AN INVESTIGATION INTO THE VIABILITY OF COCCOID CELLS OF CAMPYLOBACTER

DETECTION OF mRNA BY REVERSE-TRANSCRIPTASE-PCR AS AN INDICATOR OF VIABILITY IN CAMPYLOBACTER JEJUNI

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LIST OF ABBREVIATIONS

aa  amino acid
AO  Acridine orange
A\textsubscript{260}  Absorbance at 260nm
A\textsubscript{280}  Absorbance at 280nm
bp  base pair
CCDA  \textit{Campylobacter} charcoal differential (deoxycholate) agar
cDNA  complementary DNA
CFU  colony forming units
dATP  2' - deoxyadenosine triphosphate
dCTP  2' - deoxycytosine triphosphate
(d)dH\textsubscript{2}O  (double) distilled water
dGTP  2' - deoxyguanosine triphosphate
DMPC  dimethyl pyrocarbonate
DNA  deoxyribose nucleic acid
DNase I  deoxyribonuclease I
dNTP  2' - deoxynucleotide triphosphate
ds  double stranded
dTTP  2' - deoxythiamine triphosphate
FA  fatty acid
GlcNAc  N- acetyl glucosamine
h  hour(s)
kPa  kilo Pascals
LPS  lipopolysaccharide
min  minute(s)
mRNA  messenger ribose nucleic acid
OD\textsubscript{600}  Optical density at 600nm
PCR  Polymerase Chain Reaction
rDNA  ribosomal deoxyribose nucleic acid
RNA  ribose nucleic acid
RNase A  ribonuclease A
rRNA  ribosomal ribose nucleic acid
RT-PCR  Reverse Transcription Polymerase Chain Reaction
sec  second(s)
spp.  species
\textit{Taq}  \textit{Thermus aquaticus} DNA Polymerase
Tm  Melting point
tRNA  transfer ribose nucleic acid
UV  Ultraviolet
VNC  Viable but nonculturable
v/v  volume per volume
w/v  weight per volume
xg  times gravity
Abstract

In response to unfavourable environmental conditions, a number of bacterial species are shown to enter a viable but nonculturable (VNC) state. This phenomenon has led to the recognition of the inherent limitations of the standard microbiological cultural method and has resulted in the development of novel techniques to determine microbial viability. In this study, emphasis was placed on the analysis of viability by molecular amplification of the human and animal enteric bacterium, *Campylobacter jejuni*, which has been one of the paradigm organisms for the study of the VNC response. This gastrointestinal organism is of particular interest due to its high incidence in New Zealand and its ability to cause diarrhoeal infections in persons who consume contaminated poultry or untreated water. Due to the medical significance of the pathogen, it is essential to establish whether the VNC state is indeed living and, as such, may have a role in the contamination or infection cycle.

In this thesis, the relationship between the detection of mRNA and cellular viability in *C. jejuni* was investigated in cultures aerobically incubated below the bacterium's optimum growth temperature. Reverse transcription-PCR (RT-PCR) methods were developed for detecting mRNA from the *lpxA*, *dnaJ*, *hupB*, and *ciaB* genes and rRNA from the 23S rDNA gene. Total RNA from *C. jejuni* was isolated and, following DNase treatment, the RNA was amplified by both PCR and RT-PCR with primers specific for each gene. The levels of expression differed markedly with respect to prolonged incubation time, but the results indicated that coccoid cell forms of *Campylobacter* retain structural integrity and a degree of protein synthesis for up to two weeks under the experimental conditions of this study. This research demonstrated that either the *lpxA* or *dnaJ* genes may provide suitable targets for development of a specific method for detection of viable *C. jejuni* based on RT-PCR amplification.

The usefulness of the *lpxA* mRNA species as a viability determinant was validated in an artificially killed *C. jejuni* culture. Following a twenty minute heat treatment at 65°C, the *lpxA*-specific amplification product could be detected in the culture for 96 h post-heating. In contrast, aerobic plate counts (once an indication of viability) demonstrated loss of culturability within five minutes of heat-shock induction. These results further demonstrate that nonculturability and non-viability are not synonymous. Thus, this study supports the usefulness of RT-PCR amplification of mRNA as a sensitive method for specific detection of viable bacteria and indicates this method may prove beneficial in the detection of *C. jejuni* in clinical and environmental samples.
CHAPTER I

Campylobacter BACTERIOLOGY

As it takes two to make a quarrel, so it takes two to make a disease, the microbe and its host.

Charles Chaplain (1856-1941)

1.1 INTRODUCTION.

This chapter will cover a brief introduction to Campylobacter spp. and introduces the concept of the 'viable non-culturable' (VNC) coccoid state. Clinical aspects of campylobacteriosis, an acute infective diarrhoeal disease of humans caused by thermophilic species of this genus will be discussed. In particular, the potential role of VNC cells of Campylobacter as causal agents of this illness will be examined.

1.2 DESCRIPTION OF THE GENUS.

1.2.1 BASIC MORPHOLOGY AND PHYSIOLOGY.

Formerly in the family Spirillaceae (Smibert, 1984), the gastrointestinal pathogen Campylobacter, and a closely related genus Arcobacter, are now included in the family Campylobacteraceae (Van Damme, et al., 1991; Van Damme and Goosens, 1992; both cited from Nachamkin, 1992). The generic name, Campylobacter, is derived from the Greek words meaning "curved rod" (Skirrow, 1977). Members of the genus are observed as being Gram negative, non spore-forming spiral or helical rods 0.2 to 0.9μm wide by 0.5 to 5μm long (Nachamkin, 1992). Campylobacter exhibits 'corkscrew'-like motility by means of a single polar unsheathed flagellum present at one or both ends of

1 Cited from Talley and O'Connor, 1996.

2 Gk. adj. campylo curved; Gk. n. bacter rod; M.L. masc. n. Campylobacter a curved rod (Smibert, 1984).
Chapter I

Campylobacter Bacteriology

the cell. The genus *Campylobacter* includes 18 species and sub-species that replicate over a wide range of temperatures, with an optimum growth range of 30-37°C. Exceptions to this are the four thermophilic species - *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*, which have an optimum replication temperature of 42°C (Ketley, 1995; Nachamkin, 1995). Members of this genus are recognised animal and human enteropathogens. The most frequently isolated *Campylobacter* spp. with respect to human enteric infections are *C. jejuni*\(^3\) and *C. coli*, with infrequent isolation of *C. lari*, *C. upsaliensis* and *C. fetus* (Skirrow, 1990; Nachamkin, 1992; Lacey, 1993). All species within the genus are capnophilic (requiring carbon dioxide concentrations of 3-10%) and strictly micro-aerophilic requiring low oxygen tension (3-15%) for replication (Smibert, 1978; Ketley, 1995).

Under certain environmental conditions that are unfavourable for growth, cells of *Campylobacter* are observed to change from the characteristic vibroid morphology to a coccoid form (Rhoades, 1954; cited from Bovill and Mackey, 1997). This transition also involves a concomitant change in culturability from a viable culturable to a viable but non-culturable (VNC) state (Rollins and Colwell, 1986; Roszak and Colwell, 1987). More detail regarding the physiology and biology of the VNC state will be presented in Chapter II, but there is a great deal of medical interest in the possible role of the non-culturable form in the transmission of infection.

\(^3\) *Vibrio (Campylobacter)* *jejuni*. (Jones, Orcutt and Little, 1931) (Smibert, 1984). The name *jejuni* is derived from the source of first isolation (the jejunum of calves with diarrhoeal disease) (Rees, *et al.*, 1993).
1.2.2 THE PATHOGENICITY OF CAMPYLOBACTER SPP.

*Campylobacter* spp. have been suggested to induce disease by three mechanisms that are shared with other enteric pathogens (Cotran, *et al.*, 1994): (i) toxin-induced disease (similar to that induced by *Vibrio cholerae*) producing a watery diarrhoea; (ii) invasion and proliferation within intestinal epithelial cells resulting in cellular death, which manifests itself clinically as bloody diarrhoea with inflammatory cells in the stools; and (iii) translocation, whereby the organism penetrates the intestinal mucosa to multiply within the lamina propria and mesenteric lymph nodes. This may produce an enteric fever without frank dysentery or instigate bacteraemia.

The infectious cycle of *Campylobacter* has been hypothesised by Ketley (1995). In summary: On ingestion, campylobacters enter the intestine via the stomach, surviving the acid barrier, to colonise the distal ileum and colon. This process is designated infection as a non-resident disease-causing microorganism has established itself in the body. After adhesion and colonisation of the mucosal blanket, campylobacters perturb the normal absorptive capacity of the gastrointestinal tract by damaging epithelial cell function either by cell invasion or by the controversial production of toxin(s) or both. It is this proliferation within the epithelium which produces the symptoms associated with this disease (Harvey, *et al.*, 1996) (refer to Section 1.3.3). However, infection with *Campylobacter* does not necessarily lead to disease. Asymptomatic carriage has been reported (Wallis, 1994).

1.2.3 THE POTENTIAL VIRULENCE OF THE VNC STATE.

Experimental evidence regarding the infectivity of non-culturable cells of *Campylobacter* and other bacterial pathogens is controversial. One obvious difficulty in elucidating whether VNC cells are a potential hazard arises due to the inability to culture VNC cells from environmental sources. Routine microbiological methods do not facilitate growth (on agar media) and microscopic techniques do not distinguish the
subset of viable, potentially virulent cells from dead cells. Therefore experimentation into the potential virulence and pathogenicity of these cells has proven problematic.

Some researchers suggest coccolid forms may act as potential vehicles for the transmission of disease (Jones, et al., 1991; Saha, et al., 1991; Colwell, et al., 1996), whilst others believe the VNC state is degenerative and poses no infectious risk (Moran and Upton, 1987b; Eaton, et al., 1995).

However epidemiological findings indicate the distribution of certain infectious diseases is at variance with the ability to culture causal organisms from implicated environmental sources or reservoirs (Colwell, et al., 1985). The possibility that these apparent discrepancies can be attributed to the failure of culture-based systems to detect VNC forms of the organisms concerned presents a potentially serious challenge to public health microbiology.

1.3 *Campylobacter jejuni* AND HUMAN INFECTION.

In the last thirty years, *Campylobacter jejuni* subspecies *jejuni* (referred to as *C. jejuni* in this thesis) has emerged from obscurity as a veterinary pathogen (King, 1957; cited from Blaser and Reller, 1981) to recognition as a leading cause of sporadic enterocolitis in human beings (Skirrow, 1977; Skirrow, 1990; Nachamkin, 1995). First isolated from diarrhoeal stools of humans in 1972 (Dekeyser et al., 1972; Butzler et al., 1973), *Campylobacter* spp. are the most common enteric bacterial pathogens reported and in many industrialised countries (including New Zealand) have overtaken *Salmonella* spp. as the principal cause of bacterial gastroenteritis (McNicholas, et al., 1995; Nachamkin, 1995; The New Zealand Public Health Report, 1996-1999). Notification levels in New Zealand from 1980 to 1998 of these two gastrointestinal pathogens are presented in Figure 1.1.
Figure 1.1: Campylobacteriosis (●) and Salmonellosis (○) notifications in New Zealand by year, 1980-1998 (Adapted from the New Zealand Public Health Report, 1999).


1.3.1 *Campylobacter* INCIDENCE.

In New Zealand *Campylobacter* enteritis (campylobacteriosis) was made notifiable in 1980 (Faoagali, 1984). Campylobacteriosis is caused by two closely related species - *C. jejuni* and *C. coli*, of which there are more than 100 heat stable and heat labile serotypes (refer to Section 1.3.5). In most parts of the world, including New Zealand, *C. jejuni* is the predominant species accounting for 80 to 90% of all reported cases of the disease (Skirrow, 1990; McNicholas, *et al.*, 1995; Yates, 1998).

The incidence of campylobacteriosis in New Zealand is one of the highest in the industrialised world. At present, rates of campylobacteriosis in New Zealand are at least three times higher than rates in comparable countries (Table 1.1).

| Table 1.1: International rates of campylobacteriosis (Adapted from the New Zealand Public Health Report, February 1999). |
|---|---|
| **Country (year)** | **Rate per 100 000 population** |
| New Zealand (1997) | 244.5 |
| Australia (1997) | 98.0 |
| United States (1997) | not notifiable |
| Canada (1996) | 42.7 |
| England and Wales (1997) | 96.9 |
| Scotland (1997) | 108.0 |

In New Zealand, rates of infection have increased from a national average in 1981 of 14 per 100 000 population to 317 per 100 000 notified cases in 1998 (Brieseman, 1994; The New Zealand Public Health Report, February 1999). Since 1993 notifications of campylobacteriosis in this country have consistently exceeded 230 cases per 100 000 population per annum (Hudson, 1997; Withington and Chambers, 1997). 1998 was a record year for campylobacteriosis in New Zealand with the highest number of cases notified in any year since reporting began (provisional data) (The New Zealand Public Health Report, February 1999).
Infection rates in the Canterbury region are consistently higher than the national average, with upwards of 380 cases per 100,000 population notified annually (Brieseman, 1994). Despite the national increase in campylobacteriosis notifications, research could not attribute this rise in numbers to any changes in laboratory diagnostic procedures (McNicholas, et al., 1995). Proposed reasons for this upshift include a greater number of laboratories performing the testing and increased presentation of infected individuals at medical centres for testing - a phenomenon largely due to heightened practitioner and public awareness.

However, the incidence of infection, as derived from laboratory isolations, is almost certainly a significant underestimate of the true rate of this disease in the general population. This situation reflects under-reporting due to the fact not all physicians request stool cultures and, owing to cost, not all diagnostic laboratories routinely screen for *Campylobacter* spp. (Blaser, 1997). It may also result from a failure to detect VNC cells by traditional culture-based methods.

### 1.3.2 *Campylobacter* EPIDEMIOLOGY.

*Campylobacter* spp. sources of infection have been extensively studied. In New Zealand the results of a multicentre analysis of gastroenteritis induced by *Campylobacter* (MAGIC) study represents the first large scale epidemiological national survey of *Campylobacter* infections. The MAGIC study (1995) consistently found the consumption of contaminated foods (particularly poultry products) to be associated with *Campylobacter* infection. Three other principal sources were identified for the majority of the remainder of reported cases; these are the consumption of unpasteurized milk, contact with domestic and food-producing animals, and untreated water (Brieseman, 1985; Brieseman, 1990; Skirrow, 1990; Stehr-Green, et al., 1991; Eberhart-Phillips, et al., 1995). Faeces is the chief cause of contamination within these sources, as *C. jejuni* is widely distributed as normal intestinal flora in many warm-blooded vertebrates.
These include cattle (beef and dairy), sheep, pigs, and dogs (Brieseman, 1990; Ikram, et al., 1994). *Campylobacter* spp. are especially common as commensal flora in avian species (poultry, seagulls, ducks, and sparrows), an adaptation which is reflected in their high optimum temperature of 42 to 43°C (Skirrow, 1990). *Campylobacter* contamination of poultry may be transmitted via the surface of the meat as a result of faecal contamination during slaughtering. Consequent infection may then result from undercooking meat or cross contamination of 'innocent' uncooked foods (Eberhart-Phillips, et al., 1995). Secondary transmission (direct person to person) is also important but plays only a minor role (Skirrow, 1990).

### 1.3.3 CLINICAL SYMPTOMS OF CAMPYLOBACTERIOSIS.

Once established a *Campylobacter* infection can develop in several different forms, ranging from asymptomatic to severe gastroenteritis (Walker, et al., 1986; Nachamkin, 1992). Symptomatic acute inflammatory enterocolitis is usually self-limited and the histological features of the illness can be indistinguishable from those seen with 24-hour viral gastroenteritis (Blaser and Reller, 1981; Nachamkin, 1992) or those of a *Salmonella* or *Shigella* infection (Skirrow, 1990). The illness can last up to three weeks with an average incubation period of three days (range one to seven days). In about one-half of patients the onset of *C. jejuni*-associated gastroenteritis is preceded with a febrile or non-specific prodromal period, characterised by acute abdominal pain, often with fever, myalgia and general malaise. This usually progresses to the major clinical manifestation - a profuse liquid diarrhoea (often grossly bloody). Acute diarrhoea lasts on average two to three days, causing dehydration and exhaustion (Butzler and Skirrow, 1979; Ketley, 1995; Blaser, 1997). Diarrhoeic stool microscopy shows the presence of fresh blood in 25% of patients (Nachamkin, 1992), muco-pus and an inflammatory exudate with leucocytes (Ketley, 1995; Blaser, 1997). Rapidly motile flagellated *Campylobacter* may be seen in fresh faeces (Ketley 1995).
Patients generally recover in seven days without antibiotic treatment, but in up to 20% of cases a relapse or prolonged illness occurs (Blaser, 1983). Mortality directly attributable to *Campylobacter* infection is infrequent (Nachamkin, 1992; Lacey, 1993). Details of campylobacteriosis are reviewed in several past and current articles (Blaser *et al.*, 1979; Blaser and Reller, 1981; Skirrow, 1990; Allos and Blaser, 1995; Blaser, 1997).

Successful bacterial infection normally requires the ingestion of thousands to millions of bacteria (Jones, *et al.*, 1991). Human volunteer studies (Black, *et al.*, 1988) have shown flagellated *C. jejuni* produces symptomatic infection at a variety of doses ranging from 800 organisms (which caused diarrhoeal illness in 50% of the sample) to 100 million required to infect 100% of volunteers (n=111). Of the two *C. jejuni* strains tested, one was noted to produce a more severe illness than the other (Black, *et al.*, 1988). Other experimental studies concluded as few as 500 organisms could initiate a febrile diarrhoeal illness (Jones, *et al.*, 1991; Lacey, 1993; Robinson, 1991). It appears that considerable variation exists either in individual susceptibility to the organism or in the relative virulence of the infecting strains or both, and that these are important factors in the pathogenesis of campylobacter enteritis (Wallis, 1994).

Intervention is seldom required in the treatment of campylobacteriosis as the disease is largely self-limiting (Skirrow, 1990). Common therapeutic needs include the restoration of fluid loss (rehydration) and correction of metabolic and electrolytic imbalances (Hope, *et al.*, 1993). In protracted or severe cases antibiotic therapy is indicated. Erythromycin remains the treatment of choice. This agent is characterised by low toxicity, a relatively narrow spectrum of activity, ease of administration, and low cost (Allos and Blaser, 1995). However, high doses of erythromycin stearate (500 to 1000mg) may induce severe diarrhoea (British National Formulary, 1995), therefore ciprofloxacin (fluoroquinolone) has been used as an alternative treatment (Rang, *et al.*, 1995). In recent years bacterial resistance to this agent has increased (Lacey, 1993).
jejuni strains are almost universally resistant to cephalosporins, penicillin, vancomycin, trimethoprim and rifampicin yet the resistance of C. jejuni to erythromycin occurs at a low rate that has changed little in the past 15 years (Allos and Blaser, 1995).

1.3.4 EXTRaintestinal Campylobacter diseases.
Systemic infections can occur as a result of C. jejuni infection and although uncommon, include bacteraemia, reactive (aseptic) arthritis, bursitis, urinary tract infection, osteomyelitis, meningitis, endocarditis, peritonitis, pancreatitis, abortion, neonatal sepsis, skin rashes, cholecystitis, and Reiter’s syndrome (Blaser and Reller, 1981; Blaser, et al., 1986; Walker, et al., 1986; Nachamkin, 1992; Lacey, 1993; Yuki, et al., 1994; Allos and Blaser, 1995). The incidences of bacteraemia, resulting from C. jejuni penetration of the intestinal wall and subsequent invasion of the bloodstream, is speculated to be 0.15% of all intestinal infections (Lacey, 1993; Nachamkin, 1992). This figure may be an under-estimate of the true incidence due to the difficulty associated with recovery of C. jejuni from the blood (Lacey, 1993).

1.3.5 Post-infectional complications.
Campylobacter post-infection complications may occur and are often very severe. They include neurological disorders such as stroke, haemolytic anaemia, empyema, encephalopathy, acute motor axonal neuropathy (AMAN), Guillain-Barré Syndrome (GBS)\(^4\) and its variant Miller-Fisher Syndrome (MFS)\(^5\) - the latter two of which are acute inflammatory polyradiculoneuropathic conditions (Yuki, et al., 1994; Yuki, 1997).

GBS is an acute demyelinating paralytic disease of the peripheral nervous system and the most common cause of acute neuromuscular paralysis, with annual incidence

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\(^4\) Georges Guillain (1876-1951), Jean Alexandre Barré (1880-1967) and A. Strohl described the syndrome in 1916; the last author's name was dropped because of anti-German feeling during World War One (Talley and O'Connor, 1996).

\(^5\) In 1956, Miller and Fisher described three patients with a syndrome of acute external ophthalmoplegia, ataxia, and absent tendon reflexes (Miller - Fisher Syndrome).
internationally of 1.3 cases per 100,000 population (range 0.4 to 4) (Ropper, 1992; Hughes and Rees, 1997). Numbers of GBS notifications have doubled over the last six decades (Tomera, 1996), and cases are particularly prevalent in Asian nations (Allos, et al., 1993; Ho, et al., 1995; Yuan and Xiao, 1996). The extent to which _C. jejuni_ is involved as an etiological agent of neurological diseases is unknown, but the frequency of campylobacteriosis prior to Guillain-Barré Syndrome has been reported as probably between 26% (Rees, et al., 1995; Hughes and Rees, 1997; Withington and Chambers, 1997) and 66% (Ho, et al., 1995), and thus an antecedent infection with this organism is now regarded as the single most important predisposing factor (Salloway, et al., 1996; Yuki, et al., 1997). The high incidence of _C. jejuni_ infections and their propensity to invade tissue and to induce inflammation are also compatible with a role in the causation of GBS (Blaser, 1997).

Two independent serological classifications have been established for _C. jejuni_ and _coli_. The Lior slide agglutination technique (Lior, et al., 1982; Lior, 1984) is based on heat-labile antigens (namely the flagellar protein) whilst Penner developed the thermostable passive haemagglutination method based on the heat-stable antigens - lipopolysaccharide (LPS) and capsular polysaccharide (Penner and Hennessey, 1980; Penner, et al., 1983). Serotype Pen:19 (HS:19) appears to be over-represented among _C. jejuni_ strains isolated from GBS patients (Allos and Blaser, 1995). This serogroup is rare in sporadic cases of campylobacter enteritis (Fujimoto, et al., 1992; Yuki, et al., 1997) and analysis of the core oligosaccharide region has shown all serogroup 19 strains from patients with GBS contain terminal β-N-acetylglucosamine residues on the cell surface, whilst those non-neuropathic serogroup 19 strains do not (Kuroki, et al., 1993).

Speculation suggests that _C. jejuni_ induced GBS may arise due to immunologic cross-reactivity with _C. jejuni_ HS:19 terminal LPS antigenic regions mimicking epitopes on the human gangliosides GD1a and GM1 (Aspinall, et al., 1994; Rees, et al., 1995; Yuki et al 1997). It is believed this molecular mimicry between the infectious agent and the
gangliosides may function in the production of anti-neural antibodies. This in turn stimulates macrophages and initiates an interleukin response which may be responsible for the axonal degeneration and demyelination of neural structures (Lacey, 1993; Salloway, et al., 1996). In severe cases the disease progresses to affect respiration, eye movement, deglutition, or autonomic function (Desforges, 1992), and may result in disability or fatal paralysis (1 to 8% of cases) (Ropper, 1992; Rees, et al., 1993).

An infrequent variant of GBS, MFS also demonstrates antibody cross-reactivity between human gangliosides (GT1a, GQ1b, GD2) and the terminal antigens from Pen2:Lior4 and Pen23 serostrains of *C. jejuni* (Aspinall, et al., 1996; Neisser, et al., 1997), resulting in demyelinization of cranial nerves.

1.4 THE CURRENT STUDY.

Given the serious nature of the conditions brought on by a *Campylobacter* infection (as described above), it is necessary to determine if indeed the VNC coccoid state of this bacterium has a role in the contamination cycle. This role may be as a "semi-dormant" state to survive hostile environments prior to ingestion by a suitable host or may, in fact, represent a state that maintains its infectious capability and is able itself to induce a symptomatic gastroenteritis without resuscitation to the typical rod morphology. Correlated to the research on the maintenance of virulence by *Campylobacter* spp. are studies into the continuing viability of the species. This thesis aims to investigate the latter phenomenon with a non-neuropathic *C. jejuni* HS:19 test strain (KLC 4315).
CHAPTER II

THE VNC STATE OF Campylobacter jejuni

2.1 VIABILITY = CULTURABILITY?

For many years microbiologists have accepted the view that the capacity of cells to replicate to detectable (culturable) levels reflects viability. However, evidence now shows greater than 99% of all extant microbes can not be detected by these classical enumeration techniques (Pace, 1996), and therefore as such may go undetected in the environment. Conventional techniques defined cell death as the inability of the microbe to form colonies on media previously capable of supporting growth, or the failure to make liquid media become turbid after bacterial inoculation (Barer, 1997). Recent investigations into microbial survival in natural environments has indicated that some organisms lose the ability to form colonies on appropriate media under certain conditions, yet still exhibit signs of metabolic activity and thus viability (Roszak and Colwell, 1987; Byrd, et al., 1991; Oliver, et al., 1991). This reproducible loss of culturability by many non-sporeforming bacterial species led to the description of cells in this state as 'viable but nonculturable' (VBNC or VNC) (Davies and Evison, 1991; Garcia-Lara, et al., 1991; McDougald, et al., 1998).

The proposal that a wide variety of culturable non-differentiating bacteria, upon encounter with certain environmental stresses, may enter a state in which they retain viability but fail to grow in conventional culture strikes at the core of the equation, viability = culturability (Barer, 1997). The VNC state has been extensively studied in many human pathogens, including Vibrio spp. (Linder and Oliver, 1989; Nilsson, et al., 1991), Campylobacter jejuni (Rollins and Colwell, 1986), Helicobacter pylori (Bode, et al., 1993), Legionella pneumophila (Bej, et al., 1991), Salmonella enteritidis (Roszak, et al., 1984), and Escherichia coli (Xu, et al., 1982). For a full review of species capable of forming a VNC state see (McDougald, et al., 1998).
2.2 THE VIABILITY OF VNC CELLS DEBATE.

A recent editorial referred to the term 'viable but nonculturable' as an oxymoron and a misnomer (Barer, 1997). In what is essentially a nomenclature problem, the use of the self-contradictory VNC expression has sparked debate as to whether in fact cells in this state are actually viable or not. It is questionable if the VNC phenomenon represents an alternative physiological state in the microbial life cycle or is an end-of-life process.

