CHARACTERISTICS OF *CAMPYLOBACTER JEJUNI* AND THEIR RELATIONSHIP TO DETECTION METHODOLOGY IN FOOD SYSTEMS

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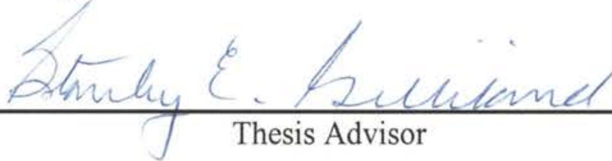

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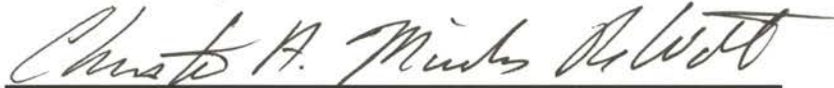

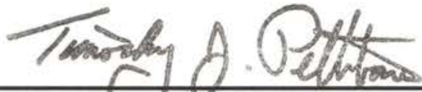

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PREFACE

The morphological change exhibited by *Campylobacter jejuni* and its influence on detection methodology was investigated in two parts: 1) methodology to manage this occurrence within the framework of detection of *C. jejuni* in food systems, and 2) the relationship between membrane fatty acid composition and the morphology of *C. jejuni*.

I wish to give enormous thanks to my committee members: Dr. P. Larry Claypool; Dr. Elizabeth Droke; Dr. Christina Dewitt and Dr. Stanley Gilliland. Also, I wish to convey a sincere thank you to my family, friends, colleagues and students.

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CHAPTER I

INTRODUCTION

Campylobacter jejuni is proving to be the leading cause of food borne illness in the world. Billions of dollars are spent each year on illnesses directly related to the consumption of food and water contaminated with this organism. Since the early 1970's the quest for understanding this organism has been made more difficult by its well-documented morphological changes. These changes have been linked to environmental stress factors such as incubation time, atmosphere, temperature, oxidation, and nutritional intricacies. Differences are also apparent among and within species. The morphology change begins with the organism in its classic spiral rod shape and then through an unknown mechanism the cells may straighten, shrink, elongate or curl resulting in a plethora of rod and round shapes. Often this morphological transition is linked with a loss of culturability, detectability or perhaps viability. The challenge is best described by the question: "Have we lost our ability to detect this organism or has this organism lost its ability to grow? And furthermore, if it has lost its ability to grow, is it still alive?" This very confusing phenomenon is discussed in terms of the "viable nonculturable" (VNC) state. When in this state, the organism remains alive but will not grow in agar or liquid media. If the VNC form is potentially pathogenic, then standard detection and enumeration techniques may underestimate the risk of infection from *Campylobacter* spp. At present, this complex interrelationship has not been sufficiently explained. So, instead

of focusing on detection of these wayward forms of *Campylobacter*, this dissertation addresses (1) management of this phenomenon within the context of detection of *C. jejuni* in food, and (2) the relationship between cellular fatty acid composition and morphology of *C. jejuni*.

CHAPTER II

REVIEW OF THE LITERATURE

History

The documentation of *Campylobacter* began as early as 1886 with sketches and notes published by Theodor Escherich, in which he described an organism found in the colons of infants who died of what was then called "cholera infantum" (Skirrow and Butzler 2000). There was little to add until a period in the early 20th century (1909 thru 1919) when sheep and cattle veterinarians became aware of a vibrio-like organism that was linked to spontaneous abortions. It was called *Vibrio jejuni* at the time and also was linked to devastating cases of diarrhea in cattle termed "winter dysentery." In 1963, the genus *Campylobacter* was proposed and the first distinctions were made between *Campylobacter fetus* and *Campylobacter bulbus* (now called *Campylobacter sputorum*) (On 2001). It was several more years before the distinction between the genera *Campylobacter* and *Vibrio* became accepted universally. In 1972, facilitated by advancements in media/isolation procedures, researchers successfully isolated *Campylobacter* from human feces (Skirrow and Butzler 2000). Only after this event, could the real accounting or risk assessments for *Campylobacter* begin.

Taxonomy

On (2001) reviewed the taxonomic status of the genus *Campylobacter* and identified 16 species and 6 subspecies. They are *Campylobacter mucosalis,*

hyointestinalis, fetus, lanienae, concisus, showae, rectus, sputorum, hominis, gracilis, upsaliensis, helveticus, coli, lari, and *jejuni*. *Campylobacter hyointestinalis* is comprised of two subspecies (*hyointestinalis* and *lawsonii*), *C. fetus* has two subspecies (*fetus* and *venerealis*), *C. jejuni* has two subspecies (*doylei and jejuni*), *C sputorum* is comprised two biovariants (*sputorum, paraureolyticus* and *faecalis*), and *C. coli* has only one variant (*hyoilei*). The genome of *Campylobacter* is reported as being relatively small consisting of 1.6-17 Mbp with a GC ratio around 30% (Simbert 1986; Ketley 1997; Kelly 2001). In this review, there is mention of both *C. jejuni* and *C. fetus* supsp. *jejuni*. These are one and the same, but the names used in the original publications are retained.

Campylobacteriosis

One of the earliest and largest reported outbreaks of enterocolitis attributed to *Campylobacter* occurred in 1978 when approximately 3000 people were sickened. The source was a community water supply (Vogt and others 1982; Tauxe and others 1988). Today, *Campylobacter* is the most common bacterial pathogen in both the United States and abroad (Friedman and others 2000). Within the genus, *C. jejuni* is responsible for the majority of *Campylobacter* enteritis cases in humans (Ketley 1997; Altekruse and others 1999; Skirrow and Blaser 2000).

Infection with *Campylobacter* begins by ingestion of the bacteria, passage through the stomach, and colonization in the distal ileum and colon (Ketley 1997; Skirrow and Blaser 2000). Reports indicate that very few (500-800) organisms are required for infection (Ketley 1997; Skirrow and Blaser 2000). The average incubation period is 3.2 days and mild symptoms are usually experienced for 3 to 4 days (Skirrow and Blaser 2000). Colonization is facilitated by the organism's motility and ability to

detect chemical gradients in the intestines (Ketley 1997). Following colonization, the organism may remain in the mucus or adhere to epithelial cells and become invasive (Ketley 1997; Skirrow and Blaser 2000).

Symptoms are minor in many cases, resulting in abdominal discomfort, diarrhea, fever, headache and dizziness. Serious consequences of infection may also arise in that *C. jejuni* is often associated with incidence of Guillian-Barré Syndrome (GBS). Infection with *Campylobacter* may stimulate an auto-immune response in the body resulting in acute neuromuscular paralysis due to demyelination (inflammation and subsequent damage to myelin) (Buzby and Roberts 1997; Altekruse and others 1999; Skirrow and Blaser 2000; Hadden and Gregson, 2001). Damage to myelin (an insulating layer of cells over the peripheral nerves) may lead to loss of axial, cranial, respiratory and peripheral muscle activity, leading to substantial immobility (Buzby and Roberts 1997). Epithelial translocation (traversing the epithelial cell barrier) followed by intracellular survival and activities are possible precursor mechanisms for this disease (Ketley 1997). *Campylobacter jejuni* enteritis occurred in 13-72% of GBS cases reported between 1984 and 2001 (Hadden and Gregson, 2001). Buzby and Roberts (1997) estimated the costs associated with GBS approached $1.3 billion, and when combined with the other consequences of food borne illness associated with *Campylobacter* the yearly costs approached $5.6 billion.

Surveillance of *Campylobacter* transmission began with only eight states in the US in 1982 and by 1983 thirty-one states joined in the effort to track outbreaks. The initial goals of the laboratories monitoring the occurrence of *Campylobacter* were to describe the epidemiology, detect and investigate the outbreaks, and to speculate on

future research needs (Tauxe and others 1988). In the first five years of surveillance, the group reported a total of 41,343 isolates to the Centers for Disease Control. Of those that were identified, 99% were *C. jejuni*. The predominant remaining pathogens that were identified were *C. fetus* and *C. coli* (Tauxe and others 1988). In the following years until 1986, there were a reported 11 water borne and 45 food borne outbreaks predominantly due to *C. jejuni*. Of the early food borne outbreaks, the largest percentage was attributed to raw milk, followed by poultry (Klein and others 1986). Between 1992 and 1994, of 21 outbreaks in England and Whales, contaminated water (n=6) was the most common source of outbreaks followed by unpasteurized milk (n=3), poultry (n=2), shellfish/poultry (n=1), pasteurized milk (n=1), bird pecked milk (n=1), meat products (n=2) and unknown (n=5) (Peabody and others 1997; Frost 2001).

The handling and/or consumption of raw chicken may be one of the leading routes for sickness related to *Campylobacter* (Yu and others 2001; Rosenquist and others 2002). Jorgensen and others (2002) evaluated raw, whole chickens for *Campylobacter* and *Salmonella* and found that 83% of the chickens were positive for *Campylobacter* (*Salmonella* was only present in 25% of the samples). The other sources that have played significant roles have been consumption of raw milk, contaminated water and contact with pets (Tauxe and others 1988). *Campylobacter* are commonly associated with the intestinal tracts and ultimately feces of many animals and transferred to the animal food product via cross contamination during the slaughter process (Ketley 1997; Rosenquist and others 2002). *Campylobacter* has been reported to exist in swine in higher numbers than either *Salmonella* or *Yersinia* species (Borch and others 1996). Borch and others (1996) also reported on a Danish study that evaluated the feces of 600 pigs from more

than 152 herds. Ninety-six percent of the fecal samples were positive for *Campylobacter*. Following the animals through slaughter and processing yielded 66% *Campylobacter* positive isolates on the carcasses before chilling, 14% one day after slaughter and 0.5% on the further processed products (ham). Cattle are also significant carriers of *Campylobacter* spp. Wesley and others (2000) found that fecal shedding of *C. jejuni* occurred in 38% of the samples. A Swedish study reported 85% of cattle fecal samples positive for *Campylobacter* (Borch and Arinder 2002). Kramer and others (2000) isolated *Campylobacter* from the following retail meats: chicken portions (n=165; 83%), lamb liver (n=70; 73%), ox liver (n= 52; 54%) and pig liver (n=71; 72%).

Of 111 outbreaks associated with *Campylobacter* spp. between the years 1978 and 1996, 99 were attributed to food while the remaining 12 were attributed to contaminated water sources (Friedman and others 2000; Food Safety Authority of Ireland 2002). The increasing prevalence of human *Campylobacter* infections can be clearly seen in Denmark. From 1980 to 1997 the number of infections rose from 1034 to 2666 registered cases annually. And in the year 2000, more than 4000 cases of campylobacteriosis were reported (Josefsen and others 2002). They estimated that the real incidence was probably twenty times higher due to lack of attention paid to milder symptoms. During this same period (1994), Norway and Sweden reported 1050 and 5529 registered human cases of food borne illness due to *C. jejuni* and *C. coli* (Borch and others 1996). Japan has reported more than 5000 cases each year between 1982 and 1999 (Ono and Yamamoto 1999). An estimated five million incidents of bacterial food borne illness occur each year in the United States and of those, 2.4 million cases per year are related to *Campylobacter* spp. (Mead and others 1999). Of these, 17.3 % result in

hospitalization and 5.5% result in death (Mead and others 1999). There has been little evidence suggesting that transmission of *Campylobacter* may occur via person-to-person contact (Buzby and Roberts 1997).

To summarize, the most common risk factors related to food borne illness due to *Campylobacter* spp. are consuming or handling undercooked or raw poultry, consuming contaminated water, consuming raw or under-pasteurized milk or dairy products, and contact with pets (i.e., cats, dogs, birds) that are shedding *Campylobacter* spp. (Food Safety Authority of Ireland 2002).

Characteristics

Campylobacter are generally characterized as Gram negative, nonsporeforming, microaerophilic, pathogenic, spiral-rod shaped bacteria with single polar flagellum that allows motility in diverse niches (Simbert 1986; Park 2002). In the spiral-rod form, the organism moves by using one or multiple flagella with a corkscrew type action. This motility, and the ability to detect chemical changes in the intestines, facilitates the process of infection (Ketley 1997). The flagella may help the organism move and/or proliferate in the mucous layer of the stomach and large intestines. They have a unique attraction to fucose, a monosaccharide found in glycoproteins and cell wall polysaccharides, and a major constituent of mucin (Hugdahl and others 1988; Park 2002).

Morphology

Campylobacter jejuni is known to undergo substantial morphological changes in broth and on agar media. Under various conditions, the organism may change from a Gram negative, spiral rod possessing a flagellum, to a round, semi-round, donut, or coccoid morphology, which may be Gram variable (observed in our laboratory). In

addition, there are a variety of intermediate forms. This morphological shift may be the result of various environmental stress factors placed on the organism in sub-optimal conditions.

Bacteria undergo, as do other living organisms, changes throughout their life cycle that are associated with age. With *Campylobacter*, it is difficult at times to separate the effect of culture age and the associated morphological changes from other environmental stress (i.e., nutrient availability) factors that challenge the culture. Ng and others (1985) used scanning electron microscopy to study the morphological forms of *Campylobacter* from a single colony-forming unit (CFU) selected from an agar medium after 48-72 hours of incubation. Photographs indicated that 1) the center of the CFU contained a population of coccoid/amorphous cells, 2) the intermediate region contained a number of "donut" or ring shaped cells with a hollow center, and 3) the outer periphery contained the normal spiral shaped cells associated with an actively growing culture. Perhaps this is an example of the confounding of culture age and nutritional status on morphology assuming a single cell produced the colony forming unit (CFU).

As *Campylobacter* ages, it takes on different forms and many of those forms are smaller than those in original culture. This pleomorphic phenomenon brought on by various conditions has been characterized as "size reduction." Byrd (2000) indicates that there are two mechanisms by which a bacterium may reduce in size: 1) Multiple genomic cells do not increase in size before dividing but subsequently become smaller after each division coupled with the transfer of at least one copy of the genome. Several strains of *Vibrio*, *Pseudomonas*, *Acinetobacter* and *Spirillum* are noted to undergo this type of reductive process. 2) Cells reduce in size without cell division and hence no increase in

cell numbers. Through either of these two processes, the cells lose some characteristic that they once possessed that made them detectable on culture media. If the cells reduce in size and do not transfer the genetic material then they may in fact be dead.

In contrast to size reduction in aged cultures mentioned by Byrd (2000), Griffiths (1993) observed increases in size of *C. jejuni* as cultures aged when grown in liquid culture. Cultures of the organism were grown in conical flasks continuously shaken (100rpm) at 37°C with a modified atmosphere. Samples were evaluated for total plate counts and morphology. Growth curves were typical with the traditional exponential phase, stationary phase (maximum log CFU/ml ≈ 9.0), and death phase. Morphological changes corresponded with different growth phases. Short, spiral rods were predominant in the exponential phase followed by a pronounced increase in cell size. For the mid-stationary phase there was an estimated 2-fold increase in size. In the late stationary/early decline phase, cells were 3 to 4 times longer than the original length, and the coccoid form was present. The late decline/death phase resulted in mostly coccoid forms. In the stationary phase there seemed to be a substantial increase in cell size preceded by cessation of septum formation, all of which leads to inhibition of cell division.

Thomas and others (1999) evaluated the morphological changes of *C. jejuni* grown in broth culture over a 72-hour incubation period at 37°C. Two *C. jejuni* strains (NCTC 11168 and 11385) were initially grown on Columbia Blood Agar plates. Cells were collected and grown in a biphasic (agar/broth) culture system (Rollins and others 1983) at 37°C for 24 hours in a modified atmosphere cabinet (5% O_2, 5% CO_2, 2% H_2 and nitrogen balance). This process was repeated (i.e., subcultured) and the new culture

was used to inoculate a final biphasic system incubated for 12 hours at 37°C. The liquid phase of this system was used to inoculate a single-phase broth system containing 100 ml of Nutrient Broth No. 2 (NB2). Samples were removed and examined for growth and morphological changes after 6, 12, 48 and 72 hours incubation at 37°C.

Typical growth curves were described containing the standard logarithmic, stationary and decline/death phase. Maximum cell populations ($\approx 10^9$ CFU/ml) were obtained within 24 hours. Scanning electron micrographs were used to examine morphological changes. The logarithmic phase was characterized by small helical, curved rods; the stationary phase had similar cells but 45% were larger, elongated, and less coiled; and the decline phase had 125% longer cells, many coccoid forms, and a loss of cellular integrity. Additionally, there were descriptions of localized cell expansions or what they called "blebs." These blebs appeared as specific budding points on the end of elongated cells. The authors suggest that this stage is intermediate to the formation of the coccoid form and once the "bleb" expands from the elongated "suspensor" cell it breaks off forming the coccoid and the suspensor cell may lose its cellular integrity.

Analyses of the inner and outer membrane proteins revealed that protein synthesis was inhibited in the late stages of exponential growth by treating cells of *C. jejuni* with chloramphenicol. There were no banding differences between treated and untreated cells on SDS PAGE gels. In addition, no banding pattern differences were detected for the outer/inner membrane proteins of the coccoid, elongated, or spiral form. The authors reported that as the cells still elongated after treatment, even in the absence of protein synthesis, the morphological shift may be accomplished by the use of intracellular

reserves or cell stretching, and that the shift is not the result of an active reproductive process but that of a passive, degenerative one.

Biochemistry

Campylobacter do not utilize carbohydrates but derive energy from amino acids that have been deaminated to tricarboxylic acid (TCA) cycle intermediates, such as citrate, cis-aconitate, α-ketoglutarate, isocitrate, succinate, fumarate, malate, and oxaloacetate. Some strains hydrolyze casein, RNA and DNA. In addition, no acid or neutral end products are produced, lipase-activity is negative, gelatin and urea are not hydrolyzed, and nitrate is reduced. (Simbert 1986).

Cell walls may contain galactose, glucose, mannose or a combination of the three (Simbert, 1986). Common fatty acids in the cells of this organism are listed as tetradecanoate (14:0), hexadecanoate (16:0), cis-9-hexadecanoate ($16{:}1^{9}$), cis or trans-9-octadecanoate (18-1) and cis-9,10-methyleneoctadecanoate ($19{:}0^{\Delta}$) (Blaser and others 1980; Simbert 1986;Weyant and others 1996). Approximately 93% of *C. jejuni* have phosphatase activity, and about 6% are positive for arylsulfatase. Antibiotic sensitivities due to chloramphenicol, dihydrostreptomycin, erythromycin, neomycin, oxytetractyclin, streptomycin, and tetracycline exist. Partial inhibition of growth may occur with novobiocin, penicillin, bacitracin, and polymyxin (Simbert 1986).

Blaser and others (1980) identified several predominant fatty acids in strains of *C. fetus* using gas-liquid chromatography (Table 1). All 13 isolates of *C. fetus* subsp. *jejuni* contained a fairly high proportion (14%) of cis-9,10-methyleneoctadecanoate ($19{:}0^{\Delta}$), but this acid was not found in any of the other subspecies tested. In addition, small amounts

of 3-hydroxytetradecanoic acid (3-OH 14:0) were detected in *C. fetus* subsp. *fetus*, *intestinalis* and *jejuni* strains at 6, 6 and 7% respectively (not shown in Table 1).

