

Abstract

BRITT, DANIELLE. Roles of the Capsule in Environmental Adaptations of Animal-derived *C. jejuni* and *C. coli* Isolates. (Under the guidance of Dr. Sophia Kathariou).

Campylobacter spp. is a food-borne pathogen that colonizes common food animals and persists in many foods and in the environment. Previous studies have shown that the capsular polysaccharide (CPS) on the cell surface of *Campylobacter* may affect its virulence and survival. The focus of this study is to determine possible roles of the capsule in environmental adaptations of animal-derived *C. jejuni* and *C. coli* isolates. The following wild type strains were used in this study with the CPS-deficient mutant counterparts produced for comparison through site-specific mutagenesis of the *kpsM* gene involved in CPS transport: two turkey isolates, *C. coli* 6979 and *C. jejuni* SC1453, along with one bovine isolate, *C. jejuni* BS142. Comparisons were made between wild type and mutant strains in terms of competence and motility, and during environmental stresses such as desiccation, freeze survival, and survival in a low-nutrient environment. Species-specific trends in appearance and motility were observed, not only between the various strains tested, but also between the wild type and mutant swarms, suggesting differences in chemotaxis. The mutant strain of *C. coli* 6979 was more competent than the wild type, showing that the protective capsule may also be a barrier that inhibits access to extraneous DNA and other cellular components. Both *C. jejuni* strains were more competent than *C. coli* 6979 and modifications will need to be implemented in order to assess differences between the wild type and mutant for those strains. The wild type exhibited an advantage over the mutant of all three strains during freeze survival and for

C. coli 6979 during survival in deionized water; however, there were more evident species-specific trends observed in the desiccation and salt tolerance assays. The mutants of both *C. jejuni* BS142 and *C. jejuni* SC1453 survived better than the wild type, while the opposite was observed for *C. coli* 6979 during the desiccation assay. The wild type strain of *C. jejuni* BS142 grew on MHA with a concentration of up to 1.2% NaCl, making this strain the most salt tolerant of the three tested. *C. jejuni* SC1453 only grew up to a concentration of 0.5% NaCl, making it the most sensitive to salt.

These results indicate that the capsule affects the competence, motility, and environmental stress response of *Campylobacter* with obvious implications in colonization and the incorporation of new genetic material into its genome. Since these characteristics are commonly associated with the survivability and pathogenicity of *Campylobacter*, future experimentation and analysis using *kpsM*-deficient mutants will give insight into the function and purpose of the capsule in virulence and environmental adaptations.

Roles of the Capsule in Environmental Adaptations of Animal-derived
C. jejuni and *C. coli* Isolates

by
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Biography

Danielle Elizabeth Britt was born in Cape Girardeau, Missouri, in 1986. Her family settled in Hawkins, Texas, through her elementary school years before moving to Perry, Georgia, where she attended and graduated from Perry High School in 2004. She attended Macon State College for two years before transferring to the University of Georgia to pursue a Bachelor's of Science in Microbiology. In May 2007, she was married to Darrell Steven Britt Jr., and she obtained her bachelor's degree from the University of Georgia the following year in May 2008. She was then accepted into the North Carolina State University graduate program, where she began her studies towards a Master's in Food Science under the guidance of Dr. Sophia Kathariou.

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Chapter 1
Literature Review

The Effects of Cell Surface Glycoconjugates on the Survival and Pathogenesis of *Campylobacter*

Background information

Campylobacter spp. is a prevalent enteric food-borne pathogen that persists in many foods as well as the environment. These bacteria are Gram-negative and microaerophilic with a rod- or spiral-shaped cell morphology. Members of this genus are non-spore-forming, sensitive to more than 3% NaCl, and have an optimum growth temperature range between 30-42°C (Dworkin and Fallow, 2006). The bacterium can be found as part of the normal gastrointestinal (GI) microflora of many animals, enabling human infection through the consumption of contaminated animal food products and water (Harris *et al.*, 1986). It is frequently isolated from the intestinal tract of a number of warm-blooded animals, most commonly poultry, as the environmental conditions in the gut appear to be extremely conducive to *Campylobacter* growth (Altekruse *et al.*, 1999). The colonization of the GI tract of dairy animals by *Campylobacter* contributes to the contamination of milk products. Furthermore, a study by Orr *et al.* (1995) reported the direct excretion of *Campylobacter* in milk, suggesting a route for the contamination of raw milk (Orr *et al.*, 1995). In an animal host, the bacterium generally acts as a commensal, eliciting an asymptomatic colonization in which the host is not harmed (Altekruse *et al.*, 1999). For this reason, most animal hosts for *Campylobacter* present few of the standard symptoms of infection that are frequently associated with human illness.

Campylobacteriosis is one of the most common causes of bacterial food-borne illness worldwide (Allos, 2001). This disease is an acute bacterial gastroenteritis, producing symptoms indicative of a GI tract infection. The most notable symptoms include diarrhea, stomach cramping, fever, chills, and vomiting (Butzler and Skirrow, 1997). There are currently 18 species categorized under the *Campylobacter* genus, with new species continuing to be identified; however, the two *Campylobacter* species most commonly associated with food-borne disease are *C. jejuni* and *C. coli* (Lastovica and Skirrow, 2000). Most reported cases of campylobacteriosis in humans are sporadic, with *C. jejuni* being responsible for over 85% of the cases of infection, while *C. coli* is largely responsible for the remaining cases (Friedman *et al.*, 2000). Since campylobacteriosis is a common cause of gastroenteritis worldwide, the study of the mechanisms underlying the virulence of this microorganism could have important implications for the treatment of the disease.

There are also a number of secondary post-infection complications that have been associated with campylobacteriosis. Guillain-Barré and Miller-Fischer syndromes are serious neurological disorders thought to be caused by an autoimmune response in the body after invasion of the pathogen (Newswanger and Warren, 2004). Guillain-Barré syndrome (GBS) is characterized by the loss of muscle coordination in the form of ascending paralysis, while the less common variation of the disease, Miller-Fischer syndrome (MFS), causes descending paralysis (Godschalk *et al.*, 2007). An acute infectious illness most commonly initiates the onset of GBS, with a preceding *C. jejuni* infection as the leading cause, accounting for 30-40% of the cases (Allos, 1997). The

bacterial cell surface lipooligosaccharides (LOS) of some *Campylobacter* serotypes have been implicated in the mimicry of human ganglioside structures. The antibodies produced against the ganglioside-like structures may then cross-react with human gangliosides to cause the neuropathies displayed in GBS and MFS patients (Koga *et al.*, 2001).

Cell surface carbohydrates

The genome of *Campylobacter* contains a variety of genes dedicated to the synthesis of surface polysaccharides. This genetic diversity helps enable the production of the many carbohydrate molecules that form structural components of the cell. Four prominent glycoconjugates present on the surface of the cell include the lipooligosaccharide (LOS), capsular polysaccharide (CPS), and O- and N-linked glycans. While the functions of these molecules range from the CPS-mediated protection from desiccation to the LOS-mediated mimicry of host cells, they are all crucial components involved in a myriad of interactions between the cell and the environment. Future research will entail the use of the mechanisms of these biological systems as targets for antimicrobial treatments in the prevention of initial animal host colonization and for prevention of disease (e.g. vaccines) or disease treatment.

During the first complete sequencing of a *C. jejuni* genome, four carbohydrate gene clusters were discovered that encode for the following principal glycan structures in the NCTC 11168 strain: lipooligosaccharide (LOS), capsular polysaccharide (CPS), and O- and N-linked glycosylation systems (Parkhill *et al.*, 2000). Each gene cluster is located at a single chromosomal locus, allowing for the coordinate regulation of a large number of genes that may be involved in the biosynthesis and export of the respective

structure. With the exception of the conserved N-linked glycan gene cluster, the loci are highly variable among strains (Szymanski and Wren, 2005). The differences in sequence can be attributed to genes that are prone to phase variation by slip strand mismatch repair (Guerry and Szymanski, 2008). These phase variation mechanisms account for the ever-changing carbohydrate phenotypes that contribute to a wide range of virulence and survival strategies. Carbohydrates on the outside of the cell are not only readily accessible by foreign molecules, but they are also involved in a wide variety of biological and pathogenic processes, making them a prime target for future developments in therapeutic and antimicrobial treatments.

Lipopolysaccharides

Lipopolysaccharide (LPS) is an abundant surface component of the outer membrane structure of many Gram- bacteria. Its main purpose is to bolster the structural integrity of the cell, meanwhile providing an additional barrier to the outside environment. The three structural components of the LPS are lipid A, core oligosaccharide, and O antigen. Lipid A acts as a membrane anchor to hold the core composed of heterogenous monosaccharides, while the O antigen is made up of glycosyl residues covalently attached to the core (Schnaitman and Klena, 1993). In a previous study carried out by Logan and Trust (1984), *C. jejuni* and *C. coli* strains were found to lack the O antigen chains characteristic of the LPS structure, indicating that the structure was actually made up of low molecular weight LOS (Logan and Trust, 1984). Since this study, O antigen-like polysaccharide repeats were characterized for a number of *C. jejuni* serotypes; however, further observations of the repeat molecules suggested that they were

not linked to the lipid A-inner core and were therefore not actually O antigens (Fry *et al.*, 1998; Fry *et al.*, 2000). This high-molecular-weight polysaccharide was then demonstrated to be genetically similar to the capsular polysaccharide found in other Gram- bacteria, indicating that the outer membrane of *Campylobacter* is made up of LOS, with some strains producing a capsule (Karlyshev *et al.*, 2000).

The LOS genes from a number of *C. jejuni* isolates have been sequenced. The ability of *Campylobacter* species to synthesize and integrate sialic acid into their LOS cores can result in the human ganglioside mimicry responsible for autoimmune response discussed earlier (Salloway *et al.*, 1996). This autoimmune response caused by the incorporation of sialic acid was demonstrated in rabbits, while a lessened immune response was reported with the use of LOS cores that lacked sialylation in mice (Godschalk *et al.*, 2007; Koga *et al.*, 2001); however, not all isolates expressed sialylated LOS cores. Some *C. jejuni* strains exhibit O-acetylation of the sialic acids or the addition of N-acetyl-quinovosamine on their LOS cores instead of sialic acid (Aspinall *et al.*, 1995; Dzieciatkowska *et al.*, 2007). The effects of these LOS core variations on *Campylobacter* interactions with host cells are not yet fully understood.

O- and N-linked glycans

Post-translationally modified proteins with either O- or N- linked glycans provide yet another form of glycoconjugate in *Campylobacter*. Glycosylation is the process of connecting carbohydrates together to form glycans that can then be attached to other molecules. The O-linked glycosylation of flagellin has been characterized in many bacteria; however, in *Campylobacter*, this modification is important in flagellin synthesis

and assembly, with mutants lacking the modification being non-motile (Goon *et al.*, 2003). An in-depth study of the flagellin glycosylation locus for *C. jejuni* 81-176 indicated that the most common modification is the addition of pseudaminic acid (Pse5Ac7Ac) (Goon *et al.*, 2003). The flagellins of *C. jejuni* and *C. coli* exhibit the highest level of bacterial protein glycosylation that has yet been observed, with a reported glycosylation at 19 serine or threonine residues in *C. jejuni* 81-176 and at 16 sites in *C. coli* VC167 (Logan *et al.*, 2002).

It has been suggested that some of these flagellin glycans are exposed on the surface of the bacterium, and may therefore affect the immunogenicity and virulence of the pathogen. In a study carried out by Guerry *et al.* (2006), a mutant *C. jejuni* 81-176 strain without the pseudaminic acid modification exhibited decreases in adherence and invasion of epithelial cells. The observed reduction in adherence and invasion could be due to the failure of the mutant strains to autoagglutinate (Guerry *et al.*, 2006). While the role of autoagglutination in the virulence of *Campylobacter* is not yet fully understood, another study carried out by Golden and Acheson (2002) supports the evidence that the lack of properly synthesized flagella that are able to carry out motility and aid in the process of autoagglutination causes reduced invasion of intestinal epithelial cells.

In an N-linked protein glycosylation system, identified for *C. jejuni* by Szymanski *et al.* (1999), the *pgl* genes encoding this mechanism affect the glycosylation of a number of proteins. Although the genes involved in this pathway appear to be mostly conserved, a wide range of proteins are affected. For this reason, a substantial amount of research ensued to give insight into the functions of the glycosylation products. The far-reaching

effects of the N-linked glycosylation system became more evident through a comprehensive analysis of some of the glycoproteins found in *C. jejuni* NCTC 11168. In a study carried out by Young *et al.* (2002), 38 different glycoproteins affected by this mechanism were identified for this bacterium alone. One example of the effect of the N-linked glycosylation pathway involves the modification of a part of the type 4 secretion system (T4SS) in *C. jejuni* 81-176. Since this secretion system helps regulate natural transformation of the strain, the loss of N-linked sites results in a reduction in competence (Larsen *et al.*, 2004). Also, the process of N-linked protein glycosylation has recently emerged as a new area of research in diagnostic tools and therapeutics. The glycosylation pathway has been implicated in the cause of several genetic disorders and, due to the fact that it is very similar in both prokaryotes and eukaryotes, bacterial systems can be used to study its mechanism (Reid *et al.*, 2008).

Capsular polysaccharides

Capsular polysaccharides (CPS) may also be found as a surface carbohydrate on bacterial cells. When present, they are part of the outermost layer of the cell, connected to the surface through covalent bonds to either phospholipid A or lipid A structures (Whitfield, 2006). The CPS structure consists of single monosaccharide units joined together by glycosidic linkages (Whitfield and Valvano, 1993). The numerous configurations by which the monosaccharides may be joined contribute to the heterogeneity in CPS structure exhibited throughout the different *Campylobacter* species (Roberts, 1996). Therefore, the diverse array of CPS structures continually reported may

not only contain different monosaccharides, but may additionally vary in how the different units are linked together to form the molecule.

Because the capsule forms the outermost layer of the cell surface, it functions in mediating contact between the cell and the environment. Although the effects of this mediation on survival and virulence have not been completely elucidated, a number of studies have highlighted the CPS as a major virulence factor involved in prevention of desiccation, adherence to surfaces, and resistance to host immunity (Roberts, 1996). Furthermore, the CPS of *C. jejuni* has been directly linked with chicken colonization, diarrheal disease in ferrets, and pathogenic interactions with human epithelial cells (Bacon *et al.*, 2001; Jones *et al.*, 2004).

Table 1.1. Possible protective mechanisms of capsular polysaccharide (Modified from Roberts, 1996).

Function	Relevance
Prevention of desiccation	Transmission and survival outside of host
Adherence	Colonization of surfaces Biofilm formation Cell-cell interactions Cell-environment interactions
Resistance to nonspecific host immunity	Prevention of complement-mediated phagocytosis and killing
Resistance to specific host immunity	Poor antibody response to capsule Steric hindrance

One possible mechanism by which CPS may prevent desiccation involves its hydrated gel-like structure acting as a protective encapsulation for the bacterium. CPSs

are composed of over 95% water and their formation can be triggered by changes in external osmolarity (Roberts, 1996). Protection from dry environments becomes especially relevant when the bacterium is traveling from one host to another. Adherence to surfaces is also an essential factor in biofilm formation and host colonization that is characteristic of many pathogens. These adherence properties are facilitated by interactions between the carbohydrate chains that make up the CPS and the external environment. While a recent study carried out by Joshua *et al.* (2006) showed no correlation between capsule-deficient mutants of *C. jejuni* and biofilm formation, the authors suggested that a modified capsular polysaccharide may be involved in the process. Another mechanism by which the CPS may aid in virulence of the pathogen is its ability to circumvent host immune responses. During the evasion of host defenses, the CPS serves as a barrier to mask other surface structures and prevent them from eliciting a stronger immune response (Howard and Glynn, 1971). The mechanism that protects the outer layer may involve steric hindrance of the CPS structure, or resistance conferred through the surface charge of the cell. A highly negatively charged cell surface may provide more resistance to components of the immune response (Moxon and Kroll, 1990).

It was not initially known that *Campylobacter* exhibited a CPS structure. A study by Chart *et al.* (1996) suggested that what was previously thought to be high molecular weight lipopolysaccharide in *C. jejuni* may actually be CPS. Karlyshev *et al.* (2000) shed more light on the subject, reporting that genome sequence data found similarity between these molecules and the structures of group II and III CPS from other bacteria.

Furthermore, this study established the CPS as the major immunogenic determinant used for serotyping. The synthesis of the CPS structure in *Escherichia coli*, a bacterium with similar capsule expression, is outlined in a paper by Whitfield and Roberts (1999): assembly first requires nucleotide diphosphosugar precursors available in the cytoplasm and concludes with a polymer that is put together at the periplasmic face of the plasma membrane. This assembly is performed through translocation reactions in which an elongated polysaccharide is moved across the plasma membrane by an ATP-binding cassette (ABC) transport system. A variety of *kps* genes, including the *kpsM* gene to be later discussed in more detail, code for the proteins that comprise the translocation structure and aid in capsule assembly. The CPS structure of the sequenced *C. jejuni* strain NCTC 11168 was found to be composed of b-D-Ribp, b-D-GalfNAc, a-D-GlcpA6(NGro), a uronic acid amidated with 2-amino-2-deoxyglycerol at C-6, and 6-O-methyl-D-glycero-a-L-gluco-heptopyranose as a side branch, further resulting in an array of modifications to the sugar repeats (St. Michael *et al.*, 2002). The variable glycerol, ethanol, and methyl modifications may account for the wide range of CPS structures attributed to *Campylobacter* spp.

More experimentation is needed to understand the methods by which the CPS aids in cell function, and recent research has highlighted a specific genomic region of interest for this purpose. The *kpsM* gene, part of a gene cluster called the *kps* locus, was found during the genome sequencing of *C. jejuni* NCTC 11168 to display sequence similarity to other bacterial genes encoding proteins involved in CPS transport (Karlyshev *et al.*, 2000). The *kpsM* gene is thought to encode a component of the ABC transporter involved

in formation of capsule (Karlyshev *et al.*, 2001; Whitfield, 2006). In a study carried out by Karlyshev *et al.* (2001), the CPS present in *C. jejuni* cells was shown to be absent in a *kpsM*-deficient mutant, showing that the *kpsM*-encoded protein is pivotal in the formation and function of the capsule. The entire *kps* locus of group 2 capsule expression is comprised of three regions and encodes several conserved proteins that make up the composition of both group 2 and group 3 capsules in *E. coli* (Whitfield, 2006) (Figure 1.1). The KpsMT protein and ABC transporter that exports the polymers of the capsule structure across the inner membrane is made up of gene products from region 3 in the *kps* loci, with the KpsM being the transmembrane component and the KpsT being the ATPase component (Whitfield and Roberts, 1999; Whitfield, 2006) (Figure 1.1).