Formation of the VNC state has been proposed by some authors to be a programmed survival strategy, perhaps comparable to the stress-induced response of differentiating bacteria - sporulation (McDougald, et al., 1998). However, to complete this analogy, VNC cells must have the ability to reverse this program and 'resuscitate' when conditions become more conducive for growth. Proponents of this theory believe VNC cells to be equally as virulent as culturable forms of the bacterium, and therefore represent a potentially undetectable source of infection (Jones, et al., 1991; Saha, et al., 1991; Colwell, et al., 1996). Studies have typically been based on introduction of the VNC cells into eukaryotic models, with proposed resuscitation to a rod form upon animal passage. Some electron microscopic observations have been reported to favour the viability of VNC cells by demonstrating a lack of ultrastructural degradation and maintenance of cell wall integrity and cellular componentry. To date, little genetic evidence has been presented to validate the viability of VNC cells.

Conversely, opponents of this theory argue that the VNC state is a moribund condition in which cells become progressively debilitated until cell death finally occurs (Moran and Upton, 1986/1987a/1987b; Beumer, et al., 1992). These cells may maintain signs of metabolic activity or respiration for some time but are unable to 'resuscitate' (Bovill and Mackey, 1997). Similar experimentation has been employed to demonstrate a lack of viability in VNC cells. Electron microscopy findings suggest degradation of the cell wall (Ng, et al., 1985b) and leakage of cytoplasmic contents (Moran and Upton, 1986). Animal passage studies with VNC cells of H. pylori and C. jejuni failed to result in the
colonisation of the gastrointestinal tract or the excretion of 'resuscitated' rods (Beumer, et al., 1992; Eaton, et al., 1995)

Tables 2.1a and 2.1b summarise some of the reports which support or refute the hypothesis that the VNC response is a programmed cellular response. Such contradictory reports may well be caused by the different induction conditions under which the VNC cells were formed, and also by the differing experimental conditions (ie., that created by laboratory and procedural variation) under which the viability assays were performed. It is important, too, to remember that the VNC response may not be a single phenomenon universal in all bacteria (Barer, et al., 1998). As such, results will be influenced by species and strain variation, as well as differing experimental conditions (Buswell, et al., 1998).
Table 2.1a: Research supporting the viability of VNC cells.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Organism</th>
<th>Study Type</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Benaissa, et al., 1996)</td>
<td><em>Helicobacter pylori</em></td>
<td>Electron microscopy</td>
<td>Preservation of ultrastructure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antigenic profiles</td>
<td>Differential expression of antigens</td>
</tr>
<tr>
<td>(Cheers and Zhan, 1996)</td>
<td>Review</td>
<td>Macrophage detection</td>
<td>Macrophage response to live bacteria</td>
</tr>
<tr>
<td>(Colwell, et al., 1996)</td>
<td><em>Vibrio cholerae</em> O1</td>
<td>Human volunteer ingestion</td>
<td>Resuscitation</td>
</tr>
<tr>
<td>(Oliver, et al., 1995a)</td>
<td><em>Vibrio vulnificus</em></td>
<td>Animal passage</td>
<td>Colonisation of mice</td>
</tr>
<tr>
<td>(Oliver, et al., 1995b)</td>
<td><em>Vibrio vulnificus</em></td>
<td>Temperature upshift</td>
<td>Resuscitation</td>
</tr>
<tr>
<td>(Bode, et al. 1993)</td>
<td><em>Helicobacter pylori</em></td>
<td>Electron microscopy</td>
<td>No ultrastructural degradation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorescent labelling</td>
<td>Presence of polyphosphate aggregates</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ability to synthesize DNA</td>
</tr>
<tr>
<td>(Jones, et al., 1991)</td>
<td><em>Campylobacter jejuni</em></td>
<td>Animal Passage</td>
<td>Colonisation of mice</td>
</tr>
<tr>
<td>(Saha, et al., 1991)</td>
<td><em>Campylobacter jejuni</em></td>
<td>Animal passage</td>
<td>Excretion of resuscitated spiral cells</td>
</tr>
</tbody>
</table>
Table 2.1b: Opponents to the theory that VNC cells are viable.

<table>
<thead>
<tr>
<th>Author</th>
<th>Organism</th>
<th>Study Type</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Bovill and Mackey, 1997)</td>
<td><em>Campylobacter jejuni</em></td>
<td>Resuscitation</td>
<td>Regrowth of residual rods - not resuscitation</td>
</tr>
<tr>
<td>(Kusters, et al., 1997)</td>
<td><em>Helicobacter pylori</em></td>
<td>Electron Microscopy</td>
<td>Ultrastructural changes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein and nucleic acid profiles</td>
<td>Protein/DNA/RNA degradation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorescent probes</td>
<td>Loss of membrane potential</td>
</tr>
<tr>
<td>(Sorberg, et al., 1996)</td>
<td><em>Helicobacter pylori</em></td>
<td>Fresh media addition</td>
<td>No resuscitation</td>
</tr>
<tr>
<td>(Eaton, et al., 1995)</td>
<td><em>Helicobacter pylori</em></td>
<td>Animal passage</td>
<td>No colonisation of gastrointestinal tract</td>
</tr>
<tr>
<td>(Ravel, et al., 1995)</td>
<td><em>Vibrio cholerae</em></td>
<td>Temperature upshift</td>
<td>Regrowth of residual rods - not resuscitation</td>
</tr>
<tr>
<td>(Beumer, et al., 1992)</td>
<td><em>Campylobacter jejuni</em></td>
<td>Simulated intestine</td>
<td>No colonisation observed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Animal passage</td>
<td>No symptoms, no cells in stool samples, no antibodies in blood</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human volunteer ingestion</td>
<td></td>
</tr>
<tr>
<td>(Moran and Upton, 1987b)</td>
<td><em>Campylobacter jejuni</em></td>
<td>Electron microscopy</td>
<td>Loss of cellular integrity</td>
</tr>
<tr>
<td>(Moran and Upton, 1986)</td>
<td><em>Campylobacter jejuni</em></td>
<td>Cytoplasmic contents</td>
<td>Leakage of cell contents/DNA Degradation of cell wall</td>
</tr>
<tr>
<td>(Ng, et al., 1985b)</td>
<td><em>Campylobacter jejuni</em></td>
<td>Electron microscopy</td>
<td>Ultrastructural degradation</td>
</tr>
<tr>
<td>(Buck, et al., 1983)</td>
<td><em>Campylobacter jejuni</em></td>
<td>Electron microscopy</td>
<td>Loss of cellular integrity Evidence of autolysis</td>
</tr>
</tbody>
</table>
2.3 STUDIES INVESTIGATING THE VNC RESPONSE.

Four types of investigations have been carried out on VNC cells: (i) analysis into the onset or formation of the VNC state; (ii) studies into cellular changes (physical and physiological) during this transition; (iii) resuscitation experiments; and (iv) detection and assessment of viability.

2.3.1 INDUCTION OF THE VNC STATE.

Frequent exposure to suboptimal growth conditions and environmental stress factors has been shown to induce nonculturability in bacteria. These conditions differ according to the organism with species and strain variations reported (Roszak and Colwell, 1987), but include such diverse factors as oxygen saturation, fluctuations in nutrient availability, salinity (osmotic stress), visible light and solar illumination, alkaline pH, antibiotic presence, desiccation, and temperature (Bode, et al., 1993; Fearnley, et al., 1996; Harvey, et al., 1996; Pommepuy, et al., 1996; Kusters, et al., 1997; McDougald, et al., 1998; Reezal, et al., 1998). Five key environmental stresses will be addressed below.

- **NUTRITION**
  The kinetics of VNC formation are affected by the nutritional state of the population (McDougald, et al., 1998). For example, it has been shown that carbon (or multiple-nutrient) and phosphorus starvation are conditions that induce the VNC response (Jiang and Chai, 1996). With temporal progression, cultures of *Vibrio parahaemolyticus* exhaust nutrient supplies and this has been shown to induce nonculturability.

- **TEMPERATURE**
  Temperature plays an important role in the induction and maintenance of the VNC state. Low temperature is the only factor required for inducing the VNC response in the estuarine bacterium, *Vibrio vulnificus*, as demonstrated in microcosms containing artificial seawater (Nilsson, et al., 1991). In the case of *V. vulnificus*, the nonculturable
state has been shown to be highly temperature dependent, with entry occurring at 5°C whilst at ambient temperature the organism exhibits a classic starvation response with no decrease in culturable count (Oliver, et al., 1991; Paludan-Muller, et al., 1996). However, this observation contradicts what has been shown for C. jejuni which exhibits prolonged culturability at low temperatures (≤ 10°C) (Rollins and Colwell, 1987; Calder, 1998).

**OXYGEN TENSION**
Aerobiosis is believed to be a key environmental stress factor. Conversion to a non-culturable state takes place more rapidly under aerobic conditions than anaerobic (Moran and Upton, 1987a/1987b). In their latter study (1987b), Moran and Upton theorised the toxic oxygen derivatives formed by cells may trigger the coccoid formation observed in C. jejuni. Addition of blood, iron compounds, charcoal, and other oxygen scavengers can overcome the development of these toxic ions. Addition of any of these neutralising agents to the media is shown to minimise the production of coccoid forms, which suggests the role of oxygen saturation in VNC induction (Moran and Upton, 1987b; Boucher, et al., 1994; Harvey, et al., 1996; Bloomfield, et al., 1998).

**CELLULAR COMMUNICATION**
Cellular triggers may have an involvement in stimulating VNC production. Cells undergoing transition to coccoid form may produce a signal which other cells can detect and respond to. Supernatants from nonculturable or starving cells of Micrococcus luteus have been shown to induce nonculturability in actively growing cells (Kaprelyants, et al., 1994; Volyakova, et al., 1994; Mukamolova, et al., 1995).

**ANTIBIOTICS**
Antibiotic supplementation is routinely used in VNC induction but some workers have found antibiotic formulations used in selective media do not influence the production of coccoid forms of C. jejuni (Moran and Upton, 1987b; Hazeleger, et al., 1995).
Nevertheless, antibiotics are still typically added to media - if not to induce nonculturability, to protect against contamination.

2.3.2 RESUSCITATION.

With respect to bacterial pathogens, interest regarding VNC cells is that they may still retain their virulence (McDougald, et al., 1998). The adherence and invasion of VNC cells has not been demonstrated therefore at present it is assumed VNC cells must initially exit their survival state and resuscitate to an actively metabolising state before they can cause infection. As such, if VNC cells are not capable of resuscitation, then they no longer pose a health risk (Ng, et al., 1985b; Sorberg, et al., 1996).

Numerous in vivo and in vitro studies have investigated the ability of VNC cells to be resuscitated. These have included addition of fresh medium or nutrients to aged cultures, transfer to fresh medium, injection into embryonated eggs, animal passage, inclusion in simulated gastrointestinal environments, human volunteer ingestion, temperature upshift or heat shock, sparging of gas through liquid culture, and the addition of supernatants from log phase cell cultures (thought to possess a growth initiator). A majority of these resuscitation studies have been attempted with Campylobacter spp. (Tables 2.1a and 2.1b).

The results from these experiments have been highly variable, with variation even noted between bacterial strains from the same species. The principal controversy with resuscitation reports stems from whether the increase in colony counts results from ‘true’ resuscitation of the VNC cells or the ability of a few residual viable cells which have escaped detection to form colonies (McDougald, et al., 1998). In most studies of resuscitation of VNC cells reported in the literature, the quantitative details of the recovery and growth have not been assessed (Weichart, et al., 1992; Barer, et al., 1993; Ravel, et al., 1995).
2.3.3 PHYSIOLOGICAL AND PHYSICAL CHANGES IN VNC CELLS.

Regardless of the inducing stimulus resulting in VNC formation, consistent physiological and physical changes occur in cells entering this state. These changes are thought to stabilise the cell through the stress. One protective mechanism observed in a majority of VNC cells (including Campylobacter) involves alterations to the structure and make-up of the cytoplasmic membrane and thickening of the cell wall (McDougald, et al., 1998). As a result of the stress, it is postulated that the cell loses its permeability function, thus, maintaining nutrients and essential ions within the cell but additionally preventing nutrient entry. The loss of permeability is speculated to be a potential reason for loss of culturability in M. luteus (Mukamolova, et al., 1995). Cells which enter the VNC state appear to maintain gross membrane integrity although changes in the fatty acid composition of the membrane and increased thickening of the peptidoglycan has been witnessed to occur in C. jejuni (Hazeleger, et al., 1995, Holler, et al., 1998). These changes are believed to render the cell wall more damage resistant and increased resistance to 10% ethanol, oxidative stress (hydrogen peroxide), and mechanical or shear stress (sonication) have been reported in V. vulnificus as a consequence (Weichart and Kjelleberg, 1996). These authors believe such an event may give the VNC cells a competitive advantage in their environment during extended exposure to stress. It is speculated that the changes could allow for increased protection against, for example, predation and virus attack which have been found to control bacterial populations in environmental ecosystems (Weichart and Kjelleberg, 1996).

Thus, it is apparent that these changes in the cell wall and membranes allow for long-term stability, persistence, and minimisation of cell maintenance requirements (Tholozoan, et al., 1999). Tholozoan and corroborators found physiological measurements for C. jejuni cells in the VNC state revealed numerous similarities with previous measurements obtained for starving cell suspensions of members of other genera. Experiments included the monitoring of cellular volume, internal pH, adenylate energy charge, intracellular potassium concentration, and membrane potential values.
Although comparison of values obtained for growing or resting-cell suspensions demonstrated a progressive decrease in the ability of VNC *C. jejuni* cells to maintain internal homeostasis, Tholozoan reports cellular death did not occur. Thus, the physiological changes witnessed in this study assist in the longevity and persistence of the *Campylobacter* cells, such that they were able to revert to a culturable pathogenic state infective in a newborn mice model (Tholozoan, *et al.*, 1999).

The VNC response may not be a single phenomenon universal in all bacterial genera, however it is evident that such changes are found in several organisms and serve to allow for cellular stability. Table 2.2 summarises some of these other changes observed during the transition from culturable to nonculturable.
Table 2.2: Stress induced physiological responses observed in cells following transformation to a VNC state and the proposed rationale for the associated changes.

<table>
<thead>
<tr>
<th>Physiological Change</th>
<th>Proposed reasoning for the change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction in size - coccoid formation</td>
<td>Reduced surface area, increased strength available with spherical shape</td>
</tr>
<tr>
<td>Cell wall thickening</td>
<td>Increased cellular stability</td>
</tr>
<tr>
<td>Condensation of the cytoplasm</td>
<td>Protection of nucleic acids</td>
</tr>
<tr>
<td>Changes in membrane fatty acids/</td>
<td>Increased cellular stability</td>
</tr>
<tr>
<td>Thickening of the peptidoglycan layer</td>
<td>Reduced potential for autolysis</td>
</tr>
<tr>
<td>Loss of permeability barrier by</td>
<td>Reduced leakage from cell (maintenance of ions and nutrients)</td>
</tr>
<tr>
<td>cytoplasmic membrane</td>
<td>Maintenance requirements</td>
</tr>
<tr>
<td>Accumulation of storage polymers (polyphosphates)</td>
<td></td>
</tr>
<tr>
<td>Conservation of ribosomal structure</td>
<td>Decreased transcription (Production of 'essential' gene products only)</td>
</tr>
<tr>
<td>Reduction in ribosome numbers</td>
<td></td>
</tr>
<tr>
<td>Decreased RNA levels and in some cases DNA</td>
<td>Initial cannibalism for nutrients</td>
</tr>
<tr>
<td>Inability to multiply</td>
<td>Reduced metabolic activity</td>
</tr>
<tr>
<td>Increased stress resistance (eg., Ethanol, Oxidative, Mechanical)</td>
<td>Prevention of further environmental stress</td>
</tr>
<tr>
<td></td>
<td>Increased protection against predation</td>
</tr>
</tbody>
</table>
2.3.3.1 THE VNC HYPOTHESES.

Recent hypotheses have been put forward to explain the formation of VNC cells and account for all the changes observed. Weichart (1997) and McDougald (1998) propose that there are two distinct phases in the formation of VNC cells (see Figure 2.1) (Weichart, et al., 1997; McDougald, et al., 1998). Stage I is characterised by transition to a VNC state with concomitant loss of culturability whilst cellular integrity and intact nucleic acids are maintained. Cells in this initial VNC state are believed to retain the potential for metabolic activity, respiration, and gene transcription, and thus viability. It is speculated, too, that such cells would continue to synthesize cell walls and be able to repair any structural damage, such that, on return to environmental conditions conducive to growth, Stage I cells could 'revive' and initiate regrowth. With extended time in the VNC state, it is postulated the coccoid cells undergo further change and enter the second stage. This stage involves gradual loss of cellular integrity with degradation of RNA and DNA, ultimately resulting in loss of viability.

Despite the predictions made by Weichart (1997) and McDougald (1998), no specific novel methods have been put forward to test this hypothesis. It is hoped this thesis may add weight to or reject this theory.
Figure 2.1: The two stages of cell degradation as suggested by (Weichart, et al., 1997; McDougald, et al., 1998). Stage I is typified by formation of a VNC state after exposure to an environmental stress factor or cellular inducer. Stage II is regarded as a degenerative stage in which the cell is no longer viable.

Environmental Stress Factor
* temperature
* osmotic pressure
* nutrient / carbon starvation
* inducer from other cells
* aerobiosis
* antibiotics

Stage I - Transition to Non-culturable state
TRAITS:
* loss of culturability
* reduction in size (coccoid formation)
* maintenance of cellular integrity
* maintenance of intact nucleic acids
* potential for resuscitation
* potential for host colonisation
* potential for gene transcription / protein synthesis
* virulence retained
CONCLUSION: * VIABLE BUT NONCULTURABLE

Extended time in the VNC state

Stage II - Onset of cellular death
TRAITS:
* gradual loss of cellular integrity
* degradation of RNA and subsequently DNA
* loss of potential for resuscitation (nonresuscitable)
* loss of virulence (avirulent)
CONCLUSION: * NONVIABLE NONCULTURABLE
2.3.4 DETERMINATION OF VIABILITY.

As mentioned previously, a key point of discussion related to the VNC debate is the determination of viability. A number of methods have been proposed to assess the viability of nonculturable cells, all of which have advantages and disadvantages. These methods assess microbial viability by one of three criteria - demonstration of metabolic activity, maintenance and integrity of cellular structures, or gene expression assays.

2.3.4.1 INDICATION OF CELLULAR METABOLIC ACTIVITY.

Methods used as indicators of the maintenance of metabolic activity are listed in Table 2.3. The latter two presented in the table are the most commonly used techniques to demonstrate maintenance of viability in VNC cells and will be addressed more fully.

<table>
<thead>
<tr>
<th>Experimental method</th>
<th>Metabolic activity observed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microautoradiography</td>
<td>Uptake of radiolabelled substrate</td>
<td>(Shahamat, et al., 1993)</td>
</tr>
<tr>
<td>Inducible enzyme assays</td>
<td><em>De novo</em> protein synthesis</td>
<td>(Nwoguh, et al., 1995)</td>
</tr>
<tr>
<td>Bioluminescent assays</td>
<td>Changes in ATP levels</td>
<td>(Ng, et al., 1985a)</td>
</tr>
<tr>
<td>Most Probable Number</td>
<td>Growth (turbidometric changes)</td>
<td>(Xu, et al., 1982)</td>
</tr>
<tr>
<td>Fluorescein diacetate staining assays</td>
<td>Enzymatic activity</td>
<td>(Chrzanoski, 1984)</td>
</tr>
<tr>
<td></td>
<td>(intracellular esterase)</td>
<td>(Tsuji, et al., 1995)</td>
</tr>
<tr>
<td>Direct Viable Counts</td>
<td>Growth (elongation of cells)</td>
<td>(Kogure, et al., 1979)</td>
</tr>
<tr>
<td>Tetrazolium salt reduction assays</td>
<td>Active electron transport chain</td>
<td>(Maki &amp; Remsen, 1981)</td>
</tr>
<tr>
<td></td>
<td>Respiratory activity</td>
<td>(Rodriguez, et al., 1992)</td>
</tr>
</tbody>
</table>
2.3.4.1.1 DIRECT VIABLE COUNTS.

The original direct viable count (DVC) method, described by Kogure, et al., 1979, is conducted by the incubation of samples with a single antibiotic (nalidixic acid (NA)) in the presence of nutrient medium (e.g., yeast extract). Although high concentrations of NA (≥ 50μg.ml⁻¹) have severe inhibitory effects on some Gram-negative bacteria (British National Formulary, 1995), a moderate concentration acts as a specific inhibitor of bacterial DNA synthesis (i.e., DNA gyrase inhibition), preventing cell division without interfering with other metabolic activities of the cell (Goss, et al., 1964). In the presence of NA and nutrients, viable Gram-negative bacteria continue to metabolise and grow without cell division, resulting in the formation of elongated cells (King and Parker, 1988). Microscopic examination permits estimation of numbers of substrate-responsive cells. Despite increased technology, the DVC method proposed three decades ago has changed little. Joux and LeBaron (1997) suggest improvement by usage of an antibiotic cocktail instead of NA alone; fluorochrome incorporation has allowed for easier visualisation of metabolically active bacteria (Kepner and Pratt, 1994), and Singh, et al., 1990 utilised Image Analysis for rapid enumeration of ‘viable’ bacteria.

2.3.4.1.2 REDUCTION OF TETRAZOLIUM SALTS.

The dehydrogenase stain assay, first proposed by Zimmermann, et al., 1978, involves the use of tetrazolium salts as cytochemical indicators of oxidative metabolism in bacteria. In particular, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) have been advanced as indicators of viability (Gribbon and Barer, 1995). Most living cells possess an active electron transport chain. A key enzyme in this chain, dehydrogenase, is employed to reduce the synthetic, water-soluble, membrane-permeable tetrazolium salts, converting them to coloured, water-insoluble formazans (Maki and Remsen, 1981). In contrast, non-viable cells do not maintain cellular respiration which is required to power the reduction of the fluorogenic indicator therefore no colorimetric change is observed (Boucher, et al., 1994; Bovill and Mackey, 1997).
2.3.4.2 MAINTENANCE OF STABLE CELLULAR STRUCTURES.
Methods that have been developed to demonstrate the maintenance of structural integrity are based on immunofluorescent (antibody) microscopy (Brayton, et al., 1987), or the epifluorescent direct count technique. The latter involves the use of fluorochrome dyes, of which 3,6-bis[dimethylamino] acridinium chloride (Acridine orange [AO]) and 4’-6-diamidino-2-phenylindole (DAPI) are most often used. These dyes act as indicators of intact nucleic acids, with AO binding RNA and both binding ds-DNA (Roszak and Colwell, 1987), thereby indicating only cells with possibly functional genomes (King and Parker, 1988). However, the distribution of dead and viable cells can not be determined by the standard technique of either AO or DAPI staining alone, because the DNA target retains its staining properties, even in non-viable cells, due to its stability (Kepner and Pratt, 1994). Therefore, the fluorochromes must be used in conjunction with the DVC (as described above), allowing for enumeration by fluorescent microscopy of the actively growing (elongating) sub-population.

2.3.4.3 DNA ANALYSIS AND PCR-BASED TECHNIQUES.
To overcome limitations observed with conventional culture-based methods, a number of immunological and molecular biology-based techniques for the rapid detection of C. jejuni have been developed in recent years, including immuno-assays, nucleic acid-based hybridisation assays, and DNA-based PCR amplification technology. The latter is the most commonly employed, showing a high degree of species- and strain-specificity with the added advantage of extreme sensitivity. Although this technique has been very successful at decreasing the time necessary for pathogen detection, it does not unequivocally demonstrate whether cells are alive or dead (Lindahl, 1993). Josephson (1993) illustrated the potential for PCR detection of non-viable cells of E. coli, Salmonella and Shigella spp, whilst Masters (1994) showed there was no relationship between viability and PCR detection of DNA targets in L. monocytogenes or E. coli that had been exposed to heat, acid, desiccation, hydrogen peroxide, or starvation (Josephson, et al., 1993; Masters, et al., 1994). In the latter study, even autoclaved cells
provided a positive PCR signal. Subsequent studies have shown the presence of false positives due to amplification of DNA from non-viable cells (Klein and Juneja, 1997), and the persistence of *C. jejuni* DNA in model seawater microcosms (Calder, 1998).

Calder demonstrated detection of *Campylobacter* specific 23S rDNA in nonculturable cells after twelve months following the loss of culturability, irrespective of the stress cells were subjected to (i.e., temperature, salinity, aeration). Because PCR analyses are based on the detection of DNA rather than intact viable cells, there exists the possibility that PCR amplification may arise from either dead or non-infectious cells. Additionally, in some instances, the condensation of the bacterial genome due to the transformation to a VNC state may result in the deletion of the target gene sequence (Weichart, *et al.*, 1997). This, too, may cause PCR detection to fail.

The disadvantages of DNA-based methods may be overcome in part by the inclusion of a pre-enrichment step that allows for organisms to multiply before PCR (Purdy, *et al.*, 1996; Niederhauser, *et al.*, 1992; cited from Klein and Juneja, 1997). This procedure increases sensitivity and helps to distinguish between samples that contain living cells and those that do not. However, the use of enrichment techniques prior to the PCR can prolong time taken for analysis, eliminating much of the high-sensitivity advantage of PCR (Sheridan, *et al.*, 1998).

An alternative method is therefore required which combines sensitivity and specificity with the ability to differentiate between viable and non-viable cells.
2.4 THE mRNA APPROACH.

A functional gene product or protein is produced by the translation of an mRNA species, which in turn has been transcribed from the bacterial genome (DNA). In starved cells, this energy-expensive process of protein synthesis is thought to be more highly regulated with only 'essential' house-keeping genes being expressed, whilst bacteria that are physiologically dead cease to produce any proteins. This hypothesis has been tested by Hood (1986), who demonstrated that in starved cells there is no net change in DNA levels, yet there is a drastic decrease in mRNA and protein levels (Hood, et al., 1986). It has therefore been suggested that the detection of mRNA species may act as an indication of ongoing bacterial metabolism, replication and protein synthesis in VNC cells (Sheridan, et al., 1998).

