THE MODE OF CELL DIVISION AND CHARACTERIZATION OF THE \textit{ftsZ} GENE OF \textit{Campylobacter jejuni} ATCC 43431

by

Shahnaz Tahihra Al Rashid

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Medical Genetics and Microbiology
University of Toronto

© Copyright by Shahnaz Tahihra Al Rashid 1998
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

0-612-34053-8
The mode of cell division and characterization of the ftsZ gene of Campylobacter jejuni ATCC43431

Shahnaz Tahihra Al Rashid

Master of Science, 1998

Graduate Department of Medical Genetics and Microbiology

University of Toronto

ABSTRACT

Campylobacter jejuni is a frequent bacterial fecal isolate in humans with diarrhea in most parts of the world (Skirrow, 1994; Walker et al., 1986). Initially identified in the early 1900's, progress has been made in its clinical identification, transmission routes, treatment of infections, physiology, phylogeny, and genetic manipulation. This study aims to identify the mode of C. jejuni ATCC 43431 cell division and characterize its ftsZ gene, which is an essential cell division gene in Escherichia coli (Dai and Lutkenhaus, 1991). Transmission electron microscopy (TEM) of C. jejuni cultures reveal that the predominant mode of cell division in logarithmic (log) and stationary phase cultures is symmetrical and asymmetrical (forming coccoid or minicells) cell division, respectively. The molecular mechanisms which dictate the modes and regulation of cell division remain to be determined. As numerous studies have shown that ftsZ is essential and plays a key role in E. coli cell division, characterization of the C. jejuni homolog in this study is an important step in elucidating the molecular mechanisms of C. jejuni cell division. After screening several C. jejuni genomic libraries, the complete C. jejuni ftsZ gene was isolated on two Lambda Gem 11 recombinant clones. The entire coding and upstream putative regulatory sequences were determined. Amino acid sequence alignment of several bacterial FtsZ proteins revealed that all of the five previously identified conserved domains are present in the C. jejuni FtsZ. Three transcription start sites were mapped by primer extension analysis. Northern blot analyses revealed that C. jejuni ftsZ is expressed as a monocistronic transcript of ~1.3 kb, and that the relative amount of ftsZ transcript is constant throughout log phase and decreases slightly in stationary phase. In addition, the partial ftsZ gene was subcloned in-frame with the glutathione-S-transferase gene (GST) of the pGEX 2T fusion vector, from which the partial FtsZ protein was expressed and purified.
AKNOWLEDGEMENTS

First and foremost, I want to express my love and deepest gratitude to my family - papa, mama, Shahin, Shahir, Nemo, and dearest David - for your unparalleled support and encouragement throughout my undergraduate and graduate studies. During the most trying times, your advice and comfort inspired me to persevere and I will continue to cherish them throughout my life. This thesis would not have been realized if it had not been for you, and after enduring my darkest moods and sleepless nights, this thesis is as much yours as it is mine.

I would also like to extend my heartfelt thanks to my supervisor, Dr. Chan, who took a chance on a second year undergraduate all the way through her Master’s degree. I will always value the opportunity you gave me to work in research, your patience, and your guidance throughout my studies. To Dr. Sherman, I want to extend my warmest appreciation for your continued feedback, help, and encouragement throughout my studies. I would also like to extend my warmest thanks to Dr. Funnell, for your insights and advice during my studies. In spirit, I also want to thank Dr. Firtel (Max), for your uplifting inspiration, support and encouragement.

I want to thank Eric H., my first lab “guru”, Hermine B., and Helena L., who watched over most of us “mad scientists”. I would also like to especially thank my lab “mateys” - Steve, Myrna and Phil D., Rocco L., Billy B., Yuwen H., Thomas W., Tin T., Sophia M., Prashanth S., Ashley S., Mark W., Angela J., and Jennifer L. for the many special times we shared (in and out of the lab) which I will always remember and treasure. And last but never least, I want to thank my dear friends Celia, Shahreen, Sherry, Margaret, Steve, Amy, and Haseena for your enthusiasm, support and encouragement, through both the good times and the tough times, as well as for your unflappable determination to get me out of “that lab!!!”. In conclusion, I would like to again thank everyone who has been with me throughout my studies and undoubtedly influenced this thesis.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>viii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1 - 39</td>
</tr>
<tr>
<td>History and clinical importance of <em>Campylobacter</em> infections</td>
<td>1</td>
</tr>
<tr>
<td>Pathogenesis of <em>C. jejuni</em></td>
<td>3</td>
</tr>
<tr>
<td>Isolation and cultivation of <em>Campylobacter</em> species</td>
<td>6</td>
</tr>
<tr>
<td>Identification and differentiation of <em>Campylobacter</em> species</td>
<td>9</td>
</tr>
<tr>
<td>Phylogeny of the genus <em>Campylobacter</em></td>
<td>13</td>
</tr>
<tr>
<td>Morphology of <em>Campylobacter</em> cells</td>
<td>16</td>
</tr>
<tr>
<td>Molecular biology of <em>C. jejuni</em></td>
<td>18</td>
</tr>
<tr>
<td>Gram negative bacterial cell cycle</td>
<td>23</td>
</tr>
<tr>
<td>Gram negative bacterial cell division</td>
<td>24</td>
</tr>
<tr>
<td>Genes and regulation of bacterial cell division</td>
<td>25</td>
</tr>
<tr>
<td>Current working model of Gram negative bacterial cell division</td>
<td>36</td>
</tr>
<tr>
<td>Objectives and long term goals of this study</td>
<td>40</td>
</tr>
<tr>
<td>Methods and materials</td>
<td>41 - 65</td>
</tr>
<tr>
<td>Strains and plasmids used in this study</td>
<td>41</td>
</tr>
<tr>
<td>Growth conditions</td>
<td>42</td>
</tr>
<tr>
<td><em>C. jejuni</em> growth curve</td>
<td>42</td>
</tr>
<tr>
<td>Transmission electron microscopy</td>
<td>43</td>
</tr>
<tr>
<td>Statistical analysis of dividing <em>C. jejuni</em> cells</td>
<td>45</td>
</tr>
<tr>
<td>DNA preparation and purification</td>
<td>47</td>
</tr>
<tr>
<td>Radioactive DNA labelling</td>
<td>47</td>
</tr>
<tr>
<td>Colony and plaque lifts</td>
<td>48</td>
</tr>
<tr>
<td>Screening of plasmid and phage libraries to isolate the complete <em>ftsZ</em> gene</td>
<td>49</td>
</tr>
<tr>
<td>PCR strategy to isolate the N-terminal coding and upstream region of <em>ftsZ</em></td>
<td>49</td>
</tr>
<tr>
<td>PCR to amplify the partial <em>ftsZ</em> to be subcloned into pGEX 2T</td>
<td>50</td>
</tr>
<tr>
<td>Analyses of recombinant clones and PCR products</td>
<td>51</td>
</tr>
<tr>
<td>Southern blot and hybridizations</td>
<td>51</td>
</tr>
<tr>
<td>Subcloning PCR products containing the N-terminal coding and upstream region of <em>ftsZ</em>, and the partial <em>ftsZ</em> gene</td>
<td>52</td>
</tr>
<tr>
<td>PCR to confirm subcloning reactions</td>
<td>53</td>
</tr>
<tr>
<td>Preparation of competent cells and transformation</td>
<td>54</td>
</tr>
<tr>
<td>Sequencing protocol</td>
<td>54</td>
</tr>
<tr>
<td>Preparation of total RNA</td>
<td>55</td>
</tr>
<tr>
<td>Primer extension analyses</td>
<td>56</td>
</tr>
<tr>
<td>Southern blot and hybridizations</td>
<td>57</td>
</tr>
<tr>
<td>Protein overexpression, isolation, and purification</td>
<td>58</td>
</tr>
</tbody>
</table>
Results

C. jejuni ATCC 43431 growth curve
Morphology and modes of C. jejuni cell division in log and stationary phase cultures
Characterization of pBXS6
Isolation and characterization of the complete C. jejuni fisZ gene
Transcription of fisZ, fliG, and flgG throughout the growth phases
Expression and purification of the GST-FtsZ fusion and partial FtsZ proteins

Discussion

C. jejuni ATCC 43431 growth curve
Mode of C. jejuni cell division
Isolation of the complete fisZ gene
Characterization of C. jejuni fisZ
Expression of C. jejuni fisZ
Expression and purification of GST-FtsZ fusion and partial FtsZ proteins

Future studies

References

Appendix 1

Identification of Campylobacter jejuni, C. coli, C. lari, and C. upsaliensis based on the glyA gene

Appendix 2

Standard normal distribution values (taken from Olson, 1987)

Appendix 3

Critical values of the X^2 distribution (taken from Olson, 1987)
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>Current list of <em>Campylobacter</em> species, known reservoirs, and disease associations</td>
<td>2</td>
</tr>
<tr>
<td>Table 2.</td>
<td>List of known cell division genes in <em>E. coli, C. crescentus, and C. jejuni</em></td>
<td>26</td>
</tr>
<tr>
<td>Table 3.</td>
<td>Bacterial strains and plasmids used in this study</td>
<td>60</td>
</tr>
<tr>
<td>Table 4.</td>
<td>Oligonucleotides designed and used in this study</td>
<td>61</td>
</tr>
<tr>
<td>Table 5.</td>
<td>Titres of <em>C. jejuni</em> ATCC 43431 cells grown at 37°C in Mueller Hinton broth</td>
<td>72</td>
</tr>
<tr>
<td>Table 6.</td>
<td>Counts of <em>C. jejuni</em> ATCC 43431 dividing cells</td>
<td>79</td>
</tr>
<tr>
<td>Table 7.</td>
<td>Grouping of <em>C. jejuni</em> cell division results to be subjected to the X^2 test</td>
<td>80</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. The phylogenetic tree of rRNA superfamily VI 15
Figure 2. The C. jejuni ATCC 43431 genomic map 22
Figure 3. A model for Z-ring formation and the series of genes involved at different stages of E. coli cell division
Figure 4. Schematic diagram of the RE profiles of pBXS6, 1.0.2, pGEX-ftsZ, 1.2.8, 1.7.1, and Lambda-ftsZ1 37, 38
Figure 5. Schematic representation of the location of oligos within pBXS6 used in this study 62, 63
Figure 6. PCR strategy to amplify N-terminal coding and upstream regions of ftsZ from the C. jejuni pBR322 DNA library 64
Figure 7. C. jejuni ATCC 43431 growth curve at 37°C in Mueller Hinton broth 65
Figure 8. TEM of C. jejuni ATCC 43431 cells at various stages of the bacterial cell cycle observed from log and stationary phase cultures 73
Figure 9. Amplification products of the PCR strategy using the C. jejuni pBR322 DNA library 74 - 78
Figure 10. Southern blot analyses of the products from the PCR strategy using the C. jejuni pBR322 DNA library 81
Figure 11. Agarose gel electrophoresis analyses of RE-digested recombinant lambda clones isolated from the Lambda Gem 11 library plaque screens 82
Figure 12. Southern blot analyses of the RE-digested recombinant lambda clones 83
Figure 13. Agarose gel electrophoresis analyses of the PCR products to confirm the subcloning of the ~1.5kb HindIII ftsZ fragment of Lambda-ftsZ1 into pBluescript II SK+ 84
Figure 14. Nucleotide and deduced amino acid sequences of the C. jejuni ftsZ gene 85
Figure 15. Alignment of the C. jejuni (Cj), E. coli (Ec), and C. crescentus (Cc) FtsZ deduced amino acid sequences 86 - 88
Figure 16. Primer extension analysis of the C. jejuni ATCC 43431 ftsZ gene 89, 90
Figure 17. Agarose-formaldehyde gel electrophoresis of C. jejuni ATCC 43431 total RNA, extracted from cultures at different time points throughout the growth phases 91
Figure 18. Northern blot analyses using ftsZ, flgF, and flgG as probes 92
Figure 19. SDS-PAGE analyses of E. coli JM101, transformants of pGEX 2T, and pGEX-ftsZ 93
Figure 20. SDS-PAGE analysis revealing GST-FtsZ in the insoluble cell fraction 94
Figure 21. SDS-PAGE analyses of GST-FtsZ solubilization 95
Figure 22. SDS-PAGE analyses of GST-FtsZ purification, thrombin cleavage, and release of purified partial FtsZ 96
Figure 23. Schematic representations of spiral and coccoid C. jejuni cells that can be observed by TEM 97

vii
LIST OF ABBREVIATIONS

α
β
γ
σ
μ/u
X²
amp
ATP
b
bp
C
CFU
Ci
CM
cpm
D
dATP
dCTP
dGTP
DNA
DNase
ds
dTTP
EDTA
FBP
g
GDP
GFP
GST
GTP
HCl
H₀
IEM
IPTG
k
l
LB
log
M
m
mol % G + C
mRNA
n
NaCl
NaOH
oligo
OM
p
PAGE
PCR

alpha
beta
gamma
sigma
micro
Chi-square
ampicillin
adenosine triphosphate
base
base pair
degree Celsius
colony forming unit
Curie
cytoplasmic membrane
counts per minute
dalton
deoxyadenosine triphosphate
deoxycytosine triphosphate
deoxyguanosine triphosphate
deoxyribonucleic acid
deoxyribonuclease
double stranded
deox thymin e triphosphate
ethylenediaminetetraacetic acid
ferrous sulfate, sodium metabisulfite, sodium pyruvate
gram
guanosine diphosphate
green fluorescent protein
 glutathione-S-transferase
 guanosine triphosphate
 hydrochloric acid
null hypothesis
immunoelectron microscopy
isopropyl-β-D-galactoside
kilo
litre
Luria Bertani
logarithmic
mole
milli
mol percentage guanidine plus cytidine
messenger ribonucleic acid
nano
sodium chloride
sodium hydroxide
oligonucleotide
outer membrane
pico
polyacrylamide gel electrophoresis
polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>peptidoglycan</td>
</tr>
<tr>
<td>ppGpp</td>
<td>guanosine tetraphosphate</td>
</tr>
<tr>
<td>RE</td>
<td>restriction enzyme / restriction endonuclease</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>sarkosyl</td>
<td>N-lauryl sarcosine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate / sodium lauryl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscope / scanning electron microscopy</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>spp.</td>
<td>species (plural)</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>subsp.</td>
<td>subspecies</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope / transmission electron microscopy</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume ratio</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-(\beta)-D-galactoside</td>
</tr>
</tbody>
</table>
INTRODUCTION

History and clinical importance of Campylobacter infections

Since the beginning of the 20th century, Campylobacter species have been recognized to be the causative agent in a multitude of human and animal infections. In 1913, Campylobacter fetus (formerly Vibrio fetus) was recognized as a cause of abortions in sheep (McFadyean and Stockman, 1913). It was not until 1947, when it was cultured from human blood, that its potential significance in human disease was appreciated (Vinzent et al., 1947; Walker et al., 1986). Over the next decade it was recognized as an opportunistic pathogen of debilitated patients (Bokkenheuser, 1970; Guerrant et al., 1978; Walker et al., 1986). In 1963, the genus Campylobacter was proposed and validated to accommodate what had until then been regarded as microaerophilic vibrios (Skirrow, 1994). By the 1970's, the number of reported cases of Campylobacter infections had risen so dramatically that required their recognition as major human pathogens. Since then, their clinical significance has been extensively reviewed (Penner, 1988; Cover and Blaser, 1989; Griffiths and Park, 1990; Butzler and Oosterom, 1991; Skirrow, 1991, 1994).

The severity of Campylobacter infections varies from asymptomatic infections, to localized or widespread systemic diseases. The most common manifestation of Campylobacter infections is gastroenteritis, ranging from watery diarrhea to inflammatory dysentery. Currently, the genus Campylobacter is comprised of 14 species (Ursing et al., 1994; On and Holmes, 1995; On, 1996; Vandamme et al., 1995), with C. fetus as the type species (listed in Table 1; adapted from On, 1996).
Table 1. Current list of *Campylobacter* species, known reservoirs, and disease associations.

<table>
<thead>
<tr>
<th>Species</th>
<th>Known source(s)</th>
<th>Disease association(s)</th>
<th>Veterinary</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter fetus</em> subsp. fetus</td>
<td>Cattle, sheep</td>
<td>Septicemia, gastroenteritis, abortion, meningitis</td>
<td>Bovine and ovine spontaneous abortion</td>
</tr>
<tr>
<td><em>Campylobacter fetus</em> subsp. venerealis</td>
<td>Cattle</td>
<td>Septicemia</td>
<td>Bovine infectious infertility</td>
</tr>
<tr>
<td><em>Campylobacter hyointestinalis</em> subsp. hyointestinalis</td>
<td>Pigs, cattle, hamsters, deer</td>
<td>Gastroenteritis</td>
<td>Porcine and bovine enteritis</td>
</tr>
<tr>
<td><em>Campylobacter hyointestinalis</em> subsp. lawsonii</td>
<td>Pigs</td>
<td>None at present</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Campylobacter concisus</em></td>
<td>Humans</td>
<td>Periodontal disease, gastroenteritis</td>
<td>None at present</td>
</tr>
<tr>
<td><em>Campylobacter mucosalis</em></td>
<td>Pigs</td>
<td>None at present</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Campylobacter sputorum</em> biovar sputorum</td>
<td>Humans, cattle, pigs</td>
<td>Abscesses, gastroenteritis</td>
<td>None at present</td>
</tr>
<tr>
<td><em>Campylobacter sputorum</em> biovar fecalis</td>
<td>Sheep, bulls</td>
<td>None at present</td>
<td>None at present</td>
</tr>
<tr>
<td><em>Campylobacter curvus</em></td>
<td>Humans</td>
<td>Periodontal disease, gastroenteritis</td>
<td>None at present</td>
</tr>
<tr>
<td><em>Campylobacter rectus</em></td>
<td>Humans</td>
<td>Periodontal disease</td>
<td>None at present</td>
</tr>
<tr>
<td><em>Campylobacter showae</em></td>
<td>Humans</td>
<td>Periodontal disease</td>
<td>None at present</td>
</tr>
<tr>
<td><em>Campylobacter gracilis</em></td>
<td>Humans</td>
<td>Periodontal disease, empyema, abscesses</td>
<td>None at present</td>
</tr>
<tr>
<td><em>Campylobacter upsaliensis</em></td>
<td>Dogs, cats</td>
<td>Gastroenteritis, septicemia, abscesses</td>
<td>Canine and feline gastroenteritis</td>
</tr>
<tr>
<td><em>Campylobacter helveticus</em></td>
<td>Cats, dogs</td>
<td>None at present</td>
<td>Feline and canine gastroenteritis</td>
</tr>
<tr>
<td><em>Campylobacter coli</em></td>
<td>Pigs, poultry, bulls, sheep, birds</td>
<td>Gastroenteritis, septicemia</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> subsp. jejuni</td>
<td>Poultry, pigs, bulls, dogs, cats, water, birds, mink, rabbits, insects</td>
<td>Gastroenteritis, septicemia, meningitis, abortion, proctitis, Guillain-Barré syndrome</td>
<td>Gastroenteritis, avian hepatitis</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> subsp. doylei</td>
<td>Humans</td>
<td>Gastroenteritis, gastritis, septicemia</td>
<td>None at present</td>
</tr>
<tr>
<td><em>Campylobacter lari</em></td>
<td>Birds, poultry, river and sea water, dogs, cats, monkeys, horses, fur seals</td>
<td>Gastroenteritis, septicemia</td>
<td>Avian gastroenteritis</td>
</tr>
</tbody>
</table>
As aforementioned, *C. fetus* was the first species described. It is an important cause of bovine and ovine abortions, as well as human diseases such as septicemia, gastroenteritis, and to a lesser extent, meningitis, peritonitis, and endocarditis. Subsequently, *C. jejuni* was isolated and cultured from human diarrheal stools (Cooper and Slee, 1971; Dekeyser *et al.*, 1972; Skirrow, 1994), and since then, has become the most frequent causative agent of *Campylobacter* gastroenteritis, with accompanying symptoms such as fever, abdominal pain and occasionally, vomiting. In a number of cases, initial *C. jejuni* gastroenteritis is followed by the development of systemic complications such as meningitis (Thomas *et al.*, 1980), septicemia (On, 1996), reactive arthritis (Ebright and Ryan, 1984), and Guillain-Barré syndrome (Kaldor and Speed, 1984; Rees *et al.*, 1995; Vriesendorp *et al.*, 1995) which can cause severe and sometimes fatal paralysis (Kuroki *et al.*, 1993).

**Pathogenesis of *C. jejuni***

Presently, the frequency of *Campylobacter* is similar to, or in some parts of the world, more common than *Salmonella* and *Shigella*, and second only to *Escherichia coli* as a bacterial etiology of gastroenteritis. The most commonly isolated *Campylobacter* species from diseased individuals is *C. jejuni*. Its high prevalence has led to considerable attempts to obtain progress in understanding the pathogenesis of *C. jejuni* gastroenteritis.

*Campylobacter* infections occur through the fecal-oral route. The predominant sources of *C. jejuni* include contaminated poultry, unpasteurized milk, meat, and water. Epidemiological studies estimate the infective dose to be between $500 - 10^9$ organisms. The potential of *C. jejuni* to cause disease is likely to be a result of both the susceptibility of the host and the relative virulence of the infecting strain (Walker, *et al.*, 1986; Wallis, 1994).
Host barriers within normal individuals that minimize the severity of diseases caused by \textit{C. jejuni} infection resulting in asymptomatic or mild infection, or preventing systemic dissemination include the gastric acid barrier, peristalsis, secretion of proteolytic pancreatic enzymes, thick mucus gel, secreted antibodies, mucosal leukocytes, an intact intestinal epithelium, and the antimicrobial metabolites of indigenous microorganisms. However, uptake of microorganisms by Peyer's patches and other intestinal lymphoid structures and translocation can allow the invader to circumvent the mucosal barrier (Walker, \textit{et al.}, 1988). This uptake and translocation of microorganisms, in addition to the virulence factors of intestinal pathogens, including \textit{Campylobacters}, enable the organisms to cause disease within the host (Walker and Owen, 1990). Therefore, numerous potential virulence traits of \textit{C. jejuni} are being elucidated.

The spiral shape and rapid, darting, flagella-powered corkscrew-like motion of \textit{C. jejuni} has been suggested to aid in both its locomotion and penetration through the viscous mucus lining of the intestinal tract (Wallis, 1994; Skirrow, 1994). Caldwell and co-workers (1985) identified several \textit{C. jejuni} strains with flagellar phase variation, in which cells can switch from aflagellate to flagellate forms. Studies using either nonmotile forms of \textit{C. jejuni} to infect rabbits, or a mixed population of motile and nonmotile forms to infect human volunteers, have shown that only motile forms are recovered from stool samples (Guerry, 1994; Black, \textit{et al.}, 1988; Caldwell, 1985). Together, these studies suggest the importance of flagellation and a role for motility in colonization. However, it was not determined if expression of other genes were affected in the motile and non-motile cells. Therefore, the role of variable flagellar expression in pathogenesis remains to be confirmed (Guerry, 1994). The role of \textit{C. jejuni} flagella as adhesins has also been suggested in colonization (McBride and Newell, 1983), as has been shown in \textit{Vibrio cholerae} (Attridge and Rowley, 1983). In addition, \textit{C. jejuni} strains can undergo flagellar antigenic variation, where strains can alter the immunological properties of its flagellin (Harris \textit{et al.}, 1987), presumably allowing the bacteria to elude the host.
immune responses.

Doyle and co-workers (1988) studied the chemotactic behaviour of \textit{C. jejuni} towards different chemical stimuli, especially the components of bile and mucin. They found that many of the components were chemo-attractants, and suggested that L-fucose may play an important role in explaining the organism's affinity to the gastrointestinal tract. The presence of additional adhesion factors on the surface of the bacterial cell, such as lipopolysaccharide (LPS) and outer membrane proteins (OMPs), have also been investigated. LPS and OMPs were found to bind to epithelial cells and to the intestinal mucus gel (McSweegan and Walker, 1986). Specific OMPs have been found in \textit{C. jejuni} that bind to epithelial cells (de Melo & Pechère, 1990) whereas intact, functioning flagella appear to be necessary for cell invasion (Grant \textit{et al.}, 1993; Skirrow, 1994). A study by Konkel, \textit{et al.} (1990) postulated that an invasive molecule of \textit{C. jejuni} could be a glycoprotein. However, \textit{C. jejuni} may express more than one invasive antigen, and these antigens could act individually or in concert to promote pathogenesis (Wallis, 1994).

The LPS and flagella of \textit{Campylobacter} species have been found to be antigenically diverse and an inductor of the humoral immune response. However, studies have shown that although this immune response prevents illness, it does not prevent infection. The late complications associated with \textit{C. jejuni} infections, such as Guillain-Barré syndrome and reactive arthritis, have been postulated to be the result of stimulation of the host humoral response to \textit{C. jejuni} antigens. However, more studies are required to confirm these associations and determine the pathogenic mechanisms involved. \textit{C. jejuni} have also been observed to attach and be taken up by M cells of the Peyer's patches into the M cell follicle (Walker \textit{et al.}, 1988). This translocation of \textit{C. jejuni} may be the mechanism of systemic spread to the blood and lymphatic system, resulting in septicemia and meningitis (Wallis, 1994).
At least two exotoxins have been isolated from *C. jejuni*: a heat-labile cytotoxic enterotoxin, and a cytotoxin. Studies of the enterotoxin reveal that it appears to be closely related in structure and function to the enterotoxins produced by *V. cholerae* and *E. coli*. Strains that cause dysentery have been demonstrated to be invasive by cell culture model systems and to elaborate the cytotoxin (Wallis, 1994). However, the pathogenic significance of these toxins has yet to be shown, as strains producing no detectable enterotoxin and minimal cytotoxic activity are fully virulent in human volunteers (Black, *et al.*, 1988) and patients do not develop neutralizing antibodies to the toxins (Skirrow, 1994). An additional potential toxin has recently been identified: a cytolethal distending toxin (CLDT)-like factor. The *C. jejuni* CLDT was observed to cause progressive cell distension and eventually cell death *in vitro*, and a hemorrhagic response in rat ligated intestinal segments *in vivo*. Three genes, *cdtA*, *cdtB*, and *cdtC*, whose products are related to *E. coli* CLDT proteins were cloned by Pickett *et al.* (1996) and found to be required for the cytolethal distending toxin activity in the *in vitro* assay. The precise role(s) that these toxins play in the virulence of *C. jejuni* strains are unknown and are currently being investigated.

**Isolation and cultivation of Campylobacter species**

*Campylobacter* species are Gram negative, non-spore-forming, microaerophilic to anaerobic bacteria requiring atmospheric concentrations of 3-10% carbon dioxide and 3-15% oxygen, with hydrogen either being required or stimulating growth of certain species. Menaquinones are the major respiratory quinones (Vandamme *et al.*, 1991). The optimum growth temperatures are between 30-42°C. They are classified as chemoorganotrophs, and derive cellular energy through the oxidation of tricarboxylic acid intermediates, and the deamination of amino acids (Hoffman and Goodman, 1982). Isolation of these microorganisms from environmental or biological (i.e. human or animal) sources requires not only selection, but also enrichment due to the fastidious, slow growing characteristics of
these cells, and the fact that some sources contain few viable cells. Detection of *Campylobacter* species in a specimen depends on the initial number of organisms, the efficiency of the collection and culturing technique, and the sampling technique.