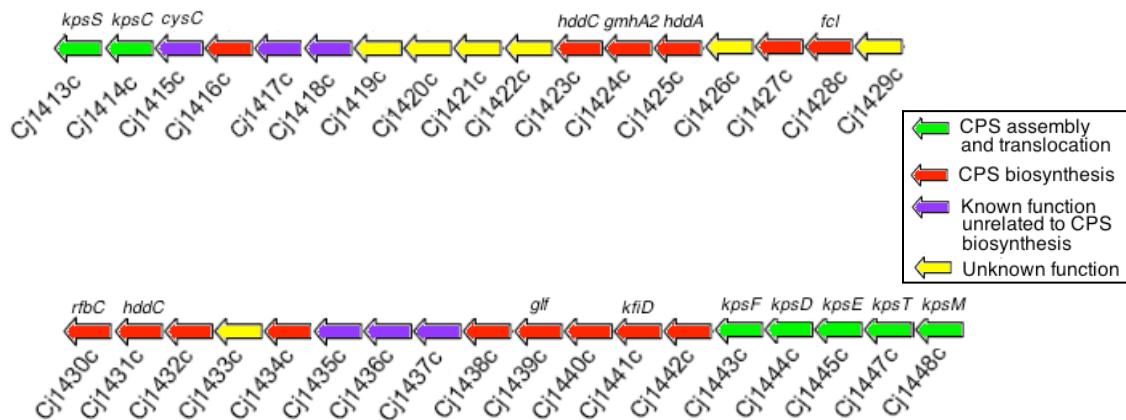


Figure 1.1. Organization of the capsular locus of *C. jejuni* NCTC 11168. CPS assembly and translocation genes are highlighted in green. Genes involved in CPS biosynthesis are highlighted in red. Genes with known function unrelated to CPS biosynthesis are highlighted in purple. Yellow denotes genes with unknown function. Size of ORFs is not drawn according to scale. This figure was created based on the NCBI gene database of NCTC 11168 and modified from Karlyshev *et al.* (2005).

CPS as a possible vaccine target

Capsular polysaccharide has proven to be a useful target for subunit vaccines in bacteria other than *Campylobacter*, and this has especially been pursued in situations in which whole-cell vaccines induce unnecessary or burdensome risk. Mai *et al.* (2003) demonstrated that a vaccine utilizing the CPS of *Salmonella* Typhi was highly effective in preventing typhoid in young children in Vietnam. Similarly, Whitney *et al.* (2003) reported on the safety of a CPS based conjugate vaccine against pneumonia for infants and high-risk children in the United States. While whole-cell vaccines for these diseases exist and are commonly used, they are unsuitable for use in young children and other high-risk groups due to potential health complications. Because *Campylobacter* infections have been linked to cases of MFS and GBS syndromes, there is also a cause for caution in using whole-cell vaccines to prevent campylobacteriosis in adults as well as children. It is thought that the link between *Campylobacter* infections and these conditions stems from human ganglioside mimicry of the LOS expressed by certain *Campylobacter* strains (Sheikh *et al.*, 1998). As Scott (1997) points out, ganglioside mimicry can be avoided by restricting the search for effective whole-cell vaccines to only those strains whose LOS has not been demonstrated to cause ganglioside mimicry and have not been implicated in cases of MFS or GBS; however, subunit vaccines remain the more attractive option, considering that this link has not been definitively confirmed to be the mechanism by which campylobacteriosis leads to these syndromes. Furthermore, even if it were definitively confirmed, it would be prudent to construct vaccines that are also effective against strains that have been implicated in MFS or GBS cases specifically

for the purpose of preventing them. Joining this with the fact that whole-cell vaccines do in many cases elicit complications in young children and other high-risk individuals, subunit vaccines should have preeminence over whole-cell vaccines.

A conjugate vaccine created from the CPS of *C. jejuni* strain 81-176 by Monteiro *et al.* (2009) was tested on mice and New World monkeys, which were challenged with the homologous strains after receiving the vaccine. The authors demonstrated that the mice developed a strong immune response after receiving the vaccine and found that the incidence of disease was reduced substantially in subsequent challenges. It was also observed that none of the monkeys treated with a sufficient dose of the vaccine exhibited diarrheal symptoms after being challenged; however, these animals were all found to have been colonized by the bacteria, and this colonization lasted for the same duration as in the non-vaccinated animals.

Motility

The flagellum of *C. jejuni* has been demonstrated as an important virulence factor in the gastrointestinal colonization of mice (Newell, 1985), humans (Black 1988), and chicks (Wassenaar *et al.*, 1993). Guerry *et al.* (1990) identified two distinct flagellin genes in *C. coli*: *flaA* and *flaB*. The expression of these genes yields the corresponding components flagellin A and flagellin B, of which the flagella of *C. jejuni* and *C. coli* are composed, and the authors showed that the flagellum is primarily composed of flagellin A. Guerry *et al.* (1991) later found that mutants deficient in either *flaA* or *flaB* were still capable of forming functional flagellar filaments, but that motility was inhibited in both cases. In particular, when *flaA* was interrupted, the resulting filament was much shorter

and motility was greatly reduced compared to wild type. The interruption of *flaB* resulted in only a slight reduction in motility compared to the wild type and did not affect the length of the filament. Recombination involving these two genes was explored by Wassenaar *et al.* (1995), who suggested that the organisms may use this mechanism to alter the immunogenic properties of the flagella in order to evade host immune responses.

Furthermore, Wassenaar *et al.* (1993) found that the actual presence of flagellin A was more important than motility in colonization of chicks by studying mutants that elaborated flagellin A but exhibited impaired motility. It was later discovered that the *maf* genes are related to biosynthesis of the flagellum as well as phase-variation of the flagellum (Karlyshev *et al.*, 2002) and that *maf5*-impaired mutants were unable to attach to surfaces to form biofilms (Joshua *et al.*, 2006) or effectively colonize chicks (Jones *et al.*, 2004). In a review of the role of flagella in *Campylobacter*, Guerry (2007) suggests that the flagellum acts as a secretion system for certain virulence-associated proteins, supporting this assertion by the fact that *C. jejuni* lacks specialized type III secretion systems such as those found to be vital to the virulence of other enteric pathogens. In a study seeking to characterize infection of *C. jejuni* by bacteriophages, Coward *et al.* (2006) found that phase-variation in CPS and flagellar expression were major factors in the effectiveness of bacteriophages. As indicated previously, the composite structure of the flagella of *Campylobacter* may be advantageous in avoiding immune responses, and it appears that *Campylobacter* may have the capacity to express a variety of different phenotypes in both its flagellum and CPS in order to avoid immune responses.

In addition to flagellar motility, surface spreading motility in *C. jejuni* strains was observed on 1.5% agar plates by Karlyshev *et al.* (2002) and was found to be absent in *kpsM*-deficient mutants, indicating that the CPS may play a role in the motility of *Campylobacter*. Surface polysaccharides excreted into the local environment have been demonstrated to play a role in swarming motility in *Salmonella enterica* (Toguchi *et al.*, 2000) and *Proteus mirabilis* (Gygi *et al.*, 1995), and are thought to facilitate this motility by providing a gel matrix to reduce friction.

Motility and CPS formation are both clearly linked to the virulence of *Campylobacter* strains. The flagella and CPS are prominent cell-surface structures, mediating a number of interactions with the host environment. One of the objectives of the present study was to further elucidate the relationship between the CPS and motility of *Campylobacter* through the observation of differences in motility between *kpsM*-deficient and wild type strains.

Competence

Competence is defined as the natural ability of a bacterium to acquire extracellular DNA from the surrounding environment and pass this DNA onto its offspring, and this capability has been observed in over 40 bacterial species across a broad spectrum of phylogeny (Lorenz and Wackernagel, 1994). The ability of *Campylobacter* to undergo natural transformation has long been known. Wang and Taylor (1990) observed competence in strains of *C. jejuni* and *C. coli*. Interestingly, they found that the bacteria more readily took up *Campylobacter* DNA than that of *E. coli*. Wilson *et al.* (2003) found that increases in cell density resulted in a decrease in natural

transformation in liquid shake cultures of 3 competent *C. jejuni* strains. Their study also found that transformation rates declined when cell growth rates increased, and this was determined by varying the amount of atmospheric CO₂ in order to vary the growth rate, with higher CO₂ concentrations allowing for higher growth rates and lower transformation rates. These results support the assertion that competence is a means by which the cell can exchange DNA during times of limited growth and low cell density in order to improve cell viability. Conversely, it may be that competence is repressed at high cell densities for the same reason, in order that no genetic modifications impede continued growth.

A study by Bacon *et al.* (2000) found that the inactivation *comB3*, a gene located on a *C. jejuni* 81-176 plasmid, impaired natural transformation compared to wild type strains. This gene encodes the ComB3 protein, which is associated with the membrane. It is plausible that the presence of this protein facilitates the process of binding the DNA to the cell surface. This study also suggested a link between competence and virulence in *C. jejuni*, as the mutant cells were less able to adhere to and invade INT407 cells. Jeon *et al.* (2008) established that the interruption of Cj1211, a homolog of *comH3* in *H. pylori*, stopped natural transformation for antibiotic resistance in *C. jejuni*.

In a review by Dubnau (1999), the mechanisms of DNA uptake for both Gram-negative and Gram-positive bacteria are described. It was noted that exogenous DNA must interact with the cell surface in order for natural transformation to occur. This fact alone suggests that surface polysaccharides may be involved in either mediating or discouraging natural transformation, and one objective of the present study was to

characterize what, if any, role the CPS may play in this interaction to affect competence in *Campylobacter*. According to the study by Jeon *et al.* (2009), the presence of capsular polysaccharide or lipooligosaccharide hindered the ability of *C. jejuni* to undergo natural transformation for antibiotic resistance genes. Though recent, this was the first study to shed light on the significance of surface polysaccharides in bacterial competence. The authors proposed two possible explanations for this behavior. Firstly, it may be that the DNA and the surface polysaccharides are both negatively charged, causing them to repel each other and subsequently preventing the transforming DNA from access to the cell surface. The other consideration is that perhaps surface polysaccharides act as a physical barrier to hinder the attachment of DNA to receptors on the outer membrane. In either case, the fact that the absence of a capsule promotes competence may suggest that one purpose of natural transformation may be to acquire advantageous traits such as capsule formation (Jeon *et al.*, 2009).

Survival in low-nutrient environments

The ability of *Campylobacter* strains to survive for extended periods of time in water has been well documented. A study in 1986 by Rollins and Colwell demonstrated that *C. jejuni* strain HC could survive for up to 4 months in stream water held in static conditions, with survival in agitated conditions decreasing logarithmically. Buswell *et al.* (1998) compared survival in water between two *Campylobacter* strains, finding that temperature and oxygenation differences had dramatic effects on survival. It was found that the two strains reacted differently to particular temperature and oxygenation conditions. Biofilm formation was also found to significantly extend the survival time of

these two strains. A study of 19 strains by Cools *et al.* (2003) found that *C. jejuni* survivability in sterile drinking water at 4°C varied widely by strain, with poultry isolates exhibiting the best survival. Three of the nineteen strains involved in the study remained culturable for over 29 days. Using Bolton broth, a medium specifically designed for resuscitating non-culturable cells, it was found that all 19 strains could be recovered after a period of 64 days. This indicates a high degree of persistence that can be obtained by viable-but-non-culturable (VBNC) cells in the environment and demonstrates that conditions exist under which these VBNC cells can again be restored to normal function. It should be noted that there is some debate as to whether the VBNC state truly exists, since some scientists propose that the mysterious “recovery” of these organisms is simply the growth of residual, culturable organisms. In either case, the ability of *Campylobacter* species to persist in some form in water for an extended period of time has been unequivocally established by these studies, which merits further inquiry into the mechanisms by which the bacteria survive in these low-nutrient stress environments. Of particular relevance to our study was what, if any, advantage the capsular polysaccharide offers in this process.

Surface polysaccharides have been demonstrated in other organisms to occur as part of the organism’s response to starvation, which suggests that their presence may have a positive impact on survival. According to Wai *et al.* (1998), low-nutrient environments induce EPS production in *V. cholerae*. It was found that the EPS played a pivotal role in attachment for biofilm formation, which has also been found to be the case for biofilm formation in *E. coli* K-12 (Danese *et al.*, 2000). Wrangstadh *et al.* (1986)

observed that marine *Pseudomonas* species elaborated an EPS in response to complete energy and nutrient starvation in a static environment of VNSS broth, but not in an agitated environment. Furthermore, the EPS did not alter the surface tension or viscosity of the starvation medium, suggesting that its intended function was not to alter the local microenvironment in these ways.

Campylobacter colonization of broiler houses resulting from a contaminated water source has been documented (Pearson *et al.*, 1993), and *C. jejuni* biofilms have been isolated from broiler house water systems (Trachoo *et al.*, 2002). Waterborne outbreaks of campylobacteriosis in humans have also been observed (Vogt *et al.*, 1982; Sacks *et al.*, 1986). Intuition from observations in other organisms suggests that the CPS may play a role in water survival via biofilm formation, nutrient conservation, or both.

Freeze-thaw stress

The freezing process itself induces dehydration stress on cells as intracellular water is removed as ice, which causes the concentration of solutes (Mazur, 1970). It has been found that the presence of trehalose improves freezing survival, as has also been demonstrated for desiccation survival, further establishing the similarities between these stress conditions (Wiemken, 1990). Park *et al.* (1998) found evidence supporting the prediction that part of the damage caused by freezing and thawing possibly resulted during thawing from a release of superoxide radicals, and this was corroborated by their study on the role of superoxide dismutase in tolerance of freeze-thaw stress in *Saccharomyces cerevisiae*. A study by Stead and Park (2000) showed the diminished capability of a SOD-deficient *C. coli* mutant to survive under freeze-thaw conditions,

indicating the presence of super-oxide anions that result from the stress process. In the same study, it was shown that a SOD-deficient, catalase-deficient double mutant did not exhibit a further reduction in survival tolerance, indicating that H₂O₂ is not formed as a result of the freeze-thaw process.

Tolerance to freezing and freeze-thawing in *Campylobacter* species at -20°C has been shown to be strain-specific, independent of strain source, and independent of the degree of cold tolerance at 4°C (Chan *et al.*, 2001). Saha *et al.* (1991) found that some freeze-thaw injured, nonculturable *C. jejuni* strains could be rejuvenated to virulent form by passage through a host, demonstrated using rats. Of the 16 strains used in the study, 7 were re-isolated after passage through the rats, and became virulent after successive passages. Lee *et al.* (1998) demonstrated the ability of *C. jejuni* 81116 to survive on the skin of chicken under freezing and thawing conditions comparable to what would be done by consumers in the home. They observed that the bacteria remained viable after freezing at both -20°C and -70°C and were able to grow quickly after thawing. Georgsson *et al.* (2005) studied the effects of varying the total time in the frozen state as well as the thawing temperature. They reported a one log₁₀ reduction immediately after freezing, with nearly constant cell counts throughout days 31-220 of frozen storage. The highest recorded log reduction after 31 days of storage was 2.87. It was found that thawing at 7°C resulted in a lower log reduction rate compared to thawing at 22°C.

Extracellular polysaccharides in *Nostoc commune* have been shown to be vital to survival and recovery of photosynthesis mechanisms following both freezing and desiccation stress (Tamaru *et al.*, 2005), indicating that surface polysaccharides may play

a role in freeze-thaw survival. One proposed mechanism by which EPS and CPS may confer desiccation resistance is by creating a hydrated microenvironment in which the bacterial cells take refuge. This cannot be the case for the dehydration induced by freezing stress since all of the water in the microenvironment is eventually frozen; therefore, if any advantage is conferred by the presence of CPS or EPS in freezing tolerance, then it must be by another, presumably more sophisticated, mechanism. On the other hand, there is support for the notion that CPS confers resistance to the oxidative stress in *Pseudomonas aeruginosa* (Sabra *et al.*, 2002), indicating that the CPS may be important to preventing oxidative damage resulting from the thawing process. In any event, one of the aims of the present study was to determine if any measurable resistance to freeze-thaw stress was gained from the presence of the capsule in *Campylobacter*.

The desiccation process and resistance

The process of desiccation for a biological system refers to the nearly absolute dehydration of or removal of water from cells through air-drying or the addition of solutes. Tolerance to desiccation, commonly termed anhydrobiosis, is highly uncharacteristic for most cells since this type of extreme water loss and metabolic suspension causes extensive damage to the structural integrity and physiological processing of the cell (Potts, 1994; Alpert, 2005). Furthermore, the ability of a biological system to survive desiccation relies heavily on the development of a variety of mechanisms to combat the phase changes associated with the drying process, and these mechanisms and survival capabilities fluctuate widely between organisms (Crowe *et al.*, 1997). Because having sufficient water available is critical to cell processes, dried cells

are in a state of suspended metabolism. Therefore, the ability of a cell to survive this environmental pressure is determined by whether the regular metabolic processes can be reactivated with the reintroduction of water (Crowe *et al.*, 1992). In light of this, desiccation tolerance may be defined as the ability to survive drying down, possibly to a level of water equilibrium between the cell and its environment, to be sustained in suspended animation for the duration of the desiccation stress, and to maintain the ability to recover normal function upon the removal of the external pressure by the addition of water.

There are three main phases that a desiccation-tolerant cell will have to withstand: drying, desiccation, and rehydration (Potts, 2001). The first stage, drying, encompasses the entire process of water loss, the rate of which is a deterministic factor in desiccation sensitivity and collective damage to a cell. The rate of drying has been shown to have an acute effect on survival, with a quick or intense rate of drying being the most deleterious. Two different studies carried out by Marshal *et al.* (1976) and Arkestein-Dijksman *et al.* (1979) both showed a decrease in the survival of rhizobia when dried rapidly. This concept was further substantiated in studies carried out by Leben *et al.* (1976), and, later, Mary *et al.* (1985), which conversely showed that bacteria are better able to survive a slower rate of drying. Furthermore, the discrepancy between the survival rates supports the hypothesis that the drying phase has the most important impact on overall survivability. There are a number of physiological responses that may be contributing to the difference in survival rates observed in the aforementioned studies. The most notable include the buildup of salts and solutes along with other osmotic stresses, the interruption

of metabolic processes, and the damage incurred to essential macromolecules in the cell (Vriezen *et al.*, 2007). A study by Record *et al.* (1998) purports that the decrease in the survival of rapidly dried *E. coli* cells could be due to the lack of time for the cells to produce protective osmolytes, a notion demonstrating that responses to desiccation are contingent upon the metabolic status of the cell immediately prior to the drying process. Overall, there is a consensus that an extended dehydration period allows the cell more time to adjust and acquire tolerance mechanisms.