Table 1. Cellular fatty acids in 29 human and animal isolates of *Campylobacter* [1].

C. fetus subsp. (# of strains)	Percentage of Total Fatty Acids 14:0	$16:1^9$	16:0	$18:1^9$	$19:0^\Delta$
fetus (6)	16	26	31	21	-
intestinalis (10)	13	25	28	28	-
jejuni (13)	12	13	29	25	14

[1] Adapted from Blaser MJ, Moss, CW, Weaver RE. 1980. Cellular Fatty Acid Composition of *Campylobacter fetus*. J. Clin Microbiol 11:448-451.

Leaper and Owen (1981) used fatty acid proportions to describe the biochemical difference between *C. fetus* and what is now *C. jejuni* (Table 2). The latter was earlier classified as *C. fetus* supsp. *jejuni*. *C. fetus* supsp. *fetus* strains contained no detectable $19:0^\Delta$, but small amounts were reported for the C. *fetus* subsp. *venerealis* . No 3-OH 14:0 was detected, in contrast to Blaser and others (1980).

Table 2. Cellular fatty acids in 50 human/animal isolates of *Campylobacter*[1].

Organism (# of strains)	Percentage of Total Fatty Acids					
	14:0	$16:1^9$	16:0	$18:1^9$	18:0	$19:0^\Delta$
C. fetus subsp. *venerealis* (2)	8.2	15.5	40.9	31.2		3.2
C. fetus subsp. *fetus* (7)	4.9	12.7	34.2	35.6		
C. coli (9)	7.0	2.1	28.5	19.1	2.3	7.0
C. jejuni (24)	7.8	3.0	32.7	26.0	1.8	7.8
Campylobacter spp. (8)	3.6	4.0	38.2	44.8		2.9

[1]Adapted from Leaper S, Owen RJ. 1981. Identification of Catalase Producing *Campylobacter* Species Based on Biochemical Characteristics and Cellular Fatty Acid Composition. Current Microbiol 6:31-35.

Hebert and others (1982) explored the cellular fatty acid composition of 128 strains of *Campylobacter* by gas-liquid chromatography of which 89 were *C. jejuni* (Table 3). As reported by Blaser and others (1980), there were trace amounts of 3-OH 14:0 (2-3%) and cis-9,12-octadecandienoate (18:2) (1%) (not shown in Table 3). The authors identified the $19:0^\Delta$ fatty acid as cis-11,12-methyleneoctadecanoic acid (lactobacillic acid).

Table 3. Cellular fatty acids in 89 strains of *Campylobacter jejuni* grouped according to similar composition.

C. jejuni (# of strains)	Percentage of Total Fatty Acids (%)							
	14:0	15:0	$16{:}1^{9}$	16:0	$17{:}0^{\Delta}$	$18{:}1^{9}$	18:0	$19{:}0^{\Delta}$
58	9	1	5	39		32	1	9
11	12	2	6	39	1	17	1	18
11	10	1	5	39	1	14	1	25
3	22	2	12	30	1	20	1	9
6	8	2	4	37		44	1	

[1]Adapted from Herbert GA, Hollis DG, Weaver RE, Lambert MA, Blaser MJ, Moss CW. 1982. Years of Campylobacters: Biochemical Characteristics and a Biotyping Proposal for *Campylobacter jejuni*. J Clin Microbiol 15:1065-1073.

As did Leaper and Owen (1981), Curtis (1983) also found four strains of *C. coli* contained amounts of the $19{:}0^{\Delta}$ fatty acid comparable to those reported for *C. jejuni* (Table 4). Again, trace amounts were found of 3-OH 14:0 ranging from 1.1 to 4.2%.

Table 4. Cellular fatty acids in 68 strains of *Campylobacter*[1].

Organism (# of strains)	Total Fatty Acids (%)								
	12:0	14:0	15:0	$16:1^{9}$	16:0	$17:0^{\Delta}$	$18:1^{9}$	18:0	$19:0^{\Delta}$
C. coli (4)		5.4		3.2	42.2		38.1	1.2	7.3
C. jejuni (6)		7.2		3.5	37.1		39.1	1.1	9.9
C. jejuni (6)		8.4		4.3	34.2		35.8	1.1	12.8
C. fetus supsp. *fetus* (28)		8.7		18.6	33.3		35.2		
C. fetus supsp. *venerealis* (6)		11.6		21.0	37.6		27.4		
C. fetus subsp. *venerealis* (4)		8.4		14.8	33.9		37.2		
C. fetus (4)		2.8	.9	29.9	32.4	2.3	18.4	1.2	
C. fetus (3)	3.8	11.2	.9	14.4	32.3	1.5	35.8		
C. sputorum (1)	6.6	8.8		24.3	25.4		31.5		
C. sputorum subsp. *bubulus* (3)		24.5		7.2	26.1		36.3		
C. sputorum subsp. *mucosalis* (3)	8.2	9.9		25.5	27.3		23.8		

[1] Adapted from Curtis MA. 1983. Cellular Fatty Acid Profiles of *Campylobacters*. Med Laborat Sci 40:333-348.

Moss and others (1984) examined 36 strains of *Campylobacter* and reported the presence of $19:0^{\Delta}$ for *C. jejuni* and *C. coli* (Table 5). Larger amounts (3-8%) of 3-OH 14:0 were found than were reported previously. The authors reported the presence of an $18:1^{\Delta 11}$ acid that accounted for a large percentage of the total fatty acids in the respective

strains as listed in Table 5. Perhaps this was actually the $18:1^9$ fatty acid, common to many strains of *Campylobacter*, that usually occurs in the proportions reported for $18:1^{\Delta 11}$ in this study.

Table 5. Cellular fatty acids in 36 strains of *Campylobacter*[1].

Organism	Percentage of Total Fatty Acids								
(# of strains)	12:0	14:0	15:0	$16:1^9$	16:0	$18:1^{11}$	$18:1^9$	18:0	$19:0^{\Delta}$
C. jejuni (5)		8	3	4	36	21	1	2	18
C. coli (5)		5		3	41	27	1	2	13
C. laridis (6)		5		6	36	45		1	
C. fetus subsp *fetus* (4)		8		17	33	32	1	1	
C. fetus subsp. *venerealis* (4)		11	1	19	38	22	1	1	
C. sputorum subpsp *bulbulus* (3)		21		7	31	27	1	1	
C. sputorum subsp. *sputorum* (2)		19	1	7	31	25	2	1	
C. sputorum supsp. *mucosalis* (2)	10	12	1	14	26	11	4	3	
C. fecalis (5)		21		7	29	29	1	1	

[1]Adapted from Moss CW, Kai A, Lambert MA, Patton C. 1984. Isoprenoid Quininone Content and Cellular Fatty Acid Composition of *Campylobacter* Species. J Clin Microbiol 19:772-776.

General growth requirements

Outside the gastro-intestinal tract, *Campylobacter* are susceptible to environmental stresses that may affect their viability and morphology. According to Park (2002), analysis of the genome sequence for *C. jejuni* revealed that this species of

bacteria lacks several proteins required for adapting to changing environments. *Escherichia coli* for example, possesses the ability to produce eight separate oxidative stress response proteins while certain strains of *Campylobacter* only have four of these proteins. Likewise, *C. jejuni* only has the capability of generating one of four osmoregulation stress proteins, one out of three stationary phase proteins (starvation), one out of three quorum sensing stress proteins, one out of two global regulation stress proteins, and three out of five heat and cold shock proteins (Park 2002). While *E. coli* did not posses all possible stress proteins (20/26) it contained more than *Campylobacter* (11/26). To say that one protein is more effective for survival than another is, however, questionable.

Campylobacter jejuni are sensitive to extreme cold or heat with a minimum growth temp of 32°C (unable to grow below 30°C) and an "optimal" growth temperature of 42°C – 45°C (Doyle and Roman 1981; Park 2001). Reported optimal growth temperatures vary and may range from 37-45°C depending upon strain variations and growth conditions (Doyle and Roman 1981; Humphry 1989). The bacteria may remain motile, and metabolically active with the ability to produce ATP, at temperatures as low as 4°C (Hazeleger and others 1998; Park 2002). It is unclear as to how, or if at all, the reproductive process takes place at or below 30°C.

Campylobacter exhibit a greater sensitivity to pH than do most other food borne bacteria (Blaser and others 1980; Park 2002). The optimal pH range is between 6.5 and 7.5 (Doyle and Roman 1981; Simbert 1986) and is strain dependant.

These organisms are considered microaerophilic because some tolerate O_2. It is common to use a mixture of 10% CO_2:85% N_2:5% O_2 to grow *Campylobacter* but ranges

of 3-15% O_2, and 3-5% CO_2 have been reported (Simbert 1986). In addition, enzymes such as catalase, oxidase and superoxide dismutase, and alkyl hydroperoxide reductase help the cells tolerate higher levels of O_2 and still remain viable (Hoffman and others 1979a 1979b; Simbert 1986; Park 2002). Some strains will grow anaerobically in the presence of fumarate alone, or in combination with formate and fumarate, and some will grow in the presence of O_2 and fumarate (Simbert 1986).

Campylobacter may also be stressed under different osmotic conditions. Reezal and others (1998) examined the growth of *Campylobacter* on media with various osmolalities. Two methods were used to change the osmolality of the media: 1) simple dilution (does not account for effect of nutrition deprivation) and 2) media reformulation (nutritional constituents undiluted, salt or glucose levels altered). The broth used contained 10g tryptone, 10g Bacto Peptone, 2 g Yeast Extract and 5.0 g NaCl per liter. The osmolality of full strength media was ≈250 mOsm, while that of the diluted and reformulated media was ≈170 mOsm. Broth was dispensed in 5.5 ml portions into 12ml test tubes that were capped but still allowed gaseous interchange with a modified atmosphere (5%O_2, 10% CO_2 and 85% N_2). Media with greater osmolality (≈250 mOsm) supported more growth than did those with lesser osmolality (≈170 mOsm). There was, however, no difference between the diluted and reformulated media, suggesting that nutrient deprivation may not be responsible for the loss of culturability at decreased osmolality.

One interesting competitive advantage that some strains of *Campylobacter* possess is the ability to use iron from hemin and hemoglobin obtained from the host (Pickett and others 1992; Park 2002). When iron sources are plentiful (i.e., when iron is

not bound by iron binding proteins), hemin and hemoglobin iron is easily captured by *Campylobacter* via microbial iron chelators called siderophores (i.e., ferrichrome and enterochelin) (Field and others 1986; Park 2002). In other instances, strains lacking in constituent siderophores have the ability to obtain iron compounds by scavenging siderophores produced by other bacteria (Baig and others 1986; Park and Richardson 1995; Richardson and Park 1995; Park 2002). The ability of *Campylobacter* to adequately bind iron is not only essential for growth but may play a major role in the organism's ability to tolerate oxidative stress (vanVliet and others 2002).

Factors Affecting Growth and Morphology

In the laboratory, *Campylobacter* spp. are extremely sensitive to the environment in which they are grown. Many studies have examined the effects of gaseous atmosphere, temperature, their interaction, and pH on growth and morphology. There is wide variation, however, among studies in the techniques used, responses measured, and conclusions made. Although there are large variations in results and conclusions, both within and among publications, and results are tedious to discuss, this variability in growth of *Campylobacter* is an important part of this dissertation. The following section of this review is intended, therefore, to provide some insight into the difficulties in culturing *Campylobacter* that impact the accuracy of current detection and enumeration methods. Because culture dynamics may differ between agar and liquid media, this discussion is divided accordingly.

Factors affecting growth on agar media

Atmosphere

Bolton and Coates (1983) evaluated the effects of O_2 and CO_2 concentration on growth of three strains of *C. jejuni* (NCTC 11168-biotype 1; NCTC 11392-biotype 2; and ATCC 3036); one strain of *C. coli* (NCTC 11352) and one other isolate noted as being nalidixic acid resistant thermophilic *Campylobacter* (NCTC 11353). The gas treatments were combinations of 0, 1.0, 2.5, 5.0, 10.0 and 15.0 % CO_2 with <0.5, 1.0, 5.0, 10.0, 15.0, and 21.0% O_2. Recovery medium was nutrient broth No. 2 (Oxoid CM67) combined with 2% agar and 5% lysed horse blood. Plate counts were determined after 48-hours incubation at 42°C for all treatment combinations, but only relative Growth Indices were reported. *C. jejuni* (NCTC 11168 and 11392) were the most tolerant strains to high levels of O_2 but growth did decline at O_2 levels of 15% and higher. *Campylobacter coli* (NCTC 11353) grew at all O_2 levels except the 21% level. *Campylobacter jejuni* (ATCC 3036) was the next least tolerant in that it failed to grow at the 15 and 21% O_2 levels. The least tolerant strain was the nalidixic acid thermophilic *Campylobacter* that did not grow above 10% O_2. Based on these results the authors concluded that the best atmosphere for growing these particular strains was 5-10% O_2 and 1-10% CO_2. There were, however, no obvious differences based on CO_2 concentrations alone. This paper did not present statistical analyses, and treatment interactions were not described, thus it is not possible to draw conclusions about the optimum combination of O_2 and CO_2 levels for growth of *Campylobacter*.

Atmosphere and temperature interaction

Wang and others (1983) collected fecal samples from 16 people known to be infected with *C. jejuni*. The isolation procedure took place under four different atmospheres (5, 10, 15 and 17 % O_2) and two different incubation temperatures (37°C and 42°C). Response variables were plate counts (colony forming units, CFU/g) and colony diameters (mm). When plates were incubated in a 5% O_2 environment, 16/16 positive *C. jejuni* isolates were found at 42°C incubation temperatures and similarly, 14/14 positive *C. jejuni* isolates were found when incubated at 37°C. Plate counts ranged from 10^4-10^8(average=10^6) and 10^4-10^7 (average=10^6) CFU/g at 42°C and 37°C respectively. Colony sizes ranged from 2mm-spreader and 0.5-10mm in size for the same temperatures. When the atmosphere was 10% O_2 the number of positive cultures detected was the same as those at the 5% O_2 levels at both temperatures. Plate counts and colony sizes at 42°C and 37°C were also very similar ranging from 10^3-10^8(average=106) and 10^3-10^7 (average=105) CFU/g and colony diameters of 2-10mm and <.5-8mm for the respective incubation temperatures. Increasing the O_2 level further (15% O_2) had only a minor effect on number of positive isolates recovered from the fecal samples. At 42°C, all (16/16) isolates were recovered whereas at 37°C, most (11/13) were recovered. The reported plate counts and colony sizes for the two incubation temperatures (42 and 37°C) were 10^3-10^8(average=10^6) and <10-10^8 (average=10^5) CFU/g and colony diameters of 2mm-spreader and <.5-12mm for the respective incubation temperatures. The final O_2 concentration of 17% did not provide much more information other than all isolates (16/16) were recovered at the 42°C incubation versus 37°C where most (10/13) isolates were recovered. The plate counts and colony sizes were 10^3-10^8(average=10^5) and <10-

10^7 (average=10^5) CFU/g and colony diameters of <0.5mm-spreader and <0.5-4mm for incubation temperatures of 42°C and 37°C respectively. Based on these results, the authors recommended that 42°C incubation should be used instead of 37°C when isolating *Campylobacter*. With such a large degree of variation, similarity in results at both temperatures, and the ambiguous meaning of the reported "spreaders," it is difficult to justify the authors' conclusion about the optimum temperature for *Campylobacter*. It appears that this publication is the basis of current recommended methodologies; i.e., FDA methods, which use a 42°C incubation temperature for the isolation of *Campylobacter* from food and water.

Lee and others (1988) evaluated the ability of *Campylobacter* to grow on un-supplemented brucella agar (George and others 1978) at various temperatures, O_2 levels, media age, and media bisulfite content. *C. jejuni* ATCC 29428 and an O_2 tolerant mutant (MC711-01) of this strain were prepared from cultures grown in brucella broth at 37°C (without agitation) under a modified atmosphere containing 6% O_2. Less than 1% of the organisms were in the coccoid form. The cultures were plated using 1) agar medium prepared from dehydrated Brucella media of various ages, 2) prepoured Brucella agar plates of various ages, and 3) freshly prepared Brucella agar with/without 0.01% sodium bisulfite. Cultures were incubated at either 37 or 42°C in atmospheres containing 1.5, 3.0, 6.0, 15.0, 21.0 or 26.0 %O_2. Both strains grew better when incubated at 42°C than at 37°C. *Campylobacter jejuni* 29428, when grown at 42°C and 15%O_2, had 176 CFU/plate compared to no growth when incubated at 37°C. The mutant strain also grew somewhat better in 15%O_2 (168 CFU/plate) at 42°C compared to 37°C incubation (130 CFU/plate). *Campylobacter jejuni* 29429 was fairly tolerant to O_2 levels of 15% but did not grow well

at the 21 and 26% levels. The mutant did well at all levels of O_2 including 26%, if plated on fresh media. While evaluating the aerotolerance of these two strains, the authors noted large variation in their results. They found that the older the media the more varied the results (i.e., plate counts).

The effect of aging dehydrated brucella media on microbial growth was examined in more detail (Lee and others 1988). Strains were plated on A) agar made from freshly opened brucella media, B) agar made from previously opened (2.5 months), un-supplemented brucella media, and C) agar made from the previously opened brucella media supplemented with 0.01% sodium bisulfite. At incubation conditions of 15% O_2/42°C, *C. jejuni* 29428 had 178, 32 and 224 CFU/plate for treatments A, B, and C, respectively. At incubation conditions of 26% O_2/42°C, the mutant *C. jejuni* MC11-01 had 175, 1 and 179 CFU/plate for the media treatments A, B and C. Similar results were obtained from rehydrated brucella agar freshly prepared, aged 1.5 months, and aged 1.5 months and supplemented with 0.01% sodium bisulfite. To verify that the sodium bisulfite was indeed the ingredient responsible for increased O_2 tolerance, the two strains were tested on D) freshly prepared brucella agar, E) brucella agar made from the individual ingredients including 0.01% sodium bisulfite, and F) brucella agar made from the individual ingredients excluding sodium bisulfite. At incubation conditions of 15% O_2/42°C, *C. jejuni* 29428 had 183, 195 and 0 CFU/plate for treatments D, E, and F, respectively. At the incubation conditions of 26% O_2/42°C, the mutant *C. jejuni* MC11-01 had 156, 112 and 0 CFU/plate for the media treatments D, E and F, respectively. The presence of fresh sodium bisulfite in the media increased the apparent ability of both strains to tolerate O_2, probably due to a decrease in O_2 concentration in the media. The

difference in O_2 tolerance between strains clearly demonstrates that this characteristic is genetic. The loss of sodium bisulfite from the aged media was due to oxidation to sodium sulfate in the presence of moisture and O_2 in the aged/open media bottles. Even more interesting is that the mutant was a derivative from the ATCC 29428 strain suggesting the organism's ability to adapt to greater O_2 concentrations.

