

ABSTRACT

Title of Dissertation: *CAMPYLOBACTER JEJUNI/COLI* – HOST
INTESTINAL EPITHELIAL CELL INTERACTION

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Campylobacter jejuni/coli have been known to be major bacterial causes of human gastroenteritis worldwide for decades. Regarding its pathogenicity, little is known yet. A better understanding of the pathogenic mechanisms will provide important information, not only for generating molecular markers to differentiate pathogenic strains versus non-pathogenic ones; but also for developing rational strategies to prevent and control *Campylobacter*-caused disease. The objectives of this study were to characterize the pathogenic abilities of various *C. jejuni/coli* retail meat isolates, including their abilities to adhere to, invade into and transmigrate across human epithelial cells, to examine the role of NF- κ B pathway in IL-8 secretion induced by *Campylobacter*, and to identify *C. jejuni*-specific adherence/invasion genes during host pathogen interaction. It was found that the adherence and invasiveness of total 43 *Campylobacter* retail meat isolates in human intestinal epithelial T84 cell model indicated that *C. jejuni/coli* present in retail meat were considerably diverse in their ability to adhere to and invade human epithelial cells. Meanwhile, eight putative virulence genes, determined by PCR, were shown to be widespread among the *Campylobacter* isolates. *C. jejuni/coli*-induced proinflammatory

cytokine Interleukin (IL)-8 secretion in polarized human colonic epithelial cells T84 was examined, and the role of NF- κ B pathway in *Campylobacter*-induced IL-8 secretion was determined. Data suggested that *C. jejuni/coli* induce basolateral-polarized secretion of IL-8 in human intestinal epithelial cells, and *C. jejuni*-induced IL-8 secretion is NF- κ B-dependent. The effort to identify *C. jejuni*-specific adherence/invasion genes during host pathogen interaction by using restriction fragment differential display PCR (RFDD-PCR) has been made. As a result, it was not successful. However this study still provides useful information and experience on the application of this technique for prokaryotic gene expression analysis during host pathogen interaction, which remains an unexplored area. In summary, *Campylobacter* retail meat isolates exhibited wide diversity in cell culture model in the ability of adherence, invasion and transmigration. As the first line defense, intestinal epithelium activates NF- κ B and secretes proinflammatory cytokine IL-8 in response to *Campylobacter* infection. Multiple virulence factors have roles in *Campylobacter*-intestinal epithelial cell interaction.

CAMPYLOBACTER JEJUNI/COLI – HOST INTESTINAL EPITHELIAL
CELL INTERACTION

by

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ATCC	American Type Culture Collection
BAM	Bacteriological Analytical Manual
CDC	Center for Disease Control and Prevention
CDT	Cytolethal distending toxin
DD	Differential display
DME/F12	Dulbecco's modified Eagle's medium and Ham's F12 medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immnosorbent assay
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
GBS	Guillian Barré syndrom
IL	Interleukin
LB	Luria-Bertani
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCCDA	Modified campy blood-free selective agar
MF	Microfilament
MH	Mueller-Hinton
MOI	Multiplicity of infection
MT	Microtubule
NF	Nuclear factor
OMP	Out membrane protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis

PGN	Peptidoglycan
PMN	Polymorphonuclear leukocyte
RAP-PCR	RNA arbitrarily primed PCR
RFDD-PCR	Restriction fragment differential display PCR
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TER	Transepithelial electrical resistance
TLR	Toll-like receptor
TPCK	N- α -p-tosyl-L-phenylalanine chloromethyl ketone
USDA	United States Department of Agriculture

CHAPTER I. LITERATURE REVIEW

Overview

Foodborne disease is caused by consumption of contaminated foods or beverages. More than 250 foodborne diseases have been described, in which most of them are infectious. An estimated approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths related to foodborne diseases occur each year in the United States (150). Known pathogens account for an estimated 14 million cases, 60,000 hospitalizations, and 1,800 deaths annually (150). *Campylobacter* is one of the most common bacterial causes of foodborne gastroenteritis in the United States, although the incidence of *Campylobacter* infection decreased by 28% from 1996 through 2003 (2). Currently, the Center for Disease Control and Prevention (CDC) estimates that about 2.4 million cases of *Campylobacter* infection, involving almost 1% of the entire population, and 124 fatal cases occur in the United States each year (74). A detailed study of 53 routinely diagnosed sporadic cases in the United Kingdom gave a figure of \$480 per case for health care and lost productivity and more than twice this sum if allowance was made for “pain and suffering” (207). Costs in United States are likely to be at least as high. Therefore, *Campylobacter* infection is consequently responsible for a major public health and economic burden (214).

Campylobacter jejuni was first isolated as *Vibrio fetus* in 1909 from spontaneous abortions in livestock (118). *Campylobacter* infections were not recognized until the development and increasingly widespread use of selective media for isolation of

Campylobacter from stool sample in the 1970s. By the 1980s, it had been determined that *Campylobacter* species are one of the most common bacterial causes of gastroenteritis worldwide. In the following decade substantial advances have been made in the classification, identification, epidemiology, diagnosis, and isolation technology, and the pathogenesis and molecular biology of *Campylobacter* species.

General characteristics of *Campylobacter*

Campylobacter species are Gram-negative slender rods with pleomorphic helical shape. The organisms are motile by means of unipolar or bipolar flagella. Most species grow best at 42°C and need microaerophilic (5% O₂, 10% CO₂, 85% N₂) condition. Biochemical reactions by which *Campylobacter* species may be differentiated are relatively few due to the inability to ferment or oxidize the usual carbohydrate substances available in the diagnostic laboratory (61, 162, 216).

The genus *Campylobacter* currently includes 15 species and 6 subspecies by rRNA sequencing (Table I-1) (184). Among 15 species, 12 species are associated with human diseases. Remarkably, 99% of reported *Campylobacter* enteritis are caused by *Campylobacter jejuni* (*C. jejuni*) followed by *Campylobacter coli* (*C. coli*) in the United States (74). *C. jejuni* is a simply referral of *C. jejuni* subsp. *jejuni*. *C. jejuni* usually carries as a commensal by a wide range of animal hosts, including chickens, cattle, pigs, sheep, dogs, and ostriches (206). *C. coli* was first isolated from pigs afflicted with infectious dysentery (57). It remains a frequently encountered species in pigs.

Table I-1. Differential reactions and characteristics for species of the genus *Campylobacter*^a

Species	Reactions and characteristics ^b										
	Catalase	Nitrate	H ₂ S (TSI)	Hippurate	Indoxyl acetate	Growth				0.1% TMAO (anaerobic)	G+C content (mol %)
						25°C	37°C	42°C	1% Glycine		
<i>C. fetus</i> subsp. <i>fetus</i>	+	+	-	-	-	+	+	(-)	+	-	33-34
<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	-	-	-	+	+	-	-	-	33-34
<i>C. Hyointestinalis</i>	+	+	+	-	-	(+)	+	+	+	+	35-36
<i>C. jejuni</i>	+	+	-	+	+	-	+	+	+	-	30-32
<i>C. coli</i>	+	+	-	-	+	-	+	+	+	-	31-33
<i>C. laridis</i>	+	+	-	-	-	-	+	+	+	+	31-33
<i>C. upsaliensis</i>	+	+	-	-	ND	-	+	+	-	-	35-36
<i>C. cinaedi</i>	(-)	+	-	-	(-)	-	+	-	+	-	37-38
<i>C. fennelliae</i>	+	+	-	-	+	-	+	-	+	-	37-38
<i>C. cryaerophila</i>	+	-	-	-	+	+	+	-	-	ND	29-30
<i>C. nitrofigilis</i>	+	+	-	-	-	+	+	-	-	ND	28-29
<i>C. sputorum</i>											
Biovar <i>sputorum</i>	-	+	(+)	-	-	-	+	+	+	d	31-32
Biovar <i>bubulus</i>	-	+	+	-	-	-	+	+	+	+	31-32
Biovar <i>faecalis</i>	+	+	+	-	-	-	+	+	+	+	32-33
Biovar	-	+	+	-	-	-	+	+	+	+	31-32
<i>paraureolyticus</i>											
<i>C. mucosalis</i>	-	+	+	-	-	+	+	+	+	-	38-39
<i>C. concisus</i>	-	+	+	-	ND	-	+	+	+	-	38-39
<i>C. pylori</i>	+	d	-	-	-	-	+	+	d	ND	36-37
<i>C. hyoilei</i>	+	+	+	-	ND	-	+	+	+	ND	34-35

^a Data were obtained from references (7, 28, 37, 75, 114, 147, 158, 169, 174, 184, 191, 192); ^b +, Positive reaction; -, negative reaction; ND, no test results found; (+), most strains positive but a low percentage negative; (-), most strains negative but some positive or weakly positive; d, different reactions.

Clinical characteristics of *Campylobacter* gastroenteritis

The clinical manifestation of patients with *C. jejuni* infection differs between developing and industrialized countries. In the industrialized world, most commonly, the usual manifestation of *Campylobacter* enteritis is a very unpleasant attack of acute diarrhea lasting about five days. The characteristic pathology is an acute inflammatory enterocolitis; stool microscopy almost invariably shows the presence of cellular exudates, and frank blood is visible in the stools of about one-quarter of patients. Fever, vomiting, and abdominal cramps are common in the early stages of the disease (175). However, in the developing countries, infection could be asymptomatic or mild noninflammatory diarrhea, predominantly affecting young children (100). But strains isolated from such children, who are symptomatic or not, produce acute inflammatory diseases in nonimmune visitors from developed countries. The explanation for the different disease expression in developing countries and developed countries is the early acquisition of immunity by children exposed to hyperendemic infection. Following gastroenteritis, late onset complications include pancreatitis, meningitis, endocarditis, septic arthritis, osteomyelitis, and neonatal sepsis. Most recently, *C. jejuni* is also been shown to associated with immunoproliferative small intestinal disease (135). Guillain-Barré syndrome (GBS) is the most important postinfectious complication of *C. jejuni*. GBS is an acute, immune system-mediated polyneuropathy, leading to ascending paralysis. GBS affects 1-2 persons per 100,000-population in the United States each year. The risk of developing GBS is increased after infection with certain *Campylobacter* serotypes in a certain geographic area, Penner type O:19 in the United States (10) and O: 41 in South

Africa (8). However, the severity of *C. jejuni* infection is not associated with an risk of GBS development (130). Carbohydrate mimicry between human ganglioside GM1 and *C. jejuni* lipooligosaccharide appears to be the key of GBS (233).

Epidemiology of *Campylobacter*

Campylobacter sporadic infections in humans far outnumber the cases linked to outbreaks (181). The age and sex distributions of *Campylobacter* infections are distinctive from other bacterial enteric pathogens. In the United States and industrialized countries, peak incidence occurs in children below one year of age and young adults, at 15-44 years old and males take preponderance among infected persons (74). The reasons for these distributions remain unknown. A distinct seasonality in *Campylobacter* transmission is present worldwide, with most showing a peak in spring (199). The timing of infection peak is weakly associated with high temperatures three months previously (199).

The most important source leading to *Campylobacter* infection in the United States and other industrialized nations remains the consumption and handling of chicken. Studies showed in many parts of the United States, Great Britain, 50%-70% of all *Campylobacter* infections have been attributed to consumption of chicken (3, 86). It should not be surprising in light of the frequency with which poultry products are consumed and the high contamination of chicken carcasses with *Campylobacter* (70%-90%) (38, 163, 235). Furthermore, seasonal variation, with peak period of

Campylobacter contamination of fresh chicken carcasses in June (152) may contribute to the seasonality of human *Campylobacter* infection. Unpasteurized milk and non-chlorinated water are also the sources of *Campylobacter* infection (35, 74, 156). Transmission of *Campylobacter* to humans is mainly via direct contact with contaminated animals or carcasses, and ingestion of contaminated food and water due to the relatively low infectious dose (500 organisms) (1). *Campylobacter* contamination of fresh produce also poses a risk to consumers (112).

The epidemiology of *Campylobacter* infections in developing countries is hyperendemic in young children and asymptomatic carriage in adults, quite different from developed countries. Nevertheless, *Campylobacter* remains one of the most common bacterial causes of diarrhea in both developed and developing countries.

Treatment and antimicrobial resistance

Campylobacter infections are generally self-limiting and do not require antibiotic treatment. Maintenance of hydration and electrolyte balance is the cornerstone of treatment for *Campylobacter* enteritis. Early antibiotic treatment can reduce the fecal shedding period effectively, but has nothing to do with the duration or severity of diarrhea, abdominal pain, or other symptoms (226). Erythromycin continues to be the drug of choice for severe or complicated enteritis (adult: 500mg b.i.d. for 5 days; children: 40mg/kg b.i.d. for 5 days). Despite decades of use, the rate of resistance of *Campylobacter* to erythromycin remains quite low (83).

Until a few years ago, ciprofloxacin, a fluoroquinolone drug, was considered as another choice, especially for the treatment of traveler's diarrhea. Unlike erythromycin, a rapidly increasing proportion of *Campylobacter* strains all over the world have been found to be fluoroquinolone-resistant (83, 197, 205). No surprisingly, this coincided with the approval and extension of the use of fluoroquinolones, such as enrofloxacin, in food animals in those countries (63). In the United States, the prophylactic treatment of poultry with fluoroquinolones plays a critical role in the problem of increasing prevalence of ciprofloxacin-resistant *Campylobacter* isolated from humans and poultry. The source of fluoroquinolone-resistant *Campylobacter* infections was the consumption of poultry colonized with resistant strains, rather than selection for *Campylobacter* resistance in the human gut after clinical treatment (102).

Isolation and detection of *Campylobacter*

According to USDA/FSIS/FDA-BAM, the isolation of *Campylobacter* from retail raw meat and poultry samples is always conducted under microaerophilic condition. Meat sample rinse solution is shaken overnight at 42°C with selective enrichment Bolton Broth, and plated on modified Campy blood-free selective agar (MCCDA). After 24-48 h of incubation at 42°C, suspected *Campylobacter* colonies, which have round, smooth with a defined edge to irregular spreading, thick to translucent, colorless to grayish appearance are identified by phase-contrast microscopy and biochemical test, including catalase and oxidase tests. However, this kind of traditional cultural detection method is slow and tedious. Therefore, several molecular-based identification methods have been

developed, such as DNA-DNA hybridization (46), species-specific polymerase chain reaction (PCR) (66), and real-time PCR (131, 232). The antigens of the *C. jejuni-C.coli* group are heterogeneous and complex. Serotyping methods for *Campylobacter* species are somewhat analogous to those for members of the family *Enterobacteriaceae*, which use tolerance to heat as a means to separate somatic, flagellar, and capsular antigens. To standardize serotyping on an international basis, the International Committee on Serotyping *Campylobacter* recommended in 1985 that the systems of Penner (134) and Lior (140), which are based on two sets of standardized strains from geographically diverse areas, be adopted and referred to as the HS and HL systems, respectively.

***Campylobacter*-host interaction**

A reasonable understanding of the general clinical, microbiological and epidemiological aspects of *Campylobacter* infection has been achieved since the selective growth media were developed in 1970s. However the mechanisms involved in pathogenesis are still not well understood partially due to the lack of suitable *in vivo* models. Based on clinical syndromes and experimental evidence, at least three mechanisms for gastrointestinal illness have been postulated: (i) intestinal colonization of ingested organisms and/or production of bacterial cytotoxin, inducing secretory diarrhea (51, 81, 106); (ii) bacterial invasion and proliferation within the intestinal epithelium (194, 231), causing cell damage and death, as well as inflammatory responses; and (iii) extraintestinal translocation (79, 127), in which the organisms cross the intestinal epithelium and migrate via the lymphatic system to various extraintestinal sites. These

possible mechanisms are not mutually exclusive, and any combination may play a role in *Campylobacter* infection depending on the host status and attributes of the infecting strain (different pathotype).

Bacterial diseases are typically the results of a complex set of interactions between the infecting bacteria and the defending host. Here, an overview of the interaction of *C. jejuni* with host cells is given as below.

Colonization and toxin production

The first visible interaction between *Campylobacter* and its host entails colonization of the mucus barrier or attachment to the eukaryotic cell surface. Since *C. jejuni* is part of the normal gut flora in many animals including avians, rodents and dogs, *C. jejuni* is certainly able to colonize the intestinal epithelium, which has also been demonstrated in many *in vivo* and *in vitro* studies (27, 64, 99, 104, 122, 170, 236). Cell adherence is a distinctly different process from invasion. The ability of *C. jejuni* to colonize the gastrointestinal tract by binding to epithelial cells has been proposed to be essential for disease production (136).

Numerous studies have been performed to describe potential *Campylobacter* adhesins that mediate binding to host cells. Reported possible adhesins include outer membrane proteins (OMPs), flagella, and glycoproteins (68, 113, 149). Also *C. jejuni* has been showed to bind to extracellular matrix components such as fibronectin, which is mediated by a conserved 37 kDa OMP, CadF (120). Flagellum has been implicated as adhesins or carriers of adhesins. Defect in FlaA, a major component of flagellum, led to

immotile bacteria and significant reduction in adhesion potential (171, 221). Mutant deficient in glycosylation was severely affected in its ability to colonize chicks (113). On the other hand, components in intestinal mucus may serve as important receptors for *C. jejuni* colonization. But the data are limited and make interpretation difficult by the multiple adherence factors of *Campylobacter* (119).

Cell adherence is not necessarily followed by cell invasion. Microorganisms adhere to the mucosa in the proximal small intestine and elaborate a toxin, resulting in a watery diarrhea, which is common in *Vibrio cholerae*. Infectious *Campylobacter* strains from patients with acute watery diarrhea, not other clinical syndromes produced cytotoxin (187), which supported that toxin production is a proposed mechanism in patients with watery diarrhea. Although our knowledge of *C. jejuni* toxins is still very limited, our understanding of one *Campylobacter* cytotoxin, cytolethal distending toxin (CDT), has been dramatically improved in recent years. CDT production was first observed by Anderson in a gastroenteritis associated *E. coli* strain in 1987 (13). In 1988, Johnson and Lior first reported CDT production by *Campylobacter* (105), which is a new heat-labile toxin cytolethal to CHO, Vero, Hela, and HEp-2 cells, termed a CLDT, and distinct from previously reported cytotoxin and cholera-like toxin. *Campylobacter* CLDT was negative in adult rabbit ligated ileal loops, suckling mouse and rabbit skin tests, and only could be neutralized by homologous rabbit antiserum. There was no correlation between CDT production and serotype or biotype or origin of the tested *Campylobacter* strains (105). The CDT produced by *C. jejuni* was shown to affect F-actin assembly within CHO (14) and cause G2 phase cell cycle block in sensitive

eukaryotic cells (225). These activities of CDT suggest possible contribution of CDT to diarrhea disease. However, the role of CDT in *C. jejuni* pathogenesis still has not been determined due to the lack of proper animal model.

Invasion and inflammatory response

Campylobacter are so-called invasive bacteria, which induce their own phagocytosis into cells that are normally nonphagocytic. The results of both *in vivo* (16, 17, 172, 194, 196) and *in vitro* (53, 65, 121) studies suggest that invasion is also a key component of *Campylobacter* pathogenesis.

Functional flagella and motility are believed to be essential for the internalization of *C. jejuni* *in vitro* (79). Either nonflagellated or nonmotile mutant of *C. jejuni* demonstrated markedly reduced levels of invasion into cultured cells (79).

Campylobacter requires nascent bacterial protein synthesis to facilitate bacterial invasion (126). Invasion was inhibited by 98% in the presence of rabbit antiserum raised against *Campylobacter* that was cultivated with INT407 cells (126). However, the ability to invade and the efficiency of invasion appear to be strain and cell line dependent (65, 119, 173, 187). Clinical isolates tend to be more invasive than nonclinical strains (122) using HEp-2 cells and extensive *in vitro* passage reduces the invasiveness of isolates (17). Everest *et al.* (65) found a significant, but not complete, correlation between strains obtained from patients with colitis and non-inflammatory diarrhea with respect to invasion of both Caco-2 and Hela cells. However, such correlation was not present in HEp-2 cells (215). Although there is still no consensus mechanism by which

Campylobacter invade host cells, the results suggested that *Campylobacter* strains have different host cytoskeletal requirements (either microfilament (MF)-dependent or microtubule (MT)-dependent) for invasion depending on the bacterial strain and host cell used (121, 160, 173).