Desiccation, the second phase of the drying process, starts when a biological system enters into a state of suspended lifelessness and ends once the rehydration recovery phase commences (Vriezen *et al.*, 2007). Most desiccation-sensitive bacteria cannot endure a cellular water content below 0.3 water/g, while true anhydrobiotes can survive an intracellular water content low enough to lose the monolayer of water around macromolecules, ceasing all cellular metabolism (Brown, 1990; Potts, 1999; Billi, 2002). A study carried out by del Maro *et al.* (2000) further defined the loss of cell viability as the cessation of gene expression and the inability to be revived into a culturable state. Under favorable conditions, some desiccation-tolerant organisms can survive in this dry state for decades or even centuries; however, as with many life-sustaining attributes, smaller organisms are more likely to survive harsh environmental conditions (Crowe *et al.*, 1992; Potts, 2001).

Rehydration is the third phase that a cell must overcome during the progression of the desiccation process. Many of the same challenges associated with the previous two stages will again become relevant, exacerbating the detriment accrued up to that point.

The total amount of damage elicited is highly dependent on a number of factors, including the rate of drying and/or rewetting, the duration of storage in the desiccated state, and the temperature during drying, storage, and rewetting. Throughout these stages and during the recovery process, much of the cell injury can be attributed to the formation of reactive oxygen species (ROS) that are unable to be combated during the metabolic arrest induced by dehydration. The appearance of ROS plays a pivotal role during the desiccation process, with the ability to cause severe structural damage to DNA, proteins, and lipids within the cell (Potts, 1994). Although a decrease in metabolic processes can slow the production of ROS, it will also inhibit both the cell's ability to remove any ROS that may be present as well as any attempt to repair the incurred damage (Potts, 1999; Billi and Potts, 2000; Stead and Park, 2000). The synthesis of superoxide dismutase (SOD) and catalase, along with other oxygen-scavenging proteins, may be employed to offset the accumulation of free radicals. Some of these enzymes have been detected in desiccated systems still fully functioning up to several years later. For example, it has been found that active Fe-SOD persists in dried Cyanobacteria after 13 years and in the cytoplasm of dried *Chroococcidiopsis sp.* after one year (Caiola *et al.*, 1996; Shirkey *et al.*, 2000). Furthermore, in a study carried out by Markillie *et al.* (1999), mutant strains of catalase- and SOD-deficient *Deinococcus radiodurans* exhibited increased sensitivity to radiation, an environmental stressor for which survival strategies are similar to those employed in desiccation (Mattimore and Battista, 1996).

In the initial stages of slow air-drying, during which there is still sufficient water activity for the cell to continue reproduction, an attempt to balance intracellular water is

made via osmotic adjustments. Membrane instability is one of the most widespread and catastrophic occurrences associated with the drying process (Black and Pritchard, 2002). The loss of membrane rigidity and function is mediated through osmotic and oxygen stresses, leading to a loss of membrane permeability and cell lysis. Many desiccation-tolerant prokaryotes allow an influx of inorganic ions and accumulate compatible solutes in order to stabilize proteins and other macromolecules in the cell (Crowe *et al.*, 1987; 1997; Bolen, 2001; Oliver *et al.*, 2002). The accumulation of compatible solutes such as trehalose and sucrose has been reported as a water and osmotic stress response in desert cyanobacteria, a biological system commonly studied in reference to desiccation tolerance (Reed *et al.*, 1986; Hershkovitz *et al.*, 1991). It is hypothesized that these two disaccharides can help to replace lost water by preventing gel formation in the membrane and the denaturation of proteins (Clegg, 1978), thereby inhibiting fatal membrane leakage and further stabilizing the structure of the dried cytoplasm (Crowe *et al.*, 1997; 1998; Potts, 2001). Alternatively, the efflux of osmoprotectants has been shown to relieve membrane tension in *E. coli* cells during osmotic stress (Ajouz *et al.*, 1998).

Desiccation survival assays

Many methods have been utilized to study the desiccation process and to assess the mechanisms of tolerance for a number of biological systems. Most of the methods to remove cell water exploit either osmotic or matric stress. In general, osmotic stress involves placing the system of interest in or near an aqueous solution or agar medium containing solute(s) that promote an efflux of water molecules out of the cell, while matric stress draws water out of the cell through altering atmospheric conditions

(Winston and Bates, 1960; Harris *et al.*, 1970; Potts, 1994). A variety of techniques have also been used to change the rate of drying, with the use of airflow in a laminar cabinet or a desiccant such as silica gel to expedite the process, whereas drying in a closed container would effect a more gradual desiccation process (Liang and Sun, 2000). While the environment of a truly desiccated cell cannot be reproduced using an osmotic stress technique, this method is commonly used to induce water stress for the convenience of being able to harvest cells and measure uptake and excretion of compounds associated with metabolism (Potts, 1994; Billi and Potts, 2002). Conversely, atmospheric desiccation allows for air-drying in an environment with a complete lack of water, providing insight into mechanisms of true anhydrobiosis by simulating a biological system unable to rely on an influx of solutes for protection (Potts, 1994). These two methods were studied by Nash *et al.* (1990), in which four lichen species were subjected to water stress in a salt solution and matric stress in a gas exchange system. Both techniques produced a marked decrease in metabolism and photosynthesis, with atmospheric desiccation being the most severe.

Past desiccation studies have made use of a variety of support systems in order to maintain and preserve cells throughout the assays, usually dependent on what type of environmental condition is most relevant to the biological system of interest. For example, many assays developed for the study of soil microbes used some type of soil or sand as the drying medium (Chen and Alexander, 1972; Roberson and Firestone, 1992; Fierer *et al.*, 2002). Chen and Alexander (1972) kept counts of viable soil bacteria over time from two collected soil samples incubated in a sterile desiccator containing CaCl₂;

in contrast, Roberson and Firestone (1992) actually inoculated soil *Pseudomonas sp.* onto a sand matrix and mineral salts growth medium in a Petri plate before drying in a desiccator containing LiCl to maintain a low relative humidity environment inside the chamber. The transforming ability of *Bacillus subtilis* was also tested on clay particles that were air-dried and then rewetted in a centrifuge tube covered by parafilm (Nannipieri *et al.*, 1997). These techniques may also be amended for use in the study of different eukaryotic organisms. Marron *et al.* (2003) used a very simple method with vials of silica gel desiccant to study the effects of desiccation on the energy metabolism of *Drosophila melanogaster*.

Membrane filters and cover slips are commonly used as convenient surfaces on which to immobilize cells for drying. Two desiccation studies by Mary *et al.* (1985; 1986) were performed by inoculating 2×10^9 *Rhizobium meliloti* cells onto membrane filters through the rapid suction of 1 mL of cell suspension from the exponential and stationary growth phases. The filters were then dried to 0% relative humidity, achieved with silica gel as the desiccant, and then resuspended in buffer by vortexing the dried filter. A more recent study by Sadowsky *et al.* (2007) harvested *Bradyrhizobium japonicum* cells by filtration onto membrane filters that were then vacuum dried and placed into Petri dishes inside a desiccator containing a potassium acetate solution in order to achieve the desired relative humidity. The use of glass cover slips to determine the survival of *Acinetobacter* spp. dried in Petri dishes over a CaCl_2 salt solution was another desiccation technique outlined by Jawad *et al.* (1996) and further utilized in two follow-up studies (Jawad and Heritage *et al.*, 1998; Jawad *et al.*, 1998). A cotton swab

was another potential surface that worked very well in a study of anaerobic and aerobic bacterial transport by Yrios *et al.* (1975). This material seemed to be more absorbent, minimizing possibly deleterious desiccation and oxidation, and produced a high recovery rate of 78% when resuspended by vortexing.

Surface polysaccharides and resistance to desiccation

Extracellular polysaccharide structures have been studied for their potential as protection against desiccation. In a study carried out by Ophir and Gutnick (1994), improved resistance to desiccation was observed in mucoid strains of *Eschericia coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* when compared to nonmucoid mutant strains. A study by Pena-Cabriales and Alexander (1979) found that suspending a species of *Rhizobium* in the polysaccharides that they produce aided in desiccation resistance. Extracellular polysaccharides have also been shown to have a measurable effect on the survival of *Rhizobium* when excreted at different relative humidity values, significantly improving the survival rate at 0% relative humidity conditions (Mary *et al.*, 1986). On the other hand, their presence negatively impacted survival at 22, 43, and 83% relative humidity. Additionally, Vanderlinde *et al.* (2009) showed that lipopolysaccharide-deficient mutants of *Rhizobium* exhibited reduced tolerance to desiccation and osmotic stress.

A more in depth study by Roberson and Firestone (1992) examined the link between desiccation conditions and exopolysaccharide production in *Pseudomonas* spp. Cultures that were desiccated while growing in a sand matrix were found to produce more extracellular polysaccharide and less protein than those that were grown in a

hydrated sand matrix. They also found that augmenting the sand matrix with the EPS caused the sand to dry more slowly, suggesting that the EPS does in fact aid in shielding the bacteria from desiccation by changing their microenvironment, helping them to retain water in the small area surrounding them. In support of this hypothesis, a study by Hill *et al.* (1997) observed that the EPS prevents the fusion of phosphatidylcholine membrane vesicles when dried at 0% relative humidity. Further research involving *Nostoc commune*, a well-studied desiccation-tolerant cyanobacterium, found that the EPS plays a vital role in preserving the ability of the bacterium to carry out photosynthesis following desiccation and rehydration (Tamaru *et al.*, 2005). It was found that laboratory strains producing only a marginal amount of EPS were more sensitive to desiccation, as were wild-type strains after removing the EPS physically by washing.

Exopolysaccharides and capsular polysaccharides have been shown in a number of organisms to have similar properties. Bonet *et al.* (1993) studied the production of EPS and CPS under varying conditions for *Aeromonas salmonicida*. The absence of glucose, phosphate, magnesium chloride, or trace minerals from the growth medium inhibited the production of both CPS and EPS. The production of both polysaccharides began at the end of the logarithmic growth phase, and the relative amounts of each were not affected by the C/N ratio or temperature changes of 15°C to 20°C.

The water-binding activity of surface polysaccharides may contribute to the desiccation tolerance of certain organisms. Water composes over 95% of the CPS structure and its formation can be triggered by changes in external osmolarity (Roberts, 1996). The production of capsule-like colanic acid, a key exopolysaccharide involved in

biofilm formation in *E. coli*, changes in response to osmotic stress, with obvious implications for desiccation resistance (Whitfield, 2006). While the regulation of surface polysaccharide expression is not fully understood, its formation creates a hydrated gel coating around the outer layer of the cell that could shield against desiccation and other environmental disturbances.

In conclusion, the pervasiveness of *Campylobacter* in the food supply and its associated disease burden makes research into the prevention of contamination and outbreaks relevant to current worldwide food safety issues, warranting further investigation. The position and structure of the CPS on the outside of the cell facilitates access to the molecule; therefore, ongoing studies into the interplay between the CPS and the survivability of the organism will serve as a foundation from which methods of disease prevention may arise. Furthermore, the CPS is not only readily accessible by foreign molecules, but it is also involved in wide variety of biological and pathogenic processes, making it a prime target for future developments in therapeutic and antimicrobial treatments.

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Chapter 2

Roles of the Capsule in Environmental Adaptations of Animal-derived

***C. jejuni* and *C. coli* Isolates**

Abstract

Campylobacter spp. is a food-borne pathogen that colonizes many food animals, persisting in many foods and in the environment. The capsule on the cell surface of *Campylobacter* may affect its virulence and survivability. The objective of this study is to determine possible roles of the CPS in environmental adaptations of animal-derived *C. jejuni* and *C. coli* isolates. Wild type strains of *C. coli* 6979, *C. jejuni* BS142, and *C. jejuni* SC1453 were compared to CPS-deficient mutant counterparts in terms of competence, motility, and during environmental stresses such as desiccation, freeze survival, and survival in a low-nutrient environment. The mutant strains were produced through site-specific mutagenesis of the *kpsM* gene involved in CPS transport. The results of this study suggest species-specific trends in the effects of capsule on competence, motility, and desiccation resistance of *Campylobacter*, with the CPS conferring overall protection and resistance throughout many of the assays. These characteristics have been associated with the survivability and pathogenicity of *Campylobacter*, therefore future experimentation and analysis of *kpsM*-deficient mutants will give insight into the function and purpose of the capsule in virulence and environmental adaptations.

Introduction

Campylobacter spp. is the one of the leading bacterial agents implicated in food-borne illness throughout the world (Allos, 2001). Human infection typically occurs as a result of *Campylobacter*'s persistence in the gut of animals used for human consumption or through a contaminated water supply (Harris *et al.*, 1986). *C. jejuni* and *C. coli* strains are primarily responsible for *Campylobacter* infections, with a study by Friedman *et al.* (2000) reporting that 85% of cases are attributed to *C. jejuni* strains and that most of the remaining cases are caused by *C. coli* strains. In addition to diarrheal and other intestinal symptoms associated with campylobacteriosis, it is hypothesized that infection by *Campylobacter* is one of the most important antecedents in the development of MFS and GBS (Newswanger and Warren, 2004), which are characterized by descending and ascending paralysis, respectively (Godschalk *et al.*, 2007). This is thought to occur as an autoimmune response resulting from human ganglioside mimicry of certain lipooligosaccharide antigens (Koga *et al.*, 2001). Between the high incidence of gastrointestinal campylobacteriosis and the complications resulting in MFS and GBS syndromes, the worldwide disease burden of *Campylobacter* species is substantial and merits research that will eventually lead to prevention.

Evidence from previous studies indicates that surface polysaccharides are integral factors involved in many bacterial survival and pathogenicity traits. The link between CPS and virulence has been well established. Studies have demonstrated CPS-associated pathogenicity of *Campylobacter* in chickens, ferrets, and human epithelial cells (Bacon *et al.*, 2001; Jones *et al.*, 2004). Motility is vital to attachment and colonization and is a

well-recognized virulence factor in *Campylobacter* (Jones *et al.*, 2004; Bacon *et al.*, 2000). Karlyshev *et al.* (2002) discovered surface spreading motility in *C. jejuni* strains that was absent in *kpsM*-deficient mutants, indicating that the CPS was involved to some degree in motility. *Campylobacter* species are also competent organisms, and it has been shown that the presence of CPS or LOS inhibits natural transformation in *C. jejuni* (Jeon *et al.*, 2009). Desiccation survival is an important trait for conditions that may be encountered when transferring between hosts. Bacterial capsules have been identified as a key virulence factor for several pathogens, possibly by mediating adhesion to surfaces, aiding in evading and resisting immunogenic responses, and preventing desiccation (Roberts, 1996). Studies of EPS in *Nostoc commune* have suggested that surface polysaccharides are also important to freeze-thaw and desiccation survival (Tamaru *et al.*, 2005). This protection is thought to be achieved by resistance to oxidative stress conferred by surface polysaccharides (Sabra *et al.*, 2002). Production of surface polysaccharides has been linked to survival in low-nutrient environments, such as deionized water survival, in *Vibrio cholerae* (Wai *et al.*, 1998) and marine *Pseudomonas* species (Wrangstadh *et al.*, 1986). Furthermore, Wai *et al.* (1998) found that the EPS produced in *V. cholerae* was vital to biofilm formation, which may be a mechanism for enhanced survival in low-nutrient environments.

There have been few studies on possible survival advantages conferred by CPS and those that have been completed focused primarily on human isolates, such as *C. jejuni* 11168, rather than animal-derived strains. Studies with animal-derived strains would be more relevant to understanding the role of the capsule in food-borne

campylobacteriosis from contamination during slaughter and processing. Also, previous research has demonstrated the necessity of the capsule for adequate antibiotic resistance and effective colonization in chicks, further linking CPS-associated benefits with animal-derived isolates and virulence (Xiong, 2008). For these reasons, the present study seeks to determine the extent to which the CPS of *Campylobacter* aids in survival following exposure to various stresses.

Materials and Methods

Bacterial strains and culture conditions

All strains were stored long-term at -80°C in brain heart infusion broth (BHI, Becton Dickinson, Sparks, MD) supplemented with 20% glycerol. For fresh cell growth prior to experimentation, cells were streaked onto tryptic soy agar supplemented with 5% sheep blood (BAP, Remel, Lenexa, KS) and incubated microaerobically at 42°C for 36-48 hours. Cells were then grown on 1.2% Mueller Hinton agar (MHA, Becton Dickinson, Sparks, MD) microaerobically at 42°C for 36-48 hours before either being resuspended in MH broth (or DI H₂O when observing survival in deionized water) or grown up overnight in MHB. One exception, *C. jejuni* BS142, was grown up on slightly harder 1.4% agar MHA plates due to the considerable spreading of the wild type colonies. The harder agar yielded smaller and more separated colonies, making them easier to count. Dilutions of the suspensions were plated on MHA to obtain colony forming units per milliliter (CFUs/mL) at time 0 and at various time points chosen to analyze survival for each assay. The microaerobic environment was achieved using the CampyPak Microaerophilic System (BBL, Sparks, MD). The strains used for this study are listed in Table 2.1.

Table 2.1. *Campylobacter* spp. strains used in this study

Strain	Species	Source	Date of Isolation
6979	<i>C. coli</i>	Turkey (fecal)	06/22/04
6979 M1	<i>C. coli</i>	J. Xiong	05/12/08
BS142	<i>C. jejuni</i>	Dairy cow (colon)	02/25/03
BS142 M1	<i>C. jejuni</i>	J. Xiong	09/27/08
SC1453	<i>C. jejuni</i>	Turkey (cecum)	12/14/06
SC1453 M1	<i>C. jejuni</i>	J. Xiong	09/27/08
7474	<i>C. coli</i>	Turkey (fecal)	07/22/04
7474 M1	<i>C. coli</i>	J. Xiong	09/01/08
6067	<i>C. coli</i>	Turkey (water)	11/15/03
6067 M1	<i>C. coli</i>	J. Xiong	09/10/08

Construction and confirmation of CPS mutants

Capsule-deficient mutants were constructed for the animal-derived strains *C. jejuni* BS142, *C. jejuni* SC1453, and *C. coli* 6979. Each of the chosen isolates was susceptible to kanamycin. *C. jejuni* DB208 is a CPS-deficient strain that was provided by P. Guerry to serve as DNA donor. The *kpsM* gene of this strain is interrupted by a 1427 bp kanamycin (kan^r) cassette at nt 575, effected by double-crossover exchange (Bacon *et al.*, 2001). The CPS-deficient mutants strains were formed through natural transformation following the general procedure of Kim *et al.* (2006), with the resulting *kpsM::kan^r* mutant strains being identified as BS142M1, SC1453M1, and 6979M1 (Xiong, 2008). The genomic DNA of DB208, and later a PCR product from the resultant mutants, was used as the donor for the natural transformation.