Factors affecting growth and morphology in liquid media

The previous section demonstrates the sensitivity of *Campylobacter* to O_2 concentration and temperature, as well as variation among strains when grown under adverse conditions. The studies described above targeted the growth behavior of *Campylobacter* on agar plates in response to environmental stimuli. Cultures are grown (enriched) in broth or liquid media, however, when attempting to isolate *Campylobacter* from food and water. Therefore, growth requirements determined on solid agar plates should be interpreted with caution because culture dynamics may not be the same in broth versus agar media. The pre-enrichment and enrichment in steps recommended by the Food and Drug Administration (FDA) (see later section) incubate broth cultures at temperatures and O_2 levels that may have been based on studies of growth in agar media. As the research to be presented in this dissertation targets the growth of *Campylobacter* in broth culture, the following section is intended to give some insight on the growth requirements of *Campylobacter* in a broth/liquid media, and on factors that may contribute to changes in morphology and culturability.

Atmosphere

George and others (1978) evaluated various media types in order to improve the growth of various species of *Campylobacter*, focusing on their tolerance to O_2. *Campylobacter* was either plated directly on agar plates or grown in broth culture (50ml). Sixty-four isolates were grown in brucella broth at 37°C under microaerophilic conditions (6%O_2, 2.5%CO_2 and 92.5%N_2). The cultures were then A) surface plated on either 1) brucella agar or 2) FBP agar (brucella agar supplemented with .025% $FeSO_4 \cdot 7H_20$, .025% sodium metabisulfite and .025% sodium pyruvate) or B) inoculated into 50 ml of either 1) brucella broth or 2) FBP broth (brucella broth supplemented with .2% $FeSO_4 \cdot 7H_20$, .025 sodium metabisulfite and .05% sodium pyruvate). Plates were incubated at 37°C for 4 days with atmospheres of either 6% O_2, 17%O_2, or 21%O_2 (each with a constant level of CO_2 (2.5%) with the remainder being N_2. Broth cultures were grown statically at 37°C in milk dilution bottles (with no mention of bottle caps or closure apparatus). Actual plate counts were not reported but a relative Growth Index was used to evaluate the effects of the media supplements, atmospheric conditions and strain variations. The 64 strains were made up of *C. fetus* subsp. *intestinalis* (n=38); *C. fetus* subsp. *jejuni* (n=18); and *C. fetus* subsp. *fetus* (n=8). Of these three groups, *C. fetus* subsp. *jejuni* seemed to grow the best over all conditions, followed by *C. fetus* subsp. *intestinalis* and *C. fetus* supsp. *fetus*. Generally, FBP supplementation in growth media increased the overall ability of strains to grow at the higher O_2 levels (17 and 21%), perhaps because the supplements acted as O_2 sequestering agents.

All 18 strains of the *C. fetus* subsp. *jejuni* grew on both the plain brucella agar and the supplemented FBP agar. When the O_2 concentration was increased to 17%, all 18 strains grew on the supplemented FBP agar but only 13 strains grew on the brucella agar alone. This trend was the same for the 21% O_2 level, in that all 18 strains grew on the FBP agar but only 7 grew on the unsupplemented agar medium. One strain of *C. fetus* subsp. *intestinalis* was so sensitive to O_2 that it would only grow in an atmosphere containing 1% O_2. Broth cultures grown in the presence of 21% O_2, also appeared to do better when grown in FBP broth. Of all the strains, 63 of 64 produced significantly turbid suspension after 72 hours incubation whereas 48 strains did not grow as well in unsupplemented broth. The use of turbidity values as an indicator of growth may not be as accurate due to the accumulation of dead cells and debris and the associated loss of culturability after 72 hours incubation at 37°C, but their use as a qualitative tool is acceptable.

Rollins and others (1983) described a biphasic (agar/broth) culture system in tissue culture flasks (TCF) for the rapid cultivation of *Campylobacter*. The use of the TCF was not the focus of their methods, and TCF caps were not fitted with semi-permeable membranes but were placed on "loosely." It is presumed that the authors were aware of the effect of the atmosphere in which the TCFs were incubated and thereby placed the caps accordingly. The authors were very careful in describing culture production and maintenance techniques. The experiments were performed in 25 cm^2 tissue culture flasks containing a biphasic media system (a 4-ml solidified brucella agar layer with 6 ml of brucella broth on top). There were two biphasic media systems: 1) un-supplemented brucella agar and broth, and 2) supplemented consisting of brucella agar

with 0.025% each of ferrous sulfate, sodium pyruvate and sodium metabisulfate, and brucella broth with 0.2%, 0.025%, and 0.05% of the above ingredients, respectively. There were four treatments consisting of the two systems incubated in either air or modified atmosphere (10%CO_2;5%O_2;85%N_2): 1) unsupplemented system in air; 2) unsupplemented system in modified atmosphere; 3) supplemented system in air; and 4) supplemented system in modified atmosphere. Each treatment was incubated at 37 and 42°C. Unfortunately, turbidity readings (OD_{625}) were the main response variable used to compare treatments, but qualitative assessment of their results are as follows. At the highest peak turbidity for all treatments, cells were mostly in the curved-rod form. The unsupplemented system in air needed a higher inoculum (>105 CFU/ml) to attain the same optical density as the unsupplemented system in modified atmosphere that was inoculated with much lower numbers (1-10 CFU/g). The supplemented system in air showed similar peak absorbencies to that of the unsupplemented system in modified atmosphere, suggesting that the supplement provided an increased aerotolerance. The supplemented system in modified atmosphere showed the higher peak absorbencies. The authors found that growth at 42 and 37 in this system were identical thus the higher temperature did not enhance growth. There was mention of cells adhering to the agar layer after removal of the broth. This may have lead to problems with enumeration because all colony-forming units may not have been recovered. In all cases, the authors were aware of the extent to which their cultures had changed morphology but they described this change as "gross contamination." We are now aware that this "contamination" might be the various forms of *Campylobacter*. Plate counts were alluded to, but those data were not shown, presumably due to the reported "large

variation" among duplicate plates. The authors stated that they were able to obtain consistently good growth in their biphasic media system. This is one of a few papers that addresses the difficulty and unpredictability of growing *Campylobacter*. Unfortunately, it has not been used or studied to the extent it deserves.

Morgan and others (1987) worked with *Campylobacter pylori* in liquid media. Although this organism is now *Helicobacter pylori* (Vandamme 2000), these experiments may provide information relative to the growth characteristics of *Campylobacter* because the two organisms are closely related. Several experiments examined the use of reducing supplements incorporated into brucella broth as a means of enhancing the growth of *C. pylori* in liquid culture. *C. pylori* was grown in 1) brucella broth alone, 2) brucella broth plus Fetal Calf Serum (FCS), 3) brucella broth plus Vitox (Oxoid product: 67.4% w/w solution of L-cysteine hydrochloride hydrate), or 4) brucella broth plus Vitox and FCS. In addition, the researchers evaluated shaking and stationary conditions on growth in liquid media. The isolates were initially grown on GCHI chocolate agar supplemented with various antibiotics. The plates were incubated for 3-5 days at 37°C in Gas Pak jars with modified atmosphere supplied from Campy-Paks. The gas generating envelopes (Campy-Paks) were replaced every 48 hours. Microscopic examinations were used to confirm morphology characteristics of inoculum. Broth studies were carried out using 10 ml of the test broth in 50 ml sterile Erlenmeyer flasks fitted with a porous stopper or a loosely fitted screw cap. The cultures were incubated in a closed hood system flushed with a mixture of 10%CO_2, 5%O_2 and 85% N_2.

Unfortunately, no data were provided, but the authors stated that brucella broth plus Vitox (1%) and FCS (10%) supported growth of the organisms only if shaken (150

rpm) and that if the cultures were grown statically, there were no detectable cells after 5 days of incubation at 37°C. The authors suggested that it was imperative that gas be adequately dispersed throughout the broth medium. Brucella broth alone or containing Vitox did not support growth after 48 hours of incubation at 37°C. Prolonged (up to 5 days) incubation resulted in a further loss of viability and an increased number of coccoid forms. Further experiments concluded that Vitox was not needed for the growth of *C. pylori*. Fetal Calf Serum was necessary at the 1% level and anything less resulted in a loss of culturable cells. This particular supplement most likely acted as a reducing agent and provided the cultures with increased tolerance to O_2.

Morgan and others (1987) evaluated the growth of *Campylobacter* in liquid media. When the culture was grown statically at 37°C in a Gas Pak jar with a modified atmosphere (Campy-Pak) the organism lost viability (plate counts) after 5 days of storage. Good growth could be achieved, however, when the broth culture was grown in a flask connected to a continuous gas system (10%CO_2, 5%O_2 and 85% N_2) and shaken at 150 rpm. When inoculated with 10^2-10^5 CFU/ml, it was possible to reach $\geq 10^8$ CFU/ml after 24, 48 and 72 hours of incubation. This article points out the importance of an adequate gas supply coupled with a shaking motion for the successful culture of *Campylobacter*.

Temperature

Doyle and Roman (1981) isolated 5 strains of *C. fetus* supsp. *jejuni* from human stool samples and subsequently prepared them on blood agar plates (brucella agar and 5% defibrinated sheep blood). Plates were incubated for 3 days at 42°C in a modified atmosphere of 5% O_2, 10% CO_2 and 85% N_2. Cells were collected and placed into a

semisolid agar and incubated for 24 hours at 42°C. These cells were then transferred to 500-ml flasks containing 100 ml of brucella broth supplemented with 0.3% sodium succinate and 0.01% cysteine hydrochloride. Flasks were flushed with the modified atmosphere and incubated 16-18 hours at 42°C in a gyrating water bath (100 gyrations/min). These cells were used for growth studies after being diluted to approximately 6.0×10^3 CFU/ml. The growth studies were performed in 16x125mm test tubes with screw-capped tops with no mention of agitation. The pH of the media (brucella broth + 0.1% agar) was adjusted to various levels ranging from 3.0 to 9.5. Tubes were incubated at temperatures between 4 and 47°C. Samples were plated on brucella agar containing 5% sheep blood, and CFU/ml were used to measure responses. All five isolates grew well between pH 5.5 and 8.0. The authors were hesitant to identify a maximum or optimum pH, however, due to the confounding drop in pH levels brought on by the growing culture.

Rates of growth for the 35 and the 37°C incubation were nearly identical. This was also true for the 42 and 45°C temperatures. The authors concluded that 42 and 45°C were optimal incubation temperatures, with 42°C being the best. According to the graphical presentation of CFU/ml, however, it appears that their conclusion was based on growth rates up to 24 hours. At that time, cultures incubated at either 42 or 45°C achieved populations similar ($\approx 5.0 \times 10^8$ CFU/ml) to those obtained after 48 hours of incubation at 35 or 37°C. After 24 hours of incubation at 42 or 45°C, however, there was little further increase in numbers of CFU, and after 48 hours, CFU/ml declined. On the other hand, cultures incubated at 35 and 37°C continued to increase after 48 hours. At 72 hours, the 42 and 45°C data points are about 1 log cycle lower ($\approx 1.0 \times 10^8$ CFU/ml) than

those at 35 and 37 °C (≈ 1.0×10^9 CFU/ml). These observations suggest that at the higher temperatures there may be a decline in culturable *Campylobacter* after 24 hours. Extrapolating from the graph indicates that after 72 hours there were roughly 7.0×10^8 CFU/ml and 3.0×10^9 CFU/ml when cultures were incubated at 35°C and 37°C, respectively. Based on these observations, it may be misleading to suggest that the optimum incubation temperature for broth is 42°C.

Humphrey (1989) recovered *C. jejuni* from three naturally contaminated samples (chicken skin, chicken carcass scald water, and fresh river water) with and without a pre-enrichment process. All three samples were pre-enriched in selective broth or non-selective broth at 37°C for 4 hours, or not pre-enriched at all. There were 20 replications for each sample and pre-enrichment procedure. For the river water, scald tank water and chicken skin, respectively, the number of positive samples for the non-selective broth were 16, 18 and 10, selective broth 16, 14 and 12, and the non-pre-enriched 4, 8 and 6. Clearly the isolation rate was affected by enrichment procedure, but it is difficult to differentiate the effect of temperature and/or pre-enrichment since these two treatment combinations were not evaluated. It was shown that 37°C was a more favorable growth temperature for *Campylobacter* compared to 43°C. It is still unclear, however, as to the benefit of the "pre-enrichment period." The optimum time and temperature for enrichment are not yet fully understood. The authors, based on these findings, made the conclusions that a 4-hour pre-enrichment at 37°C is optimal for the isolation of *Campylobacter*. Again, some details were omitted from the paper by the authors "for clarity purposes" so some conclusions are presented without verifiable data. In closing, an interesting comment within the "Statistical Methods" section states "Despite every

effort to standardize media, growth and experimental conditions, there were, at times, variations in the response of the organisms to the stress treatments and in their subsequent growth patterns on the various media."

The coccoid morphology of *C. jejuni* has been associated with lack of recoverability or the VNC form. Hazeleger and others (1995) indicated that the morphological shift from the rod to the coccoid was dependant upon temperature and nutrient availability. *Campylobacter jejuni* was inoculated into nutrient rich media (BHI-brain heart infusion broth) and nutrient poor media (PPBS-50mM potassium phosphate buffer containing 0.85% saline and PPB-50 mM potassium phosphate buffer) and stored at 4, 12 and 25°C for various periods. Cells stored at 4°C remained culturable for a longer period of time for both the nutrient rich and poor media. Cells from the nutrient poor broth were culturable on plating media longer (70 days), however, than were those from the nutrient rich media (≅25 days). The authors suggested that cells have a slower metabolic rate in the nutrient poor media than in nutrient rich media. An odd shift in detection occurred when cells were stored in PPB in that the 12°C temperature led to an increased detection period (> 40 days) compared to that at 4°C (≅25 days) or 25°C (< 5 days). The authors indicated that this was consistent with all strains tested. Later, Hazeleger and others (1998) determined that the minimum growth temperatures for two strains of *C. jejuni* (104 and 33560) occurred between 31 and 32°C.

Holler and Martin (1998) evaluated the production of the coccoid morphology of *C. coli* SP10 (pig isolate) when stored at 4, 10, 20 and 37°C. Using light microscopy, the percentage of coccoid cells present after 51 days of storage at 4, 10, 20 and 37°C were 4,

7, 94 and 98% respectively. In addition, several different sizes of the coccoid form were identified.

Thomas and others (2002) evaluated the morphological changes of *Campylobacter* spp. (*C. jejuni, C. coli and C. lari*) stored at 10 and 20°C in an aquatic model system. The model was a river sediment layer (50 mm) and the addition of fresh river water (150 mm depth) to a fermentation system. Morphological changes (visualized by light microscopy) were evident with increased storage time at both temperatures. By the third day, cells for all three species began to elongate (2.41 μm) two times greater than their initial size (1.19μm) and there were frequent occurrences of cells greater than 6μm for *C. coli*. It was noticed that *C. jejuni* and *C. lari* lost their spiral shape more rapidly than *C. coli*. After 14 days of storage *C. coli, C. jejuni* and *C. lari* contained 57, 53 and 56 % coccoid forms when stored at 10°C and 71, 69 and 72% coccoid when stored at 20°C respectively. All three continued to change and after 60 days of storage contained 81, 73 and 76% coccoid form at 10°C and 91, 86, and 89 % coccoid form when stored at 20°C for the three strains respectively. The remaining cells were described as a variety of spiral and elongated rods.

Fatty acids are not only valuable for identification of *Campylobacter*, they may be useful in understanding membrane structure, fluidity and function with respect to the morphological shift from the rod to the coccoid form. The ability of organisms to adapt to temperature changes has been linked to changes in fatty acid structure and length. An inverse relationship has been proposed between temperature and the degree of unsaturation as well as fatty acid chain length (Linder and Oliver 1989; Eaton and others 1981). Hazeleger and others (1995) determined fatty acid profiles of various forms of *C.*

jejuni when grown at different temperatures. This objective was to determine if changes in fatty acids of different proportions reflected changes in the cytoplasmic membrane associated with the morphological shift from the rod to the coccoid form. Spiral suspensions (n=11) were collected from cells grown in 500 ml volumes of brain heart infusion broth at 37°C for 20-28 hours. Coccoid suspensions were collected from cells that had formed at 4 (n=10), 12 (n=10) and 25°C (n=7). Fatty acid profiles of coccoid cells formed at the lowest temperatures (4 and 12°C) did not show marked differences from fatty acid profiles of the spiral cells. Coccoid cells that formed at 25°C, however, displayed different fatty acid profiles from those of the spiral cells. The authors reported a significant increase in hexadecanoate (16:0) and octadecanoate (18:0) with a concomitant decrease in tetradecanoate (14:0), cis-9-hexadecanoate (16:1) and the cyclic cis-9,10-methyleneoctadecanoate ($19{:}0^{\Delta}$).

Linder and Oliver (1989) evaluated the fatty acid composition of *Vibrio vulnificus* when cells were stored for 26 days at 5°C. *Vibrio* exhibits similar morphological changes to that of *Campylobacter* in response to low temperatures. Fatty acid profiles from cells incubated at 5°C for 26 days showed little change in composition until the cells became nonculturable (24 days). At this time there was a concomitant decrease in 16:0 and 16:1 and an increase in 19:0, 20:0 and 22:1 fatty acids. Saturated fatty acids increased from 49 to 69.1% and unsaturated fatty acids decreased from 45 to 17% of total fatty acids. In both studies, time/temperature/morphology treatment combinations were not completely tested. It is not possible to determine, therefore, whether cellular fatty acid profiles changed due to one or all of those variables. It is interesting that the same type of morphological changes occur in these two different organisms (*Campylobacter* and

Vibrio) but temperature does not cause changes in fatty acid profiles in *Vibrio* as it does in *Campylobacter*.

Atmosphere and temperature interaction

Humphrey (1986) indicated that a pre-enrichment step of 2 hours at 37°C is needed to recover *C. jejuni* from water and milk samples. He suggested that incubation temperatures higher than 37°C might not be advantageous for the recovery of injured cells or cells stressed by the addition of antibiotics during the enrichment process. Pre-enrichment procedures recommended by FDA (Hunt and others 2001) for the isolation of *Campylobacter* are similar to those presented in this paper except that the FDA recommends 4hours at 37°C for dairy products.

Humphrey (1989) investigated growth responses of temperature-injured *C. jejuni* as influenced by various media supplements and incubation temperatures. These results are applicable to the pre-enrichment process for the isolation of *C. jejuni* from food and water. Two strains of *C. jejuni* (B and F) were grown on Nutrient Agar (Oxoid No.2), harvested and subjected to high (50°C for 30 min) or low (-20°C for three days) temperatures in order to induce temperature-related cellular injury. Cultures were then plated on various selective agars containing quenching agents, hydrogen peroxide (H_20_2) or rifampicin. Rifampicin is an antibiotic used in preenrichment and enrichment liquid media and plating media for isolation of *Campylobacter*. Hydrogen peroxide is thought to be toxic to cells due to its highly oxidative nature, and it may build up and eventually inhibit growth. There were no more details regarding this media or its preparation.

