Characterization of *Campylobacter jejuni* growing in biofilms under different conditions

By

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Abstract

The viability of *C. jejuni* biofilms growing in different conditions was investigated by conventional plate counts and microscopy after nucleic acid staining. Differences were detected for each condition tested. However, total biofilm cells grown at 42°C microaerobically and at 22°C aerobically were not significantly different, which might indicate that *C. jejuni* biofilms grown in the natural environment might contribute to food contamination. Further, it was demonstrated that viable but not culturable (VBNC) cells might exist in *C. jejuni* biofilms, since both biofilm and planktonic cells in the 22°C aerobic condition had great differences in their cell counts obtained by plate counting and microscopy. Protein profiles of *C. jejuni* biofilms grown in different conditions were also determined and different protein patterns were observed by 1D SDS-PAGE. Therefore, we concluded that *C. jejuni* underwent physiological changes when growing in its biofilm mode.

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Chapter One

Literature Review

1.1. Introduction

Campylobacter jejuni is a gram-negative, slender, curved and motile rod bacterium. It is a microaerophilic organism which has a requirement for reduced levels of oxygen. It is relatively fragile and sensitive to environmental stress (e.g. 21% oxygen, drying, heating, disinfectants, acidic conditions) (Nachamkin and Blaser 2000). Now the bacterium is recognized as an important enteric pathogen. Surveys have shown that *C. jejuni* causes more diseases than *Shigella* spp. and *Salmonella* spp. combined (Nachamkin and Blaser 2000). *Campylobacter* bacteria are widespread in warm-blooded animals for food production, and the food production areas may easily become contaminated during processing (Nachamkin and Blaser 2000). Although large scale outbreaks of campylobacteriosis have been linked to water, the consumption of food contaminated with *Campylobacter* has also been recognized as an important source of infection (Cloak and others 2001). The principal foods associated with outbreaks are poultry and poultry products (Cloak and others 2001). Usually, *C. jejuni* is isolated from the gastrointestinal tracts and carcasses of ruminants and it has also been isolated at low levels from bulk packed red meats and retail ready meats (Dykes and Moorhead 2001). Raw milk and non-chlorinated water

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may also be a source of infections (Nachamkin and Blaser 2000). It also can be found in seafood and vegetable type foods (Nachamkin and Blaser 2000).

Since *C. jejuni* is a bacterium, susceptible to many stresses (Solomon and Hoover 1999), it is not easy for this foodborne pathogen to survive during and after food processing. Therefore, it is surprising that campylobacteriosis manifests one of the most common bacterial foodborne disease. In addition, the understanding of the physiology and virulence of *C. jejuni* which can help to develop control strategies is lacking even after the publication of the complete genome sequence for *C. jejuni* NCTC 11168 (Park 2002). It is possible that biofilms are a niche for *Campylobacter jejuni*.

Biofilm is the slime of microorganisms that are attached at an interface associating with extracellular polymeric substances (EPS) they produce (Mattila-Sandholm and Wirtanen 1992). A biofilm can protect producer microbes from hostile environments. That is because microbes in biofilms are no longer growing actively and the effect of many antibiotics is based on inhibition of active growth of the microbes. A permeability barrier formed by EPS also reduces the efficiency of antibiotics. In addition, biofilm can act as a trap for nutrients that are beneficial for bacterial growth. Biofilm will cause problems with cleaning and hygiene and can also cause energy losses and blockages in condenser tubes in many areas.

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1.2. *Campylobacter jejuni*

1.2.1. Characteristics of *Campylobacter jejuni*

C. jejuni is a Gram-negative spiral microaerophilic bacterium. It has limited capacity for growth in the environment. In addition to its microaerophilic nature, it has minimum growth temperatures between 32 and 36°C and requires complex nutritional factors. However, despite these growth limitations, this pathogen is frequently isolated from the gastrointestinal tracts of animals and from a wide range of other environmental sources, including food and surface waters. It is recognized as a major cause of bacterial secretory-type diarrhea and enteritis world-wide. The ability of *C. jejuni* to adapt its responses to a variety of environments and the corresponding stresses is paramount to the infective and contamination cycle of this bacterium.

The knowledge of the mechanisms governing the environmental regulation of gene expression in *C. jejuni* has been very limited until the availability of the *C. jejuni* genome sequence. It is clear that *C. jejuni* can recognize and respond to many environmental induced stresses (Nachamkin and Blaser 2000), such as heat stress, iron limitation, oxidative stress and presence of bile.

In response to heat shock, *C. jejuni* can synthesize at least 24 proteins immediately following heat shock. Some of these are GroELS, DnaJ, and Lon protease. It should be emphasized that *dnaJ* mutants have severely retarded growth at 46°C and are unable to colonize chickens. This suggests that the heat shock response plays an important role in both thermotolerance and colonization. Besides,

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the ability of bacteria to respond to environmental signals depends not only on their ability to synthesize new proteins, but also on the rapid reversal of the process. One such response involves the targeted hydrolysis of regulatory proteins. The Clp energy-dependent proteases are related to the regulation of proteolysis in many bacteria during heat shock and adaptation to other stresses. ClpB is confirmed to be heat-inducible in *C. jejuni* (Thies and others 1999) by Northern blot analysis. Additionally, Northern and RNA dot blot experiments confirmed heat induction of the *C. jejuni lon* gene (Thies and others 1998), revealing a maximum 6-8-fold increase in the level of specific mRNA. Under the cold shock situation, it is apparent that *C. jejuni* does not contain genes encoding any characterized cold shock proteins by analysis of its genome sequence (Nachamkin and Blaser 2000). On the other hand, this bacterium is still motile and able to consume oxygen, produce ATP, and synthesize proteins at 4°C (Nachamkin and Blaser 2000). This indicates that vital cellular processes are still functioning. Therefore, the gene expression of adaptation and regulation during cold shock is likely regulated by a different mechanism (Nachamkin and Blaser 2000).

Iron is essential for the growth of *C. jejuni. C. jejuni* is likely to meet widely fluctuating levels of this essential element during its life cycle. Thus, in order to survive, it must maintain iron homeostasis by the differential expression of iron uptake and storage systems. A fur-like iron-responsive genetic regulator was first identified in *C. jejuni.* A comparison of the protein profiles of the parental strain and a Fur-deficient mutant has demonstrated that the expression of at least seven proteins is iron responsive and regulated by Fur (Nachamkin and Blaser 2000). Also, inactivation of the gene encoding the Fur homologue by allelic exchange has confirmed that this

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protein plays a key role in iron-responsive gene regulation in *C. jejuni*. (Nachamkin and Blaser 2000). In order to maintain iron homeostasis within the cell during iron starvation, *C. jejuni* induces the expression of a number of systems for transporting iron, in the form of siderophores and iron-containing host-derived compounds, and this is mediated by Fur at the transcriptional level (Nachamkin and Blaser 2000). The *C. jejuni fur* gene is unique, since it does not have its own promoter, but is located in an operon that includes the downstream housekeeping genes *lys*S and *gly*A. Neither of these promoters are regulated in response to iron, and the presence of both promoters in front of *fur* results in higher expression of the *lac*Z reporter than with either promoter alone. Expression from two distal promoters might be a mechanism for regulating the level of the *C. jejuni* Fur protein in response to unknown stimuli (Vliet and others 2000).

The microaerophilic nature of *C. jejuni* species implies an inherent sensitivity to oxygen. Therefore, cellular defenses against the damaging effects of oxidative stress play an important role in the survival of *C. jejuni* during exposure to air. Analysis of the genome sequence indicates that the key regulators of oxidative stress defense enzymes in *E. coli* and *S. typhimurium* (SoxRS, OxyR, and SlyA) are not present in *C. jejuni*. (Nachamkin and Blaser 2000). Therefore, additional factors must be involved in the regulation of the oxidative stress response. The identification of a second Fur homologue, termed PerR, in the *C. jejuni* genome sequence shows a role for this protein as a global regulator of the *Campylobacter* response to oxidative stress, which includes the regulation of AhpC and KatA expression. (Nachamkin and Blaser 2000) Futhermore, studies show that *C. jejuni* contains three proteins active in reactive

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oxygen species (ROS) inactivation (Vliet and others 2001): the iron-containing superoxide dismutase SodB, the peroxide stress defense proteins catalase (KatA) and alkyl hydroperoxide reductase (AhpC). ROS which have the ability to damage DNA can be formed during normal (micro) aerobic metabolism during transmission when the cells are exposed to atmospheric oxygen concentrations, or during contact with the human immune system. Examination of the nucleotide sequence downstream of the *ahp*C gene reveals the presence of motility-associated genes and upstream of *ahp*C a putative ferredoxin *fdx*A can be found. A mutation of *fdx*A reduces *C. jejuni* aerotolerance (Nachamkin and Blaser 2000). However, unlike in the *C. jejuni ahp*C mutant, a reduction in peroxide stress resistance of the *fdx*A mutant is not found. Thus, it cannot be predicted in which aspect of oxidative stress resistance the FdxA protein is involved. However, given the absence of an AhpF homolog in *C. jejuni*, FdxA might be involved in reducing the AhpC protein. Consequently, it is likely that FdxA plays an important role in survival of *C. jejuni* on food sources and in the environment. The FdxA protein is a novel component of the oxidative stress defense of *C. jejuni*, and further unraveling of the regulation and mechanisms of oxidative stress resistance of *C. jejuni* will give insight into the metabolism of this important pathogen.

Campylobacter genes lack the typical *E. coli* δ^{70} consensus sequences. Therefore, *C. jejuni* must have a different sequence to form the Campylobacter δ^{70} recognition sequence. In fact, the *C. jejuni* promoter region for many housekeeping genes is unlike the equivalent sequences encountered in other bacteria. It consists of three conserved regions centered 10, 16, and 35 bp upstream of the transcriptional start site (Nachamkin and Blaser 2000). The -10 site closely matches the typical δ^{70} *E.*

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coli promoter site, the -16 region is more often observed in the promoters of gram-positive bacteria and -35 site is very different from the corresponding regions of both *B. subtilis* and *E. coli*. Characterization of the *rpo*D gene which encodes the primary δ factor of *C. jejuni* revealed some interesting features. For example, all known δ^{70} proteins contain a conserved region of 10 amino acids in subregion 4.2 except those of *C. jejuni* and *Helicobacter pylori*. Analysis of the completed genome sequence of *C. jejuni* reveals that only three δ factors which parallel the situation in *H. pylori* are present. These are δ^{70}, δ^{54} and δ^{28}. The presence of a δ^{54} recognition sequence in the promoter sequence of the *fla*B gene which encodes a subunit of the *Campylobacter* flagellin suggests that expression of *fla*B is regulated by δ^{54} (Nachamkin and Blaser 2000). Inactivation of the *C. jejuni rpo*N gene results in the abolition of *fla*B expression which confirms the dependency of *fla*B expression on δ^{54}. Other factors necessary for flagellum formation are also thought to be dependent on δ^{54}. However, the role of δ^{54} in the environmental regulation of gene expression in *C. jejuni* is poorly defined (Nachamkin and Blaser 2000).

The exact function of δ^{28} in *C. jejuni* remains to be elucidated. It is likely that it plays a role in the regulation of flagellin gene expression and motility. The promoter of the *fla*A gene, encoding the major structural component of the flagellin, contains a consensus δ^{28} recognition sequence. Therefore, it is possible that expression of FlaA and other proteins involved in flagellum formation is dependent on δ^{28} (Nachamkin and Blaser 2000). Recent studies show that the *fla*A δ^{28} promoter fusion on pRY*luxCDABE* is an effective reporter of promoter activity (Allen and Griffiths 2001)

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and it has been demonstrated that the *C. jejuni fla*A δ^{28} promoter responds to enteric environmental signals and chemotactic effectors.

In addition, the *Campylobacter* flagella are recognized to play an important role in pathogenicity. *Fla*A and *Fla*B , two tandemly arranged flagellin genes, are transcribed from δ^{28} and δ^{54}-dependent promoters respectively. This suggests that differential expression of the two genes may lead to the production of different types of flagella. Such a mechanism may allow the cell to adapt to changes of environmental parameters such as pH, viscosity, or the presence of neutralizing antibodies.

The genome of *C. jejuni* encodes three δ factors which are the same type as in *H. pylori*. *H. pylori* is not known to reside extensively in the external environment and is exposed to only a limited range of environments. In contrast, *C, jejuni* is exposed to and can tolerate a much greater diversity of environments than *H. pylori*. Moreover, *C. jejuni* lacks specific δ factors, such as RpoS, which regulates the expression of genes involved in the resistance to stationary phase and starvation, and δ^{32} which regulates the heat shock response. Those features suggest that alternative regulatory mechanisms must exist in campylobacters. It is also possible that despite the limited number of δ factors types in *C. jejuni*, the two alternative factors (δ^{54} and δ^{28}), play wider regulatory roles than those in *E. coli* and other bacteria.

1.2.2. *Campylobacter jejuni* in food industry

A significant objective of *C. jejuni* research is to remove or at least reduce it from the poultry production chain. Control of *C. jejuni* infection can be implemented at

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three practical levels, at the poultry farm, during poultry meat processing and during meat handling and cooking by the end consumers.

At the farm level, enhancing biosecurity is a primary intervention strategy to prevent the entrance of the pathogen into the broiler house from the environment. However, this approach is relatively empirical, focusing on simple intervention measures such as using closed houses, providing clean feed and water, eliminating rodents and wild birds, restricting the staff and equipment entering the houses, improving hygiene procedures by poultry house worker, concentrating on changing of outer clothes and efficient dipping of boots. Reports show that *Campylobacter* infection in broilers can be significantly reduced after strict hygienic measures are implemented (Nachamkin and Blaser 2000). However, it is extremely difficult to maintain such measures on the farm. Often, infection will be returned to the original level after a study of intervention strategies is finished. For example, data on effect of improved biosecurity on flock positivity, including boot dipping and changing of outer clothing, in the United Kingdon showed that the measures to reduce the infection only appear to delay the onset of colonization of *campylobacters* (Nachamkin and Blaser 2000). Biosecurity only can provide a preliminary barrier since the organisms are too ubiquitous in the environment to be totally excluded.

Competitive exclusion has been a relatively successful method to control some bacterial infections, particulary salmonellosis, in poultry. However, the development of a competitive exclusion agent for *C. jejuni* is difficult. It is surprising that the strategies successfully controlling *Salmonella* infection in poultry are generally ineffective against *Campylobacters* (Nachamkin and Blaser 2000).

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At poultry meat processing, the application of Hazard Analysis Critical Control Points (HACCP) principles are required during all steps of producing, processing , and distribution in the production of poultry meat products (Nachamkin and Blaser 2000). During poultry processing, several procedures are suggested to minimize *Campylobacter* contamination. These include counterflow water systems during scalding and chilling, and regular rinsing and washing of equipment to reduce cross-contamination. Chlorine or other bacterial control treatments such as trisodium phosphate or lactic acid can be used to disinfect carcasses and related contact surfaces. Slaughter *Campylobacter*-free flocks in the morning, followed by the positive flocks at the end of the day is also recommended (Nachamkin and Blaser 2000). A very accurate surveillance system is needed to practice logistical slaughter. In the United States and several other countries, the packaged fresh or frozen poultry products can be irradiated at 1.5 to 3.0 kGray as an effective treatment to eliminate *Campylobacter* from end products.

Other recognized food-related sources of human campylobacteriosis, such as raw milk and contaminated water can be avoided by consuming only properly heat-treated milk and drinking water only from approved sources. Using chlorinated water to wash contaminated vegetables can render these vegetables negative for *Campylobacter* (Nachamkin and Blaser 2000).

