Proteomic analysis of the cold stress response in *Campylobacter jejuni*

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Abbreviations Used

1D	One-dimensional
2-D DIGE	Two-dimensional differential gel electrophoresis
2DE	Two-dimensional electrophoresis
ATP	Adenosine triphosphate
BA	Blood agar
BHI	Brain-heart infusion
CDC	Centers for Disease Control and Prevention
CDT	Cytolethal distending toxins
CSD	Cold shock domain
CSP	Cold shock protein
Da	Dalton
EFSA	European Food Safety Authority
ESI	Electrospray ionization
ESR	Environmental Science and Research
EU	European Union
G2/M	Pre-mitotic and mitotic phase
GC	Guanine-cytosine
iTRAQ	Isobaric tag for relative and absolute quantitation
LC	Liquid chromatography
mRNA	Messenger ribonucleic acid
MALDI	Matrix-assisted laser desorption/ionization
MLST	Multilocus sequence typing
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCTC	National Collection of Type Cultures
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PNPase	Polynucleotide Phosphorylase

rpm	Round per minute
rRNA	Ribosomal ribonucleic acid
RO	Reverse osmosis
spp.	Species (multiple)
SCX	Strong Cationic Exchange
SDS	Sodium dodecyl sulfate
SFA	Saturated fatty acids
SVS	Statens Veterin×re Serumlaboratorium (Danish Veterinary Laboratory), Denmark
TCA	Tricarboxylic acid cycle
TCS	Two-component signal transduction system
TOF	Time of flight
UFA	Unsaturated fatty acids
UK	United Kingdom
USA	United States
UV	Ultraviolet
VBNC	Viable but non-culturable

Abstract

Campylobacter jejuni is recognised as one of the most important food-borne pathogens, as it is responsible for causing more cases of gastroenteritis than any other identified bacterial pathogen. Despite the importance of *C. jejuni* as a human pathogen, little is known about how it copes with different stress factors and survives in the environment. To control the prevalence of food-borne pathogens in food, low temperatures have been widely applied as a practical intervention during food processing and storage. To ensure the cold temperature intervention works optimally, it is important to understand the cold stress response mechanisms employed by *C. jejuni*. There are a number of integrated genomic/transcriptomic studies that have made progress towards discovering the genes involved in *C. jejuni* cold tolerance and provided the first step to decodinge the mechanisms employed by *C. jejuni* to adapt to low temperatures. However, proteomic studies, which reflectthe main components of the physiological metabolic pathways of cells, in *C. jejuni*'s cold shock and adaptation at refrigeration temperatures, are somewhat lacking. The aim of this study, therefore, was to investigate the cold stress responses of this pathogen using iTRAQ labelling comparative proteomic analysis. Through comparing the alterations in protein expression in three *C. jejuni* strains during cold shock and cold adaptation, the study found this pathogen dramatically altered its protein expression in response to cold shock. An increased level of ribosomal proteins and other proteins related to protein synthesis in cold-shocked *C. Jejuni* cells suggested that protein synthesis in *C. jejuni* was more active when it processed a cold shock response, compared with growing at an optimal temperature. Furthermore, the proteins involved in energy production were present at higher levels in cold-shocked cells. This suggested that*C. Jejuni* exhibited an enhanced demand for energy in response to cold shock. The comparative proteomic study also revealed the strategies used by different *C. jejuni* strains to respond to cold stress. This may provide an explanation for the strain-dependent variability of this pathogen for cold tolerance.

Chapter 1 Introduction

Campylobacter is a genus of Gram-negative bacteria that are microaerophillic, predominantly spiral-shaped and motile. Since the connection between *Campylobacter* and human diarrhoea was revealed in the 1970s, *Campylobacter* has emerged as a major food-borne pathogen and has rapidly became known as one of the most common causative agents of bacterial gastroenteritis in humans around the world. The symptoms of a *Campylobacter* infection normally start with cramping pain in the abdomen, followed by watery diarrhoea. Some cases may develop serious complications like septicaemia, meningitis and polyneuropathic disorders such as the Guillain-Barre syndrome (Skirrow, 1994).

Although a number of species in the genus *Campylobacter* are able to infect humans, the majority of human *Campylobacter* infections are caused by *Campylobacter jejuni*, which accounts for approximately 90% of campylobacteriosis cases (Healing et al., 1992). Similar to other species in the genus *Campylobacter*, *C. jejuni* is a microaerophile with a narrow range of growth temperatures, from 31 to 45^{o}C (Hazeleger et al., 1998; Park, 2002). It is extremely sensitive to environmental conditions and cannot proliferate outside of a host.

Most food-borne pathogens are relatively robust organisms, since they need to be resistant to environmental stresses and survive harsh conditions before they reach the human gastrointestinal tract and cause an infection. In this context, *C. jejuni* presents an interesting conundrum: generally regarded asa fragile bacterium, *C. jejuni* causes more cases of human gastroenteritis than any other enteric bacterial pathogens (USFDA, 2003).

It is important to understand the stress response mechanisms employed by *C. jejuni* to cope with various environmental stresses, since efficient interventions are reliant on knowledge of microbial stress response mechanisms. Major environmental stresses for *C. jejuni* are cold stress, heat stress, aerobic stress, Ultraviolet light (UV)

stress and acid stress**.** Among them, the cold stress response of *C. jejuni* is the one that has attracted many researchers' attention, as low temperature is a widely used intervention to control bacterial growth in foods. The ability of *C. jejuni,* a leading food-borne pathogen, to survive in cold temperatures is obviously of relevance to food safety and public health.

In comparison with other enteric bacteria, *C. jejuni* appears to have very limited capacity for regulating gene expression in response to environmental stresses (Park, 2002). What is more, many key regulators of stress defence systems found in other enteric bacteria are absent in this pathogen. These include the major cold-shock protein, CspA, which acts as an RNA chaperone to allow more efficient protein translation at cold temperatures (Qoronfleh et al., 2000), the oxidative stress defences, SoxRS and OxyR, and the sigma factor, RpoS, for stress-defence gene regulation under hostile environmental conditions (Schwab et al., 2005).

Despite lacking many of the classical bacterial stress responses, *C. jejuni* has been found it is widely distributed in the environment outside the host and survives for extended periods at low temperatures. This pathogen has been demonstrated to maintain its motility, oxygen consumption, protein synthesis and survival at 4^{o}C (Lazaro et al., 1999). In addition, *C. jejuni* strains have also been found to exhibit a sudden decrease in growth rate from maximum to zero within a few degrees below the minimum growth temperature (Hazeleger et al., 1998). Survival, and a dramatic decrease in growth rate at low temperatures, suggests that *C. jejuni* does elicit a cold shock response that regulates gene expression.

Previous studies have also found there is substantial variability among *C. jejuni* strains for tolerance to cold (Jones et al., 1991; Terzieva et al., 1991; Chan et al., 2001; Xiong, 2009). However, it is still not fully understood what causes the strain-dependent variability of this pathogen for cold tolerance. Researchers have made progress in unravelling the cold stress response mechanisms of *C. jejuni* through discovering the genes involved incold tolerance (Stintzi and Whitworth, 2003; Moen et al., 2005). However, due to protein post transcriptional modification, genomics

and transcriptomics have their limitations in reflecting the expression of proteins, which are the actual functional molecules in the cell.

In order to analyze the protein abundance influenced by temperature downshift and to more precisely study cold stress response mechanisms in *C. jejuni*, we conducted a series of cold survival ability assessments and gel-free proteomic analysis. Our study focuses on how *C. jejuni* cold stress tolerance is affected in the different strains, which proteins are up- or down-expressed during processing a cellular cold stress response and whether the protein abundance under cold stress is correlated with the abundance of mRNA found in the previous transcriptomic studies (Stintzi and Whitworth, 2003; Moen et al., 2005)

This project was designed to:

- ✧ Evaluate the effect of refrigeration on the survival of three different strains of *C. jejuni*.
- ✧ Analyze and compare the cold stress response on a proteomic scale in three different strains of *C. jejuni* using iTRAQ labelling proteomic analysis.

Chapter 2 Literature Review

2.1 History and taxonomy of *Campylobacter*

The first record concerning *Campylobacter* spp. was believed to be written in 1886 by Theodore Escherich who described non-culturable spiral-shaped or *Vibrio*-like bacteria in stool samples of children with diarrhoea (Kist, 1986). In 1913, these microorganisms were first isolated by McFadyean and Stockman from aborted bovine foetuses (Kist, 1986). They had been classified in the genus *Vibrio* at that time and they were believed to cause abortion in cattle. Later in 1927 and 1944, other species of this genus were isolated from the faeces of cattle and pig with diarrhoea, and named *Vibrio jejuni* and *Vibrio coli*, respectively (Vandamme, 2000; Vandamme et al., 2010). However, microbiologists found those microorganisms are different with true *Vibrio* spp. in DNA base GC content. These microorganisms have lower GC content (29-36 mol %) than *Vibrio* (40-53 mol %). In 1963, Sebald and Veron first proposed the genus *Campylobacter* based on this genus having a lower GC base composition, non-fermentative metabolism and microaerophilic growth requirements. Since then, the genus *Campylobacter* has been distinguished from the "true" *Vibrio* spp. (On, 2001).

Nowadays, it is generally accepted that the *Campylobacter* genus belongs to the epsilon subdivision of the *Proteobacteria* classification based on characterisation of its 16S rRNA sequences (Garrity et al., 2005). Other members of this subdivision include *Arcobacter*, *Helicobacter* and *Wolinella* genera. The classification of the genus *Campylobacter* is given in Table 2.1.

Table 2.1 Classification of the genus *Campylobacter*

Domain	Bacteria
Phylum	Proteobacteria
Class	Epsilonproteobacteria
Order	*Campylobacterales*
Family	*Campylobacteraceae*
Genus	*Campylobacter*

Since the inception of the genus *Campylobacter* in 1963, more and more *Campylobacter*-like organisms have been isolated from a variety of human, animal, and environmental sources and some of those species have been described and added to the *Campylobacter* genus. Currently, the genus consists of 21 species with a further seven subspecies (CDC, 2010, Dec 16) classified by comparing of the 16S rRNA gene sequences. *Campylobacter* spp. today were well known as bacteria pathogens causing the largest number of cases of diarrhoea in humans (Allos, 2001).

The first time a connection was made between this pathogen and human diarrhoea was in 1973 when Butzler et al. compared bacteria recovered from diarrhoea patients' stools and stools from people without diarrhoea. A total of 900 stools from diarrhoea patients and 1000 stools from people without diarrhoea had been examined. The "related vibrios" were recovered from 13 stools from 1000 people without diarrhoea, indicating a carrier state, as well as 56 stools from patients with diarrhoea, pointing to the link between the microorganism and the disease (Butzler et al., 1973). With the development of selective growth media in the 1970s, the correlation between this pathogen and human diarrhoea had been revealed. *Campylobacter* spp. have been generally been recognized as a cause of human disease since 1980, although they have been known to cause disease in animals since the early 1900s.

Among the 21 species and seven subspecies in the genus, *C. jejuni*, *C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus* are most

commonly isolated from human and animal diarrheal specimens (On, 2001). In the case of human *Campylobacter* infections, *C. jejuni* and its subspecies are responsible for approximately 90% of the cases of campylobacteriosis (Frost et al., 1999). All the work described in this thesis focused on *C. jejuni* with the following lineage: Super-kingdom: Bacteria, Phylum: proteobacteria, Subphylum: δ/ε subdivisions, Class: Epsilonproteobacteria, Order: *Campylobacterales*, Family: *Campylobacteraceae*, Genus: *Campylobacter*, Species: *Campylobacter jejuni*.

2.2 Morphological and biochemical characteristics of *C. jejuni*

C. jejuni is a Gram-negative, non-spore forming; slender spirally curved rod bacterium that is approximately 0.2 to 0.8 μm wide and about 0.5 to 5μm long (Vandamme, 2000). They also appear as S-shaped and V-shaped gull-wings when two or more cells form short chains. Although normally a curved-rod shape, other forms of *C. jejuni* such as spherical or coccoid occur and appear in response to stress or deleterious conditions (Fitzgerald et al., 2008; Debruyne et al., 2008) (Figure 1). Like most species in this genus, *C. jejuni* is motile and has a corkscrew-like motion, since it has a polar flagellum at each end of a cell.

The members of *Campylobacter* spp. are considered to be fastidious microorganisms, as they require a microaerophilic atmosphere for optimal growth (Prescott and Munroe, 1982; Fitzgerald et al., 2008). The optimal atmosphere for cultivation of *C. jejuni* contains 5-10% oxygen and 1-10% carbon dioxide (Luechtefeld et al., 1982; Bolton and Coates, 1983). Similar to all species in the genus, *C. jejuni* has a rather narrow range of growth temperatures, between 30^{o}C and 45^{o}C (Hazeleger et al., 1998; Park, 2002), with an optimal growth temperature of 42^{o}C (Park, 2002). *Campylobacter* spp. neither ferment nor oxidize carbohydrates; instead, they obtain energy from amino acids, or tricarboxylic acid cycle intermediates (Vandamme, 2000). Therefore, nutrient-rich media are often used for culturing *Campylobacter.* Oxidase activity is present in all species in the genus except for *C. gracilis*. Most species cannot hydrolyse hippurate, except for *C. jejuni, C. jejuni subsp. Doylei* and *C. avium* (Vandamme and Goossens, 1992; Rossi et al., 2009). Therefore, hippurate

hydrolysis has become the most widely used biochemical test to identify *C. jejuni* and differentiate it from other similar species (Hwang and Ederer, 1975).

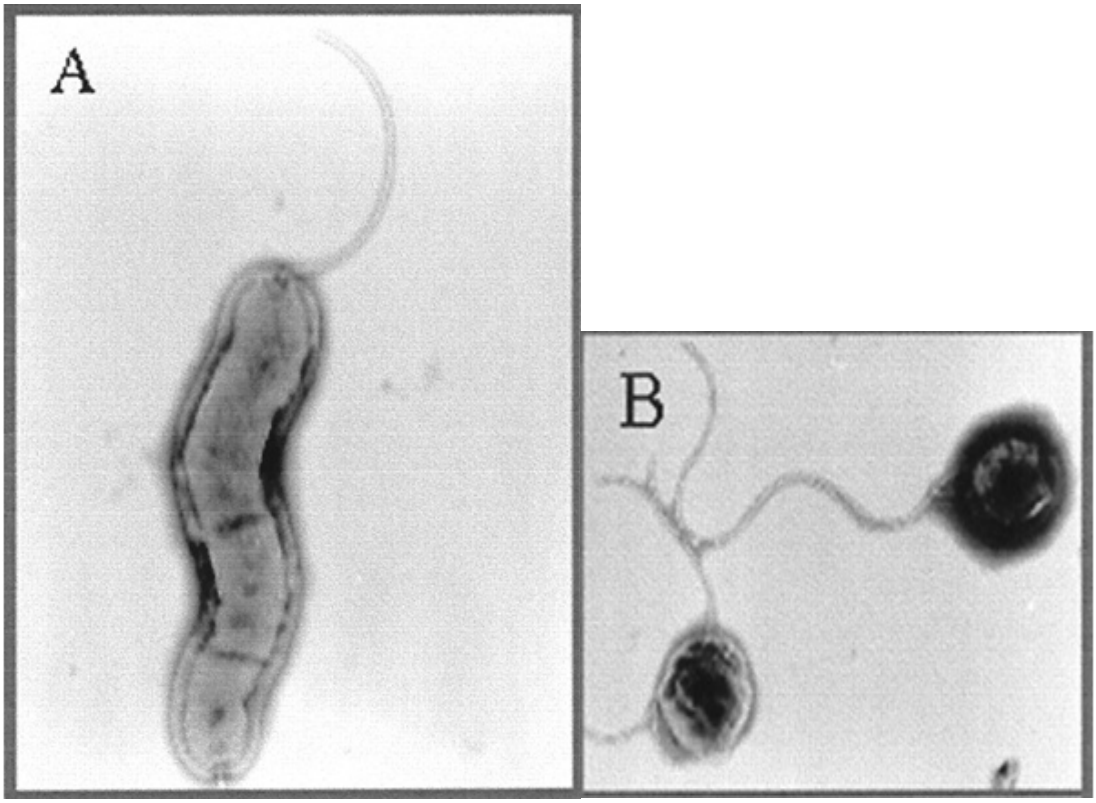


Figure 2.1 *C. jejuni* spiral (A) and coccoid (B) form

Under transmission electron microscopy, adapted from Lazaro et al., 1999.

2.3 *C. jejuni* as a pathogen

2.3.1 Clinical significance of *Campylobacter* infections

Campylobacter species are regarded as the most important zoonotic enteric bacterial pathogens of humans in developed countries, since they cause more cases of food-borne gastroenteritis each year than any other bacterial pathogen (Silva et al., 2011). Although there are a number of species in the genus *Campylobacter*, such as *C. jejuni*, *C. coli*, *C. upsaliensis*, *C. lari* and *C. fetus*, that are able to cause human campylobacteriosis, the majority of reported human campylobacteriosis cases in developed countries are attributed primarily to *C. jejuni.* It is responsible for 90% of human *Campylobacter* infections (Sheppard et al., 2009; Healing et al., 1992).

Campylobacter infections can be initiated by the consumption of as few as 500 bacterial cells (Blaser and Engberg, 2008). The average incubation period of campylobacteriosis is longer than for most other intestinal bacterial infections. The mean incubation period of campylobacteriosis is 3.2 days and the range can be from one to eight days (Blaser, 1997; Butzler, 2004; Blaser and Engberg, 2008). The range of severity of a *Campylobacter* infection is variable from asymptomatic to severe. The majority of *Campylobacter* infections are confined to local acute gastroenteritis characterized by nausea, abdominal cramps, diarrhoea and fatigue. Some patients may also vomit.

The symptoms of campylobacteriosis in developed countries are usually more severe than in developing countries (Oberhelman and Taylor, 2000). In developed countries, the typical clinical features of *Campylobacter* infection are acute gastroenteritis with diarrhoea, fever and abdominal cramps (Allos, 2001). The acute gastroenteritis normally lasts 2-3 days, but it may persist for one week or longer. In developing countries, the common symptom of campylobacteriosis is a milder form of gastroenteritis which is characterized by watery, non-bloody, non-inflammatory diarrhoea. Since people in developing countries have earlier exposure to *Campylobacter* that results in higher *Campylobacter*-specific antibody levels, the

clinical symptoms of campylobacteriosis in developing countries are less severe and asymptomatic infections are common (Padungton and Kaneene, 2003).

A *Campylobacter* infection is generally self-limiting and resolves within three to four days after the initial onset (Seal et al., 2007). Fifteen to twenty-five percent of patients may have longer relapses, which can last for several weeks (Allos, 2001; Blaser, 1997; Blaser and Engberg, 2008). After the clinical symptoms disappear, *Campylobacter* cells can still be found from patients' faeces if the patients did not receive antibiotic treatment (Blaser and Engberg, 2008).

Complications of *Campylobacter* infections in humans are rare. However, the complications can be serious and fatal (Skirrow and Blaser, 2000). Based on the site, complications can be divided into two groups: gastrointestinal tract complications and those complications arising secondary to the gastroenteritis. The gastrointestinal tract complications include cholecystitis, pancreatitis and massive gastrointestinal haemorrhages (Allos, 2001; Butzer, 2004). The latter group of complications, which is more frequently reported in the literature, includes bacteraemia, meningitis, endocarditis, septic arthritis, osteomyelitis, abortion and neuromuscular paralysis (Denton and Clarke, 1992; Allos, 2001; Blaser and Engberg, 2008). The most important post-infectious complication of campylobacteriosis is Guillain-Barre Syndrome (GBS), an acute immune-mediated neuromuscular paralysis that may lead to respiratory muscle compromise and death (Seal et al., 2007). Infection with *Campylobacter* commonly precedes GBS. An estimated 20 to 50% of patients with GBS symptoms have had a preceding *Campylobacter* infection (Bart et al., 2008).

2.3.2 Pathogenesis of *C. jejuni*

The pathogenesis of a *C. jejuni* infection involves both host- and pathogen-specific factors. The health status and age of the host and *Campylobacter* specific humoral immunity from previous exposure affect the clinical outcome of a *Campylobacter* infection. Despite its importance as a human pathogen, many virulence determinants of *C. jejuni* have not yet been clearly elucidated. This is partly due to

the lack of an ideal animal model to evaluate the pathogenesis and virulence of this pathogen (Young et al., 2007)

As a food-borne bacterial pathogen, *C. jejuni* has to survive in human stomach acid and highly alkaline bile secretions before it colonizes the distal ileum and colon. Following colonization of the mucus blanket and adhesion to the intestinal cell surfaces, *Campylobacter* reduces the normal absorptive capacity of the intestine by damaging epithelial cell function through direct cell invasion and/or toxin production or, indirectly, by inducing inflammatory reactions in the host (Wooldridge and Ketley, 1997). Thus, flagella-mediated motility, bacterial adherence to intestinal mucosa, invasive capability and the ability to produce toxins have been identified as virulence factors for this pathogen.

The flagellum is an important virulence determinant, which not only provides motility for colonization of *C. jejuni* on the small intestine, but also plays different roles under different chemotactic conditions. Having flagella is essential for this pathogen to survive in the various ecological niches encountered in the gastrointestinal tract. The *Campylobacter* flagellum is composed of two highly homologous flagellins, FlaA, which is the major one and, FlaB, the minor one. They are encoded by two flagellin genes, *fla*A and *fla*B. The *fla*A gene seems to be essential for the invasion of epithelial cells and is responsible for the expression of adherence, colonization of the gastrointestinal tract and invasion of the host cells, consequently, arresting the immune response (Guerry, 2007).

Another important virulence factors of *C. jejuni*, the cytolethal distending toxin (CDT), which is widely distributed among Gram-negative bacteria and the best characterized of the toxins produced by *Campylobacter* CDT holotoxin; it comprises three subunits encoded by the *cdt*A, *cdt*B and *cdt*C genes and causes eukaryotic cells to arrest in the G2/M phase of the cell cycle, preventing them from entering mitosis and, consequently, leading to cell death (Ge et al., 2008). In contrast to CdtB, the roles of CdtA and CdtC are still rather unclear and require further investigation. CdtA and CdtC are thought to be essential for CdtB delivery into the host cell, being responsible for binding the CDT holotoxin to the cell membrane (Lara-Tejero and

Galan, 2001). Subsequently, the CdtB active subunit, which has DNaseI-like activity, induces DNA damage in the host by breaking its double strand (Ge et al., 2008). In fact, to be functionally active, all three *cdt* gene products must be present.

2.4 Epidemiological aspects of *C. jejuni*

2.4.1 Prevalence of *Campylobacter* infections

Although *Campylobacter* spp. have been generally recognized as a cause of human disease only since 1980, they soon became the leading bacterial cause of food-borne illness in industrialized countries. Based on the Community Zoonosis Reports of the European Food Safety Authority (EFSA), since *Campylobacter* overtook *Salmonella* as the main cause of food poisoning in Europe in 2005, campylobacteriosis has been the most commonly reported zoonosis in the European Union (EU). In 2011, campylobacteriosis was the principal cause of zoonotic disease in humans, with 220,209 reported confirmed cases in the EU and with an EU notification rate of 44.2 cases per 100,000 inhabitants (EFSA and ECDC, 2013).

Food-borne campylobacteriosis is the third most common food-borne disease and accounts for 9% of a total of 9.4 million episodes of food-borne illness in the USA in 2010 (Scallan et al., 2011). The Centres for Disease Control and Prevention (CDC) in the USA used data from active and passive surveillance and other sources to estimate that there were 845,000 cases food-borne illness every year. In 2010, alone, there were 8460 hospitalizations and 76 deaths caused by *Campylobacter* (Scallan et al., 2011).

In the UK, *Campylobacter* is considered the most common cause of food borne illness, responsible for 321,000 estimated cases in England and Wales in 2008, with more than 15,000 hospitalizations and 76 deaths (Silva et al., 2011). Due to the huge health burden caused by campylobacteriosis, the UK Government increased the priority of “innovation strategy for *Campylobacter*” to tackle this food-borne disease (Food Standards Agency, 2010).

In New Zealand, since campylobacteriosis first became notifiable in 1980, there has been a steady annual increase in the number of reported cases up until 2007. In May 2006, New Zealand's campylobacteriosis epidemic reached the highest point when the annualized national notification rate exceeded 400 per 100,000 for the first time, based on 15,553 cases in the preceding 12 months (Baker et al., 2006a). The rate was one of the highest reported by any country, being more than three times higher than in Australia and 30 times higher than in the USA over the same period (Baker et al., 2006b). Although the campaign to reduce contamination of *Campylobacter* in food has resulted in campylobacteriosis trending down nation-widein last five years, the incidence rate of human campylobacteriosis in New Zealand still remains one of the highest in industrialized countries. There were still 6, 692 notified cases in 2011 in New Zealand, equal to 151.9 cases per 100,000 population (ESR, 2012), which is 10 times higher than the USA with 14.3 reported cases per 100, 000 population in 2011 (CDC, 2012).

2.4.2 Transmission of *C. jejuni*

Despite very specific growth requirements, *C. jejuni* is widespread in the environment. *C. jejuni* is a commensal organism found in avian species including domestic poultry, as well as cattle and sheep (Newell, 2002; Devane et al., 2005). The most common hosts of *C. jejuni* are avian species. It is believed that the higher body temperature of avian species provides optimum growing conditions for this pathogen. *C. jejuni* also can be found in river water and causes water-associated outbreaks (Alary and Nadeau, 1990; Hanninen et al., 2003; Richardson et al., 2007). As this organism is unable to grow outside an animal host, the sources of *C. jejuni* in water are believed to be from animals' faeces and sewage effluent (Wong et al., 2006).

There are many potential transmission routes leading to *Campylobacter* infections in humans. The main three routes are ingestion of contaminated food, direct contact with pets or other animals, and drinking contaminated milk and water. Before 1990, raw milk and water were considered to be the main sources for human campylobacteriosis (Mohan, 2011). With more epidemiological studies revealing the

strong correlation between the consumption of chicken meat and *Campylobacter* infections in the 1990s and early 2000s (Butzler and Oosterom, 1991; Tauxe et al., 1997; Corry and Atabay, 2001; Nadeau et al., 2002), the role of chicken in the transmission of human campylobacteriosis have been brought to light. Now, it is generally accepted that ingestion of undercooked meat, especially poultry and poultry products, is the main source of campylobacteriosis in humans (Kwan et al., 2008). An overview of possible sources and transmission routes of *C. jejuni* is illustrated in Figure 2.2.

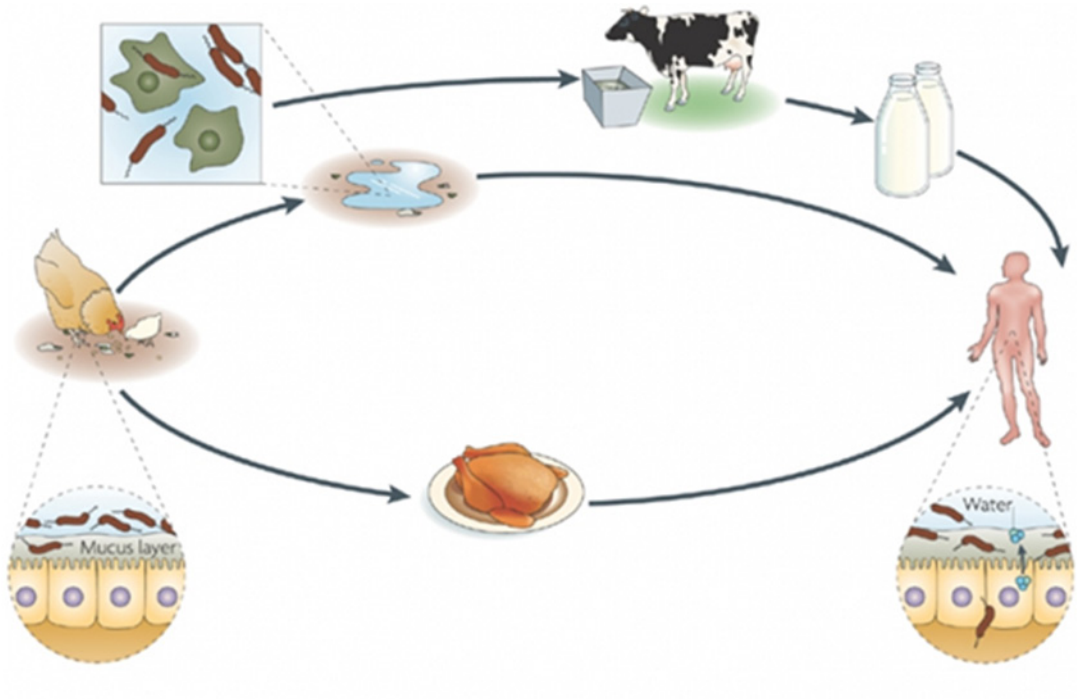


Figure 2.2 Possible sources of *C. jejuni* contamination

This figure is adapted from Young et al., 2007.

Epidemiological evidence suggests that 50 to 70% of human *Campylobacter* infections are related to consumption of contaminated poultry and poultry products (Altekruse et al., 1999; Allos, 2001). Poultry, as the main source of campylobacteriosis, has not only been identified by traditional epidemiological case control studies, but has also been confirmed by modern molecular epidemiological methods. In New Zealand, attribution models were adapted to MultiLocus Sequence Typing (MLST) surveillance data to quantify the contribution of selected sources to the human *Campylobacter* infection burden. These studies revealed that, between 2005 and 2008, poultry was the leading source of human campylobacteriosis, causing an estimated 58–76%, of notified cases (Mullner et al., 2009). A Scottish study compared genotypes of 5674 clinical isolates of *C. jejuni* with 5837 isolates from potential human infectious sources to quantify the attribution of different sources to human campylobacteriosis. It was confirmed that chicken meat was the principal source of *Campylobacter* infection in humans, which accounted for 58% and 78% of *C. jejuni* infections (Sheppard et al., 2009).

Although *Campylobacter* infection is one of the most common food-borne illnesses in developed countries, outbreaks of *Campylobacter* infections are infrequent (Friedman, 2000). The majority of human campylobacteriosis cases are sporadic. Outbreak of *Campylobacter* infection has different epidemiological characteristics from sporadic cases. While the majority of sporadic cases of campylobacteriosis are related to ingestion of undercooked meat, most of the reported outbreaks have been associated with contaminated water (Jacobs-Reitsma, 2000).

2.5 Survival of *C. jejuni* outside of host

C. jejuni colonizes the gastrointestinal tract of birds, animal and humans. In order to survive and cause infection, the bacterium needs to overcome challenges that occur in the environment between the animal and the human hosts. Those challenges include high oxygen exposure, below minimum growth temperature, desiccation and other stress factors (Murphy el al., 2006). Unlike other food-borne pathogens, *C. jejuni* is a fragile organism that is unable to grow at the levels of oxygen found in the air and hence unable to multiply outside the animal host. Despite its inability to grow

outside the host and its apparent sensitivity to stress conditions, this pathogen survives an environment external to the host and is regarded as the most frequent bacterial agent causing gastrointestinal illness in the industrialized world (CDC, 2000).

Food is now recognized as the most frequently implicated vehicle in the transmission of *C. jejuni* to humans in developed countries. Poultry and poultry products are generally accepted as a primary source of human *C. jejuni* infections. Poultry flocks are infected with *C. jejuni*, presumably from the environment, within the first weeks of life and become colonized at high levels of 10^6-10^7 CFU/g in the caecum (Corry and Atabay, 2001). Contamination of chicken meat with bacteria from the intestines can occur during routine procedures at the slaughter house such as defeathering, evisceration or carcass chilling. These activities were found to be responsible for contamination of the meat with this pathogen (Bashor et al., 2004). The levels of *C. jejuni* isolated from retail poultry products vary greatly in different countries, from 14.8% in Belgium to 87.6% in New Zealand (Table 2.2).

Table 2.2 Prevalence of *C. jejuni* in retail poultry meat in various countries

(Molecular biologically determined only) (Adapted from Suzuki and Yamamoto, 2009)

Countries	No. of references	No. of samples	Prevalence of *C. jejuni* (%)	References
New Zealand	1	205	87.6	Wang et al., 2007
Australia	1	30	83.3	Abu-Halaweh et al., 2005
Italy	1	30	74.6	Parisi et al.,2007
Canada	1	749	52.4	Valdivieso-Garcia et al., 2007
UK	2	120	50.7	Bolton et al., 2002
Japan	1	424	49.1	Suzuki and Yamamoto, 2008
Senegal	1	168	48.3	Cardinale et al., 2003
Barbados	1	94	46.6	Workman et al., 2005
USA	4	797	46.3	Cui et al., 2005; Dickins et al., 2002; Fitch et al., 2005; Oyarzabal et al., 2007
Germany	3	221	32.3	Adam et al., 2006; Alter et al., 2004; Kullmann and Hager, 2002
Korea	2	552	32.0	Han et al., 2007; Hong et al., 2007
Spain	1	51	30.1	Mateo et al., 2005
Thailand	2	87	15.7	Meeyam et al., 2004; Padungtod and Kaneene, 2005
Belgium	1	612	14.8	Ghafir et al., 2007

The reported prevalence of *C. jejuni* on raw meat products from other food animal species tended to be lower than those reported on poultry. The prevalence of *C. jejuni* in retail beef has ranged from 0–20% worldwide on the basis of culture and biochemical or molecular identification of species; the average isolated rate from retail beef samples is less than 5% (Bohaychuk et al., 2006; Cloak et al., 2001; US FDA, 2006; Wong et al., 2007). The frequency of *C. jejuni* isolated from retail pork is 9.1%, and from lamb is 6.9% (Wong et al., 2007). This pathogen has also been found to survive in seafood and vegetables, as there have been a number of *Campylobacter* outbreak cases related to seafood and vegetable consumption (Wilson and Moore, 1996; Jacobs-Reitsma, 2000), but such outbreaks are rare.

Aquatic environments are reservoirs for *C. jejuni* (Bolton et al., 1982; Thomas et al., 1999; Levin, 2007). Studies worldwide examining rivers and waterways show that there is significant contamination by *C. jejuni* in water, with sources being sewage outflows, direct faecal deposition and pasture runoff (Eyles et al., 2006; Jones, 2001; Obiri-Danso and Jones, 1999; Savill el al., 2001; Sopwith et al., 2008). This pathogen has also been found to survive in raw milk where it has caused outbreaks (Korlath et al., 1985; Hutchinson et al., 1985).

2.6 General survival mechanisms of *C. jejuni*

C. jejuni is generally considered to be fragile compared to other food-borne pathogens (Cason and Berrang, 2002), as this organism requires special growing conditions, like microaerobic conditions, and has a narrow growth temperature, ranging from 30^{o}C to 45^{o}C. It is also sensitive to other stress factors, such as sunlight and desiccation.