Cold-injured cells were plated on agar containing either rifampicin or H_20_2 and incubated at either 37 or 43°C. Un-injured cells were grown on nutrient agar at the two

temperatures for 24 hours, then plated on agar containing rifampicin or H_2O_2 and incubated at 37 or 43°C. The data were reported as IC_{50} values which are the concentration of either rifampicin (μg/ml) or H_2O_2 (μmol/L) required to inhibit 50% of the cells. When uninjured cells of *C. jejuni* strain B were incubated at 37°C and 43°C, the rifampicin IC_{50} were 17.5 and 16.0 μg/ml, respectively, whereas if the cells were injured the IC_{50} were only 10.6 and 7.5μg/ml. The effect of incubation temperature was even more pronounced with *C. jejuni* strain F. The IC_{50} of rifampicin for uninjured cells of *C. jejuni* strain F incubated at 37°C and 43°C was 12.5 and 5.1 μg/ml, respectively. Cold-injured cells were greatly affected by rifampicin at both incubation temperatures but much more so at 43°C (IC_{50} = 1.4 μg/ml) than at 37°C (IC_{50} = 5.2 μg/ml). Similar results were obtained with *C. jejuni* strain B. The detrimental effect of H_2O_2 was also compounded with higher incubation temperatures. When uninjured cells of *C. jejuni* strain B were incubated at 37°C and 43°C, the IC_{50} of H_2O_2 was 185 and 160 μmol/L, respectively, whereas with the cold-injured cells, the IC_{50} of H_2O_2 was only 123 and 90 μmol/L, respectively. Similar results were obtained with *C. jejuni* strain F. Uninjured cells incubated at 37°C and 43°C required 168 and 58 μmol/L H_2O_2 to inhibit 50% of the cells. Again, injured cells were greatly affected by H_2O_2 at both temperatures but much less at 37°C (78 μmol/L) than 43°C (31 μmol/L). Effects of quenching agents on subsequent growth of cold-injured *C. jejuni* strain F were presented graphically (no numerical data). Growth of cold-injured cells on nutrient agar containing O_2 quenching supplements (blood, etc.) was about 7 times greater when incubated at 37°C than at 43°C. When cold-injured cells were grown on an unsupplemented agar, however, growth at 37°C was 80 times greater than at 43°C. No data were shown for cells injured by heating

but the authors suggested that the effects were similar to those with frozen cells. These studies suggest that recovery of injured cells is much more likely at 37°C than at 43°C.

Identification of the Viable Nonculturable (VNC) State

The VNC state has been defined as that condition when organisms have for some reason lost their ability to grow on specific media (i.e., lost their culturability) but still remain viable. Techniques other than standard plating methodology must be used to identify the difference between living and dead bacteria. These methods can be divided into two main categories 1) in vitro biochemical assays and 2) in vivo passage models.

In vitro biochemical assays

The acridine orange direct count (AODC) staining technique uses 3,6-bis(dimethylamino) acridinium chloride (Hobbie and others 1977) and has been used to detect total bacteria however, it cannot distinguish between live and dead cells (Huq and others 2000). The Direct Viable Count (DVC) method (Kogure and others 1979) was developed to detect living marine organisms. In the DVC method, cultures are first stimulated by the addition of yeast extract and then inhibited such that protein synthesis continues in the absence of DNA synthesis, resulting in enhanced cellular elongation. These cells are noted as being larger than normal and thus distinguishable from dead cells.

Rollins and Colwell (1986) used three methods (plated counts, DVC, and AODC) to measure the survival and VNC state of *C. jejuni* over extended periods of time in artificial growth media and natural aquatic environment. The three media environments were placed in tissue culture flasks or Erlenmeyer flasks containing a biphasic (agar/broth) media, treated (filter-sterilized and aged) stream water, or natural stream

water. The vessels were kept stationary or shaken (150 rpm) and incubated for various time periods at 4, 25 and 37°C. Both temperature and aeration impacted the culturability and detectability of *Campylobacter*. For example, a 50 ml sample of treated stream water was held stationary in a 125 ml Erlenmeyer flask at 4, 25 and 37°C. At 4°C, culturable (plate counts) *C. jejuni* were found 120 days after inoculation. Higher temperatures of 25 and 37°C resulted in recovery of culturable organisms (plate counts) for only 28 and 10 days respectively, clearly indicating the reduced culturablity when stored at higher temperatures. Based on glutamate utilization and CO_2 evolution data at 27 and 37°C, higher metabolic activity at the higher temperatures may have caused of the loss of culturability. Rapid declines in plate counts were seen in the water microcosms (shaken at 150 rpm and stationary) as well as in the tissue culture flask/biphasic media system (shaken at 150 rpm). When the cultures were incubated at 37°C in the tissue culture flask/biphasic media system and not disturbed, counts remained high ($\cong$8.0 $\log_{10}$CFU/ml) for up to 12 days. It was not possible to determine whether or not any modified atmosphere was applied to the test samples. When comparing the three methods used for detection (plate counts, DVC and AODC), there was little difference between DVC and AODC numbers while plate counts rapidly declined.

Medima and others (1992) found that 6 strains of *C. jejuni* stored in an aqueous environment at 15 and 25°C declined to <1 CFU/ml in 2-6 days. Six of 7 strains tested lost viability (DVC) as quickly as they lost culturability (plate counts) possibly suggesting that the use of DVC as a tool to measure the VNC state is either strain dependant or not a reliable tool.

The DVC method was used for detection of temperature stressed cells of *Campylobacter* (Höller and Martin 1998). The objective was to determine if temporary cold stress affected cellular elongation following treatment with nalidixic acid. Cultures were grown in broth culture for 48 hours at 37°C. They were then diluted and stored for 12 hours at 37°C or 48 hours at 4, 10 and 20°C. Various concentrations of nalidixic acid were tested in order to determine the minimum inhibitory level. Cultures were then placed in buffer solution with the appropriate amount of nalidixic acid and incubated at 37°C for various times and analyzed for DVC and plate counts. The level of nalidixic acid needed for inhibition was different for each strain tested. In addition, cells that were stressed by low temperatures (i.e., 4, 10 and 20°C) did not elongate (respond to the yeast extract/nalidixic acid) as did those cells that were grown at 37°C and never exposed to lower temperatures. Therefore, the author concluded that the DVC assay might not be reliable if organisms have been injured. In order to use DVC it was necessary to know beforehand 1) concentration of nalidixic acid needed for each strain 2) what type of injury the organism experienced and to what extent. For all practical purposes this information is usually not clear. Furthermore, evaluation of cellular viability using a method such as this is very subjective.

Bovill and Mackey (1997) held cells of *C. jejuni* in sealed-shaking flasks at 37°C for 4-6 weeks. The flasks were gassed with a 10%CO_2:85%N_2:5%O_2 mixture on a continuous basis until the stationary phase of growth was reached (2.0 x 10^9 CFU/ml), then allowed to sit for 2-4 weeks until the counts reached 10^3-10^6 CFU/ml. The cultures were then regassed with the above mentioned gas mixture and plate counts increased almost to their stationary phase level. The number of "vibroids" or rod shapes increased

dramatically upon regassing. The authors suggested that, because they did not form colonies on plates just prior to regassing, the cells may have been injured or in the VNC state, and that upon regassing those cells were resuscitated. There is, however, the possibility that a few viable-culturable spiral shaped cells remained in the system and grew upon re-gassing. These conditions however may just provide a means of retaining culturability rather than regaining it.

In vivo passage models

In attempts to show that emergence of the coccoid form and the concomitant decline in detectable (plate counts) numbers is actually linked with the so called VNC form, several researchers have used animal models as indicators of viability. The use of animal models has given varied results. Medema and others (1992) pointed out that to fully realize the rate of transmission of *C. jejuni* and associated illnesses, the organisms must have the ability to regain their culturability under favorable conditions. Favorable conditions are found especially in the GI tracts of humans and other animals, as indicated by the greater rate of isolation from feces than from other sources.

Two models of study were used by Medema and others (1992): 1) embryonated eggs, and 2) one-day old chicks. Embryonated eggs were inoculated with *Campylobacter* suspensions (<1 CFU/ml plate count and <0.1 CFU/ml MPN) and incubated for 7 days at 37°C. Allantoic fluid was then plated on mCCDA for detection of culturable *Campylobacter*, but none were found. In the chicken model, eggs that were free of parasites, viral pathogens, various *Salmonella* spp. and mycobacteria were incubated and hatched under controlled/sterile conditions. One-day old chicks were orally dosed with *C. jejuni* (<0.1 CFU/ml plate count; <0 .01 CFU/ml MPN; 6.0×10^6 CFU/ml AODC and

1.8×10^5 CFU/ml DVC). The chick's caecal contents were plated after 7 days and incubated at 37°C for 48 hours. Again, no colonies of *Campylobacter* were found on mCCDA plates.

Cappelier and others (1999) used chicken and mouse models to examine the recovery of the VNC form from three strains of *C. jejuni*. Cells grown on agar plates were resuspended in 500 ml of filter sterilized water such that the final concentration of *C. jejuni* was approximately 10^8 CFU/ml. This water microcosm was incubated at 4°C for 30 days while shaking at 100 rpm in an attempt to produce VNC organisms. Viable and culturable cells were detected on spread plates while VNC cells were detected using microscopic examination of cells stained with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). This particular method distinguishes "metabolically" active cells through fluorescent microscopy. The dye is taken up by the cell, reduced by the electron transport system, and then fluoresces red. The cells showing the fluorescent CTC formazan crystals are thought to be still viable since cellular enzymatic activity has not stopped at the time of the assay. Animals were inoculated with 0.2 ml of the water suspension (chicks) or 50ml of a concentrated cells suspension made from the 30 day old water microcosm (mice). For both models, there were both positive (similar volumes of *C. jejuni* inoculum-10^7 CFU/ml) and negative (similar volumes of sterile distilled water) controls. Chick caecal/intestine samples were taken on day 2, 4 and 7, resuspended in basal medium, and either plated directly or after enrichment. Mouse stomach and intestines were processed similarly after 4, 20 and 44 hours. The recovery rate for chicks inoculated with the VNC water microcosm was 1/69 (direct plating) and 8/69 (enriched sample). The positive control (chicks inoculated with 10^7CFU/ml viable and culturable

C. jejuni) yielded 26 positive of 69 total (direct plating) and 36 positive of 69 total (enriched sample). The mouse model yielded many more positive animals inoculated with the VNC water microcosm than the chick model. The results reported were for both direct plating and enrichment methods. When inoculated with the VNC microcosm there were 45/135 positive mice. Mice inoculated with the viable and culturable suspension were almost all positive (122 positive for *C. jejuni* of 126 total mice). Perhaps the higher rate of recovery for the mouse model was due to the administration of a concentrated cell suspension of the VNC water microcosm compared to the chick inoculum.

The viability of *C. jejuni* and *C. coli* was studied by Ng and others (1985). These organisms were grown in broth for 24 hours at 37°C. The cultures of *C. jejuni* and *C. coli* contained 99% spiral shaped forms (9.14 and 9.28 log CFU/ml, respectively), and no coccoid shaped cells were observed by phase contrast microscopy. Plating resulted in 8.41 and 8.49 log CFU/ml. These plates were incubated for 5, 7 and 10 days and on each day, cells were collected and made into standardized cell suspensions equivalent to a McFarland #2 standard. After 5 days of incubation, the predominant morphology was coccoid (10^9 coccoid/ml) and approximately 10^6-10^7 spirals/ml for both strains. Day 7 cell suspensions again contained 1.0×10^9 coccoid/ml and 1.9×10^6 spirals/ml and resulted in 6.7×10^5 CFU/ml when plated. After 10 days, the collective results showed 2.2×10^9 coccoid and 5.6×10^6 spirals/ml with plate counts of 5.1×10^6 CFU/ml. Conclusions were that the loss of roughly 10^2 CFU/ml can be associated with the high incidence of the coccoid form, suggesting that this form is not viable and that propagation requires the presence of the spiral morphology.

One possible route to solving the VNC problem associated with laboratory methods may be the development of a test that actually detects this form. That has not been done at this time. However, more importantly, with respect to research in the area of detection, a protocol is needed that addresses the VNC/morphology state such that we prevent the formation of the coccoid form and thus limit variability associated with its existence. We are dealing, in many cases with information from research that has had very little control over the cultivation of this organism. Lack of controls in the beginning of experimentation may lead to questionable if not erroneous results and conclusions.

Current Methodology for Isolation of *Campylobacter* from Food and Water

The United States Food and Drug Administration through the Center for Food Safety & Applied Nutrition have created the Bacteriological Analytical Manual (BAM) detailing methodology for the detection of various organisms. Specifically, the detection of *Campylobacter* species in food and water (Hunt and others 2001) will be the focus of this section. The FDA methods are complicated, tedious and expensive. Most of the following text is a cursory summary of areas relevant to this paper. Further details of specific methodology can be found in the FDA Bacteriological Analytical Manual: Chapter 7: The Isolation of Campylobacter Species from Food and Water.

Sample preparation

Sample collection and preparation depends on the type of food product to be analyzed. In general, representative samples are weighed out in 25-gram portions (50 grams for vegetables) and aseptically placed into appropriate enrichment vessels. These vessels may be either sterile stomacher bags (400 ml or larger), or 250-500 ml Erlenmeyer flasks.

Pre-enrichment

The pre-enrichment/enrichment media are one and the same and consist of *Campylobacter* Enrichment Broth (a.k.a. Bolton Broth) mixed with lysed horse blood and a mixture of antibiotics (sodium cefoperazone, trimethoprim lactate, vancomycin and cyclohexamide). Pre-enrichment periods are either 4 hours (if sample age $\leq$ 10 days; dairy products) at 37°C or 5 hours (if sample age $\geq$ 10 days; water; shellfish). The 5-hour pre-enrichment may be partitioned with 3 hours at 30°C and 2 hours at 37°C under microaerophilic conditions. During pre-enrichment, the broth/sample should be shaken either 50-60 rpm (bubbler system) or 175-200 rpm for gassed bags or flasks. If the bubbler system (non-shaking) method is used then the entire 5 hour pre-enrichment is done at 37°C. It is presumed that anaerobes may proliferate at 30°C and would interfere with the growth and isolation of *Campylobacter*.

Enrichment

The enrichment period for shaking samples (except shell fish and dairy products) is 23-24 hours. Shellfish should be incubated an extra 4 hours while dairy products should be incubated for 48 hours. The microaerophilic gas (5% O_2, 10%CO_2 and 85% N_2) must be supplied in both the pre-enrichment and the enrichment process. This may be accomplished by using a bubbler system which supplies gas in a continuous fashion or bags/flasks which can be gassed and sealed or placed inside a larger chamber (i.e., BBL Anaerobe Jar) that is charged with gas generated from envelopes or pouches (Campy-Paks or Anaerobe Paks) and then sealed.

1) Bubbler system: Enrichments are placed into a double bag system (with a small amount of water between the bag containing the sample and the outside bag to

enhance heat transfer) and placed into support baskets. A pipet is inserted into the broth sample, bags are tightly cinched with a twist-tie, the pipet is connected to the gas system, and gas pressure is adjusted to 2-3 bubbles/sec entering the broth solution. This is commonly done in a water bath.

a. Shaking: 50-60 rpms
b. Non-Shaking: note pre-enrichment incubation specifications of time and temperature.

2) Gassed and sealed metalized poly pouches, or evacuated and gassed Erlenmeyer flasks: On an individual basis, a pipet is inserted into the headspace above the sample broth, and gas is turned on until bag fills. This is repeated two times, ending with one last gas flush before sealing the bag. This is commonly done in an incubator with a shaking platform. Shaking should be done at 175-200 rpm.

3) Anaerobe jar system: This type of system usually consists of various sizes of jars (BBL, Difco, or Oxoid) that are specially designed to maintain a tight seal after being 1) flushed with gas or 2) charged with gas from special gas generating systems (i.e., Campy-Pak). The former system has a gas valve fused into the jar such that the entire system, once sealed with the samples inside is evacuated and gassed several times ending with a final gas flush. The valve is pinched off and the system is incubated. If using the bag system for enrichment, then it is specified that the bags are left open "loosely". If using an Erlenmeyer flask system it is presumed that the top of the flask is slightly closed in some way. There was a mention of a foam cap but the details of this were omitted. Needless to say, there should be some type of covering such that the modified

atmosphere can interact with the culture while somehow preventing contamination. The specific mention of shaking this type of system was not in the procedures. It can only be assumed that the shaking would be similar to that of the gassed and sealed metalized poly pouches or evacuated and gassed Erlenmeyer flasks and set at 175-200 rpm.

Immediately following the pre-enrichment procedure, the temperature is raised to 42°C except if *C. fetus* is suspected then temperature should remain at 37°C. If using the bubbler (non-shaking) method then the incubation time is 28-29 hours except in the case of shellfish, which are incubated for 48 hours. Additionally, if *C. fetus* is a target then samples should be incubated for 48 hours in any of the shaking method or 52 hours if using the bubbler (non-shaking) method.

An important note in the procedures suggests that "if incubating in anaerobe jars, reduce the volume/flask or bag to 125 ml by dividing the enrichment into two parts." It is thought that the gas does not penetrate into a larger volume sufficiently to provide proper growth of *Campylobacter*. This statement suggests that a technique (or methodology) that addresses this and other problems that affect the handling and management of *Campylobacter* is needed.

Implications to Detection in Food and Water

Understanding the route of infection by *Campylobacter* is clouded by the production of the coccoid form and VNC state. The production of the coccoid form is dependant upon many interrelated factors, often stress related, and dominated by strain variation (Figure 1). It is unclear as to whether or not the coccoid form can cause disease on its own accord, must first revert back to the rod form, or is not an infectious entity. It is known that *Campylobacter* is ubiquitous in the environment and exists there in the

viable culturable (VC) state. It is not known, however, if this organism exists in viable nonculturable (VNC) or nonviable nonculturable (NVNC) states in the environment (Figure 1a). It is known that *Campylobacter* exists in the VC state in food and water. It is not known, however, if it exists in a VNC state in food and water (Figure 1b). Finally, it is known that *Campylobacter* is in the VC state after ingestion and fecal shedding by either humans or animals. It is not known, however, if an organism in the VNC state can resuscitate and reenter the VC state, or if it passes harmlessly through the system in a NVNC state (see figure 3c). Testing for the presence of a VNC form (in vitro or in vivo) is usually confounded by not knowing for certain that there was not at least one organism in the VC state that was able to survive, adapt and grow when provided with a favorable environment. In addition, just because an organism is in the NVNC state, is it assumed that all metabolic processes cease? If they do not, then what validity can be placed on assays that measure cellular activity and link it to viability, or animal studies that inoculate with an assumed total VNC population?

Culturable *Campylobacter* can be recovered from food and water as well as from infected humans and animals; however, in vitro studies indicate that the majority of growth on agar plates consists of the helical rod form while a variety of morphological forms are present in broth culture. Very little has been done with respect to growing *Campylobacter* in broth at optimum temperatures (35-42°C) and measuring both growth (plate counts) and morphological characteristics. It is important to keep in mind that time and temperature is extremely critical when growing *Campylobacter* for experimental purposes or isolating it from food or water. With this in mind, our efforts in this dissertation are focused more on the prevention of the formation of the coccoid form

(regardless of its viability) when working with in vitro analysis to detect *C. jejuni* in food products and water.