Cell invasion is the primary mechanism of colon damage and diarrhea disease caused by *C. jejuni*, which was shown in infant *Macaca mulatta* model (196). Clinical evidence also showed existence of intestinal epithelial invasion in cases with bloody mucus and inflammatory cells in the stool. Bacterial epithelium invasion triggers host response to transform infected cells into strongly proinflammatory cells, which recruit acute inflammatory cells, primarily polymorphonuclear leukocytes (PMN), to the site. Interleukin-8 (IL-8) is one of the principal inflammatory mediators attracting and activating PMNs, neutrophils in particular (47, 94). Recent study showed that many strains of *Campylobacter* can induce secretion of IL-8 by INT 407 cells (90). The induction of IL-8 secretion required live cells of *Campylobacter* and appears to be associated with cell invasion (90) or cytolethal distending toxin (91). However, similar response can be induced by killed *Campylobacter* as well as live bacteria and do not depend on CDT in THP-1, human monocytes (109).

In addition to triggering IL-8 production and an acute inflammatory response, internalized *C. jejuni* can be maintained within endosomal vacuoles inside epithelial cells (53, 121, 196). Intracellular bacterial numbers began to decrease after internalization in HEp-2 cells due to phagosome-lysosome fusion (53), but increased in the absence of

gentamicin in the culture medium after 21h and persisted at the high level for >96h (121). The intracellular survival time observed in vitro is considerable and would allow for translocation to occur.

Intestinal translocation

Bacterial translocation enables the movement of viable bacteria across the epithelial barrier, where further extraintestinal dissemination can occur. Although animal models are necessary for studying intestinal translocation, polarized epithelial cell lines provide a simple and controlled experimental alternative to animal models. Polarized human colon carcinoma cells with differentiated apical and basolateral surfaces separated by tight or occluding junctions show the structural resemblance to the naïve intestine. *Campylobacter* has been observed to translocate across a polarized epithelial cell monolayer (34, 127). Different from *Salmonella* (71), *C. jejuni* can penetrate from the apical to the basolateral surface of polarized Caco-2 without a large scale loss in transepithelial electrical resistance (TER) (34, 127), which indicates the tight junction integrity. The transepithelial route remains undefined. Either transcellular pathway, or paracellular pathway or both routes that *Campylobacter* may take is strain-dependent (34, 127). Some strains also appear to transcytose without invasion (65) or have different rates of transepithelial trafficking (87). However, the role of translocation in *Campylobacter*-mediated disease requires further characterization due to limited data.

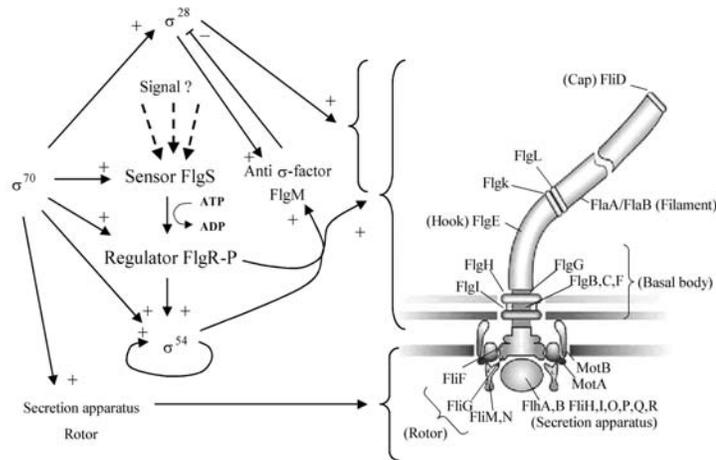
Molecular pathogenesis of *Campylobacter*

The completion of *C. jejuni* NCTC11168 whole genome sequence is a landmark in understanding of *Campylobacter* pathogenesis (178). During the last few years, research at the genome level opens the way for application of novel molecular techniques, like DNA microarray, resulting in novel and important insights into basic research on *Campylobacter*, a problematic pathogen with a small circular chromosome of 1.6-1.7 megabases. Genome-wide comparison showing gene diversity in *C. jejuni* is largely restricted to a small number of genomic regions, leaving large portions of genome stable (180, 212). These plasticity regions include locus for flagellin modification, protein glycosylation, capsule biosynthesis, as well as LOS biosynthesis (180, 212). The substantial progress in the understanding of virulence machinery of *C. jejuni* has been made and a detailed review follows.

Flagella

Campylobacter jejuni contains one or bi-polar flagella that cause the typical darting motility, and give moist appearance to colonies on agar plates. Like other bacteria, the flagellum assembly apparatus of *C. jejuni* is composed of a basal body, hook, and filament (Fig. I-1). The flagella filament is composed of two proteins, encoded by *flaA* and *flaB*; however, *flaA* is more preferential and expressed at higher level than *flaB*, which is regulated by σ^{54} promoter, and subject to environmental regulation (11). Motility and expression of *flaA* gene are essential for colonization of animal and cause disease (31, 222, 229).

Figure I-1. Model of *C. jejuni* flagellum apparatus and its regulation (229)



Moreover, the expression of *flaA* gene is necessary for maximal invasion of eukaryotic cells and for the translocation of *C. jejuni* across polarized cells (79, 221). The whole *fla* regulon in *C. jejuni* is regulated by FlgS/FlgR two component signal transduction system (229). Phosphorylated FlgR is needed to activate RpoN-dependent genes of which the products form the flagellum σ complex (229). *flgS*, *flgR* and *rpoN* are all early flagella genes and regulated by σ^{70} , and their mutants are unflagellated (229). FliD forms the cap structure at the tip of the external filament and plays essential role in polymerization of flagellin. FliD-defect mutant lacks flagella and is nonmotile (101). Similarly, defect in nonfilament structural components, hook protein FlgE2 and basal body proteins FlgB and FlgC, failed to synthesize FlaA and exhibited nonmotile phenotype (125).

Adhesion

A variety of putative *C. jejuni* adhesins have been identified. The *C. jejuni* genome sequence has clarified no pilus structures encoded on chromosome (178). Instead, flagella play a crucial role in adhesion as stated before. Fibronectin-binding

protein CadF, encoded by *cadF*, is required for binding of *C. jejuni* to intestinal epithelial cells (160). This interaction also involves host cell cytoskeleton polymerization and tyrosine phosphorylation signaling pathway, which promotes the internalization of *C. jejuni* (160). PEB1(182), (a homolog of Gram-negative ABC transport systems, involving amino acid transport) is another putative major cell binding factor. Recently, glycosylation system is implicated to be important for attachment to epithelial cells and colonization of chicks (113). *pglH* mutant, deficient in ability to glycosylate a number of *C. jejuni* protein except LOS and capsule, had significantly reduced ability to adhere to the Caco-2 cells and severely affected ability to colonize chicks (113).

Invasion

The invasiveness of this organism has been studied in a variety of cell lines, including the intestinal epithelial-derived cell lines INT407 and Caco2. Several new bacterial proteins have been shown to be required for invasion (126). Secreted CiaB protein, with similarities to type III secreted proteins, was translocated into cytoplasm of the host cell, and its null mutant was deficient in the secretion of other proteins (123). Therefore, CiaB protein secretion required functional flagella export apparatus (125). Recent study described that another protein, FlaC, in *C.jejuni* TGH9011, is dominantly found in the extracellular milieu (208). *flaC* mutant didn't affect motility and flagellum morphology, but was deficient in invasion of Hep-2 cells. Furthermore, flagella basal body gene *flgF* mutant or hook gene *flgE* mutant couldn't secret FlaC (208). All these indicated that FlaC is a protein secreted by flagella export apparatus and may play a role in cell invasion. Homologs of a type IV secretion system has been identified on a 37-kb

plasmid pVir in *C. jejuni* 81-176 (20), Type IV system, like *cag* pathogenicity island of *Helicobacter pylori*, is also used to inject bacterial protein into the cytosol of targeted cells by pathogens. However, only 10% of 58 fresh clinical *C. jejuni* isolates harbor the plasmid-encoded *VirB11* gene (20), and transfer of this plasmid to *C. jejuni* NCTC11168 did not reproduce the same phenotype, which suggested that pVir is not responsible for all *C. jejuni*-induced pathogenic effect. Recent report (217) supported that the pVir plasmid was significantly associated with the occurrence of blood in patient stool, a marker of invasive infection; but not associated with the greater occurrence of diarrhea, fever, pain, vomiting, or need for patient hospitalization, which in turn explained the low prevalence of the plasmid among strains.

No consensus has been established on the host factors involved in *Campylobacter* invasion yet. However, studies showed cytochalasin-D treated host cells dramatically decreased the number of *Campylobacter* internalization (122), suggesting actin polymerization is required for invasion. While the opposite studies reported cytochalasin-D insensitive invasion, and no involvement of microfilaments, but microtubules (173). Confusingly, there were reports on clathrin-independent endocytosis (228) and clathrin-coated pits (173) in *C. jejuni* invasion, as well as another controversial report showing no involvement of all previously indicated mechanisms in *C. jejuni* invasion of Caco-2 cells (195).

Toxin production

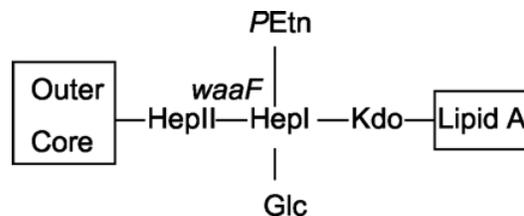
Historically, a variety of toxic activities have been attributed to *C. jejuni*. However, availability of genome sequence clarified *cdt* genes are the only identifiable toxin genes (178). It is clear that CDT toxin is a tripartite holotoxin, with CdtB as the active moiety and CdtA and CdtC necessary for the delivery of the enzymatically active subunit (133). CDT affected epithelial cells undergo cytodistension and cell cycle arrest in G₂/M phase (132). However, it is not yet clear what role CDT plays in *Campylobacter* infection *in vivo*.

Surface structures

All *C. jejuni* strains produce a surface-located glycolipid termed lipooligosaccharide (LOS), which has gained a lot attention due to a postulated role in the elicitation of GBS following *C. jejuni* infection. Now it is clear that carbohydrate mimicry of [Galβ1-3GalNAcβ1-4 (NeuAca2-3) Galβ1-] between *C. jejuni* LOS and human ganglioside GM1 causes GBS (233). Molecular mimicry of ganglioside by LOS is in part dependent on the presence of N-acetylneuraminic acid (sialic acid) in both structures. Sialic acid terminates oligosaccharide chains on mammalian and microbial cell surfaces through sialyltransferase CstII, playing critical roles in recognition and adherence (45). N-acetylneuraminic acid synthetase genes (*neuA*, *neuB* and *neuC*) involved in sialylation of LOS, and insertional mutagenesis of the *neuB* gene resulted in a LOS lacking N-acetylneuraminic acid (139). It has been proposed that *C. jejuni* lipooligosaccharide is composed of two covalently linked domains: lipid A, a hydrophobic anchor, and a nonrepeating core oligosaccharide consisting of an inner and

an outer core (15) (Fig. I-2). Two outer core mutants, lacking *N*-acetylneuraminic acid and terminal *N*-acetylgalactosamine respectively, didn't show any invasion deficiency (15), while a *waaF* mutant of *C. jejuni* 81-176, encoding heptosylthansferase II, involved in the addition of the second heptose displayed dramatic decrease in invasion of INT407 cells (111).

Figure I-2. Schematic showing the molecular structure of the LOS and highlighting the inner-core region. Hep, heptose; *PEtn*, phosphoethanolamine. Kdo, 3-deoxy-D-manno-2-octulosonic acid (111)



The analysis of *C. jejuni* genome sequence identified a group of genes (*kps*) involved in capsular polysaccharide biosynthesis (178). These genes located in one of the plasticity region of the chromosome (180) could serve as the molecular base of Penner serotyping and be involved in phase variation.

Campylobacter-induced cell signaling pathways and chemokine induction

Host cell signal transduction plays an important role in *Campylobacter* internalization. Inhibitors of host protein tyrosine phosphorylation significantly reduced invasion of Caco-2 cells (228). Biswas D *et al* (30) also reported that exposure of INT-407 cells to protein kinase inhibitors resulted in decreased invasion of these cells by *C. jejuni* in a dose-dependent manner, suggesting that protein phosphorylation is a prerequisite for *C. jejuni* internalization.

Infection by *C. jejuni* may lead to activation of the local immune response. IL-8 is a potent chemokine involved in neutrophil attraction and can be induced by *C. jejuni* (90, 91). The early-response transcription factor NF- κ B triggers the expression of genes associated with cellular immune and inflammatory responses. Co-culture of Hela cells with viable *C. jejuni* lead to the activation of the transcription factor NF- κ B (154), and a heat-stable cell free-extract of *C. jejuni* was reported to be able to activate NF- κ B through I κ B α degradation and DNA binding (154).

In vitro cell culture methods for investigating *Campylobacter* pathogenesis

Studying the mechanisms of *Campylobacter* pathogenesis is complicated by the lack of simple animal models that can mimic the disease seen in humans. Different animal models have been tried to delineate the mechanisms of *Campylobacter* infection, including ferret (26, 27, 72, 85), hamster (4, 98), mice (24, 70, 209), rabbit (39, 40), guinea pig (211), pig (33, 142, 218), chicken (194, 198), and monkey (107). However, *Campylobacter* only colonize chicken gut, but cannot cause infection; on the other hand, *Campylobacter* can cause much higher lethal rate, up to 53% (40), in rabbit model than in humans, which is only 0.05 per 1000 infections (8). For other animal models, although they develop some similar disease process as seen in humans, like leukocytic filtration, diffuse edema, mild to moderate diarrhea etc., no correlation can be found in virulence, or no association between model animal virulence and other symptoms (24), or no antigen-specific immune response can be detected (26, 72). Recently, New World Monkey was used to study *C. jejuni* infection and immunity (107). Intragastrically infection of

monkey with *C. jejuni* causes dose-related diarrhea and robust immune response (107). This model may be helpful for studying anti-*Campylobacter* vaccine efficacy. These animal models have been very useful in dissection the nature of host-bacterial interaction during infection. However, there is still no simple animal model that can resemble the etiology, course, pathology, and immunology of *Campylobacter* infection in humans. Instead of animal model, in vitro cell culture methods provide a useful alternative to investigate the interactions between *Campylobacter* and the host epithelium during infection. And no surprisingly, most our knowledge comes out from these experiments. Currently, studies on host-pathogen interactions in vitro usually use non-polarized epithelial culture. And apparently, the use of non-polarized cell lines has become a standard approach for studying *Campylobacter* pathogenesis. Different from unpolarized epithelium, polarized epithelial culture is actually the one that mimics the mucosal epithelium which is highly polarized with the apical membranes facing the luminal contents and basolateral membranes interfacing with the underlying cells in the lamina propria. The apical and basolateral membranes are very biochemically distinct with respect to transport functions and cellular localization of surface components such as Toll-like receptors, which are type I transmembrane proteins that appear to respond to different stimuli (19, 76, 164). Many pathogens have evolved different mechanisms to overcome epithelial barrier and establish infection (50). These include disruption of epithelial barrier function, transcytosing from one membrane domain to the other, or inducing cell movement such as neutrophil recruitment. When studying these processes *in vivo*, animal models often fail to or only partially mimic the disease observed in

humans and present a complex system in which many variables cannot be controlled. Therefore, in vitro transepithelial models that permit the study of a relevant biological surface have been developed, to integrate not only interactions between bacteria and epithelial cells but also, under certain conditions, to integrate a third cell type, such as neutrophils or dendritic cells. Such models are particularly useful for studying the bacteria-host relationship as it would occur in the microenvironment of the human epithelium to enhance our understanding of the unique strategies by which pathogenic bacteria exploit host cells to overcome the initial epithelial hurdle (143).

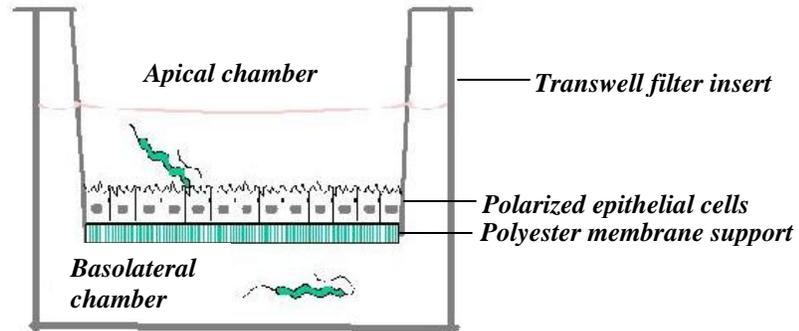
Choice of cultured epithelial cell line and multiplicity of infection

The in vitro gentamicin resistance assay has been frequently used as a standard method to measure cell penetration by bacteria. It is based on the principle that the aminoglycoside antibiotic gentamicin has a limited ability to penetrate eukaryotic cells. Internalized bacteria are thus protected and can be counted. Use of the gentamicin resistance assay has shown that invasiveness varies considerably depending on the strain (29, 87), but other variables like epithelial cell line and multiplicity of infection as well.

In vitro cell culture methods of measuring *Campylobacter* invasion have been done in a variety of cell lines with differing outcomes. Cell lines of human intestinal epithelium origin are considered to be most appropriate for studying *Campylobacter* invasion (119), although HEp-2, Int 407, HeLa and Caco-2 cells are those most commonly used (53, 65, 69, 173). Int 407 cells were originally cultured from human

embryonic intestine and developed into their present epithelial phenotype after 60 passages in culture (88). The HEP-2 cell line came from tumors produced after injection of rats with epidermoid carcinoma tissue. Both Int 407 and HEP-2 cell lines have no difference from the Human Negroid cervix epithelioid carcinoma cell line HeLa and be considered as derivatives of HeLa. Caco-2 cell line is a human colon adenocarcinoma that can differentiated into columnar cell monolayer on Transwell filter inserts with enterocytic morphology characterized by the formation of a brush border containing numerous microvilli, the presence of occluding junctions exclusively at the apical surface and a tall, more compact cell shape (92). Although similar characteristics have been observed for plastic-grown cells, the support medium and coating may cause Caco-2 cells to exhibit different features (56). T84 cell line is also a human colon adenocarcinoma that has been well characterized for use a model system for intestinal epithelial transport (Fig. I-3). It has been widely used for studying other enteric pathogenic bacteria (78, 95, 144, 146, 185) although not so popular in *Campylobacter* (159). And another advantage of T84 cell is that it has been well documented for Toll-like Receptors (TLRs) expressions, locations on cell surface and their trafficking pathway as well (41, 42, 89, 155). Anyway, it is important to understand the implications of using variable cell types for in vitro studies such as the gentamicin resistance assay. The state of epithelial cell growth and differentiation at the time of bacterial inoculation will also differ depending on the length of the culturing period post seeding. The resulting cell types may harbor different bacterial receptors or adopt different mechanisms required for bacterial invasion, thus influencing the resulting invasion phenotypes.

Figure I-3. *In vitro* T84 transepithelial cell model



The number of bacteria in the inoculum or multiplicity of infection (MOI) is another regulating factor in the ability of *Campylobacter* to invade. Studies have shown that the invasion efficiency (percentage of the inoculum that is internalized) is highest at low MOI (0.02), decreasing gradually as the MOI increases (97, 161). In order to minimize variability and to achieve maximal invasion levels for assay reproducibility, some laboratories prefer to use a high MOI, which is also demonstrated by Hu and Kopeko (97) that the invasion process appears to be saturated after 2 h incubation at MOI of 200. However, for comparing invasion ability among different *Campylobacter* strains, high MOI is not quite successful as low MOI. Hence a smaller MOI is required to achieve higher invasion efficiency so that different *Campylobacter* strains can be differentiated at their abilities of penetrating epithelial cells.