Compared to *Campylobacter*, most other enteric pathogens, such as *Salmonella* spp. and *Escherichia coli*, have more comprehensive survival mechanisms that help these bacteria survive outside the host before they enter human gastrointestinal tract to cause an infection. Compared with those bacteria, *C. jejuni* lacks many key regulators in its stress defence systems. The key regulators found in *Salmonella* spp. and *E. coli,* but absent in *C. jejuni*, include the oxidative stress defence SoxRS and OxyR, the

osmoprotectants BetAB, GbsAB, OtsAB and ProP, the rpoS-encoded sigma factor stationary phase responsive genes, the major cold shock protein, CspA, the leucine-responsive regulator (Lrp) and the alternative sigma factor, RpoH, that regulates the heat-shock response in *E. coli* (Murphy et al., 2006).

Absence of the commonly occurring survival mechanisms would seem to make it harder for this pathogen to survive and pose a threat to humans. However, *C. jejuni* has been reported to survive in food, such as poultry products and red meat, milk, water and in the environment (Park, 2002). Therefore, survival mechanisms other than those commonly found in other bacteria may be important for *C. jejuni*.

2.6.1 Viable but non-culturable states of *C. jejuni*

When encountering environmental stresses, such as low nutrient availability, fluctuations in temperature and pH or upon entry into the stationary phase, many bacteria can enter a viable but non-culturable (VBNC) state. It has been proposed that *Campylobacter* cells can enter the VBNC state under stress conditions (Rollins and Colwell, 1986). In the VBNC state, a *C. jejuni* cell can change its morphology from a motile curved-rod shape to a coccoid form.

The VBNC state is a survival mechanism used by many bacteria in response to environmental stresses (Oliver, 1993; Colwell and Huq, 1994; Kell et al., 1998; Barer and harwood, 1999). In the VBNC state, pathogens are alive and capable of initiating an infection in humans or colonizing the gut of warm-blooded animals and birds, but its ability to grow in nutrient media is lost.

However, the VBNC state in *Campylobacter* remains the subject of controversy. Many studies found the reversion from coccoid to spiral form occurs in *C. jejuni* following animal passage (Rollins and Colwell, 1986; Saha et al., 1991; Pearson et al., 1993; Tholozan et al., 1999). The morphological reversion in *Campylobacter* cells means infection or colonizing occurs. In contrast, some studies have shown that these results are not always reproducible (Medema et al., 1992; Hazeleger et al., 1995). This conflict may result from strain differences and animal variation (Jones et al., 1991; Medema et al., 1992).

Regardless of the VBNC state of *C. jejuni*to maintain its ability to infectan animal or not, the proposed VBNC state is physiologically important for survival. As proposed by Rollins and Colwell (1986), the VBNC state allows a *Campylobacter* cell to maintain its physical activities until environmental conditions become favourable for growth and cell division. It plays an important role in *C. jejuni*'s survival in the environment.

2.6.2 Two-component regulatory system of *C. jejuni*

C. jejuni has several two-component regulatory systems that are not generally found in other enteric bacteria (Murphy et al., 2006). Those two-component regulatory systems appear to be involved in stress defences through regulating sets of genes to respond to changing environments.

The two-component signal transduction system (TCS) is made of a sensory histidine kinase, located in the cytoplasm with a response regulator located in the cytoplasmic membrane. There are nine response regulators, six histidine kinases and five two-component regulator systems in the genome of *C. jejuni* 11168 (Parkhill et al., 2000). The two-component regulatory system starts when histidine kinase senses a specific environmental stimulus and phosphorylates the response regulators. Phosphorylation causes the response regulator's conformation to change, usually by activating an attached output domain, which then leads to the stimulation (or repression) of expression in the target genes (Stock et al., 1989).

Three of the five TCSs in *C. jejuni* are associated with colonization (Murphy et al., 2006). The RacR-RacS system is responsive to temperature changes. Bras et al. (1999) found that racR mutants and wild type *C. jejuni* have the same growth rate at 37 ^{o}C, but the racR mutant demonstrated a decreased growth rate when the temperature increased to 42^{o}C. A TCS involved in oxidative stress resistance in *C. jejuni* has been proposed to involve the genes Cj0889c (histidine kinase) and Cj0890c (response regulator). Mutants of this TCS system have increased sensitivity to peroxide and paraquat stress and showed sensitivity to atmospheric levels of oxygen (Garosi et al., 2003). Although how two-component regulators being involved in

Campylobacter stress global response is still waiting to be revealed, the importance of the two-component regulators in *Campylobacter* survival is undoubtable.

2.7 Survival at low temperatures

C. jejuni has a relatively narrow growth temperature, ranging from 30°C to 45°C (Hazeleger et al., 1998; Park, 2002). In the natural environment outside a host, it can be assumed that *C. jejuni* survives, rather than grows. It was determined that *C. jejuni* survived for only a few days when incubated at 20°C in surface water, but survival was prolonged to several weeks and months at 4°C in the dark (Buswell et al., 1998; Terzieva and McFeters, 1991; Rollins and Colwell, 1986). The motility, oxygen consumption and protein synthesis of *C. jejuni* still occurs during its long term survival at low temperatures (Hazeleger et al., 1998). Due to refrigeration and freezing being widely used for food preservation, the ability of *C. jejuni* to survive at low temperature is of obvious relevance to food safety and public health. The cold stress response in *Campylobacter* has attracted many researchers' attention. A number of studies have investigated how *C. jejuni* responds to low temperatures, and several cold stress response factors in this pathogen have been proposed. The main findings of these studies follow.

To examine the importance of the VBNC state in *C. jejuni* survival at low temperatures, Lazaro and co-workers (1999) investigated morphological changes in *C. jejuni* after long-term exposure to low temperatures. The study used an electron fluorescence microscope combined with cell metabolic activity detective methods and a DNA maintenance assay to observe morphological changes and investigate the correlation between morphological changes and the availability of *C. jejuni* during long-term cold exposure.

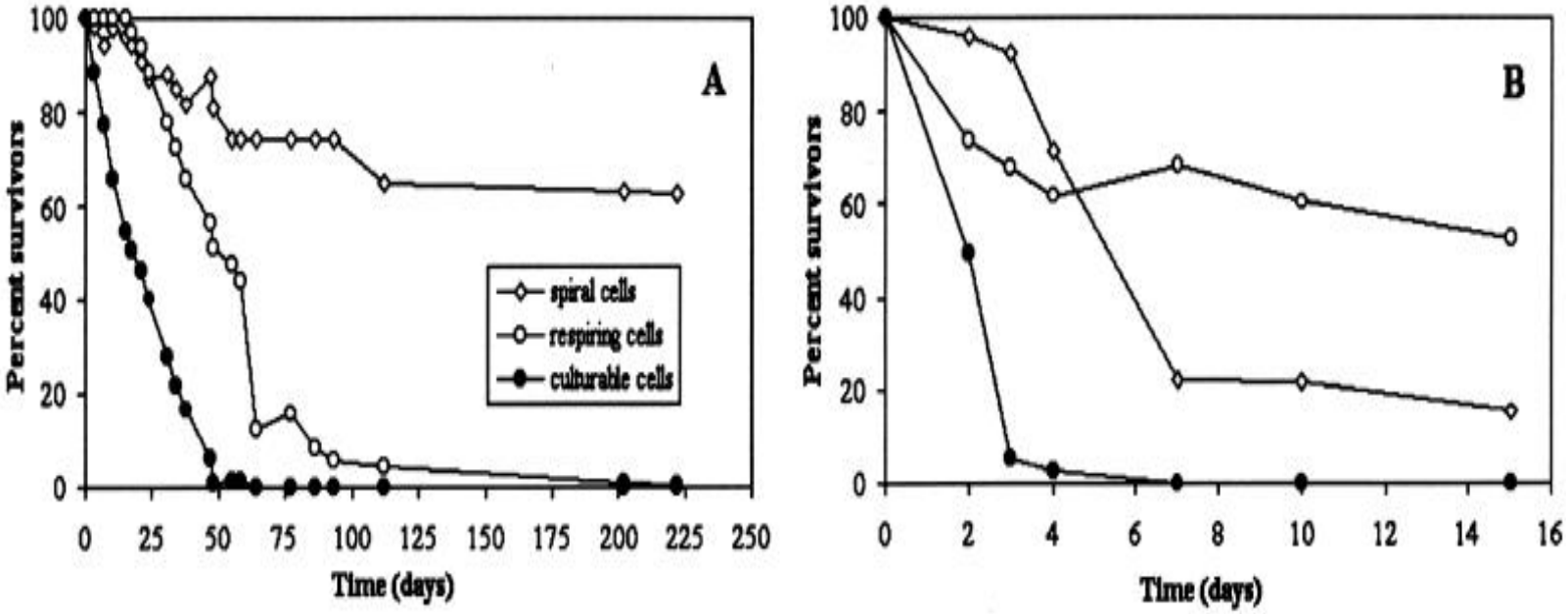


Figure 2.3 Survival curves of *C. jejuni* during incubation in PBS at 4°C (A) and 20°C (B)

The figure is adapted from Lazaro et al., 1999. In the paragraph, ◊ indicates spiral cell; ○ represents respiring cell and ● indicate the culturable cell.

As shown in Figure 2.3, the study found the *C. jejuni* used in the study survived much longer at 4^{o}C than at 20^{o}C. At 4^{o}C, *C. jejuni* was able to remain culturable for up to 50 days, while the culturability only remained for seven days at 20^{o}C. The cell integrity and respiratory activity of *C. jejuni* were maintained much longer than their culturability. In fact, survival continued for up to seven months at 4 ^{o}C based on signs of viability other than its culturablility. The study also found there was no association between culturablility and the morphological change from a spiral to coccal shape in *C. jejuni*. Both the spiral and coccal forms contain VBNC cells. The study also observed that *C. jejuni* cells became spheroid more quickly when kept at room temperature rather than at 4^{o}C. Two-dimensional (2D) gel electrophoresis was conducted in this study to compare the protein profile of the culturable and VBNC cells. The comparison showed both up- and down expression of proteins in these two different states, which suggests the transition to nonculturable cells is an active process, instead of a degenerative response without protein synthesis (Hazeleger et al., 1995).

This study revealed that the VBNC is an important state to maintainthe survival of *C. jejuni* at low temperatures. At 4^{o}C, survival of this pathogen continued for up to five months after the cell lost its culturability. However, the transition to a nonculturable cell was not associated with a morphological change from a spiral to a coccal shape.

2.8 Strain dependence of cold tolerance for *C. jejuni*

A wide range of phenotypic and genotypic diversity at strain level has been reported for *C. jejuni*. In terms of cold tolerance, studies found survival of this pathogen in water varies markedly among the different strains (Jones et al., 1991; Terzieva et al., 1991). To examine the impact of strain variability in cold tolerance of this pathogen, Chan et al. (2001) placed different strains of *C. jejuni* at 4^{o}C for two weeks to compare their viability.

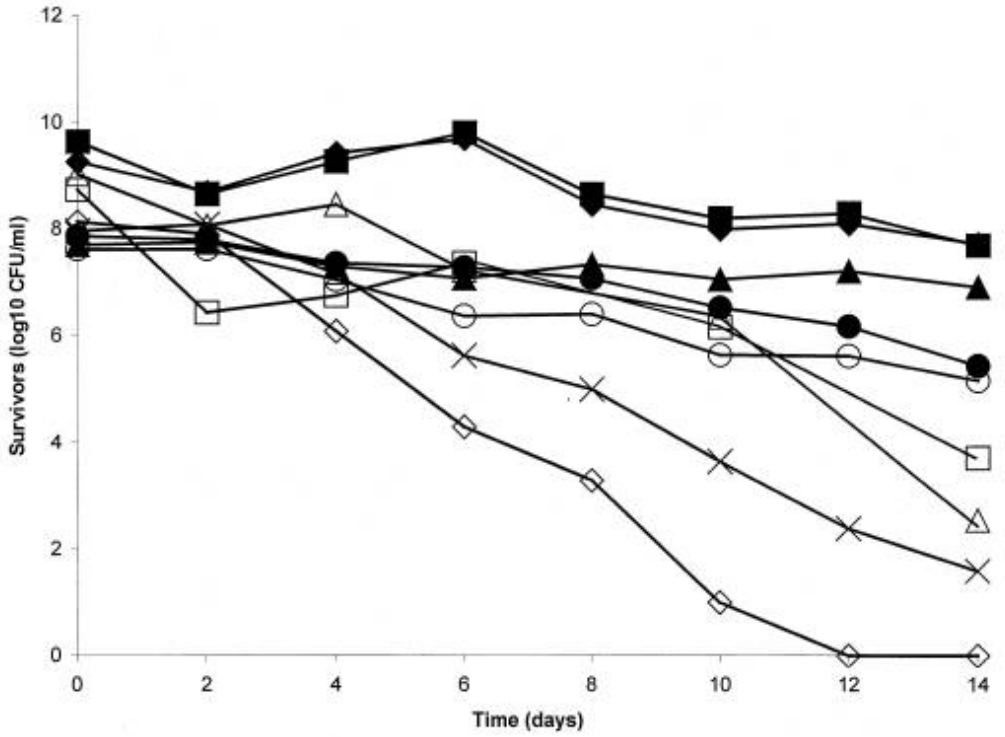


Figure 2.4 Survival curves of representative clinical and poultry-derived *C. jejuni* strains at 4°C

The figure is adapted from Chan et al., 2001. Five poultry isolates were Δ, ◊, x, o and □. Four clinical isolates were ■◆● and ▲.

As presented in Figure 2.4 by Chan et al. (2001), *C. jejuni* strains vary noticeably in terms of their cold tolerance. Chan et al. found four clinical isolates had only limited viability losses during the 14-day survey period. Conversely, five poultry-derived strains had a marked or intermediate loss of viability. Based on this observation, Chan et al. suggested that environmental stress conditions, such as low temperature, may act as a selective filter, in which only those stress tolerant strains that were able to survive with an adequate numbers can infect human. This hypothesis was supported by On et al. (2006), who found a correlation between the genomic content of *C. jejuni* and its capacity for environmental survival and suggested the correlation may help to explain why most strains with a higher survival potential are commonly found from human diarrhoea. In addition, as *Campylobacter* strains exhibited pronounced variation in cold tolerance, cold stress response studies should choose their strain carefully.

2.9 Searching for cold stress response mechanisms

2.9.1 Transcriptomic approach

Although many studies observed survival of *C. jejuni* after long-term cold exposure and maintenance of its physiological activity in the form of oxygen consumption, catalase activity, protein synthesis and ATP generation (Hazeleger et al., 1998; Lazaro et al., 1999), the mechanisms used by this pathogen to adapt to cold temperature remain unrevealed.

In order to address this question, Stintzi and Whitworth (2003) conducted a genome-wide transcriptomic analysis to investigate the response of *C. jejuni* to a temperature downshift from the optimal growth temperature of 42^{o}C to 37^{o}C, 32^{o}C, 10^{o}C and 4^{o}C. The transcriptomic analysis results showed that the transcript abundance of 218 genes, a total 13% of the genome, was significantly altered when the temperature went down. Among them, 55 genes had their transcript abundance increased when the temperature dropped from 42^{o}C to 10^{o}C and 5^{o}C. The proteins decoded by those 55 genes were likely required for the response of *C. jejuni* to low temperatures. These 55 genes can be divided into four different groups based on the function of

the proteins they encode for. Group 1 genes are involved in energy metabolism. Group 2 genes are associated with oxidative defence. The genes in the Group 3 encode for the transporters of amino-acids, carbohydrates and organic acids, which work as crytoprotectant molecules. Group 4 genes encode for proteins with unknown functions.

At 4^{o}C, *C. jejuni* had been found to strengthen its aerobic respiration and the tricarboxylic acid cycle for a higher energy demand. The Group 1 genes involved in energy metabolism were up-regulated. The proteins encoded by these up-regulated energy metabolism genes are responsible for the cell's tricarboxylic acid cycle, oxidative phosphorylation, glycolysis and gluconeogenesis (Moen et al., 2005). Those up-regulated genes are *gltA* (citrate sythase), *acnB* (aconitase hydratase), *icd* (isocitrate dehydrogenase), *oorABC* (2-oxoglutarate: acceptor oxido-reductase), *sucC* (succinyl CoA synthase), *sdhABC* (succinate dehydrogenase), *fumC* (fumarate hydratase), and *mdh* (malate dehydrogenase) (Stintzi and Whitworth, 2003).

The Group 2 genes related to oxidative defences have been found up-regulated at 4 ^{o}C. The reason may be that *Campylobacter* cells face severe aerobic stress in cold temperatures. Since oxygen solubility increases with decreasing temperature, a liquid medium at 4^{o}C contains more dissolved oxygen than at 42^{o}C. Moreover, at low temperatures, aerobic respiration and the tricarboxylic acid cycle in *Campylobacter* cells will be favoured and be used to produce energy. As the result, free radicals are assumed to be abundant in the cells during cold shock. The up-regulated oxidative defence genes are cytochrome C551 peroxidase gene (CJo358) and the superoxide dismutase (*sodB*), both which are responsible for the removal of free radicals.

The study also found that Group 3 genes which encoded for amino acids, carbohydrates and organic acid transporters were up-regulated at 4^{o}C. These genes are pebC (ABC-type amino acid transporter ATP-binding protein), Cj0919c-Cj0920c (putative ABC-type amino acid transporter permease protein), *sdaC* (serine transporter), Cj0903c (putative amino acid transport protein), *peb1A* (probable ABC-type amino acid transporter periplasmic solute-binding protein), *dctA* (putative C4-dicarboxylate transport protein), *dcuB* (putative anaerobic C4-dicarboxylate

transporter), *kgtP* (alpha-ketoglutarate permease), *dcuA* (putative anaerobic C4-dicarboxylate transporter) and *lctP* (L-lactate permease). Amino acids, carbohydrates and organic acids can function as cryoprotectant molecules in *E. coli* to protect cell viability at low temperatures. It is assumed that the transporters with increasing transcription are involved in the acquisition and uptake of cryoprotectant molecules that play an essential role in *C. jejuni* survival at low temperature (Stintizi and Whitworth, 2003).

Stintizi and Whitworth also found that, except for these 55 genes that increased expression at 10^{o}C and 5^{o}C in the study, there were another 27 genes that increased transcript abundance significantly in response to a temperature decrease from 42^{o}C to the four colder temperatures. The abundance of the transcripts from those 27 genes suggests an essential role for their product in the bacteria's adaptation to lower temperatures. In addition to genes of unknown function, these 27 genes include three signal transduction proteins (Cj1189c, Cj1223c and Cj1492c), five membrane proteins and transporters (Peb4, p19, Cj0175c, Cj0982c and Cj0654c) and two protein PssA (CDP-diacylglycerol-serine O-phosphatidyltransferase) and SodB (superoxide dismutase). The increasing gene expression of the three sets of two-component regulatory system (Cj1189c, Cj1492c and Cj1223c) suggests that they have an important role in *C. jejuni* cold adaption. The specific role in cold adaption of Peb4, p19 and Cjo175 has not yet been revealed. As Peb4 is a major antigen and p19 and Cj0175c are iron acquisition proteins, the increased level of transcripts encoding for these three proteins suggest this antigen and iron may play an important role in adaptation to cold temperature. The Cj0982c gene and the Cj0981 gene are both involved in the acquisition of cryoprotectants.

The study conducted by Stintizi and Whitworth (2003) identified potential mechanisms that could contribute to the cold adaptation of *C. jejuni*. These included acquisition or biosynthesis of cryoprotectant molecules, strengthening of aerobic respiration for energy needs, enhancement of the oxidative defence system and up-regulation of several two-component systems, which could be useful for sensing environmental changes and eliciting a proper response. This genome-wide

transcriptomic analysis of *C. jejuni* cold adaption provided a global gene expression profile at the transcriptional level, but this is only the first step in elucidating the survival mechanisms of *C. jejuni* at low temperatures. Further study of those cold up-regulated genes and their encoding proteins will be necessary for gaining a better understanding the cold survival mechanisms of this pathogen.

2.9.2 Genetic engineering approach

Genetic engineering technologies, that introduce mutations at a target gene to generate a knockout gene, have been used to investigate the role of certain factors in *C. jejuni* cold shock adaption and cold survival mechanisms. Haddad et al. (2009) proposed that long-term survival of *C. jejuni* at low temperatures is dependent on polynucleotide phosphorylase (PNPase) activity. PNPase is a major component of *E. coli* degradosome, which degrades RNA from 3' to 5' (Carpousis, 2007; Regonesi et al., 2004). In other bacteria, it has shown multiple biological functions including adaption to low temperatures (Stintzi and Whitworth, 2003). PNPase selectively degrades the mRNAs of stress-response proteins when cells process stress adaptations. The selective degradation of stress-response proteins mRNAs can prevent over production of these proteins, which is deleterious to cells (Yamanaka and Inouye, 2001).

To analyse the role of PNPase in the survival of *C. jejuni* at low temperatures, two deletion-derivative strains with the *pnp* gene inactivated have been created to compare their growth characteristics with the wild-type strains at different temperatures. The study found that the derived strains had a lower viability at 4^{o}C, of up to 3 log cfu/ml (Figure 2.5). The difference in viability clearly demonstrated the involvement of PNPase in the long-term survival of *C. jejuni* at 4^{o}C.

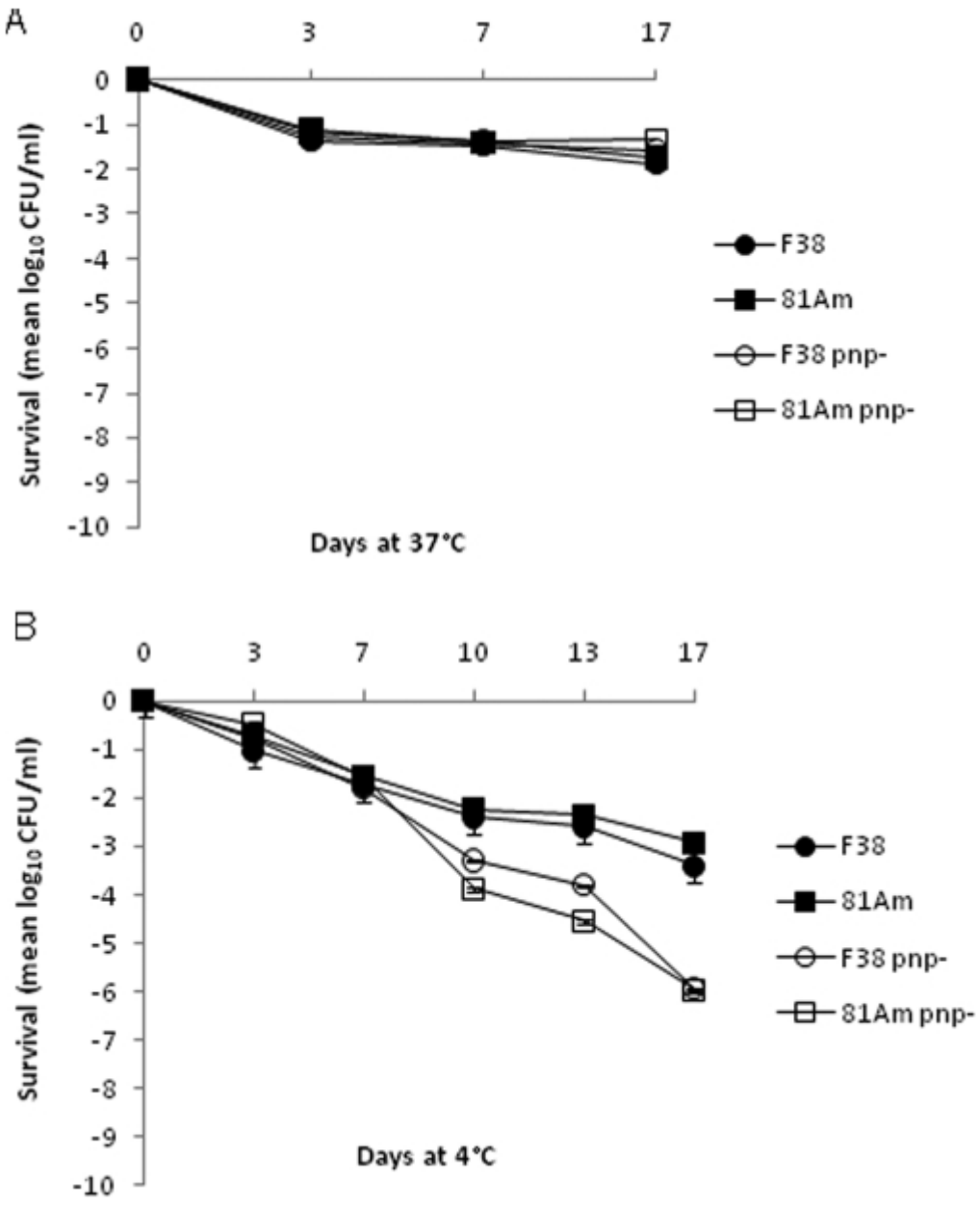


Figure 2.5 Survival of two *C. jejuni* wild types strains (F38 and 81Am) and their *pnp* mutant derivatives (F38pnp and 81Ampnp)

At 37°C (A) and 4°C (B) in the ambient atmosphere (modified from Haddad et al., 2009). The values plotted are means ± standard deviations (error bar).

2.9.3 Fatty acid composition analysis approach

As well as the transcriptomic and genetic approaches, fatty acid composition analysis has also been applied to search for potential *C. jejuni* cold-shock adaption and cold survival mechanisms. One of the most important cold exposure tolerance mechanisms for a wide range of mesophilic bacteria is a change in fatty acid composition, particularly in the outer membrane (Sinensky, 1974). Maintaining membrane homoviscosity is critical for maintaining cell integrity and viability.

Hugheset al. (2009) conducted a fatty acid composition analysis to examine the effects of 24 h exposure to refrigeration conditions on the whole-cell fatty acid compostion of *C. jejuni*. The study found cold exposure had a very small effect on the ratio of unsaturated fatty acids (UFA)/ saturated fatty acids (SFA) in *C. jejuni*, and thus, is unlikely to affect the membrane fluidity. A common bacterial response to a reduction in temperature is to change the composition of membrane fatty acids, for example, by increasing the amount of UFA and cyclic fatty acids, to increase the fluidity of the cell membrane. However, a change in the amount of UFA and cyclic fatty acids did not occur in *C. jejuni* during cold exposure.

Hughes et al. (2009) suggested that *C. jejuni* may not be required to increase the amount of UFA and cyclic fatty acids for cold adaption, as *Campylobacter* cells contain large amounts of cyclic fatty acids that increase in fluidity at low temperatures and increase membrane stability at higher temperatures (Dufourc et al., 1984). The presence of a large amount of cyclic fatty acids within the cell membrane suggests that *C. jejuni* may use this distinct mechanism to allow fluidity to be maintained in the cell membrane without altering its composition.

Through the three different approaches above, a number of potential mechanisms that could contribute to the cold adaptation and long term cold survival have been identified. Transcriptomic analysis, genetic engineering and fatty acid composition analysis all have exhibited their usefulness as tools to elucidate the potential mechanisms employed by *C. jejuni* to respond to cold stress. However, limitations are also obvious in these three approaches. Transcriptomic analysis study of mRNA,

which serves as an intermediate between DNA and proteins rarely take part in physiological metabolic activities. Alteration of fatty acids in membranes may play important role in the *Campylobacte*r cold shock response, but lipids only count for a very small percentage of cell contents and have very limited biological functions. Genetic engineering technology is very useful to confirm functions of a gene but is unable to search for potential global stress response mechanisms.

2.10 Proteomic study of *Campylobacter*

2.10.1 General microbial proteomic applications

Proteomics is the large-scale study of the proteome; "a full complement of expressed proteins from the genome of a given cell, tissue or organism at a particular point of time" (Mitton and Kranias, 2003). Proteomics provides a comprehensive approach to separate, identify, characterize and quantify proteins and provide information about protein abundance, location, modification and protein-protein interactions in a given biological system (He and Chiu, 2003; Stoughton and Friend, 2005). After genomics and transcriptomics, proteomics is considered as the next step in the study of biological systems. As proteins are the main components of the physiological metabolic pathways of cells and their composition is positively correlated with changes of cellular functions, proteomics is considered as a better method than genomics for cellular bio-physiological studies. Furthermore, the proteomic method is able to determine the time course of protein behaviour and describe the dynamics of protein alteration. In addition, proteomics has an advantage over genomics because post translational modification of proteins can only be studied by proteomics. Due to these advantages, proteomics has been used in a wide range of biological system studies.

Microbiology is considered as an important pillar of biological science. Microorganisms have relatively reduced genomes, but they also represent biological complexity and exquisite diversity (Burg et al., 2011). Most new biotechnologies have their first application in microbiology and use microorganisms as interesting experimental models to develop their strategies (Armngaud, 2012). In proteomics,

new tools and strategies are developing through several model microorganisms. Meantime, the ongoing progress in proteomic tools and strategies is opening new perspectives in the study of microbial biophysiology. Nowadays, proteomics has been widely applied to search for key cellular players, virulence factors, stress response mechanisms and vaccine target proteins in microbiology.

2.10.2 Development of microbial proteomic technology

A typical microbial proteomic experiment includes three steps: (i) the separation and isolation of proteins from a cell line; (ii) the acquisition of protein structural information to identify and characterze proteins; and (iii) database use (Graves and Haystead, 2002). Protein separation can be divided into protein-centric (gel-based) approaches, such as two-dimensional electrophoresis (2DE); peptide-centric strategies, based upon liquid chromatography (LC); and hybrid approaches, such as 'slice and dice' SDS-PAGE/LC, that incorporates elements of both (Issaq et al., 2002).

The final separation method determines the method used for the identification of proteins. For gel-based approaches, single protein bands or spots are generally excised and subjected to peptide mass mapping/fingerprinting, where the individual protein is digested with a protease and the resulting mixture analysed by MALDI-TOF and ESI-TOF mass spectrometry (MS). For peptide-centric approaches, which convert an entire protein mixture to peptides by tryptin digestion and then separate those peptides by LC, protein identification is achieved by the identification of those separated peptides through MS (Issaq et al., 2002).

In the 1990s, 2DE was the mainstream protein separation method for microbial proteomic study. With several important technological breakthroughs in mass spectrometry in the first decade of this century, protein quantification through MS then became possible. These developments in MS made gel-free shotgun proteomics increasingly used and is pushing out 2DE proteomics.

The shotgun concept refers to a gel-free method consisting of a high-pressure LC coupled to tandem mass spectrometry and database searching software. The advantages of the shotgun gel-free proteomics approach over 2DE are: i) shotgun

proteomics can not only identify proteins on proteome scale but also is able to quantify proteins at the same time; ii) shotgun proteomics has a higher resolution power to resolve the proteins with extreme isoelectric point and masses, which cannot be resolved with 2DE; iii) a complex mixture containing hundreds different proteins can be comprehensively analysed by shotgun proteomics, as the shotgun approach has higher resolution power to cope with larger dynamic ranges of protein mixtures;and iv) membrane-associated proteins, which are under-represented in 2DE, can be readily detected bythe shotgun approach. Due to the advantages over 2DE, the shotgun gel-free approach has increasingly gained popularity in the study of microbial proteome.

2.10.3 Proteomic applications at *Campylobacter*

The central issues of *Campylobacter* proteomic analysis are unravelling the pathogenic mechanisms and stress response mechanisms of this pathogen. Current proteomic research method in the study of *Campylobacter*'s stress response mostly rely on fluorescence two-dimensional differential gel electrophoresis (2-D DIGE) to quantify the variation of bacterial proteins in an electrophoresis process, and then establish statistically valid thresholds for assigning quantitative changes between cells growing in different conditions.

To gain a better understanding of the mechanisms employed by *Campylobacter* to colonize chickens' small intestines and to determine colonization-associated factors at the process of *C. jejuni* colonization, Seal et al. (2007) had used a proteomic approach, which combines 2D DIGE with MALDI TOF/TOF, to identify differences in protein synthesis between two *C. jejuni* isolates. One was a robust chicken gastrointestinal colonizing *C. jejuni* isolate, and the other was a poor colonizing strain. The comparative proteomic analysis detected several potentially important proteins involved in the colonization of this pathogen in chicken. They included several metabolism-related proteins and three potential virulence factors: a putative serine protease, a putative amino peptidase P and a branched outer membrane-fibronectin binding protein. The identification of those proteins was, potentially,

involved with *Campylobacter* colonization in chicken, facilitating the deciphering of colonization mechanisms of this pathogen in chicken.

A similar proteomic analysis was applied to detect the temperature-dependent virulence factors of *C. jejuni* (Zhang et al., 2009). *Campylobacter* behaves differently in different environments. This organism colonizes asymptomatically in chickens, at a temperature of 42^{o}C, but causes diarrheal diseases in humans, at a temperature of 37^{o}C. It is presumed that there is a subset of proteins regulated by temperature that may be virulence factors and be responsible for pathogenicity. Zhang et al. utilized 2D DIGE with MALDI TOF/TOF to investigate the difference ofprotein expressionin *Campylobacter* at 37^{o}C and 42^{o}C. The comparative proteomic analysis found 18 proteins with different expression at the two temperatures; 13 proteins were up-regulated and five proteins were down-regulated at 37^{o}C. The difference expression of these proteinsin the two different temperatures provided the basis for further investigation into the pathogencity roles of these proteins in *C. jejuni*.

As well as pathogenesis, a stress response is the other central issue of *Campylobacter* proteomic study. To evaluate the response of *Campylobacter* to high pressure and subsequent recovery at the molecular level, Bieche and colleagues conducted comparative proteomic analysis to detect alterations in protein expression caused by an ultra-high pressure treatment and subsequent recovery. The 2D protein profile of *Campylobacter* cells without ultra-high pressure treatment was used as a control to compare with the other three protein profiles, which were from the cells after being treated with ultra-high pressure 0 hour, 1 hour and 2 hours. Through comparing the protein profiles, the study identified the 22 proteins being repressed by the high pressure treatment and 23 proteins appeared in higher abundance and so might play an important role in protecting and the recovery from the high pressure treatment (Bieche et al., 2012). Those high pressure repressed proteins are mostly involved in energy metabolism and chemotaxis. Those higher abundance proteins are related to oxidative, cold-, heat- and NaCl-stress responses of *Campylobacter.* This finding provides an insight into how ultra-high pressure

treatment injures *Campylobacter* and how this pathogen recovers from injuries caused by the high pressure.

2.10.4 Searching for cold shock proteins in *C. jejuni*

To ensure their survival, mesophilic bacteria process cold shock responses to react to rapid temperature downshifts. During the cold shock response, cells stop growing and the protein production machinery of bulk proteins in the cell becomes transiently inhibited. Meanwhile, a specific set of genes is induced by the temperature downshift and results in high levels of newly synthesized cold-induced proteins, which are also called cold shock proteins (Csps). Csps have been shown to be essential for the efficient adaptation to low temperatures and they help the cell survive in temperatures lower than optimum growth temperature by a reorganisation of the bacterial DNA transcription and mRNA translation (Thieringer et al., 1998).