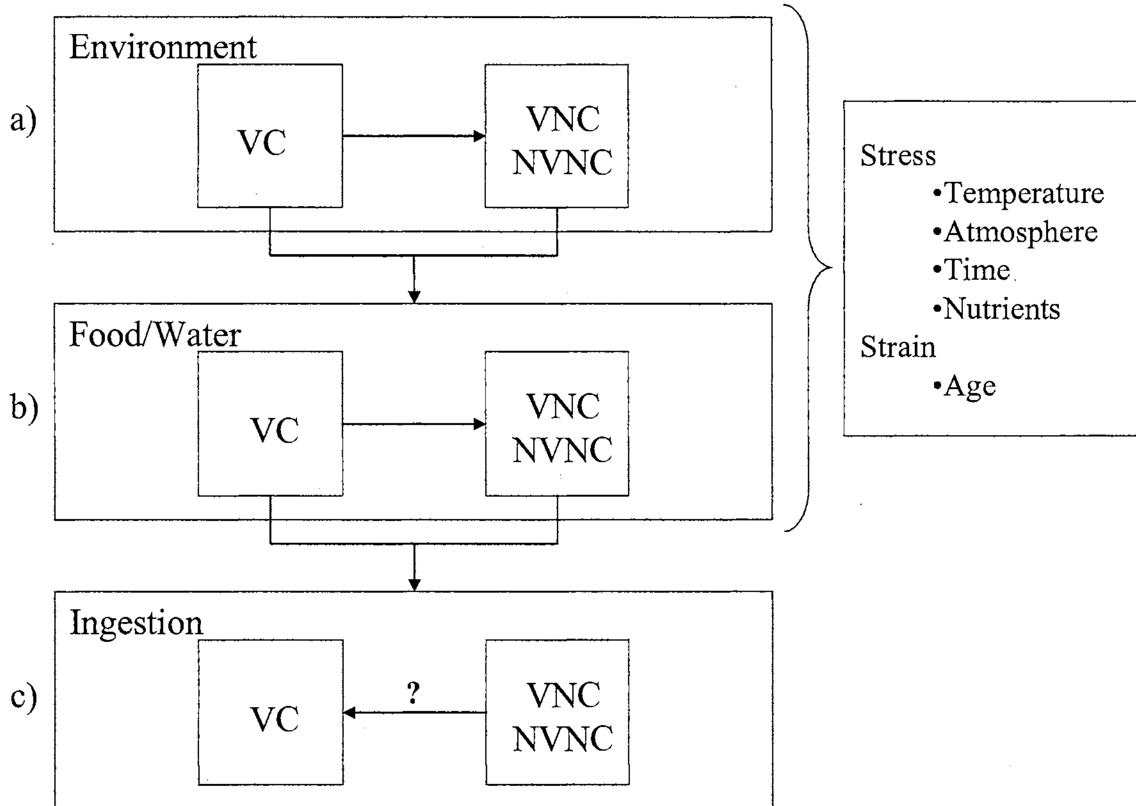

Figure 1. Interface between Viable/Culturable, Viable/NonCulturable (VNC) or a NonViable/NonCulturable (NVNC) state of *Campylobacter* and the environment, food/water, or human/animal system as well as possible interfering or inducing factors.

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CHAPTER III

IMPROVED TECHNIQUES FOR CULTURING *CAMPYLOBACTER*

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ABSTRACT

Growth and morphology of *Campylobacter jejuni* when grown under various conditions in Bolton broth at 37°C were studied. Enumeration was on campylobacter agar containing charcoal and deoxycholate (CCDA) using spiral-plating techniques. Percentages of coccoid cells were determined microscopically. Consistent growth and maintenance of the helical-rod form of *C. jejuni* was dependent upon (1) the type of growth vessel used and, more importantly, a relatively large surface-to-volume ratio ($250mm^2/ml$); and (2) exposure to a modified atmosphere (10% CO_2, 85% N_2 and 5% O_2). When *C. jejuni* was incubated for more than 24 hours, there was a large percentage (36 to 75%) of transformation to the coccoid form and fewer colony-forming units compared to incubation up to 24 hours. Strains of the organism behaved differently with respect to subculturing. In general, however, two successive subcultures may be performed without a change in growth or morphology. Decreased concentrations of colony forming units of *C. jejuni* commonly attributed to the production of the "viable but non-culturable" form may also be due to cellular "clumping" as evidenced by scanning electron micrographs. Clumping was associated with inadequate surface-to-volume ratio and gas exchange in the growth medium. Techniques to improve growth and maintenance of *C. jejuni* in pure culture and detecting it in food products are described.

INTRODUCTION

Campylobacter jejuni, first detected in humans in 1973, has been linked to millions of incidences of food-borne illnesses (Altekruse 1999). The Food and Drug Administration indicated that *C. jejuni* may be the leading cause of food borne illness in the United States (U.S. Food and Drug Administration 1992). The United States Department of Agriculture (USDA) estimated that the annual cost associated with *Campylobacter* infections ranged from $1.5 to $8.0 billion (Buzby and Roberts 1997). *Campylobacter* species are common inhabitants of the gastrointestinal tract of many domestic animals and thus food products of animal origin are frequently contaminated with *C. jejuni*. For example; live broilers, hens, turkeys and ducks harbor *Campylobacter* in the range of 10^5-10^9 CFU/g leading to the high incidence of illness due to the consumption or handling of raw poultry or related products (Jacobs-Reitsma 2000). Illnesses associated with *Campylobacter* have been attributed to other undercooked meats, raw milk, and untreated water. Ingestion of only a few bacteria (500) may cause illness (Skirrow and Blaser 2000).

Many researchers have reported difficulties growing C*ampylobacter* in pure culture and suggested that it is even more difficult to isolate *Campylobacter* from food products (Rollins and others 1983, Rollins and Colwell 1986; Aquino and others 1996; Reezal and others 1998). Cells of *Campylobacter* undergo a morphological transition marked by the physical change from a helical rod shape to a coccoid shape (Moran and Upton 1986; Griffiths 1993). The coccoid form may represent a viable but non-

culturable (VBNC) state (Rollins and Colwell 1986; Saha and others 1991; Griffiths 1993; Bovill and Mackey 1997). This transformation may occur when cultures are exposed to unfavorable atmospheres, temperatures, light, UV radiation, and hydrogen peroxide (Juven and Rosenthal 1985; Moran and Upton 1987). Furthermore, Griffiths (1993) indicated that *C. jejuni*, when grown in liquid culture, changed morphology at different stages of growth. Ng and others (1985) showed that different forms were present in different parts of a colony. They suggested that the center of the colony contained coccoid shapes while the perimeter contained mostly helical rods. Another "donut" shaped cell was attributed to a raised section of the same colony. A closely related organism, *Helicobacter pylori*, also exhibits this transformation, along with a marked cell wall modification (Costa and others 1999). Formation of the coccoid form also has been found in older cultures and related to variable growth conditions such as type of growth media and temperature (Jones and others 1991; Hazeleger and others 1995, 1998; Reezal and others 1998; Jeffrey and others 2000).

Adding to the intricacy of working with *Campylobacter* is a requirement for modified atmosphere for growth (Juven and Rosenthal 1985; Morgan and others 1987). The recommended atmosphere ranges from 3-15% oxygen and 2-10% carbon dioxide (Hodge and Krieg 1994; Ketley 1997). If oxygen concentrations become too high (>15%) there is a detrimental effect on *Campylobacter*. Hodge and Krieg (1994) indicated that *C. jejuni's* tolerance of oxygen was dependent upon the type of growth media. They concluded that media high in protein sources should be avoided. When cultures of *Campylobacter* are subjected to an aerobic environment instead of a microaerophilic one, they quickly change to the coccoid form (Karmali and others 1981).

Another divergence that may add unnecessary variability is how cultures are prepared prior to use in experiments. Growing the culture in liquid media is necessary, however, in order to achieve a consistently active culture (Morgan and others 1987). Repeated and consistent subculturing of most microorganisms in liquid media prior to experimentation is desirable in order to provide maximum numbers and consistent behavior of the culture. It is not well documented what effect, if any, subculturing has on the growth and morphology of *Campylobacter*. Rollins and others (1983) suggested that subculturing may be problematic but did not expand on the discussion. Because the method under which *Campylobacter* is grown influences its physical transformation, culture methodology must be standardized in order to have accurate and repeatable results within and among laboratories. As growth of *Campylobacter* is so sensitive to environmental conditions, experiments often provide results that are more qualitative than quantitative.

Currently, there are two views of the meaning of the viable but nonculturable state: 1) dead or 2) dormant and can be revived by exposure to favorable conditions (Jones and others 1991; Park 2002). In any case, once the organism changes morphology, enumeration and/or recovery from either broth media or food products become more difficult and subject to error. The objective of this study was to develop and evaluate improved methodology for growing *C. jejuni.*

MATERIALS & METHODS

Source, Maintenance and Preparation of Cultures

Two strains of *C. jejuni* (ATCC 29428 and 33560) were purchased from the American Type Culture Collection (ATCC; Manassas, Va, 20108). The strains were grown according to the ATCC directions using Bolton Broth (Oxoid LTD; Baskingstoke, Hamshire, England) at 37°C. The cultures were then vacuum dried and stored at –20°C until needed (stock cultures).

Prior to each experimental replication, stock cultures were rehydrated in 10 ml of freshly prepared Bolton broth that was aseptically transferred into sterile 50 ml tissue culture flasks (TCF) (Falcon® Brand; Becton Dickinson Labware, Franklin Lakes, NJ) equipped with a vented cap (0.2 μm pore size). The re-suspended culture was incubated in a Gas-Pak chamber (BBL) which was charged with a mixture of CO_2 (10%), O_2 (5%) and N_2 (85%) generated from a Campy-Pak (BBL® Campypak Plus; Becton Dickinson Microbiology Systems, Cockeysville, MD) microaerophilic gas generating system. The container was placed on an orbital benchtop shaker (Lab-Line®, Model 4626) set at 80 rpm and incubated for 18 to 24 hours at 37°C. Prior to use, the cultures were Gram stained and examined microscopically to determine morphological integrity.

Experimental Treatments and Procedures

<u>Effect of growth vessels</u>

Different sizes and/or shapes of growth vessels were used to determine effects of different surface-volume (S:V) ratios on the growth of *C. jejuni* and the formation of the

coccoid form. For the inoculum, each strain was spiral plated onto freshly prepared campylobacter agar with charcoal and deoxycholate (CCDA) (Oxoid LTD, Baskingstoke, Hampshire, England) plates and incubated for 48 hours at 37°C under modified atmosphere conditions (Campy-Pak) inside a Gas-Pak chamber. Well-isolated colonies of each strain were transferred to freshly prepared Bolton broth in separate tissue culture flasks and incubated for 18-24 hours under modified atmosphere conditions on an orbital benchtop shaker set at 80 rpm. Each resulting culture was diluted in fresh sterile Bolton broth to achieve approximately 3-4 log colony forming units/ml. The inoculated broth was aseptically dispensed as indicated in Table 6.

All three treatment combinations for each strain were placed in one Gas-Pak jar, charged with the modified atmosphere (Campy-Pak), and incubated at 37°C with agitation. The cultures were removed from incubation after 18-24 hours and analyzed for plate count and morphology. Three replications of the above procedure were done on different days.

Effect of incubation time

The effect of incubation time on growth and extent of morphological change in *C. jejuni* was determined when incubated at 37°C in the vented TCF. The procedure described above for **Source, Maintenance and Preparation of Cultures** was repeated for 24, 48, and 72-hour growth times for both strains. The cultures were removed from incubation at the appropriate time and analyzed for growth and morphology. There were three replications of the above procedure, and replications were done on different days.

Effect of modified atmosphere

The objective was to determine the effect of atmosphere modification on the growth of the two strains of C. jejuni. For these experiments, cultures of the two strains of C. jejuni prepared as described in Source, Maintenance and Preparation of Cultures were used to inoculate TCF (3 for each culture) containing 10 ml of Bolton broth to achieve approximately 4-5 log colony forming units/ml. Two of the vented TCF (one for each culture) were sealed with silicon and Parafilm M® (American National Can; Neenah, Wis). These, along with one TCF (vented) for each strain were placed in a Gas-Pak jar and charged with a Campy-Pak. The two remaining vented TCF (one for each strain) were placed along side the Gas-Pak jar onto the orbital shaker and incubated 18-24 hrs at 37°C with agitation at 80 rpm. The samples were analyzed for the amount of growth and morphology. Five replications of the experiment were done on different days.

Effect of subculturing

To determine the effect of successive subculturing on growth and extent of morphological change in *C. jejuni* when incubated at 37°C in the vented TCF the procedure described above for Source, Maintenance and Preparation of Cultures was repeated for the original (0) culture for both strains. For successive subcultures, Bolton broth was inoculated at approximately 4-5 log colony forming units/ml. Successive subcultures 1, 2 and 3 were made after incubation for 18-24 hrs, under modified atmosphere at 37°C on an orbital benchtop shaker at 80 rpm. The samples were analyzed for amounts of growth and morphology. Three replications of the experiment were done on different days.

Analytical Procedures

Enumeration

In all cases, *C. jejuni* was enumerated using a spiral plater (dwScientific; Don Whitley Scientific, Shipley, England). Appropriate dilutions were made using 0.1% peptone dilution blanks followed by spiral plating onto pre-poured plates of a selective blood free agar (CCDA) containing no additional selective agents. Samples were plated in 50 μl aliquots in a logarithmic mode setting. Plates were inverted and incubated for 48 hours at 37 °C in a Gas-Pak (BBL) chamber charged with a mixture of CO_2 (10%), O_2 (5%) and N_2 (85%) (Campy-Pak). Counts were made using an automatic plate reader (Protocol; Synoptics Ltd., Cambridge, UK) and related software. There were duplicate plates for each treatment combination, and a minimum of three readings was performed for each plate. Means of plate counts from duplicate plates for each treatment combination in each replication were used in statistical analyses.

Determination of percentage coccoid cells

Cells were prepared according to Bovill and Mackey (1997) and counted using a 10 x 10 mm grid inserted into the eyepiece of a Nikon Eclipse 600. Percentages of coccoid cells were based on observation of 1000 cells.

Scanning electron microscopy

Culture suspensions were centrifuged and washed repeatedly (3X) with sterile phosphate buffer. The cell pellet was resuspended in 0.2 ml of a mixture containing 4% paraformaldehyde, 0.1% glutaraldehyde (1N) and phosphate buffer (0.1 M, pH 7.0), and stored at 2 °C until examined. Cells were washed three times with 0.1 M phosphate buffer solution, and placed onto polylycine-coated cover slips (Mazia and others 1975). After fifteen minutes, the excess was removed and the remaining cells were washed three times with 2% phosphate buffered glutaraldehyde. Following this, the samples were dehydrated using a 50, 70, 95 and 100% ethanol treatment (20 minutes for each treatment). Samples were critical point dried and placed on aluminum stubs. Each sample was sputter coated for 3 minutes with gold/palladium coat in a Balzer 010 evaporator. Samples were observed using a JEOL JXM 6400 scanning electron microscope.

Statistical Methods

The MIXED procedure of SAS (SAS® Institute Inc 1999-2001) was used to analyze the data in each experiment. Means were separated using the Least Squares Means and PDIFF options.

Effect of growth vessels

The experimental design was a split-plot in a randomized complete block design with a 2x3 factorial arrangement of treatments (main unit treatment factor = strain; sub-unit treatment factor = growth vessel; block = replications).

Model: $y_{ijk} = \mu + \alpha_i + b_k + d_{ik} + \beta_j + \alpha\beta_{ij} + e_{ijk}$

Where y_{ijk} = k^{th} response of A_iB_j

μ = overall mean

α_i = strain main effect (A_i)

b_k = block k effect $b_k \sim N(0,\sigma_b^2)$

d_{ik} = random error due to the kth experimental unit in Strain (A_i)

β_j = growth vessel main effect (B_j)

$\alpha\beta_{ij}$ = strain*flask interaction effect

e_{ijk} = subplot random error, $e_{ijk} \sim N(0, \sigma_e^2)$

Effect of incubation time

The experimental design was a split-plot in a randomized complete block design with a 2x3 factorial arrangement of treatments (main unit treatment factor = incubation time; sub-unit treatment factor = strain; block = replications).

Model: $y_{ijk} = \mu + \alpha_i + b_k + d_{ik} + \beta_j + \alpha\beta_{ij} + e_{ijk}$

Where y_{ijk} = k^{th} response of A_iB_j

μ = overall mean

α_i = incubation time main effect (A_i)

b_k = block k effect $b_k \sim N(0,\sigma_b^2)$

d_{ik} = random error due to the kth experimental unit in Time (A_i)

β_j = strain main effect (B_j)

$\alpha\beta_{ij}$ = incubation time*strain interaction effect

e_{ijk} = subplot random error, $e_{ijk} \sim N(0, \sigma_e^2)$

Effect of modified atmosphere

The experimental design was a split-plot in a randomized complete block design with a 2x3 factorial arrangement of treatments (main unit treatment factor = strain; sub-unit treatment factor = atmosphere; block = replications).

Model: $y_{ijk} = \mu + \alpha_i + b_k + d_{ik} + \beta_j + \alpha\beta_{ij} + e_{ijk}$

Where y_{ijk} = k^{th} response of A_iB_j

μ = overall mean

α_i = strain main effect (A_i)

b_k = block k effect $b_k \sim N(0,\sigma_b^2)$

d_{ik} = random error due to the kth experimental unit in Strain (A_i)

β_j = atmosphere main effect (B_j)

$\alpha\beta_{ij}$ = strain*atmosphere interaction effect

e_{ijk} = subplot random error, $e_{ijk} \sim N(0, \sigma_e^2)$

Effect of subculturing

The experimental design was a split-plot in a randomized complete block design with a 2x4 factorial arrangement of treatments (main unit treatment factor = subculture; sub-unit treatment factor = strain; block = replications).

Model: $y_{ijk} = \mu + \alpha_i + b_k + d_{ik} + \beta_j + \alpha\beta_{ij} + e_{ijk}$

Where y_{ijk} = k^{th} response of A_iB_j

μ = overall mean

α_i = subculture main effect (A_i)

b_k = block k effect $b_k \sim N(0,\sigma_b^2)$

d_{ik} = random error due to the kth experimental unit in subculture (A_i)

β_j = strain main effect (B_j)

$\alpha\beta_{ij}$ = subculture*strain interaction effect

e_{ijk} = subplot random error, $e_{ijk} \sim N(0, \sigma_e^2)$

RESULTS & DISCUSSION

Effect of Growth Vessels

There was a tendency for an interaction ($p=0.099$; Table 7) in that There was no difference in amount of cell growth between strains but there were differences among vessels ($p<0.0001$) and a tendency for an interaction ($p=0.099$; Table 7). With both strains, there was less growth in test tubes (TT) than in either tissue culture flasks (TCF) or Erlenmeyer flasks (EF) ($p<0.03$). There was no difference in amount of cell growth between TCF and EF with strain 29428 but with strain 33560, growth was less in EF than TCF ($p<0.03$).