Objectives

Campylobacter spp. emerged as a concern in the late 1970s and the current position of Food Safety and Inspection Service (FSIS) in regard to the presence of

Campylobacter spp. in food is essentially the same as that for *Salmonella* spp. in raw meat and poultry. Basic and applied research on *Campylobacter* spp. has been, and is being conducted in a large extent to better understand and control *Campylobacter* spp. in food-producing animals, which appear to be the major reservoir. The major goal of this study is to determine the pathogenicity of *C. jejuni/coli* isolated from chicken meat products and identify the genes that are involved in.

Objectives of the project are:

- 1) To determine the abilities of various *C. jejuni/coli* retail meat isolates to adhere to, and invade the intestinal epithelial monolayer.
- 2) To identify the genes differentially expressed in *C. jejuni/coli* strains during their interaction with host epithelial cells.
- 3) To examine the abilities of *C. jejuni/coli*-host epithelial cell interaction to induce proinflammatory interleukin-8 secretion and to explore its mechanism.

In the following chapters (II, III, and IV), three studies are presented that were conducted to fulfill the goal of this project. Firstly, gentamicin resistance assay were applied to determine the adherence and invasiveness of the *Campylobacter* retail meat isolates in vitro; secondly, differential display was used to identify the genes differentially expressed in *C. jejuni* strain during the bacteria-host cell interaction; and thirdly, enzyme linked immunosorbent assay (ELISA) for IL-8 and Western-blot were taken to examine the proinflammatory IL-8 secretion abilities among *Campylobacter* isolates and explore the mechanism to induce IL-8 secretion

CHAPTER II: ADHERENCE AND INVASION OF CAMPYLOBACTER JEJUNI/COLI ISOLATED FROM RETAIL MEAT TO HUMAN INTESTINAL EPITHELIAL CELLS

ABSTRACT

The abilities of 34 *Campylobacter jejuni* and 9 *Campylobacter coli* isolates recovered from retail meats to adhere to and invade human intestinal epithelial T84 cells were examined and compared with a well-characterized human clinical strain, *C. jejuni* 81-176, in order to better assess their pathogenic potential. These *Campylobacter* retail meat isolates exhibited a wide range of adherence and invasion abilities with a few of the isolates adhering to and invading into T84 cells at levels close to those of *C. jejuni* 81-176. There was a significant correlation between the adherence and invasion abilities of the *Campylobacter* isolates. The presence of eight putative virulence genes that are potentially responsible for adherence and invasion, or encode cytolethal distending toxin in the *Campylobacter* isolates was determined using PCR. All *Campylobacter* isolates possessed *flaA*, *cadF*, *pldA*, *cdtA*, *cdtB*, and *cdtC*, and most (91%) also contained *ciaB* gene. However, *virB11* gene, carried by a virulence plasmid pVir, was absent in almost all the *Campylobacter* isolates. Our findings indicated that *C. jejuni* and *C. coli* present in retail meat were considerably diverse in their ability to adhere to and invade human intestinal epithelial cells, and that the putative virulence genes were widespread among the *Campylobacter* isolates. This suggests that despite of the presence of the putative

virulence genes, only some, but not all, *Campylobacter* strains isolated from retail meat can effectively invade human intestinal epithelial cells *in vitro*.

INTRODUCTION

Campylobacter is one of the leading causes of acute bacterial diarrhea worldwide (150). Numerous surveys have revealed retail meat and raw milk as sources of human infection (12). Particularly, a high percentage of poultry products is contaminated with *Campylobacter* (74, 163, 235).

A general body of knowledge that has been developed thus far regarding the pathogenicity of *Campylobacter* indicates that the organisms cause disease by at least three mechanisms: (i) intestinal colonization of ingested organisms, and production of bacterial cytotoxin (51, 81, 106), inducing diarrhea, (ii) bacterial invasion (194, 231), causing damage to mucosal surface cells of jejunum, ileum and colon, and (iii) extraintestinal translocation (79, 127), in which the organisms cross the intestinal epithelium and migrate via the lymphatic system to various extraintestinal sites. These possible mechanisms are not mutually exclusive, and *Campylobacter* may make use of any combination of the mechanisms depending on the host status and attributes of the infection strain. The molecular basis of pathogenicity of *Campylobacter* has not been fully elucidated. Several virulence factors, however, have been identified based on *in vitro* (53, 65, 122, 193) and *in vivo* (31, 196, 237) studies. For example, *flaA*, encoding flagellin (221), and *cadF*, encoding a protein that interacts with a host extracellular matrix protein fibronectin (160), have been shown to be required for *Campylobacter*

adherence and colonization to the host cell surface. Other genes including *ciaB* (124, 189), *pldA* (189), and genes of pVir plasmid (21) are involved in host cell invasion. The genes of *cdtA*, *cdtB* and *cdtC* (133, 188) are responsible for the expression of *Campylobacter* cytolethal distending toxin (CDT), which induces the proinflammatory cytokine production of epithelial cells (91) and causes host cell cycle arrest, cell distention and eventually cells death (225).

The ability of *C. jejuni* to adhere to and invade the epithelial cells of the gastrointestinal tract is important for the development of *Campylobacter*-mediated enteritis (183, 196). The adherence and invasion of *C. jejuni* to host cells has been studied using a variety of cell lines (54, 97, 119, 173). Human colonic epithelial cell line T84 has been widely used to assess the ability of enteric bacteria to adhere to and invade the epithelium. *C. jejuni* strain 81-176, a well-studied human clinical strain (128), has been demonstrated to have a relatively high invasion efficiency to several human intestinal epithelial cell lines, including T84 (97, 173), and to cause illness in human volunteers (31).

Despite frequent contamination of retail meat with *Campylobacter*, it remains unclear whether all *Campylobacter* in retail meat are pathogenic to humans and which factors specifically contribute to their pathogenicity. Methodologies on specifically identifying and distinguishing pathogenic strains of *Campylobacter* from non-pathogenic ones are needed for food safety surveillance. The present study was undertaken to characterize the adherence and invasion abilities of *Campylobacter* isolated from retail

meat using the T84 cell culture model and determine the prevalence of eight putative virulence genes in these isolates.

MATERIALS AND METHODS

Bacterial strains. Forty-three *Campylobacter* (34 *C. jejuni* and 9 *C. coli*) isolates were selected based on their distinct DNA fingerprinting profiles of pulsed-field gel electrophoresis (PFGE) (Fig.II-1) from 378 *Campylobacter* isolates that were recovered from retail raw meats collected in the Washington, D.C. area from June 1999 to July 2000 (235). The 43 isolates were recovered from chicken (n=36), turkey (n=4), pork (n=2), and beef (n=1) samples. The isolates had undergone two passages before used for the analyses. A well-characterized human clinical strain, *C. jejuni* 81-176 (kindly provided by Dr. P. Guerry-Kopecko at Naval Medical Research Center, Bethesda, MD) (82, 171), and a noninvasive *E. coli* laboratory strain, DH5 α , were used as positive and negative controls for all the analyses, respectively.

Bacterial growth conditions. *Campylobacter* bacteria were routinely grown on Mueller-Hinton (MH) agar (Difco Lab, Livonia MI) containing 5% (v/v) citrated sheep blood at 37 °C under a microaerophilic atmosphere (85% N₂, 10% CO₂, and 5% O₂). *E. coli* DH5 α was cultured aerobically on Luria-Bertani (LB) agar (Sigma, St Louis, MO) at 37 °C. All bacteria were subcultured for 18 h before used in experimental assays. The bacteria were harvested from the plates with phosphate-buffered saline (0.01 M sodium phosphate, pH 7.4, and 0.15 M NaCl) and diluted to a concentration of $\sim 10^8$ CFU/ml,

which was determined using a spectrophotometer (SmartSpec™ 3000; BioRad, Hercules, CA) at a wavelength of 600 nm. Titration and serial dilution analyses showed that an absorbance of 0.1 corresponded to 4.09×10^8 CFU/ml *Campylobacter* bacteria.

Cell culture. T84 cells (human colonic epithelial cell line, ATCC CCL-248) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate (Invitrogen, San Diego, CA) supplemented with 5% (v/v) fetal bovine serum, penicillin and streptomycin (5% DME/F12), as recommended by the American Type Culture Collection. Cells were seeded onto 24-well tissue culture plates (Costar, Cambridge, MA) at 2×10^5 cells/well and grown for 24 h under 5% CO₂ at 37 °C before adherence and invasion assays.

Adherence and invasion assay. The adherence and invasion assays were performed using T84 cells cultured in 24-well tissue culture plates as previously described (119). T84 cells were rinsed five times with prewarmed invasion media (5% DME/F12 without penicillin and streptomycin) and inoculated with 10^6 CFU bacteria in 1 ml invasion medium per well, with the multiplicity of infection (MOI) about 10-20. The number of inoculated bacteria was determined simultaneously on MH-blood agar plates. T84 cells were incubated with bacteria for 3 h in a humidified, 5% CO₂ incubator at 37 °C to allow bacteria to adhere and invade. The cells were then washed five times with PBS to remove bacteria that have not yet adhered and lysed with 1% saponin (Sigma) for

5 min to release cell-associated bacteria. Serial dilutions of the lysates were plated on MH-blood agar plates. After incubation at 37 °C for 48 h, *Campylobacter* colonies on MH-blood agar plates were enumerated to determine the number of cell-associated bacteria.

To determine the number of bacteria that had been internalized, T84 cells that were incubated for 3 h with bacteria were washed three times with PBS and incubated for an additional 2 h with 5% DME/F12 containing 100 µg/ml gentamicin (Sigma) to kill extracellular bacteria. After incubation, cells were washed three times with PBS to remove gentamicin and lysed with 1% saponin to release intracellular bacteria. The serial dilutions of the lysates were plated on MH-blood agar plates. The number of CFU on MH-blood agar plates was counted as the number of bacteria invaded into T84 cells. Each assay was performed in triplicate wells and repeated at least three times. The number of adhered or internalized bacteria was plotted as a percentage of the starting viable inoculum. Control studies were conducted to verify that a 2-h exposure of the 43 *Campylobacter* isolates to 100 µg/ml of gentamicin resulted in 100% killing (data not shown). Time course analyses (0 to 4 h) were carried out to determine the optimal length of time for the incubation of T84 cells with *Campylobacter* (data not shown).

The correlation between the abilities of the *Campylobacter* isolates to adhere to and invade into T84 cells was analyzed by linear regression using SPSS 10.0 version software (SPSS Inc., Chicago, IL) in order to calculate Spearman correlation coefficient.

Detection of virulence genes. PCR was used to detect eight *Campylobacter* genes that are associated with virulence in the genomic and plasmid DNA of the *Campylobacter* isolates. The eight genes include *flaA*, *cadF*, *ciaB*, *pldA*, *virB11*, *cdtA*, *cdtB*, and *cdtC*. PCR primers specific for these genes were designed based on the gene sequence information of GeneBank database and previous published studies (Table II-1). Conserved sequences of each gene were selected. Two different primer sets were used for each gene. Template DNAs for PCR were extracted by a boiling method described previously (157). Fresh cultures of *Campylobacter* isolates and the negative control strain (*E. coli* DH5 α) were suspended in 1 ml saline and boiled at 100 °C for 10 min. After centrifugation at 14,000 \times *g* for 2 min, the supernatants were collected and stored at -20 °C until use. For detecting *virB11*, both genomic DNA and plasmid DNA from each *Campylobacter* isolate were used as PCR templates. PCR was carried out using AmpliTaq Gold polymerase (Roche Molecular Biochemicals, Mannheim, Germany) with 30 cycles of amplification in a GeneAmp PCR system 9600 thermocycler (Perkin-Elmer, Foster City, CA). The cycling program included denaturation at 94 °C for 1 min, annealing at a temperature specific to each primer pair for 45 s, and extension at 72 °C for 45 s. PCR products were analyzed by 2% agarose gels, stained with ethidium bromide, and visualized with Gel Doc 1000 imaging system (BioRad).

RESULTS

The adherence and invasion abilities of *Campylobacter* retail meat isolates.

To test the pathogenic property of *Campylobacter* retail meat isolates, the adherence and invasion abilities of 43 *Campylobacter* isolates from retail raw chicken, turkey, beef and pork to human intestinal epithelial cells T84 were analyzed using gentamicin resistant assay (119). Fig. II-2A shows the adherence and invasion abilities of each isolate, and Fig. II-3 displays the distribution of the adherence and invasion abilities of 43 *Campylobacter* raw meat isolates tested. After a 3-h incubation, the 43 *Campylobacter* isolates adhered to T84 cells at a range of 0.002-3.115% of the starting viable inoculum (Fig. II-2A), compared to 1.790% adherence level of the positive control strain *C. jejuni* 81-176. A half of the isolates adhered to the cells at levels ranging from 0.002 to 0.170%, which are less than one tenth of that of *C. jejuni* 81-176, whereas five isolates (12%) adhered to the cells at levels ranging from 0.590 to 3.115% (Fig. II-3), which are close to that of *C. jejuni* 81-176. One of the isolates from a chicken meat sample, *C. coli* C54, showed the greatest adherent ability among all strains tested. Its adherent level (3.115%) was even greater than that of *C. jejuni* 81-176 (1.790%). No significant difference ($p>0.05$) between *C. jejuni* and *C. coli* strains in their abilities of adherence to T84 cells was detected. The non-pathogenic *E. coli* DH5 α also showed a high level of adherence, similar to *C. coli* C54. This suggests that a high adherent ability of bacteria to host epithelium does not necessarily lead to infection.

After a 3-h incubation, the percentages of the *Campylobacter* isolates resistant to extracellular gentamicin treatment were found to be in the range of 0.001-0.235% of the

starting viable inoculum (Fig. II-2A). The invasion abilities of the 43 raw meat isolates were all lower than that of *C. jejuni* 81-176 (0.315%). Among the 43 meat isolates, 18 isolates (42%) showed invasion levels below 0.004%, which was similar to that of the non-invasive negative control *E. coli* DH5 α (0.001%), but much lower than that of *C. jejuni* 81-176. All of the four turkey isolates belonged to this group. Six raw meat isolates had the invasion levels above 0.040%, within one tenth of the invasion level of *C. jejuni* 81-176. The remaining 19 isolates (56%) had invasion levels between 0.004% and 0.040% (Fig. II-2A). *C. coli* C54, which was isolated from a chicken sample, had the highest level of invasion (0.235%) among the strains tested, similar to that of *C. jejuni* 81-176 (0.315%). Compared to *C. jejuni*, *C. coli* isolates demonstrated a similar range of invasion levels, however, six out of nine *C. coli* isolates tested showed invasion levels lower than 0.004%. These results indicate that the abilities of *Campylobacter* meat isolates to adhere to and invade T84 cells vary in a wide range, and only a low percentage of the isolates have the adherence and invasion levels similar to the well-characterized human clinical strain *C. jejuni* 81-176.

Correlation between the adherence and invasion abilities of *Campylobacter* retail meat isolates. To test the interrelationship between *Campylobacter* adherence and invasion events, we determined the correlation coefficient between the *Campylobacter* adherence and invasion variables using the method of linear regression (Fig. II-4). The correlation coefficient (r) between the adherence and invasion efficiency of the *Campylobacter* retail meat isolates was found to be 0.543 with $P < 0.01$, indicating a

significantly positive relationship between the invasion and adherence variables. However, there were a few exceptions. *Campylobacter* isolates C114, J385, and C49 showed strong adherence but poor invasion abilities, whereas J237, J5 and J57 exhibited relatively low adherence but moderate invasion abilities. This suggests that the invasion abilities of *Campylobacter* retail meat isolates be associated with their adherent abilities, but the adherence of *Campylobacter* to the host epithelium does not necessarily lead to invasion.

Presence of the putative virulence genes. Even though the genes that determine the infectivity of *Campylobacter* have not been well studied, several genes have been found to be involved in *Campylobacter* adherence and invasion of host cells. To test whether the relative adherence and invasion levels of the *Campylobacter* isolates are related to the presence of particular virulence genes, the presence of eight putative virulence genes in the genomic and plasmid DNA of the 43 retail meat isolates were determined by PCR using two different pairs of primers for each gene (Table II-1). As showed in Fig. 1B, the majority of the *Campylobacter* isolates possessed seven of the eight virulence genes examined, including *flaA* (100%), *cdtA* (100%), *cdtB* (100%), *cdtC* (100%), *ciaB* (91%), *cadF* (100%) and *pldA* (100%). However, *virB11* gene of pVir plasmid was not detected in most of the isolates except for chicken meat isolate C114 (Fig. II-2B). All eight virulence genes were detected in positive control strain *C. jejuni* 81-176. Genes *cdtA*, *cdtC*, *cadF* and *pldA* in some of these isolates were detected by

only one of the two pairs of primers used (Fig. II-2B). This may be due to variability and random point mutations of the genes.

DISCUSSION

Campylobacter is one of the major food-borne pathogens and widely spread in retail meat, in particular the poultry products. However, little is known about the pathogenicity of these food-contaminating strains and the molecular basis of *Campylobacter* pathogenicity. In this study, we examined the ability of *Campylobacter* strains isolated from raw meat products to adhere to and invade human intestinal epithelial line T84 cells, and determined the association between the presence of certain virulence genes and the adherence and invasion abilities of *Campylobacter* isolates from retail meats. Our results revealed that *Campylobacter* strains with adherence and invasion abilities similar to those of the human clinical strain *C. jejuni* 81-176 were present in retail meat. We found that 6 out of 43 tested isolates had invasion abilities close to *C. jejuni* 81-176, which is similar to a recent report that 12 of the 42 isolates from fecal samples of food and companion animals and beef carcasses were capable of invading epithelial cells (137). The adherence and invasion abilities of the retail meat isolates varied considerably, from levels similar to that of *C. jejuni* 81-176 to levels thousand fold lower than that of *C. jejuni* 81-176. However, more than half of the retail meat isolates tested exhibited the invasion efficiency 100 fold lower than that of *C. jejuni* 81-176, indicating that not all *Campylobacter* strains that contaminate meat products are able to effectively invade human intestinal epithelial cells.

Because animal models that completely mimic *Campylobacter* infections in humans are not available, the cell culture model using human colonic epithelial cells provides us a useful tool to evaluate the abilities of *Campylobacter* food isolates to adhere to and invade the human intestinal epithelium. In particular, T84 cell line has been widely used for studies of pathogenicity of many human enteric pathogens, including *Salmonella typhimurium*, *Helicobacter pylori*, and enteropathogenic *E. coli* (49, 144, 167). Even though the process of *Campylobacter* adhering to and invading into human intestinal epithelial cells T84 in culture does not exactly mimic the process in vivo, the cell culture model allowed us to determine the relative adherence and invasion abilities of the retail meat isolates in comparison to the well studied human clinical strain *C. jejuni* 81-176. To ensure that the cell culture model was functioning properly, we carried out inoculation dose and time course studies (data not shown). These studies showed that the binding of *C. jejuni* to T84 cells was time-dependent and saturable. Even though MOI about 10 instead of 100 was used in this study the invasion and adherence levels of the human clinical strain *C. jejuni* 81-176 reported here are similar to previously published studies (119), (159, 195).

Colonization or adherence of microbial pathogens to mucosal surfaces is the primary step of infection and appears to be a prerequisite for invasion in most cases (9, 116, 117, 227). In this study, we analyzed the interrelationship between adhesion efficiency and invasion efficiency of *Campylobacter* retail meat isolates by statistical analyses. A positive correlation between the adhesion and invasion events was observed, which suggests that host invasion efficiency of *Campylobacter* is at least partially

dependent on its adherence ability. Interestingly, not all of the tested strains agreed to this correlation. Some of the isolates that adhered to T84 cells efficiently invaded T84 cells poorly. This suggests that the adherence of *Campylobacter* to host cells facilitate its invasion into host cells, but adherence itself does not necessarily lead to invasion.

