EXPRESSION OF OXIDATIVE STRESS RESPONSE GENES IN *Campylobacter jejuni* BIOFILMS

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ABSTRACT

EXPRESSION OF OXIDATIVE STRESS RESPONSE GENES IN *Campylobacter jejuni* BIOFILMS

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Campylobacter jejuni is a Gram-negative microaerophilic bacterium that is widely believed to be the number one cause of food borne gastroenteritis and diarrhea caused by bacteria. The ability to form biofilms may provide protection from oxygen by increasing the expression of genes responsible for oxidative stress protection. The genes that were tested were the *Alkyl Hydroperoxide Reductase C (ahpC)*, *Ferrodoxin (fdxA)*, *Catalyse (katA)*, and *Super Oxide Dismutase (sodB) genes, 16S rRNA* and *Gyrase (gyrA)* genes. When *C. jejuni* planktonic and biofilms cells were tested in microaerobic conditions there was no statistically significant differences in the expression of *ahpC (p=0.139), katA (p=0.065), or sodB (p=0.136).* There were differences in *fdxAfdxA (p=0.008)*, *gyrA (p=0.048)*, and *16S rRNA (p=0.002).* Two methods of qRT-PCR were tested; there was no statistically significant difference between using an ABI Prism 7000, or the Cepheid Smart Cycler (p=0.776). There were also no differences between the use of either a One-Tube or Two Tube RT-PCR protocol (p=0.388). Differences were found in the *ahpC* (p=0.007), *fdxAfdxA* (p<0.001), and *sodB* (p<0.001) genes of *C. jejuni* when grown in aerobic or microaerobic biofilms.

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Chapter 1: Literature Review

1.1 Introduction.

Infection from *Campylobacter* is the leading cause of bacterial gastroenteritis and diarrhea in the developed world (Allos, 2001). Of all *Campylobacter* infections 95% are caused by the species *Campylobacter jejuni* and *Campylobacter coli* (Blaser et al, 1983). This makes *C. jejuni* a very important human pathogen. Despite its importance to human health, much of *C. jejuni's* physiology remains unknown. The lack of information on *C. jejuni* is due to its fastidious growth requirements, initial difficulties in genetic manipulations, and differences in interstrain infectious abilities (Crushellet al., 2004). Therefore, studies seeking to uncover the mechanisms that *C. jejuni* uses to survive in the environment are crucial to our understanding of this microorganism.

1.2 History.

Campylobacters have been observed since 1886 (Park, 2002). The date when they were first positively identified is questionable due to culture and morphological techniques used at the time. What is known is that in1972 *Campylobacters* were recognized as a significant cause of illness in humans (Dekeyser et al, 1972). *Campylobacters* were reported to cause disease in humans before that time, but they were rarely reported as a major cause of illness. Cases of infection in humans were thought to be caused by *Vibrio fetus*, which is now known as *Campylobacter fetus* (Allos, 2001). The genus *Campylobacter* was not formally adopted until 1973 (Blaser et al., 1983). By the 1980's *Campylobacter* was considered the most common cause of diarrhea caused by bacteria in the world (World Health Organization, 2000).

1.3 General Physiology of *Campylobacter jejuni.*

C. jejuni are Gram-negative spiral rod shaped bacteria. They are very motile, using their bipolar flagella to move in a corkscrew darting motion (Blaser et al., 1983). The resulting rapid spiral darting motility of *Campylobacter* is suggested to facilitate movement in thick mucus (Shigematsu et al., 1998). This may allow *Campylobacter* to stay active in the gastrointestinal track. There is significant variation between *C. jejuni* isolates. Currently, based on o-antigen typing, over 60 serotypes have been reported (Wassenaar and Blaser, 1999). This level of variance between isolates makes *C. jejuni* one of the most diverse species of enteric bacteria. Many reasons have been given for the level of diversity seen between populations of *C. jejuni* (Parkhill et al., 2000). The main mechanism for this diversity includes the hypervariability seen in the genome. Another mechanism is *C. jejuni's* ability to take up exogenous DNA (Wassenaar and Blaser, 1999). *C. jejuni* is a microaerophilic organism. The optimal atmosphere for its growth is an oxygen level of 5%, a carbon dioxide level of 10% with the balance of nitrogen (Park, 2002).

The optimal growth temperature of *C. jejuni* is 42°C, which is the temperature of the avian gut, but it also grows well at 37°C. *C. jejuni* also has a minimum growth temperature of around 32°C (Hazeleger et al, 1998). Interestingly, once the temperature falls a few degrees below 32°C, *C. jejuni's* growth rate is reduced to zero. However, it can remain biologically active at temperatures as low as 4°C. This activity below optimum growth temperature can be seen by the detection of considerable production and usage of adenosine triphosphate (ATP) at temperatures as low as 4 ^{o}C (Hazeleger et al.,

1998). However, polymerase chain reaction (PCR) analysis could not detect any genes that code for cold shock proteins in the *C. jejuni* genome (Parkhill et al., 2000).

C. jejuni can also enter a viable but non-culturable state (VBNC). The VBNC state is detected by the change from spiraled rod phenotype to a coccoid phenotype (Buck et al., 1983). Numerous stressors, such as prolonged exposure to oxygen, low temperatures, and nutrient starvation, can cause the change to a VBNC state in *C. jejuni*. Also, the different forms of stress cause *C. jejuni* to enter into a VBNC state at different times (Tangwatcharin et al., 2006). There is some debate in the literature as to whether the coccoid morphology is a true VBNC state or if it is just a degenerate form of *Campylobacter* growth (Park, 2002).

1.4 Pathophysiology of *Campylobacter jejuni*.

Campylobacteriosis is a highly variable illness. The majority of cases present with non-specific symptoms, which include headache, myalgia, fever, and chills (Blaser, 1997). The major clinical symptoms defining the disease are an acute inflammatory abdominal disease with symptoms that include sharp abdominal pain that can mimic appendicitis, and watery diarrhea that can become bloody (Blaser, 1997). The incubation period ranges from 24 to 72 hours with an average of 48 hours, and the disease is typically self-limiting lasting 5 to 7 days. Some cases of Campylobacteriosis last for up to 16 days, and over 20% of people who are infected with *Campylobacter* will relapse a few days after remission of the primary infection (Wassenaar and Blaser, 1999). Although most individuals infected with *C. jejuni* do not have serious complications associated with infection, this is not the case with immune compromised persons. Many of these individuals require antibiotics and have several relapses before *C. jejuni* can be cleared

from their system (Blaser, 1997). Unfortunately, *C. jejuni* has been associated with more serious complications. Cardiac, multi-organ complication and cellulites due to *C. jejuni* bacteriemia have been reported (Blaser, 1997; Hannu et al., 2005; Monselise et al., 2004). In addition, *C. jejuni* infection can trigger post infection sequelae including the Guillian-Barré Syndrome (GBS), Miller-Fisher Syndrome (MFS), and Reactive Arthritis (Nachamkin, 2002; Smith, 2002; Willison and O'Hanlon, 1999). GBS is demyelinating disease of the peripheral nervous system. MFS is a clinical variant of GBS affecting the crainial nerves first, specifically the oculomotor fibers (Nachamkin et al., 1998). Although the mechanisms for *C. jejuni* induced reactive arthritis are largely unknown, *C. jejuni* seems to trigger GBS and MFS by molecular mimicry (Willison and O'Hanlon, 1999).

The lipopolysaccharides produced by *C. jejuni* are structurally similar to the human gangliosides, present in axonal nerve cells (Moran, 1997; Yuki, 1997). This molecular mimicry can cause macrophages to invade axonal nerve cells at the nodes of ranvier thus causing the demylanation of motor neurons in GBS, and motor sensory neurons in MFS leading to the paralysis seen in both syndromes (Hughes and Cornblath, 2005; Winer, 2001).

1.5 Epidemiology of *C. jejuni* infections.

Most *C. jejuni* outbreaks are reported as sporadic cases and not as large outbreaks. However, *C. jejuni* has been implicated in some wide spread outbreaks, usually linked to a common source (Richards, 2005). An example was the contaminated water supply in Walkerton Ontario Canada in 2000. Although the major contaminant in this waterborne

outbreak was *Escherichia coli O157:H7*, *C. jejuni* was the second highest contaminant in the water (Richards, 2005).

C. jejuni is suspected to cause an estimated number of 2.1 to 2.4 million cases per year in the United States alone (Altekruse et al., 1999). This accounts for roughly 46% of laboratory confirmed cases of bacterial gastroenteritis. This number is two times higher than *Salmonella* species, and 7 times higher than the average for *E. coli* (Allos, 2001). In developing countries it is estimated that 40,000 to 60,000 per 100,000 children under the age of five will be infected with *Campylobacter* and contract Campylobacteriosis (Coker, Isokpehi, Thomas, Amisu, and Obi, 2002). This number is staggering when compared to the number of infections in developed countries of roughly 300 cases per 100,000 individuals, for both adults and children (Tauxe and Blake, 1992). However, the number of adults in developing countries who become ill is comparable to developed nations (Coker et al., 2002).

Humans are typically infected through, direct contact with animals and contaminated food and water sources. Transmission from human to human is considered extremely rare (Blaser, 1997). One of the highest odds ratios of developing illness is the consumption of poultry prepared at a commercial food establishment (Friedman et al., 2004).

Campylobacter infections generally have a seasonal pattern, with peaks during the summer months (Altekruse et al., 1999; Jones and Telford, 1991; Jones, 2001). *Campylobacter* infections tend to double in late spring and early summer, they then wane during July and August (Samuel et al., 2004). However, *Campylobacter* infections remain high until December, when levels remain constant until the following spring (Altekruse et al., 1999). Although the reason for this seasonal trend in *Campylobacter*

infections is largely unknown, the elevated incidences of Campylobacteriosis in England seems correlated with an increase of *Campylobacter* in sewage waters, particularly around animal processing plants (Jones, 2001). There have also been many sporadic cases of *Campylobacter* infections linked back to the ingestion of undercooked barbeque food (Istre et al., 1984). Therefore, an increase in careless food handling may be a contributing factor to the higher incidence of infections during the summer months.

Normally, the rate of complications such as GBS and MFS associated with *C. jejuni* is low. GBS is thought to occur in 1 to 2 cases of *C. jejuni* infection for every hundred thousand cases, and MFS occurs in 25 to 30% of GBS cases (Hughes and Cornblath, 2005). However, 38% of patients with GBS have had serological evidence of *C. jejuni* infection (Allos, 1997). GBS affects males more frequently than females, and usually has a low fatality rate, but high morbidity due to delays in diagnosis (Willison and O'Hanlon, 1999).

1.6 Importance of *C. jejuni* in the food industry.

The transmission of *C. jejuni* through the food supply, and the ubiquitous nature of *C. jejuni* in livestock populations is the primary concern to the food industry. *C. jejuni* is commonly found as part of the normal microbial flora of many domestic farm animals and household pets (Penner and Aspinall, 1997; Wassenaar and Blaser, 1999). Typically, infection in most animals results in a harmless symptomatic colonization of the intestinal tract. However, infection in humans results in disease (Wassenaar and Blaser, 1999). The symptomatic colonization of farm animals makes isolating infected animal, and therefore limiting the spread of illness, extremely difficult.

The difficulty in limiting the persistence of *C. jejuni* is best demonstrated by the isolation rates of *C. jejuni* from the retail food supply. *C. jejuni* has been isolated from upwards to 50% of chicken, 37% of turkey, 46% of duck, and upwards to 12% from other food types (Whyte et al., 2004). However, other studies have found that the rate of *C. jejuni* in poultry carcasses was as high as 85% (Andrews, 1998). Similar studies have shown infection rates close to 100% for boiler flocks, and pigs, and around 60% of cattle (Bryan and Doyle 1995, Moore et al., 2003, Orr et al., 1995). The high colonization in these animals can contribute to the high levels of *C. jejuni* found on the carcasses sold in the retail market, as the bacteria are released from the intestinal tract during the slaughtering process. In fact, *C. jejuni* infection is largely considered an occupational illness among poultry handlers (Jones and Telford 1991). Due to the high incidence of *C. jejuni* in animal reservoirs, particularly in retail environments, this organism is of high concern for the food industry, which spends considerable effort to reduce *C. jejuni* incidence in order to increase the safety of the food supply.

1.7 Campylobacter and Biofilm growth.

1.7.1. *Campylobacter* persistence.

Campylobacters are common in the in the food supply, and are the leading cause of bacterial food borne illness (Allos, 2001). Although *C. jejuni* causes more incidences of disease than *E. coli*, or *Salmonella*, the organism is thought to be less robust than these other pathogens (Park, 2002). The notion of reduced robustness compared to other common food borne pathogens comes from experimental data showing that *C. jejuni* has a lower tolerance for environmental stressors such as oxidative stress, changes in pH or salt concentration (Doyle and Roman, 1982; Murphy et al., 2003; Yamasaki et al., 2004).

C. jejuni also lacks many of the typical environmental stress response genes found in other food borne pathogens (Park et al, 2000; Park, 2002; Parkhill et al., 2000). This includes genes that are thought to act as important regulators of an overall stress response. Genes like *oxyR, soxRS, rpoS, rpoH*, and *cspA* are absent in the *C. jejuni* genome (Table 1.1). Therefore, anecdotal evidence indicates that *C. jejuni* has evolved to specifically fill a niche, and lacks many of the mechanisms typically thought to be critical for survival in the environment (Park, 2002). Despite this apparent lack of stress response mechanisms, *C. jejuni* remains one of the infectious food borne pathogens most frequently causing disease. *C. jejuni* must survive outside of its intestinal niche, and persist in the environment as a part of its infectious cycle since direct human to human transmission is extremely rare (Blaser, 1997). *C. jejuni's* ability to grow in biofilms has been proposed as one mechanism by which Campylobacters counter environmental stress (Trachoo and Frank, 2002; Trachoo et al., 2002).

1.7.2. Characteristics of Microbial Biofilms.

Biofilms are defined as matrix-enclosed bacterial populations that are adherent to each other and/or surfaces or interfaces (Costerton et al., 1995). The matrix is comprised of carbohydrate rich exracellular polymeric substances, or EPS (Branda et al., 2005). The structures of microbial biofilms are as diverse as the number of bacteria that can be found in the environment. Most commonly biofilms form when motile bacteria attach to a surface and become sessile; they then form microcolonies, which start producing the EPS (Donlan 2002). From this point, the bacteria are firmly bound to the attachment surface (Costerton, 1995). Many mature biofilms form a towering, or mushroom shaped three-dimensional structure (Donlon 2002). Mature biofilms also possess different mechanisms

to release cells to colonize other areas and form other biofilms (Hall-Stoodley and Stoodley 2005).

Prior to forming a biofilm certain criteria for the bacteria must be met. This includes a minimum level of cell density is required prior to biofilm development (Suntharalingam and Cvitkovitch 2005). This requires an innate communication strategy between bacteria in close proximity to one another. Communication is accomplished by quorum sensing. In Gram-negative bacteria, quorum sensing is handled by the use of N-acyl hormoserine lactones (AHL). These signals are auto inducers (AI) and they can also act as transcription factors (Moat et al., 2002). AI's of many bacteria are linked to a *Lux* type of feedback system that can regulate gene expression (Elvers and Park, 2002; Moat et al., 2002). The *Lux* system is separated into two types of proteins. The two proteins are LuxI and LuxR. The LuxI will act as an AI and bind to a LuxR protein. This complex will then transcriptionally activate genes, as well as *LuxI* itself (Moat et al., 2002). *Campylobacters* use this type of quorum sensing (Elvers and Park, 2002).

Although years of research in microbiology has focused on studying planktonic cultures of bacteria, it is thought that biofilms are ubiquitous in nature, and many if not most bacteria will form biofilms (Costerton et al., 1995). In fact, many human pathogens have been shown to form biofilms (Tremoulet et al 2002; Parsek and Singh 2003; Hall-Stoodley and Stoodley 2005).

The biofilm mode of growth allows for greater environmental stress resistance, including a greater resistance to antibiotics for many bacteria (Fux et al 2005.) Although this resistance to environmental stressors had originally been attributed to the encapsulation of bacteria in the biofilm, thus limiting exposure to the stressor, other

research has shown that biofilm growth itself can up-regulate genes associated with virulence and stress response (Costerton et al., 1992; Sampathkumar et al., 2006; Branda et al., 2005).

1.7.3 *Campylobacter's* Mechanisms of Biofilm formation.

It is documented that *C. jejuni* form biofilms in the environment which increases their survival (Trachoo et al., 2002; Golden and Acheson 2002; Misawa and Blaser 2000). *C. jejuni's* growth in biofilms may cause an increase in stress gene expression. This increase in expression is thought to occur through *C. jejuni's* AI, which is also a *Lux* gene communication strategy (Elvers and Park 1998). When Karen Elvers along with Simon Park analyzed the complete genome of *C. jejuni* NCTC 11168, they discovered that it coded for a protein that had 74% homology with the *luxS* gene of *Vibio harveyi,* and only showed 40% homology to the *luxS* gene *of Helicobacter pylori.* (Park 2001; Elvers and Park 1998; Parkhill et al., 2000). The *luxS* gene in *Campylobacter* was found to produce AI-2 like signaling activity, but not AI-1 activity (Elvers and Park 1998).

Although the exact function of *luxS* in *Campylobacter* is unknown, it has been suggested to regulate some of the metabolic activity in the cell. Specifically, there is some evidence that the *luxS*-signaling molecule may contribute to the regulation of genes involved in oxidative stress response (Elvers and Park 1998). Since quorum sensing is important to the formation of biofilms, the *luxS* pathway may be important in *C. jejuni* to initiate the phenotypic changes that go along with biofilm formation. These phenotypic changes include EPS production and change to a sessile state of mobility.

C. jejuni has been shown to form at least three different types of monolayer biofilms, aggregates, pellicles, and attached biofilms. *C. jejuni* biofilms can be formed

with shaking at speeds of 50 RPM, and form strongly attached on glass at the liquid-gas interface within 3 to 6 days of growth. Traditionally, aggregates in suspension were not thought to be biofilms; however, they are structurally indistinguishable from attached biofilms when viewed with scanning electron microscopy (Joshua et al 2006). *C. jejuni* biofilms also tend to form unattached biofilms during the stationary phase of growth.

1.8 Oxidative Stress and Reactive Oxygen Molecules.

1.8.1 Biochemistry of Reactive Oxygen Species Molecules.

Campylobacters are microaerophilic organisms, and thus use molecular oxygen in their metabolic pathways. Therefore, *C. jejuni* must encounter atmospheric oxygen during its infectious cycle. Molecular oxygen can create a wide verity of stresses on microorganisms, from generating superoxide radical (Gregory and Fridovich 1973), as well as hydrogen peroxide and hydroxyl ions (Berlett and Stadtman 1997). These reactive molecules are capable of causing major structural damage to critical cellular components and ultimately affect the survival of the microorganism (Fridovich 1997). Oxygen can inhibit even microorganisms that thrive in oxygen if the pressure is high enough (Gottlieb 1971). To fully understand the toxic effects of oxygen we must look at its chemical effects on bacteria.

Oxygen is itself a very reactive molecule, because of its ability to participate in many redox type reactions (Fridovich 1998). The damaging power to cellular components is related to the reducing power of different types of oxygen species in the cell (Imlay 2003). The reducing power of molecular oxygen itself is rather weak, and does not damage components of the cell directly. Moreover it is how the cell deals with oxygen that is the problem. Therefore, it will be necessary to compare and contrast the

mechanisms of damage between anaerobic and aerobic microorganisms. These mechanisms include the generation of and damage caused by the superoxide anion, hydrogen peroxide, and hydroxyl radicals.

1.8.1.1 The Superoxide Anion.

Campylobacter jejuni requires oxygen as a terminal electron acceptor in the electron transport chain to generate adenosine triphosphate (ATP). Through the generation of ATP as energy oxygen free radicals will be produced (Imlay 2003). These oxygen free radicals are also known as the superoxide anion. It is named the superoxide anion due to the extra unpaired electron that it has acquired in it outer valence shell (Bruce 2001). Since *C. jejuni* cannot avoid oxygen completely it generates the superoxide anion, it is suggested that this occurs when a molecule of oxygen is reduced by $NADH_2$ transferring an electron to the oxygen (Imlay 2003).

The superoxide anion has greater reducing power than molecular oxygen and can thus react with more biological molecules. It is the damage the superoxide anion enacts on iron-sulphur clusters of some enzymes that creates most of the problems. The deactivation of the enzyme happens due to the superoxide anion's attraction to the catalytic iron (Imlay 2003). The reaction causes the oxidation of the cluster, making it unstable and therefore causing its degradation. This degradation causes the enzyme, and therefore its pathway, to become inactive. Although there are a variety of molecules that can be affected by the superoxide anion it does not affect deoxyribonucleic acid (DNA) or polyunsaturated lipids (Fridovich 1998).

It is estimated that *Escherichia coli* will convert 0.1% of the oxygen utilized for energy production to the superoxide anion; this would be enough to accomplish a

production rate of 5μM per second (Imlay and Fridovich 1991). However, due to the superoxide scavenging ability of enzymes such as superoxide dismutase, the steady level of the superoxide anion in *E. coli* is normally only 0.1nM per cell. Even at this level the superoxide anion concentration is enough to reduce the half-life of many cellular proteins (Imlay 2003).

1.8.1.2 Hydrogen Peroxide.

Like the superoxide anion, hydrogen peroxide is extremely toxic to cells, as it is used as defense mechanism by the macrophages to fight bacteria like *H. pylori*, especially if they lack catalase (Basu et al 2004, Demple et al 1986). Hydrogen peroxide is produced by the cell's attempts to scavenge the superoxide anion, or by the reaction of the superoxide anion with the iron-sulphur clusters (Imlay 2003, Hoffman 1979). Only 1μM of hydrogen peroxide is required to inhibit the growth of *E. coli* (Barlett and Stadtman 1997). Hydrogen peroxide can mediate oxidation reactions to cause protein carboxylation, and like the superoxide anion, it can oxidize iron-sulphur containing proteins (Imlay 2003). However, hydrogen peroxide is most destructive to the cell when it comes in contact with iron II, because it can participate in the Fenton reaction to create the even more reactive hydroxyl radicals (Barlett and Stadtman 1997). The Fenton reaction is likely to occur because of liberated iron from the iron-sulphur clusters.

