Genes were cloned into both pT18 and pT25, two plasmids that provide different domains of adenylate cyclase. We tested for interactions between all Cts proteins. Two different protein-protein interactions among Cts proteins were identified (Figure 2.3). One was between CtsE-T18 and T25-CtsE, which is consistent with observations that GspE proteins in other type II transport systems form multimers, [211, 220, 221] suggesting that CtsE may form a multi-monomer complex.

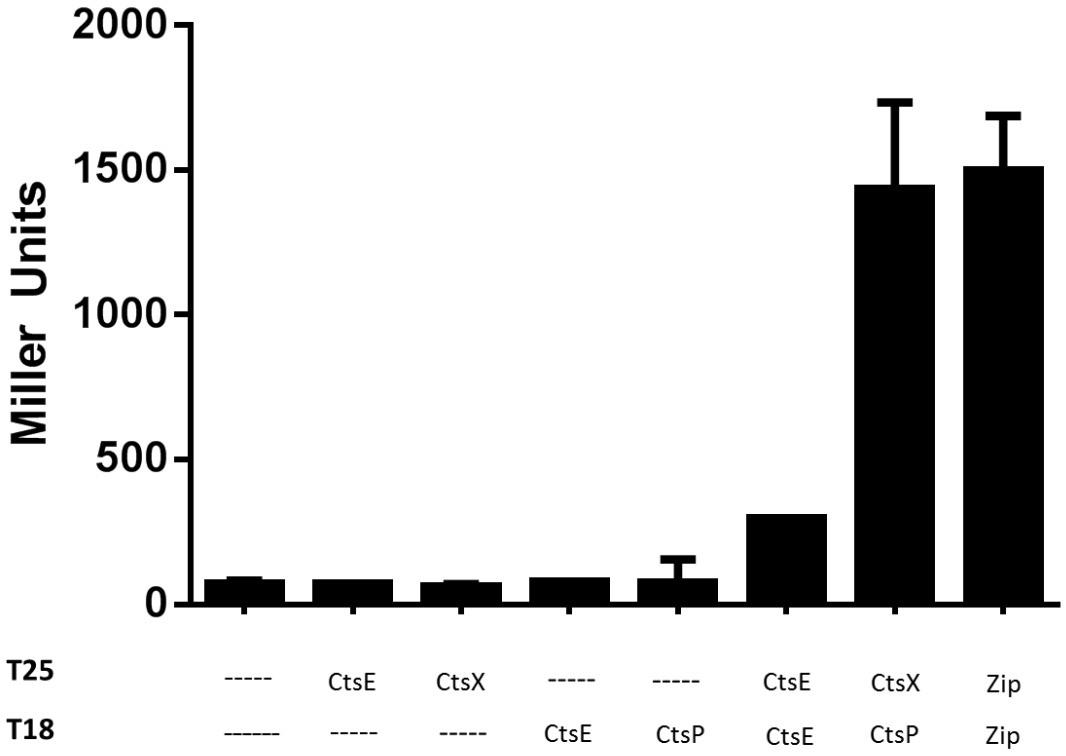

Figure 2.3. β-galactosidase assays of bacterial two-hybrid system interactions. When fused to proteins that interact, the T25 and T18 fragments of adenylate cyclase are able to associate leading to cAMP synthesis resulting in transcription of *lacZ*. For a positive control, T25 and T18 are fused to a leucine zipper (zip) [219]. Activities represent the average of three samples from one experiment which was repeated at least three times with similar results. Error bars indicate standard deviation.

The other interaction we identified was between CtsP-T18 and T25-CtsX (Figure 2.3); *ctsX* is encoded immediately downstream of *ctsP* [5]. Based on Kyte-Doolittle hydropathy analysis, CtsX is predicted to be a membrane protein by virtue of a single putative transmembrane helix from amino acid 21 to 40. To test this experimentally, we expressed a His-CtsX fusion protein in *C. jejuni* DRH212. Cells were fractionated into soluble and membrane fractions as discussed above for CtsE and CtsP, and fraction purity was assessed by isocitrate dehydrogenase assays (data not shown). His-CtsX was detected by immunoblotting with an anti-His monoclonal antibody. As predicted, the majority of the His-CtsX fusion protein was located in the membrane fraction (Figure 2.2C).

The membrane topology of CtsX was experimentally determined using β-galactosidase and alkaline phosphatase fusions to the CtsX coding sequence. Alkaline phosphatase is active only when transported outside of the cytoplasm [222], whereas β-galactosidase is active in the cytoplasm. Portions of the CtsX coding sequence at different positions on both sides of the predicted transmembrane region of CtsX were fused in frame with *lacZ* and *phoA* reporter genes in pTrcLacZ and pTrcPhoA [223].

The first 14 amino acids of CtsX directed high level β-galactosidase activity but low level alkaline phosphatase activity indicating that the N-terminus of the protein resides in the cytoplasm (Figure 2.4). In contrast, fragments of CtsX including the putative transmembrane domain directed low β-galactosidase levels, but high alkaline phosphatase activities (Figure 2.4)., consistent with there

being a transmembrane domain in the protein as predicted by the Kyte-Doolittle plot. Based on these data, we predict that small portion of the N terminus of CtsX is localized to the cytosol, with the remainder of the protein within the periplasmic space.

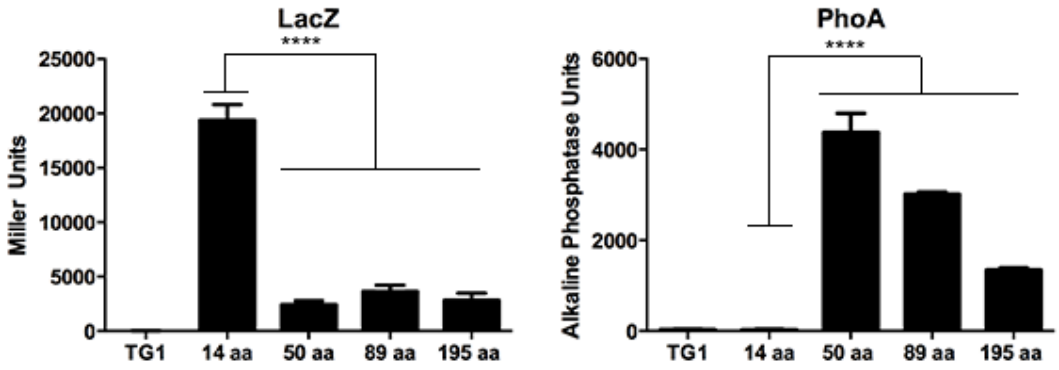

Figure 2.4. Topology studies of CtsX. Reporter protein activity levels of *E. coli* TG1 expressing four fragments of *ctsX* in a translational fusion with *lacZ* (left) or with *phoA* (right). Data represent the average of three samples from one experiment that was repeated at least three times with similar results. Error bars indicate standard deviation. Stars represent p-values calculated using Student's unpaired t test.

2.3.4 Localization of CtsP in the absence of other Cts proteins

Because CtsP lacks an obvious membrane localization signal and interacts with CtsX, which is membrane localized, we hypothesized that membrane localization of CtsP depends on CtsX. However, CtsP-FLAG localized to the membrane fraction in mutant cells lacking *ctsX*, (Figure 2.5A), as well as in mutants lacking other transformation genes including *ctsF*, *ctsE*, *ctsD*, *ctsR*, and *ceuB* ([5]; Figure 2.5B and data not shown). To test whether membrane localization of CtsP requires any *C. jejuni* proteins, we expressed CtsP-FLAG in *E. coli* JM101. Membrane localization was still observed (Figure 2.5C), suggesting either that

membrane localization is an intrinsic feature of CtsP, or that a protein with which it interacts and co-localizes is also present in *E. coli*.

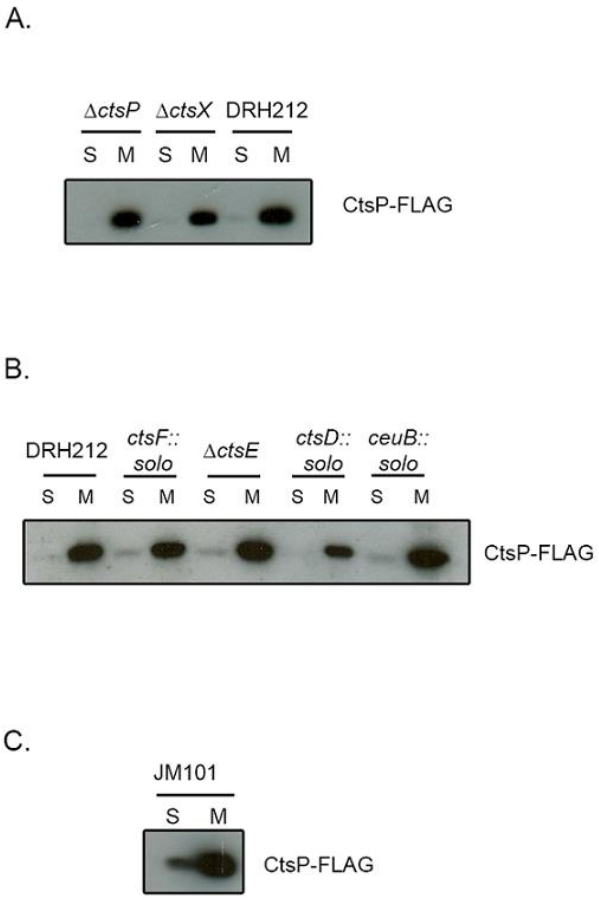

Figure 2.5. Localization of CtsP in the absence of other Cts proteins. Localization of CtsP-FLAG. (A & B) Cellular fractions of DRH212 or *cts* mutants or (C) cellular fractions from *E. coli* JM101 expressing CtsP-FLAG from pECO102 were separated by 12% SDS-PAGE. The presence of the CtsP-FLAG fusion protein was detected by immunoblotting using a monoclonal antibody against the FLAG epitope. S, soluble fraction, M, membrane fraction.

To rule out that the overexpressed protein is simply insoluble and thus pellets with membranes in the fractionation experiments described above, we tested membrane association of CtsP using a membrane flotation assay. Membrane flotation assays utilize a sucrose gradient to separate the membranes from any insoluble proteins that will pellet during ultracentrifugation. Using this

assay, we observed that while some CtsP does become insoluble (in fraction 6), the majority is located in membrane containing fractions (fractions 2-5). This is true when CtsP-FLAG is expressed in wild type cells, as well as in Δ*ctsP,* Δ*ctsX,* or Δ*ctsE* strains. Even when CtsP-FLAG is expressed in *E. coli*, it is primarily localized to the membrane containing fraction and not the insoluble fraction (Figure 2.6).

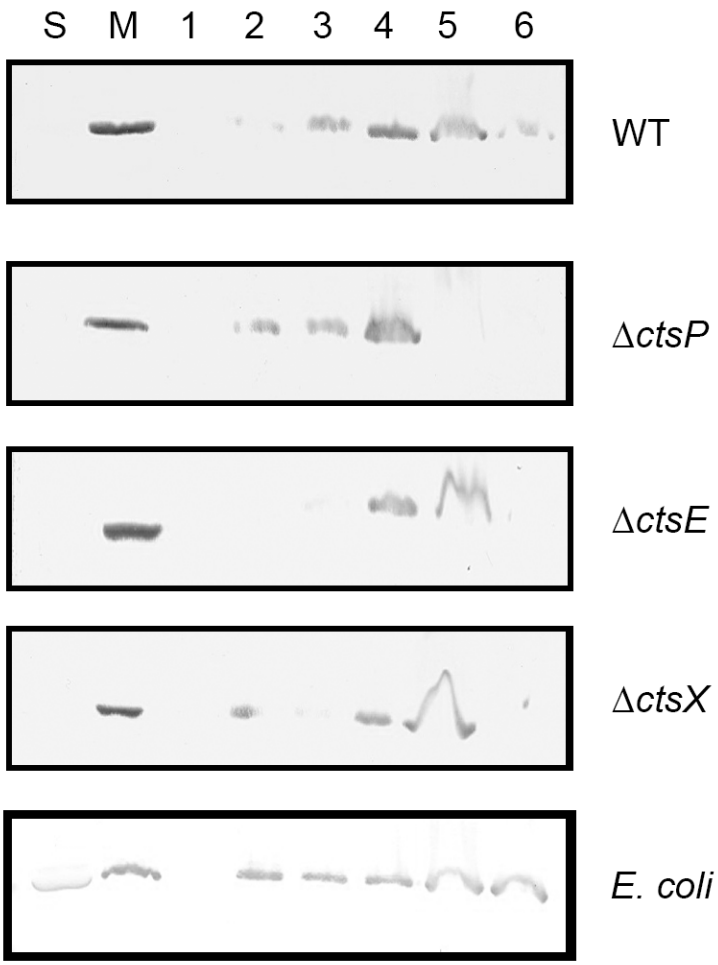

Figure 2.6. Membrane flotation of CtsP-FLAG. Soluble and membrane fractions of DRH212, *cts* mutants (Δ*ctsP,* Δ*ctsE*, and Δ*ctsX*), and *E. coli* containing the fusion protein CtsP-FLAG expressed on pECO102 were obtained. The membrane fractions were resuspended in a sucrose gradient to separate the membranes from the insoluble material, subjected to ultracentrifugation, and fractions were taken moving sequentially down the gradient (numbered 1-6

where 1 is the top of the gradient and 6 contains the insoluble proteins). These fractions were separated via 12% SDS-PAGE. The presence of the CtsP-FLAG fusion protein was detected by immunoblotting using a monoclonal antibody against the FLAG epitope. S, soluble fraction, M, membrane fraction.

2.3.5 Membrane Extractability of CtsP and CtsX

Given its fractionation with the membrane of both *C. jejuni* and *E. coli*, we hypothesized that CtsP is a peripheral membrane protein, perhaps associating with the membrane by interacting with the polar head of the phospholipid bilayer. In contrast, CtsX, with its more obvious transmembrane domain and the fusion protein's ability to localize alkaline phosphatase to the periplasmic space (Figure 2.4), appears to be an integral membrane protein. To further characterize CtsP and CtsX as integral or peripheral membrane proteins, we analyzed the avidity of their association with *C. jejuni* membranes using different extraction procedures commonly used to release peripherally associated membrane proteins [224].

Membranes of *C. jejuni* expressing CtsP-FLAG or His-CtsX were treated with high salt (1 & 2 M NaCl, 1 M KCl), urea (3 M, 5 M) or extremes of pH (Na_2CO_3 pH 3.0, pH 11.0) for 30 minutes at 4°C as described in Materials and Methods. Control membranes were treated with 10 mM Tris, pH 8.0. CtsP-FLAG was partially extracted from the membranes in high salt (1 & 2 M NaCl, 1 M KCl) while CtsX remained predominantly membrane associated (Figure 2.7). Treatment of the membranes with urea (3 M, 5 M) nearly completely solubilized CtsP-FLAG while His-CtsX was only partially extracted by this treatment (Figure 2.7). Membrane treatment with 0.5 M Na_2CO_3 at pH 11 also extracted CtsP-FLAG into the soluble fraction (Figure 2.7). In contrast, Na_2CO_3 treatment was

not sufficient to affect His-CtsX localization. It remained in the membrane fraction after (Figure 2.7). These results suggest that while His-CtsX is an integral membrane protein, the more ready extraction of CtsP-FLAG suggests that it is not integral but peripheral to the membrane, interacting with it perhaps through an as-yet undefined mechanism.

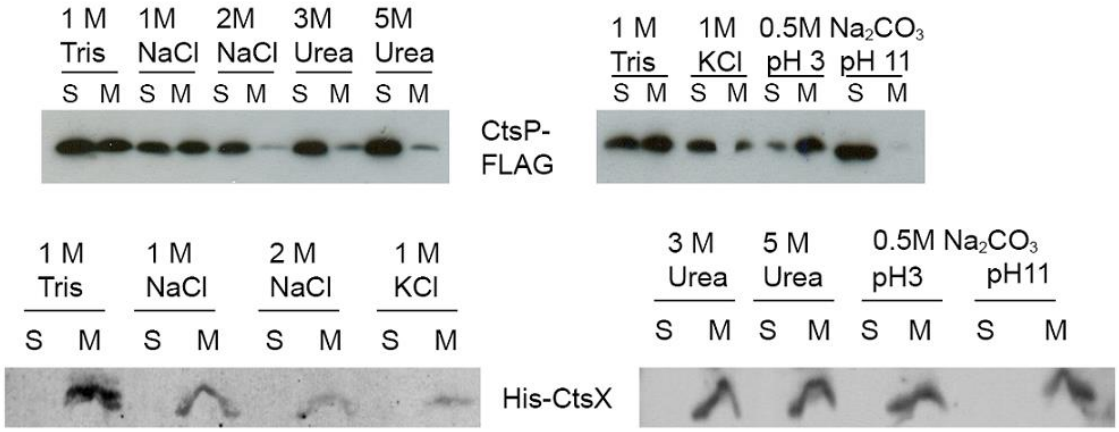

Figure 2.7. Membrane extractability of CtsP-FLAG and His-CtsX. Membranes were incubated with the indicated reagents for 30 min at 4°C and sedimented by ultracentrifugation. Separated fractions were examined by 12% or 15% SDS-PAGE. Fusion proteins were detected by immunoblotting with anti-FLAG M2 antibody for CtsP-FLAG and with an anti-6x His antibody for His-CtsX. S, soluble fraction after washes, M, membrane fraction.

2.4 Discussion

The goal of this study was to characterize two putative NTPases/NTP binding proteins involved in natural transformation of *C. jejuni*. One of these, CtsE, belongs to a family of well-studied NTPases involved in transport of macromolecules in a number of systems including type II secretion, type IV secretion, and type IV pilus biogenesis [183, 201]. Members of this family have

ATPase activity *in vitro* [207-211], although the exact ATP-dependent step in transport is unclear. Given the homology of CtsE to other members of this family and the importance of an intact Walker box A for CtsE to function in natural transformation, it is likely that CtsE has ATPase activity critical for its role in transformation.

The other putative NTPase/NTP binding protein, CtsP, has little homology to other NTPases and cannot be assigned to a defined family. CtsP Walker box A mutants were all unstable in *C. jejuni*, but a stable mutant protein lacking Walker box B was unable to correct the transformation defect of a Δ*ctsP* mutant. We suggest that CtsP may bind and hydrolyze nucleotides and that this property is necessary for natural transformation of *C. jejuni*, although further work is needed to determine whether CtsP has ATPase activity.