The underlying principle of this approach is that mRNA is rapidly turned over in living bacterial cells, with most species having a very short half-life of ≤ 2 minutes (King and Sclessinger, 1987). Bacterial mRNA is extremely susceptible to enzymatic attack and is broken down rapidly by endogenous ribonucleases (RNases) - a process that is important in the control of gene expression (King and Schlessinger, 1987). Detection of mRNA species that have a short half-life may therefore be a good indicator of living cells or those recently dead at the time of sampling.

To date, few studies have specifically investigated the relationship between detection of microbial mRNA and cellular viability (Table 2.4).
Table 2.4: Investigations into the detection of mRNA as an indicator of viability.

<table>
<thead>
<tr>
<th>Author</th>
<th>Organism</th>
<th>Target mRNA / Gene Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Bej, et al., 1991)</td>
<td><em>Legionella pneumophila</em></td>
<td><em>mip</em> (macrophage infectivity potentiator)</td>
</tr>
<tr>
<td>(Patel, et al., 1993)</td>
<td><em>Mycobacterium leprae</em></td>
<td><em>hsp</em> (71 kDa heat shock protein)</td>
</tr>
<tr>
<td>(Bej, et al., 1996)</td>
<td><em>Vibrio cholerae</em></td>
<td>Housekeeping gene</td>
</tr>
<tr>
<td>(Jou, et al., 1997)</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>85B (α-antigen)</td>
</tr>
<tr>
<td>(Klein and Juneja, 1997)</td>
<td><em>Listeria monocytogenes</em></td>
<td><em>iap</em> (extracellular protein P60)</td>
</tr>
<tr>
<td>(Narikawa, et al., 1997)</td>
<td><em>Helicobacter pylori</em></td>
<td><em>hly</em> (58 kDa Listeriolysin)</td>
</tr>
<tr>
<td>(Sails, et al., 1998)</td>
<td><em>Campylobacter spp.</em></td>
<td><em>prf</em> (27.1kDa regulatory protein)</td>
</tr>
<tr>
<td>(Sheridan, et al., 1998)</td>
<td><em>Escherichia coli</em></td>
<td>Urease mRNA</td>
</tr>
<tr>
<td>(Hellyer, et al., 1999a/b)</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Species specific protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regulatory gene</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>rpoH / groEL</em> (stress response)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>tufA</em> (housekeeping protein)</td>
</tr>
</tbody>
</table>

Investigations into the relationship between mRNA production and cellular viability have also been performed on the parasitic organisms *Cryptosporidium parvum* (Stinear, et al., 1996) and *Giardia lambia* (Abbaszedegan, et al., 1997). In the former study, Stinear developed a RT-PCR which was able to detect heat shock protein mRNA from *C. parvum* oocysts spiked into environmental water samples. In the latter, amplification of heat shock-induced mRNA from *Giardia* cysts was indicative of viability, whilst false-positive results were obtained with PCR technology.

2.4.1 APPLICATIONS OF mRNA AS A VIABILITY DETERMINANT.

Of the investigations into the detection of mRNA as an indicator of viability, none of the afore-mentioned studies (Table 2.4) analyse target gene expression levels over time. Typically, previous work in this expanding field has centred on the methodology of the technique and has yet to be used to analyse changes in cells with temporal progression. Most mRNA detection methods have been employed for one of two applications:
Determination of the relationship between the detection of mRNA and cellular viability in artificially killed model systems to facilitate potential practical application of such an assay in the detection of viable cells in foodstuffs, clinical and environmental samples. Bej (1991, 1996) used RT-PCR to examine *L. pneumophila* and *V. cholerae* exposed to heat or starvation, respectively, and observed specific mRNA only in samples that contained viable cells detected by culturing (Bej, *et al.*, 1991; Bej, *et al.*, 1996). Similarly, Patel (1993) successfully assessed the viability of heat-killed *M. leprae*, detecting a heat-shock protein mRNA in living cells (Patel, *et al.*, 1993). Recently, Klein and Juneja (1997) described a method for the specific detection of viable *L. monocytogenes* cells - a study which was validated in the detection of this pathogen in meat products. Moreover, Sails (1998) and Sheridan (1998) have reported a correlation between mRNA presence and viability in heat-killed cultures of *Campylobacter* spp. and *E. coli* respectively.

The second application has been in the assessment of drug therapies. Hellyer (1999a) initially validated the use of 85B mRNA as a marker for bacterial viability by qualitative *in vitro* RT-PCR in an effort to discriminate between drug-sensitive and drug-resistant strains of *M. tuberculosis* (Hellyer, *et al.*, 1999a). In a second study, the approach was continued to test bacterial susceptibility to a number of chemotherapeutic agents (Hellyer, *et al.*, 1999b). A RT-strand displacement amplification assay was used, with success, to assess chemotherapeutic efficacy in patients with pulmonary tuberculosis.
2.4.2 PROBLEMS WITH THE mRNA APPROACH.

Currently, the major drawbacks associated with the mRNA approach to examining microbial viability are the low copy numbers, instability and the enzymatic degradation of the mRNA itself, as well as the difficulty in finding a transcript that is constitutively expressed and species-specific. The former can be overcome by the use of RNase-inhibitory chemicals and adherence to laboratory precautions (see Materials and Methods - Section 3.5.1), yet the choice of suitable mRNA targets can be more laborious.

For any cell to be viable, it must be capable of cellular metabolism, maintain structural integrity and replicate DNA to pass on to progeny (Koch, 1988). Thus monitoring the expression of genes in some of these essential pathways should provide insight into the "life status" of the cell. Some potentially suitable target genes for amplification are presented in Table 2.5.

**Table 2.5 :** Some critical features of a cell which are be necessary for maintenance of viability and the genes which encode them.

<table>
<thead>
<tr>
<th>Essential cellular process</th>
<th>Indicator gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy / ATP production (eg., Glycolysis)</td>
<td>Glycolytic enzymes (eg., phosphofructokinase pfk)</td>
</tr>
<tr>
<td>Membrane integrity</td>
<td>Lipid biosynthetic enzymes (eg., lpxA)</td>
</tr>
<tr>
<td></td>
<td>Fatty acid biosynthetic enzymes (eg., fabZ)</td>
</tr>
<tr>
<td>Cell envelope / wall integrity</td>
<td>Peptidoglycan enzymes</td>
</tr>
<tr>
<td>Stress response</td>
<td>Stress proteins (eg., rpoS)</td>
</tr>
<tr>
<td>Chaperonin expression</td>
<td>Heat shock proteins (eg., dnaJ)</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Ribosomal genes (eg., 23S rRNA)</td>
</tr>
<tr>
<td>DNA replication</td>
<td>Replication genes (eg., DNA Polymerase)</td>
</tr>
</tbody>
</table>
2.4.3 GENE TARGETS.

Previous work on the detection of viable cells by RT-PCR has demonstrated the need for a 'true' indicator of viability. Klein and Juneja (1997) attempted to detect *L. monocytogenes* through the expression of three genes - two of which encoded virulence functions (*hly*, *prfA*). In times of stress, it is postulated that energy-expensive protein synthesis is limited and only 'essential' genes are expressed (i.e., those involved in the maintenance of viability). Virulence genes may not be included in this subset of proteins expressed on induction of stress. Indeed, Klein and Juneja (1997) found the level of expression of their third gene target (a 'housekeeping' protein shown to be essential for viability) was considerably higher and concluded that this *iap* gene would be a suitable target in foodstuff analyses.

In this thesis, five genes were selected which represent different cellular processes to be studied in the test organism. These represented essential 'house-keeping' genes which would be produced constitutively under normal growth as well as stress conditions (*lpxA* and 23S rRNA); two genes with functional roles during normal growth which may be upregulated on induction of stress (*dnaJ* and *hupB*); and a gene which encodes a virulence factor that would only be expected to be expressed in the presence of host (eukaryotic) cells (*ciaB*). Further information on each of these genes is ensuing in this thesis but it is anticipated that the five chosen genes will provide an index of viability for *Campylobacter jejuni* KLC 4315 under the induction conditions of this research.
2.5 VIABILITY DETERMINANTS FOR *Campylobacter jejuni*.

2.5.1 LIPID A AND CELLULAR VIABILITY.

2.5.1.1 THE GRAM-NEGATIVE CELL WALL.

The cell envelope of Gram-negative eubacterial cells consists of a double membrane - the inner (cytoplasmic) membrane and the outer membrane which is on the environmental side of the mesh-like peptidoglycan layer (Figure 2.2). The region between the two is termed the periplasmic space. Like the cytoplasmic membrane, the outer membrane is a lipid bilayer made up of phospholipids and proteins, but also contains a unique constituent, lipopolysaccharide (LPS) (Schlessinger, *et al.*, 1993). It is the LPS component of the outer membrane that gives many Gram-negative enterobacteria their pathogenic properties (Nikaido, 1973; cited from Crowell, *et al.*, 1986; Raetz, 1990). LPS are described as biologically active, endotoxic, immunogenic glycolipids (Raetz, 1996; Reeves, *et al.*, 1996) and consist of three covalently linked domains:

- the proximal lipid A (endotoxin), which functions as the hydrophobic anchor for the LPS in the outer membrane and is the bioactive component responsible for some of the pathophysiology associated with several Gram-negative infections (Raetz, 1986)
- the core which connects the proximal and distal domains is composed of a phosphorylated non-repeating region of sugars, particularly residues of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO), L-glycero-D-manno-heptose, hexose sugars and aminohexoses (Moran, *et al.*, 1991).
- the distal, hydrophilic O-antigen. This immunogenic repeating oligosaccharide varies greatly from strain to strain and protrudes into the external milieu.

In enteric and non-enteric bacteria the innermost region of the LPS appears to be essential for bacterial survival. This region consists of lipid A and at least two residues of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) from the core (Raetz, 1986). The role of lipid A in bacterial viability will be reviewed in this thesis.
Figure 2.2: Schematic molecular representation of the *Escherichia coli* envelope. Ovals and rectangles depict sugar residues. Circles represent the polar headgroups of the phospholipids. MDO are membrane-derived oligosaccharides, and KDO is 3-deoxy-D-manno-octulosonic acid. KDO and heptose make up the inner core of the LPS. The minimal LPS required for growth of *E. coli* consists of the lipid A domain and at least two KDO (Adapted from Raetz, 1990).
2.5.1.2 LIPID A BIOSYNTHESIS AND lpxA.

The first committed step of lipid A biosynthesis in *E. coli* and *Salmonella typhimurium* involves the transfer of a fatty acid, R-3-hydroxy-myristic acid, from an acyl carrier protein (ACP) to the 3 Carbon-OH group of the glucosamine ring of UDP-N-acetyl glucosamine (UDP-GlcNAc) (Coleman and Raetz, 1988). The cytoplasmic enzyme, UDP-GlcNAc acyltransferase, catalyzes this transfer (Figure 2.3). It is encoded for by the *lpxA* gene (Coleman and Raetz, 1988; Galloway and Raetz, 1990). This gene has been cloned and sequenced in *E. coli* (Coleman and Raetz, 1988) and more recently, Ibbitt (1997) sequenced and characterised the putative *lpxA* gene from *C. jejuni* F38011. Ibbitt proposed that *C. jejuni* possesses an enzyme that is functionally analogous to LpxA from *E. coli* and complementation analysis confirmed this hypothesis.

2.5.1.3 lpxA AND VIABILITY.

Mutational analyses suggest *lpxA* mRNA may be a good candidate as a target for viability studies by RT-PCR. The conditional lethality of *E. coli* mutants defective in *lpxA* gene expression demonstrates that lipid A is essential for cell viability (Galloway and Raetz, 1990). A temperature-sensitive (ts) mutation in *lpxA* was shown to make *E. coli* extremely susceptible to hydrophobic antibiotics (Vogel and Jahnig, 1986; cited from Nikaido, 1996). The (ts) phenotype could be corrected by *lpxA*\(^+\) -bearing plasmids which overproduce the UDP-GlcNAc acyltransferase enzyme (Raetz, 1987; Ibbitt, 1997). Other mutagenesis studies show even partial inhibition of lipid A synthesis causes profound hypersensitivity to antibiotics, like erythromycin, that are normally excluded by the outer membrane (Vuorio and Vaara, 1992; cited from Raetz, 1996). It has been theorised that the large polar head group of lipid A may favour strong non-covalent interactions with adjacent molecules, particularly in the presence of divalent cations. In other words, a monolayer of lipid A is suggested to aid in bacterial resistance to intestinal detergents and other environmental stresses (Raetz, 1986). The susceptibility of mutants defective in *lpxA* to antibiotics, indeed, demonstrates the protective function of an intact cellular membrane.
Figure 2.3: Enzymatic synthesis of lipid A in *Escherichia coli*. \( R \) designates a \( \beta \)-hydroxymyristoyl moiety, having the \( 3R \) stereochemistry, and \( U \) represents uridine. The structure shown at the bottom is the minimal one that appears to be required for cell growth, but "wild-type" LPS is much more complex than this due to the addition of different core and O-antigenic sugars (Adapted from Raetz, 1990).
2.5.1.4 THE \textit{lpxA} OPERON.

In \textit{E. coli}, the \textit{lpxA} gene is part of a cluster of 11 genes organised into a complex operon which has been termed the macromolecular synthesis II operon (Schnaitman and Klena, 1993). Ibbitt (1997) demonstrated upstream of the \textit{lpxA} gene of \textit{C. jejuni} is a dehydrase involved in fatty acid biosynthesis (Figure 2.4). The \textit{fabZ} gene encodes for $\beta$-hydroxyacyl-ACP dehydrase (Mohan, et al., 1994). Because fatty acid biosynthesis is an essential mechanism for prolonged microbial survival (Cronan and Rock, 1996), the primer sequences chosen to prime the \textit{lpxA} gene were extended to include a partial sequence of the \textit{fabZ} gene and the small intragenic region between the two. Mutations in the \textit{fabZ} gene have reduced enzymatic activity and lack the ability to suppress mutations in lipid A biosynthesis, thus signifying the role of the \textit{fabZ} gene product in cellular viability.

\textbf{Figure 2.4:} Physical map of the 3' end of \textit{fabZ} and the \textit{lpxA} gene in \textit{Campylobacter jejuni} isolate KLC4315. The large arrows show the direction of transcription. Solid boxes indicate the hybridisation positions of the primer pair and small arrows indicate direction of extension for PCR. The 308bp amplicon generated using primers 98-04 and 96-25 is shown. Scale: 1cm = 100 bp (Adapted from Ibbitt, 1997).
2.5.2 THE STRESS RESPONSE OF C. jejuni.

2.5.2.1 PRODUCTION OF HEAT-SHOCK PROTEINS (HSP).

One of the most highly conserved responses in biology is the induction of stress proteins by potentially injurious stimuli. Initially called heat shock proteins (hsp) because they were described in fruit-fly (Drosophila spp.) larvae after slight elevations of temperature (Gross, 1996), these molecules appear to be essential to cell survival in all species subjected to injury or stress (Neidhardt, et al., 1984; Lindquist and Craig, 1988). It has been proposed that upon the induction of stress, increased transcript numbers of hsp mRNA appear in the cell (Lindquist, 1986). After translation, this subset of proteins function to protect the organism from potentially lethal damage by 'rescuing' unfolded or aggregated polypeptides and refolding these heat- or stress-denatured proteins back to an active conformation, whilst accelerating the proteolysis of proteins denatured beyond repair (Craig, 1993). However, a theory has been put forward that the heat-shock response may also be instrumental in the ability of bacteria to exist over a wide temperature range as well as survival in hostile environments (Streips & Polio, 1985).

Recent evidence demonstrates hspS are expressed constitutively in non-stressed cells (at non-physiological temperatures) where they serve a vital role in normal cell metabolism by acting as 'molecular chaperones' or chaperonins (Hemmingsen, et al., 1988; Agard, 1993; Konkel, et al., 1998). Under non-stress conditions, hsp have a role in de novo intracellular protein folding and translocation, chaperonin-assisted post-translational assembly of oligomeric protein structures, targeting of proteins to their final destination, and the proteolysis of potentially deleterious, misfolded proteins (Konkel, et al., 1998).
2.5.2.2 *dnaJ* AND THE HEAT-SHOCK RESPONSE.

The *dnaJ* gene has been characterised in *E. coli*. The DnaJ gene product belongs to the Hsp 40 protein family - members of which have been shown to have roles in nascent polypeptide chain stabilisation, folding and assembly, and the regulation of the heat shock response (Langer, *et al.*, 1992; Mayhew and Hartl, 1996). Through recombinant plasmid technology with *E. coli* transformants, Konkel (1998) characterised a hsp gene from *C. jejuni*, encoding a 41-kDa protein - the functional homologue of the *dnaJ* gene from *E. coli* (Konkel, *et al.*, 1998). It is speculated heat-shock genes may be good targets for mRNA-based viability studies (Patel, *et al.*, 1993; Klena, 1999; Konkel, 1999c). As the proteins serve functional roles in both the stressed and non-stressed environment, gene expression would be expected to persist in cells that are maintaining viability through times of stress. If a cell is not viable, it can not produce new heat-shock mRNA when exposed to environmental stress (Abbaszadegan, *et al.*, 1997). This hypothesis will be examined with the *dnaJ* gene of *C. jejuni*.

2.5.3 *hupB* AND ITS ROLE IN DNA BINDING.

Most of the genetic information of the bacterial cell is contained within a bacterial chromosome composed of a single DNA macromolecule. The area occupied by this DNA is referred to as the nucleoid region. Within the nucleoid, the DNA is maintained in a highly condensed, coiled form by ionic interactions with histone-like proteins. One such polypeptide, HU, has been found in the *E. coli* nucleoid. It is composed of two non-identical subunits (HU- α and HU- β) and has a role in stimulating *in vitro* DNA replication, conformational changes, and DNA recombinational repair (Storts and Markovitz, 1988). In 1986, a 460-bp segment of the *E. coli* chromosome was sequenced and found to contain *hupB*, the gene encoding HU- β (Kano, *et al.*, 1986; cited from Storts and Markovitz, 1988).

Mutational analysis has shown deletion of *hupA* and *B* genes coding for HU is not lethal, although a strain carrying mutations in both genes grows slowly, making frequent
errors in nucleoid segregation, and can produce a high percentage of DNA-less daughter cells with aberrant nucleoid structure (Schmid and Von Freiesleben, 1996).

The hupB gene in C. jejuni was chosen as a target for viability due to its necessity in DNA folding (Klena, 1999; Konkel, 1999c). It is deemed by the author to play an important, if not more so, role when cells are stressed. In cells under stress, the histone-like protein may be required to play a role in nucleoid condensation and continued folding of the DNA for replication upon induction of conditions conducive to growth.

2.5.4 THE VIRULENCE GENE ciaB.

To be a successful pathogen Campylobacter must be able to: gain entry to a susceptible host, avoid destruction by the immune system, adhere to host tissue, colonise, replicate and transmit progeny to a new host. The molecular mechanisms by which C. jejuni causes infection are not well understood (Walker, et al., 1986; Wallis, 1994; Ketley, 1995), but most experimental work has been based on expression studies of the genes involved in binding and internalisation of the bacterium.

Konkel (1999b) identified a C. jejuni gene termed ciaB (Campylobacter invasion antigen B) (Konkel, et al., 1999b). The deduced aa sequence of CiaB exhibited identity to a number of secreted invasion and antigenic proteins from Gram-negative bacteria. Functional analysis of the ciaB gene product showed a role in internalisation of cultured cells. In vitro binding and internalisation revealed that while binding of C. jejuni ciaB null mutants (ciaB-) was indistinguishable from that of the parental isolate there was a significant reduction in the efficiency of internalisation. Konkel (1999b) concluded the gene product, encoded for by ciaB, has a role in the secretion process upon co-cultivation with host cells and in efficient entry and internalisation of the bacteria by non-professional phagocytic cells (Konkel, et al., 1999b).
The *ciaB* gene was chosen as a negative marker for mRNA-based viability studies. As a virulence gene, *ciaB* expression should be limited only to when in the presence of potential host cells. If no methods of inducing expression (e.g., eukaryotic cells) are in solution, results of *ciaB* expression in the broth cultures of *C. jejuni* should be negative. This should again validate the statement that the choice of mRNA target is important. A gene encoding a virulence factor would not seem likely to reflect the viability of the organism under study.

### 2.5.5 THE rRNA APPROACH.

Research has indicated ribosomal RNA (rRNA) may be an appealing target for nucleic acid amplification-based detection. A universal constituent of bacterial ribosomes, rRNA is involved in the process of protein synthesis and is, therefore, present in high copy numbers ($10^3$-$10^4$ molecules per actively growing cell) (Scheu, *et al.*, 1998; cited from McKillip, *et al.*, 1998). For this reason rRNA has been extensively studied in microbial systems and used in a number of functional analyses:

- Due to a growing interest in phylogenetic relationships in science, extensive bacterial rRNA sequence information is currently available - even with respect to *Campylobacter* (*Kim, et al.*, 1993; *Van Camp, et al.*, 1993; *Kim, et al.*, 1995). rRNA is used as a target for evolutionary studies due to its typical mosaic structure of phylogenetically conserved and variable regions, and structural stability - a feature which is conferred by nucleoside modifications (Bjork, 1996). Because they are genetically stable, 16S rRNA and 23S rRNA or their corresponding genes (rDNA) are very useful for detecting bacterial taxonomic groups (Woese, 1987, Amann, *et al.*, 1995).

- Genotyping of bacteria has involved many molecular biological techniques, including 16S and 23S rRNA-PCR. Initial research on PCR-RFLP analysis of the large subunit (23S) rRNA confirmed their value as targets in species-specific PCR identification.
assays but not for subtypic discrimination within *C. jejuni* (Iriate and Owen, 1996). Subsequent studies suggest amplification of the internal spacer region between the 16S and 23S rRNA genes may provide this level of discrimination (Christensen, *et al.*, 1999; Payne, *et al.*, 1999).

- Recently, rRNA has been trialed as a suitable indicator of cellular viability (Engstrand, *et al.*, 1992; Van der Vliet, *et al.*, 1994; cited from Sheridan, *et al.*, 1998; Ichikawa, *et al.*, 1996; Uyttendaele, *et al.*, 1997; McKillip, *et al.*, 1998). The two former studies concentrate on an alternative amplification system to PCR - NASBA® (isothermal transcription based nucleic acid amplification), and will be discussed below. The NASBA process involves the concerted action of three enzymes (reverse transcriptase, RNase H, and T7 RNA polymerase) as well as two target sequence-specific oligonucleotide primers (one of which bears a bacteriophage T7 promoter sequence) to amplify a RNA target sequence more than $10^8$ fold within 90 minutes incubation at 41°C.

In a comparison of the NASBA® technique (for detection of 16S rRNA) and the PCR technique (for the detection of 16S rDNA), the presence of intact RNA was found to be a valuable indicator of viability (Van der Vliet, *et al.*, 1994). Recently the NASBA® system using a 16S rRNA target sequence was developed with success for detection of pathogenic campylobacters (*C. jejuni*, *C. coli*, and *C. lari*) (Uyttendaele, *et al.*, 1994; Uyttendaele, *et al.*, 1995). However, in a later study Uyttendaele demonstrated that the NASBA® nucleic acid amplification system cannot be used for differentiating viable and non-viable *C. jejuni* (Uyttendaele, *et al.*, 1997). Positive detection signals were observed in *C. jejuni* cultures which had been subjected to antibiotic treatments (nalidixic acid and chloramphenicol) and complete 16S rRNA or the target sequence for amplification was stable for five hours following a ten minute heat-kill treatment at 100°C. Therefore, by the NASBA® method the correlation between viability and cellular rRNA content is debatable.
23S rRNA genes of *C. jejuni* (termed *rrl* by (Christensen, *et al.*, 1999)) will be investigated in this thesis by cDNA-linked PCR amplification. As the amount of new protein synthesised is dictated by the availability of rRNA, the expression of this molecule should exceed that of the mRNA species tested. Due to the increased stability and role in mRNA translation, the analysis of rDNA expression over the continuum of *Campylobacter* cellular life should give an indication of the level of protein synthesis at different time intervals. The Therm primers, described by Eyers (1993), will be employed as they are species-specific and readily available in this laboratory (Eyers, *et al.*, 1993).

2.6 THE CURRENT STUDY.

2.6.1 OBJECTIVES OF THIS RESEARCH.

In view of the potential epidemiological importance of VNC cells, it is necessary to establish unequivocally that they exist as viable entities. This thesis proposes to test the hypothesis that specific 'essential life' RNA molecules can be used to determine the life/death state of a prokaryotic cell, *Campylobacter jejuni*. The overall aim of this research is to develop a sensitive method to discriminate between viable and non-viable cells of *C. jejuni* based on amplification of mRNA by RT-PCR technology. The method needs to be reliable, robust, inexpensive, and thus suited for routine use for analysis of environmental samples. This aim will permit fulfilment of the following objectives:

- Determination of whether nonculturability is the equivalent of bacterial cellular death or if VNC cells represent a viable state, which is potentially transmissible.

- Establishment of an assay to determine a suitable target gene as an indicator of cellular viability. Such a genetic marker could then be included in RT-PCR analyses of environmental samples to detect viable *C. jejuni*.
Attempts to study the stability of the established gene target in a heat-treated *C. jejuni* culture. Detection of mRNA post-death on a presence/absence basis will be taken as an indication of the suitability of such a marker as a viability determinant.

For the purpose of this study, live or viable cells are those considered initially capable of cell division and/or growth (as determined firstly by colony forming ability and later by direct viable count) and those capable of constitutive gene transcription (mRNA production).
3.1 BACTERIAL STRAINS.

Table 3.1 shows the bacterial strains used in this study and their source.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter jejuni</em> KLC 4315</td>
<td>Penner HS:19 Serovar (faeces, human enteritis)</td>
<td>ESR/KSC, Porirua, N.Z.</td>
</tr>
<tr>
<td>KLC 4132</td>
<td>NZRM 1958 [NCTC 11168] (faeces, human enteritis)</td>
<td>ESR/KSC, Porirua, N.Z.</td>
</tr>
</tbody>
</table>

*Campylobacter jejuni* KLC 4315 [(Heat-stable) HS:19] was the predominant test strain used for this research. *C. jejuni* NZRM 1958 was used as a reference strain. The recent availability of the published genomic sequence of this *Campylobacter* strain NCTC 11168 allowed for primer design and sequence analysis (Sanger, *et al.*, 1998).

3.2 MEDIA.

Solutions and media used in this study were prepared as described in Appendix I.

For culturability assays and daily manipulations, strains were cultured on solid agar media, which included *Campylobacter* Charcoal Differential (deoxycholate) agar (CCDA) (Oxoid CM739) with the addition of cefoperazone or Nutrient broth #2 (NB#2A) Media (Oxoid CM67) containing bacteriological agar (2%) (Gibco BRL).