In order to confirm the presence of the kan^r cassette in the *kpsM* gene, PCR of the gDNA of each second-generation mutant strain was carried out by targeting the primers *kpsMT_F2* and *kpsMT_R*. The product of the PCR was compared with the *kpsMT* fragment amplified from the corresponding parent strains. The PCR products purified from the electrophoresis gel of both the parent strains and the second-generation mutants using the indicated primers were sequenced at the Genome Research Laboratory of North Carolina State University.

Forward primer:

kpsMT_F2: 18 mer 5'- ATGCCTGAAGGGATTCT -3'

Reverse primer:

kpsMT_R : 20 mer 5'- CCTATACTACAATTTTCAGG -3

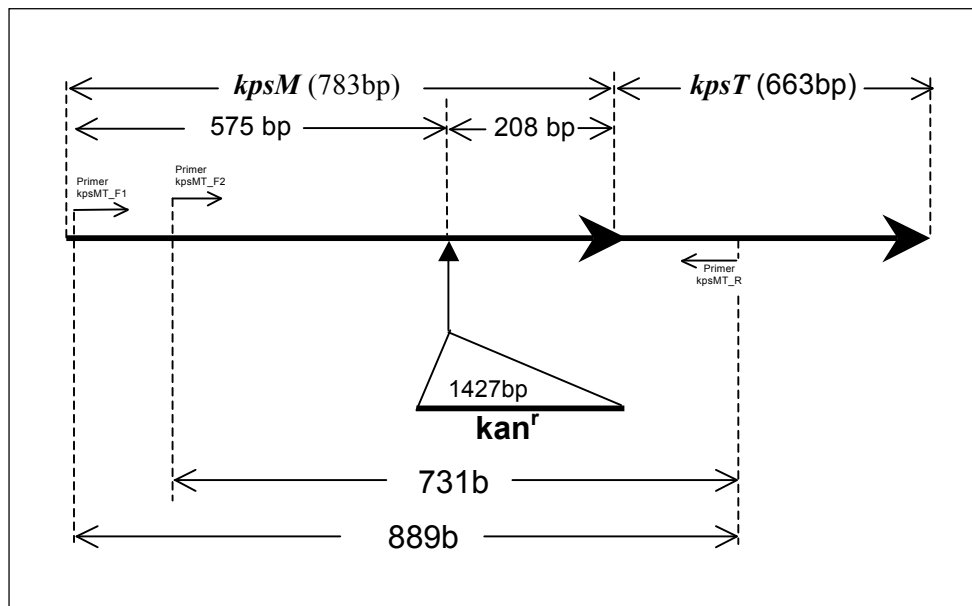


Figure 2.1. *Campylobacter* spp. *kpsM* mutant construction. Modified from Xiong, 2009.

Motility assessments and swarming assay

A soft agar assay previously described by Guerry *et al.* (1991) was modified and utilized to test for soft agar swarming of wild type and mutant strain pairs of interest. Colonies from both wild type and mutant strains were picked with a sterile loop and inoculated into 5 mL of Mueller Hinton (MH) broth separately. The inoculated tubes were incubated microaerobically at 42°C overnight and grown to an optical density (OD₆₀₀) of 0.1. Two microliters of wild type and mutant strains were then spotted in duplicate onto MHA with 0.4% agar. The soft agar plates were incubated for 24-48 hours and the appearance of the resultant swarms were compared in appearance and measured in centimeters.

Competence (transformation assay to nalidixic acid resistance)

A natural transformation assay previously described by Wiesner *et al.* (2003) was modified and utilized in order to test the difference in competence between wild type and mutant strains. The nalidixic acid transformation frequency was tested for wild type strains and their respective mutants using a nalidixic acid-resistant donor, *C. coli* 7725. Colonies from both wild type and mutant strains were picked with a sterile loop and inoculated into 3 mL of MHB separately and incubated microaerobically at 42°C overnight to an OD₆₀₀ of 0.1. The suspensions were then diluted and dilutions were plated onto both MHA and MHA + 100 ng of gDNA from donor *C. coli* 7725. To prepare plates with gDNA, 100 ng of the gDNA of *C. coli* 7725 was spread onto MHA and allowed to dry immediately prior to the plating of the dilutions of the recipient cultures. Following incubation for 36-48 hr microaerobically at 42°C, 20 colonies from both MHA w/out

DNA and MHA +DNA were then patched onto MHA and MHA + nalidixic acid (20 µg/ml) to determine frequency of nalidixic acid-resistant colonies.

Survival in deionized water and relative fitness

Cells were grown on MHA microaerobically at 42°C for 36-48 hours. For survival in deionized water, the cells were resuspended into 10 mL of pre-chilled (4°C) DI H₂O in capped tubes in duplicate and kept stored at 4°C throughout the assay. For the relative fitness in dH₂O, equal volumes (5 mL) of the wild-type and mutant cell suspensions were combined and stored as just described. Dilutions of the suspensions were plated on MHA to obtain CFUs/mL at time 0, day 3, day 5, day 7, and day 14. The mixtures for relative fitness assessments were plated on both MHA and MHA with kanamycin (50 µg/ml) at each time point to determine relative survival, as the mutant strains contain a kanamycin resistance cassette.

Freeze-thaw survival with and without cold shock

Cells were grown on MHA microaerobically at 42°C for 36-48 hours. The cells were resuspended into 5 mL of MHB and then 0.5 mL of each sample was aliquoted into 8-10 microcentrifuge tubes. The tubes were stored at -20°C throughout the assay. At selected times (day 1, 3, 5, and 7) tubes were removed in duplicate and thawed for 2 minutes in a room-temperature waterbath prior to dilutions and plating on MHA. To assess freeze-thaw survival in the presence of cold shock, cells suspensions were stored on ice for 4 hours prior to freezing while control suspensions were incubated at 42°C for 4 hours prior to freezing.

Desiccation and salt tolerance

For the desiccation tolerance and salt tolerance assays, cells were grown on MHA microaerobically at 42°C for 36-48 hours. Two to three colonies were then resuspended into 1mL of MHB with either a sterile loop or cotton swab. Two to three colonies were picked from the same plate with a cotton swab in duplicate, the swabs were placed in a closed petri dish and incubated in the dark at either 25°C or 4°C for 2 hours. After the 2 hours of incubation, the inoculated cotton swabs were resuspended in 1mL of MHB and the surviving CFU/mL were estimated for each swab at both time 0 and at 2 hours. The time 0 resuspension was also used to carry out the salt tolerance assay. Three microliters of each wild type and mutant resuspension were spotted in duplicate onto MHA with varying NaCl concentrations (0.5, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, and 4.5%). The plates were incubated microaerobically at 42°C for 48 hours and the resultant bacterial growth was compared.

Results

Confirmation of CPS mutants by PCR

Using the previously described primers, *kpsMT_F2* and *kpsMT_R*, PCR fragments were obtained that confirmed that the *kpsM* mutation was present in the mutants and absent in the wild type strains (Figure 2.2). The size of the PCR fragment for the wild type strains was expected to be 731 bp whereas for the mutant strains the expected size of the PCR products was 2158 bp. The observed PCR products had the expected size (Figure 2.2).

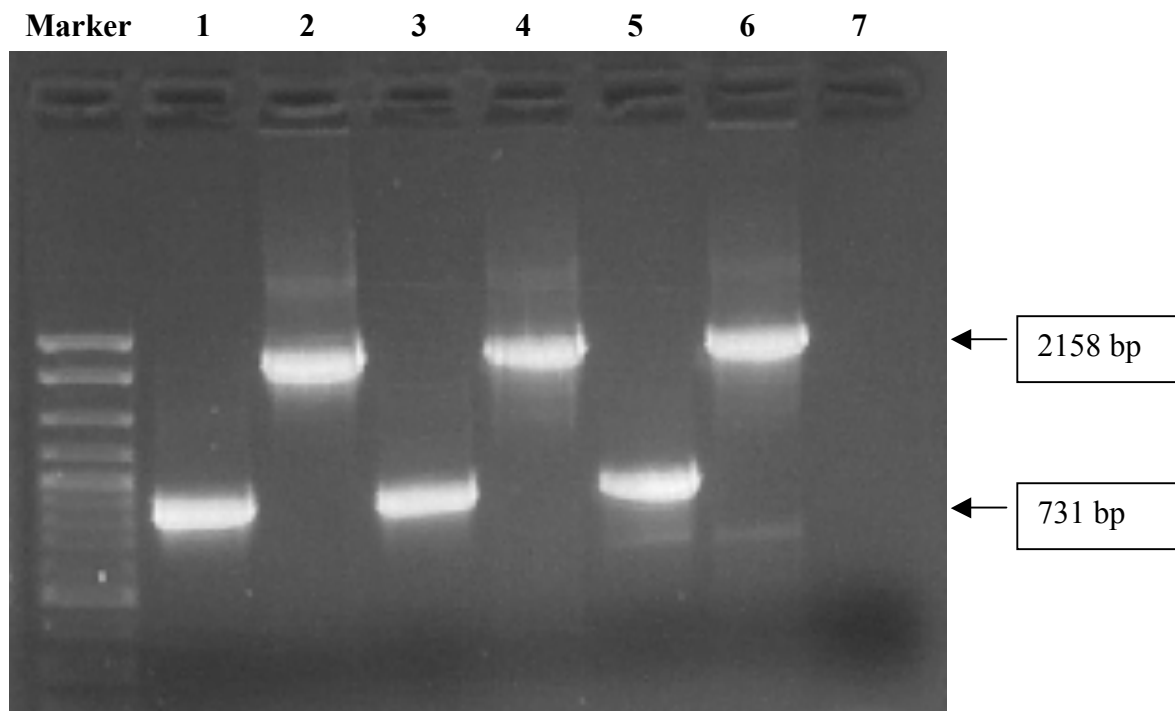


Figure 2.2. PCR assay with primers *kpsMT_F2* and *kpsMT_R*. Confirmation of presence or absence of kanamycin (Kan^r) cassette inserted into *kpsM* gene. (1.) *C. coli* 6979 (2.) *C. coli* 6979 M1 (3.) *C. jejuni* BS142 (4.) *C. jejuni* BS142 M1 (5.) *C. jejuni* SC1453 (6.) *C. jejuni* SC1453 M1 (7.) Negative control.

Swarming assays reveal strain-specific impact of *kpsM* inactivation

Swarming assays showed three types of results: Swarming was not visibly affected in the *kpsM* mutant of *C. coli* 6979 (Figure 2.3). In the case of both *C. jejuni* strains (BS142 and SC1453) the *kpsM* mutants produced smaller swarms on soft agar plates than their parental counterparts. And in the case of *C. coli* 7474 and *C. coli* 6067, the *kpsM* mutants produced larger swarms than their corresponding wild types. These results suggest a trend in motility at the species-level, and also highlight important differences in capsule-associated motility between the strains. We also noticed distinct differences in appearance of the swarms, suggesting differences in chemotaxis between parental strains. Furthermore, although we were able to recover mutants using PCR-based natural transformation for *C. coli* 6979, *C. jejuni* BS142, and *C. jejuni* SC1453, we did not for *C. coli* 7474. Additional assays to obtain a more directed mutation for *C. coli* 7474 from its PCR product were also unsuccessful; however, in each of these additional assays, *C. coli* 6979 was used as a control and produced *kpsM* deficient PCR-based mutations. Therefore, although competent, *C. coli* 7474 did not as readily transform as other isolates. In order to obtain a precise *kpsM* mutant for *C. coli* 7474, a more sensitive assay is required.

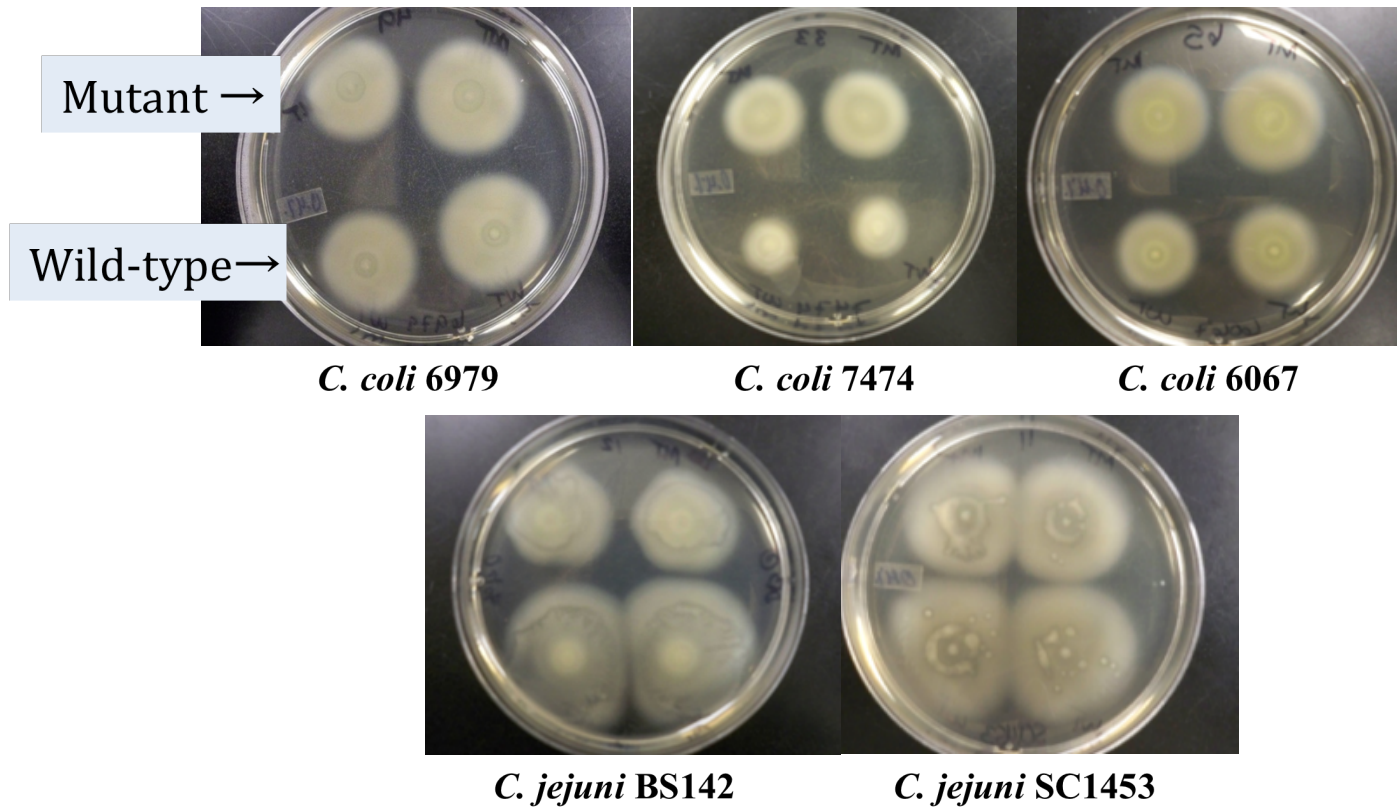


Figure 2.3. Motility swarms of *Campylobacter* spp. wild type strains and CPS mutants on 0.4% soft agar (MHA). The figure represents one of three independent experiments.

Assessment of competence of *kpsM* mutants

The *kpsM* mutant strain of *C. coli* 6979 was more competent than its wild type counterpart, with the average transformation frequency for the mutant being 19 out of 20 and the average for the wild type being 16 out of 20 (Table 2.2). The mutant for this strain was more readily able to naturally transform. A high transformation frequency was observed for *C. jejuni* strains BS142 and SC1453, yielding an average transformation frequency of 20 out of 20 for both wild type and mutant strains.

Table 2.2. Competence assay. Average transformation frequencies (number of positive growth patches out of 20 and spontaneous mutant growth patches out of 20) from two independent experiments.

Strain	MHA with Nalidixic Acid (20 µg/ml)	Spontaneous Mutants
<i>C. coli</i> 6979	16/20	0/20
	16/20	0/20
<i>C. coli</i> 6979 M1	19/20	0/20
	20/20	2/20
<i>C. jejuni</i> BS142	20/20	0/20
	20/20	0/20
<i>C. jejuni</i> BS142 M1	20/20	0/20
	20/20	0/20
<i>C. jejuni</i> SC1453	20/20	0/20
	20/20	0/20
<i>C. jejuni</i> SC1453 M1	20/20	0/20
	20/20	0/20

Survival in deionized water and relative fitness

There was a significant difference in survival at 4°C in deionized water between the wild type and mutant strain of *C. coli* 6979 with a consistent trend of the wild type performing better throughout both trials (Figure 2.4a). The mutant had a greater overall reduction in survival; however, the rate of reduction from day 3 through day 14 levels out to that of the wild type. This trend is not consistent with results from the second trial. Although the wild type continues to perform better than the mutant in the subsequent assay, the difference in the rate of survival between the two strains occurs at a later time point (Figure 3.1). There was also an initial log reduction of greater than 2 log₁₀ observed within the first 3 days and a greater than 4 log₁₀ reduction observed after 14 days for both the wild type and mutant strain (Figure 2.4b). The survival of both strains was also examined when maintained together in one suspension. The decrease in survival over time for the mixture was comparable to that of their survival when observed independently, with the wild type maintaining a slight competitive advantage throughout the assay (Figure 2.5a). The percentage of each strain as a part of the total mixture over time may also be viewed in Figure 2.5b.

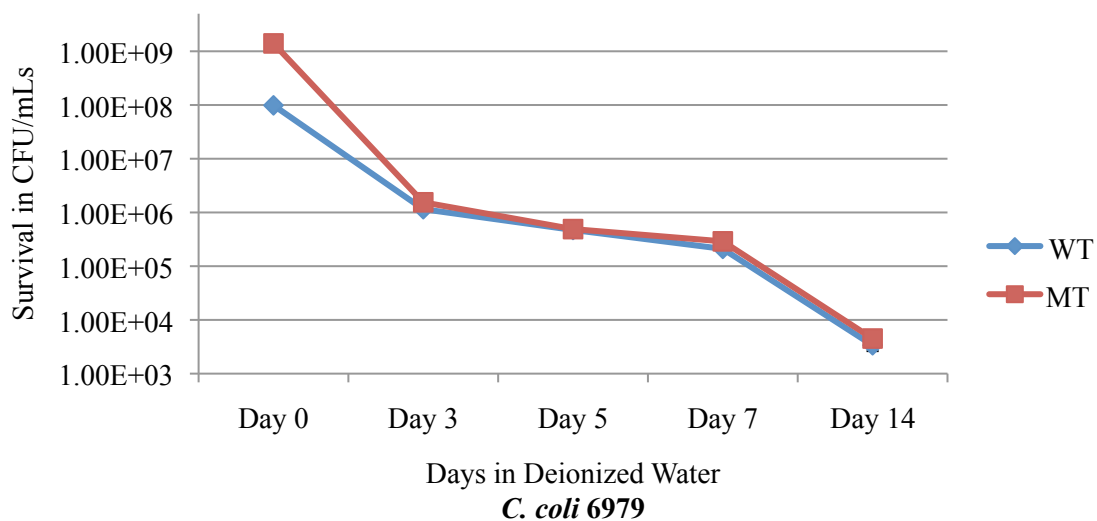


Figure 2.4a. Survival of *C. coli* 6979 wild type and mutant strains in DI H₂O at 4°C from day 0 through day 14. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4). See Table 3.1a (Trial 1) for raw data.