Since some specimens contain very small numbers of viable organisms, several transport media have been formulated to maintain all viable *Campylobacter* cells but inhibit growth of irrelevant bacteria that may be present within the specimen. Specimens with a large *Campylobacter* load may also be stored in transport medium, although this has been shown to increase the growth of competing contaminants within the specimen. The inclusion of a low concentration of selective agents such as antibiotics can be used to decrease this problem. *Campylobacter* species have been found to survive longer in specimens stored at 4°C and shorter at 10°C to 30°C. They are also sensitive to drying and atmospheric oxygen, and inhibited by acidic environments (lower than pH 5.0, organic acids are more inhibitory than inorganic acids) and sodium chloride at high concentrations (≥2%). Reports on the existence of viable but nonculturable forms of *Campylobacter* species further complicates their isolation (Rollins and Colwell, 1986). Therefore, optimal isolation of all *Campylobacter* species from either biological or environmental specimens is achieved by processing specimens immediately to prevent cell death. However, if specimens are to be stored, optimum isolation is achieved when storage is at 4°C under microaerophilic conditions, in a transport medium.

Since *C. jejuni* may have a low infective dose, it is important that sensitive and specific techniques are developed for its isolation from food, water, and environmental specimens. *C. jejuni* cells that have been damaged by heating or freezing show increased sensitivity to antibiotics and to hydrogen and oxygen radicals and seem to be less able to tolerate elevated growth temperatures compared with undamaged cells (Nachamkin *et al.*, 1991). Therefore, prior to cultivation in selective media, enrichment methods have been developed for these specimens. Enrichment methods include
incubation of specimens at $37^\circ$C for 2 to 5 hours under microaerophilic atmospheres, in buffered media or media containing oxygen-quenching agents (Nachamkin et al., 1991).

The isolation and cultivation of Campylobacter species is optimal in atmospheres with 10% carbon dioxide, 3-10% oxygen, 8% nitrogen, and 10% hydrogen. Although the optimal growth temperatures for the various species and strains vary, most species are able to grow at $37^\circ$C. Therefore, incubation at $37^\circ$C allows isolation of most Campylobacter species, while incubation at $42^\circ$C is optimal for growth of C. jejuni, C. coli, C. lari, and C. upsaliensis (thermophilic campylobacters). Numerous isolation protocols including solid and semisolid selective media and filtration methods have been developed.

Basal media that have been shown to be consistent and support most species include brucella agar (BBL, Microbiology Systems, Difco Laboratories) and Mueller Hinton agar (Difco, Oxoid). Tenover and Patton (1987) showed that 58% of examined C. jejuni isolates are auxotrophic for at least one amino acid (Walker et al., 1986). These basal media contain nutritionally-rich ingredients such as peptic digest of animal tissue and infusion from beef, respectively, and are able to support the fastidious Campylobacter species. All selective media for Campylobacter species use antibiotics to inhibit growth of unwanted organisms. Among them, cephalosporins such as cefoperazone and polymyxins such as polymyxin B both inhibit gram negative bacteria present in fecal flora, while vancomycin and rifampin inhibit gram positive bacteria, and cycloheximide and amphotericin B inhibit yeasts. Variable effects of antibiotics in combination and with different basal media have been observed. For example, Bopp et al. (18) showed that trimethoprim was inactive in both blood agar base and brucella agar, but had activity when used in combination with other antimicrobial agents such as polymyxin B and vancomycin. Nutritional supplements such as blood, charcoal, and/or FBP (ferrous sulfate, sodium metabisulfite, sodium pyruvate) are also incorporated into basal
media. The mechanism of action of these supplements to enhance growth is unclear, but it has been suggested that they are able to detoxify oxygen derivatives such as hydrogen peroxide and superoxide anions. Storage of media prior to inoculation has led to decreased isolation. Studies have suggested that dehydration and accumulation of toxic oxygen derivatives in the media may be the cause of the decreased survival of *Campylobacter* on stored versus freshly prepared media. Therefore, the incorporation of the nutritional supplements and the use of fresh media aids in *Campylobacter* survival and isolation. And if stored, the shelf life of media is lengthened when kept at 4°C under microaerophilic atmospheres (Nachamkin et al., 1991).

The filtration method involves the isolation through filters and cultivation of *Campylobacter* species on selective media. This method has been successful in the isolation of *C. upsaliensis*. However, filtration is relatively insensitive, only detecting the presence of \( \geq 10^5 \) CFU/g of feces (Nachamkin et al., 1991).

**Identification and differentiation of *Campylobacter* species**

Over the last 30 years, the identification and differentiation of *Campylobacter* species has been difficult due to the constant recruitment of species into the genus, the similar phenotypic characteristics between species and the constant emergence of atypical strains within species. In addition, *Campylobacter* species are generally biochemically unreactive, unable to ferment or oxidize carbohydrates. This has rendered the standard identification scheme of the family *Enterobactereaceae* inadequate for the identification and differentiation of the *Campylobacter* species. However, several phenotypic tests have been developed to identify and differentiate the growing number of species. Alternatively, molecular methods based on the genomic content of *Campylobacter* species have also been developed.
Phenotypic tests include antimicrobial susceptibility tests, temperature tolerances, growth requirements, and biochemical tests. The size of inoculum, composition of basal media, growth temperatures and atmospheres are factors that have been found to affect the results of these tests. Since no standard methodologies for these tests have been established, variable results from these tests have been obtained in several studies (Lastovica et al., 1994; On, 1994, 1996). For example, the hippuricase activity of C. jejuni is unique within the Campylobacter genus. However, there are atypical or mutant strains of hippuricase-negative C. jejuni (Totten et al., 1987) which were misidentified as C. coli due to their lack of hippuricase activity.

Nevertheless, several tests required for the identification of Campylobacter species have been established. In addition to their characteristic morphology, motility and Gram negative reaction, all Campylobacter species have oxidase activity, and do not hydrolyze gelatin and urea. Other phenotypic characteristics include colony morphology including swarming and pigmentation. The remaining tests are as follows: growth at 15°C, 25°C and 42°C; susceptibility to nalidixic acid and cephalothin determined by diffusion tests with Mueller Hinton agar; catalase activity; acid production from glucose by oxidation and fermentation; nitrate and nitrite reduction; hydrogen sulfide production in triple sugar iron agar (Roop et al., 1984) and iron-bisulfite-pyruvate (FBP) agar (Barrett et al., 1988); hippurate and indoxyl acetate hydrolysis; tolerance to 1.5% and 3.5% NaCl (sodium chloride); and growth in the presence of 1% glycine, 0.04% triphenyltetrazolium chloride, and 0.1% trimethylamine-N-oxide hydrochloride (Ursing et al., 1994).

The problems associated with the standard phenotypic tests for identifying Campylobacter species have led to the development of several procedures which objectively evaluate test data (On, 1996). One of the first strategies was described by Bolton et al. (1984) in which a dual-purpose
identification and biotyping scheme was developed for *C. jejuni, C. coli, C. fetus*, and *C. lari*. In this scheme, each isolate is assigned a specific numerical code based on its ability to grow in twelve inhibitory compounds such as antibiotics, dyes, and chemicals, along with hippurate hydrolysis and growth at 25°C. Positive and negative test results are each given a numerical value, which in turn, provides a numerical code specific to the isolate. Since this scheme was developed to identify and biotype *C. jejuni, C. coli, C. fetus*, and *C. lari*, the discovery of new *Campylobacter* species has limited the value of this procedure. Subsequently, computerized schemes, in which both cluster and principal coordinate analyses of phenotypic tests were used to identify isolates based on their similarity to strains of known identity (On, 1994; On and Holmes, 1995). Probability matrices for *Campylobacter* species identification were also developed. These matrices comprise of the results of numerous tests for a large number of strains within a species expressed as percentages. Evaluations of these schemes indicate that most species are readily differentiated, while discrimination between strains remains problematic. Although this is not ideal, the use of computerized identification matrices is effective since it may be revised quickly and easily if data on new strains or species become available. With the adoption of standardized methodologies for performing the aforementioned tests, identification results will be more consistent (On, 1996).

Molecular methods to identify and differentiate *Campylobacter* species include chromosomal DNA G+C content, which ranges from 30-46%, DNA restriction endonuclease (RE) profiles and restriction fragment length polymorphisms. Cellular components such as their major respiratory quinones (menaquinones), major fatty acids (tetradecanoic acid, hexadecanoic acid, hexadecenoic acid, and octadecenoic acid), and protein electrophoretic profiles have also been used in their identification and differentiation (On, 1996).

Serotyping, phage typing, and plasmid profiles have largely been used in epidemiological studies to
determine outbreak strains of *Campylobacter* species. Two serotyping schemes have been developed by Penner and Lior which utilize the antisera raised against heat stable and heat labile antigens, respectively, in agglutination assays (Penner and Hennessy, 1980; Penner *et al.*, 1983; Lior *et al.*, 1982, 1989). Several commercial kits have been developed utilizing antibody-mediated agglutination of campylobacterial antigens as a means of identification of *Campylobacter* species (Campyslide, BBL Microbiology Systems; Meritec-Campy, Meridian Diagnostics; Microscreen, Mercia Diagnostics).

Diagnostic probes using antibodies and oligonucleotides (oligos) or DNA fragments have also been developed to detect specific antigens and chromosomal sequences, respectively. Among the antibody probes are those which target the flagella and externally expressed components of the *Campylobacter* cells, while the DNA probes that have been developed are based on rRNA (ribosomal RNA) sequences, specific genes or species-unique sequences of the *Campylobacter* species' genomes. Among the latest methods of identification is the use of the polymerase chain reaction (PCR). Several studies have developed methods to amplify species-specific sequences from *Campylobacter* genomic DNA or directly amplify genomic sequences from biological and environmental specimens, thereby detecting and identifying the presence of various *Campylobacter* species. These methods not only accelerate the detection of *Campylobacter* species, but are also sensitive, capable of detecting as few as ten cells under optimal amplification conditions. Some specimens contain inhibitors of the PCR and a diverse load of contaminating species, which may lead to false negative and positive results, respectively. Therefore, genomic DNA is more often used as the template in PCRs, necessitating the isolation and growth of *Campylobacter* species for definitive identification. In the pursuit of developing a tool for identifying and differentiating the species, we developed a method combining the sensitivity of the PCR and specificity of oligo probes to detect *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* based on the glyA gene (Al Rashid *et al.*, in preparation; Appendix 1). Due to
the sensitivity of the PCR, specimens can be diluted to decrease the presence of PCR inhibitors. With the species-specific probes, only specific PCR products corresponding to the target species are detected and differentiated from non-specific amplification products from contaminating organisms. Recently, we used our strategy to affirm that the identity of the newly described *C. hyoilei* species is in fact *C. coli* (Vandamme, *et al.*, 1997).

**Phylogeny of the genus Campylobacter**

Historically, the taxonomic position of the genus *Campylobacter* was determined by their morphological and phenotypic characteristics. *Campylobacter* species were formerly classified as *Vibrio* species because of their morphological similarity. The nomenclature of Véron and Chatelaine (1973) on the taxonomy of *Campylobacter* species was officially accepted in 1980 (Skerman *et al.*, 1980).

In 1987, Romaniuk and Trust compared the partial 16S rRNA sequences from six *Campylobacter* strains and found that they formed a previously undescribed eubacterial group, which was only distantly related to the other Gram negative bacteria. Subsequently, Lau *et al.* (1987) concluded that the *Campylobacter*-Wolinella rRNA cluster was related to the phylum of the purple photosynthetic bacteria which is now known as the *Proteobacteria* (Murray *et al.*, 1990). Further study of the taxonomic structure of the genus by Thompson *et al.* (1988) using partial 16S rRNA sequences revealed that the *Campylobacter* species can be divided into three major rRNA homology groups: the first group comprised of the “true” campylobacters *C. fetus*, *C. hyointestinalis*, *C. sputorum*, *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. concisus* and *C. mucosalis*; the second group contains *C. pylori*, *C. fennelliae*, *C. cinaedi*, and *Wolinella succinogenes*; and the third group consists of *C. nitrofigilis* and *C. cryaerophila* (Vandamme *et al.*, 1991). Furthermore, in another phylogenetic
study, Paster and Dewhirst (1988) found a close relationship between *W. curva*, *W. recta*, *Bacteroides gracilis*, and *B. ureolyticus* on one hand, and the “true” campylobacters on the other.

Therefore, to study the genotypic coherence and phylogenetic relationships within the *Campylobacter* species and with other Gram negative bacteria, Vandamme *et al.* (1991) used 23S rRNA-DNA hybridization (De Ley and De Smedt, 1975), DNA-DNA hybridization (De Ley *et al.*, 1970), and the immunotyping technique of Falsen (1983). Their results led to the proposal that *Campylobacter* species and their related organisms belong to the same phylogenetic group which they named rRNA superfamily VI, that the “true” campylobacters together with *W. curva* and *W. rectus* (renamed *C. curvus* and *C. rectus*, respectively) be members of the genus *Campylobacter*, that the second rRNA homology group constitute a new genus *Arcobacter*, with renamed *Arcobacter nitrofigilis* and *A. cryaerophilus*, and that the genus *Helicobacter* consist of *H. pylori*, *H. cinaedi*, and *H. fennelliae*. Vandamme and De Ley (1991) also proposed the family *Campylobactereaceae* to encompasses the genera *Campylobacter* and *Arcobacter* due to a considerable number of their common genotypic and phenotypic features which differentiate them from the other genera within rRNA superfamily VI. Furthermore, phylogenetic studies using complete 16S rRNA sequences and their structural signatures (Lane *et al.*, 1992), and complete 23S rRNA sequences and their secondary structure models (Van Camp *et al.*, 1993; Olsen *et al.*, 1994; Trust *et al.*, 1994) have consistently shown that the genus *Campylobacter* belongs to the epsilon subdivision of the *Proteobacteria*. Figure 1 is a representation of the phylogenetic relationships between *Campylobacter* species and between the genera within rRNA superfamily VI (Vandamme *et al.*, 1991; Falsen *et al.*, 1991; Linton *et al.*, 1994; Bourke, 1995).
Figure 1. The phylogenetic tree of rRNA superfamily VI.


*The relative positions of the two most recently described Campylobacter species was extrapolated from 16S rRNA sequence similarity adapted from Linton, et al. (1994).

Figure taken from Bourke (M.D. thesis, 1995).
Morphology of *Campylobacter* cells

The average *Campylobacter* cell dimensions range from 0.5um to 5um in length and 0.2um to 0.9um in width. They have a characteristic rapid, darting corkscrew-like motion by means of a single or occasionally multiple unsheathed flagella at one or both polar ends. *Campylobacter* cell envelope layers include the outer membrane, a very thin peptidoglycan (PG) layer, and cytoplasmic membrane (CM). In addition, *C. fetus* has a protein crystalline surface layer (S-layer) which has been suggested to protect the organism during infection. Intracellular structures have been observed including dense bodies which are thought to be polyphosphate storage granules, and additional polar membranes underlying the flagella.

*Campylobacter* species exhibit pleomorphism within a culture. *Campylobacter* cells may appear as spiral, curved and S-, V- or comma- shaped slender rods, which occur in short or long chains, and as spherical or coccoid cells. The rod-shaped forms have been found to predominate in young cultures while the latter coccoid forms are present in older cultures. A study of *C. jejuni* morphology while growing in broth demonstrated that log phase cultures consisted of short and spirally curved rods, mid-log cells were twice this length, and late stationary phase consisted of coccoid or minicells and elongated cells that were between three to four times the length of log phase cells. Older cultures contained mainly coccoid forms or minicells (Griffiths, 1993).

In addition to the age of cultures, the composition and type (agar, broth and semisolid) of media, and temperature and atmospheric conditions such as moisture, oxygen and toxic oxygen derivatives all exert their effects on cell growth and morphology. The conversion or formation of coccoid cells is accelerated in solid media cultures than in semisolid or broth cultures, as well as in stored versus freshly prepared media. Increased exposure to atmospheric oxygen has been suggested to accelerate
coccoid cell formation on solid versus semisolid or liquid media. Dehydration and/or the accumulation of toxic oxygen derivatives in stored media have been suggested to increase coccoid cell production since lower rates of coccoid cell production have been observed in media which have been stored at 4°C in a microaerophilic environment than when stored at room temperature in air. Exposure to light has a negligible effect on morphology, and although exposure to ultraviolet light has been shown to decrease cell viability, it does not increase the frequency of coccoid formation or conversion. The components of media such as whole or lysed blood, containing catalase, peroxidase and superoxide dismutase, or chemicals such as FBP and charcoal which contribute to *C. jejuni* survival by detoxifying the oxygen-derivatives present in the media, also delay the occurrence of coccoid cells.

The *C. jejuni* coccoid forms or minicells vary in size, may or may not possess flagella, and studies analyzing various cytoplasmic components such as protein, RNA, and DNA contents reveal that they are different from the rod forms (Moran and Upton, 1986, 1987a, 1987b). Whether these coccoid cells are viable but nonculturable cells, or non-viable and degenerate forms of the rods remains to be determined. The mechanisms involved in their generation remain unknown. Since these cells accumulate under stressed conditions, i.e. the presence and accumulation of toxic products and nutrient depletion, this may be an environmental response: either minicells are actively formed which contain the minimum apparatus for viability to subsequently grow and divide when more favourable growth conditions arise, or coccoid forms are the result of blebbing of cytoplasm to release nonessential cell components (such as flagella and excess cytoplasmic contents) so that the viable rod forms are able to maintain a minimal set of cell machinery, or coccoid forms are a degenerate conversion from the rod forms by loss of components within the cell that are responsible for maintaining cell shape and viability. Therefore, whether coccoid forms are a result of *C. jejuni* cell degeneration or differentiation is still unknown. In the event that minicells are actively formed, the
regulators and machinery involved in this polar cell division may be distinct from those involved in normal (midcell) division, or a combination of factors from both cell division processes. However, the molecular mechanisms of *C. jejuni* cell division and minicell formation remain to be elucidated.

**Molecular biology of *C. jejuni***

Both conjugative and nonconjugative plasmids have been identified in *Campylobacter* species. However, these plasmids cannot replicate in *E. coli*, and vice versa (Guerry *et al.*, 1994). Plasmids specifying antibiotic resistance, including tetracycline, kanamycin and chloramphenical (Trieu-Cuot *et al.*, 1985; Taylor *et al.*, 1986) have been isolated, and are transferable between members of the *Campylobacter* species (Taylor *et al.*, 1981, 1987).

Several genetic transfer mechanisms into *Campylobacter* cells have been identified. These include plasmid conjugation (Labigne-Roussel *et al.*, 1987), electroporation (Miller *et al.*, 1988), and natural transformation (Wang and Taylor, 1990). Although *C. jejuni* has bacteriophages, no system of phage transduction has been developed (Taylor, 1992; Guerry *et al.*, 1994). Although not as efficient as electroporation, natural transformation frequencies were found to be higher when transferring linear DNA fragments versus plasmids, and highest when transferring genetic material between homologous *Campylobacter* species. Suicide and shuttle vectors have also been constructed to facilitate transfer between *C. jejuni* and *E. coli*. These have allowed for site-specific and gene replacement mutagenesis using the suicide vectors, as well as for *C. jejuni* genes cloned into *E. coli* to be returned to *C. jejuni* on autonomously replicating plasmids.

*Campylobacter* species have a chromosomal DNA G+C content ranging from 30 to 46 mol% (Vandamme *et al.*, 1991) and have genome sizes ranging from 1.2 to 2 megabases. Several
macrophysical and genetic maps have been constructed to study the genomic organization of *C. jejuni* ATCC 43431. At present, over 20 genes have been isolated, characterized, and mapped (Figure 2). These include house-keeping genes, biochemical pathway genes, structural genes, possible virulence genes, and cell division genes.

Difficulties in expressing *Campylobacter* genetic elements in *E. coli*, and vice versa (Calva *et al*., 1989; Tompkins, 1991) have also been encountered. This may be due to different DNA modification systems which exist between the two organisms (Labigne-Roussel *et al*., 1987), differences in promoter sequence utilization, and/or codon usage differences. However, several *C. jejuni* genes have been successfully expressed in *E. coli* from the *Campylobacter* promoter, based on studies which showed that *C. jejuni* gene expression occurred in the absence of an *E. coli* promoter and in the presence of *C. jejuni* 5' chromosomal sequences similar to *E. coli* promoter sequences, indicating that there exists similar expression elements between these bacteria.

The *glyA* gene, encoding serine hydroxymethyl transferase (SHMT), was the first *C. jejuni* ATCC 43431 chromosomal gene to be sequenced and its putative promoter sequences characterized (Chan *et al*., 1988; Chan and Bingham, 1990). As noted previously, the *glyA* sequence was used to derive an identification method for *Campylobacter* species (Appendix 1).

Among the *C. jejuni* genes which have been characterized are the two tandemly arranged flagellin genes, *flaA* and *flaB* (Nuijten *et al*., 1990; Logan *et al*., 1989; Khawaja *et al*., 1992) which have been mapped onto the chromosome (Kim *et al*., 1993). Based on primer extension analysis and comparison to other bacterial promoter sequences (Guerry *et al*., 1990), each gene contains its own, different promoter which suggests that they are subject to differential regulation: a putative recognition site for the alternative sigma factor $\sigma^{28}$ (Helmann and Chamberlin, 1988) is present.
upstream of \textit{flaA} and a putative promoter consensus sequence for \(\sigma^{24}\) has been identified upstream of \textit{flaB} (Wassenaar et al., 1994; Guerry et al., 1990; Nuijten et al., 1990). Since no gene or protein homolog of any of the known bacterial sigma factors has been isolated from \textit{C. jejuni}, the recognition sequences and promoter functions remain to be further analysed. Antigenic and phase variation have been reported, although the mechanisms involved and their functional significance is not yet understood. Recently, a third flagellin gene, \textit{flaC}, was cloned, mapped and found to be expressed as a single transcript (Chan et al., unpublished data). Its expression may be iron-regulated since the promoter region contains iron-responsive elements (\textit{fur}-boxes). Additional flagellar structural genes have also been cloned and sequenced, \textit{flgF} and \textit{flgG} (Chan et al., unpublished data). The \textit{C. jejuni} ATCC 43431 \textit{ftsZ} gene was identified upstream of the \textit{flgF} gene (Chan et al., unpublished data; this study).

A major oxidative stress gene, \textit{sodB}, from \textit{C. jejuni} has been cloned. It encodes iron superoxide dismutase which catalyses the breakdown of superoxide radicals to hydrogen peroxide and gaseous oxygen, which are important for protecting the cell under oxidative damage conditions (Pesci et al., 1994). Since the frequency of coccoid cell formation is increased under stress conditions, such as increased oxygen pressure, the expression of these genes in \textit{C. jejuni} may also play a role in the regulation of its cell division. The \textit{C. jejuni recA} gene, whose product is involved in DNA repair and recombination, was also cloned. An isogenic \textit{recA} mutant displayed an increase in sensitivity to ultraviolet light and a defect in generalized recombination as determined by natural transformation frequencies (Guerry et al., 1994). Further study of these genes in the context of cell division may reveal associations between cell growth and division in \textit{C. jejuni}.
Recently, a *tig* gene encoding a protein that shares 31% identity with the amino acid sequence of trigger factor from *E. coli* was cloned from *C. jejuni*. The trigger factor in *E. coli* is now thought to be a chaperone involved in cell division (Griffiths *et al.*, 1995).

The *ftsA* gene of *C. jejuni* was isolated in a screen for genes that are strongly expressed when cultures enter stationary phase (Griffiths *et al.*, 1996). The *ftsA* gene of *E. coli* is an essential cell division gene. However, the function of FtsA in cell division is still unknown. As compared to the *E. coli* *ftsA* gene, several differences in the structure and organization of the *C. jejuni ftsA* were observed. The *ftsQ* gene is not directly upstream of *ftsA* in *C. jejuni*. Instead, an open reading frame designated *fagA* (*fts*-associated gene A) was identified which had no similarity to any protein available in the database at that time.

Since the molecular mechanisms of *C. jejuni* cell division and minicell formation remain unknown, further experiments to elucidate these mechanisms can be designed in light of studies on cell division in the Gram negative organisms, *E. coli* and *Caulobacter crescentus*. 
Figure 2. The *Campylobacter jejuni* ATCC 43431 genomic map.
Gram negative bacterial cell cycle

During the bacterial cell cycle, three major cellular processes occur: cell growth, chromosome replication, and cell division. Cell growth is affected by environmental conditions and nutrient availability, which are sensed by sensor and signalling systems of the cell. Growth is characterized by an increase in cell mass or volume, and cell dimensions such as length or diameter, depending on the shape of the cell. Cell growth encompasses the synthesis of cytoplasmic components, and various cell structures such as the flagella, pili and/or extracellular layer (capsule or S-layer). As the bacterial cell grows, additional cell envelope is synthesized to enclose the increasing cytoplasmic volume.

Based on studies in E. coli, de novo protein synthesis is required prior to chromosome replication. Initiation of chromosome replication is precisely timed in the cell cycle and occurs when a critical mass-to-origin ratio (initiation mass) is attained (Lutkenhaus, 1990). This coupling of replication to cell growth is mediated by several systems including the DnaA protein (critical in initiation), the dam methylation system, adenosine triphosphate (ATP), and guanosine tetraphosphate (ppGpp). Following initiation, elongation and termination of chromosome replication, a few minutes of postreplication protein synthesis is required (Donachie, 1993). This period of protein synthesis precedes chromosome decatenation (or separation) and nucleoid segregation (or partition), and may be identical to the period that has been described as a prerequisite for cell division. The period of postreplication protein synthesis may be needed for the synthesis of specific partition protein(s), or to allow cells to reach the critical cell length for partition (Donachie, 1993). Following nucleoid segregation, cell division or cytokinesis occurs with additional cell envelope synthesized preferentially at the division site, usually at midcell, forming the invaginating septum (ingrowth of the cell wall) and CM. Division concludes with cell separation giving rise to two viable daughter cells.
These processes are coupled, and temporally and topologically coordinated so that the cell divides into two viable daughter cells. Although the mechanisms that coordinate them are still not well understood, in recent years various aspects of cell division and its coordinate regulation in *E. coli* and *C. crescentus* have been elucidated.

**Gram negative bacterial cell division**

As aforementioned, many factors play important roles in cell growth, division and its regulation in each bacterial species. These factors include metabolic or growth requirements, environmental pressures (such as temperature, nutrition, and variability of these conditions), bacterial shape or morphology, the structure and organization of the multiple cell envelope layers as well as external layers (capsule or S-layer), and cell structures (flagella or pili). Conditions that sustain the growth of the bacterial culture and maintain cell integrity and viability are essential for the processes of growth and division.