It has been generally accepted that bacterial virulence is multi-factorial and determined by the expression of virulence genes. To determine the interrelationship between the abilities of *Campylobacter* retail meat isolates to adhere/invade human intestinal epithelial cells and virulence genes that they carry, the prevalence of the putative virulence genes *flaA*, *cadF*, *ciaB*, *pldA* and *virB11* and toxin genes *cdtA*, *cdtB*, *cdtC* among the 43 *Campylobacter* retail meat isolates was determined by PCR. Even though the CDT has not been shown to be directly involved in host epithelial invasion, its role in inducing proinflammatory cytokine secretion of host epithelial cells (91) and inhibiting cell cycles (225) could influence the invasion and adherence ability of *Campylobacter*. Using two different pairs of PCR primers that target conserved sequences for each gene, we were able to detect six out of the eight putative virulence genes in all of the 43 *Campylobacter* isolates. The gene of *ciaB* was undetectable in four isolates with very low invasion ability. In some of the retail meat isolates, the putative virulence genes were detected with one of the two PCR primer sets used, suggesting the presence of variability and/or random mutations in these genes. The high prevalence of these genes in *Campylobacter* retail meat isolates with wide ranges of adherence and invasion efficiency may also suggest that additional genes be involved in the process. Our finding is consistent with previous studies that showed the high prevalence of the

same seven putative virulence genes in *Campylobacter* strains isolated from Danish turkeys, pigs and cattle (22, 23). In addition to *C. jejuni* 81-176, only one of the 43 retail meat isolates, which had an invasion ability thousand times lower than *C. jejuni* 81-176, was positive for *virB11* gene. Bang *et al.* (22, 23) also reported a relatively low prevalence for *virB11* gene in *Campylobacter* isolates compared to the prevalence of other putative virulence genes tested. The prevalence of *virB11* among the food isolates has not been well studied. Our study did not find a general correlation between the invasion efficiency of *Campylobacter* retail meat isolates and the putative virulence genes present in their genomic DNA. There are several possible explanations. First, even though some of the low invasive strains contain most of virulence genes in their genome, they may fail to express these genes when they interact with host cells or they may express inactive variants of the virulence genes. Secondly, virulence genes may have redundant and overlapping functions, thus missing one or two of these genes may not significantly affect the invasion ability of *Campylobacter*. Thirdly, besides these eight virulence genes, additional genes are required for host invasion. Further studies using the approaches of DNA microarray and mutagenesis are required to identify additional virulence genes of *Campylobacter*, determine which virulence genes are expressed upon host cell contact, examine the variability of virulence genes, and explore how different virulence genes work together to establish infection.

Table II-1. *Campylobacter* virulence genes and primer sequences for PCR identification.

Target gene	Primers	Sequence (5' to 3')	Product (bp)	GeneBank Accession#	Reference
<i>C. jejuni</i>	<i>cadF</i> -278	ATGGTTTAGCAGGTGGAGGA	252	AF104302,	This study
<i>cadF</i>	<i>cadF</i> -510	GAGCTGGACGAGTATCAGCA		AF104303	
<i>C. coli</i>	<i>cadF</i> -310	GGTTTAGCTGGTGGGGGATA	525	CJ11168X5,	
<i>cadF</i>	<i>cadF</i> -815	CAGAAAGACGGGCTGAAAGC		CJU87559	
<i>cadF</i>	<i>cadF-F2B</i>	TTGAAGGTAATTTAGATATG	400		(120)
	<i>cadF-R1B</i>	CTAATACCTAAAGTTGAAAC			
<i>cdtA</i>	<i>cdtA</i> -284	TCCTTTGGGGCGTTCATTTG	534	AF038283,	This study
	<i>cdtA</i> -798	ACCGCTGTATTGCTCATAGG		U51121	
<i>cdtB</i>	<i>cdtB</i> -1094	TCATCCGCAGCCACAGAAAG	203		
	<i>cdtB</i> -1277	ACCCTATCAGGCCTTGAAAG			
<i>cdtC</i>	<i>cdtC</i> -1630	TCCAAC TAGCGCAACTCAAG	493		
	<i>cdtC</i> -2103	GCTCCAAAGGTTCCATCTC			
<i>cdt</i> genes	GNW	GGNAA YTG GATH TGGGGNTA			(67)
	WMI1	RTTRAARTCNCCYAADATCATCC			
	VAT2	GTNGCNACBTGGAAYCTNCARGG			
	LPF-D	DAAYTGNACDTANCCDAANGG			
<i>ciaB</i>	<i>ciaB</i> -652	TGCGAGATTTTTTCGAGAATG	527	AJ312325,	This study
	<i>ciaB</i> -1159	TGCCCGCCTTAGAACTTACA		AF114831,	
	<i>ciaB</i> -403	TTTTTATCAGTCCTTA	986	CJ11168X3	(52)
	<i>ciaB</i> -1373	TTTCGGTATCATTAGC			
<i>C. jejuni</i>	<i>pldA</i> -361	AAGAGTGAGGCGAAATTCCA	385	AJ786391,	This study
<i>pldA</i>	<i>pldA</i> -726	GCAAGATGGCAGGATTATCA		Y11031,	
<i>C. coli</i>	<i>pldA</i> -539	GCTTATAGTTTTGGCTCATTAG	637	AL139078	
<i>pldA</i>	<i>pldA</i> -1154	TACAACAAACGCTTACAAAGAC			
<i>pldA</i>	<i>pldA</i> -U	AGATGAATTATTTYTACCT	600		
	<i>pldA</i> -L	TTGTTRTARTCTATAAGGCT			
<i>flaA</i>	<i>flaA</i> -440	TCCAAATCGGCGCAAGTTCA	217	AF345999,	This study
	<i>flaA</i> -637	TCAGCCAAAGCTCCAAGTCC		AF140252	
<i>virB11</i>	<i>virB11</i> -235	TGTGAGTTGCCTTACCC	395	AF472533	This study
	<i>virB11</i> -612	TTATTTATCGCCGTTTGA			
	<i>virB</i> -232	TCTTGTGAGTTGCCTTACCCCTTTT	494		(52)
	<i>virB</i> -710	CCTGCGTGTCTGTGTTATTTACCC			

Figure II-1. *SmaI*-digestion patterns of the *Campylobacter* retail meat isolates tested in this study. Genomic DNAs were digested with *SmaI* and analyzed by pulsed-field gel electrophoresis followed by the clustering analysis.

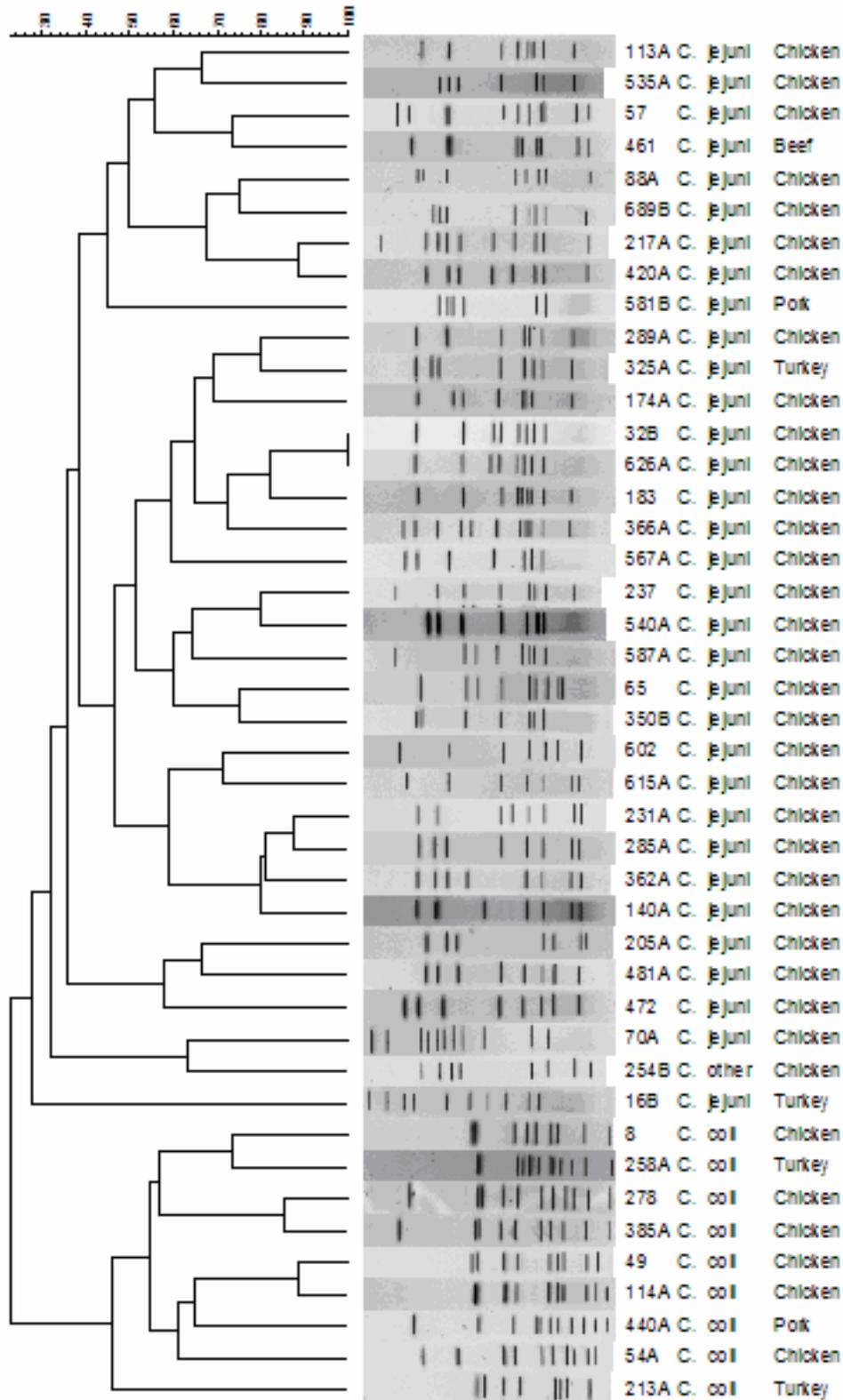


Figure II-2. The ability of *Campylobacter* retail meat isolates to adhere to and invade human intestinal epithelial cells T84 and the distribution of virulence genes among these isolates. (A) Forty-three of *Campylobacter* strains isolated from retail chickens, turkey (*), pork (†), beef (‡), and the humans clinical isolate *C. jejuni* 81-176 were tested for their abilities to adhere to and invade into T84 cells using a gentamicin resistant assay (119). The data were plotted as percentages of the starting viable inoculum. Shown are the means of percentages of cell (\pm S.D.) associated and involved in invasion based on three independent experiments. (B) The presence of putative virulence genes, *flaA*, *cadF*, *ciaB*, *pldA*, *virB11*, *cdtA*, *cdtB*, and *cdtC* in the genomes and plasmids of different *Campylobacter* isolates was detected by PCR using two different pairs of primers for each gene. The * indicates genes that were detected by only one of the two pairs of PCR primers.

Figure II-3. Distribution of adherence and invasion abilities among 43 *Campylobacter* strains isolated from retail meat. The experiments were performed as described in Figure 1A. The adherence (A) and invasion efficiency (B) of *Campylobacter* retail meat isolates was expressed as percentages of the starting viable inoculum and plotted as the function of the number of strains.

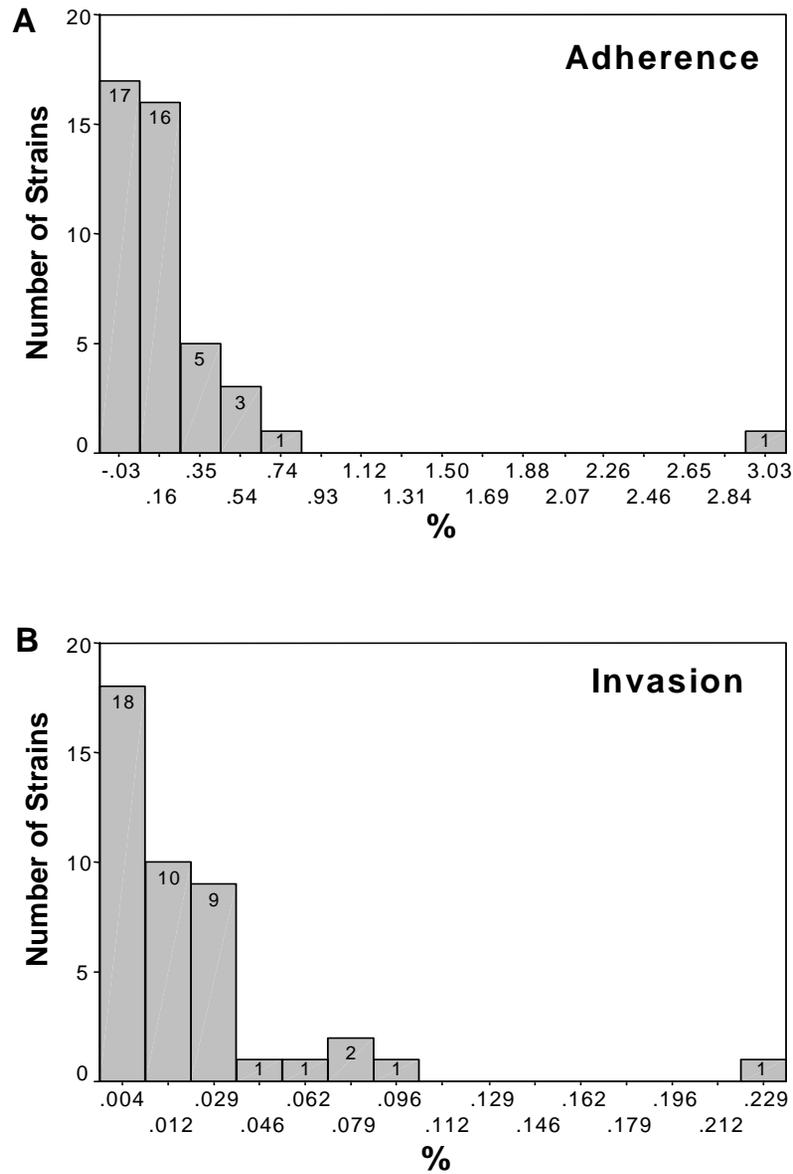
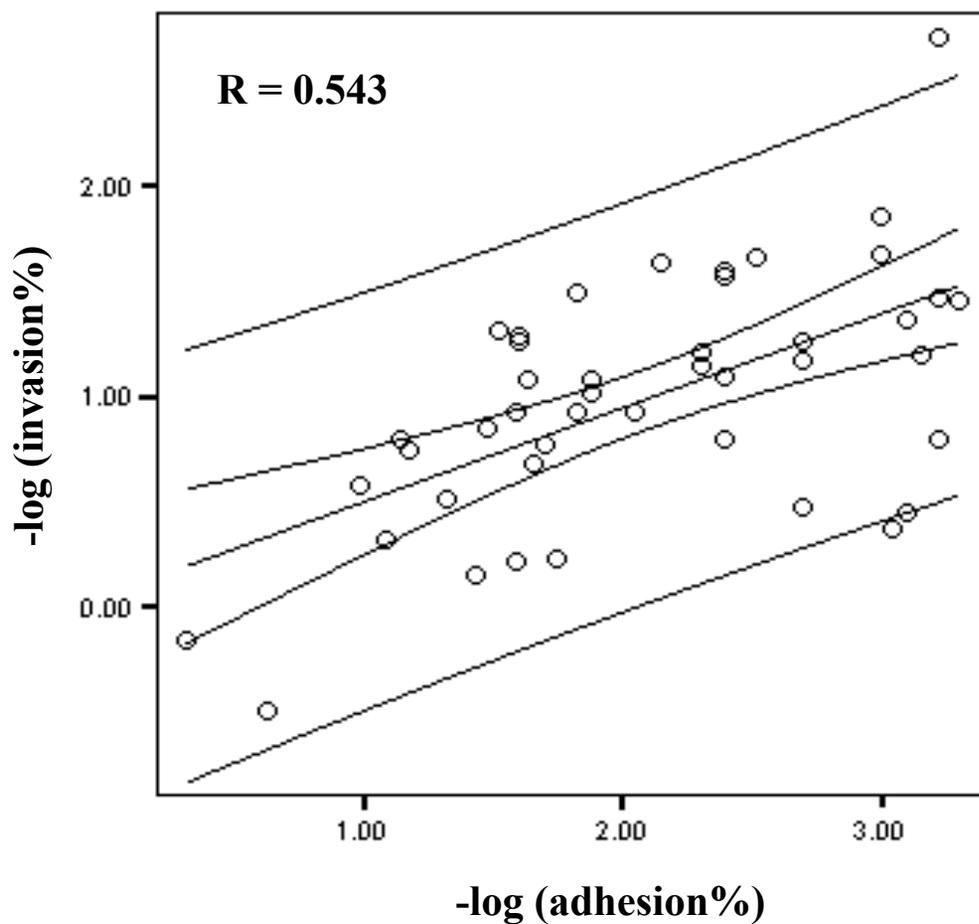


Figure II-4. The correlation between the invasion and adherence abilities of 43 *Campylobacter* strains from retail meat. The correlation between the invasion and adherence abilities of *Campylobacter* isolates as analyzed by linear regression using SPSS 10.0 version software. Shown is the plot of $-\log(\text{invasion \%})$ versus $-\log(\text{adherence \%})$ of each strain. The central line represents regression line [$-\log(\text{adhesion \%}) = 0.06 + 0.45 * [-\log(\text{invasion \%})]$]; the inner sidelines near the central line represent 95% mean prediction interval; and the outer sidelines represent 95% individual prediction interval



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**CHAPTER III. IDENTIFICATION OF COLONIZATION AND
INVASION-RELATED GENES IN *CAMPYLOBACTER JEJUNI* BY
RESTRICTION FRAGMENT DIFFERENTIAL DISPLAY**

ABSTRACT

Campylobacter jejuni is considered as an invasive bacterium, which is the most common bacterial cause of foodborne diarrhea illness worldwide. In vitro cell culture model gives useful information on the potential role of different genes in pathogenesis. Gene expression in *Campylobacter jejuni* 81-176 during infection was compared to the expression of the control strain by restriction fragment differential display PCR (RFDD-PCR). This method amplifies cDNA restriction fragments under stringent PCR conditions, enabled by the use of specific primers complementary to ligated adaptor sequences. Total twenty gene fragments having increased expression in T84-associated *C. jejuni* 81-176 were identified. All these fragments had homology to stable bacterial ribosomal RNAs, which means false positives. However, this study may provide some useful information for future prokaryotic gene expression analysis during host pathogen interaction based on no such report on *Campylobacter* host cell interaction studying yet, so far.

INTRODUCTION

Campylobacter jejuni is one of the most common causes of human acute bacterial enteritis in industrialized countries. Human campylobacteriosis is considered primarily a food-borne disease. *C. jejuni* is a common gut commensal in most food producing animals and birds, and fecal contamination of meat during processing is a well-recognized route of transmission to humans. In contrast to *Salmonella* spp., *Campylobacter* spp. does not grow or compete well with other bacteria at common food abuse temperatures. And it appears that the level of *Campylobacter* spp. in contaminated foods is governed only by the extent of initial contamination and that temperature abuse is not a significant factor. However, the mechanisms by which *C. jejuni* colonizes and invades the intestinal tract after consumption and the genes that are involved in the processes are poorly understood. In vitro cell culture model have enabled some invasion factors to be identified by using different mutants; these factors include flagellin (171, 221), fibronectin-binding protein CadF (159), *Campylobacter*-invasion antigen Cia (190), and cytolethal distending toxin CDT (188). However, such studies are highly dependent on the presumed properties of genes and thus do not generally lead to the identification of *C. jejuni*-specific colonization and invasion factors.