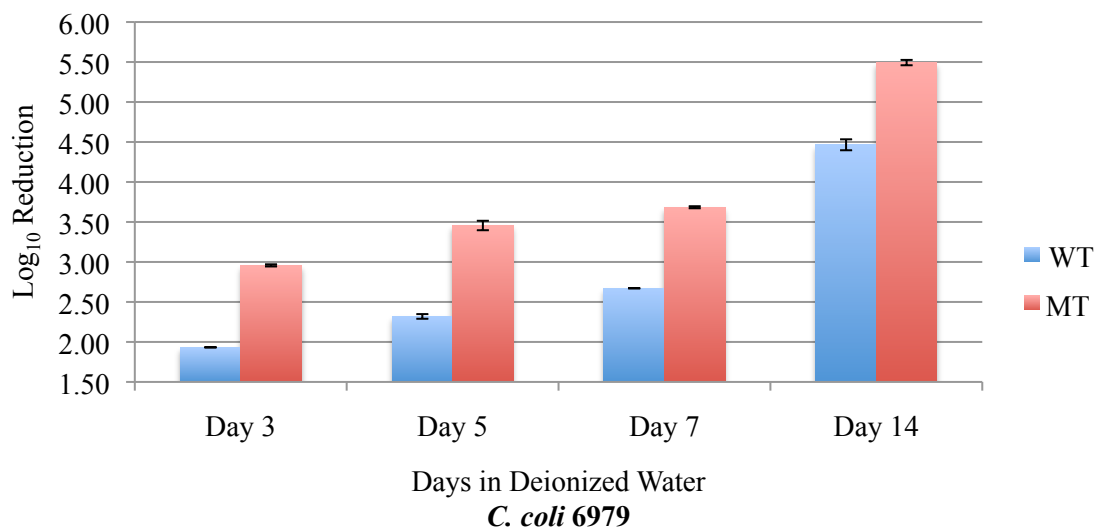


Figure 2.4b. Survival of *C. coli* 6979 wild type and mutant strains in DI H₂O at 4°C after 14 days. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.1a (Trial 1) for raw data.

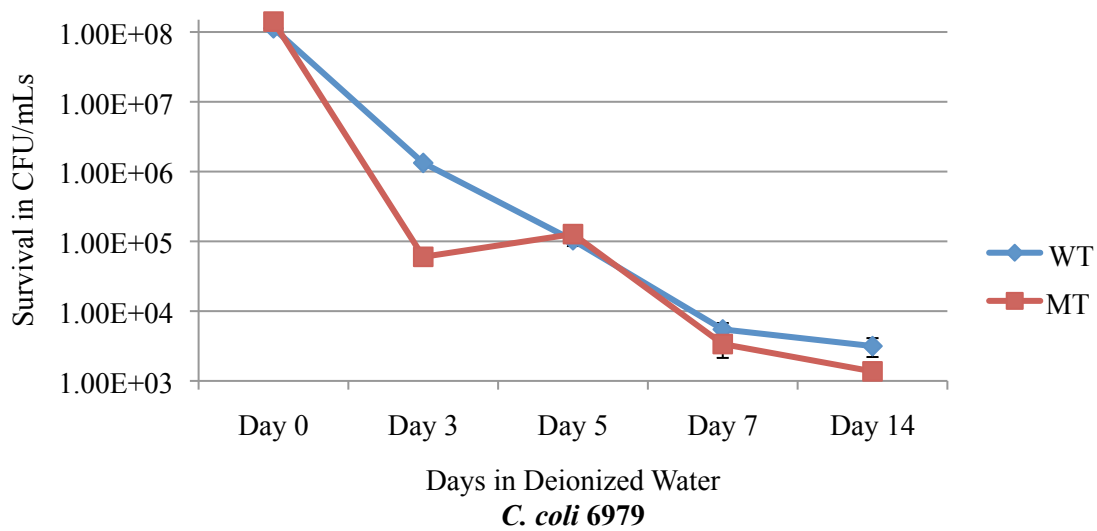


Figure 2.5a. Relative fitness of *C. coli* 6979 wild type and mutant strains in DI H₂O at 4°C for a time period of 14 days. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4).

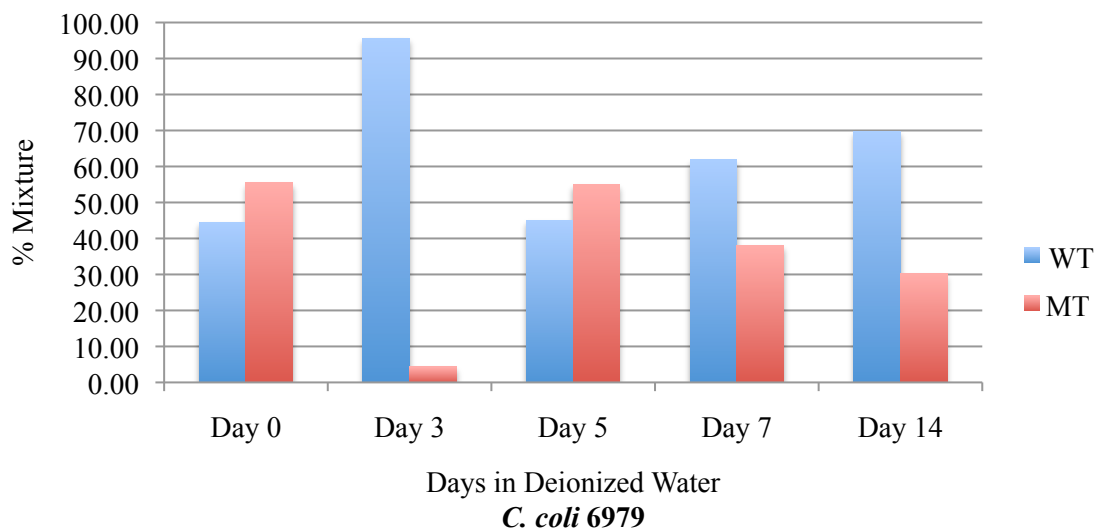


Figure 2.5b. Relative fitness of *C. coli* 6979 wild type and mutant strains in DI H₂O at 4°C for a time period of 14 days. This figure is one representation of two independent experiments. Each bar represents the mean of two replicates and the amount of cells present as a percentage of the total mixture at each time point.

Freeze-thaw survival with and without cold shock treatment

Both *C. coli* 6979 and its *kpsM* mutant had a large initial decrease in survival after being frozen for three days and then thawed (Figure 2.6a and 2.6b). The decrease in survival was not markedly different when the cells were thawed following longer periods at -20°C (five and seven days), suggesting that the loss in viability was associated primarily with the thawing event. Within the first three days of being frozen there was a loss of between 4 and 6 log₁₀ with a subsequent decrease of between 1 and 2 log₁₀ by day seven. *C. coli* 6979 exhibited the highest decrease in overall survival and the least difference in survival between its wild type and mutant counterpart after seven days of frozen storage (Figure 2.6a; Figure 2.6b). A difference in freeze-thaw tolerance between the wild type and the *kpsM* mutant was observed for *C. jejuni* BS142, with the wild type surviving better than the mutant (Figure 2.7a and 2.7b). The same trend was observed for the other *C. jejuni* strain that was tested, *C. jejuni* SC1453 (Figure 2.8a and 2.8b). Very little difference was observed between the *C. coli* 6979 and its *kpsM* mutant when freezing was preceded by cold shock (Figure 2.9a and 2.9b). Cold shock did not appear to enhance the freeze-thaw tolerance of the bacteria (Figure 2.9a and 2.9b). There was a lot of variation between the two cold shock freeze survival trials with neither indicating any significant protection conferred with or without a cold shock prior to freezing. The dramatic loss in viability upon freezing and thawing may have made it difficult to detect subtle differences between strains.

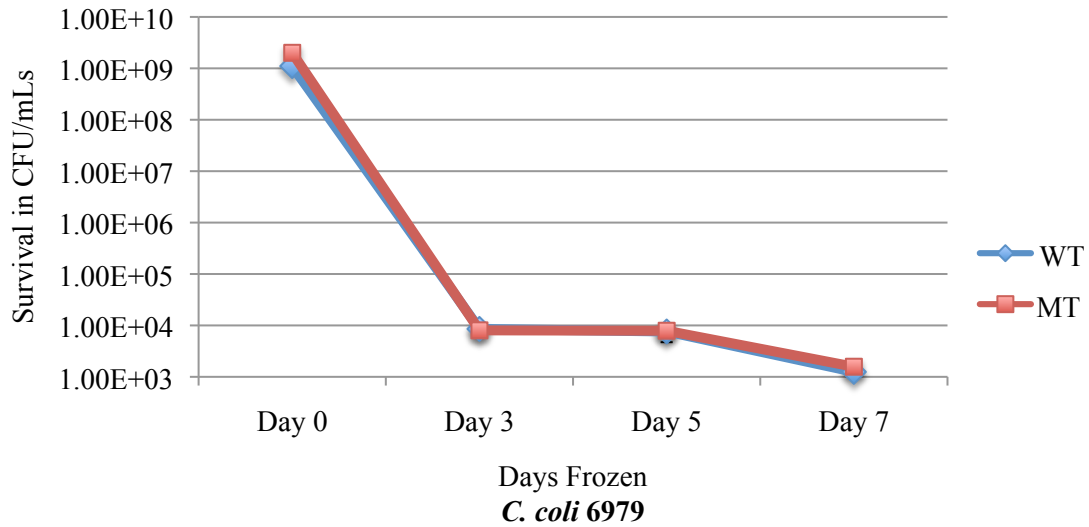


Figure 2.6a. Survival of *C. coli* 6979 wild type and mutant strains frozen at -20°C after 7 days. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4). See Table 3.2 (Trial 1) for raw data.

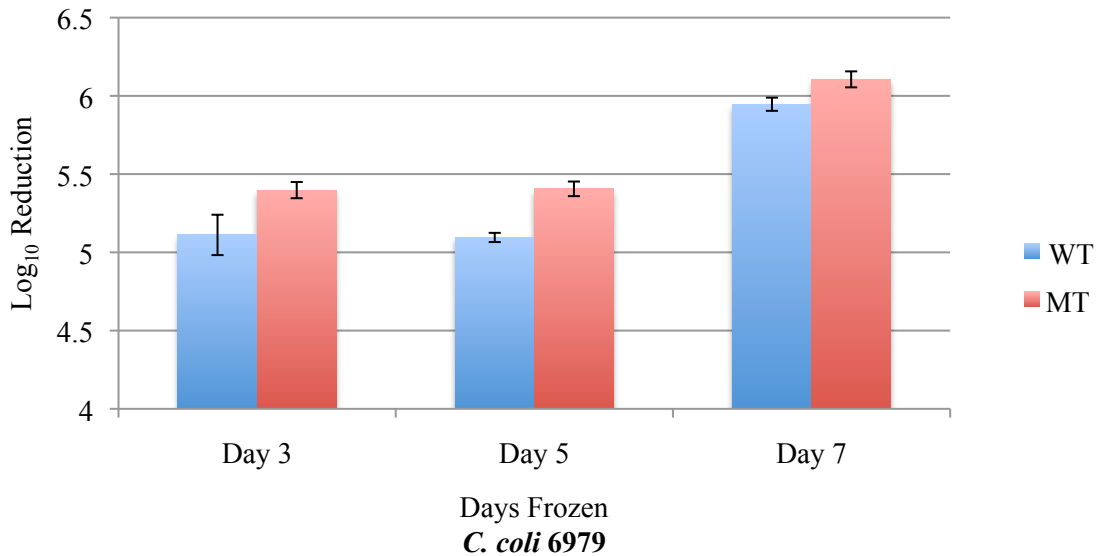


Figure 2.6b. Survival of *C. coli* 6979 wild type and mutant strains at -20°C after 7 days. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.2 (Trial 1) for raw data.

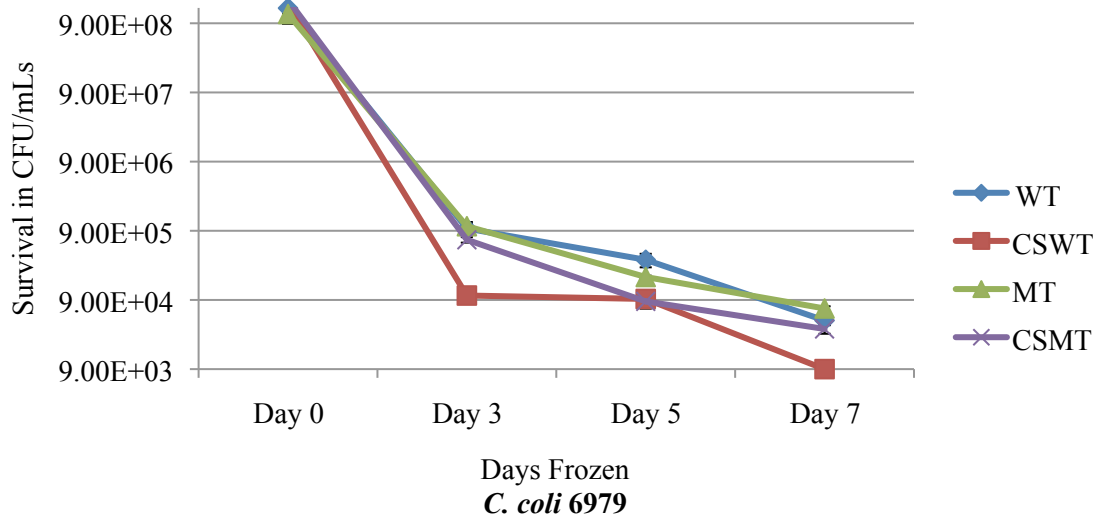


Figure 2.7a. Survival of *C. coli* 6979 wild type and mutant strains cold-shocked and frozen at -20°C after 7 days. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean ($n = 4$). See Table 3.5 (Trial 1) for raw data.

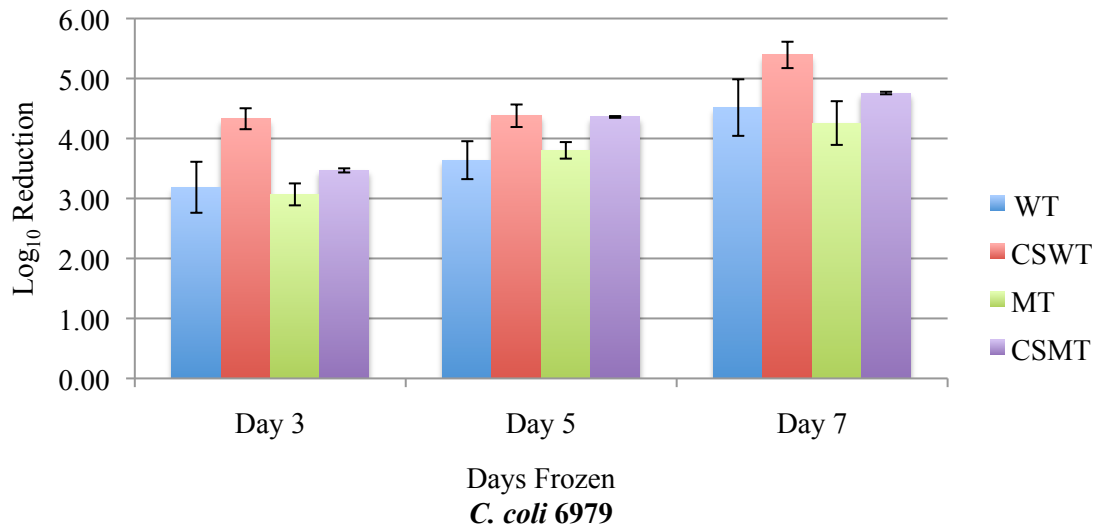


Figure 2.7b. Survival of *C. coli* 6979 wild type and mutant strains cold shocked and frozen at -20°C after 7 days. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean ($n = 4$). See Table 3.5 (Trial 1) for raw data.

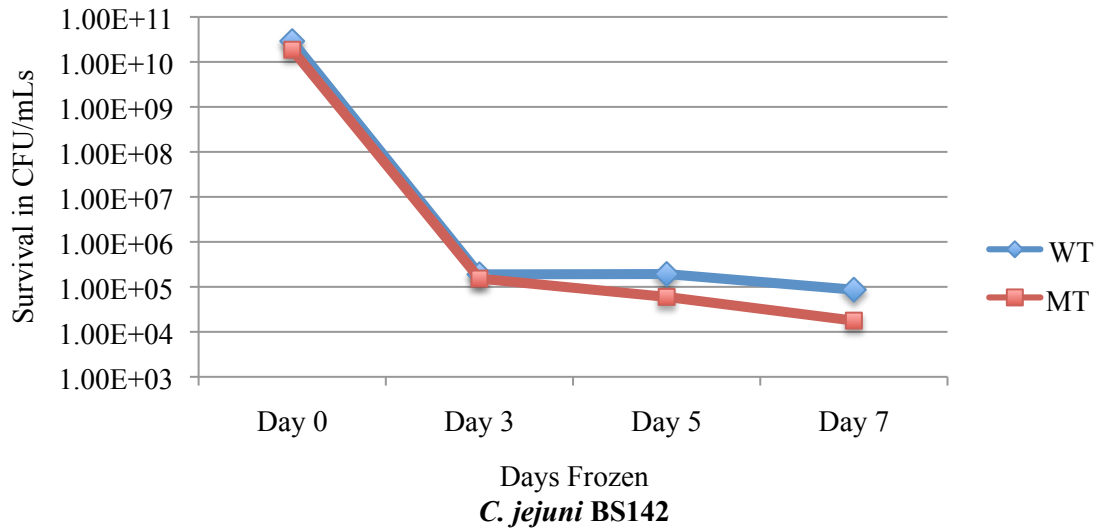


Figure 2.8a. Survival of *C. jejuni* BS142 wild type and mutant strains frozen at -20°C after 7 days. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4). See Table 3.3 (Trial 1) for raw data.