Gram negative bacteria encompass a wide range of very different bacteria, which assume a diverse range of shapes, such as, spherical (coccus), straight short rod (bacillus), long filamentous rod, curved or vibrioid, spirally curved, helical or spiral, stalked, budding, or appendaged (prosthecate), which may possess pili (or fimbriae) and/or flagella, and which may have an external layer in addition to the cell envelope. The straight short rod *E. coli* undergoes symmetrical division giving rise to two identical daughter cells, while the stalked *C. crescentus* undergoes asymmetrical division giving rise to two morphologically distinct cell types with different developmental fates: a motile, flagellated swarmer cell and a non-motile stalked cell (Gober and Marques, 1995).
Genes and regulation of bacterial cell division

Cytokinesis begins when the cell reaches a critical length and completes the period of postreplication protein synthesis (reviewed in Cooper, 1991). It requires the coordinated ingrowth of all three cell envelope layers: CM, PG layer, and outer membrane (OM). In Gram negative bacteria, circumferential invagination of the CM is closely coupled to the ingrowth of the cell wall components (PG layer and OM) at the division site, resulting in the separation of the cytoplasm into two compartments. Cytokinesis, or septation, involves the gene products required for this coordinated ingrowth. These gene products may be activators, inhibitors, or regulators of cell division. During cell growth, the envelope is synthesized to enclose the enlarging cytoplasm by the random incorporation of new envelope material throughout the cell (Cooper, 1991). However, at the onset of cell division, there is a shift to the preferential incorporation of cell envelope components and synthesis at a specific location in the cell. Thus, cell division genes also include those encoding the proteins required for sensing the correct critical time which is appropriate for septation, in determining the division site and influencing the shift to the preferential envelope synthesis at this site, and in regulating these processes.

Examination of cell division mutants have enabled the identification and characterization of many cell division genes. Several genes have been identified by the isolation of temperature-sensitive mutants. These mutants form filaments with regularly spaced nucleoids at the non-permissive temperature, thus were given the designation fits for filamenting temperature sensitive. Table 2 lists the genes that have been isolated from *E. coli*, *C. jejuni*, and *C. crescentus* and a brief description of their characteristics and putative functions in cell division.
Table 2. List of known cell division genes in *E. coli*, *C. crescentus*, and *C. jejuni*.

**E. coli**

*ftsZ* essential division gene; part of a cluster of division genes *ftsQ, ftsA* and *ftsZ*: GTP-binding, GTPase; has homology to eukaryotic tubulin; localizes to the division site and forms a Z-ring prior to visible invagination; appears to be required for initiation of septation; *ftsZ* transcript levels oscillate throughout the cell cycle, while *ftsZ* levels are abundant and relatively constant

*zipA* essential division gene; integral CM protein; interacts with FtsZ; localizes to the division site and assembles into a ring structure prior to visible invagination

*ftsW* CM protein; required for expression of PBP2

*ftsA* has homology to eukaryotic actin; interacts with FtsZ; ratio of FtsA to FtsZ is critical for the proper regulation of division: may interact with PBP3 (FtsI)

*ftsI* integral membrane protein; transglycosylase and transpeptidase; encodes PBP3 which is responsible for septal PG synthesis; may interact with FtsA

*ftsQ* transmembrane protein; may interact with FtsZ, FtsA, and PBP3; may be involved in coupling division and PG physiology

*ftsL* integral membrane protein; may be involved in coupling division and PG physiology

*ftsJ* its expression is heat-inducible; not essential for growth, but important in division

*ftsH* integral CM protein; essential for cell viability; may be involved in folding, assembly or topogenesis of PBP3

*ftsY* CM-associated protein

*ftsE* CM-associated, ATP-binding protein

*ftsX* CM-associated protein

*ftsN* transmembrane protein; may interact with FtsA, PBP3, and FtsQ; may affect PG physiology during division in response to intracellular signals

*ftsK* mutants are blocked at a very late stage of division; may be involved in septal PG synthesis and cell-cell separation

**C. crescentus**

*ftsZ* essential for division; forms the Z-ring at constriction site; FtsZ are abundant in stalked cells, while swarmer cell are almost devoid of FtsZ; intracellular concentrations of FtsZ parallels pattern of DNA synthesis

*ftsA* formerly named *divE*: present upstream of *ftsZ*

*divJ* essential for division

*divK* essential regulator of cell differentiation and division

*divL* essential for division

**C. jejuni**

*ftsA* organization of genes different than in *E. coli* in that, *ftsA* is not downstream of *ftsQ*; *ftsA* transcription is maintained in stationary phase possibly by its most distal promoter

*ftsZ* this study; *ftsZ* transcript levels are relatively constant during log phase and decrease in stationary phase

26
Over the last 20 years, studies have cumulatively shown that the *E. coli* *ftsZ* gene plays a key role in cell division and its regulation. This gene is part of the division and cell wall (*dcw*) cluster located at minute 2.5 on the *E. coli* genetic map (Ayala *et al.*, 1994). *FtsZ* acts in concert with the other division genes in this cluster which include *ftsL* (*mraR*), *ftsI* (*pbp3*), *ftsW*, *ftsQ*, *ftsA*, and *envA*. The *ftsZ* gene was identified and characterized through the isolation of conditional lethal mutants which were blocked in cell division, but not affected in DNA replication or segregation (Lutkenhaus *et al.*, 1980; Yi *et al.*, 1985). It is an essential cell division gene (Dai and Lutkenhaus, 1991). It has been sequenced and encodes a 40-kilodalton (kD) hydrophilic protein (Yi and Lutkenhaus, 1985). *FtsZ* is a GTP/GDP-binding protein with weak GTPase activity (de Boer *et al.*, 1992b; RayChaudhuri and Park, 1992; Mukherjee *et al.*, 1993). Recently, using proteolysis and the yeast two-hybrid system, Wang *et al.* (1997) showed that the N-terminal domains were responsible for GTP/GDP binding, GTPase activity and *FtsZ-FtsZ* interactions which result in polymerization and Z-ring formation. However, genetic studies have demonstrated that truncated *FtsZ* (missing the C-terminal) cannot substitute for full length *FtsZ*. Using the two-hybrid system, *FtsZ* was shown to interact with *FtsA*, and that the truncated *FtsZ* cannot interact with *FtsA*. Thus, these observations suggest that the *FtsZ-FtsA* interaction is essential for septation and occurs between *FtsA* and the C-terminal of *FtsZ*.

As compared to *fts* mutants that have an indented morphology at midcell, which indicated that septation is arrested, *ftsZ* mutants have a smooth morphology, which indicated that *FtsZ* is involved at an earlier stage of division prior to visible invagination. Immunoelectron microscopy (IEM) studies confirmed these observations by localizing *FtsZ* throughout the cell cycle. These studies showed that *FtsZ* forms a cytokinetic ring (Z-ring) at the future division site prior to visible invagination, and remains at the leading edge of the invaginating septum throughout cell division (Bi and Lutkenhaus, 1991). *In vivo* studies using *FtsZ-GFP* (green fluorescent protein) fusion constructs confirmed that Z-rings are formed...
and localized at the division site prior to septation, and remain at the leading edge of the invaginating septum throughout cell division (Ma et al., 1996).

Since the invagination of the PG layer is accomplished by the synthesis of new cell wall material in a circumferential ring at the division site, and based on the IEM and fluorescence microscopy observations, the Z-ring may be acting as a cytoskeletal element and driving the invagination of the CM and synthesis of the new cell wall. It also suggests that FtsZ assembly may be a point at which division is regulated (Lutkenhaus, 1993; Lutkenhaus and Addinall, 1997). Since it has a GTP-binding site similar to that of eukaryotic tubulin and has a weak GTPase activity, these activities may be involved in the assembly of the FtsZ ring. *In vitro* studies have shown that FtsZ polymerizes into similar structures formed by tubulin (Mukherjee and Lutkenhaus, 1994). These similarities of FtsZ with eukaryotic tubulin support the observations that FtsZ may function as a cytoskeletal element during division.

Studies have shown that FtsZ is the target of endogenous cell division inhibitors, SulA and MinCDE (Bi and Lutkenhaus, 1993, 1991, 1990a, 1990b; de Boer et al., 1992a, 1989, 1988; Huang et al., 1996). This linkage between FtsZ and the cell division inhibitors has revealed that these proteins are required to ensure the correct placement of the septum between the separated daughter nucleoids and possibly to ensure that septation occurs only after replication is completed.

When a cell is stressed, for example by DNA damage, or an interruption or block in DNA replication, or heat shock, cell division is blocked. In most cases, this is mediated through the induction of stress-response systems such as the SOS pathway or the heat-shock response which produce cell division inhibitors. In the SOS response, SulA inhibits cell division by binding to FtsZ and preventing FtsZ localization to the division site (Lutkenhaus, 1983; Bi and Lutkenhaus, 1993, 1990a; Dai et al., 1994;
In the MinCDE system, the MinC protein is a cell division inhibitor which is normally activated by MinD. MinCD prevents division at all potential division sites (midcell and poles) in the cell. However, the target of MinCD is unknown. Studies have indicated that MinCD could be interacting with FtsZ (Bi and Lutkenhaus, 1993, 1990b; de Boer et al., 1992a, 1989, 1988). However, one attempt to demonstrate an interaction of FtsZ and MinCD failed (Huang et al., 1996). Other studies have shown that MinE binds to MinD and disrupts the MinCD complex to relieve its inhibitory effects. This observation was recently corroborated by a study (Raskin and de Boer, 1997) using a MinE-GFP fusion protein. This study showed that a MinE ring formed near the middle of young cells, which required MinD but was independent of MinC. The MinE ring also formed in nondividing cells in which FtsZ function was inhibited. Together, these observations suggest that MinE is bound to a target at midcell and would topologically restrict MinCD inhibition and allow FtsZ ring to form at midcell (Raskin and de Boer, 1997). Thus, septation is prevented only at the cell poles and not at midcell (de Boer et al., 1992a, 1990, 1989, 1988; Bi and Lutkenhaus, 1990b; Pichoff et al., 1995; Zhao et al., 1995; Huang et al., 1996).

An early indication that FtsZ may play a pivotal role in cell division came from experiments which studied the effects of \textit{ftsZ} gene dosage. Increasing intracellular levels of FtsZ results in a minicell phenotype, in which small anucleate cells form due to divisions occurring at the cell poles in addition to normal midcell divisions (Ward and Lutkenhaus, 1985). In contrast, reduced levels of FtsZ results in a delay or inhibition of cell division depending on the level that FtsZ is reduced (Dai and Lutkenhaus, 1991; Tétarte and Bouche, 1992).

Regulation of \textit{ftsZ} expression has also been studied. Five promoters have been identified for \textit{ftsZ}, three within the \textit{ftsA} gene and two upstream of the \textit{ftsQ} gene. Overall, \textit{ftsZ} expression detected according to FtsZ levels is induced during entrance into stationary phase and is inversely dependent on growth rate
(Aldea et al., 1990). This expression is characteristic of regulation by gearbox promoters. However, among the five promoters that were identified for *ftsZ*, only one displayed a gearbox behaviour (Aldea et al., 1990). Another study (Garrido et al., 1993) has shown that transcription of *ftsZ* oscillates during the *E. coli* cell cycle, during which *ftsZ* mRNA levels are maximal at the initiation of DNA replication, and that cell cycle-dependent transcription is due to the two proximal promoters. Several factors have been shown to regulate *ftsZ* expression. Among these are the gene products of *sdiA* (Wang et al., 1991) and *rcsB* (Gervais et al., 1992) which regulate *ftsZ* transcription, and a 53-nucleotide RNA molecule, encoded by *dicF*, which is an antisense regulator of *ftsZ* translation (Tétarte and Bouche, 1992).

In *C. crescentus*, the *ftsZ* gene was recently identified and studied (Quardokus et al., 1996). The levels of FtsZ throughout the cell cycle, within stalked and swarmer cells were studied. These were shown to be different from the *E. coli* FtsZ levels, which remain relatively high throughout the cell cycle (Dai and Lutkenhaus, 1992). In contrast, *C. crescentus* FtsZ levels in stalked cells increase as DNA replication begins, and reaches a peak just before cell division. Immediately after division, the resulting swarmer cell is almost devoid of FtsZ, while FtsZ remains in the stalked cell. Therefore, the regulation of FtsZ levels coincides with the cell cycle and DNA replication (Quardokus et al., 1996).

Recently, the *zipA* gene was identified and found to be an essential component of septation (Hale and de Boer, 1997). ZipA is a novel integral CM protein which was found to interact with FtsZ *in vitro*. Overexpression of ZipA inhibits cell division resulting in the formation of smooth, filamentous cells which indicates that cell division was blocked before the onset of cell wall invagination. This cell division inhibition is suppressed by FtsZ overexpression, which suggests that the specific interaction of FtsZ and ZipA observed *in vitro*, also occurs *in vivo* (Hale and de Boer, 1997). The dependence of septation on the presence of ZipA was observed when *E. coli* cells were unable to septate and formed
smooth filamentous cells due to a depletion of ZipA. Additionally, examination of a ZipA-GFP fusion localized ZipA at the division site prior to visible invagination. These observations indicate that ZipA acts at a very early stage of cell division, and that ZipA and FtsZ may localize and act at the same stage of division. Since ZipA remains at the leading edge of the invaginating septum throughout cell division and interacts with FtsZ, ZipA could be involved in the assembly and function of the Z-ring (Hale and de Boer, 1997).

In addition to ZipA, the gene product of *ftsW* has also been suggested to be involved in the initiation stage of cell division via an interaction with FtsZ that stabilizes the Z-ring (Khattar et al., 1994, 1997). The *E. coli* FtsW protein contains many hydrophobic domains, which suggests that it is an integral membrane protein. It shows high homology with the RodA protein (Ikeda et al., 1989). RodA functions in lateral elongation during cell growth to determine cell shape, and is located in the CM (Ikeda et al., 1989). It is required for the expression of the enzymatic activity of PBP2, which synthesizes the PG in the step of initiating cell elongation (Ikeda et al., 1989). If RodA functions in forming the rod shape of the cell by positively affecting the enzymatic activities of PBP2 (encoded by *pbpA*), the structurally similar FtsW protein, mutation in which causes a defect in cell division, might affect PBP3 (encoded by *ftsI*, responsible for septum PG synthesis) or some other PBP ensuring septum formation (Ikeda et al., 1989).

In contrast to the smooth morphology of *ftsZ, zipA* (Hale and de Boer, 1997), some *ftsW* (Khattar et al., 1997), and some *ftsQ* (Carson et al., 1991) mutants, the indented morphology of *ftsI* and *ftsA* mutants suggest that they are arrested at a later stage of division. Thus, PBP3 and FtsA act at a later stage of septation than FtsZ, ZipA, and possibly FtsW and FtsQ. FtsA is a 45kD protein, which also has homology to a eukaryotic protein, actin. The FtsA ATP-binding domain has homology to that of actin, DnaK and hexokinase, other ATP-binding proteins (Bork et al., 1992). Cytoplasmic FtsA can
bind ATP and is phosphorylated, but FtsA is unable to bind ATP and is unphosphorylated when associated with the inner surface of the CM (Pla et al., 1990; Sanchez et al., 1994). Mutagenesis studies showed that phosphorylation and ATP binding may not be essential for FtsA function, however, and they may have a regulatory role on the action of FtsA during septation (Sanchez et al., 1994). The ratio of FtsA to FtsZ is critical for the proper regulation of the cell division, and not the levels of the individual proteins (Dai and Lutkenhaus, 1992; Dewar et al., 1992). Immunofluorescence microscopy using anti-FtsA antibodies, and TEM using FtsZ-GFP and FtsA-GFP localization within living E. coli cells, have shown that FtsA localization depends on FtsZ localization (Addinall and Lutkenhaus, 1996; Ma et al., 1996). Collectively, these observations indicate that FtsA interacts with FtsZ. This interaction was recently supported by results of the yeast two-hybrid system in which a direct interaction between FtsA and FtsZ was detected (Wang et al., 1997). Additionally, studies have suggested that FtsA interacts with PBP3 (Tormo and Vicente, 1984; Tormo et al., 1986; Dai and Lutkenhaus, 1992). Therefore, FtsA may function in the constriction process together with FtsZ, in stabilizing the Z-ring, in signalling of the constriction process to PBP3, or in the recruitment of other division proteins to the septum. However, these possibilities remain to be determined.

PBP3, FtsQ, FtsL, and FtsN are transmembrane proteins (Carson et al., 1991; Dai et al., 1993; Guzman et al., 1992). Each of these proteins has a non-cleavable, N-terminal signal sequence which acts to transport the main body of the protein to the periplasmic space and to anchor the protein to the CM (Dai et al., 1993). The ftsI or pnpB gene encodes PBP3 which is specifically required for septal PG biosynthesis (Dai et al., 1993; Nanninga, 1991; Spratt, 1975). It has both transglycosylase and transpeptidase activities, where the latter is inhibited by certain beta-lactams and is encoded by its periplasmic domain (Donachie, 1993; Pogliano et al., 1997). As aforementioned, studies have suggested that FtsA interacts with PBP3 (Dai and Lutkenhaus, 1992; Tormo et al., 1986). Since FtsA is a peripheral CM protein and interacts with FtsZ, FtsA could be the signalling system between FtsZ
and PBP3, to initiate septal PG synthesis. However, the direct interaction between PBP3 and FtsA has yet to be shown. A recent study by Pogliano et al. (1997) showed that Z-ring constriction requires the transpeptidase activity of PBP3. In addition, by examining Z-ring formation in newly formed daughter cells versus filamentous cells, their observations indicated that PBP3 is not required for initial Z-ring assembly, but it is required for the subsequent assembly of additional Z-rings.

The roles of FtsQ, FtsL, and FtsN are still unclear. The *E. coli* *fisQ* gene has been sequenced and its termination codon was found to overlap the start codon of *ftsA*. The FtsQ level in the cell is lower than FtsZ and FtsA (Yi et al., 1985; Carson et al., 1991; Pla et al., 1990; Wang and Gayda, 1992). Carson et al. (1991) showed that the *ftsQ1* mutant forms smooth filaments at 42°C but indented filaments at 37°C, and proposed that FtsQ is required throughout septation. Although the role of FtsQ is still unknown, studies have suggested that it functionally interacts with PBP3, FtsA and FtsZ (Descoteaux and Drapeau, 1987: Dai and Lutkenhaus, 1992) and proposed that FtsQ either plays a role in stabilizing the Z-ring or link FtsZ to a penicillin-insensitive PG synthetic system (PIPS) which is thought to act before PBP3 (Nanninga, 1991: Dai and Lutkenhaus, 1992).

The *ftsL* (*mraR*) gene, encoding FtsL, is essential in cell growth and division, and has been suggested to have a role in coupling cell division and PG physiology (Dai et al., 1993; Guzman et al., 1992). The *ftsN* gene encodes FtsN which appears to interact with FtsA, PBP3, and FtsQ (Dai et al., 1993). FtsN is an integral membrane protein with a bitopic topology, consisting of an N-terminal cytoplasmic domain, transmembrane segment, and periplasmic domain. Dai et al. (1996) showed that only the periplasmic domain was essential and FtsN is functional as long as this domain is exported to the periplasm. Recently, Addinall et al. (1997) showed that FtsN is localized to the septum through its periplasmic domain and its localization depends upon other cell division proteins such as PBP3, FtsQ, FtsA, FtsZ, FtsW, ZipA, and FtsL. Due to their transmembrane topology, the FtsQ, FtsL, and FtsN
proteins have the potential to affect PG physiology during cell division in response to intracellular signals (Dai et al., 1993).

The \textit{ftsK} gene was identified from a mutant blocked at a very late stage of division. The mutant phenotype was specifically suppressed by the deletion of \textit{dac}, which encodes the PBP5 protein involved in PG synthesis. This suggests that it is involved in PG synthesis during septation. In addition, FtsK has been suggested to be involved in cell-cell separation (Begg et al. 1995; Diez et al. 1997).

The \textit{ftsH} and \textit{ftsJ} genes have also been isolated and mapped to the minute 69 region on the genetic map (Tomoyasu et al. 1993a). The \textit{ftsH} gene is essential for cell viability in \textit{E. coli} (Tomoyasu et al. 1993a). The deduced amino acid sequence of FtsH reveals that it has two hydrophobic stretches characteristic of membrane spanning, a putative ATP-binding sequence, and a significant homology to the domain found in members of a novel, eukaryotic family of putative ATPases which function in protein transport pathways, cell division cycle, and gene expression. Recently, the FtsH protein was shown to be an integral CM protein which spans the membrane twice and to have a large cytoplasmic carboxy-terminal which contains the ATP-binding domain (Tomoyasu et al. 1993b). Evidence from recent studies suggests that FtsH is involved in the localization processes of some envelope proteins: folding, assembly or topogenesis for PBP3, and membrane translocation for beta-lactamase (Tomoyasu et al. 1993a). The \textit{ftsJ} gene is not essential for cell growth but is important in cell division, since a deletion mutant of \textit{ftsJ} formed filaments (Ogura et al., 1991). FtsJ has significant amino acid sequence homology to the protein encoded by \textit{YCF4} of \textit{Saccharomyces cerevisiae}. However, the function of this gene is unknown, thus no role has been suggested for FtsJ (Tomoyasu et al. 1993a).

\textit{pbpA} encoding the PBP2 protein, which is involved in PG synthesis during cell growth, has been recently implicated in cell division since its inactivation results in cell division inhibition. However, the
inhibition was relieved by FtsZ overexpression. The cell division inhibition was reversible when the level of ppGpp was increased at a high temperature and with a required de novo protein synthesis. This investigation lead to the hypothesis that the nucleotide ppGpp activated ftsZ expression, and thereby coupled cell division to protein synthesis (Vinella et al., 1993).

In addition to the ftsZ gene, the ftsA (divE) gene of C. crescentus has also been identified upstream of ftsZ (Ohta et al., 1997). Several other genes that affect cell division of C. crescentus have also been identified by isolating division mutants which were designated div. Similarly, they were categorized according to their morphology. Among these genes are divJ, divK, divL, and crA (Ohta et al., 1992; Hecht et al., 1995; Sommer and Newton, 1991; Quon et al., 1996). Although their precise roles in cell division and its regulation in C. crescentus have yet to be determined, they have been shown to be essential in division.

Several genes involved in flagellar biogenesis have also been found to affect cell division. In C. crescentus, the flIL, flIM, flIQ, flIR, rpoN, and flbD flagellar genes expressed early in the cell cycle are not only required for flagellar biogenesis, but are also for cell division (Dingwall et al., 1992; Yu and Shapiro, 1992; Zhuang and Shapiro, 1995). Mutants of these genes display a filamentous phenotype, indicating that flagellar assembly events are required for the proper coupling of cell growth to cell division (Gober and Marques, 1995).

Upon completion of septum formation, a covalently bonded double layer of PG, the EnvA protein splits the layers apart. Both over- and under-production of EnvA are lethal. Until the layers are split apart, the OM cannot invaginate, as observed in the envA.22 mutant in which the OM balloons out around the septal site (Donachie, 1993). Normally, the OM invaginates as the ingrowing septal layers are split, so that the OM is tightly apposed to the new cell poles. It is not known what causes the OM to follow the
ingrowth of the septum in this way. However, this membrane is covalently linked to the PG layer by
the Braun lipoprotein, at one end, and at the other end, embedded in the OM (Donachie, 1993).
Therefore, the constant formation of these new bonds may keep the OM attached to the PG layer
during cell division. But, there exists mutants of the lpp gene (defective in lipoprotein) that have an
OM layer that is only loosely associated with the PG layer, but these mutants can divide successfully.
Thus, the OM appears to be capable of self-sealing around new cell poles without the necessity of a
direct linkage to the PG layer (Donachie, 1993).

Current working model of Gram negative bacterial cell division

Under suitable conditions, a cell will grow until it reaches initiation mass at which point the
chromosome is replicated, followed by cell division which gives rise to two viable daughter cells. The
sensing and signalling systems that trigger and regulate cell division remains to be an area that is highly
investigated. Based on E. coli studies, my current view of cell division is diagrammed in Figure 3
(adapted from Lutkenhaus and Addinall, 1997; Hale and de Boer, 1997; Rothfield and Justice, 1997).
In this model, once the chromosomes have been separated, a cell cycle cue initiates division. FtsZ and ZipA may localize to the division site defined by an as yet unknown receptor, or ZipA may be localized at the division site and is the receptor for FtsZ (Hale and de Boer, 1997). FtsZ is thought to first localize at one point of the division site, and then polymerize to form the Z-ring through bidirectional growth (Addinall and Lutkenhaus, 1996). Two possibilities have been proposed for the mechanism of cell envelope invagination. In both cases, the Z-ring drives invagination by pulling the CM inward. CM invagination is coordinated with septal PG synthesis. The OM, in turn, invaginates passively through its covalent linkage with the PG layer by the Braun lipoprotein. In the first possibility, ZipA by interacting with FtsZ, connects the Z-ring to the CM and may simply act to transduce the force generated by the Z-ring to the lipid bilayer. In the second possibility, ZipA may be actively involved in the contraction of the Z-ring and/or in the coordination of the activity of FtsZ in the cytoplasm with that of the PG-synthesizing machinery in the periplasm (Hale and de Boer, 1997). Septum formation also requires other Fts proteins. It has been suggested that Z-ring formation leads to the recruitment of other Fts proteins (Addinall and Lutkenhaus, 1997). According to morphological and genetic studies, FtsW localization and involvement may precede those of FtsA, FtsQ, and PBP3. PBP3 is involved in septal PG synthesis, possibly in association with FtsW. Subsequently, FtsL, FtsN, and FtsK are involved in septation, where both FtsN and FtsK act during the late stages of division. FtsK has been suggested to also be involved in cell-cell separation. Upon completion of septum formation, EnvA is involved in the separation of the covalently-bonded PG double-layer, which is followed by cell-pole formation, and finally, cell separation giving rise to two identical and viable daughter cells.

In the figure, R represents the unknown receptor at the division site, which could be ZipA.
Z-ring formation

Z-ring constriction cell separation
Several cell division proteins and their interactions have been characterized. However, the mechanisms by which these proteins interact, when they interact and function, and how each protein and protein complex functions have not been established. An important area to be explored is how these cell division mediators interact with mediators of cell growth and chromosome replication. Since FtsZ has been shown to be essential and one of the earliest proteins involved in cell division, it may be a point at which division is coordinated with growth and chromosome replication. This coordination may involve protein-protein interactions with FtsZ, or the regulation of FtsZ levels. For example, FtsZ could be the target of mediators that sense or signal the different growth phases of the cell or status of chromosome replication. As previously mentioned, the FtsZ ring may also act as a cytoskeletal element, driving the invaginating septum during division. Since FtsZ is a critical cell division protein, studying how FtsZ levels are regulated and how it interacts with other proteins, will lead to a more comprehensive understanding of cell division.