The ability to define genes that may be expressed differentially during colonization and invasion will allow the molecular mechanisms of host-pathogen interaction to be elucidated. Two approaches at the RNA level are currently applicable: 1) methods that select genes based on their differential expression under varying conditions

(during colonization and invasion or not), such as RAP-PCR (141) (219) or cDNA subtraction hybridization and differential library screens (179); 2) to define expression patterns of sequence-defined genes by mRNA analysis by northern hybridization, ribonuclease protection or RT-PCR. The availability of entire genome sequence of *C. jejuni* (178) makes any gene in theory may be investigated for expression by mRNA analysis.

Differential display (DD) technique was developed in the early 1990s for eukaryotic cell study by integrating two of the most simple, powerful and commonly used molecular biological methods; namely, PCR and DNA sequencing by gel electrophoresis (138). Numerous applications have been done for the identification of differentially expressed genes. Unlike microarray, DD does not require any previous knowledge of mRNA or gene sequences, making it an 'open' system that is applicable to any eukaryotic organism. However, some of the limitations in DD-PCR, such as the restriction to visualization of the 3' end of the transcripts only, low annealing temperature during PCR amplification and a high number of false positive (55), have encouraged the development of new and improved techniques. Furthermore, the original method displays polyadenylated transcripts and can therefore only be applied to eukaryotic RNA. Welsh and McClelland (224) reported a related method known as RNA arbitrarily primed PCR (RAP-PCR) using only primers of random sequence (44, 80, 220). Bachem (18) made a further improvement to use differential display technique based on cDNA amplified fragment length polymorphism (AFLP). Another DD variant method is developed as so-called restriction fragment differential display PCR (RFDD-PCR) (80).

The technology is based on digesting cDNA with endonucleases followed by adaptor ligation and PCR amplification with specific primers at high-stringency PCR amplification conditions. Thus the method can be equally applied to both eukaryotic and prokaryotic system. But so far, no application in related bacteria-host cell interaction study has been reported yet, especially on *Campylobacter*. This study may provide useful information on application of differential display technology in *Campylobacter*-host cell interaction study.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Campylobacter jejuni* 81-176 was kindly provided by Dr. P. Gurry-Kopecko, from Naval Medical Research Center, Bethesda, MD. It was routinely grown on Mueller-Hinton (MH) agar (Difco Lab, Detroit, MI) containing 5% (v/v) citrated sheep blood at 37 °C under a microaerophilic atmosphere (85% N₂, 10% CO₂, and 5% O₂), and subcultured for 18 h before used in experimental assays. The bacteria cells were harvested from the plates with phosphate-buffered saline (PBS; Difco) and diluted to a concentration of $\sim 1.2 \times 10^9$ CFU/ml, which was determined using a spectrophotometer (SmartSpecTM 3000; BioRad, Hercules, CA) at a wavelength of 600 nm. Titration and serial dilution analyses showed that an absorbance of 0.1 corresponded to 4.09×10^8 CFU/ml of *Campylobacter*.

Cell culture. T84 cells (human colonic epithelial cell line, ATCC CCL-248) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate (Invitrogen, San Diego, CA) supplemented with 5% (v/v) fetal bovine serum, penicillin and streptomycin (5% DME/F12), as recommended by the American Type Culture Collection (ATCC). Cells were seeded into T-75 flask (Costar, Cambridge, MA) at 2.4×10^6 cells/ml and grown for 24 h under 5% CO₂ at 37 °C before experiment.

Infection protocol. T84 cells were rinsed five times with prewarmed invasion media (5% DME/F12 without penicillin and streptomycin) and inoculated with $\sim 1.2 \times 10^9$ CFU bacteria in 1 ml invasion medium per flask, with the multiplicity of infection (MOI) about 500. The number of inoculated bacteria was determined simultaneously on MH-blood agar plates. T84 cells were incubated with bacteria for 4 h in a humidified, 5% CO₂ incubator at 37 °C to allow bacteria to adhere and invade.

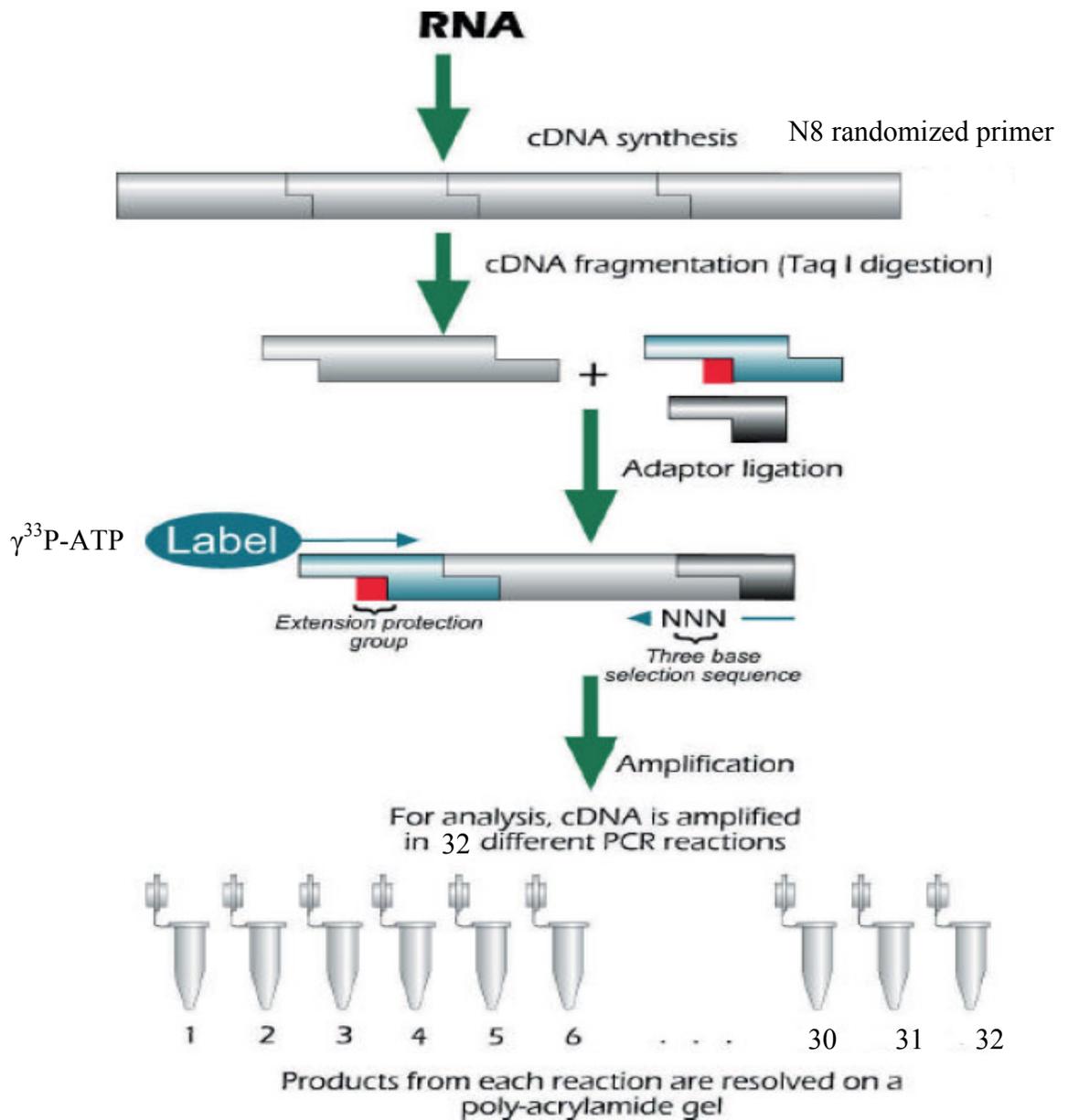
Isolation of bacterial and cellular total RNA. Bacterial cells were washed four times with chilled 1× PBS to remove those that were not yet adhered, and 5 ml filter-sterilized 2% saponin / RNAlater (1:1 volume ratio) was added to allow cell lysis for 5 min to release host cell-associated bacteria and stabilize bacteria RNA at the same time. After vigorously vortexing, cell debris was removed by centrifugation for 4 min at 300×g, 4 °C. The rest of cell lysate was pelleted at 9000 ×g, 4 °C for 10 min. The cell pellets

were resuspended in 100 μ l of lysozyme-containing TE buffer (1mg/ml) by vortexing and incubated for 5 min at RT. Bacterial and cellular total RNA were isolated using RNeasy Mini kit (Qiagen Inc., Valencia, CA), and contaminated DNA was removed by on-column DNase digestion kit (Qiagen Inc., Valencia, CA). The quality and quantity of total RNA were assessed by Nanodrop, ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE) and RNA 6000 Nano lab-on chip electrophoresis (Agilent Technologies, Inc., Palo Alto, CA).

Enrichment of bacterial RNA. The bacterial RNA was further enriched from total RNA by using MICROBEnrich™ kit (Ambion, Inc., Austin, TX) to remove mammalian ribosomal RNAs and mRNA. The quality and quantity of enriched bacterial RNA were compared to those of total RNA by RNA 6000 Nano lab-on chip electrophoresis (Agilent Technologies, Inc., Palo Alto, CA).

RFDD-PCR. The analysis was carried out as described in detail in the protocol from the displayPROFILE™ kit developed by Qbiogene, Morgan Irvine, CA (Fig. III-1). Briefly, 500 ng enriched total RNA was reverse-transcribed using 0.75 μ M N₈ randomized primer and 100 units reverse transcriptase in 25 μ l volume. The reaction was incubated at 42 °C for 2 h. For second-strand synthesis, a 50 μ l mix consisting of buffer, dNTPs, 12 U DNA polymerase I, and 0.8 U RNase H was added. The reaction mixture was incubated at 16 °C for 2 h and subsequently phenol/chloroform extracted, precipitated by ethanol and dissolved in 20 μ l H₂O. Total 10 μ l of cDNA was checked on an agarose gel for cDNA smear between 100 and 2000 bp.

Figure III-1. Schematic outline of the RFDD-PCR technology. Adopted from displayPROFILE kit application manual of Qbiogene.



The remaining 10 μl cDNA was digested with *TaqI* endonuclease at 65 °C for 2 h.

The RFDD-PCR template was completed by ligating the digest to adaptor mix (a standard

adaptor and an EP adaptor containing an extension protection group) at 37 °C for 3 h using T4 DNA ligase. The incubation was carried out at 37 °C and not the standard 16 °C to maintain the *TaqI* endonuclease activity during the ligation. This prevented religation of cDNA *TaqI* fragments, but not adaptor ligation, since the latter did not re-establish the *TaqI* site.

The template was PCR amplified using a 0-extension primer complementary to the EP adaptor in combination with a 3-extension primer recognizing the standard adaptor and the three nucleotides adjacent to the *TaqI* site. The 0-extension primer was kinase-labelled with [γ -³³P]dATP (MP biomedical, Irvine, CA). All PCR reactions were carried out in a 20 μ l volume using 0.2 μ l template and standard concentration of dNTPs and primers, using the following “touch down” PCR-amplification profile: initial denaturation 94 °C, 1 min, then for the first 10 cycles: 94 °C, 30 s; 60 °C with touchdown by 0.5 °C for each cycle until 55 °C is reached; 72 °C, 1 min, and for the last 25 cycles: 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min.

The complete analysis was performed using radioactive labelling. For each template, 32 PCR reactions using the [γ -³³P]dATP-labelled 0-extension primer in combination with the 32 different 3-extension primers (NNA/G or NNC/T) were performed. The PCR products were resolved on a standard 6% polyacrylamide sequencing gel and dried onto Whatman paper. Bands that are indicated to be differentially expressed were quantified on a phosphorimager (Bio-Rad, Hercules, CA). The dried gel was lined up with markings on the film and the fragments were excised

from the gel. The gene fragments were eluted in 50 μ l Tris-EDTA buffer and reamplified using the same PCR conditions and primers as in the initial PCR reaction.

Sequence analysis of differentially amplified bands. Each reamplified fragments were cloned into the blue/white cloning vector pGEM-T-easy (Promega, Madison, WI) according to the manufacturer's instructions. Two clones from each cloning were submitted for sequencing (Macrogen, Korea). Using the sequence assembly program Sequencher (Gene Code Corporation, Ann Arbor, MI), the nucleotide sequences obtained were aligned to assemble larger contiguous sequences (contigs). These sequences were compared to protein and nucleic acid sequence databases using the BLAST alignment programs.

RESULTS AND DISCUSSION

Extraction of RNA from intracellular *C. jejuni*. Any RNA extraction method must be rapid enough to prevent degradation of short half-life mRNA (~2 min), and must prevent artifactual in transcription during the bacterial recovery and extraction processes. DD technology requires pure bacterial RNA. Otherwise the preponderance of host RNA would effectively compete for the available random primers, then reducing the specific activity of the bacterial component. So it is critical to purify the bacterial RNA from the much more abundant host RNA. We have extended pure bacterial RNA extraction method to rapidly isolate *Campylobacter* total RNA from epithelial cell infection models

free from most of host RNA (Fig. III-2) through differential lysis with simultaneous protection from degradation of the bacterial mRNA and removing polyadenylated mRNAs along with eukaryotic rRNAs by magnetic beads, derivatized with an oligonucleotide that hybridizes to the capture oligonucleotide and to the polyadenylated 3' ends of eukaryotic mRNAs. The resulting RNA will contain total bacterial RNA, and other small RNAs (5S, tRNAs) from the eukaryotic RNA population.

Use of RFDD-PCR for analysis of *C. jejuni* colonization/invasion-related gene expression. The RFDD-PCR technology relates gene expression to the concentration of the corresponding cDNA amplified by PCR, as in traditional DD-PCR. Double-stranded cDNA was synthesized from total RNA and digested with the *TaqI* restriction enzyme. Following digestion and adaptor ligation, the template was amplified using a 0-extension primer combined with a 3-extension primer. The 3-extension primer binds to the specially designed adaptor, which has three extra bases (NNN) that extend past the adaptor junction. These three bases determined which subpopulation of cDNA fragment would be amplified in the reaction. Thus, each PCR reaction only amplified a specific subset of cDNA fragments that had a specific three-base sequence upstream of the *TaqI* restriction site. To amplify all subsets, which provide a complete cross section of all the expressed sequences, only 64 for eukaryotes, and 32 for prokaryotes different primers are necessary. Visualization of the PCR products was obtained by labeling of the 0-extension primer. Fragments with two 3-extension primers will be amplified if the three-

base extension matches the sequence adjacent to the *TaqI* restriction sites at both ends, but will not be labeled by the 0-extension primer.

The purpose of the analysis was to identify constitutive changes in gene expression during infection with epithelial cells. Therefore, RNA for RFDD-PCR analysis was isolated without co-culture with epithelial cells of wide-type strains as control. The RFDD-PCR products were analyzed with the radioactively labeled 0-extension primer (Fig. III-3). The 32 radioisotopic profiles of the 'induced' J81-176 strain and 'control' J81-176 strain were compared on the radiogram. The PCR products having at least fivefold difference in expression were considered in the analysis. In total, twenty fragments met the above criteria. These fragments were excised from the gel; DNA was eluted, and re-amplified to get the same size PCR products. The cloned fragments were sequenced and databases were searched for homology. It turns out that they all originated from the *Campylobacter* ribosomal RNA genes.

Reasons of failure to isolate differentially expressed gene fragments. Several reasons can lead to fail to isolate differentially expressed gene fragments. Taken together, there are two major ones. First of all, the yields of *Campylobacter* RNA are much lower than host epithelial cell RNA in extracted total RNA. The number of internalized *Campylobacter* increase with greater bacterial concentrations but even with the most invasive strains only one to three bacteria were internalized per cell (29, 30), not to mention the other average strains, which means bacterial RNA yields in total RNA mixture are hundred times lower than eukaryotic RNA (200). Therefore, purifying total

RNA from a mixture of eukaryotic cells and bacteria could result in a vast excess of eukaryotic cellular RNA and very little bacterial RNA. Too little *Campylobacter* RNA is present for generating adequate signals in RFDD-PCR. Secondly, even after enrichment of bacterial RNA, expression profile of bacterial mRNA is also embedded in the noise of bacterial ribosomal RNA. In principle, the 16 S and 23 S rRNAs can be extracted from the total RNA by hybridization to beads coated with conserved 16 S or 23 S rDNA sequences. But in practice because the stable RNAs are 20-50 times more abundant than all combined mRNAs, a 1% difference in the efficiency of removal of stable RNA between the 'control' and 'induced' RNA constitutes a difference larger than that of the expression of many mRNAs. Therefore, bacterial differential display must be practiced on the total RNA population, which leads to generation of many false positive in weak signal vs. strong noise environment.

RFDD-PCR as a prokaryotic gene expression technology. There are currently a number of different technologies available for studying gene expression in both eukaryotic and prokaryotic organisms (84, 234) and several aspects require consideration in the choice of method. For instance, it should be clarified how reproducible the method is, what comprehensive coverage the method provides, whether the method is dependent on the organism being studied and, finally, whether the method can detect low-abundance transcripts. Differential display should, in principle, be a very useful tool to identify genes in bacteria that lack a genetic system or for which a 'reverse genetics' approach has

failed. However, there are only limited number of reports and has not been adopted widely by microbiologists, not to mention for studying bacteria host interactions.

Several factors have contributed to the poor development of bacterial differential display. Firstly, because prokaryotic mRNAs lack stable poly(A) tails there is no straightforward way to reverse-transcribe mRNA at the exclusion of stable RNA species which can potentially overwhelm the experiment (166). A second factor, which makes contribution, is that this technique generates false positive frequently, which is partly due to the density of sampling of the mRNA population. An aliquot of reverse transcription reaction is used as a DNA template for PCR amplification in a second tube after the synthesis of the first cDNA strand from the total RNA pool by this reaction in a first tube, which in largely lower the density of sampling. In turn, it enhances the chance of getting sequences of contaminating background DNA, which are false positives. An increase in the density of sampling of the mRNA population should allow the multiple sampling of differentially expressed messages.

Recently, cDNA array hybridization techniques have become increasingly used for the study of eukaryotic and prokaryotic gene expression (234). Gene fragments are spotted down on a surface and different probes prepared from RNA samples are hybridized onto the cDNA arrays to visualize differences in expression levels. The main restriction to the application of micro-arrays is the requirement of access to extensive genome sequence information in order to synthesize the gene fragment arrays for the

specific organism, which in *Campylobacter*, would not be a problem due to the availability of complete genome sequence (178).

Figure III-2. Bacterial and cellular total RNA were isolated using RNeasy Mini kit. The bacterial RNA was further enriched from total RNA by MICROBEnrich kit. Ribosomal RNAs before and after the enrichment by removing eukaryotic RNA showed by RNA 6000 Nano assay (eukaryotic ribosomal RNA including 18S and 28S rRNA; prokaryotic ribosomal RNA including 16S and 23S rRNA).