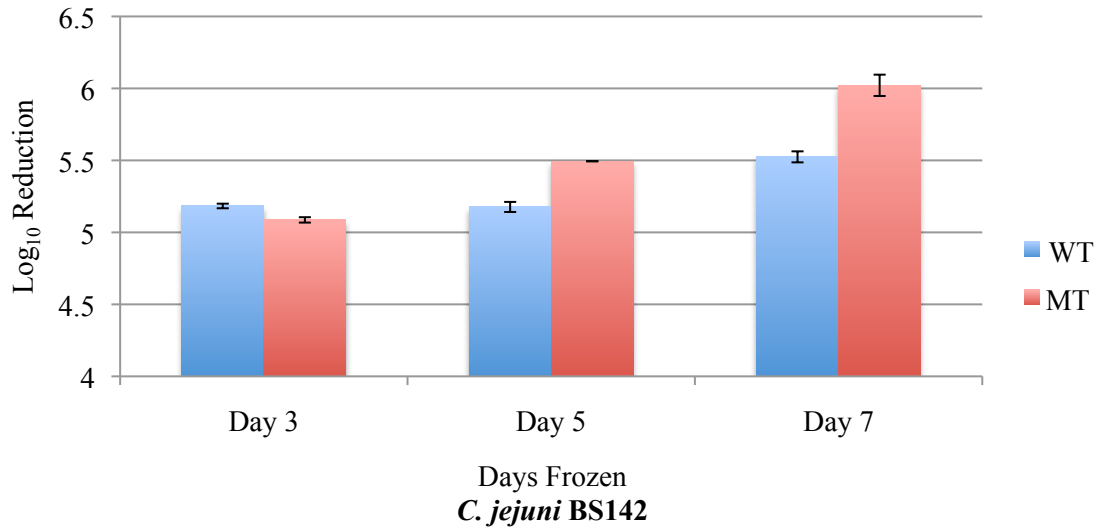


Figure 2.8b. Survival of *C. jejuni* BS142 wild type and mutant strains cold-shocked and frozen at -20°C after 7 days. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.3 (Trial 1) for raw data.

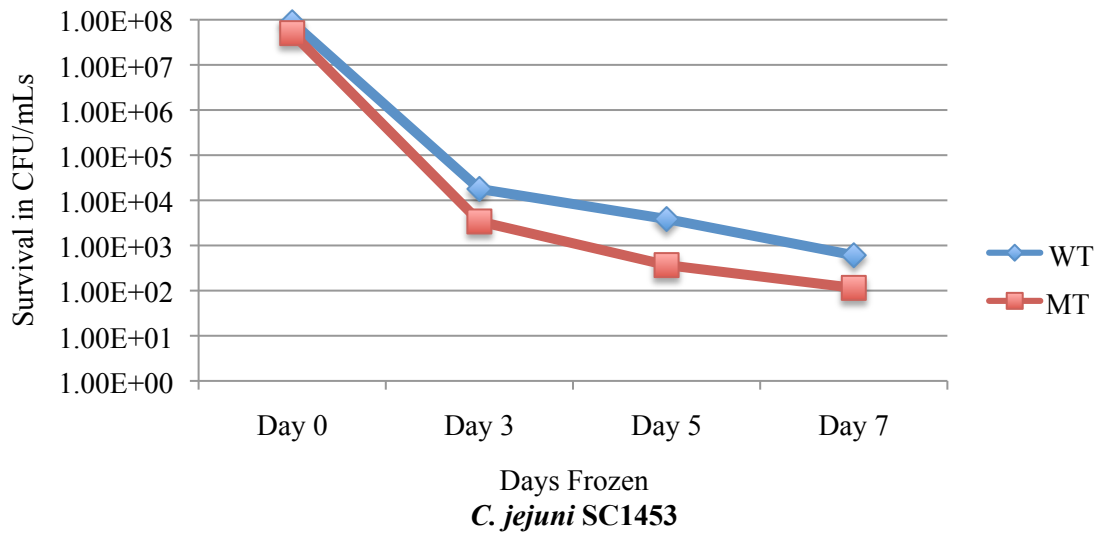


Figure 2.9a. Survival of *C. jejuni* SC1453 wild type and mutant strains frozen at -20°C after 7 days. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4). See Table 3.4 (Trial 1) for raw data.

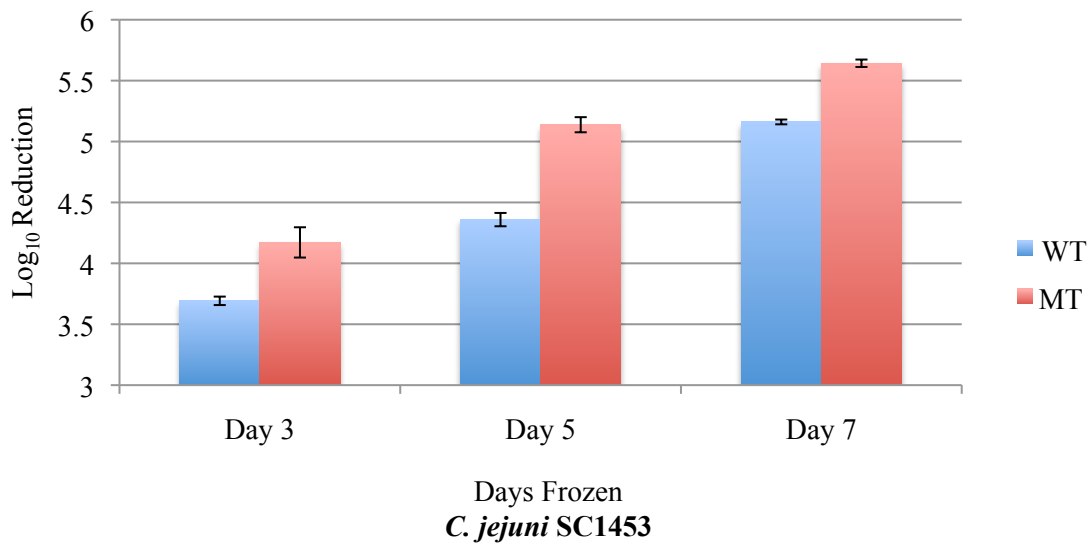


Figure 2.9b. Survival of *C. jejuni* SC1453 wild type and mutant strains frozen at -20°C after 7 days. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.4 (Trial 1) for raw data.

Desiccation and salt tolerance

The desiccation tolerance and salt tolerance of *Campylobacter* were tested to observe the effects of matric stress and water stress on survival and to examine any possible CPS impact on resistance. Desiccation survival at 4°C was significantly better than when bacteria were dried at room temperature, with a strain-dependent drop in survival 1 to 4 log₁₀ at 4°C and 4 to 6 log₁₀ at 25°C (Figure 2.10 and 2.11). Also, marked species-specific trends in survival were consistently observed. The two *C. jejuni* strains exhibited the largest difference in survival at both temperatures between wild type bacteria and their corresponding *kpsM* mutant. For each *C. jejuni* strain, the mutant survived better than the wild type, while the opposite was observed for *C. coli* 6979 during the desiccation assay. The wild type for *C. jejuni* SC1453 was the most sensitive to the desiccation process at both temperatures, while *C. coli* 6979 had the most sensitive mutant strain (Figure 2.10 and 2.11). Furthermore, although loss of survival was lower at 4°C, differences in survival between wild type strains and their respective mutants were more readily noted at this temperature (Figure 2.10).

The salt tolerance assay indicated that *C. jejuni* BS142 was the most salt tolerant strain tested, with the wild type growing on MHA with a concentration of up to 1.2% NaCl (Figure 2.12). Both the wild type and mutant strains of *C. coli* 6979 grew on MHA with up to 0.7% NaCl; however the mutant counterpart colonies had visibly less growth at this concentration. *C. jejuni* SC1453 wild type and mutant strains only grew up to 0.5% NaCl, making it the most sensitive to salt (Figure 2.12). These results suggest the maximum salt concentration on which the three *Campylobacter* strains can grow, but not

the maximum on which they may survive. Although the strains only produced colonies on plates with added salt up to the concentrations previously described, surviving and highly motile cells were observed at higher concentrations, even though visible growth was lacking. Live, motile cells were observed under microscopic examination on 0.7%, 1.0%, and 1.4% NaCl MHA plates for *C. jejuni* SC1453, *C. coli* 6979, and *C. jejuni* BS142 respectively (data not shown). Furthermore, microscopic examination of the strains at varying concentrations showed the progression of the effects of increasing salt on the cells, with the cells aggregating together prior to becoming coccoid in morphology (Figure 2.13)

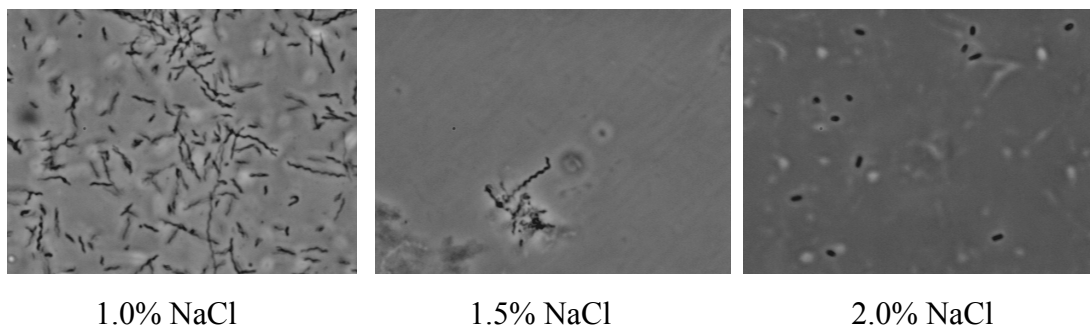


Figure 2.10. Morphology of *C. jejuni* BS142 wild type cells from varying concentrations of NaCl MHA agar media using phase contrast microscopy.

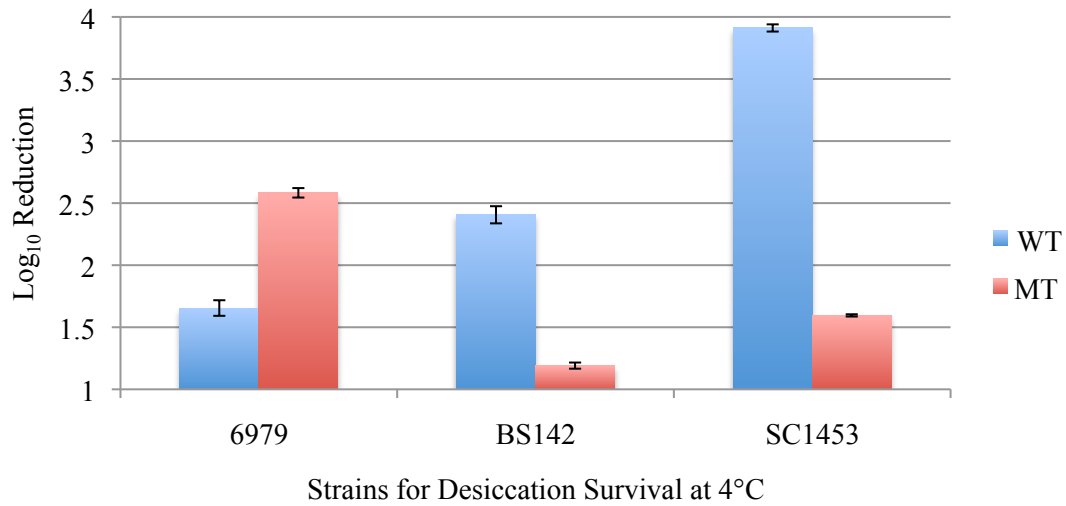


Figure 2.11. Survival of wild type and mutant strains after desiccation at 4°C for a 2 hour time period. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Tables 3.6a, 3.7a, and 3.8a (Trial 1) for raw data.

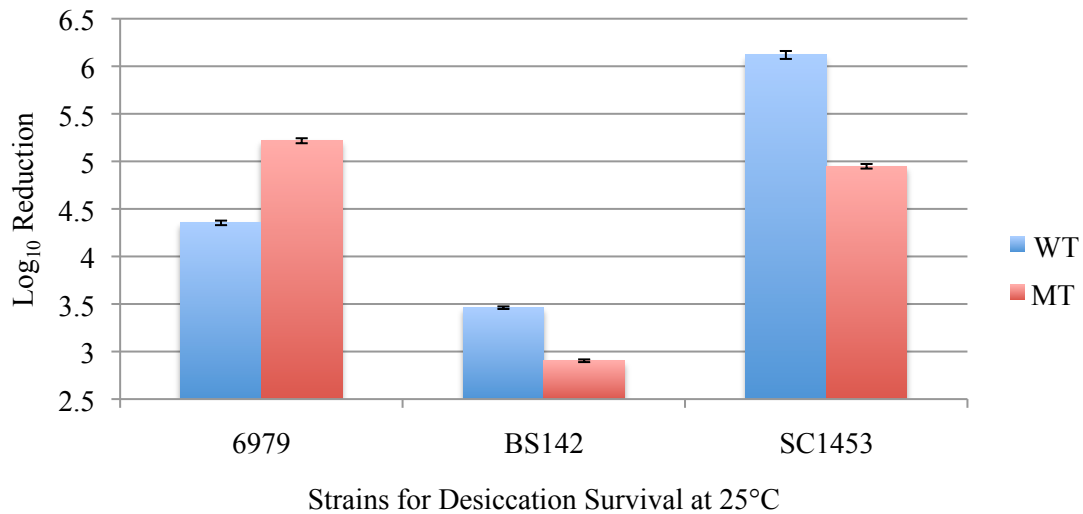


Figure 2.12. Survival of wild type and mutant strains after desiccation at 25°C for a 2 hour time period. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Tables 3.6b, 3.7b, and 3.8b (Trial 1) for raw data

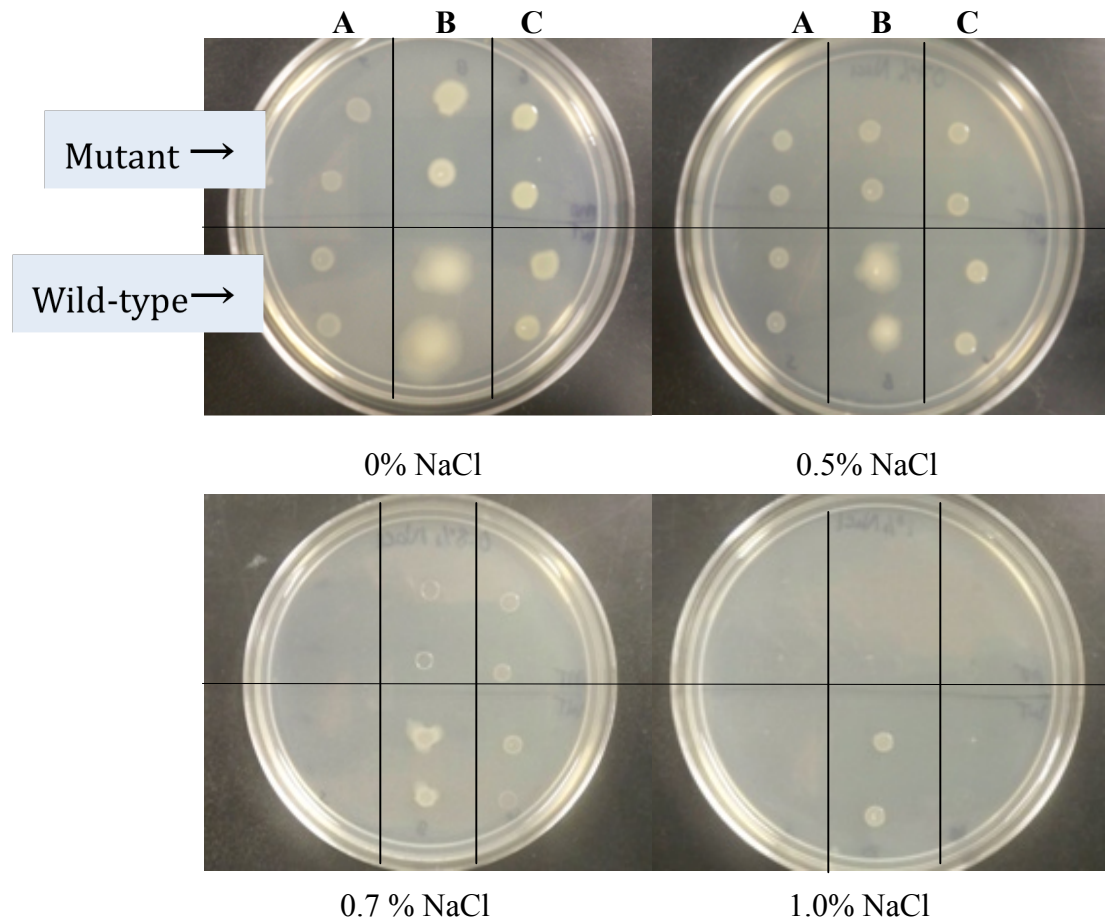


Figure 2.13. Salt tolerance assay. Growth of both wild type and mutant bacterial cells (in duplicate) on MHA containing varying amounts of NaCl. (A.) *C. jejuni* SC1453 (B.) *C. jejuni* BS142 (C.) *C. coli* 6979.

Discussion

The role and mechanisms of CPS function in *Campylobacter* have not yet been fully studied. Through the use of CPS-deficient mutants we were able to further characterize the phenotypic differences between wild type strains of *Campylobacter* and those lacking CPS. The CPS may be a contributing factor in the motility and surface spreading ability of *Campylobacter*. Not only have surface polysaccharides been shown to reduce friction and facilitate motility in other organisms (Gygi *et al.*, 1995; Toguchi *et al.*, 2000), but a study by Karlyshev *et al.* (2000) showed that the surface spreading ability of *C. jejuni* was absent in *kpsM*-deficient strains. In this study, the motility of both *C. jejuni* and *C. coli* strains were tested and observed in a soft agar motility assay. The growth and the formation of the swarms were different for each strain and there were also notable differences in their appearance between the wild type and mutant swarm formation for certain strains. Inactivation of *kpsM* was associated with reduced swarming in both tested *C. jejuni* strains, BS142 and SC1453, whereas swarming was not affected in *C. coli* 6979. Notably, there was a clear halo that visibly surrounded the mutant swarms, indicating that although there was no capsule formation, there may indeed be some other structure or carbohydrate produced and secreted from the cell when the capsule is not present. Further characterization of this material could give insight into mechanisms of survival when the capsule is inactive. These may imply an impact of CPS in the swarming assay that varies depending on species (*C. jejuni* vs. *C. coli*) and strain. The two *C. jejuni* strains tested in this study suggest that CPS is required for normal swarming in this species.