1.8.1.3 Hydroxyl Radicals.

As stated above, the hydroxyl radicals are formed by the Fenton reaction. This reaction oxidizes free iron II into iron III and creates a negatively charged hydroxyl ion and a hydroxyl free radical (Barlett and Stadtman 1997). Hydroxyl ions are extremely reactive and can cause hydrolytic damage to DNA. This damage can either add electrons

to unsaturated bases or to the sugar backbone thereby causing DNA lesions, or DNA cleavage. The hydroxyl radicals can also cause the oxidation of the protein backbone. The cleavage of both DNA and peptide bonds will cause extensive damage to the cell. Even a ten-minute exposure to mM levels of hydrogen peroxide will create enough hydroxyl radicals to cause DNA damage that heavily mutates or kills most bacteria (Imlay 2003). Therefore, microorganisms require some defensive mechanisms against these reactive oxygen species (ROS).

1.8.2. *Campylobacter jejuni* Defense Mechanisms for ROS.

To prevent the detrimental attacks of ROS molecules, *C. jejuni* possesses defense mechanisms to enhance survival in the presence of oxygen. *C. jejuni* possesses several genes to deal with oxidative stress that are identical to those in *E. coli* and other bacteria. Defense against oxidative stress in *C. jejuni* includes Super Oxide Dismutase *(sodB)*, Alkyl hydroperoxide reductase *(ahpC)*, and a Catalase *(KatC)* (Park 2000). However, *C. jejuni* also lack several stress genes that are present in *E. coli* (Table 1.1).

1.8.2.1. Mechanisms of Action and Control of *sodB*.

Unlike *E. coli* and many other bacteria, which contain *sodB* and a magnesium linked *Sod* (*sodA*), *C. jejuni* only contains the former (Park 2000). Therefore, *C. jejuni* only codes for one known superoxide savaging protein, which limits its protection from oxidative stress. *sodB* uses two superoxide anions and two hydrogen atoms to produce oxygen and hydrogen peroxide. The *sodB* gene of *C. jejuni* is very well characterized. It has been mapped and consists of a 662 base pair (bp) open reading frame that encodes a 220 amino acids protein (Pesci et al 1994). *C. jejuni's sodB* gene is 64% similar to *H. pylori* and 59% similar to *E. coli*.

An experiment exposing *C. jejuni* to increased superoxide anions after the addition of an externally produced Sod protein showed that greater concentrations of the Sod protein improved *C. jejuni* survival in the presence of superoxide anions (Hoffman et al 1979b). The ability of *sodB* conferring oxidative resistance to *C. jejuni* was confirmed by Purdy et al (1999). Compared to its parent strain, a *sodB* knockout mutant of *C. jejuni* showed a marked decrease in growth in the presence of methyl viologen (a superoxide producing chemical). The mutant also showed a 25,000 fold lower survival to H_2O_2 stress than the parental strain. Likewise, the mutant showed a marked decrease in its ability to colonize chicks. However, the addition of a plasmid that contained a *sodB* gene returned the characteristics of the parental strain to the mutant (Purdy et al 1999). Current research shows that *sodB* is a significant contributor to the survival of *C. jejuni;* however, it is not the only gene that protects *C. jejuni* from ROS molecules.

1.8.2.2. Mechanisms of Action and Control of *ahpC*.

The *ahpC* gene, like *sodB*, has been well mapped and sequenced in *C. jejuni.* The *ahpC* gene is located near the *fdxAfdxA* gene in *C. jejuni* (Baillon, et al., 1999; Van Vliet et al., 2001). The two genes are separated by a 204 bp intergenic region. The *katA* gene is also located close by, just 444 bp down stream of the *ahpC* gene. The *ahpC* gene in *C. jejuni* shares good homology with the *ahpC* sequences from *H. pylori, E. coli*, and *Legionella pneumophila* (Baillon et al., 1999). *ahpC*, like *katA*, has been shown to be regulated by a fur homologue designated *PerR* (Van Vliet et al., 1999). This *PerR* regulator seems to repress the expression of *ahpC* when iron levels are elevated.

ahpC is known for its ability to reduce organic peroxides to alcohols in various bacteria (Barlett and Stadtman, 1997; Olczak, Olson, and Maier, 2002). In *E. coli*

however, hydrogen peroxide is the sole substrate of *ahpC*, and mutants that lacked both *ahpC* and a catalase had lower survival to hydrogen peroxide stress, than mutants that lacked only catalase. The latter had similar survival to the parental strain (Seaver and Imlay, 2001). Therefore, *ahpC* may act to help eliminate hydrogen peroxide from *C. jejuni* which is produced by the activities of *sodB* and other processes. *ahpC* eliminates hydrogen peroxide by converting it to water by using hydrogen from NADH.

Predictably, the scavenging of hydrogen peroxide should confer some resistance to oxidative stress for *C. jejuni.* This hypothesis has been proven experimentally using a *ahpC* knock out mutant (Baillon et al., 1999). Growth under atmospheric oxygen conditions, at optimal growth temperature, had a 1000 fold reduced survival in the mutant than in the wild type. Therefore, *ahpC* is another enzyme that is required in *C. jejuni's* defense to oxidative stress. Although survival to oxidative stress was affected by the inactivation of *ahpC*, survival to the addition of hydrogen peroxide was not changed (Baillon et al., 1999). Therefore, like *E .coli, C. jejuni* must possess more the one gene that deals with the scavenging of hydrogen peroxide.

1.8.2.3. Mechanisms of Action and Control of *fdxA*.

An interesting candidate may be the iron induced Ferridoxin *(fdxA)*. As stated above, the *fdxA* gene is separated from the *ahpC* gene by a 204-nucleotide spacer. However, unlike *ahpC*, expression of *fdxA* is up-regulated in response to elevated iron availability (Van Vliet et al., 1999). The gene product from the *fdxA* gene is a predicted 94 amino acid protein with a hypothesized molecular mass of 9.9 kDa. This gene is similar to the 2[Fe-4S] family of Ferridoxins from other bacteria. FdxA is used as a reducing protein in *E-coli.* It is interesting that the *fdxA* start codon resembles the *C.*

jejuni σ^{70} consensus sequence (Van Vliet et al., 2001), which may indicate a method of this gene's transcription or regulation. However, there is little known about the role of *fdxA* in the oxidative stress response.

When an *fdxA* knock out mutant was subjected to atmospheric oxygen, it demonstrated a reduced survival rate from the parent strain after about 4 hours (van Vliet et al., 1999). Although this reduction in survival was not as pronounced as for the *ahpC*, or the *sodB* knock out mutants, there is still a significant decrease in the survival of the *C. jejuni fdxA* mutant in comparison to its parental strain. Like for *ahpC*, the *fdxA* mutant did not show a significant reduction in survival when exposed to hydrogen peroxide stress alone. However, this still does not answer the question of how *fdxA* may work. There may be a relationship between *fdxA* and *ahpC*. Interestingly, in other bacteria, like *E. coli*, *ahpC* reduces hydrogen peroxide to water and then is reduced itself by NADH to become active again (Baillon et al., 1999). *AhpF* usually carries out the reduction of *ahpC* utilizing NADH. This does not occur in *C. jejuni* because *C. jejuni* lacks the *AhpF* subunit. Therefore, *fdxA's* predicted action in *C. jejuni* thus far is to reduce *ahpC* (Van Vliet et al., 1999). This would make sense from the aspect that the two genes are located very close to each other in the *C. jejuni* genome. However, the theory falls short because *ahpC* is repressed by an increased availability of iron, and *fdxA* is induced by an increased availability of iron. Hence the action and exact role of *fdxA* in *C. jejuni's* oxidative defense are still largely unknown. However, since the action of neither *ahpC* nor *fdxA* can fully explain the scavenging of hydrogen peroxide, there may be yet another gene that could confer some resistance to oxidative stress by dealing with hydrogen peroxide.

1.8.2.4. Mechanisms of Action and Control of *katA*.

The scavenger of hydrogen peroxide that may be involved in *C. jejuni's* defense against oxidative stress is *katA*. The *katA* gene is 1425 bp long, and codes for Catalase a protein with 417 amino acids. Like *ahpC, katA* is down-regulated in the presence of iron (Van Vliet et al., 1999). The *perR* promoter carries out this regulation. This would make sense since both *ahpC* and *katA* are scavengers of hydrogen peroxide. Day et al (2000) showed that a *C. jejuni katA* knockout mutant could not break down hydrogen peroxide as effectively as the wildtype. The *katA* mutant also had a sharp decrease in H_2O_2 resistance when compared to the wildtype. The mutant dropped to about 0% survival within 15 minutes when exposed to hydrogen peroxide. On the other hand the wildtype held at 60% survival for over an hour (Day et al., 2000). It would seem that *katA* is important in protecting *C. jejuni* from the effects of hydrogen peroxide, just like *ahpC*.

1.9.0. Genetic Methods for Studying Gene Expression.

1.9.1. Northern Blotting.

One of the first methods for studying differences in gene expression was Northern Blotting, which was first described in 1977 (Alwine, Kemp, and Stark, 1977). Like Southern Blotting, Northern Blotting requires the transfer of nucleic acids from a poly acrylamide matrix or agarose matrix to a nitrocellulose membrane post electrophoresis (Alberts 2004). The difference is that Northern Blotting utilizes RNA, opposed to Southern Blotting, which utilizes DNA. In a Northern blot, RNA is separated based on molecular weight by electrophoresis and then transferred to a nitrocellulose membrane where it is hybridized by a labeled probe that is specific to the gene of interest. After the hybridization, the signals from the probe are measured and quantified by comparing the

signal strength between samples, and/or a set of standards (Calvet, 1991). One of the main advantages of Northern blots is that they give information regarding the size of the mRNA studied (Kozian and Krischbaum, 1999). Even twenty years after its introduction, Northern blotting is still seen as a reliable method for the quantification of gene expression and is often used as a validation technique for other methods used to quantify gene expression (Bustin, 2000).

However, there are still limitations with the technique of Northern blotting. The first limitation is that only transcripts with a known sequence can be analyzed due to the necessity of creating cDNA or RNA probes (Kozian and Krischbaum, 1999). More concerning is the limitation on Northern Blotting's sensitivity. In comparison to other techniques, Northern Blotting requires a larger amount of RNA and may not be as well suited to the accurate quantification of rare mRNA transcripts (Bustin, 2000; Melton et al., 1984).

1.9.2. Polymerase Chain Reaction.

The polymerase chain reaction (PCR) is a powerful technique used throughout molecular biology for gene analysis. PCR is a procedure for the enzymatic amplification of DNA sequences using multiple cycles of DNA denaturation, oligonucleotide primer annealing, and primer extension by commercial DNA polymerases (Mullis et al., 1986). Kerry Mullis first described PCR in 1984, and since that time many different PCR chemistries and methods have been developed to increase the flexibility of the technique (Edwards and Saunders, 2001; Markoulatos et al., 2002; Mullis et al., 1986; Ririe et al, 1997). PCR is a highly sensitive technique requiring in theory only one copy of DNA in a sample to create a measurable product after 20 cycles. The sensitivity of PCR comes

from its exponential increase in the copies of the regions flanked by the primers along with its copy accuracy with a misincorporation rate of less then 1 in 700 bases within 20 cycles (Scharf et al., 1986).

1.9.2.1 Quantitative PCR (qPCR).

Quantitative PCR (qPCR), also known as real-time PCR, involves adding a fluorescent label to the PCR reaction so that one can analyze the reaction kinetics in real time. Most of the chemistries utilized for qPCR involve the incorporation of fluorescent markers into the synthesized DNA that is read either after the primer annealing step (for fluorescently labeled probes) or the primer extension step (for interchelating dyes) (Ginzinger, 2002). The idea being that the increase in fluorescence is proportional to the increase in the amount of DNA in the sample.

Fluorescent probes are usually labeled with a fluorescent molecule and a quencher molecule so to eliminate fluorescence from the probe prior to incorporation into the DNA. The quencher is either released from the probe once it is incorporated, or the primer has a specific conformation prior to incorporation, and incorporation causes a conformational change in the probe that in turn increases the distance between the fluorescent molecule and the quencher to the appropriate distance to cause fluorescence (Bustin, 2002; Ginzinger, 2002). Likewise, interchelating dyes like SYBR Green I, do not have much fluorescence unless they attach to the major groves of double stranded DNA (dsDNA) (Ponchel et al., 2003). By virtue of their respective chemistries, there are pros and cons to both methods of fluorescence.

SYBR Green I has the advantage of being easy to incorporate into an already existing assay, and has less cost associated with it than other techniques (Ponchel et al.,

2003). However, this comes at the expense of specificity. Since SYBR Green I attaches to the major grove of dsDNA, it will also attach and produce a signal for primer dimers and for non-specific products (Bustin, 2000). Also, incorporation depends on the size of the amplicon. Since a larger amplicon will incorporate proportionally more SYBR Green, it will have a stronger signal than a smaller amplicon. Therefore, all SYBR Green assays require the addition of a melting curve step to be preformed at the end of the assay to confirm that there was only one product contributing to the signal (Ririe et al., 1997; Varga and James, 2006).

The major advantage of fluorescently labeled probes is that they only incorporate one fluorescent molecule into a DNA strand. They also allow for multiplex reactions due to the ability to add different primers with different fluorescent signals (Bustin, 2000). The down side to using fluorescent probes is their higher cost compared to regular primers. Also, if they are used for an existing assay, the assay will have to be optimized again (Bustin, 2002).

Regardless of the chemistry used, qPCR allows quantification of the DNA originally present in a sample, or the comparative quantification of DNA present in two samples (Ginzinger, 2002). The greater sensitivity of qPCR compared to traditional PCR comes from an accurate determination of the reaction kinetics. When using traditional PCR, the quantification is done after the reaction is completed, or at the end point, regardless of the quantification methodology used. Quantification in qPCR is performed in real-time and uses the point at which the reaction crosses a pre-determined threshold during the exponential phase of the reaction designated the crossing point, or cycle threshold (Ct)(Ginzinger, 2002). This gives DNA quantification by qPCR an advantage

over other techniques of enzymatic amplification, because the kinetics of the reaction can be calculated for the entire reaction, not just from the end point where the kinetics may be changing due to reagent limitation (Liu and Saint, 2002). qPCR can detect a two fold change in the amount of DNA in the sample, where end point PCR run on a gel will require a ten fold change in concentration to detect a change (Goerke et al., 2001; Orlando et al., 1998).

1.9.2.2 Reverse-Transcription PCR (RT-PCR).

Although powerful for the amplification of DNA, RNA cannot be used as a template in PCR, because PCR uses DNA polymerases that will not enzymatically amplify RNA (Bustin, 2000). Therefore, prior to amplification by PCR, RNA must be subjected to a reverse transcription step to convert it into cloned or copy DNA (cDNA) using reverse transcriptase (Wang and Brown, 1999). Thus, PCR utilizing a reverse transcription step prior to amplification is called Reverse-Transcription PCR (RT-PCR). RT-PCR is a highly sensitive method for the detection and quantification of mRNA's. However, it is not without disadvantages. The first problem is that reverse transcriptases can interfere with the sensitivity of the DNA polymerase used in the PCR (Sellner et al., 1992). This problem is usually overcome by maintaining an adequate ratio of DNA polymerase to reverse transcriptase, or by purifying the cDNA prior to its addition to a PCR. Another challenge with RT-PCR is related to the reverse transcriptase itself, which is therefore the least reliable step in a RT-PCR reaction.

Due to the inefficiency of reverse transcriptase to convert RNA to cDNA, there can be a difference between the copy numbers generated between samples containing the same amount of the mRNA transcript (Bustin, 2002). The type of reverse transcriptase

used in the reaction can further compound this problem of inefficiency (Stahlberget al., 2004).

Combining RT-PCR with qPCR allows for quantification of RNA in the sample and is known as quantitative RT-PCR (qRT-PCR). However, the largest difficulty with qRT-PCR is the lack of universal standards in the use of the technique (Skern et al., 2005). The results obtained from qRT-PCR can be dramatically affected by assay conditions, by the quality of the RNA used (which can be affected by the method of extraction), and by the methods used for data analysis (Bustin and Nolan, 2004). Unfortunately, there are a multitude of enzymes, kits, chemistries, assay set up, and data analysis models to choose from, and this variety of methods can cause two different laboratories to come up with different results for the same experiment (Eleaume and Jabbouri, 2004; Lekanne Deprez et al., 2002; Liu and Saint, 2002; Peirson et al., 2003; Shiao, 2003; Stahlberg et al., 2004; Swillens et al., 2004).

Even with these problems, qRT-PCR is becoming increasingly popular for gene expression studies and is considered to be far more sensitive than traditional gene expression protocols such as Northern blotting. The method is also widely used to validate microarray results (Klein, 2002).

1.9.3. Microarray.

One of the newest techniques to study gene expression is the use of microarrays. A microarray is glass slide, or other similar surface, which is coated with spotted RNA or DNA array and hybridized with two probes that are labeled with different colour fluorescent dyes. A stimulating laser then scans the assay and the resulting image is analyzed (Shalon et al., 1996; Yadetie et al., 2004). Each probe is generated from cDNA

that has been generated using RT-PCR technology; one colour probe is used for the control sample and a different colour for the test sample. Usually Cyanine dyes (Cy3 or Cy5) are used for the fluorescent probes (Zhao and Bruce, 2003). The methods used to produce microarrays allow for a multitude of genes to be analyzed simultaneously. Therefore, the microarray technique is a shotgun type approach to gene expression analysis. Like the other techniques, microarrays are not without problems and challenges.

Just like qRT-PCR techniques, microarrays require validation experiments to substantiate the claims that are made. Also, microarrays are prone to false positive results just like qRT-PCR techniques (Venkatasubbarao, 2004). Another problem with microarrays is that they are a relative technique (Venkatasubbarao, 2004). Unfortunately, microarrays can only be used to perform comparative tests of gene expression. Although qRT-PCR and microarrays tend to share many of the problems described above, both techniques are frequently used because they require less starting material than the more traditional method of Northern blotting.

1.10 Goals of this study.

The goals of the current study are to determine if biofilm growth confers a survival advantage to *C. jejuni* 16-2R by up regulating its oxidative stress genes during the late stationary growth phase. To determine gene expression, qRT-PCR will be used to study the expression levels of *ahpC, fdxA, katA,* and *sodB* of *C. jejuni* 16-2R after 48-hours of growth in planktonic growth conditions, or biofilm growth conditions. The expression levels of *ahpC, fdxA, katA*, and *sodB* will also be compared for *C. jejuni* 16-2R biofilms that were grown aerobically and microaerobically (CO_2 10%, O_2 5%, and N_2 85%). We will also test the difference of results obtained with a commercial one-tube

RT-PCR kit using AMV reverse transcriptase and a two-tube RT-PCR procedure using M-MLV reverse transcriptase.

Table 1.1: Typical Stress response genes in bacteria. Species chosen are *Campylobacter jejuni* (a Gram negative bacterium), *Escherichia coli* (example of a typical Gram negative bacterium), and *Bacillus subtilis* (example of a typical Gram positive bacterium). Table is taken from Park 2002.

Oxidative Stress gene	Function	C. jejuni	E. coli	B. subtilis
soxRS	Positive regulators of the response to superoxide stress	-	+	-
oxyR	Positive regulator of the response to peroxide stress	-	+	-
perR	Negative regulator of the response to peroxide stress	+	+	+
sodB	Iron cofactored superoxide dismutase	+	+	+
sodA	Manganese cofactored superoxide dismutase	-	+	+
katA	HPII, catalase	+	+	+
katG	HPI, catalase	-	+	-
ahpC	Alkyl Hydroperoxide reductase	+	+	+

Chapter 2: Establishment of Growth Conditions and qRT-PCR Protocols.

2.1. Introduction.

The mechanisms *Campylobacters* use to survive under environmental stress are poorly understood (Moen et al., 2005). *Campylobacter jejuni* is frequently found to grown in biofilms in the environment, and this mode of growth is often associated with resistance to environmental stresses and chemical sanitation methods (Costerton, 1995; Trachoo and Frank, 2002; Trachoo et al., 2002). Therefore, growth in biofilms may confer some protection against environmental stress, and thus allow *C. jejuni* to survive outside of their ideal growth conditions.

C. jejuni may have a higher up regulation of oxidative stress genes in the stationary phase of growth due to an ability to mount a stringent response that can cause an increase in its resistance to different stressors (Gaynor et al., 2005). Stringent response is a global stress response that alters gene expression pathways to enhance survival under unfavorable conditions (Gaynor et al., 2005). The *C. jejuni* stringent response required for the transmission of antibiotic resistance and low CO_2, and high O_2 survival regulated by *SpoT*. *SpoT* is a gene that encodes for a protein that hydrolyses (p)ppGpp, and thus causes a signalling cascade that up regulates stress response genes (Mittenhuber, 2001). Due to the increased environmental survival of *C. jejuni* in biofilms, there may be a higher stringent response, and therefore greater oxidative stress gene expression in *C. jejuni* biofilms than in planktonic growths.

Biofilms have been shown to enhance survival of other bacteria by means of increased protein and gene expression (Fux et al., 2005; Tremoulet et al., 2002; Vandercasteele et al., 2003). If biofilms cause an increased expression of environmental

resistance genes of other bacteria then there should be a difference in the expression pattern of *C. jejuni*. There has been some evidence for differential gene and protein expression patterns between *C. jejuni* planktonic growth and biofilm growth (Dykes et al., 2003; Sampathkumar et al., 2006; Stintzi, 2003). Dykes et al (2003) found that over 50 different proteins were up-regulated in *C. jejuni* when grown in biofilms compared to planktonic cells. Sampathkumar et al (2006) found that immobilized growths of *C. jejuni* had higher *katA* gene expression then planktonic cells when in exponential growth phase. Finally Stintzi et al (2003) found that *C. jejuni* changed its gene expression profile in response to temperature variation.

Different techniques have been used to attempt to ascertain genetic changes between different experimental conditions. Among these techniques are protein assays, micro arrays, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (O'Connor et al., 2005; Resch et al, 2005; Thomas et al., 2006). Each method has been useful in investigating bacterial lifestyle differences when grown in biofilms. However, each method of investigation requires different optimization procedures.

This study will focus on the optimization of *C. jejuni* 16-2R conditions for the growth and harvest of planktonic and biofilms in late stationary phase, development and optimization of a PCR and RT-PCR assay for four genes believed to be involved in the *C. jejuni* oxidative stress response.

2.2 Materials and Methods.

2.2.1. Bacterial Cultures.

Campylobacter jejuni (*C. jejuni*) strain 16-2R, a meat isolate, was selected for this study because it has been shown form biofilms on glass wool under both microaerobic

and aerobic conditions (Zhou, 2005). The organism was maintained at -80°C in an ultra freezer (Thermo electric) contained in a 2 mL mixture of Trypticase Soy Broth (TSB) (BBL) and 50% glycerol. Frozen Culture (100μL) was streaked on Campy-Line agar (Line, 2001) and plates were incubated for 48 hours at 42°C in a microaerobic environment (5% O_2, 10% CO_2, 85% N_2). A microaerobic environment was created using a gas pack jar (BBL) and Campy packs (BBL). Revived cultures were then subcultured on Campy-Line agar plates, and incubated for 48 hours prior to use in experiments. Inoculating a 10 mL TSB (BBL) containing *Campylobacter* growth supplement (Oxoid) with a single colony from a fresh 48-hour growth plate was the procedure used to prepare broth cultures. Broth cultures were grown under the same conditions as the growth plates, but with the addition of shaking at 100 rpm.