Csps form a highly conserved family of structurally related nucleic acid-binding proteins. These small proteins with a molecular mass of approximately 7.4 KDa comprise the typical cold shock domain (CSD). Since the first cold-induced protein, CspA, found in *E. coli* (Goldstein et al., 1990), there have been eight homologous proteins revealed in *E. coli*, named in alphabetical order from CspB to CspI. In *Bacillus subtilis*, three proteins homologous to *E. coli* CspA have been found to be induced upon a temperature downshift (Willimsky et al., 1992; Graumann et al., 1996). As the CSD is the most evolutionary conserved nucleic acid-binding domain within prokaryotes and eukaryotes (Wolffe, 1994; Graumann and Marahiel, 1998), Csps which contain CSD have been found in more than 50 Gram-negative and Gram-positive bacteria species (Graumann and Marahiel, 1998).

There have been a number of attempts to search for Csps in *C. jejuni* through different methods including the proteomic approach. Hazeleger et al. (1998) compared cold exposure (4^{o}C and 20^{o}C) *C. jejuni* protein profiles with protein profile from the cells without cold exposure to find cold-induced proteins. This comparison showed the total numbers of protein bands produced seemed to be similar for all

temperatures, and no cold induction of a specific protein was observed. Hazeleger et al. (1998) used low molecular protein separation gels to research cold-induced protein band in the 7 kDa region, which is the expected size for Csps homologues. However, there was no cold-induced band observed in the cold exposure *C. jejuni* protein profiles. The other proteomic approach launched by Lazaro et al. (1999) also failed to find any cold induced protein in the 7 kDa region.

The main reasons for previous proteomics attempts failing to find major cold induced proteins in *C. jejuni* are that these studies (Hazeleger et al., 1998; Lazaro et al., 1999) mainly focused on the 7 kDa area. With more cold-induced proteins being revealed from different organisms, the range in molecular weight of these cold-induced proteins has been extended greatly (Ferhan, 2000), so cold shock protein research should not only focus on the low molecular proteins but also on other proteins as well. What is more, these previous proteomicstudies relied on 2DE, which has a number of limitations for protein separation. For example, when a protein mixture has large range of proteins in the same molecule weight, 2DE will not be able to identify low abundant proteins, which are masked by the most abundant proteins. Moreover, the sub-sequenced protein identification and comparisons with 2DE are relatively time-consuming and labour intense. Lazaro et al. (1999) found the differences in protein synthesis in *C. jejuni* under cold stress, but there were no further comparative studies about the differences in protein synthesis in *C. jejuni* processing cold stress response, presumably due to the time constraints of 2DE proteomics analysis.

With developments in microbial proteomics technology, gel-free shotgun proteomics with higher resolution power provides a better chance to illustrate the protein biosynthesis alteration in *C. jejuni* influenced by temperature downshifts. Although gel-free proteomics has gained its popularity in microbiology, there have still been no gel-free proteomic approaches applied in the study of *C. jejuni*. To my knowledge, the present iTRAQ labelling proteomic analysis is the first to apply gel-free proteomics in the study of *C. jejuni*.

Chapter 3 Materials and Methods

3.1 Bacterial strains

All *C. jejuni* strains used in this study were supplied by ESR, New Zealand. Three *C. jejuni* strains were selected for this comparative proteomic analysis, namely: *C. jejuni* NCTC 11168, SVS 5001 and SVS 5141 (Table 3.1). In this study, *C. jejuni* NCTC 11168 was used as a reference strain, as this strain has been fully sequenced and extensively studied. Both *C. jejuni* SVS 5001 and SVS 5141strains are associated with a Danish waterborne outbreak; the former is a human diarrhoeal isolate and the latter was recovered from the water that was the source of the outbreak. Previous genomics studies based on DNA microarray technology suggested that these two Danish waterborne outbreak strainsdeserved further investigation, in particular their cold tolerance (On et al., 2006).

Table 3.1 Three *C. jejuni* strains used in the study

Strain	Description
C. jejuni NCTC 11168	The first C. jejuni strain which was sequenced and annotated in 2000 (Parkhill et al., 2000).
C. jejuni SVS 5001	Danish waterborne outbreak strain, isolated from human faeces.
C. jejuni SVS 5141	Same Danish waterborne outbreak strain, isolated from water.

3.2 Preparation of bacterial starter cultures

The protocol used in this study to grow the bacterial culture was modified from the method used by Bieche et al. (2012). The three strains were stored in glycerol broths at -80^{o}C. Prior to the experiment, all strains were inoculated onto Columbia base agar containing 5% defibrinated sheep blood (Oxoid) and incubated at 42^{o}C for 48 hours in a microaerobic incubator (10% CO_2, 5% O_2 and 85% N_2) to recover from frozen storage.

After 48 h incubation for recovery, the bacterial growths of each strain on the plates were washed by 20 ml of Brain-Heart Infusion Broth (BHI) broth and harvested into individual bottles. The optical density (OD) of the 20 ml suspension was measured using a spectrophotometer. To prepare an inoculum broth, the 20 ml suspension was adjusted to OD 1.0 (or its equivalent) at 540 nm by adding sterile BHI broth. A 5 ml sample of the adjusted suspension was taken and inoculated into duplicate BHI broth bottles (500 ml each). The starter cultures were then incubated for 24 h at 42^{o}C in arotary shaker set at 120 rpm under microaerobic conditions to promote log phase cultured cells as a starter culture. The starter cultures of the three *C. jejuni* strains were prepared using a similar method.

3.3 Cold stress exposure

For each strain, the two 500 ml bottle starter cultures were separated into twenty 50ml sterilized tubes. Two tubes of 50 ml starter cultures were used immediately for viability assessment and cell protein extraction after separation. The protein extracts from the liquid cultures without cold exposure were used as non-stressed references (0 h) to compare with protein samples extracted from cold stressed cells.

The remaining 18 tubes of liquid culture for each strain were placed in a rotary shaker set at 15 rpm at 4^{o}C under microaerobic conditions for up to 8 days. During the 8 days of cold incubation, viability assessment and cell protein extractions were performed by sampling at 5 different time points, i.e. after 6h, 1 day, 2 days, 6 days and 8 days. The experimental plan for the viability assessment and cell protein extraction is shown in Table 3.2. At each time point, duplicate cultures of each strain were removed out from the 4^{o}C incubator and viable cell counts undertaken and whole cell protein extracts were prepared.

Table 3.2 Timeline for viability assessment and preparation of cell protein extracts

Time	Experiments
Two days for the strains recover	Took the three strains out of -80^{o}C storage and recovered them on BA at 42^{o}C for 48h.
One day for starter culture preparation	Harvested the bacterial growths and inoculated them to BHI for 24h incubation.
0 h and 6 hour cold exposure cultures sampling	1. Liquid cultures separation for the three strains and start cold exposure. 2. Viability assessment of 0 h and 6 h samples of the three strains. 3. Protein extraction for 0 h and 6 h samples of the three strains.
Day 1 sampling of cold exposure cultures	1. Viability assessment of day 1 samples of the three strains. 2. Protein extraction of day 1 samples of the three strains.
Day 2 sampling of cold exposure cultures	1. Viability assessment of day 2 samples of the three strains. 2. Protein extraction of day 2 samples of the three strains.
Day 6 sampling of cold exposure cultures	1. Viability assessment of day 6 samples of the three strains. 2. Protein extraction of day 6 samples of the three strains.
Day 8 sampling of cold exposure cultures	1. Viability assessment of day 8 samples of the three strains. 2. Protein extraction of day 8 samples of the three strains.

3.4 Assessment of viability

The viability assessment was conducted using the method of Miles and Misra (1938). The procedure of viability assessment in this study is as follows: Viable cells in the cultures were enumerated by serial dilution (10^{-8}) using BHI broth as the diluent and plating in triplicate on Blood Agar (BA) plates. Three BA (Oxoid) plates were divided into eight equal sectors, and 20µl of the appropriate dilutions were dropped into each sector (Miles and Misra, 1938). After 48h of microaerobic incubation at 42^{o}C, colonies were counted in the sector where the highest number of full-size discrete colonies can be seen (usually sectors containing 2-20 colonies were counted).

The viability assessments were performed using the above described method for the starter culture and the cultures sampled after 6 h, 1 day, 2 days, 6 days and 8 days of cold (4^{o}C) exposure. The starter liquid culture without cold exposure is regarded as 0 h sample (non-stressed cells sample).

The purity of the starter culture and each aliquot of cold exposure culture were examined by Gram staining and streaking on plates. All cultures used in this study had been confirmed without contamination.

3.5 Preparation of protein sample

The protein extraction method described by Kalmokoff et al. (2006) was used in this study. For each *C. jejuni* strain, two 50 ml liquid cultures were used for whole-cell protein extraction at each time point. The procedure of protein extraction in this study was as follows: the cultures in the broth were centrifuged at 4,000g at 4^{o}C for 10 min and washed two times in Phosphate Buffered Saline (PBS) buffer (pH 7.4). After the washed cells were recovered by centrifugation, the bacterial pellet was suspended in 1 ml of lysis buffer ((Protease inhibitor cocktail of aprotinin, 7 M urea, 2 M thiourea, 4.0% w/v CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 1% DTT (Dithiothreitol) and 0.2% Biolytes)) and sonicated on ice six times for 20 s at 20 s intervals until the suspension became clear. Following sonication, the protein samples were solubilised by incubation of the lysate on ice for 1 h. Thecell debris wasthen discarded after centrifugation at 12,000g at 4^{o}C for

15 min. The supernatants containing the total protein were stored at -80^{o}C until further analysis.

Protein extraction was performed on these three strains immediately after viability assessment at each time point. Similar to viability assessments sampling, the starter liquid culture was regarded as 0 h sample. The protein extractions from cold exposure cultures for each strain were completed after cold incubation 6 h, one day, two days, six days and eight days.

3.6 Protein quantification

After obtaining protein extracts from *C. jejuni* cells, the protein concentrations of the protein samples were assayed using a modified Bradford assay (Ramagli, 1999). Please refer to Appendix 1 for the *C. jejuni* cells protein extract quantification.

3.7 Acetone precipitation of protein samples

Acetone precipitation of protein samples was performed after the protein quantification assay. As the lysis buffer used in this study contains a high concentration of urea that disturbs the following 1D electrophoresis analysis, a protein precipitation needs to be done to get rid of urea from the protein extraction samples. In addition, protein precipitation can be useful to adjust the samples protein concentration.

According to the protein concentration of samples, different amounts of protein extracts that contained 20 µg proteins were added to a 1ml tube, and then a four times the sample volume of cold (-20^{o}C) acetone were added to each sample. The tube with acetone and sample mixture was vortexed gently, and then incubated at -20^{o}C for more than two hours. After incubation at -20^{o}C, the samples were centrifuged for 20 min at 15,000g at 4^{o}C. The supernatant in the tube was discarded without dislodging the protein pellet. The protein pellet was then air dried for 30 min at room temperature. Next, 20 µl of 40mM Tris buffer (pH 7.0) was added to each tube to resuspend the 20 µg protein pellet resulting in a 20 µl solution of protein sample with a protein concentration of approximately 1mg/ml.

3.8 1D electrophoresis for whole-cell proteins

1D PAGE electrophoresis was performed to obtain 1D protein profiles for all samples. The NuPAGE electrophoresis system (Invitrogen Life Technologies, 2010) was used in this study according to manufacturer's instructions.

The protein samples containing 20 µg of proteins were mixed with 6 µl of 4× loading buffer (Invitrogen), and then were heated at 95^{o}C for 10 min. After 10 minof protein heat denaturation, the protein mixtures were loaded into the wells of a NuPAGE Novex 4-12% Bis-Tris gel (10 wells). Each gel needed to have one separate well to load 5 µl protein molecular weight standards (Invitrogen). After loading samples and standards, the gels were run in NuPAGE MES Running buffer (Invitogen) at 200 volts for 50 min. After electrophoresis was complete, the gels were taken out from cassette and were ready for staining.

3.9 Gel staining

The gel Coomassie staining method used in the study was modified from the NuPAGE Technical Guide (Invitrogen Life Technologies, 2010). After electrophoresis, the precast gel was opened by a gel knife then washed by reverse osmosis (RO) water. After washing, the gel was transferred in to a container that contained 80 ml of protein fixing solution (40% methanol and 10% acetic acid) and the gel was kept in the fixing solution for 30 min. The gel was then carefully transferred to a staining container that contained 80ml of Coomassie blue dying reagent solution (0.1% Coomassie blue G-250, 40% methanol and 10% acetic acid). The gel in the staining container was heated by microwave full-power three times for 15 s at 15 s intervals. After heating, the staining container with the gel was removed from the microwave and shaken on an orbital shaker for 30 min at room temperature. The gel was kept in the container while the Coomassie blue dying reagent solution was discarded. After decanting the Coomassie blue, the gel was rinsed once by RO water then 80 ml of destaining solution (10% ethanol and 7.5% acetic acid) was added. The gel with destaining solution was then heated three times in a microwave on full-power for 15

s at 15 s intervals. After microwaving, the gel with destaining solution was shaken overnight at room temperature on an orbital shaker.

3.10 Gel imaging and processing

After gel staining, the 1D SDS gels were scanned. The protein banding patterns from different samples in the same gel were then compared by naked eye to check the density of the protein bands.

3.11 Quantitative analysis of protein expression using iTRAQ labelling proteomics

The iTRAQ labelling proteomic analysis was carried out in the AgResearch proteomic laboratory, Lincoln, New Zealand. The procedure of iTRAQ proteomic analysis of *C. jejuni* cold stress responses included sample protein quantitation and purification, digestion of samples, labelling the protein digest, Strong Cationic Exchange fraction, liquid chromatography-mass spectrometry and iTRAQ data analysis.

3.11.1 Sample protein quantitation and purification

Protein quantification of the extracts was carried out using a 2D Quant kit. After obtaining the correct concentration of cell extracts, 80 µg of proteinwas taken from each sample for MeOH/$CHCl_3$ precipitation. After obtaining the protein pellets, they were resuspended in 60 µl 0.5M TEAB (triethylammonium bicarbonate buffer), then reduced with 20µl 100 mM TCEP (tris 2-carboxyethyl phosphine hydrochloride) in 0.5M TEAB. After reduction, the samples were alkylated with 20µl 150mM IAM (iodoacetamide) in 0.5M TEAB.

3.11.2 Digestion and labelling of the samples

Trypsin (10 µg) was added to each alkylated sample for protein digestion and then the samples were incubated at 37^{o}C overnight. After incubation, the digests were driedand each was resuspended in 20 µl 0.5M TEAB. iTRAQ labelling was followed the iTRAQ labelling protocol provided with the iTRAQ reagents-8plex kit (AB Sciex Pte. Ltd).

3.11.3 SCX (Strong Cationic Exchange) fraction

After iTRAQ labelling, a small amount of iTRAQ labelled and dried digest was transferred into a new Eppendorf tube and resuspended in 0.1% TFA (trifluoroacetic acid). The tube was then put into a conditioned C18 empore disc for shaking for 3 h. After shaking, the empore disc was washed with 0.1% TFA containing 5% ACN (acetonitrile). After washing, 50% ACN in 0.1% TFA was used to elute the peptides from the empore disc. Then, dried down the empore disc and prepared 40 µl 0.1% formic acid for SCX fractionation. The SCX fractionation was performed using high-pressure liquid chromatography. Empore-purified each fraction from the SCX fractionation step (flow through, 1%, 5%, 10%, 20%, 30%, 40%, 60%, 80% and 100%) as mentioned above.

3.11.4 LC-MS/MS (liquid chromatography-mass spectrometry)

LC-MS/MS was performed on a nanoAdvance UPLC coupled to a maXis impact mass spectrometer equipped with a CaptiveSpray source (Bruker Daltonik, Bremen, Germany). Samples (2µl) were loaded on a C18AQ nano trap (Bruker, 75 µm × 2 cm, C18AQ, 3 µm particles, 200 Å pore size). The trap column was then switched in line with the analytical column (Bruker Magic C18AQ, 100 µm × 15 cm C18AQ, 3 µm particles, 200 Å pore size). The column oven temperature was 50^{o}C. The elution gradient was from 0% to 40% in 90 min at a flow rate of 800 nl/min. Solvent A was LCMS-grade water with 0.1% FA and 1% ACN; solvent B was LCMS-grade ACN with 0.1% FA and 1% water.

Samples were measured in auto MS/MS mode, with a mass range of m/z 50-2200. One MS was followed by 10 MS/MS of the most intense ions. The acquisition speed was 2 Hz in MS and 10 or 5 Hz in MS/MS mode depending on precursor intensity. Precursors were selected in the m/z 400-1400 range; with charge states of 2-5 (singly charge ions were excluded). Active exclusion was activated after one spectrum for 0.3 min.

3.11.5 iTRAQ data analysis

Peak list files (mgf format) were generated using DataAnalysis (Bruker), concatenated and submitted to an in-house Mascot server (v2.4) (Matrix Science, UK). The following search parameters were used: Taxonomy *Campylobacter jejuni*; Enzyme semitrypsin; Cysteine modification carbamidomethyl; MS tolerance 0.02 Da; MS/MS tolerance 0.1 Da; one missed cleavage; instrument specificity ESI-QUAD-TOF. Mascot iTRAQ parameters included variable iTRAQ8plex (N-term, K, Y), with reporter ions defined as appropriate for the experiment.

Peptides with Mascot score below 20, and proteins with fewer than two peptides were discarded. Only unique peptides were used for quantitation. Normalization was based on division by channel sum.

The ratio for each protein in a sample was calculated as the signal of that sample divided by the average signal for that protein across all samples from that strain. Specifically, for each protein in these samples, the formula is as follows:

$$S1 = \frac{Spl1}{\left(\frac{Spl1+Spl2}{2}\right)}$$

Where S1 is the quantitative ratio of a protein in Sample 1, and Spl1, 2 are the intensity signals of that protein in Samples 1, 2.

$$S2 = \frac{Spl2}{\left(\frac{Spl1+Spl2}{2}\right)}$$

Chapter 4 Viability of Three *C. jejuni* Strains at 4°C

4.1 Methods

Previous studies have found refrigeration storage leads to prolonged survival of *C. jejuni* (Buswell et al., 1998; Terzieva and McFeters, 1991; Rollins and Colwell, 1986) and the survival ability of *C. jejuni* at 4°C is strain-dependant (Chan et al., 2001). To investigate the strain-dependant cold tolerance, two Danish waterborne outbreak *C. jejuni* strains (SVS 5141 and SVS 5001) and one *C. jejuni* reference strain (NCTC 11168) were exposed to 4°C (recommended food storage temperature) to examine their survival ability under cold stress.

The three *C. jejuni* strains were grown in BHI broth at 42°C under microaerobic conditions for 24 h. These cultures were transferred into fresh BHI broths and placed in an incubator set at 4°C for eight days and microaerophilic conditions were maintained. During cold exposure, a conventional microbial viability assessment (Miles and Misra, 1938) was performed at 0 h, 6 h, day 1, day 2, day 6 and day 8.

4.2 Viability assessment results

The 4°C cold storage caused the number of culturable cells to decline at all the three *C. jejuni* strains. The percentage of cell death rates of each strain at different time points and their average cell death rates during cold exposure are shown in Table 4.1 (Day 2 *C. jejuni* NCTC 11168 viability data is missing).

Table 4.1 Cell death rate of the three strains at different time points

Strain / Time points	NCTC 11168	SVS 5001	SVS 5141
6 h	53.5%	28.1%	41.9%
24 h	55%	26.1%	53.5%
48 h		54.2%	61.6%
6 day	57%	52.3%	50%
8 day	55%	52.3%	50%
Average	55.1%	42.6%	51.4%

The viability assessment results showed that there was no appreciable decrease in the number of viable cells following eight days of incubation at 4^{o}C for all three strains of *C. jejuni* used in this study. Although the three strains had nearly forty to fifty percent of decline in viable cell count during cold storage, the viable cell counts of all strains still remained around 10^{8}cfu/ml during the low temperature incubation period. To better display the dynamics of the viable count changes for the three strains, a survival curve figure (Figure 4.2) was created basing on viable cell count of these strains at the different time points.

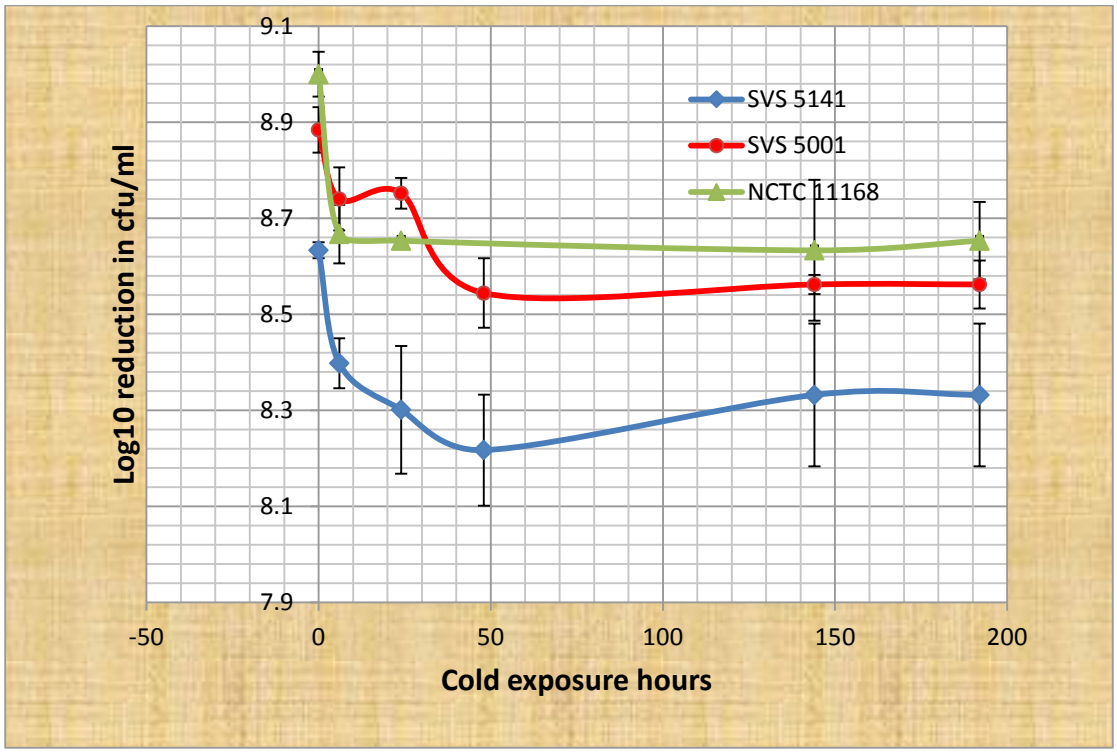


Figure 4.1 Survival curve of three *C. jejuni* strains SVS 5141, SVS 5001 and NCTC 11168 at 4°C under microaerophilic conditions for eight days

The values plotted are means ± standard deviation (error bars).

Figure 4.2 shows that NCTC 11168 and SVS 5141 has a relatively steep decrease in cell viability during the first 6 h of incubation at 4°C. The cell death rates of the two strains after 6 h cold exposure were 53.5% and 41.9%, respectively. After the first 6 h decrease in cell viability, the viable cell count of *C. jejuni* NCTC 11168 became stable. Its death cell rate remained between 53.5% and 57% during the eight days of cold exposure. The viability of *C. jejuni* SVS 5141 continued to decline for 48 h and the cell death rate reached its highest point of 61.6%, after 48 h cold exposure. Theviable cell count of SVS 5141 then increased at Day 6 and remained stable until Day 8. The cell viability of *C. jejuni* SVS 5001 fluctuated for the first two days. The death cell rate of SVS 5001 in the first 6 h was relatively low when compared with the other two

strains that suffered a large loss in their cell viablility in the first six hours of cold exposure. The viable cell count of this strain remained at a similar level after 24 h cold exposure, and then had the other clear decrease in viable cell count at 48 h when the death cell rate reached 54.2%. After the clear decrease in viable cell count at 48 h, there was no obvious change in viable cell count over the following six days.

4.3 Discussion

The cold survival ability of *C. jejuni* varied noticeably between strains (Chan et al., 2001). The differences in the cold survival ability of *C. jejuni* had been found to be related to variation in the genomic content. Generally, clinical isolates strains are more tolerant to cold stress than food-derived strains (Chan et al., 2001). In this study, two clinically isolated strains, *C. jejuni* NCTC 11168 and SVS 5001, appeared tolerant to 4^{o}C and had only limited viability loss during the eight days of cold exposure. A previous survival study had also found these two strains appeared to have a long survival at room temperature under aerobic conditions than the other chicken isolated strains (On et al., 2006). The information obtained from these two studies suggested the two clinical strains, NCTC 11168 and SVS 5001, have high survival potential.

Previous epidemiological studies had found *C. jejuni* strains with higher survival potential were more commonly isolated from human diarrhoea than those strains that were sensitive to environmental stresses. The connection between virulence determinant and survival potential in *C. jejuni* has not been elucidated. It was presumed that these strains with high survival potential were able to survive in adequate number unders multiple environmental stresses and reach the human gastroenteritis tract to cause infection.

The viability assessment found *C. jejuni* SVS 5141, hada lower average rate of cell death during eight days of cold storage compared withthe other two strains. The average rate of cell death for SVS 5001 during eight days cold storage was 42.6%, while SVS 5141 had 51.4% and NCTC 11168 had 55.1% average death cell rate in this eight days cold stress response experiment. There were no statistically significant

differences between the group means of cell death rates between the three strains,as determined by one-way ANOVA (F=2.07, P=0.17) (Appendix 2). SVS 5001 was isolated froma waterborne outbreak patient's diarrhoeal faeces; SVS 5141 was recovered from the water that was the source of the same outbreak. It was presumed these two strains have very similar genomic content and phenotype characters. Hence, It was not surprising to see these two strains appeared to have similar longevity at 4^{o}C under microaerobic conditions. The only difference between SVS 5141 and SVS 5001 was that SVS 5001 had infected a human. It will be interesting to compare cold survival ability and cold response in proteome scale for these two closely-related strains.

The survival curve showed the steepest decrease in cell viability for these three strains all happened at the first six hours of cold exposure. This indicated that the first six hours of cold exposure has a more significant impact on cell cultivability in the culture medium. In *E. coli*, the most frequently studied cold stress response Gram-negative bacteria model, synthesis of housekeeping proteins and cell division ceased after the first four hours of cold exposure. Meantime, the synthesis of cold-induced proteins in *E. coli* dramatically increased. At the end of four hours of cold exposure, the synthesis of the cold-induced proteins in *E. coli* decreased; cells become acclimated to low temperature and growth resumed (Jones et al., 1987). The influence of cold shock at cellar protein synthesis of *C. jejuni* has not been elucidated. However, from angle of viability assessment, a cold shock had a profound impact on cell function which, remarkably, reduced this pathogen's ability to be cultivated on nutritional medium.

Chapter 5 Proteomic Study Cold Shock and Adaptation in *C. jejuni* NCTC 11168

5.1 *C. jejuni* NCTC 11168 as a reference strain

C. jejuni NCTC 11168 was originally isolated from a clinical faeces sample in 1977 (Skirrow, 1977). In 2000 it became the first fully sequenced genome in the *C. jejuni* species (Parkhill et al., 2000). The genome of *C. jejuni* NCTC 11168 is 1.64 million base pairs long and contains approximately 1699 predicted genes, which are predicted to encode for 1654 proteins and 54 stable RNA species (Parkhill et al., 2000).

The genomic sequence of *C. jejuni* NCTC 11168 has provided a valuable resource for *Campylobacter* post-genomic study. Many transcriptomic and proteomic studies have used *C. jejuni* NCTC 11168 as a reference strain for comparative analysis (Stintzi and Whitworth, 2003; On et al., 2006; Kalmokoff et al., 2006; Birk et al., 2012; Zhang et al., 2012).

Previous studies have found there was substantial variability among *C. jejuni* strains in cold tolerance and clinical isolates, such as *C. jejuni* NCTC 11168, tended to have more tolerance to cold stress than environmental isolates (Chan et al., 2001; On et al., 2006). In this proteomic study, *C. jejuni* NCTC 11168 was used as the reference strain to study what caused the strain-dependent variability of this pathogen under cold conditions.

5.2 Proteomics analysis of *C. jejuni* NCTC 11168

This section reports the proteomics analysis completed to explore the cold stress responses in *C. jejuni* NCTC 11168. 1DE and iTRAQ labelling proteomics analyses that were conducted according to the methods described in Sections 3.8 and 3.11, respectively.

5.2.1 1D protein profile of *C. jejuni* NCTC 11168 during cold shock and adaptation

1D electrophoresis protein profiles of *C. jejuni* NCTC 11168 cells collected at different time-points are shown in Figure 5.1. In this study, six hours of cold exposure is considered as cold shock to *C. jejuni* NCTC 11168. All the other longer periods of cold exposure, such as one day, two days, six days and eight days of cold exposure, were considered as a cold adaptation period for this pathogen.

Cold-shocked *C. jejuni* NCTC 11168 cells have a unique 1D protein profile, which were different from the protein profile of cold adapted cells and cells without cold exposure. As shown in the Figure 5.1, two red circles in the 6 h cold shock protein banding pattern contained two down-regulated protein bands with molecular weights ～10 kDa and ～23 kDa. Two 6 h cold shock protein bands in black circles were up-regulated proteins with molecular weights～40 kDa and ～55 kDa. The four protein bands, above, in the six-hour cold shock protein profile were different from all other protein banding patterns. Except for the four unique protein bands, the six-hour cold shock protein banding pattern had two protein bands that appeared different from the 0 h protein profile but were similar to all cold adapted protein profiles. As shown in Figure 5.1, two cold shock protein bands, in yellow circles, were up-regulated compared with the 0 h 1D protein banding pattern; however, those two yellow circled protein bands, whose molecular weights were ～11 kDa and ～19 kDa, had similar densities to their counterparts in the cold adapted cells.

The Day 1, Day2, Day 6 and Day 8 cold adapted *C. jejuni* NCTC 11168 cells have similar 1D protein banding patterns. Compared with 6h cold shock cells' protein profile, those cold adapted cell protein profiles shared more similarities with the 0h cell protein profile. There were only two bands in the cold adapted cell protein profiles that appeared different from 0 h cell protein profile. They were the ～11 kDa and ～19 kDa protein bands, circled by a yellow line, as shown in Figure 5.1. Those two bands appeared up-regulated in all cold adapted cells (Day 1, Day 2, Day 6 and Day 8), but their intensity decreased successively with longer cold exposure times.

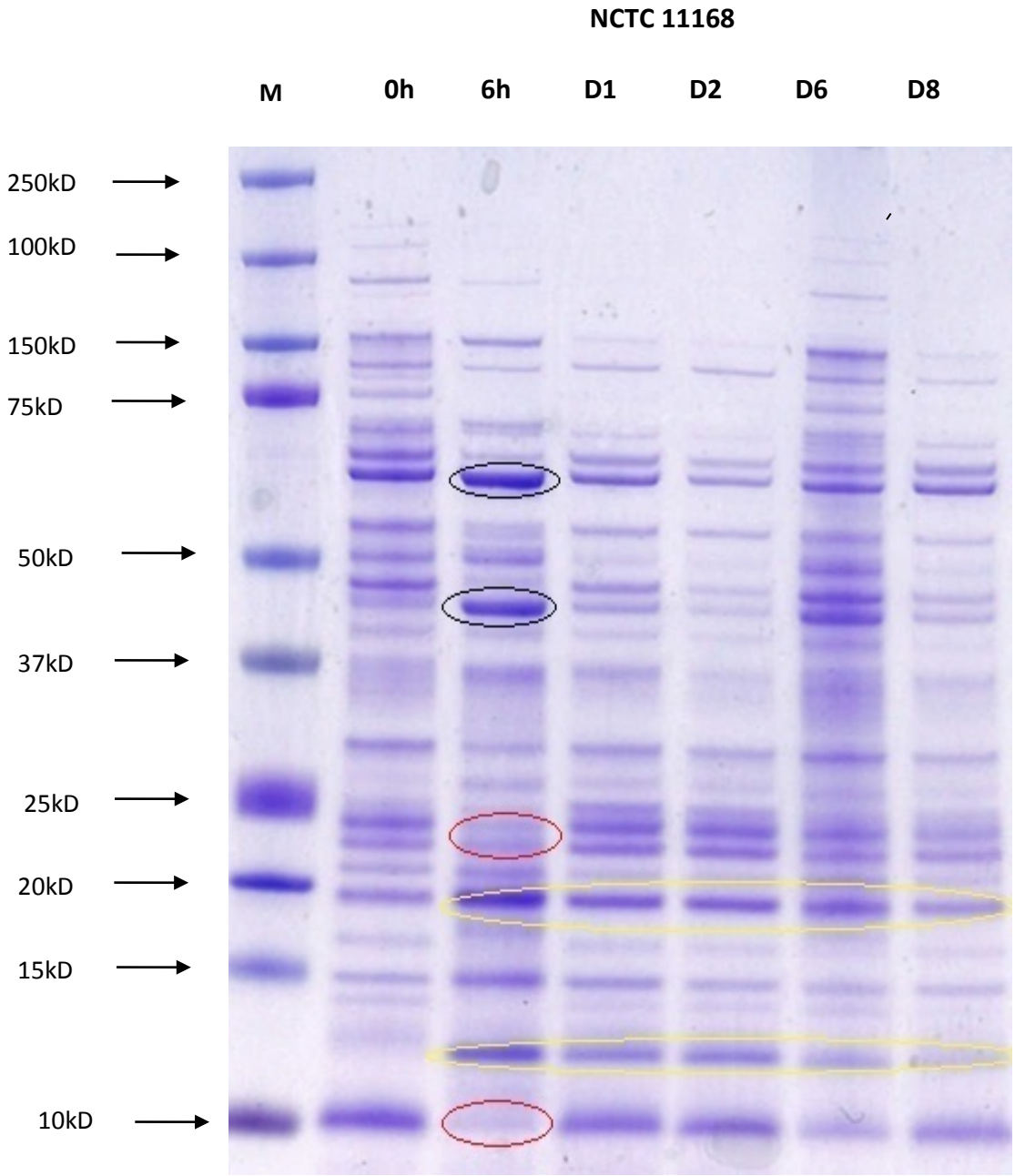


Figure 5.1 1D protein profiles of *C. jejuni* NCTC 11168 collected at 0 h, 6 h, Day 1, Day 2, Day 6 and Day 8

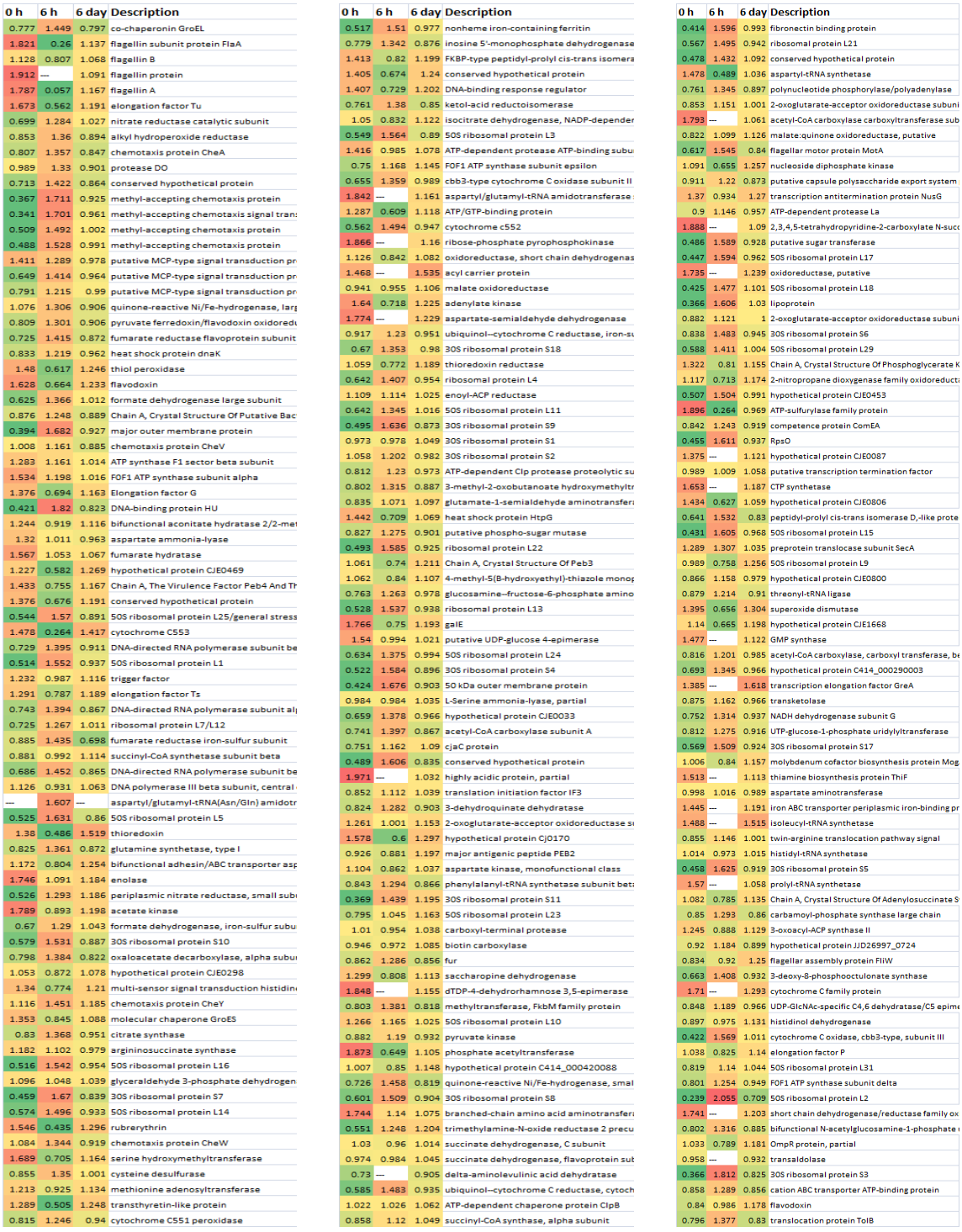
M is the marker.