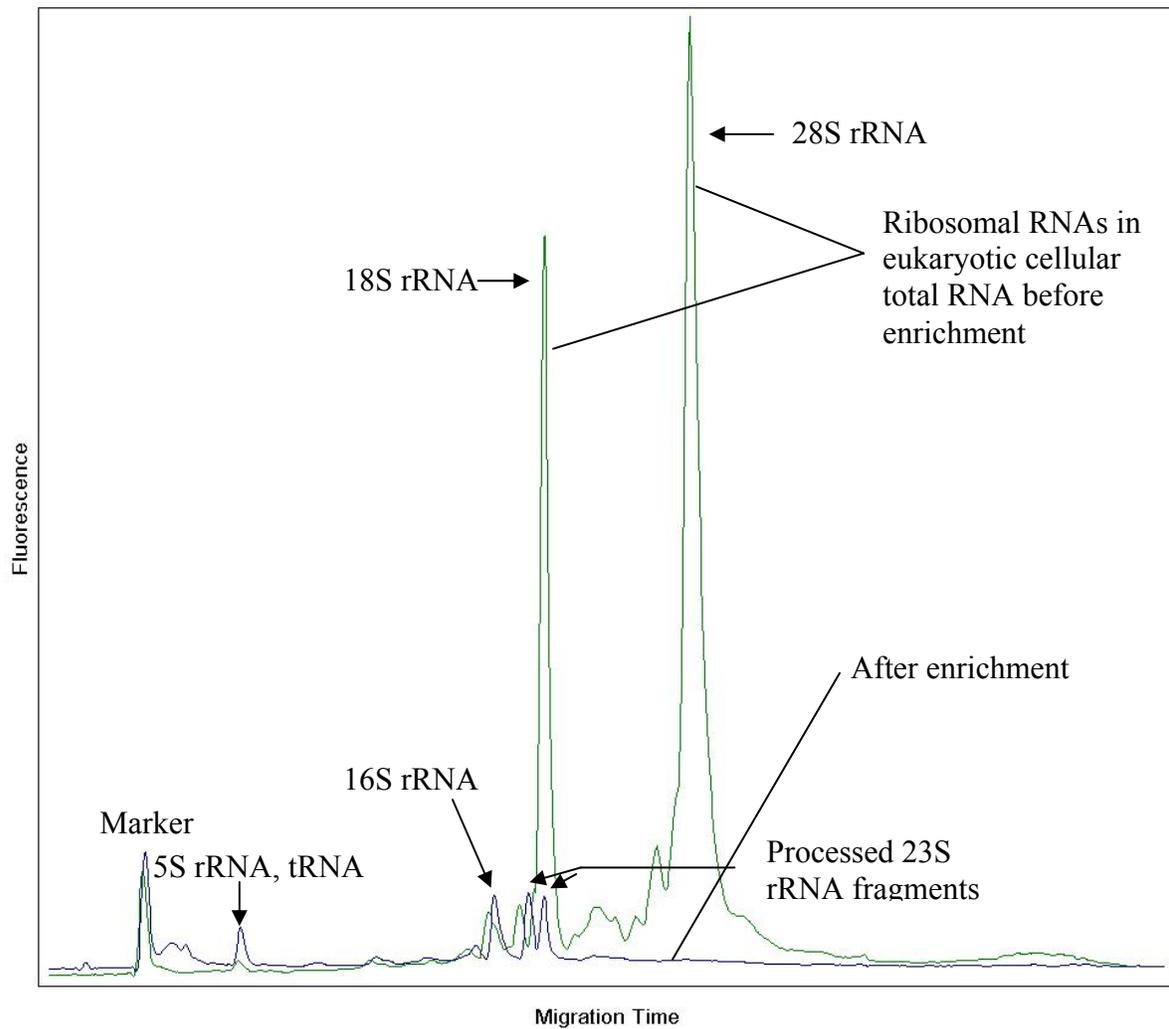
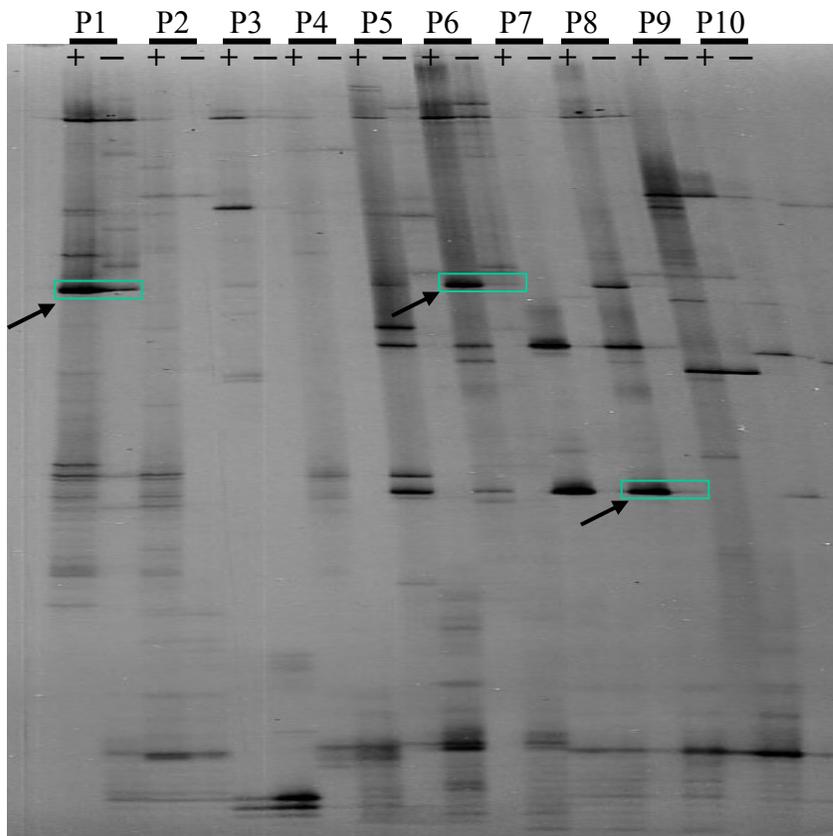


Figure III-3. Example of amplified RFDD-PCR fragments, image taken by PharosFX plus imager system (Bio-Rad, CA). The DNA products of RFDD-PCR reactions using the RNA of T84-associated *C. jejuni* 81-176 (+) and *C. jejuni* 81-176 in culture (-) were analyzed in adjacent wells by 6% acrylamide gel, and signals were visualized with ImageQuant software. Each pair of RFDD-PCR reaction was driven by one of the thirty-two selection primers. Rectangle indicated DNA bands with 5-fold different radioactivities in both T84 cell associated bacteria and bacteria in culture which were subsequently cut out, reamplified, cloned into sequencing vector and sequenced. The products of ten representative pairs of RFDD-PCR reactions (out of 32) are partially presented.



**CHAPTER IV. *CAMPYLOBACTER JEJUNI/COLI* INDUCES
POLARIZED SECRETION OF INTERLEUKIN-8 IN HUMAN
INTESTINAL EPITHELIAL CELLS**

ABSTRACT

Campylobacter is one of the leading causes of acute inflammatory bacterial diarrhea. *Campylobacter* colonizes and infects the intestinal epithelium. The intestinal epithelium provides a physical barrier to bacterial infection and also functions as a sensor of bacterial infection by secreting proinflammatory cytokines. This study examined *Campylobacter*-induced proinflammatory cytokine interleukin (IL)-8 secretion in polarized human colonic epithelial cells T84. *Campylobacter* induced IL-8 but not TNF- α secretion in polarized T84 cells. More significantly, no matter whether the bacteria were inoculated from the apical or basolateral surface, the polarized epithelial cells secreted IL-8 predominantly to the basolateral side where the epithelial cells were in contact with leukocytes *in vivo*. There was no statistically significant correlation between the IL-8 secreting levels and the invasion or transcytosis abilities of *Campylobacter*. Treating the epithelial cells with conditioned supernatants (bacteria supernatant-T84 co-culture supernatants) induced the IL-8 secretion to the level similar to that induced by the bacterial inoculation, suggesting that live, intact bacteria are not required for the induction of IL-8 secretion. Treating the conditioned supernatants with protease K, heat,

DNase I, or polymyxin B led to different levels of reduction of IL-8 secretion, suggesting that the multiple bacterial factors are involved in the induction of IL-8 secretion.

Furthermore, *C. jejuni* induced I κ B α degradation, and NF- κ B inhibitor TPCK blocked *Campylobacter*-induced IL-8 secretion. These results demonstrated that *C. jejuni* induced basolateral-polarized secretion of IL-8 in human intestinal epithelial cells, and that *C. jejuni*-induced IL-8 secretion was NF- κ B-dependent.

INTRODUCTION

The *Campylobacter jejuni* and *Campylobacter coli* are among the most frequent causes of foodborne bacterial gastroenteritis in humans worldwide (213). Infections caused by *Campylobacter* mostly manifest acute, self-limiting enteritis with diarrhea, fever, vomiting, and abdominal cramps (48, 175). A localized acute inflammatory response, which can lead to tissue damage, has been observed from both animal studies (176, 177) and clinical cases (32). The pathogenic mechanisms responsible for human acute intestinal infection of *Campylobacter* are not fully understood.

Epithelium is the initial defensive line against pathogenic threats. It provides a physical barrier for bacterial infection. In addition, it can function as a sensor of bacterial infection for the immune system. The interaction of bacteria with the intestinal epithelium could induce the secretion of a panel of proinflammatory cytokines, which provide the earliest warning signs for the immune system (145, 202). One of the major proinflammatory cytokines secreted by the intestinal epithelial cells is interleukin-8 (IL-

8) (59, 60, 110), a chemoattractant that recruits neutrophils to the infected site. The infection of a human embryo intestinal epithelial cell line INT407 *in vitro* by *C. jejuni* induced secretion of IL-8 (90). The bacterial factors that induce IL-8 secretion of epithelial cells remain to be identified. Live, intact *Campylobacter* (90) or *Campylobacter*-secreted cytolethal distending toxin (CDT) (91) has been shown to be able to induce IL-8 secretion. A recent report showed that *C. jejuni*-induced IL-8 secretion required *C. jejuni* gene products that were expressed upon contacting with epithelial cells and the activation of MAP kinase ERK in epithelial cells (223). Flagellin of enteric bacteria, such as *Salmonella*, has been shown as a major inducer of IL-8 (77). However, the flagellin of *C. jejuni* appeared to be a poor stimulator of IL-8 (223). Recent studies have shown that common microbial components, such as lipopolysaccharide (LPS) (186), bacterial DNAs that contain unmethylated CpG motif (5), and peptidoglycan (PGN) (204), can evoke epithelial IL-8 production by activating Toll-like receptors (TLRs), a family of pattern-recognition receptor that bind to conserved microbial structures and activate the innate immunity (6, 151).

Most studies thus far have been done using unpolarized epithelial cells. However, the intestinal epithelial cells *in vivo* are polarized with the apical surface faces the intestinal lumen. Two surfaces of polarized epithelial cells express different proteins and different TLRs (19, 164). It has been shown *in vitro* that *Campylobacter* colonized, invaded and transmigrated across polarized intestinal epithelial cells (127, 159). It is unknown whether the interaction of *Campylobacter* with polarized human intestinal epithelial cells will induce IL-8 production, and if so, whether the colonization, invasion,

and transmigration of *Campylobacter* are associated with the induction of IL-8 secretion in polarized epithelial cells.

Nuclear factor (NF) κ B is the major transcriptional regulator of proinflammatory cytokines in the intestinal epithelial cells (62, 165) and the main downstream target of TLR signaling pathway (6). The interaction of mucosal pathogens including *Salmonella* (93), *Neisseria* (168), *Shigella. Flexneri* (58), *Escherichia coli* (201) with epithelial cells activate NF- κ B. *C. jejuni* has been shown to activate NF- κ B in HeLa cells and human colonic epithelial cells (HCA-7) (154). *C. jejuni*-induced secretion of proinflammatory chemokines GRO α , γ IP-10, and MCP-1 has been shown to be NF- κ B dependent (96). Gastroenteritis caused by *C. jejuni* was more severe in NF- κ B deficient mice than in wild type mice (73), further supporting the importance of NF- κ B in the host responses to *C. jejuni* infection. However, the relationship between *Campylobacter*-induced NF- κ B activation and IL-8 secretion remains to be established.

In this study, we examined *Campylobacter*-induced IL-8 secretion in polarized human intestinal epithelial cells, and demonstrated that *C. jejuni* induced a polarized IL-8 secretion in polarized human colonic epithelial cells (T84), and that this polarized IL-8 secretion required the activation of NF- κ B and involved multiple bacterial factors whose production was stimulated by contacting with the epithelial cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Five *Campylobacter jejuni* and three *C. coli* chicken meat isolates were routinely grown on Mueller-Hinton (MH) agar (Difco Lab, Detroit, MI) containing 5% (v/v) lysed horse blood at 37 °C under a microaerophilic atmosphere (85% N₂, 10% CO₂, and 5% O₂). Human clinical strains *C. jejuni* 81-176 (82, 171) and NCTC11168 (210) were kindly provided by Dr. P. Guerry-Kopecko at Naval Medical Research Center (Bethesda, MD) and Dr. Alain Stintzi at Ottawa institute of systems biology, respectively. The eight *C. jejuni* and *C. coli* isolates used in this studies were selected based on their distinct DNA fingerprinting profiles of pulsed-field gel electrophoresis (PFGE) from 378 *Campylobacter* isolates that were recovered from retail raw meats in the Washington, D.C. area from June 1999 to July 2000 (235).

Cell culture. T84 cells (human colonic epithelial cell line, ATCC CCL-248) were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate (Invitrogen, San Diego, CA) supplemented with 5% (v/v) fetal bovine serum, penicillin and streptomycin (5% DME/F12), as recommended by the American Type Culture Collection, Manassas, VA. To establish polarized monolayers, T84 cells seeded onto transwells (0.33 cm²) with 3.0- μ m pore-size clear polyester membranes (Corning Costar Corp., Cambridge, MA). The medium was changed every other day. The polarization of the epithelial monolayers was monitored by transepithelial resistance measured by Millicell-ERS (Millipore, Billerica, MA). The

transepithelial resistance of T84 epithelial monolayers normally reaches 1,400 Ω /cm² or above after two weeks culture.

Inhibitors. *N*- α -p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a NF- κ B inhibitor (Sigma-Aldrich, St. Louis, MO) (36) was stored in dimethyl sulfoxide (DMSO) at -20°C. Cells were preincubated for 30 min with TPCK (50 μ M), and the inhibitor was included in the inoculation medium. Cycloheximide (148), an inhibitor of eukaryotic but not prokaryotic protein synthesis, and polymyxin B (103, 204), which binds and neutralizes LPS, were purchased from Sigma-Aldrich.

Analyses for IL-8 secretion. Polarized T84 monolayers cultured on transwells (resistance > 1,400 Ω /cm²) were washed three times with PBS and two times with the invasion medium (5% DME/F12 without antibiotics) at 37°C. Bacteria were cultured for 18 h and harvested from the plates with PBS. T84 monolayers were treated by one of the following conditions: (a) inoculation of *Campylobacter*, 4 \times 10⁶ CFU (multiplicity of infection (MOI) \sim 10) in the invasion medium either from the apical or basolateral chamber of transwells, (b) addition of conditioned supernatants generated from the apical and basolateral culture media to the apical and basolateral culture chambers, and (c) addition of polymyxin B-treated DNA extract of *C. jejuni* 81-176, and (d) addition of TNF- α (1-100 ng/ml) to the basolateral chamber. The epithelial cells were incubated for times indicated in a 5% CO₂ incubator at 37°C. The culture media from the apical and basolateral chamber were individually collected and centrifuged for 20 min at 13,000xg

to remove bacteria. A cocktail of protease inhibitors (Sigma-Aldrich) was added to the supernatant to prevent proteins from degradation. The amount of IL-8 and TNF- α in the supernatants was determined by enzyme-linked immunosorbent assay (ELISA). Mouse anti-human IL-8 monoclonal antibody (mAb) and mouse anti-human TNF- α mAb (BD Pharmingen) as capturing antibodies, biotinylated mouse anti-human IL-8 and anti-human TNF- α mAb (BD Pharmingen, San Diego, CA) as detecting antibodies, and streptavidin-alkaline phosphatase (Southern Biotech. Assoc. Inc., Birmingham, AL) and substrate *p*-nitrophenylphosphate disodium (pNPP, Sigma-Aldrich) to visualize bound antibodies. Recombinant human IL-8 and TNF- α (BD Pharmingen) with known concentrations were used to established standard curves.

Preparation of conditioned supernatants from the *Campylobacter*-epithelial cell co-culture. Conditioned media were prepared using a previously described procedure with minor modifications (77). Typically, polarized T84 epithelial monolayers were washed three times with PBS and two times with invasion medium, and were allowed to equilibrate in the invasion medium for 30 min at 37°C. *C. jejuni* 81-176 suspension (around 4×10^6 CFU) in 0.1 ml invasion medium was inoculated apically and incubated at 37°C for 4 h. After the 4 h-incubation, the apical and basolateral media were collected individually and centrifuged for 20 min at 13,000xg, and the supernatants were stored at -20°C for future use. The same amount of bacteria was suspended in 0.1 ml invasion medium and incubated alone for 4 h at 37°C. The cultures were centrifuged to remove bacteria, and the supernatant was collected and stored at -20°C. To inactivate

heat-sensitive proteins, the collected basolateral conditioned supernatant was boiled for 20 min. To remove most of protein factors, the conditioned supernatant was treated with protease K (100 µg/ml, Invitrogen) at 56°C overnight, followed by a 20 min-incubation at 100°C to inactivate protease K. To remove bacterial DNA, the conditional supernatant was treated with DNase I (10U/ml) (New England Biolab, Ipswich, MA) 37°C for 2 h respectively. The treated supernatants were analyzed using SDS-PAGE (12%) and agarose gel to confirm the removal of proteins by protease K and bacterial DNA by DNase I (data not shown). The supernatant and *C. jejuni* 81-176 DNA extract were incubated with polymyxin B (100 µg/ml) to neutralize LPS.

Adherence, internalization, and transcytosis assays. The adherence, invasion, and transcytosis of *Campylobacter* in polarized epithelial cells were analyzed as previously described (159). Typically, polarized T84 epithelial monolayers were washed three times with PBS and two times with invasion medium. *Campylobacter* suspension (around 4×10^6 CFU, MOI ~ 10) in 0.1 ml invasion medium was inoculated apically and incubated at 37°C for 4 h. The transepithelial resistance was checked before and after the incubation with the bacteria. After the 4 h-incubation, bacteria, which transmigrate across the cell monolayer into the basolateral medium, were enumerated by plating 10-fold serial dilutions of the basolateral medium on MH-blood agar plates. Cell-associated bacteria were released by lysing T84 cells with 1% saponin after washes. After incubation for 5 min and vortex vigorously for 2 min, serial dilutions of the lysates were plated on MH-blood plates. To determine internalized bacteria, T84 epithelial

monolayers were washed and incubated with gentamicin (100 µg/ml) in invasion medium at both the apical and basolateral chambers for 2 h to kill extracellular bacteria. Then cells were washed and lysed. Each assay was performed in triplicate wells and repeated at least three times. The number of adhered, internalized or translocated bacteria was plotted as a percentage of the starting viable inoculum.

Analysis for NF-κB activation. The activation of NF-κB was analyzed by following the degradation of NF-κB inhibitor IκB-α. Epithelial cells T84 were incubated with *C. jejuni* 81-176 (MOI ~10) in the presence of protein synthesis inhibitor, cyclohexamide (10 ng/ml, Sigma-Aldrich), which block protein synthesis, for varying lengths of times. By the end of the incubation, the epithelial cells were washed with cold PBS and lysed with a lysis buffer (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM MgCl₂, 50 mM NaF, 1 mM Na₃VO₄, 10 mM Na₄P₂O₇, and 1% protease inhibitor cocktail) at 4°C. After centrifugation at 13,000xg for 30 min at 4°C to remove cell debris and bacteria, the protein concentration of supernatant was determined by using BCA protein assay (Pierce, Rockford, IL). Equal amounts (20 µg) of cell lysates were subjected to SDS-PAGE and Western blot, probed with rabbit anti-IκB-α antibody (Santa-Cruz Biotechnology, Santa Cruz, CA). The blots were stripped and reprobed with mouse antibody to α-tubulin (R&D Systems, Minneapolis, MN) as loading controls.