Transformation in *C. jejuni* involves a number of proteins with similarity to those required for pilus biogenesis and natural transformation in *Neisseria gonorrhoeae* and *Vibrio cholerae*[5, 225-227], [228, 229]. In these species, transformation is facilitated by the production of a pilus that is produced and retracted through the power of two ATPases. Transformation in *N. gonorrhoeae* requires the activity of two ATPases, PilF and PilT [225, 230] and in *V. cholerae* it requires PilB and PilT [228]. Both *N. gonorrhoeae* ATPases are members of the PulE – VirB11 superfamily [183, 201]. PilF is required for elaboration of the pilus while PilT is required for retraction [225, 231]. As noted, while CtsE appears to fall into this family, CtsP does not. However, unlike these two systems, *C. jejuni* has never been shown to produce a pilus (1) and lacks a homolog to the major

structural subunits found in these systems, PilE in *N. gonorrhoeae* and PilA in *V. cholerae.* Instead, *C. jejuni* contains a number of genes that may encode pseudo-pilins: *ctsG, ctsT,* and *Cjj81176_1096*; two of these have been shown to function in transformation [5]. Whether a pilus or pseudo-pilus is responsible for DNA binding or uptake across the outer membrane, requiring the actions of CtsE and CtsP, has yet to be determined. In the *V. cholerae* transformation system, pseudopilins are thought to initiate pilus formation while the major pilin subunit, PilA, is then responsible for the pilus structure [228]. Loss of any of these proteins leads to approximately a three-log decrease in transformation efficiency [228]. Similarly to the *V. cholerae* system, the pseudopilins in *C. jejuni* may be sufficient to induce transformation. A second possibility is that unlike in the *V. cholerae* system, the pseudopilins might also form enough of a pilus-like structure to facilitate transformation even without the presence of a major pilin subunit.

CtsE appears to be predominantly cytosolic in *C. jejuni*, whereas CtsP is predominantly membrane-associated. A CtsE homologue, ComGA, involved in transformation of *Bacillus subtilis* localizes to the membrane in that species, where it behaves as a peripheral membrane protein [232]. In some other bacteria, CtsE homologues (generally termed GspE proteins) become membrane localized by interacting with an integral membrane protein generally termed GspL [233, 234] but no GspL homologue was identified in the *C. jejuni* genome sequence. It is difficult to imagine how CtsE could play a role in the early stages of transformation unless it interacts with proteins at the membrane. In the *Vibrio*

cholerae system, a GFP tagged version of the CtsE homolog, PilB, forms dynamic foci at the membrane that transiently overlap with other components of the *Vibrio* transformation apparatus [228]. Perhaps similarly to the *Vibrio* system, a transient, or very weak, association of CtsE occurs with the membrane and this could have been disrupted during fractionation; this might account for the small amount of CtsE-FLAG observed in the membrane fraction.

Unlike CtsE, CtsP localizes to the membrane in *C. jejuni*, where it may interact with other components of the transformation machinery. By bacterial two-hybrid analysis we detected interaction between CtsP and CtsX, another protein necessary for efficient *C. jejuni* transformation. CtsX is encoded immediately downstream of CtsP in a putative operon [5]. CtsX is an integral membrane protein with its amino terminus exposed to the cytoplasm, and residues 50 – 195 in the periplasm. Interaction with CtsX is not necessary for CtsP localization to the membrane, nor is interaction with several other components of the transformation machinery. CtsP behaves as a peripheral membrane protein and it may associate directly with the membrane. This possibility is strengthened by the membrane localization of CtsP-FLAG observed during expression in *E. coli*. If membrane localization required interaction with another protein, it would have to be present in *E. coli* as well and would likely not be involved in natural transformation, a process that has not been described in *E. coli*. As noted above, type II secretion system GspE family members, with which CtsE shares homology, associate with the membrane through interaction with another protein, GspL. When these GspE family members are expressed in *E.*

coli cytoplasmic localization is observed [235], which differs from our observed localization of CtsP to membrane fractions in *E. coli*. Further study is needed to confirm that CtsP directly associates with the membrane. Furthermore, we were able to replicate the membrane association phenotype using membrane flotation. If this association were an artifact of the initial fractionation procedure, we would expect to see CtsP-FLAG associate predominately with the insoluble aggregate and not float with the membranes. One possibility that has yet to be discounted definitively is that the CtsP-FLAG localization (as well as that of CtsE-FLAG) is due to studying plasmid-encoded proteins, rather than the chromosomally-encoded native protein. But the behavior of the plasmid-encoded, FLAG-tagged proteins mitigates this concern, because both CtsE-FLAG and CtsP-FLAG restore transformation efficiency of their respective mutant strains to near wild type levels.

CtsX and CtsP represent novel components of the *C. jejuni* transformation machinery. Homologues of CtsX have not been identified in other transformation systems and BLAST analysis does not provide obvious clues about its function. As it resides largely in the periplasm, perhaps its C-terminus interacts with other components of the transformation machinery in that compartment. CtsX and CtsP are encoded in a putative operon between the type II secretion/type IV pilus biogenesis system homologues c*tsD, ctsE,* and *ctsF*. Given the CtsX/CtsP interaction, we hypothesize that these two proteins comprise a component of type II secretion/type IV pilus biogenesis systems specifically involved in *C. jejuni* competence. Perhaps the interaction is required

for assembly or translocation of other transformation components, or it allows assembly of a structure needed specifically for DNA uptake and not pilus biogenesis.

2.5 Materials and Methods

2.5.1 Bacterial strains and media

Bacterial strains used in this work are listed in Table 2.1. *C. jejuni* was routinely cultured on Muller Hinton (MH) agar with 10% sheep's blood in microaerophilic conditions (5% CO_2, 10% O_2, balanced with N_2) in a tri-gas incubator at 37°C. When necessary, media was supplemented with antibiotics in the following concentrations: trimethoprim (10 μg ml^{-1}), kanamycin (as noted, either 30 or 150 μg ml^{-1}), streptomycin (100 μg ml^{-1}), and chloramphenicol (as noted, either 20 or 30 μg ml^{-1}). All *C. jejuni* strains were stored at -80°C in MH broth with 20% glycerol.

Escherichia coli strains were routinely cultured at 37°C in Luria-Bertani (LB) broth or agar. When necessary, antibiotics were used at the following concentrations: ampicillin (100 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and tetracycline (12.5 μg ml^{-1}). All *E. coli* strains were stored at -80°C in LB broth with 20% glycerol.

Table 2.1. Strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics	Reference
Bacteria		
E. coli		
DH5α	*supE44* Δ*lacU*169 (Φ80*lacZ*ΔM15) *hsDr*17 *rec*A1 *end*A1*gyrA*96 *thi*-1 *rel*A1	Lab strain
JM101	F' *traD*36 *proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)*M15/Δ(*lax-proAB*) *glnV thi*	New England Biolabs
DH5α pRK212.1	contains conjugative plasmid for transferring DNA into *Campylobacter*	[236]
Sp850	*lam-, el4-, relA1, spoT1, cyaA1400(::kan), thi-1*	*E. coli* Genetic Stock Center, Yale
TG1	Δ*hsdS* Δ*(lac-proAB) supE thi F*^{0}*[traD36 proAB*$^+$ *lacI*$^{'q}$Δ*lacZM15]*	Lab strain
C. jejuni		
81-176	clinical isolate	[237]
DRH153	81-176 *astA*::*aphA3*; Kn^r	[238]
DRH212	81-176 *rpsL*^{Sm}	[238]
RSW46	DRH212 *ctsX*	[5]
RSW53	81-176 *ceuB::solo*	[5]
RSW57	81-176 *ctsD::solo*	[5]
RSW58	81-176 *ctsF::solo*	[5]
RSW115	DRH212 *ctsP*	[5]
RSW136	DRH212 *ctsE*	[5]
Plasmids		
pT18	encodes T18 fragment of CyaA	[219]
pT25	encodes T25 fragment of CyaA	[219]
pT18-Zip	encodes T18 fragment in frame with 35 aa leucine zipper	[219]
pT25-Zip	encodes T25 fragment in frame with 35 aa leucine zipper	[219]
pTrcLacZ	pTrc99A with the '*lacZ* gene (lacks codons Met 1 to Ser 7)	[223]
pTrcPhoA	pTrc99A with the '*phoA* gene (lacks codons Met 1 to Met 26)	[223]
pECO102	pRY112 derivative with *cat* promoter in *Xho*I-*BamHI* sites	[5]
pRSW176	pT18 with *ctsP* in *Xho*I-*Cla*I sites	This study
pRSW184	pT25 with *ctsX* in *Pst*I-*Bam*HI sites	This study
pRSW186	pT25 with *ctsE* in *Pst*I-*Bam*HI sites	This study
pRSW191	pT18 with *ctsE* in *Xho*I-*Cla*I sites	This study
pRSW206	pECO102 with N terminal His tag in frame with *Bam*HI site	This study
pRSW208	pECO102 with *ctsP* and a C-terminal FLAG tag	
pRSW211	pECO102 with C-terminal FLAG tag in frame with *Xho*I site	This study
pRSW223	pRSW211 with *ctsE* in *Bam*HI-*Xho*I site	This study
pRSW228	pRSW208 with E81A point mutation	This study
pRSW246	pRSW223 with K296Q point mutation	This study
pRSW248	pTrcLacZ w/coding seq for 1st 50 a.a. of *ctsX* in *Nco*I-*Xma*I site	This study
pRSW249	pTrcLacZ w/coding seq for 1st 100 a.a. of *ctsX* in *Nco*I-*Xma*I site	This study
pRSW250	pTrcPhoA w/coding seq for 1st 14 a.a. of *ctsX* in *Nco*I-*Xma*I site	This study
pRSW251	pTrcLacZ w/coding seq for 1st 14 a.a. of *ctsX* in *Nco*I-*Xma*I site	This study
pRSW252	pTrcPhoA w/coding seq for 1st 50 a.a. of *ctsX* in *Nco*I-*Xma*I site	This study
pRSW253	pTrcPhoA w/coding seq for 1st 100 a.a. of *ctsX* in *Nco*I-*Xma*I site	This study
pRSW254	pTrcPhoA w/coding seq of *ctsX* in *Nco*I-*Xma*I site	This study
pRSW255	pTrcLacZ w/coding seq of *ctsX* in *Nco*I-*Xma*I site	This study
pRSW256	pRSW211 with *ctsX* in *Bam*HI-*Xho*I sites	This study
pJMB1	pRSW206 with *ctsX* in *Bam*HI-*Xho*I	This study

2.5.2 Construction of FLAG fusion proteins

To express FLAG fusion proteins in *C. jejuni* we modified pECO102 by mutagenesis with PFU turbo [5]. Two primers were made that annealed to pECO102 and would insert the coding sequence for the FLAG tag and a stop codon downstream of the *Xho*I site. (All primers used in this study are indicated on Table 2.2.) pECO102 was amplified by PCR with these primers and the template DNA was digested with *Dpn*I. *E. coli* DH5α was transformed with the reaction mixture and clones were screened by PCR. Candidate plasmids were sequenced to confirm insertion of the FLAG sequence and one, pRSW211, was used for subsequent cloning. To construct in-frame fusion proteins with a C-terminal FLAG tag, PCR primers were designed to amplify the coding sequence of the protein of interest from the second amino acid through the last amino acid (excluding the stop codon). The 5' primer had *Bam*HI sites and the 3' primer had *Xho*I sites for in-frame insertion into pRSW211. All subsequent clones were verified by sequence determination.

Table 2.2. Oligonucleotide primers used in this study

Plasmid	Sequence	Site	Primer Name
pRSW176	5' CCC*ATCGAT*ACCCTTAATAAGCCATTCTCTAAAGCACTAAGCTTT 3'	*Cla*I	Cj1473c pT18 3'
pRSW176	5' CCG*CTCGAG*GAGTAAAATTATTCCATTTAGAGAAGAAATTTTTC 3 '	*Xho*I	Cj1473c pT18 5'
pRSW184	5' CGC*GGATCC*CTACTTTCCATCTAATTCCATTAAACC 3'	*Bam*HI	Cj1472c pT25 3'
pRSW184	5' AAAA*CTGCAG*GGCAAGAAAGAATTAAAGAGCTTGAGCTTAGG *3'*	*PstI*	Cj1472c pT25 5'
pRSW186	5' CGC*GGATCC*TCATCTTACAACCCTTAAAAGCTCATC 3'	*Bam*HI	Cj1471c pT25 3'
pRSW186	5' AAAA*CTGCAG*GGGAAAGTAGAATGGATAAAATTTTTCAAGC 3'	*Pst*I	Cj1471c pT25 5'
pRSW191	5' CCC*ATCGAT*ACTCTTACAACCCTTAAAAGCTCATCTATAC 3	*Cla*I	Cj1471c pT18 3'
pRSW191	5' CCG*CTCGAG*GGAAAGTAGAATGGATAAAATTTTTCAAGC 3'	*Xho*I	Cj1471c pT18 5'
pRSW208	5' CGC*GGATCC*AGTAAAATTATTCCATTTAGAGAAGAA 3'	*Bam*HI	Cj1473c comp strt
pRSW208	5' CCG*CTCGAG*TTATTTGTCATCATCGTCCTTATAATCCCTTAAT AAGCCATTCTCTAAAGCACT 3'	*Xho*I	Cj1473c comp stp FLAG
pRSW211	5' ATCAAGCTTATCGATACCGTCGACCTCGAGGATTATAAGGACG ATGATGACAAATAAGGGGGGCCCGGTACCCAGCTTTT 3'		pECO FLAG tag 1
pRSW211	5' AAAAGCTGGGTACCGGGCCCCCCTTATTTGTCATCATCGTCC TTATAATCCTCGAGGTCGACGGTATCGATAAGCTTGAT 3'		pECO FLAG tag 2
pRSW223	5' CGC*GGATCC*CAAAGTAGAATGGATAAAATTTTTCAAGCT 3'	*Bam*HI	Cj1471c comp strt
pRSW223	5' CCG*CTCGAG*TCTTACAACCCTTAAAAGCTCATCTAT 3'	*Xho*I	Cj1471c no stp cod

pRSW228	5’ CAAGGTCAGAGTTAAATCATTCTTGATGCAGTGGGAATG TATGATTATGCAA 3’		CtsP E81A 1
pRSW228	5’ TTGCATAATCATACATTCCCACTGCATCAAGAATGATTAA ACTCTGACCTTG 3’		CtsP E81A 2
pRSW246	5’ AAACACGCGTATAAAGTTGTGCTTTGACCACTACCTGTAG GACCGGTTAAT 3’		Cj1471c K296Q 1
pRSW246	5’ ATTAACCGGTCCTACAGGTAGTGGTCAAAGCACAACTTTA TACGCGTGTTT 3’		Cj1471c K296Q 2
pRSW248	5’ CTCAT*CCATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3’	*NcoI*	CtsX fusion 5’
pRSW248	5’ TCCC*CCCGGG*AATATTTTTTTGCTTATTATATTTTTGCAT 3’	*Xma*I	CtsX 50 aa fus 3’
pRSW249	5’ CTCAT*CCATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3’	*NcoI*	CtsX fusion 5’
pRSW249	5’ TCCC*CCCGGG*TTTAACTTCTTCTAGTTCTTTATATAACTT 3’	*Xma*I	CtsX 100 aa fus 3’
pRSW250	5’ ACAATTTCACACAGGAAACAGACCATGCAAGAAAGAATTAAA GAGCTTGAGCTTAGGTATAAATACTTTCCTGTTCTGGAAAAC CGGGCTGCTCAG 3’		Nterm 14aa PhoA1
pRSW250	5’ CTGAGCAGCCCGGTTTTCCAGAACAGGAAAGTATTTATACCTA AGCTCAAGCTCTTTAATTCTTTCTTGCATGGTCTGTTTCCTGTG TGAAATTGT 3’		Nterm 14aa PhoA2
pRSW2515’	ACAATTTCACACAGGAAACAGACCATGCAAGAAAGAATTAAA GAGCTTGAGCTTAGGTATAAATACTTTCTGGCCGTCGTTTTACA ACGGGGATCC 3’		Nterm 14aa LacZ1
pRSW251	5’ GGATCCCCGTTGTAAAACGACGGCCAGAAAGTATTTATACCT AAGCTCAAGCTCTTTAATTCTTTCTTGCATGGTCTGTTTCCT GTGTGAAATTGT 3’		Nterm 14aa LacZ2
pRSW252	5’ CTCAT*CCATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3’	*NcoI*	CtsX fusion 5’
pRSW252	5’ TCCC*CCCGGG*AATATTTTTTTGCTTATTATATTTTTGCAT 3’	*Xma*I	CtsX 50 aa fus 3’
pRSW253	5’ CTCAT*CCATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3’	*NcoI*	CtsX fusion 5’
pRSW253	5’ TCCC*CCCGGG*TTTAACTTCTTCTAGTTCTTTATATAACTT 3’	*Xma*I	CtsX 100 aa fus 3’
pRSW254	5’ CTCAT*CCATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3’	*NcoI*	CtsX fusion 5’
pRSW254	5’ TCCC*CCCGGG*CTTTCCATCTAATTCCATTAAACCATA 3’	*Xma*I	CtsX full fus 3’
pRSW255	5’ CTCAT*CCATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3’	*NcoI*	CtsX fusion 5’
pRSW255	5’ TCCC*CCCGGG*CTTTCCATCTAATTCCATTAAACCATA 3’	*Xma*I	CtsX full fus 3’
pRSW256	5’ CGC*GGATCC*CAAGAAAGAATTAAAGAGCTTGAGCTT 3’	*Bam*HI	Cj1472c comp strt
pRSW256	5’ CCG*CTCGAG*CTTTCCATCTAATTCCATTAAACCATA 3’	*Xho*I	Cj1472c no stp cod
pJMB1 *Bam*HI	5’ CGC*GGATCC*AGTAAAATTATTCCCATTTAGAGAAGAA 3’	*Bam*HI	CtsX comp st +
pJMB1 +*Xho*I	5’ CCG*CTCGAG*TTACCTTAATAAGCCATTCTCTAAAGA 3’	*Xho*I	CtsX comp stop

2.5.3 Construction of Walker Box point mutants

Point mutants were constructed in the Walker boxes of *ctsE* and *ctsP* by PFU mutagenesis (Stratagene). Both *ctsE* and *ctsP* were cloned into pRSW211 to create C-terminal FLAG fusions, pRSW223 (CtsE-FLAG) and pRSW208 (CtsP-FLAG). The conserved lysine residue (K296) of the Walker box A of CtsE-FLAG was mutated by changing the coding sequence from AAA to CAA resulting in an amino acid change to a glutamine (K296Q); this plasmid was designated pRSW246. The conserved glutamic acid residue (E81A) of the Walker box B of

CtsP-FLAG was mutated by changing the coding sequence from GAA to GCA resulting in an amino acid change to an alanine (E81A); this plasmid was designated pRSW228. DH5α. pRK212.1 was transformed with these constructs and conjugations were performed as described previously [236, 239]. pRSW208 and pRSW228 were conjugated into DRH212 Δ*ctsP* (RSW115), while pRSW223 and pRSW246 were conjugated into DRH212 Δ*ctsE* (RSW136) [5]. Transconjugants were verified by PCR. Expression of the fusion proteins was detected in whole cell lysates by SDS-PAGE and western blot analysis as discussed below.