Competence, or the ability to pick up extraneous genetic material from the environment, is another important factor affecting the virulence and survivability of *Campylobacter*, as it is a process of DNA exchange that can lead to the acquisition of advantageous traits. Previous studies have suggested that *Campylobacter* is highly competent, especially within its own species, and that the CPS may be a possible inhibitor to the transformation process (Wilson *et al.*, 2003; Jeon *et al.*, 2009). The results of this study lend some support to this hypothesis. While the wild type and mutant of both *C. jejuni* strains tested could be transformed to nalidixic acid resistance with similar frequencies, the mutant strain of *C. coli* 6979 was more competent than its wild type counterpart. The average transformation frequency for the mutant was 19 out of 20 and 16 out of 20 for the wild type strain of *C. coli* 6979. The results show that both *C. jejuni* strains were more competent than the *C. coli* strain tested, but they also suggest that the mutant for *C. coli* 6979 was more readily able to be naturally transformed than the wild type, as also observed in previous studies. Possible explanations for this phenomenon involve the CPS acting as a physical barrier between the cell and its environment, therefore inhibiting contact with other genetic material. This hindrance may even involve a repelling mechanism between the negatively charged CPS and the negatively charged DNA molecules. It may also be a method by which cells that lack a CPS are attempting to acquire the genes necessary to produce one. Further testing utilizing a more defined assay and additional strains will provide more insight into the complexity of competence in *Campylobacter* and any roles the CPS may play in transformation.

Survival in a low-nutrient environment is critical to the movement of an organism from one host to another and is of particular relevance for *Campylobacter* in the form of waterborne outbreaks with and without the involvement of an intermediate animal host. The presence of surface polysaccharides has been shown to positively impact survivability in many organisms, even increasing in production under low-nutrient conditions (Wrangstadh *et al.*, 1986; Wai *et al.*, 1998; Danese *et al.*, 2000). The survival of wild type and mutant strains of *C. coli* 6979 was observed in a low-nutrient environment (deionized water at 4°C) for a time period of two weeks. For this strain and under these conditions, the wild type did perform better than the mutant lacking the CPS. These results suggest that some protection was conferred by the CPS to the surviving cells. The protection of the CPS detected in this assay lends support to previous research, implying some inherent protection and involvement of the capsule in these types of conditions. The large reduction in survival of the mutant within the first three days of trial one suggests that the protection elicited by the CPS is most effective in the initial stress response. The differences in survival between the wild type and mutant strain observed in the first trial were delayed in the second trial. The lag in time before the CPS-associated advantages in survival were detected could be due to the higher initial mutant starting concentration in the second trial. Similar findings of better performance by the wild type were also observed in the relative fitness assay. Even though there was a similar and substantial decrease in survival when both the wild type and mutant were assessed together, the wild type retained a slight competitive advantage during the assay. In both assays, the presence of live cells after 14 days of storage with no nutrients available

shows the endurance of *Campylobacter*, a bacterial species that is notably fragile. This demonstrates *Campylobacter's* ability to survive the types of conditions indicative of what the organism may encounter before and after coming into contact with a host, making the study of any contributing factors worthy of further investigation.

Survival under conditions of freezing and thawing is also of importance, not just environmentally, but in reference to the processing of food commonly used for products associated with contamination by *Campylobacter*. The process of freezing and subsequent thawing has been shown to lead to the concentration of protective solutes as well as the release of damaging superoxide anions, particularly while thawing (Mazur, 1970; Park *et al.*, 1998). This process produces effects similar to those of desiccation. Studies carried out by Stead and Park (2000) and by Weimken (1990) showed a decrease in freeze-thaw survival without intrinsic capabilities of superoxide protection and that an increase in compatible solutes can help shield the cell from freezing and desiccation processing, further establishing similarities between the two processes and bacterial tolerance to the associated stresses. As with any environmental stress, the interplay of injurious processes and possible mechanisms of protection can become problematic when performing an assay and evaluating results.

The results obtained during the freeze-thaw survival assay lend further support to the protective capacity of the capsule, with the wild types for all three strains performing better than their corresponding mutant. The overall decrease in survival was similar for the three strains, with the differentiation between the wild type and mutant being the most distinct for *C. jejuni* SC1453. Possibly due to the increased sensitivity of this strain in

many assays when compared to either *C. jejuni* BS142 or *C. coli* 6979, *C. jejuni* SC1453 has not performed as well as a whole and is the slowest growing of the three strains. As with many of the stressors utilized in this study, the initial response and loss of survival was quite extensive. The subsequent assessments for the freeze-thaw survival assay at days five and seven yielded a decrease in survival similar to that observed at day 3, suggesting that most of the loss in survival was due either to the initial freezing process or to the thawing process as opposed to a longer time period of frozen storage. With much of the damage attributed to the thawing process in previous research, the process of thawing the cells after an initial freezing step is likely eliciting the most injury. This conclusion was also observed when carrying out preliminary testing. The cells were injured to an undetectable state when frozen and thawed more than once. Furthermore, although previous studies have intimated that an initial cold shock prior to freezing may elicit some protection, no such conclusions may be drawn from the results presented in this study. There were no significant differences in survival observed, perhaps due to variation and sensitivity of the cells during the two hour cold shock storage on ice. During this time period, the cells would have been subjected to a more oxygen-rich atmosphere than optimal for the bacterium's survival and growth.

The results of the salt tolerance assay contribute evidence to the sensitivity of *C. jejuni* SC1453 and the mutant strains when compared to their respective wild type counterparts. The mutants for all three strains were more sensitive to salt than their corresponding wild type strains, with the *C. jejuni* BS142 wild type strain being the most salt tolerant when compared to its mutant and the other strains tested. *C. jejuni* SC1453

was the most susceptible to salt, a sensitivity that may be associated with its slower growth. Notably, the maximum salt concentrations for the growth of each strain does not necessarily correspond to their survival. All three strains were alive and motile at a salt concentration somewhat higher than they were able to grow; however, the trend of their growth and survival remained the same.

The desiccation assay yielded results that were unexpected in that the mutant for both *C. jejuni* strains, BS142 and SC1453, performed better than their wild type counterparts. As the desiccation process can have a similar effect on cells as freezing, and both *C. jejuni* wild type strains had stronger phenotypic characteristics in other assays (e.g. motility, competence, freeze-thaw survival), these results were unanticipated. Desiccation tolerance, especially when assessed at room temperature, was the most severe environmental condition utilized to test the strains in this study. There was a massive decrease in survival in a very short time period which may not have allowed for the full development and progression of survival for the strains to come to fruition; however, under these conditions and during the short time period that was assessed, there seems to be a strain-dependent trend for the mutant of *C. jejuni* to survive better than the wild type. Although, there are likely a variety of factors contributing to this outcome, it may be that the assay is so taxing that initially the mutant is able to survive better because there is no energy expenditure in capsule production and the sugars that are then present inside the mutant cells can be utilized for sustainability. Also, the mutant cells seemed to aggregate more than the wild type cells when visualized after the desiccation process via phase contrast microscopy. The lack of capsule and possible secretion of an alternative

carbohydrate structure, discussed previously in reference to the motility assay, may be contributing to an aggregation, biofilm-like survival mechanism in which the cells can be protected without a capsule. The desiccation process may be such a detrimental stressor to *Campylobacter* that the aggregation survival strategy was able to show through, while also providing enough protection to the mutant cells so as to minimize the differences seen between the wild type and mutant survival during other assays.

One potential source of variation throughout the assays could be due to the cells being resuspended from plate growth. Difficulties stemmed from the colonies tending to stick together and the need to resuspend a large number of colonies into a relatively small amount of liquid, making consistent initial starting inoculums challenging to optimize between trials. *Campylobacter* is highly sensitive to a number of different stresses. As a result, designing an assay to observe the effects of a particular stress on *Campylobacter* must involve consideration of multiple factors that may be difficult to control. Most notably, inherent sensitivities of *Campylobacter* to oxygen may produce atmospheric variations during experimentation. This is another substantial factor that likely contributed to disparities within and between trials.

The large differences observed between the strains for motility and competence, as well as past research regarding its effects on colonization, strongly support the CPS as a virulence determinant. It may be that the CPS has more association with facilitating entry into a host and evading host immune response than with overall survivability and environmental endurance of the organism. Whatever the case may be, the capsular polysaccharide associated with the outer surface of *Campylobacter* cells did confer

overall protection and resistance to environmental stress throughout many of the assays tested in this study, further asserting its position as a factor that aids in survivability and pathogenicity.

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Appendix

Additional Data and Graphs

Deionized Water

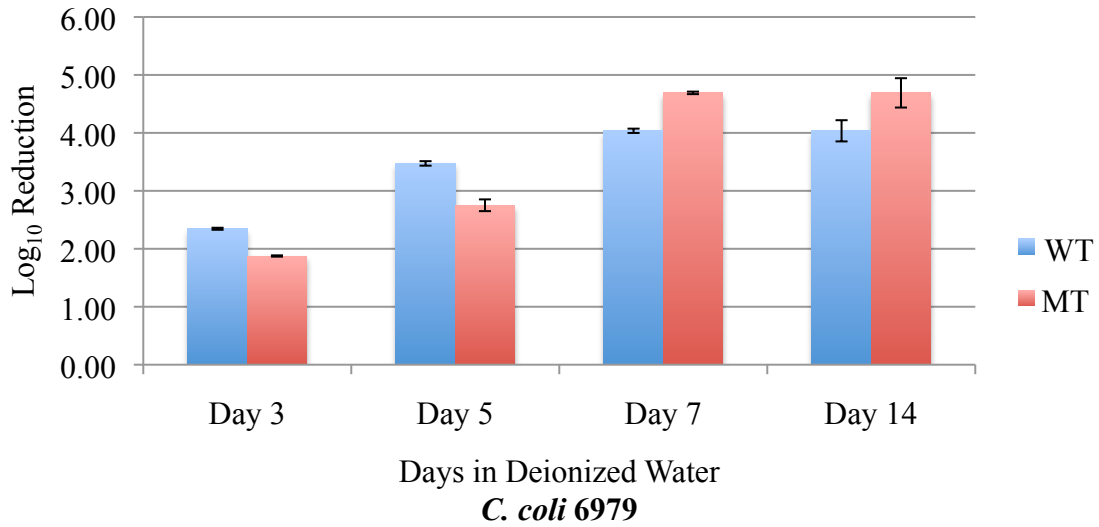


Figure 3.1. Survival of *C. coli* 6979 wild type and mutant strains in DI H₂O at 4°C from day 0 through day 14. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4). See Table 3.1b (Trial 2) for raw data.

Table 3.1a. Average survival in deionized water in CFU/mL over 14 days.

Trial 1	Day 0	Day 3	Day 5	Day 7	Day 14
WT	1.13E+08	5.10E+05	3.80E+04	1.04E+04	3.23E+03
MT	1.41E+08	1.88E+06	2.50E+05	2.88E+03	2.00E+03
Mix		1.39E+06	2.31E+05	8.85E+03	4.53E+03
K50		6.00E+04	1.27E+05	3.38E+03	1.37E+03

Table 3.1b. Average survival in deionized water in CFU/mL over 14 days.

Trial 2	Day 0	Day 3	Day 5	Day 7	Day 14
WT	9.85E+07	1.15E+06	4.73E+05	2.10E+05	3.38E+03
MT	1.40E+09	1.55E+06	4.90E+05	2.90E+05	4.50E+03
Mix		9.98E+05	1.22E+06	2.12E+05	3.60E+03
K50		5.65E+05	1.44E+05	5.50E+04	1.48E+03

Freeze-Thaw

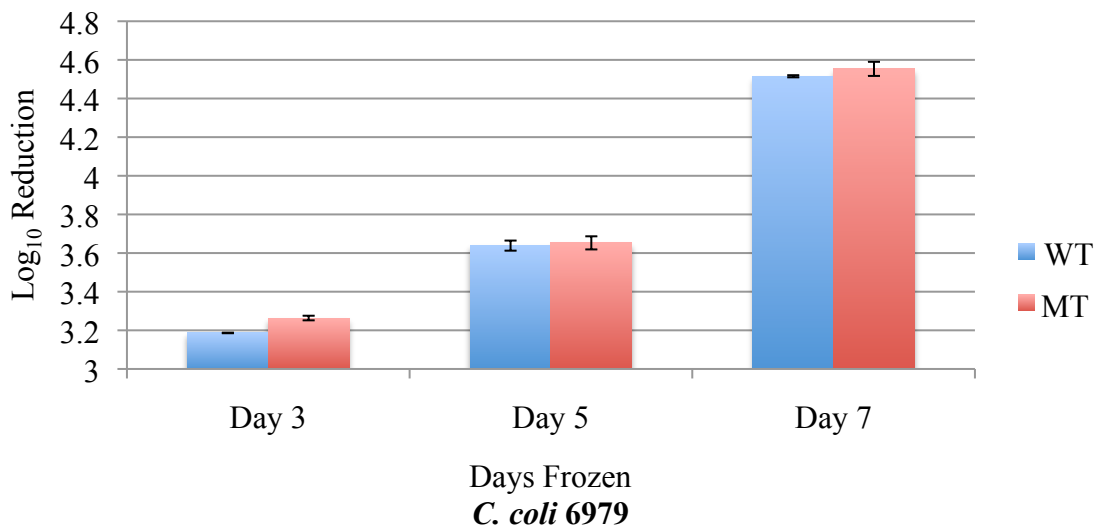


Figure 3.2. Survival of *C. coli* 6979 wild type and mutant strains frozen at -20°C after 7 days. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4). See Table 3.2 (Trial 2) for raw data.

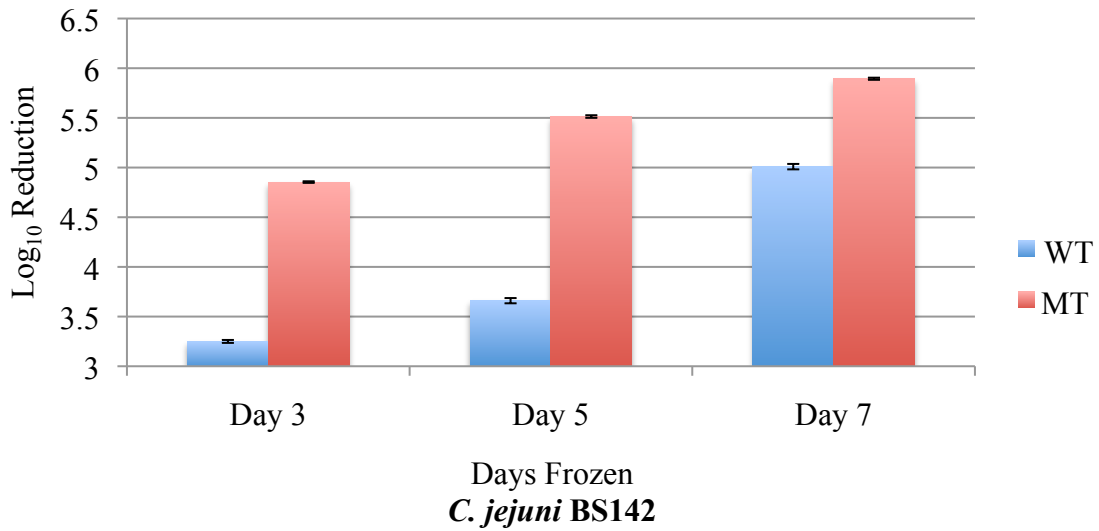


Figure 3.3. Survival of *C. jejuni* BS142 wild type and mutant strains frozen at -20°C after 7 days. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4). See Table 3.3 (Trial 2) for raw data.

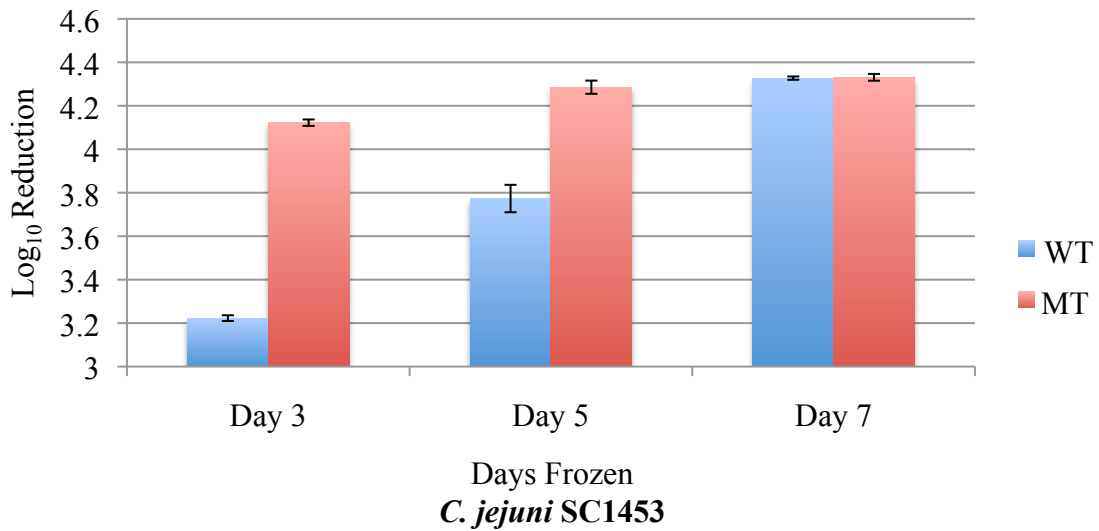


Figure 3.4. Survival of *C. jejuni* SC1453 wild type and mutant strains frozen at -20°C after 7 days. This figure is one representation of two independent experiments. Error bars denote the standard deviation of the mean (n = 4). See Table 3.4 (Trial 2) for raw data.

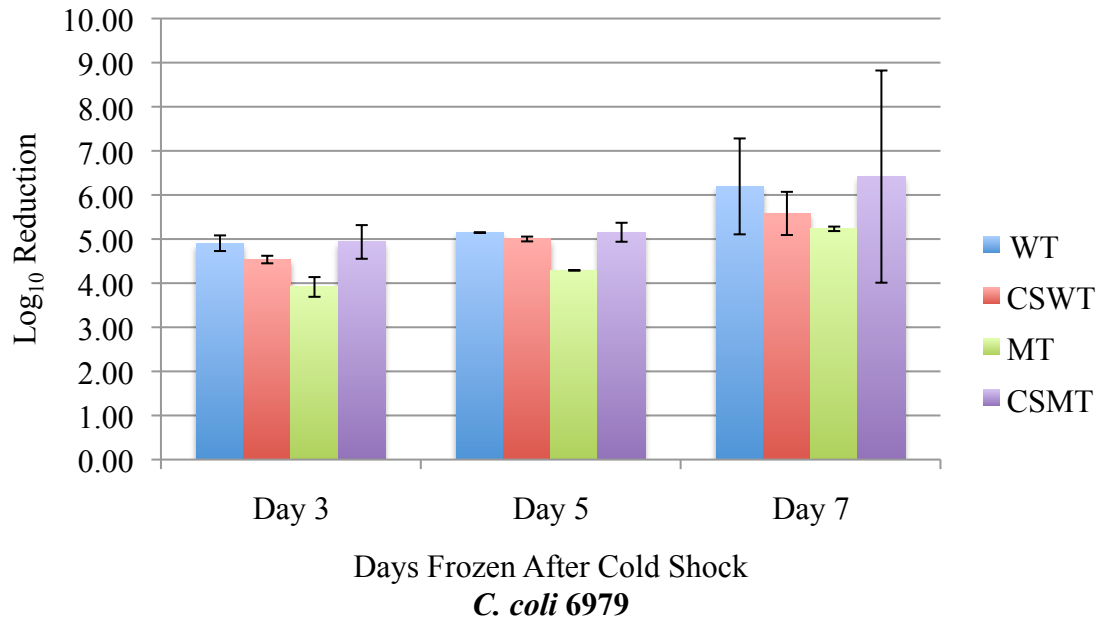


Figure 3.5. Survival of *C. coli* 6979 wild type and mutant strains after initial 2 hour cold shock and freezing at -20°C after 7 days. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.5 (Trial 2) for raw data.

Table 3.2. Average survival of *C. coli* 6979 in CFU/mL after freezing over 7 days.

	Day 0	Day 3	Day 5	Day 7
Trial 1				
WT	1.11E+09	8.55E+03	8.88E+03	1.25E+03
MT	2.01E+09	8.03E+03	7.88E+03	1.57E+03
Trial 2				
WT	1.49E+09	9.70E+05	3.43E+05	4.55E+04
MT	1.23E+09	6.68E+05	2.73E+05	3.43E+04

Table 3.3. Average survival of *C. jejuni* BS142 in CFU/mL after freezing over 7 days.

	Day 0	Day 3	Day 5	Day 7
Trial 1				
WT	2.64E+09	1.58E+06	4.45E+05	1.24E+05
MT	8.25E+08	6.23E+04	4.28E+04	3.85E+04
Trial 2				
WT	8.80E+07	1.79E+04	3.85E+03	6.08E+02
MT	5.05E+07	3.40E+03	3.68E+02	1.15E+02

Table 3.4. Average survival of *C. jejuni* SC1453 in CFU/mL after freezing over 7 days.

	Day 0	Day 3	Day 5	Day 7
Trial 1				
WT	2.90E+10	1.90E+05	1.93E+05	8.65E+04
MT	1.87E+10	1.53E+05	5.98E+04	1.78E+04
Trial 2				
WT	2.40E+09	1.35E+06	5.25E+05	2.35E+04
MT	1.68E+10	2.35E+05	5.15E+04	2.15E+04

Table 3.5. Average survival of *C. coli* 6979 in CFU/mL after cold-shock and freezing over 7 days.

Trial 1				
	Day 0	Day 3	Day 5	Day 7
WT	3.40E+08	4.23E+03	2.43E+03	2.18E+02
CSWT	7.05E+08	2.06E+04	7.03E+03	1.85E+03
MT	1.80E+08	2.19E+04	9.20E+03	1.05E+03
CSMT	6.45E+08	7.50E+03	4.53E+03	2.48E+02
Trial 2				
WT	1.49E+09	9.70E+05	3.43E+05	4.55E+04
CSWT	2.23E+09	1.04E+05	9.30E+04	9.00E+03
MT	1.23E+09	1.05E+06	1.93E+05	6.78E+04
CSMT	1.96E+09	6.68E+05	8.58E+04	3.43E+04

Desiccation

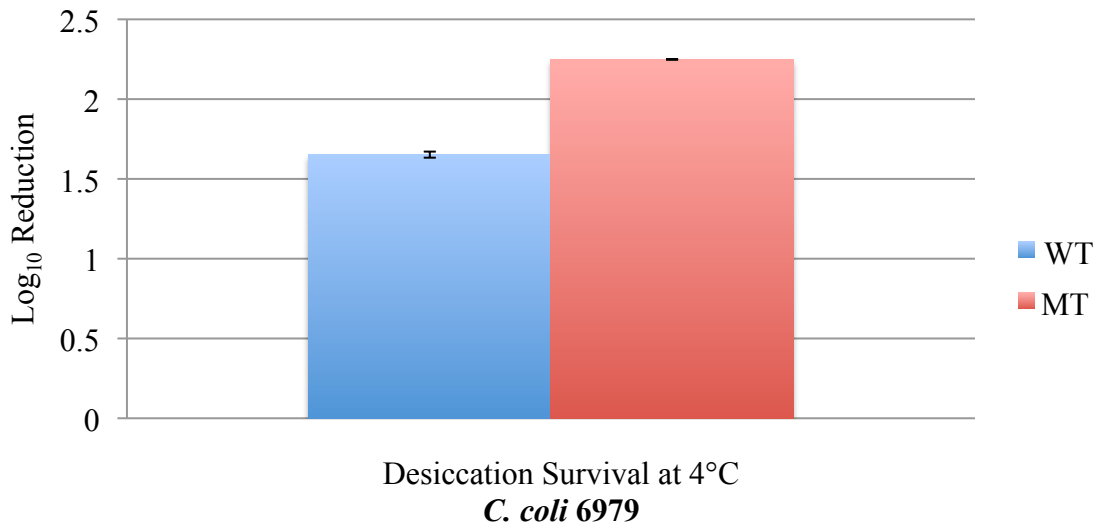


Figure 3.6a. Survival of *C. coli* 6979 after desiccation at 4°C for a 2 hour time period. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.6a (Trial 2) for raw data.

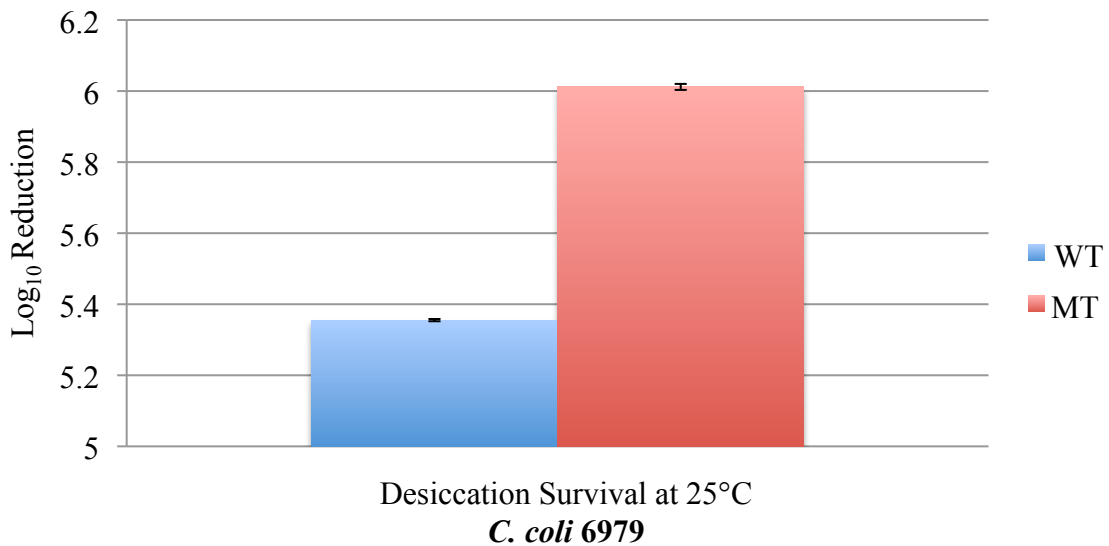


Figure 3.6b. Survival of *C. coli* 6979 after desiccation at 25°C for a 2 hour time period. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.6b (Trial 2) for raw data.

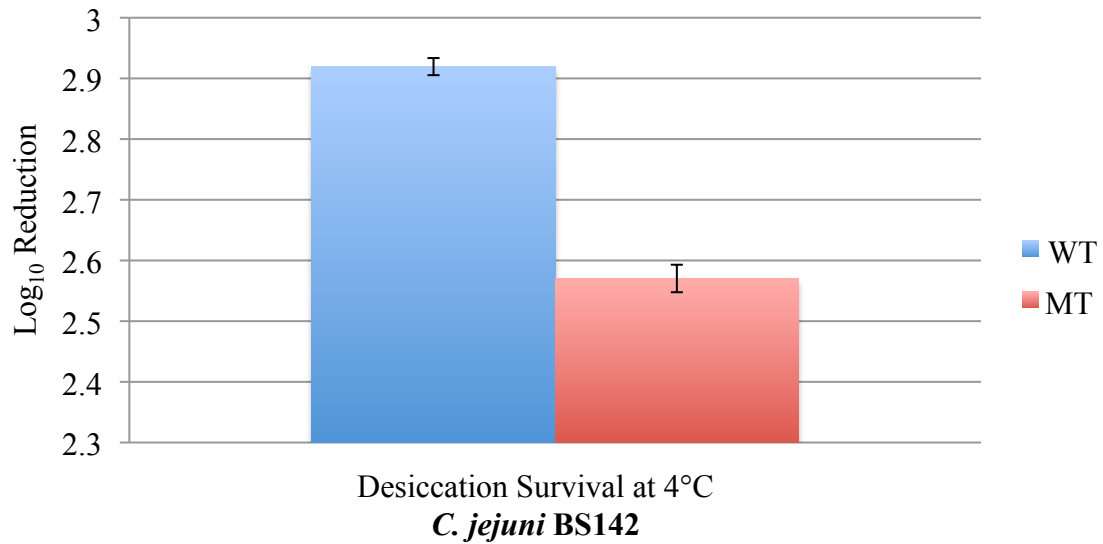


Figure 3.7a. Survival of *C. jejuni* BS142 after desiccation at 4°C for a 2 hour time period. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.7a (Trial 2) for raw data.

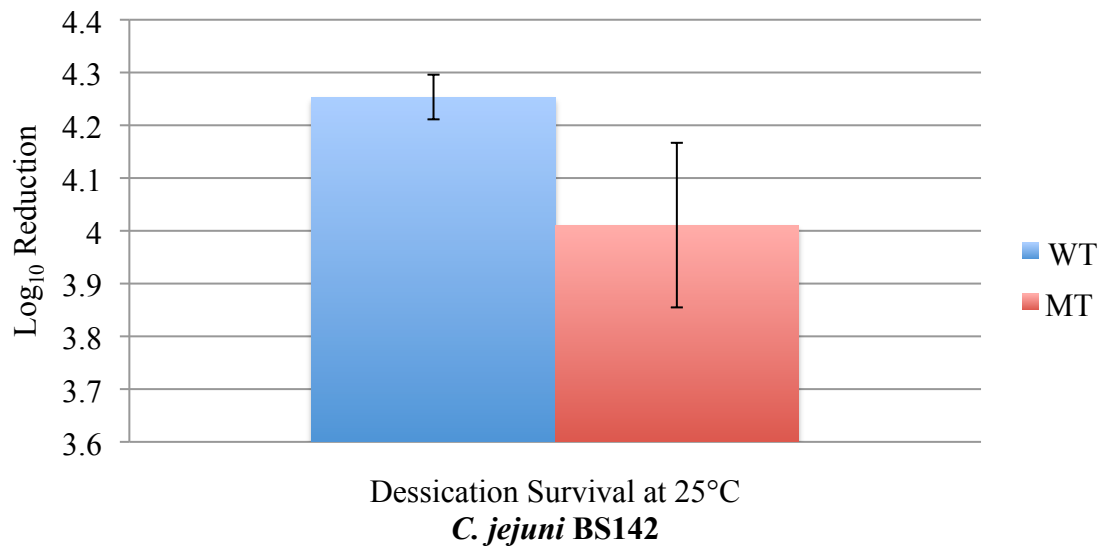


Figure 3.7b. Survival of *C. jejuni* BS142 after desiccation at 25°C for a 2 hour time period. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.7b (Trial 2) for raw data.

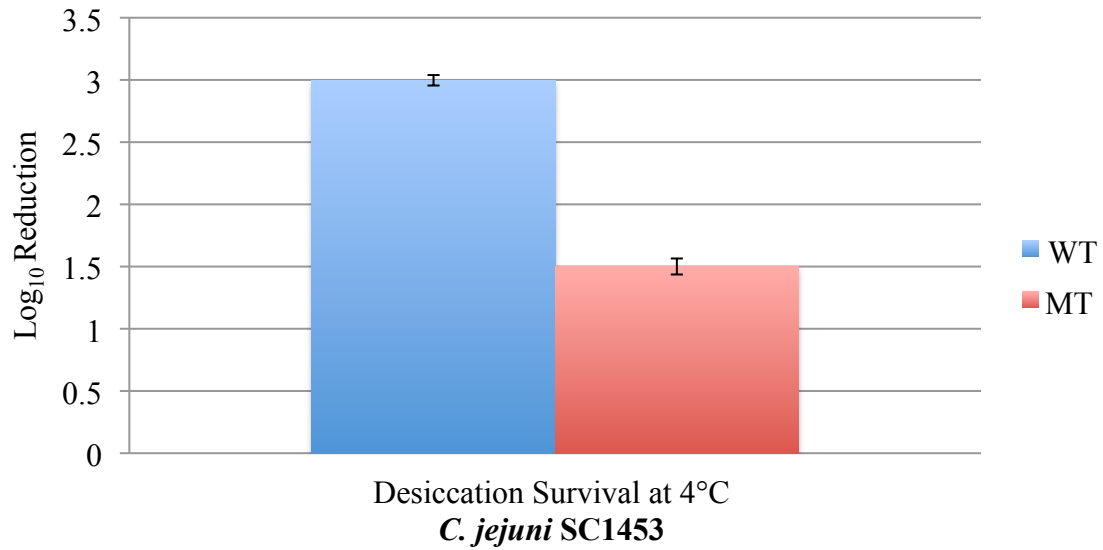


Figure 3.8a. Survival of *C. jejuni* SC1453 after desiccation at 4°C for a 2 hour time period. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.8a (Trial 2) for raw data.

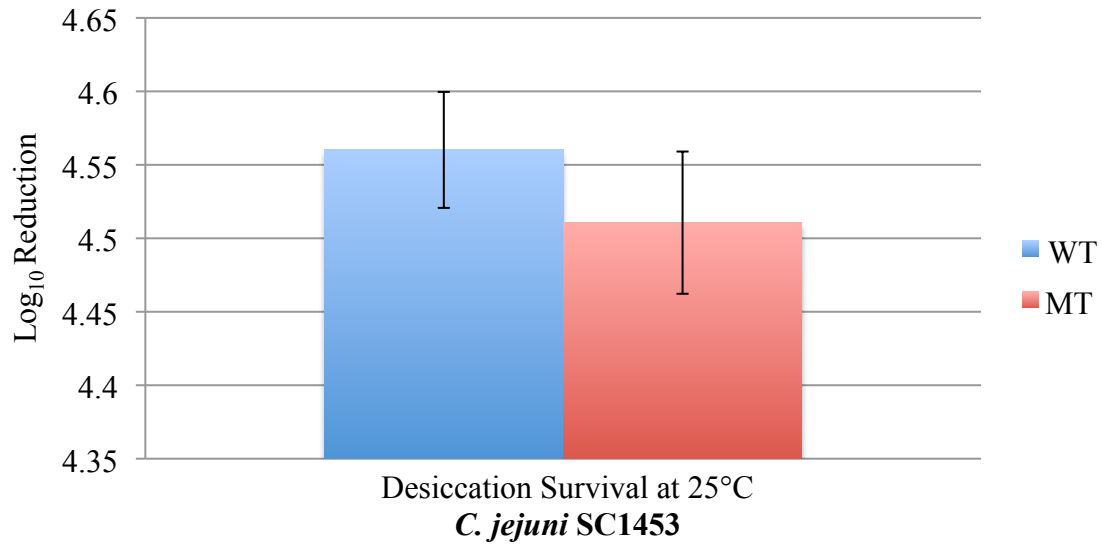


Figure 3.8b. Survival of *C. jejuni* SC1453 after desiccation at 25°C for a 2 hour time period. Each bar represents the mean of one trial in duplicate. Error bars denote the standard deviation of the mean (n = 4). See Table 3.8b (Trial 2) for raw data.

Table 3.6a. Average survival of *C. coli* 6979 in CFU/mL after desiccation at 4°C for 2 hours.

Trial 1			Trial 2		
	0 hr	2 hr		0 hr	2 hr
WT	2.90E+08	6.45E+06	WT	2.88E+08	4.98E+06
MT	4.70E+08	2.65E+06	MT	6.70E+08	1.75E+06

Table 3.6b. Average survival of *C. coli* 6979 in CFU/mL after desiccation at 25°C for 2 hours.

Trial 1			Trial 2		
	0 hr	2 hr		0 hr	2 hr
WT	2.90E+08	1.28E+03	WT	2.88E+08	1.28E+04
MT	4.70E+08	4.58E+02	MT	6.70E+08	4.07E+03

Table 3.7a. Average survival of *C. jejuni* BS142 in CFU/mL after desiccation at 4°C for 2 hours.

Trial 1			Trial 2		
	0 hr	2 hr		0 hr	2 hr
WT	2.17E+08	8.50E+05	WT	1.87E+08	2.25E+05
MT	3.05E+08	1.97E+07	MT	2.05E+09	5.50E+06

Table 3.7b. Average survival of *C. jejuni* BS142 in CFU/mL after desiccation at 25°C for 2 hours.

Trial 1			Trial 2		
	0 hr	2 hr		0 hr	2 hr
WT	2.17E+08	1.21E+04	WT	1.87E+08	6.45E+04
MT	3.05E+08	2.98E+04	MT	2.05E+09	2.56E+06

Table 3.8a. Average survival of *C. jejuni* SC1453 in CFU/mL after desiccation at 4°C for 2 hours.

Trial 1			Trial 2		
	0 hr	2 hr		0 hr	2 hr
WT	3.95E+07	3.98E+04	WT	8.15E+08	1.00E+05
MT	5.25E+08	1.66E+07	MT	3.95E+08	1.00E+07

Table 3.8b. Average survival of *C. jejuni* SC1453 in CFU/mL after desiccation at 25°C for 2 hours.

Trial 1			Trial 2		
	0 hr	2 hr		0 hr	2 hr
WT	3.95E+07	1.09E+03	WT	8.15E+08	6.20E+02
MT	5.25E+08	1.62E+04	MT	3.95E+08	4.45E+03