For induction of the coccoid form of *Campylobacter* and viability assays, cells were cultured in undiluted NB#2 (25g.L⁻¹), supplemented with polymyxin B sulphate and rifampicin (Table 3.2).
3.2.1 ANTIBIOTIC SUPPLEMENTS.

Table 3.2: Antibiotic supplements for solid and liquid media.

<table>
<thead>
<tr>
<th>Antibiotic/Supplement</th>
<th>Abbreviation</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium cefoperazone</td>
<td>Cef</td>
<td>32mg.L(^{-1})</td>
</tr>
<tr>
<td>Polymyxin B sulphate</td>
<td>PmB</td>
<td>5000 iU.L(^{-1})</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Rif</td>
<td>10mg.L(^{-1})</td>
</tr>
</tbody>
</table>

3.2.2 STERILISATION TECHNIQUES.

All experimental work was performed in an Email Air Handling Biological Safety Cabinet Class II with decontamination by microbiocidal UV light and surface sterilisation with ethanol prior to experimentation.

3.2.2.1 AUTOCLAVING.

Heat sterilisation of all media, glassware, and equipment by autoclaving was carried out at 121\(^\circ\)C, at a pressure of 103.4 kPa for 20 min.

3.2.2.2 FILTER STERILISATION.

Antibiotics and reagents that cannot withstand autoclaving were filter sterilised using sterile 0.22\(\mu\)m cellulose acetate membrane filters (Millipore Type GS).

3.2.2.3 IMPLEMENTS.

All implements (spatulas, glass rods, forceps) used were either autoclaved or surface sterilised with 70% ethanol between samples to prevent cross contamination.
3.3 BACTERIOLOGICAL METHODS.

3.3.1 ROUTINE CULTURE MAINTENANCE.

Strains used for daily manipulations were maintained on solid culture medium supplemented with antibiotics as required and stored at 4°C for up to several weeks. Cultures streaked onto solid media were grown at 37°C in a water-jacketed CO₂ incubator (Nuaire™) in a microaerophilic OCN environment (5% Oxygen, 10% Carbon Dioxide, 85% Nitrogen). All cultures maintained on solid media were Gram-stained periodically to assess culture purity.

3.3.2 LONG TERM STORAGE.

Long term storage of selected strains was achieved by harvesting bacterial growth from CCDA plates using Brain Heart Infusion (BHI) broth (Merck), containing sterile glycerol as a cryoprotectant (BDA) (final concentration of 20%), with a sterile glass rod. Aliquots (1.5 ml) of cell suspensions were aseptically transferred into sterile 2ml Nunc cryo-tubes containing six (2.5 to 3.5 mm diameter) glass beads (BDA). Cryotubes were stored at -80°C until required. To recover Campylobacter from -80°C, bacterial suspensions were thawed and a single glass bead rotated on the surface of a CCDA plate. Plates were subsequently incubated at 37°C for 48 h.

3.3.3 HARVEST OF Campylobacter CELLS.

High concentrations of Campylobacter cells (≥ 10⁸ CFU.ml⁻¹) were obtained by streaking Campylobacter onto CCDA followed by 48 h incubation in microaerobic conditions at 37°C. CCDA plates were scraped using 5ml physiological saline (phosphate buffered saline (PBS) - see Appendix I) and a sterile glass spreader. The cell suspension was centrifuged (3 700 x g) (Eppendorf 5403 Centrifuge) at 4°C for 7 min in a 30ml Oak Ridge tube. Supernatant was discarded, taking care not to excessively aerate the sample and the pellets were gently washed in 5ml of PBS. After centrifugation, the pellets were resuspended in 3ml of PBS. The cell recovery after resuspension (for one plate of growth) was between 10⁷ and 10⁹ CFU. ml⁻¹.
3.3.4 INDUCTION OF THE 'VNC' STATE.

The effects of aging, temperature, aerobiosis, and starvation on the viability and morphology of *C. jejuni* were assessed. The morphologic transition rate of the bacterium from rod to coccoid was determined by light and phase contrast microscopy and the loss of culturability was monitored by aerobic plate (culturable) counts. The experimental protocols followed are described later in this chapter (Sections 3.3.6 and 3.3.8).

3.3.4.1 TEMPERATURE-INDUCED NONCULTURABILITY.

Initial attempts to induce the nonculturable state of *C. jejuni* KLC4315 centred on experimental evidence that the formation of the coccoid form occurred fastest at or around the organism's optimum growth temperature (Rollins and Colwell, 1986). 150ml of NB#2 was dispensed (in triplicate) into labelled sterile Erlenmeyer flasks under aseptic conditions. Total growth was scraped from five CCDA plates (as described in Section 3.3.3) and transferred into each liquid culture. Cultures were maintained at 37°C in an oxygen-limited atmosphere. At regular intervals the cultures were assessed for loss of culturability. Conversion to a predominantly coccoid population and subsequent loss of culturability was found to exceed three weeks under these conditions - a temporal progression which was deemed too long for this research.

3.3.4.2 ANTIBIOTIC-INDUCED NONCULTURABILITY.

Three antibiotics were trialed in an effort to reduce the length of time required for a population to reach a state of nonculturability (Table 3.3).
Table 3.3: Antibiotic formulations added to liquid culture of *C. jejuni* to achieve nonculturability.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymyxin B sulphate</td>
<td>5000i.U.L(^{-1})</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>10mg.L(^{-1})</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>10mg.L(^{-1})</td>
</tr>
<tr>
<td>Polymyxin / Rifampicin</td>
<td>5000i.U.L(^{-1}) / 10mg.L(^{-1})</td>
</tr>
<tr>
<td>Polymyxin / Rifampicin / Trimethoprim</td>
<td>5000i.U.L(^{-1}) / 10mg.L(^{-1}) / 10mg.L(^{-1})</td>
</tr>
</tbody>
</table>

Antibiotics and cell inoculum were added to 150ml NB#2 and the cultures incubated at 37°C for extended periods of time. With prolonged culture, loss of culturability was observed in just over two weeks in the sample with polymyxin B and rifampicin supplementation. The time course, however, was insufficient for efficient and rapid assessment of gene expression over the cell cycle.

3.3.4.3 AERATION AND TEMPERATURE - INDUCED NONCULTURABILITY.

The antibiotic 'cocktail' of rifampicin and polymyxin B was tested in conjunction with varied temperature and oxygenation parameters. After the addition of these antibiotics to 150ml NB#2, the total growth collected from five CCDA plates of the test strain was transferred to the nutrient broth. Cotton bungs and foil caps were replaced to maintain aseptic conditions and to prevent evaporation after the removal of samples. Broth cultures were incubated in a dark, aerobic environment at 25°C for an extended time. Loss of culturability was achieved within a week. This method of inducing nonculturability was employed for all further experimentation and all enumeration and RNA work was achieved on a culture established by this technique. Bacterial samples were taken aseptically at regular intervals from the broth to assess culturability, viability, morphology, total bacterial numbers and for RNA extraction.
3.3.5 BACTERIAL GROWTH MONITORING.

3.3.5.1 TURBIDOMETRIC ANALYSIS.

Growth was monitored by turbidometric measurements made at regular intervals at a wavelength of 600nm (λ₆₀₀ / OD₆₀₀) using an Hitachi U-2000 spectrophotometer. Triplicate 1ml aliquots of the liquid broth cultures (set up by the method in Section 3.3.4.3) were blanked against uninoculated NB#2, the figures arithmetically averaged and the data graphed.

3.3.5.2 MOST PROBABLE NUMBER ANALYSIS (MPN).

In the five-tube MPN procedure (Atlas, 1988), five replicate tubes were used for each dilution level (10⁰, 10⁻¹, 10⁻²). The first five replicate tubes were inoculated with an undiluted 5ml aliquot of the Campylobacter broth culture. Two ten-fold serial dilutions were made with five replicate tubes at each dilution. The diluent was freshly prepared NB#2. Positive controls with identical media inoculated with dilutions of the cultured cells and three negative controls without inoculum were included at every sampling time. Tubes were incubated at 37°C for 48 h and then checked for changes in turbidity. The pattern of positive and negative results were used to estimate the concentration of bacteria in the original sample by comparison of the observed results with a reference MPN table of statistical probabilities for obtaining those results (refer to Atlas (1988)).

3.3.6 CULTURABLE COUNTS.

3.3.6.1 QUANTIFICATION OF CULTURABLE COUNTS.

100μl aliquots were removed immediately following inoculation of the broth with the Campylobacter test strain, serially diluted in PBS and spread plated (in triplicate) onto non-selective media (NB#2) or selective media (CCDA containing cefoperazone). Plates were incubated at 37°C under micro-aerobic conditions for two days. Subsequent samples were withdrawn and processed in the same manner as the initial sample at daily intervals or whenever aliquots for RNA extraction were removed. This provided a direct measure into the level of culturability of the bacterial population at the time intervals tested.
3.3.6.2 PLATE COUNTS.

*Campylobacter* colonies are typically flat, glossy and effuse, pinkish-grey colonies, with a tendency to spread along the direction of the tracks of the inoculating wire (Butzler and Skirrow, 1973). Colonies with this typical *Campylobacter* species appearance were counted and recorded as culturable counts. Values from each triplicate set were arithmetically averaged. Representative colonies were confirmed as *C. jejuni* by Gram-stain.

3.3.6.3 FILTRATION AND PLATE COUNTS.

Following the procedure of Jones, *et al.* (1991), when plate counts fell below the level of detection (10 cells.ml\(^{-1}\)), 10 to 30ml aliquots of the undiluted broth sample were filtered through membrane filters (0.2\(\mu\)m pore size, 25mm diameter; Millipore), which were then placed on solid media with the cells facing upwards. Colonies were counted following incubation as described above. This reduced the lower detection limit to 0.03 to 0.1 CFU.ml\(^{-1}\). When less than 0.1 cell per ml was culturable, the culture was considered to be in the nonculturable state.

3.3.7 VIABLE COUNTS.

3.3.7.1 TOTAL DIRECT COUNTS.

Total direct counts were made by haemocytometric measurements under the light microscope (Olympus BH2) by the method of Atlas (1988). A drop of inoculated broth was placed on the haemocytometer and a coverslip mounted on top. The counting chamber employed had a total area of 1/400 mm\(^2\) with a depth of 0.1 mm. The slide was viewed and the average number of cells determined in an area delimited by the grid.

3.3.7.2 ACRIDINE ORANGE DIRECT COUNTS (AODC).

At regular intervals along the cell cycle, 5 to 10ml broth samples were withdrawn and processed by the direct viable count method described by Hobbie, *et al.* (1977) and
Kogure, *et al.* (1979), with minor modifications (Singh, *et al.*, 1990). This technique was employed to measure total bacterial cell numbers. Withdrawn samples were heated for 5 min in a 100°C water bath, and cooled rapidly by immersion in ice-cold water. Heat-treated samples were passed through a filter (pore size, 0.2μm; diameter, 25mm; Millipore), which had been stained overnight in 0.2 % Nigrosin. The membrane were moistened with filter-sterilized water, and a low filtration pressure applied to ensure uniform spreading of the bacteria on the filter. Retained bacteria were washed with 5ml water and immediately fixed with Formalin (final concentration 4%), allowing 1 to 2 min contact. Fixed bacteria were washed with 5ml water and stained with 3 to 5ml of 0.02% Acridine Orange solution (allowing 2 to 3 min contact). 5ml of wash water was finally filtered through to remove unbound stain. The wash water, formalin solution and staining solution were all filtered through 0.2μm-pore-size membranes before use. Each stained membrane was air dried and placed on a drop of immersion oil on a glass slide. Another drop of oil was then placed on the filter, and a cover slip firmly pressed over it. The slides were examined by laser scanning confocal microscopy (Biorad/Olympus).

### 3.3.7.3 ACRIDINE ORANGE DIRECT VIABLE COUNTS (AODVC).

The acridine orange direct viable count method was utilised to quantify levels of viable bacterial cells. For AODVC, 150ml broth samples were enriched with 0.025% (w/v) yeast extract and 0.002% (w/v) nalidixic acid (final concentration 10μg.ml⁻¹) and maintained in darkness at 25°C. After a 4 h incubation, the DVC of the culture was determined by the epifluorescent technique, described in Section 3.3.7.2. Only those particles that were elongated or fattened to the size and shape of freshly cultured cells, and that fluoresced reddish orange, were counted. Counting were performed within 5 min after preparation. Before determination of DVC, the corresponding total direct count of the sample was observed.
3.3.8 MICROSCOPY.

3.3.8.1 LIGHT MICROSCOPY.

Conventional light microscopy was used to visualise both culturable and non-culturable forms of C. jejuni KLC 4315. A sample for examination consisted of a loopful of bacterial growth from an agar culture or 10μl of bacterial suspension. Heat fixed smears of samples were prepared in duplicate. Cells were stained with carboyl fuchsin for 5 min or by the Gram staining method. Bacterial morphology was studied with an Olympus BH2 standard microscope at a magnification of x1000 under oil immersion. Microscopic determination of the ratio of bacillary to coccoid forms of cells over an extended time period was conducted using the Olympus BH2 microscope integrated with an Image Analysis Metamorph Computer Package: Image-1/Metamorph, version 2.75. The percentage of coccoid forms was estimated in ten fields of vision per smear; on average 600 bacteria were counted. The mean of the coccoid estimations for each sample was recorded.

3.3.8.2 PHASE CONTRAST MICROSCOPY.

Wet-mount preparations of C. jejuni KLC 4315 were examined under phase-contrast microscopy for typical Campylobacter motility.

3.3.8.3 TRANSMISSION ELECTRON MICROSCOPY.

The use of a transmission electron microscope (TEM) permitted the cellular morphology of C. jejuni KLC 4315 cells to be more closely examined over time.

Cells were negatively stained with phosphotungstic acid and examined on a transmission electron microscope (JEOL). Micrographs produced from these images failed to develop sufficiently to draw conclusions - yet observations were made from the screen print-out.
3.4 DNA MANIPULATION.

3.4.1 PREPARATION OF TOTAL GENOMIC DNA.
The quick preparation method of Pospeich and Neumann (1995) was used for the extraction of *C. jejuni* chromosomal DNA. Briefly, a 48 h streak culture of *C. jejuni* was scraped from CCDA medium using 5ml ddH₂O and a sterile glass rod. A 1.5ml aliquot was dispensed into an Eppendorf centrifuge tube. Cells were harvested by centrifugation (Eppendorf 5403 Centrifuge) at 4°C for 3 min at 6 500 x g. Supernatant was removed and the cell pellet resuspended in 250μl SET buffer (75mM NaCl, 25mM EDTA, 20mM Tris-Cl; pH 7.5) (Appendix I) with the addition of lysozyme to a concentration of 1mg.ml⁻¹. Cell lysates were incubated at 37°C for 45 min. After the addition of 27μl of 10% SDS and 15μl Proteinase K (1mg.ml⁻¹), whole cell lysates were incubated for 2 h at 55°C with periodic inversion. One-third volume of 5M NaCl (Appendix I) and an equal volume of chloroform was added and the tube inverted several times to mix before incubation at ambient temperature for 30 min. Suspensions were centrifuged at 4°C (14 800 x g; 15 min) and the upper aqueous phase carefully transferred to a new sterile Eppendorf tube for deproteinisation with equal volumes of phenol/chloroform. After inversion of the tube and subsequent centrifugation for 1 min the aqueous phase was transferred to a fresh 1.5ml Eppendorf. Nucleic acids were extracted with two additional phenol/ chloroform extractions as described above. To remove traces of phenol, the resultant supernatant containing DNA (and trace amounts of RNA) was extracted with one volume of chloroform, inverted to emulsify and centrifuged (7 500 x g; 5 min) at 4°C before supernatant transferral to a sterile Eppendorf.

DNA was precipitated with two volumes of cold 100% (v/v) ethanol and one-tenth volume of 3M Sodium acetate (Appendix I). After gentle inversion, the DNA was collected by centrifugation at 4°C for 30 sec at 17 400 x g. Supernatant was discarded and the DNA was washed with 1ml cold 70% (v/v) ethanol. Ethanol was decanted away and the DNA dried at ambient temperature by inverting the 1.5ml Eppendorf. Genomic DNA was resuspended in 50μl TE buffer (10μM Tris-Cl, 1μM EDTA; pH 8.0) (Appendix I) and stored at -80°C until required.
3.4.2 ESTIMATION OF GENOMIC DNA.

Quantitation and purity of DNA was determined by absorption spectroscopy. A 1ml blank of 8mM NaOH was initially used to zero the spectrophotometer (Hitachi U-2000) before determining the absorbance at 260nm ($A_{260}$) of the sample. An $A_{260}$ of 1 indicates 50μg.ml$^{-1}$ of double-stranded DNA (Sambrook, et al., 1989).

The ratio of $A_{260}/A_{280}$ estimates the purity of the nucleic acid sample. Ratios of 1.8 to 1.9 indicate highly purified preparations of DNA. Contaminants that absorb at 280nm, including protein and RNA, will lower this ratio (Sambrook, et al., 1989).

3.4.3 GEL ELECTROPHORESIS OF DNA.

3.4.3.1 AGAROSE GEL ELECTROPHORESIS.

For agarose gel electrophoresis, *ultra* Pure agarose (Gibco BRL) or SeaKem LE agarose (FMC Bioproducts) dissolved in 1x TAE buffer (Appendix I) was used. This method was employed to determine the integrity of native DNA and for the visualisation of DNA and cDNA products after PCR amplification. The agarose content varied between 0.8% for large amplicon fragments (greater than 500bp) to 3% for small (less than 500bp) PCR products. 10μl of DNA was mixed with 2μl of 6x Bromophenol blue gel dye (Appendix I) or 5x nucleic acid sample loading buffer (Biorad) for loading into the agarose gel. A molecular weight marker (Biorad EZ Load™ 100bp molecular ruler) was used as a standard to determine amplicon sizes.

DNA fragments were separated by exposure to an electrical current of 50-100 volts/cm$^2$ for 1 to 2.5 h in a Biorad Mini-Sub™ or DNA sub cell apparatus (Jordan Scientific Co.). Ethidium bromide (0.5μg. ml$^{-1}$) (Appendix I) in 1x TAE was used to stain DNA located in the agarose gel (10 to 20 min). DNA bands were visualised using a Sigma T2210 UV transilluminator (302nm) and gels were photographed using Polaroid P/N type 667 film.
3.4.3.2 POLYACRYLAMIDE GEL ELECTROPHORESIS.

Non-denaturing polyacrylamide gel electrophoresis was employed for the separation of fragments of ds DNA (Sambrook, et al., 1989). Prior to the electrophoresis, solutions of 30% Acrylamide, 1x TBE buffer, and 10% Ammonium persulphate were prepared (Appendix I). The glass plates and spacers were cleaned with warm detergent water (Pyroneg) and rinsed well, first in tap water and then in double-distilled water. Plates were sprayed with 95% ethanol and set aside to dry. To arrange the electrophoresis apparatus, spacers were fixed between the glass plates with petroleum jelly and the edges sealed with agarose. To cast the polyacrylamide gel, a 5% acrylamide solution was required. This consisted of 16.6ml of 30% Acrylamide, 62.7ml water (ddH2O), 20ml 5x TBE, 0.7ml 10% Ammonium persulphate, and 35μl TEMED (N, N', N'-tetramethylethylene-diamine) (Life Technologies). The mixture was swirled and drawn into a 50-ml syringe. The syringe was inverted to expel any air before introduction into the space between the two glass plates. The space was filled almost to the top, the comb inserted immediately and clamped in place with a bulldog paper clip. Polymerization of the acrylamide took an hour at ambient temperature. To set up for electrophoresis, the comb was removed, the wells immediately rinsed with water to prevent polymerization of any remaining acrylamide in the well, and the gel transferred to the tank unit. The reservoirs of the tank were filled with 1x TBE and the wells flushed out with the same buffer. 5μl of DNA was mixed with 1μl of 6x Bromophenol blue gel dye (Appendix I) and the mixture loaded into the well. The fragments were exposed to a voltage gradient of 8V.cm⁻¹. For detection of DNA post-electrophoresis, the gel was stained in ethidium bromide for 30 min and viewed under UV transillumination.
3.5 RNA MANIPULATION.

3.5.1 MAINTENANCE OF RNase-FREE CONDITIONS.

To obtain good preparations of prokaryotic mRNA, it is necessary to minimize the activity of RNases liberated during cell lysis. To create and maintain an RNase-free environment, the following precautions were taken during pretreatment (Sambrook, et al., 1989).

3.5.1.1 GENERAL HANDLING.

Aseptic techniques were employed when working with RNA. Prior to RNA isolation, the working area was UV sterilised for 30 min to 1 h. The inside of the biological safety cabinet and all instruments and bottles of solutions entering this environment were then surface sterilised with 95% ethanol.

As dust particles and the hands of the investigator are potentially major sources of RNase contamination, powder-free gloves were worn and changed frequently and tubes were kept closed during the preparation of materials and solutions used for the isolation and analysis of RNA and during manipulations involving RNA.

3.5.1.2 DISPOSABLE PLASTICWARE.

Sterile, disposable polypropylene tubes were used throughout experimentation as they were essentially RNase-free. Certified aeroshield filter pipette tips (Robbins Scientific and Axygen™ Scientific) were also employed to guard against contaminating aerosols.

3.5.1.3 NON-DISPOSABLE PLASTICWARE.

Non-disposable plasticware was treated before use to ensure it was RNase-free by rinsing in 0.1M NaOH and 1mM EDTA, followed by a rinse in RNase-free H₂O (0.1% (v/v) DMPC-H₂O) (as prepared in 3.5.1.5).

3.5.1.4 GLASSWARE.

General laboratory glassware is often contaminated with RNases therefore any glassware used for RNA work was cleaned with detergent, thoroughly rinsed in DMPC-treated water, and dry heat sterilised by ovenbaking at > 240°C overnight.
3.5.1.5 SOLUTIONS.

Solutions (including water) were treated with 0.1% (v/v) dimethyl pyrocarbonate (DMPC) (Sigma), a strong but not absolute inhibitor of RNases (Fedorcsak and Ehrenberg, 1966; cited from Sambrook, et al., 1989) (Appendix I). DMPC was used instead of another RNase inhibitor - DEPC (diethyl pyrocarbonate); due to its better availability and cost. After overnight treatment with DMPC, solutions were autoclaved to remove traces of the chemical which might otherwise modify purine residues in the RNA by carboxymethylation. As DMPC also reacts with amines, it could not be used directly to treat Tris buffers. When preparing Tris buffers, water was first treated with DMPC, autoclaved and this water was used to dissolve Tris as required.

3.5.1.6 ELECTROPHORESIS TANKS.

The electrophoresis tank was cleaned with detergent solution (Pyroneg), rinsed in water, dried with ethanol and filled with a solution of 3% H₂O₂. After 10 min at ambient temperature, the tank was rinsed thoroughly with RNase-free water.

3.5.2 EXTRACTION OF RNA.

Several methods of extracting RNA were tested. These included hot- (Barry, et al., 1992) and cold-phenol (TRIzol) (Savill, 1997) extractions. The most reproducible results were obtained using the Qiagen RNeasy Mini Kit protocol for isolation of total RNA from bacteria. This protocol also gave the greatest yield (w/v) of total RNA from the same starting volume.

Total RNA was extracted from cells at measured points along the continuum of cell cycle - from the exponential, stationary, viable but nonculturable, to metabolic death phases. The experimental procedure followed is described below. All steps of the RNeasy protocol were performed at ambient temperature although centrifugation was at 4°C. The fore-named kit contained four reagents - three buffers (RLT, RWI and RPE) and RNase-free water.
Briefly, a 3ml aliquot (Section 3.3.4.3), containing approximately $10^8$ cells, was withdrawn and centrifuged at $5000 \times g$ for 5 min at 4°C. Supernatant was discarded and the harvested cells were incubated in 100μl TE buffer (10mM Tris-Cl, 1mM EDTA; pH 8.0) containing lysozyme at a concentration of 400μg.ml$^{-1}$. A 5 min incubation period at ambient temperature was sufficient for complete digestion of the bacterial cell wall prior to lysis. Lysis was initiated with 350μl of guanidinium isothiocyanate (GITC)-containing lysis buffer (Buffer RLT) and 14.5M β-mercaptoethanol (β-ME) (at a concentration of 10μl.ml$^{-1}$) with vortexing to mix. These two compounds are known inactivators of RNases - GITC has a denaturing role and β-ME breaks the intramolecular disulphide bridges which impart stability to the RNase structure (Sela, et al., 1957; cited from Sambrook, et al., 1989). After addition of 250μl of 100% (v/v) ethanol, the lysate sample (approximately 700μl) was loaded onto an RNeasy mini spin column. Centrifugation for 15 sec at 8 000 x g resulted in total RNA adsorption to the spin column membrane. To remove contaminants, three wash steps were performed with the buffers supplied with the kit - firstly 700μl Buffer RWI and then two subsequent 500μl Buffer RPE washes. After the final wash, the RNeasy column was centrifuged at 8 000 x g for 2 min to dry the membrane and remove residual ethanol which may interfere with subsequent reactions. The final step was to pipette 50μl of RNase-free water directly onto the membrane and centrifuge for 1 min at 8 000 x g thus ready to use RNA was eluted in water. The eluted RNA sample was immediately frozen at -80°C until required.

The bacterial RNA isolation protocol by Qiagen resulted in high yields of RNA (between 10 to 15μg), yet there was potential for DNA contamination. To ensure a sample was completely free of contaminating DNA, a number of control digests were performed on each extracted RNA sample prior to the PCR. Eluted RNA samples were subjected to DNase I, RNase A or DNase I/RNase A digests (as described below) and 1μl volumes of each of these were analysed separately by both PCR and RT-PCR.
3.5.3 DNase DIGESTION OF EXTRACTED RNA.

The method of DNase digestion to remove contaminating DNA from the eluted RNA was adapted from Savill (1997) as described below.

A DNase digestion mixture, consisting of 10μl RNA in RNase-free H₂O, 4.4μl DMPC-H₂O, 2μl 10x PCR Buffer (Boehringer Manheiem), 1.6μl MgCl₂ (25mM) (Boehringer Manneheim) and 1μl DNase I (32 U.μl⁻¹) (Life Technologies), was incubated at ambient temperature for 15 min. To terminate the reaction 0.5μl of 50mM EDTA was added.