2.2.2 Growth, Harvest, and Enumeration of Planktonic and Biofilm Cells.

Planktonic cultures were grown in 100 mL media bottles containing 20 mL of TSB with *Campylobacter* growth supplement (Oxoid). The medium was inoculated with 1.5 mL of a 48-hour broth culture (~10^7 CFU/mL) using aseptic technique. The cultures were grown under microaerobic or aerobic conditions (Room air) for 48 hours at 42°C with shaking at 100 rpm. A series of ten fold dilutions was prepared using Buffered Saline (PBS) from the 48-hour cultures and the dilutions were plated on Campy-Line agar plates by a 6x6 drop plate method (Chen et al., 2003). Plates were incubated under microaerobic conditions for 48-hours and colonies counted using the method set out by (Chen et al., 2003). Planktonic cultures were harvested by transferring 9 mL of TSB medium containing cultured cells to a 50 mL centrifuge tube (Fisher). The cultures were then collected at the bottom of the centrifuge tube by centrifugation at 3000 X g for 10

minutes. The supernatant was poured off leaving the cells in a pellet at the bottom of the tube. To develop biofilms of *C. jejuni* strain 16-2R, the protocol established by Zhou (2005) was used: Biofilms were grown on sterilized glass wool (0.1g) added to 20 mL of TSB with *Campylobacter* growth supplement. Inoculation and growth conditions were the same as for planktonic cells with shaking at 100 rpm.

Biofilm cells were harvested after removing the supernatant containing the planktonic cells and washing the glass wool three times with 25 mL of Phosphate Buffered Saline (PBS). Five grams of sterile glass beads (Sigma) and with 10 mL of Brucella broth (BBL) supplemented with Tween 20 (1 mL Tween per L of Brucella broth) were added to the washed glass wool and vortexed for 2 minutes to remove the biofilm cells from the 0.11g of glass wool. Enumeration and centrifugation of the collected biofilm cells was the same as planktonic cells. Growth data generated from 48-hour drop plate counts of biofilm and planktonic growths were analyzed for any statistical significance using ANOVA.

2.2.3 Extraction of Nucleic Acids.

DNA was extracted from 48-hour microaerobic broth cultures using the Wizard Genomic DNA Purification and Extraction kit (Promega). The manufacturer's instructions for Gram-negative bacteria were followed. Extracts were run on a 1% agarose gel and quantified using the Genequant II (Pharmacia) to ascertain quality and quantity of the DNA. DNA was stored at 4°C for short-term (7 days) use and at -80°C for long-term storage (over 7 days).

RNA was extracted from all *Campylobacter jejuni* 16-2R samples (microaerobic, aerobic, planktonic and biofilm growth) using the High Pure RNA extraction kit (Roche),

following the manufacturer's instructions for Gram-negative bacteria. RNA was also run on a 1% Agarose gel and on the Genequant II to ascertain its quality and quantity. A polymerase chain reaction (see section 2.2.4.2 for conditions) using the primers described in section 2.2.4.1 was performed with the RNA extract to ensure that there was no carry over DNA contamination. If the RNA was contaminated with DNA, then an additional DNAse I digest was performed to eliminate the carry over DNA under the following conditions: Eight μL of RNA was incubated at 37°C for 30 minutes with 1U of RNAse free DNAse 1 (Promega) and 1X incubation buffer followed by the addition of 1μL of stop solution (20mM EGTA pH 8.0) (Promega) and a incubation of 67°C for 10 minutes followed by placing the sample on ice for 5 minutes.

RNA was used immediately (within 3 hours), or was stored in 20 μL aliquots at -80°C if it was to be used in future experiments.

2.2.4. Primer Design, and Optimisation of Polymerase Chain Reactions.

2.2.4.1. Primer Design.

Primers were developed for the following genes of interest using sequence data for the published *Campylobacter jejuni* NCTC 11168 (#AL111168) from Genebank: *ahpC, fdxA, katA, sodB*, and *gyrA*. The DNA Man software version 5.0 (Lynnon BioSoft) and the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) were used to ensure primers were sequence specific to *Campylobacter*, were free of secondary structure, had minimal homology to each other, and had their melting temperatures (Tm) within one degree of each other.

2.2.4.2 Optimization of PCR Conditions.

Each primer set was tested to ensure that they were specific for their target using a *Campylobacter jejuni* 16-2R DNA template (Figure 2.2). PCR reactions (50 μL) contained final concentrations of approximately 1 μg of purified *Campylobacter jejuni* 16-2R DNA, 1X Buffer (Fermentas), 1.5 mmol $MgCl_2$ (Fermentas), 200 μM of each deoxynucleoside triphosphate (Promega), 0.5 μM of each primer and 1 Unit of Taq polymerase (Fermentas). PCR was carried out on a Hybaid PCR Sprint thermocycler. Cycling conditions were as follows: initial denaturation at 94.0°C for 5 minutes, followed by 10 cycles of 94.0°C for 30 seconds, 55.0°C for 30 seconds, and 72°C for 45 seconds, followed by 20 cycles of 94.0°C for 30 seconds, 55.0°C for 30 seconds, and 72.0°C for 45 seconds plus one second per cycle. A final step of 72.0°C seconds for 5 minutes was carried out.

For each assay, a master mix containing all of the reagents required for the number of reactions with the exception of the DNA was prepared for each gene target. Mixing was carried out under sterile conditions and care was taken to ensure that there was no cross contamination of samples. For each assay, a negative control was included, which consisted of all of the materials for the reaction, but instead of a template sterile DNA/RNA free double distilled water was used.

The optimal annealing temperature, magnesium and primer concentrations were assessed for each primer set following the protocol set out by (Kramer and Coen, 2001) using freshly prepared *Campylobacter jejuni* 16-2R planktonic DNA as a template. Amplification was confirmed by running the PCR product on a 1% agarose gel containing ethidium bromide visualized under UV light. An image of the gel was obtained using the Syngene photodocumentation system and software.

2.2.4.3. Reverse Transcription PCR.

Reverse transcription PCR (RT-PCR) was carried out using the Titan one tube RT-PCR kit (Roche) following the manufacturer's instructions. RT-PCR (50μL) contained the final concentrations per reaction: 200μM of each deoxynucleoside triphosphate (Promega), 0.4μM of each primer, 5mM DTT solution (Roche), 10 units of RNase inhibitor (RNasin® Ribonuclease inhibitor from Human placenta) (Promega), and 40ng of RNA template, 1X RT-PCR buffer (Roche), 1.5 mM MgCl2, and 1μL of enzyme mix (Roche). Cycling conditions were as follows: a reverse transcription step of 52.0°C for 30 minutes followed by a denaturation step of 94.0°C for 2 minutes, followed by 10 cycles of 94.0°C for 30 sec, 55.7°C for 30 seconds, and 68.0°C for 45 seconds were performed. The initial 10 cycles were followed by 25 cycles of 94.0°C for 30 sec, 55.7°C for 30 seconds, and 68.0°C for 45 seconds plus five seconds per cycle. RT-PCR was run on a Hybaid PCR sprint thermocycler.

2.2.5 qRT-PCR Methods.

2.2.5.1 qRT-PCR.

Quantitative RT-PCR (qRT-PCR) used the same conditions as RT-PCR. The exception was that 2.5μL of water was replaced with 2.5μL of 10X SYBR Green (Applied Biosystems) for a final concentration of 0.5X per reaction. Samples were loaded into 0.2mm optically pure strip tubes (Applied Biosystems) and run on an ABI 7000 prism (Applied Biosystems) thermocycler as described above.

2.2.5.2 Creation of external standards.

The external standards were created by amplifying the gene target of interest by PCR, then purifying the product with the Wizard PCR clean up kit (Promega). After clean up,

the product was run on a 1% Agarose gel to ensure that no secondary products were produced. The gene product was quantified using the Genequant II. Standards were then produced by serial dilution of the purified PCR product. The number of copies in a quantity of PCR product was calculated using the information from the ABI technical bulletin entitled "Creating standard curves with genomic or plasmid DNA templates" (ABI 2003). The known copies of control target were calculated by the following formula:

Number of copies = (weight of DNA in ng x Avogadro's number)/(length of amplicon in bp x ng/g x g/mole of bp) (Equation 1).

Where Avogadro's number is 6.022×10^{23}, ng/g = 1×10^{9}, and g/mole of bp was based on the average weight per mole of each base pair which is 650g/mole.

Following calculation from equation 1, the standards were created using 10 times serial dilutions of the known copy number DNA in sterile DNA/RNA free water. Following dilution 1µL of the each copy number standard was used in individual qPCR reactions, after which a standard curve of Threshold cycle vs. Copy number was created. The 10^{7}, 10^{6}, 10^{5}, and 10^{4} dilutions were used in this assay.

2.2.6 Comparative Analysis of Oxidative Gene Expression in Biofilm and Planktonic Cells.

2.2.6.1 qRT-PCR Set-up for Comparative Oxidative Gene Expression.

Biofilm and Planktonic cells were grown, enumerated, and harvested as described in section 2.2.1 and section 2.2.2. RNA was extracted as described above. *ahpC, fdxA, katA*, and *sodB* genes were amplified using the qRT-PCR procedure described previously

in section 2.2.3. For each assay the gene being studied, and the internal reference genes were run in triplicate. The *16S* ribosomal RNA (rRNA) and *gyrA* were amplified as internal reference genes. A set of external standards of known copy number was amplified along side the assay to allow an estimation of gene copy number set up as described in section 2.2.5.

2.2.6.2 Analysis of qRT-PCR Comparison of Oxidative Gene Data.

Relative cycle thresholds (Ct) were obtained by comparing the Ct generated from the 16S rRNA reactions from the planktonic cell RNA and the 16S rRNA reactions from the biofilm cell RNA. If the *16S* rRNA Ct values for planktonic and biofilm RNA extracts were equal, then the gene of interest Ct results were compared to each other directly. However, if the *16S* rRNA values for planktonic and biofilm RNA extracts differed by 1 Ct then the gene of interest Ct values were normalized prior to comparison. Normalization was carried out as per the ABI technical guide entitled "Guide to performing relative quantification of gene expression using real time PCR" (ABI 2004). Data was also compared to each other using gene copy number data generated from a standard curve of known copy number. Copy numbers were standardized to the *16S* rRNA using the following formula provided by Applied Biosystems.

Normalized target Ct = Gene target Ct/ Internal standard Ct (Equation 2).

To calculate a relative fold increase the values from equation 2 were substituted into equation 3:

Fold increase = Biofilm normalized sample/ planktonic normalized sample (Equation 3).

Differences between copy numbers and Ct's was analyzed using ANOVA.

2.3 Results.

2.3.1 Bacterial growth and survival.

Planktonic and Biofilm cultures were both started with initial inocula of 1.5 mL from a 48-hour planktonic culture of approximately $5x10^7$ CFU/mL. Planktonic cells of *Campylobacter jejuni* 16-2R grew well in microaerobic conditions to $3.09x10^8$ CFU/mL (Figure 2.1). Planktonic *C. jejuni* 16-2R also survived in an aerobic environment with an average of a one-log reduction to $3.64x10^7$ CFU/mL in growth between micoraerobic growth and aerobic survival. A similar trend was seen in biofilm formation. Biofilms were recovered from microaerobic conditions ($2.58x10^6$ CFU/g of glass wool) in greater CFU/g of glass wool then from aerobic growth conditions ($1.33x10^6$ CFU/g of glass wool) (Figure 2.1).

2.3.2 Extraction of DNA and PCR Primer Optimization.

An average of 500 μg/mL of DNA was extracted from an average of 10^7 CFU/mL of planktonic *C. jejuni* 16-2R growths. The specificity of the primers from a BLAST search of short nucleotide sequences showed that the primers for *C. jejuni* genes *ahpC, fdxA, gyrA, katA*, and *sodB* were all specific to *C. jejuni*. The primers for *ahpC, fdxA, gyrA*, and *katA* were each specific to their respective *C. jejuni* gene target and all had bit scores of 42.1 and e-values of $2x10^{-3}$. SodB was specific to *C. jejuni* Superoxide Dismutase B with a bit score of 38.2 and an e-value of $1.9x10^{-3}$ for both forward and reverse primers. The 16S primers were equally specific for the 16S genes of many bacteria with a bit score of 40.1 and e-value of $6x10^{-3}$.

These primers designed specifically for the *C. jejuni ahpC, fdxA, gyrA, katA, sodB*, and *16S rRNA* (Table 2.1), were able to amplify targets that corresponded to the expected target size (Figure 2.2). However, the polymerase chain reaction (PCR) was optimised for $MgCl_2$ concentration (Figure 2.3), annealing temperature (Figure 2.4), and primer concentration (data not shown). The optimal PCR conditions were a $MgCl_2$ concentration of 1.5 mM, a primer concentration of 0.3 μM, and an annealing temperature that resulted in a strong signal for the specific product with little or no primer dimmer, and had a noticeable decrease in reported signal at the next higher temperature (assessed by visual inspection of the 1% agarose gel). The annealing temperature that looked to be an optimal balance was determined to be 55.7°C. Figure 2.4 shows the primer annealing temperature for *katA*, all other genes used in this study showed the same annealing temperature results as *katA* (data not shown).

2.3.3 PCR Copy Number Standards.

The optimalized PCR conditions produced average amplicon weights between 130.0μg/mL to 152.0 μg/mL for each target after a post PCR clean-up step was performed. Based on Equation 1, the average copy number of purified PCR product of each gene target was approximately $3.00x10^{11}$ copies per μL. qPCR results of a 10 times serial dilution showed that there was an average of 3.38 cycles between dilutions with a liner slope where copy number was inversely proportional to threshold cycle. The R^2 value of this correlation was 0.99 (Figure 2.5) with an equation of $y = 4E+10e^{-0.6068x}$.

2.3.4 Extraction of RNA and RT-PCR optimization.

RNA was extracted from an average of 10^7 CFU/mL *C. jejuni* 16-2R microaerobic planktonic and biofilm growths. The High Pure RNA kit (Roche) was able to extract

between 150 to 300 ng of RNA from *C. jejuni*. Higher levels of RNA were extracted from planktonic growths of *C. jejuni*. Figure 2.6 shows an example of good quality RNA extracted from planktonic and biofilm growths. Amplifiable DNA was also extracted along with the RNA. The contaminating DNA was eliminated following an additional DNase I treatment (Figure 2.7).

Following preparation of the DNA-free RNA, RT-PCR was carried out using the primers listed in Table 2.1 and the Titan One-Tube RT-PCR system (Roche). mRNA and rRNA targets were amplifiable in both planktonic and biofilm growths with the exception of *gyrA* (Figure 2.8). *gyrA* was weakly amplified in biofilms and no band was visible from planktonic growths. Likewise, 40ng of RNA could be visualized in real-time during the qRT-PCR assay using the ABI Prism 7000 real-time thermocycler.

2.3.5 qRT-PCR results of *C. jejuni 16-2R* oxidative gene expression from planktonic and biofilm microaerobic growth conditions at 42°C.

The qRT-PCR showed that *ahpC, fdxA, gyrA, katA, sodB*, and *16S* gene targets all produced sigmoidal curves consistent with the exponential, linear, and plateau phases of PCR amplification (data not shown). Melting curves confirmed that the amplified targets were of the correct size and had expected melting temperatures (data not shown). *ahpC, fdxA, gyrA, katA, sodB*, and *16S* gene targets also showed amplification of the correct fragment size when the product was run on a 1% agarose gel.

Figure 2.9 shows at which cycle the different gene targets crossed the detection threshold. There was no statistically significant difference at the 95% confidence interval for the threshold cycles of planktonic and biofilm *ahpC, katA*, or *sodB* gene targets. These targets had p-values of 0.139, 0.065, and 0.136 respectively. However, the cycle

thresholds of *fdxA* were significantly different between planktonic and biofilm growth with a p-value of 0.008. *fdxA* was up regulated in biofilm growth. Interestingly, the *gyrA* values also differed with a p-value of 0.048, a higher expression found in biofilms.

After constructing a standard curve as described in section 2.2.5.2, the threshold cycles were compared and using equation 1 the copy number of target transcripts for *ahpC, fdxA, gyrA, katA*, *sodB,* and *16S rRNA* were determined (Figure 2.10). Statistically significant results were for *fdxA* and *gyrA* with p-values of 0.02 and 0.05 respectively. Again, biofilm growth produced the higher expression levels. After solving for equations 2 and 3, the fold increases in copy number were 1.77±0.11 for *fdxA* (p=0.004), and a 4.54±0.70 for *gyrA* (p=0.036). The *16S rRNA* also showed a statistically significant difference ($p<0.001$) however, the difference was less then 0.3 Ct between biofilm and planktonic samples.

2.4 Discussion.

Under microaerobic growth conditions at 42°C for 48 hours, growth of planktonic *C. jejuni* 16-2R was similar to other strains of *C. jejuni* (Hazeleger et al., 1998; Kelly et al., 2001). Typically, *C. jejuni* shows no phenotypic changes after entering stationary phase (Kelly et al., 2001). The apparent lack of a stationary phase response to stress has been traditionally studied using changes in temperature, and aeration using different media, and exposing *C. jejuni* to the stress after the entry into stationary phase (Kelly et al., 2001; Martinez-Rodriguez and Mackey, 2005). Kelly et al (2001) reported that *C. jejuni* was less resistant to aeration after entry into stationary phase, but other authors have found that *C. jejuni* has the ability to mount an adaptive tolerance response to aerobic stress after entry into a stationary phase. For example Murphy et al. (2003) used

extended growth times of up to three days in the presence of oxygen to produce an aerobic tolerant culture of *C. jejuni*. They found that subculturing in aerobic conditions an aerobic tolerant culture was produced that grew similarly to the microaerobic cultures. Likewise in this study, *C. jejuni* 16-2R was able to survive well when exposed to aerobic conditions, and was able to be recovered and cultured after prolonged stationary phase (Figure 2.1). *C. jejuni* 16-2R was also able to form biofilms in aerobic conditions. The apparent increase in *C. jejuni* biofilm cells when compared to survival in oxygen shows some support of a greater oxidative stress response from *C. jejuni* in biofilms than in planktonic growths.

Amplification of *ahpC, fdxA, gyrA, katA, sodB*, and *16S* from *C. jejuni* DNA showed that *C. jejuni* 16-2R contained each of the genes being studied. Furthermore, the amplification from these genes by RT-PCR from both planktonic and biofilm growths showed that *ahpC, fdxA, gyrA, katA, sodB*, and *16S* rRNA are actively transcribed in *C. jejuni* 16-2R after 48 hours of growth. This is encouraging considering the number of hyper variable regions in the *C. jejuni* genome, and *C. jejuni's* genetic and phenotypic variance (Gaynor et al., 2004; Parkhill et al., 2000). Since RNA is an unstable molecule and therefore short lived in the cell, the active expression of the genes of interest in *C. jejuni* 16-2R demonstrates that it transcribes products that are able to produce proteins directly related to oxygen survival in planktonic and biofilm growth in late stationary phase (Frenandez and Singh, 1976; Hirashima et al., 1973).

When analyzed using qRT-PCR, differences in the gene expression pattern between planktonic and biofilm grown *C. jejuni* 16-2R (Figure 2.9 & Figure 2.10) seemed to be present. Average values for biofilm growths showed up-regulation of *sodB,*

fdxAfdxA, and *gyrA*, but down-regulation of *katA*, and *ahpC*. However, the variance between some of the RNA samples was too large to establish any statistical significance in the differences in expression for *sodB, katA*, and *ahpC*.

There was a 1.77 fold up regulation of *fdxA* in biofilms. Similar differences in gene regulation have been reported with an average of a 1.55 fold change in gene expression for *C. jejuni's* response to heat stress (Stintzi, 2003). The up-regulation of *fdxA* with a down-regulation in *ahpC* and *katA* can be explained by its importance as a regulator of iron uptake, and that it is under an opposite transcriptional control than *ahpC* and *katA* (Van Vliet, Wooldrige and Ketley, 1998; Van Vliet et al., 1999; Van Vliet, Rock, Madeleine and Ketley, 2000; Van Vliet et al., 2001). An increase in the transcription of *fdxA* is often associated with a decrease in the transcription of *ahpC* and *katA* (Palyada et al., 2004; Van Vliet et al., 2001).

In this study *ahpC* and *katA* were down regulated in biofilm growth although this was statistically not significant. However other researchers have found that *C. jejuni* does up-regulate *katA* transcripts, and *AhpC* protein production when grown in immobilized colonies (Sampathkumar et al., 2006). The difference in the results may be explained by the differences in methodology for studying gene expression. Sampathkumar found that the *KatA protein* was up regulated during the log phase of *C. jejuni* NCTC 11168 immobilized growth. It appears that *C. jejuni* has a greater resistance to environmental stress prior to establishment in stationary phase. Also, there may be differences in *katA* gene expression between log phase and stationary phase (Martinez-Rodriguez and Mackey, 2005).

The greater resistance to environmental stress during log phase seems to disagree with the increase in resistance due to a stringent response in stationary phase. Therefore, *ahpC, katA, and sodB* gene expression may not be affected by an increase in *spoT* expression, or the stringent response may be elicited only during log phase. The assumption that a stringent response would be elicited in stationary phase came from evidence that *spoT* was required for stationary phase survival (Gaynor et al., 2005). An investigation into *spoT* expression during log and stationary phases between planktonic and biofilm growth may solve some of these questions.

Another explanation may come from strain differences. *C. jejuni* NCTC 11168 is a clinical isolate were *C. jejuni* 16-2R is a meat isolate. Genotypic differences between isolates may come from the hypervariable regions in the *C. jejuni* genome since most of the genes coding for stress survival lay in those areas (Parkhill et al., 2000). These hypervariable regions cause phenotypic changes in *C. jejuni* that have been overlooked by researchers for long periods of time. Hence a recent discovery that the published genome for *C. jejuni* NCTC 11168 differs from the original clinical isolate causing noticeable differences in phenotype, and gene expression patterns between the two NCTC 11168 cultures (Gaynor et al., 2004).