2.5.4 Construction of His fusion proteins

To express a 6x-His tagged version of CtsX, genomic DNA from *C. jejuni* strain 81-176 was used as a template to amplify *ctsX* from the second codon to the stop codon using a forward primer with a *Bam*HI site and a reverse primer with *Xho*I site flanking the coding sequence. This fragment was cloned into the *Bam*HI and *Xho*I sites of pBW206, a derivative of pECO102 that contains a N-terminal 6x-His tag immediately upstream of the *Bam*HI site, creating JMB1. This plasmid was transformed into DH5α pRK212.1 and transferred by conjugation into the Δ*ctsX* strain as described previously [236, 239]. Transconjugants were verified by PCR and the stability of the fusion proteins was assessed using SDS-PAGE and Western blot analysis from whole cell lysates.

2.5.5 Transformation assays

Transformation assays were performed as described previously. [5, 193]. Briefly, *C. jejuni* was grown 16 – 18 hours on MH agar plates supplemented with

appropriate antibiotics. Cells were resuspended in MH broth to an optical density at 600 nm of 0.5 and 500 μl aliquots were added to 13 mm test tubes containing one ml solidified agar. After incubation for three hours at 37°C in 5% CO_2, one microgram of DRH153 (81-176 *astA::aphA3*) DNA was added and the cultures were incubated for an additional four hours [238]. The number of transformants and the total number of bacteria were determined by dilution plating on MH agar with appropriate antibiotics. Transformation efficiency represents the number of transformants per total number of bacteria per microgram of DNA. Transformations were conducted in triplicate and the transformation efficiency represents the average of the three samples from one experiment.

2.5.6 Cell Fractionations

Cell fractionation was carried out as previously described for *C. jejuni* with a few modifications [218]. *C. jejuni* was cultured on MH agar plates under microaerophilic conditions at 37°C for 16 – 18 hrs. Cells were resuspended from plates in MH broth and centrifuged (10,000 x g, 10 min at 4°C) and the resulting pellet was resuspended in 10 mM HEPES pH 7.4. After one freeze-thaw cycle in a dry ice-ethanol bath, cells were sonicated with a micro-tip in a Branson digital sonifier at 30% amplitude six times for 10 seconds. Cell debris was pelleted by centrifugation (10,000 x g, 10 min at 4°C in a Sorvall tabletop centrifuge) and the supernatants were subjected to ultracentrifugation to pellet membranes (100,000 x g, 70 min at 4°C with a SW41 rotor). The supernatant was removed and saved as the soluble fraction; the pellet (membrane fraction) was washed once in 10 mM HEPES and subsequently resuspended in an appropriate volume of 10 mM

Tris-HCl pH 8.0. The soluble fraction contains cytoplasmic and periplasmic contents while the membrane fraction contains both inner and outer membranes. The same procedure was followed for fractionation of *E. coli*. Protein concentrations were determined with the BioRad protein assay or with the ThermoScientific BCA assay per manufacturer's instructions. Equal concentrations of proteins were then analyzed by SDS-PAGE and Western blot analysis.

2.5.7 Membrane Flotation

C. jejuni was cultured on MH agar plates under microaerophilic conditions at 37°C for 16 – 18 hrs. Cells were resuspended from plates in MH broth and centrifuged (Sorvall tabletop centrifuge, 10,000 x g, 10 min at 4°C) and the resulting pellet was resuspended in P Buffer (100 mM sodium phosphate pH 7.6, 50 mM $MgCl_2$, and 10 mM EDTA). Cells were lysed by passage through a French press. Lysates were incubated with DNase for 30 minutes and centrifuged (5000 rpm for 5 min at 4°C) to remove cell debris. The supernatant was subjected to ultracentrifugation (100,000 x g, 1 hour, at 4°C, SW41 rotor), and a sample of the supernatant, containing the cytosolic and periplasmic proteins, was collected. The pellet was resuspended in P buffer and centrifuged (100,000 x g, 1 hour, at 4°C, SW41 rotor). The supernatant was discarded and the pellet was resuspended in P buffer and mixed with 81% sucrose (dissolved in P buffer) to a final concentration of 71%. The sample was overlaid with 52% and 42% sucrose and subjected to ultracentrifugation (18 hour at 100,000 x g, at 4°C, SW41 rotor). After ultracentrifugation, the presence of the membrane bands was noted.

Fractions (1.8 ml) were taken, starting from the top of the sample and labeled 1-6 sequentially. The sucrose was diluted in P buffer and proteins were precipitated using 2% deoxycholate and trichloroacetic acid (TCA) overnight at 4°C. After precipitating the proteins, fractions were subjected to ultracentrifugation (1.5 hour, at 100,000 x g, at 4°C, SW41 rotor). Pellets from each fraction were washed with acetone to remove residual TCA and subsequently with 100% ether to remove lipids. Pellets were vacuum dried and resuspended in water to be tested in the isocitrate dehydrogenase assay or resuspended in 6X sample buffer for SDS-PAGE and immunoblot analysis.

2.5.8 SDS-PAGE and Immunoblotting

To detect FLAG fusion proteins, samples were diluted 1:1 with 2X SDS sample buffer and boiled for five minutes before loading onto 12% polyacrylamide gels for separation by SDS-PAGE. For His-CtsX containing samples, samples were diluted 1:6 with 6X SDS sample buffer and were separated on a 15% polyacrylamide gel. Samples were normalized by protein concentration. Proteins were transferred to nitrocellulose membranes (Hoefer semi-dry, 200 mAmps for 2 hours) and, after blocking with 5% milk in TBS, membranes were probed with primary antibody. For FLAG-tagged fusion proteins, an anti-FLAG M2 monoclonal antibody peroxidase conjugate (Sigma) at 1:1000 was used. This was developed using the Western Lighting Chemiluminescence reagent plus (Perkin-Elmer Life Sciences). For the His-tagged fusion protein, THE™ His-tag antibody (Genscript) at 1:1500 was used. A rat anti-mouse IgG1-HRP conjugated antibody (Southern Biotech) was used at

1:3500 for the secondary antibody and blots were developed using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific).

2.5.9 Isocitrate Dehydrogenase assays

To demonstrate efficient fractionation, fractions were tested for the presence of isocitrate dehydrogenase, a cytoplasmic protein. Isocitrate dehydrogenase activity was assayed as described previously [218]. The reaction mixture contained 100 μl 10 mM $MgCl_2$, 100 μl 50 mM Tris-HCl pH 8.0, 100 μl 10 mM NADP, 550 - 600 μl distilled H_2O, and 0 – 50 μl of the cell fraction. The reaction was started by the addition of 100 μl of 50 mM sodium isocitrate and the optical density at 340 nm was read at 15 second intervals for three minutes. The specific activity of isocitrate dehydrogenase was calculated using an absorption coefficient of NADPH of 6.22 mM^{-1} cm^{-1} at 340 nm. The percent (of total unfractionated sample) of isocitrate dehydrogenase activity present in each fraction was calculated. For all fractionations, a minimum of 90% of the isocitrate dehydrogenase activity was present in the cytoplasmic fraction.

2.5.10 Identification of interacting proteins with the bacterial-two hybrid system

A bacterial two hybrid system was used to identify potential interactions among Cts proteins. This system is based on functional complementation between two fragments of the catalytic domain of adenylate cyclase from *Bordetella pertussis* [219]. Genes *ctsE*, *ctsP*, *ctsX*, *ctsF*, *ctsD*, *ctsR*, *ctsG*, *ctsW*, and *dprA* were PCR amplified from the coding sequence of the second amino acid through the stop codon and cloned into the *Pst*I and *Bam*HI sites of pT25 to

generate in-frame T25 fusion proteins. The same genes were amplified with primers containing *Xho*I and *Cla*I sites from the second amino acid to the last amino acid, excluding the stop codon, and inserted into the *Xho*I and *Cla*I sites of pT18 to create in-frame fusion proteins to the adenylate cyclase T18 domain. The insertions in these plasmids were verified by PCR and confirmed by sequencing.

E. coli strain Sp850 (*cya*-) was transformed with all combinations of Cts-pT18 and Cts-pT25 plasmids and transformants were screened for protein-protein interactions by growth on LB agar plates at 30°C with 100 μg ml^{-1} ampicillin, 30 μg ml^{-1} chloramphenicol, 40 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 0.5 mM isopropyl-β-D-thiogalactopyranoside (ITPG). The negative control contained Sp850 with pT18 and pT25 and the positive control was Sp850 with pT18-Zip and pT25-Zip [219]. Assays were done in triplicate and the data represent the average of three biological replicates. Standard deviation is indicated.

2.5.11 Topology analysis of CtsX

LacZ and PhoA Fusions of *ctsX* were made using pTrcLacZ and pTrcPhoA [223]. Translational fusions were generated at amino acid residues 14, 50, and 89 and 195 (the final amino acid in full length CtsX protein). These sites were chosen after analysis of CtsX hydrophobicity by several computer programs (Kyte Doolittle, EMBL) which predicted a transmembrane segment at roughly amino acids 22 – 42. PCR products were cloned into the *Nco*I and *Xma*I sites in pTrcLacZ and pTrcPhoA. Plasmids were screened by PCR and verified

by sequencing. *E. coli* strain TG1 was transformed with these plasmids to carry out alkaline phosphatase assays and β-galactosidase assays using standard methods [222]. Strains were assayed in triplicate and the data represent the average of three biological replicates. Standard deviations are indicated.

2.5.12 Membrane extractability of CtsP and CtsX fusion proteins

For membrane solubility studies, cells were fractionated as described previously. Purified membranes were diluted 1:1 into either a) 10 mM Tris-HCl pH 8.0, b) 1 M NaCl, c) 2 M NaCl, d) 3 M urea, e) 5 M urea, f) 0.5 M Na_2CO_3 pH 3.0, g) 0.5 M Na_2CO_3 pH 11.0 or h) 1 M KCl. and rocked on a nutator mixer for 30 minutes at 4°C. Membranes were subsequently collected by ultracentrifugation (1 hour, 100,000 x g, at 4°C, SW41 rotor) and were resuspended in 50 μl of 10 mM Tris, pH 8.0. The supernatant was removed and precipitated by addition of 400 μl of acetone, incubation at -20°C for one hour, and centrifugation at 16,000 x g at 4°C. Precipitated proteins were air dried for 30 minutes and resuspended in 50 μl of 10 mM Tris-HCl pH 8.0. Equal volumes of soluble and membrane fractions were resuspended in SDS sample buffer and loaded onto polyacrylamide gels for separation by SDS-PAGE and Western blot analysis to detect CtsX or CtsP as described above.

2.6 Notes

This work was in colloraboration with Dr. Rebecca Erfut and has been published and can be cited as follows:

Beauchamp, J. M., R. S. Erfurt and V. J. DiRita (2015). "Characterization and localization of the Campylobacter jejuni transformation system proteins CtsE, CtsP, and CtsX." J Bacteriol **197**(3): 636-645.

CHAPTER III

IDENTIFICATION OF METHYLATION DEPENDENT DNA SELECTION IN *CAMPYLOBACTER JEJUNI* NATURAL TRANSFORMATION

3.1 Abstract

Campylobacter jejuni, a leading cause of bacterial gastroenteritis in the developed world, is one of approximately sixty-five identified naturally competent bacteria. This trait may contribute to the large amount of diversity seen between sequenced strains of *C. jejuni.* The mechanisms underlying how DNA is recognized for uptake and recombination are not well characterized in *C. jejuni*. Although *C. jejuni* can readily be transformed by *C. jejuni* derived DNA, it is poorly transformed by the same DNA derived from *E. coli* or via PCR. Our work indicates that methylation plays an important role in marking *C. jejuni* transforming DNA. DNA derived from a mutant lacking a specific DNA methyltransferase, which we term *Campylobacter* transformation system methyltransferase (*ctsM*) transforms wild type *C. jejuni* at levels several orders of magnitude lower than DNA containing the CtsM mark. Both *ctsM* mutant and wild type become transformed to the same levels when provided DNA from wild type (CtsM+). The *ctsM* mutant cannot be transformed by its own DNA, i.e. DNA lacking the specific methylation. However, it otherwise grows like wild type, with no evidence of its own DNA being restricted due to loss of the methyltransferase.

We confirmed that the DNA purified from the *ctsM* mutant lacks methylation at the methyltransferase recognition site RAATTY. These data indicate that the presence of the methyl mark at this site enhances transformability of the DNA. This extends to DNA derived from *E. coli* DNA and methylated *in vitro* at RAATTY; the *in vitro* methylated DNA is sufficient to transform *C. jejuni* whereas otherwise identical unmethylated DNA is not. We also demonstrate that only one methylated site is required to allow for transformation. We also determine that selection occurs inside the cell, not at the outer membrane. Here we demonstrate a mechanism of DNA selection in *C. jejuni* that is distinct from DNA selection by other competent bacteria.

3.2 Introduction

Natural transformation, the ability of bacterial cells using dedicated machinery to transport DNA into the cytosol and recombine it into the genome, is a widespread trait. As a mechanism of horizontal gene transfer, it may allow bacteria to acquire new genes to better adapt to their environment. Over 65 species of both Gram negative and Gram positive bacteria are naturally competent, expressing protein complexes that span all cellular compartments to acquire exogenous DNA and transport it into the cell for recombination with the chromosome [27].

One such bacterium is *Campylobacter jejuni*, a leading cause of bacterial gastroenteritis worldwide. *C. jejuni* often colonizes the avian intestinal tract; a common source of infection is consumption of contaminated poultry [198]. The ability to readily acquire DNA from the environment may contribute to the

extensive genetic diversity observed among strains of *C. jejuni*. Horizontal gene transfer *in vivo* has been demonstrated during experimental infection of chicks, a natural host for this pathogen [3].

C. jejuni is exceedingly selective in the DNA it uses for natural transformation, using DNA originating from closely related strains. Many bacteria actively select the DNA used for transformation. One such mechanism for selection involves the use of DNA uptake sequences (DUS) [119, 120, 240]. These are sequences of ten to twelve base pairs repeated frequently throughout the genome. Uptake sequences have not been identified in *Campylobacter*. Evidence indicates that DNA selection does not appear to be solely sequence-based because a given piece of DNA transforms *C. jejuni* when the DNA is isolated from *C. jejuni* but not from *E. coli* [193].

DNA methyltransferases (MTases) play many roles in biology; a key one in bacteria is to identify foreign DNA through restriction modification systems (RM systems). RM systems expressed in many different bacteria protect from phage attack. DNA MTases methylate specific sequences of bacterial DNA to protect the DNA from restriction enzyme (REase) degradation. Incoming phage DNA lacks the methylation pattern of its host. Cognate REases therefore degrade phage DNA without affecting the methylated bacterial DNA. These RM systems play a role in transformation in *Helicobacter pylori,* a close relative of *C. jejuni.* The HpyAXII RM system degrades unmethylated plasmid and chromosomal DNA during natural transformation [112]. Other RM systems degrade incoming DNA lacking the proper methylation motifs as well as decreasing the length of

integrated DNA [113, 114]. Similarly, increasing numbers of NlaIV restriction sites has an inhibitory effect on transformation in *Neisseria meningitidis* [116]. A negative correlation between the RM systems and transformation has also been demonstrated in *Pseudomonas stutzeri* [115]. In all of these cases, incoming DNA lacking the appropriate methylation motifs is then degraded and therefore cannot complete the transformation process by recombining onto the chromosome. In *C. jejuni* strain 11168, the RM system encoded by Cj1051c, plays a role in DNA selection of plasmid DNA [194]. When this system is deleted, transformation of plasmids derived from other *Campylobacter* strains is enhanced [194]. However, strains lacking this system are still unable to be transformed by *E. coli* derived plasmids [194]. While this system is important in strain 11168, it is not widely distributed throughout *Campylobacter* strains nor does it completely explain the DNA discrimination observed as *E. coli* derived plasmids are still non-transformable. Therefore, it cannot be the sole determinant of DNA selection in *C. jejuni.*

A large number of DNA MTases have been identified among the numerous sequenced *C. jejuni strains.* These DNA MTases tend to differ among strains. Using Single-Molecule Real-Time (SMRT) sequencing, active DNA MTases and the sequences they methylate were identified [144, 149-153]. We chose to examine the role of the single conserved methylation motif RA^{m6}ATTY in the laboratory strain DRH212, a streptomycin resistant derivative of 81-176. We hypothesized that recognition of methylation of this motif would allow *Campylobacter* to distinguish DNA from close relatives from DNA from other

species, as the methylated motif has been found in all *Campylobacter* SMRT sequenced strains to date. This methylation mark is conferred by a single DNA MTase, Cjj81-176_0240, conserved in *Campylobacter* species but not in other closely related genera. It lacks a cognate restriction enzyme, putting it into the class of orphan methyltransferases, similar to the *dam* enzyme of *E. coli* and others. We hypothesized this MTase is important for DNA selection in natural transformation.

This study demonstrates that the motif, RA^{m6}ATTY functions as an uptake sequence in *C. jejuni.* DNA lacking methylation at this motif transforms *C. jejuni* at levels orders of magnitude lower than DNA carrying the methyl mark. We term this orphan methyltransferase (Cj81-176_0240) *Campylobacter* transformation system methyltransferase, *ctsM*. CtsM methylated DNA or DNA methylated *in vitro* to introduce the RAATTY site is required for transformation into *C. jejuni.* Methylation of this motif enables *E. coli* or PCR-derived DNA to serve as a high-efficiency substrate for transformation in comparison to similar DNA lacking the RAATTY methylation mark. Furthermore, DNA localization studies suggest that the selection for methylated DNA occurs inside the cell.