After the addition of 500μl isopropanol (IPA), the DNA digest was extracted by freezing for 1 h at -80°C before centrifugation at 4°C (17 400 x g; 10 min). The DNA was washed with 500μl 70% (v/v) ethanol, centrifuged and the pellet air-dried before resuspension in 10μl DMPC-H₂O. Storage was at -80°C.

3.5.4 RNase DIGESTION OF EXTRACTED RNA.

To ensure the DNase I digest had gone to completion, thus eliminating any chance of DNA going through into the PCR, a RNase A digestion was performed. The method of Savill (1997) was employed. 10μl of undigested or DNase-digested RNA was added to the RNase digest mixture consisting of 7.6μl 0.1% (v/v) DMPC-H₂O, 2μl 10x PCR Buffer (Boehringer Maheiem) and 0.4μl RNase A (concentration 10mg.ml⁻¹) (Sigma).

Digests were incubated at 37°C for 30 min before the addition of 0.5μl of 50mM EDTA and extraction with IPA as for the DNase digest. The digested product was then stored at -80°C.
3.5.5 RNA QUANTIFICATION AND PURITY.

The concentration and purity of RNA was determined by measuring the absorbance (with the UV lamp on) at 260nm ($A_{260}$) and 280nm ($A_{280}$). Cuvettes were washed with 0.1M NaOH, 1mM EDTA and RNase-free H$_2$O to ensure they were free of RNase activity. DMPC-H$_2$O was used as a blank control. 10µl of the RNA sample was diluted in 990µl DMPC-H$_2$O and the $A_{260}$ measured. When measured in H$_2$O an $A_{260}$ value of 1 is equal to 40µg.ml$^{-1}$ of RNA. The ratio of $A_{260}$ to $A_{280}$ in 10mM Tris chloride (pH 7.5) provided an estimate of the purity of the RNA. Pure RNA has an $A_{260}/A_{280}$ of 1.8-2.1 in this buffer (Qiagen RNeasy Mini Kit Information booklet).

3.5.6 ELECTROPHORESIS OF RNA.

The integrity of the RNA (10µl samples) was examined by horizontal gel electrophoresis (12V.cm$^{-1}$) in 1% agarose (TBE buffer) (Appendix 1). The gels were stained in ethidium bromide and visualised by UV transillumination.

3.6 REVERSE TRANSCRIPTION.

3.6.1 ONE-STEP RT-PCR WITH THE TITAN™ SYSTEM.

Reverse transcription of RNA to cDNA was initially attempted using the Titan™ One-tube RT-PCR system. Although the one-tube system minimized hands on contamination, the Titan method lacked the ability to incorporate random priming into the RT reaction. Reverse transcription was therefore performed according to a second technique.
3.6.2 TWO-STEP RT-PCR WITH SUPERSCRIPT II AND Taq DNA POLYMERASE.

Two step RT-PCR was performed with the Life Technologies kit, as per the manufacturer's instructions. First strand cDNA synthesis was conducted by adding 5μl DMPC-H₂O, 5μl digested RNA in RNase-free H₂O (5μg total RNA) and 1μl random primers (30ng.μl⁻¹) (Life Technologies) to a nuclease-free microcentrifuge tube. The pre-incubation mixture was heated to 70°C for 10 min, cooled on ice and briefly centrifuged before 4μl First Strand Buffer, 2μl 0.1M Dithiothreitol (DTT) and 1μl 10mM dNTP mix was added. The sample was subjected to an incubation step of 25°C for 10 min and 2 min at 42°C before 1μl (50U) Superscript™ RNase H⁻ Reverse Transcriptase was added and the RT reaction continued at 42°C for a further 50 min. The reaction was inactivated by heating to 70°C for 15 min thus producing cDNA which could be used as a template for amplification in PCR. The random primed cDNA was stored at -80°C until required for PCR amplification with Taq DNA polymerase.

3.7 POLYMERASE CHAIN REACTION (PCR).

Amplification of specific targets (genes) using genomic DNA and cDNA was performed in a 50μl reaction mixture in 0.5ml thin-walled PCR tubes in an automatic thermal cycler (Corbett Research FTS-320 Thermal Sequencer). Taq polymerase, buffers and dNTPs were purchased from Gibco-BRL and primers were purchased from Amrad-Pharmacia Biotech (lpxA / fabZ ;23S rDNA Therms I and II) and from Life Technologies (dnaJ; hupB; ciaB) (Table 3.4).

3.7.1 OLIGONUCLEOTIDE PRIMER DESIGN.

The selection of the primers was based on computer alignments (Blast sequences from Genebank) of the C. jejuni gene sequences. Oligonucleotide primers specific for Campylobacter were available for the lpxA gene (Ibbitt, 1997), the hupB and ciaB genes (Konkel, et al., 1999), and for the 23S rDNA genes (Eyers, et al., 1993). A pair of primers was designed using the computer programme Prophet, based on the published sequence of a heat shock protein (dnaJ) (Konkel, et al., 1998).
Table 3.4: PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Tm</th>
<th>Primer Nucleotide Sequence</th>
<th>Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-25 (F)</td>
<td>64.8</td>
<td>GTGCCTGAGATAGGC</td>
<td>fabZ llpxA</td>
</tr>
<tr>
<td>98-04 (R)</td>
<td>53.8</td>
<td>TACAGGATGATGACC</td>
<td>lpxA</td>
</tr>
<tr>
<td>97-05 (F)</td>
<td>52.7</td>
<td>TATCCAATACCAACATTAG</td>
<td>23S rDNA(^b)</td>
</tr>
<tr>
<td>97-06 (R)</td>
<td>61.5</td>
<td>CGGTACGGGCAACATTAG</td>
<td>23S rDNA(^c)</td>
</tr>
<tr>
<td>98-22 (F)</td>
<td>68</td>
<td>TGGAACAGGGCTAAAGATG</td>
<td>dnaJ</td>
</tr>
<tr>
<td>98-23 (R)</td>
<td>68</td>
<td>CCTGACAATCAGGCAAGT</td>
<td>dnaJ</td>
</tr>
<tr>
<td>99-04 (F)</td>
<td>63</td>
<td>CAAATTTAGATGATGCAATGG</td>
<td>ciaB</td>
</tr>
<tr>
<td>99-05 (R)</td>
<td>62</td>
<td>AATTCACAATCTCAGGCAATGG</td>
<td>ciaB</td>
</tr>
<tr>
<td>99-06 (F)</td>
<td>60</td>
<td>GATTTCCATTTCCATTAGTGG</td>
<td>hupB</td>
</tr>
<tr>
<td>99-07 (R)</td>
<td>62</td>
<td>TTTGATTTTCTTCTGTGC</td>
<td>hupB</td>
</tr>
</tbody>
</table>

a = Primers bind and are transcribed in either the forward (F) or reverse (R) direction.  
b = Therm I (Eyers et al, 1993).  
c = Therm II (Eyers et al, 1993).

3.7.2 PCR AMPLIFICATION OF DNA TARGETS.

Prior to PCR set up, stock primers (300pmol.\(\mu l^{-1}\)) were diluted 1:30 in DMPC-treated water to give a final concentration of 10pmol.\(\mu l^{-1}\). dNTPs were diluted in RNase-free water to give a final concentration of 10mM each (ddA, ddC, ddG, ddT) (Appendix I) - the dNTP mixture was diluted 1:5 to give a concentration of 2mM per reaction.

For the PCR mix, 1\(\mu l\) of template DNA (approximately 1ng.ml\(^{-1}\)) was added to 4\(\mu l\) of the dNTP mix (final concentration 2mM) and 0.5\(\mu l\) of each 10pmol primer. To this was added 5\(\mu l\) of 10x PCR buffer (with 15mM Mg\(^{2+}\)), 3\(\mu l\) of 10mM MgCl\(_2\) (final Mg concentration 3mM) and DMPC-treated H\(_2\)O up to a volume of 49.75\(\mu l\). 1.25U of Taq DNA polymerase was added to each tube bringing the volume to 50\(\mu l\). The mixture was pulsed briefly in a microfuge at 17 400 \(x\) g to collect all components of the reaction and then overlaid with 30\(\mu l\) of sterile paraffin oil.

PCR amplification was initiated with a three stage programme of 30 repeated cycles. Each amplification cycle consisted of a denaturation step (94°C for 1 min), an annealing step (50°C or 55°C for 1 min) and an extension of PCR product for 1 min at 72°C. A
The final cycle, in which the denaturing/annealing conditions were the same, used an extension time of 5 min to ensure that all PCR products initiated were able to be completed. The programme concluded with a soak step that incubated the PCR products at 4°C before removal from under the paraffin oil and storage at 4°C or -20°C. 10μl from each PCR reaction was loaded into wells in a 2% agarose gel, subjected to an electrical field (50-100 V/cm²) and stained with ethidium bromide for visualisation under UV to observe PCR amplicon sizes.

3.7.3 PURIFICATION OF PCR FRAGMENTS.
PCR fragments were purified using the QIAquick spin column (Qiagen). This resulted in 50μl of DNA with a concentration of approximately 50ng.μl⁻¹.

3.7.4 SEQUENCING OF PCR PRODUCTS.
Purified PCR products were sent to the Waikato DNA Sequencing Facility. Samples were quantified, and the nucleotide sequences obtained using an ABI Prism 377 DNA Sequencer (Perkin Elmer). Results were received via e-mail and also as an electropherogram printout. Sequencing acted as a check to ensure that the primer sets used were indeed amplifying the gene of interest.

3.8 CELLULAR DEATH.
*C. jejuni* cells (10⁸ CFU.ml⁻¹) were cultured in 150ml NB#2 (25gL⁻¹) with antibiotics (polymyxin B / rifampicin) for two days at 25°C before subjecting the cells to a 20 min heat treatment at 65°C. 4ml aliquots were withdrawn at the following time intervals: 0, 1, 3, 5, 10, 15, 20 min (at which time the cells were removed from the heat and returned to ambient temperature), 1 h, 3, 5, 24, 48, 96 and 216 h. From any aliquot removed, 100μl was plated on CCDA plates to determine cellular viability. The remaining cells were frozen at -80°C for RNA extraction. mRNA and rRNA was extracted and cDNA produced and visualised as described above (Section 3.5-3.7).
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3.9 EXPERIMENTAL CONTROLS.

3.9.1 RNA EXTRACTION CONTROLS.

A positive RNA extraction control consisted of RNA isolated, by the above method, from viable, freshly harvested Campylobacter jejuni (48 h CCDA plate cultures). Light microscopic counting (haemocytometry) revealed the population was predominantly spiral at 48 h. A 48 h CCDA plate culture was scraped using a sterile glass spreader and 3ml PBS. A 1.5ml aliquot was centrifuged (4°C; 5 000 x g; 5 min) and the protocol continued as in Section 3.5.2.

A negative RNA extraction control was performed by autoclaving (121°C; 103.4 kPa; 20 min) a one-day-old broth culture of C. jejuni (as established in Section 3.3.4.3). Total RNA was extracted from a 3ml aliquot of the heat-killed culture immediately after the autoclave treatment.

3.9.2 PCR CONTROLS.

For each RNA sample analysed, two PCR controls were employed:

- A negative PCR control (water blank) to ensure decontamination of PCR reagents
- A positive PCR control (DNA) to ensure the PCR amplification was functioning.

3.9.3 SAMPLE CONTROLS.

To ensure the DNase digestion had gone to completion and that no contaminating DNA was remaining in the RNA sample, RNase and RNase/DNase digestions were performed on each RNA sample (as described in Sections 3.5.3-3.5.4).

The use of numerous controls has permitted the following results and conclusions to be made.
CHAPTER IV
RESULTS

4.1 PRELIMINARY EXPERIMENTS - VNC INDUCTION.

Preliminary experimentation centred on induction of the VNC state of *Campylobacter jejuni* KLC 4315. Due to the temporal constraints of a Masterate project, induction had to occur in a short time frame (i.e., approximately one week). Prior research has indicated an ability of *C. jejuni* to maintain itself in liquid culture (Rollins and Colwell, 1986; Griffiths, 1993; Hazeleger, *et al.*, 1995). Incubation in liquid media at 37°C in an oxygen-limited atmosphere has resulted in conversion of *Campylobacter* cultures from a typically vibroid morphology to a predominantly coccoid form in 5 to 12 days (Rollins and Colwell, 1986; Griffiths, 1993). However, results from preliminary experiments performed in the current study suggest that conversion to coccoid morphology and subsequent loss of culturability (as determined by dilution plate counts) of *C. jejuni* KLC 4315 in NB#2 at 37°C was not as rapid as has been reported for other strains in the literature (data not shown). The loss of culturability of KLC 4315 (initial concentration $10^8$ CFU.ml$^{-1}$) exceeded three weeks under these conditions (data not shown).

4.1.1 ANTIBIOTIC-INDUCED NONCULTURABILITY.

To decrease the apparent time necessary for the morphological conversion of KLC 4315, antibiotic treatments were trialed. Several studies have suggested the addition of antibiotics to media accelerates the formation of the VNC state in related organisms (Bode, *et al.*, 1993; Calder, 1998). However, although antibiotic use is well documented in enrichment broths and selective media (Humphrey, 1986), Moran and Upton postulated that antibiotic addition is insufficient to encourage the transition to nonculturable coccoid form alone (Moran and Upton, 1986).
In the current study, the addition of antibiotic 'cocktails' was trialed using C. jejuni KLC 4315 as the test strain. Antibiotics that have been shown to be most effective include polymyxin B sulphate, rifampicin, trimethoprim, and cefoperazone. Polymyxin B is active against many Gram-negative bacteria and may be inhibitory to some strains of C. jejuni (Nachamkin, 1997); trimethoprim usually inhibits Proteus species as well as other Gram-negative bacteria, and rifampicin and cefoperazone are effective against Gram-positive bacteria and are primary inhibitors of enteric bacterial flora (especially Pseudomonas sp.) (Corry, et al., 1995; Nachamkin, 1997). The four antibiotics target Campylobacter spp. by different mechanisms (Singleton and Sainsbury, 1978; The Merck Index):

- Polymyxin B binds strongly to the bacterial cell membrane - apparently via phosphate groups - causing an increase in the permeability of the membrane to small molecules.
- Rifampicin specifically inhibits bacterial DNA-dependent RNA polymerase; it has no action on the equivalent eukaryotic enzyme.
- With an affinity for bacterial dihydrofolate, the inhibitory effects of trimethoprim prevent the reduction of dihydrofolate to tetrahydrofolate - a key step in folic acid metabolism.
- A cephalosporin, cefoperazone acts by inhibition of the formation of cross-links in the bacterial peptidoglycan layer.

The results of these experiments are presented in Figure 4.1. At 37°C in the presence of rifampicin and polymyxin B at sub-MIC levels, the rate of transition to coccoid morphology from rod morphology was faster than in the (no-antibiotic) control. Culturability in all antibiotic-treated cells declined much more rapidly than in controls. The antibiotic supplement of rifampicin and polymyxin B (final concentrations of 10mg.L⁻¹ and 5000 i.U.L⁻¹ respectively) was determined to be the best treatment tested for induction, yet complete loss of culturability still required over two weeks.
Figure 4.1: Survival curve for *C. jejuni* KLC 4315 at 37°C in NB#2 liquid broth with antibiotic formulations - no antibiotic control (●); polymyxin B (●); rifampicin (●); trimethoprim (●); polymyxin B and rifampicin (●); polymyxin B, rifampicin and trimethoprim (●). Data points represent mean plate counts from three independent experiments.
4.1.2 TEMPERATURE / AEROBIOSIS - INDUCED NONCULTURABILITY.

To encourage loss of culturability, variation of two environmental parameters, thought to be important in the induction of the VNC state, was trialed. Hazeleger provided evidence of conversion of \textit{C. jejuni} to a VNC state at 25°C in non-microaerophilic conditions in under ten days (Hazeleger, \textit{et al.}, 1994; Hazeleger, \textit{et al.}, 1995). Under these conditions, with the addition of the two afore-mentioned antibiotics, a similar time-frame for complete loss of culturability was observed for KLC 4315 in this research (Figure 4.2).

The nonculturable response of \textit{C. jejuni} KLC 4315 on NB#2A and CCDA is shown in Figure 4.2. Total direct counts and most probable number values also plotted on the graph will be discussed later in this chapter. At daily intervals, 100μl aliquots were withdrawn from the liquid culture and serially diluted in PBS. Aerobic plate counts on CCDA and NB#2A were performed. After ca. seven days, plate counts from triplicate liquid cultures had decreased to $\leq 1 \text{ CFU.ml}^{-1}$ on CCDA. Prolonged incubation of these cells on either selective CCDA or non-selective NB#2A media did not alter the result. Once the limits of detection by dilution plate count were exceeded, 10 and 30 ml broth suspensions were filtered through a 0.22μm cellulose acetate membrane filter (Millipore) and the inverted filter incubated at 37°C for 48 h before observation of any colonies. This reduced the lower detection limit to 0.03 to 0.1 CFU.ml$^{-1}$.

Results presented in this thesis have demonstrated that loss of culturability is shown to be dependent on the growth media used. Of the two media trialed, platability of the test organism KLC 4315 fell more rapidly on the non-selective NB#2A. Additionally, culturable cell numbers were observed for an extended period of time (ca. 2 days) on CCDA compared to NB#2A (Figure 4.2). Possible reasoning for this observation is proposed in the discussion (Section 5.4.1).
Figure 4.2: Survival curve of *C. jejuni* KLC 4315 maintained in stationary supplemented NB#2 at 25°C. Datum points are mean values from replicate experiments. Comparison of plate counts on NB#2A (●) and CCDA (●); total direct count by haemocytometric analysis (∗); and most probable number viable cell enumeration (●) as indices of viability and culturability.

### 4.1.3 SUMMARY OF PRELIMINARY EXPERIMENTS.

Preliminary experiments were based on induction of the VNC state. Transition to a nonculturable form was best observed at lower temperatures of incubation (25°C), therefore this temperature was used for all further studies involving the morphological transition of the coccoid forms of *C. jejuni* KLC 4315. Prolonged *C. jejuni* culture was performed in liquid media (NB#2) for up to six months at 25°C under non-microaerophilic conditions, without supplementation by fresh medium.
4.2 BACTERIAL ENUMERATION METHODS.

A number of standard microbiological techniques were employed to monitor changes in (total and viable) cell numbers over the conversion to coccoid VNC state.

4.2.1 BACTERIAL GROWTH MONITORING BY TURBIDOMETRY.

Figure 4.3 shows the growth curve for the test strain as determined by optical density readings at a wavelength of 600 nm. As assessed by turbidometry, the bacterial mass increased exponentially from day one to day two, then increased slightly for the next 48 h before reaching a plateau level. The growth curve correlates with the previous culturability results (Figure 4.2). The highest plate count was obtained at the first peak OD$_{600}$ and as culturability fell, the turbidity readings of the culture were observed to level off.

Figure 4.3: Growth curve for *C. jejuni* KLC 4315 established by turbidometry. Datum points are mean values from replicate experiments.
4.2.2 TOTAL CELL COUNTS.

The total bacterial count is a measurement of the total number of cells in a certain culture volume as the methodology is unable to distinguish viable from non-viable. Counts were performed by daily haemocytometric readings using an Image Analysis package. Computer-assisted viewing using the programme Metamorphosis permitted increased magnification onto an output screen, allowing for easier visualisation and subsequent enumeration of a number of sample fields. The results from total count analysis are presented earlier in this chapter in Figure 4.2.

Throughout the sampling period, total bacterial numbers (as determined by haemocytometry) remained relatively constant whilst the number of culturable organisms gradually decreased. An initial increase was observed which corresponded with an increase in culturable cell numbers, followed by a small net decrease - reflective of a level of autolysis.

4.2.3 MOST PROBABLE NUMBER DETERMINATION (MPN).

Most probable number (MPN) enumeration of bacterial cells is a statistical method based on probability theory. Serial dilutions of the C. jejuni inoculated NB#2 were performed in conjunction with plate counts in an effort to reach a point of extinction (when not even one cell would be deposited into one of the multiple tubes at a certain dilution level). The established criterion for this research to indicate whether a particular dilution tube contained the bacterium relied on the development of turbidity in liquid growth medium.

Results of the MPN analysis are presented on the culturability curve (Figure 4.2). During the ten-day time-course of the experiment a level of extinction was not achieved, indicating complete 'die-off' (0 CFU.ml⁻¹) of C. jejuni had not occurred and actively growing and replicating cells may still have been present. A decrease in MPN values was observed but it remained between one (in a day-one culture) and five log cycles higher than plate counts (at the conclusion of the experiment).
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4.2.4 ACRIDINE ORANGE DIRECT VIABLE COUNTS (AODVC).

The acridine orange direct viable count (AODVC) method of Singh, *et al.* (1990) was attempted with KLC 4315. The epifluorescent technique is reported to give a more accurate estimate of total (including non-viable and VNC) cell numbers (Kepner and Pratt, 1994). Samples were removed at different stages along the continuum of the cell cycle and tested for physiological responsiveness upon the addition of nalidixic acid and nutrients. Under epifluorescent laser-scanning microscopy, the total counts of cells stained by the acridine orange dye and the direct viable count of elongated cells were enumerated. The literature suggests the difference between total direct counts (ie., measuring live and dead cells) and plate counts (ie., measuring culturable cells) can be upwards of three log cycles, while DVC (ie., measuring viable cells) are always at least one log higher than plate counts (Rollins and Colwell, 1986; Jiang and Chai, 1996).

A 0.22\(\mu\)m cellulose acetate filter (Millipore) was stained for 48 h in 0.2% Nigrosin in an effort to provide a suitably dark background for epifluorescent study, and although results have been reported with this technique it did not appear sufficient for visualisation of the acridine orange stained *Campylobacter* cells. Therefore, results were not obtained from AODVC analysis with *C. jejuni* KLC 4315. The AODVC method was not pursued due to time constraints.

The AODVC technique was also to be employed in this research to gain insight into rates of cellular metabolism. Acridine orange binds to both DNA and RNA with an excitation maximum of approximately 470nm (Kepner and Pratt, 1994). AO-stained single-stranded nucleic acids emit orange-red fluorescence, whilst double-stranded DNA tends to fluoresce green *in vivo*. Roszak and Colwell (1987) reported that a high RNA:DNA ratio indicates active metabolism, whereas a low ratio is indicative of metabolic inactivity. Ratios in KLC 4315 were to be explored but as mentioned above had to be abandoned due to limitations with time.
4.3 CHANGES IN CELL MORPHOLOGY.

4.3.1 MORPHOLOGICAL CHANGES.

As shown in Figure 4.4, morphological changes (as determined by microscopic observation) paralleled changes in culturability. All the test systems were inoculated with homomorphous (morphologically uniform, spiral) *C. jejuni* in the logarithmic phase of growth. In fresh preparations examined by phase-contrast microscopy, the rod-shaped bacteria (with a size of approximately 0.2 to 0.3 μm wide by 2.1 to 2.5 μm long) showed a characteristic rapid to-and-fro movement. Repeated examination by light microscopy revealed the expected gradual transition to a predominance of coccoid forms that is common to both *Helicobacter* and *Vibrio* species (Colwell, *et al.*, 1985; Ogg, 1962; cited from Rollins and Colwell, 1986; Boucher, *et al.*, 1994). These nonculturable cells of *C. jejuni* were small cocci with a diameter of 0.3 to 0.7 μm. As observed by Griffiths (1993), the rod cell of *C. jejuni* initially elongated during the transition to coccoid morphology. Cell length was witnessed to increase and in some cases double (particularly during the decline phase as the population lost culturability. A proposed rationale for this observation will be discussed in Section 5.2.5.

4.3.2 POPULATION CHANGES.

The ratio of rods:cocci in liquid culture of *C. jejuni* was determined with the assistance of image analysis technology. Unfortunately, computer-assisted computation of the relative ratios was not possible due to the small size of the *Campylobacter* bacteria. Instead, duplicate smears were prepared at daily intervals and ten fields of vision counted manually for each smear. Increased magnification on an output screen allowed for better visualisation of culture proportions. On average, 600+ bacterial cells were recorded as being coccical, spiral or intermediary in morphology. The relative proportions of coccoid forms of *C. jejuni* KLC 4315 produced in liquid media are shown in Figure 4.4.
Figure 4.4 Proportions of different morphological forms in liquid culture of *C. jejuni* KLC 4315. The percentages of bacillary, intermediary, and full coccoid forms were determined by light microscopy.

Rod morphology (●); Coccoid morphology (●); Intermediary (●).

The original bacterial inocula consisted of 100% helical *C. jejuni* cells. The percentage of coccoid forms increased with prolonged incubation. Analysis of seven-day-old samples of the test strain demonstrated the presence of almost 100% spherical bacteria. However, during the time frame under study, there existed a continuum of morphological types. Intermediary structures have been reported for *C. jejuni* (Ng, et al., 1985b; Moran and Upton, 1987b). Under light microscopy, a few U-shaped and doughnut-shaped forms were observed. The latter are thought to be intermediates between U-shaped and completely coccoid forms (Ng, *et al.*, 1985b; Benaissa, *et al.*, 1996).
4.3.3 GRAM-STAIN DIFFERENCES.
Gram-stains were performed on rod and coccoid forms of *C. jejuni* KLC 4315. Plate cultures, consisting mainly of organisms with spiral morphology, gave the typical Gram-negative reaction, whereas aged cultures containing predominantly coccoid forms were lightly stained with the saffranin counterstain. A similar observation was made by Moran and Upton (1986), who postulated the difference was an indication of cell wall changes occurring during the morphological conversion.

4.4 DNA ANALYSES.
Total genomic DNA extracted from broth samples, incubated at 25°C for six months, was compared (in its ability to serve as a template for PCR amplification) to DNA extracted from logarithmic-phase cells (48 h plate cultures of *C. jejuni* KLC 4315). Log-phase cells maintained intact chromosomal DNA, while the VNC cells contained partially degraded DNA, as shown in Figure 4.5, suggesting the loss of viability of a significant portion of the population.

**Figure 4.5:** Electrophoretic image of chromosomal DNA extracted from logarithmic-phase and VNC cells of *C. jejuni* KLC 4315 and amplified with the lpxA primers 98-04/96-25. lane 1. 100bp molecular weight marker: lane 2. Log-phase cell DNA (1 ng); lane 3. 6-month-old nonculturable cell DNA (1 ng). lane 4: Reference strain DNA (*C. jejuni* NCTC 11168); lane 5: No template (-) control.
4.5 THE GENE EXPRESSION ASSAY.