Unfortunately, no statistically significant difference could be observed in *sodB* gene expression between planktonic and biofilm growth due to the sample variance. Although *sodB* seemed to have a large increase in its expression when *C. jejuni* was grown in biofilms, the variance between samples was as much as 3.8 cycles for the biofilm samples. This is unfortunate because other authors have described an increase in the expression of *sodB* proteins between planktonic and biofilm growth in other bacteria

(Beloin and Ghigo, 2005; Sauer et al., 2002; Tremoulet et al., 2002). The large difference between the threshold cycles in *sodB* biofilms may be partially explained by the numerous pitfalls of RT-PCR. In particular the inherent differences in interassay variability, and unreliability of the reverse transcription step in converting the entire gene target in RNA to cDNA target. This topic is described in greater detail in section 3.4.

The last genes that were studied were the *16S* rRNA and *gyrA* genes. These genes were selected as "house keeping" genes, or internal standards. The *16S* rRNA functioned well as an internal standard because it was in such abundance over the other gene targets. Although there was a significant difference between the planktonic and biofilm expression of the *16S* rRNA, it was due to a very low level of variance between *16S* samples. The difference observed between the *16S* transcripts of biofilm and planktonic cells less than 0.3 cycles. Nolan, 2004). We concluded that the 16S rRNA can be used as an internal standard for *C. jejuni* gene expression comparisons. We also studied *gyrA* to see if it could be used as a suitable internal standard. *gyrA* seems to be affected by biofilm growth in *C. jejuni* 16-2R. Although there was a slight difference between the *16S* rRNA transcript, the difference observed in *gyrA* was greater than the observed difference for *fdxA*. *gyrA* has been shown to act as a good internal standard in other bacteria for gene expression studies (Desroche et al., 2005). However, for *C. jejuni* mutations of the *gyrA* gene have been reported (Beckmann et al., 2004). These mutations may make *gyrA* an unsuitable candidate for use as an internal standard, and may be contributing to the over 4-fold difference in gene expression.

Another possible explanation to the wide variance between biofilm and planktonic samples may be in the how *C. jejuni* grows in biofilms. Recently it was reported that C.

jejuni forms biofilms at the liquid-gas interface, and will form aggregates in suspension. Although these aggregates were not thought to be biofilms, they have now been reported to have no structural differences between traditional biofilms and these aggregates (Jones et al., 2006). Therefore, it is possible that biofilm cells were co-harvested along with the planktonic cells introducing an inherent error in the assay. Some method of truly separating planktonic cells from biofilms that form in suspended aggregates should be designed before undertaking any other planktonic and biofilm studies.

2.5 Conclusions.

Biofilm growth allows for a greater survival of *C. jejuni* 16-2R during oxygen exposure based on the reduced number of viable cells recovered from planktonic than for biofilm growths after exposure to oxygen. qRT-PCR has shown that there is no statistically significant difference in the gene expression of *ahpC*, *katA*, or *sodB* during planktonic growth or biofilm growth of *C. jejuni* 16-2R. Therefore, any extra defense to oxygen stress of *C. jejuni* cells growing in biofilms may be attributed to the up-regulation of *fdxA*. However, the impact of *fdxA* up regulation is largely unknown.

gyrA had a statistically significant difference in expression between planktonic and biofilm growth conditions. This may be attributed to mutations in the *gyrA* gene or an as of yet unreported increase in activity in biofilm cells. Therefore *gyrA* is not a suitable choice for use as a normalizing gene during *C. jejuni* gene expression studies that compare planktonic cells to biofilm cells.

Table 2.1. Primers used to investigate the oxidative stress response in *Campylobacter jejuni*.

Primer[a]	Gene	Sequence (5' to 3')	Position[b]
Cj-ahpC-f	Alkyl Hydroperoxide Reductase C	TTATGCAGAAGCATTGCCCT	348
Cj-ahpC-r	Alkyl Hydroperoxide Reductase C	TGCCAAGATATTCAGCCACG	577
Cj-fdxA-f	Ferridoxin A	TGCGTTGAATGCGTAGGACAT	130
Cj-fdxA-r	Ferridoxin A	TGCAAAAACTGGAGTGTCCCC	282
Cj-katA-f	Catalyse A	TCCAAGCAATATCGTTCCTGG	933
Cj-katA-r	Catalyse A	TTCATAGCACCAGCGACATTG	1103
Cj-sodB-f	Superoxide Dismutase B	TGTGGCGGTTCATGTCAAA	262
Cj-sodB-r	Superoxide Dismutase B	GCGTGCATTGCGATGATCT	534
Cj-gyrA-f	Gyrase A	TGCTAAAGTGCGTGAAATCGG	2133
Cj-gyrA-r	Gyrase A	ATTCTCCAGCATTGGTGCG	2296
16s-f	16s rRNA	CCTACGGGAGGCAGCAG	341
16s-r	16s rRNA	CCGTCAATTCCTTTGAGTTT	907

Primers were designed using DNA Man version 5.0 software (Lynnon Biosoft), with the exception of the 16S universial primers. 16S universial primers were taken from Watanabe et al (2001).

[a]f, forward primer; r, reverse primer

[b]Corresponding to the numbering in the specific gene. Gene sequences taken from Campylobacter jejuni NCTC 11168.

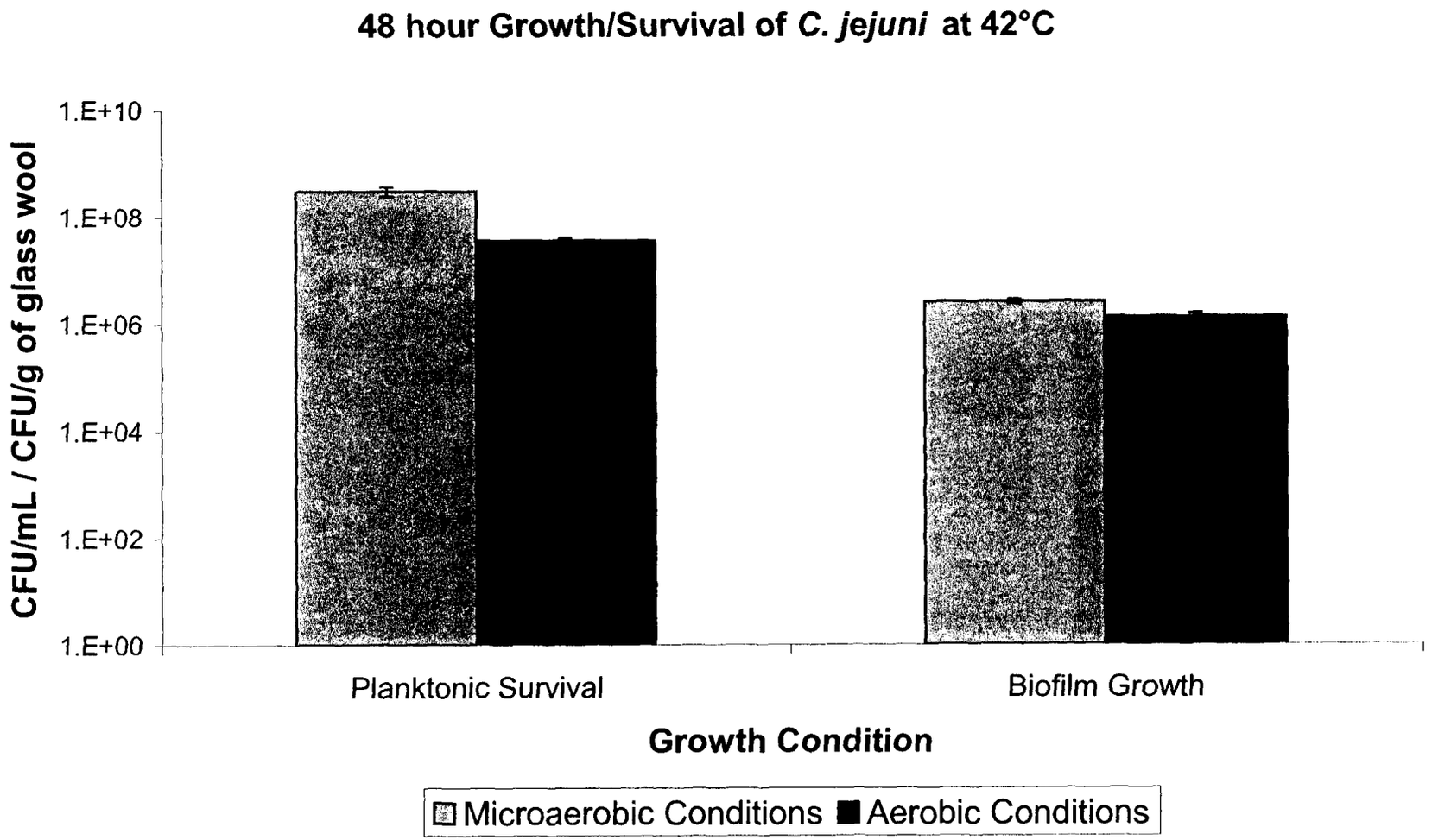

Figure 2.1 Growth/Survial data for *Campylobacter jejuni* 16-2R. Biofilms were extracted from 0.11g of glass wool and suspended in 10mL of Brucella broth with 1% Tween. Both planktonic survival and biofilm formation were assessed by drop plating on Campy-Line agar and incubating for 48 hours at 42°C in microaerobic conditions. Error bars represent the standard error of the mean.

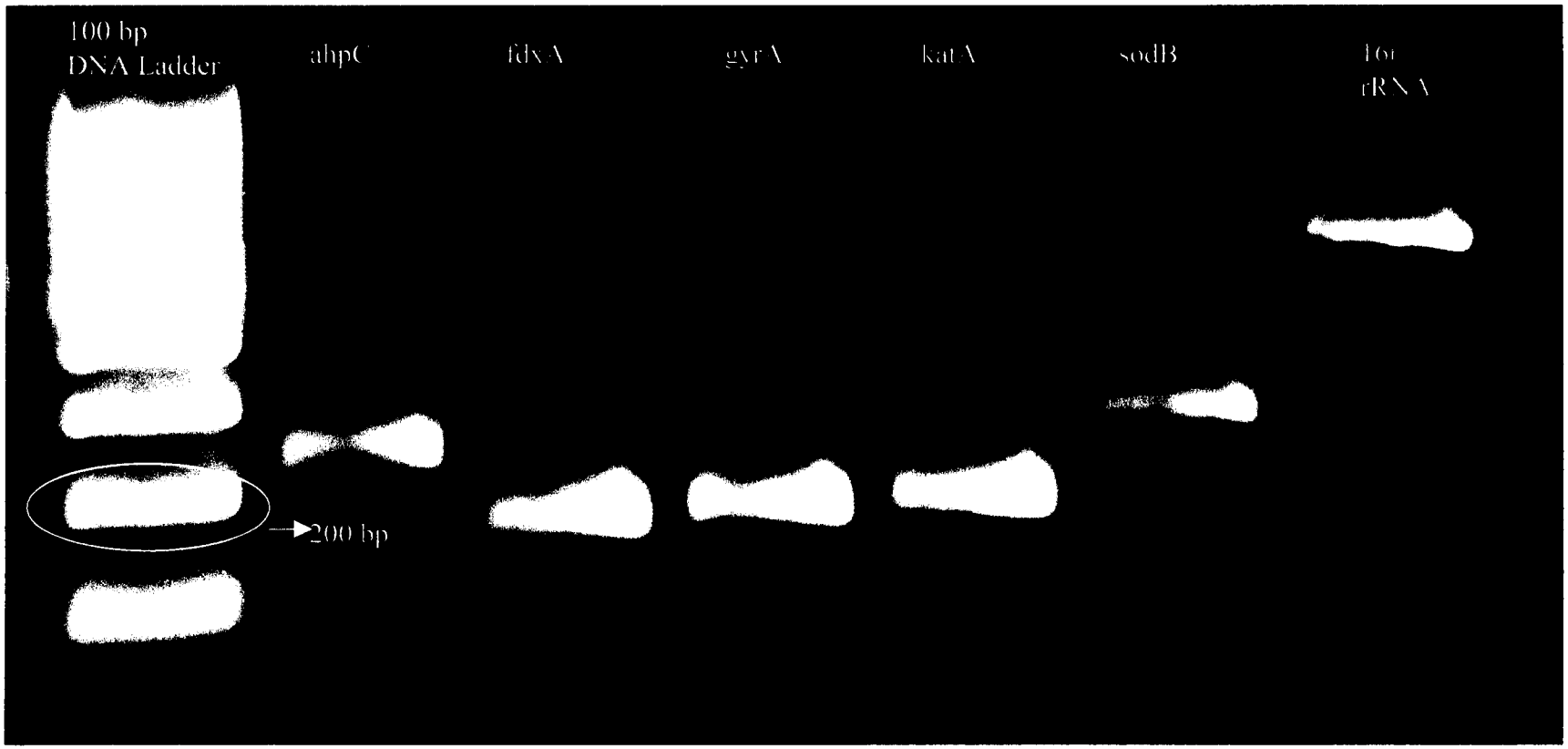

Figure 2.2 *Alkyl Hydroperoxide Reductase C (ahpC)*, *Ferridoxin (fdxA)*, *Gyrase (gyrA)*, *Catalase (katA)*, *Super Oxide Dismutase (sodB)*, and *16S* PCR products from *Campylobacter jejuni* 16-2R DNA on a 1% Agarose gel containing ethidium bromide excited under UV light. Lane 1: 100bp Ladder, Lane 2: *ahpC*, Lane3: *fdxA*, Lane 4: *gyrA*, Lane 5: *katA*, Lane 6: *sodB*, Lane 7: *16S*.

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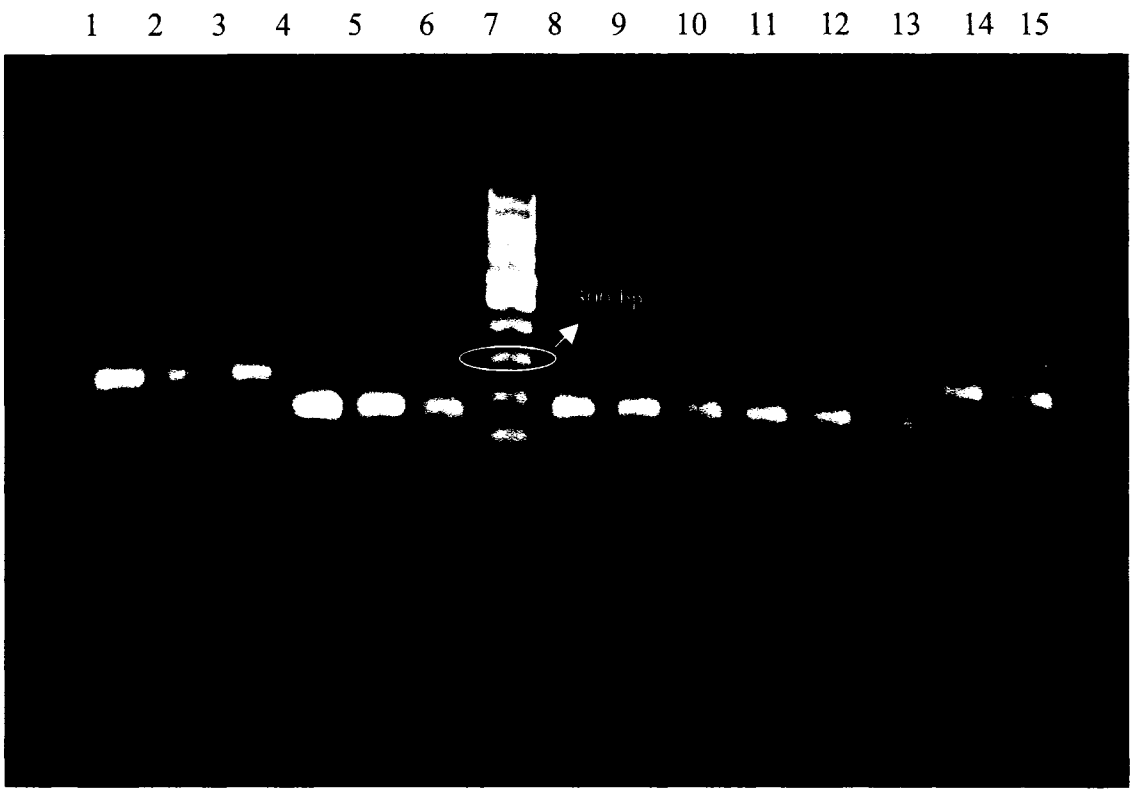

Figure 2.3 $MgCl_2$ optimization with DMSO of Alkyl Hydroperoxide Reductase (*ahpC*) (lanes 1-3), *Catalase (katA)* (lanes 4-6), *Ferridoxin (fdxA)*(lanes 8-10), *Gyrase (gyrA)* (lanes 11-13), and *Super Oxide Dismutase (sodB)* (lanes 14 & 15) on a 1% Agarose gel containing ethidium bromide excited under UV light. Products in lanes 1, 4, 8, 11, & 14 were amplified using 1.5mM of $MgCl_2$. Products in lanes 2, 5, 9, 12, & 15 were amplified using 3.0mM of $MgCl_2$. Products in lanes 3, 6, 10, & 13 were amplified using 4.5mM $MgCl_2$. 5µL of crude PCR product was loaded in lanes 1-6, & lanes 8-15. Lane 7 contained 1µL of a 100bp DNA ladder (Fermentas) and 4µL of water. *ahpC, katA, fdxA, gyrA, & sodB* were amplified from *Campylobacter jejuni* 16-2R

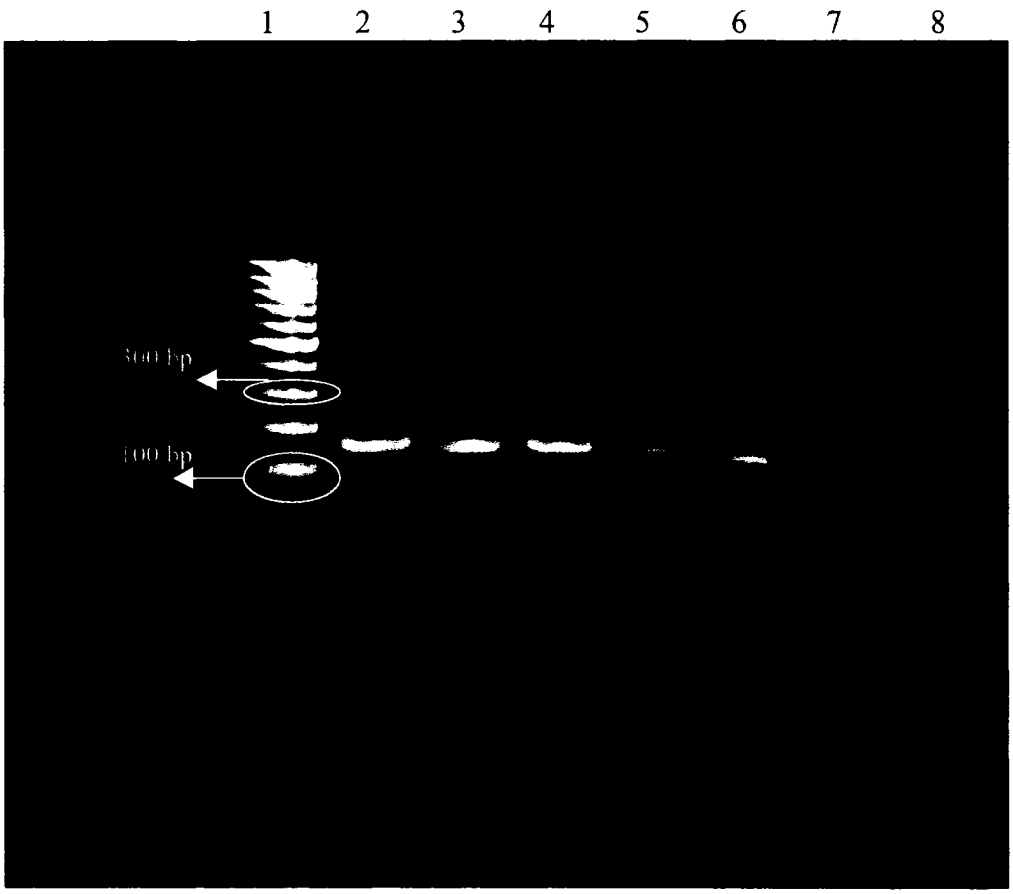

Figure 2.4 *Catalase (katA)* primer annealing temperature on a 1% Agarose gel containing ethidium bromide excited under UV light. Lanes 2, 3, 4, 5, 6, 7, 8 contain 5μL of crude PCR product amplified from *Campylobacter jejuni* 16-2R DNA. Lane 1 contains 1μL of 100bp DNA ladder (Fermentas) and 4μL of water. The annealing temperatures of each lane are as follows: Lane 2: 53.6°C, Lane 3: 55.0°C, Lane 4: 56.7°C, Lane 5: 58.2°C, Lane 6: 60.2°C, Lane 7: 61.8°C. Lane 8: Negative control.