Improved growth with vented TCF may be related to the relatively large surface:volume (S:V) ratio available for growth (250 mm^2/ml) especially when compared to a standard TT (150 mm length, 14 mm diameter). In the TT containing 10 ml of broth the surface-to-volume ratio is only 15.4 mm^2/ml. Thus, growing the culture in a traditional test tube may not be optimum due to the lack of surface-to-volume ratio needed by the culture to maximize atmosphere exchanges. When using an Erlenmeyer flask (EF), growth may be better due to the larger surface:volume ratio, however capping or closing the flask in order to minimize contamination may still lead to inadequate gas exchange. The vented TCF has a screw-top cap that contains a semi-permeable membrane (<0.2μm pore size) providing efficient gas exchange while preventing contamination.

In preliminary studies, we found it desirable to slowly agitate the culture (about 60-90rpm), which enhances the gas exchange by increasing the mixing of the medium within the TCF. Need for agitation was alluded to in previous research where *Campylobacter* was grown in broth in culture jars and continuously agitated under microaerophilic conditions (Griffiths 1993). The authors assumed this allowed enough oxygen to be incorporated into the media. It may also provide a means to reduce the build up of toxic oxygen byproducts (i.e., hydrogen peroxide). Moran and Upton (1987) showed that the build-up of hydrogen peroxide and subsequent dissociation products enhanced the development of the coccoid form. They associated this conversion with the lack or loss of the superoxide dismutase enzyme commonly found in actively metabolizing cells.

There was no difference in percentage of coccoid forms between strains and there was a tendency for differences among vessels ($p=0.119$) with no interaction (Table 7). For both strains, the percentage of coccoid cells increased significantly from 6.8 and 4.5 in TCF to 31.5 and 24.8 in TT for strains 29428 and 33560, respectively ($p<0.05$). Percentage of coccoid cells found in EF was intermediate between TCF and TT.

It is desirable to prevent the morphological shift from the rod to the coccoid form. Our research indicates that a higher numbers of CFU of *Campylobacter* are obtained with the majority of the morphology of cells in the rod shape, when using the vented TCF compared to test tubes (Table 7). Optimal conditions can be attained while maintaining the integrity of the culture, maximizing culture activity and consistency of growth by using a vented TCF. Rollins and others (1983) indicated a biphasic culture system containing brucella agar and brucella broth, supplemented with ferrous sulfate, sodium metabisulfite and sodium pyruvate, and incubated under a gas mixture of 5 %O_2, 10%

CO_2 and 85% N_2 was best for growth of the organism. They used TCF but did not compare to growth in other vessels and concluded that the key to successful growth was the media used. Additionally, it is also important to address the atmospheric gas exchange and adequate surface-to-volume ratios for improved growth. In our research, TCFs with vented closures when used with media specific for *Campylobacter* (i.e., Bolton Broth) led to drastically improved culture integrity and growth compared to other systems. When using other common types of growth vessels, (i.e., TT or EF) the percentage coccoid forms increased while numbers of colony forming units decreased, hence producing the perceived viable but nonculturable (VNBC) cells.

Effect of Incubation Time

There were differences in plate counts between strains ($p=0.014$) and incubation times ($p<0.0001$) but no interaction (Table 8). After 48 hours incubation, there was a 2.0 and 3.2 log reduction compared to those at 24 hours for strains 29428 and 33560, respectively. Counts were further decreased by 3.4 and 5.8 log cycles after 72 hours for both strains, respectively. Even though there was no interaction ($p=0.186$), the difference between strains became greater at the two longer incubation times (72 hours).

Strains tended to be different in percentages of coccoid forms ($p=0.089$) and there were differences for each among incubation times ($p<0.0002$) (Table 8). The morphology of both strains changed markedly with increasing incubation time. After 24 hours of incubation, the percentage of coccoid cells was approximately 13 and 10% for strains 29428 and 33560, respectively. After 48 and 72 hours the coccoid form increased to 36 and 52%, respectively, for strain 29428, and 56 and 75%, respectively, for strain 33560. It is important to realize the extent of morphological changes of *C.*

jejuni over time. These data suggest that excessive incubation periods (greater than 24 hours) do not support the maintenance of viable cells detectable by plate count. Based on our findings, the optimum incubation time when working with *C. jejuni* appears to be 24 hours or less.

Effect of Modified Atmosphere

There was an interaction between strain and atmosphere for cell growth ($p=0.022$; Table 9). For both strains, growth was greatest for VM, intermediate for NVM, and least for VA. The interaction occurred in the NVM treatment, where growth of strain 29428 was less than that of strain 33560 ($p<0.05$). There was no difference between strains in percentage of coccoid form, but a large difference among atmospheres ($p<0.0001$). There was no difference between NVM and VA but both treatments resulted in large increases ($p<0.0001$) in percentage of coccoid forms compared to VM.

Both strains responded in a similar fashion to changes in atmosphere with respect to growth and the production of coccoid form. Counts were greatest when grown in vented TCF in the modified atmosphere environment (VM) compared to either non-vented flasks (NVM) or vented flasks in an air environment (VA). In addition, the formation of the coccoid form was dramatically higher when the strains were not exposed to the modified atmosphere (treatments NVM and VA). *C. jejuni* 29428 transformed 77% when grown in a non-vented TCF (NVM) and 94% when grown in a vented TCF exposed to air (VA). *C. jejuni* 33560 responded in a similar fashion with 89% transforming to the coccoid in non-vented TCF (NVM) and 84 percent in air (VA).

From these data, it is apparent that *Campylobacter* requires modified atmosphere during growth. The ideal atmosphere according to the literature is 3-15% oxygen and 2-

10% carbon dioxide. If, however, oxygen concentrations become too high (>15%) there is a detrimental effect on *Campylobacter* (Hodge and Krieg 1994). Our findings support these reports.

Effect of Successive Subculturing

There was an interaction between strain and subculture for growth ($p<0.0001$; Table 10). Growth of both strains remained the same for two successive subcultures. The amount of growth in 24 hours for strain 33560 was over 2 log cycles lower in the third successive subculture than in subcultures 1 and 2. Strain 29428 did not exhibit any decline in growth in the third subculture compared to the first two. There was an interaction between strain and subculture with respect to formation of the coccoid form ($p=0.0026$; Table 10). Strain 29428 appeared to be fairly stable in terms of percentages of coccoids (7% after two subcultures and 15% upon the third subculture). However, for strain 33560, the percentage of coccoid forms (45%) increased significantly after the second subculture ($p<0.06$) and even more after the third subculture (86% coccoid). It appears that there is a tendency for percentage coccoid to be related to growth; however, it is interesting to note that upon the second successive subculture, strain 33560 maintained relatively high numbers (9.57 log CFU/ml) even though it transformed to 45% of the coccoid form. This may suggest that transformation to the coccoid form does not necessarily result in loss of viability that is associated with the VBNC aspect of *C. jejuni*.

Morphological Observations

The "classic" morphologies of helical rod and coccoid forms of *C. jejuni* are illustrated in Figure 2-A. We have, however, observed many other cell forms/shapes. Based on scanning electron micrographs it appears that the helical rod produces either at the very least one coccoid cell and then generates a "spent" cell/debris (Figure 2-B), or a multitude of cells that become massed together into what may result in only one colony-forming unit (Figure 2-C and D). These other forms may explain some of the reported loss of viability previously attributed to the production of the viable but nonculturable coccoid form. Some of these forms are similar to those reported earlier (Ng and others 1985; Thomas and others 1999) but include other forms that have not been documented previously.

CONCLUSION

The growth and maintenance of *C. jejuni* is enhanced by the use of vented tissue culture flasks (TCF) in conjunction with an atmosphere of 10% CO_2, 5% O_2 and 85% N_2 compared to test tubes and Erlenmeyer flasks. Improved growth and decreased appearance of coccoid cells with vented TCF may be related to the surface-volume ratio of growth media. In addition, the TCF's screw-top cap contains a semi-permeable membrane (<0.2μm pore size) allowing for increased gas exchange while preventing contamination. It also helps ensure that the pathogen is not inadvertently spread to other samples or to the person conducting the assays. A modified atmosphere (10% CO_2, 5% O_2 and 85% N_2) was needed for adequate growth and maintenance of the helical-rod form when using TCFs. Incubation time of *C. jejuni* in vented TCF was also critical. Incubation for 24 hours produced higher counts and fewer coccoid cells than did longer times of incubation. When incubation was 48 or 72 hours, however, counts decreased and *C. jejuni* transformed to a large percentage of coccoid cells. There was a strain-to-strain variation in response to subculturing. Strain 29428 did not show any loss in viability or great increase in percentage coccoids after three successive subcultures when grown in TCF under a modified atmosphere (10% CO_2, 5% O_2 and 85% N_2). Strain 33560 drastically declined in growth following the third subculture, and transformed to 45 and 86% coccoid after the second and third subculturing respectively. The combination of vented TCF, modified atmosphere, ≤ 24-hour incubation time and limited

subculturing is critical to the maintenance of viable cultures and improved enumeration methodology. Deviations from these procedures may give rise to an increase in the viable but nonculturable state of *Campylobacter*. A recommended enumeration protocol is in the **Appendix.**

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APPENDIX

Protocol for Growing *Campylobacter jejuni* at 37°C

1. Remove stock culture and aseptically transfer into freshly prepared (<2days) sterile Bolton Broth in vented tissue culture flask.

2. Place flask into GasPak chamber and charge with modified atmosphere (CampyPak). (10% CO_2, 5%O_2 and 85%N_2)

3. Quickly seal chamber and place on orbital benchtop shaker (80 rpm for small (50ml) flasks and 60 rpm for larger (250ml) flasks. Broth should gently move inside flask but not wet the semi-permeable membrane in the vented cap.

4. Limit incubation time to no more than 24 hours.

5. Remove culture and plate (spiral-plate[a]) immediately onto freshly prepared CCDA plates[b].

6. Incubate plates inverted for at least 48 hours in a GasPak chamber charged with modified atmosphere (CampyPak). (10% CO_2, 5%O_2 and 85%N_2)

7. Remove plates and aseptically select a well-isolated colony and transfer to freshly prepared (<2day) sterile Bolton Broth in a vented tissue culture flask. This is the recommended starting culture for pre-planned experiments.

8. Repeat steps 2-4[c].

9. Always check for morphological integrity of culture. If large percentages of coccoid forms are present discard and re-isolate.

[a]Spiral-plating techniques and associated low plating volumes (50 μl) are advantageous for elimination of colony "spreading" and irregularity.

[b]Plates should be allowed to dry (inverted) for 1-2 days. If excessive moisture is present allow longer drying. Check plates for contamination.

[c]Unless strain behavior is well known, do not subculture more than twice from the original culture. Make serial dilutions of the culture so that the inoculum level is approximately 10^4-10^5 CFU/ml.

Table 6. Growth vessels related surface:volume (S:V) ratios used for evaluating the growth of *C. jejuni*.

Container	Volume of Broth (ml)	S:V Ratio (mm^2:ml)
TCF[1]	10	250
EF[2]	10	139
TT[3]	10	15

[1]Falcon Tissue Culture Flask (50ml, 0.2µm vented cap).
[2]Pyrex Erlenmeyer Flask (50ml, perforated aluminum foil cap).
[3]Fisherbrand Test Tube (150mm length, 14mm diameter, loosely fitted screw cap).

Table 7. Growth and morphological changes of two strains of *Campylobacter jejuni* incubated in Bolton broth with different surface to volume ratios in a modified atmosphere environment at 37°C for 24 hours.

C. jejuni strain (ATCC)	Growth Vessel[1]	S-V Ratio[2] (mm^2/ml)	Growth[3] (Log CFU/ml)	Morphology[3] (% Coccoid)
29428	TCF	250.0	9.43^{ab}	6.8^{e}
	EF	138.5	9.19^{bc}	12.8^{ef}
	TT	15.4	8.25^{d}	31.5^{f}
33560	TCF	250.0	9.68^{a}	4.5^{e}
	EF	138.5	9.13^{c}	16.3^{ef}
	TT	15.4	8.12^{d}	24.8^{f}
SEM			0.097[4] (df=9.17)	7.09[5] (df=12)
p values:				
Strain (S)			.825	.824
Vessel (GV)			<.0001	.119
S*GV			.099	.881

[1]TCF: Falcon Tissue Culture flask (size: 50ml, .2μm vented cap).
EF: Pyrex Erlenmeyer flask (size: 50ml)
TT: Fisherbrand test tube (size: 150mm length, 14mm diameter)

[2]S-V Ratio: <u>S</u>urface-<u>V</u>olume Ratio of the various vessels calculated based on 10 ml media volume

[3]Data are the means from three replications.

[4]Standard Error of the Mean (SEM) and associated degrees of freedom (df) for S*GV.

[5]Standard Error of the Mean (SEM) and associated degrees of freedom (df) for GV.

[abcd] Interaction (S*GV) means without a common letter in superscript groups are different ($p < 0.03$).

[ef] Vessel (V) means within strain without a common letter in superscript group are different ($p < 0.05$)

Table 8. Growth and morphology of two strains of *Campylobacter jejuni* grown in tissue culture flasks[1] and incubated for 24, 48 and 72 hours in modified atmosphere at 37°C.

C. jejuni strain (ATCC)	Incubation Time (hrs)	Growth[2] (Log CFU/ml)	Morphology[2] (% Coccoid)
29428	24	8.91[a]	13.0[d]
	48	6.92[b]	35.7[e]
	72	5.48[c]	51.8[e]
33560	24	8.69[a]	9.5[d]
	48	5.51[b]	55.6[e]
	72	2.85[c]	74.8[e]
SEM[3]		0.432	6.14
		(df = 12)	(df = 12)
p values			
Strain (S)		0.014	0.089
Time (T)		<0.0001	<0.0002
S*T		0.186	0.286

[1]Falcon Tissue Culture flask (size: 50ml, .2μm vented cap) containing 10 ml Bolton broth.
[2] Data are the means from three replications.
[3]Standard Error of the Mean (SEM) and associated degrees of freedom (df) for T.
[abc] Time (T) means within strain without a common letter in superscript groups are different ($p < 0.0001$).
[de] Time (T) means within strain without a common letter in superscript groups are different ($p < 0.07$).

Table 9. Growth and morphology of two strains of *Campylobacter jejuni* grown in tissue culture flasks[1] exposed to different atmospheric environments at 37°C for 24 hours.

C. jejuni strain (ATCC)	Atmosphere[2]	Growth[3] (Log CFU/ml)	Morphology[3] (% Coccoid)
29428	VM	8.50[a]	8.2[e]
	NVM	4.68[b]	76.7[f]
	VA	2.82[d]	93.5[f]
33560	VM	9.27[a]	18.9[e]
	NVM	7.58[c]	88.6[f]
	VA	3.30[d]	83.9[f]
SEM		0.468[4] (df = 22.3)	4.23[5] (df = 24)
P values			
Strain (S)		.015	.422
Atmosphere (A)		<.0001	<.0001
S*A		.022	.801

[1]Falcon Tissue Culture flask (size: 50ml, .2μm vented cap) containing 10 ml Bolton broth.

[2]Atmospheres were:

VM = Vented tissue culture flask in modified atmosphere chamber containing a modified atmosphere (10% CO_2, 85% N_2 and 5% O_2).

NVM = Non-vented tissue culture flask in modified atmosphere chamber.

VA = Vented tissue culture flask exposed to air (no modified atmosphere).

[3] Data are the means from five replications.

[4]Standard Error of the Mean (SEM) and associated degrees of freedom (df) for S*A.

[5]Standard Error of the Mean (SEM) and associated degrees of freedom (df) for A.

[abcd] Interaction (S*A) means without a common letter in superscript groups are different (p <.05).

[ef] Atmosphere (A) means within strain without a common letter in superscript groups are different (p = <.0001) .

Table 10. Growth and morphology of two strains of *Campylobacter jejuni* grown in tissue culture flasks[1] and subcultured three times (24 hour intervals) from original culture in modified atmosphere at 37°C.

C. jejuni strain (ATCC)	SubCulture	Growth[2] (Log CFU/ml)	Morphology[2] (% Coccoid)
I (29428)	0 (original)	9.64^{a}	7.6^{d}
	1	9.64^{a}	6.0^{d}
	2	9.44^{a}	7.0^{d}
	3	9.60^{a}	15.5^{d}
II (33560)	0 (original)	9.22^{a}	7.4^{d}
	1	9.41^{a}	13.1^{d}
	2	9.57^{a}	45.1^{e}
	3	7.25^{b}	86.1^{e}
SEM[3]		0.173 (df = 14.6)	9.92 (df = 11.2)
p values			
Strain (S)		<.0001	.0479
SubCulture (SC)		<.0001	.0004
S*SC		<.0001	.0026

[1]Falcon Tissue Culture flask (size: 50ml, .2µm vented cap).

[2] Data are the means from three replications.

[3]Standard Error of the Mean (SEM) and associated degrees of freedom (df) for S*SC.

[ab] Interaction (S*SC) means without a common letter in superscript groups are different ($p < 0.0001$).

[de] Interaction (S*SC) means without a common letter in superscript groups are different ($p < 0.06$).