RESULTS

Polarized T84 cells basolaterally secrete IL-8 but not TNF- α after apical exposure to *C. jejuni* 81-176. *Campylobacter*-induced IL-8 and TNF- α secretion in polarized human colonic epithelial cells T84 was determined. *C. jejuni* 81-176 was inoculated apically at a MOI of 10:1. Secretion of IL-8 was detected at the basolateral media 6 h post inoculation, the earliest point tested (Fig. IV-1). By 24 h, the concentration of IL-8 in the basolateral media reached to 100 pg/ml. In contrast, there was no significant amount of IL-8 detected in the apical media (Fig. IV-1). This indicates that *Campylobacter*-induced IL-8 secretion is basolaterally polarized. In addition, no significant increase in TNF- α secretion was detected in both the apical and basolateral media 24 h post the inoculation (Fig. IV-1).

Increased IL-8 secretion following *Campylobacter* infection is not mediated by secreted TNF- α . To test whether the IL-8 secretion by polarized T84 cells was induced by the small amount of TNF- α induced by *Campylobacter*, we determined the IL-8 secreting level of polarized T84 cells that were treated with different concentrations of TNF- α for 24 h. Figure IV-2 showed that the basolateral secretion of IL-8 by T84 cells increased in TNF- α dose-dependent manner. Induction of IL-8 to 100 ng/ml in the basolateral medium required more than 50 ng/ml TNF- α , which was much higher than the levels of TNF- α induced by *Campylobacter* (10 pg/ml, Fig. IV-1), suggesting that increased IL-8 secretion following *Campylobacter* infection is not primarily mediated by secreted TNF- α .

Campylobacter*-induced IL-8 secretion is independent of *Campylobacter

invasion and transcytosis abilities. To test the interrelationship between the IL-8 secretion induction and invasion/transcytosis abilities of *Campylobacter*, we determined the adherence, invasion, transcytosis, and IL-8 induction abilities of eight *Campylobacter* retail chicken meat isolates and two human clinic isolates that were previously shown different invasion abilities in non-polarized T84 cells(97, 108). The ten *Campylobacter* strains displayed similar adherence abilities, but different invasion and transcytosis abilities (Fig. IV-3). Human clinical strain *C. jejuni* 81-176 displayed the highest invasion and transcytosis efficiencies among ten isolates tested. The invasion ability of *C. jejuni* 65 was 40 fold less than that of *C. jejuni* 81-176, and no transcytosis was detected with *C. jejuni* 587 and 602 (Fig. IV-3). When inoculated from the apical surface of polarized T84 cells, all the ten strains of *Campylobacter* were able to induce IL-8 secretion primarily to the basolateral media despite of their differences in the invasion and transcytosis efficiencies (Fig. IV-4A). Human clinic strain *C. jejuni* 81-176 that has the highest invasion and transcytosis efficiencies among the ten strains tested (Fig. IV-3) induced a higher level of IL-8 secretion than eight other strains (Fig. IV-4A). However, no significant correlation was found between *Campylobacter*-induced IL-8 secretion with either *Campylobacter* invasion or transcytosis efficiency when analyzed by linear regression. This suggests that the invasion and transmigration of *Campylobacter* is not essential for the induction of IL-8 secretion.

***Campylobacter*-induced polarized IL-8 secretion from T84 cells is independent of *Campylobacter* entry direction.** To test whether the inoculation direction affects the polarity of *Campylobacter*-induced IL-8 secretion, we compared the IL-8 secreting levels induced by apical inoculation of *C. jejuni* 81-176 with those induced by basolateral inoculation of *C. jejuni* 81-176. Both apical and basolateral inoculation of the polarized T84 epithelial cells with eight *Campylobacter* retail meat isolates and two human clinic isolates resulted in a substantial increase in IL-8 secretion compared to uninfected T84 cells (Fig. IV-4B). The levels of IL-8 induced by the basolateral inoculation of *Campylobacter* were either similar to or slightly higher than those induced by the apical inoculation. Similar to the apical inoculation, the basolateral inoculation induced IL-8 secretion primarily to the basolateral media, indicating that IL-8 was preferentially secreted at the basolateral surface no matter whether *Campylobacter* were inoculated from the apical or basolateral surface.

Multiple bacterial factors are involved in IL-8 induction. To test whether the induction of IL-8 secretion requires live *Campylobacter*, polarized T84 cells were treated apically or basolaterally with the bacteria-free apical and basolateral conditional supernatants. The bacteria-free conditional supernatants were generated from the apical or basolateral media of polarized T84 cells that were inoculated with *C. jejuni* 81-176 apically for 4 h. In addition, polarized T84 cells treated with bacteria culture supernatant apically was used as a control. As shown in Figure IV-5, regardless of the type of the conditional supernatant that T84 cells were incubated with, a significant amount of IL-8

was induced in a basolaterally polarized manner. This suggests that live, intact bacteria are not required for IL-8 secretion. The levels of IL-8 induced by the apical and basolateral conditioned supernatants were similar, but higher than that induced by bacterial culture supernatant (Fig. IV-5), suggesting that bacterial factors secreted upon *Campylobacter*-T84 cells interaction are involved in the induction of IL-8 secretion. To test which bacterial factors in the conditioned media contribute to the induction of IL-8 secretion, we treated the basolateral conditioned supernatant with DNase to remove bacterial DNA, ligands for TLR 9, polymyxin B to remove LPS, ligands for TLR4, protease K to remove proteins, and heat to denature proteins. SDS-PAGE and agarose gel analyses showed that most of DNA and proteins were degraded by DNase and protease K treatment (data not shown). The effects of different treatments on the ability of the basolateral-conditioned supernatants to trigger IL-8 secretion were determined. Both boiling alone and protease K treatment plus boiling significantly decreased IL-8 secreting level, while protease K plus boiling was most effective in reducing IL-8 secretion among all the treatments with a 70% reduction of the basolateral secretion of IL-8 (Fig. IV-6). This suggests that bacterial secreted proteins play roles in IL-8 induction. While DNase or polymyxin B alone did not significantly change the ability of the basolateral-conditioned supernatant to induce IL-8 secreting, incubating polarized T84 cells with polymyxin B-treated DNA extract from *C. jejuni* 81-176 significantly increased IL-8 secretion (Fig. IV-6). Here, the polymyxin B treatment was used to remove LPS contraindication in *Campylobacter* DNA extract. This result suggests that both *Campylobacter* DNA and LPS are involved in the induction of IL-8. Taken together,

multiple secreted factors of *Campylobacter* appear to be involved in the induction of IL-8 secretion.

***Campylobacter* induces IL-8 secretion is NF- κ B-dependent.** NF- κ B is an important regulator of IL-8 expression (62, 165). To determine whether *Campylobacter*-induced IL-8 secretion is dependent NF- κ B activation, we first test whether *Campylobacter* can induce the activation of NF- κ B. The activation of NF- κ B requires the degradation of I κ B- α , an inhibitory subunit of the complex by the proteasome (153). The degradation of I κ B- α was followed by Western blot (Fig. IV-7A) using TNF- α treated T84 cells as a positive control. TNF- α treatment (10ng/ml) significantly decreased I κ B α level at 30 min (Fig. IV-7A). Inoculation of *C. jejuni* 81-176 induced the degradation of I κ B- α in a time-dependent manner. After incubated with the bacteria for 120 min, the amount of I κ B- α in the cell lysate decreased to the level similar to that of TNF- α -treated T84 cells. This indicates that *Campylobacter* inoculation induces NF- κ B activation in T84 cells. Next, we tested the effect of a proteasome inhibitor TPCK, which was shown to be able to effectively inhibit I κ B degradation (43, 230), on *Campylobacter*-induced IL-8 secretion. T84 cells were pretreated with TPCK (50 μ M) for 30 min before the inoculation and during the inoculation in both the apical and basolateral media. The TPCK treatment reduced the IL-8 secretion to a level similar to that of control T84 cells that were not inoculated with the bacteria (Fig. IV-7B). This

result indicates that the NF- κ B activation is required for *Campylobacter*-induced secretion of IL-8.

DISCUSSION

The interaction of enteric pathogens, including *Campylobacter*, with human intestinal epithelial cells induced proinflammatory cytokine secretion (90, 91, 145, 202), which initiates the local inflammation. One of the early cytokines that were induced by enteric bacteria is IL-8 (59, 60, 110) that chemoattracts neutrophils and T cells to infection sites (115, 129). Studies presented here show that *C. jejuni* induced NF- κ B activation and a basolaterally dominant IL-8 secretion in polarized human colonic epithelial cells (T84), and this polarized IL-8 secretion requires the activation of NF- κ B and involves multiple bacterial factors.

Similar to other enteric pathogens, the interaction of *C. jejuni* and *C. coli*, with polarized human intestinal epithelial cells T84 induces basolaterally polarized secretion of IL-8, which provides a signal for the transmigration of subepithelial neutrophils to the apical surface (25, 144, 145, 203). Furthermore, we found that the polarity of IL-8 secretion was independent of *Campylobacter*-inoculation sites, suggesting that the basolateral secretion of IL-8 does not require the transmigration of *C. jejuni* from the apical to basolateral sides. Our observation that *Campylobacter* isolates that failed to transcytose across polarized T84 cells were able to trigger the basolateral secretion of IL-8 further supports this notion. This ensures the induction of inflammatory responses

despite the varying abilities of infecting *Campylobacter* strains to transmigrate. The basolateral secretion of IL-8 independently of inoculation directions suggests that such polarized IL-8 secretion is the intrinsic property of the epithelial cells. The underlying mechanism for the polarized secretion of IL-8 by epithelial cells is unknown.

Compare to previously published studies (90, 91), the levels of IL-8 induced by *Campylobacter* showed in this study appears to be relatively low. There are several possible explanations. First, human colonic epithelial cells T84 cells that can form polarized monolayer when grown on filter were used to mimic intestinal epithelium instead of non-polarized human embryo intestinal epithelial cell INT407. Secondly, a ratio of bacteria to epithelial cells at 10 to 1 was used in this study instead of 100 to 1 used by most of previous studies (90, 91). The IL-8 secretion level has been shown to be dependent of inoculation doses of *Campylobacter*. Considering the dose effect, the IL-8 secreting levels showed here are comparable to those previously reported for *Campylobacter* (90).

A previous study demonstrated a correlation between the levels of IL-8 secretion induction and adherence and/or invasion by *Campylobacter* using a group of human clinical strains, a set of mutants of *C. jejuni* 81-176 (90), and human embryo intestinal epithelial cells INT407 as the model. In this study, we compared the IL-8 secreting levels induced by a group of chicken meat isolates with similar adherent abilities but different invasion and transcytosis abilities using polarized human intestinal epithelial cells T84. We didn't find any significant correlation between IL-8 secretion levels and

invasion or transcytosis efficacies (90). Because the strains used here have similar adherent abilities, our observation does not exclude the possible correlation between the levels of IL-8 secretion induction and adherence of *Campylobacter* to T84 cells.

The studies presented here showed that bacteria-free conditioned supernatants generated from basolateral and apical media of T84 cells that were inoculated with *C. jejuni* 81-176 for 4 h induced IL-8 secretion to the level similar to that induced by direct bacterial inoculation, which indicates that live, intact bacteria are not required for induction of IL-8 secretion and that the induction of IL-8 secretion in T84 cells involved molecules secreted by *C. jejuni* 81-176. The conditioned supernatants were more effective than the bacterial culture supernatant in the induction of IL-8 secretion, suggesting that the interaction between the bacteria and T84 cells induces the secretion of bacterial factors that are important for IL-8 induction. This is consistent with previous studies of other enteric pathogens (25, 60). Interestingly, the basolateral and apical conditioned supernatants induced IL-8 secretion to similar levels, despite that there was a much fewer number of bacteria in the basolateral media than that in the apical media since only less than 1% of *Campylobacter* inoculated transmigrated from the apical into the basolateral chamber. This may be due to a rapid transcytosis of *Campylobacter*-secreted factors independent of the bacterial transcytosis. The transcytosis of purified LPS and the association of LPS transcytosis with IL-8 secretion induction have been demonstrated previously (25), which support this hypothesis.

In this study, we further tested which secreted bacterial factors are important for IL-8 induction. It was found that protein factors appear to be the main elements involved in IL-8 induction since the treatment of protease K plus boiling abolished 70% of IL-8 basolateral secretion. One of the protein factors is likely to be cytolethal distending toxin (CDT), which has been shown to be able to induce IL-8 secretion in the absence of live, intact bacteria (91). Another potential candidate is flagellin. However, *Campylobacter* flagellin was found to be a weak stimulator for IL-8 secretion (223). The treatment with protease K plus boiling did not completely reduce the IL-8 secretion to the control level, suggesting the involvement of additional factors besides proteins. Treating polarized T84 cells with *Campylobacter* DNA alone significantly increased IL-8 secretion, suggesting that *Campylobacter* DNA, a potential ligand of TLR9, is important factor for IL-8 induction. Purified LPS, a TLR4 ligand, has been shown to be able to transcytose across polarized T84 cells and induce IL-8 secretion (25). However, no significant effect was found when polarized T84 cells treated with polymyxin B, which binds and neutralizes LPS, or DNase, which degrades DNA, alone. This implicates that IL-8 secretion can be induced by multiple bacterial factors.

In common with other enteric pathogens, *C. jejuni*-induced IL-8 secretion is NF- κ B pathway dependent, which demonstrated by the induction of I κ B degradation by *C. jejuni* inoculation and the inhibition of *Campylobacter*-induced IL-8 secretion by a NF- κ B inhibitor. Mellits et al. previously showed that viable *C. jejuni* and boiled cell-free extract of *C. jejuni* activated both NF- κ B and IL-8 secretion in HeLa and human colonic epithelial (HCA-7) cells (154). A recent report by Hu and Hickey showed that *C. jejuni*

81-176 up-regulated the expression of mRNAs of proinflammatory chemokines MCP-1, MIP-1 α and γ IP-10 at the NF- κ B dependent manner (96). Our results provide evidence directly connecting *Campylobacter*-induced NF- κ B activation and IL-8 secretion. How *Campylobacter* triggers NF- κ B activation remains to be elucidated. Further studies are required to examine the role of secreted bacterial factors individual or in combination in NF- κ B activation and IL-8 secretion induction and to understand the molecular mechanism by which *Campylobacter* triggers NF- κ B activation.

Figure IV-1. Apical exposure of *Campylobacter* induces the basolateral secretion of IL-8 but not TNF- α in polarized human intestinal epithelial T84 cells. T84 cells were cultured on transwells until the transepithelial resistance (TER) reaches 1400 Ω/cm^2 . *C. jejuni* 81-176 was inoculated from the apical chamber at MOI ~10 and incubated at 37°C for 0, 6, and 24 h. For the 24 h time point, the cells were washed at 6 h to remove unattached bacteria and cultured in fresh medium for another 18 h. The supernatants from the apical and basolateral chambers were collected separately, the bacteria were removed by centrifugation, and protease inhibitors were added to prevent protein degradation. The concentrations of IL-8 and TNF- α were determined by ELISA. Shown are the averages (\pm S.D.) of three independent experiments.

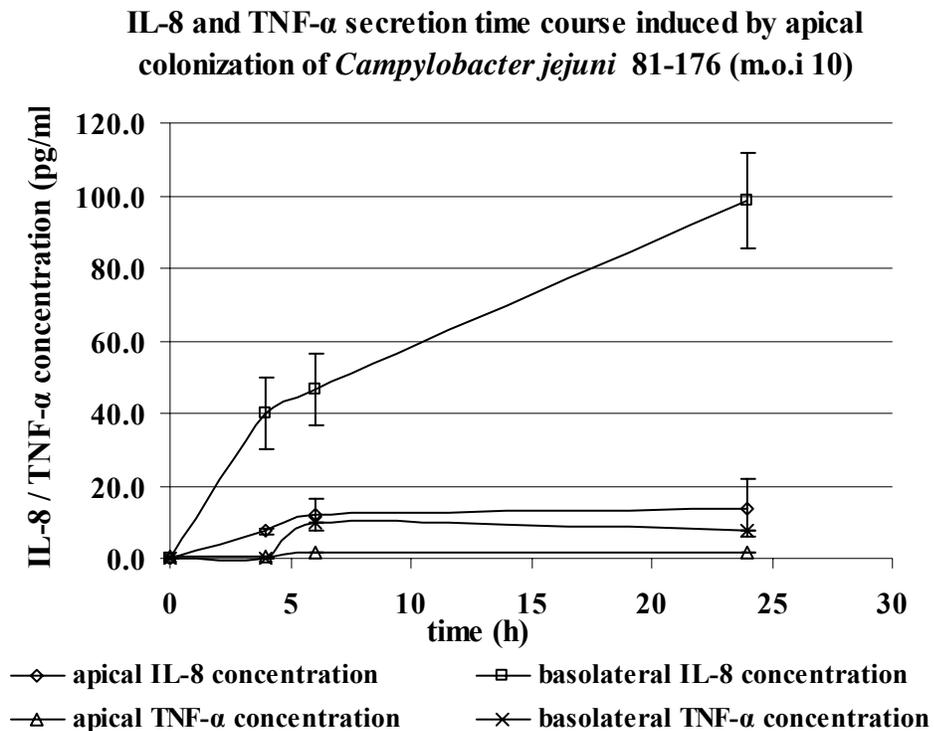


Figure IV-2. TNF- α -induced IL-8 secretion in polarized T84 cells. Polarized T84 cells were incubated with varying concentrations of TNF- α in the basolateral chamber for 4 h at 37°C. The treatment of TNF- α did not significantly changed TER of epithelial monolayers. The basolateral media were collected and the concentrations of IL-8 were determined using ELISA.

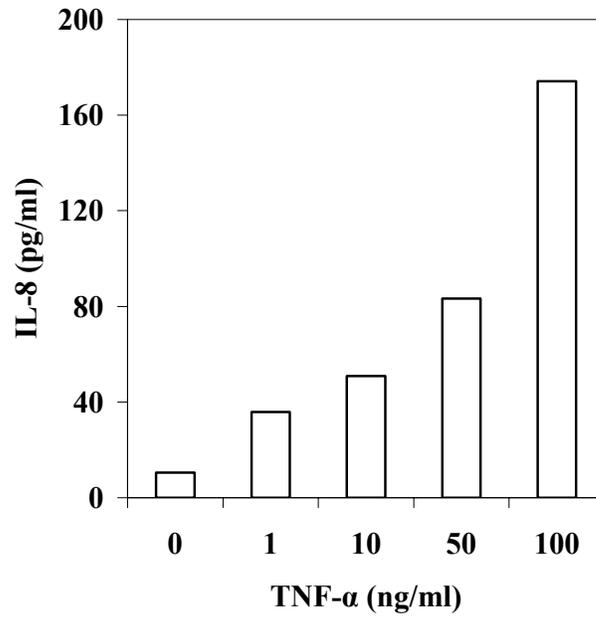


Figure IV-3. The adherence, invasion and transcytosis abilities of different *Campylobacter* isolates in polarized human intestinal epithelial cells. T84 cells were inoculated with different chicken and human clinic isolates of *Campylobacter* (*C. coli* and *C. jejuni*) from the apical chamber of transwells at MOI ~10 and incubated at 37°C for 4 h. The medium from the basolateral chamber was collected to determine the number of transcytosed bacteria. T84 cells were washed and lysed to determine the number of host cell-associated bacteria. Parallel transwells were treated with 100 µg/ml gentamicin, washed and lysed to determine the number of internalized bacteria. The data are presented as a percentage of the inoculums. Shown are averages (±S.D.) of three independent experiments with triplicate samples. Human clinical strain J81-176 was used as positive control.

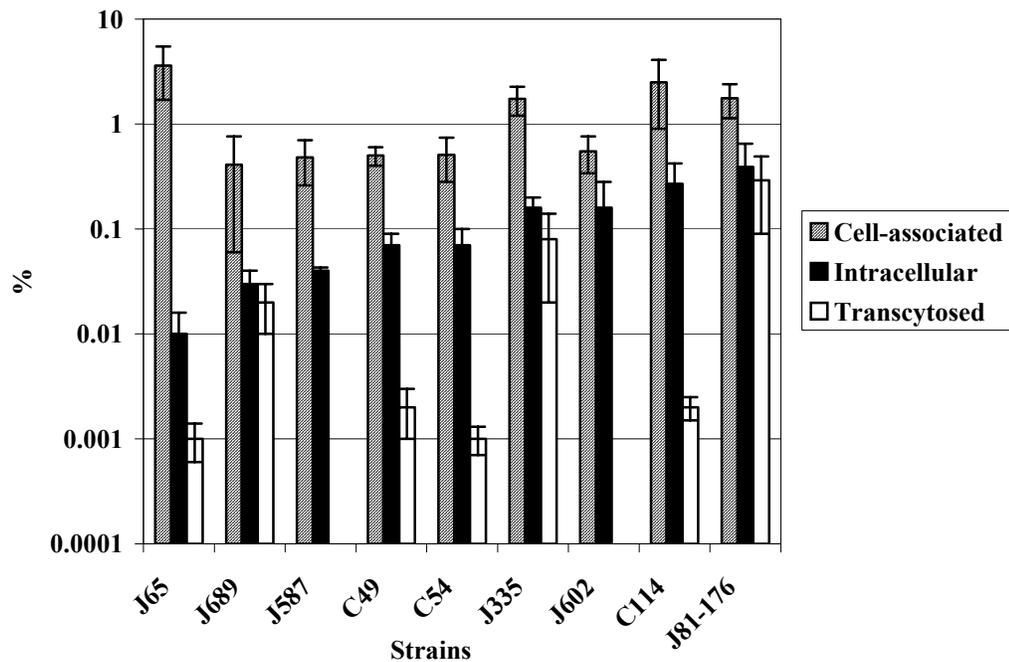


Figure IV-4. Comparison of IL-8 secretion induced by different *Campylobacter* isolates inoculated apically and basolaterally. Polarized T84 cells were inoculated from either the apical (A) or basolateral (B) side with one of eight chicken meat isolates and two human clinic isolates of *Campylobacter* (*C. coli* and *C. jejuni*) at MOI ~10 and incubated for 24 h. At 6 h, T84 cells were washed and changed into fresh medium. The apical and basolateral media were collected at 24 h, and the concentrations of IL-8 were measured using ELISA. Shown are averages (\pm S.D.) of three independent experiments. Human clinical strain J81-176 was used as positive control.

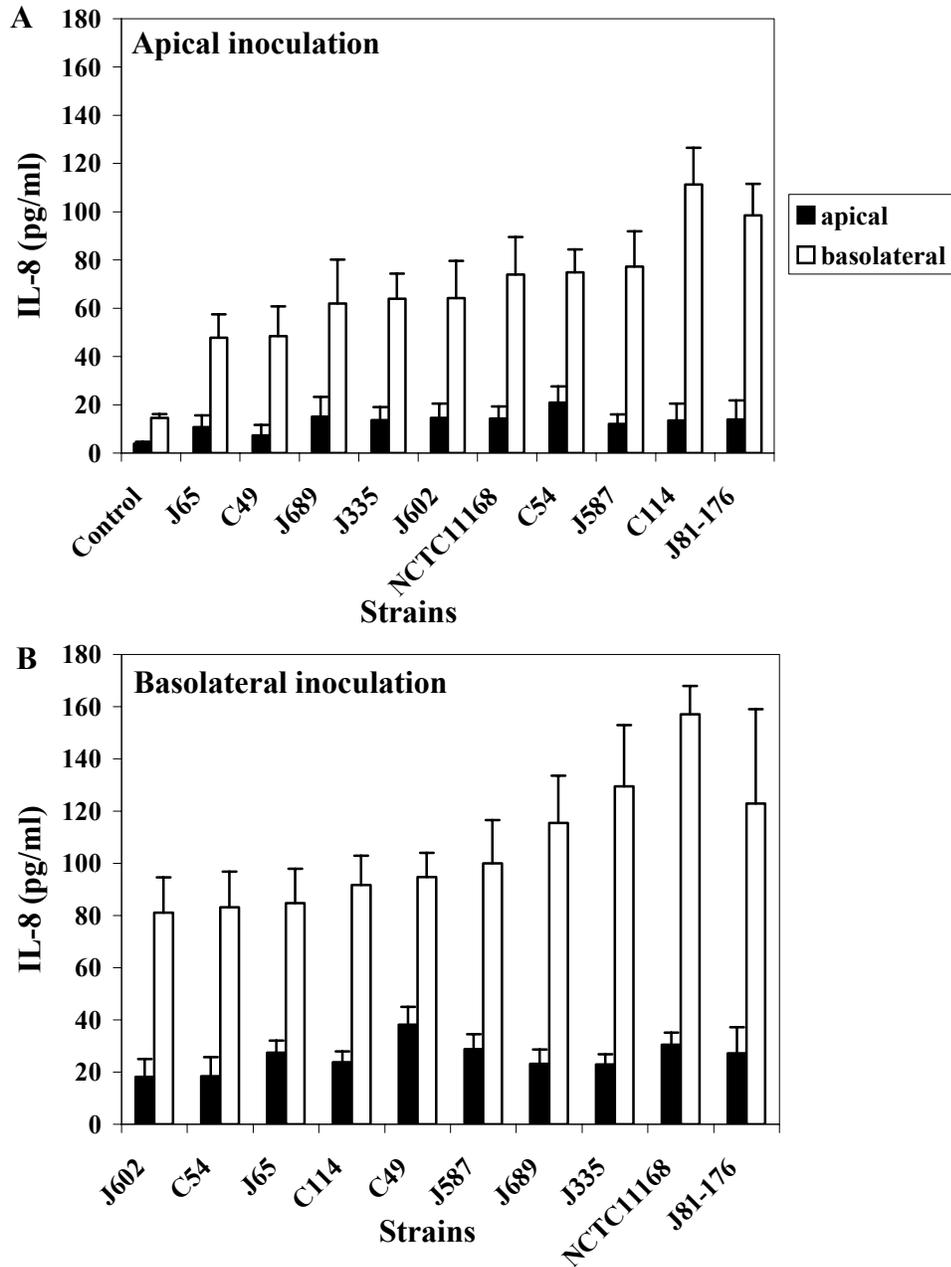


Figure IV-5. IL-8 secretion induced by *Campylobacter* culture media and conditioned supernatants. Polarized T84 cells were incubated for 24 h with bacteria-free medium that were generated from *C. jejuni* 81-176 cultured in the invasion medium for 4 h or the apical or basolateral medium of polarized T84 cells that were inoculated with *C. jejuni* 81-176 for 4 h. The apical and basolateral media were collected, and the IL-8 levels were determined by ELISA. Shown are the averages (\pm S.D.) of three independent experiments.

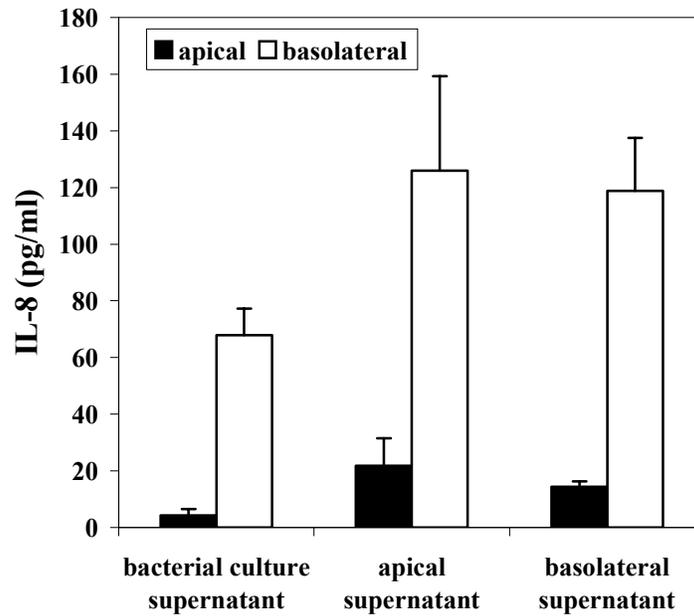


Figure IV-6. The effect of different treatments on the abilities of the conditioned supernatant to induce IL-8 secretion. The basolateral-conditioned supernatant was pretreated with proteinase K (100 $\mu\text{g}/\text{ml}$) overnight, followed by a 20 min-incubation at 100°C, DNase I (10U/ml) or polymyxin B (100 $\mu\text{g}/\text{ml}$) at 37°C for 2 h. Polarized T84 cells were incubated with the pretreated conditioned supernatants or *C. jejuni* 81-176 DNA extract (25 $\mu\text{g}/\text{ml}$) in the presence of polymyxin B (50 $\mu\text{g}/\text{ml}$) in the basolateral chamber at 37°C for 24 h. The apical and basolateral media were collected, and IL-8 concentrations were determined by ELISA. The data represent the mean (\pm S.D.) of three independent experiments and are expressed as a percentage of untreated basolateral conditioned supernatant control.

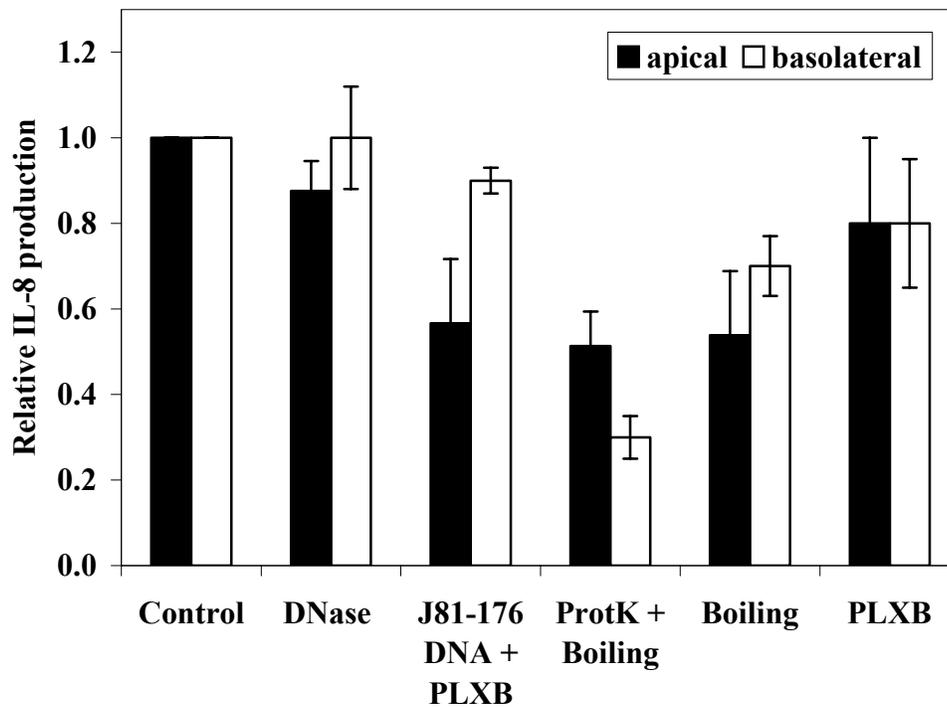
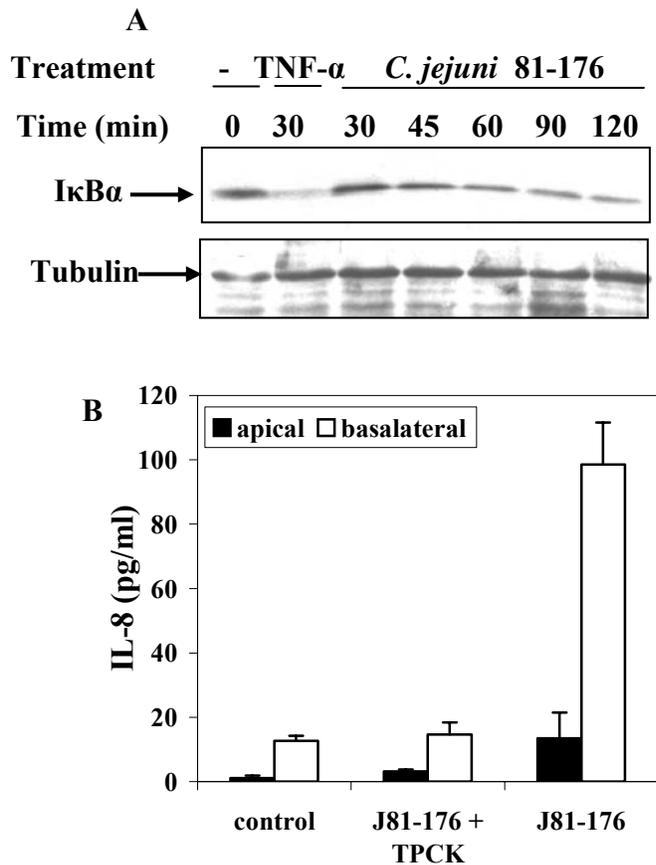


Figure IV-7. *Campylobacter*-induced IL-8 secretion is dependent on NF- κ B activation. A. T84 cells were incubated with *C. jejuni* 81-176 (MOI ~10) in the presence of cycloheximide for varying lengths of time. The cells were washed and lysed, and the cell lysates were analyzed using SDS-PAGE and Western blot, probing for I κ B α . The blots were stripped and reblotted for tubulin. Shown are the representative results of three independent experiments. B. Polarized T84 cells were incubated with *C. jejuni* 81-176 in the presence or absence of 50 mM TPCK at 37°C for 24 h. The apical and basolateral media were collected, and the IL-8 concentrations were determined by ELISA. The data represent the mean (\pm S.D.) of three independent experiments.



CHAPTER V. GENERAL DISCUSSION AND FUTURE WORK

Campylobacter jejuni/coli are common enteric pathogens, which cause human enteritis worldwide. Although campylobacteriosis can be transmitted through different means, the principal vehicle of human campylobacter infection is raw or undercooked meat. Raw meat can be contaminated with campylobacters, and poultry is by far the most important source, especially broiler chickens. Despite frequent contamination of retail meat with *Campylobacter*, it remains unclear whether all *Campylobacter* in retail meat are pathogenic to humans and which factors specifically contribute to their pathogenicity. Methodologies on specifically identifying and distinguishing pathogenic strains of *Campylobacter* from non-pathogenic ones are needed for food safety surveillance.

We examined 43 *C. jejuni/coli* isolates with distinct DNA fingerprinting profiles of PFGE recovered from retail meats. Their abilities to adhere to and invade human intestinal epithelial T84 cells were characterized and compared with a well-characterized human clinical strain, *C. jejuni* 81-176, in order to better assess their pathogenic potential. In the meanwhile, the presence of eight putative virulence genes that are potentially responsible for adherence and invasion, or encode cytolethal distending toxin in *Campylobacter* isolates was determined using PCR. Our data indicated that *C. jejuni* and *C. coli* present in retail meat were considerably diverse in their ability to adhere to and invade human intestinal epithelial cells. There was a significant correlation between the adherence and invasion abilities of the *Campylobacter* isolates. The putative virulence genes were widespread among the *Campylobacter* isolates. All *Campylobacter* isolates

possessed *flaA*, *cadF*, *pldA*, *cdtA*, *cdtB*, and *cdtC*, and most (91%) also contained *ciaB* gene except *virB11* gene, which was found only in one isolate, C114. *VirB11* gene, carried by a virulence plasmid pVir, has been showed associated with greater occurrence of blood in patient stool. However, C114 showed extremely low level of invasion ability, one possible reason could be this isolate fail to express the gene when they interact with host cells or it may express inactive variant of the *virB11* gene.

The prevalence of putative virulence genes among the *Campylobacter* isolates was high, but there was no clear correlation between the presence of the genes and the adherence or invasion abilities of the isolates. In order to understand the role of the genes in the *Campylobacter* pathogenesis, studies on the expression of these virulence genes in *Campylobacter* with or without interaction with host cells are needed. In addition, deletion mutation studies will also provide insight on the association of these genes with adherence or invasion abilities of *Campylobacter*, and on the potential redundancy or overlapping functions of these genes.

We also applied restriction fragment differential display PCR (RFDD-PCR) technique to identifying additional genes responsible for host adherence or invasion. Gene expression profiles in *C. jejuni* 81-176 were compared with or without contact with T84 cells. In the study, we extended pure bacterial RNA extraction method to rapid isolation of campylobacterial total RNA from epithelial cell infection models. However, we was not able to identify any differentially expressed genes of *C. jejuni* due to the false positive of all the isolated gene fragments, which were from stable bacterial ribosomal RNAs. In the future, DNA microarray will be a more successful alternative approach to identify *Campylobacter* specific genes associated with colonization and invasion due to

the completion of *C. jejuni* and *C. coli* genome sequences. Firstly, microarray uses either synthetic oligonucleotide or cDNA fragment as probes, where each sequence is unique and displays minimal cross-hybridization to related sequences. Hence the probes on a microarray would ideally be a comprehensive representation of the expressed fraction of the genome. Unlike RFDD-PCR, even using total RNA, this method can overcome the massive presence of ribosomal RNA and identify the real expression signals. Secondly, genomic hybridization between pathogenic and non-pathogenic strains or between strains with or without interaction with host cells using DNA microarray can sort out the genes related to pathogenesis in *Campylobacter*.

During *Campylobacter*-host cell interaction, the intestinal epithelium provides a physical barrier to bacterial infection and also functions as a sensor of bacterial infection by secreting proinflammatory cytokines. *C. jejuni/coli*-induced proinflammatory cytokine Interleukin (IL)-8 secretion in polarized human colonic epithelial cells T84 was examined in our study. *C. jejuni/coli* induced IL-8 but not TNF- α secretion in polarized T84 cells. Significantly, no matter whether the bacteria were inoculated from the apical or basolateral surface, the polarized epithelial cells secreted IL-8 predominantly to the basolateral side where the epithelial cells had contact with leukocytes *in vivo*. Treating the epithelial cells with bacteria-free conditioned supernatants from *Campylobacter*-T84 culture induced the IL-8 secretion to the level similar to that induced by the bacterial inoculation, suggesting that live, intact bacteria are not required for the induction of IL-8 secretion. Treating the conditioned supernatants with protease K, heat, DNase I, or polymyxin B led to different levels of reduction of IL-8 secretion, suggesting that the multiple bacterial factors are involved in the induction of IL-8 secretion. Furthermore, *C.*

jejuni induced I κ B α degradation, and NF- κ B inhibitor TPCK blocked *Campylobacter*-induced IL-8 secretion. These results demonstrated that *C. jejuni* induced basolateral-polarized secretion of IL-8 in human intestinal epithelial cells, and that *C. jejuni*-induced IL-8 secretion was NF- κ B-dependent. In addition, protease-treated supernatant inhibited 70% of IL-8 secretion, which had the greatest effect on IL-8 production. However, it was not clear what the protein factors were, and through which receptors *Campylobacter* induced host cells to secrete IL-8. In future studies, CDT and flagella antibodies can be used to treat the supernatants individually or together to determine if they are associated with IL-8 secretion. Additionally, protease-treated supernatants can be compared with untreated supernatants using two dimensional gel electrophoresis to identify potential proteins, which can be subsequently sequenced by mass spectrum to determine their amino acid sequences. The amino acid sequences can be converted to DNA sequences to predict the possible open reading frames. Mmutagenesis studies of these predicted genes can be used to confirm the functions of the genes. Furthermore, antibodies to host cell surface receptors can be used to block the receptors to elucidate cell surface receptors through which *Campylobacter* activates NF- κ B pathway and induces IL-8 secretion.

In summary, our studies demonstrated the pathogenic potential among *Campylobacter* isolates from retail meats regarding adherence and invasiveness in *in vitro* model; how the host epithelial cells reacted in response to *Campylobacter* infection; and what the players were involved in these processes. Yet, there is still a lot of work that needs to be done in order to elucidate what makes some *Campylobacter* isolates more pathogenic than others; and which group(s) of genes is actually involved in *C. jejuni* colonization and invasion in genetic and molecular level. Taking advantage of the

availability of the complete genome sequence of *Campylobacter*, research strategies such as DNA microarrays, reverse genetics etc. should make possible to delineate fundamental questions about *Campylobacter*-host interaction.

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