To the end consumer, the risk of getting campylobacteriosis can be reduced by always treating raw poultry, beef and pork as if they were contaminated and handle them properly. This includes wrapping fresh meats in plastic bags to prevent blood from dripping on other foods, refrigerating foods promptly, to limit exposure at room

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temperature, washing cutting boards and counters immediately after use to prevent cross contamination with other foods, avoiding consumption of raw or uncooked meats, ensuring the correct internal cooking temperature, avoiding foods containing raw eggs and raw milk, and washing hands before and after food preparation, and after handling pets (Nachamkin and Blaser 2000).

1.3. Microbial biofilms

1.3.1 Definition of biofilms

Biofilm is the slime of micro-organisms that are attached to an interface. When bacteria adhere to a surface which is in an aqueous environment, these bacteria will produce a slimy, glue-like substance which anchors them to that surface. Biofilm can form on the surface of all types of materials (e.g. metals, plastics and tissues) and it can consist of a single species or many species of bacteria, as well as fungi, algae, protozoa, debris and corrosion products.

1.3.2 Mechanism of biofilm formation

Biofilm formation is a dynamic process which involves a series of steps (Kumar and Anand 1998). First, bacteria along with other organic and inorganic molecules adsorb to the surface forming a conditioning film. The transport of adsorbing particles is due to diffusion or flow of liquid. Molecules gather at the surface which leads to a

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higher nutrient concentration, also termed conditioning film. The physico-chemical properties of the surface are changed because of this conditioning film. Microorganisms preferably attach to a conditioned surface.

The second step is the adhesion of cells to the conditioned surface. This adhesion is separated into two stages: a reversible adhesion and an irreversible adhesion. The first stage involves initial weak interactions which are developed between the bacterial cell and the substratum. Van der Waals attraction forces, electrostatic forces and hydrophobic interactions affect the reversible adhesion process. In this stage, the bacteria show Brownian motion and they can be removed easily, just by merely rinsing. The second stage is a crucial step in biofilm development. Despite repulsive forces preventing the bacterial cells from making direct contact with the surface, the contact still happens since protrusions of the bacteria, such as flagella, pili and exopolysaccharides can overcome the barrier and help the cell to reach a stable area where microcolonies start to form and a biofilm begins to grow. In this irreversible stage, dipole-dipole interactions, hydrogen, ionic and covalent bonding and hydrophobic interactions are involved. Polymeric fibrils form a bridge between bacterial cells and the substratum which makes the removal of the bacteria difficult. Stronger forces such as scrubbing or scraping are required to remove these irreversibly attached cells.

The third step involves the formation of microcolonies. After the irreversible attachment, the cells continue to grow and divide by using the nutrients in the conditioning film and the surrounding fluid environment and this leads to formation of microcolonies. At the same time, the attached cells produce extracellular polymeric

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substances (EPS) that help the cells to adhere to the surface and anchor the colony. After the primary colonizers attach, grow, divide and produce the EPS, the microenvironment of the surface and the biofilm changes.

The next step is the formation of a mature biofilm. Bacterial cells continue to attach to the substratum and the associated biofilm or EPS. Biofilm growth is usually slow and it will take several days to reach a few millimeters thickness depending on the organism and culture conditions. The composition of biofilms can be variable depending on the different microorganisms present and different nutritional requirements. Biofilms do not exist as a uniform layer on the substratum. Other organic and inorganic solutes can attach to the original biofilm and increase its size. The structure and physiology of the biofilm is affected significantly by the interactions of various microorganisms during the initial stages of biofilm formation. The initial colonizing bacteria may encourage the colonization of physiologically compatible species. In addition, mixed species biofilms are often thicker and more stable than the monospecies biofilms.

The last step of a biofilm cycle is the detachment and dispersal of biofilms. The bacterial cells can detach or be sloughed off by fluid dynamics or shear effects of the bulk fluid. The released bacterial cells may be transported to a new location and form a new biofilm.

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1.3.3. Characteristics of biofilms

In natural environments, the bacterial lifestyle occurs normally as a complex group of organisms attached to a surface called a biofilm (Corona-Izquierdo and Membrillo-Hernández 2002). Biofilms are relevant in medical, industrial and environmental settings. Biofilm-associated bacteria generally possess increased resistance to antimicrobial agents (Corona-Izquierdo and Membrillo-Hernández 2002). Different environmental conditions exist at surfaces and within biofilms through the generation of chemical and physical gradients to which bacteria will respond, such as pH, ion concentration, osmolarity, viscosity, nutrient availability and gas exchange rates (Pratten and others 2001). The need for bacteria to interact and bind to a surface also affects the way in which the bacteria adapt to the different environmental conditions (Pratten and others 2001). Indeed, the attachment of bacteria to surfaces has been shown to trigger the upregulation of gene expression and the production of specific proteins (Pratten and others 2001). The study of genetic determinants of biofilm formation by using *E.coli* as a model indicates that mutant cells lacking flagella (type I pili) (*fliC* or *flhD*) or affected in motility (*mot AB*) are severely impaired in the first steps of biofilm formation (Corona-Izquierdo and Membrillo-Hernández 2002). However, little is known about the genes and signals involved in the expansion and disruption of biofilms in *E. coli* (Corona-Izquierdo and Membrillo-Hernández 2002).

As indicated by Corona-Izquierdo and Membrillo-Hernández (2002), the master regulator of stationary phase-specific gene expression in *E. coli* is the product of the *rpoS* gene. RpoS controls the synthesis of more than 50 proteins during the transition

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from the exponential to the stationary phase of growth, starvation and osmotic shock (Corona-Izquierdo and Membrillo-Hernández 2002). Increasing evidence has suggested that RpoS may play an important role during the exponential phase of growth and may function as a negative regulator (Corona-Izquierdo and Membrillo-Hernández 2002). Nevertheless, the role RpoS may play in biofilm metabolism is still unclear. Experiments have shown that an *rpo*S mutant produced higher amounts of biofilm than its wild-type counterpart. The reason for negative regulation of biofilm formation exerted by RpoS during exponential growth is unknown. A clue to the mechanism is that the increased production of biofilm by the *rpo*S mutant may be mediated by an extracellular factor that is produced during the exponential phase of growth in an *rpo*S mutant (Corona-Izquierdo and Membrillo-Hernández 2002).

Many of the genes encoding the virulence factors for *Staphylococcus aureus* are controlled by the accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*) (Pratten and others 2001). This regulation may be affected by the environment where the bacteria are grown. *S. aureus* strains containing mutations inactivating *agr* and *sar* were used to determine whether the presence of these genes influenced the attachment of the bacterium to a surface. Strains harbouring reporter constructs of the *agr* and *sar* operons were also used to determine their expression in biofilms. The study showed that the *sarA* mutant strain adheres better to glass than the *agrA* mutant or the wild type. Also, there is an increased adherence to fibronectin-coated glass for all three strains compared to glass (Pratten and others 2001). So, these adhesion studies demonstrate that *agr* and *sar* have pleiotrophic effects on the surface expression of molecules responsible for binding to different substrata (Pratten

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and others 2001). In the biofilms, the expression of the *agr* and *sar* reporter fusions was significantly higher in the deepest layers, the region where the greatest numbers of bacteria were observed (Pratten and others 2001).

Coagulase-negative staphylococci (CoNS) are among the most common causes of hospital-acquired infection in the intensive care unit (ICU). The treatment of staphylococcal infection is increasingly problematic. Although most staphylococcal infections result in acute disease, bacterial persistence and recurrent infections are also observed, especially in patients with indwelling medical devices (Fitzpatrick and others 2002). Production of CoNS biofilm, which is composed of sessile bacterial cells embedded in a protective extracellular polysaccharide matrix is considered to be an important pathogenic determinant in prosthetic device-related infections (Fitzpatrick and others 2002). Once formed, biofilm are resistant to antimicrobial chemotherapy and host immune responses, and can be very difficult to treat clinically (Fitzpatrick and others 2002). Reports suggest that polysaccharide intercellular adhesion (PIA) is encoded by the *ica* (intercellular adhesion) operon. The *icaADBC* operon encodes the enzymes required for PIA biosynthesis and a number of groups have isolated *S. epidermidis* biofilm-negative strains with transposon insertions mapping to the *ica* locus (Fitzpatrick and others 2002).

Vibrios are an important component of marine biofilms (Wai and others 1999). Within the biofilm, bacteria can access trapped and adsorbed nutrients, engageing favorable metabolic transactions with other members of the biofilm and be protected form predators and from natural environmental oxidants. Studies on how *V. cholerae* responds to starvation stress reveals that *V. cholerae* responses include changes in

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morphology, macromolecular synthesis and development of stress resistant cells, influenced by the nature of the starvation stress (Wai and others 1999). However, little is known about the role of *V. cholerae* in forming biofilms and how biofilms improve survival of this organism in a nutrient deprived environment (Wai and others 1999). Furthermore, the knowledge of molecular genetic mechanisms affecting biofilm formation by *V. cholerae* is poor (Wai and others 1999).

1.3.4. Biofilms in food industry

The attachment of the bacteria to food products or product contact surfaces causes serious hygienic problems and economic losses (Kumar and Anand 1998). When biofilm is formed, the microbes are protected against sanitizers and disinfectants (Zottola 1994, Kumar and Anand 1998, Mattila-Sandholm and Wirtanen 1992). The biofilms also reduce heat treatment and sterilization effects (Kumar and Anand 1998). Floors, waste water pipes, bends in pipes, rubber seals, conveyor belts, stainless steel surfaces are other common areas for biofilm formation (Kumar and Anand 1998). Also, biofilm contributes to a significant reduction of the performance of membrane technologies such as ultrafiltration (UF) and reverse osmosis (RO) which are widely used in food industries (Kumar and Anand 1998). Even a small degree of adsorption can lead to pore blockage and filter clogging and cause reduced permeate flux and reduced product yields (Kumar and Anand 1998). Special attention should be given to the most commonly used food contact surface material, stainless steel. It is widely used in the food industry because of its chemical and physical stability,

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cleanability and the high resistance to corrosion (Kumar and Anand 1998, Zottola and Sasahara 1994). The microtopography of stainless steel is composed of cracks and crevices that is unlike its macroscopic appearance. These cracks and crevices allow foodborne pathogens and spoilage microorganisms to attach to the stainless steel surface (Kumar and Anand 1998, Zottola and Sasahara 1994).

Can biofilm formation be avoided in the food industry? An effective cleaning and sanitation program implemented in the process from the very beginning can inhibit the bacterial cells from attaching to the surface and thus prevent biofilm formation. A good food processing design which includes equipment, materials accessories, layout and construction will help to reduce bacterial adhesion. Electropolishing stainless steel is also an effective way to smoothen the surface and reduce biofilm formation.

Biofilm can be eliminated by adopting mechanical, physical, chemical and biological control methods. Also, traditional mechanical methods such as brushing should not be neglected (Kumar and Anand 1998). Recently, a newer physical method, low electrical currents in combination with antibiotics was successfully used for removal of biofilm (Kumar and Anand 1998). Chemical methods are most commonly used. Chelators containing detergents, such as, EDTA, ethylene glycol-bis, or EGTA can help to remove biofilms (Kumar and Anand 1998). Some sanitizers, such as, peracetic acid, chlorine, iodine and hydrogen peroxide, may even depolymerize EPS, thus enabling the detachment of the biofilms from surfaces. Antimicrobials have been shown to play a major role in preventing bacterial colonization (Kumar and Anand 1998). Among the newer biological means devised for biofilm control, Nisin is well

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known and the most applied antimicrobial peptide (Kumar and Anand 1998). It is an effective inhibitor for many food pathogens and spoilage bacteria. Enzymes have been reported as effective in dissolving the EPS which form the biofilm matrix. A mixture of enzymes which consisted of protease, α-amylase and β-glucanase is effective in cleaning biofilm in paper pulp manufacture (Kumar and Anand 1998). Endo H had the unique property to remove bacteria from glass and cloth surfaces in buffer and detergent solutions (Kumar and Anand 1998). Very recently, an exopolysaccharide-degrading enzyme derived from a streptomyces isolate was reported to control biofilm formation in food industries (Kumar and Anand 1998).

1.4. *Campylobacter jejuni* biofilms

Dykes and others (2003) indicated that *Campylobacter jejuni* biofilm cells are less resistant to stress than cells not grown on a surface (planktonic cells). Experiments with *C. jejuni* planktonic and biofilm cells in phosphate buffered saline (PBS) showed that culturability of both biofilm and planktonic cells decreases rapidly at higher storage temperatures. For example, either planktonic cells or biofilm cells can not be detected by plate counting after 1 day storage at 37°C, while they can be detected at 4°C until 14 days. However, no cells of either type can be cultured after 14 days even at 4°C. The growth pattern of planktonic and biofilm cells during storage is apparently different. The numbers of biofilm cells are significantly lower than those of planktonic cells at any given day or storage temperature. The reduced survival ability of cells grown in biofilms may be explained by the suggestion that campylobacters

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lack many of the adaptive stress-resistance responses that are common in other bacteria (Dykes and others 2003).

Hydrophobicity experiments by Dykes and others (2003) showed that planktonic cells have an initial adherence to xylene of 15% and this decreases significantly during storage in PBS and reaches 0% after 7 days. Biofilm cells initially show <3% adherence to xylene and this does not change significantly during storage in buffer. A high percentage of hydrophobicity is usually required for virulence in enteric pathogens, so the result of the experiment indicated that biofilm *C. jejuni* cells are probably less virulent than their planktonic counterparts.

Despite the induction of some putative stress proteins in biofilm cells, the biofilm cells appears more sensitive to stress conditions than planktonic cells (Dykes and others 2003). An experiment conducted in a two-stage continuous mixed culture aquatic biofilm model using serine as the carbon source demonstrated that carbon load significantly reduces the survival of *C. jejuni* survival in aquatic environment (Buswell and others 1999).

Water quality is an important factor for the survival of *C. jejuni* in biofilms (Buswell and others 2001). *C. jejuni* biofilm cells persist 2 to 4 times longer in poultry house mains supply water than in a bore hole water source. In poultry house mains supply, the presence of *C. jejuni* biofilm cells were detected by using specific rRNA probes long after they were culture negative that also can indicate a viable but non culturable status (Buswell and others 2001).

C. jejuni is recognized as the most prevalent food-borne bacterial enteric pathogen. The understanding of the ability of *C. jejuni* to adapt to environmental

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conditions is only beginning. Sequencing of the whole *C. jejuni* genome has paved the way to improved knowledge of the mechanisms of the *C. jejuni* response to the environment. Despite the ability of *C. jejuni* to survive in a diverse range of environments, including the gastrointestinal tract of animals and humans, food, and oligotrophic environments such as surface waters, its capacity for regulating gene expression in response to changes in environmental conditions appears to be limited in comparison to other bacteria (Nachamkin and Blaser 2000).

In addition, a large number of distinct frameshift mutator elements are observed by analysis of the genome sequence. A general solution for *Campylobacter* survival may lie in the operation of mutational systems to diversify the antigenic and phenotypic properties of individual cells within propagating populations (Nachamkin and Blaser 2000).

1.5. Methods to analyze biofilms

1.5.1. Plate counting

There are two important methods to enumerate bacteria. Most probable number (MPN) and direct plating. The direct plating includes "pour", "drop", "spread", and "spiral" plating. Conventional MPN methods are sensitive, but not precise and the procedure is labor intensive (Chen and others 2003), while the drop plate method is economical and used widely in microbiology research laboratories. Direct plating is an effective technique for isolation and enumeration of *Campylobacters* (Line, 2001).