The role of FtsZ in Gram negative cell division has been studied mainly in the rod-shaped, symmetrically dividing E. coli cell. However, cell division occurs asymmetrically in C. crescentus, and its FtsZ levels throughout the cell cycle are different compared to the levels in E. coli (Quardokus et al., 1996; Dai and Lutkenhaus, 1992). This suggests that different molecular mechanisms of cell division are used. Since the mode of C. jejuni cell division has not been established, this study is the first to examine cell division in this spiral organism. Characterization of the fisZ gene will initiate studies into its role in C. jejuni cell division and also facilitate the identification of FtsZ-interacting proteins.
OBJECTIVES AND LONG TERM GOALS OF THIS STUDY

The objectives of this study were to determine the mode of *Campylobacter jejuni* ATCC 43431 cell division and to characterize its *ftsZ* gene. The predominance of spiral rod-shaped cells in young cultures versus coccoid cells in older cultures has been observed, but the mechanisms giving rise to these morphologically distinct cells remain unknown. It has been postulated that the coccoid cells are degenerate forms of the spiral cells. This has led to the hypothesis that different modes of cell division occur under favourable and unfavourable growth conditions: symmetrical or asymmetrical cell divisions, respectively. TEM was used to examine *C. jejuni* cells in log and stationary phase cultures to determine the predominant mode of cell division.

Since two modes of cell division were observed from *C. jejuni* log and stationary phase cells, the role of FtsZ in each division event needed to be elucidated. The *C. jejuni ftsZ* gene was isolated and characterized in this study to facilitate studies into its role in and the regulation of *C. jejuni* cell division. Based on DNA and deduced amino acid sequences, site-specific mutagenesis can be used to design and generate temperature sensitive mutants. These mutants will allow the study of *ftsZ* functional domains and the role of *ftsZ* in each mode of cell division. Similar to *E. coli* studies, shuttle vectors in *C. jejuni* can be used to study the dosage effects of *ftsZ*.

The partial FtsZ was purified to generate anti-FtsZ antibodies. Using IEM studies, the antibodies will be used to localize FtsZ throughout the cell cycle of symmetrically and asymmetrically dividing log and stationary phase cells, respectively. The anti-FtsZ antibodies will also facilitate the isolation of other division proteins that interact with FtsZ during the different modes of cell division. Therefore, determining the modes of cell division and characterizing its *ftsZ* gene will facilitate future investigations to elucidate the molecular mechanisms of *C. jejuni* cell division.
METHODS AND MATERIALS

Strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 3. My thesis examines the mode of cell division and the ftsZ gene of Campylobacter jejuni ATCC 43431. E. coli JM101 was used as the host of plasmids pBluescript II SK+, pBR322, pGEX 2T and the respective recombinant plasmids, while E. coli LE392 was used to propagate Lambda GEM 11 recombinant phage clones.

The C. jejuni unamplified pBR322 recombinant DNA library is the original ligation mixture of Sau3AI-digested genomic DNA and BamHI-digested pBR322 vector. The C. jejuni pBR322 library consists of JM101 transformants and the Lambda GEM 11 library consists of phage particles that have been amplified. pBXS6 is a recombinant pBluescript II SK+ clone isolated previously (Chan et al., unpublished data), containing the partial ftsZ gene and downstream flgF and flgG genes. 1.0.2, 1.2.8, and 1.7.1 are recombinant pBluescript II SK+ clones which contain the partial ftsZ, partial flgF and complete flgG genes, respectively, subcloned from HindIII fragments of pBXS6 (Figure 4). The pGEX 2T recombinant clone, pGEX-ftsZ, contains the partial ftsZ gene (PCR amplified from pBXS6 using primers PCRF3 and PCRF4) fused in-frame at the 3' end of the glutathione-S-transferase gene. Lambda-ftsZ1 and Lambda-FtsZ2 are unique recombinant Lambda GEM 11 clones isolated from the Lambda GEM 11 library, which contain the complete coding and upstream sequences of ftsZ. Schematic diagrams representing the recombinant clones used in this study are shown in Figure 4.
Growth conditions

Mueller Hinton agar plates and broth were used to grow and maintain *C. jejuni* cultures. These cultures were grown in a 37°C incubator with a ∼5-10% CO₂ atmosphere. All *E. coli* strains were grown aerobically at 37°C. *E. coli* JM101 cultures were grown in Luria Bertani (LB) broth to obtain competent cells, while recombinant plasmid transformants were grown on LB+amp (LB supplemented with 100μg/ml ampicillin) agar plates and broth. *E. coli* LE392 cultures and LE392-infected with recombinant Lambda GEM 11 phage cultures were grown on LB plates and broth. *E. coli* JM101 was maintained on minimal agar plates (supplemented with thiamine) while LE392 was maintained on LB agar plates at 4°C. In addition, frozen stocks of bacteria in LB containing 15% glycerol were stored at -70°C. The recombinant phage lysates were maintained in lambda diluent (with a drop of chloroform) at 4°C.

*C. jejuni* growth curve

An isolated *C. jejuni* colony was picked and grown in 10 ml broth for ~24 hours. An aliquot of this broth culture was re-inoculated at a dilution of 1/1000 and grown in fresh broth. Each growth curve was plotted based on at least six time points that were titred. Each titre, expressed as colony forming units per ml (CFU/ml), was calculated by averaging the colony counts of duplicate plates from three dilutions of the culture at each time point: 0 to 10 hours, 10^1, 10^2, 10^3 dilutions; 11 to 24 hours, 10^3, 10^4, 10^5 dilutions; and 25 to 49 hours, 10^4, 10^5, 10^6 dilutions. The growth curve was also plotted for the culture from which total RNA was extracted at the different time points.
Transmission electron microscopy (TEM)

Stationary phase broth cultures of *C. jejuni* were initially fixed by adding paraformaldehyde (0.8% final concentration) and incubating for 5 minutes. The cells were then centrifuged at 4°C, 3000 rpm for 15 minutes to obtain a pellet. The supernatant was removed and the pellets were fixed in the fixative solution at room temperature (RT) for 45 minutes. The pellets were further fixed at 4°C for ~16 hours, followed by centrifugation at 4°C, 3000 rpm for 15 minutes to obtain a firm pellet. The pellets were incubated in a 50mM sodium cacodylate buffer at RT for 5 minutes, after which it was removed and replaced with fresh buffer. Three cacodylate buffer washes were followed by a fixation in 1% osmium tetroxide (in 0.1M Sorensen, pH 7.0) at RT for 2 hours. Subsequently, the pellets were washed three times in cacodylate buffer, each wash at RT for 10 minutes. Then, using 1% uranyl acetate (in 30% ethanol), the pellet was simultaneously fixed and stained at RT for 1 hour. The pellet was then dehydrated in 4 steps, each step consisting of two incubations at RT for 10 minutes: first in 50% ethanol, second in 70% ethanol, third in 95% ethanol, and fourth in 100% ethanol. The dehydration was followed by stepwise infiltration of the pellet with LR white plastic resin at RT: first in 50% plastic/50% ethanol for 30 minutes, second in 75% plastic/25% ethanol for ~16 hours, third in 100% plastic for 2.5 hours, and fourth in 100% plastic for 2 hours. Finally, the pellets were transferred into beam capsules with 100% plastic and baked at 60°C for 12 to 20 hours.

Log phase *C. jejuni* cultures were also fixed while in broth with paraformaldehyde (0.8% final concentration), and centrifuged at 4°C, 3000 rpm for 15 minutes to obtain a pellet. The pellets were incubated in fixative solution at RT for 10 minutes, then in fresh fixative solution at RT for 45 minutes, which was then incubated further at 4°C for ~16 hours. Following another round of centrifugation, the firm pellets were washed three times in 0.1M phosphate buffer, pH 7.2 at RT for 5 minutes, and a fourth time in the phosphate buffer at 4°C for 1 hour. Subsequently, the pellets were
fixed in 1% osmium tetroxide at RT for 1 hour, and washed once in the phosphate buffer at RT for 10 minutes, then, twice in distilled water (H₂O) at RT for 10 minutes, and once in H₂O at RT for 6 minutes. Then, the pellet was simultaneously fixed and stained using 1% uranyl acetate at RT for 40 minutes and washed with H₂O at RT for 10 minutes. The pellet was then dehydrated in 4 steps (all steps at RT): first in 50% ethanol (once for 10 minutes and once for 5 minutes), second in 70% ethanol (twice for 5 minutes), third in 95% ethanol (twice for 5 minutes), and fourth in 100% ethanol (three times for 10 minutes). The dehydration was followed by stepwise infiltration of the pellet with LR white plastic resin: first in 50% plastic/50% ethanol at RT for 30 minutes, second in 75% plastic/25% ethanol at RT for 30 minutes, third in 100% plastic at RT for 10 minutes, and fourth in 100% plastic at 4°C for ~20 hours. Finally, the pellets were transferred into beam capsules with 100% plastic and baked at 60°C for ~48 hours.

Once the beam capsules had hardened, they were carved and thin-sectioned. The sections were placed onto copper grids. The plate culture cells and stationary phase cells were stained with 5% uranyl acetate for 10 minutes followed by three H₂O rinses for 15 minutes each, and stained with lead citrate for 10 minutes followed by three H₂O rinses for 15 minutes each. The log phase cells were stained with 5% uranyl acetate for 5 minutes followed by five H₂O rinses for 5 minutes each, and stained with lead citrate for 5 minutes followed by five H₂O rinses for 5 minutes each. All H₂O rinses were followed with a final 30 second H₂O rinse. The C. jejuni cells within the sections were viewed using a Phillips 300 transmission electron microscope. Cells were photographed using 35 mm film, which were then developed on Kodak paper.
Statistical analyses of dividing *C. jejuni* cells

For this study, the mode of *C. jejuni* cell division in log and stationary phase cultures is inferred based on the morphology of 100 dividing cells as observed by TEM. Each dividing cell that was enumerated and involved in the statistical analyses was classified as follows: (i) a cell is considered dividing symmetrically if both daughter cells are longer than coccoid or minicells, regardless of whether none, one or both poles are visible; and (ii) a cell is considered dividing asymmetrically if one daughter cell is coccoid, regardless of whether one or both poles are visible. In addition, an assumption is also made, that the same number of false coccoid daughter cells (cells that are cross-sectioned, thus appear coccoid) are observed from both log and stationary phase cultures.

To obtain a statistically significant inference of the mode of *C. jejuni* cell division, three null hypotheses (H₀) were designed:

(i) to determine if symmetrical division occurs in log cultures, the H₀ = the expected probability of symmetrical and asymmetrical division in log phase each occurs 50% of the time. Thus, it will test whether the probability for a division to occur symmetrically is \( p = 0.5 \) (follows the binomial distribution), if division occurs based on chance instead of a specific biological process;

(ii) to determine if asymmetrical division occurs in stationary cultures, the H₀ = the expected probability of asymmetrical and symmetrical division in log phase each occurs 50% of the time. Thus, it will test whether the probability for a division to occur asymmetrically is \( p = 0.5 \) (follows the binomial distribution), if division occurs based on chance instead of a specific biological process; and

(iii) to determine if the same mode of cell division occurs in log and stationary cultures, the H₀ = the ratio of the numbers of symmetrically and asymmetrically dividing cells in log and stationary phase is the same.
The appropriate Chi square ($X^2$) tests were applied to test each $H_0$ and determine the cell division mode(s) within this study's log and stationary cultures. Several statistical terms are defined as follows: $sp$, sample proportions of the specified category being tested; $sq$, sample proportions of the opposite category; $k$, number of categories (symmetrical or asymmetrical mode of division); $n$, total number of observational units (number of dividing cells); $o$, number of observational units per category; $e$, number of expected units per category; (df), degrees of freedom; and $\alpha$, significance level. Each of the tests are tested at the 5% (0.05) significance level, and if the sample $X^2$ value is equal or greater than the critical $X^2$ value, the $H_0$ is rejected, while if the sample $X^2$ value is less than the critical $X^2$ value, the $H_0$ is accepted.

The (i) and (ii) $H_0$ were tested using the following $X^2$ goodness-of-fit test:

$$X^2_{o} = \sum_{i=1}^{k} [(o - e)^2 + e] \text{ with (} k - 1 \text{) degrees of freedom and } k = 2, \text{ and at the critical value of } \alpha = 5\% (0.05) \text{ significance level (Appendix 2).}$$

Then, the 95% confidence limits were calculated as follows:

$$sp \pm (z_{0.02}) (\sigma_{sp})$$

in which $z_{0.02}$ is the standard deviation units based on the standard normal distribution (Appendix 3), and

$\sigma_{sp}$ is the sample standard deviation calculated as follows:

$$\sigma_{sp} = (spsq + n)^{0.5}.$$ 

The (iii) $H_0$ was tested using the following $X^2$ test of homogeneity of populations:

$$X^2_{o} = \sum_{i=1}^{k} [(o - e)^2 + e] \text{ with (} k_A - 1 \text{)(}k_B - 1 \text{) degrees of freedom which accounts for the categories of log (} k_A = 2 \text{) and stationary (} k_B = 2 \text{) phase, and at a critical value of } \alpha = 5\% (0.05) \text{ significance level (Appendix 2).}$$
DNA preparation and purification

Genomic DNA was prepared from *C. jejuni* using the DNAzol reagent (Gibco BRL Life Technologies) according to the manufacturer's recommendations. Plasmid DNA was prepared using the alkaline lysis method (Sambrook *et al.*, 1982). Sequencing grade plasmid DNA was obtained by further purifying the plasmid DNA as follows: 30ul of a polyethylene glycol solution (20% PEG-8000, 2.5M NaCl) was added to 50ul alkaline lysis-prepared plasmid DNA, mixed, and incubated on ice for 1 hour, followed by centrifugation at 13000rpm, 4°C for 20 minutes, then a brief 70% ethanol wash of the pellet, and finally, resuspending the purified DNA pellet in H₂O. Phage DNA was prepared using the rapid phenol/chloroform method (Grossberger, 1987).

DNA fragments were purified from agarose gels using the GENECLEAN II® kit (BIO 101 Inc.) and the QIAEX® II Gel Extraction kit (QIAGEN Inc.) according to the manufacturer's recommendations. Oligos designed for sequencing the *ftsZ* gene, the PCRs, and the primer extension reactions, were synthesized either in the Expedite Nucleic Acid Synthesis System (Beckmann synthesizer) using Millipore 0.2um columns and purified according to the manufacturer's recommendations, or by ACGT Corporated, or by the Hospital for Sick Children's oligo synthesizer facility. The oligos and respective sequences are listed in (Table 4) which also indicates the use of the oligo. The locations of each oligo are shown in Figure 5.

Radioactive DNA labelling

Oligos were end-labelled using γ-³²P ATP (4500Ci/m mole, ICN Biomedicals Canada Ltd.) to be used in the primer extension reaction. In each end-labelling reaction, 40 pmoles of oligos were labelled in a 20ul reaction mix of kinase buffer (70mM Tris.Cl pH 7.6, 10mM MgCl₂, 5mM dithiothreitol),
50uCi γ-32P ATP, and 10U T4 Polynucleotide Kinase (New England Biolabs, Inc.) at 37°C for 30 minutes. Each reaction was terminated by heat inactivation at 65°C for 20 minutes.

Double-stranded (ds) DNA fragments were labelled in nick translation reactions using α-32P dATP (3000Ci/mmole, ICN Biomedicals Canada Ltd.). In each nick translation reaction, approximately 0.01ug - 0.10ug of dsDNA template was labelled in a 50ul reaction mix of 50mM Tris.Cl pH 7.8, 5mM MgCl2, 10mM 2-mercaptoethanol, 10ug/ml nuclease-free BSA, 0.2mM dGTP, 0.2mM dTTP, 0.2mM dCTP, 50uCi α-32P dATP, and a mix of 20U DNA polymerase I/200pg DNase I (Gibco BRL Life Technologies) at 14°C for 1 hour. Each reaction was terminated with 5ul of 0.5M EDTA pH 8.0.

All the labelled oligos and dsDNA fragments were purified from unincorporated nucleotides by centrifugation through a Sephadex G-50 column equilibrated in STE (10mM NaCl, 10mM Tris.Cl pH 8.0, 1mM EDTA pH 8.0). The amount of radioactivity incorporated was quantified using a liquid scintillation counter (Beckmann Instruments).

Colony and plaque lifts

The C. jejuni pBR322 library of JM101 transformants was plated out on LB-amp, while the Lambda GEM 11 library of phage particles was mixed with E. coli LE392 cells and pour plated onto LB agar plates. All colonies and plaques were transferred onto Colony/Plaque Screen™ membranes (DuPont NEN* Research Products) according to the manufacturer's recommendations. Briefly, the colonies/plaques on the membranes were treated with 0.5N NaOH (sodium hydroxide) for 2 minutes (twice) and with 1M Tris.Cl pH 7.5 for 2 minutes (twice). The DNA from the colonies/plaques were fixed by air drying the membranes.
Screening of plasmid and phage libraries to isolate the complete \textit{ftsZ} gene

The \textit{HindIII} fragment of pBXS6 containing the partial \textit{ftsZ} gene was used to probe the plasmid and phage libraries for clones containing the complete or partial and upstream region of the gene. Colony/plaque lift membranes were briefly soaked in 2 X SSC (0.3M NaCl, 0.03M disodium citrate), and then prehybridized in 50% deionized formamide, 1% ultra pure sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 1M NaCl at 42°C for 30 minutes to 2 hours. The hybridization solution was identical with the addition of denatured 100ug/ml sheared salmon sperm DNA and \textit{ftsZ} probe at a specific activity of 1.5 X 10^5 cpml/ml. Following hybridization at 42°C for 16 to 24 hours, the membranes were washed in 2XSSC at RT, for 10 minutes, twice. The positive clones were visualized by autoradiography using X-ray films (X-OMAT AR, Kodak Scientific Imaging Film) exposed to the membranes at -70°C.

\textbf{PCR strategy to isolate the N-terminal coding and upstream region of \textit{ftsZ} from the unamplified pBR322 plasmid DNA library}

To obtain the complete and upstream regions of \textit{ftsZ}, a PCR strategy was designed to amplify, identify, and isolate fragments from the \textit{C. jejuni} unamplified pBR322 DNA library that contain the N-terminal coding and upstream regulatory region of the \textit{ftsZ} gene (Figure 6). Oligos F1 and F2 were designed from within \textit{ftsZ}, complementary to its coding strand. In the PCR, the F2 oligo would be extended to obtain the N-terminal coding and upstream region of \textit{ftsZ}. Oligos BamHI A and BamHI B derived from pBR322 were used to target sites flanking the BamHI RE site in which all the \textit{C. jejuni} genomic fragments are inserted in pBR322. F2 was paired with BamHI A and BamHI B (F2-BamHI A, F2-BamHI B) to amplify fragments from clones with various orientations of insertions.
PCR products from clones with the identical partial ftsZ insert as pBXS6 are of expected sizes 502bp from F2-BAmHI A and 516bp from F2-BamHI B. The PCR products possibly containing the N-terminal coding and upstream region of ftsZ were detected by the radiolabelled F1 oligo in Southern blot hybridization.

Each 50ul PCR included 0.4ug pBR322 DNA library (covers ~4X10^10 times the genome), 10 pmoles F2 primer, 10 pmoles (BamHI A or BamHI B) primer, 10 mM dGTP, 10 mM dATP, 10 mM dTTP, 10 mM dCTP, 1mM MgCl_2, amplification buffer (10mM Tris.Cl, 50mM KCl, and 0.01% gelatin, pH8.3), and 2.5U Taq DNA polymerase (Boehringer Mannheim). The PCR consisted of an initial melting step at 95^0C for 3.5 minutes, followed by 30 cycles, each consisting of a melting step at 95^0C for 1.5 minutes, an annealing step of 50^0C for 1 minute, and an extension step of 72^0C for 1.5 minutes. A final extension step of 72^0C for 10 minutes was included and the reaction was stored at -20^0C.

**PCR to amplify the partial ftsZ gene to be subcloned into the pGEX 2T fusion vector**

In a 50ul PCR, 10 ng pBXS6 was used as the template with 10 pmoles PCRF3 primer, 10 pmoles PCRF4 primer, 10mM dGTP, 10mM dATP, 10mM dTTP, 10mM dCTP, 2 mM MgCl_2, amplification buffer (10mM Tris.Cl, 50mM KCl, and 0.01% gelatin, pH8.3), and 2.5U Taq DNA polymerase (Boehringer Mannheim). The PCR consisted of an initial melting step at 95^0C for 3.5 minutes, followed by a set of 5 cycles (95^0C for 1.5 minutes, 42^0C for 1 minute, 72^0C for 1.5 minutes), and a set of 25 cycles (95^0C for 1.5 minutes, 55^0C for 1 minute, and 72^0C for 1.5 minutes). Following the 25 cycles, a final extension of 72^0C for 10 minutes was included and the reaction was stored at -20^0C.
Analyses of recombinant plasmid and phage clones, and PCR products

A RE map of pBSX6 was determined (Figure 4) and used as a comparison to the recombinant clones obtained from the plasmid and phage library screens. The RE digests consisted of using ~0.1ug DNA in a 20ul reaction of 1 X RE buffer and 1U RE at the appropriate incubation temperature for 1 hour. Subsequently, 10ul of each reaction was resolved by electrophoresis in 0.7% to 1% agarose gels. From each PCR, one tenth of the products was resolved in a 1% agarose gel. Ethidium bromide was used to stain DNA, which was then visualized under ultraviolet light illumination.

Southern blot and hybridizations

To map ftsZ within the recombinant plasmid and phage clones, and to identify amplified fragments that contain the ftsZ N-terminus coding and upstream region, the agarose gels that were used to resolve the fragments were transferred onto GeneScreen Plus (DuPont NEN Research Products) membranes by vacuum transfer using the LKB 2016 VacuGene Vacuum Blotting System (Pharmacia LKB Biotechnology). The transfer procedure consists of 15 minutes depurination (0.2N HCl), 20 minutes of denaturation (1.5M NaCl, 0.5M NaOH), 20 minutes of neutralization (1M Tris.Cl pH 5.0, 2M NaCl), and 1 hour of transfer (20 X SSC - 3M NaCl, 0.3M disodium citrate), under a constant vacuum pressure of 50 cm.H2O.

After the transfer, the membranes were air-dried at RT for at least 12 hours or at 37°C for at least 1 hour. Prior to hybridization, the membranes were briefly soaked in 2 X SSC, and prehybridized in 50% deionized formamide, 1% ultra pure SDS, 10% dextran sulfate, and 1M NaCl at 42°C for 30 minutes to 2 hours. The hybridization solution was identical with the addition of denatured 100ug/ml sheared salmon sperm DNA and ftsZ probe at a specific activity of 1.5 X 10^5 cpm/ml. Following
hybridization at 42°C for 16 to 24 hours, the membranes were washed in 2XSSC at RT, for 10 minutes, twice. The membranes were then exposed to X-ray film at -70°C.

Subcloning the N-terminal coding and upstream region of ftsZ, and the partial ftsZ gene into plasmid vectors and transformation of JM101

To subclone the N-terminal coding and upstream region, as well as the partial coding region of ftsZ, fragments from the recombinant phage clones and amplification products thought to contain the ftsZ regions of interest, were gel purified and mixed with linearized pBluescript II SK+ and pBR322 with compatible ends. In the case of the vector having compatible ends, these were initially dephosphorylated using calf intestinal alkaline phosphatase to prevent self-ligation and promote ligation with inserts. Ligation reactions contained insert to vector mol ratios of 3:1, 2:1, 1:1, and 1:2. Appropriate negative and positive controls for the ligation reactions were also included. Negative controls include the ligation reactions without insert, and either with (+) or without (-) ligase: single or double RE-digested vector (-) ligase (to ensure that each RE completely digested the vector), single RE-digested, dephosphorylated vectors (+) ligase (to ensure complete dephosphorylation), for double RE-digested vectors (+) ligase (to ensure both REs completely digested the vector). Positive controls include the ligation reactions without insert and with single RE-digested, linearized plasmid (+) ligase (to ensure enzyme activity and optimal conditions for ligase activity).

In identical ligation reactions, the partial ftsZ PCR products were digested with RE's and mixed with similarly digested pGEX 2T to subclone the partial ftsZ gene in-frame with the glutathione-S-transferase (GST) gene.

The ligation reactions consisted of buffer (50mM Tris.Cl pH7.6, 10mM MgCl₂, 1mM ATP, 5%w/v
polyethylene glycol-8000), and 1U T4 DNA ligase (Gibco BRL Life Technologies), in addition to the vector and insert DNA. Reactions were carried out at 15°C for 12 to 16 hours, and terminated by heat inactivation at 65°C for 20 minutes.

**PCR to confirm subcloning reactions success and amplification of encoding N-terminus and upstream region of ftsZ for sequencing**

Since the fragments were inserted into pBluescript II SK+, primers that targeted the vector and flanking the multiple cloning site were paired with the F2 primer that targeted ftsZ and used in PCRs to determine whether the insert was subcloned. The PCR products of the recombinant clone containing the N-terminal coding and upstream region of ftsZ were gel purified and used as a template for DNA sequencing.

In the 50ul PCR, 1/10 of each ligation reaction was used as the template with 10 pmoles of pBluescript T7 primer and FG6 (T7-FG6), or pBluescript Reverse primer and FG6 (R-FG6). The other components of the PCR included 10mM dGTP, 10mM dATP, 10mM dTTP, 10mM dCTP, 1.5mM MgCl₂, amplification buffer (10mM Tris.Cl, 50mM KCl, and 0.01% gelatin, pH8.3), and 2.5U Taq DNA polymerase (Boehringer Mannheim). The PCRs for both primer combinations included the initial melting at 95°C for 3.5 minutes, followed by 30 cycles of 95°C melting for 1.5 minutes, annealing temperatures of 42°C for T7-FG6 and 45°C for R-FG6, and extension at 72°C for 1.5 minutes, and completed by a final extension at 72°C for 10 minutes.
Method to prepare competent *E. coli* JM101 cells and transformation

The rubidium chloride and calcium chloride method (Sambrook *et al*., 1982) was used to prepare competent *E. coli* cells. The competent cells were stored at 4°C for up to 2 days. After the ligation reactions were terminated, the reactions were diluted 5 X in T10E1 (10mM Tris.Cl, 1mM EDTA pH8.0), mixed with competent cells, and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds and immediately placed on ice. LB+amp broth was added to the mix, which was incubated at 37°C for 1 hour. For each transformation, two controls were included: a positive control to determine if the cells were competent (using the same amount of vector used in the ligation reactions), and a negative control (using H2O or diluted ligation mix without the DNA) to ensure that the selective plates were effective and there are no contaminating bacteria present.

Following the 37°C incubation, the mixtures were plated on LB+amp plates. In addition, for colour selection, the plates were supplemented with isopropyl-1-thio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) to differentiate colonies harboring recombinant and non-recombinant pBluescript II SK+ plasmids. A known pBluescript II SK+ recombinant clone was also included as a positive control. After overnight growth of the plated transformants at 37°C, the plates were incubated at 4°C to enhance the difference between the blue colonies which contain non-recombinant versus the white colonies which contain the recombinant pBluescript II SK+ plasmids.