3.3 Results

3.3.1 One methylation motif is conserved among all SMRT sequenced strains of *Campylobacter.*

Campylobacter species have a large number of annotated DNA MTases [160]. Introduction of SMRT sequencing has enabled easy identification of all adenosine methylation motifs as well as some cytosine methylation motifs [144].

These methylation motifs can be paired with their corresponding MTase genes using both bioinformatics and genetic mechanisms. To date ten *Campylobacter* strains have been sequenced using this method, including four *C. jejuni* isolates [149-153]. These strains include laboratory, animal, and human isolates (Table 3.1). Of the thirty-four methylation motifs identified, only one methylation motif and cognate DNA MTase was conserved among all ten of these strains. Five methylation motifs were found in more than one strain, but the majority of methylation motifs were only found in one strain. The diversity of methylation patterns indicates that even though two strains might be closely related, their DNA will have different patterns of methylation.

The single conserved methylation motif is RA^{m6}ATTY. Not only is this methylation motif found in all SMRT sequenced strains of *Campylobacter*, it is over-represented in the *C. jejuni* genome (Fig 3.1). Of the 26,876 RAATTY sites in the genome, 26,609 sites were found to be methylated [149]. Because *C. jejuni* has a small genome, on average this results in a RAATTY motif approximately every sixty base pairs, nearly 70 times more frequent than is predicted for a six-base motif. This is significantly more than the other methylation motifs identified or for another six base pair recognition sequence, GKAAYG, for which 1,236 or 1,259 sites were methylated (Fig 3.1) [149]. We hypothesized that recognition of methylation of this methylation motif, which has been found in all SMRT sequenced *Campylobacter* strains to date, would allow *Campylobacter* to identify DNA from close relatives from other species for use in transformation.

In 81-176, this methylation mark is conferred by CJJ81176_0240, which we term *C*ampylobacter transformation system methyltransferase, *ctsM* [149]. This D12 class N6 adenine specific DNA MTase is an orphan MTase, meaning it has no cognate restriction enzyme. Using a BLAST search, we determined that this gene is highly conserved among *Campylobacter* species but has a more limited conservation outside this genus. We hypothesized that this particular MTase would play a role in DNA selection during transformation for two reasons. First, its high degree of conservation within *Campylobacter* indicates an important role in a variety of habitats (it is active in laboratory, human and animal isolates), while its lack of conservation outside the genus suggests it might help discriminate *Campylobacter* DNA from non-*Campylobacter* DNA. Second, its lack of a cognate restriction enzyme as an orphan DNA MTase indicates that it might play a unique role in the biology of this bacterium outside of restriction-modification per se.

Recognition Sequence	**Laboratory Strains**		**Human Isolate**	**Sheep Isolate**	***C. coli***	***C. peloridis***	***C. subantarcticus***		**UPTC NCTC**	***Campylobacter spp.***
	81-176 [149]	11168 [149]	F38011 [150]	IA3902 [153]	BfR-CA-9557 [151]	[152]	LMB 24374 [152]	LMG 24377 [152]	11845 [152]	RM16704 [152]
RA<u>A</u>TTY	+	+	+	+	+	+	+	+	+	+
G<u>A</u>TC	-	-	-	-	+	+	+	+	+	-
TA<u>A</u>YN$_5$TGC	+	+	-	+	+/-	-	-	-	-	-
CAAGA<u>A</u>	-	-	-	-	+	+	-	-	-	-
GCA<u>A</u>GG	+	+/-	+/-	+	-	-	-	-	-	-
G<u>A</u>GN$_5$GT	-	+	-	+/-	-	-	-	-	-	-
CA<u>A</u>YN$_6$ACT	+	-	-	-	-	-	-	-	-	-
GGRC<u>A</u>	+	-	-	-	-	-	-	-	-	-
G<u>A</u>GN$_5$RTG	-	-	+	-	-	-	-	-	-	-

AN$_4$CGNAAATTY	-	-	+	-	-	-	-	-	-	-
CTAN$_8$TAYC	-	-	+	-	-	-	-	-	-	-
GAAGAA	-	-	-	+	-	-	-	-	-	-
RCATC	-	-	-	-	+	-	-	-	-	-
GGGTDA	-	-	-	-	+	-	-	-	-	-
DACATTGB	-	-	-	-	+	-	-	-	-	-
TAAAN$_5$TTTA*	-	-	-	-	+	-	-	-	-	-
CACN$_5$TTTA*	-	-	-	-	+	-	-	-	-	-
CAAYN$_7$TTYG #	-	-	-	-	+	-	-	-	-	-
CRAAN$_7$RTTG #	-	-	-	-	+	-	-	-	-	-
GGRAT	-	-	-	-	-	+	-	-	-	-
TAAYN$_6$RTTG	-	-	-	-	-	+	-	-	-	-
ACCTA $	-	-	-	-	-	+	-	-	-	-
ATCN$_7$TCC	-	-	-	-	-	-	+	-	-	-
GAYN$_7$TYTC	-	-	-	-	-	-	+	-	-	-
GNCGAA	-	-	-	-	-	-	+	-	-	-
AACCA	-	-	-	-	-	-	+	-	-	-
GGAAY $	-	-	-	-	-	-	+	-	-	-
GAGG	-	-	-	-	-	-	-	+	-	-
GTRGAG	-	-	-	-	-	-	-	+	-	-
CCAAT	-	-	-	-	-	-	-	+	-	-
GWCAT	-	-	-	-	-	-	-	+	-	-
GCGAA	-	-	-	-	-	-	-	-	+	-
CAYN$_6$RTG	-	-	-	-	-	-	-	-	+	-
GANTC	-	-	-	-	-	-	-	-	-	+
GCATC	-	-	-	-	-	-	-	-	-	+
CGAN$_8$ACA	-	-	-	-	-	-	-	-	-	+

Table 3.1: **Identified methylation motifs in *Campylobacter* strains.** Methylation motifs were determined using SMRT sequencing. Ambiguous nucleotides are denoted using IUPAC ambiguity codes. +/- indicates the motif contains one nucleotide difference between the two strains. Underlining indicates the methylated base. Stars (*) and pound signs (#) indicate double stranded motifs where the methylation occurs in different spots on each strand. Dollar signs ($) indicate approximately 50% of DNA was methylated.

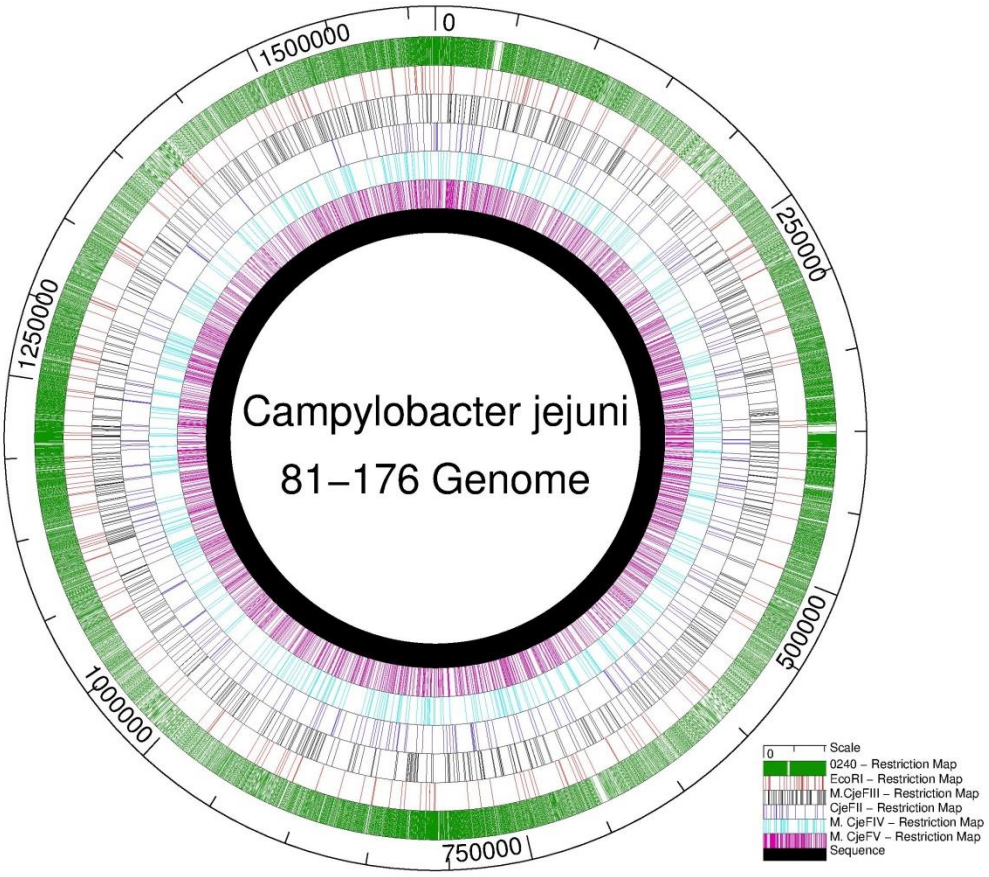

Figure 3.1 Distribution of all possible methylation motifs in 81-176. CtsM (RAATTY) sites are indicated in green. For comparison, EcoRI (GAATTC) sites are in red. M. CjeFII (CAAYN$_6$ACT) sites are in dark blue, M. CjeFIII (GCAAGG) sites are in black, M. CjeFIV (TAAYN$_5$TGC) are in light blue, and M. CjeFV (GGRCA) sites are in fuschia.

To test the role of *ctsM* in transformation, we constructed an insertion-deletion allele and assayed for loss of methylation by testing the sensitivity of DNA from the mutant strain to restriction by EcoRI or ApoI. The recognition site of each enzyme overlaps the RA^{m6}ATTY site predicted to be the substrate of CtsM, and neither can cleave DNA when that site is methylated. DNA from the mutant strain could be digested by both EcoRI and ApoI, whereas DNA from the wild type strain could not. (Fig 3.2).

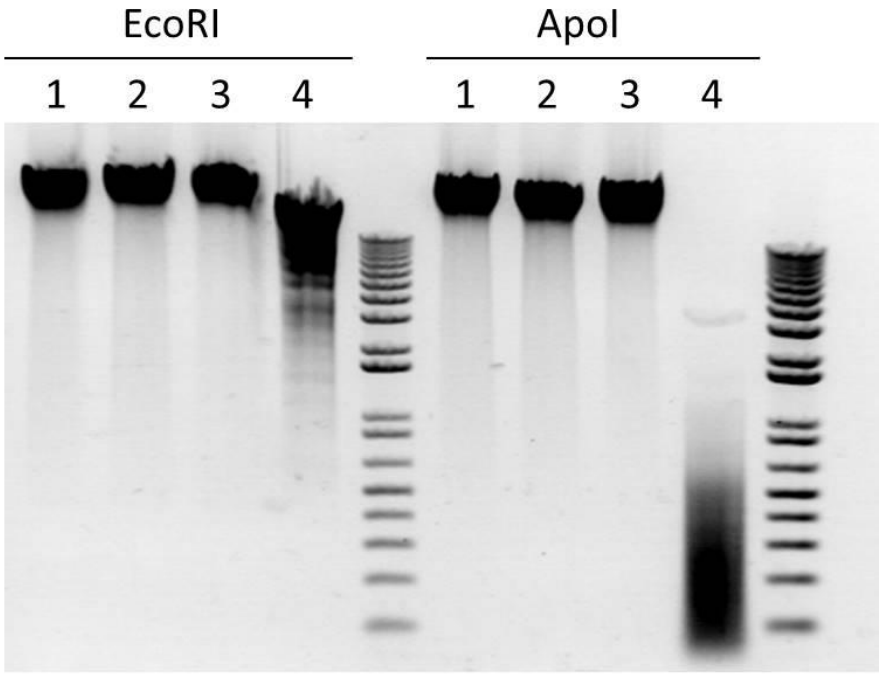

Figure 3.2 DNA from *ctsM*::kan is degraded by ApoI and EcoRI. gDNA was purified from either DRH153 cells or *ctsM*::kan mutants. The DNA was then either mock digested (lanes 1 and 2) or digested (lanes 3 and 4) with the restriction enzymes EcoRI (left) or ApoI (right). The DNA was then electrophoresed on an agarose gel and visualized using ethidium bromide.

3.3.2 Loss of RA^{m6}ATTY methylation negatively affects transformation

Having confirmed that DNA methylation was disrupted in *ctsM*::kan, we tested whether the methylation at RAATTY is required for transformation. gDNA was purified from a strain carrying a marker for kanamycin resistance in the *astA* gene or from the *ctsM*::kan mutant strain. Both were used in separate transformation assays to transform our wild type strain DRH212. The mutant gDNA lacking methylation at the RAATTY site transformed to levels approximately 10,000 times lower than gDNA cells wild type for *ctsM*, indicating that the RA^{m6}ATTY methylation mark is critical for the ability of gDNA to serve as a substrate in transformation (Fig 3.3).

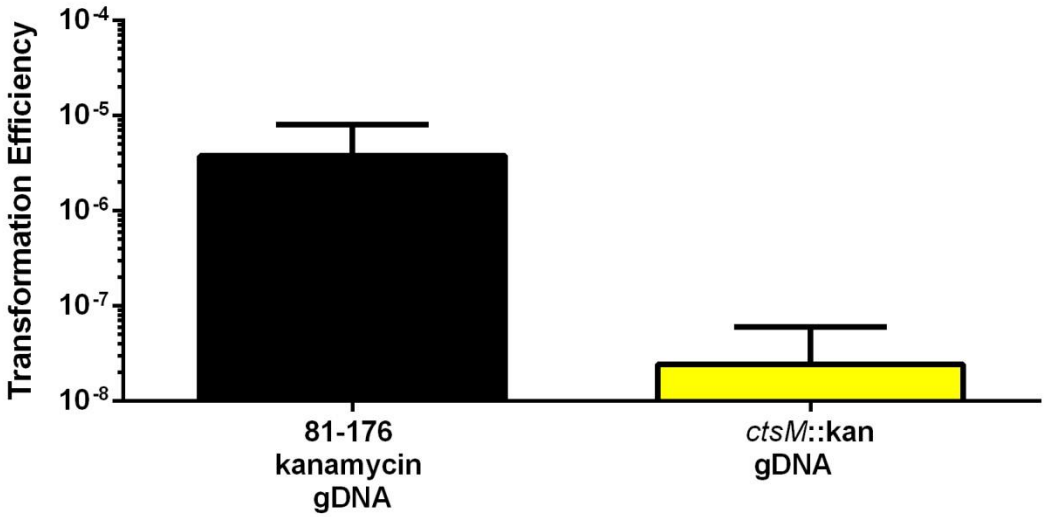

Figure 3.3 DNA lacking CtsM methylation is a poor substrate for transformation. DNA was purified from either DRH153 or the *ctsM*::kan mutant. This DNA was then used to transform DRH212 cells. Transformation efficiency was calculated as described in the Materials and Methods section. Results are from three biological replicates and each biological replicate included three technical replicates. Errors bars indicate standard deviation.

We then tested the ability of the *ctsM* mutant strain to be transformed, using gDNA from cells containing different antibiotic resistance marker cassettes or alleles (kanamycin and chloramphenicol cassettes at the *astA* locus, or a *gyrA256* allele encoding resistance to naladixic acid). Purified gDNA from these different antibiotic resistance mutants was used in transformation assays with wild type and *ctsM* mutant *C. jejuni*. No difference in transformation frequency was detected using these gDNAs as substrates (Fig 3.4A), indicating that the MTase does not affect the ability of the cell to be transformed, just the ability of DNA to act as a substrate in transformation. The *ctsM* mutants were unable to be transformed with their own gDNA, i.e. DNA lacking the methylation at RAATTY, similar to wild type cells (Fig 3.4B), indicating that as in wild type cells, those lacking CtsM require the incoming DNA to be methylated at the RAATTY site for natural transformation

Collectively our data suggest that the *ctsM* is required for methylation at the RAATTY site, and, while not required for cells to be competent for transformation, is required for DNA to serve as a substrate for transformation.

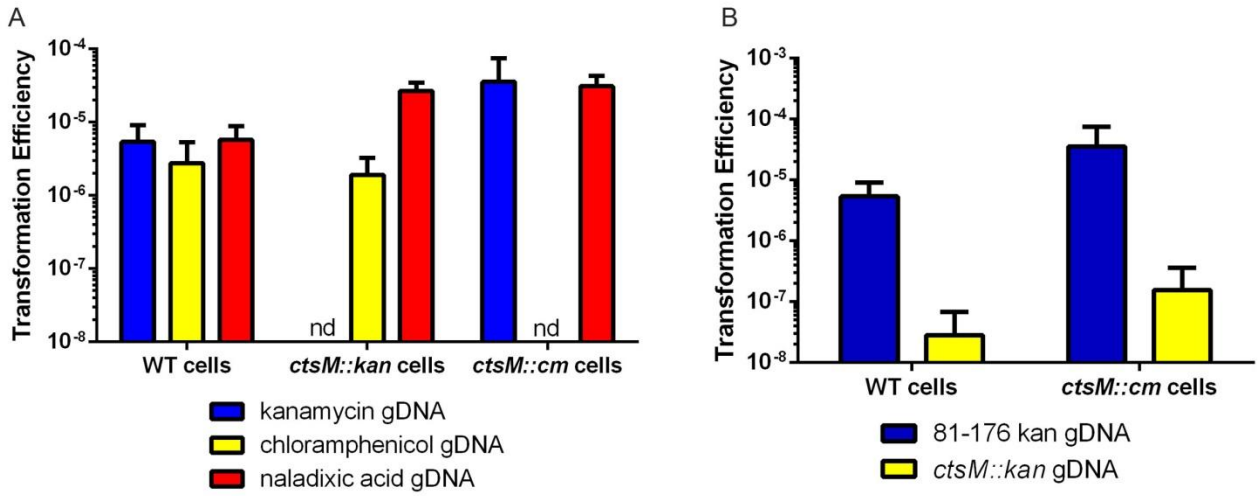

Figure 3.4 *ctsM* mutants are transformed with WT frequency by WT gDNA but not their own gDNA A) WT DNA with three distinct antibiotic resistance markers (kanamycin, chloramphenicol, and naladixic acid) was purified and used to transform DRH212, *ctsM*::kan, or *ctsM*::cm cells. Transformation efficiency was calculated as previously described. n.d. indicates that transformation efficiency was not determined because the cells already contained the antibiotic resistance being used to transform cells. B) DRH154 cells or *ctsM*::cm cells were transformed with either DRH153 gDNA or DNA from the *ctsM*::kan gDNA and transformation efficiency was measured as previously described. Data represents three biological replicates. Each biological replicate contained three technical replicates. Errors bars indicate standard deviation.

3.3.3 Methylation at RAATTY is required for transformation

The *ctsM* mutant strain produces gDNA that i) that serves as a poor substrate for natural transformation and ii) is more readily digested by enzymes (EcoRI and ApoI) that are inhibited by methylation at the site recognized by this methyltransferase. To test directly whether the methylation mark provided by CtsM is required for transformation, we took advantage of the fact that EcoRI MTase (M. EcoRI) recognizes the same a subset of RAATTY sites, GAATTC. Commercially obtained M. EcoRI was used to methylate gDNA from the *ctsM*::kan strain. As controls, we used two other commercially available MTases, M. TaqI and M. HpaII, which methylate the sites TCG^{m6}A and C^{m6}CGG respectively.

After treatment with the MTases, purified gDNA from *ctsM*::kan was protected from digestion by each cognate restriction enzyme, confirming the gDNA had been successfully methylated. We then measured the transformation efficiency of *C. jejuni* when provided these methylated gDNAs as substrates, in comparison to transformation with wild type gDNA (Fig 3.5). The gDNA that was methylated by M. EcoRI transformed as well as gDNA from wild type *C. jejuni*,

with a frequency approximately 1000 times higher than gDNA from the *ctsM* mutant that had not been methylated by M.EcoRI, or that was methylated by M. TaqI or M. HpaII.

These results indicate that methylation of the RAATTY site is required for transformation. Further, while the M. EcoRI site overlaps with and methylates the same site as CtsM, the site is more specific (GAATTC versus RAATTY) than the site recognized by the *Campylobacter* enzyme. Given that fewer overall sites will be methylated by M. EcoRI than by CtsM, our data also suggest that not every RA^{m6}ATTY site needs to be methylated for transformation to occur.

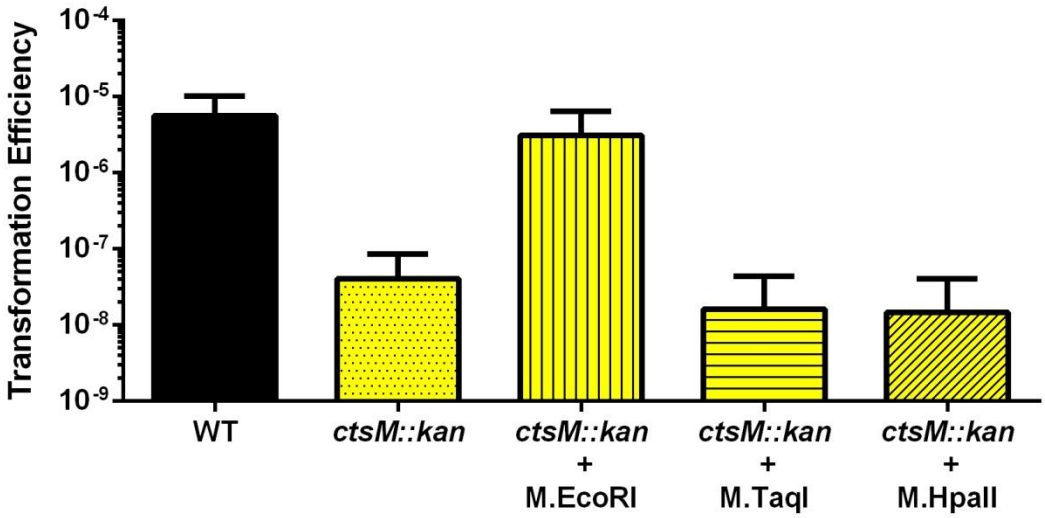

Figure 3.5 *In vitro methylation* at the CtsM site RAATTY but not at other sites significantly enhances gDNA substrate capability. DNA was purified from either DRH153 or *ctsM*::kan cells. *ctsM*::kan DNA was then either mock methylated or *in vitro* methylated with EcoRI MTase, TaqI MTase, or HpaII MTase. EcoRI MTase methylated GA^{m6}ATTC; TaqI methylates TCG ^{m6}A; and HpaII methylates C ^{m6}CGG. DRH212 cells were transformed with the methylated DNA. Transformation efficiency was calculated as previously described. Data represents three biological replicates. Each biological replicate contained three technical replicates. Errors bars indicate standard deviation.

3.3.4 *In vitro* methylation by M. EcoRI is sufficient to allow *E. coli* derived plasmids and PCR generated products to transform *Campylobacter*

Relative to gDNA purified from wild type *C. jejuni*, DNA from lab strains of *E. coli* or derived *in vitro* by polymerase chain reactions (PCR) is a poor substrate for *Campylobacter* transformation. The mechanism for this selection has been unclear. As we had shown that methylation of RAATTY was necessary for transformation, we next wanted to determine whether this methylation is also sufficient for transformation.

We constructed antibiotic cassettes flanked with the *C. jejuni astA* sequence in order to allow for homologous recombination; these were cloned into pUC19, which is unable to replicate in *C. jejuni*. The resulting plasmids have three EcoRI sites, two in *astA* and one in the pUC19 vector (Fig 3.6A). We purified these plasmids from *E. coli*, methylated them with M. EcoRI, confirmed their methylation using restriction digest, and assessed their ability to transform DRH212 by selecting for the antibiotic cassette (Fig 3.6B). Plasmids treated with M.EcoRI transformed *C. jejuni* at frequencies similar to gDNA, and at frequencies 1000 times greater than unmethylated DNA of the same origin.

We also tested the ability of methylated PCR products to transform *C. jejuni*. We designed primers to amplify two different PCR products from the plasmids described above. The first contains the homologous DNA and the kanamycin cassette (astAkan), and has two closely spaced EcoRI sites in the *astA* sequence (Fig 3.6A). The second is larger and has a third EcoRI site derived from the multiple cloning site of the pUC19 backbone (Fig 3.6A). These

fragments were amplified and methylated with M. EcoRI. After confirming methylation using restriction digests we tested if they could serve as efficient DNA transformation substrates for DRH212 (Fig 3.6C). Both products transformed DRH212 at frequencies much higher than their non-methylated cognate fragments. The larger product, containing three EcoRI sites, transformed about 10 times better than the smaller product with only two sites. This is most likely due to the increased amount of homologous DNA contained in the larger fragment, which has been demonstrated to greatly increase transformation frequency in *V. cholerae* (Ankur Dalia, personal communication).

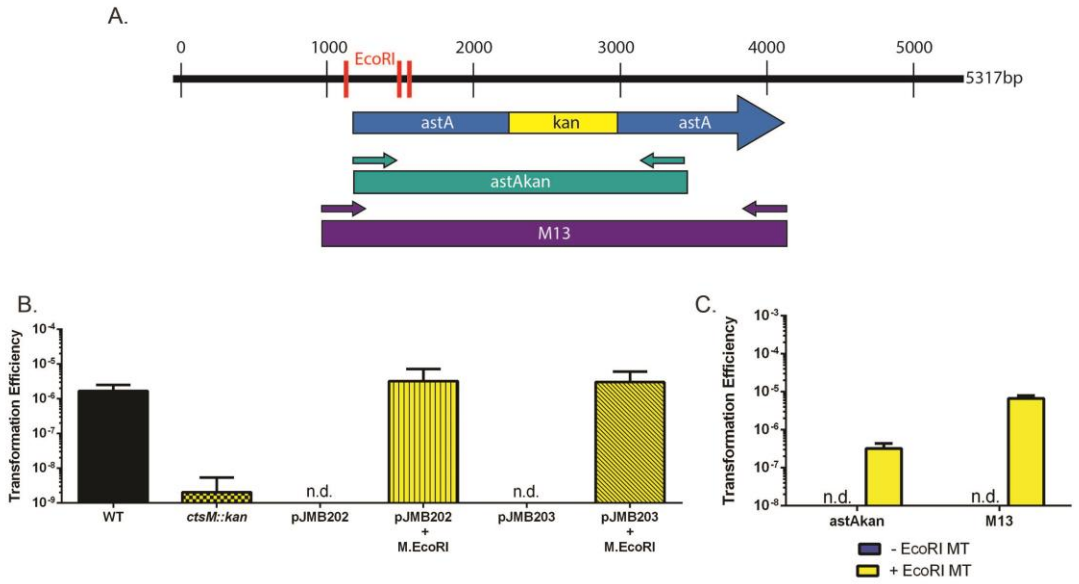

Figure 3.6 *In vitro* methylation by M.EcoRI allows for transformation of plasmid and PCR products. A) Plasmid map of pJMB202 with pertinent features denoted. Kan^R refers to a kanamycin resistance cassette. It is inserted in *astA.* Small arrows refer to primer pairs. These were used to amplify the PCR products astAkan and M13 indicated in green and purple rectangles respectively, used in Part C. pJMB203 is identical except contains a chloramphenicol resistance gene insertion instead of a kanamycin resistance gene insertion. B) DRH212 cells were transformed with various types of DNA as indicated. C) PCR products were created by amplifying a fragment from as indicated. These products were then methylated *in vitro* using EcoRI MTase. These DNA fragments were then used to transform DRH212 cells. Transformation efficiency was calculated as previously described. Data represents three biological replicates. Each biological replicate contained three technical replicates. Errors bars indicate standard deviation.

3.3.5 One methylated RAATTY site is sufficient for transformation

In order to determine the exact requirements for transformation, we performed site directed mutagenesis on pJMB203 to generate plasmids pJMB204-209, either adding (JMB204) or eliminating (JMB205-209) EcoRI sites (Fig 3.7A). This allowed us to determine the number of methylated RAATTY sites required for transformation as well as the location of the sites relative to the recombining DNA. We determined that one site is sufficient to allow for transformation and that the site does not need to be located on the DNA that will ultimately recombine (Fig 3.7B). The site can be at least one kilobase away without significantly impacting transformation efficiency.

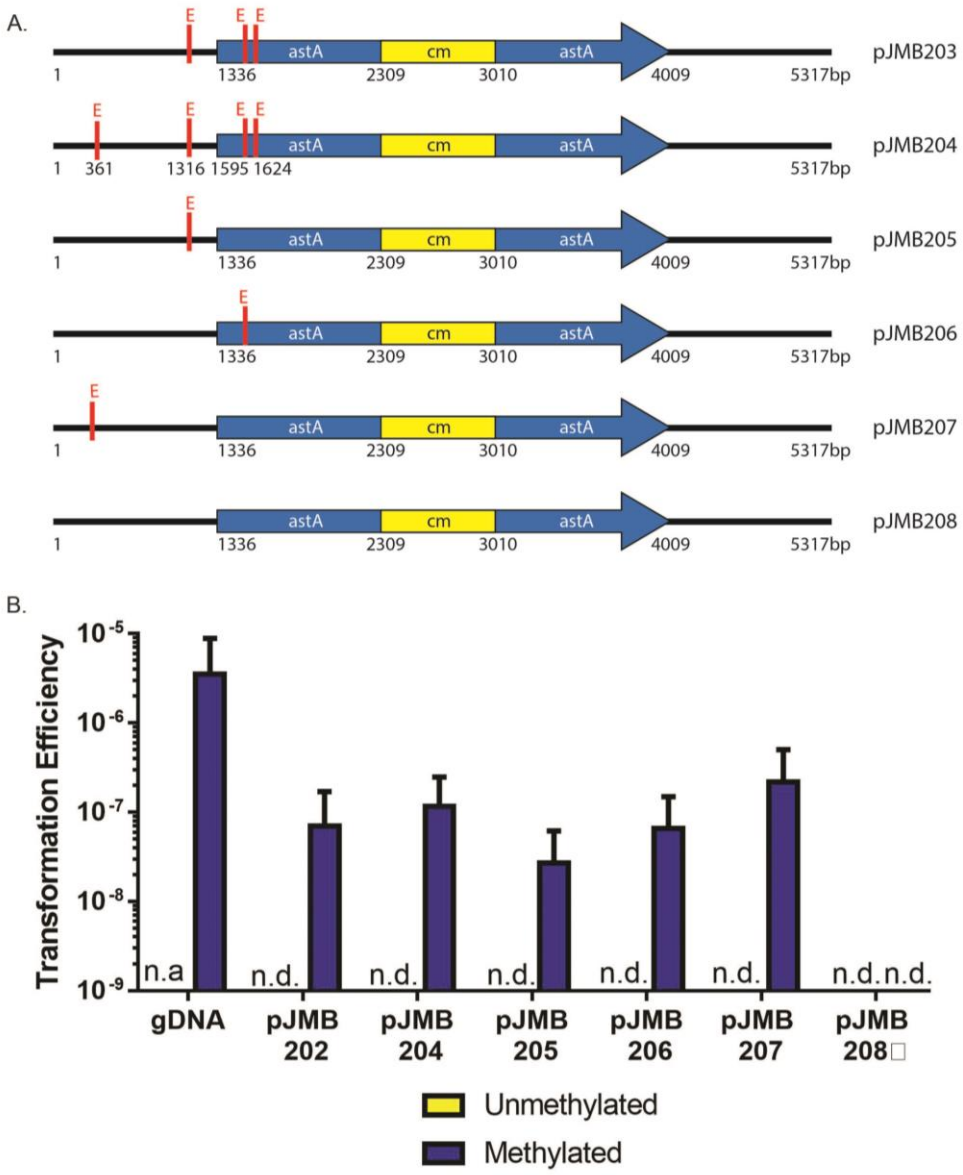

Figure 3.7 One methylated EcoRI site is sufficient for transformation. A. Diagram of plasmids used. EcoRI sites (E) are in red. The exact sites of the EcoRI sites are indicated for pJMB204. The plasmids are identical except for the location and number of EcoRI sites. B. DRH212 cells were transformed with different plasmids as indicated and transformation efficiency was measured as noted previously. Errors bars indicate standard deviation.

3.3.6 DNA lacking RAATTY methylation is internalized

Having identified that methylation at RAATTY is necessary and sufficient for transformation, we wanted to determine when during transformation

methylated DNA is selected. To determine this, we performed a DNA binding and uptake assay. This assay differentiates DNA that has been internalized by the cell (become DNase resistant) from cell associated DNA (DNA that has either been bound on the surface of the cell or has been internalized).

We sought to determine if the mechanism by which methylated versus unmethylated is recognized for transformation includes binding of the methylated DNA to the surface of the cell prior to being taken up. We carried out DNA binding and uptake assays as describe in Materials and Methods. We observed equivalent amounts of DNA from wild type and DNA from *ctsM*::kan becoming DNase resistant indicating that lack of methylation does not preclude DNA from becoming internalized (Fig 3.8).

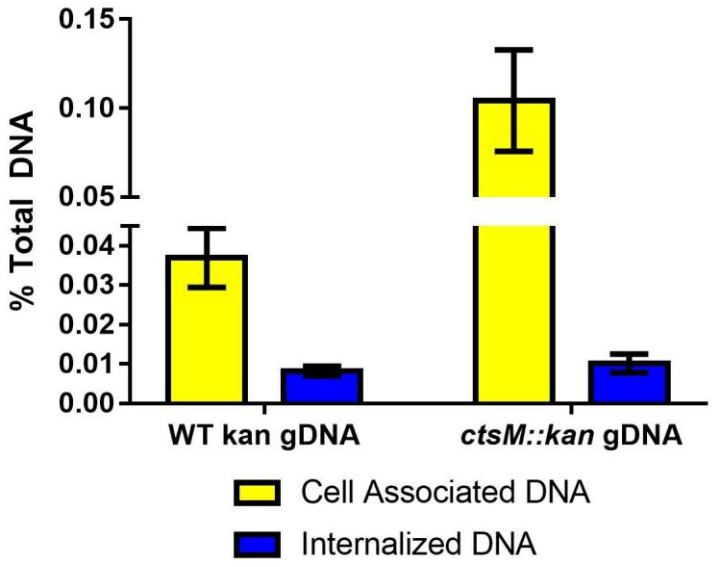

Figure 3.8 DNA lacking RAATTY methylation becomes internalized. DRH212 cells were incubated with either DRH53 (kan^R) or *ctsM::kan* DNA for one hour. They were centrifuged, washed, split and either mock treated or treated with DNase. Mock treated cells contain the cell associated DNA (yellow) and DNase treated cells have internalized DNA (blue). After DNase incubation, cells were washed and DNA was purified. The DNA was quantified via qPCR using primers to the kanamycin cassette. Total DNA was also quantified and the amount of

DNA was normalized to total DNA. Data represent four biological replicates. Each biological replicate contained three technical replicates. Errors bars indicate standard error.

3.4 Discussion

The goal of this study was to understand how *C. jejuni* selects DNA during natural transformation. We identified a methylation motif, RA^{m6}ATTY, which plays a critical role in the mechanism by which selection takes place. When this site is methylated, the DNA will transform cells at a much higher frequency than when the DNA is not methylated. The 26,609 methylated RAATTY sites are distributed fairly equally around the genome with the exception of three places where there are significantly fewer sites (Fig 3.1). These sites map to the ribosomal gene cluster. 81-176 has three copies of this gene cluster and each 5 kb cluster contains only five RAATTY sites compared to the average of 40 sites in the 5 kb flanking each cluster. This dearth of methylation might affect the ability of the sites to be exchanged during natural transformation or might affect regulation of these genes.

Not all of the sites need to be methylated in order for the DNA to be a substrate for transformation. This was demonstrated using *in vitro* methylation using the EcoRI MTase which methylates the more specific, and therefore less frequent, site GAATTC. Furthermore, DNA that was previously untransformable such as *E. coli* derived DNA or PCR generated DNA can be *in vitro* methylated using this MTase and then successfully transform *C. jejuni*. The PCR products contain no other methylation, so this single methylation motif is sufficient for

transformation. Using identically sized plasmids containing different numbers of EcoRI sites, we showed that that one methylated EcoRI site, anywhere on the plasmid was sufficient for transformation. This argues strongly against a restriction based system because both the plasmids and the PCR products contained unmethylated RAATTY sites that would be targeted by a restriction enzyme.

This is the first identification of methylation-dependent DNA selection process during natural transformation. This selection process is distinct from bacteria such as *H. pylori, N. meningitidis, and P. stutzeri* which use RM systems to degrade DNA that lacks the correct methylation motif [112-116]. CtsM is an orphan MTase lacking a cognate restriction enzyme. Furthermore, insertional inactivate of this gene did not which would not be possible if there were an unidentified restriction enzyme. This enzyme would degrade the chromosomal DNA that was lacking the correct methylation, resulting in cell death. Also, we were able to use plasmid DNA that contained seven unmethylated RAATTY sites to transform the cells. If a restriction enzyme existed, it would degrade the plasmid at these sites, eliminating transformation.

While we were unable to determine the precise mechanism for *C. jejuni* recognition of the DNA that it uses in transformation, we were able to provide evidence that selection occurs once the DNA is inside the cell as DNA lacking RAATTY methylation and fully methylated gDNA became equally DNase resistant. These data lead to three hypotheses the mechanism of DNA selection. 1) Selection occurs in the periplasm before the DNA is transported into the

cytosol. 2) Transportation into the cytosol is dependent on methylation. 3) Recombination is dependent on this methylation motif.

In addition to identifying the DNA selection motif and opening up questions about the mechanism through which *C. jejuni* recognizes this methyl mark for transformation, we have provided a potentially powerful tool for genome editing in *C. jejuni.* To introduce DNA into *C. jejuni*, it is only necessary to methylate it *in vitro* with M. EcoRI, and then successfully transform *C. jejuni*. This may enable transformation of *E. coli* plasmids or PCR products to make mutations on the chromosome However we were able to transform WT *C. jejuni* with both plasmid and PCR derived DNA after methylation.

Cells lacking CtsM transform as well, if not better, than WT cells. This DNA MTase and its cognate methylation motif are conserved among the PacBIo sequenced *Campylobacter* strains. These strains are diverse and range from laboratory *C. jejuni* isolates to the more distantly related *C. subantarcticus* and *C. peloridis* isolates. Of more than thirty-five identified methylation motifs, this was the only methylation motif that is conserved among all SMRT sequenced strains thus far. If this is the sole selection requirement for DNA in *Campylobacter* competence, we would predict that this methylation motif will be found in all naturally competent strains of *Campylobacter* in order to allow the strain to be transformed with its own DNA. As we were able to insertionally inactivate the DNA MTase, it cannot be required for growth *in vitro,* though its strict conservation throughout the genera indicates it might play other roles.

Orphan MTases have been a growing field of study and are now recognized to play diverse roles in gene regulation. For example Dam MTases found in Gammaproteobacteria regulate the timing DNA replication and CcrM MTases found in Alphaproteobacteria regulate cell cycle progression [163-166, 170-172]. Because orphan MTases are more conserved at the genus, family, and class level than MTases in RM systems, it is hypothesized that they have a functional role distinct from host genome protection [147]. Among over 230 diverse bacteria, the largest novel class of orphan MTases was those that methylated RAATTY [147]. These orphan MTases are found in *Campylobacter*, *Akkermanisia muciniphilia*, three Spirochaeta species, and three Treponema species [147]. In *Spirochaeta smaragdinae*, RAATTY sites preceded transcriptional regulators and were not methylated leading to the suggestion that that these orphan MTases play a role in gene regulation [147]. Hypomethylation of RAATTY was also identified in *C. jejuni* in front of rRNAs as well as the flagellar genes FlgK and a flagellar basal-body rod protein [153].

In conclusion, we have identified a novel methylation-dependent DNA selection mechanism in *C. jejuni*. RAATTY methylation is required to allow for transformation and when this site is methylated, previously non-transformable substrates become capable of transforming *C. jejuni*.

3.5 Materials and Methods

3.5.1 Bacterial strains and media

Bacterial strains used in this work are listed in Table 3.2. *C. jejuni* was routinely cultured on Muller Hinton (MH) agar with 10% sheep's blood in

microaerophilic conditions (5% CO^2, 10% O^2, balanced with N^2) in a tri-gas incubator at 42°C. When necessary, media was supplemented with antibiotics in the following concentrations: trimethoprim (10 µg ml^{-1}), kanamycin (150 µg ml^{-1}), naladixic acid (30 ug ml^{-1}) and chloramphenicol (15 µg ml^{-1}). All *C. jejuni* strains were stored at -80°C in MH broth with 20% glycerol.

Escherichia coli strains were routinely cultured at 37°C in Luria-Bertani (LB) broth or agar. When necessary, antibiotics were used at the following concentrations: ampicillin (100 µg ml^{-1}), chloramphenicol (30 µg ml^{-1}), kanamycin (50 µg ml^{-1}), and tetracycline (12.5 µg ml^{-1}). All *E. coli* strains were stored at -80°C in LB broth with 20% glycerol.

Table 3.2: Strains and Plasmids used in this study

Strain or Plasmid	Relevant Characteristics	Reference
Bacteria		
E. coli		
DH5α	*supE44* Δ*lacU*169 (Φ80*lacZ*ΔM15) *hsDr*17 *rec*A1 *end*A1*gyrA*96 *thi*-1 *rel*A1	Lab strain
GM2163	*dam-, dcm-, camR*	New England Biolabs
DH5α pRK212.1	contains conjugative plasmid for transferring DNA into *Campylobacter*	[236]
C. jejuni		
81-176	clinical isolate	[237]
11168	Clinical isolate	
DRH153	81-176 *astA*::*aphA3*; Kan^r	[238]
DRH154	81-176 *astA::cat,* Cm^R	[238]
DRH212	81-176 *rpsL*^{Sm}	[238]
JMB200	DRH212 *ctsM*::kan, Kan^R	This study
JMB202	DRH212 NA^R	This study
JMB204	DRH212 *ctsM*::cm, Cm^R	This study
Plasmids		
pUC19	Amp^R	New England Biolabs
pEC0102	pRY112 derivative with *cat* promoter	[5]
pJMB200	pUC19 *ctsM*::kan, Kan^R	This study
pJMB201	pUC19 *ctsM*::cm, Cm^R	This study
pJMB202	pUC19astAkan, Kan^R	This study
pJMB203	pUC19astAcm, Cm^R	This study
pJMB204	pJMB203 + new EcoRI site at 4053, Cm^R	This study
pJMB205	pJMB203 only 1 EcoRI site in MCS, Cm^R	This study
pJMB206	pJMB203 only 1 EcoRI, in *astA,* Cm^R	This study
pJMB207	pJMB203 only 1 EcoRI site at 4053 (away from insert), Cm^R	This study
pJMB208	pJMB203 no EcoRI sites, Cm^R	This study

3.5.3 Construction of *ctsM*::kan and *ctsM*::cm mutants

To create insertional mutants we used Gibson assembly. We designed primers according to the NEB Assembly Tool. (All primers used in this study are indicated in Table 3.3). We amplified 500bp upstream and downstream of *ctsM* as well as either kanamycin cassette or a chloramphenicol cassette from

DRH153 or DRH154 respectively. These fragments were purified using the Qiagen PCR purification kit and quantified using the Qubit 2.0. We also digested pUC19 with BamHI and SalI. The digested plasmid was purified using the Qiagen PCR purification kit and quantified using the Qubit 2.0. The PCR fragments and the digested plasmid were then mixed in equimolar ratios according the NEB Gibson Assembly Master Mix instructions and incubated at 50°C for 60 minutes. They were then transformed into NEB 5-alpha Competent E. coli (High Efficiency) cells according to the manufacturer's instructions. Cells were then plated on LB kanamycin or LB chloramphenicol. All subsequent clones were verified by sequence determination. Plasmids were transformed into *C. jejuni* using electroporation and plated on MH agar with appropriate antibiotics. gDNA was purified and the creation of the mutant was confirmed using PCR.

Table 3.3: Primers used in this study

Number	Name	Sequence (5'-3')	Description
145	*ctsM* kan/CM GA 1	ATT CGA CGT CGG TAC CCG GGT GTG TTG GCG ATG ATG GC	Used to make *ctsM*::kan and *ctsM*::cm insertional mutants
146	*ctsM* Kan GA 2	TCA TTT TAG CCA TAA TTT AAA ACC TTT AAA AGC CTT ATC CAC	Used to make *ctsM*::kan insertional mutant
147	*ctsM* Kan GA 3	GGT TTT AAA TTA TGG CTA AAA TCA CAA TAT CAC	Used to make *ctsM*::kan insertional mutant
148	*ctsM* Kan GA 4	AGC ACA AAA TCT AAA ACA ATT CAT CCA GTA AAA TAT AAT ATT TTA TTT TTC	Used to make *ctsM*::kan insertional mutant
149	*ctsM* Kan GA 5	ATT GTT TTA GAT TTT GTG CTA AAA TTA TAT TTA TAA AAA GG	Used to make *ctsM*::kan insertional mutant
150	*ctsM* Kan/CM GA 6	CAA GCT TGC ATG CCT GCA GGC TTT TAT TGA GAC TAA TCT TAC TCA TTT TTA AAA C	Used to make *ctsM*::kan and *ctsM*::cm insertional mutants
151	*ctsM* CM GA 2	TTA ACT TGG AAA TTT AAA ACC TTT AAA AGC CTT ATC CAC	Used to make *ctsM*::cm insertional mutant
152	*ctsM* CM GA 3	GGT TTT AAA TTT CCA AGT TAA TTG CGT GAT ATA G	Used to make *ctsM*::cm insertional mutant
153	*ctsM* CM GA 4	AGC ACA AAA TTG CGC CCT TTA CTT CCT AAA G	Used to make *ctsM*::cm insertional mutant
154	*ctsM* CM GA 5	AAA GGG CGC AAT TTT GTG CTA AAA TTA TAT TTA TAA AAA GG	Used to make *ctsM*::cm insertional mutant
183	pUC19astA F	GGC ACT TTT GGC GGG TTC TAC C	Used to create astAkan PCR fragment
184	pUC19astA R	GGA TCA TAA TCA ACG CTA TTT ACA TGA GCC C	Used to create astAkan PCR fragment
185	M13 F	TGT AAA ACG ACG GCC AGT	Used to create M13 PCR product
186	M13 R	CAG GAA ACA GCT ATG ACC	Used to create M13 PCR product

187	astA1 F GA	AGT GAA TTC GAG CTC GGT ACC CGG GAT GAG ACT TAG CAA AAC TCT TTG	Used to make pJMB202 and pJMB203
188	astA1 cm R GA	CAA TTA ACT TGG AAA CAA CAT TAC CAT TCT CAT C	Used to make pJMB203
189	CM F GA	TGG TAA TGT TTC CAA GTT AAT TGC GTG ATA TAG	Used to make pJMB203
190	CM R GA	ATC TCC AAT CAT CAA TGC GCC CTT TAG TTC C	Used to make pJMB203
191	astA 2 CM F GA	TAA AGG GCG CAT TGA TGA TTG GAG ATT GTA TGA G	Used to make pJMB203
192	astA 2 R GA	TAC GCC AAG CTT GCA TGC CTG CAG GTT ATT TTT TAG GAT TGA ATG CTT GAT C	Used to make pJMB202 and pJMB203
193	astA 1 Kan R GA	TCA TTT TAG CCA TTC ATT TTA GCC ATA ACA ACA TTA C	Used to make pJMB202
194	Kan F GA	ATG GCT AAA ATG AAT GGC TAA AAT GAG AAT ATC AC	Used to make pJMB202
195	Kan R GA	ATC TCC AAT CAT CCT AAA ACA ATT CAT CCA GTA AAA TAT AAT ATT TTA TTT TC	Used to make pJMB202
196	astA2 Kan F GA	TGA ATT GTT TTA GGA TGA TTG GAG ATT GTA TGA G	Used to make pJMB202
197	Kan qPCR F	GAA AGC TGC CTG TTC CAA AG	Used for binding and uptake assay to amplify off kan fragment
198	Kan qPCR R	GAA AGA GCC TGA TGC ACT CC	Used for binding and uptake assay to amplify off kan fragment
79	CtsX RT F	GCATTTGGAGCAAAAGATTC	Used for binding and uptake assay to amplify off chromosome
80	CtsX RT R	GTGTATTTTCCTGCACCG	Used for binding and uptake assay to amplify off chromosome
199	a1317cF	ACG ACG GCC AGT GAC TTC GAG CTC GGT AC	Used for SDM to eliminate EcoRI site in pUC19 MCS
201	g1594	CAA ATG GTC AAA CTA TTT CTT ATA ATG TCA ATT CTA AAA TGG CTA AGA CTT ATG	For SDM. Remove 1st EcoRI site in *astA*
203	a1624c F	AAA ATG GCT AAG ACT TAT GGT GGC ATT CCT ATT TTT GGA CTT TAT CC	For SDM. Remove 2nd EcoRI site in *astA*
205	c4053g F	GTG AAA TTG TTA TCC GCT CAG AAT TCC ACA CAA CAT ACG AG	For SDM. Add EcoRI site in plasmid backbone.
207	*ctsM* GA comp 1	AGC TCG GTA CCC GGG GAT CCA AAG AAA ATC CTT CTT TTT TAA AAG AG	Used to make ctsM complementaiton plasmid
208	*ctsM* GA comp 2	TTG CAT GCC TGC AGG CTC GAG TTT TTA ATT TTT AAC TAA AAT ATA AAG	Used to make ctsM complementaiton plasmid

3.5.3 Construction of pJMB202 and pJMB203

To create these plasmids, we used Gibson assembly. We designed primers according to the NEB Assembly Tool. (All primers used in this study are indicated in Table 3.3). We amplified the first 975 nucleotides of *astA* from 81-176, either the kanamycin or chloramphenicol cassette from DRH153 and DRH154 respectively, followed by the remainder of *astA.* These fragments were purified using the Qiagen PCR purification kit and quantified using the Qubit 2.0. We also digested pUC19 with BamHI and SalI. The digested plasmid was

purified using the Qiagen PCR purification kit and quantified using the Qubit 2.0. The PCR fragments and the digested plasmid were then mixed in equimolar ratios according the NEB Gibson Assembly Master Mix instructions and incubated at 50°C for 60 minutes. They were then transformed into NEB 5-alpha Competent E. coli (High Efficiency) cells according to the manufacturer's instructions. Cells were then plated on LB kanamycin or LB chloramphenicol. All subsequent clones were verified by sequence determination.

3.5.4 Construction of pJMB204-209

To create these plasmids, we used site directed mutagenesis of pJMB203. This plasmid has three EcoRI sites. We used the QuikChange Lightning Site-Directed Mutagenesis Kit from Agilent and followed the manufacturer's instructions to design primers to either add a new EcoRI site on the plasmid backbone, greater than 1 kb from the insert, or to eliminate EcoRI sites so there was only one site left on the plasmid. We made single nucleotide changes in all cases and the resulting sites are not capable of being recognized by either EcoRI or CtsM.

3.5.5 gDNA purification

Campylobacter cells were grown on MH agar plates with appropriate antibiotics as previously described. For transformation assays, a small amount of cells (1-2 passes with an inoculating loop) was scraped off the plate and resuspended in 300µL filter sterilized genomic lysis buffer (50 mM Tris Base pH 7.5, 50 mM EDTA, 1% SDS, 10 mM NaCl). The cells were heated at 95°C for 10 minutes. 150 µL of Epicenter MPC Protein Precipitation Reagent was added and

cells were vortexed for twenty seconds. They were then incubated on ice for five minutes and spun at 14,000 rpm for 15 minutes. After centrifugation, the supernatant was transferred to a clean 1.5 ml tube and 750 µL of isopropanol was added. This was mixed by inversion and centrifuged again at 14,000 rpm for five minutes. The pellet was then washed twice with 70% ethanol. After washing, all ethanol was removed and the pellet was dried in a SpeedVac for 10 minutes and then resuspended in 100 µL of dH_2O. The concentration was determined using the Qubit 2.0.

3.5.6 Transformation assays

Transformation assays were performed as described previously with minor changes [5, 193]. Briefly, *C. jejuni* was grown 16 – 18 hours on MH agar plates supplemented with appropriate antibiotics. Cells were resuspended in MH broth to an optical density at 600 nm of 0.5 and 500 µl aliquots were added to 13 mm test tubes. Unless otherwise indicated, one microgram of DNA (genomic, plasmid, or PCR product) was added to the tubes and the cultures were incubated for four hours at 37°C in 5% CO_2. The number of transformants and the total number of bacteria were determined by dilution plating on MH agar with appropriate antibiotics. Biphasic growth conditions and initial 3h incubation at 37°C in 5% CO_2 were found to not be necessary for transformation (See Appendix A). Transformation efficiency represents the number of transformants per total number of bacteria per microgram of DNA. Transformations were conducted in triplicate and repeated at least three times. Transformation efficiency represents the average of three experiments.

3.5.7 *In vitro* methylation

DNA MTases (M. EcoRI, M. HpaII, M. TaqI) were purchased from NEB. DNA was then methylated according to instructions using the appropriate buffer and SAM. After incubation, the DNA was purified. Plasmid and PCR fragments were purified using the Qiagen PCR purification kit. Genomic DNA was purified using ethanol precipitation. After purification, the DNA was quantified using the Qubit 2.0 and the methylation status was tested using appropriate restriction enzyme digestion. Methylation was deemed complete if the DNA was protected from degradation after a one hour digest as determined by gel electrophoresis.

3.5.8 qPCR Binding and Uptake assays

C. jejuni was grown for sixteen to eighteen hours on MH agar plates supplemented with appropriate antibiotics. Cells were resuspended in MH broth to an optical density at 600 nm of 0.5 and 200 μl aliquots were made. One microgram of DNA was added to these cells and they were incubated at 37°C for one hour. The cells were pelleted and resuspended in fresh MHB to remove DNA not associated with the cell. Half the samples were placed on ice and the other half had 10 μl DNase added and were incubated an additional thirty minutes at 37°C. All cells were then pelleted and washed again. DNA was prepared as previously described. DNA was quantified using a Qubit. 20 ng of DNA was added to a qPCR reaction. Primers are indicated in Table 3.3.

CHAPTER IV

CONCLUSION AND PERSPECTIVES

4.1 Conclusions

The goal of my thesis was to investigate to different parts of natural transformation in *C. jejuni.* In Chapter II, I examined the role of three proteins involved in the competence machinery, CtsE, CtsP, and CtsX. In Chapter III, I examined how *C. jejuni* selected the DNA it used in transformation.