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Usually, *Campylobacters* are cultured on a *Campylobacter* specific agar medium under 42°C microaerobic (5% O_2, 10% CO_2 and 85% N_2) conditions for 48 hours. Brucella-reducing (BR) agar, *Campylobacter* charcoal differential agar (CCDA), Mueller Hinton agar, Columbia sheep blood agar (CBA), and *Campylobacter* blood-free selective agar (modified-CCDA) are commonly used to isolate and enumerate the *Campylobacter* cells. However, new agars such as Campy-Line agar (CLA) and Campy-Line blood agar (CLBA) have been developed recently. These two new media are translucent and triphenyltetrazolium chloride (TTC) results in colonies of deep-red to magenta color which facilitates *Campylobacter* enumeration.

1.5.2. Microscopy

Fluorescent molecules can absorb light at one wavelength and emit it at another longer wavelength. A fluorescence microscope is designed to detect the cells stained with fluorescent dyes. This microscope is nearly the same as an ordinary light microscope except that the illuminating light is from a very powerful source and passed through two sets of filters. Only the wavelengths that excite the particular fluorescent dye can pass the first filter. The second filter blocks out this light and lets the wavelengths emitted by the fluorescent dye pass. However, the images produced by this conventional fluorescence microscope are blurred by the presence of fluorescent structures above and below the plane of focus. The confocal microscope can achieve crisp optical sections by removing the out-of-focus information. The basic arrangement of the optical components of the confocal fluorescence microscope is

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similar to that of the standard fluorescence microscope except that a laser is used to illuminate a small pinhole whose image is focused at a single point in the specimen. The fluorescence emitted from the illuminated specimen is collected and brought to an image at a suitable light detector. A second pinhole is placed in front of the detector which is "confocal" with the illuminating pinhole. Thus, the rays emitted from the illuminated point in the specimen precisely come to a focus. The light from this point in the specimen converges on this aperture and enters the detector. On the other hand, the light from regions out of the plane of focus of the spotlight is also out of focus at the pinhole aperture, and this light is thus largely excluded from the detector. Furthermore, a two-dimensional image can be built up by sequentially collecting each point in the plane of focus data through scanning across the field in a raster pattern.

There are three main types of fluorescent nucleic acid stains which include stains for all cells, stains for metabolically active cells, and stains for membrane damaged cells. DAPI (4,6-diamidino-2-phenylindo), acridine orange (AO), Sytox green and Syto 9 are stains that label all bacteria in a bacterial population. CTC (5-cyano-2,3-ditolyltetrazolium chloride) will stain metabolically active bacteria, while propidium iodide (PI) is only able to penetrate bacteria with damaged membranes. It is possible to use two different types of dyes together to simultaneously monitor the active (live) and inactive (dead) bacteria.

Chae and Schraft (2000) used acridine orange to stain cells followed by confocal laser scanning microscopy (CLSM) to explore viability of different *L. monocytogenes* strains in 2- and 4-day-old-biofilms. Metabolic active cells are able to reduce CTC to CTF, the red fluorescing formazan crystals of CTC. So, CTC can be

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used as a fast method for monitoring the metabolic activity of bacteria by combination with CLSM. CTC has been used by Bartosch and others (2003) to visualize and quantify in situ actively respiring microorganisms on natural stone. CTC-DAPI double staining is used frequently to measure the activity of cells. The viable but non-culturable state in four strains of *L. monocytogenes* during the starvation in microcosm water has been demonstrated by CTC-DAPI double staining (Besnard and others 2000). Since the ability of starved bacterial cells to survive in hostile conditions is thought to be the result of a starvation protein synthesis, Cappelier and others (2000) used direct count to determine the percentage of viable cells by CTC-DAPI double staining to demonstrate that protein synthesis exists in starved *C. jejuni* cells. Furthermore, Federighi and others (1998) also used CTC-DAPI double staining and found the existence of non-coccoid viable but non-culturable *C. jejuni* cells in microcosm water. Live/Dead stain is also used in bacterial viability assays. It provides two color fluorescence based on cell membrane permeability. Viable bacteria with intact cell membranes stain fluorescent green (Syto 9), while dead bacteria with damaged membranes stain fluorescent red (propidium iodide). Perrot and others (1998) demonstrated that gel immobilization improves the survival of *Escherichia coli* under temperature stress in nutrient-poor natural water by using Live/Dead stain combined with confocal laser scanning microscopy.

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1.5.3. Protein expression

The ability of cell survival in different living conditions can be analysed by the protein profiles of individual cells in a given living condition. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful method to detect the protein profiles of a cell.

Proteins can be separated into different bands depending on their molecular weights by SDS-PAGE. The proteins obtained from the cell extract are treated with SDS which gives them a negative charge. Samples are loaded in wells at one end of the gel, which contains a microscopic network of pores. Under the influence of an electric field, the proteins move through the gel toward the positive electrode. Proteins with smaller molecular weights migrate faster than larger ones and thus different molecular weight proteins become separated. Over several hours, the proteins are spread out across the gel according to their size. Protein bands on polyacrylamide gels are invisible before the gels are stained. One method of staining the gel is Coomassie blue stain. Silver stain is a more sensitive method which is commonly used for staining SDS-PAGE gels.

The phenotypic differences among 2 and 18 hour old biofilm and planktonic *Bacillus cereus* cells grown in the presence and absence of glass wool (Oosthuizen and others 2002) were analysed by 2-D gel electrophoresis and proteomic analysis methods. The results indicated that the sigma 54 modulation protein family plays an important role in the biofilm phenotype regulation and that there are significant band differences in the extracellular protein of 18 h old cultures in the presence and

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absence of glass wool. Sauer and Camper (2001) used silicone tubing as a surface to grow biofilm by attachment of *Pseudomonas putida*. They demonstrated that *P. putida* has a global change in gene expression during the early biofilm development. Through analysis of 2-D gel electrophoresis, it revealed 45 differences in the protein profiles between planktonic cells and sessile cells.

Images of two-dimensional protein electrophoresis of *C. jejuni* biofilm and planktonic revealed that there are apparent differences in the protein profiles in these two types of cells (Dykes and others 2003). Molecular weights of biofilm proteins are in the range of 45 to 55 kDa, this is opposed to planktonic cells, which shows no proteins in this range. There are two unique protein spots in planktonic cells and five in biofilm cells. Moreover, five protein spots are up-regulated two-fold in planktonic cells while seven are up-regulated two-fold in the biofilm cells.

Although the specific protein spots have not been sequenced in the experiment by Dykes and others (2003), some of them could be tentatively identified based on descriptions in other studies. The protein spots with the molecular weights of 25.2 and 24.5 kDa which are upregulated two-fold and unique to the planktonic cells were likely to be surface associated proteins. The protein spots of 45.7 and 14.4 kDa which are also upregulated two-fold in planktonic cells may be stress-associated proteins. In addition, the 12.3 and 10.4 kDa spots in biofilm cells are also suspected to be stress-associated proteins.

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1.6. Objectives of thesis

Exquisite research of planktonic *C. jejuni* cells (i.e. cells growing free floating and not attached to a surface) can not explain why this fastidious bacterium has been able to become a leading foodborne disease pathogen. The reason for presence of *C. jejuni* in the finished food products and for the outbreaks caused by it may be revealed by researching the *C. jejuni* biofilm mode.

The survival of both biofilm and planktonic *C. jejuni* cells growing in optimal and not optimal conditions was investigated in this thesis by direct viable count method using fluorescent nucleic acid staining combined with confocal microscopy. Direct viable counting can enumerate the viable but not culturable cells which exist widely in biofilm mode cells. Thus, the exact numbers of cells that contribute to virulence can be more accurately estimated than by plate counting.

Protein profiles of biofilm and planktonic *C. jejuni* cells which were grown in optimal and not optimal conditions were also studied in this thesis. Unique, up-regulated, and down-regulated protein bands among the biofilm and planktonic cells under different living conditions were investigated. This would help to understand the physiological changes of the cells due to the environmental changes.

The findings could guide the food safety regulation in the food processing industry if the optimal and non-optimal living conditions of the *C. jejuni* biofilm cells are better understood.

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Chapter Two

Investigation of the viability of *C. jejuni* biofilm and its planktonic counterpart in different growth conditions

2.1. Introduction

C. jejuni is one of many species and subspecies within the genus *Campylobacter*, family *Campylobacteraceae.* The genus *Campylobacter* comprises 15 species, 12 of which are associated with human disease and *C. jejuni* and *C. coli* together cause over 95% of *Campylobacter* infections in humans (Park 2002). Now, *C. jejuni* is recognized as a leading foodborne pathogen and poultry meat, raw milk and water are the main transmission routes (Federighi and others 1998). For example, 69% of chickens bought from US supermarkets were found to be contaminated with *C. jejuni,* at levels of 10^2 to 10^5 CFU per carcass (Park 2002).

As indicated in the previous chapter, *C. jejuni* is susceptible to a variety of environmental conditions. It does not grow below the temperature of 30°C, it is microaerophilic and it is sensitive to drying, high-oxygen and low pH. Thus, it is surprising that the organism can survive for a long time outside the host and that the pathogen survives in food products after processing. Nevertheless, *C. jejuni* is frequently involved in foodborne disease outbreaks.

Biofilms are matrix-enclosed bacterial populations adhered to a surface or to each other (Poulsen 1999). In nature and food systems, microorganisms attach to

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surfaces conditioned with nutrients, ions and other organic material, which enhances their viability and growth and may lead to biofilm formation (Poulsen 1999). Parts of the biofilm may detach from the surface to locate on the food products during processing which will cause the risk for infection (Jessen and Lammert 2003). Biofilms may express an increased resistance to disinfectants, thus, this risk may become even more serious (Jessen and Lammert 2003).

The lack of understanding about how *C. jejuni* causes foodborne disease and the concept of biofilms providing protection for pathogens from environmental stress and antimicrobial agents were the driving force for investigating *C. jejuni* biofilms.

The objective of this study is to determine the ability of *C. jejuni* to survive as planktonic and biofilm cells under different conditions which included different temperatures, and different atmospheric environments.

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2.2. Materials and methods

2.2.1. Bacterial strain and culture conditions

The strain *C. jejuni* 16-2R, an isolate from poultry meat, was used in all experiments described in this thesis. A stock culture, prepared by mixing equal parts of *C. jejuni* grown in trypticase soy broth (TSB, Becton, Dickinson and Company) with *Campylobacter* growth supplement (Oxoid Limited) and 50% glycerol, was stored at -80°C. Before each use, the stock culture of *C. jejuni* was activated by transferring twice onto Campy-Line agar (CLA) (Line 2001) and incubating at 42°C for 48 h under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). The microaerophilic environment was generated using the CampyGen microaerobic jar or a Sanyo O_2/CO_2 incubator. A fresh *C. jejuni* culture was cultivated in TSB with *Campylobacter* growth supplement at 42°C for 48 h in a microaerophilic environment for preparation of biofilm and associated planktonic *C. jejuni* cells.

2.2.2. Preparation of biofilm cells and associated planktonic cells

2.2.2-1. Biofilm formation

A 0.1 g aliquot of glass wool (Cat No. 3950, Fisher Scientific) was placed in a 100 ml bottle and autoclaved at 121°C for 15 min. After the glass wool and bottles were completely cooled to room temperature, 15 ml TSB with *Campylobacter* growth

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supplement were added to each bottle. Each bottle was inoculated with 100 µl of a 48 h *C. jejuni* culture and incubated for 48 h with agitation (100rpm) at either 42°C microaerobic or 22°C aerobic condition.

2.2.2-2. Collection of biofilm associated planktonic cells

After biofilms were developed, the supernatant was collected as biofilm associated planktonic cells.

2.2.2-3. Detachment and collection of biofilm cells

After removing the growth medium, the glass wool was washed 3 times with 25 ml sterile phosphate-buffered saline (PBS). Then, the biofilm cells were detached and collected as follows: Fifteen ml Brucella broth (Becton Dickinson and Company) with 0.1% Tween 80 (Fisher Scientific) and 7.5 g autoclaved glass beads (600 µm diameter, Sigma) were poured into each bottle containing the glass wool. Biofilm cells were detached by vortexing the glass beads at high speed for 2 min. The liquid phase was then collected as biofilm cells.

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2.2.3. Plate counts

2.2.3-1. Campy-Line media preparation

Campy-Line media (CLA) (Line 2001) was prepared by mixing 21.5 g Brucella agar, 0.25 g ferrous sulfate, 0.1 g sodium bisulfite, 0.25 g pyruvic acid, 0.5 g α-ketoglutaric acid, 0.3 g sodium carbonate, 1.5 g yeast extract and 500 ml distilled water. After completely dissolving all components, the mixture was autoclaved for 15 min at 121°C and cooled in a 50°C water bath to 50°C ± 5°C. Two supplements, hemin and 2,3,5-triphenyltetrazolium chloride (TTC) were added. A stock solution of hemin was prepared by dissolving 0.5 g hemin powder in 10 ml of 1 N sodium hydroxide and adding 90 ml distilled water to this solution. The solution was fully mixed and autoclaved for 15 min at 121°C and kept at 4°C. One ml hemin solution was added to the cooled medium. The second supplement, TTC solution was prepared as a 20% stock solution in distilled water. This solution was slightly heated to completely dissolve the powder, filter sterilized with a 0.22 μm filter and stored at 4°C. This solution will recrystallize at refrigeration temperatures. A gentle heating is needed to redissolve before each use. An amount of 0.5 ml of this TTC solution was added to the cooled medium. After all supplements were added to the medium, the pH was adjusted to 7.4 ± 0.2 with 10 N sodium hydroxide. The media was dispensed into sterile petri dishes which were stored at 4°C.

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2.2.3-2 Plate counts

Plate counts were determined by either spiral plating or drop plating. For spiral plate counts, samples were serially diluted in PBS and plated on CLA by the Autoplate® 4000 (Spiral Biotech). After 48 h incubation at 42°C in a microaerobic environment, dark red pinpoint colonies were enumerated at appropriate dilution according to the Autoplate® User Guide and referred to the original samples.

For drop plate counts, serial decimal dilutions in PBS were prepared and 5 drops of 10µl of each dilution plated onto CLA. The plates were incubated at 42°C in a microaerobic environment for 48 h. Colonies counted at suitable dilutions were used to calculate colony forming units.

2.2.4. Direct viable counts

2.2.4-1. Procedures for fluorescent nucleic acid staining

Planktonic or biofilm *C. jejuni* cells were harvested by centrifugation (Centra CL3R, Thermo IEC) at 1800 x g, 4°C for 30 min. The harvested cell pellets were resuspended in 1 ml sterile distilled water (PBS will shine viewed under microscope) and 10 fold serial dilutions were made from this 1 ml suspension. These dilutions used for analysis were pelleted again by centrifugation. Supernatant was removed and the cell pellets were stained with either Dapi, or Live/Dead stain.

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2.2.4-1-1. Procedures for Dapi staining

Cell pellets were suspended in 1 ml Dapi working solution (0.001mg/ml). The mixture was incubated for 30 min at room temperature in the dark and then filtered onto a 0.2µm pore size polycarbonate black membrane filter (Cat No. GTBP02500, Millipore). The membrane was transferred to a slide and air-dried. Non-fluorescent immersion oil was mounted on the membrane and a coverslip added. Prepared slides could be stored at -30°C for observation at a later time. The blue fluorescence of Dapi could be observed by an IX 51 inverted microscope (Olympus) using Narrow UV as light source. The maximum of excitation and emission for Dapi bound to dsDNA is 358nm and 461nm respectively. Thus, narrow UV tube was used. Images of the samples were saved via a digital camera (Roper Scientific Photometrics) sending the images to Imagepro software.