5.2.2 Proteomic alteration of *C. jejuni* 11168 during cold shock and adaptation

5.2.2.1 A proteome map of C. jejuni NCTC 11168

In the present study, three *C. jejuni* NCTC 11168 cell protein extracts collected at 0 h, 6 h and after 6days of cold storage were analyzed using iTRAQ labelling proteomic approach, as described in Section 3.11. The iTRAQ labelling proteomic analysis identified 236 proteins in *C. jejuni* NCTC 11168 in this cold stress response experiment. Those 236 proteins found at NCTC 11168 with 0 h, 6 h and 6 days of cold exposure are shown in Figure 5.2. All identified proteins are shown by number and different colours. As shown in Figure 5.2, the red colour indicates those proteins' ratios are greater than the unity. The green colour indicates those proteins' ratios are less than unity. The blank indicates protein that could not be identified at that time point.

C. jejuni NCTC 11168 cells without cold exposure had 235 proteins identified. Protein extracted from this strain after 6 days of cold storage had the same number of proteins. The number of proteins in *C. jejuni* NCTC 11168 with 6 h cold shock was 214, which included one cold-induced protein (aspartyl/glutamyl-tRNA amidotransferase, A subunit) that was not found in cells without cold exposure.



0 h	6 h	6 day	Description
0.777	1.449	0.797	co-chaperonin GroEL
1.821	0.26	1.137	flagellin subunit protein FlaA
1.128	0.807	1.068	flagellin B
1.912	---	1.091	flagellin protein
1.787	0.057	1.167	flagellin A
1.673	0.562	1.191	elongation factor Tu
0.699	1.284	1.027	nitrate reductase catalytic subunit
0.853	1.36	0.894	alkyl hydroperoxide reductase
0.807	1.357	0.847	chemotaxis protein CheA
0.989	1.33	0.901	protease DO
0.713	1.422	0.864	conserved hypothetical protein
0.367	1.711	0.925	methyl-accepting chemotaxis protein
0.341	1.701	0.961	methyl-accepting chemotaxis signal trans
0.509	1.492	1.002	methyl-accepting chemotaxis protein
0.488	1.528	0.991	methyl-accepting chemotaxis protein
1.411	1.289	0.978	putative MCP-type signal transduction pr
0.649	1.414	0.964	putative MCP-type signal transduction pr
0.791	1.215	0.99	putative MCP-type signal transduction pr
1.076	1.306	0.906	quinone-reactive Ni/Fe-hydrogenase, larg
0.809	1.301	0.906	pyruvate ferredoxin/flavodoxin oxidored
0.725	1.415	0.872	fumarate reductase flavoprotein subunit
0.833	1.219	0.962	heat shock protein dnaK
1.48	0.617	1.246	thiol peroxidase
1.628	0.664	1.233	flavodoxin
0.625	1.366	1.012	formate dehydrogenase large subunit
0.876	1.248	0.889	Chain A, Crystal Structure Of Putative Bac
0.394	1.682	0.927	major outer membrane protein
1.008	1.161	0.885	chemotaxis protein CheV
1.283	1.161	1.014	ATP synthase F1 sector beta subunit
1.534	1.198	1.016	FOF1 ATP synthase subunit alpha
1.376	0.694	1.163	Elongation factor G
0.421	1.82	0.823	DNA-binding protein HU
1.244	0.919	1.116	bifunctional aconitate hydratase 2/2-met
1.32	1.011	0.963	aspartate ammonia-lyase
1.567	1.053	1.067	fumarate hydratase
1.227	0.582	1.269	hypothetical protein CJE0469
1.433	0.755	1.167	Chain A, The Virulence Factor Peb4 And Th
1.376	0.676	1.191	conserved hypothetical protein
0.544	1.57	0.891	50S ribosomal protein L25/general stress
1.478	0.264	1.417	cytochrome C553
0.729	1.395	0.911	DNA-directed RNA polymerase subunit be
0.514	1.552	0.937	50S ribosomal protein L1
1.232	0.987	1.116	trigger factor
1.291	0.787	1.189	elongation factor Ts
0.743	1.394	0.867	DNA-directed RNA polymerase subunit al
0.725	1.267	1.011	ribosomal protein L7/L12
0.885	1.435	0.698	fumarate reductase iron-sulfur subunit
0.881	0.992	1.114	succinyl-CoA synthetase subunit beta
0.686	1.452	0.865	DNA-directed RNA polymerase subunit be
1.126	0.931	1.063	DNA polymerase III beta subunit, central
---	1.607	---	aspartyl/glutamyl-tRNA(Asn/Gln) amidotr
0.525	1.631	0.86	50S ribosomal protein L5
1.38	0.486	1.519	thioredoxin
0.825	1.361	0.872	glutamine synthetase, type I
1.172	0.804	1.254	bifunctional adhesin/ABC transporter asp
1.746	1.091	1.184	enolase
0.526	1.293	1.186	periplasmic nitrate reductase, small subu
1.789	0.893	1.198	acetate kinase
0.67	1.29	1.043	formate dehydrogenase, iron-sulfur subu
0.579	1.531	0.887	30S ribosomal protein S10
0.798	1.384	0.822	oxaloacetate decarboxylase, alpha subu
1.053	0.872	1.078	hypothetical protein CJE0298
1.34	0.774	1.21	multi-sensor signal transduction histidin
1.116	1.451	1.185	chemotaxis protein CheY
1.353	0.845	1.088	molecular chaperone GroES
0.83	1.368	0.951	citrate synthase
1.182	1.102	0.979	argininosuccinate synthase
0.516	1.542	0.954	50S ribosomal protein L16
1.096	1.048	1.039	glyceraldehyde 3-phosphate dehydrogen
0.459	1.67	0.839	30S ribosomal protein S7
0.574	1.496	0.933	50S ribosomal protein L14
1.546	0.435	1.296	rubrerythrin
1.084	1.344	0.919	chemotaxis protein CheW
1.689	0.705	1.164	serine hydroxymethyltransferase
0.855	1.35	1.001	cysteine desulfurase
1.213	0.925	1.134	methionine adenosyltransferase
1.289	0.505	1.248	transthyretin-like protein
0.815	1.246	0.94	cytochrome C551 peroxidase

0 h	6 h	6 day	Description
0.517	1.51	0.977	nonheme iron-containing ferritin
0.779	1.342	0.876	inosine 5'-monophosphate dehydrogenase
1.413	0.82	1.199	FKBP-type peptidyl-prolyl cis-trans isomera
1.405	0.674	1.24	conserved hypothetical protein
1.407	0.729	1.202	DNA-binding response regulator
0.761	1.38	0.85	ketol-acid reductoisomerase
1.05	0.832	1.122	isocitrate dehydrogenase, NADP-depender
0.549	1.564	0.89	50S ribosomal protein L3
1.416	0.985	1.078	ATP-dependent protease ATP-binding subu
0.75	1.168	1.145	FOF1 ATP synthase subunit epsilon
0.655	1.359	0.989	cbb3-type cytochrome C oxidase subunit II
1.842	---	1.161	aspartyl/glutamyl-tRNA amidotransferase
1.287	0.609	1.118	ATP/GTP-binding protein
0.562	1.494	0.947	cytochrome c552
1.866	---	1.16	ribose-phosphate pyrophosphokinase
1.126	0.842	1.082	oxidoreductase, short chain dehydrogenas
1.468	---	1.535	acyl carrier protein
0.941	0.955	1.106	malate oxidoreductase
1.64	0.718	1.225	adenylate kinase
1.774	---	1.229	aspartate-semialdehyde dehydrogenase
0.917	1.23	0.951	ubiquinol--cytochrome C reductase, iron-su
0.67	1.353	0.98	30S ribosomal protein S18
1.059	0.772	1.189	thioredoxin reductase
0.642	1.407	0.954	ribosomal protein L4
1.109	1.114	1.025	enoyl-ACP reductase
0.642	1.345	1.016	50S ribosomal protein L11
0.495	1.636	0.873	30S ribosomal protein S9
0.973	0.978	1.049	30S ribosomal protein S1
1.058	1.202	0.982	30S ribosomal protein S2
0.812	1.23	0.973	ATP-dependent Clp protease proteolytic su
0.802	1.315	0.887	3-methyl-2-oxobutanoate hydroxymethyltr
0.835	1.071	1.097	glutamate-1-semialdehyde aminotransferi
1.442	0.709	1.069	heat shock protein HtpG
0.827	1.275	0.901	putative phospho-sugar mutase
0.493	1.585	0.925	ribosomal protein L22
1.061	0.74	1.211	Chain A, Crystal Structure Of Peb3
1.062	0.84	1.107	4-methyl-5(B-hydroxyethyl)-thiazole monop
0.763	1.263	0.978	glucosamine--fructose-6-phosphate amino
0.528	1.537	0.938	ribosomal protein L13
1.766	0.75	1.193	galE
1.54	0.994	1.021	putative UDP-glucose 4-epimerase
0.634	1.375	0.994	50S ribosomal protein L24
0.522	1.584	0.896	30S ribosomal protein S4
0.424	1.676	0.903	50 kDa outer membrane protein
0.984	0.984	1.035	L-Serine ammonia-lyase, partial
0.659	1.378	0.966	hypothetical protein CJE0033
0.741	1.397	0.867	acetyl-CoA carboxylase subunit A
0.751	1.162	1.09	cjaC protein
0.489	1.606	0.835	conserved hypothetical protein
1.971	---	1.032	highly acidic protein, partial
0.852	1.112	1.039	translation initiation factor IF3
0.824	1.282	0.903	3-dehydroquinate dehydratase
1.261	1.001	1.153	2-oxoglutarate-acceptor oxidoreductase su
1.578	0.6	1.297	hypothetical protein CjO170
0.926	0.881	1.197	major antigenic peptide PEB2
1.104	0.862	1.037	aspartate kinase, monofunctional class
0.843	1.294	0.866	phenylalanyl-tRNA synthetase subunit beta
0.369	1.439	1.195	30S ribosomal protein S11
0.795	1.045	1.163	50S ribosomal protein L23
1.01	0.954	1.038	carboxyl-terminal protease
0.946	0.972	1.085	biotin carboxylase
0.862	1.286	0.856	fur
1.299	0.808	1.113	saccharopine dehydrogenase
1.848	---	1.155	dTDP-4-dehydrorhamnose 3,5-epimerase
0.803	1.381	0.818	methyltransferase, FkbM family protein
1.266	1.165	1.025	50S ribosomal protein L10
0.882	1.19	0.932	pyruvate kinase
1.873	0.649	1.105	phosphate acetyltransferase
1.007	0.85	1.148	hypothetical protein C414_000420088
0.726	1.458	0.819	quinone-reactive Ni/Fe-hydrogenase, smal
0.601	1.509	0.904	30S ribosomal protein S8
1.744	1.14	1.075	branched-chain amino acid aminotransferi
0.551	1.248	1.204	trimethylamine-N-oxide reductase 2 precu
1.03	0.96	1.014	succinate dehydrogenase, C subunit
0.974	0.984	1.045	succinate dehydrogenase, flavoprotein sut
0.73	---	0.905	delta-aminolevulinic acid dehydratase
0.585	1.483	0.935	ubiquinol--cytochrome C reductase, cytoch
1.022	1.026	1.062	ATP-dependent chaperone protein ClpB
0.858	1.12	1.049	succinyl-CoA synthase, alpha subunit

0 h	6 h	6 day	Description
0.414	1.596	0.993	fibronectin binding protein
0.567	1.495	0.942	ribosomal protein L21
0.478	1.432	1.092	conserved hypothetical protein
1.478	0.489	1.036	aspartyl-tRNA synthetase
0.761	1.345	0.897	polynucleotide phosphorylase/polyadenylase
0.853	1.151	1.001	2-oxoglutarate-acceptor oxidoreductase subuni
1.793	---	1.061	acetyl-CoA carboxylase carboxyltransferase sub
0.822	1.099	1.126	malate:quinone oxidoreductase, putative
0.617	1.545	0.84	flagellar motor protein MotA
1.091	0.655	1.257	nucleoside diphosphate kinase
0.911	1.22	0.873	putative capsule polysaccharide export system
1.37	0.934	1.27	transcription antitermination protein NusG
0.9	1.146	0.957	ATP-dependent protease La
1.888	---	1.09	2,3,4,5-tetrahydropyridine-2-carboxylate N-succ
0.486	1.589	0.928	putative sugar transferase
0.447	1.594	0.962	50S ribosomal protein L17
1.735	---	1.239	oxidoreductase, putative
0.425	1.477	1.101	50S ribosomal protein L18
0.366	1.606	1.03	lipoprotein
0.882	1.121	1	2-oxoglutarate-acceptor oxidoreductase subuni
0.838	1.483	0.945	30S ribosomal protein S6
0.588	1.411	1.004	50S ribosomal protein L29
1.322	0.81	1.155	Chain A, Crystal Structure Of Phosphoglycerate K
1.117	0.713	1.174	2-nitropropane dioxygenase family oxidoreducta
0.507	1.504	0.991	hypothetical protein CJE0453
1.896	0.264	0.969	ATP-sulfurylase family protein
0.842	1.243	0.919	competence protein ComEA
0.455	1.611	0.937	RpsO
1.375	---	1.121	hypothetical protein CJE0087
0.989	1.009	1.058	putative transcription termination factor
1.653	---	1.187	CTP synthetase
1.434	0.627	1.059	hypothetical protein CJE0806
0.641	1.532	0.83	peptidyl-prolyl cis-trans isomerase D,-like prote
0.431	1.605	0.968	50S ribosomal protein L15
1.289	1.307	1.035	preprotein translocase subunit SecA
0.989	0.758	1.256	50S ribosomal protein L9
0.866	1.158	0.979	hypothetical protein CJE0800
0.879	1.214	0.91	threonyl-tRNA ligase
1.395	0.656	1.304	superoxide dismutase
1.14	0.665	1.198	hypothetical protein CJE1668
1.477	---	1.122	GMP synthase
0.816	1.201	0.985	acetyl-CoA carboxylase, carboxyl transferase, be
0.693	1.345	0.966	hypothetical protein C414_000290003
1.385	---	1.618	transcription elongation factor GreA
0.875	1.162	0.966	transketolase
0.752	1.314	0.937	NADH dehydrogenase subunit G
0.812	1.275	0.916	UTP-glucose-1-phosphate uridylyltransferase
0.569	1.509	0.924	30S ribosomal protein S17
1.006	0.84	1.157	molybdenum cofactor biosynthesis protein Mog
1.513	---	1.113	thiamine biosynthesis protein ThiF
0.998	1.016	0.989	aspartate aminotransferase
1.445	---	1.191	iron ABC transporter periplasmic iron-binding pr
1.488	---	1.515	isoleucyl-tRNA synthetase
0.855	1.146	1.001	twin-arginine translocation pathway signal
1.014	0.973	1.015	histidyl-tRNA synthetase
0.458	1.625	0.919	30S ribosomal protein S5
1.57	---	1.058	prolyl-tRNA synthetase
1.082	0.785	1.135	Chain A, Crystal Structure Of Adenylosuccinate S
0.85	1.293	0.86	carbamoyl-phosphate synthase large chain
1.245	0.888	1.129	3-oxoacyl-ACP synthase II
0.92	1.184	0.899	hypothetical protein JJD26997_0724
0.834	0.92	1.25	flagellar assembly protein FliW
0.663	1.408	0.932	3-deoxy-8-phosphooctulonate synthase
1.71	---	1.293	cytochrome C family protein
0.848	1.189	0.966	UDP-GlcNAc-specific C4,6 dehydratase/C5 epime
0.897	0.975	1.131	histidinol dehydrogenase
0.422	1.569	1.011	cytochrome C oxidase, cbb3-type, subunit III
1.038	0.825	1.14	elongation factor P
0.819	1.14	1.044	50S ribosomal protein L31
0.801	1.254	0.949	FOF1 ATP synthase subunit delta
0.239	2.055	0.709	50S ribosomal protein L2
1.741	---	1.203	short chain dehydrogenase/reductase family ox
0.802	1.316	0.885	bifunctional N-acetylglucosamine-1-phosphate
1.033	0.789	1.181	OmpR protein, partial
0.958	---	0.932	transaldolase
0.366	1.812	0.825	30S ribosomal protein S3
0.858	1.289	0.856	cation ABC transporter ATP-binding protein
0.84	0.986	1.178	flavodoxin
0.796	1.377	0.83	translocation protein TolB

Figure 5.2 236 proteins of *C. jejuni* NCTC 11168 identified by iTRAQ labelling proteomic analysisin the cold exposure experiment

Colours indicate the amounts of identified protein. Green is a ratio of protein less than unity, red is a ratio of protein greater than unity, blank indicates theprotein cannot be identified at that time point.

5.2.2.2 Proteins significantly change expression during cold exposure

In this study, protein extracted from cells without cold exposure was used as the reference standard. The amount of protein in the 0 h protein extract was considered to be one. Compared with the 0 h protein extract, a protein that changed its expression more than two times after cold exposure was regarded as a protein with a significant change in expression. There werea total of 102 proteins with significant changesin *C. jejuni* NCTC 11168 under cold stress. 56 proteins were up-regulated after 6 h cold shock and 19 of those 56 proteins were up-regulated in 6 days cold adapted cells. 24 proteins decreased their expression and 22 proteins stopped synthesis in 6 h cold-shocked cells. The 22 proteins that disappeared in 6 h cold-shocked *C. jejuni* NCTC 11168 cells were all re-synthesised and their amounts were back to normal levels after 6 days of cold storage. All 235 proteins found in 0 h cell extracts appeared after six days of cold storage. No identified protein decreased its expression after 6 days of cold storage when compared with 0 h cells' extracts.

Table 5.1 presents all significantly changed *C jejuni* NCTC 11168 proteins under cold stress found in this study. A total of 56 proteins were up-regulated after 6 h cold shock. Twenty-two proteins were up-regulated after six days of cold storage. Twenty-eight proteins of 56 up-regulated proteins in six hour cold-shocked cells were up-expressed more than 2 fold, but less than 3-fold. Twenty proteins of the 56 up-regulated proteins were up-expressed more than 3-fold but less than 4-fold. Eight proteins of the 56 up-regulated proteins in the six hours of cold-shock cells were up-expressed more than 4 times. They were two chemotaxis proteins, two ribosomal proteins, three cell envelope proteins and one DNA binding protein. The highest up-regulated protein was 50S ribosomal protein L2, which was up-expressed 8.6-fold. In the 6 day's cold adapted cells, 22 proteins were up-expressed. Twenty-one of the 22 proteins were up-expressed more than two times, but less than three times. Only one protein was up-expressed more than 3-fold, which is 50S ribosomal protein L2 with 3.2 times over expression. Overall, 22 proteins were up-regulated in both cold shock and after 6 days of cold adaption; 38 proteins were only up-regulated after six hours of cold shock conditions.

The down-regulated proteins and proteins that stopped synthesis in 6h cold shock of *C. jejuni* NCTC 11168 cells are also shown in Table 5.1. The iTRAQ proteomic analysis had detected 24 down-regulated proteins in NCTC 11168 cells after six hours of cold shock. Eighteen of those 24 proteins decreased more than two times but less than three times. Six proteins decreased more than 3-fold but less than 8-fold. They were one translation factor (Tuf), one tRNA binding protein (AspS), one oxidative stress defence protein (Rbr), one energy metabolism protein (Cyf), one intermediary metabolism protein and one flagellin subunit protein FlaA. Only one protein decreased more than 8-fold. It was Flagellin A (partial) with a notable 31.3-fold decrease in its expression. No proteins were down-regulated after six days of cold shock. Twenty-two proteins stopped synthesis after six hours of cold shock, but re-synthesized after six days of cold adaption. The amount of those re-synthesised protein in 6 days sample had not significant change comparing with the 0 hours sample.

Table 5.1 *C. jejuni* NCTC 11168 proteins significantly affected by cold stress

Accession number	Protein name	Theoretical Mw (Da)	Cold shock (6 h)	Cold adaptation (6 day)
Up-regulated proteins				
gi|121613277	methyl-accepting chemotaxis protein	72901	+4.7	+2.5
gi|121613238	methyl-accepting chemotaxis signal transduction protein	73191	+5.0	+2.8
gi|121613238	methyl-accepting chemotaxis protein	72546	+2.9	
gi|121613017	methyl-accepting chemotaxis protein	77220	+3.1	+2.0
gi|419589392	putative MCP-type signal transduction protein	48552	+2.2	
gi|384447762	DNA-directed RNA polymerase subunit	156229	+2.1	
gi|57237529	50S ribosomal protein L1	25031	+3.0	
gi|283953694	50S ribosomal protein L5	20083	+3.1	
gi|57236893	30S ribosomal protein S10	11665	+2.6	
gi|57237362	50S ribosomal protein L25/general stress protein Ctc	19506	+2.9	
gi|57238707	50S ribosomal protein L16	16365	+3.0	
gi|57237544	30S ribosomal protein S7	17681	+3.6	
gi|57238704	50S ribosomal protein L14	13354	+2.6	
gi|57238730	50S ribosomal protein L3	20824	+2.9	
gi|57237954	30S ribosomal protein S18	10322	+2.0	
gi|86149254	ribosomal protein L4	22273	+2.2	
gi|57237528	50S ribosomal protein L11	15127	+2.1	
gi|57238502	30S ribosomal protein S9	14128	+3.3	
gi|68248462	ribosomal protein L22	15885	+3.2	
gi|57169062	ribosomal protein L13	15864	+2.9	
gi|57238703	50S ribosomal protein L24	8335	+2.2	
gi|57238607	30S ribosomal protein S4	23895	+3.0	
gi|57238606	30S ribosomal protein S11	13942	+3.9	+3.2
gi|57238700	30S ribosomal protein S8	14795	+2.5	
gi|57168772	ribosomal protein L21	11602	+2.6	
gi|57238609	50S ribosomal protein L17	13240	+3.6	+2.2
gi|57238698	50S ribosomal protein L18	13288	+3.5	+2.6
gi|57238706	50S ribosomal protein L29	7029	+2.4	
gi|633730	RpsO	10206	+3.5	+2.1
gi|57238696	50S ribosomal protein L15	14019	+3.7	+2.3
gi|57238705	30S ribosomal protein S17	9600	+2.7	
gi|57238697	30S ribosomal protein S5	15787	+3.6	+2.0
gi|57238711	50S ribosomal protein L2	30515	+8.6	+3.0
gi|153952472	30S ribosomal protein S3	26067	+5.0	+2.3
gi|57238510	cytochrome C oxidase, cbb3-type, subunit III	31370	+3.7	+2.4
gi|218563111	formate dehydrogenase large subunit	104573	+2.2	
gi|419641626	periplasmic nitrate reductase, small subunit	19251	+2.5	+2.3
gi|419622743	cytochrome c552	69795	+2.7	
gi|86151404	trimethylamine-N-oxide reductase 2 precursor	93857	+2.3	+2.2
gi|57238056	ubiquinol--cytochrome C reductase, cytochrome C1 subunit	41617	+2.5	
gi|57238512	cbb3-type cytochrome C oxidase subunit II	25078	+2.1	
gi|57238139	quinone-reactive Ni/Fe-hydrogenase, small subunit	39846	+2.0	
gi|57237388	flagellar motor protein MotA	28279	+2.5	
gi|85036689	major outer membrane protein	45632	+4.3	+2.4
gi|86152514	50 kDa outer membrane protein	53832	+4.0	+2.1
gi|57237913	lipoprotein	18559	+4.4	+2.8

gi|283955143	3-deoxy-8-phosphooctulonate synthase	29826	+2.1	
gi|57237741	DNA-binding protein HU	10268	+4.3	
gi|57238192	nonheme iron-containing ferritin	19531	+2.9	
gi|57236997	peptidyl-prolyl cis-trans isomerase D,-like protein	57475	+2.4	
gi|4704599	fibronectin binding protein	32429	+3.9	+2.4
gi|86150017	putative sugar transferase	89945	+3.3	
gi|57237048	hypothetical protein CJE0033	26440	+2.1	
gi|86150082	conserved hypothetical protein	43511	+3.3	
gi|86150649	conserved hypothetical protein	16323	+3.0	+2.3
gi|57237459	hypothetical protein CJE0453	30726	+3.0	
Down-regulated proteins				
gi|57238619	ATP/GTP-binding protein	40355	-2.1	
gi|57237524	elongation factor Tu	43623	-3.0	
gi|57236953	aspartyl-tRNA synthetase	66676	-3.0	
gi|86148961	heat shock protein HtpG	69640	-2.0	
gi|86150249	thiol peroxidase	18771	-2.4	
gi|57168680	thioredoxin	11501	-2.9	
gi|86150520	rubrerythrin	25023	-3.6	
gi|419635264	superoxide dismutase	25056	-2.1	
gi|86150091	flavodoxin	17224	-2.5	
gi|57238028	cytochrome C553	11002	-5.6	
gi|218562328	acetate kinase	44429	-2.0	
gi|419641974	serine hydroxymethyltransferase	46079	-2.4	
gi|86149461	ATP-sulfurylase family protein	44829	-7.2	
gi|3413445	galE	37224	-2.4	
gi|86150126	flagellin subunit protein FlaA	59075	-7.00	
gi|56806980	flagellin A	11704	-31.4	
gi|88597146	phosphate acetyltransferase	56356	-2.9	
gi|153952659	adenylate kinase	21432	-2.3	
gi|57237017	transthyretin-like protein	15910	-2.6	
gi|57237475	hypothetical protein CJE0469	21025	-2.1	
gi|86150070	conserved hypothetical protein	29837	-2.0	
gi|86149608	conserved hypothetical protein	39053	-2.1	
gi|218561850	hypothetical protein Cj0170	28767	-2.6	
gi|57237008	hypothetical protein CJE0806	27951	-2.3	
Stopped biosynthesis proteins				
gi|57237051	cytochrome C family protein	39104		6 day
gi|57237336	transaldolase	37122		6 day
gi|121612363	oxidoreductase, putative	64074		6 day
gi|57238069	aspartyl/glutamyl-tRNA amidotransferase subunit B	53256		6 day
gi|57237342	transcription elongation factor GreA	18056		6 day
gi|317511751	isoleucyl-tRNA synthetase	106086		6 day
gi|57238257	prolyl-tRNA synthetase	65053		6 day
gi|57237746	ribose-phosphate pyrophosphokinase	33848		6 day
gi|57236939	CTP synthetase	60765		6 day
gi|86150584	GMP synthase	57397		6 day
gi|86148986	acyl carrier protein	8592		6 day
gi|57237500	acetyl-CoA carboxylase carboxyltransferase subunit alpha	34473		6 day
gi|13509099	aspartate-semialdehyde dehydrogenase	21405		6 day
gi|57238618	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	42635		6 day
gi|86150038	dTDP-4-dehydrorhamnose 3,5-epimerase	21245		6 day
gi|455428	flagellin protein	59652		6 day

gi|57237820	delta-aminolevulinic acid dehydratase	36733		6 day
gi|86150511	thiamine biosynthesis protein ThiF	30047		6 day
gi|57237180	iron ABC transporter periplasmic iron-binding protein	37406		6 day
gi|419641488	short chain dehydrogenase/reductase family oxidoreductase	28161		6 day
gi|317511289	highly acidic protein, partial	45096		6 day
gi|57237099	hypothetical protein CJE0087	49254		6 day
Cold-induced proteins found in 6h cold-shocked cells only				
gi|283954623	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase, A subunit	49712	6 h	

5.2.2.3 Functional classification of significantly changed C. jejuni NCTC 11168 proteins

The 102 proteins with significant alteration during cold exposure were grouped by functional classification according to the Sanger Centre *C. jejuni* functional database. Table 5.2 presentsthe functional classifications of the significantly changed proteins in NCTC 11168 in response to cold stress. Based on their functionality, those 102 significantly changed proteins at cold shock conditions were divided into 16 different groups. The 56 up-regulated proteins were classified into 10 different functional categories. The 24 down-regulated proteins were grouped into 10 functional categories. The 22 stopped-synthesis proteins belonged to 11 different functional categories.

There were 10 different functional categories corresponding to these 56 up-regulated proteins. Fifty percent of up-regulated proteins, 28 of 56, were ribosomal proteins, responsible for translating mRNA to protein. The second largest functional category of up-regulated protein was involved with energy metabolism. Eight up-regulated proteins were involved in energy metabolism. Most of them participated in bacterial anaerobic respiration. The third and fourth largest functional protein groups had the same number of proteins. They were cell envelope and chemotaxis and cell motility proteins. Those two groups both had five up-regulated proteins.

Twenty-four down-regulated proteins were grouped into 10 functional categories. Excluding six down-regulated proteins with unknown functions, the largest functional group in down-regulated protein was oxidative stress, which had four down-regulated proteins. The second and third largest functional categories in down-regulated protein were general intermediary metabolism and cell envelope. They both have three down-regulated proteins.

The 22 proteins whose synthesis stopped at 6 hours of cold shock were divided into 11 different functional groups. The largest functional group was genetic information processing, which had four proteins. The second largest functional category in those stopped-synthesis proteins was metabolism of purine, pyrimidine, nucleoside and

nucleotide. The remanining functional categories had only two or one proteins, including two stopped-synthesis proteins with unknown function.

Table 5.2 Functional grouping of proteins significantly changed in *C. jejuni* NCTC 11168 in response to cold temperatures

Functional classification	Cold shock cells (6h)			Cold adapted cells (6day)
	Up-regulated proteins	Down-regulated proteins	Protein that stopped synthesis	Up-regulated proteins
Chemotaxis and motility	5			3
Genetic information processing	1	2	4	
Ribosomal protein	28			8
Energy metabolism	8	2	2	3
Cell envelope	5	3	2	3
Adaptation and atypical condition	2			
Pathogenicity	1			1
General intermediary metabolism	1	3	1	
Molecular chaperone	1	1		
Oxidative stress defence		4	1	
Fatty acid biosynthesis		1	2	
Purine, pyrimidine, nucleoside and nucleotide		1	3	
Amino acid metabolism			2	
Transport/binding		1	1	
Cofactors and vitamins synthesis			2	
Unknown function	4	6	2	1
Total number	56	24	22	19

5.3 Discussion

5.3.1 Changes in 1D protein banding patterns under cold stress

Our study found the 1D protein profile of the *C. jejuni* NCTC 11168 used in the study do not show obvious change after Day 1, Day 2, Day 6 and Day 8 of 4^{o}C cold exposure. However, 6 h of 4^{o}C cold exposure caused visible alterationsin the 1D protein profile of NCTC 11168, when compared with its protein profile without cold exposure.

Hazeleger et al. (1998) had used an isotopic labelling technique and 1D protein electrophoresis to study *de novo* protein synthesis of *C. jejuni* ATCC 33560 under cold temperatures. Hazeleger and his colleagues found *C. jejuni* ATCC 33560 only synthesized 9% of proteins at 4^{o}C, compared with at 37^{o}C; however, excluding decrease in the density of protein bands, the 1D protein profile of *C. jejuni* ATCC 33560 exposed to 4^{o}C was similar to the protein profile of cells growing at 37^{o}C. Our study also found that the *C. jejuni* NCTC 11168 cells exposed to 4^{o}C for 24 and 48 h, six and eight days, have a similar 1D protein profile to cells without 4^{o}C cold exposure. The 1D protein electrophoresis results suggested that *C. jejuni* NCTC 11168 did not alter its protein expression noticeably during cold adaptation.

The study found six hour cold shock caused avisible change in the NCTC 11168 1D protein banding pattern. This was the first time 1D protein electrophoresis had identified the alteration of *C. jejuni* protein synthesis under cold stress. As 1D protein electrophoresis is not a sensitive enough technique to reveal small protein alterations, it is assumed that *C. jejuni* NCTC 11168 had altered its protein expression tremendously when processing the cold shock response.

5.3.2 Comparative proteomic analysis of cold stress response in *C. jejuni* NCTC 11168

There were 102 proteins with significant changes (more than a 2-fold increase or decrease) during cold exposure and one cold-induced protein,which cannot be found in the cell without cold exposure, had been identified by iTRAQ labelling proteomic

analysis in *C. jejuni* NCTC 11168. In response to six hours of cold shock, 56 proteins were up-regulated, 24 proteins decreased their expression and 22 proteins stopped synthesis in *C. jejuni* NCTC 11168 cells. What is more, six hours of cold shock was found to induce the expression of one protein, GatA (Aspartyl/glutamyl-tRNA amidotransferase subunit A), which was notable to be found in 0 h and cold adapted cells. GatA is responsible for translational fidelity. Anderson et al. (2006) reported glutamyl amidotransferase increased nine times in *Staphylococcus aureus* in response to genotoxic chemical exposure, but did not see the expression of this protein affected by cold stress. The role of GatA in *C. jejuni* to response cold stress requires further study. After six days cold storage, the alteration of protein expression became less conspicuous. There were only 19 proteins with significantly changed in 6 day cold adapted cells extraction. All 19 proteins were up-regulated proteins.

As shown in Table 5.2, those 102 proteins were categorised in 16 groups based on their function. The most notable up-regulated protein group was that of the ribosomal proteins. Twenty-eight of 56 up-regulated proteins in cold shocked NCTC 11168 cells and eight of 22 up-regulated proteins in cold adapted NCTC 11168 cells were ribosomal proteins, which were responsible for protein translation. Over expression of such a large number of ribosomal proteins suggested that *C. jejuni* NCTC 11168 increased its capability for biosynthesis of certain proteins to cope with cold stress. Our observation was the opposite of the findings of Stintzi and Whitworth (2003), who found a temperature downshift significantly decreased transcript abundance of ribosomal protein genes. The reason behind this result in transcript and protein abundance, in the case of *C. jejuni* cold response, was a result of the different experimental time points. Stintzi and Whitworth (2003) tested *C. jejuni* NCTC 11168 transcript alterations after 10 min of cold exposure. The shortest cold exposure time in our experiment was six hours. I assumed that *C. jejuni* decreased its protein synthesis at the first 10 min cold exposure and increases its capability for biosynthesis of protein after six hoursof cold exposure.

Cold induced ribosomal proteins have been reported in both *E. coli* (Joens et al., 1992) and *B. subtilis* (Graumann et al., 1996). However, the numbers of cold-induced

ribosomal protein in *E. coli* and *B. subtilis* were very small, compared with *C. jejuni* NCTC 11168. A temperature downshift only induced ribosomal proteins S1, S6, L7/L12 in *E. coli* and S6, L7/L12 in *B. subtilis* (Joens et al., 1992; Graumann et al., 1996). These four ribosomal proteins did not change their expression in *C. jejuni* NCTC 11168 in our cold exposure study. This suggested that the strategy used by *C. jejuni* NCTC 11168 to cope with cold stress was very different from the one used by *E. coli* and *B. subtilis.*

Eight up-regulated proteins in cold shock cells and three up-regulated proteins in cold adapted cells were related to energy metabolism. The over expression of proteins involved in energy metabolism suggested more energy was needed by *C. jejuni* NCTC 11168 in response to cold stress. This finding was in agreement with the studies of Stintzi and Whitworth (2003) and Moen el al. (2005), who showed that *C. jejuni* genes encoding enzymes involved in energy metabolism increased transcription in response temperature downshifts. However, up-regulated metabolism genes found in Stintzi and Whitworth's study were all involved in aerobic respiration. In our study, four of eight up-regulated energy metabolism proteins (FdhA ((formate dehydrogenase large subunit)), ZP_14173510 ((small subunit of periplasmic nitrate reductase)), cytochrome c552 and trimethylamine-N-oxide reductase 2 precursor) in cold shock cells and two of three up-regulated energy metabolism proteins (ZP_14173510 and trimethylamine-N-oxide reductase 2 precursor) in cold adapted cells were found to be involved in anaerobic respiration. This suggested that *C. jejuni* NCTC 11168 relied more on aerobic respiration in the first 10 min of cold exposure, but after six hours of 4^{o}C cold exposure, the anaerobic respiration pathway become more favourable.

The third and fourth largest functional groups of up-regulated proteins both have five proteins. They are chemotaxis and cell motility proteins and cell envelope protein. The category of chemotaxis and cell motility contained five up-regulated proteins in cold shock cells and three up-regulated proteins in cold adapted cells. They were all methyl-accepting chemotaxis proteins, which is responsible for signal transduction. It has been reported that chemotaxis proteins were involved in molecular thermosensing in *E. coli* (Nara et al., 1996) and cold shock induced

fourmethyl-accepting chemotaxis proteins in *Shewanella oneidensis* (Gao et al., 2006). The increasing biosynthesis of chemotaxis proteins in *C. jejuni* NCTC 11168 in response cold stress suggested these methyl-accepting chemotaxis proteins may also be involved in cold signal transduction and cold stress response regulation in *C. jejuni*.

There were five up-regulated proteins in cold-shocked cells and three up-regulated proteins in cold adapted cells belonging to cell envelope proteins. They were PorA (major outer membrane protein), Omp50 (50 kDa outer membrane protein), KdsA (lipopolysaccharides synthesis enzyme), MotA (flagellar motor protein) and a lipoprotein. Expression of PorA and Omp50 had increasedat 42^{o}C, compared with 36^{o}C and 31^{o}C (Dedieu et al., 2002; Dedieu et al., 2008). Antimicrobials had also been found to induce PorA and Omp50 in *C. jejuni*, and it was believed the increase expression of those two cell envelope proteins was an adaptive response to increase surface polysaccharides (Xia et al., 2013). The mechanism behind up-regulation of PorA and Omp50 in response to cold temperature could be the same response to antimicrobials. PorA, Omp50, KdsA, MotA and the lipoprotein are all involved in cell membrane construction. This suggested that *C. jejuni* NCTC 11168 increased biosynthesis of cell membrane constructed proteins to protect cell walls from low temperatures in response to cold stress.

The largest functional group of down regulated proteins in cold-shocked *C. jejuni* NCTC 11168 cells was the oxidative stress defence protein. There were four oxidative stress defence proteins found that had decreased their expression during cold shock more than 2-fold. They were Tpx (Thiol peroxidise), Trx (Thioredoxin), Rbr (Rubrerythrin) and Sod (Superoxide dismutase). Those proteins were involved in oxidative defence and catalysed the conversion of hydrogen peroxide to oxygen and water. The decreased expression of oxidative stress proteins was in agreement with the over-expression of anaerobic respiration proteins found in this study in both cold shock and cold adapted *C. jejuni* NCTC 11168 cells. Our finding about decreasing oxidative stress proteinsin *C. jejuni* under cold stress was opposite to the finding of Stintzi and Whitworth (2003), who observed two genes encoding proteins involved in the oxidative stress defence were up-regulated. Since Stintzi and Whitworth tested *C. jejuni* transcript alteration after 10 min of cold exposure, the opposite

results of the two studies could be that *C. jejuni* NCTC 11168 relied more on aerobic respiration and up-regulate oxidative stress defence proteins at the first 10 min of cold exposure, but after six hours of 4^{o}C cold exposure, anaerobic respiration pathway become favourable and cell decreases oxidative stress defence proteins.

The other functional group worth mentioning in down regulated proteins was cell envelope proteins. According to the Sanger Center *C. jejuni* functional database, flagellar proteins are belonging to cell envelope protein. Our study disclosed flagellin A and its partial protein had notable decrease in cold shock *C. jejuni* NCTC 11168, with 7-fold and 31.3-fold decrease in their expression respectively. Our finding was in agreement with the observation of Stintzi and Whitworth (2003), who had found the transcription of flagellar protein genes in *C. jejuni* NCTC 11168 decreased when temperatures downshifted from 42^{o}C to 4^{o}C. The notable decrease in flagellar protein expression suggested the cell deprioritises it movement in favour of survival in response to cold.

The largest functional group of these proteins that stopped synthesis in cold shock conditions was genetic information processing. There were four genetic information processing proteins, which were responsible for transcription and translation. Together with two down-regulated genetic information processing proteins, there were a total of six genetic information processing proteins that significantly reduced their biosynthesisin response to cold shock. Our study has identified 28 up-regulated ribosomal proteins and one up-regulated genetic information processing protein in cold-shocked *C. jejuni* NCTC 11168 cells. As ribosomal proteins are involved in mRNA translation, ribosomal proteins are regarded as a subgroup of genetic information processing proteins. Overall, in response to six hours of cold shock, *C. jejuni* NCTC 11168 increased 29 genetic information processing proteins and decreased 6 genetic information processing proteins. This suggested hat *C. jejuni* NCTC 11168 cells selectively produced protein enzymes to response cold shock.

The second largest functional group of these stop synthesis proteins was purine, pyrimidine, nucleosides and nucleotidemetabolism proteins. This functional category has three stop synthesized proteins and one down-regulated protein. They are PrsA

(ribose-phosphate pyrophosphokinase), PyrG (Cytidine triphosphate synthetase), GuaA (Guanosine monophosphate synthetise) and Adk (Adenylate kinase). Those four proteins are related to the metabolism of purine, pyrimidine, nucleoside and nucleotide and biosynthesis of DNA. This suggested that *C. jejuni* NCTC 11168 cells slowed down their DNA synthesis during cold shock.

5.3.3 Conclusion

This study found that *C. jejuni* managed its protein expression in very different ways to respond to cold shock and cold adaptation. *C. jejuni* NCTC 11168 reprogrammed its protein biosynthesis dramatically to respond to cold shock. It significantly altered expression of 102 of 235 identified proteins to respond 6 h cold shock. Twenty-eight ribosomal proteins, eight energy metabolism proteins, five chemotaxis proteins and five cell envelope proteins have been found increase their expression during cold shock. Meanwhile, four oxidative stress defence proteins were down-regulated. Four genetic information processing proteins and three DNA biosynthesized proteins stopped their expression during cold shock.

Compared with 6 h cold shock, six days cold storage caused fewer changes in protein expression in *C. jejuni* NCTC 11168. Only 19 proteins were up-regulated in response to six days cold exposure. This suggested *C. jejuni* NCTC 11168 dramatically altered its physiological characteristics in response to cold shock. In the cold shock stage, it increased protein synthesis and energy metabolism, while slowing down its DNA synthesis. After the first few hours of cold shock, *C. jejuni* NCTC 11168 cells entered a cold adaptation period where its physiological characteristics may have changed back to normal, as its proteomic profile in cold adaptation period was similar to the proteomic profile of cells without cold exposure.

During cold exposure, the most notable up-regulated protein group in *C. jejuni* NCTC 11168 was ribosomal protein. Over expression of a large number of ribosomal proteins, suggesting that *C. jejuni* NCTC 11168 might increase its capability for protein biosynthesis to cope with cold stress. Cold induced ribosomal proteins have been reported in both *E. coli* (Joens et al., 1992) and *B.subtilis* (Graumann et al., 1996). However, the numbers of cold-induced ribosomal protein in *E. coli* and *B.*

subtilis were very small, compared with *C. jejuni* NCTC 11168. A temperature downshift only induced four ribosomal proteins in *E. coli* and *B. subtilis* and those four ribosomal proteins did not change their expression in *C. jejuni* NCTC 11168. This suggested that the strategy used by *C. jejuni* NCTC 11168 to cope with cold stress was very different from the one used by *E. coli* and *B. subtilis*.

The over expression of proteins involved in energy metabolism suggested more energy was needed by *C. jejuni* NCTC 11168 in response to cold stress. Our study found, apart of up-regulation of energy metabolism proteins involved in aerobic respiration, *C. jejuni* NCTC 11168 increased its energy metabolism proteins related to anaerobic respiration. This suggested that *C. jejuni* NCTC 11168 rely on both aerobic respiration and anaerobic respiration pathway to provide energy in cold condition.

Chapter 6 Proteomic study of Cold Shock and Adaptation in Two Waterborne Outbreak *C. jejuni* strains

6.1 *C. jejuni* SVS 5001 and SVS 5141

C. jejuni SVS 5001 and SVS 5141 were isolated from a Danish waterborne outbreak in 1996 (Engberg et al, 1998). The former was a human diarrhoeal isolate; the latter was recovered from the water that was the source of the outbreak. Due to the similar phenotypic characteristics and different origins of these two strains, they have been used in number of subtyping studies to examine the sensitivity of subtyping methods (Siemer et al, 2004; Kokotovic and On, 1999; On and Harrington, 2000).

A previous survival study had found the clinical isolate, *C. jejuni* SVS 5001, has higher survivability at room temperature than strains isolated from bovine, turkey and chicken (On et al, 2006). As SVS 5001 has the potential for longer survival at low temperatures that do not permit growth, this human diarrhoeal isolate and the water origin strain from the same waterborne outbreak, SVS 5141, were selected for this comparative proteomics analysis to elucidate their cellular cold stress responses mechanisms.

6.2 Proteomic analysis of *C. jejuni* SVS 5001 and SVS 5141

This section reports the proteomics analysis of cold stress responses in *C. jejuni* SVS 5001 and SVS 5141. 1D protein profile analysis and iTRAQ labelling proteomics analysis for these two strains' cold stress response was conducted as described in Sections 3.8 and 3.11, respectively.

6.2.1 1D profile of *C. jejuni* SVS 5001 and SVS 5141 during cold shock and adaptation

Whole-cell protein extractions of *C. jejuni* SVS 5001 and SVS 5141 were collected at 0 h, 6 h, Day 1, Day 2, Day 6 and Day 8 during the 4^{o}C storage. After protein qualification, protein extraction samples from the two strains collected at different time-points were used to run 1D electrophoresis to compare their protein profiles. In this analysis, six hours of cold exposure was considered as cold shock; 24h, 48h, 6 day and 8 day cold exposure were considered as cold adaptation periods for *C. jejuni* SVS 5001 and SVS 5141.

As there was a shortage of a six-hour cold shock *C. jejuni* SVS 5001 protein extraction sample, the 1D electrophoresis for SVS 5001 only had five time-point samples; they were samples from 0 h, day 1, day 2, day 6 and day 8. Figure 6.1 displays the protein profiles of *C. jejuni* SVS 5001 collected at these five time-points. The protein profiles of this *C. jejuni* strain exposed at 4^{o}C for 1 day, 2 days, 6 days and 8 days appeared identical. Those cold exposure cell protein profiles were similar to the protein profile of the cells without cold exposure.

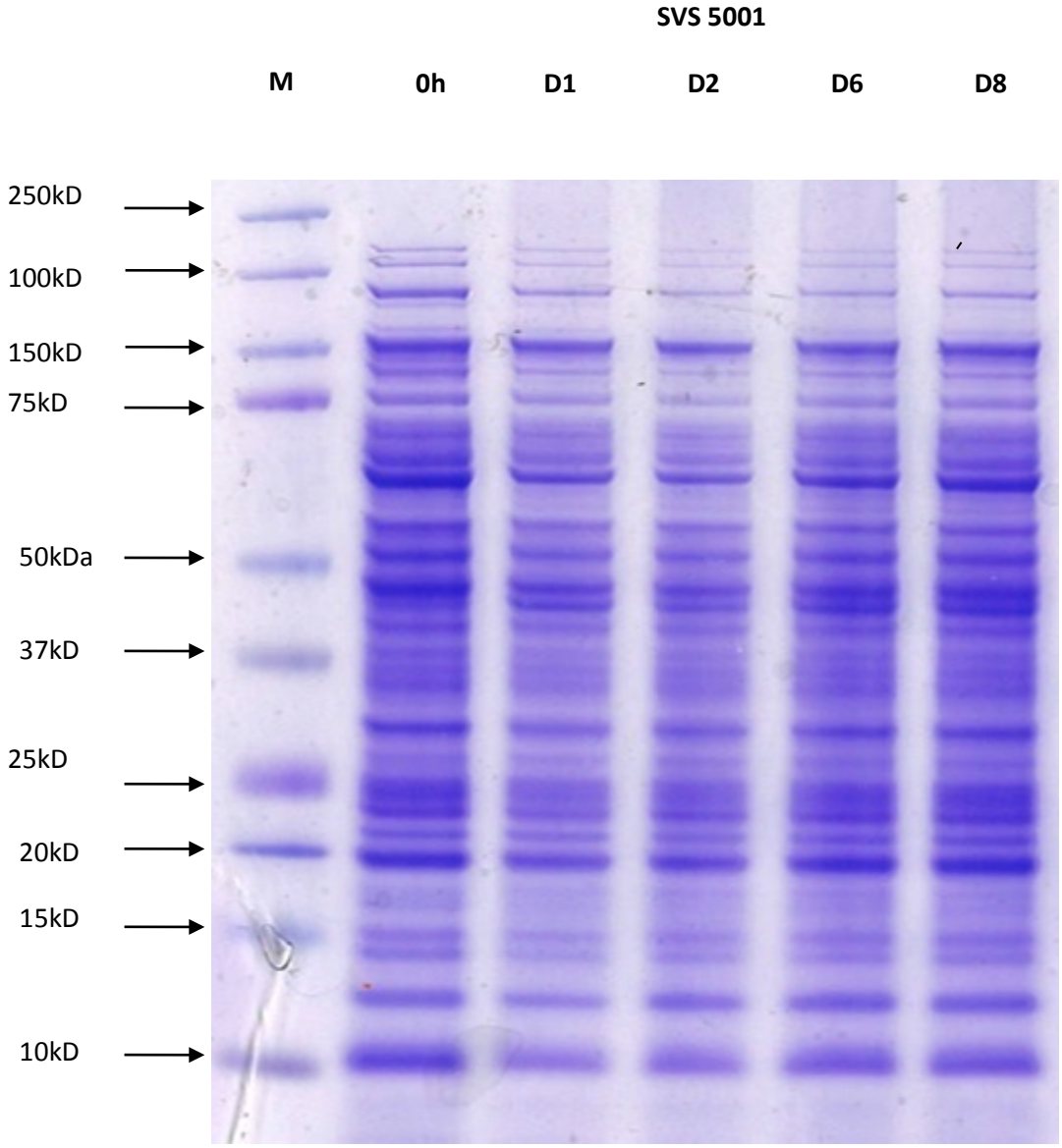


Figure 6.1 1D Protein profiles of *C. jejuni* SVS 5001 collected at 0 h, and Day 1, Day 2, Day 6 and Day 8

(M is the marker).

The 1D protein profiles of *C. jejuni* SVS 5141 collected at different time-points are shown as Figure 6.2. 1D protein profile of this strain with 1 day, 2 days, 6 days and 8 days of cold exposure appear identical. This is the same as the protein banding pattern of *C. jejuni* SVS 5001, these four cold adapted *C. jejuni* SVS 5141 cells' protein profiles do not have obvious visual alterations compared with the protein profile of cell without cold exposure. In contrast to those cold adapted cell protein profiles, the six hours of cold shock *C. jejuni* SVS 5141 protein profiles appeared different from the protein profile of the cell without cold exposure. As shown in Figure 6.2, there were five clear visual changes in the protein profile of cold-shocked *C. jejuni* SVS 5141. These five obvious variations in protein bands circled in red and yellow lines. The five circled protein bands at six hours of cold storage cell protein profiles all appeared down-regulated. The three red circles contained protein bands that were abundant at the protein profile of cells without cold exposure and all cold adapted cell protein profiless, but disappeared from the six hours of cold-shocked cells' protein profiles; the molecular weights were ～10kD, ～14kD and ～75kD. The other two down-regulated protein bands, which are circled by a yellow line, were much less intense than the same protein bands in the protein profiles of cells without cold exposure and all cold adapted cells. The molecular weight of these two protein bands were ～23kD and～28kD.

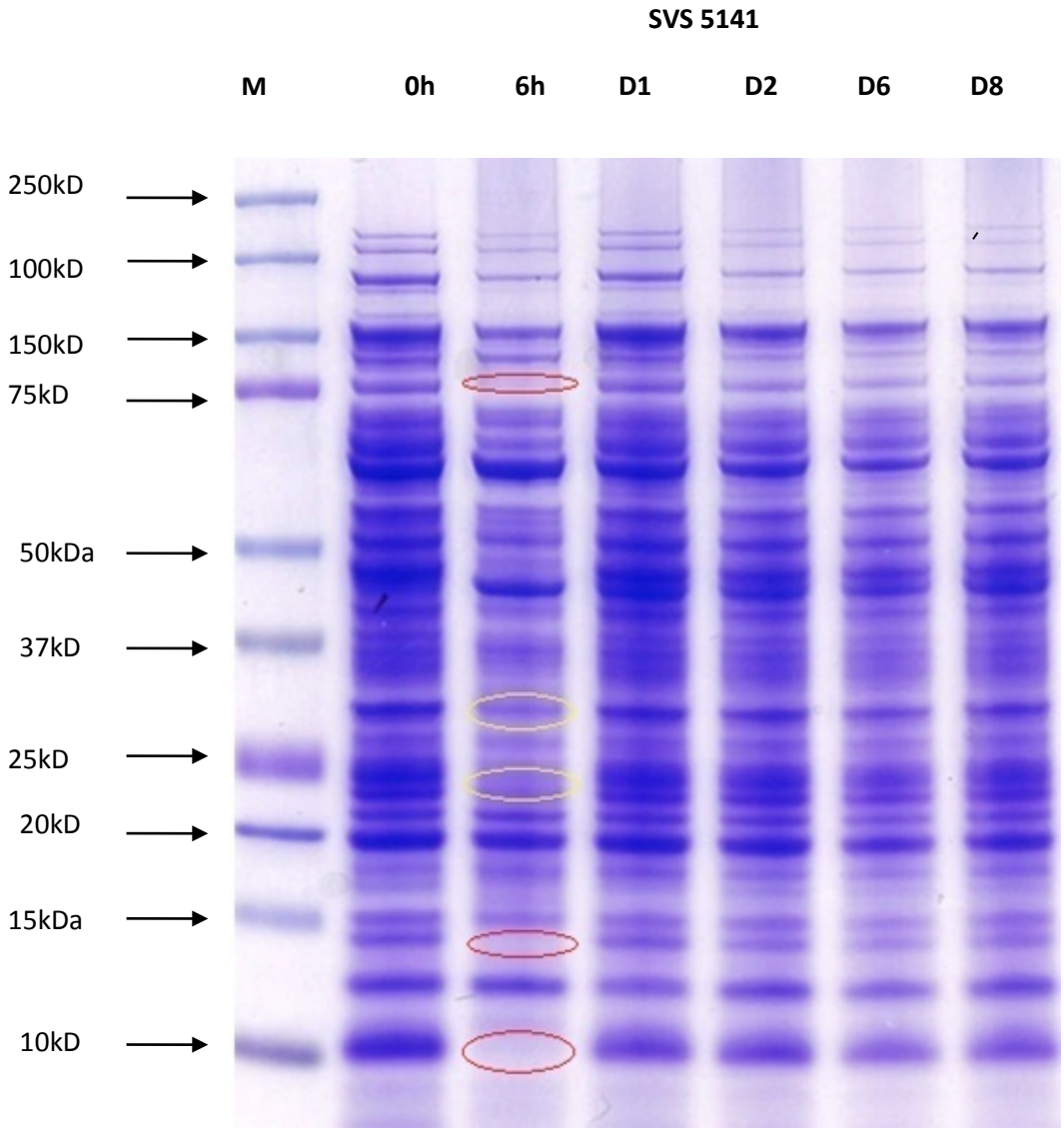


Figure 6.2 1D Protein profiles of *C. jejuni* SVS 5141 collected at 0 h, 6 h and Day 1, Day 2, Day 6 and Day 8

(M is the marker).

6.2.2 Different protein expression in *C. jejuni* SVS 5001 and SVS 5141 under cold stress

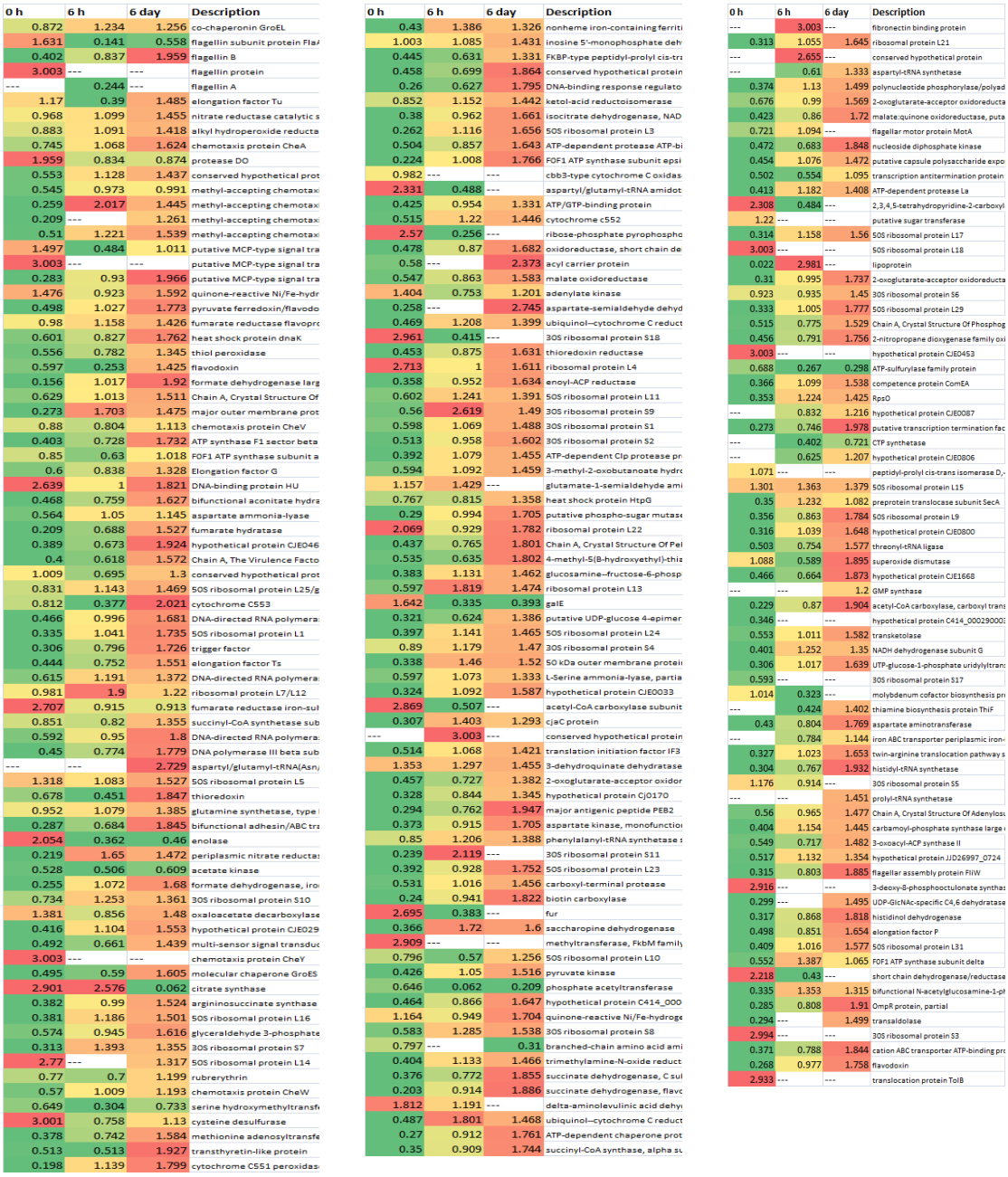
iTRAQ labelling gel-free proteomic analysis was used to study protein quantitative changes under cold stress in proteome scale for *C. jejuni* SVS 5001 and 5141. Five protein extraction samples were selected for this comparative proteomic analysis. They are whole-cell protein extractions from 0 h, 6 h and 6 days of 4^{o}C storage of *C. jejuni* SVS 5001 and 0 h, 6 days 4^{o}C storage of *C. jejuni* SVS 5141.

In comparing study protein quantitative changes effected by cold exposure, the protein samples extracted from cells without cold storage were regarding as the standard. The protein samples extracted from cold storage cells were compared with the standard. If the ratio of quantity variation for one identified protein is more than two times, this protein was considered as a protein with significant expression changes during cold storage.

6.2.2.1 Proteome map of C. jejuni SVS 5001

In the present study, iTRAQ labelling comparative proteomic analysis had identified 227 proteins in *C. jejuni* SVS 5001. Figure 6.3 shows the amounts of those 227 proteins displayed by ratios and with different colours. The red colour indicated the ratio of a protein is greater than unity; the green colour indicated the ratio of a protein less than unity; blank indicated protein that could not be identified at that time point.

The 0 h cold exposure cells had 214 proteins identified. After six hours of 4^{o}C cold exposure, *C. jejuni* SVS 5001 stopped synthesizing 21 proteins; meantime ten cold-induced proteins that cannot be found in the cells without cold storage started to synthesize. The protein numbers in the six hour cold-shocked SVS 5001 cells was 203. After six days of cold exposure, the number of proteins identified in *C. jejuni* SVS 5001 was reduced to 195. Compared with the 0 hours sample, there were 28 proteins that stopped biosynthesis and nine proteins that could not be found in the 0 hours sample were induced by six days of cold exposure.



0 h	6 h	6 day	Description
0.872	1.234	1.256	co-chaperonin GroEL
1.631	0.141	0.558	flagellin subunit protein FlaA
0.402	0.837	1.959	flagellin B
3.003	---	---	flagellin protein
---	0.244	---	flagellin A
1.17	0.39	1.485	elongation factor Tu
0.968	1.099	1.455	nitrate reductase catalytic s
0.883	1.091	1.418	alkyl hydroperoxide reducta
0.745	1.068	1.624	chemotaxis protein CheA
1.959	0.834	0.874	protease DO
0.553	1.128	1.437	conserved hypothetical prot
0.545	0.973	0.991	methyl-accepting chemotaxi
0.259	2.017	1.445	methyl-accepting chemotaxi
0.209	---	1.261	methyl-accepting chemotaxi
0.51	1.221	1.539	methyl-accepting chemotaxi
1.497	0.484	1.011	putative MCP-type signal tra
3.003	---	---	putative MCP-type signal tra
0.283	0.93	1.966	putative MCP-type signal tra
1.476	0.923	1.592	quinone-reactive Ni/Fe-hydr
0.498	1.027	1.773	pyruvate ferredoxin/flavodo
0.98	1.158	1.426	fumarate reductase flavopro
0.601	0.827	1.762	heat shock protein dnaK
0.556	0.782	1.345	thiol peroxidase
0.597	0.253	1.425	flavodoxin
0.156	1.017	1.92	formate dehydrogenase larg
0.629	1.013	1.511	Chain A, Crystal Structure Of
0.273	1.703	1.475	major outer membrane prot
0.88	0.804	1.113	chemotaxis protein CheV
0.403	0.728	1.732	ATP synthase F1 sector beta
0.85	0.63	1.018	F0F1 ATP synthase subunit a
0.6	0.838	1.328	Elongation factor G
2.639	1	1.821	DNA-binding protein HU
0.468	0.759	1.627	bifunctional aconitate hydra
0.564	1.05	1.145	aspartate ammonia-lyase
0.209	0.688	1.527	fumarate hydratase
0.389	0.673	1.924	hypothetical protein CJE046
0.4	0.618	1.572	Chain A, The Virulence Facto
1.009	0.695	1.3	conserved hypothetical prot
0.831	1.143	1.469	50S ribosomal protein L25/g
0.812	0.377	2.021	cytochrome C553
0.466	0.996	1.681	DNA-directed RNA polymera
0.335	1.041	1.735	50S ribosomal protein L1
0.306	0.796	1.726	trigger factor
0.444	0.752	1.551	elongation factor Ts
0.615	1.191	1.372	DNA-directed RNA polymera
0.981	1.9	1.22	ribosomal protein L7/L12
2.707	0.915	0.913	fumarate reductase iron-sul
0.851	0.82	1.355	succinyl-CoA synthetase sub
0.592	0.95	1.8	DNA-directed RNA polymera
0.45	0.774	1.779	DNA polymerase III beta sub
---	---	2.729	aspartyl/glutamyl-tRNA(Asn
1.318	1.083	1.527	50S ribosomal protein L5
0.678	0.451	1.847	thioredoxin
0.952	1.079	1.385	glutamine synthetase, type
0.287	0.684	1.845	bifunctional adhesin/ABC tra
2.054	0.362	0.46	enolase
0.219	1.65	1.472	periplasmic nitrate reducta
0.528	0.506	0.609	acetate kinase
0.255	1.072	1.68	formate dehydrogenase, iro
0.734	1.253	1.361	30S ribosomal protein S10
1.381	0.856	1.48	oxaloacetate decarboxylase
0.416	1.104	1.553	hypothetical protein CJE029
0.492	0.661	1.439	multi-sensor signal transduc
3.003	---	---	chemotaxis protein CheY
0.495	0.59	1.605	molecular chaperone GroES
2.901	2.576	0.062	citrate synthase
0.382	0.99	1.524	argininosuccinate synthase
0.381	1.186	1.501	50S ribosomal protein L16
0.574	0.945	1.616	glyceraldehyde 3-phosphate
0.313	1.393	1.355	30S ribosomal protein S7
2.77	---	1.317	50S ribosomal protein L14
0.77	0.7	1.199	rubrerythrin
0.57	1.009	1.193	chemotaxis protein CheW
0.649	0.304	0.733	serine hydroxymethyltransfe
3.001	0.758	1.13	cysteine desulfurase
0.378	0.742	1.584	methionine adenosyltransfe
0.513	0.513	1.927	transthyretin-like protein
0.198	1.139	1.799	cytochrome C551 peroxidas

0 h	6 h	6 day	Description
0.43	1.386	1.326	nonheme iron-containing ferriti
1.003	1.085	1.431	inosine 5'-monophosphate deh
0.445	0.631	1.331	FKBP-type peptidyl-prolyl cis-tra
0.458	0.699	1.864	conserved hypothetical protein
0.26	0.627	1.795	DNA-binding response regulato
0.852	1.152	1.442	ketol-acid reductoisomerase
0.38	0.962	1.661	isocitrate dehydrogenase, NAD
0.262	1.116	1.656	50S ribosomal protein L3
0.504	0.857	1.643	ATP-dependent protease ATP-bi
0.224	1.008	1.766	F0F1 ATP synthase subunit epsi
0.982	---	---	cbb3-type cytochrome C oxidas
2.331	0.488	---	aspartyl/glutamyl-tRNA amidot
0.425	0.954	1.331	ATP/GTP-binding protein
0.515	1.22	1.446	cytochrome c552
2.57	0.256	---	ribose-phosphate pyrophospho
0.478	0.87	1.682	oxidoreductase, short chain de
0.58	---	2.373	acyl carrier protein
0.547	0.863	1.583	malate oxidoreductase
1.404	0.753	1.201	adenylate kinase
0.258	---	2.745	aspartate-semialdehyde dehyd
0.469	1.208	1.399	ubiquinol--cytochrome C reduct
2.961	0.415	---	30S ribosomal protein S18
0.453	0.875	1.631	thioredoxin reductase
2.713	1	1.611	ribosomal protein L4
0.358	0.952	1.634	enoyl-ACP reductase
0.602	1.241	1.391	50S ribosomal protein L11
0.56	2.619	1.49	30S ribosomal protein S9
0.598	1.069	1.488	30S ribosomal protein S1
0.513	0.958	1.602	30S ribosomal protein S2
0.392	1.079	1.455	ATP-dependent Clp protease pr
0.594	1.092	1.459	3-methyl-2-oxobutanoate hydr
1.157	1.429	---	glutamate-1-semialdehyde ami
0.767	0.815	1.358	heat shock protein HtpG
0.29	0.994	1.705	putative phospho-sugar mutase
2.069	0.929	1.782	ribosomal protein L22
0.437	0.765	1.801	Chain A, Crystal Structure Of Pel
0.535	0.635	1.802	4-methyl-5(B-hydroxyethyl)-thia
0.383	1.131	1.462	glucosamine--fructose-6-phosp
0.597	1.819	1.474	ribosomal protein L13
1.642	0.335	0.393	galE
0.321	0.624	1.386	putative UDP-glucose 4-epimer
0.397	1.141	1.465	50S ribosomal protein L24
0.89	1.179	1.47	30S ribosomal protein S4
0.338	1.46	1.52	50 kDa outer membrane protei
0.597	1.073	1.333	L-Serine ammonia-lyase, partia
0.324	1.092	1.587	hypothetical protein CJE0033
2.869	0.507	---	acetyl-CoA carboxylase subunit
0.307	1.403	1.293	cjaC protein
---	3.003	---	conserved hypothetical protein
0.514	1.068	1.421	translation initiation factor IF3
1.353	1.297	1.455	3-dehydroquinate dehydratase
0.457	0.727	1.382	2-oxoglutarate-acceptor oxidor
0.328	0.844	1.345	hypothetical protein CJ0170
0.294	0.762	1.947	major antigenic peptide PEB2
0.373	0.915	1.705	aspartate kinase, monofunctio
0.85	1.206	1.388	phenylalanyl-tRNA synthetase
0.239	2.119	---	30S ribosomal protein S11
0.392	0.928	1.752	50S ribosomal protein L23
0.531	1.016	1.456	carboxyl-terminal protease
0.24	0.941	1.822	biotin carboxylase
2.695	0.383	---	fur
0.366	1.72	1.6	saccharopine dehydrogenase
2.909	---	---	methyltransferase, FkbM family
0.796	0.57	1.256	50S ribosomal protein L10
0.426	1.05	1.516	pyruvate kinase
0.646	0.062	0.209	phosphate acetyltransferase
0.464	0.866	1.647	hypothetical protein C414_000
1.164	0.949	1.704	quinone-reactive Ni/Fe-hydroge
0.583	1.285	1.538	30S ribosomal protein S8
0.797	---	0.31	branched-chain amino acid ami
0.404	1.133	1.466	trimethylamine-N-oxide reduct
0.376	0.772	1.855	succinate dehydrogenase, C sul
0.203	0.914	1.886	succinate dehydrogenase, flavc
1.812	1.191	---	delta-aminolevulinic acid dehy
0.487	1.801	1.468	ubiquinol--cytochrome C reduct
0.27	0.912	1.761	ATP-dependent chaperone prot
0.35	0.909	1.744	succinyl-CoA synthase, alpha su

0 h	6 h	6 day	Description
---	3.003	---	fibronectin binding protein
0.313	1.055	1.645	ribosomal protein L21
---	2.655	---	conserved hypothetical protein
---	0.61	1.333	aspartyl-tRNA synthetase
0.374	1.13	1.499	polynucleotide phosphorylase/polyad
0.676	0.99	1.569	2-oxoglutarate-acceptor oxidoreducta
0.423	0.86	1.72	malate:quinone oxidoreductase, puta
0.721	1.094	---	flagellar motor protein MotA
0.472	0.683	1.848	nucleoside diphosphate kinase
0.454	1.076	1.472	putative capsule polysaccharide expo
0.502	0.554	1.095	transcription antitermination protein
0.413	1.182	1.408	ATP-dependent protease La
2.308	0.484	---	2,3,4,5-tetrahydropyridine-2-carboxyl
1.22	---	---	putative sugar transferase
0.314	1.158	1.56	50S ribosomal protein L17
3.003	---	---	50S ribosomal protein L18
0.022	2.981	---	lipoprotein
0.31	0.995	1.737	2-oxoglutarate-acceptor oxidoreducta
0.923	0.935	1.45	30S ribosomal protein S6
0.333	1.005	1.777	50S ribosomal protein L29
0.515	0.775	1.529	Chain A, Crystal Structure Of Phosphog
0.456	0.791	1.756	2-nitropropane dioxygenase family oxi
3.003	---	---	hypothetical protein CJE0453
0.688	0.267	0.298	ATP-sulfurylase family protein
0.366	1.099	1.538	competence protein ComEA
0.353	1.224	1.425	RpsO
---	0.832	1.216	hypothetical protein CJE0087
0.273	0.746	1.978	putative transcription termination fac
---	0.402	0.721	CTP synthetase
---	0.625	1.207	hypothetical protein CJE0806
1.071	---	---	peptidyl-prolyl cis-trans isomerase D,
1.301	1.363	1.379	50S ribosomal protein L15
0.35	1.232	1.082	preprotein translocase subunit SecA
0.356	0.863	1.784	50S ribosomal protein L9
0.316	1.039	1.648	hypothetical protein CJE0800
0.503	0.754	1.577	threonyl-tRNA ligase
1.088	0.589	1.895	superoxide dismutase
0.466	0.664	1.873	hypothetical protein CJE1668
---	---	1.2	GMP synthase
0.229	0.87	1.904	acetyl-CoA carboxylase, carboxyl trans
0.346	---	---	hypothetical protein C414_000290003
0.553	1.011	1.582	transketolase
0.401	1.252	1.35	NADH dehydrogenase subunit G
0.306	1.017	1.639	UTP-glucose-1-phosphate uridylyltrans
0.593	---	---	30S ribosomal protein S17
1.014	0.323	---	molybdenum cofactor biosynthesis pr
---	0.424	1.402	thiamine biosynthesis protein ThiF
0.43	0.804	1.769	aspartate aminotransferase
---	0.784	1.144	iron ABC transporter periplasmic iron-
0.327	1.023	1.653	twin-arginine translocation pathway s
0.304	0.767	1.932	histidyl-tRNA synthetase
1.176	0.914	---	30S ribosomal protein S5
---	---	1.451	prolyl-tRNA synthetase
0.56	0.965	1.477	Chain A, Crystal Structure Of Adenylos
0.404	1.154	1.445	carbamoyl-phosphate synthase large
0.549	0.717	1.482	3-oxoacyl-ACP synthase II
0.517	1.132	1.354	hypothetical protein JJD26997_0724
0.315	0.803	1.885	flagellar assembly protein FliW
2.916	---	---	3-deoxy-8-phosphooctulonate synthas
0.299	---	1.495	UDP-GlcNAc-specific C4,6 dehydratase
0.317	0.868	1.818	histidinol dehydrogenase
0.498	0.851	1.654	elongation factor P
0.409	1.016	1.577	50S ribosomal protein L31
0.552	1.387	1.065	F0F1 ATP synthase subunit delta
2.218	0.43	---	short chain dehydrogenase/reductase
0.335	1.353	1.315	bifunctional N-acetylglucosamine-1-ph
0.285	0.808	1.91	OmpR protein, partial
0.294	---	1.499	transaldolase
2.994	---	---	30S ribosomal protein S3
0.371	0.788	1.844	cation ABC transporter ATP-binding pro
0.268	0.977	1.758	flavodoxin
2.933	---	---	translocation protein TolB

Figure 6.3 227 proteins of *C. jejuni* SVS 5001 identified by iTRAQ labelling proteomic analysis in this cold exposure experiment

Colour indicates amount of identified protein. Green is a ratio of protein less than unity, red is a ratio of protein greater than unity and the blank indicates protein cannot be identified at that time point.

6.2.2.2 Proteins with significant alteration in C. jejuni SVS 5001 during cold exposure

In response to cold stress, *C. jejuni* SVS 5001 significantly altered expression of 197 proteins. There were only 30 proteins without significant changes during cold exposure. Table 6.1 presents those 197 of *C. jejuni* SVS 5001 proteins that were altered significantly during cold exposure.

Proteomic analysis found 85 and 138 proteins were up-regulated in six hour of cold shock cells and six days of cold storage cells, respectively. Eighty-two proteins were both up-regulated in cells with 6 h cold shock and six days of cold exposure. Eighty-five proteins' expression was up-regulated in six hours of cold shock *C. jejuni* SVS 5001 cells. Forty-two of them increased 2-fold; 25 of the 85 increased 3- fold; nine proteins increased four times, six proteins had a five to eight time increase. One lipoprotein had a tremendous 135-fold increase in cold shock *C. jejuni* SVS 5001 cells. One hundred thirty eight proteins were up-regulated in six-day cold adapted SVS 5001 cells. Thirty of 138 proteins increased two times; 40 of them had a 3-fold increase; 26 proteins increased four times; 19 proteins increased five times; 12 of the 138 proteins had a six times increase; five proteins increased 7-fold and five proteins had a eight to twelve times increase. The highest increased rate of protein expression had been seen in six-day cold adapted SVS 5001 cells was 12.3-fold, which was in the formate dehydrogenase large subunit of SVS 5001 cells.

Cold stress had induced 12 proteins that cannot be found in 0 hour *C. jejuni* SVS 5001 cells. Six hours of cold shock had induced nine proteins; six day of cold storage had induced nine proteins. Six proteins had been induced by both six hours of cold shock and six days of cold storage.

The down-regulated proteins and proteins that stop synthesis under cold stress in *C. jejuni* SVS 5001 are also showed in Table 6.1. There were 24 proteins that decreased their expression in six-hour cold-shocked SVS 5001 cells, 11 proteins decreased their expressions in six days of cold adapted cells. Eight protein's expressions decreased both in six hours of cold shock and six days of cold storage cells. Twenty and 28 proteins stopped their synthesis in six-hour cold shock cells and six days of cold

adapted cells, respectively. Expression of 14 protiens ceased in both six hours of cold shock and six days of cold exposure.

Table 6.1 *C. jejuni* SVS 5001 protein alteration as affected by cold stress

Accession number	Protein name	Theoretical Mw (Da)	Cold shock (6 h)	Cold adaptation (6 day)
Up-regulated proteins				
gi|86149534	chemotaxis protein CheA	85349		+2.2
gi|218563153	methyl-accepting chemotaxis signal transduction protein	73191	+7.8	+5.6
gi|121613238	methyl-accepting chemotaxis protein	72546	None	+6.0
gi|121613017	methyl-accepting chemotaxis	77220	+2.4	+3.0
gi|419619379	putative MCP-type signal transduction protein	40704	+3.3	+7.0
gi|57237338	chemotaxis protein CheW	19504		+2.1
gi|384441131	Elongation factor G	76983		+2.2
gi|419629625	DNA-directed RNA polymerase subunit beta'	169643	+2.1	+3.6
gi|218562793	elongation factor Ts	39758		+3.5
gi|57238608	DNA-directed RNA polymerase subunit alpha	37734		+2.2
gi|384447762	DNA-directed RNA polymerase subunit beta	156229		+3.0
gi|415729844	DNA polymerase III beta subunit, central domain protein	28735		+4.0
gi|57237410	DNA-binding response regulator	25614	+2.4	+6.9
gi|205356548	translation initiation factor IF3	14527	+2.1	+2.8
gi|419632940	polynucleotide phosphorylase/polyadenylase	79322	+3.0	+4.0
gi|57237527	transcription antitermination protein NusG	20183		+2.2
gi|57236924	competence protein ComEA	8847	+3.0	+4.2
gi|40217918	putative transcription termination factor	47265	+2.7	+7.3
gi|407941670	threonyl-tRNA ligase	69916		+3.1
gi|419648839	histidyl-tRNA synthetase	47897	+2.5	+6.4
gi|57238249	elongation factor P	21199		+3.3
gi|57237529	50S ribosomal protein L1	25031	+3.1	+5.2
gi|57238707	50S ribosomal protein L16	16365	+3.1	+3.9
gi|57237544	30S ribosomal protein S7	17681	+4.5	+4.3
gi|57238730	50S ribosomal protein L3	20824	+4.3	+6.3
gi|57237528	50S ribosomal protein L11	15127	+2.1	+2.3
gi|57238502	30S ribosomal protein S9	14128	+4.7	+2.7
gi|57237722	30S ribosomal protein S1	62827		+2.5
gi|153952052	30S ribosomal protein S2	30449		+3.1
gi|57169062	ribosomal protein L13	15864	+3.1	+2.5
gi|57238703	50S ribosomal protein L24	8335	+2.9	+3.7
gi|57238606	30S ribosomal protein S11	13942	+8.9	None
gi|57238712	50S ribosomal protein L23	10561	+2.4	+4.5
gi|57238700	30S ribosomal protein S8	14795	+2.2	+2.6
gi|57168772	ribosomal protein L21	11602	+3.4	+5.3
gi|57238609	50S ribosomal protein L17	13240	+3.7	+5.0
gi|57238706	50S ribosomal protein L29	7029	+3.0	+5.3
gi|633730	RpsO, 30S ribosomal protein S15	10206	+3.5	+4.0
gi|57236974	50S ribosomal protein L9	16272	+2.4	+5.0
gi|57237163	50S ribosomal protein L31	7757	+2.5	+3.9
gi|419640337	pyruvate ferredoxin/flavodoxin oxidoreductase	132355	+2.1	+3.6
gi|86150091	flavodoxin	17224	-2.4	+2.4
gi|218563111	formate dehydrogenase large subunit	104573	+6.5	+12.3
gi|283955551	ATP synthase F1 sector beta subunit	50885		+4.3
gi|419637637	bifunctional aconitate hydratase 2/2-methylisocitrate	93523		+3.5

	dehydratase			
gi|148926719	fumarate hydratase	50947	+3.3	+7.3
gi|57238028	cytochrome C553	11002	-2.2	+2.5
gi|419641626	periplasmic nitrate reductase, small subunit	19251	+7.5	+6.7
gi|86149326	formate dehydrogenase, iron-sulfur subunit	24717	+4.2	+6.6
gi|57238440	glyceraldehyde 3-phosphate dehydrogenase A	36691		+2.8
gi|57237413	cytochrome C551 peroxidase	37021	+5.8	+9.1
gi|317509581	isocitrate dehydrogenase, NADP-dependent	86588	+2.5	+4.4
gi|384447418	F0F1 ATP synthase subunit epsilon	13825	+4.5	+7.9
gi|419622743	cytochrome c552	69795	+2.4	+2.8
gi|148926276	malate oxidoreductase	44170		+2.9
gi|57238058	ubiquinol--cytochrome C reductase, iron-sulfur subunit	18332	+2.6	+3.0
gi|153951531	2-oxoglutarate-acceptor oxidoreductase subunit OorC	20141		+3.0
gi|283955815	pyruvate kinase	53954	+2.5	+3.6
gi|86151404	trimethylamine-N-oxide reductase 2 precursor	93857	+2.8	+3.6
gi|57237495	succinate dehydrogenase, C subunit	31983	+2.1	+4.9
gi|419648255	succinate dehydrogenase, flavoprotein subunit	67163	+4.5	+9.3
gi|57238056	ubiquinol--cytochrome C reductase, cytochrome C1 subunit	41617	+3.7	+3.0
gi|57238266	succinyl-CoA synthase, alpha subunit	30216	+2.6	+5.0
gi|57238263	2-oxoglutarate-acceptor oxidoreductase subunit OorB	31758		+2.3
gi|86149076	malate:quinone oxidoreductase, putative	50953	+2.0	+4.1
gi|153951934	2-oxoglutarate-acceptor oxidoreductase subunit OorA	41211	+3.2	+5.6
gi|317455537	Chain A, Crystal Structure Of Phosphoglycerate Kinase	43921		+3.0
gi|419645546	NADH dehydrogenase subunit G	94588	+3.1	+3.4
gi|57237111	F0F1 ATP synthase subunit delta	20497	+2.5	
gi|57237336	transaldolase	37122	None	+5.1
gi|57238563	flavodoxin	22239	+3.7	+6.6
gi|3290034	flagellin B	59220	+2.1	+4.9
gi|85036689	major outer membrane protein	45632	+6.2	+5.4
gi|330689730	Chain A, The Virulence Factor Peb4	28359		+3.9
gi|86152514	50 kDa outer membrane protein	53832	+4.3	+4.5
gi|148926946	major antigenic peptide PEB2	24485	+2.6	+6.6
gi|57237913	lipoprotein	18559	+135	None
gi|57237957	flagellar assembly protein FliW	14892	+2.6	+6.0
gi|153952664	bifunctional N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase	48372	+4.0	+3.9
gi|881376	OmpR protein, partial	22640	+2.8	+6.7
gi|205355989	putative 80hosphor-sugar mutase	48938	+3.4	+5.9
gi|121613200	glucosamine—fructose-6-phosphate aminotransferase	67687	+3.0	+3.8
gi|37719580	putative UDP-glucose 4-epimerase	35556		+4.3
gi|57238142	2-nitropropane dioxygenase family oxidoreductase	39995		+3.9
gi|57238656	transketolase	70001		+2.9
gi|57238554	UTP-glucose-1-phosphate uridylyltransferase	30831	+3.3	+5.4
gi|419622287	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	37683	None	+5.0
gi|57238192	nonheme iron-containing ferritin	19531	+3.2	+3.1
gi|148926924	heat shock protein dnaK	67432		+2.9
gi|419658906	trigger factor	50970	+2.6	+5.6
gi|57238092	molecular chaperone GroES	9452		+3.2
gi|57237122	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	20547		+3.0
gi|419641014	ATP-dependent chaperone protein ClpB	95538	+3.4	+6.5
gi|86150249	thiol peroxidase	18771		+2.4

gi|57168680	thioredoxin	11501		+2.7
gi|419694122	thioredoxin reductase	34000		+3.6
gi|86148986	acyl carrier protein	8592	None	+4.1
gi|57238437	enoyl-ACP reductase	29961	+2.7	+4.6
gi|419641319	biotin carboxylase	49485	+3.9	+7.6
gi|283955572	acetyl-CoA carboxylase, carboxyl transferase, beta subunit	31300	+3.8	+8.3
gi|57237499	3-oxoacyl-ACP synthase II	43103		+2.7
gi|57237383	nucleoside diphosphate kinase	15161		+3.9
gi|329666276	Chain A, Crystal Structure of Adenylosuccinate Synthetase	46529		+2.6
gi|148926982	carbamoyl-phosphate synthase large chain	122342	+2.9	+3.6
gi|86149847	argininosuccinate synthase	45778	+2.6	+4.0
gi|317510573	methionine adenosyltransferase	40926		+4.2
gi|13509099	aspartate-semialdehyde dehydrogenase	21405	None	+10.6
gi|283954138	aspartate kinase, monofunctional class	42771	+2.5	+4.6
gi|121612631	saccharopine dehydrogenase	45561	+4.7	+4.4
gi|419622789	aspartate aminotransferase	43692		+4.1
gi|57238611	histidinol dehydrogenase	46687	+2.7	+5.7
gi|284055744	Chain A, Crystal Structure of Putative Bacterioferritin	17574		+2.4
gi|57237749	bifunctional adhesin/ABC transporter aspartate/glutamate-binding protein	28243	+2.4	+6.4
gi|57238619	ATP/GTP-binding protein	40355	+2.2	+3.1
gi|146386525	Chain A, Crystal Structure of Peb3	26351		+4.1
gi|86149797	cjaC protein	27835	+4.6	+4.2
gi|148925618	putative capsule polysaccharide export system periplasmic protein	58916	+2.4	+3.2
gi|419645158	preprotein translocase subunit SecA	98244	+3.5	+3.1
gi|88596055	twin-arginine translocation pathway signal	72004	+3.1	+5.1
gi|57237149	cation ABC transporter ATP-binding protein	33050	+2.1	+5.0
gi|86149225	3-methyl-2-oxobutanoate hydroxymethyltransferase	30354		+2.5
gi|57237728	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme	20427		+3.4
gi|415730403	multi-sensor signal transduction histidine kinase	25368		+2.9
gi|57236972	ATP-dependent protease ATP-binding subunit HslU	49747		+3.3
gi|57237197	ATP-dependent Clp protease proteolytic subunit	21807	+2.8	+3.7
gi|419627400	carboxyl-terminal protease	48990		+2.7
gi|283954636	ATP-dependent protease La	90364	+2.9	+3.4
gi|88597680	aspartate ammonia-lyase	52166		+2.0
gi|317511439	L-Serine ammonia-lyase, partial	36633		+2.2
gi|86150500	conserved hypothetical protein	20593	+2.0	+2.6
gi|57237475	hypothetical protein CJE0469	21025		+5.0
gi|57237305	hypothetical protein CJE0298	32085	+2.7	+3.7
gi|57237017	transthyretin-like protein	15910		+3.8
gi|86149608	conserved hypothetical protein	39053		+4.1
gi|86150182	oxidoreductase, short chain dehydrogenase/reductase family	28233		+3.5
gi|57237048	hypothetical protein CJE0033	26440	+3.4	+4.9
gi|218561850	hypothetical protein Cj0170	28767	+2.6	+4.1
gi|283955175	hypothetical protein C414_000420088	16753		+3.6
gi|57237003	hypothetical protein CJE0800	25980	+3.3	+5.2
gi|57238518	hypothetical protein CJE1668	22100		+4.0
gi|153951812	hypothetical protein JJD26997_0724	41387	+2.2	+2.6
Protein biosynthesizing at cold stress only				

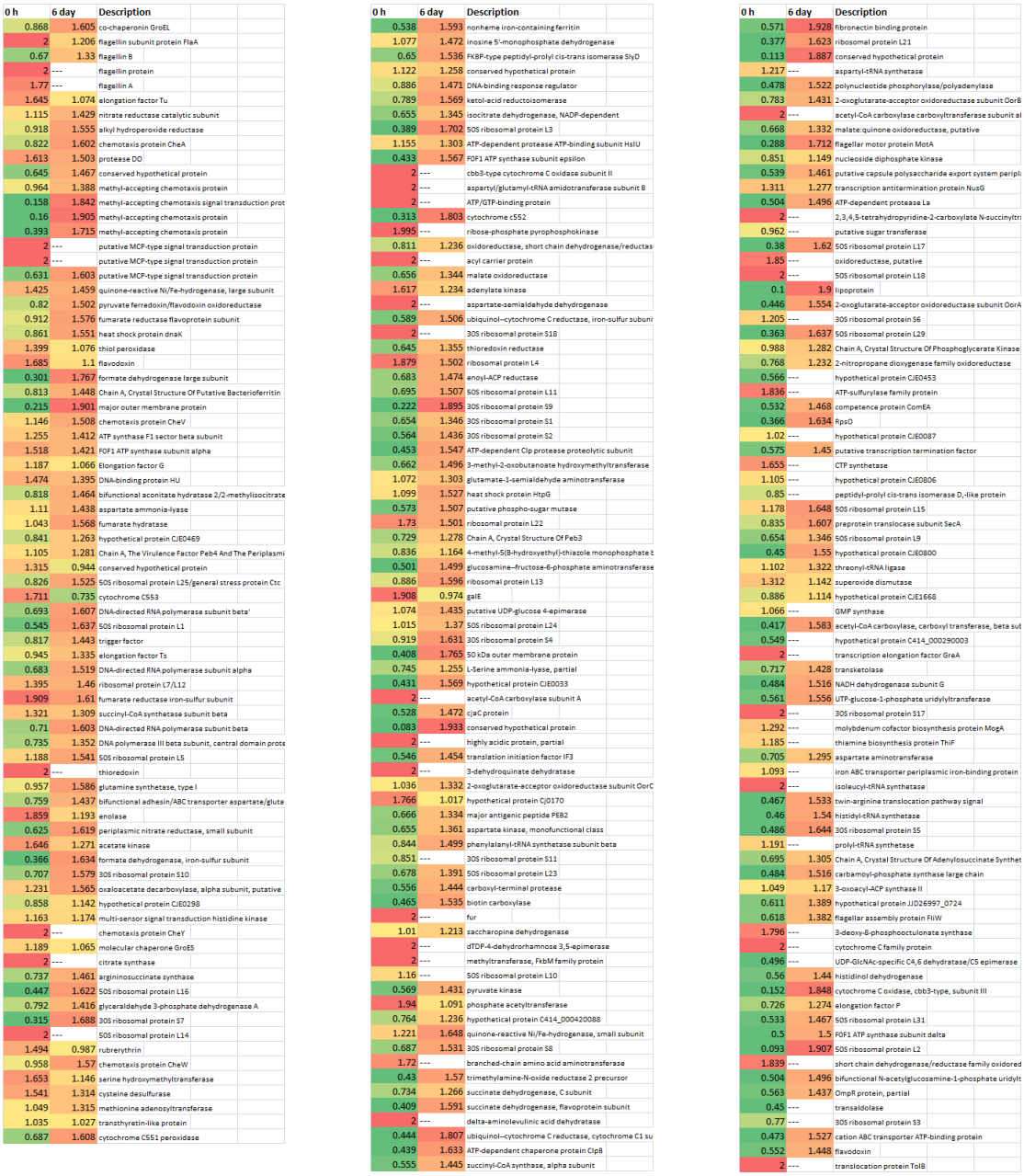
gi|283954623	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase, A subunit	49712		6d
gi|57236953	aspartyl-tRNA synthetase	66676	6h	6d
gi|57238257	prolyl-tRNA synthetase	65053		6d
gi|56806980	flagellin A	11704	6h	
gi|57236939	CTP synthetase	60765	6h	6d
gi|86150584	GMP synthase	57397		6d
gi|57237180	iron ABC transporter periplasmic iron-binding protein	37406	6h	6d
gi|86150511	thiamine biosynthesis protein ThiF	30047	6h	6d
gi|86150082	conserved hypothetical protein	43511	6h	
gi|86150649	conserved hypothetical protein	16323	6h	
gi|57237099	hypothetical protein CJE0087	49254	6h	6d
gi|57237008	hypothetical protein CJE0806	27951	6h	6d
Down-regulated proteins				
gi|419638103	putative MCP-type signal transduction protein	48561	-3.09	
gi|57237524	elongation factor Tu	43623	-3	
gi|57238069	aspartyl/glutamyl-tRNA amidotransferase subunit B	53256	-4.78	None
gi|475890	fur	18175	-7.04	None
gi|57238704	50S ribosomal protein L14	13354	None	-2.1
gi|57237954	30S ribosomal protein S18	10322	-7.13	None
gi|86149254	ribosomal protein L4	22273	-2.71	
gi|68248462	ribosomal protein L22	15885	-2.23	None
gi|86150091	flavodoxin	17224	-2.36	+2.39
gi|57238028	cytochrome C553	11002	-2.15	+2.49
gi|57237465	fumarate reductase iron-sulfur subunit	28282	-2.96	-2.96
gi|86149479	enolase	45253	-5.67	-4.47
gi|57238690	citrate synthase	48109		-46.8
gi|86150126	flagellin subunit protein FlaA	59075	-11.6	-2.92
gi|3413445	galE	37224	-4.9	-4.18
gi|57237741	DNA-binding protein HU	10268	-2.64	
gi|419641974	serine hydroxymethyltransferase	46079	-2.13	
gi|86149461	ATP-sulfurylase family protein	44829	-2.58	-2.31
gi|57237923	acetyl-CoA carboxylase subunit A	54834	-5.66	None
gi|88597146	phosphate acetyltransferase	56356	-10.4	-3.09
gi|57237746	ribose-phosphate pyrophosphokinase	33848	-10.	None
gi|57237300	cysteine desulfurase	43191	-3.96	-2.66
gi|419633975	branched-chain amino acid aminotransferase	34036	None	-2.57
gi|57238618	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	42635	-4.77	None
gi|153951811	molybdenum cofactor biosynthesis protein MogA	20371	-3.14	None
gi|86150607	protease DO	50976	-2.35	-2.24
gi|419641488	short chain dehydrogenase/reductase family oxidoreductase	28161	-5.16	None
Proteins stopped synthesis under cold stress				
gi|121613238	methyl-accepting chemotaxis protein	72546	None	+6.0
gi|57238000	chemotaxis protein CheY	14428	None	None
gi|419589392	putative MCP-type signal transduction protein	48552	None	None
gi|57238069	aspartyl/glutamyl-tRNA amidotransferase subunit B	53256	-4.8	None
gi|475890	fur [Campylobacter jejuni]	18175	-7.0	None
gi|57238704	50S ribosomal protein L14	13354	None	-2.1
gi|57238698	50S ribosomal protein L18	13288	None	None
gi|57237954	30S ribosomal protein S18	10322	-7.1	None
gi|57238606	30S ribosomal protein S11	13942	+8.9	None
gi|57238705	30S ribosomal protein S17	9600	None	None

gi|57238697	30S ribosomal protein S5	15787		None
gi|153952472	30S ribosomal protein S3	26067	None	None
gi|57238512	cbb3-type cytochrome C oxidase subunit II	25078	None	None
gi|57237336	transaldolase	37122	None	+5.1
gi|455428	flagellin protein	59652	None	None
gi|57237388	flagellar motor protein MotA	28279		None
gi|57237913	lipoprotein	18559	+135	None
gi|283955143	3-deoxy-8-phosphooctulonate synthase	29826	None	None
gi|57237119	translocation protein TolB	44711	None	None
gi|57236997	peptidyl-prolyl cis-trans isomerase D,-like protein	57475	None	None
gi|86150084	methyltransferase, FkbM family protein	33582	None	None
gi|86150017	putative sugar transferase	89945	None	None
gi|419622287	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	37683	None	+5.0
gi|57237746	ribose-phosphate pyrophosphokinase	33848	-10.0	None
gi|86148986	acyl carrier protein	8592	None	+4.1
gi|57237923	acetyl-CoA carboxylase subunit A	54834	-5.7	None
gi|13509099	aspartate-semialdehyde dehydrogenase	21405	None	+10.6
gi|419633975	branched-chain amino acid aminotransferase	34036	None	-2.6
gi|57238618	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	42635	-4.8	None
gi|57237694	glutamate-1-semialdehyde aminotransferase	46517		None
gi|57237820	delta-aminolevulinic acid dehydratase	36733		None
gi|153951811	molybdenum cofactor biosynthesis protein MogA	20371	-3.1	None
gi|57237459	hypothetical protein CJE0453	30726	None	None
gi|283954942	hypothetical protein C414_000290003	44381	None	None
gi|419641488	short chain dehydrogenase/reductase family oxidoreductase	28161	-5.2	None

6.2.2.3 A proteome map of C. jejuni SVS 5141

Because of a shortage in the six hour cold shock *C. jejuni* SVS 5141 cell extraction sample, only 0 h and six days of cold exposure protein samples from *C. jejuni* SVS 5141 were able be analyzed by the iTRAQ labelling proteomic approach. The iTRAQ labelling comparative proteomic analysis had identified 235 proteins in 0 h and six days of cold exposure *C. jejuni* SVS 5141 cells. As shown in Figure 6.4, amount of those 235 proteins is displayed in ratios and different colours. The red colour indicated the ratio of a protein is greater than unity; the green colour indicated the ratio of a protein less than unity; blank indicated protein that could not be identified at that time point.

There were 235 proteins identified by iTRAQ labelling proteomic analysis from the cell without cold exposure. After six days of cold storage, 54 proteins stopped biosynthesis, the number of proteins in *C. jejuni* SVS 5141 with six days of cold exposure had reduced to 181. The six days of cold exposure did not induce any protein that could not be found in *C. jejuni* SVS 5141 cells without cold storage.



0 h	6 day	Description
0.868	1.605	co-chaperonin GroEL
2	1.206	flagellin subunit protein FlaA
0.67	1.33	flagellin B
2	---	flagellin protein
1.77	---	flagellin A
1.645	1.074	elongation factor Tu
1.115	1.429	nitrate reductase catalytic subunit
0.918	1.555	alkyl hydroperoxide reductase
0.822	1.602	chemotaxis protein CheA
1.613	1.503	protease DO
0.645	1.467	conserved hypothetical protein
0.964	1.388	methyl-accepting chemotaxis protein
0.158	1.842	methyl-accepting chemotaxis signal transduction prot
0.16	1.905	methyl-accepting chemotaxis protein
0.393	1.715	methyl-accepting chemotaxis protein
2	---	putative MCP-type signal transduction protein
2	---	putative MCP-type signal transduction protein
0.631	1.603	putative MCP-type signal transduction protein
1.425	1.459	quinone-reactive Ni/Fe-hydrogenase, large subunit
0.82	1.502	pyruvate ferredoxin/flavodoxin oxidoreductase
0.912	1.576	fumarate reductase flavoprotein subunit
0.861	1.551	heat shock protein dnaK
1.399	1.076	thiol peroxidase
1.685	1.1	flavodoxin
0.301	1.767	formate dehydrogenase large subunit
0.813	1.448	Chain A, Crystal Structure Of Putative Bacterioferritin
0.215	1.901	major outer membrane protein
1.146	1.508	chemotaxis protein CheV
1.255	1.412	ATP synthase F1 sector beta subunit
1.518	1.421	F0F1 ATP synthase subunit alpha
1.187	1.066	Elongation factor G
1.474	1.395	DNA-binding protein HU
0.818	1.464	bifunctional aconitate hydratase 2/2-methylisocitrate
1.11	1.438	aspartate ammonia-lyase
1.043	1.568	fumarate hydratase
0.841	1.263	hypothetical protein CJE0469
1.105	1.281	Chain A, The Virulence Factor Peb4 And The Periplasmi
1.315	0.944	conserved hypothetical protein
0.826	1.525	50S ribosomal protein L25/general stress protein Ctc
1.711	0.735	cytochrome C553
0.693	1.607	DNA-directed RNA polymerase subunit beta'
0.545	1.637	50S ribosomal protein L1
0.817	1.443	trigger factor
0.945	1.335	elongation factor Ts
0.683	1.519	DNA-directed RNA polymerase subunit alpha
1.395	1.46	ribosomal protein L7/L12
1.909	1.61	fumarate reductase iron-sulfur subunit
1.321	1.309	succinyl-CoA synthetase subunit beta
0.71	1.603	DNA-directed RNA polymerase subunit beta
0.735	1.352	DNA polymerase III beta subunit, central domain prote
1.188	1.541	50S ribosomal protein L5
2	---	thioredoxin
0.957	1.586	glutamine synthetase, type I
0.759	1.437	bifunctional adhesin/ABC transporter aspartate/gluta
1.859	1.193	enolase
0.625	1.619	periplasmic nitrate reductase, small subunit
1.646	1.271	acetate kinase
0.366	1.634	formate dehydrogenase, iron-sulfur subunit
0.707	1.579	30S ribosomal protein S10
1.231	1.565	oxaloacetate decarboxylase, alpha subunit, putative
0.858	1.142	hypothetical protein CJE0298
1.163	1.174	multi-sensor signal transduction histidine kinase
2	---	chemotaxis protein CheY
1.189	1.065	molecular chaperone GroES
2	---	citrate synthase
0.737	1.461	argininosuccinate synthase
0.447	1.622	50S ribosomal protein L16
0.792	1.416	glyceraldehyde 3-phosphate dehydrogenase A
0.315	1.688	30S ribosomal protein S7
2	---	50S ribosomal protein L14
1.494	0.987	rubrerythrin
0.958	1.57	chemotaxis protein CheW
1.653	1.146	serine hydroxymethyltransferase
1.541	1.314	cysteine desulfurase
1.049	1.315	methionine adenosyltransferase
1.035	1.027	transthyretin-like protein
0.687	1.608	cytochrome CS51 peroxidase

0 h	6 day	Description
0.538	1.593	nonheme iron-containing ferritin
1.077	1.472	inosine 5'-monophosphate dehydrogenase
0.65	1.536	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD
1.122	1.258	conserved hypothetical protein
0.886	1.471	DNA-binding response regulator
0.789	1.569	ketol-acid reductoisomerase
0.655	1.345	isocitrate dehydrogenase, NADP-dependent
0.389	1.702	50S ribosomal protein L3
1.155	1.303	ATP-dependent protease ATP-binding subunit HslU
0.433	1.567	F0F1 ATP synthase subunit epsilon
2	---	cbb3-type cytochrome C oxidase subunit II
2	---	aspartyl/glutamyl-tRNA amidotransferase subunit B
2	---	ATP/GTP-binding protein
0.313	1.803	cytochrome c552
1.995	---	ribose-phosphate pyrophosphokinase
0.811	1.236	oxidoreductase, short chain dehydrogenase/reductas
2	---	acyl carrier protein
0.656	1.344	malate oxidoreductase
1.617	1.234	adenylate kinase
2	---	aspartate-semialdehyde dehydrogenase
0.589	1.506	ubiquinol--cytochrome C reductase, iron-sulfur subuni
2	---	30S ribosomal protein S18
0.645	1.355	thioredoxin reductase
1.879	1.502	ribosomal protein L4
0.683	1.474	enoyl-ACP reductase
0.695	1.507	50S ribosomal protein L11
0.222	1.895	30S ribosomal protein S9
0.654	1.346	30S ribosomal protein S1
0.564	1.436	30S ribosomal protein S2
0.453	1.547	ATP-dependent Clp protease proteolytic subunit
0.662	1.496	3-methyl-2-oxobutanoate hydroxymethyltransferase
1.072	1.303	glutamate-1-semialdehyde aminotransferase
1.099	1.527	heat shock protein HtpG
0.573	1.507	putative phospho-sugar mutase
1.73	1.501	ribosomal protein L22
0.729	1.278	Chain A, Crystal Structure Of Peb3
0.836	1.164	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate t
0.501	1.499	glucosamine--fructose-6-phosphate aminotransferase
0.886	1.596	ribosomal protein L13
1.908	0.974	galE
1.074	1.435	putative UDP-glucose 4-epimerase
1.015	1.37	50S ribosomal protein L24
0.919	1.631	30S ribosomal protein S4
0.408	1.765	50 kDa outer membrane protein
0.745	1.255	L-Serine ammonia-lyase, partial
0.431	1.569	hypothetical protein CJE0033
2	---	acetyl-CoA carboxylase subunit A
0.528	1.472	cjaC protein
0.083	1.933	conserved hypothetical protein
2	---	highly acidic protein, partial
0.546	1.454	translation initiation factor IF3
2	---	3-dehydroquinate dehydratase
1.036	1.332	2-oxoglutarate-acceptor oxidoreductase subunit OorC
1.766	1.017	hypothetical protein CJ0170
0.666	1.334	major antigenic peptide PEB2
0.655	1.361	aspartate kinase, monofunctional class
0.844	1.499	phenylalanyl-tRNA synthetase subunit beta
0.851	---	30S ribosomal protein S11
0.678	1.391	50S ribosomal protein L23
0.556	1.444	carboxyl-terminal protease
0.465	1.535	biotin carboxylase
2	---	fur
1.01	1.213	saccharopine dehydrogenase
2	---	dTDP-4-dehydrorhamnose 3,5-epimerase
2	---	methyltransferase, FkbM family protein
1.16	---	50S ribosomal protein L10
0.569	1.431	pyruvate kinase
1.94	1.091	phosphate acetyltransferase
0.764	1.236	hypothetical protein C414_000420088
1.221	1.648	quinone-reactive Ni/Fe-hydrogenase, small subunit
0.687	1.531	30S ribosomal protein S8
1.72	---	branched-chain amino acid aminotransferase
0.43	1.57	trimethylamine-N-oxide reductase 2 precursor
0.734	1.266	succinate dehydrogenase, C subunit
0.409	1.591	succinate dehydrogenase, flavoprotein subunit
2	---	delta-aminolevulinic acid dehydratase
0.444	1.807	ubiquinol--cytochrome C reductase, cytochrome C1 su
0.439	1.633	ATP-dependant chaperone protein ClpB
0.555	1.445	succinyl-CoA synthase, alpha subunit

0 h	6 day	Description
0.571	1.928	fibronectin binding protein
0.377	1.623	ribosomal protein L21
0.113	1.887	conserved hypothetical protein
1.217	---	aspartyl-tRNA synthetase
0.478	1.522	polynucleotide phosphorylase/polyadenylase
0.783	1.431	2-oxoglutarate-acceptor oxidoreductase subunit OorB
2	---	acetyl-CoA carboxylase carboxyltransferase subunit al
0.668	1.332	malate:quinone oxidoreductase, putative
0.288	1.712	flagellar motor protein MotA
0.851	1.149	nucleoside diphosphate kinase
0.539	1.461	putative capsule polysaccharide export system peripl
1.311	1.277	transcription antitermination protein NusG
0.504	1.496	ATP-dependent protease La
2	---	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltr
0.962	---	putative sugar transferase
0.38	1.62	50S ribosomal protein L17
1.85	---	oxidoreductase, putative
2	---	50S ribosomal protein L18
0.1	1.9	lipoprotein
0.446	1.554	2-oxoglutarate-acceptor oxidoreductase subunit OorA
1.205	---	30S ribosomal protein S6
0.363	1.637	50S ribosomal protein L29
0.988	1.282	Chain A, Crystal Structure Of Phosphoglycerate Kinase
0.768	1.232	2-nitropropane dioxygenase family oxidoreductase
0.566	---	hypothetical protein CJE0453
1.836	---	ATP-sulfurylase family protein
0.532	1.468	competence protein ComEA
0.366	1.634	RpsO
1.02	---	hypothetical protein CJE0087
0.575	1.45	putative transcription termination factor
1.655	---	CTP synthetase
1.105	---	hypothetical protein CJE0806
0.85	---	peptidyl-prolyl cis-trans isomerase D,-like protein
1.178	1.648	50S ribosomal protein L15
0.835	1.607	preprotein translocase subunit SecA
0.654	1.346	50S ribosomal protein L9
0.45	1.55	hypothetical protein CJE0800
1.102	1.322	threonyl-tRNA ligase
1.312	1.142	superoxide dismutase
0.886	1.114	hypothetical protein CJE1668
1.066	---	GMP synthase
0.417	1.583	acetyl-CoA carboxylase, carboxyl transferase, beta sut
0.549	---	hypothetical protein C414_000290003
2	---	transcription elongation factor GreA
0.717	1.428	transketolase
0.484	1.516	NADH dehydrogenase subunit G
0.561	1.556	UTP-glucose-1-phosphate uridylyltransferase
2	---	30S ribosomal protein S17
1.292	---	molybdenum cofactor biosynthesis protein MogA
1.185	---	thiamine biosynthesis protein ThiF
0.705	1.295	aspartate aminotransferase
1.093	---	iron ABC transporter periplasmic iron-binding protein
2	---	isoleucyl-tRNA synthetase
0.467	1.533	twin-arginine translocation pathway signal
0.46	1.54	histidyl-tRNA synthetase
0.486	1.644	30S ribosomal protein S5
1.191	---	prolyl-tRNA synthetase
0.695	1.305	Chain A, Crystal Structure Of Adenylosuccinate Synthet
0.484	1.516	carbamoyl-phosphate synthase large chain
1.049	1.17	3-oxoacyl-ACP synthase II
0.611	1.389	hypothetical protein JJD26997_0724
0.618	1.382	flagellar assembly protein FliW
1.796	---	3-deoxy-8-phosphooctulonate synthase
2	---	cytochrome C family protein
0.496	---	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase
0.56	1.44	histidinol dehydrogenase
0.152	1.848	cytochrome C oxidase, cbb3-type, subunit III
0.726	1.274	elongation factor P
0.533	1.467	50S ribosomal protein L31
0.5	1.5	F0F1 ATP synthase subunit delta
0.093	1.907	50S ribosomal protein L2
1.839	---	short chain dehydrogenase/reductase family oxidored
0.504	1.496	bifunctional N-acetylglucosamine-1-phosphate uridylt
0.563	1.437	OmpR protein, partial
0.45	---	transaldolase
0.77	---	30S ribosomal protein S3
0.473	1.527	cation ABC transporter ATP-binding protein
0.552	1.448	flavodoxin
2	---	translocation protein TolB

Figure 6.4 235 proteinsof *C. jejuni* SVS 5141 identified by iTRAQ labelling proteomic analysisin the cold exposure experiment

Colours indicate the amountsof identified protein. Green is for a ratio of protein less than unity, red is for a ratio of protein greater than unity, the blank indicates protein cannot be identified at that time point.

6.2.2.4 Proteins of C. jejuni SVS 5141 significantly affected by cold stress

Compared with *C. jejuni* SVS 5001, alteration of protein expression in *C. jejuni* SVS 5141 in response to six days of cold storage was less conspicuous. The iTRAQ labelling proteomic analysis found 86 proteins were up-regulated, 54 proteins stopped synthesis and only one protein was down-regulated after six days of cold storage. The proteins of *C. jejuni* SVS 5141 affected by six days of cold exposure are shown in Table 6.2.

Thirty-nine of those 86 up-regulated proteins increased two times; 25 of them had a 3-fold increase; nine proteins had a four times increase; four proteins increased 5-fold; two proteins increased eight times, five proteins increased more than 10-fold but less than 20-fold, and only two proteins had more than a 20-times increase after six days of cold storage. The two proteins with more than 20-fold increase were 50S ribosomal protein L2 and one conserved hypothetical protein.

Table 6.2 Protein in *C. jejuni* SVS 5141 affected by six days of cold storage

Accession number	Protein name	Theoretical Mw (Da)	Cold adaptation (6 day)
Up-regulated proteins			
gi|218563153	methyl-accepting chemotaxis signal transduction protein	73191	+11.7
gi|121613238	methyl-accepting chemotaxis protein	72546	+11.9
gi|121613017	methyl-accepting chemotaxis protein	77220	+4.4
gi|419619379	putative MCP-type signal transduction protein	40704	+2.5
gi|419629625	DNA-directed RNA polymerase subunit beta'	169643	+2.3
gi|57238608	DNA-directed RNA polymerase subunit alpha	37734	+2.2
gi|384447762	DNA-directed RNA polymerase subunit beta	156229	+2.3
gi|205356548	translation initiation factor IF3	14527	+2.7
gi|419632940	polynucleotide phosphorylase/polyadenylase	79322	+3.2
gi|57236924	competence protein ComEA	8847	+2.8
gi|40217918	putative transcription termination factor	47265	+2.5
gi|419648839	histidyl-tRNA synthetase	47897	+3.4
gi|57237529	50S ribosomal protein L1	25031	+3.0
gi|57236893	30S ribosomal protein S10	11665	+2.2
gi|57238707	50S ribosomal protein L16	16365	+3.6
gi|57237544	30S ribosomal protein S7	17681	+5.4
gi|57238730	50S ribosomal protein L3	20824	+4.4
gi|57237528	50S ribosomal protein L11	15127	+2.2
gi|57238502	30S ribosomal protein S9	14128	+8.5
gi|57237722	30S ribosomal protein S1	62827	+2.1
gi|153952052	30S ribosomal protein S2	30449	+2.6
gi|57238712	50S ribosomal protein L23	10561	+2.1
gi|57238700	30S ribosomal protein S8	14795	+2.2
gi|57168772	ribosomal protein L21	11602	+4.3
gi|57238609	50S ribosomal protein L17	13240	+4.3
gi|57238706	50S ribosomal protein L29	7029	+4.5
gi|633730	RpsO	10206	+4.5
gi|57236974	50S ribosomal protein L9	16272	+2.1
gi|57238697	30S ribosomal protein S5	15787	+3.4
gi|57237163	50S ribosomal protein L31	7757	+2.8
gi|57238711	50S ribosomal protein L2	30515	+20.5
gi|218563111	formate dehydrogenase large subunit	104573	+5.9
gi|419641626	periplasmic nitrate reductase, small subunit	19251	+2.6
gi|86149326	formate dehydrogenase, iron-sulfur subunit	24717	+4.5
gi|57237413	cytochrome C551 peroxidase	37021	+2.3
gi|317509581	isocitrate dehydrogenase, NADP-dependent	86588	+2.1
gi|384447418	F0F1 ATP synthase subunit epsilon	13825	+3.6
gi|419622743	cytochrome c552	69795	+5.8
gi|148926276	malate oxidoreductase	44170	+2.1
gi|57238058	ubiquinol--cytochrome C reductase, iron-sulfur subunit	18332	+2.6
gi|283955815	pyruvate kinase	53954	+2.5
gi|86151404	trimethylamine-N-oxide reductase 2 precursor	93857	+3.7
gi|419648255	succinate dehydrogenase, flavoprotein subunit	67163	+3.9
gi|57238056	ubiquinol--cytochrome C reductase, cytochrome C1 subunit	41617	+4.1
gi|57238266	succinyl-CoA synthase, alpha subunit	30216	+2.6
gi|153951934	2-oxoglutarate-acceptor oxidoreductase subunit OorA	41211	+3.5

gi|419645546	NADH dehydrogenase subunit G	94588	+3.1
gi|57238510	cytochrome C oxidase, cbb3-type, subunit III	31370	+12.2
gi|57237111	F0F1 ATP synthase subunit delta	20497	+3.0
gi|57238563	flavodoxin	22239	+2.6
gi|86152514	50 kDa outer membrane protein	53832	+4.3
gi|57237913	lipoprotein	18559	+19.0
gi|153952664	bifunctional N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase	48372	+3.0
gi|881376	OmpR protein, partial	22640	+2.6
gi|57237388	flagellar motor protein MotA	28279	+5.9
gi|57237957	flagellar assembly protein FliW	14892	+2.2
gi|148926946	major antigenic peptide PEB2	24485	+2.0
gi|85036689	major outer membrane protein	45632	+8.8
gi|57238192	nonheme iron-containing ferritin	19531	+3.0
gi|4704599	fibronectin binding protein	32429	+3.4
gi|205355989	putative phospho-sugar mutase	48938	+2.6
gi|121613200	glucosamine--fructose-6-phosphate aminotransferase	67687	+3.0
gi|57238554	UTP-glucose-1-phosphate uridylyltransferase	30831	+2.8
gi|57237122	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	20547	+2.4
gi|419641014	ATP-dependent chaperone protein ClpB	95538	+3.7
gi|419694122	thioredoxin reductase	34000	+2.1
gi|57238437	enoyl-ACP reductase	29961	+2.2
gi|419641319	biotin carboxylase	49485	+3.3
gi|283955572	acetyl-CoA carboxylase, carboxyl transferase, beta subunit	31300	+3.8
gi|148926982	carbamoyl-phosphate synthase large chain	122342	+3.1
gi|283954138	aspartate kinase, monofunctional class	42771	+2.1
gi|57238611	histidinol dehydrogenase	46687	+2.6
gi|86149797	cjaC protein	27835	+2.8
gi|148925618	putative capsule polysaccharide export system periplasmic protein	58916	+2.7
gi|88596055	twin-arginine translocation pathway signal	72004	+3.3
gi|57237149	cation ABC transporter ATP-binding protein	33050	+3.2
gi|57237197	ATP-dependent Clp protease proteolytic subunit	21807	+3.4
gi|419627400	carboxyl-terminal protease	48990	+2.6
gi|283954636	ATP-dependent protease La	90364	+3.0
gi|86149225	3-methyl-2-oxobutanoate hydroxymethyltransferase	30354	+2.3
gi|86150500	conserved hypothetical protein	20593	+2.3
gi|57237048	hypothetical protein CJE0033	26440	+3.6
gi|86150082	conserved hypothetical protein	43511	+23.3
gi|86150649	conserved hypothetical protein	16323	+16.7
gi|57237003	hypothetical protein CJE0800	25980	+3.4
gi|153951812	hypothetical protein JJD26997_0724	41387	+2.3
Down-regulated and stopped-synthesis proteins			
gi|419638103	putative MCP-type signal transduction protein	48561	None
gi|419589392	putative MCP-type signal transduction protein	48552	None
gi|57238000	chemotaxis protein CheY	14428	None
gi|57238069	aspartyl/glutamyl-tRNA amidotransferase subunit B	53256	None
gi|475890	fur	18175	None
gi|57236953	aspartyl-tRNA synthetase	66676	None
gi|57237342	transcription elongation factor GreA	18056	None
gi|317511751	isoleucyl-tRNA synthetase	106086	None
gi|57238257	prolyl-tRNA synthetase	65053	None
gi|57238704	50S ribosomal protein L14	13354	None
gi|57237954	30S ribosomal protein S18	10322	None
gi|57238606	30S ribosomal protein S11	13942	None

gi|153951193	50S ribosomal protein L10	17775	None
gi|57238698	50S ribosomal protein L18	13288	None
gi|57237952	30S ribosomal protein S6	14689	None
gi|57238705	30S ribosomal protein S17	9600	None
gi|153952472	30S ribosomal protein S3	26067	None
gi|57238028	cytochrome C553	11002	-2.33
gi|57238690	citrate synthase	48109	None
gi|57238512	cbb3-type cytochrome C oxidase subunit II	25078	None
gi|57237051	cytochrome C family protein	39104	None
gi|57237336	transaldolase	37122	None
gi|455428	flagellin protein	59652	None
gi|56806980	flagellin A	11704	None
gi|283955143	3-deoxy-8-phosphooctulonate synthase	29826	None
gi|86150038	dTDP-4-dehydrorhamnose 3,5-epimerase	21245	None
gi|57237119	translocation protein TolB	44711	None
gi|86150084	methyltransferase, FkbM family protein	33582	None
gi|86150017	putative sugar transferase	89945	None
gi|86149461	ATP-sulfurylase family protein	44829	None
gi|419622287	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	37683	None
gi|57236997	peptidyl-prolyl cis-trans isomerase D,-like protein	57475	None
gi|57168680	thioredoxin	11501	None
gi|86148986	acyl carrier protein	8592	None
gi|57237500	acetyl-CoA carboxylase carboxyltransferase subunit alpha	34473	None
gi|57237746	ribose-phosphate pyrophosphokinase	33848	None
gi|57237923	acetyl-CoA carboxylase subunit A	54834	None
gi|57236939	CTP synthetase	60765	None
gi|86150584	GMP synthase	57397	None
gi|13509099	aspartate-semialdehyde dehydrogenase	21405	None
gi|57237076	3-dehydroquinate dehydratase	17755	None
gi|419633975	branched-chain amino acid aminotransferase	34036	None
gi|57238618	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	42635	None
gi|57238619	ATP/GTP-binding protein	40355	None
gi|57237180	iron ABC transporter periplasmic iron-binding protein	37406	None
gi|57237820	delta-aminolevulinic acid dehydratase	36733	None
gi|153951811	molybdenum cofactor biosynthesis protein MogA	20371	None
gi|86150511	thiamine biosynthesis protein ThiF	30047	None
gi|317511289	highly acidic protein, partial	45096	None
gi|121612363	oxidoreductase, putative	64074	None
gi|57237459	hypothetical protein CJE0453	30726	None
gi|57237099	hypothetical protein CJE0087	49254	None
gi|57237008	hypothetical protein CJE0806	27951	None
gi|283954942	hypothetical protein C414_000290003	44381	None
gi|419641488	short chain dehydrogenase/reductase family oxidoreductase	28161	None

6.2.3 Functional classification of significantly changed proteins in *C. jejuni* SVS 5001 and SVS 5141 under cold stress

All proteins with significant expression changed (more than 2-fold) in *C. jejuni* SVS 5001 and 5141 during cold exposure have been grouped by functional classification according to the Sanger Centre *C. jejuni* functional database.

6.2.3.1 Functional grouping of significantly changed protein in C. jejuni SVS 5001

As shown in Table 6.3, the 6 h cold shock caused 85 proteins' expression increased in *C. jejuni* SVS 5001. Those 85 up-regulated proteins were grouped into 14 functional categories. The largest group of up-regulated proteins contained 21 proteins and was responsible for energy metabolism. The second largest up-regulated protein group was ribosomal proteins and contained 17 up-regulated proteins. Eight up-regulated proteins belonged to the cell envelope protein category, which was the third largest up-regulated protein group in six-hour cold-shocked *C. jejuni* SVS 5001 cells. Twenty-four proteins reduced their biosynthesis significantly and 21 proteins stopped their biosynthesis after six hours of cold shock. The 24 down-regulated proteins corresponded to 13 different functional categories. The three largest functional categories in the down-regulated proteins were energy metabolism, ribosomal proteins and genetic information processing. The 21 stopped-synthesis proteins were grouped into nine different functional categories. Ribosomal protein, cell envelope and chemotaxis were the three largest functional categories of stopped-synthesis proteins in six hours of cold- shocked *C. jejuni* SVS 5001 cells.

Six days of 4^{o}C cold storage had significantly altered protein expression in *C. jejuni* SVS 5001 cells. After six days of cold storage, 138 proteins were up-regulated; 11 proteins were down-regulated; 28 proteins stopped biosynthesis, meantime nine proteins that could not be found in cells without cold exposure were induced. Functional classification of the significantly changed proteins in six days of cold exposure *C. jejuni* SVS 5001 cells is shown in Table 6.3. These 138 up-regulated proteins were grouped into 18 different functional categories. The three largest functional categories of up-regulated protein were energy metabolism, ribosomal

proteins and genetic information processing, which contained 30, 18 and 15 up-regulated proteins, respectively. The 11 down-regulated proteins were grouped into seven different functional categories and the largest functional category of down-regulated protein was energy metabolism, which contained three down-regulated proteins. The 28 stopped biosynthesis proteins in six days of cold storage *C. jejuni* SVS 5001 cells were grouped into 12 functional categories. The three largest functional categories of those stopped-biosynthesis proteins were ribosomal proteins, cell envelope and cofactor and vitamins synthesis, which contained six, five and three stopped-synthesis proteins, respectively. The nine induced proteins only found in six days of cold storage *C. jejuni* SVS 5001 cells corresponded to five different functional categories; and the largest functional category of the cold-induced proteins was chemotaxis and mobility, which contained three cold-induced proteins.

Table 6.3 Functional grouping of significantly changed proteins in *C. jejuni* SVS 5001 in response to cold stress

Functional classification	Cold shock cells (6h)				Cold adapted cells (6 day)			
	Upregulated proteins	Down regulated proteins	Protein that stop synthesis	Cold induced protein	Up-regulated proteins	Down regulated proteins	Protein that stop synthesis	Cold induced protein
Chemotaxis and mobility	3	1	3	1	6		2	3
Genetic information processing	7	3			15		2	
Ribosomal protein	17	3	4		18	1	6	
Energy metabolism	21	4	2		30	3	1	
Cell envelope	8	2	3	1	8	2	5	
Adaptation and atypical condition	1	1			1			
Pathogenicity				1				
General intermediary metabolism	3	2	3		7	1	2	
Molecular chaperone	2		1		5		1	
Oxidative stress defence					3			
Fatty acid biosynthesis	3	2	1		5	1	1	
Purine, pyrimidine, nucleoside and nucleotide	1	1		1	3		1	2
Amino acid metabolism	4	2	2		7	2	1	
Signal transduction					1			
Transport/binding	7			1	9			1
Cofactors and vitamins synthesis		1		1	2		3	1
Degradation of macromolecule	2	1			4	1		
Degradation of small molecular					2			
Unknown function	6	1	2	4	12		3	2
Total number	85	24	21	10	138	11	28	9

6.2.3.2 Functional grouping of significantly changed proteins in C. jejuni SVS 5141

Compared with *C. jejuni* SVS 5001, the six days at 4^{o}C cold storage had less impact on protein expression in *C. jejuni* SVS 5141. The six days of cold storage caused SVS 5141 increased expression of 86 proteins, decreased one protein's expression and stopped biosynthesis of 54 proteins. Functional classification of the significant changed proteins in six days of cold exposure *C. jejuni* SVS 5141 is shown in Table 6.4. The 86 up-regulated proteins corresponded to 17 different functional categories. The four largest functional categories of those up-regulated proteins were energy metabolism, ribosomal proteins, cell envelope and genetic information processing, which contained 19, 19, 8 and 8 up-regulated proteins, respectively. Those 54 stopped synthesis proteins in six days of cold storage *C. jejuni* SVS 5141 cells were grouped into 15 functional categories. The three largest functional categories of stopped synthesis proteins were ribosomal proteins, genetic information processing proteins and cell envelope proteins. They contained 8, 6 and 5 stopped-synthesis proteins respectively. The only down-regulated protein found in six days of cold storage *C. jejuni* SVS 5141 belonged to the energy metabolism category.

Table 6.4 Functional grouping significantly changed proteins in *C. jejuni* SVS 5141 in response to cold temperatures

Functional classification	Cold adapted cells (6 day)		
	Up-regulated proteins	Down regulated proteins	Proteins that stopped synthesis
Chemotaxis and mobility	4		3
Genetic information processing	8		6
Ribosomal protein	19		8
Energy metabolism	19	1	4
Cell envelope	8		5
Adaptation and atypical condition	1		
Pathogenicity	1		
General intermediary metabolism	3		4
Molecular chaperone	2		1
Oxidative stress defence	1		1
Fatty acid biosynthesis	3		2
Purine, pyrimidine, nucleoside and nucleotide	1		4
Amino acid metabolism	2		4
Signal transduction			1
Transport/binding	4		1
Cofactors and vitamins synthesis	1		3
Degradation of macromolecules	3		
Unknown function	6		7
Total number	86	1	54

6.3 Discussion

6.3.1 1D protein banding pattern changes in *C. jejuni* SVS 5001 and SVS 5141 under cold stress

The 1D electrophoresis results showed *C. jejuni* SVS 5001 cells with 24 hours, 48 hours, six days and eight days of 4^{o}C cold exposure have similar 1D protein profiles; moreoever, those similar four 1D protein profiles of cold adapted *C. jejuni* SVS 5001 cells appeared identical to the 1D protein profiles of SVS 5001 cells without cold exposure. In contrast to 1D electrophoresis, the iTRAQ labelling proteomic analysis results showed six days of cold exposure caused dramatic alterations in protein expression in *C. jejuni* SVS 5001 (see table 6.3). iTRAQ proteomic analysis identified that 138 proteins increased their expression and 11 proteins decreased their expression in the six days of cold storage SVS 5001 cells. The differences between the results of 1D electrophoresis analysis and iTRAQ labelling proteomic analysis in this cold stress response study suggested the 1D protein electrophoresis technique was not sensitive enough to use alone to examine the alternations in cellular protein expression. 1D protein electrophoresis could only confirm alterations of protein expression, but unable to conclude there was no protein expression changes between the two identical 1D protein profiles.

The 1D protein electrophoresis analysis results suggested that six hours of cold shock caused more significant alternations of protein expression in *C. jejuni* SVS 5141 than 24 hours, 48 hours, six days and eight days of cold storage. Due to a sample shortage, the six-hour cold shock *C. jejuni* SVS 5141 cells' proteomic analysis did not procede. Because of the lack of proteomic analysis data about six-hour cold shock *C. jejuni* SVS 5141, conclusions about 6 hours of cold shock cause more significant protein expression alterations in *C. jejuni* SVS 5141 than six days of cold storage does cannot be made.

6.3.2 Comparative proteomic analysis of *C. jejuni* cold shock responses

In the present study, only two cold shock protein extraction samples were processed by iTRAQ labelling comparative proteomic analysis. They were the protein extraction samples from six hour cold-shocked *C. jejuni* SVS 5001 and *C. jejuni* NCTC 11168. To reveal the difference of strain-dependent cold shock response mechanisms, the cold shock proteomic profiles of the two strains have been compared.

6.3.2.1 Up-regulated proteins in two cold shock C. jejuni strains

As shown in Table 6.5, *C. jejuni* SVS 5001 increased expression of 85 proteins in response to cold shock. The three largest functional groups of up-regulated protein in *C. jejuni* SVS 5001 were the same as in *C. jejuni* NCTC 11168, but in a different order. In cold shocked *C. jejuni* SVS 5001 cells, the largest functional group was energy metabolism, which contained 21 up-regulated proteins; the second largest functional group was ribosomal proteins, which had 17 proteins; the third largest functional group was cell envelope proteins, which containedeight up-regulated proteins. In cold-shocked *C. jejuni* NCTC 11168, the three largest functional groups were ribosomal proteins (with 28 up-regulated proteins), energy metabolism proteins (with 8 up-regulated proteins) and cell envelope proteins (with 5 up-regulated proteins).

Table 6.5 Difference in protein expression of *C. jejuni* NCTC 11168 and SVS 5001 in response to cold shock

Functional classification	NCTC 11168				SVS 5001			
	Up-regulated proteins	Down regulated proteins	Protein that stop synthesis	Cold induced protein	Up-regulated proteins	Down regulated proteins	Protein that stop synthesis	Cold induced protein
Chemotaxis and mobility	5				3	1	3	1
Genetic information processing	1	2	4	1	7	3		
Ribosomal protein	28				17	3	4	
Energy metabolism	8	2	2		21	4	2	
Cell envelope	5				8	2	3	1
Adaptation and atypical condition	2				1	1		
Pathogenicity	1							1
General intermediary metabolism	1	3	1		3	2	3	
Molecular chaperone	1	1			2		1	
Oxidative stress defence		4	1					
Fatty acid biosynthesis		1	2		3	2	1	
Purine, pyrimidine, nucleoside and nucleotide		1	3		1	1		1
Amino acid metabolism			2		4	2	2	
Signal transduction								
Transport/binding		1	1		7			1
Cofactors and vitamins synthesis			2			1		1
Degradation of macromolecule					2	1		
Unknown function	4	6	2		6	1	2	4
Total number	56	24	22		85	24	21	10

Both *C. jejuni* NCTC 11168 and SVS 5001 increased expression of proteins involved in energy metabolism suggesting that more energy was needed by *C. jejuni* in response to cold shock. This was in agreement with the study of Stintzi and Whitworth (2003) and Moen el al. (2005). Comparing up-regulated energy metabolism proteins in cold-shocked *C. jejuni* SVS 5001 and *C. jejuni* NCTC 11168, five energy metabolism proteins were found being up-regulated in both strains. These five proteins were FdhA (formate dehydrogenase large subunit), ZP_14173510 (small subunit of periplasmic nitrate reductase), cytochrome c552, trimethylamine-N-oxide reductase 2 precursor and PetC (ubiquinol--cytochrome C reductase). Apart from PetC, four of them were involved in anaerobic respiration. This suggested that both *C. jejuni* SVS 5001 and *C. jejuni* NCTC 11168 activated their anaerobic respiration to produce energy in cold shock conditions. In addition to increasing four anaerobic respiration-related proteins, *C. jejuni* SVS 5001 increased expression of eight proteins involved in the TCA cycle in response to cold shock. The TCA cycle was an important aerobic respiration pathway. Increasing expression of TCA cycle related protein and anaerobic respiration-related proteins in cold-shocked *C. jejuni* SVS 5001 suggested this strain increased both anaerobic and aerobic respiration to fulfil higher energy demand in cold shock conditions. Our study found that six hours of cold exposure did not induce any protein related to the aerobic respiration pathway in *C. jejuni* NCTC 11168, but caused increased expression of four anaerobic pathway related proteins. This suggested that *C. jejuni* NCTC 11168 used different mechanisms from *C. jejuni* SVS 5001 to product energy to respond six hours of cold shock. Instead of activating both anaerobic and aerobic pathways, *C. jejuni* NCTC 11168 relied more on its anaerobic pathway to provide energy in six hours of cold shock conditions.

C. jejuni SVS 5001 cell significantly increased its ribosomal protein expression in response to six hours of cold shock. Overall, 17 ribosomal proteins were up-regulated. Twelve were ribosomal proteins from the 50S ribosomal subunit (50S ribosomal protein L1, L3, L9, L11, L13, L16, L17, L21, L23, L24, L29 and L31) and five were up-regulated ribosomal proteins from the 30S ribosomal subunit (30S ribosomal protein S7, S8, S9, S11 and S15). In cold shock conditions, *C. jejuni* NCTC 11168 cell increased its ribosomal proteins' production. All 28 ribosomal proteins

were found to increase their expressions in cold shocked *C. jejuni* NCTC 11168 cells. Seventeen of them were from the 50S ribosomal subunit (50S ribosomal protein L1, L2, L3, L5, L10, L11, L13, L14, L15, L16, L17, L18, L21, L22, L24, L25 and L29) and the other 11 up-regulated ribosomal proteins were from the 30S ribosomal subunit (30S ribosomal protein S3, S4, S5, S7, S8, S9, S10, S11, S15, S17 and S18). Fourteen ribosomal proteins increased their expression in both strains in response to cold shock. Cold inducing ribosomal proteins had also been found in *Bacillus subtilis*, in which three ribosomal proteins, L7, L12 and S6, were induced by cold shock (Graumann et al., 1996). Ribosomal proteins played critical roles in protein biosynthesis. A previous study also had found ribosomes were able to act as sensors of cold shock in *E. coli* (VanBogelen and Neidhardt, 1990). The up-regulation of larger numbers of ribosomal proteins in cold shock conditions suggested that *C. jejuni* might enhance the protein translation process in response to cold shock and ribosomes might play an important role rather than in protein translation in response to cold stress, such as acting as sensors of cold shock.

Cell envelope proteins were the third largest up-regulated protein category in cold-shocked *C. jejuni* SVS 5001. A total of eight cell envelope proteins significantly increased their expression in response to cold shock. They were PorA (major outer membrane protein), Omp50 (50kDa outer membrane protein), OmpR (major outer membrane protein synthesis regulator), lipoprotein, Peb2 (a major antigen), GlmU (an enzyme catalyse cell wall synthesis), FliW (a flagellar assembly protein) and FlaB (flagellin B protein). The last two proteins are involved in the assembly of flagellae, but they are categorized into cell envelope proteins according to the Sanger Center *C. jejuni* functional database. *C. jejuni* NCTC 11168 increased five cell envelope proteins' expression to response cold shock. Three of the five envelope proteins had increased in both strains in response to cold shock. They were PorA (major outer membrane protein), Omp50 (50kDa outer membrane protein) and lipoprotein. Expression of PorA and Omp50 in *C. jejuni* had also been found to increase at a 42^{o}C growing temperature, compared with 36^{o}C and 31^{o}C (Dedieu et al., 2002; Dedieu et al., 2008). Moreover, Xia et al. (2013) found PorA and Omp50 can be induced by antimicrobials and believed the increased expression of those two cell envelope proteins was an

adaptive response to increase surface polysaccharides. The cell envelope protein was important for cell wall and plasma membrane structural integrity. Increasing expression of those envelope proteins in response cold shock in *C. jejuni* suggested these proteins played important roles in retaining cell integrity in cold shock conditions. This was especially so for lipoproteins, which maintained the cell membrane a biologically functional fluid phase in response to low temperature, increased 135.5-fold in cold-shocked *C. jejuni* SVS 5001 cells.

6.3.2.2 Down-regulated and stopped-synthesis proteins in cold-shocked C. jejuni

The number of down-regulated protein and stopped-synthesis proteins in cold-shocked *C. jejuni* SVS 5001 cells were similar with the number of correlated proteins in cold-shocked *C. jejuni* NCTC 11168 cell. *C. jejuni* SVS 5001 significantly reduced 24 proteins and stopped synthesizing 21 proteins in response to cold shock. *C. jejuni* NCTC 11168 reduced 24 proteins' expression and stopped synthesizing 22 proteins.

The functional categories of reduced protein and stopped-synthesis proteins in those two cold shock strains were quite different. The largest functional group of down regulated protein in cold shock *C. jejuni* NCTC 11168 cells was oxidative stress defence, which matched our finding in which *C. jejuni* NCTC 11168 did not increase any protein involved in aerobic respiration pathway in response to six hours of cold shock. In cold-shocked *C. jejuni* SVS 5001 cells, the largest functional group of down regulated protein was energy metabolism. There were no down-regulated oxidative stress defence proteins in the cold-shocked *C. jejuni* SVS 5001 cells. The largest functional group of stopped synthesis protein in cold shocked *C. jejuni* NCTC 11168 cells was genetic information processing, which included three amino acid tRNA proteins (GatB, IleS and ProS) and one transcription elongation factor (GreA). *C. jejuni* SVS 5001 did not stop synthesizing any genetic information processing proteins under cold shock conditions. The largest functional group of stopped-synthesis proteins in cold-shocked *C. jejuni* SVS 5001 cells was ribosomal protein, which included 50S ribosomal proteins, L14, L18 and 30S, and ribosomal proteins, S3, S17. These four ribosomal proteins were all up-regulated in cold-shocked *C. jejuni* NCTC

11168 cells. The huge differences in down-regulated proteins and stopped-synthesis protein between these two strains in response to cold shock suggested that the two strains used very different strategies to cope with cold shock.

6.3.2.3 Cold shock induced protein that cannot be found in C. jejuni without cold stress

Ten cold-induced proteins in cold-shocked *C. jejuni* SVS 5001 cells were not present in cells without cold exposure. In *C. jejuni* NCTC 11168, there was only one cold-induced protein that was not present in cells without cold exposure. The 10 cold-induced *C. jejuni* SVS 5001 proteins that were not present in the 42^{o}C growing cells belonged to seven different functional categories. Four of those 10 proteins have unknown function, the rest of the six proteins belonged to six different functional categories: chemotaxis, cell envelope, pathogenicity, transport/binding, cofactor and vitamin synthesis and purine, pyrimidine nucleoside and nucleotide. The cold-induced protein that was only present in cold-shocked *C. jejuni* NCTC 11168 celss was GatA (subunit A of aspartyl/glutamyl-tRNA amidotransferase), which regulated translational fidelity.

The cold-induced protein that was not present in 42^{o}C was not needed by *C. jejuni* under normal growing temperatures, but was essential for *C. jejuni* to survive at cold temperatures. The difference between the two strains in expression of cold-induced protein reminded us that these two strains used very different strategies in response to cold shock.

6.3.2.4 Conclusions

The proteomic comparative study found both *C. jejuni* NCTC 11168 and SVS 5001 increased expression of large numbers of ribosomal and cell envelope proteins in response to cold shock. Cold shock inducing ribosomal proteins have also been seen in *E. coli* (Joens et al., 1992) and *B. subtilis* (Graumann and Marahiel, 1999). However, the numbers of cold-induced ribosomal protein in *E. coli* and *B. subtilis* were very small, compared with *C. jejuni*. A temperature downshift only induced ribosomal proteins S1, S6, L7/L12 in *E. coli* and S6, L7/L12 in *B. subtilis* (Joens et al., 1992; Graumann et al., 1996). These four ribosomal proteins did not change their

expression in *C. jejuni* at our cold exposure study. A large number of ribosomal proteins (28 in *C. jejuni* NCTC 11168, 7 in *C. jejuni* SVS 5001) increased their expression in *C. jejuni* to response cold shock suggesting that ribosomal proteins played an important role rather than protein translation in cold shock response of *C. jejuni*. Cell envelope proteins played important roles in remaining cell structural integrity and diffusion of small molecular. Both two strains increased the number of cell envelope proteins in response to cold shock, such as PorA, Omp50 and Lipoprotein, suggesting that the two strains might employ similar cold response mechanisms to regulate their cell envelopes.

The two strains of *C. jejuni* have very different protein profiles in their cold-induced energy metabolism protein. *C. jejuni* SVS 5001 increased expression of 21 energy metabolism proteins in response to cold shock. *C. jejuni* NCTC 11168 increased fewer energy metabolism proteins in cold shock, which were eight. In addition to variation in the numbers of proteins increased, the respiration pathway of the increased energy metabolism proteins was different between the two strains. *C. jejuni* SVS 5001 increased both anaerobic and aerobic respiration pathway proteins to enhance the cell's energy generation in cold shock conditions. *C. jejuni* NCTC 11168 only increased expression of the proteins that were involved in anaerobic respiration. Other evidence regarding *C. jejuni* NCTC 11168 relying on anaerobic respiration rather than aerobic respiration to provide energy at cold shock was the reduction of four oxidative stress proteins' expression in cold-shocked *C. jejuni* NCTC 11168 cells. The two strains managed their protein expression in two different modus to fulfil energy requirements in cold-shocked condition suggesting that the mechanism of energy metabolism employed by *C. jejuni* might contribute to the strain-specific differences in cold tolerance of this pathogen.

6.3.3 Comparative analysis of two closely related *C. jejuni* strains' cold adaption

C. jejuni SVS 5001 and *C. jejuni* SVS 5141 were isolated from the same waterborne outbreak. Although the two isolates had different origins, the phenotypic characteristics of them were similar. Comparative analysis of the two strains' cold-

adapted protein profile revealed the difference in the two isolates' cold adaptation mechanisms.

As shown in the Table 6.6, to adapt to cold temperature, the clinically isolated *C. jejuni* SVS 5001 altered its protein expression more substantially than the environmentally isolated *C. jejuni* SVS 5141, probably due to its readaptation to a warm-blooded environment. After six days of cold storage, *C. jejuni* SVS 5001 increased 138 proteins' expression, decreased 11 proteins' expression, stopped synthesis of 28 proteins and started to synthesize nine proteins that could not be found in the cells growing at 42^{o}C. The alteration of protein expression in six days of cold exposure *C. jejuni* SVS 5141 was less notable. In cold adapted *C. jejuni* SVS 5141 cells, 86 proteins were up-regulated, only one protein was down-regulated and the biosynthesis of 54 proteins was stopped. Six days of cold storage did not induce any protein that could not be found in *C. jejuni* SVS 5141 growing at 42^{o}C.

Table 6.6 Difference in protein expression of *C. jejuni* SVS 5001 and SVS 5141 in response to 6 days cold exposure

Functional classification	SVS 5001				SVS 5141			
	Up-regulated proteins	Down regulated proteins	Protein that stop synthesis	Cold induced protein	Up-regulated proteins	Down regulated proteins	Protein that stop synthesis	Cold induced protein
Chemotaxis and mobility	6		2	3	4		3	
Genetic information processing	15		2		8		6	
Ribosomal protein	18	1	6		19		8	
Energy metabolism	30	3	1		19	1	4	
Cell envelope	8	2	5		8		5	
Adaptation and atypical condition	1				1			
Pathogenicity					1			
General intermediary metabolism	7	1	2		3		4	
Molecular chaperone	5		1		2		1	
Oxidative stress defence	3				1		1	
Fatty acid biosynthesis	5	1	1		3		2	
Purine, pyrimidine, nucleoside and nucleotide	3		1	2	1		4	
Amino acid metabolism	7	2	1		2		4	
Signal transduction	1						1	
Transport/binding	9			1	4		1	
Cofactors and vitamins synthesis	2		3	1	1		3	
Degradation of macromolecule	6	1			3			
Unknown function	12		3	2	6		7	
Total number	138	11	28	9	86		54	

6.3.3.1 Up-regulated proteins in two cold adapted C. jejuni strains

The three largest functional groups of up-regulated protein in cold adapted *C. jejuni* SVS 5001 were same as in *C. jejuni* SVS 5141, and in the same order. In cold adapted *C. jejuni* SVS 5001, the largest functional group of up-regulated protein was energy metabolism protein, which had 30 up-regulated proteins; the second largest functional group was ribosomal protein, which had 18 proteins; the third largest functional group was genetic information processing protein, which had 15 up-regulated proteins. In cold-adapted *C. jejuni* SVS 5141, the three largest functional groups were energy metabolism proteins (with 19 up-regulated proteins), ribosomal proteins (with 19 up-regulated proteins) and genetic information processing proteins (with eight up-regulated proteins).

Comparing the study of up-regulated energy metabolism proteins in cold-*adapted C. jejuni* SVS 5001 and *C. jejuni* SVS 5141 found 18 energy metabolism proteins were up-regulated in both strains. Six proteins responsible for electron transport and five proteins involved in anaerobic respiration increased their expression in both strains after six days cold of exposure. The five anaerobic respiration proteins were FdhA ((formate dehydrogenase large subunit), FdhB (formate dehydrogenase, iron-sulfur subunit), small unit of periplasmic nitrate reductase, cytochrome c552 and trimethylamine-N-oxide reductase 2 precursor. Four proteins that participated in the TCA cycle increased in both cold adapted strains. They were SucD (succinyl-CoA synthase, alpha subunit), OorA (2-oxoglutarate-acceptor oxidoreductase subunit), NADP-dependent isocitrate dehydrogenase and malate oxidoreductase. The increased expression of proteins involved in electron transport and the TCA cycle to respond to cold stress found in this study matched the previous *C. jejuni* cold shock transcriptomic study, in which the transcript abundance of genes encoding enzymes involved in the TCA cycle all increased in response to cold stress (Stintzi and Whitworth, 2003). Large numbers of energy metabolism proteins increased in both strains to adapt to cold temperatures suggesting the two closely related *C. jejuni* strains employed similar mechanisms to fulfil their energy needs for cold adaption.

Both strains increased large numbers of ribosomal proteins to adapt cold temperatures. *C. jejuni* SVS 5001 increased expression of 18 ribosomal proteins. *C. jejuni* SVS 5141 increased expression of 19 ribosomal proteins. Sixteen ribosomal proteins increased in both cold adapted strains. They were ten 50s ribosomal proteins (L1, L3, L9, L11, L16, L17, L21, L23, L29 and L31) and six 30S ribosomal proteins (S1, S2, S7, S8, S9 and S15). The identical up-regulated ribosomal protein profiles in the two cold adapted strains emphasized the similarity of the cold adaption mechanisms used by the two strains.

The third largest functional group of up-regulated protein in both cold-adapted strains was genetic information processing. *C. jejuni* SVS 5001 increased expression of 15 proteins involved in genetic information processing. *C. jejuni* SVS 5141 increased expression of eight proteins that participated in genetic information processing. Seven genetic information processing proteins increased in both strains to adapt to cold temperatures. They were DNA-directed RNA polymerase subunit RpoA and RpoB (catalyse the transcription of DNA), InfC (translation initiation factor), Pnp (responsible for mRNA processing and degradation), ComEA (repair DNA and remove exogenous DNA), HisS (histidyl-tRNA synthetise) and a putative transcription termination factor.

6.3.3.2 Down-regulated and stopped-synthesis proteins in cold adapted C. jejuni

The present proteomic analysis found *C. jejuni* SVS 5001 reduced 11 proteins' expression and stopped biosynthesis of 28 proteins to adapt to cold temperatures. *C. jejuni* SVS 5141 reduced only one protein's expression, but stopped biosynthesis of 54 proteins to adapt to six days of 4^{o}C cold exposure. For easier comparative analysis the alteration of the proteome in the two cold-adapted *C. jejuni* strains, the down-regulated proteins and the proteins that stopped synthesis in response to cold adaption were all regarded as cold-repressed proteins.

The three largest functional groups of cold-repressed protein in cold adapted *C.jejuni* SVS 5001 were ribosomal protein (seven cold-repressed proteins), cell envelope protein (seven cold-repressed proteins) and energy metabolism protein (four cold-

repressed proteins). In cold adapted *C. jejuni* SVS 5141, the four largest functional groups of cold-repressed protein were ribosomal protein (eight cold-repressed proteins), genetic information processing protein (six cold-repressed proteins), cell envelope proteins (five cold-repressed proteins) and energy metabolism proteins (five cold-repressed proteins).

Six ribosomal proteins had been found repressed by cold in both *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141. They were two 50s ribosomal proteins (L14 and L18) and four 30S ribosomal proteins (S3, S11, S17 and S18). Ribosomal proteins play a critical role in process of translating mRNA into protein. Reducing expression or stopped biosynthesis of certain numbers of ribosomal proteins corresponded to large numbers of proteins being repressed in those two cold-adapted *C. jejuni* strains. Meanwhile, the biosynthesis of six genetic information processing proteins that stopped in cold-adapted *C. jejuni* SVS 5141 was in agreement with stopped biosynthesis of 54 proteins.

Cell envelope related proteins were the second and third largest groups of cold-repressed proteins in cold adapted *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141, respectively. Four cell envelope related proteins had been found repressed by cold in both strains. They were Fla (flagellin protein), FlaA (flagellin A), TolB (translocation protein, responsible for cell envelope integrity) and KdsA (3-deoxy-8-phosphooctulonate synthase, responsible for outer membrane biogenesis). Although being involved in cell motility, Fla and FlaA are considered as cell envelope proteins, since they are both structural constituents of cell membranes. While the two strains decreased expression of the above cell envelope proteins, eight cell envelope proteins had increased their expression in both of the two cold adapted *C. jejuni* strains. This suggested *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141 processed the reconstruction of cell membranes to adapt to cold storage.

Energy metabolism proteins were the third and fourth largest functional groups of cold-repressed protein in cold adapted *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141, respectively. *C. jejuni* SVS 5001 reduced expression of three energy metabolism proteins (FrdB, Eno and GlA) and stopped synthesis of one energy metabolism

protein (CcoO) to adapt to six days of cold storage. In six days of cold storage *C. jejuni* SVS 5001, biosynthesis of four energy metabolism proteins (GltA, CcoO, Tal and one cytochrome C family protein) stop, and one energy metabolism protein's (Cyf) expression had decreased. Two energy metabolism proteins, GltA and CcoO, had been repressed by six days of cold storage in both strains. GltA (citrate synthase) is a pace-making enzyme in the first step of the TCA cycle. *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141 both have high concentrations of GltA when they were grown at 42^{o}C. The expression of GltA decreased dramatically, to -46.7 times, in *C. jejuni* SVS 5001 after six days of cold storage. In *C. jejuni* SVS 5141, six days of cold storage completely suspended biosynthesis of GltA. A decrease of this important TCA cycle pace-making enzyme in cold adapted *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141 was different from the increased expression of this protein in cold-shocked *C. jejuni* NCTC 11168. This suggested that *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141 used similar metabolism mechanisms to adapt to cold, which were different from *C. jejuni* NCTC 11168.

6.3.3.3 Conclusions

C. jejuni SVS 5001 and *C. jejuni* SVS 5141 were isolated from the same waterborne outbreak. The difference between the two strains was the former had infected humans from consuming the water from which SVS 5141 had been recovered. The previous study found clinically-isolated *C. jejuni* strains tended to be significantly more likely to remain viable following cold exposure than poultry-derived strains (Chan et al., 2001). It was believed that cold tolerant strains have better survival ability in the environment，therefore, remaining viable after environmental exposure to cause human infections. The present proteomics analysis of two closely related strains compared the alterations of protein quantity during cold adaption to examine how a human infection affected cold adaption mechanism of this pathogen.

The viability assessment results showed *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141 had similar survival curves at eight days of cold incubation (Figure 4.2), and the one-way ANOVA statistical test determined there were no statistically significant differences between the average death cell rates of the two strains. Comparative proteomics

analysis found similar of protein expression in the two strains during cold adaptation. To adapt to cold temperatures, both *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141 increased a large number of proteins. *C. jejuni* SVS 5001 increased a total of 135 proteins' expression during six days of cold adaption. The number of increased proteins in cold-adapted *C. jejuni* SVS 5141 was 86. In comparison with the 19 up-regulated proteins in six days of cold adapted *C. jejuni* NCTC 11168, the two strains were still in a state of vigorous increased protein expression after six days of cold exposure.

The up-regulated proteins in *C. jejuni* SVS 5141 appeared to be a subset of up-regulated proteins in *C. jejuni* SVS 5001. For example, 30 and 19 energy metabolism proteins increased expression in cold-adapted *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141, respectively; 18 were found to have increased expression in both strains. A similar situation was found in the genetic information processing proteins. Fifteen and eight genetic information processing proteins increased their expression in cold adapted *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141, respectively; seven were found to have increased expression in both strains. The similarity of up-regulation in both *C. jejuni* SVS 5001 and *C. jejuni* SVS 5141 suggested that these two closely related *C. jejuni* strains employed similar cold adaption mechanisms.

The differences between the two strains' protein expression during cold adaptation were also quite obvious. To adapt to cold temperatures, the clinically-isolated strain, *C. jejuni* SVS 5001, increased more proteins' expression than its closely related water-originating strain did. This suggested that adaptation in the clinically-isolated, SVS 5001, was considerably more major after six days of cold exposure compared with the strain isolated from an already cool environment-water.

Protein expression was an energy cost process. To fulfil energy requirements for increasing 135 proteins' expression, *C. jejuni* SVS 5001 increased 30 energy metabolism proteins to enhance its energy production. Eighteen of the 30 up-regulated energy metabolism proteins in the cold-adapted *C. jejuni* SVS 5001 were also found to have increased expression in *C. jejuni* SVS 5141. The other 12 proteins, which increased in the clinically-isolated strain only, were six TCA cycle proteins,

three electron transport proteins, three glycolysis proteins and one ATP-proton motive force protein. A total of ten proteins involved in TCA cycle increased in *C. jejuni* SVS 5001 to adapt to six days of 4oC cold storage. In *C. jejuni* SVS 5141, there were only four up-regulated proteins involved in the TCA cycle. *C. jejuni* SVS 5001 was the only strain with increased protein expression of oxidative defence proteins in this proteomic study. Compared with the water-isolated strain, the clinically-isolated *C. jejuni* SVS 5001 relied more on aerobic reparation to provide energy during cold adaptation.

This comparative study suggested that an infection did not significantly increase cold-tolerance of *C. jejuni*, but made this pathogen take a longer time to enter the stage of cold adaption; moreoever, an infection caused a huge proteomic alteration in *C. jejuni* and affected this pathogen's energy metabolism pathways and oxidative defence systems.

Chapter 7 Final Remarks

7.1 General discussion

Despite being generally regarded as a fragile bacterium; *C. jejuni* has established itself as the world's leading cause of food-borne gastroenteritis. How this fragile microorganism survives outside of host and, subsequently, infects humans has attracted many researchers' attentions. There are many environmental stresses encountered by *C. jejuni* while it lives outside of host. To survive harsh conditions, this pathogen need to react simultaneously to a wide variety of stresses. Among them, the cold stress response of *C. jejuni* is the one of stress response systems worth further study. To better understand how this leading food-borne pathogen responds to cold will be useful for food industry in developing better intervention strategies to reducethe prevalence of *C. jejuni* in food and therefore improve food safety and protect public health.

Bacterial cold survival ability is known to be dependent upon bacterial cold stress response systems, which govern the expression of large numbers of genes and, consequently, maintain the stability of cellular equilibrium under cold conditions (Digel et al., 2008). Alteration of gene expression in a bacterium under cold stress is, therefore, a key to interpreting the cold stress response mechanisms employed by the bacterium. To decipher gene expression changes under cold stress, several strategies have been described, including comparative genomics, transcriptomics, 1DE MS, 2DE proteomics (On et al., 2006; Stintzi and Whitworth, 2003; Lazaro et al., 1999; Zhang et al., 2009).

Two approaches have been employed in this study, namely, 1DE and iTRAQ labelling gel-free proteomics. 1DE was used for roughly comparing the protein banding profiles of all samples and selecting meaningful samples for further iTRAQ labelling proteomic analysis. The iTRAQ proteomic analysis is a MS-based approach for the relative quantification of proteins, relying on the derivatization of primary amino groups in intact proteins using isobaric tags for relative and absolute quantification

(Wiese et al., 2007) and is presented here. *C. jejuni* protein extracts were labelled with isobaric taps, then identified and comparative quantified through liquid chromatography-MS and iTRAQ data analysis.

To my knowledge, this was the first description of iTRAQ labelling proteomics of *C. jejuni*, although many other 2DE proteomics of *C. jejuni* have been described (Kalmokoff et al., 2006; Zhang et al., 2009; Liu et al., 2012). The present iTRAQ labelling proteomics has identified 236 proteins from three *C. jejuni* strains. The genome of *C. jejuni* NCTC 11168 contains approximately 1699 predicted genes, which is predicted to encode 1654 proteins (Parkhill et al., 2000). In this study, only 236 proteins were identified and the coverage of the identification was 14.2%. A previous approach to obtain comprehensive proteomic profiles of *C. jejuni* NCTC 11168 through 2-DE/MS combined 2-DLC/MS/MS had identified 195 proteins and achieved 11.8% coverage for protein identification (Zhang el al., 2013). Other recent research used in-gel digestions combined with nanoflow LC-MS/MS and achieved a 86% coverage of identification (Liu et al., 2012). The method of protein sampling and the protein identification was not likely to cause such significant difference in the number of protein identification, as the three studies all used whole-cell lysates for protein preparation and LC-MS/MS for protein identifed. However, the numbers of LC-MS/MS replicated havea significant impact on the coverage of protein identification. Liu et al. (2012) detected 1428 *C. jejuni* proteins and achieved 86% coverage of identification through replicating the LC-MS/MS experiment 126 times. Moreover, the protein digestion method could have considerable impact on the coverage of protein identification. Liu et al. (2012) and this study both used whole-cell protein extraction for protein digestion, and achieved a higher coverage in protein identification than Zhang et al. who excised the entire visualized spots on 2-DE gels for protein digestion.

Apart from protein identification and proteomic map construction for *C. jejuni*, the research performed during this project has analysed and compared the alteration of protein expression in three different *C. jejuni* strains under cold stress. The research revealed differences in the impact of cold shock and cold adaptation in *C. jejuni*

protein expression, and the various mechanisms used by different *C. jejuni* strains to fulfil their energy demands to respond to cold stress. The study also found a human infection changed the cold stress response protein profile of *C. jejuni*. The most important findings from this project follow.

This study found that *C. jejuni* managed its protein expression in very different ways to respond to cold shock and cold adaptation. *C. jejuni* NCTC 11168 reprogrammed its protein biosynthesis dramatically to respond to cold shock. It significantly altered expression of 104 of 235 proteins identified to respond after six hours of cold shock. Twenty-eight ribosomal proteins, eight energy metabolism proteins, five chemotaxis proteins and five cell envelope proteins have been found increase their expression during cold shock. Meanwhile, four oxidative stress defence proteins were down-regulated. Four genetic information processing proteins and three DNA biosynthesized proteins stopped their expression during cold shock. Compared with six hours of cold shock, six days of cold storage caused fewer changes in protein expression in *C. jejuni* NCTC 11168. Only 19 *C. jejuni* NCTC 11168 proteins were up-regulated in response to six days of cold storage. This suggested *C. jejuni* NCTC 11168 dramatically altered its physiological characteristics in response to cold shock. While in the cold shock stage, it increased protein synthesis and energy metabolism, while slowing down its DNA synthesis. After the first few hours of cold shock, *C. jejuni* NCTC 11168 cells entered a cold adaptation period where its physiological characteristics may have changed back to normal, as its proteomic profile in cold adaptation period was similar to the proteomic profile of cells without cold exposure.

Through comparing protein expression alterations in *C. jejuni* SVS 5001 and NCTC 11168 during their cold shock period, this study found that the two strains have very different profiles for their cold-induced energy metabolism proteins. *C. jejuni* SVS 5001 increased both anaerobic and aerobic respiration pathway proteins in response to cold shock, while *C. jejuni* NCTC 11168 only increased expression of the proteins involved in anaerobic respiration. The two strains managed their protein expression in two different modes to fulfil energy requirements in cold shock conditions

suggesting that the mechanisms of energy metabolism employed by *C. jejuni* might contribute to the strain-specific differences in cold tolerance of this pathogen.

This study also found that both *C. jejuni* NCTC 11168 and *C. jejuni* SVS 5001 increased expression of large numbers of ribosomal proteins and cell envelope proteins in response to cold shock. Ribosomal proteins played critical roles in protein biosynthesis. A previous study also found that ribosomes were able to act as sensors of cold shock in *E. coli* (VanBogelen and Neidhardt, 1990). The cell envelope protein was important for cell wall and plasma membrane structural integrity. The increased ribosomal protein and cell envelope proteins in the two strains suggested *C. jejuni* might employ similar cold response mechanisms to regulate its ribosome and cell envelope proteins in response to cold shock.

C. jejuni SVS 5001 and *C. jejuni* SVS 5141 were isolated from the same waterborne outbreak. The difference between the two isolates was the former had infected humans; the later was a water-isolated environmental source. Through comparing their protein expression in the cold adaptation stage, our study found the clinical isolate altered its protein expression more substantially than the environmental source isolate. To adapt to cold temperatures, *C. jejuni* SVS 5001 increased a total of 135 proteins' expression. The number of increased proteins in cold adapted *C. jejuni* SVS 5141 was 86. The up-regulated proteins in *C. jejuni* SVS 5141 appeared to be a subset of the up-regulated proteins in *C. jejuni* SVS 5001. A previous study had suggested better survival ability resulted in higher chancea of infecting humans (On et al., 2006). Our study found infection caused huge protein expression changes in *C. jejuni* in response to cold stress, which affected this pathgen's energy metabolism and oxidative defences.

7.2 Conclusions

The present study was the first to describethe construction of a proteomic map for *C. jejuni* through iTRAQ labelling proteomics. The proteomic map constructed using iTRAQ labelling approach contained 236 proteins and achieves 14.2% of protein identification coverage. Apart from protein identification and proteomic map

construction for *C. jejuni*, the study focused on comparative analysis of protein expression alteration in three different *C. jejuni* strains under cold stress. The present comparative proteomics has led to a better understanding of ways in which *C. jejuni* response to cold shock and adapts to low temperatures. Through comparative analysis, the study found cold shock and cold adaptation in *C. jejuni* are two completely different stages from the angle of their proteomic profiles. Moreover, the study found different *C. jejuni* strains used similar mechanisms to regulate its ribosome and cell envelope in response cold stress, but they employed various mechanisms to fulfil their energy demands. This study also disclosed that a human infection can causehuge alteration in protein expression in *C. jejuni* under cold stress, and the proteomic alteration affected its energy metabolism and oxidative defences.

7.3 Future directions

The present comparative proteomic analysis has not only presented a comprehensive protein map for the three *C. jejuni* strains, but also revealed protein expression alterations affected by cold shock and cold adaptation. The work presented in this study could be continued in a number of ways.

An obvious way is continuing to study cold stress response mechanisms employed by all *C. jejuni* strains to cope with cold. For example, these three strains all increased their cell envelope proteins and ribosomal proteins in response to cold stress. The large numbers of ribosomal protein that increased in all strains in response to cold stress is worth a more in depth investigation. That such a large number of bacterial ribosomal proteins increased their expression in response to cold stress was, to my knowledge, the first time this has been described.

Another line of research could be to focus on the differences in the cold stress response mechanisms employed by different *C. jejuni* strains. The present study found *C. jejuni* SVS 5001 activated both anaerobic and aerobic respiration pathways to fulfil its increasing energy demands in response cold shock, while *C. jejuni* NCTC 11168 only increased expression of the proteins that were involved in anaerobic

respiration when it encountered cold shock. The differences inenergy metabolism in the two strains under cold shock is worth to study further, as it may be the key to interpreting why cold tolerance in *C. jejuni* is strain-dependent.

This study indicated that passage through humans from a cooler environmental origin caused proteomic alteration in cold shock and cold adapted *C. jejuni* cells. To confirm the relationship between human infections, the cold survival ability of *C. jejuni,* and re-adaptation to other environments, more isolates are need for comparative study, to determine if the pathways involved are the same or different. The other findings suggest differing protein responses; yet the diversity of such reponses is as yet undetermined.

There is a long way to go towards fully interpreting the cold stress response of *C. jejuni.* The iTRAQ labelling proteomics is one tool that may lead to new insights into this complex stress response.

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Appendix 1:Protein Quantification

Protein quantification for *C. jejuni* whole-cell protein extract used in this study was a modified Bradford assay (Ramagli, 1999).The protocol of this modified Bradford assay is as follow:

Firstly, a standard curve was prepared by using a dilution series (0.05-0.5mg/ml) of ovalbumin. The preparation of the series of ovalbumin shows as Table Appendix 1. Standard ovalbumin (2mg/ml) was diluted with lysis buffer (which had been used at protein extraction) to get 50µl protein mixture containing 5 to 50µg of protein, and then added 25µl 0.1N HCl and 25 µl water into the 50µl protein mixture to make several dilutions of ovalbumin standard containing from 5 to 50µg of protein in 100µl solution (Table Appendix 1).

Table Appendix 1. Preparation of a series diluted ovalbumin standards for the modified Bradford assay.

Vial	Volume of 50% lysis buffer (the lysis buffer /water ratio is 1:1)	Volume of 1mg/ml ovalbumin (the 2mg/ml standard ovalbumin/lysis buffer ratio is 1:1)	0.1N HCl	Distilled water	Final ovalbumin concentration
A	0 µl	50 µl	25 µl	25 µl	0.5 mg/ml
B	10 µl	40 µl	25 µl	25 µl	0.4 mg/ml
C	20 µl	30 µl	25 µl	25 µl	0.3 mg/ml
D	25 µl	25 µl	25 µl	25 µl	0.25 mg/ml
E	30 µl	20 µl	25 µl	25 µl	0.2 mg/ml
F	35 µl	15 µl	25 µl	25 µl	0.15 mg/ml
G	40 µl	10 µl	25 µl	25 µl	0.1 mg/ml
H	45 µl	5 µl	25 µl	25 µl	0.05 mg/ml
I	50 µl	0 µl	25 µl	25 µl	0 mg/ml=blank

Then, 3.5 ml of Coomassie Brilliant Blue G-250 dye reagent was added to the dilutions of ovalbumin standard and the contents were vortexed gently to mix. The absorbance of the dilution series of ovalbumin were measured at 595 nm wavelength using spectrometer (PG T60U UV Visible spectrophotometer). The absorbance reading finished in 5 min after adding Coomassie Blue G-250 dye reagent.Basing on the absorbance reading of the serial protein solutions, a standard curve and a formula for the standard curve was created.

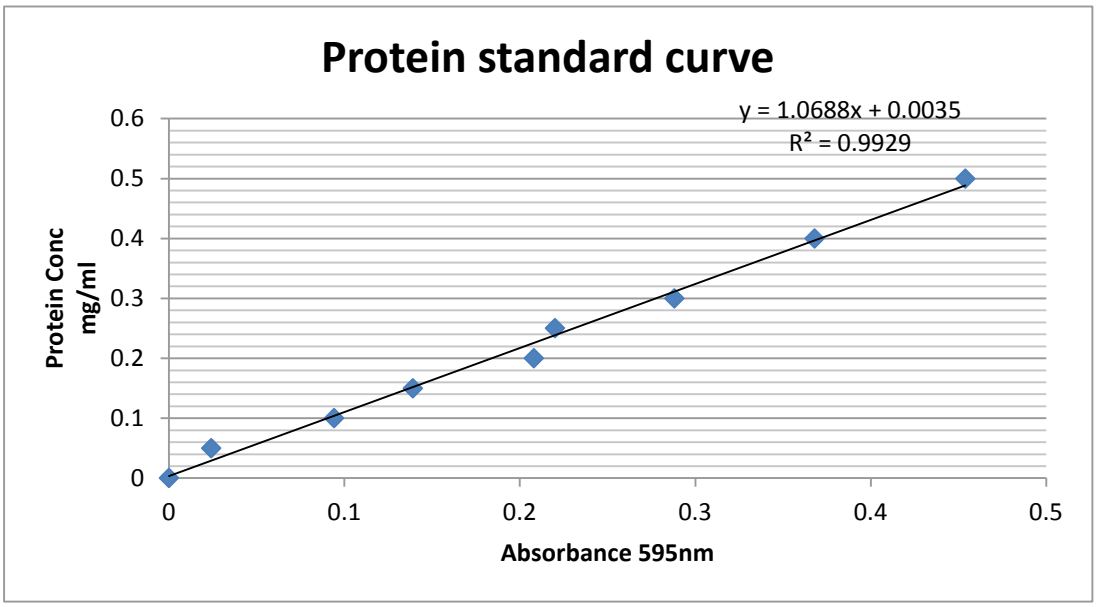

Figure Appendix 1. Protein concentration standard curve

The concentration of the protein extraction samples were examined based on the formula for the standard curve. 25µlsamples mixed with 25µl 0.1NHCl and 50µl waters, and then added3.5 ml of Coomassie Brilliant Blue G-250 dye reagent and vortex mixed gently. The absorbance of sample was read at 595 nm wavelength and reading finished in 5 min after adding Coomassie Brilliant Blue regent.Amounts of *C. jejuni*whole cell protein in samples were determined through interpolating the sample's absorbance onto the formula of the standard curve.

Appendix 2: One-way ANOVA Statistical Test Cell Death Rate

We had calculated the death cell rate at different time points for the three strains with percentage, the results are shown at the table 4.1. To compare cell death rates of the three strains at this 8 day cold exposure experiement, One-way ANOVA statistics test were performed. The statistics test results show as the following table.

Table Appendix 1. Comparation of 3 strains' death cell rate (percentage)

	NCTC 11168	SVS 5001	SVS 5141
1	53.5	28.1	41.9
2	55	26.1	53.5
3	57	54.2	61.6
4	55	52.3	50
5		52.3	50
n	4	5	5
X	55.125	42.600	51.400
s	1.436	14.188	7.117
$\mathbf{X}_{ave}$	49.321		

source	df	SS	MS	F	P-value
treatments	2	382.216	191.108	2.0731	0.1722
error	11	1014.048	92.186		
total	13	1396.264			

P value of this one-way ANOVA test is 0.1722, which is greater than 0.05. There are no statistically significant differentces between the three strains' average death cell rate as determined by one-way ANOVA (P=0.1722).