Sequencing the complete and upstream region of *ftsZ*

pBXS6 and the PCR products containing the N-terminal coding and upstream region of *ftsZ* were sequenced by the Sanger dideoxy sequencing method. The Sequenase™ Version 2.0 DNA Sequencing kit (United States Biochemicals) with α-35S-dATP and the Cycle Sequencing kit
(Pharmacia Biotech) with $\alpha^{32}$P-dATP, were used according to the manufacturer's recommendations. Prior to electrophoresis, the sequencing reactions were heated at 80°C (Sequenase reactions) and 100°C (cycle sequencing reactions) for 5 minutes.

The sequencing products were then resolved by electrophoreses in a 6% sequencing polyacrylamide (PA, for 100ml: 42g urea, 19ml 30% acrylamide, 15ml 2%bis-acrylamide, 20ml 5X TBE [per L: 54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA pH8.0], and H$_2$O to 100ml) gel for the appropriate length of time to obtain the ftsZ and upstream sequences. After electrophoresis, the gel was washed in a solution of 10% acetic acid and 10% methanol, transferred onto 3mM Whatman filter paper, and dried at 80°C for 1 hour. Dried sequencing gels were exposed to X-ray film at RT or -70°C, for either $^{35}$S- or $^{32}$P-containing gels, respectively, for visualization.

**Preparation of total RNA**

Prior to all RNA work, all H$_2$O was treated with diethyl pyrocarbonate (DEPC-H$_2$O), plasticware was autoclaved or single-use and disposable, glassware was baked at 150°C for at least 12 hours, and electrophoreses apparatus were treated with 3% hydrogen peroxide for 30 minutes and rinsed with DEPC-H$_2$O.

*Clostridium jejuni* total RNA used in the primer extension reaction was extracted using the hot phenol method as follows (Aiba, *et al.*, 1981). A broth culture was centrifuged at 5000rpm, 4°C for 10 minutes. The cell pellet was resuspended in a lysis solution of 0.02M sodium acetate pH5.5, 0.5% SDS, and 1mM EDTA pH8.0. Total RNA was extracted by adding an equal volume of hot phenol (equilibrated in 0.02M sodium acetate pH5.5) to the cell suspension. This was mixed at 60°C for 5 minutes, followed by centrifugation at 10000rpm, RT for 2 minutes. The upper aqueous phase containing total RNA
was transferred to a fresh tube and was subjected to two more rounds of similar extraction. Finally, the total RNA was precipitated with 0.3M sodium acetate pH5.5 and 3 volumes of 100% ethanol, incubated at -70°C for at least 1 hour. Then, the RNA solutions were centrifuged at 13000rpm, 4°C for 30 minutes. The RNA pellets were washed briefly with 75% ethanol and air dried at RT. Each pellet was resuspended in DEPC-H2O and the total RNA content and purity was estimated (at OD260, OD280 and their ratio of OD260/280) using a spectrophotometer. Samples were stored in aliquots of 10ug and 25ug of total RNA in the sodium acetate and ethanol solutions at -70°C.

Total RNA samples from time points throughout the C. jejuni growth cycle were extracted using the RNeasy® kit (QIAGEN Inc.) according to the manufacturer’s recommendations. The quantity and purity, aliquots, and sample storage were identical to those for RNA samples extracted using the hot phenol method.

**Primer extension analyses**

Prior to its use in the primer extension reaction, each of the purified labelled oligos (F1 and PE-2) were diluted to a concentration of 1ng/ul. Several primer extension reactions were done, each containing different ratios of total RNA and primer (ug): 25:0.5, 25:1, 25:3, and 75:1. In a 10ul volume, the primers were annealed to their target RNA in a hybridization mix consisting of 5mM Tris.Cl pH8.3, 100mM potassium chloride, primer, total RNA, and ~15U RNAguard ribonuclease inhibitor (Pharmacia Biotech). The hybridization mix was incubated at 75 - 80°C for 3 minutes, followed by a brief centrifugation at 10000rpm, and an incubation at 41°C for 75 minutes to allow primer annealing. Then, 24ul of extension mix containing 10mM Tris.Cl pH8.3, 1.4mM magnesium chloride, 14mM dithiothreitol, 0.7mM dGTP, 0.7mM dATP, 0.7mM dTTP, and 0.7mM dCTP, and 1ul (~12U) of AMV reverse transcriptase (Pharmacia Biotech) was added to the hybridization mix.
Each primer extension reaction was incubated at 41°C for 1 hour. The primer extension products were precipitated in 0.3mM sodium acetate pH5.5 and 3 volumes of 100% ethanol at -70°C for at least 30 minutes. Finally, an aliquot of 1/3 of each reaction was resolved on a 6% sequencing PA gel adjacent to sequencing reactions using the corresponding primers.

**Northern blot and hybridizations**

*C. jejuni* total RNA samples from the time points 0, 10, 12, 18, 24, 36 and 48 hours after inoculation were resolved on 1.5% agarose/formaldehyde gels. The protocols and solutions used were as recommended by GeneScreen Plus® manual (DuPont NEN® Research Products). Northern transfer of the gels onto GeneScreen Plus® membranes was by capillary transfer in 10X SSPE (Sambrook *et al.*, 1982) and by vacuum transfer using the vacuum blotting apparatus LKB 2016 VacuGene Vacuum Blotting System (Pharmacia LKB Biotechnology). The molecular weight marker used was the RNA ladder from Gibco BRL, which was electrophoresed alongside each set of RNA samples.

Transcripts of *ftsZ*, *flgF*, and *flgG* were detected by Northern hybridizations: each set of RNA samples on a membrane was probed with either the HindIII fragment of *ftsZ*, a NsiI-HindIII fragment of *flgF*, and a BglII-ScaI fragment of *flgG* (Figure 4). The Northern hybridization protocols and solutions used were also as recommended by GeneScreen Plus® manual. Following the hybridizations and washings, the probed membranes were exposed to X-ray film at -70°C for an appropriate amount of time to visualize the resolved bands of the various RNA transcripts.
Protein overexpression, isolation and purification

Large amounts of purified partial FtsZ protein were prepared as follows. *E. coli* JM101 transformants of pGEX-ftsZ were grown in LB-amp broth at 37°C for 16 hours. An aliquot of this overnight culture was re-inoculated into fresh LB+amp broth at a 1/100 dilution and grown at 37°C for 2-3 hours into exponential phase. Subsequently, fusion protein overexpression was induced by adding 0.1mM IPTG and continuing growth at 37°C for 2-3 hours. The cells were pelleted by centrifugation at 5000rpm, 4°C for 15 minutes. Pellets were washed in ice cold 1 X phosphate buffered saline (PBS, 140mM NaCl, 2.7mM KCl, 10mM Na₂PO₄, 1.8mM KH₂PO₄, pH7.3) once and resuspended in 1 X PBS.

The cell suspension was passed through a French press twice at a pressure of 16000 lb/in² (pounds per square inch) to lyse the cells. The lysed cell suspension was centrifuged at 3000 rpm, 4°C for 15 minutes. Following separation from the pellet, the supernatant was re-centrifuged at 5000rpm, 4°C for 10 minutes. Both pellets were resuspended and pooled in ice cold 1 X PBS. At this point, an aliquot from the supernatant and pellet was kept for analysis by SDS-PAGE. Then, dithiothreitol (5mM DTT final concentration) and N-laurylsarcosine (1.5% sarkosyl final concentration) were added to the pellet suspension. The mixture was sonicated for 1 minute in a "cup horn" sonicated waterbath. The soluble (supernatant) and insoluble (pellet) fractions were separated by centrifugation at 7500 rpm, 4°C for 20 minutes. The fractions were separated, and at this stage, an aliquot from the soluble and insoluble fractions was kept as a test sample to be analysed by SDS-PAGE. Then, Triton X-100 (stock 10% in STE) was added to the soluble fraction at a final concentration of 2%. The mixture was then vortexed for 5 seconds. To purify the fusion protein from the other *E. coli* proteins, 50% v/v Glutathione bead suspension was added (20ul bead suspension per ml of sample) to the rest of the soluble suspension. Altogether, 7 washes were
performed using ice cold 1 X PBS according to the manufacturer's recommendations to obtain the purified fusion protein. An aliquot of the final resuspension of GST-FtsZ fusion protein bound to the beads was also taken to be analyzed for the purity of the washes and isolation by SDS-PAGE. Finally, the partial \textit{ftsZ} was cleaved from the GST portion of the fusion protein using \( \sim 4 \text{CU} \) (cleavage units) thrombin per ml 1 X PBS suspension (\( \sim 50 \text{ug} \) GST-FtsZ) at RT for 6-16 hours. Centrifugation at 5000 rpm, \( 4^\circ \text{C} \) for 15 minutes separated the partial FtsZ which was released into the supernatant, away from the GST moiety that was bound to the glutathione beads. An aliquot from both fractions was taken to be analyzed by SDS-PAGE so as to determine whether all the fusion protein was cleaved.

All protein samples taken during the isolation and purification process were analyzed by 0.1% stacking-10% separating SDS-PA gel electrophoresis as previously described (Ausubel \textit{et al.}, 1989; Laemmli, 1970). The expression levels of the fusion protein, whether it was in the soluble or insoluble cell fractions, the efficiency of sarkosyl solubilization, and the size and quality of the purified fusion and cleaved partial FtsZ proteins were determined.
Table 3. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni ATCC 43431</td>
<td>TGH 9011, serotype reference strain for O:3</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>E. coli JM101</td>
<td>▲(lac-pro) thi rpsL supE endA sbcB hsdR F’[ traD36 proAB lacR Z▲M15 ]</td>
<td>Yanisch-Perron, 1985</td>
</tr>
<tr>
<td>E. coli LE392</td>
<td>F, hsdR574, (r_, m_), supE44, supF58, lacY, or ▲(lacI/zy)6, galK2, galT22, metB1, trpR55</td>
<td>Murray, 1977</td>
</tr>
<tr>
<td>pBR322</td>
<td>4.361 kb, AmpR, TetR, cloning vector</td>
<td>Bolivar, et al., 1977</td>
</tr>
<tr>
<td>pBluescript II SK+</td>
<td>2.961 kb, AmpR, lacZα, cloning vector</td>
<td>Strategene</td>
</tr>
<tr>
<td>pGEX 2T</td>
<td>4.948 kb, AmpR, GST gene fusion vector</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pBXS6</td>
<td>pBluescript II SK+ clone containing flicFG and the partial ftsZ gene from C. jejuni</td>
<td>This study</td>
</tr>
<tr>
<td>1.0.2</td>
<td>HindIII fragment from pBXS6 containing the partial ftsZ gene</td>
<td>This study</td>
</tr>
<tr>
<td>1.2.8</td>
<td>HindIII fragment from pBXS6 containing the flicF gene</td>
<td>This study</td>
</tr>
<tr>
<td>1.7.1</td>
<td>HindIII fragment from pBXS6 containing the flicG gene</td>
<td>This study</td>
</tr>
<tr>
<td>pGEX-ftsZ</td>
<td>The partial ftsZ gene fused at the 3’ end, in-frame with GST</td>
<td>This study</td>
</tr>
<tr>
<td>Lambda-ftsZ1</td>
<td>C. jejuni lambda genomic clone containing the complete ftsZ gene</td>
<td>This study</td>
</tr>
<tr>
<td>Lambda-ftsZ2</td>
<td>C. jejuni lambda genomic clone containing the complete ftsZ gene</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 4. Oligonucleotides designed and used in this study.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence (5’ - 3’)</th>
<th>Use in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>GCA TAC CAG CGC CCA AAC C</td>
<td>library PCR strategy, ftsZ sequencing</td>
</tr>
<tr>
<td>F2</td>
<td>CCA ATC TAA AAG CAT CTT TAA TGC CTG CT</td>
<td>library PCR strategy, ftsZ sequencing</td>
</tr>
<tr>
<td>F4</td>
<td>AGG TTT GGC CGC TGG TAT GCT TCC TG</td>
<td>ftsZ sequencing</td>
</tr>
<tr>
<td>F6</td>
<td>AGC AGG CAT TAA AGA TGC TTT TAG ATT GG</td>
<td>ftsZ sequencing</td>
</tr>
<tr>
<td>F7</td>
<td>GCT ATA GAA GAG GCT CTT TCA AAC G</td>
<td>ftsZ sequencing</td>
</tr>
<tr>
<td>F8</td>
<td>GAA GTA ACT ATT ATT GCG ACA GGG</td>
<td>ftsZ sequencing</td>
</tr>
<tr>
<td>F9</td>
<td>CTT TCT TGC GTC GTC AAA TGG A</td>
<td>ftsZ sequencing</td>
</tr>
<tr>
<td>FG4</td>
<td>CCA TTG ACG ACG CAA G</td>
<td>ftsZ sequencing</td>
</tr>
<tr>
<td>FG5</td>
<td>CTT GTA TAC TAT TTT CTG CTG CCG</td>
<td>ftsZ sequencing</td>
</tr>
<tr>
<td>FG6</td>
<td>CAA GTA AGA TAG ACA CCA TAC C</td>
<td>ftsZ sequencing, PCRs to check ftsZ subclones</td>
</tr>
<tr>
<td>FG7</td>
<td>GCT TGT GCA ATA ACT GGA GTG GCA CCC G</td>
<td>ftsZ sequencing</td>
</tr>
<tr>
<td>PCRF3</td>
<td>GAG GAT CCA TTG CAC AAG CAG C</td>
<td>amplify ftsZ to subclone into pGEX 2T</td>
</tr>
<tr>
<td>PCRF4</td>
<td>CAA TGA ATT CCG CCA GTT GC</td>
<td>amplify ftsZ to subclone into pGEX 2T</td>
</tr>
<tr>
<td>PE-1</td>
<td>CGG GAT CCG AAA TAG CTT GTG CAT CAG TAT TCG CTG</td>
<td>ftsZ sequencing, primer extension analyses</td>
</tr>
<tr>
<td>BamH1A</td>
<td>ATG CGT CCG GCG TAG A</td>
<td>pBR322 primer in library PCR strategy</td>
</tr>
<tr>
<td>BamH1B</td>
<td>TAC TTG GAG CCA CTA TCG ACT ACG CTA</td>
<td>pBR322 primer in library PCR strategy</td>
</tr>
<tr>
<td>T7</td>
<td>GGC CTA ATA CGA CTC ACT ATA G</td>
<td>pBluescript II SK+ primer in PCRs to check ftsZ subclones</td>
</tr>
<tr>
<td>Reverse</td>
<td>AAC AGC TAT GAC CAT G</td>
<td>pBluescript II SK+ primer in PCRs to check ftsZ subclones</td>
</tr>
</tbody>
</table>
Plasmid pBXS6 is a recombinant pBluescript II SK+ clone containing a ~4.8kb region of the C. jejuni genome inserted in its BamH1 and Xba1 sites. The ftsZ gene was identified on this clone while sequencing upstream of the flgF gene (Chan et al., unpublished data). The insert encompasses the partial ftsZ gene and downstream flgF and flgG genes.

The following conventions are used:

- vector sequences: solid cages,
- genomic sequences: thin black lines, and
- genes: shaded cages.
Figure 5. Schematic representation of the locations of oligos within pBXS6 used in this study. The sequence and purpose of each oligo in this study are listed in Table 4.
Figure 6. PCR strategy to amplify fragments from any recombinant clones containing the N-terminal coding and upstream regions of *ftsZ* within the unamplified *C. jejuni* pBR322 DNA library.

In this strategy, F2 primer from within *ftsZ* is paired with either pBR322 BamHI A or pBR322 BamHI B primers in two separate PCRs. Fragments containing *ftsZ* sequences are subsequently detected with the F1 oligo probe via Southern blot hybridization. Amplification products containing the identical partial *ftsZ* fragment as pBXS6 are of expected sizes, 502bp from F2-BamHI A and 516bp from F2-BamHI B. Thus, larger products should contain the N-terminal coding and upstream *ftsZ* regions.
RESULTS

*C. jejuni* ATCC 43431 growth curve in Mueller Hinton broth

In order to study the modes of cell division from *C. jejuni* log and stationary phase cells and to study *ftsZ* expression throughout the growth phases, three series of growth curves were plotted. Based on these growth curves, log and stationary phase cells were isolated and examined by TEM. The final, Series 4, growth curve represents the culture from which total RNA was extracted at different time points. The values and plots of the growth curves are shown in Table 5 and Figure 7, respectively.

Morphology and modes of *C. jejuni* cell division in log and stationary phase cultures

The morphology of *C. jejuni* cells observed by TEM are shown in Figure 8, plate a. These figures show the several angles at which a cell can be viewed by TEM: cross-sectioned (cells appear coccoid), longitudinally-sectioned (spiral morphology of *C. jejuni* cells is revealed), and irregularly-sectioned (cells appear in several shapes representing the angle at which each cell is sectioned). Due to the various shapes that are observed by TEM, the exact position of a septum within a dividing cell cannot be determined. Thus, dividing cells that were enumerated and involved in the statistical analyses were placed in two categories: (i) a cell is considered dividing symmetrically if both daughter cells are longer than coccoid or minicells, regardless of whether none, one or both poles are visible; and (ii) a cell is considered dividing asymmetrically if one daughter cell is coccoid, regardless of whether one or both poles are visible. In addition, the assumption was made that the same number of false coccoid (cross-sectioned) daughter cells are observed from both log and stationary phase cultures. Several stages of symmetrical cell division are shown in Figure 8, plate b. In contrast, asymmetrical cell divisions are shown in Figure 8, plate c. The electron micrographs also
demonstrate that both symmetrical and asymmetrical cell divisions occur with the coordinate invagination of the envelope layers.

Among approximately 3000 C. jejuni cells from log and stationary phase cultures, 100 dividing cells were enumerated. In log cultures, symmetrical division was observed from 20 cells which had centrally located invaginations with respect to the visible cell poles, while symmetrical division was assumed in 79 cells in which only one or none of the poles were visible, but both daughter cells were longer than coccoid or minicells. Asymmetrical division was observed in 1 cell in which one daughter cell was coccoid and one daughter cell was at least three times the length of the minicell (the pole was not visible). In stationary cultures, symmetrical division was assumed in 5 cells in which the both daughter cells were longer than coccoid cells, while asymmetrical division was observed in 95 cells in which one daughter cell was of coccoid morphology and one daughter cell was at least 3 times the length of the coccoid cell (Table 6). These results were grouped in three tables to be used in the appropriate $X^2$ tests of the three $H_0$ (Table 7).

The following calculations were used to test the $H_0$:

(i) \[ X^2_{0.05} = \left( \frac{99-50}{2} \right)^2 \left( \frac{50}{2} \right) + \left( \frac{1-50}{2} \right)^2 \left( \frac{50}{2} \right) \]

\[ \equiv 96.04 \]

Compared to the critical value of $X^2_{0.05}$ (2-1 df) = $X^2_{0.05}$ (1 df) = 3.841, the $X^2_{0.05}$ of the sample is greater than the critical value, thus the $H_0$ is rejected. Therefore, the sample proportion of 0.99 is statistically significant, and may represent the true proportion (the general proportion) of symmetrically dividing cells within log cultures. The 95% confidence limits for the true proportion of symmetrical cell division in log cultures (based on the sample proportion
calculation) is

\[ sp \pm (z_{0.02})(\sigma_p) = 0.99 \pm (1.96) [(0.99 \times 0.01)+100]^{0.5} = 0.99 \pm 0.02. \]

Therefore, with 95% confidence, the *true proportion* of symmetrically dividing cells in log cultures is between 97% and 100%.

(ii) \[ X^2_{0.05} = [(5-50)^2 + 50] + [(95-50)^2 + 50] \]

\[ = 81 \]

Compared to the critical value of \( X^2_{0.05} (2-1 \text{ df}) = X^2_{0.05} (1 \text{ df}) = 3.841, \)

the \( X^2_{0.05} \) of the sample is greater than the critical value, thus the \( H_0 \) is rejected. Therefore, the sample proportion of 0.95 is statistically significant, and may represent the *true proportion* of asymmetrically dividing cells within stationary cultures. The 95% confidence limits for the *true proportion* of symmetrical cell division in stationary cultures is

\[ sp \pm (z_{0.02})(\sigma_p) = 0.95 \pm (1.96) [(0.95 \times 0.05)+100]^{0.5} = 0.95 \pm 0.04. \]

Therefore, with 95% confidence, the *true proportion* of asymmetrically dividing cells in stationary cultures is between 91% and 99%.

(iii) \[ X^2_{0.05} = [(99-52)^2 + 52] + [(5-52)^2 + 52] + [(1-48)^2 + 48] + [(95-48)^2 + 48] \]

\[ \equiv 177 \]

Compared to the critical value of \( X^2_{0.05} ( [2-1][2-1] \text{ df}) = X^2_{0.05} (1 \text{ df}) = 3.841, \)

the \( X^2_{0.05} \) of the sample is greater than the critical value, thus the \( H_0 \) is rejected. This indicates that the difference between the proportions of symmetrical and asymmetrical divisions within log and stationary phase samples are statistically significant. **Therefore, there is a difference in the predominant modes of division in log and stationary phase cultures.**
Characterization of the pBXS6 recombinant clone

Figure 4 shows the RE map of the pBXS6 recombinant clone containing the partial ftsZ gene. I determined the complete sequence of the partial ftsZ fragment from pBXS6 and used the sequence to design primers for the pBR322 library PCR strategy, amplification of the partial ftsZ gene to be subcloned into the pGEX 2T fusion vector system, and checking of subcloning reactions (Figure 5). Based on the RE sites and nucleotide sequences (flgF and flgG sequences were previously determined, Chan et al., unpublished data), I isolated and purified the partial ftsZ, flgF, and flgG genes from 1.0.2, 1.2.8, and 1.7.1 (Figure 4), respectively, to be used as probes in subsequent screening and hybridization experiments. The HindIII ftsZ fragment of 1.0.2 was used as a probe to detect ftsZ sequences and transcripts. The NsiI-HindIII flgF fragment of 1.2.8 and ScaI-BglII flgG fragment of 1.7.1, were used to detect flgF and flgG transcripts, respectively.

Isolation and characterization of the complete C. jejuni ftsZ gene

The pBR322 library PCR strategy was used to amplify fragments containing the N-terminal coding and upstream ftsZ regions. The F2-BamHI A and F2-BamHI B primer combinations generated PCR products ranging from approximately 0.2 to 2.0 kb in length (Figure 9). PCR products in the range of 1kb, which possibly contained the N-terminal coding and upstream ftsZ regions, were identified by Southern blot analysis (Figure 10). However, although attempts were made to subclone these fragments, no E. coli transformants were isolated following the ligation reactions.

Alternatively, several recombinant clones were identified and isolated from the pBR322 and Lambda Gem 11 genomic library. The RE profiles and Southern blots (probed with the HindIII ftsZ fragment of 1.0.2) of these clones were characterized and compared to pBXS6, respectively. All the pBR322
recombinant clones contained identical partial \textit{ftsZ} fragments (data not shown). However, Southern blot analyses of two recombinant Lambda Gem 11 clones (subsequently named Lambda-\textit{ftsZ}1 and Lambda-\textit{ftsZ}2) revealed an internal and identical \textasciitilde1.5kb \textit{Hind}III fragment containing \textit{ftsZ} (Figures 11 and 12). When compared to the pBXS6 \textit{ftsZ} fragment, the size of the \textit{ftsZ} fragment of Lambda-\textit{ftsZ}1 and Lambda-\textit{ftsZ}2 suggests that they contain the complete coding and upstream regulatory regions of \textit{ftsZ}.

The \textasciitilde1.5kb \textit{ftsZ} \textit{Hind}III fragment of Lambda-\textit{ftsZ}1 was subcloned into pBluescript II SK+, as evidenced by the PCR reactions (Figure 13). However again, no transformants were obtained following ligation reactions. Therefore, these PCR fragments were used as templates in subsequent sequencing reactions to obtain the complete nucleotide sequence of the \textit{ftsZ} coding and upstream regulatory regions (Figure 14). Since these were PCR products, sequences were obtained from products of several PCRs. The sequences were compared to ensure that there were no errors introduced by Taq polymerase during the PCRs. The FtsZ amino acid sequences were deduced and compared to those of the \textit{E. coli} and \textit{C. crescentus} FtsZ (Figure 15).

Four preparations of \textit{C. jejuni} total RNA were used with the FG6 oligo in four primer extension reactions. The primer extension analyses revealed that \textit{C. jejuni} \textit{ftsZ} is transcribed from three start sites (Figures 14 and 16). Based on the three start sites that were mapped, two putative promoters were identified (Figure 14). The relative level of each primer extension product indicates that the product with its start site furthest upstream of the putative start methionine codon is most abundant.

**Transcription of \textit{ftsZ}, \textit{flgF}, and \textit{flgG} throughout the \textit{C. jejuni} growth phases**

Total RNA of \textit{C. jejuni} was extracted from broth cultures at different time points, which correspond
to different growth phases (as shown in Figure 7). Equal amounts of total RNA extracted from each
time point was resolved on two separate 1.5% agarose/formaldehyde gels (Figure 17). Northern blot
hybridizations (Figure 18) revealed that \textit{ftsZ} is expressed on a \textasciitilde{}1.3kb transcript, \textit{flgF} on a \textasciitilde{}1.7kb
transcript, and \textit{flgG} on two transcripts, \textasciitilde{}0.9kb and \textasciitilde{}1.8kb. The \textit{ftsZ} transcript remains relatively
constant throughout log phase, but starts to decrease upon entry into stationary phase. In contrast,
the relative levels of \textit{flgF} \textasciitilde{}1.7kb, and \textit{flgG} \textasciitilde{}0.9kb and \textasciitilde{}1.8kb transcripts fluctuate throughout the
growth phases. The levels of each transcript increase to a peak at mid-log phase (10 hours), then
decrease, then increase again to another peak at entry into stationary phase (24 hours), and eventually
decrease as the culture progresses into stationary phase.

Expression and purification of the GST-\textit{FtsZ} fusion and partial \textit{FtsZ} proteins

The pGEX-\textit{ftsZ} clone was initially sequenced to determine the correct in-frame insertion of the
partial \textit{ftsZ} gene downstream of the GST gene. Expression of the GST-\textit{FtsZ} fusion protein was
induced by IPTG (Figure 19), and following cell lysis, was found in the insoluble fraction (Figure
20). The fusion protein was subsequently solubilized by sarkosyl and Triton X-100, and purified
(Figures 21 and 22). Finally, thrombin cleavage and centrifugation was used to purify the partial
\textit{FtsZ} protein (Figure 22).
Table 5. Titres of *C. jejuni* ATCC 43431 cells grown at 37°C in Mueller Hinton broth. The colony forming units per ml (CFU/ml) at each time point are the averaged values of colony counts from duplicates of three dilutions.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Titre (X10^6 CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Series 1</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>6.8</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>39.5</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>27.5</td>
</tr>
<tr>
<td>36</td>
<td>23.5</td>
</tr>
<tr>
<td>41</td>
<td>76.8</td>
</tr>
<tr>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td>49</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 7. *C. jejuni* ATCC 43431 growth curve at 37°C in Mueller Hinton broth.