2.2.4-1-2. Procedures for Live/Dead staining

Cell suspensions were stained using Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Cat No. L-7012) according to the manufacturer's instructions. Equal volumes of SYTO 9 dye and Propidium iodide were combined in an eppendorf tube and mixed thoroughly. Three µl of dye combination was added into each 1ml of diluted sample. The stained samples were vortexed and then kept in the dark at room temperature for 15 min. The samples were mounted onto black membranes as described above and analysed with a confocal microscope FV300 (Olympus)

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equipped with argon ion laser (excitation wavelength 488nm). The excitation/emission maxima for Live/Dead stain are about 480nm/500nm for SYTO 9 stain and 490nm/635nm for propidium iodide. Thus, the strongest green fluorescent (live cells) signals were obtained by the confocal microscope through the filter setting which combined setting a mirror and short pass barrier 530nm. By setting the detection-dichroic splitter 630 nm and short pass 660nm, the strongest red fluorescent (dead cells) signals could be detected.

2.2.4-2 Method for microscopy count

Ten microscopic fields per membrane were randomly chosen and each field was captured to take the image in Imagepro software. Samples were diluted to no more than 200 cells on each image. And then, cell numbers were counted manually in each image and the average cell number of these ten fields was calculated. The numbers of *C. jejuni* planktonic and biofilm cells per cm^2 could be extrapolated by,

$$N= (S \cdot N_1/S_1) \cdot D$$

where, S is the area of the black membrane

N_1 is the mean of cell numbers of ten microscopy fields

S_1 is the area of the microscopic field enumerated

D is the fold of dilution.

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2.3. Results

2.3.1. Evaluation of biofilm development by *C. jejuni* in different environments by standard plate counts

Development of biofilm cells and associated planktonic cells at 42°C and 22°C in both aerobic and microaerophilic conditions over 5 days is shown in Figures 2.1 (a) and (b) and Table 2.1 (a), (b), (c), (d). Planktonic *C. jejuni* grew best at 42°C microaerobic (42M) reaching over 5 $\log_{10}$ cfu/ml for all time points. The growth of 42M biofilm cells was good and only one sample was below the detection limit, on day 5.

There existed a large variability for cell counts at 42°C aerobic (42A), 22°C microaerobic (22M) and 22°C aerobic (22A). Some planktonic cells grew at 42A, but they were clearly lower than for 42M. Only one of the 4 biofilms had countable cells with 1.8 $\log_{10}$ cfu/ml at 42A. Like for 42A, some planktonic cells grew at 22M, but they were lower than for 42M. Only 3 of 8 biofilm samples were countable with range of 2.2 - 3.5 $\log_{10}$ cfu/ml. 22A planktonic cells grew similar to 22M, but more biofilm cells were countable.

Thus, 42°C microaerobic and 22°C aerobic were the two conditions selected for all further experiments.

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2.3.2. Comparison of 2 day old biofilm and planktonic cells growing in 42°C microaerobic and 22°C aerobic conditions.

Both 2 day old biofilm and planktonic cells grown at 42M and 22A were enumerated by drop plating to get culturable counts (log_{10} cfu/ml) and by microscopy using either Live/Dead stain or Dapi stain to get viable counts (log_{10}cells/ml). As shown in Figure 2.2, under 22°C aerobic conditions, no biofilm cells could be cultured (<1.3 log_{10} cfu/ml), while 42M biofilm cells reached 4.3 log_{10} cfu/ml. However, the total biofilm cells which were stained by the Live/Dead kit or Dapi and observed by confocal microscopy had no significant difference ($P>0.05$) between the two conditions. For planktonic cells, the plate count numbers for 42M cells were significantly higher than those of 22A. 42M cells were at 8.6 log_{10} cfu/ml and 22A cells only at 2.5 log_{10} cfu/ml. For 22A, microscopic counts of total cells for both biofilm cells and planktonic cells were significantly higher than numbers obtained by plate counting. Within 22°C aerobic conditions, the cell numbers obtained by plate counting and microscopy were significantly different for both biofilm cells and planktonic cells. However, under 42°C microaerobic conditions, these two methods did not yield different cell counts.

As indicated in Table 2.3 and Figure 2.3, 22°C aerobic biofilm *C. jejuni* cells had the highest percentage and the mean area of clumpings by the microscopy which were 54.54 $\pm$ 4.04 % and 615.23 $\pm$ 130.08 μm^2 respectively. Planktonic cells at 42M got the least mean area of clusters (200.10 $\pm$ 43.8 μm^2).

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2.3.3. Estimation of viable cells by nucleic acid staining

A preliminary growth curve of *C. jejuni* under optimal conditions showed *C. jejuni* growth at exponential stage from the start of culture until reaching a peak at 36 h (Data not shown). 42M planktonic *C.jejuni* growing at 12 h and 24 h was selected to stain by Live/Dead stain. In this second experiment, the stationary phase was reached at 12 h and the percentage of live cells at 12 h and 24 h were 22.4% and 8.7% respectively (Table 2.2).

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2.4. Discussion

Evaluation of biofilm development by *C. jejuni* in different environments by standard plate counts indicated that 42°C microaerobic was the optimal condition for *C. jejuni* which agreed well with most literature reports. *C. jejuni* growing under 42°C aerobic and 22°C microaerobic conditions have not been reported in the literature. Specifically, Trachoo and others (2002) reported that *C. jejuni* on PVC coupons with different biofilm producing bacteria, such as *Pseudomonas sp*, gram-positive biofilm cultures isolated from a chicken house in northeastern Georgia, USA, and a gram-negative bacterium, could not be detected by plate counts at 23°C aerobic after 4 day incubation. Among the other 3 non-optimal conditions tested, *C. jejuni* biofilms growing at 22°C in an aerobic environment produced the highest colony forming units per ml. *C. jejuni* is a thermophilic bacterium. It could be speculated that cells growing under 42°C aerobic condition should perform better than growing at 22°C aerobic condition. However, our results showed that *C. jejuni* yields more colony forming units in 22A than in 42A. Therefore, 42°C microaerobic (42M) and 22°C aerobic (22A) were the two conditions selected for all further experiments and 2 day old biofilms were used throughout.

For biofilms grown at 22°C aerobically, many samples were below the detection limit (Fig. 2.1b). Cells in these samples might be in the form of viable but not culturable cells (VBNC). Direct viable count methods were introduced to study the VBNC cells. Live/Dead nucleic acid staining and Dapi staining were used in the experiments of comparison of 2 day old biofilm and planktonic cells growing in 42°C

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microaerobic and 22°C aerobic conditions for the total cell counts. As indicated in the results (Fig. 2.2), it was determined that the total biofilm cells of 42M and 22A were not different from each other ($P>0.05$). Thus, *C. jejuni* biofilms might contribute to food contamination in the natural condition (22°C aerobic is the normal working environment and biofilm is the microbial existing form in the natural environment). It was also quite noticeable that both biofilm and planktonic cells in the 22°C aerobic condition had great differences in their $\log_{10}$ cfu/ml by plate counts and microscopy. Environmental microbiologists have reported differences between bacterial counts conducted by culture and by microscopy for many years during the working on water samples (Cappelier and others 2000). These observations demonstrated that the viable but non-culturable (VBNC) state existed in bacteria and a number of pathogenic bacteria have been reported to enter the VBNC state, such as, *Escherichia coli*, *Salmonella enteritidis*, *Vibrio cholerae*, *C. jejuni* and *Legionella pneumophila* (Cappelier and others 2000). One might conclude that VBNC cells were present when *C. jejuni* were growing in 22°C aerobic condition.

Federighi and others (1998) studied *C. jejuni* VBNC cells by using 36 strains suspended in microcosm water at 4°C over 30 days. Spread plate counts, direct viable count (using Ciprofloxacin exposure and acridine orange staining where twice elongated cells were counted as viable cells) and CTC-DAPI double staining were used in that investigation. The data of one particular strain (number 79) showed that the total cell count (stained by DAPI) was constant (8 $\log_{10}$ cells/ml) throughout the experiment period (30 days). However, culturable cells could not be enumerated after 14 days by spread plate counts. Thus, the VBNC state was reached after 14 days of

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starvation in microcosm water. On the other hand, an average cell number of 6 $\log_{10}$ cfu/ml was found by CTC staining after 14 days, which indicated that a small proportion (1%) of the total cells in the sample were actually respiring cells. The viable cell number obtained by direct viable counts was very similar to that of CTC staining. Federighi and others (1998) also indicated that the time required to reach the VBNC state is dependent on the strain and experimental conditions, tending to be shorter when the incubation temperature is higher. This was reported by Rowe and others (1998) as well. For example, incubation of the microcosm at 37°C produced a loss of culturability of *C. jejuni* in 3 days as opposed to the 14 days at 4°C. There still exists considerable controversy about the VBNC form of *C. jejuni* cells, in particular whether this type of cells can revert to a culturable state. Federighi and others (1998) conducted an experiment by using a newborn mice model inoculated with 40 ml of VBNC suspensions *per os*; the rates of recovery were 31.6% for strain 79 (six positive animals out of 19 inoculated). However, the recovery results may be variable due to the fate of *C. jejuni* cultures and VBNC suspensions or the type of methods used for viability and recovery assessment (Federighi and others 1998).

Live/Dead stain was used in this study to compare 42°C microaerobic and 22°C aerobic conditions. However, the cell numbers obtained by Live/Dead staining did not seem to reflect the real live and dead cells since sometimes the number of dead cells in biofilms (stained by PI) was higher than total cells (Data not shown). To explore this further and to test the double staining, we used *C. jejuni* cells growing under 42°C microaerobic condition, the optimal condition. Surprisingly, only 22% and 8% of total cells stained "live" in a 12 h and 24 h culture respectively. Thus, in our experiment of

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comparison among 42M and 22A biofilm and planktonic cells only perceived total cells were evaluated. Further experiments would be needed to find a method for distinguishing viable and non viable *C. jejuni*.

Rowe and others (1998) developed an image system for the study of VBNC forms of *C. jejuni* and this method was used to determine the comparative resistance of the culturable and VBNC forms to the disinfectants which were quaternary ammonium compounds (QACs) and QACs combined with glutaraldehyde, amphoterics and chlorine. The report indicated that the culturable *C. jejuni* cells were significantly more resistant than the VBNC population at all the contact times, except for chlorine which contradicts the results by Trachoo and others (2002). Trachoo and others (2002) evaluated the effectiveness of chemical sanitizers against *C. jejuni* in mixed-culture biofilms on PVC coupons after treatment with chlorine, quaternary ammonia, peracetic acid (PAA) and a PAA/peroctanoic acid mixture (PAA/POA). They concluded that *C. jejuni* present in biofilms was sensitive to all sanitizers and chlorine was the most effective of the sanitizers tested. Therefore, Trachoo and others (2002) recommended to use chlorine in the water delivery systems of poultry houses when designing *C. jejuni* control programs.

Trachoo and others (2002) determined the ability of *C. jejuni* to integrate into preexisting biofilms in a low-nutrient growth medium under normal atmospheric conditions. The effects of temperature (12°C and 23°C) and type of biofilm producer (3 gram positive bacteria isolated from a chicken house and one gram negative bacterium, *Pseudomonas sp.*) on the attachment of *C. jejuni* to a PVC surface were evaluated. Their experiments showed that biofilm environments appeared to protect *C.*

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jejuni from environmental stress which might be explained by its association with biofilm exopolymeric substance since greater survival was observed in biofilms having greater surface coverage. When *C. jejuni* was incubated at 20°C in normal atmospheric conditions, the organism was migrating through a semisolid gel to the most favorable concentration of oxygen (Trachoo and others 2002). Some of the *Campylobacter* cells in the *Pseudomonas* biofilm were embedded in the biofilm, as opposed to being on the surface and biofilms also concentrated nutrients which enhanced the survival of *C. jejuni*. This is the reason why *C. jejuni* can survive in 22°C aerobic conditions, despite its sensitivity to oxygen.

As observed in the images, both 22°C aerobic and 42°C microaerobic biofilm cells had large clumps. Highest percentage of clumps and clump size were observed in biofims at 22A and the clumps may be important for *C. jejuni* survival in this suboptimal condition. One clump was enumerated as one cell when biofilm cells were counted by microscopy. If the number of cells in the clumps was known, the exact cells in the clumps enumerated and converted to the total cells, the number of biofilm cells would be much higher. The number of cells in the clumps and clump sizes should be investigated in future research to determine the role of clumping in enhancing survival of *C. jejuni* bioifilms under aerobic conditions.

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Table 2.1 (a). Plate counts of *C. jejuni* biofilm and associated planktonic cells growing under 42°C microaerobic condition

		2 days		4 days		5 days	
	Inoculum (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)
Sample 1	ND	4.85	>5.6	1.9	>5.6	ND	ND
Sample 2	ND	5.61	>5.6	3.02	>5.6	ND	ND
Sample 3	ND	4.5	>5.6	ND	ND	<1.3	7.33
Sample 4	ND	2.83	>5.6	ND	ND	3.69	7.39

Table 2.1 (b). Plate counts of *C. jejuni* biofilm and associated planktonic cells growing under 42°C aerobic condition

		2 days		4 days		5 days	
	Inoculum (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)
Sample 1	ND	<1.3	<1.3	<1.3	2.62	ND	ND
Sample 2	ND	<1.3	<1.3	<1.3	<1.3	ND	ND
Sample 3	ND	<1.3	4.88	ND	ND	1.79	4.26
Sample 4	ND	<1.3	4.77	ND	ND	<1.3	4.2

ND: Not determined

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Table 2.1(c). Plate counts of *C. jejuni* biofilm and associated planktonic cells growing under 22°C microaerobic condition

		2 days		4 days	
	Inoculum (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)
Sample 1	ND	2.15	>5.6	3.45	>5.6
Sample 2	ND	<1.3	>5.6	3.2	>5.6
Sample 3	4.32	<1.3	<1.3	<1.3	2.87
Sample 4	4.32	<1.3	<1.3	<1.3	2.79
Sample 5	6.8	<1.3	<1.3	<1.3	2.58
Sample 6	6.8	<1.3	<1.3	<1.3	2.5
Sample 7	6.08	<1.3	3.58	<1.3	<1.3
Sample 8	6.08	<1.3	3.64	<1.3	<1.3

Table 2.1 (d). Plate counts of *C. jejuni* biofiim and accociated planktonic cells growing under 22°C aerobic condition

		2 days		4 days	
	Inoculum (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)	Biofilm cells (log_{10} cfu/ml)	Planktonic cells (log_{10} cfu/ml)
Sample 1	ND	2.38	>5.6	3.43	>5.6
Sample 2	ND	2.85	>5.6	3.79	>5.6
Sample 3	4.32	<1.3	<1.3	<1.3	<1.3
Sample 4	4.32	<1.3	<1.3	<1.3	<1.3
Sample 5	6.8	<1.3	3.89	<1.3	<1.3
Sample 6	6.8	<1.3	3.9	<1.3	<1.3
Sample 7	6.08	<1.3	3.7	<1.3	<1.3
Sample 8	6.08	4.2	4.44	<1.3	<1.3

ND: Not determined

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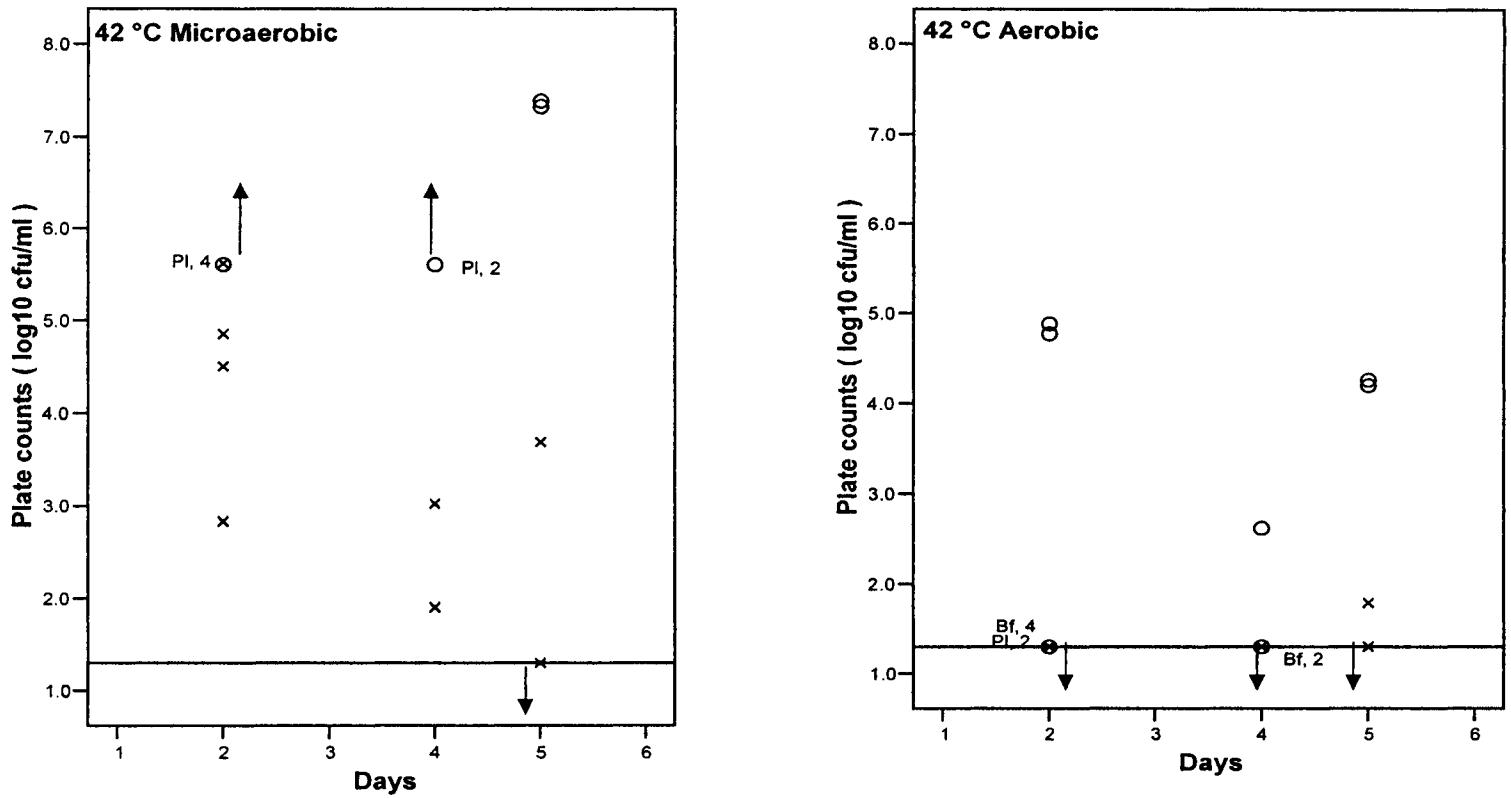
Figure 2.1 (a). Plate counts of *C. jejuni* growing under 42°C microaerobic and 42°C aerobic conditions



○ represents planktonic cells;

× represents biofilm cells;

↑ is above and ↓ is below the detection limit of 1.3 $\log_{10}$ cfu/ml and 5 $\log_{10}$ cfu/ml respectively; The line indicates the detection limit;

Data points with overlaying samples are annotated to indicate sample type (Bf = biofilm and Pl = planktonic) and number of samples. Thus, a data point marked Pl, 2, represents two planktonic cell samples

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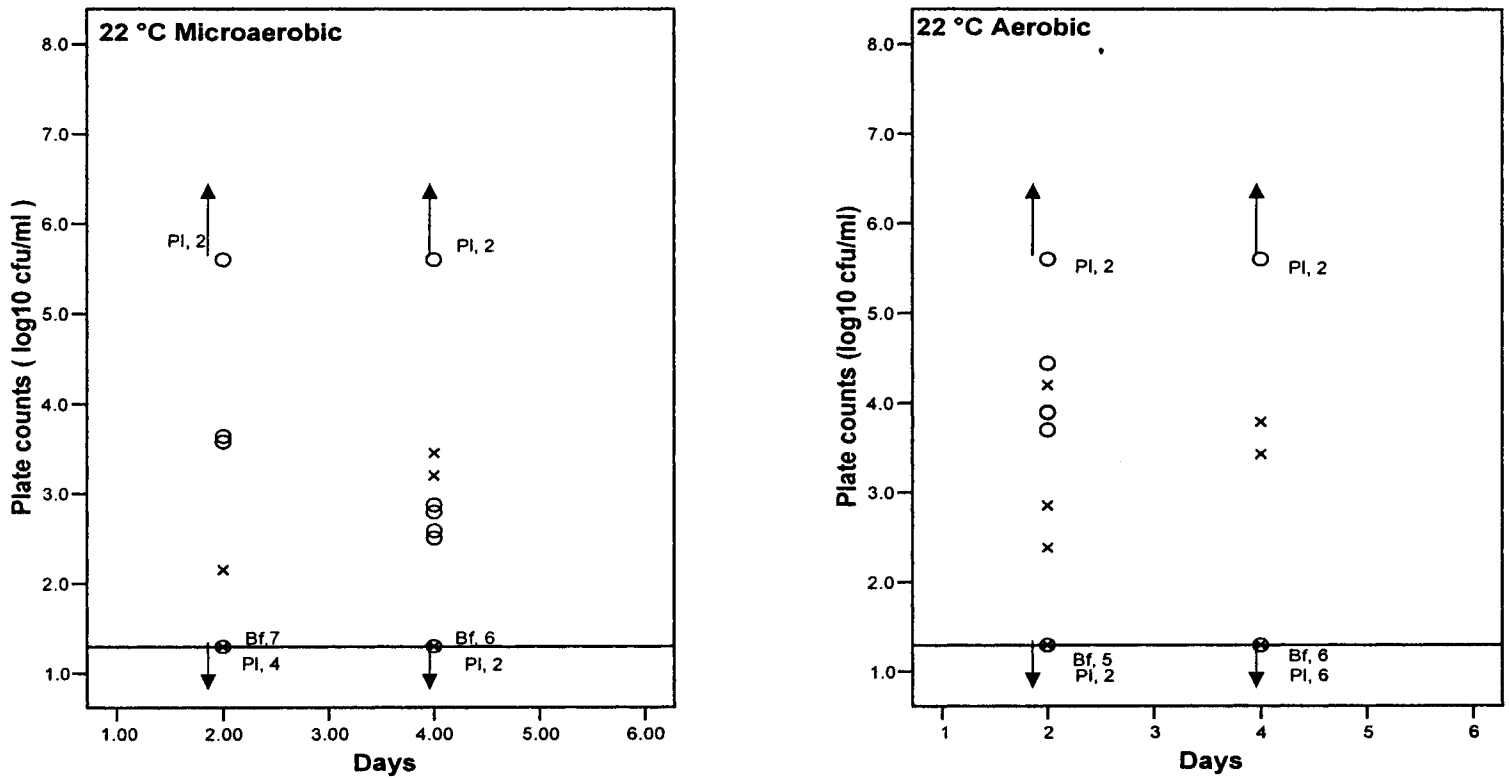
Figure 2.1 (b). Plate counts of *C. jejuni* growing under 22°C microaerobic and 22°C aerobic conditions



Legend see figure 2.1 (a).

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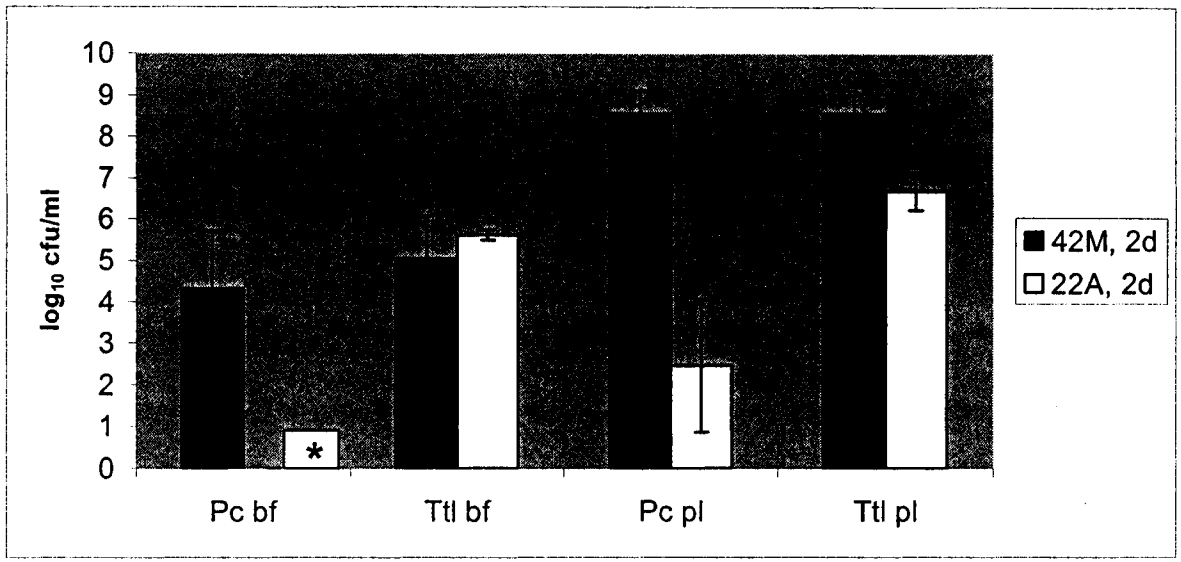
Figure 2.2. Cell numbers of *C. jejuni* biofilm and associated planktonic cells growing under 42°C microaerobic and 22°C aerobic conditions for 2 days



[*] was below the detection limit
42M, 2d was 42°C microaerobic, 2 days
22A, 2d was 22°C aerobic, 2 days
Pc bf was *C. jejuni* biofilm cells enumerated by plate counts
Ttl bf was *C. jejuni* biofilm cells enumerated by microscopy after staining with either Dapi or Live/Dead stain
Pc pl was *C. jejuni* planktonic cells enumerated by plate counts
Ttl pl was *C. jejuni* planktonic cells enumerated by microscopy after staining with either Dapi or Live/Dead stain

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Table 2.2. Live/Dead stain for 42°C microaerobic planktonic cells

		Microscopy			
	Plate count (log_{10} cfu/ml)	Total cells (log_{10} cells/ml)	Dead cells (log_{10} cells/ml)	Live cells (log_{10} cells/ml)	Percentage of live cells %
12h	8.75	8.69	8.58	8.04	22.4
24h	8.76	8.15	8.11	7.09	8.7
36h	8.43	ND	ND	ND	ND

ND: Not determined

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Table 2.3. Average percentage and mean area of cell clusters of *C. jejuni* for each treatment

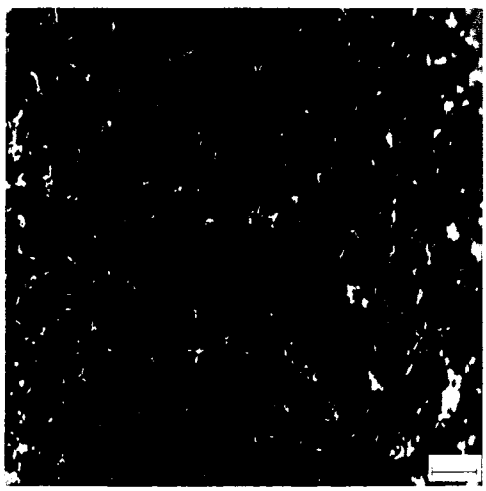
	Percentage of cluster cells (%) ±SD	Mean area of clusters (μm^2)±SD
42°C microaerobic, planktonic (n=30)	31.30 ± 7.05 [a]	200.10± 43.8 [c]
42°C microaerobic, biofiim (n=30)	29.86± 4.88 [a]	382.73 ± 37.18 [d]
22°C aerobic, planktonic (n=30)	47.90 ± 17.28 [a]	340.67 ± 82.07 [e]
22°C aerobic, biofilm (n=30)	54.54 ± 4.04 [b]	615.23 ± 130.08 [d]

n is number of images

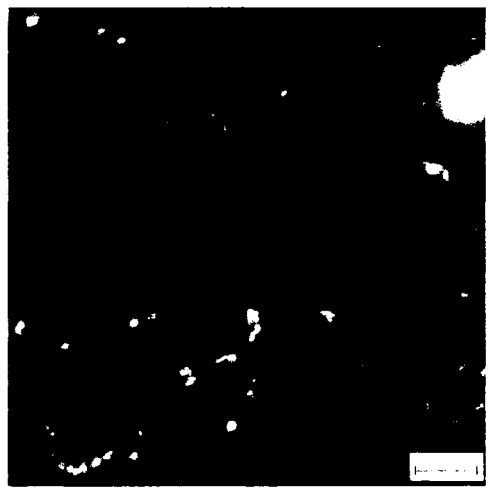
a, b, c, d, e indicates the statistic difference, same letter represents no significant difference at $P<0.05$

Figure 2.3. Images of planktonic and biofilm *C. jejuni* growing for 2 days under 42°C microaerobic and 22°C aerobic conditons

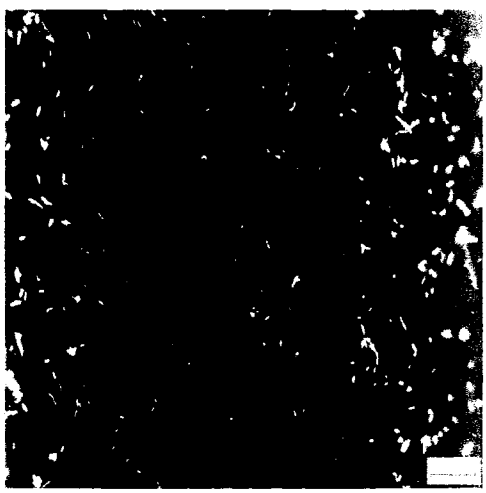
(A) 42°C microaerobic, planktonic cells



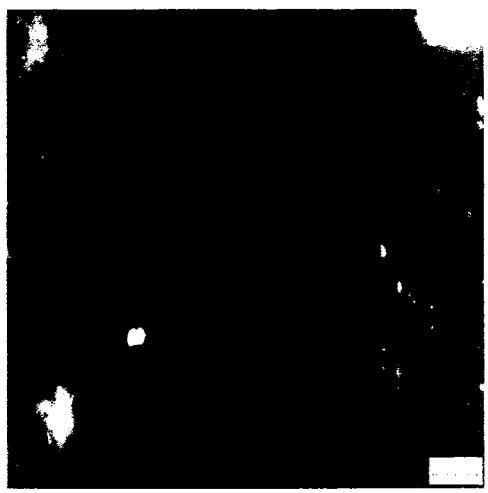
(B) 42°C microaerobic, biofilm cells



(C) 22°C aerobic, planktonic cells



(D) 22°C aerobic biofilm cells



Each scale bar on the images represents 10 µm.

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Chapter Three

Protein profiles of *C. jejuni* biofilm cells and associated planktonic cells in different growth conditions

3.1 Introduction

Bacteria in the biofilm form display specific physiological features that are different from microorganisms growing in the planktonic state (Vilain and others 2004). Cell surface structures, cell wall composition and virulence, exopolysaccharides and capsular polysaccharide and protein profiles also undergo changes when a bacterial cell switches from planktonic to biofilm mode of growth (Asha and others 2004). In particular, biofilm cells are extremely resistant to environmental stresses such as exposure to antimicrobial agents, including antibiotics, biocides, and toxic pollutants which is the crucial problem for the cleaning of food industrial equipment subject to biofilms (Vilain and others 2004).

C. jejuni is a leading cause of foodborne disease in developed countries which was widely reported in recent years. Its features of unusual sensitivity to environmental stress compared to other foodborne pathogens, lack of adaptive mechanisms and the minimal capacity for recognizing and responding to environmental stress should not allow this organism to survive in the food chain. *C. jejuni* may undergo a variety of physiological changes during biofilm development. Survival in biofilms might explain why this bacterium with fastidious growth

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requirements could become the primary causative agent of foodborne gastroenteritis in industrialized countries.

The aim of this study is to investigate physiological changes of *C. jejuni* growing in 42°C microaerobic and 22°C aerobic conditions, in biofilm and planktonic state, by analyzing protein profiles through 1D SDS-PAGE.

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3.2 Materials and methods

3.2.1. Preparation and counting of biofilm and associated planktonic cells

Bacterial strain and culture conditions, preparation of biofilm and associated planktonic cells, plate counts and direct viable counts (Total cells were counted by Dapi staining) were the same as in the previous experiments described in Chapter 2.

3.2.2. Protein extraction

3.2.2-1. Protein extraction from planktonic cells

3.2.2-1-1. Protein extraction from planktonic cells by conventional method

The conventional method for protein extraction was based on standard Laemmli methods. Planktonic cell suspension was centrifugated at 1,800 x g for 20 min (Thermo IEC) to collect the pellet of planktonic cells. After discarding the supernatant, 250 µl TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) was added to the pellet and the suspension sonicated on ice at highest speed for 1 min (Fisher Scientific, sonic Dismembrator or Branson Sonic, Sonifier® cell disruptor). A 250 µl volume of lysis buffer (1.5 % Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and a few crystals of bromthymol blue) was added into the sonicated samples. The suspension was centrifugated at 21,000 x g for 30 min at 4°C to spin down the cell

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debris. Supernatant was collected and heated in a 95 - 100°C waterbath for 90 seconds. The sample was processed immediately or kept at -30 °C until further experiments (determination of protein concentration and SDS-PAGE). A 20µl aliquot of the sample could be loaded into the wells of the SDS-gel.

3.2.2-1-2. Protein extraction from planktonic cells directly into CHAPS

The procedures were the same as described in 3.2.2-1-1. , except that 250 µl CHAPS buffer (7M urea, 2M thiourea, 4% CHAPS, 1% DTE) was used instead of TE buffer.

3.2.2-1-3. Protein extraction from planktonic cells according to Trémoulet and others (2002) with modifications

Proteins of planktonic cells were extracted according to Trémoulet and others (2002) with modifications. Planktonic cells were spinned down by centrifugation at 1800 x g for 30 min at 4°C followed by discarding the supernatant. One ml Tris buffer (20mM of Tris, 5mM of EDTA and 5mM of $MgCl_2$, pH 7.5) was added to the planktonic cell pellets. Cells were sonicated on ice (Fisher Scientific, sonic Dismembrator, Model 100) three times for 2 min at the highest frequency. The suspension was centrifuged (Sorvall® RC-5B refrigerated superspeed centrifuge) at 14,000 x g for 10 min at 4°C to remove unbroken cells and cell debris. The supernatant was transferred to an acetone resistant tube for protein precipitation. Four volumes of cold acetone were

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added to 1 volume of each protein sample solution. The compounds were mixed and kept at -20°C overnight and then centrifuged at 4°C for 15 min at 15,000 x g. The supernatant was discarded and the pellet air dried to eliminate any acetone residue. Ten µl CHAPS buffer (7 M urea, 2M thiourea, 4% CHAPS, 1% DTE) and 10 µl lysis buffer (1.5 % Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and a few crystals of bromthymol blue) were added into each sample pellet. The colored solution was ready for loading in the wells of the SDS-gel.

3.2.2-2. Protein extraction from biofilm cells

3.2.2-2-1. Protein extraction from biofilm cells by conventional method

The same steps used for protein extraction from planktonic cells (3.2.2-1-1) could be adopted to extract biofilm cells obtained using glass beads and sonication according to the procedures described in section 2.2.2.

The proteins of the biofilm cells could also be extracted directly from the glass wool. A given weight of glass wool was moved to an eppendorf tube and buffer added in a 1:10 ratio (0.1 g glass wool need 500 µl TE buffer and 500 µl lysis buffer). The conditions of sonication and removing the cell debris were the same as described in section 3.2.2-1-1.

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3.2.2-2-2. Protein extraction from biofilm cells directly into CHAPS

Just like described above (3.2.2-2-1), two methods could be used to lyse biofilm cells. For biofilm cells obtained with glass beads and sonication, the steps to extract biofilm cell protein by directly into CHAPS were the same as described in section 3.2.2-1-2. For extraction directly from glass wool, CHAPS and lysis buffer were added in a 1:10 ratio as described above (3.2.2-2-1).

3.2.2-2-3. Protein extraction from biofilm cells by Trémoulet and others (2002) with modifications

The glass wool with attached biofilms was moved to an acetone resistant centrifuge tube (Teflon or polypropylene). Ten ml Tris buffer was added for each 1 g of glass wool. The biofilm cells were sonicated (Sonifier® cell disruptor, Branson Sonic) three times for 2 min with tubes in the ice using continuous mode. All liquid was squeezed from the glass wool which was then removed. The resulting suspension was centrifuged in a Sorvall centrifuge at 14,000 x g for 10 min at 4°C to remove unbroken cells and cell debris as described for planktonic cells (section 3.2.2-1-3) after removal of glass wool. The supernatant was collected and processed immediately (determination of protein concentration and SDS PAGE) or kept at -30°C until further experiments.

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3.2.3 Protein concentration determination

The protein concentration was determined using the Bio-Rad Protein Assay (Dye Reagent Concentrate, Cat No. 500-0006, Bio Rad Laboratories, Hercules, CA) which is based on the method of Bradford. Bovine serum albumin (BSA) was used as standard. The procedures for planktonic and biofilm cells were the same except that protein extracts from planktonic cells were diluted 10 times in distilled water before each assay, while extracts of biofilm cells were tested directly.

Eight hundred µl of five dilutions of BSA were prepared in the eppendorf tubes which were representative of the protein concentration of 2, 4, 6, 8 and 10 µg per ml after adding 200 µl dye reagent. Eight hundred µl of test samples were also pipeted to the eppendorf tubes. Two hundred µl of dye reagent concentrate (Bio-Rad Laboratories) was added to each tube, vortexed and incubated at room temperature for 10 min. OD values were measured in a Novaspec®II spectrophotometer (Biochrom) at a wavelength of 600nm.

A standard curve was plotted according to the absorbance readings of BSA by using Excel software. A linear regression equation was used to fit the linear standard curve. The sample protein concentrations were determined by interpolating the standard curve.

CHAPS did react with the Protein Assay due to its own dark red color. Lysis buffer was also dark brown in color due to bromthymol blue. Therefore, protein concentration could not be done when CHAPS and lysis buffer were present.

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3.2.4. One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

3.2.4-1. Gel preparation

The glass-plate sandwich (Model P8DS, Owl Separation System Inc, Portsmouth, NH) with 2 spacers in a one side sealed plastic bag was locked in the casting stand according to the manufacturer's instructions. To prepare the 15% acrylamide separating gel, 4.95ml 30% acrylamide solution, 2.5 ml 4X Tris·Cl (1.5M Tris-HCl pH8.8), 0.1 ml 10% SDS solution and 2.4 ml water were added into a 125 ml side arm flask. The mixture was degassed for about 5 min under vacuum followed by adding 50 µl 10% ammonium persulfate (AMPS) and 3.5 µl TEMED. The separating gel solution was immediately pipeted into the casting assembly along an edge of one of the spacers to the level of 2.5 cm from the top of the glass. One ml of water-saturated n-butanol was gently applied to cover the top of the gel by layering against the edge of one and then the other of the spacers. The gel was left for about 1 h at room temperature for polymerization.

After the polymerization of the separating gel, the water-butanol overlay was poured off by tilting the casting stand and the gel surface was rinsed with 1X Tris·Cl/SDS, pH8.8 by using the same manner as applying the water-butanol overlays.

Like the preparation of the separating gel, 1 ml of 30% acrylamide solution, 1.25 ml 4X Tris·Cl (0.5 M Tris-HCl, pH 6.8), 0.05ml 10% SDS solution and 2.7 ml water were added into the side arm vacuum flask to prepare 5 ml of 4% stacking gel.

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The solution was degassed as described for the separating gel before catalysts (25µl 10% AMPS and 2.5 µl TEMED) were added. After swirling gently, the stacking solution was filled onto the top of the glass sandwich at once. A 10-well comb was inserted in the center of the sandwich. The stacking gel solution would polymerize after 30-45 min at room temperature.

3.2.4-2. Running the gel

The treated biofilm and planktonic protein samples were loaded after the comb was carefully removed. The gel sandwiches were attached to the buffer chamber according to the manufacturer's instructions. One time SDS electrophoresis buffer (3g of Tris, 14.4 g of glycine, 10 ml of 10% SDS solution in 1 liter of deionized water) was filled in both upper and lower buffer chamber of the electrophoresis apparatus. A power supply was then connected to the cell and run at constant current of 15mA or at constant voltage of 70V. After the bromophenol blue tracking dye had reached the bottom of the separating gel, the electrophoresis could be stopped by disconnecting the power supply.

3.2.4-3. Staining the gel by silver staining and Coomassie Blue staining

The gel was removed from between the glass plates after electrophoresis and submerged in Coomassie blue stain working solution (1% coomassie Blue R-250) for at least 1h followed by placing in destaining solution I (50% methanol, 10% acetic

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acid) for 1h and then moving the gel into destaining solution II (5% methanol and 7% acetic acid). The gel was destained until the blue protein bands stood out and the background was clear (overnight). Once the gel was completely destained, it could be photographed using a Syngene Gel Documentation system (Fisher Scientific).

Gels were also treated by silver staining (Bio-Rad) according to the protocol provided by the supplier. After the gel was immersed in oxidizer working solution for 5 min, it was washed by large volumes of water within 15 min and then kept in the silver reagent working solution for 20 min. After the silver staining, the gel was quickly rinsed in water (maximum 30 seconds). Then, the developer working solution was poured onto the gel. The solution was quickly poured off and the fresh developer was added when a brown or smokey precipitate appeared. This step was repeated if precipitate appeared again until the solution remained clear. Developer solution was changed every 5 min until the desired intensity was obtained. Acetic acid (5%) for 15 min was used to stop the developing. The gel was photographed by a Syngene Gel Documentation System as described for Coomassie Blue stained gels.

3.2.5. Estimate of unknown protein's molecular size

The relationship between the relative mobility and log molecular weight is linear. Therefore, the molecular size of unknown protein can be estimated by comparing the relative mobility of the molecular size of protein marker (MBI Fermentas, #SM0431).

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3.3. Results

3.3.1. Total protein present in *C. jejuni* cells grown under different conditions: 42°C microaerobic biofilm, 42°C microaerobic planktonic, 22°C aerobic biofilm and 22°C aerobic planktonic

Biofilm cells contained more protein (14.4 ± 17.8 pg) than planktonic cells (1.11 ± 1.17 pg) under the condition of 42°C microaerobic (Table 3.1), although this difference was not significant (P=0.156) due to the large standard deviation. In the 22°C aerobic environment, individual biofilm cells had on average significantly more protein (74.4 ± 38.2 pg) than planktonic cells (12.1 ± 6.25 pg, P=0.017, Table 3.1). Planktonic cells grown at 22°C aerobically contained significantly more protein than cells grown at 42°C microaerobically (P=0.010). Biofilm cells, grown at 22°C aerobically had significantly higher protein contents than those grown at 42°C microaerobically (P=0.012, Table 3.1).

3.3.2. Comparison protein profiles between 2 day old biofilm and planktonic cells growing in 42°C microaerobic and 22°C aerobic conditions

Total protein patterns of the whole cell lysate for 42°C microaerobic biofilm and planktonic cells, and 22°C aerobic planktonic cells are shown in Fig 3.1. Approximately 15 bands were observed for the protein extracts of planktonic cells grown at 42M in a gel stained by coomassie blue (Fig 3.1 A). A significant difference

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could be observed for the protein bands of 42M biofilm cells (Fig 3.1 B). Unlike the planktonic cells, 42°C microaerobic biofilm cells expressed only 5 bands (75.9 kDa, 66.2 kDa, 60.0 kDa, 37.0 kDa and 25.0 kDa). The other protein bands observed for planktonic cells seemed down regulated in the biofilms. Protein bands of 22°C aerobic planktonic cells could not be visualized by coomassie blue staining. After silver staining, 4 bands could be seen (66.2 kDa, 57.5 kDa, 38.0 kDa and 36.0 kDa, Fig 3.1 C). No protein bands could be seen for 22°C aerobic biofilm cells irrespective of protein extract method and the level of protein concentration and the type of staining.

3.3.3. Comparison protein profiles between 2 day old biofilm present and non-biofilm present 42°C microaerobic planktonic cells

As indicated in Fig 3.2, the 2 day old biofilm present and non-biofilm present 42°C microaerobic planktonic cells revealed bands having different molecular weights. Between the marker range of 66.2 kDa and 116.0 kDa, planktonic cells with biofilm present had at least 6 protein bands, while only 3 bands could be observed in the cells without biofilm present.

3.3.4. Effectiveness of silver staining

Fig 3.3 shows two images of proteins from 42°C microaerobic planktonic cells stained by coomassie blue staining and silver staining. It could be obviously observed that silver staining was more sensitive than coomassie blue staining.

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3.4. Discussion

Protein profiles of biofilm and planktonic *C. jejuni* grown under 42°C microaerobic and 22°C aerobic conditions analyzed by one-dimensional protein electrophoresis were compared. Different protein patterns were observed that could be the result of *C. jejuni* changing its physiology upon growing in the different environments. The investigation of the physiology of *C. jejuni* by 1D SDS-PAGE has not been reported. However, Dykes and others (2003) determined the differential expression of proteins between 37°C microaerobic *C. jejuni* cells grown in planktonic and biofilm modes by 2D SDS-PAGE. The ranges of protein bands of 42°C microaerobic planktonic and biofilm cells in this study somewhat differed from the ranges of protein spots of 37°C microaerobic planktonic and biofilm cells reported by Dykes and others (2003). Many protein bands of 42°C microaerobic planktonic cells were shown above 45.7 kDa by this experiment, while 45.7 kDa was the highest molecular weight of protein spots of 37°C microaerobic planktonic cells detected by Dykes and others (2003). For the biofilm cells, we could not detect any proteins below 25 kDa, however the experiment by 2D SDS-PAGE traced 4 protein spots ranging from 9.3 kDa to 13.1 kDa. In addition, the most notable feature of the two-dimensional protein profiles of 37°C microaerobic biofilm cells was a cluster of six proteins in the 45 to 55 kDa range, which is similar to this work where most of the proteins also gathered above 45 kDa (Fig 3.1). Two dimensional gel electrophoresis (2DE) can resolve proteins in a higher resolution. Therefore, 2DE analysis for biofilm and

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planktonic *C. jejuni* growing in other non-optimal conditions, such as 22°C aerobically should be performed.

Although it is common to use proteomic analysis to investigate differential protein expression of bacteria during biofilm formation, most studies use high-resolution two dimensional gel electrophoresis instead of 1D SDS-PAGE. However, Oosthuizen and others (2002) separated the 2 h and 18 h old planktonic and biofilm proteins of *Bacillus cereus*, a dairy-assocated toxigenic bacterium, by 1D SDS-PAGE. There were no different protein profiles between planktonic and biofilm cells after 2 h of growth. However, distinct protein bands could be identified between the cultures grown in the presence and in the absence of glass wool after 18 h incubation. Furthermore, total protein, S-layer protein and lipopolysaccharides (LPS) of biofilm cells of *Aeromonas hydrophila*, a common fish pathogen, were analysed through 1D SDS-PAGE and compared with that of planktonic cells by Asha and others (2004). This report indicated that about 15 proteins were repressed in the whole cell lysate of biofilm cells and 3 new proteins were expressed compared to planktonic cells. Moreover, the S-layer proteins were lost and LPS showed an additional high molecular weight band in biofilm cells compared to its planktonic counterpart.

Similar observations were also made with other bacteria by 2-D gel electrophoresis. There existed 45 differences in the protein profiles of planktonic and biofilm cells of *Pseudomonas putida* and 15 proteins were up-regulated, while 30 proteins were down-regulated after it attached to the surface of silicone for 6 h (Sauer and Camper 2001). Proteomic analysis of *Listeria monocytogenes* by Trémoulet and others (2002) revealed that 31 proteins varied significantly between planktonic and

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biofilm cells. Twenty-two proteins were up-regulated and 9 were down-regulated. *P. aeruginosa* under iron limited conditions or developed on glass wool and starved cells of *A. hydrophila* and *Vibrio. sp* also presented changes in protein profiles (Asha and others 2004).

Oosthuizen and others (2002) applied both one dimensional (1D) and two dimensional (2D) gel electrophoresis to reveal the phenotypic differences between 2- and 18-h-old biofilm and planktonic *B. cereus* cells. The protein profiles obtained with the two methods were different. For the 2 h old *B. cereus* cells grown in the presence and absence of glass wool no differences were observed between the protein profiles and only a few proteins could be detected by one dimensional SDS-PAGE. However, the 2D protein patterns of 2 h old planktonic and biofilm or the planktonic cells growing in the presence of glass wool were distinctively different. Fifteen protein spots were uniquely expressed in the biofilm mode of growth after 2 h. The proteomes of planktonic cells and planktonic cells with glass wool present respectively produced 1 and 2 unique protein spots. Furthermore, both the biofilm cells and the glass wool present planktonic cells produced a number of proteins for which expression was up-regulated compared to that of planktonic cells. For the 18 h old cells, three unique bands were identified in both planktonic and combined glass wool present planktonic and biofilm cultures by 1D SDS-PAGE. On the other hand, comparison of 2D protein spots of 18 h old cultures indicated that a single unique protein was present in the planktonic proteome and seven unique proteins were owned by each biofilm and glass wool present planktonic proteomes. The experiment of Oosthuizen and others (2002) demonstrated that 2D presented a higher resolution and different protein profiles could

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be detected by conducting two different dimensional gel electrophoresis, which again implied that it would be necessary to analyse *C. jejuni* protein expression grown in non-optimal conditions by performance of 2D gel electrophoresis.

As shown in Fig 3 (A), a high resolution of protein bands of 42M planktonic cells was achieved by loading approximately 28 µg of proteins. We tried to also prepare 28µg of proteins for the other three conditions in order to get visible protein bands. However, it was very difficult to get the same amount of proteins from biofilm cells when using similar volumes as for planktonic cultures. By increasing the culture volume, in theory, approximately 114 µg of proteins of 42M biofilm cells could be obtained, but these were quite dilute in a large volume of water. Acetone precipitation was required to concentrate these proteins, but only a small fraction of the proteins could be recovered and loaded in the gel. For the same reason, smaller amounts of protein were obtained for *C. jejuni* growing at 22A in both biofilm and planktonic modes. Only 7 µg proteins of 22A planktonic cells were obtained and much slighter protein bands could be observed after silver staining which is a more sensitive staining. We even could not recover sufficient amounts of proteins from 22A biofilm cells to express visible protein bands.

Our results indicated that the proteome of planktonic *C. jejuni* cells is modified when grown in the form of biofilm, or in non-optimal conditions, and even in its planktonic mode when biofilm is present. This proved that *C. jejuni* underwent a variety of physiological changes during biofilm development or under environmental stress. Understanding the role of these specific proteins during the biofilm development should permit a better understanding of the mechanisms sustaining the

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proliferation and the resistance of bacteria on abiotic surfaces (Trémoulet and others 2002). Dykes and others (2003) tentatively identified the different protein spots between the biofilm and planktonic 37°C microaerobic *C. jejuni*, but they were not sequenced, and no other papers reported the identification of unique proteins of *C. jejuni* under various conditions. Therefore, future work might focus on identities of these proteins separated by 2D gel electrophoresis.

Protein identification of other planktonic bacteria and their biofilm courterparts have been reported. Sauer and Camper (2001) identified the selected 2-D gel protein spots of *P. putida* which were down-regulated after 6 h of attachment. N-terminal sequence analysis revealed that the proteins with reduced levels were membrane protein (NlpD), transport protein (PotF2), and the amino acid metabolism proteins (NI^b, AnsB, ArcA, ArcB and GlyA3). In addition, the excised specific protein spots of *B. cereus*, which varied reproducibly in cellular concentration as a consequence of changes in the growth conditions were subjected to N terminal sequencing and identified with high confidence (Oosthuizen and others 2002). These proteins were catabolic ornithine carbamoyltransferase and L-lactate dehydrogenase. YhbH, a member of the sigma 54 modulation protein family which is strongly induced in response to environmental stresses and energy depletion through δ^B and δ^H were observed at increased levels within 2 h in both attached cells and planktonic cultures of *B. cereus* growing in the presence of glass wool (Oosthuizen and others 2002). Furthermore, some *L. monocytogenes* proteins that are modified by growth conditions were also identified (Trémoulet and others 2002). They included dehydrogenase

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(pdhD), 30S ribosomal protein S2 (rpsB), 6-phosphofructokinase (pfk), and the flagellin protein (flaA).

Protein identification could help understand the physiological changes of a cell exposed to a certain growth condition. In the study of Sauer and Camper (2001), proteins of *P. putida* involved in amino acid metabolism, such as, AsnB, ArcA, ArcB and GlyAd, were down-regulaed following initial attachment, and both planktonic and biofilm cultures were cultivated with glutamate as the sole carbon source. The results suggested that in addition to alterations in structural components of cells after initial adhesion to a surface, bacteria may also undergo metabolic changes. Also, NlpD, the outer membrane lipoprotein which is believed to have a cell wall lytic function was down-regulated following the initial attachment of *P. putida*, which indicated that membrane proteins had a substantial influence on attachment and may also play a role in early biofilm development. Further, Oosthuizen and others (2002) found that the YhbH protein was up-regulated in both biofilm and glass wool present planktonic cells of *B. cereus* compared to its planktonic levels. The authors concluded that this protein played an important role in the switch from the planktonic phenotype to the biofilm phenotype (Oosthuizen and others 2002). This may also indicate its dependence on both δ^B and δ^H, since it displayed significant sequence identity of YvyD of *B. subtilis* which is δ^B and δ^H dependent (Oosthuizen and others 2002). As well, the up-regulation of YhbH in planktonic cells with glass wool present may indicate that these cells sensed the same future stress as the attached cells and started to induce a general stress response (Oosthuizen and others 2002). Again, Trémoulet and others (2002) proved that the proteome of *L. monocytogenes* was

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greatly influenced by biofilm development on a surface and specific genes may be induced under such conditions. One protein indentified which decreased as a result of biofilm growth was flagellin which further substantiates the well known fact that flagella are directly implicated in the initial adhesion of *L. monocytogenes*. Trémoulet and others (2002) also reported that two key enzymes involved in global carbon metabolism, pyruvate dehydrogenase (PdhD) and 6-phosphofructokinase were among the proteins with increased expression as a result of biofilm formation. PdhD catalyses the overall conversion of pyruvate to acetyl-Coa and CO_2 and 6-phosphofructokinase is the key control step of glycolysis and is primarily regulated by intracellular ATP levels. This finding indicated that biofilm development could affect the central metabolism of *L. monocytogenes*. Moreover, the levels of ribosomal proteins YvyD and rpsB were increased in biofilms of *L. monocytogenes*. These two proteins may act as sensors to detect physical or chemical changes in the environment of biofilms since Yvyd is known to be induced under various stress conditions, such as starvation, heat, ethanol and salt stress in *B. subtilis*. Additionally, two proteins known to be increased in oxidative stress conditions were also up-regulated in biofilm conditions.

In this thesis, different protein extraction methods were tested to obtain the protein bands of *C. jejuni* growing under different conditions. As indicated in the results (section 3.3.5), the proteins of 42°C microaerobic planktonic cells could be successfully visualized by the traditional method using the treatment buffer containing Tris SDS, glycerol, 2-mercaptoethanol and bromthymol blue followed by either coomassie blue staining or silver staining. However, using this method neither 42°C

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microaerobic biofilm cell proteins nor proteins of 22°C aerobic planktonic and biofilm cells could be visualized after electrophoresis, even when 10 times increased cell volumes were used. Protein bands of 42°C microaerobic planktonic and biofilm cells and 22°C aerobic planktonic cells were visible after extracting directly into CHAPS buffer which contains urea, thiourea, CHAPS and DTE. However, the bands of 22°C aerobic planktonic cells could only be observed after silver staining. It was not possible to visualize the protein bands of 22°C aerobic biofilm cells by any of above methods. Cultures growing under different conditions need different methods to extract proteins which may indicate that the properties of cells and proteins are different for each condition.

C. jejuni planktonic cells grown at 42°C microaerobically had the highest total protein levels and the protein bands of 42°C microaerobic planktonic cells could be observed without raising the total protein amounts. For the other conditions, several methods were tested to increase the protein volume. First, we enlarged the amount of cells by growing 10 fold volumes of cultures, which was an effective method that was performed throughout the experiments in getting protein bands of *C. jejuni* growing under different conditions. The protein bands of 22°C aerobic planktonic cells were only visible when 10 times culture volumes and silver staining were used (Fig 3.1) Acetone precipitation was also used to concentrate proteins of *C. jejuni*. The protein bands of 42°C microaerobic biofilm cells shown in Fig 3.1 were obtained after acetone precipitation. However, the proteins of 22°C aerobic biofilm cells could not be visualized by any of the methods described above. Biofilm cells grown at 22°C aerobic had the lowest total protein concentrations compared to the cells grown under other

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conditions (Table 3.1). This result might indicate that the protein volume should be further increased in future research of the proteome of 22°C aerobic *C. jejuni* biofilms.

We also evaluated the cell concentration of *C. jejuni* of each growing condition by microscopy along with measuring the protein concentration. Therefore, the level of protein per cell could be calculated. Biofilms grown at 22°C aerobic had the highest levels of protein per cell, while 42°C microaerobic planktonic cells had the least. This was obviously due to the difference in cell number in the samples which was much larger between the two conditions than the difference of total protein. This difference may be due to cell clumping observed by microscopy (see previous chapter). Determining the actual number of cells represented in each clump would be needed to determine whether protein concentration per cell is increased under stress conditions.

Protein profiles of planktonic cells grown with biofilm present and without biofilm present (42°C microaerobic) obtained by one dimensional SDS-PAGE were compared. The protein profiles of these two conditions were different, which agrees with the conclusions for *B. cereus* reported by Oosthuizen and others (2002). As well, the results of silver staining evaluation showed that silver staining is more sensitive than Coomassie Blue staining and this has been reported in the literature before.

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Table 3.1. Culturability, total cell numbers, protein amount for extracted by Trémoulet and others (2002) of biofilm and planktonic *C. jejuni* growing in 42°C microaerobic and 22°C aerobic conditions.

	42M, biofilm	42M, planktonic	22A, biofilm	22A, planktonic
Plate counts (log_{10} cfu/ml)	6.21 ± 0.13	7.91 ± 0.44	<1.3	<1.3
Total cell counts (log_{10} cells or clumps/ml)	7.07 ± 0.69	8.43 ± 0.75	5.56 ± 0.40	6.41 ± 0.18
Total protein (µg)	89.36 ± 21.07	141.58 ± 90.63	24.68 ± 7.81	32.91 ± 26.38
Protein pg/cell or clump	14.4 ± 17.8[a]	1.11 ± 1.17[a]	74.4 ± 38.2[b]	12.1 ± 6.25[c]

42M: 42°C microaerobic
22A: 22°C aerobic
a, b, c, d indicates the statistic difference
Same letter represents no significant difference at $P<0.05$?
One clump is counted as one cell in the microscopic images as indicated in Chapter 2.

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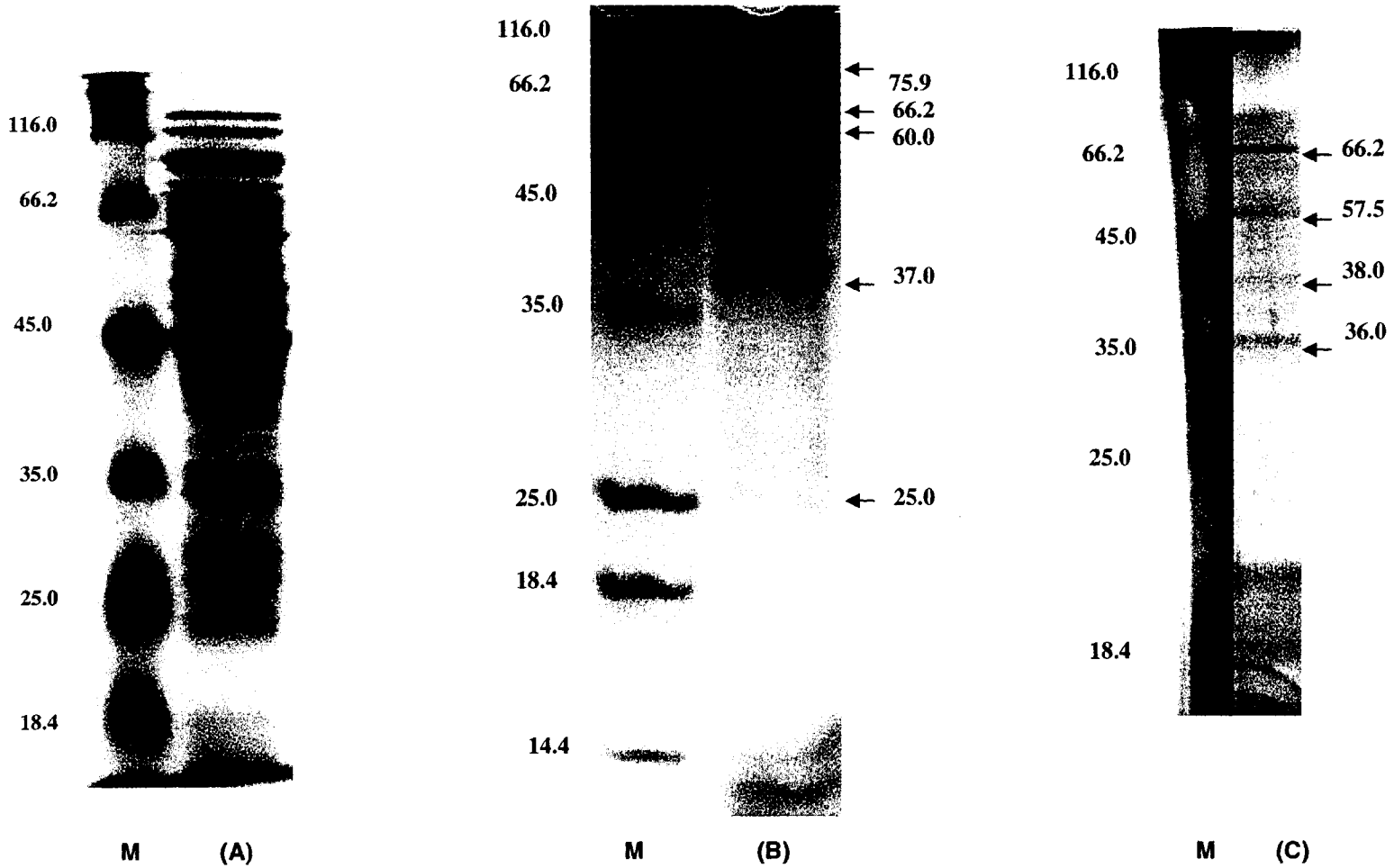
Figure 3.1. SDS-PAGE of *C. jejuni* for 42°C microaerobic planktonic and biofilm cells and 22°C aerobic planktonic cells



(A) is representative for 42°C microaerobic planktonic cells, was extracted by conventional method and stained by coomassie blue. Approximately 28 µg of proteins were loaded.
(B) 42°C microaerobic biofilm cells, extracted by 3.2.2-2-3 and stained by coomassie blue. In theory, approximately 114 µg of proteins were loaded. However, control experiments showed that some of the proteins would be lost when suspended in 20 µl loading buffer.
(C) 22°C aerobic planktonic cells, was extracted directly by CHAPS and stained by silver stain. Approximately 7 µg of proteins were loaded.
M indicates the marker (MBI, Fermentas, # SM0431) and the unit of molecular weight was kDa.

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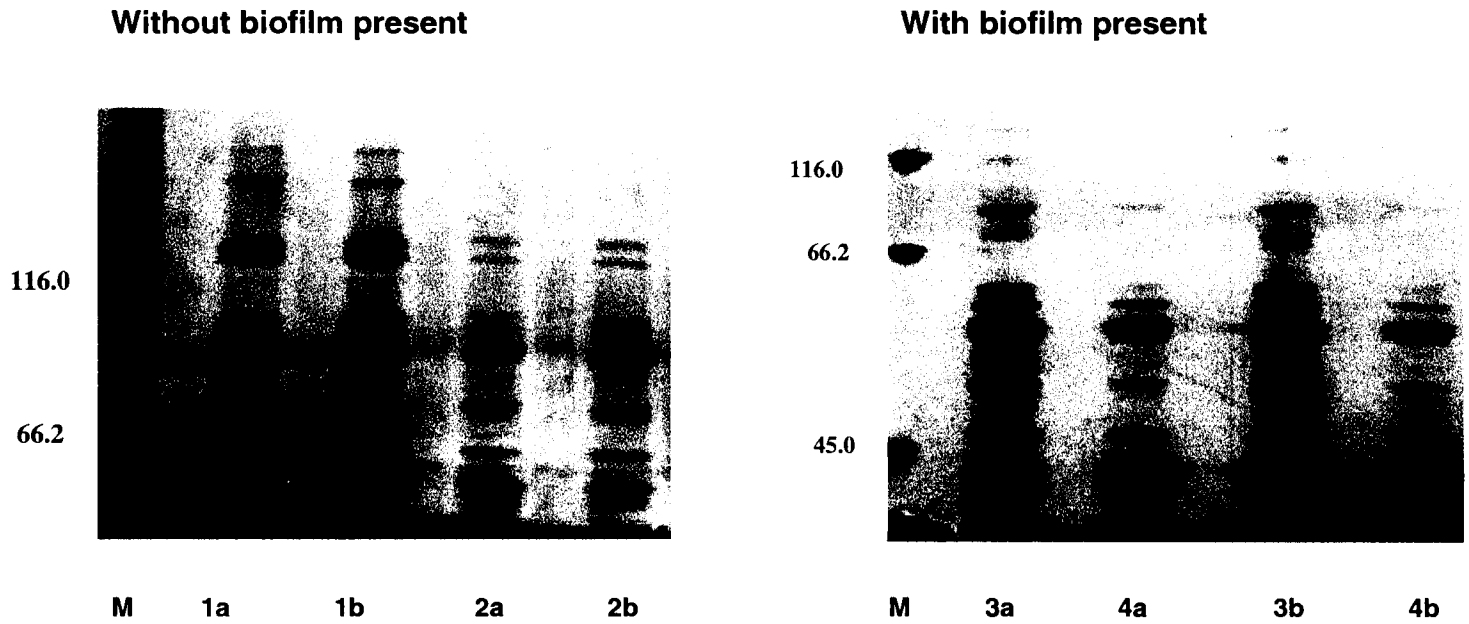
Figure 3.2. Comparison of protein profiles for planktonic cells grown at 42°C microaerobic with and without biofilm present



1a, 1b, 2a, 2b were 42°C microaerobic planktonic cells without biofilm present. Samples 1a+1b and 2a+2b were identical, samples 2a+2b were 10 fold dilutions of samples 1a+1b

3a, 3b, 4a, 4b were 42°C microaerobic planktonic cells with biofilm (glass wool) present. Samples 3a+3b and 4a+4b were identical, samples 4a+4b were 10 fold dilutions of samples 3a+3b.

Both gels were stained by silver stain.

M indicates the marker (MBI, Fermentas, #SM0431) and the marker unit was kDa.

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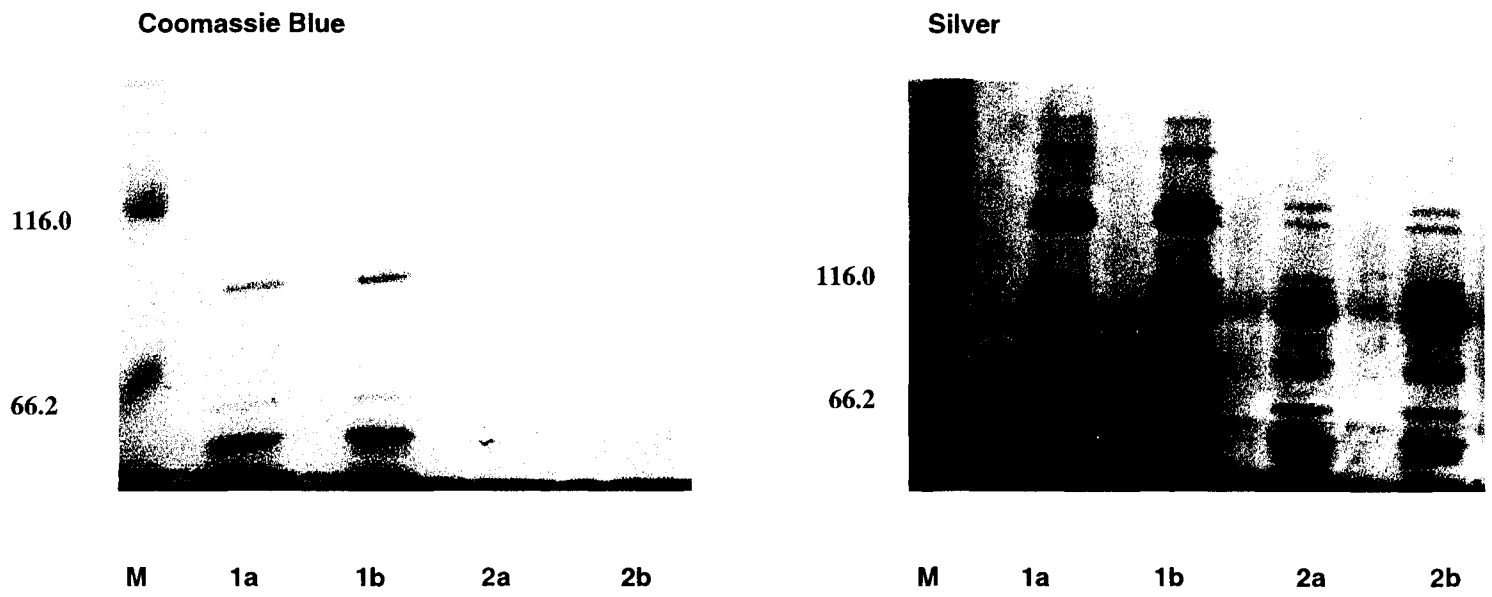
3.3. Comparison of Coomassie Blue staining and silver staining



Samples 1a+1b are duplicates of a protein extract from planktonic cells (42°C, microaerobic).

Samples 2a+2b are ten fold dilutions of 1a+1b.

M indicates the protein marker (MBI, Fermentas, #SM0431) and the marker unit was kDa.

Chapter Four

Summary and Conclusions

In recent years, "emerging" pathogens such as *Listeria monocytogenes*, *Bacillus cereus*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, and *Campylobacter jejuni* have gained widespread importance as agents of foodborne disease instead of the traditional three human foodborne pathogens of concern: *Staphylococcus aureus*, *Salmonella,* and *Clostridium botulinum* (Solomon and Hoover 1999). *C. jejuni* has made an astounding leap to the top over the past ten years to be considered the leading cause of acute bacterial gastroenteritis in the United States and perhaps in the world since its first recognition as a cause of foodborne gastrointestinal disease in 1977. It has caused one to seven million cases of enteritis per year in the US, resulting in 100 to 500 deaths (Solomon and Hoover 1999). As we know, *C. jejuni* is extremely susceptible to a wide variety of antimicrobial treatments, food processing methods, environmental stresses and it also is difficult to culture and maintain in the laboratory.

Biofilms have been of considerable interest in the context of food hygiene (Kumar and Anand 1998). Due to the presence of an exopolysaccharide matrix which may act to various degrees as a diffusion barrier, molecular sieve and adsorbent, it is well established that bacterial biofilms exhibit a higher resistance to antimicrobial treatments than individual cells grown in suspension (Kumar and Anand 1998). In addition, several molecular signals and growth phase regulated genes, such as *rpoS*

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are involved during biofilm formation (Corona-Izquierdo and Membrillo-Hernández 2002). We hypothesed that biofilms might be the niche to protect *C. jejuni* against adverse environments.

In this study, Glass wool was selected to form biofilm throughout all the experiments as it provides a large surface-to-volume ratio and allows separation of the biofilm biomass from the surrounding planktonic cells for further characterization (Oosthuizen and others 2002). First of all, the culturability of *C. jejuni* grown in 42°C microaerobic, 42°C aerobic, 22°C microaerobic and 22°C aerobic was investigated using standard plate counts. Our results agreed well with other literature that 42°C microaerobic was the optimal condition for *C. jejuni.* However, large variability existed among the other three conditions. The highest biofilm cell counts within the three non-optimal conditions were for *C. jejuni* grown at 22°C aerobically. *C. jejuni* is a thermophilic bacterium. It could be speculated that cells growing under 42°C aerobic condition should perform better than growing at 22°C aerobic condition. However, our results showed that *C. jejuni* yields more colony forming units in 22A than in 42A. Thus, 42°C microaerobic and 22°C aerobic were the two conditions selected for all other experiments.

We assumed that *C. jejuni* entered a viable but non-culturable (VBNC) state due to the low plate counts of 42°C microaerobic biofilm cells and 22°C aerobic planktonic and biofilm cells. A bacterium in the VBNC state is defined as a cell which is metabolically active, but incapable of undergoing the cellular division in or on a medium normally supporting growth of that cell (Besnard and others 2000). Traditional culture methods are inefficient in detecting VBNC cells. The activity of VBNC cells is

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routinely studied by microscopy methods (Federighi and others 1998). Secondly, Live/Dead and Dapi nucleic acid stain were used to stain 42°C microaerobic and 22°C aerobic planktonic and biofilm cells. Stained cells were observed by the FV300 Olympus confocal microscope or the IX 51 Olympus inverted microscope. Under the 42°C microaerobic condition, both planktonic and biofilm cell counts were not significantly different ($P>0.05$) by plating and by microscopy. However, counts were significantly lower ($P<0.05$) by plate counting than by microscopy for the 22°C aerobic condition. The biofilm cell numbers obtained by microscopy were not significantly different ($P>0.05$) between 42M and 22A which might explain that biofilm *C. jejuni* cells could contribute to *C. jejuni* contamination under normal working conditions. In addition, clumps were observed in the images of biofilm cells, especially for 22A biofilm cells. Therefore, in order to determine the role of clumping in enhancing survival of *C. jejuni* biofilms, the number of cells in clumps and the clump sizes should be investigated in future research. Further, Live/Dead stain was used to estimate viable cells. However, our results did not seem to reflect the real live and dead cells. A method to distinguish VBNC *C. jejuni* cells would be needed for further experiments. Live/Dead only used with other bacteria, such as *E. coli* (Perrot and others 1998), but not with *C. jejuni.*

C. jejuni may undergo a variety of physiological changes during biofilm development, which might explain why this fastidious bacterium could become the primary causative agent of foodborne gastroenteritis in industrialized countries. Proteomic analysis by 1D SDS PAGE was used in this study to characterize physiological changes of *C. jejuni* growing in biofilms at 42°C microaerobic and 22°C

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aerobic conditions. Protein contents of cells grown in 42°C microaerobic biofilm, 42°C microaerobic planktonic, 22°C aerobic biofilm and 22°C aerobic planktonic mode were investigated. Differences of protein profiles of 2 day old biofilm and planktonic *C. jejuni* developed in 42°C microaerobic and 22°C aerobic environments were compared in our experiments. The profiles of 2 day old 42°C microaerobic planktonic *C. jejuni* grown in the presence and absence of a biofilm were compared as well. The effectiveness of different protein extraction methods and different staining methods for gels were also evaluated.

We measured the protein amount of cells grown in each experimental condition. Biofilm cells contained more protein than their planktonic counterparts. Viable but not culturable cells which exit in biofilm mode cells might have the increased amount of proteins. However, no reports have indicated to date whether protein amount in cells is related to cell viability which is required for virulence. Significant differences of protein profiles were observed not only between biofilm and planktonic cells of *C. jejuni*, but also between two growing conditions which were 42°C microaerobic and 22°C aerobic. Furthermore, 42°C microaerobic planktonic cells expressed different protein patterns when growing in broth containing glasswool with biofilms compared to growing in pure broth without biofilms. Our results supported the conclusions of other researches studying the biofilm proteome of *Listeria monocytogenes, Bacillus cereus,* and *Pseudomonas putida*, which was that cells undergo phenotypic changes during biofilm development. Testing different protein extraction methods showed that CHAPS buffer should be applied when extracting biofilm proteins. Our work on commassie

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blue staining and silver staining confirmed other reports that silver staining is more sensitive.

As 2-D electrophoresis allows the separation of several hundred proteins in a single gel, this technique has become an important tool for proteomic studies investigating cellular physiology (Trémoulet and others 2002). Also, protein patterns of 2 h and 18 h old biofilm and free floating *Bacillus cereus* analyzed by 1-D SDS PAGE and 2DE were different (Oosthuizen and others 2002). Thus, 2-D electrophoresis is strongly recommended to further study the *C. jejuni* biofilm proteome. In addition, no reports have identified the specific proteins expressed by *C. jejuni* during its biofilm formation and understanding the role of these unique proteins could help to reveal the mechanisms of the capacity of *C. jejuni* surviving in different environments. Therefore, the identification of the modified *C. jejuni* biofilm proteins after 2DE might be a major objective for these *C. jejuni* proteomic studies.

Controlling microorganisms is essential in food processing for providing safe, wholesome food to consumers. This thesis demonstrated that *C. jejuni* growing in biofilms presented different survival capacity and different physiological features compared to its planktonic counterpart under different conditions. New strategies should be implemented to control *C. jejuni* biofilms in the food industry.

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