CtsE, CtsP and CtsX were found to be involved in transformation in *C. jejuni* based on a transposon screen. Based on homology, we hypothesized CtsE and CtsP were ATPases. We proved that they were NTPases and that their NTPase activity was required for transformation by making point mutations that disrupted their Walker boxes. When these point mutants were expressed in *C. jejuni*, the cells were unable to be transformed. However, we cannot conclusively state that they are ATPases as we did not do *in vitro* assays testing for ATP cleavage. In other systems, similar ATPases associate with the membrane in order to power the production or retraction of the pilus. In the membrane localization studies I performed, we saw localization to the membrane for both

proteins, but the localization had features that were distinct from other systems. CtsE primarily localized to the cytosol, though a small percentage was seen at the membrane. We also determined that CtsE oligomerizes. CtsP strongly associated with the membrane but it did this without requiring any other individual Cts protein. We did see that it interacted with CtsX, which has no overall homology to other proteins. CtsX is a single-pass transmembrane protein required for transformation. We determined that the N terminus of the protein is in the cytosol and the C terminus is in the periplasm. However, we still do not know the specific role of this protein in competence.

From the first identification of competence in *C. jejuni*, it has been observed that *C. jejuni* is selective in the DNA that it uses for transformation [193]. However, the basis of that selection has been unclear until now. I identified a new DNA uptake sequence, methyl-RAATTY. This sequence is methylated *in vivo* by the orphan MTase CtsM but can be methylated *in vitro* by M. EcoRI. When DNA is not methylated at this sequence, it fails to transform *C. jejuni* cells. We determined this was true for both laboratory isolates we tested, 81-176 and 11168. However, the presence of CtsM is not required for transformation. In cells lacking CtsM, the same substrate specificity was demonstrated. Furthermore, I demonstrated that we could take DNA that has never been a transformation substrate, DNA derived from *E. coli* or from PCR, *in vitro* methylate this site, and convert the DNA into transformation substrate. I also determined that only one methylated RAATTY site was required, and that that site could be at least a one kilobase away from the DNA that will ultimately

recombine onto the chromosome. Finally, I determined that DNA lacking methylation at RAATTY sites is transported into the cell to the same degree that fully methylated DNA is.

This work has demonstrated that *C. jejuni* functions similarly to other competence systems in that it has two ATPases that are required for transformation and that it encodes a mechanism for selecting the DNA that it uses in transformation. However, when taken as a whole, the *C. jejuni* competence system has many unique characteristics that separate it from other competence systems. As discussed previously, the Cts system is not the sole transformation system encoded in the genome. To our knowledge, no other bacterium has been found that contains two separate transformation systems. Furthermore, within the Cts system, there are many unique features. The localization of CtsE and CtsP differ from previously studied competence ATPases. At the sequence level, CtsP does not look like other competence retraction ATPases and has been placed in unique class of ATPases [183]. CtsX has no homology to other proteins but is still required. We initially hypothesized that CtsX might functionally replace a protein found in other systems that the Cts system was missing, but we have yet to identify any missing protein in this system. The mechanism of DNA selection is distinct as well. Other systems use uptake sequences, but these are not methylated. Furthermore in other systems with uptake sequences, selection occurs on the outer membrane where the receptor protein ComP recognizes the DUS and allows the DNA to enter the cell [95]. As DNA lacking RAATTY methylation enters the cells as well as fully

methylated DNA, this cannot be the mechanism of selection. In other cases where methylation is important, there is a cognate restriction enzyme to degrade DNA lacking the methylation. However, there is no restriction enzyme present to perform this function.

4.2 Perspectives

My work has answered a number of questions about natural transformation in *C. jejuni* but there are still many questions yet to be answered. There are new questions brought to life by my research, both regarding the transformation machinery itself and DNA selection. There are also more general questions about the regulation of transformation.

In terms of the competence machinery, we still have not addressed the individual roles of the two ATPases. We know that they are each required and that their individual ATPase functions are required, but we do not know how or when that energy is used by the system. In other systems, these ATPases power the production and retraction of a pilus [29, 73, 212]. *C. jejuni* has not been demonstrated to produce a pilus and does not encode the gene for the major pilin subunit. However, pseudopili have been demonstrated to work in competence and *C. jejuni* does encode to pseudopilins [5, 241]. We also do not know if CtsE transits to the membrane upon a signal, such as the presence of DNA, or if the amount we detect at the membrane is sufficient to power the system. We found that CtsP has a strong association with the membrane independent of other individual components but we do not know the mechanism of that interaction. It is possible that it can interact with multiple components of

the system and that when we removed individual components of the system, there were still other interacting partners present to facilitate the interaction. To test this hypothesis, we would need to make strains lacking multiple components of the system. However, when I expressed *ctsP* in *E. coli*, it still interacted with the membrane. This indicates it might not be a Cts specific mechanism. Finally we do not know the role of CtsX. The primary amino acid sequence does not have homology to other proteins. However, if we use Phyre 2 or programs that predict homology based on predicted structure, we do identify an interesting area of homology towards the C terminus. This region, predicted to be in the periplasm, looks likes chaperones involved in Type III secretion. This leads us to hypothesize that CtsX might be involved in stabilizing the competence machinery or helping periplasmic proteins fold properly.

As previously discussed, the uptake sequence we identified is different from other uptake sequences. While I have tested it in two lab strains of *C. jejuni*, we do not know if this mechanism will hold true for all *C. jejuni* strains, particularly environmental isolates, or if it will work for more distantly related strains such as *C. coli.* CtsM is conserved and has been found to be active in distantly related strains such as *C. subantarticus.* Competence has not been studied in *Campylobacter* species other than *C. jejuni* and *C. coli,* so we do not know if these species are naturally competent and if they are, how selective they are in the DNA they use. I also have yet to determine the mechanism of selection. Based on the DNA binding and uptake assays that I performed, the DNA lacking RAATTY methylation is transported into the cell similarly to fully

methylated DNA. This means selection must occur inside the cell. Based on this, there are a number of possibilities for the mechanism of selection. 1) There is a protein in the periplasm that binds to and recognizes methyl-RAATTY and this binding event allows transport of the DNA into the cytosol. 2) All of the DNA is transported into the cytosol, independent of methylation state, and then only properly methylated DNA recombines onto the chromosome. 3) There is a methylation specific restriction enzyme that is always trapped in the periplasm. This is the least likely of the three hypotheses because *ctsM* insertional mutants do not appear to have any growth defects. If such a restriction enzyme exists, it would target the chromosomal DNA in these cells anytime it erroneously remained in the cytoplasm and would kill the cell. To determine the mechanism of specificity, I have attempted to purify perplasmic fractions and to test if there is an enrichment of DNA lacking RAATTY methylation in this fraction. This would indicate the DNA is trapped in the periplasm and would point to the first hypothesis. A pulldown assay looking for proteins that specifically bind RAATTY methylated DNA could also help address this question. Finally, orphan MTases have been implicated in gene regulation in other systems. As this MTase is highly conserved among *Campylobacter* species, it is likely that it plays a role in more than transformation. There are conspicuous regions of either hypomethylation or the lack of possible methylation motifs. These include 16S and 23S ribosomal RNAs, flagellar proteins including FlgK and a flagellar basal-body rod protein, FtsZ, and central metabolism proteins including SucC and

SucD [153]. This indicates that CtsM might be involved in peigentic regulation similar to CcrM and Dam. This could be assessed by RNAseq.

There are also a number of outstanding questions about transformation in *C. jejuni* that my research did not touch upon. We still do not know the function of the Vir transformation system. It is likely that it is important in some yet to be determined environmental condition, though why the Cts system would not be sufficient is not clear. The regulation of competence is not clear. It seems like the Cts system is constitutively active, but there are environmental conditions where the system is more active—higher temperatures, microaerophilic conditions [1, 2]. Furthermore, translation inhibitors eliminate transformation [1]. All of these indicate that there is some regulation that happens and that it is likely post-transcriptional. RNA-seq data from our lab indicate that different environmental conditions such as chicken colonization, mid-log growth or exponential growth, do not significantly impact transcription of the Cts operon [242]. We also do not know if all cells are competent such as in *S. pneumoniae* or if only a subset of cells are actively competent such as in *B. subtilis.* In species such as *V. cholerae,* an environmental signal is needed to induce competence. While *C. jejuni* seems to be constitutively active, the number of transformants produced is lower than in other species. Therefore, there might be an environmental signal that upregulates the system to increase overall transformation efficiency.

The work presented in this thesis addressed some mechanistic questions regarding DNA selection and the roles of individual Cts proteins in *C. jejuni*

transformation. However, there are many outstanding questions that remain to be answered.

APPENDIX A

EVALUATION OF BIPHASIC TRANFORMATION PROTOCOL

The published protocol for *C. jejuni* natural transformation is time intensive. Cells, grown overnight on plates, are resuspended to an OD of 0.5. 0.5 ml of that culture is added to a biphasic tube (a tube with 1 ml MH agar in the bottom) and allowed to incubate for three hours at 37°C and 5% CO_2 and then 1 µg of DNA is added. After the addition of DNA, the cells are returned to the previous conditions and incubated for an additional four hours and then dilutions are plated on antibiotic containing media to measure transformants. However, there was no indication why this protocol was used and what the effects on transformation efficiency were if these parameters were changed. Therefore, I wanted to test three different parts of this protocol for their effect on transformation efficiency. First I tested the role of the biphasic tubes. Second, I tested the role of the pre-DNA incubation period. Third, I tested the role of the post-DNA incubation period.

As the logic behind all of these steps is not included in any of the publications that used this protocol, I can only speculate to the reason behind them. Biphasic growth is often used when cell density affects the phenotype being tested, such as production of lantibiotics in *S. pneumoniae* [243]. However, the cultures are not stabbed into the agar nor are the mixed into warm agar. It is possible that aggregation could occur when the cells fall out of suspension which

happens because the tubes are not shaking in the incubator. Cell density does affect competence in other systems so it is possible this was a way to account for that. However, we now know that *C. jejuni* is constitutively competent, so this would have no effect. When I kept the rest of the transformation protocol the same but performed the protocol in regular tubes as opposed to biphasic tubes, I saw no difference in transformation efficiency (Fig A1). Based on this data, I eliminated this step.

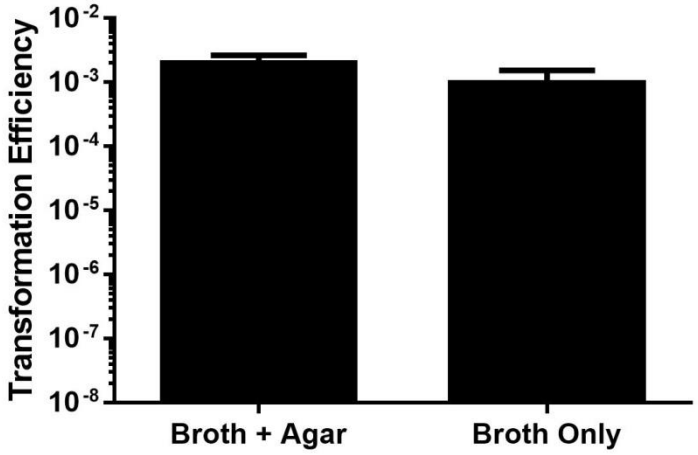

Figure A1. Biphasic tubes do not affect transformation efficiency. WT *C. jejuni* was transformed with kan^r gDNA in either biphasic tubes (broth + agar) or in regular tubes (broth only). Transformation efficiency was calculated as before. This was done in triplicate. Student's t test was done to compare the two samples and no difference was determined.

I then tested the role of the pre-DNA incubation phase. I took identical cultures and incubated them at 37°C and 5% CO_2 for between thirty minutes and 3 hours. I then added DNA and allowed the cells and DNA to incubate for an additional four hours before I plated for transformants. Again, I can only speculate as to why this step was included. In some bacteria, competence is triggered by stress and *C. jejuni* does prefer higher CO_2 conditions [37]. Perhaps

the idea was to stress the bacteria by placing them in a low carbon dioxide environment which would then induce them to upregulate competence genes. When I performed this experiment, I saw no difference between the cells that I preincubated at 37°C and 5% CO_2 and those that I did not (Fig A.2). Therefore I eliminated this step as well.

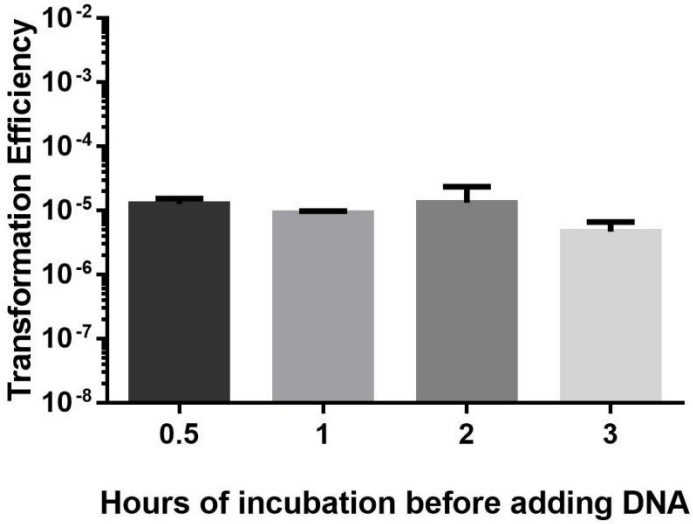

Figure A2. Preincubation in low CO_2 does not affect transformation efficiency. WT *C. jejuni* cells were transformed with kan^r gDNA. Before the addition of gDNA, the placed in biphasic tubes and incubated for various lengths of time at 5% CO_2 and 37°C. After the addition of 1 ug of DNA, the cells were further incubated for 4 hours in the previous conditions. Transformation efficiency was calculated as before. This was done in triplicate. One way ANOVA with Dunnett's multiple comparison test were performed and no statistical difference was determined for any of the samples.

Finally, I tested the role of the post DNA incubation phase. Again, no reasoning was included for the length of this period but most transformation protocols do include an incubation period after adding DNA. It does take some time for the bacteria to bind to the DNA, transport it through both membranes, and then recombine it onto the chromosome. As we measure transformation by the number of bacteria that become resistant to the antibiotic cassette provided,

increasing this time should allow for more transformants. When I varied the post-incubation period from 30 minutes to the normal 4 hours, I saw an increase in transformants as I increased the incubation time (Fig A3).

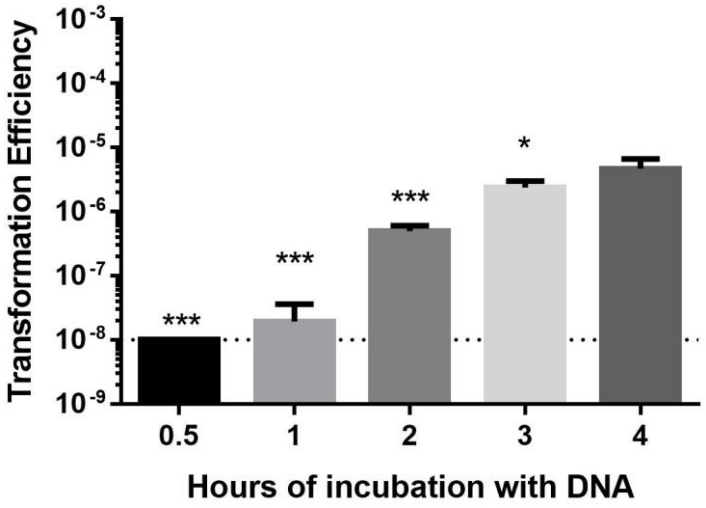

Figure A3. Incubation time in the presence of DNA affects transformation efficiency. WT *C. jejuni* was transformed with kan^r gDNA. Cells were preincubated at 5% CO_2 and 37°C for three hours before the addition of 1ug of gDNA. Cells were then incubated at the previous conditions for various lengths of time before being plated to determine transformants. Dotted line indicates limit of detection. Transformation efficiency was calculated as before. One way ANOVA and Dunnett's multiple comparison test was performed and all samples were compared to the 4-hour incubation time point. All of the time points were significantly lower than the 4-hour time point.

Based on these experiments, I modified the transformation assay such that I grew the cells overnight on plates, made a 0.5 OD suspension. I aliquoted 0.5 ml of that suspension to tubes and then added 1 µg of DNA to the cells and allowed them to incubate at 37°C and 5% CO_2 for four hours and then I made serial dilutions and plated for transformants. When I directly compared this method to the original protocol, in every case but one I saw no difference between the original protocol and the new protocol. When I did see a difference

(WT), there was a higher transformation efficiency with the new protocol (Fig A4).

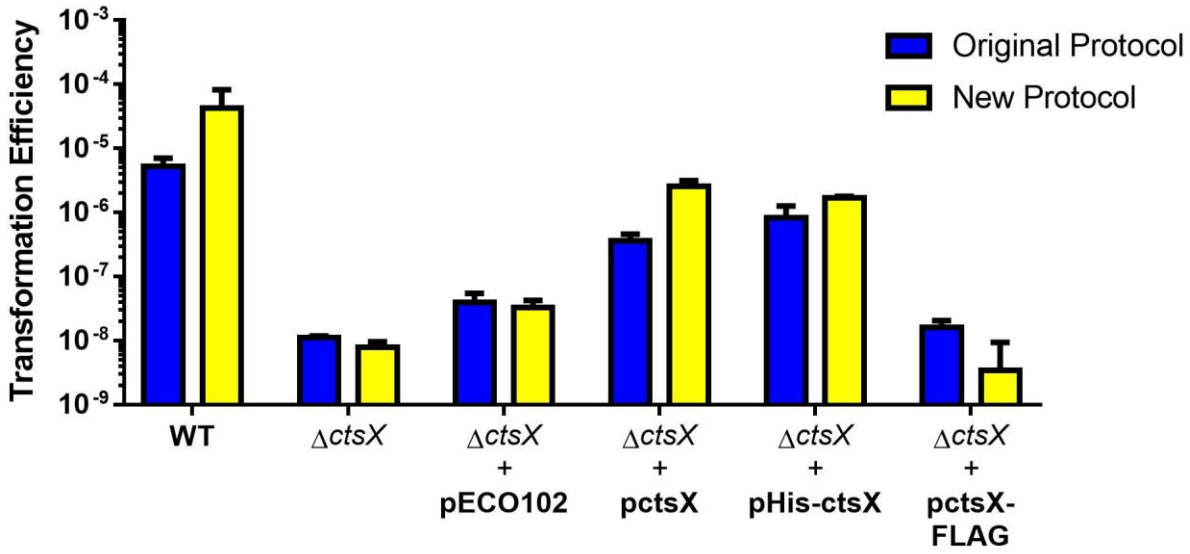

Figure A4. The modified transformation protocol works as well or better than the original protocol. Different strains of *C. jejuni* were transformed with kan^r gDNA using either the original transformation protocol as previously described or a new protocol in which I skipped the pre-DNA incubation step and did not use biphasic tubes. Transformants were calculated as previously described. This experiment was done in triplicate. Data was analyzed by two-way ANOVA with Tukey's multiple comparison tests. The only statistical difference was between the WT strains with a p-value of 0.02.