Series 1 to 4 growth curves are plotted according to the values shown in Table 2. Total RNA was extracted from time points 0, 10, 12, 18, 24, 36, and 48 hours from the Series 4 growth curve.
Figure 8. TEM of *C. jejuni* ATCC 43431 cells at various stages of the bacterial cell cycle observed from log and stationary phase cultures. Cell division was observed to occur with the coordinated ingrowth of the cell envelope layers.

Plate a. Morphology of *C. jejuni* cells sectioned at different angles.

Plate b. Symmetrical cell division.

Plate c. Asymmetrical cell division giving rise to coccoid or minicells.
Plate b (ii).
Table 6. Counts of *C. jejuni* ATCC 43431 dividing cells.

<table>
<thead>
<tr>
<th></th>
<th>Log phase divisions</th>
<th>Stationary phase divisions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symmetric</td>
<td>Asymmetric</td>
</tr>
<tr>
<td>Visible poles</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Number of cells</td>
<td>20</td>
<td>46</td>
</tr>
<tr>
<td>Number of cells</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 7. Grouping of *C. jejuni* cell division results to be subjected to the $X^2$ test. The expected number of units per category ($e$) are in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>Cell divisions during log phase</th>
<th></th>
<th>Cell divisions during stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Symmetric</td>
<td>Asymmetric</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asymmetric</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>Total ($n =$)</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

b. Cell divisions during stationary phase

<table>
<thead>
<tr>
<th></th>
<th>Symmetric</th>
<th>Asymmetric</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>(50)</td>
<td>(50)</td>
</tr>
<tr>
<td>Total ($n =$)</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

c. Cell divisions during log phase | Cell divisions during stationary phase

<table>
<thead>
<tr>
<th></th>
<th>Symmetric</th>
<th>Asymmetric</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>99</td>
<td>1</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>(52)</td>
<td>(48)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>95</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>(52)</td>
<td>(48)</td>
<td></td>
</tr>
<tr>
<td>Total ($n =$)</td>
<td>100</td>
<td>100</td>
<td>200</td>
</tr>
</tbody>
</table>
Figure 9. Amplification products of the PCR strategy using the *C. jejuni* pBR322 DNA library.

F2-BamH1 A PCR products were resolved in a 1% agarose gel and visualized by ethidium bromide staining illuminated under UV light.

Lane m: molecular weight markers;
Lane 1: no DNA - PCR negative control;
Lanes 2, 3, 4, 5: PCRs using 1, 2, 3, and 4 mM MgCl₂, respectively;
Lanes 6, 7: pBR322 and pBluescript II SK+ DNA - negative controls for hybridization; and
Lane 8: pBXS6 DNA - positive control for hybridization.

Lane 8: pBXS6 DNA - positive control for hybridization.
Figure 10. Southern blot analyses of the products from the PCR strategy using the C. jejuni pBR322 DNA library.

The F1 oligo was used as a probe in the Southern blot hybridization and the lanes correspond to those of Figure 9.

expected size for identical \textit{ftsZ} fragment
Figure 11. Agarose gel electrophoresis analyses of RE-digested recombinant lambda clones isolated from the Lambda Gem 11 library plaque screens.

DNA in odd-numbered lanes were digested with HindIII, while DNA in even-numbered lanes were digested with SalI and HindIII.

Lanes 1, 2: Lambda-ftsZ1
Lanes 3, 4: Lambda 2
Lanes 5, 6: Lambda 3
Lanes 7, 8: Lambda 4
Lanes 9, 10: Lambda 5
Lane m: HindIII-digested Lambda DNA - molecular weight marker;
Lanes 11, 12: Lambda-ftsZ2
Lanes 13, 14: Lambda 7
Figure 12. Southern blot analyses of the RE-digested recombinant lambda clones.

The *HindIII* fragment containing the partial *ftsZ* of PBXS6 was used as a probe in the Southern blot hybridization. An ~1.5kb *HindIII* fragment was identified in both Lambda-ftsZ1 and Lambda-ftsZ2, and according to the RE profiles (data not shown) it is an internal fragment. Thus, both these clones contain the complete genomic *ftsZ* gene and additional upstream regions. The lanes correspond to those of Figure 11.
Figure 13. Agarose gel electrophoresis analyses of the PCR products to confirm the subcloning of the ~1.5kb *Hind*III *ftsZ* fragment of Lambda-*ftsZ* into pBluescript II SK+.

Since the *Hind*III *ftsZ* fragment can be inserted in two orientations, a combination of the FG6-Reverse in one set of PCRs and FG6-T7 primers in another set of PCRs were used. In all the PCRs, an ~1.1kb product was generated.

Lane 1: no DNA - PCR negative control;
Lanes 2, 4, 6, 8: FG6-Reverse primer products generated from ligations in which the vector was dephosphorylated with 0.2U, 0.5U, 1U, and 0U of alkaline phosphatase, respectively;
Lanes 3, 5, 7, 9: FG6-T7 primer products generated from ligations in which the vector was dephosphorylated with 0.2U, 0.5U, 1U, and 0U of alkaline phosphatase, respectively;
Lane 10: 100bp ladder (Pharmacia) - molecular weight marker;
Lane 11: *Hind*III-digested Lambda DNA - molecular weight marker.
Figure 14. Nucleotide and deduced amino acid sequences of the *C. jejuni* *ftsZ* gene.

The following designations are used to describe various features within the gene:
- Met and TAA indicate the methionine start and stop codons, respectively
- ▼ indicates a transcription start site
- SD indicates the putative Shine-Dalgarno sequence
- underlined sequences are the putative -10 and -35 promoter sequences
- underlined arrowhead sequences are inverted repeats
- bolded amino acid sequences are the conserved domains found in FtsZ proteins
AGC GGT GAA AAT GCT ATA GAA GAG GCT CTT TCA AAC GCT ATA GAA TCT CCT TTG CTT GAT GGA ATG GAT ATT AAA
SGENAIEEALESNAIESPLLDGMDIK
GGT GCC AAG GGA GTT ATT TTA CAT TTT AAA ACA AGT TCT AAT TGC TCT TTG TTT GAA ATT TCG GCA GCA GCA AAT
GAGAVIKIHFKTSNSCSSLFEIISAAN
AGT ATA CAA GAA ATT GTT GAT GAA AAT GCT AAG ATT ATT TTT GGT TCT ACC ACA GAT GAT AGC ATG GAA GAT AGA
SIIQELVIDENAKIKIFFGSGSTTDSDMEDR
GGT GAA GTA ACT ATT ATT GCG ACA GGG TTT GAA GAT AAA GAT ACA GTA GCT AAA AAA TCT ACC GAA GAA GCT CAA
VEVTIIAATGFGEDKDTVAKKSTEEAQ
GCT TCT AAG ACA AAT CCT TAT TTA AGT CTT AAA AAG GTA AGC GGT GGT TAC GAT GAA GAT ATT ACA AGC GGA ATT
ASKTPYLSSLKVKVSGGYDEEIMAQI
GAA ACA CCA ACT TTC TTG CGT CGT CAA ATG GAT TAA AAT TGA TTT TTA AAG CAA GAT TGA GTC AAA TCT TGC TTT
ETPTFLRRRQMD*Stop
TTA TAG TTT AAA ATT CTC TCC TAA TAA TCT TTT AAA TAT TTA TCT CTC TCT GTC TTT AAA AGT TGG
Figure 15. Alignment of the *C. jejuni* TGH 9011 (Cj), *E. coli* (Ec), and *C. crescentus* (Cc) FtsZ amino acid sequences.

The amino acid sequences were aligned using the Clustal V program. All five of the conserved domains of FtsZ are bolded.
Figure 16. Primer extension analysis of the *C. jejuni* ATCC 43431 *ftsZ* gene.

Using the PE-1 primer, three transcription start sites, P1, P2 and P3, were mapped. The P1 primer extension product appears to be present at a higher level than P2 and P3 products. The corresponding sequence generated using the PE-1 primer is also shown (lanes G, A, T, C).

Lanes 1, 2: primer extension reactions from total RNA of 30-hour cultures;
Lanes 3, 4: primer extension reactions from total RNA of 50-hour cultures.
Figure 17. Agarose-formaldehyde gel electrophoresis of *C. jejuni* total RNA extracted from cultures at different time points throughout the growth phases.

There is 4ug and 2ug of total RNA per lane, in gels (a) and (b), respectively. Lane designations for both (a) and (b) gels are identical.
Lane m: molecular weight marker;
Lane 1: total RNA extracted from the inoculum culture;
Lane 2: total RNA from 10 hour culture;
Lane 3: total RNA from 12 hour culture;
Lane 4: total RNA from 18 hour culture;
Lane 5: total RNA from 24 hour culture;
Lane 6: total RNA from 36 hour culture;
Lane 7: total RNA from 48 hour culture.
Figure 18. Northern blot analyses using *fisZ*, *flgF*, and *flgG* as probes.

Gel (a) in Figure 17 was transferred onto GeneScreen Plus membrane and probed with *fisZ* producing the resulting (a) autoradiograph. Duplicate gels of (b) in Figure 17 were similarly treated and probed with *flgF* and *flgG* producing the resulting (b) and (c) autoradiographs, respectively. Lane designations are identical to those in Figure 17.
Figure 19. SDS-PAGE analyses of *E. coli* JM101, transformants of pGEX 2T, and pGEX-ftsZ.

To confirm and distinguish the expression of the GST-FtsZ fusion protein, aliquots of cultures before and after IPTG induction were analysed. For each culture, two cell densities were lysed (low in odd-numbered lanes and high in even-numbered lanes), and resolved by SDS-PAGE. As compared to JM101 cells, JM101/pGEX 2T cells contain the ~26kDa GST protein and JM101/pGEX-FtsZ cells contain the ~54kDa GST-FtsZ fusion protein.

Lane m : molecular weight markers;
Lanes 1, 3 : uninduced JM101;
Lanes 2, 4 : induced JM101;
Lanes 5, 7 : uninduced JM101/pGEX 2T;
Lanes 6, 8 : induced JM101/pGEX 2T;
Lanes 9, 11 : uninduced JM101/pGEX-ftsZ;
Lanes 10, 12 : induced JM101/pGEX-ftsZ.
Figure 20. SDS-PAGE analysis revealing GST-FtsZ in the insoluble cell fraction.

Following cell lysis, soluble and insoluble fractions were separated by centrifugation. The GST-FtsZ fusion protein was detected in the insoluble fraction.

Lane m: molecular weight marker;
Lanes 1, 2: soluble cell fractions;
Lanes 3, 4: insoluble cell fractions.
Figure 21. SDS-PAGE analyses of GST-FtsZ solubilization.

Lysed cell suspensions were treated with different concentrations of sarkosyl and Triton X-100 to solubilize GST-FtsZ. Following treatment, the soluble and insoluble fractions were separated by centrifugation and analyzed. Although different treatments were used, similar amounts of residual GST-FtsZ remained in the insoluble fraction.

Lane m: molecular weight marker;
Lanes 1, 2, 3: insoluble fraction of induced JM101/pGEX-ftsZ treated with 1% sarkosyl + 1% Triton, 1.5% sarkosyl + 2% Triton, 2% sarkosyl + 3% Triton, respectively;
Lane 4: insoluble fraction of uninduced JM101/pGEX-ftsZ treated with 1.5% sarkosyl + 2% Triton;
Lanes 5, 6, 7: soluble fraction of induced JM101/pGEX-ftsZ treated with 1% sarkosyl + 1% Triton, 1.5% sarkosyl + 2% Triton, 2% sarkosyl + 3% Triton, respectively;
Lane 8: soluble fraction of uninduced JM101/pGEX-ftsZ treated with 1.5% sarkosyl + 2% Triton.
Figure 22. SDS-PAGE analyses of purification of GST-FtsZ, thrombin cleavage and release of purified partial FtsZ.

Following solubilization, GST-FtsZ was purified from other cell proteins by using the glutathione beads which specifically bind to the GST moiety. Then, the GST-FtsZ-beads were washed. Each wash was analyzed to ensure that the fusion protein remains bound to the beads and loss of the protein was minimal. Finally, the partial FtsZ was cleaved from the GST moiety and centrifuged to separate the GST-bound beads from the partial FtsZ.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>m</td>
<td>molecular weight markers;</td>
</tr>
<tr>
<td>1, 10</td>
<td>soluble fraction containing GST-FtsZ;</td>
</tr>
<tr>
<td>2-8</td>
<td>supernatant of washes following binding of GST-FtsZ to glutathione beads;</td>
</tr>
<tr>
<td>9, 11</td>
<td>final resuspension of GST-FtsZ bound to glutathione beads;</td>
</tr>
<tr>
<td>12</td>
<td>supernatant containing partial FtsZ following thrombin cleavage and centrifugation.</td>
</tr>
</tbody>
</table>

Lane m: molecular weight markers; Lanes 1, 10: soluble fraction containing GST-FtsZ; Lanes 2 through 8: supernatant of washes following binding of GST-FtsZ to glutathione beads; Lanes 9, 11: final resuspension of GST-FtsZ bound to glutathione beads; Lane 12: supernatant containing partial FtsZ following thrombin cleavage and centrifugation.
DISCUSSION

*C. jejuni* ATCC 43431 growth curve

According to the growth curves plotted under the current conditions (Figure 7), the average doubling time during log phase is ~2.7 hours. *C. jejuni* cultures enter mid-log phase after ~6 to 10 hours, and enter stationary phase after ~24 hours. Therefore, total RNA extracted at all time points in Series 4 (except at 6 hours), represents expression of genes during log, late log and stationary phases of the *C. jejuni* culture.

A previous study showed a similar growth curve of *C. jejuni* cultures in broth (Griffiths, 1993). In both the previous and this study, since the initial inoculum was transferred to an identical medium, thus no lag phase was observed, and the cultures entered mid-log phase after ~6.5 to 10 hours of growth, respectively. However, in this study the *C. jejuni* cultures enter stationary phase after ~24 hours at a titer of ~10^8 CFU/ml and a decline phase was observed after ~36 to 48 hours. The previous study indicated that *C. jejuni* cultures entered stationary phase after only ~11 hours at a titer of ~10^9 CFU/ml and started the decline phase after ~23.5 to 30 hours of growth. As previously mentioned, several factors may contribute to these differences. Griffiths (1993) used *C. jejuni* NCTC 11168 grown in Nutrient broth No. 2 (Oxoid CM67), while *C. jejuni* ATCC 43431 grown in Mueller Hinton broth was employed in the present study. In addition, while Griffiths (1993) incubated the cultures in a jar with 10% v/v H₂, 10% CO₂ in N₂ atmosphere (BOC Ltd.) which was shaken at 37°C, in this study, cultures were incubated in a chamber enriched with 5% CO₂ which was unshaken at 37°C. Thus, the differences in the strain, growth medium and atmosphere each could have contributed to the observed differences in the growth curves.
Mode of *C. jejuni* cell division

TEM is a method to study the morphology as well as the cellular structures and organization of a cell. However, processing such as fixing, dehydrating, and embedding the cells are steps that have been found to alter normal physiological morphologies and cause cell lysis. Therefore, to obtain a good representation of *C. jejuni* morphology, several methods and chemicals for fixing, dehydrating and embedding the cells were investigated (data not shown). The combination of treatments were optimized based on the observation of minimizing cell envelope distortion and cell lysis. Since previous studies have shown that *C. jejuni* cells are sensitive to oxygen levels, which promote coccoid formation, exposure to the atmosphere was also minimized during the processing of cells.

Besides processing, the different morphologies of a cell observed by TEM are also a consequence of the angles at which the cell is embedded with respect to the sectioning plane. Cells are cross-sectioned (c-sect), longitudinally-sectioned (l-sect), and irregularly-sectioned (i-sect) revealing a number of representations of its shape (as shown in Figure 23). For example, c-sect cells with their spherical appearance cannot be differentiated from the true coccoid or minicells. Furthermore, since *C. jejuni* are spirally-curved, parts of cells may curve in and out of the sectioning plane causing certain regions within these cells to be observed as in and out of focus, respectively. The calculation of cell length based on TEM would be inaccurate in consideration of several factors such as the infrequency of observing both cells poles and that i-sect cells can be mistaken as whole cells. This phenomenon also affects the determination of the exact position of the division point within the cell with respect to the poles. Thus, the division point is an estimation when viewed by TEM.
Figure 23. Schematic representations of spiral and coccoid *C. jejuni* cells that can be observed by TEM.

The orientation of the cells with respect to the sectioning plane are shown. These indicate that cell dimension measurements are not accurately shown in TEM due to the spiral shape of *C. jejuni* and various sectioning planes. In addition, false coccoid and false asymmetrical cell division can be observed. Thus, to analyze the log and stationary phase samples, it was assumed that the same number of false observations occur in both samples.
Since previous studies have shown that spiral-shaped cells predominate in log cultures while coccoid-shaped cells form the majority in stationary cultures, it remains to be determined whether there is a difference in the mode of cell division that occurs in different growth phases of cultures, or if coccoid cells arise from degeneration of the spiral cells.

Therefore, in this study, two modes of cell division have been defined:

(i) symmetrical cell division which includes all divisions forming rod-shaped cells longer than coccoid or minicells: these may result from divisions which occur precisely (constriction or division point exactly central/equidistant from the poles) or imprecisely (constriction not exactly central but still forming a spiral or curved rod morphology), and

(ii) asymmetric division which gives rise to coccoid or minicells.

Based on these definitions, cell types were distinguished and enumerated as follows: dividing cells with none, one or both poles visible and daughter cells longer than coccoid cells were considered symmetrically dividing, while dividing cells with one or both ends visible and one daughter cell having a coccoid morphology were considered asymmetrically dividing. These criteria were used to choose the 100 dividing cells that would be used to determine the mode of C. jejuni cell division in both log and stationary phase cultures. Based on the proportions of the different dividing cells within these samples, three $H_0$ were designed to obtain a statistically significant inference of the mode of C. jejuni cell division in log and stationary phase cultures. All three $H_0$ were rejected and indicated that the sample proportions of symmetrical divisions in log phase, asymmetrical divisions in stationary phase, and the difference between the proportions of symmetrical and asymmetrical divisions that occur in log and stationary phase are all statistically significant. Therefore, within 95% confidence limits, 97% to 100% of dividing cells within log cultures divide symmetrically, 91% to 99% of dividing cells within stationary cultures divide asymmetrically, and there is a statistically
significant difference in the mode of cell division employed by dividing cells depending on whether these cells are in log or stationary phase. Further studies should work to reveal the mechanism of regulation of symmetrical and asymmetrical cell division in log and stationary phase cultures.

Isolation of the complete *ftsZ* gene

Two strategies were used concurrently to isolate the complete *ftsZ* gene. In one strategy, the PCR was employed (Figure 6). Together with the unamplified pBR322 DNA library as template, a combination of primers from within *ftsZ* (F2) and pBR322 (BamHIA and BamHIB) were used to amplify fragments from recombinant clones containing the N-terminal coding and upstream regulatory region of *ftsZ*. Since non-specific priming by the F2 oligo could occur during the PCR, the F1 oligo was used as a probe in Southern blot hybridization to detect fragments containing internal *ftsZ* sequences (Figures 9 and 10). Since ~500bp products are those expected from identical partial *ftsZ* fragments, the PCR products in the range of 1 kb were thought to contain the missing N-terminal coding and upstream *ftsZ* regions. These PCR products were isolated, gel purified, and subcloned into pBluescript II SK+ and pBR322. However, no *E. coli* transformants were isolated following the ligations.

In another strategy, the radiolabelled partial *ftsZ* was used to screen the pBR322 and Lambda Gem 11 genomic libraries (covering 65X the genome size). Multiple pBR322 and Lambda Gem 11 recombinant clones with the identical partial *ftsZ* fragment were isolated. However, two Lambda Gem 11 recombinant clones (Lambda-ftsZ1 and Lambda-ftsZ2) containing the complete *ftsZ* and upstream regulatory region were isolated.

Restriction enzyme analysis of the Lambda-ftsZ1 and 2 revealed that both recombinant phage clones
had the identical internal HindIII fragment containing the complete coding and upstream regulatory region of ftsZ (Figure 4). The ~1.5kb HindIII fragment of Lambda-ftsZ1 was isolated and subcloned into pBluescript II SK+. However, no E. coli transformants were isolated following the ligation reactions. The ligations were checked by PCR with a combination of an internal ftsZ primer, FG6, together with each of the pBluescript II SK+ T7 and Reverse primers. The PCRs yielded products which confirmed that the ftsZ-containing fragments were subcloned (Figure 13).

The inability to isolate a recombinant pBR322 clone from the library, and to subclone the complete ftsZ gene into pBluescript II SK+ suggests that there is a bias against a transformant to harbor a recombinant clone containing the complete C. jejuni ftsZ gene. The presence of a complete and functional C. jejuni ftsZ may be toxic to the transformed E. coli cells. Possible explanations could be that an increased copy number and therefore, expressed level of ftsZ is lethal to E. coli as has been shown in E. coli ftsZ studies, or that the complete C. jejuni FtsZ may be interfering with the function of the E. coli FtsZ and therefore, interfering with E. coli cell division. Future studies can be done to confirm whether the increased levels of C. jejuni ftsZ is toxic in E. coli. Different methods can be employed: observe if C. jejuni ftsZ (present on a plasmid) can complement an E. coli temperature-sensitive mutant at the non-permissive temperature, or observe the effects of different levels of the C. jejuni ftsZ on wild-type E. coli by subcloning the gene into a temperature-sensitive-replicating plasmid whose copy number is altered at different temperatures.

Characterization of C. jejuni ftsZ

The nucleotide sequence of the entire coding and upstream regulatory region of ftsZ determined on both strands is shown in Figure 14. Based on the primer extension analyses, three transcription start sites of ftsZ were mapped (Figure 16). These start sites are 23, 26, and 96 nucleotides upstream of
the first Met codon. Since the total RNA used in the analysis was extracted from stationary and
decline phase cultures, the three start sites may reflect the regulatory mechanisms that are used to
promote transcription of ftsZ during these phases. As has been shown for the C. jejuni ftsA gene
(Griffiths et al., 1997), there may be additional start sites used in log phase. Primer extension
analyses using total RNA extracted from log phase cultures will reveal whether there are additional
start sites that are utilized. Comparing the E. coli σ^70 (TAtaaT) -10 and (TTGaca) -35 consensus
sequences and the four ftsZ promoters (TTTAAC_p1, TTTCAT_p2, TATGCT_p3, TACAAT_p4) -10 and
(TCGGCG_p1, TTGCAG_p2, TTGCGC_p3, CTGCCT_p4) -35 sequences, to the C. jejuni ftsZ promoter
(TAAAAAT_p1, TACAAT_p2) -10 and (TAGTGT_p1, TTGATG_p2) -35 sequences, revealed that the
putative upstream (p2) C. jejuni ftsZ promoter sequences has a better match than the putative
proximal promoter (p1). Therefore, the observed levels of transcripts may be due to the strengths of
each promoter. However, this remains to be confirmed. One way to confirm this is to mutagenize
the promoter sequences and use reporter assays to examine the effects of the mutations.

Based on the nucleotide sequence, a putative Shine-Dalgarno sequence was determined and a long
inverted repeat was also identified downstream of the ftsZ coding sequence, which may be a
mechanism used to terminate ftsZ transcription (Figure 14). The amino acid sequence was deduced
and aligned to the amino acid sequences of other known bacterial FtsZ sequences. All five of the
conserved domains are present within the C. jejuni FtsZ (Figure 15). In particular, the G-box
(GGGTGTG) which has been shown to be important for GTP-binding, GTPase activity and
polymerization of FtsZ, is identical between C. jejuni and E. coli FtsZ. This is another indication
that C. jejuni FtsZ could interfere with the normal functions of E. coli FtsZ, either by interfering with
the E. coli FtsZ polymerization, or by depleting the GTP pool, thus resulting in its toxicity to E. coli
transformants.
Expression of *C. jejuni* *ftsZ*

Total RNA was extracted from various points in the *C. jejuni* growth cycle. Northern blot analyses (Figure 18) was used to determine the size and relative levels of *ftsZ* transcripts. Although primer extension analyses revealed three transcription start sites which would correspond to three transcripts, the agarose gel electrophoresis and autoradiography of the resulting Northern blot hybridization was not sufficient to resolve RNA fragments differing by 2 to 73 nucleotides. Thus, *ftsZ* was observed to be expressed on a ~1.3kb transcript. Its transcript levels remain relatively stable throughout log phase and decreases upon entry into stationary phase.

As mentioned earlier, several flagellar genes have been identified that affect cell division (Dingwall *et al.*, 1992; Yu and Shapiro, 1992; Zhuang and Shapiro, 1995; Goer and Marques, 1995). Since only the transcription start site of *flgF* (Chan *et al.*, unpublished data) and *ftsZ* (this study) were available, the putative σ^70^ promoter sequences were compared. By comparing the putative promoter sequences of *flgF* (TAAAAT, TAAACAG -10 and (TTGGAA, TTCAAA -35 (Chan *et al.*, unpublished data), to those of *ftsZ* (TAAAAT, TACAAAT -10 and (TAGTGT, TTGATG -35, the -10 promoter sequences displayed a high sequence identity, while the -35 promoter sequences showed less identity. Therefore, the presence of *flgF* and *flgG* downstream of *ftsZ* may be of significance, in that these genes may be coordinately regulated. The relative levels of *flgF* and *flgG* transcripts were examined to determine whether *ftsZ* is expressed on the same transcript as *flgF* and *flgG*, and whether there is any correlation between the expression of *ftsZ* and its adjacent downstream genes. From the Northern blot analyses, *ftsZ* is expressed on a ~1.3kb transcript, while *flgF* is expressed on a ~1.7kb transcript and *flgG* is expressed on two transcripts, ~1.8kb and ~0.9kb. Thus, the *ftsZ* transcript is monocistronic. However, the ~1.7/1.8kb transcript probably encodes both
flgF and flgG, and the ~0.9kb flgG transcript could either be transcribed from its own promoter, or a cleavage product of the larger transcript. Primer extension analyses of flgG could determine whether it can be transcribed independently and help identify putative promoter sequences.

The Northern blot results of C. jejuni ftsZ reveal that ftsZ transcript levels remain relatively constant throughout log phase and begins to decrease upon entry into stationary phase. In contrast, the E. coli ftsZ transcript levels oscillate throughout the growth curve, reaching a maximum at approximately the same time as initiation of DNA replication (Garrido et al., 1993). In C. crescentus stalked cells, FtsZ protein levels start increasing at initiation of DNA replication and is maximal immediately prior to cell division (Quardokus et al., 1996). FtsZ rapidly disappears in the motile swarmer cells. Thus, in cultures of rapidly growing and dividing cells, C. crescentus FtsZ levels are maximal. Based on the assumption that the levels of transcripts correspond to the levels of protein, if applied to the case of C. jejuni, this study shows that FtsZ levels are maximal during division. However, this correlation does not occur in E. coli in which the levels of FtsZ protein is relatively constant throughout the cell cycle (Dai and Lutkenhaus, 1992), although the transcript levels oscillate. In this instance, it has been suggested that the FtsZ proteins are stable, with a long half life, and only as the critical level drops does the cell signal the expression or transcription of E. coli ftsZ (Garrido et al., 1993).