RNA was isolated from *C. jejuni* cultures at regular intervals, reverse-transcribed and amplified with each of the five primer sets. This method aimed to detect levels of gene expression of the chosen genes over the continuum of the cell cycle, with a particular objective to assess for a good marker or indicator of viability in KLC 4315.

4.5.1 RNA EXTRACTION.

Several methods of RNA extraction were trialed, including hot- and cold-phenol extractions and the Qiagen Rneasy Mini Kit. The hot-phenol technique of (Barry, *et al.*, 1992) failed to yield any RNA which could be used in downstream applications (eg., RT-PCR amplifications). A comparison of the Qiagen RNA extraction kit and the TRIzol reagent (Life Technologies) was performed. Although both systems were rapid and easy to use, the Qiagen system was more efficient at isolation of RNA from small numbers of cells (data not shown) and the most reproducible results were obtained using this kit. However, some DNA contamination was present in the RNA samples. To avoid subsequent amplification of the DNA, a short DNase treatment step was incorporated prior to the RT-PCR (refer to Materials and Methods - Section 3.5.3).

The quality of the total cellular RNA extracted was monitored by gel electrophoresis, but, surprisingly, the efficiency of extraction of the total RNA was not a reliable guide to the success of the subsequent RT-PCR of mRNA (data not shown).

4.5.2 DEVELOPMENT OF THE RT-PCR PROTOCOL.

No significant difference was observed between the two different reverse transcriptase enzymes trialed (see Materials and Methods - Section 3.6.1 and 3.6.2). However, in the one-tube method, all the reverse-transcribed RNA (cDNA) is available for amplification, whereas in the two-tube method, only a subsample of cDNA is amplified. For the purpose of this project, the latter RT-PCR regimen was deemed more suitable.
With the two-step method, the substrate could be randomly primed with hexamers in the Superscript II-catalysed reverse transcription reaction thus allowing the cDNA produced to be probed with a number of different specific primer sets in the PCR step. The one-step Titan™ system did not have the facility to incorporate random primers into its RT-PCR amplification.

4.5.3 OPTIMISATION OF THE RT-PCR ASSAY.

4.5.3.1 PRIMER ANNEALING.

The primers for amplification of the *lpxA*, *dnaJ*, 23S rRNA, *hupB* and *ciaB* genes were chosen such that the theoretical primer melting points for each primer pair were similar, allowing for an annealing temperature of 50°C (for *lpxA*) or 55°C (for the remainder). Varied annealing temperatures from these resulted in the observation of increased non-specific amplification products - an undesired by-product of a non-optimised PCR system.

4.5.3.2 MAGNESIUM OPTIMISATION.

Experiments were conducted to determine the optimal magnesium concentration for RT-PCR amplification for each of the primer pairs. Reverse transcription was performed using the Superscript II enzyme as recommended by the manufacturer, except that the magnesium concentration was changed to 3mM.

Increasing the magnesium concentration to 3mM resulted in an increase in amplification with inhibition of non-specific binding products (data not shown), therefore in subsequent experiments RT-PCRs were performed with 3mM magnesium.
4.6 EXPERIMENTAL CONTROLS.

The RT-PCR method is not valid without an assurance of the absence of DNA. To ensure that RNA, and not DNA, was being amplified and detected, isolated RNA was subjected to DNase and RNase treatments. The following results confirm that the isolation and amplification were specific for mRNA and that DNA was not being carried over into the PCR. Table 4.1 indicates the expected PCR or RT-PCR results of nuclease treatments on the extracted RNA.

Table 4.1: PCR and RT-PCR of controls performed on RNA extracted from Campylobacter cells.

<table>
<thead>
<tr>
<th>Control (RNA treatment)</th>
<th>PCR</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNased sample</td>
<td>-</td>
<td>- / + a</td>
</tr>
<tr>
<td>RNased sample</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNased/RNased</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a = The DNased/ RT-PCR sample may give a positive or negative result, depending on whether the gene encoded for by the mRNA is being expressed or not.

Figure 4.6a demonstrates the ipxA controls performed for the RNA sample extracted from a one-day-old NB#2 liquid culture. RNA preparations typically contained small amounts of contaminating chromosomal DNA. Untreated, on PCR amplification, this contaminating DNA is amplified to give a PCR product of the correct size (308 bp) (Lane 1). After treatment with DNase I and PCR amplification, no product was observed (Lane 2) - indicating the removal of DNA sequences from the extracted RNA sample. To further demonstrate that the RT-PCR product derived only from mRNA, the extracted RNA and DNase I-treated RNA preparations were subjected to treatment with RNase A. The RNased sample on PCR and RT-PCR gave an amplicon (Lanes 3 and 6). This is due to amplification of contaminating DNA. Control samples which had been subjected to RNase and DNase failed to give an amplicon on both PCR and RT-PCR (Lanes 4 and 7) - an indication that the DNase digestion had gone to completion and there was no nucleic acid left in the sample.
Lane 5 represents the RNA sample of study - a DNA-digested mRNA preparation which has been reverse-transcribed and PCR amplified with the \textit{lpxA} primer set. If the gene of choice is being expressed at a certain time period an amplicon will be observed as in Lane 5. In contrast if the gene is not being expressed, one would not expect to see a RT-PCR product.

Extraction and PCR controls were performed with every sample and with each genetic marker. Standard PCR gave positive results for RNA extracted from viable cells (log-phase 48 hour plate cultures) or heat-killed (autoclaved) cells (data not shown). In contrast, RT-PCR resulted in amplification of RNA extracted from spread plates of \textit{C. jejuni} KLC 4315 (Lane 9) but did not give a signal for RNA extracted immediately following autoclaving of a 48 hour broth culture of the test strain (Lane 8).

Lanes 10 and 11 represent the negative and positive PCR controls respectively. The negative control is a water blank and the positive control is PCR-amplified genomic DNA. These are incorporated, respectively, to ensure there is no DNA contamination of PCR reagents and that the PCR reaction is proceeding as it should.

Controls confirmed no contaminating DNA was present in the DNase-treated RNA samples, eliminating the chance of false positive results in the subsequent RT-PCR analysis. For each RNA sample for each gene set, the above controls were performed. Thus, the use of DNase I and RNase A established that mRNA was the template and if an amplicon was observed in the DNased/ reverse-transcribed sample, gene expression was assumed for that time interval.
Figure 4.6a: Agarose gel electrophoresis of day-one \(lpxA\) control PCR products stained with ethidium bromide. Lanes M denote 100-bp ladders (Bio/rad): lane 1, the 308-bp target sequence of undigested RNA from a day-one culture of \(C. jejuni\) KLC 4315 \((10^8 \text{ CFU.ml}^{-1})\); lanes 2 to 7 represent \(C. jejuni\) RNA extracted from a one-day old culture and treated with: lane 2, DNase I digested but not RTed: lane 3, RNase A digested but not RTed: lane 4, digested sequentially with DNase I and RNase A but not RTed: lane 5, DNase I digested, RTed: lane 6, RNase A digested, RTed: lane 7, DNase I and RNase A digested, RTed: lane 8, RNA extracted from killed \(C. jejuni\); lane 9, RNA extracted from plated \(C. jejuni\); lane 10, PCR reagent control without template; lane 11, \(C. jejuni\) DNA (1 ng).

Figure 4.6b: RT-PCR detection of \(lpxA\) mRNA from \(C. jejuni\) cells \((10^8 \text{ CFU.ml}^{-1})\) maintained in a dark, microaerobic environment at 25°C. Lanes M contain a 100 bp molecular weight marker (Bio/rad). Lanes 1 to 14 denote mRNA extracted from the broth culture at marked intervals. The PCR-positive (lane +) and negative (lane -) controls contain \(C. jejuni\) DNA and sterile water, respectively.
4.7 DETECTION OF mRNA AND rRNA IN LIQUID CULTURE.

4.7.1 EXPRESSION OF THE lpxA GENE PRODUCT.

After 14 days of induction, all RNA samples from predominantly coccoid forms were negative for lpxA mRNA (Figure 4.6b). No samples were taken in the time period between 14 days and 21 days therefore mRNA may have been completely translated to the enzyme UDP-N- acetylglucosamine transferase during this time-frame. With extended time, the intensity of the cDNA band decreased, such that at three weeks no PCR product was observed.

4.7.2 rRNA STABILITY AND DETECTION AS EVALUATED BY RT-PCR.

To corroborate the results of the lpxA gene expression experiments and to evaluate the ability of rRNA to support efficient nucleic acid amplification, RT-PCR amplification of 23S rRNA from broth cultures of C. jejuni KLC 4315 was performed. Again, samples taken at various times, corresponding to different cellular stages along the continuum of bacterial death, were analysed. The primer set (Therm I/Therm II) used as targets a region of the 23S rRNA, generating a 220 bp amplicon (Eyers, et al, 1993).

23S rDNA amplicons were observed by agarose gel electrophoresis for the length of the experiment - reflecting the stability of the molecule. Little decrease in the intensity of the band was seen, even at three months post-inoculation.
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Figure 4.7a: Agarose gel electrophoresis of day-one 23S rRNA control PCR products stained with ethidium bromide. Lanes M denote 100-bp ladders (Biorad): lane 1, the 222-bp target sequence of undigested RNA from a day-one culture of C. jejuni KLC 4315 (10^8 CFU.ml^-1): lanes 2 to 7 represent C. jejuni RNA extracted from a one-day old culture and treated with: lane 2, DNase I digested but not RTed: lane 3, RNase A digested but not RTed: lane 4, digested sequentially with DNase I and RNase A but not RTed: lane 5, DNase I digested, RTed: lane 6, RNase A digested, RTed: lane 7, DNase I and RNase A digested, RTed: lane 8, RNA extracted from killed C. jejuni: lane 9, RNA extracted from plated C. jejuni: lane 10, PCR reagent control without template; lane 11, C. jejuni DNA (1 ng).

Figure 4.7b: RT-PCR detection of 23S rRNA from C. jejuni cells (10^8 CFU.ml^-1) maintained in a dark, microaerobic environment at 25°C. Lanes M contain a 100 bp molecular weight marker (Bio/rad). Lanes 1 to 14 denote mRNA extracted from the broth culture at marked intervals. The PCR-positive (lane +) and -negative (lane -) controls contain C. jejuni DNA and sterile water, respectively.
4.7.3 dnaJ EXPRESSION IN STRESSED Campylobacter CELLS.

C. jejuni cells were maintained for extended periods of time in an aerobic environment without addition of fresh media, thus, putting them under nutritional and oxidative stresses. Expression of the dnaJ gene was monitored on a positive/negative basis over the length of the experiment (Figure 4.8a and 4.8b). The former shows the controls performed for the dnaJ gene set (as described for IpxA in Section 4.8). The latter is the result of gene expression studies by mRNA-based RT-PCR, demonstrating that the dnaJ gene product is produced for the first ten days under the conditions trialed (Lanes 2 to 9).

Due to the relatively small size of the dnaJ amplicon (ca. 100bp), polyacrylamide gel electrophoresis was employed in an attempt to increase visible observation of the fragment. However, little difference was observed between this technique and that of agarose gel electrophoresis. Additionally, a non-specific band was observed (ca. 500 bp) in some, but not all, of the amplified mRNA samples.
Figure 4.8a: Agarose gel electrophoresis of day-one dnaJ control PCR products stained with ethidium bromide. Lanes M denote 100-bp ladders (Bio/rad): lane 1, the ca.100-bp target sequence of undigested RNA from a day-one culture of C. jejuni KLC 4315 (10^8 CFU.ml⁻¹); lanes 2 to 7 represent C. jejuni RNA extracted from a one-day old culture and treated with: lane 2, DNase I digested but not RTed; lane 3, RNase A digested but not RTed: lane 4, digested sequentially with DNase I and RNase A but not RTed: lane 5, DNase I digested, RTed: lane 6, RNase A digested, RTed: lane 7, DNase I and RNase A digested, RTed: lane 8, RNA extracted from killed C. jejuni: lane 9, RNA extracted from plated C. jejuni: lane 10, PCR reagent control without template; lane 11, C. jejuni DNA (1 ng).

Figure 4.8b: RT-PCR detection of dnaJ mRNA from C. jejuni cells (10^8 CFU.ml⁻¹) maintained in a dark, microaerobic environment at 25°C. Lanes M contain a 100 bp molecular weight marker (Bio/rad). Lanes 1 to 7 denote mRNA extracted from the broth culture at marked intervals. The PCR-positive (lane +) and negative (lane -) controls contain C. jejuni DNA and sterile water, respectively.
4.7.4 *hupB* EXPRESSION AND DNA CONGREGATION.

The results of *hupB* gene expression studies are summarised in Figure 4.9b (with the day 1 controls shown in Figure 4.9a). Lanes 2 to 5 represent the one- to four-day-old cultures of *C. jejuni*. Expression of the *hupB* gene is evidenced in these first four samples yet is not seen in any samples past this time point. In other words, *hupB* gene expression does not exceed the first four days post-inoculation.

4.7.5 THE EXPRESSION OF THE VIRULENCE GENE *ciaB*.

The gene encoding the invasive antigen of *C. jejuni* -*ciaB*- was not expressed during the time-course of experimentation (Figure 4.10a and 4.10b). This was predicted as *ciaB* was included as a negative genetic marker, which would only be expressed in the presence of host eukaryotic cells.

4.7.6 SUMMARY OF GENE EXPRESSION STUDIES IN *C. jejuni*.

Of the four gene sets targeted for mRNA RT-PCR, *lpxA* persisted for the longest period of time (two weeks). The 23S rRNA was found to be present for the entire experimental time-frame (three months) - a result that was anticipated and has been extensively reported in the literature (refer to Section 5.4.3). Thus, *C. jejuni* cultures were exposed to an artificial form of cellular death (heat-shock), in an endeavour to demonstrate the stability and suitability of *lpxA* mRNA as a suitable indicator of cellular viability.
Figure 4.9a: Agarose gel electrophoresis of day-one *hupB* control PCR products stained with ethidium bromide. Lanes M denote 100-bp ladders (Bio/ rad); lane 1, the c 200-bp target sequence of undigested RNA from a day-one culture of *C. jejuni* KLC 4315 (10^8 CFU.ml^-1); lanes 2 to 7 represent *C. jejuni* RNA extracted from a one-day old culture and treated with: lane 2, DNase I digested but not RTed; lane 3, RNase A digested but not RTed; lane 4, digested sequentially with DNase I and RNase A but not RTed; lane 5, DNase I digested, RTed; lane 6, RNase A digested, RTed; lane 7, DNase I and RNase A digested, RTed; lane 8, RNA extracted from killed *C. jejuni*; lane 9, RNA extracted from plated *C. jejuni*; lane 10, PCR reagent control without template; lane 11, *C. jejuni* DNA (1 ng).

![Agarose gel electrophoresis of day-one *hupB* control PCR products stained with ethidium bromide.](image1)

Figure 4.9b: RT-PCR detection of *hupB* mRNA from *C. jejuni* cells (10^8 CFU.ml^-1) maintained in a dark, microaerobic environment at 25°C. Lanes M contain a 100 bp molecular weight marker (Bio/ rad). Lanes 1 to 14 denote mRNA extracted from the broth culture at marked intervals. The PCR-positive (lane +) and PCR-negative (lane -) controls contain *C. jejuni* DNA and sterile water, respectively.

![RT-PCR detection of *hupB* mRNA from *C. jejuni* cells.](image2)
Figure 4.10a: Agarose gel electrophoresis of day-oneciaB control PCR products stained with ethidium bromide. Lanes M denote 100-bp ladders (Bio/rad): lane 1, the ca. 500-bp target sequence of undigested RNA from a day-one culture of C. jejuni KLC 4315 (10^8 CFU.ml^-1); lanes 2 to 7 represent C. jejuni RNA extracted from a one-day old culture and treated with: lane 2, DNase I digested but not RTed; lane 3, RNase A digested but not RTed; lane 4, digested sequentially with DNase I and RNase A but not RTed; lane 5, DNase I digested, RTed; lane 6, RNase A digested, RTed; lane 7, DNase I and RNase A digested, RTed; lane 8, RNA extracted from killed C. jejuni; lane 9, RNA extracted from plated C. jejuni; lane 10, PCR reagent control without template; lane 11, C. jejuni DNA (1 ng).

Figure 4.10b: RT-PCR detection ofciaB mRNA from C. jejuni cells (10^8 CFU.ml^-1) maintained in a dark, microaerobic environment at 25°C. Lanes M contain a 100 bp molecular weight marker (Bio/rad). Lanes 1 to 14 denote mRNA extracted from the broth culture at marked intervals. The PCR-positive (lane +) and negative (lane -) controls contain C. jejuni DNA and sterile water, respectively.
4.8 DETECTION OF RNA IN LIQUID CULTURE SUBJECTED TO THERMAL INJURY.

A heat-kill experiment was designed to examine the stability of RNA species in liquid cultures subjected to thermal injury. Heating of a suspension of *C. jejuni* KLC 4315 (10^8 CFU.ml^-1) at 65°C for 20 minutes killed or inactivated the bacteria (as determined by culturable counts on CCDA). No culturable cells were detected in samples after treatment or during subsequent incubation at room temperature. In fact, the culturable count fell below the limits of detection after five minutes of the heat treatment (Table 4.2).

Table 4.2: Culturability counts for *Campylobacter jejuni* KLC 4315 during a 20 minute heat treatment.

<table>
<thead>
<tr>
<th>Time (min) after heat-shock induction</th>
<th>Culturable Count (CFU.ml^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0^a</td>
<td>3.3 x 10^8</td>
</tr>
<tr>
<td>1</td>
<td>4.1 x 10^6</td>
</tr>
<tr>
<td>2</td>
<td>6.3 x 10^4</td>
</tr>
<tr>
<td>3</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>4.1</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>20^b</td>
<td>0</td>
</tr>
</tbody>
</table>

^a= prior to the onset of heat-shock induction  
^b= at the completion of heat-shock induction

Detection of *IpxA* mRNA in 65°C-inactivated cells is shown in Figure 4.10. mRNA transcribed from this gene was detected by RT-PCR in the cell suspension immediately after heating at 65°C (Table 4.3). However, during subsequent incubation at ambient temperature, *IpxA* mRNA species became undetectable and was absent at 216 h.
Figure 4.11: RT-PCR detection of lpxA mRNA from C. jejuni KLC 4315 cells (10^8 CFU.ml^-1) heat-treated at 65°C for 20 mins. Lanes M denote 100 bp molecular markers (Bio/rad): lanes 1 to 15 represent extracted RNA samples taken at time intervals after induction of heating.

Table 4.3: mRNA and rRNA species detected by RT-PCR after incubation of heat-killed Campylobacter jejuni at ambient temperature.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Untreated</th>
<th>1m</th>
<th>3</th>
<th>5</th>
<th>10</th>
<th>15m</th>
<th>20m</th>
<th>1h</th>
<th>3</th>
<th>5</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>96</th>
<th>216</th>
</tr>
</thead>
<tbody>
<tr>
<td>65°C/20 min</td>
<td>lpxA mRNA</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>65°C/20 min</td>
<td>23s rRNA</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

a = Y, positive RT-PCR amplification; N, negative RT-PCR amplification.
b = Time measured in minutes (first six entries); hours (last eight entries).
By contrast, the heat treatment did not lead to complete degradation of rRNA, as samples taken at time points through the length of the experiment ($t = 0\ h$ to $t = 216\ h$) yielded a positive signal after RT-PCR detection (Table 4.3). Holding the samples for an extended period of time (up to nine days after heat-treatment) at 25°C did not lead to the elimination of the RT-PCR detection signal (Figure 4.12). Thus, RT-PCR amplification of the 23S rRNA sequence enclosed by the primer set Therm I/Therm II does not correlate to the viability of *C. jejuni*.

**Figure 4.12:** RT-PCR detection of 23S rRNA from *C. jejuni* KLC 4315 cells ($10^8\ CFU.ml^{-1}$) heat-treated at 65°C for 20 mins. Lanes M denote 100 bp molecular markers (Bio/rad): lanes 1 to 15 represent extracted RNA samples taken at time intervals after induction of heating.
CHAPTER V
DISCUSSION

The most salient feature of life has been the stability of its bacterial mode from the beginning of the fossil record until today and, with little doubt, into all future time so long as the earth endures. This is truly the “age of bacteria” - as it was in the beginning is now and ever shall be.

(Stephen Jay Gould, 1994)  

5.1 MICROBIAL ADAPTABILITY AND EFFICIENCY.

Living bacterial cells are esoteric physicochemical systems that have evolved to survive and reproduce in their natural environment (Koch, 1988). In natural ecosystems, nutrient insufficiency is the most common environmental stress which microorganisms routinely encounter (Jiang and Chai, 1996). As nature exerts strong selective pressures in the course of evolution, two properties have manifested themselves in bacteria - efficiency and adaptability. The microbial cell can be viewed as being in a dynamic state, adapting readily to shifts in environmental parameters by means of a wide variety of genotypic and phenotypic accommodations.

Postgate (1967) theorised that nondifferentiating bacteria have evolved a resistance to starvation and stress. This adaptation process involves a substantial reduction in size, thereby achieving minimal maintenance requirements and limited turnover of cellular components - without the complete loss of activity that is observed in spores. In these bacterial species, the cost of maintaining metabolic activity throughout prolonged starvation appears to be balanced by the selective advantage of immediate growth ability upon addition of nutrients or removal of stress (Weichart and Kjelleberg, 1996). Termed

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6 Quote cited from (Neidhardt, 1996).
the starvation response, this is one adaptation microorganisms have evolved in an effort to withstand the life of 'feast and famine' they lead.

However in the last two decades, a second related phenomenon has been observed. In these systems, the introduction of stress (be it environmental or nutritional) results in the appearance of a viable but nonculturable phenotype. This so-called VNC state has been suggested to represent an escape strategy or a second survival mechanism to the starvation response (Colwell, et al., 1985; Roszak and Colwell, 1987; Oliver, et al., 1991; McDougald, et al., 1998). As such, a substantial amount of information on the starvation response has been accredited to occurring in VNC cells as well. Much research now abounds on why and how certain organisms lose their culturability in stressful environments and the degree of relatedness of this phenomenon to both the starvation response and the stationary-phase of the bacterial life cycle.

5.2 LOSS OF CULTURABILITY.

In this thesis, results consistent to those presented in the literature were observed with respect to loss of culturability of the test strain KLC 4315. Although this research did not aim to find the correlation coefficients or the statistical significance of environmental parameters on the formation of the VNC state of Campylobacter, time, temperature, and aeration were shown to be dominant factors in determining the rate at which culturability is lost. For a review of statistical analyses on the VNC conversion rate in this genus, refer to Calder (1998).

The results of culturability studies undertaken in this thesis are summarised below.

5.2.1 TIME.

Results from the current study demonstrated that the length of time following inoculation of C. jejuni KLC 4315 into liquid broth was a principal factor in determining the onset of the VNC response. The transition to VNC is known to occur as a normal event in the stationary phase of growth and when exponentially growing cells
are transferred to starvation conditions (Rollins and Colwell, 1986). At the two temperatures tested (37°C and 25°C), increased loss of culturability was correlated to temporal progression. With extended incubation time, the platability of the culture fell such that it reached a level of extinction (when no culturable organisms were present). This result is supported by a number of workers investigating the loss of culturability in several organisms using a variety of culture media (Rollins and Colwell, 1986; Garcia-Lara, et al., 1991; Jones, et al., 1991; Beumer, et al., 1992; Oliver, 1995; Sorberg, et al., 1996; Calder, 1998).

5.2.2 TEMPERATURE.

The temperature that broth cultures of KLC 4315 were incubated was shown to affect the temporal effect on the rate at which culturability was lost. In contrast to results observed by Rollins and Colwell (1986), a state of nonculturability was achieved at a faster rate at 25°C rather than 37°C (seven days as compared to over two weeks). This is postulated to be related to the enzymatic activity within the cell. At the moderate temperature (25°), the metabolic functions may be reduced, resulting in transformation to a survival state at a faster rate. In contrast, at 37°C there was little change in culturability over a two to three week period, reflecting the organism's ability to grow at this temperature (ie., just below its optimum of 42°C (Nachamkin, 1995)).

Temperature has been shown to determine the rate at which culturability is lost by a number of workers using different organisms and media (Blaser, et al., 1983; Rollins and Colwell, 1986; Jones, et al., 1991; Nilsson, et al., 1991; Oliver, et al., 1991; Oliver, et al., 1996; Calder, 1998). It is evident from these studies that there is a high degree of variability between genera, species and strains, and as such, any conclusions relating to temperature-induced nonculturability only pertain to the strain examined. This is best illustrated with low temperature incubation studies. Oliver (1996) demonstrated a temperature downshift to below 10°C results in rapid conversion (within 24 h) of Vibrio vulnificus to VNC form, whilst the organism exhibited no loss of culturability at room
temperature for a month. This observation contradicts what has been seen for *C. jejuni* which exhibits prolonged culturability in microcosms or culture media at low temperature (< 10°C) (Rollins and Colwell, 1986; Calder, 1998).

### 5.2.3 AERATION.

Broth cultures of *C. jejuni* KLC 4315 lost culturability at a faster rate in aerobic environments. This is consistent with observations made by Moran and Upton (1987a/1987b). *Campylobacter* spp. are known to be sensitive to atmospheric oxygen concentrations (Ketley, 1995), and therefore, loss of culturability in aerated environments is expected to be more rapid than under the microaerophilic conditions favoured by this fastidious organism. It is likely that this factor acts in conjunction with other environmental parameters either to accelerate entry into a nonculturable state or to prolong culturability. For example, Rollins and Colwell (1986) put forward the suggestion that slow-moving, low oxygenated stream waters or aquifers with cold temperatures may permit *Campylobacter* overwintering or survival in a culturable state.

### 5.2.4 CULTURABILITY CURVE.

The culturability curve for the test strain incubated at 25°C (presented in Figure 4.2) could be divided into three stages. During stage I (0 to 1 day), the number of culturable cells was initially seen to increase, in some cases, by an order of a one log; stage II (1-4 days) corresponded to a rapid decrease in the culturable cell fraction; and stage III (4-7 days) was characterised by a stabilisation in the loss of culturability rate.

The initial increase in culturable cell numbers (Stage I), usually observed within 24 to 48 h post-inoculation, is a phenomena which has been reported previously (Calder, 1998; Ekweozor, *et al.*, 1998). Calder (1998) demonstrated an increase in *C. jejuni* colony numbers often by an order of magnitude in freshwater and seawater microcosm studies, whilst Ekweozor (1998) reported transient increases of 0.2 to 2 log units in *C. jejuni* held at low temperature. It is postulated that the increase in culturable cell number
may arise from autolysis of a number of cells on stress induction and the utilisation of
the nutrients from dead cells by cells which are better adapted to the stress environment.
With extended time and nutrient exhaustion, the culture enters stage II. Following the
initial rapid decrease in the numbers of culturable cells observed in this stage, the rate at
which culturability is lost also slowly decreases (Stage III). This may arise from more
persistent cells within the culture (pre-adapted to the stress) surviving, or may represent
resistances associated with different growth stages (ie., log versus stationary phase
cells).

5.2.5 MORPHOLOGICAL AND POPULATION CHANGES.
Over the seven-day period taken for *C. jejuni* KLC 4315 to lose culturability, light
microscopy observations were made. Results from this study correlate with those
observed in liquid culture by Griffiths (1993). Rod cells of *Campylobacter* were
observed to elongate (in a similar response as to that seen on nalidixic acid incubation)
before the gradual transition to coccoid morphology. Griffiths (1993) postulated this
observation was due to cessation of septum formation in rod campylobacters - a
phenomenon reported in cells prior to entering stationary-phase. The resultant inhibition
of cell division is due to differential gene expression, which can also be indirectly
altered by environmental or nutritional stimuli.

Associated with the loss of culturability is a change in the KLC 4315 culture to
predominantly coccoid morphology. 100% coccoid populations (as determined by light
microscopic observations) of the test strain were nonculturable. In fact, the culture lost
culturability at seven days post-inoculation - at a time when less than 10% of the
population were spiral morphology. This result is subject to investigator bias (ie., the
bias introduced by an individual's interpretation of what actually constitutes a countable
morphological form). To partially address the problem of subjectivity involved in
identifying bacterial cells as one morphology or the other, intermediate counts were also
recorded (ie., those cells not resembling a coccus or rod). With the inclusion of
intermediary counting, the steady decline in rod cell numbers and increase in coccal counts could be witnessed more accurately. Physiological and morphological variation is accepted in this study as the culture is an asynchronous population.

Transmission electron microscopy of the morphological forms of *C. jejuni* KLC 4315 was attempted. Original results can not be verified by pictorial data, due to the non-development of micrographs. Temporal constraints provided limited opportunity to re-address this problem, however some observations were made. Analysis of coccoid cells of the test strain showed no evidence of protoplast shrinkage, cell wall stretching, surface creasing, and flagella fragmentation previously observed with this organism (Ng, *et al*., 1985b; Jones, *et al*., 1991). The majority of coccal cells of KLC 4315 maintained an apparently intact, although asymmetric, membrane structure. TEM revealed a condensed cytosol in these cells. As described by Rollins and Colwell (1986) 'ghost' cells or spheroplasts were occasionally observed - a phenomenon which could not be detected under light microscopy. The spheroplasts retained their circular appearance, yet appeared to lack any cytosolic components, presumably rendering them non-viable. However, no degenerative changes were detected in most coccal samples taken at the onset of complete loss of culturability (seven days post-inoculation).

### 5.3 THE NONCULTURABLE STATE - Viable or Non-viable?

The pivotal question at present appears to be what significance VNC cells have in the environment with regard to ecology, epidemiology, or pathogenesis (Barer, *et al*., 1993). In this respect, the most controversial issues are whether VNC cells can persist and whether they can regain culturability and infectivity. To date, studies of the physiology of VNC cells have been hampered by almost complete shutdown of activity in these cells, which is reflected in the paucity of reports on the physiology of the VNC state. In spite of over fifteen years of research in this area, there is no consensus on the physiological basis for, or the medical significance of VNC cells.
5.4 DETECTION OF VIABILITY.

5.4.1 VIABILITY-CULTURABILITY ASSAYS.

The terms ‘viable’ and ‘living’ have proved difficult to define because there is no unique attribute common to all things deemed ‘alive’. In microbiology, viability is generally defined by the ability of an organism to multiply to form colonies on agar plates or visible turbidity in broth (Barer, 1997; Sheridan, et al., 1998). In recent years, the plate count method has come under increasing criticism because it is inefficient, at best, for enumerating viable bacteria present in environmental and clinical systems (Roszak and Colwell, 1987). The concept that Campylobacter and a number of other enteropathogens can enter a nonculturable state strikes at the heart of microbiology as the ability to recover organisms on agar remains its cornerstone (Bloomfield, et al., 1998).

Termed the ‘great plate count anomaly’ (Amann, et al., 1995), this phenomenon was witnessed in the test strain maintained for extended time in NB#2 at 25°C. Aerobic plate counts showed that at day seven post-inoculation the culture had entered a state of nonculturability. By conventional methodology using the traditional equation culturability = viability, this C. jejuni culture would be expected to contain no living cells. Most Probable Number (MPN) analysis and Total Direct Counts (TDC) demonstrate this not to be the case (refer to Results - Sections 4.2.2 -4.2.3).

Throughout the entire process of entry into the nonculturable state, no changes in total direct counts by haemocytometry were observed. The TDC method is a measure of total (viable and nonviable) bacterial cells as it can not distinguish between the two. However, MPN analysis detects increased broth turbidity as a measure of growth therefore only detecting the viable fraction of the population. An MPN assay, also called the dilution extinction or fraction-negative method, dissects a bacterial population into multiple samples of dilution, ultimately with one or less than one cell per sample. This approach allows the recovery of single cells and their subsequent growth can be
followed with high accuracy. Visual MPN analysis of *C. jejuni* KLC 4315 broth cultures failed to reach a level of extinction during the two week time course of the experiment (ie., a point when no viable cells were detectable). These results conflicted with those observed with plate counts by demonstrating retention of viability. A decline in numbers was detected over the experiment but MPN counts were consistently over one log cycle higher than plate counts. A possible reason for this was the non-selectivity of the MPN medium, thus preventing potential growth inhibition, in comparison to the cefoperazone-supplemented agar plates. Although no extinction level was reached with the MPN experiment, the results were not taken as definitive. The MPN index relies on the elimination of experimenter bias and error. In the current study, this could not be validated. This was because MPN analyses rely on establishing a clear line between turbidity and non-turbidity in small inoculated volumes. In the case in question, increased turbidity in the samples was not able to be directly correlated with negative controls, as these too appeared turbid in nature. Quantitative spectroscopy, measuring the turbidity in the serially diluted samples against a reference non-inoculated culture, demonstrated little difference in optical density.

The definition of nonculturability clearly depends on the efficiency of the media at recovering damaged or inactive organisms. Two conventional *Campylobacter* media were employed and contrasting results were observed with respect to the loss of culturability on agar plates. Plate counts on the selective CCDA remained the highest of the two media tested. Reduced CFUs were the most apparent on the nonselective NB#2A, and nonculturability was exhibited first on this medium. The proposed rationale for this observation is the increased aerotolerance of *C. jejuni* on CCDA, due to the presence of charcoal and other oxygen-quenching compounds in this medium.

Aerotolerance, the ability of a microaerophilic organisms to grow in atmospheres of increased oxygen tensions, has been reported to be enhanced by the addition of supplements to campylobacter media (Moran and Upton, 1987b). These authors
concluded that compounds which enhance the aerotolerance of *C. jejuni* do so by quenching toxic oxygen derivatives (e.g., superoxide anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide), formed as a result of auto-oxidation, photochemical oxidation, and/or microbial metabolism. Such compounds include blood, charcoal, iron compounds, or a supplement of ferrous sulphate, sodium metasulphite, and sodium pyruvate - many of which contain an active superoxide anion scavenging enzyme, superoxide dismutase (SOD). The addition of one of these compounds to a medium has been shown to maintain the culturability and characteristic rod morphology of *C. jejuni* (Moran and Upton, 1987b).

### 5.4.2 MAINTENANCE OF ‘ESSENTIAL’ FEATURES.

Cellular integrity and the presence of nucleic acids, ribosomes, and machinery for protein synthesis may be considered necessary but not sufficient for viability (Weichart, *et al.*, 1997). Since replication involves the co-ordinated activity of biosynthetic, homeostatic, and energy-conserving systems, there is no *a priori* reason to suppose that any single indicator of metabolic activity will correlate with viability (Sheridan, *et al.*, 1998). Nevertheless, the detection of DNA by PCR or hybridisation (Calder, 1998), the detection of rRNA by hybridisation (Amann, *et al.*, 1995), the detection of protein synthesis by means of inducible enzyme activity (Nwoguh, *et al.*, 1995), ultrastructural analysis by electron microscopy (Ng, *et al.*, 1985b) and the incorporation of vital dyes and AODVC counts (Singh, *et al.*, 1986; King and Parker, 1988; Bianchi and Guiliano, 1996) are techniques which identify cells as potentially viable. Metabolic indicators such as membrane potential (Tholozan, *et al.*, 1999), the ability to generate reducing power (Rodriguez, *et al.*, 1992; Pyle, *et al.*, 1995), or the ability to undertake DNA synthesis (Bode, *et al.*, 1993) have also proved useful determinants of viability of cells in the natural environment. Based on these methods, it is apparent that VNC cells maintain certain characteristics of viable cells, such as the potential for metabolic activity and respiration as well as cellular integrity. However, the detection limits of these techniques do not always permit a reliable determination of viability in naturally
starved or stressed populations (Weichart, et al., 1997). A more sensitive approach has been suggested - the use of nucleic acids as viability indicators, which would have the added advantage of specificity.

5.4.3 NUCLEIC ACID-BASED PCR AMPLIFICATION.

Sheridan (1998) proposed that the presence of nucleic acid (DNA, rRNA, tRNA, or mRNA) in bacterial cells might be useful as a viability determinant if (i) it is present only in viable cells, (ii) the kinetics of its disappearance is related to loss of viability, and (iii) it disappears from cells soon after death (Sheridan, et al., 1998).

While DNA is the target nucleic acid most commonly used for amplification reactions, recent studies have brought into question the association between DNA and cellular viability (Josephson, et al., 1993; Masters, et al., 1994; Calder, 1998). In general, investigators have found that DNA is stable enough to be amplified by PCR many days after cell viability has been lost. Similar results were witnessed in two instances in this thesis:

- After autoclaving a broth culture of C. jejuni \(10^8\) CFU ml\(^{-1}\), DNA was extracted immediately and was shown to suitably act as a substrate for PCR amplification whilst the isolated mRNA could not.

- DNA was isolated from a six-month old culture of KLC 4315. PCR amplification with the \(lpxA\) primers (98-04 and 96-25) and subsequent electrophoretic imaging demonstrated that although a smear pattern was observed, an amplicon was produced. This demonstrated the persistence of this molecule.

DNA, therefore, has been dismissed as an appropriate target for gene expression studies due to its relative stability and violation of Sheridan's third criteria - disappearance from cells soon after death. In contrast, due to the presence of the \(2'\)-hydroxyl group in the ribose sugar, the phosphodiester bonds of RNA molecules are more susceptible to hydrolysis than those of DNA, particularly in the presence of divalent cations (eg.,
magnesium and calcium) (Lindahl, 1993). This decreases the stability of the RNA molecule, potentially making it a better target.

Ribosomal RNA was initially believed to be an appealing target for nucleic acid amplification-based detection of viability. Due to its ubiquitous nature and high copy number in microbial systems, targeting this molecule would indicate the physiological state of the cell by examining the status of the ribosomes. A review of the literature suggested rRNA may be more closely related with cellular viability than DNA is (Rosenthal and Iandolo, 1970; Tomlins and Ordal, 1971), yet more recent research disputes this claim (Uyttendaele, et al., 1997; Sheridan, et al., 1998; this study). These authors have reported that like DNA, rRNA is extremely stable (King and Schlessinger, 1987) with high copy number and, therefore, may give an overinflation of cell numbers thus may not be a suitable target for discrimination between viable and non-viable cells.

Little information is available on the persistence of tRNA in injured or dead cells. Davis (1986) demonstrated tRNA persisted for longer periods of time than rRNA in cells of *E. coli* that had died of phosphate starvation, thus indicating tRNA is unlikely to be a good determinant of viability (Davis, et al., 1986).

A number of workers have investigated the usefulness of various mRNA transcripts for determining viability in pathogenic bacteria (see Table 2.4). Potential problems exist with choosing mRNA as a viability determinant - it is typically present in low copy numbers, is unstable, and there may be difficulty in finding a transcript that is constitutively expressed and species-specific (McKillip, et al., 1998). However, such problems can be overcome by selection of an appropriate methodology and compliance with certain precautions to eliminate the possibility of RNase contamination. The choice of mRNA as an indicator of viability is advantageous in that the potential for protein synthesis, important for active growth, can be monitored. This research indicates that it is potentially possible to monitor specific gene expression at different times along the
continuum of cellular life. Of particular interest is the potential for multiple pathway monitoring. mRNA species, encoding both proteins involved in metabolic activity (ie., respiration, protein synthesis, or energy production) and in structural integrity, can be monitored on a presence/absence basis through RT-PCR technology at regular intervals over the cell cycle. This may eliminate the need for other specific methods as criteria for viability (see Section 5.2.2), as major genes in these processes can instead be targeted by mRNA-based RT-PCR methodology.

5.5 VIABILITY DETERMINANTS IN *C. jejuni*.

Five functional proteins were chosen to be assessed in KLC 4315 - those representing the processes of outer membrane maintenance, protein synthesis, stress response, DNA supercoiling and virulence. The objective of the research was to analyse the expression of these genes over the cell cycle, with the aim in mind to find a genetic marker for *C. jejuni* whose expression would signify the viability of a population and when not expressed, would be indicative of cellular death. Each of the potential viability determinants are discussed below.

5.5.1 MAINTENANCE OF STRUCTURAL INTEGRITY.

The bacterial envelope plays a crucial role in facilitating responses to environmental changes (Linder and Oliver, 1989). It is well-recognised that loss of more than half of total LPS from the Gram-negative envelope results in loss of the outer membrane permeability barrier and membrane integrity, ultimately resulting in cell death (Costerten, *et al.*, 1974; Coleman and Leive, 1979; Xu, *et al.*, 1982). Structural integrity of the *C. jejuni* outer membrane was monitored by RT-PCR of the *lpxA* gene, which encodes the enzyme involved in the rate-limiting step in the production of the major component of the outer leaflet, lipid A. Only recently characterised in *C. jejuni* (Ibbitt, 1997), studies with *E. coli* have illustrated that the *lpxA* gene product is essential for the production of LPS as null mutations in the gene are lethal (Galloway and Raetz, 1990). In *C. jejuni*-seeded NB#2 maintained at 25°C, *lpxA* expression was visualised up to 14
days post-inoculation - seven days after apparent 'die-off' as determined by CCDA plate counts (ie., when plate counts were zero). This demonstrates that de novo protein synthesis of the enzyme involved in the catalysis of lipid A precursors was maintained for a period of two weeks in Campylobacter populations which were composed of predominantly coccoid VNC cells. The inference from this is that VNC cells of C. jejuni produced under the conditions of this study retain an intact cell membrane and the capability of repairing any damage - properties associated with and deemed necessary for viability.

5.5.2 MAINTENANCE OF PROTEIN SYNTHESIS ABILITY.

rRNA has been suggested as an indicator of viability in Mycobacterium smegmatis (Van der Vliet, et al., 1994). The authors found in cells exposed to rifampicin and ofloxacin, the presence of 16S rRNA, as detected by the nucleic acid sequence-based amplification (NASBA®) method, corresponded to viable cell counts and a lack of signal coincided with loss of viability. However in the current study, a 23S rRNA signal was observed in C. jejuni liquid culture for the time frame over which the experiments were conducted, indicating rRNA was not likely to be a suitable target molecule for monitoring viability. Although the RT-PCR band intensity decreased somewhat over the three-month post-inoculation period, a level of extinction for ribosomal RNA expression was not achieved. This result is not without reason. Ribosomal RNA is a relatively stable molecule due to nucleotide changes (Bjork, 1996) and its close association with ribosomal proteins as the major intramolecular component of bacterial ribosomes. The tight ribosomal configuration may confer on the rRNA a high level of resistance to endogenous degradative enzymes. Additionally, amplification of only a portion, not the entire, rRNA molecule has been reported to produce an amplicon (Uyttendaele, et al., 1997).

Of the five gene targets tested in this research, rRNA was chosen for its ubiquity, stability, and constitutive expression. For protein synthesis to transpire, the bacterium
has a requirement for intact ribosomal structure before translation of mRNA species can occur. As in *E. coli* approximately two-thirds of the ribosome is rRNA (Neidhardt, *et al.*, 1996), intact rRNA potentiates ribosomal integrity. Expression of 23S rRNA is, therefore, a measure of the protein-synthetic ability of a cell. The discussed results for *C. jejuni* KLC 4315 demonstrate mRNA transcription (see Section 5.3.1) and mRNA translation (see Section 5.3.2) is possible in 14-day-old coccoid populations.

5.5.3 INDUCTION OF THE STRESS RESPONSE.

The well-characterised heat shock response appears to be universal among the prokaryotic and eukaryotic kingdoms. Organisms produce a specific subset of highly conserved, coregulated proteins in response to elevated temperatures as well as a variety of other physiological stress factors, such as anoxia, ethanol, or heavy metal exposure (Neidhardt, *et al.*, 1984; Lindquist and Craig, 1988; Willimsky, *et al.*, 1992). However, more recent evidence suggests these proteins may also play a role in normal functioning of a cell under non-stress conditions, as molecular chaperone proteins (Mayhew and Hartl, 1996) involved in cytosolic protein folding. One of the most characterised hsp is encoded by the *dnaJ* gene. The DnaJ protein has been demonstrated to bind non-native polypeptides and prevent protein aggregation in *E. coli* (Langer, *et al.*, 1992).

Recent mutagenesis studies of the *dnaJ* gene in *C. jejuni* has indicated a role in thermotolerance, with *dnaJ* mutants severely retarded in their ability to form colonies at 46°C (Konkel, *et al.*, 1998). Konkel and collaborators hypothesised that on confrontation with environmental stresses an increase in synthesis of specific stress-related proteins might result. Due to their hurried production, this may result in an increase in misfolded or unfolded polypeptides within the bacterial cell. Konkel (1998) postulated increased synthesis of hsp would be necessary for proper folding of these polypeptides and, therefore, crucial for cellular survival. This hypothesis was tested in *C. jejuni* by constructing a null mutation in the *dnaJ* gene, coding for such a hsp (Konkel, *et al.*, 1998). *dnaJ* mutants generated were shown to be incapable of colonising
chickens, which suggest they were unable to overcome the stresses encountered on entering an avian host, which include changes in temperature and pH upon passage through the stomach to the caecum.

In the current study, the expression of dnaJ was monitored in C. jejuni KLC 4315 over a three month period. dnaJ mRNA was shown to be produced for the first ten days post-inoculation but not at any time intervals after this point. By day ten, plate count data shows complete loss of culturability of the culture and light microscopy findings indicate the population is entirely coccoid in morphology. Thus, it appears coccoid cells of Campylobacter are able to produce a stress response or continue with the constitutive expression of molecular chaperones for some period of time. These results corroborate the above mutagenesis study, and, if the hypothesis put forward by Konkel (1998) is correct, the suggestion that the DnaJ protein plays a role in refolding of stress-denatured and misfolded proteins and chaperonin-assisted assembly of newly synthesised polypeptides may be founded. Such an hypothesis could be tested with a repeat experiment employing a dnaJ mutant strain.

5.5.4 EXPRESSION OF A DNA-BINDING PROTEIN.
The studied mRNA species, hupB, encodes for the β subunit of the histone-like nucleoid protein HU. This protein has a role in congregation and conformational changes to the DNA (Storts and Markovitz, 1988). Mutagenesis studies have indicated deletion of the hupB gene is not lethal, however mutants consistently make more frequent errors in DNA-binding and segregation (Storts and Markovitz, 1988).

Analysis of hupB gene expression over the experimental time-period demonstrated this mRNA species was transcribed for the first four days post-inoculation. No amplicon was observed in the daily samples past this point. The author hypothesises that on introduction into the liquid broth, rod cells of Campylobacter react to the lower than optimum temperature (25°C) by inducing a response (similar to that seen with environ-
mental or nutritional stress) and start the process of DNA condensation. This interim survival strategy would involve the *hupB* gene product.

Possible explanation for the observed results come from two avenues. Firstly, it may suggest that the DNA is wound up tightly in the first four days - thus reflected in a lack of gene expression after this time point and possibly in the change in culturability of the population too. Secondly, Claret and Rouviereyaniv (1997) studied the stability of the HU protein. They determined that it is a relatively stable polypeptide and, as such, it is possible that the *hupB* gene does not need to be constitutively expressed as the protein is not readily degraded. In fact, expression of *hupA* and *hupB* genes has been shown by these workers to vary over the different growth phases of *E. coli*. Histone stability in eukaryotic systems may also prove a good comparison.

5.5.5 ANTIGENIC OR VIRULENCE FACTOR EXPRESSIONAL ANALYSIS.

An invasion antigen of *Campylobacter* is encoded by the *ciaB* gene (Konkel, *et al.*, 1999b) and was included in this study as a negative gene expression control. Only recently characterised in *C. jejuni*, direct sequence analyses revealed that the deduced CiaB amino acid sequence exhibited the greatest identity to an adhesin protein from *Mycoplasma hominis*, as well as similarity to secreted proteins from a number of pathogenic Gram-negative bacteria - specifically, SipB (*Salmonella invasion protein*) from *S. typhimurium*, IpaB (invasion plasmid antigen) from *Shigella flexneri*, and YopB from *Yersinia* spp. Functional analysis of CiaB product was performed to qualify these findings (Konkel, *et al.*, 1999b). *C. jejuni ciaB* mutants were tested for their ability to bind to (adhere) and enter cultured cell lines. Binding efficiency was found to be indistinguishable from that of the non-mutant parental isolate. However, a significant reduction in internalisation of mutants was observed when compared to the parental isolate. This data demonstrates a null mutation in this gene results in a non-invasive phenotype and indicates synthesis of functional CiaB protein is required for internalisation of the bacterium into host cells.
In the current study, *ciaB* mRNA was not induced at any time-point. This result was as predicted. CiaB expression is only activated in the presence of host (typically, mammalian) cells. The invasive phenotype associated with *ciaB* expression is likely to be due to recognition of CiaB by a host cell receptor allowing for adherence and internalisation of *Campylobacter*. As in the absence of host cells *ciaB* is not expressed, it would be an interesting follow-up experiment to add cultured cells to the media in an effort to induce *ciaB* mRNA transcription. Due to time constraints, this concept was not pursued further in this thesis.

### 5.6 RNA STABILITY DURING THERMAL INJURY.

#### 5.6.1 RNA DEGRADATION.

There is not much known about the stability of the RNA molecule outside the bacterial cell. It has been established that messenger RNA (mRNA) is a fairly unstable molecule, with most bacterial mRNAs having a very short half-life, on the order of 0.5 to 2 minutes (King and Schlessinger, 1987). Especially vulnerable to chemical degradation at alkaline pH, the rapid decay of mRNA can also be attributed to the combined action of endo- and exonucleases to convert full-length transcripts into mononucleotides (Kushner, 1996). These RNA-degrading enzymes (RNases) are ubiquitous in the bacterial cell and the environment (Farrell, 1993). Boiling at 100°C (Savill, 1997; Uyttendaele, *et al.*, 1997) may possibly denature RNases leaving RNA undegraded or only partially degraded and thus available for amplification. However, RNases are remarkably stable enzymes which maintain their tertiary configurations by virtue of four disulphide bridges that allow these enzymes to renature quickly, even after boiling (Farrell, 1993).

#### 5.6.2 PREVIOUS EXPERIMENTATION ON RNA STABILITY.

1998; Uyttendaele, et al., 1997), rather than changes in gene expression over the cellular life cycle (this study).

Early studies (ie., prior to PCR) indicated heat-shock resulted in cell death due to heat denaturation of proteins and RNA. Rosenthal and Iandolo (1970) reported on thermally induced intracellular alterations of rRNA. As a result of heating at 55°C for 15 minutes, unwinding of the 23S rRNA of Staphylococcus aureus was observed, which on cooling and reannealing resulted in net loss of helical configuration. A later study showed only minor degradation of 23S rRNA of Salmonella typhimurium after 30 minutes heating at 48°C, although there was total loss of 16S rRNA (Tomlins and Ordal, 1971).

With the onset of RT-PCR technology, continued studies into RNA stability during heat treatments took place. Uyttendaele (1997) found heating a suspension of C. jejuni cells (10^8 CFU. ml^-1) at 100°C for ten minutes killed (or inactivated) the bacteria (ie., no growth on CCDA), but did not affect 16S rRNA stability or did not lead to a complete degradation of rRNA over the five-hour post-heating period (Uyttendaele, et al., 1997). Thus it was determined nucleic acid sequence based amplification (NASBA®) of the 16S rRNA sequence did not correlate to the viability of C. jejuni.

Sheridan (1998) used RT-PCR to evaluate the stability of mRNA from one constitutively expressed E. coli housekeeping gene (tufA, a gene encoding an elongation factor) and two genes of a stress response regulon (rpoH and groEL) (Sheridan, et al., 1998). The conclusion made was that mRNA was degraded more quickly in heat-killed cells (killed at 100°C and 80°C) than bacteria killed by either 50-60% ethanol. These authors suggested mRNA disappears more rapidly from bacteria killed by treatments that do not inactivate the degradative RNase enzymes and that the overall persistence of mRNA in dead cells depends on the method used to kill the bacteria.
In 1998, a second paper was published describing an RT-PCR assay for the detection of thermophilic *Campylobacter* species (Sails, *et al.*, 1998). mRNA of *C. jejuni* NCTC 11168 could be detected up to three hours post-treatment (72°C for five minutes), but not at four hours. The mRNA species amplified by RT-PCR is the transcript of an open-reading frame adjacent to and downstream of a novel two-component regulatory gene, developed by Jackson and colleagues (1996) (Jackson, *et al.*, 1996; cited from Sails, *et al.*, 1998).

5.6.3 THE CURRENT STUDY.

In the current study, RT-PCR methods have been developed for detecting specific mRNA of *C. jejuni* in an effort to differentiate between viable and dead cells. To eliminate possible denaturation of RNases by extreme heat treatment (100°C) *C. jejuni* cells were killed by treatment with moderate heat (65°C for 20 minutes). Enumeration of the culture on CCDA plates revealed that the cell cultures had been rendered nonculturable within five minutes of heat induction. However, positive RT-PCR signals were noted for *lpxA* mRNA after bacterial ‘die-off’ occurring five minutes into the heat treatment, as determined by plate counts. Results show that mRNA was initially present in cells of *C. jejuni* inactivated by heat but subsequently disappeared on maintenance at ambient temperature. The findings correlate with that of Sheridan and collaborators (1998), who detected mRNA from *groEL*, *rpoH* and *tufA* genes initially in cells killed by heat or ethanol (Sheridan, *et al.*, 1998). The subsequent disappearance of the mRNA signal was at a rate dependent on the inactivating treatment. Conversely, Bej (1996) detected no mRNA in cells of *Vibrio cholerae* that were killed by heat or starvation (Bej, *et al.*, 1996). Similarly, Patel (1993) did not detect a heat shock protein mRNA from *Mycobacterium leprae* killed by heat treatment, probably because the sample was left at room temperature for five hours before analysis, allowing for any surviving mRNA to be degraded.
The length of time that the *lpxA* mRNA signal was observed for is longer than reported in the literature. In heat-killed cells of *E. coli* the mRNA targets became negative after 2-16 h; however, after ethanol exposure mRNA was still detectable after 16 h (Sheridan, *et al.*, 1998). Sails (1998) reported detection of mRNA up to three hours post treatment (72°C for five minutes). In contrast, the mRNA transcript from the *lpxA* gene was evident up to 96 h post-heating in this thesis.

Possible explanations for the extended detection of *lpxA* mRNA include:

- *lpxA* mRNA may still be stable at 65°C. This seems unlikely as Sails (1998) targeted a matching length RNA fragment and subjected it to a similar thermal injury programme, yet lost the signal much earlier into the experiment. To justify this hypothesis, an alternative experiment could be performed targeting the *dnaJ* gene over a heat treatment.

- 65°C may not be sufficient for killing of the entire population (although plate counts became non-detectable). To qualify this statement, total direct count and direct viable count would need to be performed.

- Although unlikely, *lpxA* may prove to be a better determinant of viability then has been tested before.

In conclusion, if *lpxA* mRNA was to be present in cells which have been rendered “dead” by some artificial means, then it would prove to not be a good marker for viability. In other words, a marker is desired that reflects the life or death phase of a population. If cells are non-viable the genetic marker would need to lose its signal soon after die-off of the culture. What constitutes a reasonable time-frame past the point of total cellular death is debatable (Sheridan, *et al.*, 1998). As mentioned previously, the expression of a mRNA species in a dying or killed culture is highly dependent on the inactivating treatment and the presence/absence of RNases. Analysis of an autoclaved broth showed that RT-PCR products disappeared immediately after autoclaving (ie., at zero time), but this could not necessarily be attributed to RNase degradation as heating
at 121°C under pressure would surely inactivate these degradative enzymes initially (Farrell, 1993).

The factors controlling mRNA longevity in dead cells in not understood (Brawerman, 1987), but presumably mRNA would disappear most rapidly from cells killed by treatments that do not inactivate the degradative enzymes. Conversely, mRNA may remain intact for longer periods of time in cells killed by treatments that also inactivate RNases or render the RNA resistant to attack. In other words, RNases may be denatured by high to extreme temperatures leaving the RNA undegraded or only partially degraded and thus available for amplification. However, as noted previously (Section 5.6.1) RNases are very stable enzymes with the ability to renature quickly (Farrell, 1993).

rRNA has been suggested as an indicator of viability. In this study, 23S rRNA signals from heated C. jejuni cells did not disappear after 216 h of incubation in broth at ambient temperature. It was not, therefore, a useful indicator of viability for the time frame over which the experiments were conducted (ie., 216 h). This result is supported by a number of workers investigating the loss of ribosomal RNA signal in several organisms after heating at a variety of temperatures (Uyttendaele, et al., 1997; McKillip, et al., 1998; Sheridan, et al., 1998).

However, the presence of an rRNA signal indicates the relative stability of either the complete 23S rRNA or the target sequence for amplification. Uyttendaele (1997) demonstrated a positive RT-PCR signal can be generated by amplification of small amounts of degradation product of the RNA enclosing the target sequence for RT-PCR. This may have occurred in the current study, with weakening of the RT-PCR detection signal over time possibly indicating gradual degradation of rRNA. This degradation of RNA is probably caused by released bacterial intracellular enzymes.
5.7 SIGNIFICANCE OF THE RESULTS.

A number of studies have been performed on maintenance of nucleic acids over time in bacterial systems. Weichart and Yamamoto found nonculturable cells of the organisms, *Vibrio vulnificus* and *Legionella pneumophila* respectively, retained intact RNA and DNA (as visualised by gel electrophoresis) which became degraded during long-term incubation (Weichart, *et al.*, 1997; Yamamoto, *et al.*, 1996). It has been reported that the PCR amplification of DNA sequences from VNC cells of *V. vulnificus* requires over 10,000 times more extracted DNA than the amplification of the same sequence in samples of growing cells (Brauns, *et al.*, 1991). It appears that the degradation of DNA in aged VNC cells is responsible for the lack of amplification of specific DNA sequences (Weichart, *et al.*, 1997). Warner and Oliver (1998) described changes in randomly amplified polymorphic DNA profiles in VNC cells of *V. vulnificus* over time. They suggested this was due to either DNA binding by proteins or chromosomal supercoiling, or a combination of the two, resulting in the alteration of the chromosome in such a manner that sequences complementary to the primer or DNA polymerase binding sites may become inaccessible.

Lack of amplification was not a finding observed with DNA extracts from cells of *C. jejuni* KLC 4315 taken over the continuum of cellular life. Over the six-month period post-inoculation DNA amplification by PCR was possible with all primer sets. This corroborates reports on the persistence of DNA in environmental settings (Josephson, *et al.*, 1993; Masters, *et al.*, 1994; Calder, 1998). However, in this thesis electrophoretic analysis demonstrated log-phase cells maintained intact chromosomal DNA which migrated as a single band with a high molecular mass, while native DNA from six-month old VNC cells yielded a smear-like DNA pattern, indicative of partial degradation. Because PCR is a sensitive molecular technique, amplicon production may have resulted from primer extension of intact or partially degraded DNA sequences.
Degradation of RNA and the release of the degradation products have been reported to occur in populations of starved or stressed bacteria (Davis, et al., 1986; Strange and Shon, 1964). In this thesis, mRNA species were monitored over a three month time period. During the length of experimentation the RT-PCR signal was noticed to decrease in intensity in all the gene sets targeted. Indeed, quantification showed a slight drop in total RNA levels. This suggests some degree of degradation, and although RNA extraction was not any harder to perform on older cells, amplification was still possible with the more stable rRNA target at three months.

Thus, the findings of the current study correlate with the stages of culturability loss, as hypothesised by Weichart (1997) and McDougald (1998) (see Figure 2.1) (Weichart, et al., 1997; McDougald, et al., 1998). These authors suggest during Stage I cells transform to a nonculturable state yet maintain viability. It is during this stage that cellular integrity and intact nucleic acids are retained - thus, the potential for metabolic activity, respiration and gene expression. The results presented in this thesis suggest that in C. jejuni maintained under the conditions of this study, Stage I exists from days 1 to 14. During this initial two week period, the cells of C. jejuni KLC 4315 convert to a nearly 100% coccoid population, with continued structural cell membrane integrity (as determined by lpxA gene expression). Genes involved in both normal cell functioning and in stress responses are activated (ie., dnaJ and hupB). It is likely that on introduction to the nutrient broth, the rod cells of C. jejuni begin a process of energy minimisation. DNA is condensed (as shown by hupB gene expression for the first four days) and the dnaJ gene product functions either as a chaperonin for protein transport and assembly, or utilises its stress capabilities and negates the effects of deleterious or misformed polypeptides. Electrophoresis indicates maintenance of intact nucleic acids (ie., intact enough that they can be used as substrates for PCR amplification) although the band intensity decreases as the incubation period became longer.
With extended time in this Stage I VNC state, McDougald (1998) postulates the coccoid cells undergo degradative changes (termed Stage II), resulting in a non-viable nonculturable phenotype - cellular death (McDougald, et al., 1998). Conclusions from the current study demonstrate loss of gene expression signals by the three week time point in all mRNA species tested. Although rRNA and DNA amplification was possible past this time point, this result is dismissed as potentially artifactual due to the prolonged stability and persistence exhibited by these two molecules. Electrophoresis of extracted RNA and DNA samples later into the experiment (i.e., at three and six months post-inoculation respectively) indicated the onset of cellular depreciation. With a lack of \textit{lpxA} and \textit{fabZ} gene expression after two weeks, it is proposed that gradual loss of cellular integrity has occurred and the cell has entered the death phase. Additionally, a lack of expression from the two stress-related genes (\textit{hupB} and \textit{dnaJ}) corroborates such a proposal. A similar phenomenon has been suggested for \textit{H. pylori}, with two diverse types of VNC cells reported (Mizoguchi, et al., 1998).

This thesis aimed to investigate the life/death state of a population and in doing so have examined 'essential' genetic products required for a cell to be alive. Davis (1986) proposed that, on stress induction, there were three predictable mechanisms of cell death, each of which resulted from the loss of an 'essential' cellular component (Davis, et al., 1986):

- The first mechanism involves irreversible damage to a vital element in the genome. This has since been dismissed as unlikely as cells have elaborate devices for protecting their DNA, including the HU protein discussed in this thesis. Additionally, studies into the persistence of DNA in artificially killed cultures (this study) demonstrate this not to be the cause of cellular death.

- The second mechanism is based on irreversible damage to the cell membrane. Such a mechanism is clearly important in organisms that autolyse readily during starvation (e.g.,
Streptococcus pneumoniae). However, the importance in enterobacteria is debatable (Davis, et al., 1986). This thesis suggests a possible link between lipid A biosynthesis (as seen by lpxA mRNA production) and the maintenance of cellular structure and viability. Indeed, non-production of lipid A precursors (shown by null mutations in lpxA) is conditionally lethal in E. coli (Galloway and Raetz, 1990). This study and others like it suggest a loss in cell membrane integrity may in fact cause cell death.

- The third mechanism involves complete loss of a certain species of macromolecule, be it most enzymes, cofactors, any kind of RNA, components of energy-transducing systems, ribosomes or RNA polymerase. These products would be restorable if their genes were intact and if necessary building blocks and energy could be provided. However, the complete loss of any protein species required for protein synthesis should be lethal. Davis (1986) suggested the degradation of ribosomal structure as a possible method for cell death as it is a well known response of bacteria to starvation (Davis, et al., 1986). The theory suggests that ribosome breakdown is initiated, resulting in redistribution of nutrients. With extended time in a stressful environment, this adaptive modification becomes a suicidal route. This proposal by Davis is partially backed up by evidence presented in this thesis. A decrease in rRNA band intensity was witnessed over the experimental time-frame (three months) and because the ribosomal structure is over two-thirds rRNA, this may correlate with decreasing ribosomal numbers and the potential loss of viability.

5.8 FURTHER WORK.

- FURTHER INDEPENDENT MEASURES OF CELLULAR VIABILITY.

Due to time and resource constraints, an independent measure of cellular viability was unable to be explored in this research. Such a method may be the continuation of direct viable count methodology, the employment of either rhodamine or similar dyes to measure the membrane potential, or the use of a commercial kit (eg., Baclight from Molecular Probes). Data supplied from any of these methods would serve as an
independent assessment of viability and may add qualification and validation to observed results.

- DECAY RATES OF mRNA.
Many workers have attempted to determine whether VNC cells retain their virulence and are able to cause an infection (see Section 2.3.2). The purpose of this study was not to determine whether these cells display retention of pathogenicity, but to determine if indeed cells in this state remain viable following loss of culturability. If VNC cells are determined to be non-viable, then concern regarding their infectious capability should prove unfounded - unless the bacterium under study causes illness through exotoxin release. If, however, the VNC proves to be a survival mechanism, characterised by metabolic inactivity or reduced activity (Roszak and Colwell, 1987) with a role in the contamination cycle, this is a potential threat to public health microbiology.

Work using mRNA as a viability determinant suggests the latter may be so (Klein and Juneja, 1997; Mizoguchi, et al., 1998; this study). Such studies rely on the detection of mRNA indicating either the cell is alive or recently dead. However, as the RNA extraction process renders the bacterium dead, there is a need to qualify the last statement with further work to characterise the decay rates of mRNA in killed cells under a range of conditions. Of particular interest, would be the expression of the dnaJ gene in heat-killed samples. This approach was not pursued in this study, purely due to time constraints, but has been considered by the Institute of Environmental and Scientific Research.

- ANALYSIS OF COCCOIDS FORMED AT DIFFERENT TEMPERATURES.
Associated with the previous point is the need to examine the viability of coccoid cells of C. jejuni formed at different temperatures. Physiological studies have suggested that coccoids formed at lower temperatures (4°C) may maintain a survival state for longer periods of time (Rollins and Colwell, 1986; Hazeleger, et al., 1994/1995/1998). As
such, the incentive to test for gene expression in these cells is justified. Protein synthesis has been observed in C. jejuni strains at temperatures as low as 4°C - indicative that vital cellular processes are still functioning. This was studied by the incorporation of $[^{35}\text{S}]$ methionine autoradiography (Hazeleger, et al 1998). Studies may indicate differential levels of gene expression in cells formed at varying temperatures.

• OBSERVATION OF OTHER Campylobacter SPECIES.
Further experimentation must take into account that the VNC response is not likely to be a single phenomenon (Barer, et al., 1998). It must be recognised that there may be several underlying causes of the failure to culture different microorganisms, or the same microorganism under different culture conditions. As such, the conversion kinetics of other C. jejuni strains may need to be studied to exclude the possibility that strain KLC 4315 is a variant with exceptional properties.

• VNC, STARVED AND STATIONARY-PHASE CELLS.
The link between the VNC state and the stationary-phase and starvation response needs to be established. The induction of genes specific for the latter two stages in the bacterial life cycle is well documented (Lange & Hengge-Aronis, 1991; Oliver, et al., 1991; Weichart, et al., 1993). The coordinated response to stationary-phase, or to carbon (or multiple nutrient) starvation has been reported to involve the induction of stimulons and regulons, some of which overlap with known stress responses (Nyström, et al., 1990). This suggests that genetic changes witnessed in starved or stationary-phase cells may be potentially compatible to changes observed in VNC cells. Indeed, the multitude of studies on the morphological and physiological changes during stationary-phase and starvation (Amy and Morita, 1983; Kjelleberg, et al., 1983; Humphrey, et al., 1983; Reeve, et al., 1984; Matin, et al., 1989; Preiss, 1989; Siegele and Kolter, 1992; Huisman, et al., 1996) mirror observations already made on a number of different organisms undergoing loss of culturability. Essentially, they represent similar phenomena - a process of physiological down-regulation in result to an environmental
stimuli. Thus, analysis of observations made in starved bacterial cultures or those entering stationary-phase may provide further insight into the role of the VNC state.

- **QUANTITATIVE AND QUALITATIVE DETERMINATIONS.**

  It has been noted that the second stage of loss of culturability involves degradation of RNA (see Figure 2.1). The application of techniques such as TaqMan technology would be useful to determine cellular metabolism, as these methodologies quantitate extracted nucleic acid levels, giving an indication of ongoing protein synthesis and metabolic activity or macromolecular degradation. Such a line of experimentation is being pursued at the Institute of Environmental and Scientific Research (ESR) (Christchurch) at present.

- **MULTI-MARKERS OF RNA.**

  Multiple-marker analyses can also be performed. Due to the random priming of the extracted RNA in the assay developed in this thesis, there is potential to probe an environmental or clinical sample for both viable and virulent organisms. The isolated RNA could be PCR-amplified with a viability marker (eg., \(lpxA\)) and a virulence marker (eg., \(ciaB\), \(cadF\) or the \(fla\) genes). When ingested with food or water *Campylobacter* cells enter the host intestine via the stomach and colonise the distal ileum and colon (Ketley, 1995). Production of flagella, which are the best-characterised virulence determinants of the *Campylobacter* spp., is necessary for adhesion and colonisation (Ketley, 1995). Waage (1999) used a seminested PCR assay for the detection of the intergenic region between the two *Campylobacter* flagellin genes, \(flaA\) and \(flaB\) (Waage, *et al.*, 1999). One of these genes could thus be used in combination with an indicator of viability. Additionally the afore-mentioned \(ciaB\) gene or the *Campylobacter* adhesion factor (\(cadF\)) gene may be of use (Konkel, *et al.*, 1997; Konkel, *et al.*, 1999a; Konkel, *et al.*, 1999b).
In Vivo Studies

As cited from (Joux, et al., 1997), "simple laboratory systems cannot, of course, capture all the richness and complexity of natural ecological systems, however, such systems do permit careful analyses of population dynamic under well-defined conditions and moreover, rigorous experimental test of mechanistic hypotheses".

Bacterial growth and survival in vivo is often very different from that seen in laboratory culture (Matin, et al., 1989). It is generally believed that bacteria in the human gut are growing very slowly or are in the stationary phase due to competition with other species for nutrients and lack of availability of essential nutritional requirements such as iron (Griffiths, 1993). Analysis of cells in vivo by the mRNA-RT-PCR methodology may provide further insight into the virulence mechanisms of Campylobacter.

Gene Sequencing.

Sequencing of random-primed cDNA may provide a better indicator of viability. Certain proteins may be only expressed in dying Campylobacter cells. Genetic and functional analyses of these proteins should lead to more definite assessment of importance of 'survival' mechanisms for C. jejuni since their regulation would be expected to be specifically coordinated unless merely a reflection of cellular degeneration.

5.9 Conclusion.

Results presented in this thesis demonstrate further evidence that nonculturability and non-viability are not synonymous. Discrepancies between the culture (plate count) method and the molecular amplification technique should probably be ascribed to inherent limitations of the cultural method. Thus, the use of mRNA-based RT-PCR may provide a better indication of the viability / non-viability of a bacterial population, if a suitable genetic determinant can be located.

The five different RNA species detected in this study were selected to represent two genes functional under normal conditions and also postulated to play a role in the stress
response (\textit{dnaJ} and \textit{hupB}); a constitutively expressed, ubiquitous gene (23s rDNA); a gene involved in infectivity of the bacterium; and a gene encoding a structural, cellular housekeeping proteins (\textit{lpxA/fabZ}). The levels of expression of these genes differed markedly, with \textit{lpxA} and \textit{dnaJ} mRNAs being detected for a longer time after inoculation into liquid media (14 and 10 days respectively). This may be correlated with the stability of the different mRNA sequences targeted by the oligonucleotide primers designed here or to the relative abundance of each mRNA in a given cell. Either way, it appears that the type of mRNA selected for detection of viable bacteria will be important.

This work demonstrates that of the four species of nucleic acid, mRNA is the most promising candidate as an indicator of viability in bacteria. Of particular concern was developing a suitable genetic marker for viability in environmental samples. Regarding the criteria proposed by Sheridan (1998) for suitable viability determinants (see Section 5.2.3), \textit{lpxA} mRNA fits all three with respect to use in \textit{C. jejuni} studies performed in this research:

- \textit{lpxA} mRNA is only present in viable cells. The author was unable to detect mRNA amplification of the target \textit{lpxA} gene by the RT-PCR assay, following exposure of the \textit{C. jejuni} cells to extreme heat (autoclaving at 121°C for 20 min) indicating the sensitivity of bacterial mRNA to rapid degradation as well as the tight association between \textit{lpxA} mRNA and cell viability.
- The kinetics of the disappearance of the \textit{lpxA} signal was related to loss of viability (as determined by \textit{lpxA} gene expression studies over time).
- \textit{lpxA} mRNA disappears from cells soon after death (as demonstrated by the heat-kill experiment).

The results indicated that either the \textit{lpxA} gene or the \textit{dnaJ} gene would provide a good target for the development of a specific method for detection of viable \textit{C. jejuni} in environmental samples based on RT-PCR amplification. These two genes appear to most closely mimic the plate count phenomenon. This approach has been taken up by ESR Christchurch for further examination and validation studies.
The work presented in this thesis consolidates the potential viability of *C. jejuni* coccoids - at least for a certain period of time. Instrumental to this observation is the necessity to qualify it by saying that this is only under the experimental guidelines established for this study. This work demonstrates that (i) for a two-week period coccoid forms of *C. jejuni* KLC 4315 retain cellular structure compatible with viability (ie., an intact cell membrane as determined by *lpxA* gene expression) and (ii) some degree of protein synthesis is still occurring early in the described experimental time-frame (as shown by *dnaJ* and *hupB* mRNA and 23S rRNA presence). Whether these coccoid forms of *C. jejuni* are viable *in vivo* and/or in *in vitro* environmental settings (ie., not within the laboratory ecosystem), whether they represent a temporary adaptation to a particular environment (ie., potentially resuscitable), and whether they are involved in the transmission of the bacterium remains open questions.
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References


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References


References


References


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APPENDIX I
MEDIA

II. i. GENERAL MEDIA.

Unless otherwise stated, all media were sterilised by autoclaving for 20 min, 121°C at 120kPa. Solutions that were labile at this temperature were filter-sterilised through a 0.22µm filter (Millipore) prior to addition to sterile media.

**Brain Heart Infusion Broth (BHI)**

Typical formula (g/L): Per litre

- Beef heart infusion: 25g
- Calf brain infusion: 20g
- Protease peptone: 10g
- NaCl: 5g
- Na₂HPO₄·12H₂O: 2.5g
- Glucose: 2g

Dissolved in dH₂O and pH adjusted to 7.4 before autoclaving.

**Nutrient Broth No. 2 (NB#2)**

(Oxoid CM 67)

Typical formula (g/L): Per litre

- Beef extract: 10g
- Peptone: 10g
- NaCl: 5g

Dissolved in dH₂O and pH adjusted to 7.4 before autoclaving.

**Nutrient Broth No. 2 Agar (NB#2A)**

(Oxoid CM 67)

Typical formula (g/L): Per litre

- Beef extract: 10g
- Peptone: 10g
- NaCl: 5g
- Bacteriological agar: 20g

Dissolved in dH₂O and pH adjusted to 7.4 before autoclaving.
I. ii. SPECIALISED MEDIA.

*Campylobacter* Charcoal Deoxycholate/Differential Agar (CCDA)  
(Skirrow, 1977) (Oxoid CM 739)

Typical formula (g/L):  

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>10g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5g</td>
</tr>
<tr>
<td>Charcoal</td>
<td>4g</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>3g</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>1g</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.25g</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.25g</td>
</tr>
<tr>
<td>Bacteriological agar</td>
<td>12g</td>
</tr>
</tbody>
</table>

Dissolved in 1 litre dH₂O, adjusting the pH to 7.4 ± 0.2 before autoclaving.
Appendix II: Buffers and Solutions

II.i. COMMON BUFFERS.

Solutions requiring sterilisation were either autoclaved for 20 min at 121°C at 120 kPa or filter-sterilised through a 0.22μm filter. All solutions were stored at ambient temperature unless otherwise stated.

6 x DNA LOADING DYE FOR AGAROSE GEL ELECTROPHORESIS

50% (v/v) Glycerol
0.25% (w/v) Bromophenol blue
0.25% (w/v) Xylene cyanol FF

Dissolved in dH₂O.

0.5M EDTA (50mM Na₂EDTA) (pH 8.0) Per litre

Na₂EDTA·2H₂O 186.1g

Dissolve in dH₂O with magnetic stirring, adjusting the pH to 8.0 with 10M NaOH.

Ethidium Bromide (10mg.ml⁻¹)

Dissolve 0.2g Ethidium Bromide in 20ml dH₂O with a magnetic stirrer. Store at 4°C in the dark.

PBS (PHOSPHATE BUFFERED SALINE) Per litre

NaCl 80g
KCl 2g
Na₂PO₄·7H₂O 11.5g
KH₂PO₄ 2g

Dissolve in dH₂O to a final volume of 1 litre, pH adjusted to 7.4.
Appendix II: Buffers and Solutions

**SET BUFFER**

75mM NaCl  
25mM EDTA  
20mM Tris-Cl (as prepared in this appendix)

50 x TAE (Tris Acetate EDTA)  
Per litre

- 2.5M Tris base  
- 0.11% (w/v) glacial acetic acid  
- 50mM Na$_2$EDTA (pH 8.0)

Dissolved in dH$_2$O to a final volume of 1 litre, pH adjusted to 8.0.

1 x TAE

20ml of 50 x TAE dissolved in ddH$_2$O to a final volume of 1 litre.

10 x TBE (Tris Borate EDTA)  
Per litre

- Tris base  
- Boric acid  
- 50mM Na$_2$EDTA (pH 8.0)

Dissolve in dH$_2$O to a final volume of 1 litre.

5 x TBE (Polyacrylamide gel electrophoresis)  
100ml of 10 x TBE dissolved in ddH$_2$O to a volume of 1 litre.

0.5 x TBE (Agarose gel electrophoresis)  
10ml of 10 x TBE dissolved in ddH$_2$O to a volume of 1 litre.

**TE BUFFER (pH 8.0)**

10mM Tris-Cl (prepared below)  
1mM EDTA (prepared above)

1 M TRIS (Tris (hydroxymethyl) aminomethane)  
Tris base  

Dissolve in 800ml H$_2$O. Adjust the pH to desired value with concentrated HCl. Add H$_2$O to a volume of 1 litre.
II. ii. SPECIFIC BUFFERS AND SOLUTIONS.

II. ii. a. CHROMOSOMAL DNA PREPARATION SOLUTIONS.

3M NaAC (Sodium Acetate) Per litre
NaAC·3H₂O 408g
Dissolve in dH₂O to a final volume of 1 litre, pH adjusted to 5.2 with glacial acetic acid.

5M NaCl (Sodium Chloride) Per litre
NaCl 295g
Dissolve in dH₂O to a final volume of 1 litre.

Phenol: Chloroform
Mix equal volumes of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1M Tris.Cl (pH 7.6). Store equilibrated solution under 0.01M Tris.Cl (pH 7.6) at 4°C in a dark glass bottle.

10 % SDS (Sodium dodecyl sulphate) Per litre
SDS 100g
Dissolve in dH₂O at 68°C to a final volume of 1 litre, adjusting the pH to 7.2 with a few drops of concentrated HCl.

II. ii. b. RNA ISOLATION SOLUTIONS.

β - ME (β- Mercaptoethanol)
Obtained as a 14.4M solution, storage is in a dark bottle at 4°C.

DMPC-TREATED WATER (Dimethyl pyrocarbonate)
Treat ddH₂O with 0.1% DMPC overnight at 37°C. Autoclave before use.