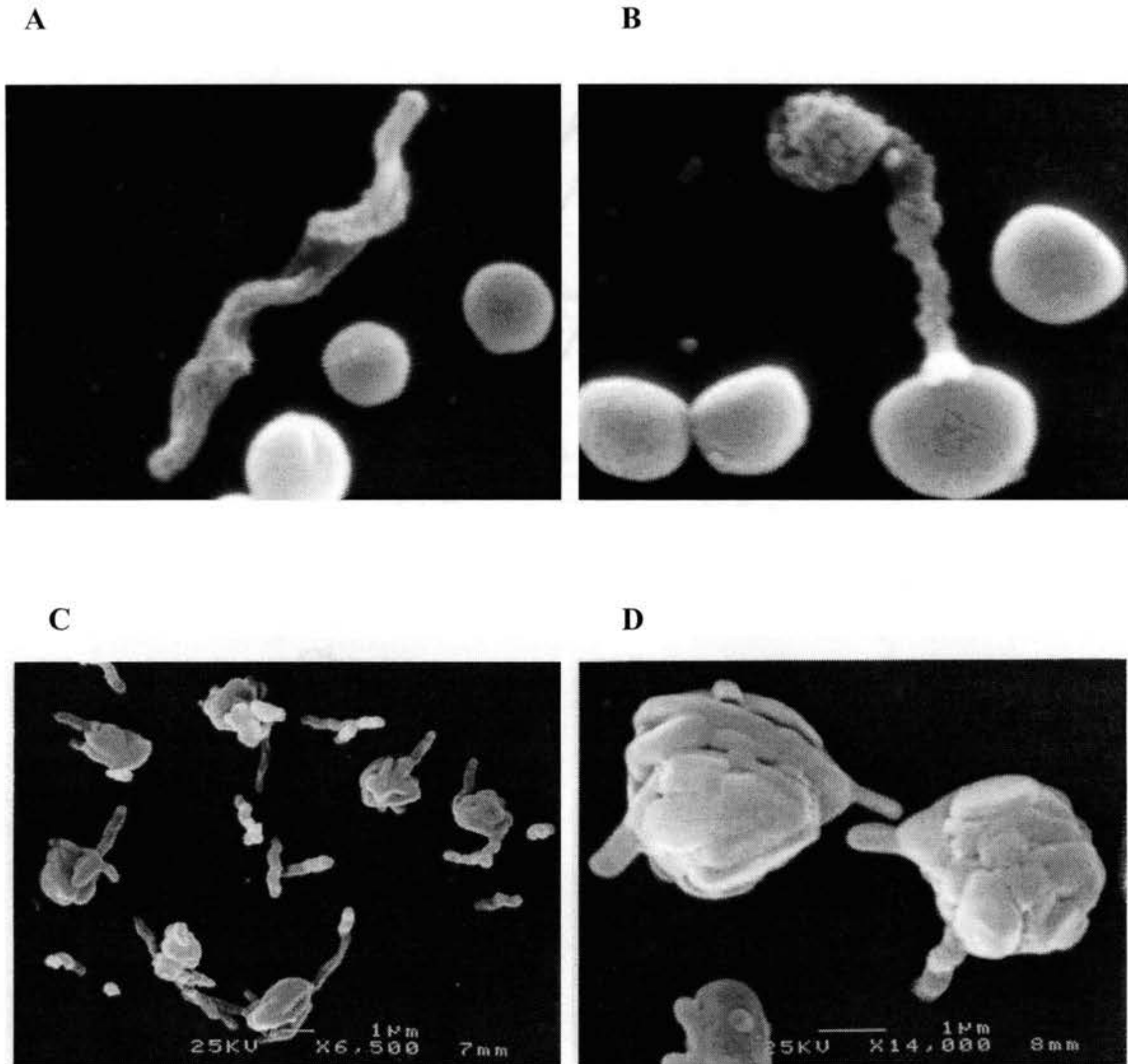

Figure 2. Scanning electron micrographs of *Campylobacter jejuni* in the helical-rod and coccoid forms (A), the production of the coccoid form (B), and intermediate forms and cell clumping (C) and (D).

CHAPTER IV

INFLUENCE OF GASEOUS ATMOSPHERE ON MORPHOLOGY AND CELLULAR FATTY ACID COMPOSITION OF *CAMPYLOBACTER JEJUNI*

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ABSTRACT

Morphology and fatty acid profiles of *Campylobacter jejuni* (ATCC 29428 and 33560) during growth under various gaseous atmosphere conditions in Bolton broth at 37°C for 72 hours were studied. Enumeration was on campylobacter agar containing charcoal and deoxycholate (CCDA) using spiral-plating techniques. Percentages of coccoid cells were determined microscopically. Following extraction, fatty acids were methylated and relative amounts of each compound was measured by gas chromatography. Most variation in percentage coccoid cells and fatty acid profiles were due to strains however, treatments often contributed to variability within strains. For strain 29428, there were no differences in percentages of coccoid cells (12 to 21%) for any gaseous treatments. Plate counts were similar among treatments (7 to 8 log CFU/ml) with exception of cells exposed to air (4 log CFU/ml). Predominant fatty acids in strain 29428 were 16:0 (33.8 to 39.6%) and $18:1^{9}$ (35.7 to 42.4%); intermediate fatty acids (1.9 to 11.5%) were 14:0, $16:1^{9}$, and $19:0^{\Delta}$; minor fatty acids found in cells from all treatments (0.4 to 3.0%) were 11:0, 12:0, 15:0 and 18:0; and $17:0^{\Delta}$ were detected in some treatments (0 to 0.4%). Percentages of coccoid cells in strain 33560 were greater than for strain 29428, and varied among treatments (13 to 87%). Plate counts varied among treatments and were least (2 log CFU/ml) when cells were exposed to air. Predominant fatty acids in strain 33560 were 14:0 (19.8 to 31.4%), 16:0 (26.2 to 33.8%), and $19:0^{\Delta}$ (18.3 to 21.9%); intermediate fatty acids (3.7 to 11.1%) were $16:1^{9}$, $17:0^{\Delta}$, and $18:1^{9}$; and

minor fatty acids (0.4 to 1.8%) were 11:0, 12:0, 15:0, and 18:0. Using correlation analysis and stepwise regression to evaluate the data, no apparent relationships were found to exist between percentages of coccoid cells and amounts of individual fatty acids either among or with strains.

INTRODUCTION

Campylobacter jejuni is a principal bacteriological agent for food borne illness in the U.S and abroad (Ketley 1997; Altekruse and others 1999; Skirrow and Blaser 2000). Poultry products, milk, and water are the primary vehicles for transmission to humans (Tauxe and others 1988; Jacobs-Reitsma 2000;Yu and others 2001; Rosenquist and others 2002). Millions of dollars are spent each year on illnesses due to this pathogen (Buzby and others 1997), not to mention costs related to testing food and water products for its presence. Detection of *Campylobacter* is often hampered by a morphological transformation from the characteristic helical-rod form to a round-coccoid form (Moran and Upton 1986; Aquino and others 1996). The rate of transformation is dependant upon many factors, including age, species, growth temperature, growth atmosphere, incubation time, subculturing, osmotic stress, and gaseous interchange between the atmosphere and media as affected by surface to volume ratio or sparging. (Rollins and others 1983; Juven and Rosenthal 1985; Moran and Upton 1987; Hodge and Krieg 1994; Reezal and others 1998; Chapter III).

Campylobacter are classifieds as Gram-negative bacteria. Their cell wall structure consists of a capsule, outer membrane, periplasmic space, peptidoglycan layer, and finally the cytoplasmic membrane (Vandemark and Batzing 1987). The outer membrane is essentially a lipid bilayer attached to the peptiglycan layer. Structural components include lipopolysaccharides and lipoproteins. The latter attaches the bilayer

to the peptidoglycan layer. Alternating N-acetylglucosamine and N-acetylmuramic acid molecules make up a two dimensional backbone, tetrapeptide tails are attached to the N-acetylmuramic acid molecules, and amino acids link these tails together (VanDemark and Batzing 1987). The lipid fraction (fatty acids) in this structure has been extracted and used to identify species and strains. In general, cellular fatty acid profiles can be unique to a given organism and may be used for the identification of Gram-negative bacteria (Weyant and others 1996).

Blaser and others (1980) identified six fatty acids within three strains of *Campylobacter* (*C. fetus* susp. *fetus*; *C. fetus* supsp. *intestinalis*; *C. fetus* subsp *jejuni*). They are: tetradecanoate (14:0); 3-hydroxytetradecanote (3-OH 14:0); cis-9-hexadecenoate ($16{:}1^9$); hexadecanoate (16:0); cis-9-octadecanoate/trans-9-octadecanoate ($18{:}1^9$); and cis-9,10-methyleneoctadecanoate ($19{:}0^\Delta$). The $19{:}0^\Delta$ fatty acid was reported only in *C. fetus* subsp. *jejuni*. Similar fatty acid profiles were reported by Leaper and Owen (1981) with the exception that 3-OH 14:0 was not detected. Alternatively, small amounts of octadecanoate (18:0) were found in *C. coli* and *C. jejuni*. The $19{:}0^\Delta$ was found in *C. fetus* subsp. *venerealis*, *C. coli* and *C. jejuni*. A very comprehensive study reported on the biochemical characteristics of *C. jejuni* included the fatty acid profile of 89 strains (Hébert and others 1982). The predominant fatty acids were 16:0 (30-39%), $18{:}1^9$ (14-32%) followed by $19{:}0^\Delta$ (9-25%) and 14:0 (8-22%). Trace amounts (1-2%) were found for 15:0 (pentadecanoate), $17{:}0^\Delta$ (cis-9,10-methylenehexadecanoate) and 18:0 fatty acids. An additional fatty acid (dodecanoate,12:0) was reported by Curtis (1983) in *C. fetus* (3.8%), *C. sputorum* (6.6%), and *C. sputorum* subsp. *mucosalis* (8.2%). The

presence of this fatty acid in *C. sputorum* subsp. *mucosalis* was confirmed by Moss and others (1984) and accounted for 10% of the total fatty acid composition of the cells.

In addition to identifying *Campylobacter* strains, fatty acid profiles may be useful in understanding membrane structure with respect to the morphological shift from the rod to the coccoid form. An organism may adapt to changes in temperature by altering its cell envelope with respect to fatty acid structure and length. Inverse relationships have been reported between temperature and level of unsaturated fatty acids and cell size (Linder and Oliver 1989; Eaton and others 1981). Linder and Oliver (1989) evaluated the fatty acid composition of *Vibrio vulnificus* when cells were stored for 26 days at 5°C. *Vibrio* exhibits similar morphological changes to that of *Campylobacter* and the production of the coccoid form. Fatty acid profiles from cells incubated at 5°C for 26 days showed little change until the cells became nonculturable (24-days). At this time there was a concomitant decrease in 16:0 and 16:1 and an increase in 19:0, 20:0 and 22:1 fatty acids. In addition, saturated fatty acids increased approximately 20% while unsaturated fatty acids declined 28%. This study indicated that in *Vibrio* the morphological change to the coccoid form was correlated with changes in membrane fatty acid composition.

Hazeleger and others (1995) determined the fatty acid composition of various forms of *C. jejuni* when grown at different temperatures. No differences in fatty acid profiles were found between spiral-rod cells and coccoid cells formed at the lowest temperatures of 4 and 12°C. On the other hand, in the coccoid cell suspension at 25°C, there was a significant increase in 16:0 and 18:0 fatty acids with a concomitant decrease in 14:0, 16:1 and $19{:}0^{\Delta}$ compared to the cell suspension containing mostly rods. In a

similar study, Höller and others (1998) evaluated *C. coli* at 4, 10 and 20°C. Little change was detected in cells incubated at either 4 or 10°C. In contrast to Hazeleger and others (1995), however, cells incubated at 20°C for 72 hours (predominantly coccoid) showed an increase in $19{:}0^{\Delta}$ from 3.8% to 20.6% and a concurrent decrease in $18{:}1^{9}$ fatty acid. Little change was noted for the two lower temperatures. It is interesting that the same type of morphological changes occur in these different organisms (*Campylobacter* and *Vibrio*) but temperature does not elicit the same response in membrane fatty acids.

In order to more clearly define the relationship between morphological changes and cell membrane structure, we evaluated the morphology and fatty acid profiles of two strains of *C. jejuni* grown under various gaseous conditions at 37°C for 72 hours.

MATERIALS & METHODS

Source, Maintenance and Preparation of Cultures

Two strains of *C. jejuni* (ATCC 29428 and 33560) were purchased from the American Type Culture Collection (ATCC; Manassas, Va, 20108). The strains were grown according to the ATCC directions using Bolton Broth (Oxoid LTD; Baskingstoke, Hamshire, England) at 37°C. The cultures were then vacuum dried and stored at –20°C until needed (stock cultures).

Prior to each experimental replication, stock cultures were rehydrated in 10 ml of freshly prepared Bolton broth that was aseptically transferred into sterile 50 ml tissue culture flasks (TCF) (Falcon® Brand; Becton Dickinson Labware, Franklin Lakes, NJ) equipped with a vented cap (0.2 µm pore size). The inoculated broth was incubated in a Gas-Pak chamber (BBL), which was charged with a modified atmosphere of CO_2 (10%), O_2 (5%) and N_2 (85%) generated from a Campy-Pak (BBL® CampyPak Plus; Becton Dickinson Microbiology Systems, Cockeysville, MD) microaerophilic gas generating system. The container was placed on an orbital bench top shaker (Lab-Line®, Model 4626) set at 80 rpm and incubated for 18 to 24 hours at 37°C. Prior to use, the cultures were Gram stained and examined microscopically to determine morphological integrity. In all cases, cells were greater than 92% rods.

Experimental Treatments and Procedures

To produce cell suspensions of *C. jejuni* containing various percentages of the coccoid morphology each strain was grown under seven different conditions (Table 11) at 37°C for 72 hours. To prepare inocula, each strain was spiral plated onto freshly prepared campylobacter agar with charcoal and deoxycholate (CCDA) (Oxoid LTD, Baskingstoke, Hampshire, England) and incubated for 48 hours at 37°C under modified atmosphere conditions CO_2 (10%), O_2 (5%) and N_2 (85%) generated from a Campy Pak (BBL® Campypak Plus; Becton Dickinson Microbiology Systems, Cockeysville, MD) inside a Gas-Pak chamber. Well-isolated colonies of each strain were transferred to freshly prepared Bolton broth in separate tissue culture flasks and incubated at 37°C for 18-24 hours under modified atmosphere conditions (Campy Pak) on an orbital bench top shaker set at 80 rpm. Each resulting culture was used to inoculate 200 ml volumes of fresh sterile Bolton broth for each of seven treatments. The vented tissue culture flask (TCF) and the partially vented Erlenmeyer flask (EF-P) were placed in individual Gas-Pak jars, charged with the modified atmosphere (Campy-Pak), and incubated in a 37°C incubator with agitation (80rpm). The other five Erlenmeyer flasks were incubated either with no gas (EF-S-N), or with one of four gas treatments using a continuous bubbler system similar to that described in the FDA-Bacteriological Analytical Manual (Hunt and others 2001). The gases were Air (A), Blood Gas (BG), and two A plus BG combinations, 50 parts A to 10 parts B (EF-A/BG) and 50 parts BG to 10 parts A (EF-BG/A). Flasks were incubated in a 37°C water bath/shaker (Precision Reciprocal Shaking Bath; Model 66802; Chicago, Ill) at 60 rpm. In order to collect enough cells for fatty acid analyses from all treatments, it was necessary to incubate for 72 hours. Flask

contents were analyzed for total plate counts, morphology and fatty acids. The experiment was replicated three times.

Analytical Procedures

Enumeration

In all cases, *C. jejuni* was enumerated using a spiral plater (dwScientific; Don Whitley Scientific, Shipley, England). Appropriate dilutions were made using 0.1% peptone dilution blanks followed by spiral plating onto prepoured plates of a selective blood free agar (CCDA) containing no additional selective agents. Samples were plated in 50 μl aliquots in a logarithmic mode setting. Plates were inverted and incubated for 48 hours at 37°C in a Gas-Pak (BBL) chamber charged with a mixture of CO_2 (10%), O_2 (5%) and N_2 (85%) (Campy-Pak). Counts were made using an automatic plate reader (Protocol; Synoptics Ltd., Cambridge, UK) and related software. There were duplicate plates for each treatment combination, and a minimum of three readings was performed for each plate. Means of plate counts from duplicate plates for each treatment combination in each replication were converted to $\log_{10}$ CFU/ml and used in statistical analyses.

Determination of percentage coccoid cells

Cells were prepared according to Bovill and Mackey (1997) and counted using a 10 x 10 mm grid inserted into the eyepiece of a Nikon Eclipse 600. Percentages of coccoid cells for each sample were based on observation of 1000 cells.

Determination of percentage fatty acids

Cellular fatty acid profiles were determined by lipid extraction, saponification and methylation (Weyant and others 1996). The fatty acid methyl esters (FAME) were analyzed on a Hewlett-Packard 5890 Gas Chromatograph equipped with an autosampler, a flame ionization detector and a capillary SPB™-1 column (30 m x 0.25 µm tube thickness; Supelco; Bellefonte, PA). The run conditions used were 150°C for 2 min, followed by increments of 4°C/min until the injector/detector temperature reached 250°C. The split ratio was 1:50 with the carrier gas being helium (0.7 ml/min) and the injection volume was 2µl. Peak identification was accomplished by comparison of retention times with a reference standard of specific bacterial FAME (Supelco, #47080-U, Bellefonte, PA). The percentage of each acid was calculated from the ratio of the area of its peak to the total area of all peaks.

Statistical Methods

General Linear Models (GLM), Pearson Correlation Coefficients (CORR), and Regression (REG) procedures of SAS (SAS® Institute Inc 1999-2001) were used to analyze the data. Coccoid counts, as a percentage of total cell counts, plant counts, and individual fatty acid peak areas, as a percentage of total peak areas, were analyzed with a model including strain (n = 2), treatment (n = 7), and the strain by treatment interaction. As there were large differences between strains, and several interactions, the data also were analyzed within strain with only treatment in the model. Treatment means within strain were separated using the Least Significant Difference (LSD) with alpha = 0.065. Correlation and stepwise regression procedures were used to investigate the relationships

between percentages of the coccoid form and fatty acids of individual replicates, both among and within strains.

RESULTS & DISCUSSION

The overall model accounted for a large proportion of the variability ($p < 0.0001$, $R^2 > 0.73$) for all dependent variables except the 11:0 ($p=0.0928$) and 15:0 ($p=0.1326$) fatty acids (Table 12). There were also strain differences ($p < 0.04$) for all variables except 11:0 and 15:0 fatty acids. There were treatment differences ($p < 0.05$) for all variables except the 12:0 and 15:0 fatty acids, but a tendency ($p=0.0698$) for treatment differences with the 15:0 fatty acid.

There were strain*treatment interactions ($p \leq 0.05$) only for % coccoid, and the 16:0, $17:0^{\Delta}$, 18:1, and $18:0^{9}$ fatty acids (Table 12). In all cases, the effect of strain accounted for most of the variation in fatty acid profiles (8 out of 10), plate counts, and % coccoid. Therefore, it would be misleading to present main effect means even if there are no interactions.

Morphology

For strain 29428, there were no differences ($p=0.7037$) in the percentage coccoid (10.0-20.6%) cells formed after 72 hours of incubation at 37°C (Table 13). There were however, treatment differences ($p=0.0206$) in coccoid formation for strain 33560 (Table 14). When the cultures were grown in a partially vented Erlenmeyer flask (EF-P) with a modified (M) atmosphere of 10%CO_2; 5%O_2; 85%N_2 the coccoid form accounted for only 13% of the total cells. When cells were grown in a tissue culture flask with similar modified (M) atmosphere, the coccoid form accounted for 86.6% of the cells (Table 14)

as illustrated in Figure 3. The other treatments were not very different ranging from 37.1% to 47.7% coccoid forms. The large differences may be attributed to the stage of growth the organism was in at the time of harvest. Based on earlier findings (Chapter III), the growth rate in the TCF is much faster (max population in 24 hours or less) than in all other treatments. Therefore, after 72 hours incubation the culture (TCF) probably entered the late stationary or early death phase resulting in more coccoid cells. We believe that the production of coccoid cells may be confounded with growth rate. Preliminary studies dictated the incubation time of 72 hours for all treatments in order to produce an adequate cell mass for all assays.

Plate Counts

When strain 29428 was grown for 72 hours at 37°C there was little difference in culturable numbers of cells based on CFU/ml due to treatment, with one exception (Table 13). Cells grown in an Erlenmeyer flask with a continuous supply of air (EF-B-A) had lower ($p<0.065$) numbers of culturable cells (4.22 log CFU/ml) on agar plates than did any of the other treatments (range of 7.30-8.33 log CFU/ml). Strain 33560 responded to EF-B-A treatment similarly with only 2.28 log CFU/ml detected on agar plates (Table 14). The other treatments ranged from 5.93 to 8.40 log CFU/ml. For both strains (29428 and 33560) the highest numbers (8.33 and 8.40 log CFU/ml, respectively) on agar plates occurred in the partially vented Erlenmeyer flasks in the modified gas (EF-P) treatment. Again, we do not know the extent to which the culture had progressed in the growth cycle. In some instances (i.e., EF-P) the culture may not have entered the stationary phase whereas in other cases (i.e., TCF) the organism may have been beyond the stationary phase of growth and entered into the death phase.

It is interesting to note that, for strain 33560 in TCF, even though 86% of cells were in the coccoid form, the plate counts remained high (7.26 log CFU/ml). Several reports correlate the presence of the coccoid form with a decline in colony forming units thus indicating the formation of viable nonculturable (VNC) cells (Ng and others 1985; Moran and Upton 1986; Rollins and Colwell 1986; Park 2002). This does not appear to be the case in the present study.

Fatty Acid Profiles

This experiment was designed to produce an array of coccoid forms to determine if there was a relationship between fatty acid profiles and change in morphology exhibited by *Campylobacter*. This was accomplished such that the strains ranged in percent coccoid cells from 10.0% to 86.8%. Again, the fatty acid profiles were unique to strain and will be discussed accordingly.

The 16:0 and $18{:}1^9$ fatty acids were predominant in strain 29428 (Table 13). The concentration of these fatty acids ranged from 33.78% to 39.62% for the 16:0 and 35.71% to 42.36% for $18{:}1^9$. The 14:0, 16:1, and $19{:}0^\Delta$ fatty acids were intermediate in concentration (1.91 to 11.47%). The smallest concentrations were found for 11:0, 12:0, 15:0 and 18:0 fatty acids (0.41 to 2.12%), while trace amounts (0.0 to .38%) were found for the $17{:}0^\Delta$ fatty acid in some treatments. There were treatment ($p<.01$) effects among 5 of 10 of the fatty acids (14:0,$16{:}1^9$,16:0,$18{:}1^9$ and 18:0) and a tendency ($p=.0897$) for difference in the 19:0Δ fatty acid as a consequence of treatment. Precision of analysis within treatments was high as indicated by the standard error of the means (SEM, Table 13).

Strain 33560 had three (14:0, 16:0 and $19{:}0^{\Delta}$) predominant fatty acids with concentrations ranging from 19.83-31.44%, 26.15-33.84%, and 18.32-21.89%, respectively. This was followed by the $16{:}1^{9}$, $17{:}0^{\Delta}$, and $18{:}1^{9}$ fatty acids with concentrations ranging from 3.71 to 11.07%. The concentrations of the remaining fatty acids (11:0, 12:0. 15:0, and 18:0) ranged from 0.40 to 1.82%. There were significant ($p<0.062$) treatment effects among 6 of 10 of the fatty acids (11:0,14:0,$16{:}1^{9}$,$17{:}0^{\Delta}$,$18{:}1^{9}$ and 18:0) and a tendency ($p<0.085$) for difference in the 15:0 and 16:0 fatty acids. Again, precision of analysis within treatments was high as indicated by the standard error of the means (SEM, Table 14).

To some extent, our findings agree with fatty acid profiles reported previously for *C.jejuni* (Blaser and others 1980, Leaper and Owen 1981; Hébert and others 1982; Curtis 1983; Moss and others 1984; Weyant and others 1996). In general, these similarities are the presence of the$19{:}0^{\Delta}$ fatty acid and predominance of the 16:0 fatty acid. On the other hand, to our knowledge, occurrence of the 11:0 and 12:0 fatty acids has not been reported for *C. jejuni*. The 11:0 and 12:0 fatty acids occurred in both strains with 33560 having slightly greater ($p < 0.0001$) concentrations of the latter fatty acid (Table 12). The $17{:}0^{\Delta}$ has been reported to occur at the 1% level (or not at all) in *C. jejuni* (Hébert and others 1982). In this study, the $17{:}0^{\Delta}$ fatty acid was detected in greater ($p <0.0001$, Table 12) concentrations (3.84-7.60%) in strain 33560 than in 29428 (0.0-.38%). This fatty acid was not detected in 4 of the 7 treatments applied to strain 29428, whereas in strain 33560 this fatty acid was present in all cases. This difference between treatment effects within strain is reflected in the strain by treatment interaction for the $17{:}0^{\Delta}$ fatty acid ($p <0.0001$, Table 12).

Hazelegar and others (1995) identified the two saturated straight-chain fatty acids (14:0 and 16:0) and two monounsaturated fatty acids ($16{:}1^9$ and $18{:}1^9$), in addition to the cyclopropane acid ($19{:}0^{\Delta}$), as the predominant fatty acids occurring in the rod form of *C. jejuni*. We tested the same strain (ATCC 33560) that was used by Hazelegar and others (1995). The treatment that produced the most coccoid cells (86.8%) had greater or equal concentrations of the above mentioned fatty acids compared to our treatment that produced the least number of coccoid cells (13.0%).

Our comparison of percentage coccoid form with individual or combinations of fatty acids did not indicate any relationships among strains (data not shown) or within strains (Table 15). Including squared, cubic, and quadratic terms in regression equations did not improve the fit, nor did the use of combinations of fatty acids. We cannot, based on our evaluation of two strains in this study, support a hypothesis that there are any significant relationships between fatty acid profiles and the rod or coccoid form. In addition, because the treatments used in this study contributed to the variation seen within strain, we conclude that fatty acid profiles lack accuracy as a tool for either identification purposes or explaining the biochemistry relating to the morphological changes exhibited by *Campylobacter jejuni*. The methodology used to grow *Campylobacter* should be standardized in order to investigate these relationships further.

CONCLUSION

There were considerable differences in coccoid formation and fatty acid profiles due to strain variation and to a lesser extent gas/growth treatment. *C. jejuni* ATCC 29428 appears to be much more stable with respect to morphological integrity than *C. jejuni* 33560. No correlations could be made between percentage of coccoid morphology and fatty acid percentages.

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Table 11. Containers and gas treatments used for the growth of *C. jejuni*.

Treatment	Container	Atmosphere	Gas Flow (ml/min)	
			Air	Blood Gas
1	Tissue Culture Flask[1]	Campy Pak[5]	-	-
2	Erlenmeyer Flask-Loose Cap[2]	Campy Pak	-	-
3	Erlenmeyer Flask-Sealed[3]	-	-	-
4	Erlenmeyer Flask[4]	Blood Gas[6]	-	5.2
5	Erlenmeyer Flask[4]	Air	5.2	-
6	Erlenmeyer Flask[4]	Air & Blood Gas	4.1	1.0
7	Erlenmeyer Flask[4]	Air & Blood Gas	1.0	4.1

[1]TCF=Tissue Culture Flask (Corning 500ml, 0.2μm vented cap).
[2]Pyrex Erlenmeyer Flask (500 ml; loose cap).
[3]Pyrex Erlenmeyer Flask (500 ml; sealed cap).
[4]Pyrex Erlenmeyer Flask (500 ml; sparged with air and/or blood gas).
[5]Campy Paks (5%O_2; 10%CO_2 and 85%N_2).
[6]Blood Gas (5%O_2; 10%CO_2 and 85%N_2).

Table 12. Individual General Linear Models Procedure (GLM) statistics.

Dependent Variable	Overall Model (Pr>F)	R^2	Source Variables (Pr>F) Strain	Trt	Str*Trt
Coccoid (%)	<.0001	.74	<.0001	.0162	.0160
Log CFU/ml	<.0001	.73	.0362	<.0001	.7462
11:0	.0928	.46	.5042	.0498	.2543
12:0	<.0001	.73	<.0001	.3555	.3092
14:0	<.0001	.93	<.0001	.0019	.3464
15:0	.1326	.43	.3723	.0698	.3412
$16:1^{9}$	<.0001	.83	<.0001	<.0001	.2215
16:0	<.0001	.84	<.0001	.0012	.0328
$17:0^{\Delta}$	<.0001	.98	<.0001	<.0001	<.0001
18:1	<.0001	.99	<.0001	.0014	.0277
$18:0^{9}$	<.0001	.79	.0003	<.0001	.0500
$19:0^{\Delta}$	<.0001	.95	<.0001	.2293	.9040

Table 13. Percentage coccoid cells, final plate counts, and cellular fatty acid composition of *Campylobacter jejuni* (ATCC 29428) grown under different atmospheric conditions for 72 hours.

Treatment		Coccoid[1]	Plate Counts[1]	Percentage (%) of Total Fatty Acids[1]									
Container[2]	Gas[2]	(%)	(Log CFU/ml)	11:0	12:0	14:0	15:0	16:1^{9}	16:0	17:0$^{\Delta}$	18:1^{9}	18:0	19:0$^{\Delta}$
TCF	M	17.9^{a}	7.39^{a}	0.94^{a}	0.52^{a}	8.90^{b}	2.12^{a}	3.91^{bc}	36.21^{b}	0.38^{a}	39.16^{b}	1.49^{b}	6.37^{a}
EF-P	M	20.6^{a}	8.33^{a}	1.34^{a}	0.67^{a}	5.45^{c}	1.38^{a}	1.91^{d}	39.62^{a}	0.00^{a}	39.48^{b}	3.02^{a}	7.12^{a}
EF-S	N	12.2^{a}	7.63^{a}	0.67^{a}	0.44^{a}	11.47^{a}	0.91^{a}	3.50^{c}	39.34^{a}	0.00^{a}	35.71^{c}	1.23^{bc}	6.74^{a}
EF-B	A	11.7^{a}	4.22^{b}	1.14^{a}	0.70^{a}	9.94^{ab}	1.60^{a}	5.10^{a}	33.78^{c}	0.00^{a}	40.03^{ab}	0.62^{c}	7.09^{a}
EF-B	BG	10.0^{a}	7.30^{a}	0.78^{a}	0.43^{a}	8.79^{b}	0.79^{a}	3.07^{c}	38.76^{a}	0.00^{a}	40.14^{ab}	1.65^{b}	5.58^{a}
EF-B	A/BG	15.4^{a}	7.75^{a}	0.65^{a}	0.41^{a}	8.96^{b}	1.41^{a}	4.94^{ab}	33.98^{c}	0.20^{a}	41.62^{ab}	1.23^{bc}	6.60^{a}
EF-B	BG/A	10.8^{a}	7.39^{a}	0.72^{a}	0.46^{a}	10.26^{ab}	0.96^{a}	4.07^{abc}	35.20^{bc}	0.20^{a}	42.36^{a}	1.20^{bc}	4.57^{a}
SEM[3]		**5.03**	**0.74**	**0.27**	**0.11**	**0.87**	**0.44**	**0.41**	**0.71**	**0.17**	**1.00**	**0.22**	**0.61**
p values: TRT (T)		**.7037**	**.0325**	**.4911**	**.3854**	**.0086**	**.3895**	**.0011**	**<.0001**	**.5696**	**.0095**	**<.0001**	**.0897**

[abc]Means in the same column without a common letter in superscript groups are different ($p < .065$).
[1]Data are the means from three replications.
[2] For container and gas treatment descriptions see table 1.
[3]Standard Error of the Mean (SEM); associated degrees of freedom (df=6) for treatment (T).

Table 14. Percentage coccoid cells, final plate counts, and cellular fatty acid composition of *Campylobacter jejuni* (ATCC 33560) grown under different atmospheric conditions for 72 hours.

Treatment		Coccoid[1]	Plate Counts[1]	Percentage of Total Fatty Acids[1]									
Container[2]	Gas[2]	(%)	(Log CFU/ml)	11:0	12:0	14:0	15:0	$16:1^{9}$	16:0	$17:0^{\Delta}$	$18:1^{9}$	18:0	$19:0^{\Delta}$
TCF	M	86.8[a]	7.26[ab]	0.56[c]	0.84[a]	23.57[bc]	1.50[a]	5.41[ab]	31.66[a]	6.14[b]	9.20[ab]	0.86[bc]	20.26[a]
EF-P	M	13.0[c]	8.40[a]	1.57[ab]	1.63[a]	19.83[c]	0.59[a]	4.09[c]	33.84[a]	3.84[c]	11.07[a]	1.59[a]	21.89[a]
EF-S	N	37.1[bc]	7.10[ab]	0.72[c]	1.40[a]	27.40[ab]	0.40[a]	3.71[c]	30.65[a]	7.26[ab]	6.04[c]	0.80[bc]	21.61[a]
EF-B	A	40.3[bc]	2.28[c]	0.72[c]	1.33[a]	24.44[bc]	1.78[a]	5.99[a]	29.50[a]	7.04[ab]	8.63[ac]	0.74[bc]	19.83[a]
EF-B	BG	45.7[b]	5.93[b]	1.69[a]	1.75[a]	26.70[ab]	1.82[a]	4.77[bc]	27.23[a]	6.75[ab]	7.31[bc]	1.41[ab]	20.58[a]
EF-B	A/BG	47.7[b]	7.29[ab]	0.81[c]	1.28[a]	22.86[bc]	0.99[a]	6.49[a]	30.58[a]	6.08[b]	10.59[a]	0.98[abc]	19.34[a]
EF-B	BG/A	38.6[bc]	5.99[b]	0.83[bc]	1.71[a]	31.44[a]	0.72[a]	6.13[a]	26.15[a]	7.60[a]	6.44[bc]	0.67[bc]	18.32[a]
SEM[3]		**11.4**	**0.66**	**0.26**	**0.29**	**2.26**	**0.37**	**0.39**	**1.69**	**0.42**	**1.07**	**0.22**	**1.52**
p values: TRT (T)		**.0206**	**.0004**	**.0463**	**.3547**	**.0576**	**.0732**	**.0009**	**.0845**	**.0004**	**.0282**	**.0618**	**.6710**

[abc]Means in the same column without a common letter in superscript groups are different ($p<.065$).

[1]Data are the means from three replications.

[2] For container and gas treatment descriptions see table 1.

[3]Standard Error of the Mean (SEM); associated degrees of freedom (df=6) for treatment (T).

Table 15. Pearson Correlation Coefficients (r) and probability levels (prob>|r|) between coccoid percentages and individual fatty acids for *C. jejuni* 29428 and 33560.

	C. jejuni	
	29428	33560
Variables	r (Prob>$\lvert r \rvert$)	r (Prob>$\lvert r \rvert$)
11:0	.09 (.69)	-.14 (.54)
12:0	-.14 (.53)	-.28 (.21)
14:0	-.41 (.07)	.03 (.90)
15:0	.10 (.66)	.24 (.29)
16:1^{9}	-.03 (.88)	.27 (.24)
16:0	.08 (.73)	-.10 (.68)
17:0$^{\Delta}$	.56 (.01)	.24 (.30)
18:1	.06 (.78)	-.08 (.74)
18:0^{9}	.29 (.20)	-.28 (.22)
19:0$^{\Delta}$	.08 (.74)	-.03 (.90)

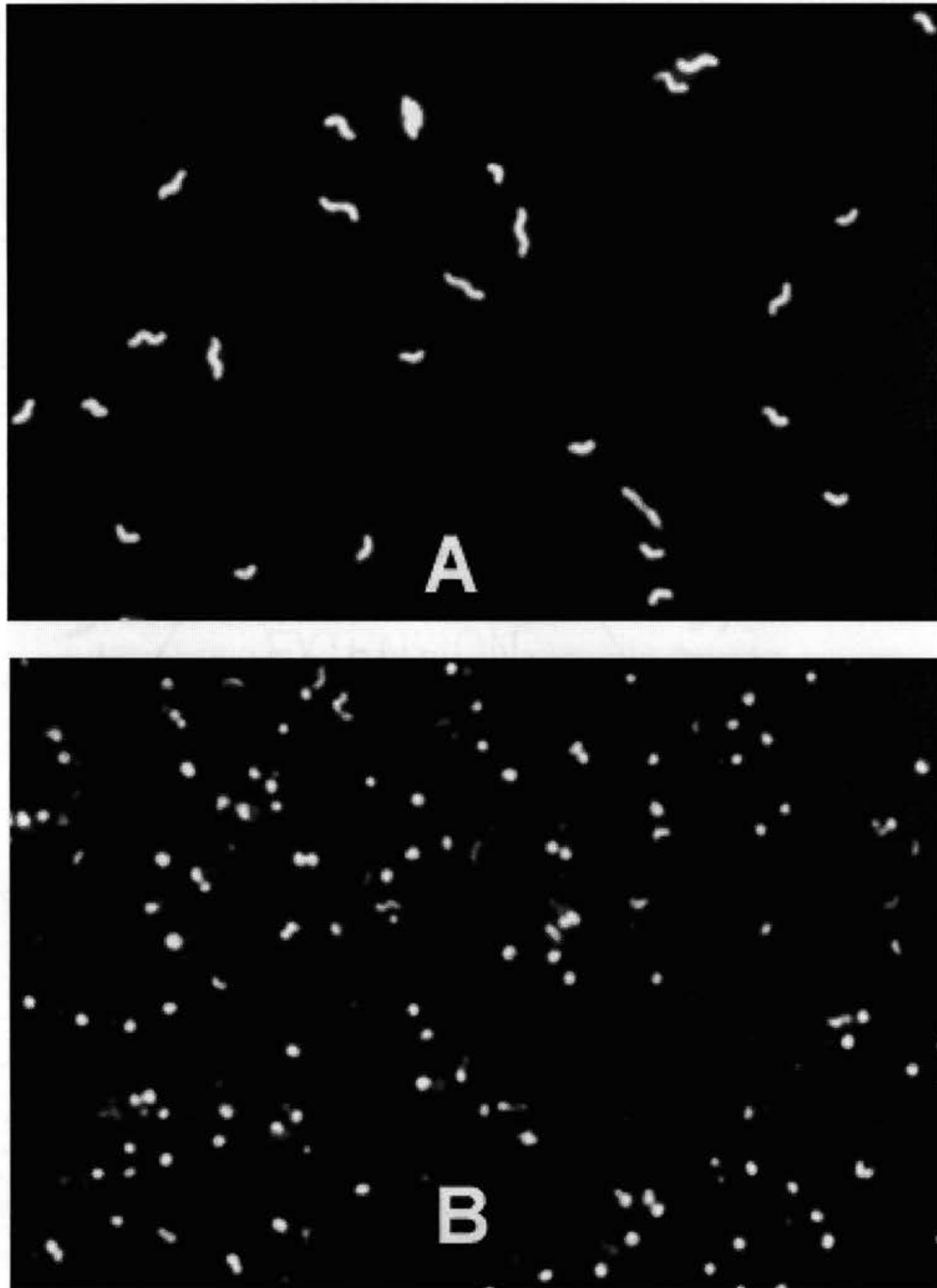

Figure 3. *Campylobacter jejuni* 33560 in the helical-rod and coccoid forms (A), the production of the coccoid form (B).

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