In C. crescentus, the levels of FtsZ peak in non-motile stalked cells immediately prior to cell division, and disappears rapidly in the motile swarmer cells which are thought to be generated in order to search for different environments to colonize. Once the swarmer cells enter an environment that is rich in nutrients or optimal for proliferation, they differentiate into stalked cells which are then able to rapidly divide. Another similarity of C. crescentus and C. jejuni can also be postulated in that as C. jejuni cells are rapidly dividing during log, during stationary phase, with nutrient depletion and accumulation of toxic by-products, cells may not completely shut down synthesis of ftsZ since it may
be needed to generate the coccoid or minicells as a mechanism of survival. As mentioned earlier, the coccoid cells may be actively formed either containing the minimal set of machinery to maintain viability and survival of the culture until more favourable conditions are encountered, or may be actively formed to expel unwanted or excess cell components. Thus, the resulting cells of either spiral or coccoid morphology are able to survive using the bare machinery to maintain viability. The culture may also contain a fraction of the coccoid cells that are non-viable degenerate forms of the spiral cells due to cell lysis or degeneration of the cell envelope layer which leads to loss of their normal spiral morphology. In observing the level of ftsZ transcripts present in stationary phase cultures, the former two possibilities may be a mechanism of C. jejuni asymmetrical cell divisions which occur in stationary cultures, in addition to symmetrical divisions that occur during log phase.

Expression and purification of GST-FtsZ fusion and partial FtsZ proteins

Since the E. coli FtsZ has been found to be essential and involved in the earliest stage of cell division, the C. jejuni homolog may also play a similar role. This study has proposed that two modes of cell division may be occurring, based on the position of the invaginating septum and the morphology of the daughter cells being formed during the division process. Therefore, the role that FtsZ plays in these two modes will further the understanding of how these divisions take place.

The GST-FtsZ fusion was highly expressed and purified by using the pGEX 2T gene fusion system. Then, the partial FtsZ protein (without the conserved D- and G-box) was released from the fusion protein by thrombin cleavage and purified from the GST-bound beads by centrifugation. According to SDS-PAGE analyses, the size of the fusion and partial FtsZ proteins are ~54 and ~27 kDa, respectively. These sizes correspond to the predicted sizes based on the amino acid content of each of the proteins. In the future, anti-FtsZ antibodies will be raised to facilitate further studies of C.
jejuni cell division. Specifically, the levels of FtsZ protein throughout the C. jejuni growth cycle will be determined. Similar IEM studies will be used to localize FtsZ within the cell and to demonstrate whether C. jejuni cell division employs FtsZ in the same manner as E. coli, and whether asymmetrical divisions utilize FtsZ at all or in a different organization at the invaginating septum.
FUTURE STUDIES

Based on TEM, the predominant mode of *C. jejuni* ATCC 43431 cell division in log and stationary phase cultures is symmetric and asymmetric cell division, respectively. Due to the spiral morphology and various angles at which each cell can be sectioned (as previously mentioned and shown in Figure 23), the precision of symmetrical division remains to be determined. These results can be further corroborated and refined by scanning electron microscopy (SEM) by which whole cells are visualized. Therefore, the dimensions of *C. jejuni* cells and the exact point of constriction, corresponding to the invaginating septum, can be measured from each cell pole, to differentiate precise or imprecise symmetric cell division.

Furthermore, the cloning and characterization of *ftsZ* will be used to elucidate the molecular mechanisms and regulation of *C. jejuni* cell division. These studies will include expression and mutational studies of *ftsZ* utilizing shuttle and suicide vectors to transform *C. jejuni* cells. Anti-FtsZ antibodies will be raised against the purified partial FtsZ and used to determine the levels of FtsZ protein throughout the growth cycle and used in IEM studies to localize FtsZ within *C. jejuni* cells throughout the cell cycle of cells from log and stationary phase cultures. IEM studies will reveal the involvement of FtsZ in both symmetric and asymmetric division. In particular, carrying out IEM studies concurrently with chromosomal DNA staining could be used to study asymmetrical division giving rise to coccoid or minicells. These studies could indicate that these cells are actively formed since FtsZ recruitment is an active process, and are possibly viable if they contain what could be chromosomal DNA. A shuttle vector containing a fusion protein of *C. jejuni ftsZ* and GFP could also be used to localize FtsZ in *vivo* and to complement the IEM studies. The anti-FtsZ antibodies will also be used to isolate proteins which interact with FtsZ and are involved in division, providing the elucidation of more molecular machinery involved in *C. jejuni* cell division.
REFERENCES


Kim, N.W., R. Lombardi, H. Bingham, E. Hani, H. Louie, D. Ng, and V. L. Chan. 1993. Fine-


Intestinal Campylobacters from Man and Animals. J. Hyg. 85:427-442.


APPENDIX 1

Identification of *Campylobacter jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* Based on the glyA Gene

Shahnaz Tahihra AL RASHID, Irene DAKUNA, David NG, Peter VANDAMME, and Voon Loong CHAN*

Department of Medical Genetics and Microbiology, University of Toronto, Fitzgerald Building, 150 College Street, Toronto, Ontario, Canada M5S 1A8.

*Corresponding author: Dr. Voon Loong Chan
Department of Medical Genetics and Microbiology
University of Toronto
150 College Street
Toronto, Ontario
Canada M5S 1A8
Degenerate oligodeoxyribonucleotides (oligos), designed by comparing the glyA gene sequences of Campylobacter jejuni (4) and Escherichia coli (25), were used in the polymerase chain reaction (PCR) to amplify a glyA fragment of approximately 640 base pairs (bps) from C. jejuni ATCC 33560, C. coli ATCC 33559, C. lari ATCC 35221, and C. upsaliensis ATCC 43954. Alignment of the DNA sequences of these glyA fragments revealed two regions which were used to develop species-specific oligo probes. Two sets of probes targeting two regions of glyA were designed to detect and differentiate C. jejuni, C. coli, C. lari, and C. upsaliensis. Using the described hybridization and washing conditions, these probes were found to be species-specific.
INTRODUCTION

Campylobacter species are common human and animal pathogens (29). Although their pathogenicity has long been known, their phylogenetic relationships, isolation, detection, identification, and classification by traditional biochemical tests, have been variable. This is largely due to their fastidious growth requirements, inability to ferment carbohydrates, and diverse growth characteristics which vary, not only between genera and species, but also within species. Thus, their large phenotypic variations have made biochemical tests unreliable as a sole method for identifying and differentiating these bacteria.

Many of the species in the genera Helicobacter and Arcobacter were once classified under the genus Campylobacter. However, the phylogenetic relationships of these bacteria have been reevaluated based on information from DNA-DNA hybridization, 23S rRNA-DNA hybridization (33, 34), and partial 16S rRNA sequences (17, 22, 31). These phylogenetic studies have led to the formation of the current classification of the Campylobacter and Vibrio organisms into Campylobacter, Helicobacter, and Arcobacter.

Alternative methods other than the conventional biochemical tests, based on molecular and genetic approaches, have been proposed to improve the identification and differentiation of these bacteria to the species level. These methods include serology (11, 23), enzymology (6, 20), cellular fatty acid compositions (10),
electrophoretic protein patterns (5, 23), random PCR-DNA fingerprinting (7, 8, 9, 35), and DNA-DNA hybridization (18, 23). A highly specific DNA-DNA hybridization method is oligo hybridization. By varying hybridization conditions such as ionic concentration and temperature, oligo probes can detect a single nucleotide difference (16).

In this paper, the partial sequence of a specific conserved and essential gene, glyA (encoding serine hydroxymethyltransferase or SHMT), was used to develop species-specific oligo probes. To identify and differentiate these closely-related species, a combined PCR-hybridization strategy was explored using these probes to target different regions within the glyA gene.

METHODS AND MATERIALS

Bacterial strains, plasmids, and growth conditions used:

Bacterial strains used in this study are listed in Table 1.

Campylobacter species (Table 1) were grown on Columbia Agar Base (Oxoid) supplemented with defibrinated horse blood (5% final concentration), incubated at 37°C from 24 to 48 hours. All species were grown in a 3L anaerobic jar under microaerophilic conditions created by the Campylobacter Gas Generating Kit (Oxoid) which generates an atmosphere containing approximately 6% oxygen and 10% carbon dioxide.
For gene cloning experiments, plasmid pBluescript II KS+ (Stratagene) and E. coli strain JM101 (26) were used. The E. coli cells were grown in Luria Bertani (LB) broth at 37°C. Competent cells were prepared by the rubidium chloride/ calcium chloride protocol and transformed by standard procedures (26). Transformants were grown on LB agar supplemented with ampicillin (100µg/ml final concentration).

**Extraction of genomic DNA:**

Genomic DNA from *Campylobacter* spp. with the strain designations LMG, RG, and BVA (Table 1) was from P. Vandamme (Gent, Belgium). Genomic DNA from the remaining bacterial species was extracted as previously described (30). Briefly, cultures grown on agar plates were scraped off and washed three times in 1XSSC (150mM sodium chloride, 15mM trisodium citrate, pH7.0). For each wash, cells were centrifuged at 5000 rpm (GSA Sorval rotor) for 5 minutes. Following centrifugation, the supernatant was decanted and the cell pellet was resuspended in 1XSSC. Finally, the washed cells were resuspended in 1XSSC containing 27% sucrose to a concentration of approximately 10⁷ cells per ml. Proteinase K was then added to a final concentration of 0.2mg/ml and incubated at 60°C for 1 hour. Genomic DNA was purified and extracted with an equal volume of buffer-saturated phenol (50mM Tris.Cl, 10mM EDTA, pH8.0) with constant slow agitation for 30 minutes at room temperature. The mixture was then chilled to 0°C and centrifuged
at 5000 rpm for 5 minutes. The phenol (top) phase was removed and the extraction was repeated. The aqueous DNA solution was dialyzed in 1.5L of T₁₀E₁ (10mM Tris.Cl, 1mM EDTA, pH8.0), three times, at 4°C for 12 hours. The DNA was then precipitated with 0.3M sodium acetate pH 5.2 and two volumes of 95% ethanol for 12 hours at -20°C. The DNA was centrifuged for 30 minutes at 7000 rpm. The DNA pellet was redissolved in T₁₀E₁ and stored at 4°C.

Polymerase chain reaction (PCR):

A 640 bp region of the glyA gene was chosen to be amplified. It is directly flanked by conserved domains identified by amino acid sequence alignment of the C. jejuni, E. coli, and available partial sequence of the rabbit SHMT homolog (4). This region also encompasses the domain implicated for binding the coenzyme, pyridoxal-5'-phosphate (25) and a domain that has been suggested to be part of the enzyme’s active site (14, 25). The sequences of the two conserved flanking domains were used to synthesize degenerate oligo primers, S₁ (5' - AA(C/T) AAA TA(C/A) GC(A/T) GAA GG(T/A) TAT - 3') and S₂ (5' - ATG CAT (C/T)AA (A/T)GG (A/T)CC (A/T)CC TTG - 3'), to amplify the region of the glyA gene of the selected species. The PCR was performed on all the Campylobacter spp., the Helicobacter spp., A. nitrofigilis, B. adolescentis, E. coli, L. casei, P. aeruginosa, and S. sonnei (Table 1) using a thermal cycler (Perkin Elmer Cetus).
The PCRs were optimized at a concentration of 1mM magnesium chloride (MgCl₂) for all species except for A. nitrofigilis, which was optimized at a concentration of 2mM MgCl₂. The components of each 100ul PCR were: 1ug of genomic DNA (except for H. pylori for which 0.5ug was used [as estimated from ethidium bromide stained agarose gels]), 20 pmoles of each primer, 20 umoles of deoxyribonucleotide triphosphates (dNTPs), amplification buffer (10mM Tris.Cl, 50mM potassium chloride, pH 8.3), and 2.5 units of Taq DNA polymerase (Promega and Boehringer Mannheim). The reaction solutions were overlaid with 100ul of mineral oil to prevent any evaporation. The samples were subjected to 30 cycles of amplification, each consisting of template denaturation at 95°C for 1.5 minutes, primer annealing at 42°C for 2 minutes, and chain extension at 72°C for 1 minute. After the 30 cycles, an additional extension step at 72°C for 5 minutes was performed. The PCR products were purified from the dNTPs by passing the reaction solution through a Sephadex G-50 spun column (equilibrated in STE [10mM Tris.Cl pH 7.5, 10mM sodium chloride, 1mM EDTA]). From each 100ul PCR, 10 ul was electrophoresed in an ethidium bromide-stained 1% agarose gel, visualized under ultra violet light illumination, and photographed.

To test the sensitivity of the PCR/hybridization method, serial dilutions of the C. jejuni ATCC 33560 genomic DNA template ranging from 1fg to 1ug were used in the PCRs.
Cloning, miniprep, and sequencing:

The PCR products of the *Campylobacter* spp. (Table 1) were subcloned into pBluescript II KS+ at the EcoRV site and subsequently used to transform *E. coli* strain JM101 competent cells. Plasmid preparations (minipreps) were obtained and purified for sequencing using the alkaline lysis method (26). The clones were sequenced by the Sanger dideoxy-chain termination method (27) using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical) according to the manufacturer’s recommendations. α-[^35]S-dATP (1000 Ci / mmole, ICN Biomedicals Canada Ltd.), and the M13 (-20) forward and reverse primers (Stratagene) of pBluescript II KS+, and the PCR primers, S1 and S2, were used for the reactions.

DNA sequence alignment, probe designs, and syntheses:

The nucleotide sequences were analyzed using the Microgenie Sequence Analysis Program Version 5 (Beckman Instruments, Inc.) and Clustal V Multiple Alignment Program (12, 13). Alignment of the partial *glyA* nucleotide sequences of *C. jejuni* ATCC 33560, *C. coli* ATCC 33559, *C. lari* ATCC 35221, and *C. upsaliensis* ATCC 43954 identified 28 bp and 32 bp regions which were used to design the species-specific oligo probes CJATC-1, CC-1, CL-1, and CU-1 (series 1 probes) (synthesized by Dalton Chemical Laboratories, Incorporated), and CJATC-2, CC-2, CL-2, and CU-2 (series 2 probes) (synthesized by ACGT Corporation).
End-labelling of the probes:

The species-specific oligos were radioactively-labelled in 20μl reactions containing 20 pmoles of the oligos, 20 pmoles of γ-32P-ATP (4500 Ci / mmole, ICN Biomedicals Canada Ltd.), kinase buffer (70mM Tris.Cl pH 7.6, 10mM MgCl₂, 5mM dithiothreitol), and 20 units of T4 Polynucleotide Kinase (Pharmacia and New England Biolabs). The reactions were incubated at 37°C for 30 minutes and stopped by heating at 65°C for 15 minutes. The radioactively-labelled probes were purified by passing the reaction solution through a STE-equilibrated Sephadex G-50 spun column.

Southern blot:

HindIII-digested lambda phage DNA, 100 bp ladder DNA (Pharmacia), and pBluescript II KS+ vector (molecular weight markers and negative controls), glyA recombinant plasmid clones (positive controls), and the PCR products from all the species examined were electrophoresed in a 1% agarose gel and transferred onto GeneScreen Plus nylon-based membranes (DuPont Canada Inc.) by vacuum transfer using the LKB 2016 VacuGene Vacuum Blotting System (Pharmacia LKB Biotechnology). The transfer procedure consists of 15 minutes of depurination (0.2N hydrochloric acid), 20 minutes of denaturation (1.5M sodium chloride, 0.5M sodium hydroxide), 20 minutes of neutralization (1.0M Tris.Cl, 2M sodium chloride, pH 5.0), and 1 hour of transfer (20XSSC - 3M sodium chloride, 0.3M...
trisodium citrate, pH 7.0) under a constant vacuum pressure of 55 cm.H_{2}O.

Southern hybridizations:

After Southern blotting, the membranes were air-dried at room temperature for 12 hours. Prior to hybridization, they were soaked in 2XSSC and prehybridized at 42^0C and 45^0C (for series 1 and 2 probes, respectively) for 30 minutes in 10ml of prehybridization solution (1% sodium dodecyl sulfate, 1M sodium chloride, 10% dextran sulfate, and 5mg/ml denatured salmon sperm DNA). Then, the labelled probe was added with a specific activity of 3x10^5 cpm/ml and the hybridization was done at 42^0C and 45^0C (for series 1 and 2 probes, respectively) for 8 to 24 hours. This was followed by two washes, each with 0.2XSSC at 60^0C and 50^0C (for series 1 and 2 probes, respectively) for 10 minutes with constant agitation. Bands were visualized by autoradiography using X-ray film (X-OMAT AR, Kodak Scientific Imaging Film) exposed to the membranes for 40 minutes to 10 hours at room temperature, and also 10 to 20 hours at -70^0C.
RESULTS

DNA sequences and alignment - species-specific oligo probes. The complete sequences were obtained by merging the sequences from both ends of the subcloned glyA fragment. Two independent glyA recombinant clones from each species were sequenced to ensure the accuracy of the sequences.

Two regions were chosen to design species-specific rather than genus-specific probes. The first set of oligo probes to detect C. jejuni, C. coli, C. lari, and C. upsaliensis were designed from the region suggested to be part of the active site of SHMT (14, 25), while the second set of oligo probes to detect the four Campylobacter spp. were designed from a region of variable sequences which is adjacent to the conserved domain implicated for binding to the coenzyme, pyridoxal-5'-phosphate (25). The sequences of the species-specific single-stranded oligo probes are shown below:

CJATC-1 5'- TTTTC CGCAC GCTCA TGTAG TAAGC TCAAC TA -3'
CC-1 5'- ATTTC CTCAT GCTCA TGTAG TAAGC TCTAC AA -3'
CL-1 5'- ATTCC CTTAT GCTCA TGGTG TAAGT TCT -3'
CU-1 5'- TTTCC CTCAC GCACA CATCG TAAGC TCA -3'
CJATC-2 5'- AAAAG TAAGA GAAAT TGCTA AAAAA GAA -3'
CC-2 5'- AAAAG TTAGG GAAAT TGCTC ATATT GTA -3'
CL-2 5'- TAAAG TTAGA GAGAT AGCAA AAGAG ATT -3'
CU-2 5'- AAAAG TAAGA GAAAT AGCAG ACATC GTT -3'
The series 1 set of probes has a $T_m$ range from 60.7°C (CL-1) to 66.9°C (for CJATC-1), while the series 2 set of probes has a $T_m$ range from 54.9°C (for CJATC-2) to 59.3°C (for CU-2). The conditions for hybridization and washing were optimized to select for species-specific hybridizations. Thus, two stringent conditions were used based on the $T_m$ of the two sets of probes.

Each probe's species-specificity was tested against the bacteria listed in Table 1. This was done by PCR amplifying the glyA fragments using genomic DNA from all the species including all the Campylobacter spp., Helicobacter spp., A. nitrofigilis, B. adolescentis, E. coli, L. casei, P. aeruginosa, and S. sonnei (Figure 2). However, no PCR products were obtained from B. adolescentis and L. casei (data not shown). In addition, pBluescript II KS+ and the recombinant plasmids that were sequenced, were used in the hybridizations as negative and positive controls, respectively.

The results of the hybridizations are shown in Figures 3 (A) to (D) for the series 1 probes and in Figures 4 (A) to (D) for the series 2 probes. The CU-1 probe is species-specific under the hybridization and washing conditions since exposure times between 40 minutes to 20 hours did not show cross-specific hybridization. While the CJATC-1, CC-1, and CL-1 probes are species-specific after exposure times between 40 minutes to 4 hours, some cross-hybridizations can be detected after 18 to 20 hours of exposure.
The CJATC-1 probe cross-hybridized to the PCR products of C. coli strains, CC-1 probe cross-hybridized to the PCR products of C. jejuni and C. upsaliensis strains, and the CL-1 probe cross-hybridized to the PCR products of A. nitrofigilis. The CC-2 probe is species-specific under the hybridization and washing conditions since no cross-hybridization to the other species' PCR products were observed after exposure times of up to 22 hours. The CJATC-2, CL-2, and CU-2 probes are species-specific after 4 hours of exposure. However, there is some cross-hybridization that was observed after exposure of 22 hours. The CJATC-2 and CL-2 probes cross-hybridized to the PCR products of C. upsaliensis strains, while the CU-2 probe cross-hybridized to the PCR products of C. jejuni strains.

**Detection of different strains and serotypes.** The ability of the probes to hybridize to different strains and serotypes of the different species was also tested. Both the CJATC-1 and CJATC-2 probes can detect all 14 C. jejuni strains and serotypes (Figures 3A and 4A). Both the CC-1 and CC-2 probes can detect all 10 C. coli strains (Figures 3B and 4B). The CL-1 probe can detect 13 of the 15 C. lari strains, while the CL-2 probe can detect 12 of the 15 C. lari strains that were tested (Figures 3C and 4C). The CU-1 probe can detect all 14 C. upsaliensis strains, while the CU-2 probe can detect 12 of the 14 strains that were tested (Figures 3D and 4D).
**Sensitivity.** The PCR using the S1 and S2 oligos were performed on serially diluted *C. jejuni* ATCC 33560 genomic DNA to determine the amplification yield and ultimately, sensitivity of this PCR/hybridization approach. The CJATC-1 probe was tested for its ability to detect the lowest amount of the PCR product. The results in Figure 5 show that the lowest amount of genomic DNA required in order to yield enough PCR product to be detected by the CJATC-1 probe is 4 picograms (4 X 10^{-12} grams).

**DISCUSSION**

Species-specific oligos targeting *C. jejuni, C. coli, C. lari,* and *C. upsaliensis* were designed from the aligned *glyA* sequences. The probes' specificities were tested by subsequent Southern hybridizations to the PCR products that were isolated from the various *Campylobacter* spp., *Helicobacter* spp., *A. nitrofigilis, E. coli, P. aeruginosa,* and *S. sonnei.*

From the hybridization results, the CU-1 probe is species-specific under these hybridization and washing conditions since exposure of up to 20 hours did not detect any cross-hybridization with any of the other species. However, the CJATC-1 probe cross-hybridized to the PCR products of *C. coli,* the CC-1 probe cross-hybridized to the PCR products of *C. jejuni* and *C. upsaliensis* strains, and the CL-1 probe cross-hybridized to the PCR products of *A. nitrofigilis* when exposed for greater than 18 hours.
The second set of probes were tested using different hybridization and washing conditions. The results demonstrate that the CC-2 probe is species-specific since exposure of up to 22 hours did not reveal any cross-hybridization to any of the other species. However, both the CJATC-2 and CL-2 probes cross-hybridized to the PCR products of some C. upsaliensis strains, and the CU-2 probe cross-hybridized to the PCR products of some C. jejuni strains with longer exposure times (for example, 22 hours).

All the cross-hybridizations were observed to be, at the most, one tenth the intensity when compared to the intensity observed from the species-specific hybridizations. Since an unknown sample may contain any one of the species, the use of both sets of probes would resolve the discrepancies due to cross-hybridizations (Table 2). For example, if an unknown sample was detected by both CJATC-1 and CC-2, since CJATC-1 cross-hybridizes weakly C. coli and CC-2 only detects C. coli, therefore the sample is determined as C. coli. However, if the unknown sample is detected by CJATC-1 but not by CC-2, then by the same deduction, the sample is determined as C. jejuni. Thus, cross-hybridizations would not be a factor for misidentification. Furthermore, cross-hybridization would not affect the identification of either C. jejuni or C. lari strains since C. jejuni probes do not cross-hybridize to the C. lari glyA PCR fragments and vice versa.
The probes could also detect different strains of their various targeted species. However, the strength of the hybridizations were varied, and some strains were not detected. This is due to the length and specificity of oligo probes. Since there are minor nucleotide sequence variations between different strains, the probe may hybridize to the target that varies by up to eight different nucleotide substitutions (as shown by the cross-hybridizations that can occur), but may not detect strains if the target sequence has more substitutions, is missing nucleotides, or has additional nucleotides. For example, when the glyA sequences of C. jejuni ATCC 43431 (4) and C. jejuni ATCC 33560 were aligned (data not shown), the sequences varied by 2 nucleotides at the CJATC-1 target sequence. However, CJATC-1 probe did detect C. jejuni ATCC 43431. In addition, a wider range of strains can be detected by using both sets of probes. For example, the CL-1 probe can detect C. lari LMG 9253, which was not detected by the CL-2 probe. Furthermore, none of the probes hybridized to the other bacterial species such as C. sputorum, E. coli, P. aeruginosa, and S. sonnei.

The sensitivity of this PCR/oligo hybridization strategy was determined by using the CJATC-1 probe targetting C. jejuni ATCC 33560 as the test species. The lowest amount of genomic DNA required to yield sufficient PCR products which could be detected by CJATC-1 was 4 picograms (4 X 10^{-12} grams). Since the C. jejuni chromosome is approximately 1.8 X 10^6 bps, 4 X 10^{-12} grams corresponds to approximately 2062 copies of template. However,
the result that was detected was of 10ul of the total 100ul reaction volume. Therefore, the PCR would be able to amplify detectable amounts of product from approximately 200 copies of template.