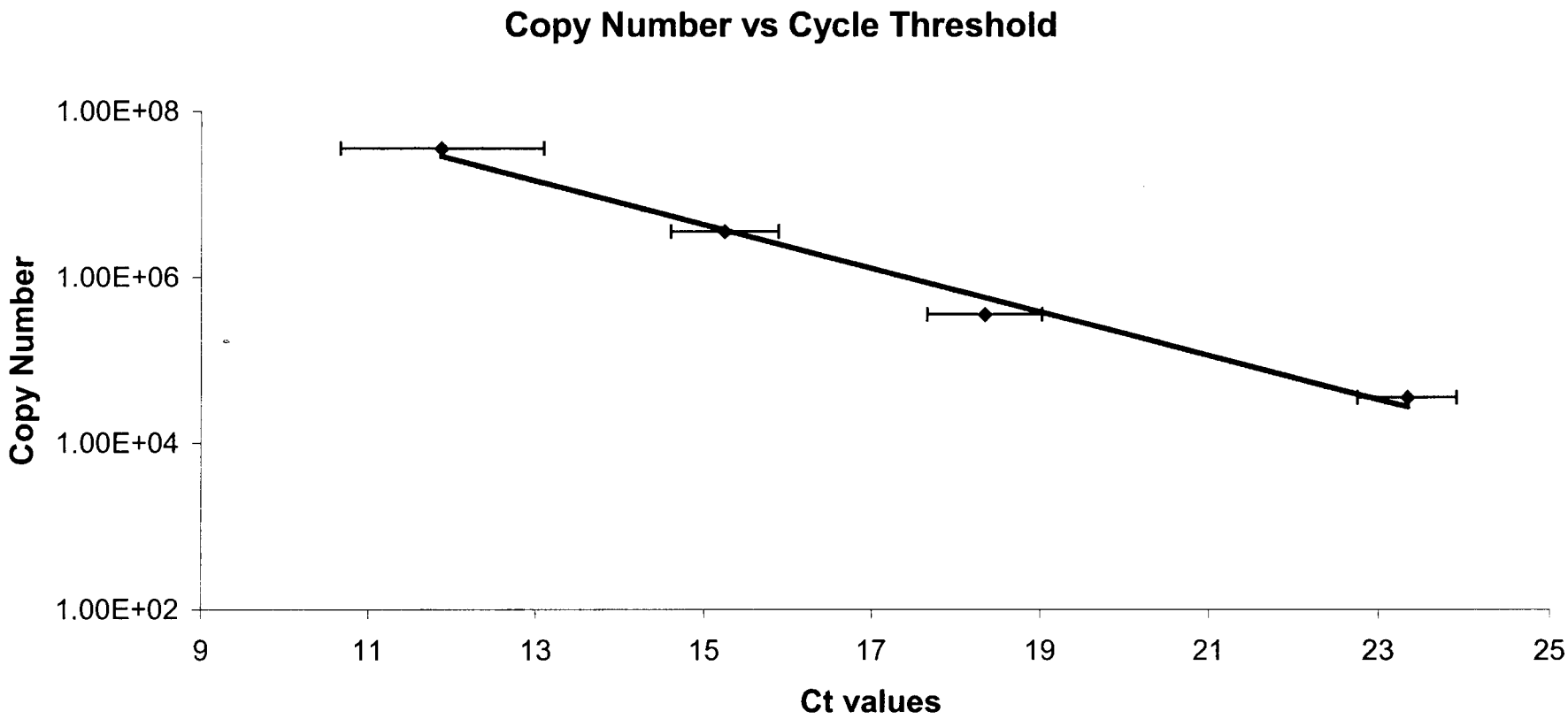

Figure 2.5 Standard Curve Generated for the ABI Prism 7000. A set of DNA standards were serially diluted to a copy number of $3.60x10^7$, $3.60x10^6$, $3.60x10^5$, and $3.60x10^4$ respectfully in three independent experiments then amplified on a ABI Prism 7000 using PCR conditions set up for a qRT-PCR one-tube assay with SYBR Green I chemistry. Error bars represent the standard error. The equation of the line generated was $y=4E+10e^{-0.6068x}$ and had an R^2 value of 0.99.

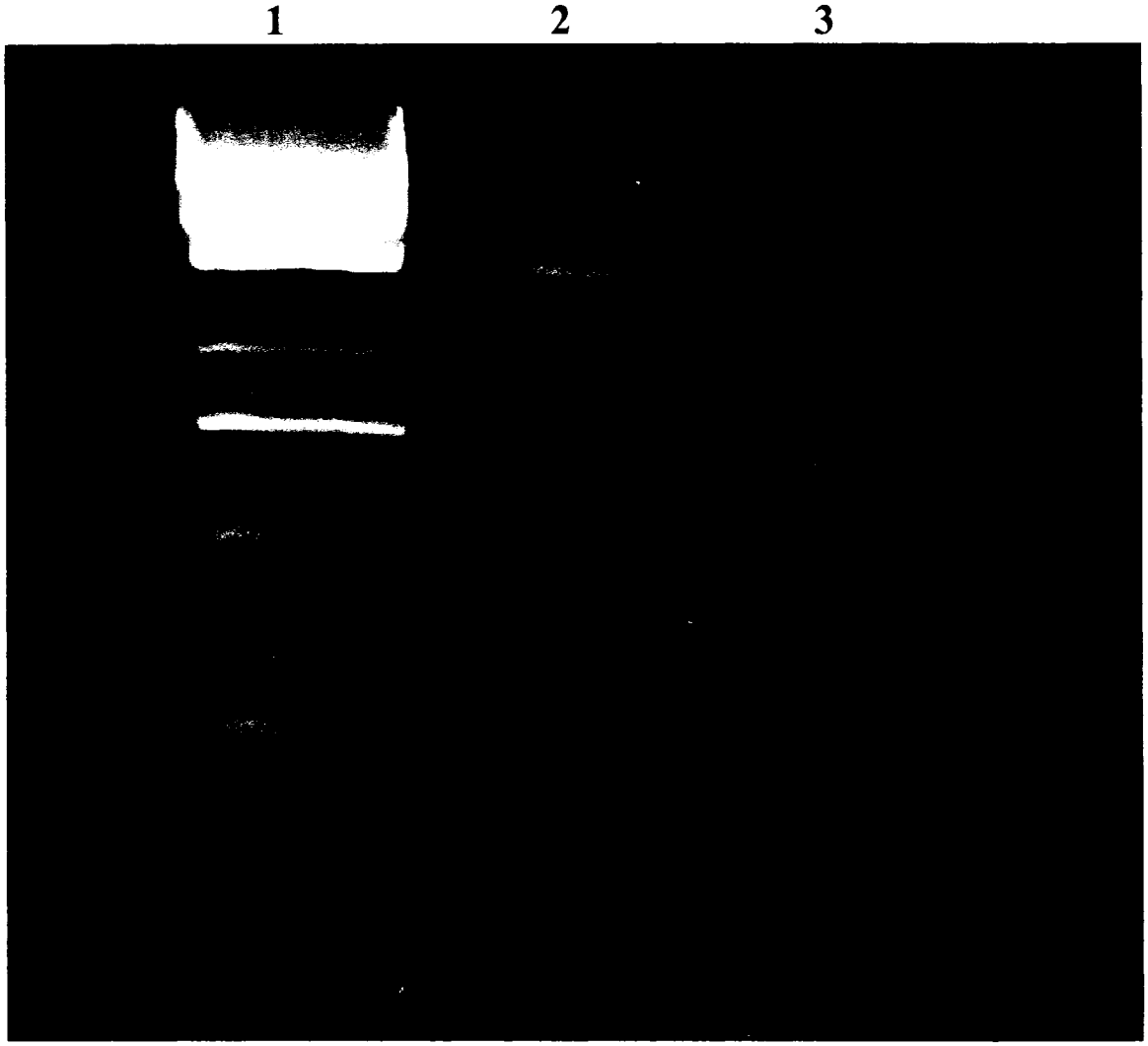

Figure 2.6 RNA extracted from *Campylobacter jejuni* 16-2R using the High pure total RNA extraction kit from Roche on a 1% Agarose gel containing ethidium bromide excited under UV light. 1μL of a 1kb DNA ladder (Fermentas) was loaded into lane 1 with 4μL of water. 5μL of an 84.5ng/μL sample of planktonic RNA was loaded into lane 2, and 5μL of a 74.1ng/μL sample of biofilm RNA was loaded into lane 3.

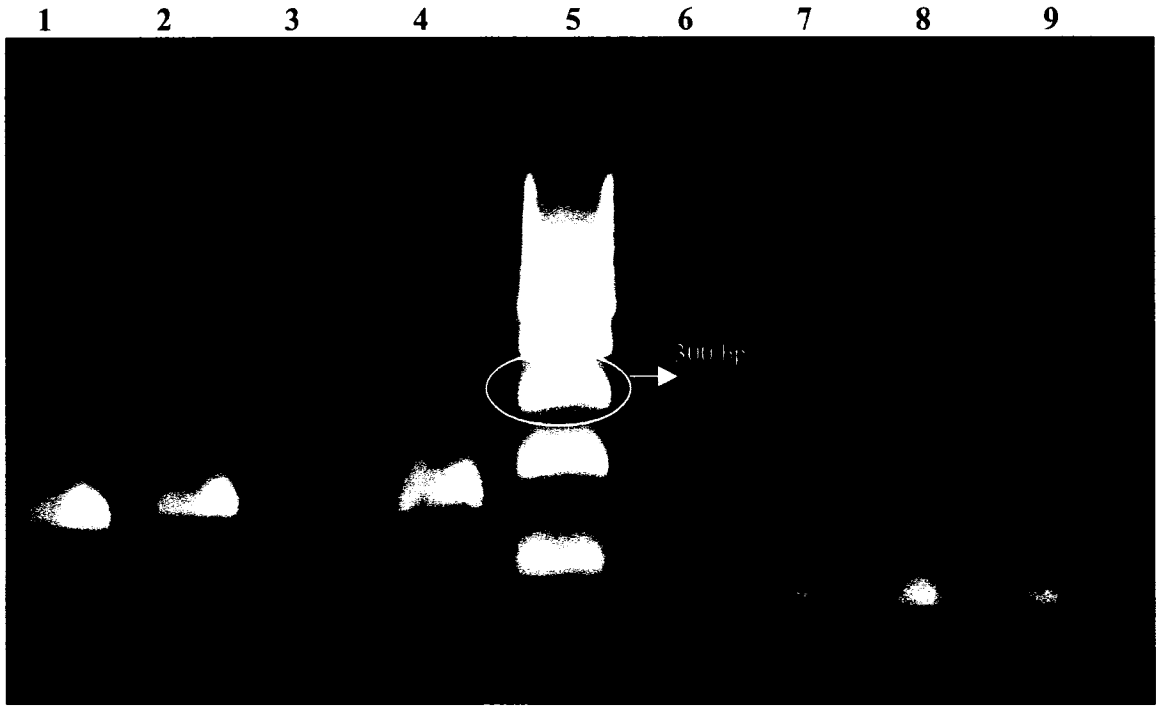

Figure 2.7 DNA contamination in the RNA samples. RNA extracted from *C. jejuni 16-2R* was used as a template for PCR with *katA* primers. Lanes 1-4: RNA extract used before DNase I treatment resulted in positive PCR signal, indicating co-extraction of DNA, along with RNA. Lanes 6-7: RNA extract used after DNase I treatment showed no PCR amplification. Lane 5: 100 bp DNA ladder.

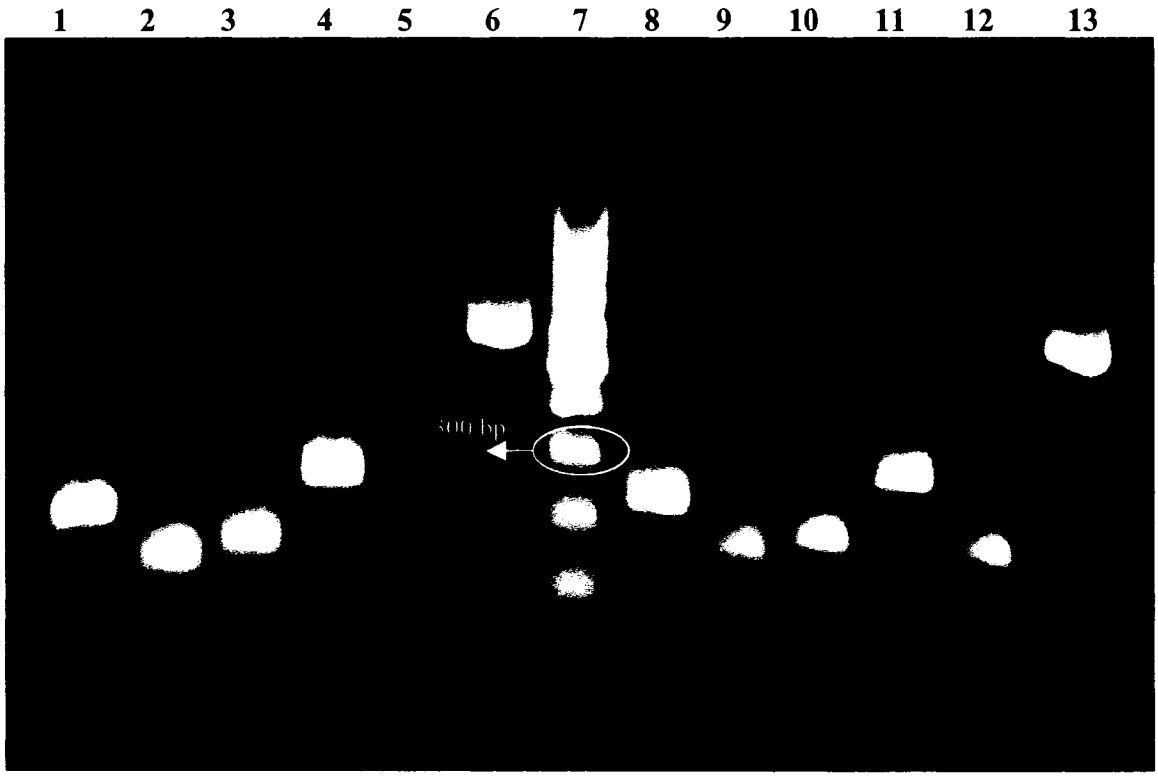

Figure 2.8 RT-PCR products from planktonic and biofilm growths of *Campylobacter jejuni* 16-2R on a 1% Agarose gel containing ethidium bromide excited under UV light. All gene targets were amplified from 2μL of total RNA extract using the Titan one-tube RT-PCR system from Roche. Lane 1: Planktonic *Alkyl Hydroperoxide Reductase (ahpC)*, Lane 2: Planktonic *Ferridoxin (fdxA)*, Lane 3: Planktonic *Catalase (katA),* Lane 4: Planktonic *Super Oxide Dismutase (sodB)*, Lane 5: Planktonic *Gyrase (gyrA)*, Lane 6: Planktonic *16S rRNA (16S)*, Lane 7: 100 bp Ladder, Lane 8: Biofilm *ahpC*, Lane 9: Biofilm *fdxA*, Lane 10: Biofilm *katA*, Lane 11: Biofilm *sodB*, Lane 12: Biofilm *gyrA,* Lane 13: Biofilm *16S.*

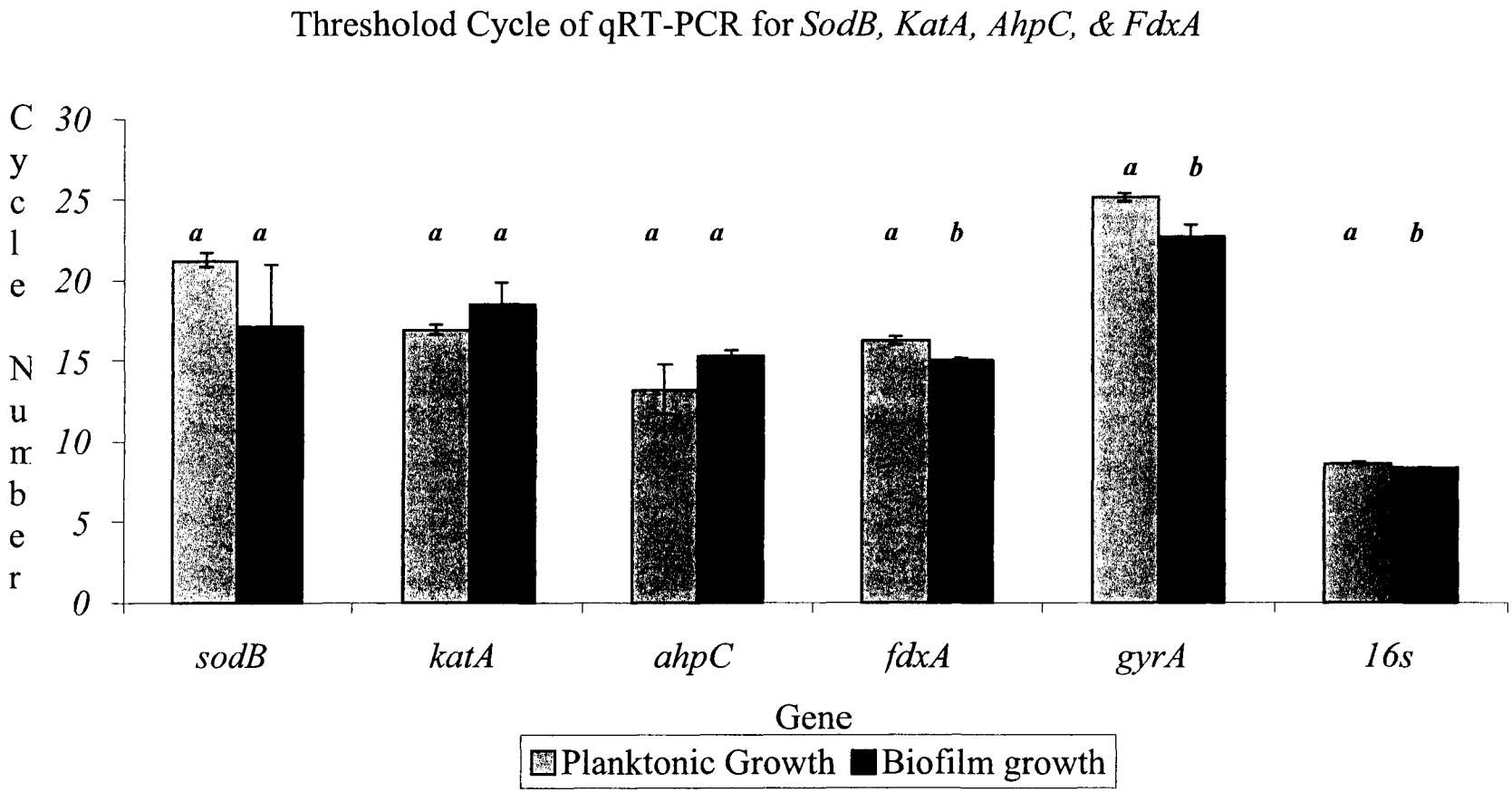

Figure 2.9 Cycle thresholds of *Superoxide Dismutase B (sodB)*, *Catalayse A (katA)*, *Alkyl Hydroperoxide Reductase C (ahpC)*, *Ferridoxin A (fdxA)*, *Gyrase A (gyrA)*, and *16S rRNA (16S)* from RNA extracted from 48 hour microaerobic biofilm growths and 48 hour microaerobic planktonic growth of *Campylobacter jejuni* 16-2R. Results generated from a qRT-PCR reaction carried out on an ABI 7000 prism real-time thermocycler. Error bars represent the standard deviation of the mean. For each gene target, bars with different letters were significantly different from the other ($P<0.05$).

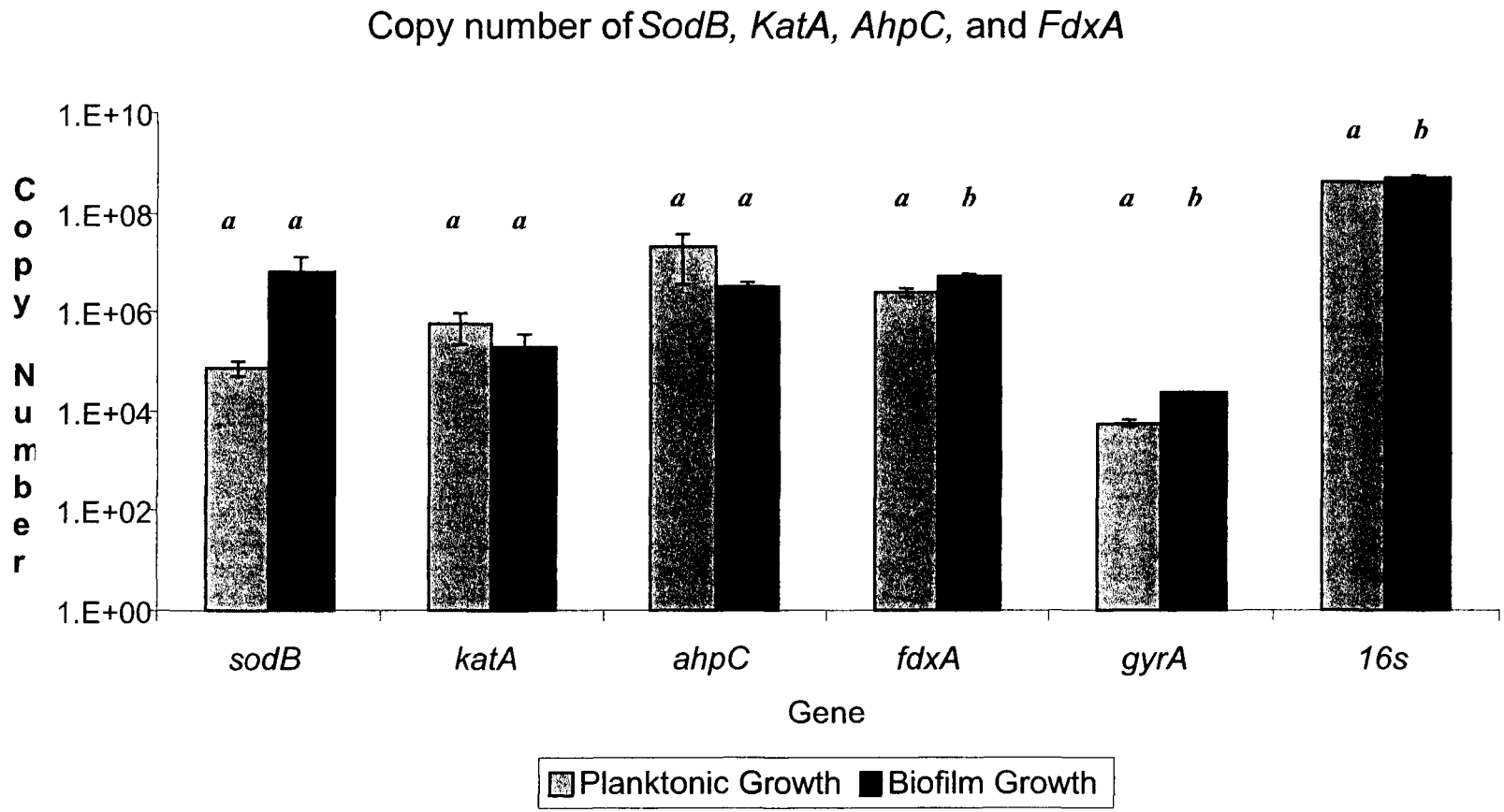

Figure 2.10 Copy numbers of *Superoxide Dismutase B (sodB)*, *Catalayse A (katA)*, *Alkyl Hydroperoxide Reductase C (ahpC)*, *Ferridoxin A (fdxA)*, *Gyrase A (gyrA)*, and *16S rRNA (16S)* from RNA extracted from 48 hour microaerobic biofilm growths and 48 hour microaerobic planktonic growth *Campylobacter jejuni* 16-2R. Results generated from a qRT-PCR carried out on an ABI 7000 prism real-time thermocycler Error bars represent the standard deviation of the mean. For each gene target, bars with different letters were significantly different from eachother ($p<0.05$).

Chapter 3: Comparison of a One and Two-Tube RT-PCR Assay

3.1 Introduction.

The use of quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) has been growing in popularity for use in gene expression studies (Bustin, 2000). However, there are a multitude of methods for performing qRT-PCR with little standardization between laboratories (Bustin and Nolan, 2004; Goerke et al., 2001). Due to the differences in manufacturers and costs between methods, many laboratories may find a need to change the methods used for gene expression studies (Bentley et al, 2005). However, there is still debate regarding convenience and sensitivity of different assay types. Therefore, this study was performed to assess if there was a difference between the sensitivity of two different types of Reverse-Transcription Polymerase Chain Reactions (RT-PCR) the Titan one-tube RT-PCR commercial kit based on the use of avian myeloblastosis virus (AMV), and a two-tube RT-PCR based on the use of Moloney murine leukemia virus (M-MLV) and Taq polymerase.

We also set out to determine if there was any difference in the use of two different Real-Time PCR platforms. The ABI Prism 7000 and the Cepheid Smart Cycler.

3.2 Methods.

3.2.1. Bacterial Cultures.

Bacterial Cultures were prepared as described in section 2.2.1

3.2.2. Growth, Harvest, and Enumeration of Planktonic and Biofilm Cells.

Growth, Harvest, and Enumeration of Planktonic and Biofilm Cells were prepared as described in section 2.2.2.

3.2.3. Extraction of Nucleic Acids.

Nucleic DNA and RNA were extracted as described in section 2.2.3.

3.2.4. Primer Design, and Optimisation of Polymerase Chain Reactions.

3.2.4.1. Primer Design.

Primers used are described in section 2.2.4.1

3.2.4.2. Reverse Transcription PCR Using a One-Tube Assay.

Reverse transcription PCR (RT-PCR) was carried out using the Titan one tube RT-PCR kit (Roche) using the manufacturer's instructions. RT-PCR (50μL) contained the final concentrations per reaction as follows: 200μM of each deoxynucleoside triphosphate (Promega), 0.4μM of each primer, 5mM DTT solution (Roche), 10 units of RNase inhibitor (RNasin® Ribonuclease inhibitor from Human placenta) (Promega), and 40ng of RNA template, 1X RT-PCR buffer (Roche), 1.5 mM $MgCl_2$, and 1μL of enzyme mix (Roche). Cycling conditions were as follows: a reverse transcription step of 52.0°C for 30 minutes followed by a denaturation step of 94.0°C for 2 minutes, followed by 10 cycles of 94.0°C for 30 seconds, 55.7°C for 30 seconds, and 68.0°C for 45 seconds were performed. The initial 10 cycles were followed by 25 cycles of 94.0°C for 30 seconds, 55.7°C for 30 seconds, and 68.0°C for 45 seconds plus five seconds per cycle. RT-PCR was run on a Hybaid PCR sprint thermocycler.

3.2.4.3. Reverse Transcription PCR Using a Two-Tube Assay.

Reverse transcription PCR (RT-PCR) was also carried out using M-MLV reverse transcriptase (Promega) using the manufacturer's instructions. RT-PCR (25μL) contained the following final concentrations per reaction: Tube 1: 50 ng of RNA, 20 units of RNase inhibitor (Promega), 20 pmol of each primer, up to 10 μL of nuclease free double

distilled H_2O. This mixture was heated to 70°C for 5 min to melt any secondary structure in the RNA template, then lowered to the specific annealing temperature of the gene specific primers for 45 seconds, and then placed on ice for 5 min. After placing on ice, the following reagents were added: M-MLV reaction buffer to a final concentration of 1X, 100 μM of each deoxynucleoside triphosphate (Promega), 200 units of M-MLV reverse transcriptase (Promega), and nuclease free double distilled H_2O to a final volume of 25μL. After gentle flicking to mix the reagents the tube was spun down, and Tube 1 was incubated at 45°C to carry out the reverse transcription step for 60 minutes. Following reverse-transcription 2μL or 4μL of product was transferred from Tube 1 to Tube 2 for PCR in a 25μL volume.

3.2.4.4 PCR Conditions.

PCR (25 μL) contained final concentrations of 500ng of purified *Campylobacter jejuni* 16-2R DNA, 1X Buffer (Fermentas), 1.5 mmol $MgCl_2$ (Fermentas), 200 μM of each deoxynucleoside triphosphate (Promega), 0.5 μM of each primer and 1 Unit of Taq polymerase (Fermentas). PCR was carried out on a Hybaid PCR Sprint thermocycler. Cycling conditions were as follows: initial denaturation at 94.0°C for 5 minutes, followed by 10 cycles of 94.0°C for 30 seconds, 55.0°C for 30 seconds, and 72°C for 45 seconds, were performed. This was followed by 20 cycles of 94.0°C for 30 seconds, 55.0°C for 30 seconds, and 72.0°C for 45 seconds plus one second per cycle. A final step of 72.0°C for 5 minutes was carried out.

For each assay, a master mix containing all of the reagents required for the number of reactions with the exception of the DNA was prepared for each gene target. Mixing was carried out under sterile conditions and care was taken to ensure that there was no cross

contamination of samples. For each assay, a negative control was included. The negative control consisted of all of the materials for the reaction, but sterile DNA/RNA free double distilled water was used instead of a DNA template.

PCR product was run on a 1% agarose Gel containing ethidium bromide visualized under a UV light to confirmation of amplification. An image of the gel was obtained using the Syngene photodocumentation system and software.

3.2.5 qRT-PCR Methods.

3.2.5.1 qRT-PCR.

quantitative RT-PCR (qRT-PCR) used the same conditions as RT-PCR. The exception was 1.25μL of a 10X SYBR Green I mix (Applied Biosystems) was added to replace some of the water for a final concentration of 0.5X per reaction. Samples were loaded into 0.2mm optically pure strip tubes (Applied Biosystems) and run on an ABI 7000 prism (Applied Biosystems) thermocycler, or loaded into optically pure tubes (Cepheid) and run on a Smart Cycler (Cepheid).

3.2.5.2 Creation of external standards.

External standards were prepared as described in section 2.2.5.2

3.2.6 Comparison between the Cepheid Smart Cycler and the ABI Prism 7000.

Standard curves of known copy numbers were compared for three independently replicated experiments using both the Cepheid Smart Cycler and the ABI Prism 7000. Samples were prepared as above, and put in a 25μL PCR reaction as described above with 1.5μL of SYBR Green in place of the same amount of nuclease free water. Negative controls were also run to rule out contamination.

To compare the ability of the Cepheid Smart Cycler and the ABI Prism 7000 to detect the cycle threshold of RT-PCR products, the 16S rRNA was amplified using the two-tube RT-PCR procedure described above.

Data were analyzed using SigmaStat version 2.03 software (SPSS inc). To test for significance, a one-way ANOVA was performed. If a significant result was obtained Turkeys test was performed to test for the difference between significant results.

3.2.7 Comparison of a One-Tube assay and a Two-Tube assay.

C. jejuni 16-2R biofilms were grown, harvested, and quantified as described above. RNA was extracted and then amplified using both the one-tube RT-PCR and the two-tube RT-PCR. For the two tube RT-PCR both 2μL and 4μL amounts of RT-Product were used in the second tube stage. Results were analysed on the ABI Prism 7000 in three independent replicate experiments.

Data were analyzed using SigmaStat version 2.03 software (SPSS inc). To test for significance, a one-way ANOVA was performed. If a significant result was obtained Turkeys test was performed to test for the difference between significant results.

3.3 Results.

Initial reaction conditions showed that many factors were required to optimize the two-tube protocol when compared to the Titan one-tube system. The two-tube M-MLV protocol was more sensitive to non-specific binding of the primers and therefore produced non-specific products during the reverse transcription step (Figure 3.1). Reduction of the primer amount and the addition of an annealing step following the RNA melting step, to eliminate of secondary structure, solved this problem (Figure 3.2). The

concentration of primers that was finally used was 20 pmol of each primer in the reverse transcription step.

After being resolved on a gel, the product of the two-tube RT-PCR was less intense than the product from the Titan one-tube PCR when 2μL of product was used from the first step of the two-tube PCR. When 4μL of product was used, the bands were just as intense as the Titan one-tube product. The two-tube RT-PCR was able to amplify *ahpC, fdxA, katA, sodB*, and the *16S* RNA targets from *C. jejuni* 16-2R biofilm cultures.

Standard curves were created using known copy numbers of PCR products on both the Cepheid Smart Cycler and the ABI Prism 7000. For this comparison, copy numbers of $2.44x10^7$, $2.44x10^6$, $2.44x10^5$, and $2.44x10^4$ were used. The average threshold cycle for each standard were 11.90±0.50, 15.69±0.37, and 18.63±0.40, and 24.00±0.29 respectively for the ABI Prism 7000. The threshold cycles for the Cepheid Smart cycler were 11.59±0.37, 14.8±0.58, and 18.65±0.29, and 24.00±0.29. The standard curve for the Cepheid had an r^2 value of 0.997, and the r^2 value for the ABI Prism was 0.995 (Figure 3.4 and Figure 3.5). There was no significant statistical difference between the threshold cycles of the standards between the ABI Prism 7000 and the Cepheid Smart cycler (p-values of 0.897, 0.298, 0.943 and 0.968).

qRT-PCR results showed a significant difference between the amplification of *ahpC* from *C. jejuni 16-2R* planktonic cells amplified with the Titan one-tube PCR system and using 2μL of product from the first step in the two-tube RT-PCR. The average difference in cycle thresholds was 1.34±0.12 cycles (p=0.031). However, *fdxA* from *C. jejuni 16-2R* biofilms showed that when 4μL of product was used in the second stage showed no statistical difference in the reactions (p=0.080). *ahpC* values had no

statistical difference when 4μL of the two-tube product was used (p=0.388). Copy numbers of *ahpC* and *fdxA* were also similar between the assays when 4μLof two- tube product was used (Figure 3.6).

3.4.Discussion.

The current study shows that there are some differences in reaction efficiencies, and reaction set up when using different reverse transcription assay protocols. Although quantitative reverse-transcription PCR (qRT-PCR) is widely utilized in gene expression studies, there is little standardization in methodology (Gabert et al., 2003). Therefore, assays must be run to determine if any differences exist between the methods used before any comparisons of results can be made with confidence. Differences to be considered include enzyme efficiency, reaction efficiencies due to reactant differences, and differences in real-time platform.

Our comparison included two different enzymes (AMV and M-MLV), two different approaches to second strand amplification (One-Tube design and Two-Tube design), and two amplification platforms (ABI Prism 7000 and Cepheid Smart Cycler). There were differences in the protocol between the Titan One-Tube system and the Two-Tube system. The Titan One-Tube RT-PCR kit did not require a primer-template incubation step prior to the incubation with the reverse transcriptase. The incubation of template with primers was deemed unnecessary, but the manufacturers indicated in the instructions provided with the kit, that it could be carried out if so desired. With the Two-Tube assay this incubation was required to avoid non-specific binding of the primers. Non-specific binding was likely caused by the lower temperature used for the reverse transcription with M-MLV reverse transcriptase, which was approximately 10°C lower

than the optimal annealing temperature of the primers. The Titan One-Tube system's reverse transcription step was approximately 5°C lower than the optimal primer annealing temperature. Although both temperatures would likely cause mis-priming, it would be more severe in the Two-Tube system. Non-specific products were effectively eliminated from the Two-Tube system by incorporating a proper annealing step prior to the incubation with the reverse transcriptase. The Two-Tube system was also more sensitive to the concentration of primers added. By its very nature, the Two-Tube's requirement for extra RNA handling makes it more labour intensive than the Titan One-Tube system.

Arguments have been made that these extra handling steps increase the chances of sample cross contamination (Bustin, 2002; Cale et al., 1998; Tobisch et al., 2003). Even with the use of master mixes, we did not find any problems with contamination between samples, as assessed by negative controls (data not shown). Although many researchers prefer to create cDNA libraries with a Two-Tube system due to the belief that cDNA is more stable than mRNA, a recent report has demonstrated that cDNA may in some cases have greater instability than mRNA (Wacker and Godard, 2005). This issue can be overcome by treating extracted mRNA and newly synthesized cDNA in a similar manner of either immediate use, or storage at –80°C.

The common practice of diluting the cDNA prior to second strand amplification is well documented in the literature, and this was exploited in this study by using only a portion of the cDNA in the second stage of the Two-Tube procedure as opposed to amplifying the total concentration of cDNA as in the one-tube reaction (Lekanne Deprez et al., 2002). As expected, when 2μL of cDNA was amplified in the second stage, the

signal as seen in a 1% agarose gel with ethidium bromide staining was weaker than the Titan One-Tube product. However, when 4μL of cDNA was amplified, the signal strength was not only comparable to the Titan One-Tube procedure when compared on a gel, but also had threshold values comparable to the Titan One-Tube kit. A dilution of cDNA (4μL) is used in a Two-Tube protocol using M-MLV and the One-Tube protocol with AMV utilizes the total cDNA synthesized in the sample. Therefore, the Two-Tube protocol utilizing M-MLV is more efficient at detecting the mRNA concentrations from *C. jejuni* 16-2R than a One-Tube system utilizing AMV since the Two-Tube protocol will detect the gene products at the same Ct values as the One-Tube protocol using a more diluted cDNA sample.

There could be a number of explanations for the higher efficiency of the Two-Tube reaction. Explanations range from differences in the availability of reagents to enzyme kinetics. Although there is an increase in the concentration of primers, and other reagents of PCR in the second stage for the Two-Tube reaction due to the transfer of product to a new tube, this increase is probably minor since the reagents of the Titan One-Tube system are in abundance. However, the Two-Tube protocol did not have the addition of dithiothreitol (DTT) to the solution. The Titan one-tube reaction calls for the addition of DTT, which is a sulfide bridge stabilizer. Lekanne Deprez et al (2002) found that addition of DTT, even in small amounts, to a real-time reaction with SYBR Green, resulted in higher Ct's, greater background florescence, and a decrease in the steepness of the amplification curves. Therefore, real-time reactions using the Titan one-tube RT-PCR kit should be tested without the addition of DTT.

Traditionally, researchers have reported that there is greater sensitivity of one-tube reactions than two tube reactions (Tobisch et al., 2003; Wacker and Godard, 2005). Many of these studies have compared assays using random hexadimers in the Two-Tube assays and gene specific primers in the One-Tube assays (Wacker and Godard, 2005). However, there are conflicting studies that also show that Two-Tube/two enzyme assays can be more sensitive than One-Tube/two enzyme assays (Battaglia et al., 1998; Bustin, 2002; Manayani et al., 2002). We have described a comparison of a One-Tube and Two-Tube assay both using gene specific primers, and have found that the two-tube assay had greater sensitivity than the one-tube assay. We also avoided the preferential amplification of one target over another by using gene specific primers to amplify one gene target during the reverse transcription step instead of using random hexadimers (Markoulatos et al 2002).

Differences in the sensitivities between the Titan One-Tube RT-PCR reaction and the two-tube RT-PCR reactions could also be explained by the differences in enzyme kinetics. The two-tube assay probably had less carry over of reverse transcriptase to the second stage of amplification, since not all of the cDNA was used in the second step. Reverse transcriptase to Taq polymerase ratio affects amplification: the greater the concentration of reverse transcriptase, the greater the inhibition of the Taq polymerase (Sellner et al., 1992). The inhibition of Taq polymerase occurs even after the reverse transcriptase has been heat deactivated. However, this was probably not an issue in our assay, since the Titan One-Tube RT-PCR kit is a commercial kit and the enzyme mix is probably within the optimal level to avoid inactivation of the Taq, with a ratio of less than 3:2 units of reverse transcriptase to Taq (Sellner et al., 1992).

A more likely explanation the difference in amplification sensitivity is the type of reverse transcriptase itself. The Titan One-Tube kit uses AMV reverse transcriptase, and the Two-Tube assay uses M-MLV reverse transcriptase. The AMV reverse transcriptase is popular in One-Tube RT-PCR due to its higher incubation temperature (Bustin, 2002). However, the M-MLV reverse transcriptase has been shown to be more sensitive than AMV by producing a higher yield of cDNA from mRNA (Stahlberg et al., 2004). In fact M-MLV conversion average is about 44% of the mRNA to cDNA, while AMV converts only an average of 2.0% (Stahlberg et al., 2004). The 20-times greater efficiency in yield was demonstrated by Stahlberg et al (2004) across a range of mRNA concentrations. Differences in efficiency would explain why our two-tube assay showed similar results to the One-Tube when only 4μL of transcribed product was used from a 25μL reaction.

There was no significant difference in the detection of an assay when we moved them between the ABI Prism 7000 and the Cepheid Smart Cycler. The multiple amplification of standard curves and two genes of interest confirmed the transition. Therefore, our results match those of Bentley et al (2005) who also compared moving a detection assay from a ABI Prism 7000 to a Cepheid Smart cycler.

3.5 Conclusions.

Switching Real-Time PCR platforms can be accomplished easily by comparing an assay on both platforms. Switching between different RT-PCR conditions is more problematic and requires knowledge of enzyme kinetics and optimization parameters to standardize protocols. We found that a two-tube RT-PCR using M-MLV reverse transcriptase was more sensitive than the Titan one-tube RT-PCR when gene specific primers were used. Therefore, the selection of RT-PCR protocols should reflect the type

of study the researcher wishes to conduct. Our results would suggest that an RT-PCR protocol using M-MLV and gene specific primers should be used when looking at rare mRNA targets.

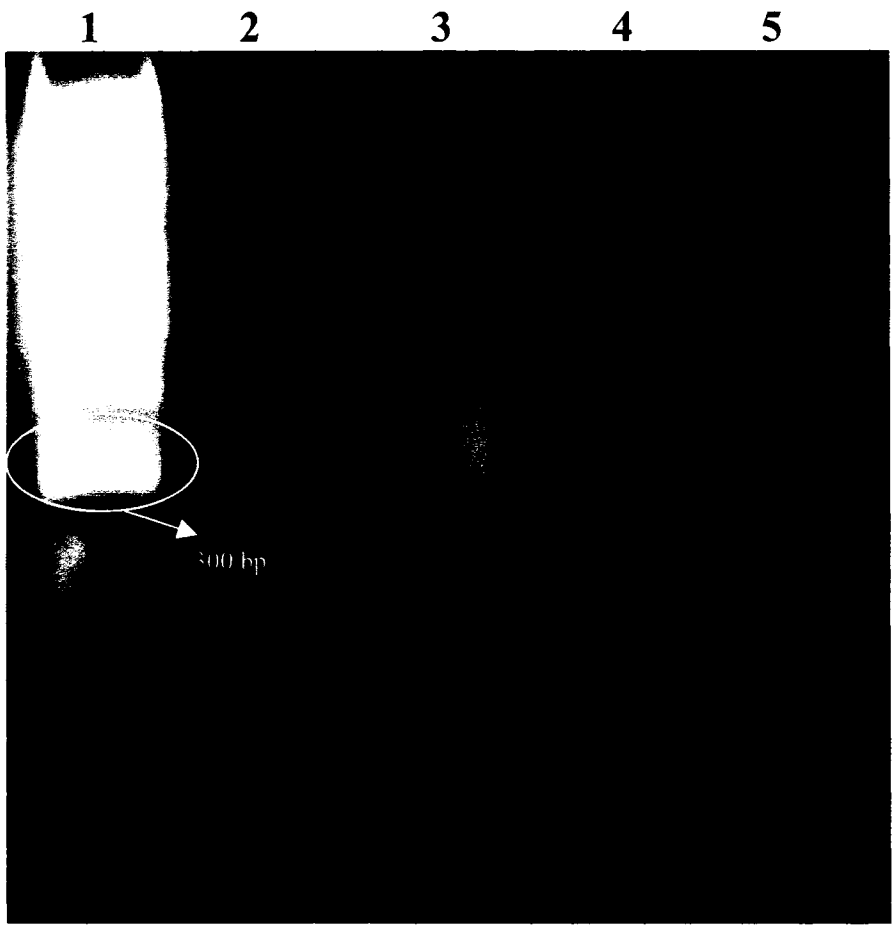

Figure 3.1 Two-Tube RT-PCR prior to optimization. 40 ng of *C. jejuni* 16-2R RNA from microaerobic biofilm RNA was amplified in a Two-tube RT-PCR reaction then run on a 1% Agarose gel with ethidium bromide staining. Lane 1: 100 bp standard, Lane 2: *ahpC*, Lane 3: *fdxA*, Lane 4: *katA*, and Lane 5: *sodB*.

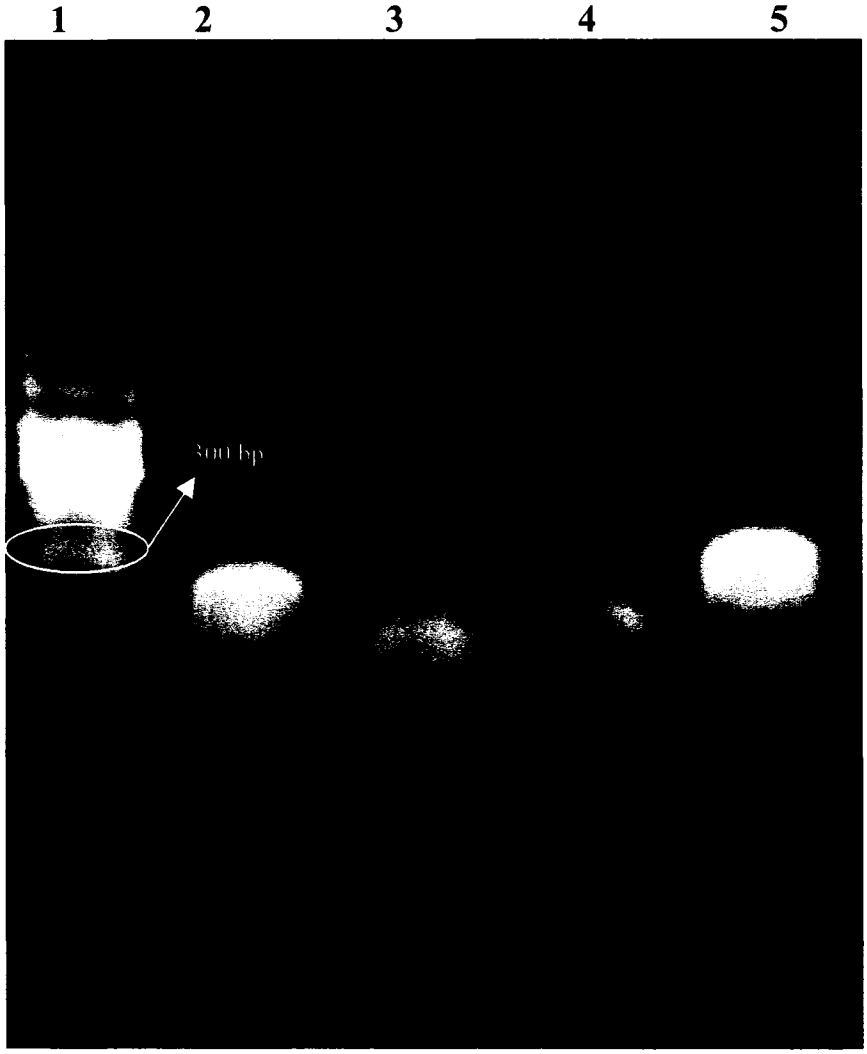

Figure 3.2 Optimized Two-Tube RT-PCR reaction. 40 ng of *C. jejuni* 16-2R RNA from microaerobic biofilm RNA was amplified in a Two-tube RT-PCR reaction then run on a 1% Agarose gel with ethidium bromide staining. Lane 1: 100 bp standard, Lane 2: *ahpC*, Lane 3: *fdxA*, Lane 4: *katA*, Lane 5: *sodB*.

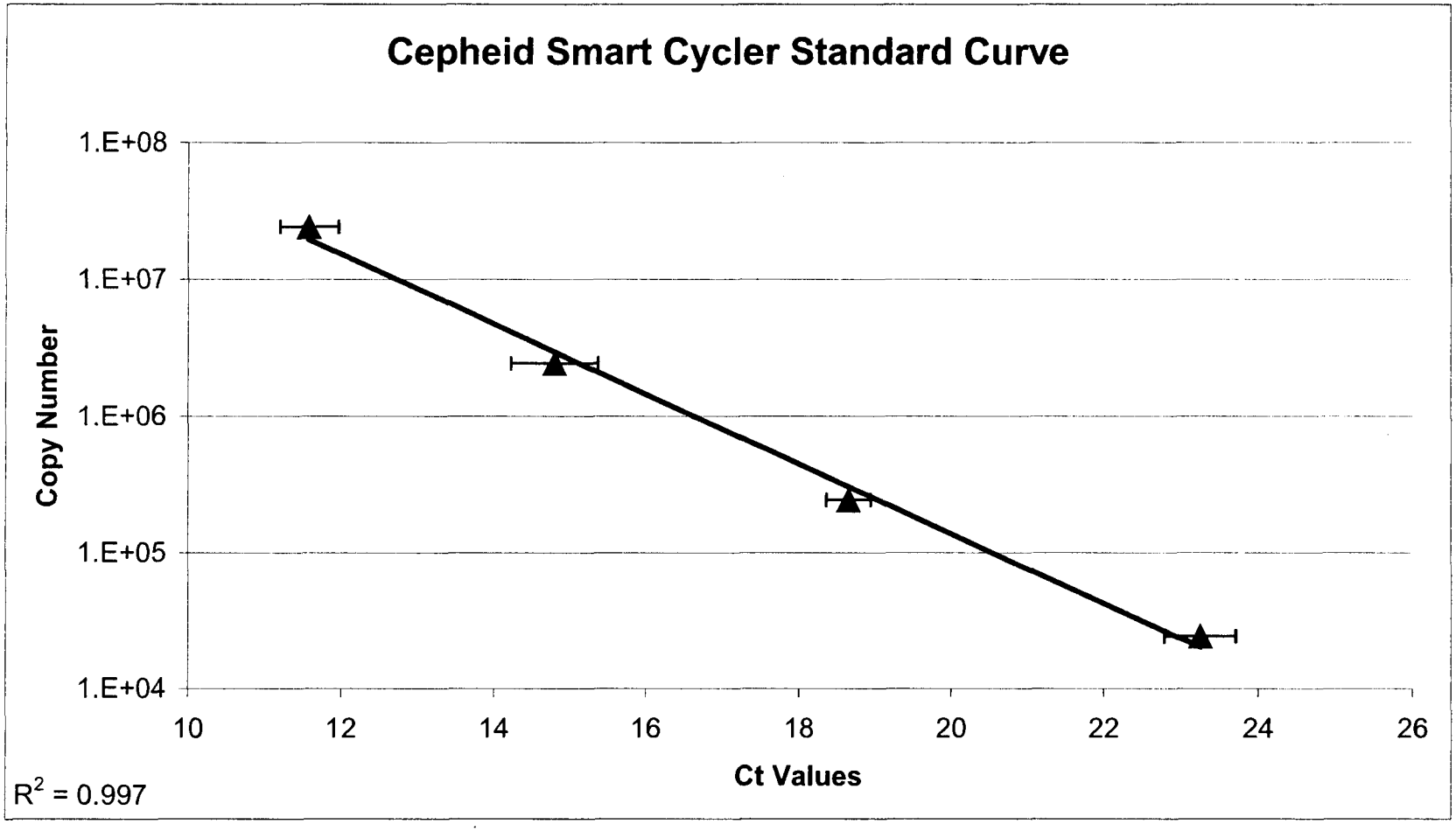

Figure 3.3 Standard Curve Generated for the Cepheid Smart Cycler. A set of DNA standards were serial diluted to 2.44e7, 2.44e6, and 2.44e5 respectfully in triplicate then amplified on a Cepheid Smart Cycler using PCR conditions set up for a qRT-PCR two-tube assay with SYBR Green I chemistry. Error bars represent the standard error. The equation of the line generated was $y=4E+10e^{-0.6502x}$ and had an R^2 value of 0.997.

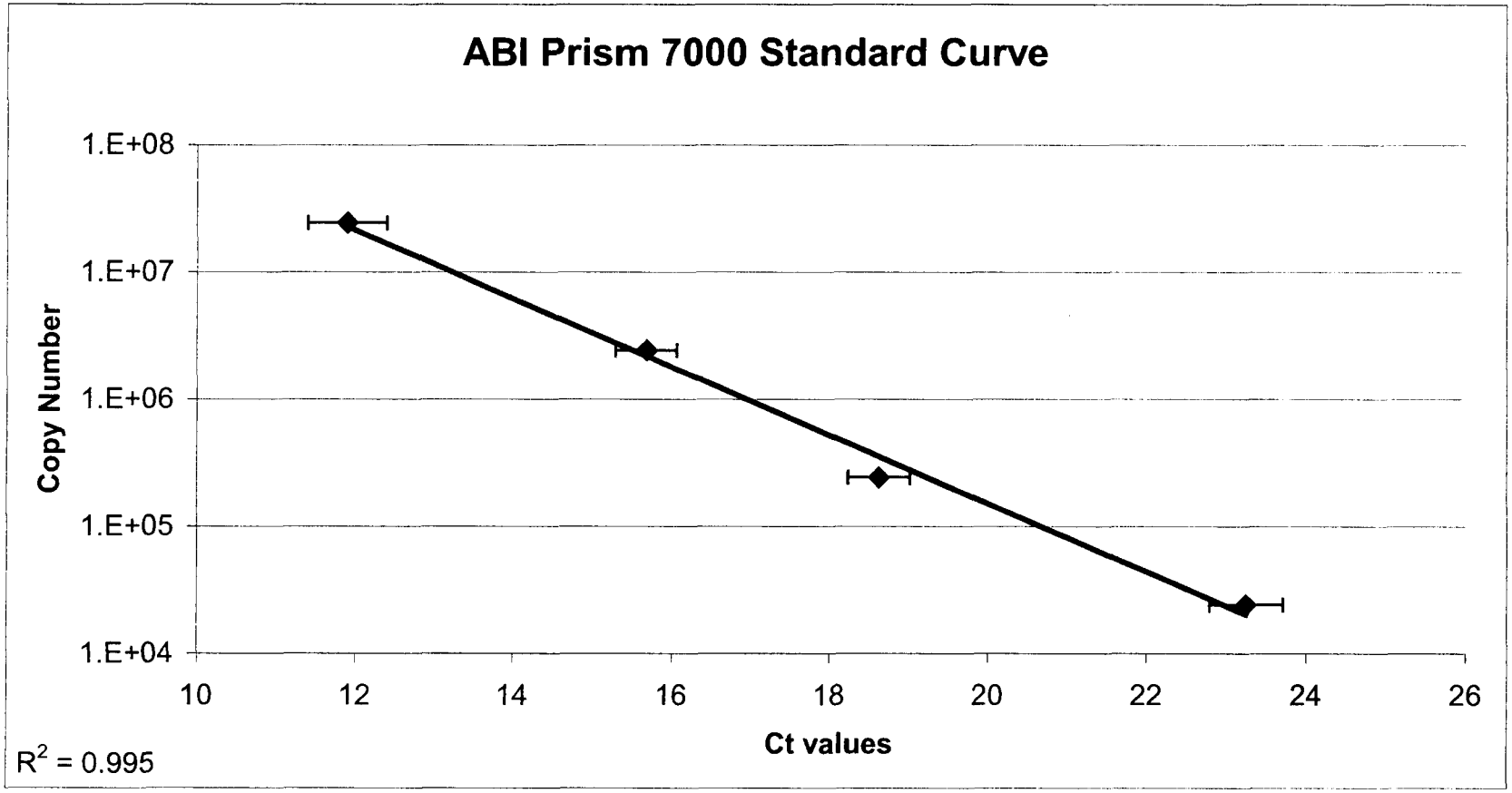

Figure 3.4 Standard Curve Generated for the ABI Prism 7000. A set of DNA standards were serial diluted to 2.44e7, 2.44e6, and 2.44e5 respectfully in triplicate then amplified on a Cepheid Smart Cycler using PCR conditions set up for a qRT-PCR two-tube assay with SYBR Green I chemistry. Error bars represent the standard error. The equation of the line generated was $y=9E+10e^{-0.6814x}$ and had an R^2 value of 0.995

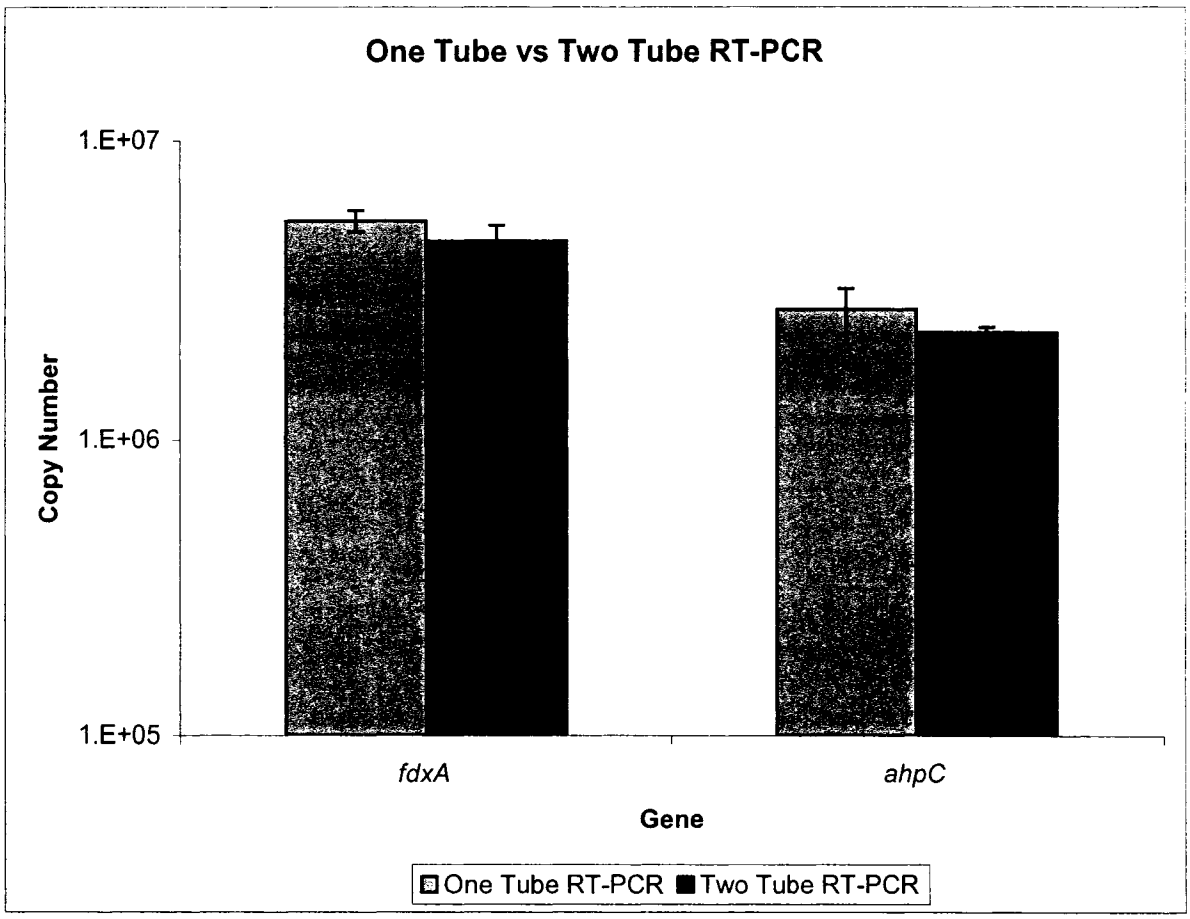

Figure 3.5 Copy number comparison of *ahpC* and *fdxA* from a One-Tube RT-PCR reaction, or Two-Tube reaction. 40ng total RNA from *C. jejuni* 16-2R microaerobic biofilm was added to the Titan One-Tube RT-PCR kit containing SYBR green I, or a Two-Tube RT-PCR reaction. 4μL of cDNA from the 25μL two-tube RT reaction was added to a 25μL PCR containing SYBR green I. Copy numbers were determined by comparing the threshold values of the RT-PCR reactions to the threshold values with standards of known target copy numbers amplified under the same conditions as the RT-PCR assay being tested.

Chapter 4: Gene Expression Comparison Between Aerobic and Microaerobic Biofilms

4.1 Introduction.

In section 2 we described the lack of difference in gene expression between *C. jejuni* biofilms and planktonic cells grown under microaerobic conditions. However, there is some speculation in the literature that bacteria in biofilms are protected by their polysaccharide matrix, and thus limiting the amount of stress that reaches the bacteria (Costerton et al., 1995; Moen et al., 2005). On the other hand, reports have been published recently proposing that stress response genes are also up-regulated in biofilms. Thus the stress must somehow either reach the bacteria or be sensed by intercellular communication strategies of biofilm cells (Branda et al., 2005; Fux et al., 2005; Sampathkumar et al., 2006).

Although this study is not setting out to solve this debate, it attempts to shed some light on whether *C. jejuni* 16-2R up-regulates its oxidative stress response genes when grown in biofilms that are exposed to atmospheric oxygen. If there is no change in the gene expression of *ahpC, fdxA, katA*, or *sodB* between biofilms grown in oxygen compared to biofilms grown in microaerobic conditions, then *C. jejuni* 16-2R may have some other method to protect itself from oxidative stress when grown aerobically in biofilms. However, if there is up-regulation in the oxidative stress genes, then it may indicate that those genes could play a role in protecting *C. jejuni* from increases in oxidative stress.

4.2 Methods.

4.2.1. Bacterial Cultures.

Cultures were prepared as described in section 2.2.1

4.2.2. Growth, Harvest, and Enumeration of Planktonic and Biofilm Cells.

Growth, harvest, and enumeration of planktonic and biofilm cells were carried out as outlined in section 2.2.2.

4.2.3. Extraction of Nucleic Acids.

Extraction of nucleic acids was carried out as per section 2.2.3, with the exception that the extraction buffer was allowed to interact with the High Pure RNA spin column for one minute prior to the final spin. This allowed for extraction of a greater amount of RNA.

4.2.4. Primer Design, and Optimization of Polymerase Chain Reactions.

4.2.4.1. Primer Design.

The primers described in section 2.2.4.1 were used for all experiments, with the exception of the *gyrA* forward and reverse primers, which were not used in this study.

4.2.4.3 Creation of external standards.

External standards were created following the procedures set out in section 2.2.5.2

4.2.4.4. Reverse Transcription PCR Using a Two-Tube Assay.

Reverse transcription PCR (RT-PCR) was also carried out using M-MLV reverse transcriptase (Promega) using the manufacturer's instructions. RT-PCR (25μL) contained the following final concentrations per reaction: Tube 1: 50 ng of RNA, 20 units of RNase inhibitor (Promega), 20 pmol of each primer, up to 10 μL of nuclease free double

distilled H_2O. This mixture was heated to 70°C for 5 min to melt any secondary structure in the RNA template, then lowered to the specific annealing temperature of the gene specific primers for 45 seconds, and then placed on ice for 5 min. After placing on ice, the following reagents were added: M-MLV reaction buffer to a final concentration of 1X, 100 μM of each deoxynucleoside triphosphate (Promega), 200 units of M-MLV reverse transcriptase (Promega), and nuclease free double distilled H_2O to a final volume of 25μL. After gentle flicking to mix the reagents the tube was spun down, and Tube 1 was incubated at 45°C to carry out the reverse transcription step for 60 minutes. Following reverse-transcription 2μL or 4μL of product was transferred from Tube 1 to Tube 2 for PCR in a 25μL volume.

4.2.4.5 PCR Conditions.

PCR (25 μL) contained final concentrations of 2μL (~200 ng) of purified *C. jejuni* 16-2R DNA, 1X Buffer (Fermentas), 1.5 mmol $MgCl_2$ (Fermentas), 200 μM of each deoxynucleoside triphosphate (Promega), 0.5 μM of each primer and 1 Unit of Taq polymerase (Fermentas). PCR was carried out on a Hybaid PCR Sprint thermocycler. Cycling conditions were as follows: initial denaturation at 94.0°C for 5 minutes, followed by 10 cycles of 94.0°C for 30 seconds, 55.0°C for 30 seconds, and 72°C for 45 seconds, followed by 20 cycles of 94.0°C for 30 seconds, 55.0°C for 30 seconds, and 72.0°C for 45 seconds plus one second per cycle. A final step of 72.0°C seconds for 5 minutes was carried out.

For each assay, a master mix containing all of the reagents required for the number of reactions with the exception of the DNA was prepared for each gene target. Mixing was performed under sterile conditions and care was taken to ensure that there

was no cross contamination of samples. For each assay a negative control was included. The negative control consisted of all of the materials for the reaction, but DNA/RNA-free double distilled water was used instead of a DNA template.

PCR products were run on a 1% agarose Gel containing ethidium bromide and visualized under a UV light for confirmation of amplification. An image of the gel was obtained using the Syngene photodocumenation system and software (Syngene) .

4.2.5 Quantitative RT-PCR Methods.

4.2.5.1 quantitative RT-PCR.

For quantitative RT-PCR (qRT-PCR), the same conditions were used as for RT-PCR. The exception was that 10X SYBR green (Applied Biosystems) was added in place of some of the water for a final concentration of 0.5X per reaction. Samples were loaded into optically pure tubes (Cepheid) and run on a Smart Cycler (Cepheid).

Data were analyzed using SigmaStat version 2.03 software (SPSS inc). To test for significance, a one-way ANOVA was performed. If a significant result was obtained Turkeys test was performed to test for the difference between significant results.

4.3. Results.

4.3.1 Bacterial growth.

Bacterial cultures were grown in biofilms under aerobic (ambient room oxygen levels) and microaerobic conditions. The results for the growth of the bacteria are discussed in section 2.3.1 and are illustrated by Figure 2.1.

4.3.2. Extraction of RNA and DNA from Microaerobic and Aerobic Biofilms.

The quantity of DNA extracted from *C. jejuni* 16-2R biofilms (approximately 10^7 CFU) was approximately 24 μg (Data not shown). The extracted DNA was used in PCR

to create the copy number standards used in determining the copy number of transcripts in the sample. The amount of RNA extracted from *C. jejuni* 16-2R biofilms was approximately 18 μg for biofilms grown under microaerobic growth conditions (approximately 10^7 CFU), and approximately 6 μg for biofilms grown under aerobic growth conditions (approximately 10^7 CFU) (Figure 4.1). For microaerobic biofilms, this extraction efficiency was increased from approximately 5.1 μg to approximately 18 μg by allowing the nuclease free water to incubate with the membrane in the spin column for a longer period of time prior to centrifugation. This was a critical step in obtaining useable quantities of RNA from aerobic biofilm samples. The efficiency of extraction for aerobically grown biofilms was increased from an immeasurable quantity using the genequant II (Pharmacia) to 6 μg.

4.3.2. RT-PCR/qRT-PCR of *C. jejuni* 16-2R oxidative stress genes from aerobic biofilms.

50 ng of *C. jejuni* 16-2R total RNA was added to each RT-PCR reaction. *ahpC, fdxA, katA, sodB, and 16S* genes amplified from *C. jejuni* 16-2R aerobic biofilms had similarly strong signals when observed on 1% agarose gels as the signals from microaerobic biofilms (Figure 4.2).

Each gene was amplified using qRT-PCR in separate experiments from at least three independently grown biofilms. Since there is a tendency for greater variance between qRT-PCR runs using the same samples than the variance observed between the different tubes in a reaction, each gene was tested individually (Bustin 2004). However, between different runs the *16S rRNA* gene's Ct remained relatively constant, with only a 1 Ct difference between test runs for a Ct of about 5 to 6 for the *16S rRNA* signal of

microaerobic biofilms, and about 7 to 8 for the *16S rRNA* of aerobic biofilm growth (data not shown). Therefore, each gene was standardized to its respective *16S rRNA* internal standard as per the equations described in section 2.2.6.2.

The standard curve used for each gene was also similar and showed good linearity. The copy standards that were used were $2.44x10^7$, $2.44x10^6$, $2.44x10^5$, and $2.44x10^4$ for each gene tested. The regression of these standards gave an R^2 value of 0.99 with the equation of the line of best fit being $y=2E+10e^{-0.6021x}$ (Figure 2.3).

Compared to microaerobic biofilms all genes, with the exception of *katA*, showed up-regulation in *C. jejuni* 16-2R aerobic biofilms. The differences in expression can be seen in Figure 2.4. *ahpC* showed the greatest difference in expression with a 5.67 fold increase in biofilms grown under aerobic conditions ($p< 0.001$). Expression of *sodB*, and *fdxA* also showed a marked increase in biofilms that were grown under aerobic growth conditions. *sodB* and *fdxA* had 2.77 ($p=0.001$) and 2.47 ($p=0.002$) fold increases in their expression levels respectively. Although expression of *katA* was also increased, the difference between aerobic and microaerobic grown biofilms was statistically not significant ($p=0.093$) (Figure 2.4).

The difference between the means for *ahpC, sodB*, and *fdxA* from aerobic and microaerobic biofilms was $2.80x10^6$, $9.73x10^5$, and $2.55x10^6$ transcripts per sample respectively ($p=0.001, 0.001, 0.002$).

When the expression levels between the genes were compared for the same growth condition, *ahpC* from aerobic biofilm growth had a statistically significant difference in its expression level when compared with *sodB* for aerobic biofilm growth of 2.13E+6 transcripts ($p=0.007$). However, there was also a significant difference between

fdxA and *sodB* ($p<0.001$) of 3.02E+6 transcripts from aerobically grown biofilms. There was no statistically significant difference between *sodB* and *katA* ($p=0.518$). When the expression pattern for the four genes tested was compared for microaerobically grown biofilms all were statistically different from each other with the exception of *sodB* and *katA* ($p=0.439$). With about 10^6 transcripts, *fdxA* had a greater expression level when compared to *ahpC, katA*, and *sodB* ($p<0.001$). Interestingly *ahpC* had a higher level of expression than *katA* ($p=0.006$), but *ahpC* was not expressed significantly greater than *sodB* ($p=0.097$).

4.4. Discussion.

Increased gene expression in response to higher oxygen exposure for the genes studied was expected. Previous studies have shown that there is a greater number of stress genes expressed under high oxygen tension than under low oxygen exposure (Moen et al., 2005). While some authors have shown an up-regulation of stress genes in *C. jejuni* in response to higher levels of oxygen, others have shown a down regulation of certain genes involved in energy metabolism in response to higher oxygen (Gaynor et al., 2004; Woodall et al., 2005). Many authors have reported changes in gene expression when *C. jejuni* is exposed to different stresses. These changes were in the order of 1.5 to 9 fold increases, although expression changes for metabolic genes has been shown to differ up to 40 fold (Moen et al., 2005; Sampathkumar et al., 2006). Therefore, our results fit within the range of gene expression reported by other studies.

Interestingly, the *16S rRNA* gene was expressed in a slightly different manner for aerobic biofilms than for microaerobic biofilms. However, we did not disregard the *16S rRNA* gene as a suitable "house keeping" gene as its regulation and use in *C. jejuni*

aerobic growth conditions has been documented by others, and has been used extensively as a normalizing gene (Klancnik et al., 2006; Woodall et al., 2005). Differences in the results obtained in this study should be validated by either microarray, or replication by another laboratory before any conclusive statements can be made regarding the oxidative gene expression seen between aerobic biofilm growth and microaerobic biofilm growth (Bustin, 2000; Bustin, 2002; Bustin and Nolan, 2004; Klein, 2002; Skern et al., 2005).

Although oxygen exposure resulted in the greatest increase in transcription of the *ahpC* gene, compared to biofilms grown under microaerobic conditions, this gene did not have the greatest transcription level in the aerobic biofilm samples. The observation that *fdxA* was further up-regulated in aerobic biofilms than any of the other oxidative stress genes studied may be due to a difference in gene regulation. (Van Vliet et al., 1999). As expained in section 2.4, *fdxA* is regulated by the amount of iron available to *C. jejuni*, and the gene is important to the oxidative stress response of the bacterium (Van Vliet et al., 2001). Up-regulation of *fdxA* has also been shown to down regulate *ahpC, katA*, and *sodB* (Palyada et al., 2004; van Vliet et al., 1999).

It is interesting that after *C. jejuni* was exposed to a greater oxidative stress, the expression level of *fdxA* was significantly higher than *sodB*. However, this was not the case for the other genes. Also, *fdxA* was the least up-regulated gene when exposed to oxygen that showed a significant difference between microaerobic and aerobic growth. Further experiments would have to be completed on the *perR* transcription factor that controls the regulation of *ahpC* and *katA*. Also, the *sodB*, and *fdxA* regulation factors would have to be identified. This would further elucidate the mechanisms of transcription for the four oxidative stress genes used in this study (Baillon et al., 1999; Day et al.,

2000; Palyada et al., 2004; van Vliet et al., 1999; Wooldrige, Williams, and Ketley, 1994).

There is some evidence that the increases in gene expression found between biofilms formed under aerobic conditions compared to biofilms formed under microaerobic conditions may translate to an increase in the amount of oxidative stress proteins present in *C. jejuni*. Holmes et al (2005) reported that changes in *ahpC* and *katA* gene expression were correlated with an increase in the amount of corresponding protein seen on a 2D-PAGE. Therefore, the increase in gene expression seen in this study could also cause an increase in the translation of the protein.

4.5. Conclusions.

Biofilm growth in aerobic growth conditions resulted in a noticeable increase in the expression levels of *ahpC*, *fdxA*, and *sodB*. However, testing whether the same trend in gene expression also occurs in planktonic *C. jejuni 16-2R* should validate this observation. Studies that looked at the expression levels of *ahpC, fdxA, katA*, and *sodB* during different growth phases of the bacteria's life cycle would also give better insight to the regulation of oxidative stress in *C. jejuni*. In addition, oxidative stress response of other strains of *C. jejuni* could be undertaken to screen for transcriptional changes. This may be a prudent step before any broad statements about oxidative gene expression levels are given, since there can be a significant genetic and phenotypic variation between different *C. jejuni* strains. (Gaynor et al., 2004; Stintzi et al., 2005).

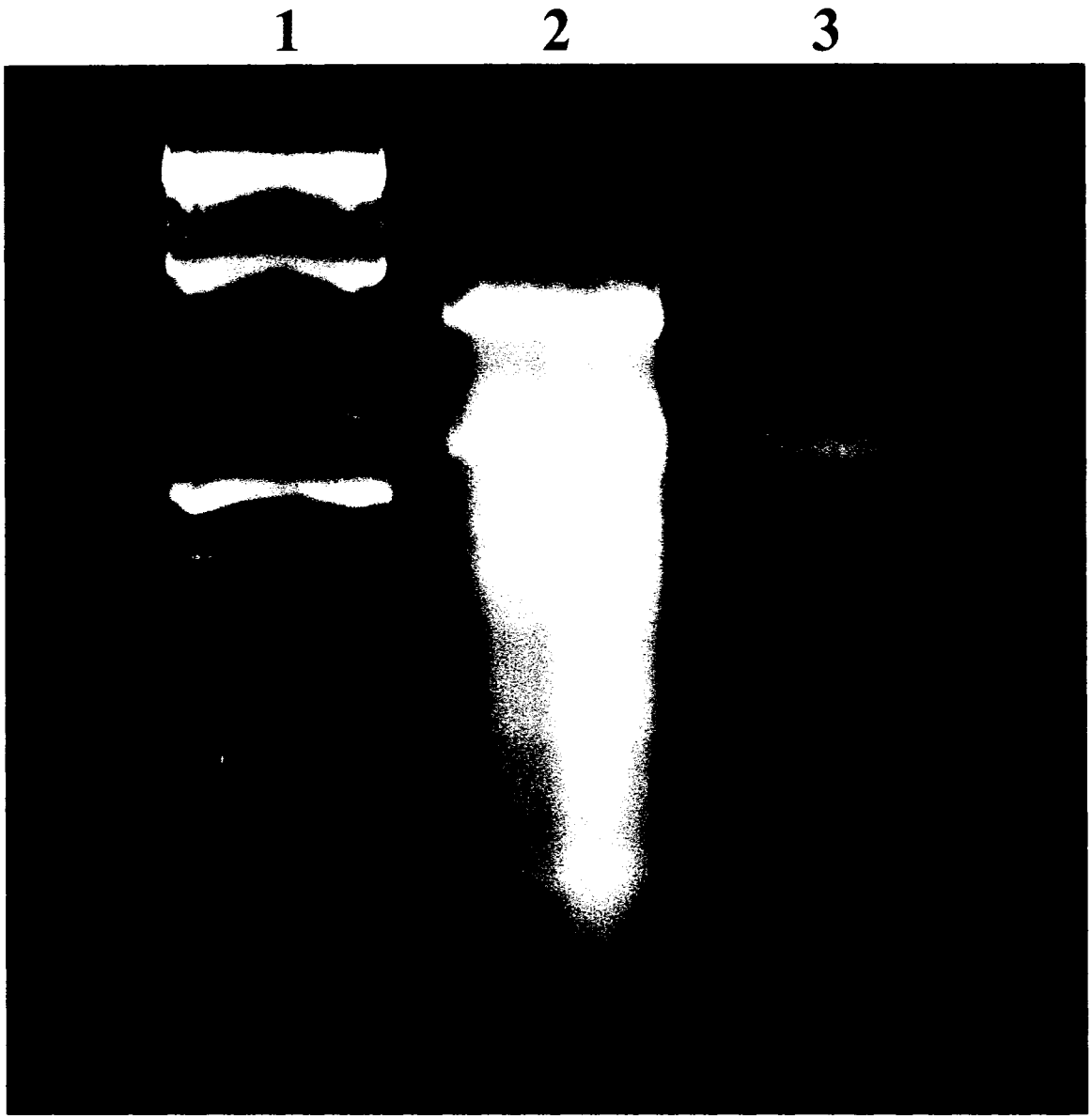

Figure 4.1 RNA extracted from *Campylobacter jejuni* 16-2R microaerobic and aerobic biofilms on a 1% Agarose gel containing ethidium bromide excited under UV light prior to dilution in RNAse free sterile water. 1μL of a 1kb DNA ladder (Fermentas) was loaded into lane 1 with 4μL of water. 5μL of a 300 ng/μL sample of microaerobic biofilm RNA was loaded into lane 2, and 5μL of a 139 ng/μL sample of aerobic biofilm RNA was loaded into lane 3. RNA was extracted from *Campylobacter jejuni* 16-2R using the High pure total RNA extraction kit from Roche.

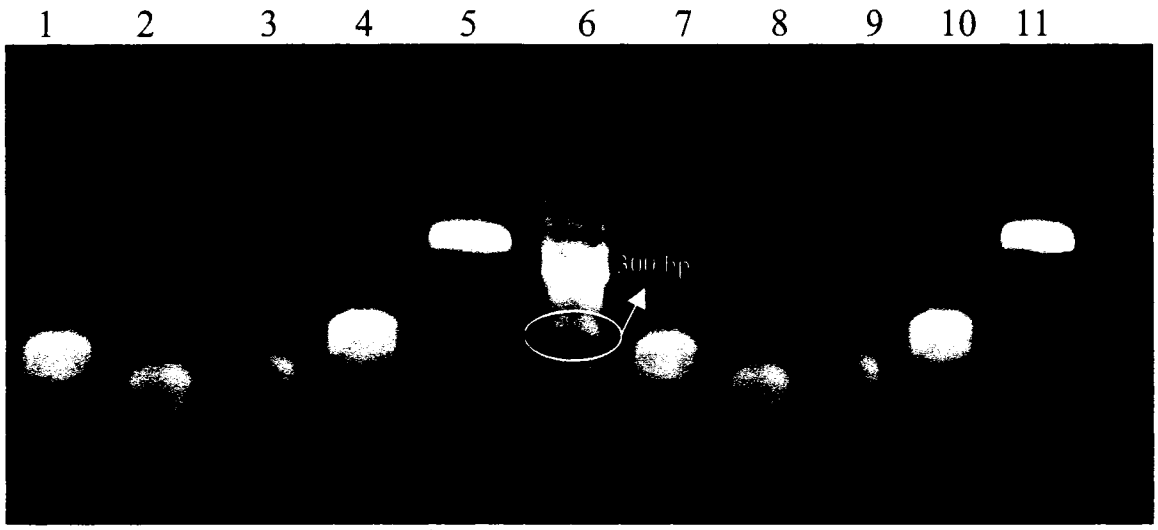

Figure 4.2. *ahpC, fdxA, katA, sodB*, and *16S* RT-PCR products from *C. jejuni* 16-2R microaerobic and aerobic biofilms. 5μL of RT-PCR product was loaded onto a 1% agarose gel and stained with ethidium bromide. Lane 1: *ahpC* from microaerobic biofilm, Lane2: *fdxA* from microaerobic biofilm, Lane 3: *katA* from microaerobic biofilm, Lane 4: *sodB* from microaerobic biofilm, Lane 5: *16S* from microaerobic biofilm, Lane 6: 100 bp DNA ladder, Lane 7: *ahpC* from aerobic biofilm, Lane 8: *fdxA* from aerobic biofilm, Lane 9: KatA from aerobic biofilm, Lane 10: *sodB* from aerobic biofilm, Lane 11: 16S from aerobic biofilm

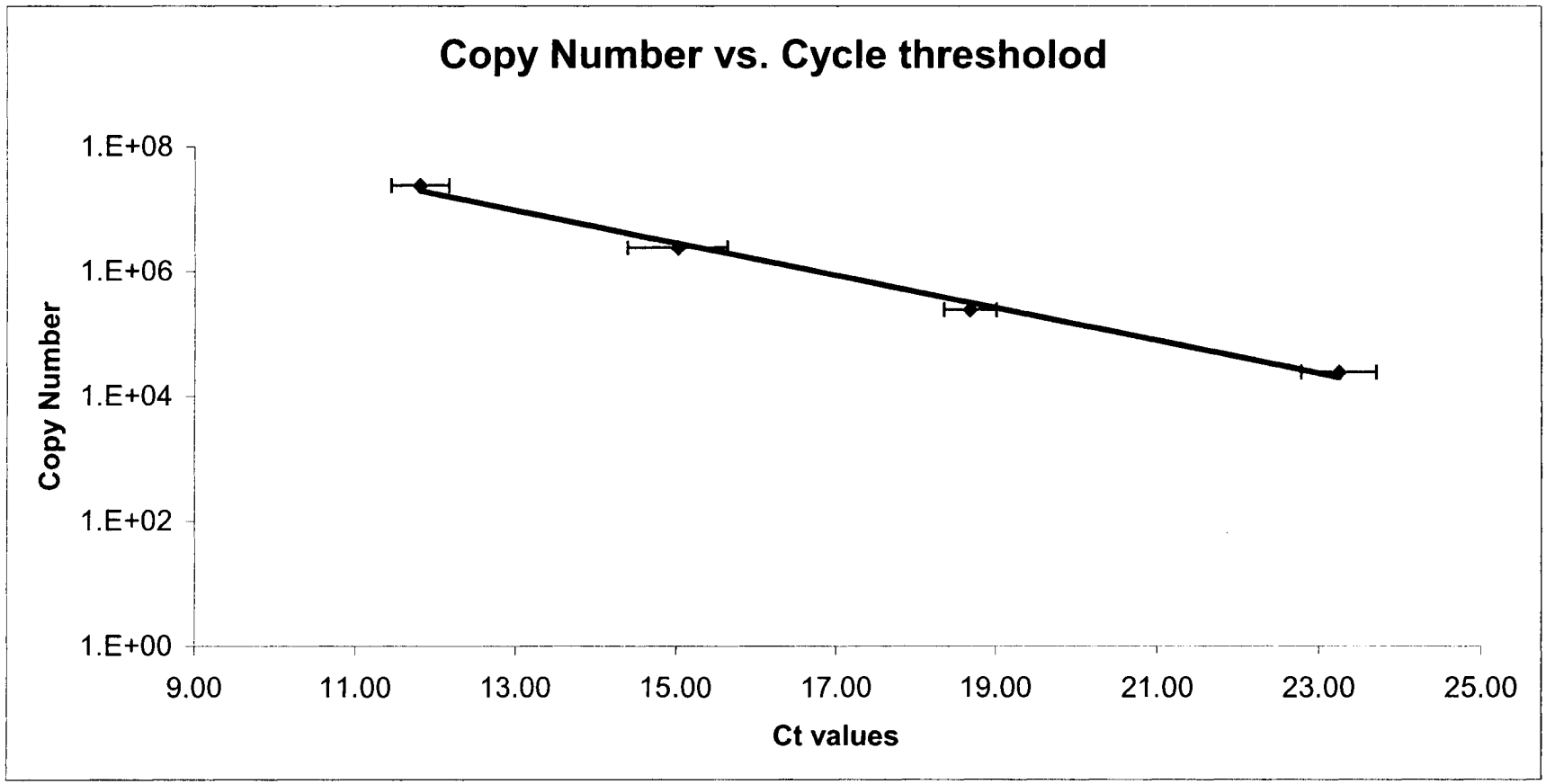

Figure 4.3 Standard Curve generated for the Cepheid Smart Cycler. A set of DNA standards were serially diluted to $2.44x10^7$, $2.44x10^6$, $2.44x10^5$, and $2.44x10^4$ respectively in triplicate, and then amplified on a Cepheid Smart Cycler using PCR conditions set up for a qRT-PCR Two-tube assay with SYBR Green I chemistry. Error bars represent the standard error. The equation of the line generated was $y=2E+10e^{-0.6021x}$ and had an R^2 value of 0.99.

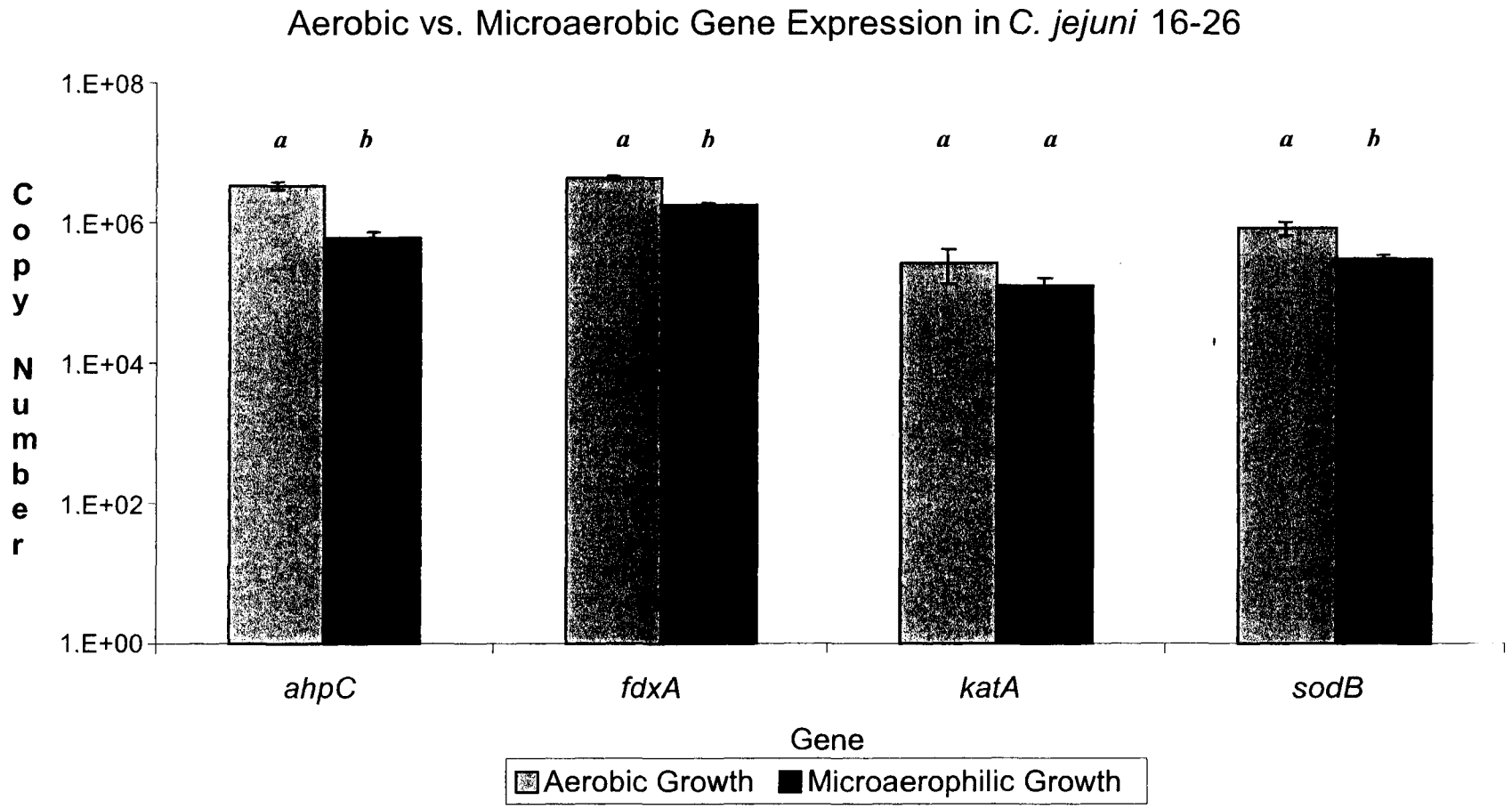

Figure 4.4 Copy numbers of Superoxide Dismutase B (SodB), Catalase A (KatA), Alkyl Hydroperoxide Reductase C (AhpC), Ferridoxin A (FdxA), Gyrase A (GyrA), and 16S rRNA (16S) from RNA extracted from 48 hour microaerobic biofilm growths and 48 hour aerobic biofilm growths of *Campylobacter jejuni* 16-2R. Results were generated from a qRT-PCR carried out on an Cepheid Smart Cycler real-time thermocycler Error bars represent the standard error of the mean. For each gene target, bars with different letters were significantly different form each other ($p<0.05$)

General Conclusions

In conclusion this research found that *Campylobacter jejuni* 16-2R is able to survive in aerobic growth conditions with a one-log reduction from $3.64x10^8$ to $3.64x10^7$ CFU/mL when grown in planktonic cultures. Likewise there was a decrease in *C. jejuni* 16-2R biofilm growths from $2.58x10^6$ to $1.32x10^6$ CFU/g of glass wool when grown in aerobic conditions. Therefore, we were able to show that biofilm growth confers resistance to oxygen exposure in *C. jejuni* 16-2R. Likewise, when *C. jejuni* planktonic and biofilms cells were tested in microaerobic conditions there were no statistically significant differences in the expression of *ahpC (p=0.139), katA (p=0.065), or sodB (p=0.136).* There were differences in *fdxA (p=0.008), gyrA (p=0.048),* and *16S rRNA (p=0.002).* This indicated that *fdxA* provides protection to *C. jejuni* 16-2R biofilms when grown in microaerobic conditions. Also, that *gyrA* is also affected by biofilm growth and is not a suitable normalizing gene for planktonic and biofilm gene expression studies in *C. jejuni* 16-2R.

Of the two methods of qRT-PCR were tested; there was no statistically significant difference between using an ABI Prism 7000, or the Cepheid Smart Cycler (p=0.776). Also, there were no differences between the Titan One-Tube and a Two-Tube RT-PCR protocol (p=0.388). However, the Two-Tube protocol was more efficient at detecting mRNA because it utilizes the reverse transcriptase M-MLV. Therefore, switching qRT-PCR enzymes can be accomplished easily by comparing an assay on both platforms. Switching between different RT-PCR conditions is more problematic and requires knowledge of enzyme kinetics and optimization parameters to standardize protocols.

Differences were found in the *ahpC* (p=0.007), *fdxA* (p<0.001), and *sodB* (p<0.001) genes of *C. jejuni* when grown in aerobic or microaerobic biofilms. Therefore, biofilm

growths of *C. jejuni* 16-2R up-regulate there oxidative stress genes in response to exposure to oxygen. Testing whether the same trends in gene expression also occur in planktonic growths of *C. jejuni* 16-2R should validate these observations. Likewise, it may be prudent to compare *C. jejuni* planktonic aerobic growth oxidative stress gene expression with the biofilm data. Future research should look at the oxidative stress gene expression between planktonic and biofilm growths during different phases of growth and on different strains of *Campylobacter* before any broad statements about oxidative gene expression are made.

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