APPENDIX B

NATURAL TRANSFORMATION OF ENVIRONMENTAL STRAINS OF *CAMPYLOBACTER*

The majority of the work that I did over the course of my thesis involved the laboratory strain 81-176 or its streptomycin resistant derivative DRH212. This is one of the best characterized *C. jejuni* strains. It was originally isolated from a patient who became ill with campylobacteriosis after drinking raw milk in 1989 [237]. We were curious about how naturally competent recent environmental isolates were, especially in comparison to 81-176. A post-doctoral fellow in the lab, Michael Taveirne, was performing a farm study looking at the prevalence of *Campylobacter* in pasture raised chickens and comparing the microbiota of those chickens with the chickens we raise in the animal facility for our colonization studies. In the course of this study as well as another study in collaboration with the University of Michigan Hospital looking at recent human isolates of *Campylobacter,* he isolated a number of chicken and human isolates. I took some of those strains and tested if they were competent.

We initially started with two chicken isolates, MTVDSCJ20 and SCC1. MTVDSCJ20 is a *C. jejuni* strain that we eventually did whole genome sequencing on. SCC1 is *C. coli* strain. These were both isolated from pasture-

raised chickens. I first tested their ability to be transformed by DNA derived from DRH212, containing either a kanamycin cassette or a point mutation conferring naladixic acid (NA) resistance (Fig B1). Neither of these strains were competent based on this experiment. There were a few colonies detected when we used NA^r gDNA. However, this is most likely because this resistance is conferred by a point mutation and we always recover spontaneous mutants naladixic acid containing plates (data not shown). As we suspected that methylation played an important role in DNA selection in transformation, this was not completely surprising. We did not have genome sequences for either of these strains at this point, but all of the sequenced strains contained the Cts system, so we speculated that the transformation defect was due to a difference in methylation between 81-176 and these two environmental strains.

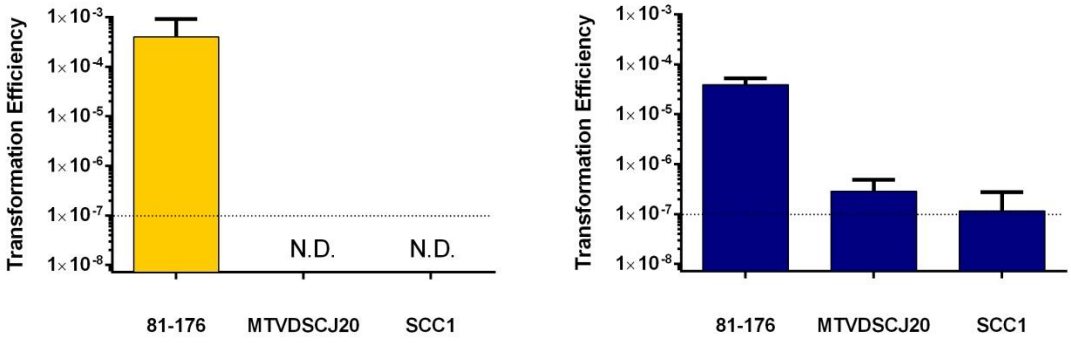

Figure B1. Environmental isolates of *Campylobacter* are not transformed with gDNA from 81-176. Different strains of *Campylobacter* were transformed with either kan^r gDNA from 81-176 (yellow) or naladixic acid resistant gDNA from 81-176 (blue). Transformation efficiencies were calculated as before. The experiment was done in triplicate. N.D. means not detected. The dotted line indicates the limit of detection.

If methylation status were affecting transformation in these strains, we hypothesized that these strains would be transformable with their own gDNA. In

order to do this, we first had to introduce antibiotic cassettes to these strains in order to measure transformation. We introduced a kanamycin resistance cassette into MTVDSCJ20. We also grew both strains on naladixic acid plates and isolated spontaneous mutants. I then purified gDNA from all of these strains. I then asked if these environmental strains would transform better with their own DNA. I also tested if the DNA from these isolates could transform 81-176 as well as a laboratory strain of *C. coli.* When I did this experiment, I saw the environmental isolates were never transformed (Fig B2). I did recover colonies when I grew the colonies on naladixic acid containing plates, but no more than my no DNA control (green and purple vs blue bars). I never saw transformants using the kanamycin resistant gDNA from MTVDSCJ20. This supported our hypothesis that methylation plays a role in transformation because DNA from MTVDSCJ20 could not transform 81-176, which we know transforms well.

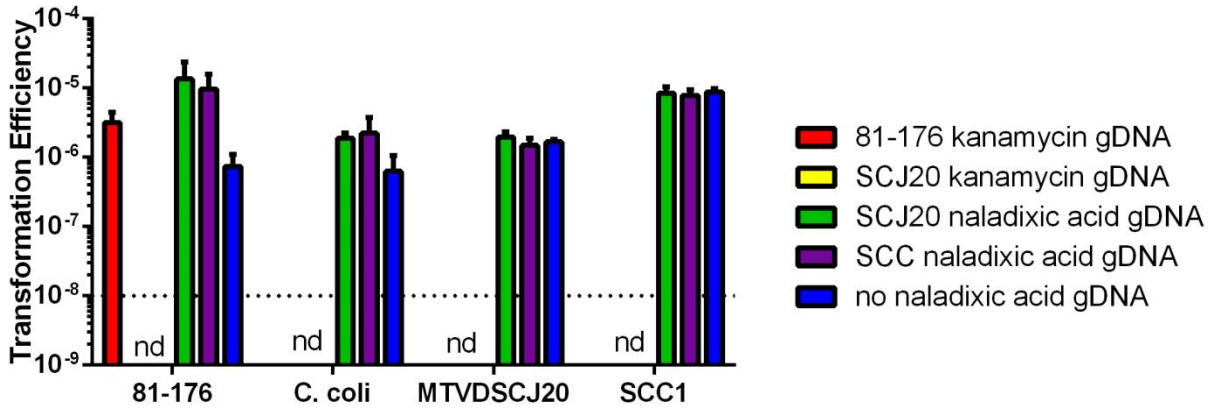

Figure B2. Environmental strains are not transformed with their own DNA. Four strains of *Campylobacter* were transformed with DNA as indicated. Transformation efficiency was measured as before. The blue bars indicates the cells were treated identically to cells that were transformed but no DNA was added and then the cells were plated on naladixic acid containing media in order to determine the rate of spontaneous mutation. The dotted line indicates the limit of detection and nd indicates no colonies were determined. No DNA controls

were also done for kanamycin resistant but no colonies were detected for any strain so that data is not plotted.

While the previous experiment indicated there might be differences in the methylation status of the DNA between 81-176 and MTVDSCJ20 because kan^r gDNA derived from MTVDSCJ20 was incapable of transforming 81-176, it did not explain the lack of transformation with their own DNA that we saw for either environmental strain. We then tested these strains, as well as other non-laboratory isolates, for their ability to produce eDNases. *Campylobacter* is capable of producing up to three eDNases and all are capable of inhibiting transformation [49, 50]. We therefore grew the cells overnight, normalized the ODs, spun them down and filtered the supernatants to remove any remaining bacteria. We then treated 81-176 gDNA with these supernatants and looked for the degradation of DNA (Fig B3). Overall, we saw three distinct phenotypes. In some cases such as our laboratory strain 81-176, there was no degradation, indicating lack of eDNase activity. We also saw complete degradation of the gDNA, indicating strong eDNase activity. This was the case for both previously tested environmental strains—MTVDSCJ20 and SCC1. Finally, we saw one case, H15, where there was some activity, indicated by smearing of the gDNA, but it was not as strong as in the other cases of activity that we saw. This data explained our transformation phenotypes—the eDNases produced by MTVDSCJ20 and SCC1 were eliminating any possible transformation that was occurring by degrading any extracellular DNA. We eventually did full genome sequencing on MTVDSCJ20. Based on these data, we determined that it has a fully intact Cts system and that it encodes one of the identified eDNases. This

indicates, as has previously been reported, that a single eDNase is sufficient to eliminate natural transformation [49, 50].

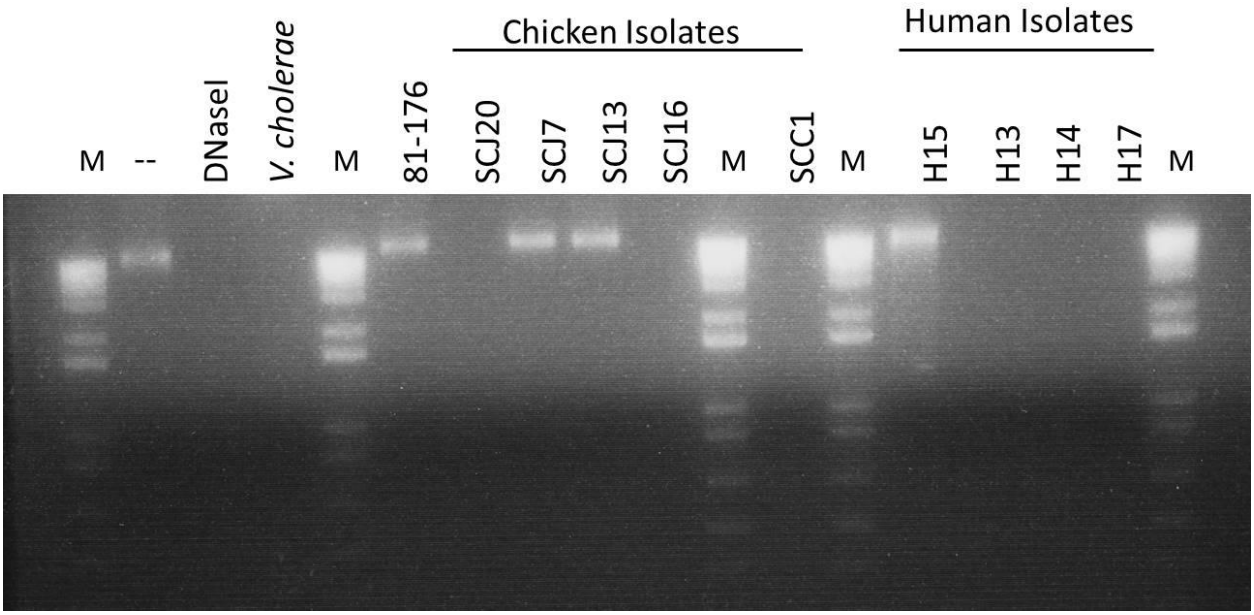

Figure B3. eDNase activity of non-laboratory isolates. A panel of non-laboratory strains were tested for their eDNase activity. gDNA was added to supernatants, incubated for an hour, and then run on an agarose gel.

Based on this experiment, we did identify other environmental isolates, SCJ7 and SCJ13 that did not have eDNase activity as well as H15, which had weak eDNase activity. We then examined transformation in these strains. We first tested these strains with 81-176 gDNA. We found for SCJ7 and SCJ13, the strains that had no eDNase activity were capable of being transformed, though they transformed less efficiently than 81-176. However, the strain that had a small amount of eDNase activity was not transformed (Fig B4).

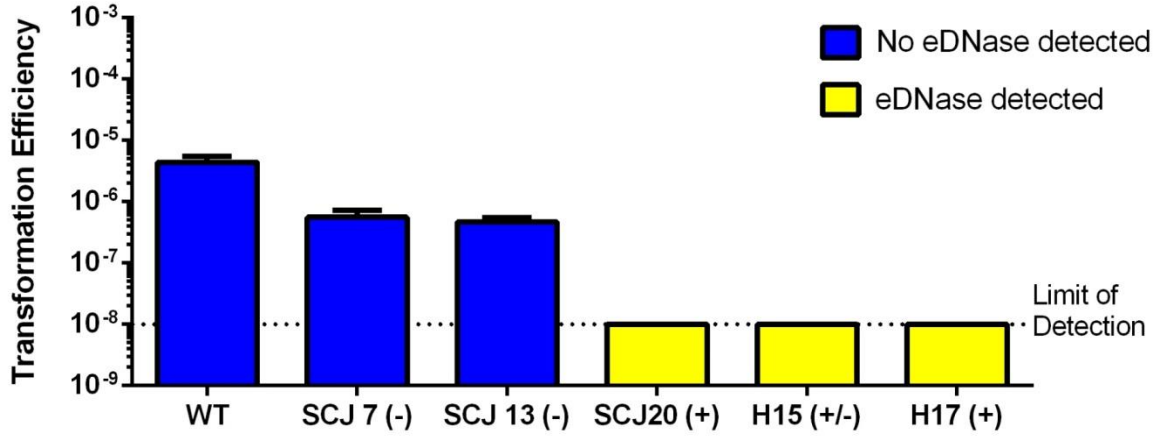

Figure B4. Environmental strains lacking eDNase activity can be transformed by 81-176 gDNA. Various *Campylobacter* strains were transformed with 81-176 kan^r gDNA and transformation efficiency was calculated as previously described. These strains are color coded based on if we detected eDNase activity in the assay described in Fig B3. Blue indicates no eDNase activity and yellow indicates some activity. This is also designated in parenthesis next to the strain name by a plus or a minus. +/- indicates weak activity was detected. The dotted line indicates the limit of detection.

While we saw no transformants for H17, we could not conclude if this was because of the presence of eDNases or because there was some methylation-dependent difference between its DNA and the DNA from 81-176. Therefore, I grew these strains on naladixic acid containing media in order to elicit spontaneous naladixic acid point mutants. I then purified the NA^r gDNA from these strains and tested the ability of this DNA to transform the environmental strains as well as 81-176 (Fig B5).

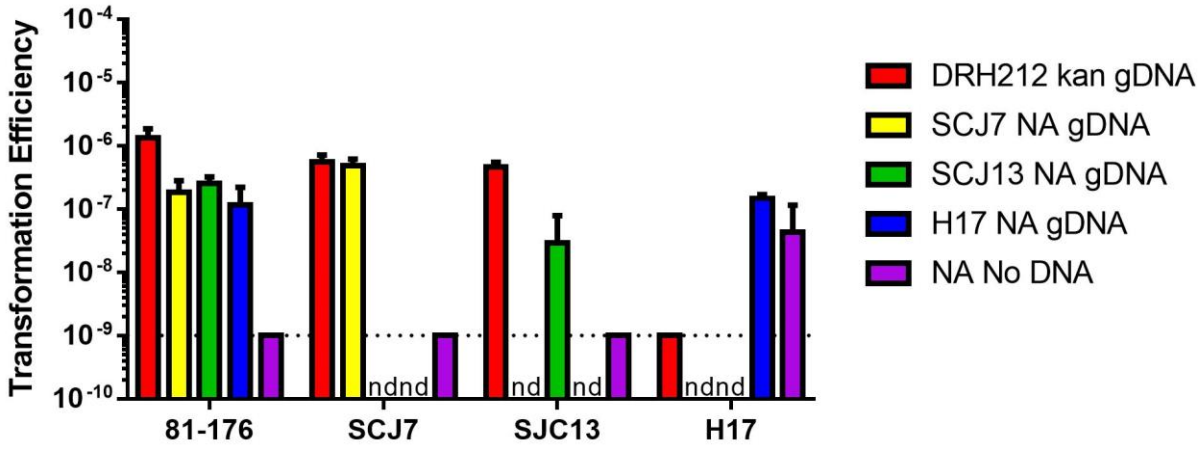

Figure B5. Environmental isolates can be transformed with their own gDNA. Various *Campylobacter* strains were transformed with the various type of DNA indicated. Transformation efficiency was calculated as previously described. I did not transform all environmental strains with all possible types of DNA. This is indicated by a nd where I did not determine transformation efficiency. The dotted line indicates the limit of detection. Where no colonies were detected, bars were set to the limit of detection.

Both of the strains lacking eDNase activity were able to be transformed with their own DNA. While colonies were detected for H17, the strain with weak eDNase activity, there were only slightly more colonies than in the no DNA control. This most likely indicates that the eDNase is strong enough to degrade the added DNA and that the colonies we are detecting are spontaneous mutants. Interestingly, neither SCJ7 nor SCJ13 were more transformable with their own gDNA. They were still less transformable than 81-176. Furthermore, their gDNA did not transform 81-176 as well as its own gDNA.

These experiments were all performed before I identified the DNA selection mechanism in *C. jejuni*. We do not have genome sequences for these isolates. However, I would hypothesize that SCJ7 and SCJ13 both have *ctsM* as

without CtsM methylation, I do not see any transformants in 81-176. Therefore, the gDNA from these strains must have RAATTY methylation. This does not explain the difference we see in the transformation efficiencies I see when using gDNA from these strains. It is likely that the genomes for these strains differ from 81-176. As mentioned previously, *C. jejuni* is known to have a great deal of genetic diversity. Therefore, it is possible that the DNA flanking the point mutant is not entirely homologous. While the exact requirements for transformation have not been determined in *C. jejuni*, decreasing the amount of homologous DNA significantly decreases the transformation efficiency in *V. cholerae* and *S. pneumoniae* [244]. This might explain the difference I observe using different DNA.

This work demonstrates that some environmental strains are naturally competent. However, many more strains of our isolates expressed eDNases than did not. As this expression correlated with the lack of transformation, this might indicate that transformation might not be favored in either the chicken or the human intestine. However, this is a very small number of strains to base such a conclusion on. This should be investigated with many more environmental isolates. Furthermore, transformation was less efficient in the environmental strains that did not have eDNase activity than in our laboratory strain, even when they were transformed by their own DNA. This indicates there is more to transformation and possibly DNA selection in environmental strains than in our laboratory strain.

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