While isotopic detection systems may have disadvantages (for example, isotopic decay, radiation exposure), this PCR/hybridization strategy can be used as a rapid diagnostic method for detecting the various Campylobacter species. Five hours could be used as the minimum exposure time for species-specific identification. This exposure time would not reveal the cross-hybridizing bands. However, as previously mentioned, the probes detect different strains of the same species with varying signal intensities. Therefore, a further exposure, such as ten hours could also be done to detect different strains without the appearance of cross-hybridizations. In addition, simultaneous use of both sets of probes would significantly reduce the likelihood of misidentifications due to cross-hybridizations and detect a wider range of strains within each species. With the current conditions and limited exposure times, all the probes that have been designed are species-specific and could identify and differentiate the Campylobacter spp. that were studied.
REFERENCES


Table 1. Reference bacteria used in this study.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ATCC, American Type Culture Collection, Rockville, USA.</td>
</tr>
<tr>
<td>1</td>
<td>supE thi Δ(lac-proAB) F'[traD36 proAB' lacI' lacZΔM15] (26)</td>
</tr>
<tr>
<td>2</td>
<td>Clinical isolates</td>
</tr>
<tr>
<td>3</td>
<td>Hippuricase negative variants (32)</td>
</tr>
<tr>
<td>4</td>
<td>Obtained from Dr. J.L. Penner, University of Toronto, Toronto, Ontario, Canada</td>
</tr>
<tr>
<td>5</td>
<td>Obtained from Dr. A. Bognar, University of Toronto, Toronto, Ontario, Canada</td>
</tr>
<tr>
<td>6</td>
<td>Obtained from Dr. P. Vandamme, Laboratorium voor Microbiologie, Belgium</td>
</tr>
<tr>
<td>*</td>
<td>Strains used for the PCR, subcloned, and sequenced to generate the species-specific oligo probes</td>
</tr>
<tr>
<td>†</td>
<td>Strains used for the PCR and Southern hybridizations to determine species-specificity of the probes</td>
</tr>
<tr>
<td>‡</td>
<td>C. jejuni type strain used to determine sensitivity of this PCR/hybridization strategy</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Strain</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>JM101</td>
</tr>
<tr>
<td></td>
<td>ATCC 9637</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>ATCC 33560 (type)</td>
</tr>
<tr>
<td></td>
<td>ATCC 43429</td>
</tr>
<tr>
<td></td>
<td>ATCC 43430</td>
</tr>
<tr>
<td></td>
<td>ATCC 43431</td>
</tr>
<tr>
<td></td>
<td>ATCC 43432</td>
</tr>
<tr>
<td></td>
<td>ATCC 43433</td>
</tr>
<tr>
<td></td>
<td>CEPA3C 2*</td>
</tr>
<tr>
<td></td>
<td>COC6-85 2*</td>
</tr>
<tr>
<td></td>
<td>INN7383 2*</td>
</tr>
<tr>
<td></td>
<td>V48 2*</td>
</tr>
<tr>
<td></td>
<td>D594 3*</td>
</tr>
<tr>
<td></td>
<td>D603 3*</td>
</tr>
<tr>
<td></td>
<td>D1916 3*</td>
</tr>
<tr>
<td><em>Campylobacter coli</em></td>
<td>ATCC 33559 (type)</td>
</tr>
<tr>
<td></td>
<td>LMG 7535 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 8530 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 9853 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 9854 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 9855 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 9856 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 9857 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 9858 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 9859 6*</td>
</tr>
<tr>
<td></td>
<td>LMG 15882 6*</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter sputorum</em> subsp. bubulus</td>
<td>ATCC 33562 (type)</td>
</tr>
<tr>
<td><em>Helicobacter cinaedi</em></td>
<td>ATCC 35683 (type)</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Clinical isolate</td>
</tr>
<tr>
<td><em>Arcobacter nitrofigilis</em></td>
<td>ATCC 33309 (type)</td>
</tr>
<tr>
<td><em>Bifidobacterium adolescentis</em></td>
<td>ATCC 15703</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
</tr>
<tr>
<td><em>Shigella sonnei</em></td>
<td></td>
</tr>
</tbody>
</table>

ATCC 33560 (type)
Table 2. Summary of the Southern hybridization results of probe specificity.
<table>
<thead>
<tr>
<th>Probes</th>
<th>Specific hybridization to:</th>
<th>Weak cross-hybridization to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJATC-1</td>
<td>C. jejuni</td>
<td>C. coli</td>
</tr>
<tr>
<td>CJATC-2</td>
<td>C. jejuni</td>
<td>C. upsaliensis</td>
</tr>
<tr>
<td>CC-1</td>
<td>C. coli</td>
<td>C. jejuni and C. upsaliensis</td>
</tr>
<tr>
<td>CC-2</td>
<td>C. coli</td>
<td>none</td>
</tr>
<tr>
<td>CL-1</td>
<td>C. lari</td>
<td>A. nitrofigilis</td>
</tr>
<tr>
<td>CL-2</td>
<td>C. lari</td>
<td>C. upsaliensis</td>
</tr>
<tr>
<td>CU-1</td>
<td>C. upsaliensis</td>
<td>none</td>
</tr>
<tr>
<td>CU-2</td>
<td>C. upsaliensis</td>
<td>C. jejuni</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Multiple nucleotide sequence alignment of the partial *glyA* sequences.

Alignment of the sequences from *C. jejuni* ATCC33560, *C. coli* ATCC33559, *C. lari* ATCC335221, and *C. upsaliensis* ATCC43954. Each single-underlined sequence corresponds to each of the species-specific probes, while the double-underlined sequences correspond to the degenerate primers used in the PCR to amplify the partial *glyA* fragment from the species used in this study.
Figure 2. PCR products of all species resolved in a 1% agarose gel and used in the Southern hybridization experiments.

The molecular weight markers are in lanes L (HindIII-digested lambda phage DNA), and M (100bp ladder). The PCR products of each bacterial strain are in the following lanes: lanes 1 to 13 are C. jejuni strains ATCC 33560, ATCC 43429, ATCC 43430, ATCC 43431, ATCC 43432, ATCC 43433, CEPA-3C, CO06-85, INN7383, V48, D594, D603, and D1916; lanes 14, 15 and 16 are C. coli ATCC 33559, C. lari ATCC 35221, and C. lari PC 637, respectively; lanes 17 to 26 are C. coli strains ATCC 33559, LMG 7535, LMG 8530, LMG 9853, LMG 9854, LMG 9855, LMG 9856, LMG 9857, LMG 9858, and LMG 9859; lanes 27 to 40 are C. lari strains ATCC 35221, LMG 8845, LMG 8844, LMG 7929, LMG 9887, LMG 9888, LMG 9889, LMG 9913, LMG 9914, LMG 9152, LMG 9253, LMG 11251, 2314 RG, and 2665 BVA; lanes 41 to 54 are C. upsaliensis strains ATCC 43954, 12030, 13064, 13950, 14013, 14080, 14506, 14510, 14526, 14529, 14530, 14532, 14967, and 15172; lane 55 to 61 are C. sputorum subsp. bubulus ATCC 33562, H. cinaedi ATCC 35683, H. pylori (clinical isolate), A. nitrofigilis ATCC 33309, E. coli ATCC 9637, P. aeruginosa ATCC 10145, and S. sonnei ATCC 11803, respectively; and finally, lanes 62 to 68 are the glyA recombinant plasmids of C. jejuni ATCC 33560, C. coli ATCC 33559, C. lari ATCC 35221, C. upsaliensis ATCC 43954, H. cinaedi ATCC 35683, H. pylori (clinical isolate), and A. nitrofigilis ATCC 33309, respectively. Lane designations are maintained for all figures.
Figure 3. Autoradiographs of the Southern hybridizations testing the species-specificity of the series 1 probes. Unless noted, no cross-hybridizations were observed on the autoradiographs after 20 hours of exposure. Lane assignments are as described in Figure 2.

(A) CJATC-1 hybridizing to C. jejuni strains. Autoradiograph after 4 hours of exposure. After 21 hours, cross-hybridization to C. coli was observed.

(B) CC-1 hybridizing to C. coli strains. Autoradiograph after 2 hours of exposure. After 20 hours, cross-hybridization to C. jejuni and C. upsaliensis was observed.

(C) CL-1 hybridizing to C. lari strains. Autoradiograph after 2 hours of exposure. After 20 hours of exposure, cross-hybridization to A. nitrofigilis was observed.

(D) CU-1 hybridizing to C. upsaliensis strains. Autoradiograph after 4 hours of exposure.
Figure 4. Autoradiographs of the Southern hybridizations testing the species-specificity of the series 2 probes. All autoradiographs were taken after 22 hours of exposure. Lane assignments are described in Figure 2.

(A) CJATC-2 hybridizing to C. jejuni strains. Cross-hybridization to C. upsaliensis was observed.

(B) CC-2 hybridizing to C. coli strains. No cross-hybridization was observed.

(C) CL-2 hybridizing to C. lari strains. Cross-hybridization to C. upsaliensis was observed.

(D) CU-2 hybridizing to C. upsaliensis strains. Cross-hybridization to C. jejuni was observed.
Figure 5. Sensitivity of the PCR/hybridization strategy.

(A) PCR products resulting from the various amounts (in µg) of template C. jejuni ATCC33560 genomic DNA used. Lanes a to g are: 1x10⁻³µg, 8x10⁻⁴µg, 6x10⁻⁵µg, 4x10⁻⁶µg, 2x10⁻⁶µg, and no DNA.

(B) Autoradiograph of the Southern hybridization using the CJATC-1 probe. The probe can detect one tenth of the PCR products from the 4x10⁻⁶µg of template DNA.
APPENDIX 2
(taken from Olson, C.L., 1987)
STANDARD NORMAL DISTRIBUTION

<table>
<thead>
<tr>
<th>$F(z_a)$</th>
<th>$z_a$</th>
<th>$F(-z_a)$</th>
<th>$z_a$</th>
<th>$F(z_a)$</th>
<th>$z_a$</th>
<th>$F(-z_a)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.10</td>
<td>0.10</td>
<td>-0.10</td>
<td>0.10</td>
<td>-0.10</td>
<td>0.10</td>
<td>-0.10</td>
</tr>
<tr>
<td>-0.05</td>
<td>0.05</td>
<td>-0.05</td>
<td>0.05</td>
<td>-0.05</td>
<td>0.05</td>
<td>-0.05</td>
</tr>
<tr>
<td>-0.02</td>
<td>0.02</td>
<td>-0.02</td>
<td>0.02</td>
<td>-0.02</td>
<td>0.02</td>
<td>-0.02</td>
</tr>
<tr>
<td>0.01</td>
<td>0.01</td>
<td></td>
<td>0.01</td>
<td></td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>0.02</td>
<td></td>
<td>0.02</td>
<td></td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>0.03</td>
<td></td>
<td>0.03</td>
<td></td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td>0.05</td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.10</td>
<td></td>
<td>0.10</td>
<td></td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.20</td>
<td></td>
<td>0.20</td>
<td></td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.30</td>
<td></td>
<td>0.30</td>
<td></td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>0.40</td>
<td>0.40</td>
<td></td>
<td>0.40</td>
<td></td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
<td></td>
<td>0.50</td>
<td></td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>0.60</td>
<td>0.60</td>
<td></td>
<td>0.60</td>
<td></td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>0.70</td>
<td>0.70</td>
<td></td>
<td>0.70</td>
<td></td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>0.80</td>
<td>0.80</td>
<td></td>
<td>0.80</td>
<td></td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>0.90</td>
<td>0.90</td>
<td></td>
<td>0.90</td>
<td></td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

A2-1
<table>
<thead>
<tr>
<th>$F(x_0)$</th>
<th>$z_0$</th>
<th>$A$</th>
<th>$F(x_0)$</th>
<th>$z_0$</th>
<th>$A$</th>
<th>$F(x_0)$</th>
<th>$z_0$</th>
<th>$A$</th>
<th>$F(x_0)$</th>
<th>$z_0$</th>
<th>$A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>.9772</td>
<td>.203</td>
<td>.0228</td>
<td>.9938</td>
<td>.250</td>
<td>.0042</td>
<td>.9986</td>
<td>.300</td>
<td>.0015</td>
<td>.9997</td>
<td>.500</td>
<td>.0025</td>
</tr>
<tr>
<td>.9788</td>
<td>.217</td>
<td>.0222</td>
<td>.9940</td>
<td>.251</td>
<td>.0040</td>
<td>.9989</td>
<td>.301</td>
<td>.0013</td>
<td>.9997</td>
<td>.501</td>
<td>.0024</td>
</tr>
<tr>
<td>.9788</td>
<td>.223</td>
<td>.0212</td>
<td>.9953</td>
<td>.253</td>
<td>.0037</td>
<td>.9987</td>
<td>.303</td>
<td>.0011</td>
<td>.9997</td>
<td>.503</td>
<td>.0022</td>
</tr>
<tr>
<td>.9793</td>
<td>.205</td>
<td>.0207</td>
<td>.9955</td>
<td>.254</td>
<td>.0035</td>
<td>.9987</td>
<td>.304</td>
<td>.0010</td>
<td>.9997</td>
<td>.504</td>
<td>.0021</td>
</tr>
<tr>
<td>.9798</td>
<td>.225</td>
<td>.0202</td>
<td>.9956</td>
<td>.255</td>
<td>.0034</td>
<td>.9986</td>
<td>.305</td>
<td>.0009</td>
<td>.9997</td>
<td>.505</td>
<td>.0020</td>
</tr>
<tr>
<td>.9803</td>
<td>.208</td>
<td>.0207</td>
<td>.9958</td>
<td>.256</td>
<td>.0032</td>
<td>.9986</td>
<td>.306</td>
<td>.0008</td>
<td>.9997</td>
<td>.506</td>
<td>.0019</td>
</tr>
<tr>
<td>.9808</td>
<td>.227</td>
<td>.0202</td>
<td>.9960</td>
<td>.257</td>
<td>.0031</td>
<td>.9986</td>
<td>.307</td>
<td>.0007</td>
<td>.9997</td>
<td>.507</td>
<td>.0018</td>
</tr>
<tr>
<td>.9813</td>
<td>.210</td>
<td>.0198</td>
<td>.9961</td>
<td>.258</td>
<td>.0030</td>
<td>.9985</td>
<td>.308</td>
<td>.0006</td>
<td>.9997</td>
<td>.508</td>
<td>.0017</td>
</tr>
<tr>
<td>.9817</td>
<td>.229</td>
<td>.0193</td>
<td>.9963</td>
<td>.259</td>
<td>.0029</td>
<td>.9985</td>
<td>.309</td>
<td>.0005</td>
<td>.9997</td>
<td>.509</td>
<td>.0016</td>
</tr>
<tr>
<td>.9822</td>
<td>.212</td>
<td>.0189</td>
<td>.9965</td>
<td>.260</td>
<td>.0028</td>
<td>.9985</td>
<td>.310</td>
<td>.0004</td>
<td>.9997</td>
<td>.510</td>
<td>.0015</td>
</tr>
<tr>
<td>.9826</td>
<td>.225</td>
<td>.0184</td>
<td>.9967</td>
<td>.261</td>
<td>.0027</td>
<td>.9985</td>
<td>.311</td>
<td>.0003</td>
<td>.9997</td>
<td>.511</td>
<td>.0014</td>
</tr>
<tr>
<td>.9830</td>
<td>.218</td>
<td>.0180</td>
<td>.9969</td>
<td>.262</td>
<td>.0026</td>
<td>.9985</td>
<td>.312</td>
<td>.0002</td>
<td>.9997</td>
<td>.512</td>
<td>.0013</td>
</tr>
<tr>
<td>.9834</td>
<td>.231</td>
<td>.0176</td>
<td>.9971</td>
<td>.263</td>
<td>.0025</td>
<td>.9985</td>
<td>.313</td>
<td>.0001</td>
<td>.9997</td>
<td>.513</td>
<td>.0012</td>
</tr>
<tr>
<td>.9838</td>
<td>.244</td>
<td>.0172</td>
<td>.9973</td>
<td>.264</td>
<td>.0024</td>
<td>.9985</td>
<td>.314</td>
<td>.0001</td>
<td>.9997</td>
<td>.514</td>
<td>.0011</td>
</tr>
<tr>
<td>.9842</td>
<td>.258</td>
<td>.0168</td>
<td>.9975</td>
<td>.265</td>
<td>.0023</td>
<td>.9985</td>
<td>.315</td>
<td>.0000</td>
<td>.9997</td>
<td>.515</td>
<td>.0010</td>
</tr>
<tr>
<td>.9846</td>
<td>.271</td>
<td>.0164</td>
<td>.9976</td>
<td>.266</td>
<td>.0022</td>
<td>.9985</td>
<td>.316</td>
<td>.0000</td>
<td>.9997</td>
<td>.516</td>
<td>.0009</td>
</tr>
<tr>
<td>.9850</td>
<td>.285</td>
<td>.0160</td>
<td>.9978</td>
<td>.267</td>
<td>.0021</td>
<td>.9985</td>
<td>.317</td>
<td>.0000</td>
<td>.9997</td>
<td>.517</td>
<td>.0008</td>
</tr>
<tr>
<td>.9854</td>
<td>.299</td>
<td>.0156</td>
<td>.9979</td>
<td>.268</td>
<td>.0020</td>
<td>.9985</td>
<td>.318</td>
<td>.0000</td>
<td>.9997</td>
<td>.518</td>
<td>.0007</td>
</tr>
<tr>
<td>.9857</td>
<td>.312</td>
<td>.0152</td>
<td>.9981</td>
<td>.269</td>
<td>.0019</td>
<td>.9985</td>
<td>.319</td>
<td>.0000</td>
<td>.9997</td>
<td>.519</td>
<td>.0006</td>
</tr>
<tr>
<td>.9861</td>
<td>.325</td>
<td>.0148</td>
<td>.9982</td>
<td>.270</td>
<td>.0018</td>
<td>.9984</td>
<td>.320</td>
<td>.0000</td>
<td>.9997</td>
<td>.520</td>
<td>.0005</td>
</tr>
<tr>
<td>.9865</td>
<td>.339</td>
<td>.0144</td>
<td>.9983</td>
<td>.271</td>
<td>.0017</td>
<td>.9984</td>
<td>.321</td>
<td>.0000</td>
<td>.9997</td>
<td>.521</td>
<td>.0004</td>
</tr>
<tr>
<td>.9868</td>
<td>.353</td>
<td>.0140</td>
<td>.9984</td>
<td>.272</td>
<td>.0016</td>
<td>.9984</td>
<td>.322</td>
<td>.0000</td>
<td>.9997</td>
<td>.522</td>
<td>.0003</td>
</tr>
<tr>
<td>.9871</td>
<td>.367</td>
<td>.0136</td>
<td>.9985</td>
<td>.273</td>
<td>.0015</td>
<td>.9984</td>
<td>.323</td>
<td>.0000</td>
<td>.9997</td>
<td>.523</td>
<td>.0002</td>
</tr>
<tr>
<td>.9874</td>
<td>.381</td>
<td>.0132</td>
<td>.9986</td>
<td>.274</td>
<td>.0014</td>
<td>.9984</td>
<td>.324</td>
<td>.0000</td>
<td>.9997</td>
<td>.524</td>
<td>.0001</td>
</tr>
<tr>
<td>.9877</td>
<td>.395</td>
<td>.0128</td>
<td>.9987</td>
<td>.275</td>
<td>.0013</td>
<td>.9984</td>
<td>.325</td>
<td>.0000</td>
<td>.9997</td>
<td>.525</td>
<td>.0000</td>
</tr>
<tr>
<td>.9880</td>
<td>.410</td>
<td>.0124</td>
<td>.9988</td>
<td>.276</td>
<td>.0012</td>
<td>.9984</td>
<td>.326</td>
<td>.0000</td>
<td>.9997</td>
<td>.526</td>
<td>.0000</td>
</tr>
<tr>
<td>.9883</td>
<td>.424</td>
<td>.0120</td>
<td>.9989</td>
<td>.277</td>
<td>.0011</td>
<td>.9984</td>
<td>.327</td>
<td>.0000</td>
<td>.9997</td>
<td>.527</td>
<td>.0000</td>
</tr>
<tr>
<td>.9886</td>
<td>.438</td>
<td>.0116</td>
<td>.9990</td>
<td>.278</td>
<td>.0010</td>
<td>.9984</td>
<td>.328</td>
<td>.0000</td>
<td>.9997</td>
<td>.528</td>
<td>.0000</td>
</tr>
<tr>
<td>.9888</td>
<td>.453</td>
<td>.0112</td>
<td>.9991</td>
<td>.279</td>
<td>.0009</td>
<td>.9984</td>
<td>.329</td>
<td>.0000</td>
<td>.9997</td>
<td>.529</td>
<td>.0000</td>
</tr>
<tr>
<td>.9891</td>
<td>.468</td>
<td>.0108</td>
<td>.9992</td>
<td>.280</td>
<td>.0008</td>
<td>.9984</td>
<td>.330</td>
<td>.0000</td>
<td>.9997</td>
<td>.530</td>
<td>.0000</td>
</tr>
<tr>
<td>.9893</td>
<td>.482</td>
<td>.0104</td>
<td>.9993</td>
<td>.281</td>
<td>.0007</td>
<td>.9984</td>
<td>.331</td>
<td>.0000</td>
<td>.9997</td>
<td>.531</td>
<td>.0000</td>
</tr>
<tr>
<td>.9895</td>
<td>.497</td>
<td>.0100</td>
<td>.9994</td>
<td>.282</td>
<td>.0006</td>
<td>.9984</td>
<td>.332</td>
<td>.0000</td>
<td>.9997</td>
<td>.532</td>
<td>.0000</td>
</tr>
<tr>
<td>.9897</td>
<td>.512</td>
<td>.0096</td>
<td>.9995</td>
<td>.283</td>
<td>.0005</td>
<td>.9984</td>
<td>.333</td>
<td>.0000</td>
<td>.9997</td>
<td>.533</td>
<td>.0000</td>
</tr>
<tr>
<td>.9899</td>
<td>.527</td>
<td>.0092</td>
<td>.9996</td>
<td>.284</td>
<td>.0004</td>
<td>.9984</td>
<td>.334</td>
<td>.0000</td>
<td>.9997</td>
<td>.534</td>
<td>.0000</td>
</tr>
<tr>
<td>.9901</td>
<td>.542</td>
<td>.0088</td>
<td>.9997</td>
<td>.285</td>
<td>.0003</td>
<td>.9984</td>
<td>.335</td>
<td>.0000</td>
<td>.9997</td>
<td>.535</td>
<td>.0000</td>
</tr>
<tr>
<td>.9903</td>
<td>.557</td>
<td>.0084</td>
<td>.9998</td>
<td>.286</td>
<td>.0002</td>
<td>.9984</td>
<td>.336</td>
<td>.0000</td>
<td>.9997</td>
<td>.536</td>
<td>.0000</td>
</tr>
<tr>
<td>.9905</td>
<td>.572</td>
<td>.0080</td>
<td>.9998</td>
<td>.287</td>
<td>.0001</td>
<td>.9984</td>
<td>.337</td>
<td>.0000</td>
<td>.9997</td>
<td>.537</td>
<td>.0000</td>
</tr>
<tr>
<td>.9907</td>
<td>.587</td>
<td>.0076</td>
<td>.9999</td>
<td>.288</td>
<td>.0000</td>
<td>.9984</td>
<td>.338</td>
<td>.0000</td>
<td>.9997</td>
<td>.538</td>
<td>.0000</td>
</tr>
</tbody>
</table>

### CRITICAL VALUES OF THE CHI-SQUARE DISTRIBUTION

<table>
<thead>
<tr>
<th>df</th>
<th>$x^2_{0.05}$</th>
<th>$x^2_{0.025}$</th>
<th>$x^2_{0.01}$</th>
<th>$x^2_{0.005}$</th>
<th>$x^2_{0.001}$</th>
<th>$x^2_{0.0005}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>11.070</td>
<td>15.086</td>
<td>18.907</td>
<td>22.626</td>
<td>24.323</td>
<td>26.151</td>
</tr>
<tr>
<td>7</td>
<td>14.292</td>
<td>18.467</td>
<td>22.875</td>
<td>26.850</td>
<td>27.924</td>
<td>29.632</td>
</tr>
<tr>
<td>9</td>
<td>17.496</td>
<td>21.596</td>
<td>26.850</td>
<td>31.074</td>
<td>31.367</td>
<td>33.079</td>
</tr>
<tr>
<td>10</td>
<td>19.090</td>
<td>23.107</td>
<td>28.840</td>
<td>33.186</td>
<td>33.079</td>
<td>34.779</td>
</tr>
<tr>
<td>11</td>
<td>20.682</td>
<td>24.582</td>
<td>30.830</td>
<td>35.298</td>
<td>34.779</td>
<td>36.469</td>
</tr>
<tr>
<td>12</td>
<td>22.275</td>
<td>26.029</td>
<td>32.819</td>
<td>37.410</td>
<td>36.469</td>
<td>38.147</td>
</tr>
<tr>
<td>13</td>
<td>23.867</td>
<td>27.450</td>
<td>34.807</td>
<td>39.522</td>
<td>38.147</td>
<td>39.816</td>
</tr>
<tr>
<td>14</td>
<td>25.459</td>
<td>28.857</td>
<td>36.795</td>
<td>41.634</td>
<td>40.816</td>
<td>41.475</td>
</tr>
<tr>
<td>15</td>
<td>27.051</td>
<td>30.246</td>
<td>38.782</td>
<td>43.746</td>
<td>42.475</td>
<td>43.123</td>
</tr>
<tr>
<td>16</td>
<td>28.642</td>
<td>31.624</td>
<td>40.770</td>
<td>45.858</td>
<td>44.123</td>
<td>44.760</td>
</tr>
<tr>
<td>17</td>
<td>30.234</td>
<td>33.001</td>
<td>42.757</td>
<td>47.969</td>
<td>45.760</td>
<td>46.398</td>
</tr>
<tr>
<td>18</td>
<td>31.826</td>
<td>34.376</td>
<td>44.744</td>
<td>49.081</td>
<td>47.407</td>
<td>48.035</td>
</tr>
<tr>
<td>19</td>
<td>33.417</td>
<td>35.749</td>
<td>46.730</td>
<td>51.193</td>
<td>48.947</td>
<td>49.672</td>
</tr>
<tr>
<td>20</td>
<td>35.008</td>
<td>37.120</td>
<td>48.717</td>
<td>53.294</td>
<td>50.484</td>
<td>51.308</td>
</tr>
<tr>
<td>21</td>
<td>36.600</td>
<td>38.489</td>
<td>50.703</td>
<td>55.396</td>
<td>51.954</td>
<td>52.946</td>
</tr>
<tr>
<td>22</td>
<td>38.191</td>
<td>39.856</td>
<td>52.689</td>
<td>57.497</td>
<td>53.421</td>
<td>54.583</td>
</tr>
<tr>
<td>23</td>
<td>39.783</td>
<td>41.221</td>
<td>54.675</td>
<td>59.598</td>
<td>54.832</td>
<td>56.219</td>
</tr>
<tr>
<td>24</td>
<td>41.374</td>
<td>42.586</td>
<td>56.660</td>
<td>61.699</td>
<td>56.222</td>
<td>57.856</td>
</tr>
<tr>
<td>25</td>
<td>42.965</td>
<td>43.950</td>
<td>58.646</td>
<td>63.790</td>
<td>57.601</td>
<td>59.492</td>
</tr>
<tr>
<td>26</td>
<td>44.556</td>
<td>45.313</td>
<td>60.631</td>
<td>65.891</td>
<td>58.971</td>
<td>61.128</td>
</tr>
<tr>
<td>27</td>
<td>46.147</td>
<td>46.676</td>
<td>62.616</td>
<td>67.992</td>
<td>60.331</td>
<td>62.764</td>
</tr>
<tr>
<td>28</td>
<td>47.738</td>
<td>48.039</td>
<td>64.601</td>
<td>69.092</td>
<td>61.691</td>
<td>64.400</td>
</tr>
<tr>
<td>29</td>
<td>49.329</td>
<td>49.402</td>
<td>66.586</td>
<td>71.193</td>
<td>63.040</td>
<td>66.036</td>
</tr>
<tr>
<td>30</td>
<td>50.920</td>
<td>50.764</td>
<td>68.571</td>
<td>73.294</td>
<td>64.390</td>
<td>67.672</td>
</tr>
</tbody>
</table>

When the number of degrees of freedom (df) is greater than 30, chi-square critical values may be approximated by

\[ x^2(df) = \left[ z_a + \sqrt{2(df - 1)} \right]^2. \]

IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved