

**Activation of the Transcription Factor NF- $\kappa$ B by**  
*Campylobacter jejuni*

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## ABSTRACT

*Campylobacter* is currently the most frequently isolated food-borne bacterial pathogen worldwide. Infections caused by *C. jejuni* may be self-limiting enteritis or chronic conditions such as Guillain–Barre’ syndrome (GBS). Although the mechanisms by which *C. jejuni* causes disease are not clearly understood, the activation of the transcription factor NF- $\kappa$ B, which controls pro-inflammatory responses, is thought to be an important contributing mechanism for initiating the host's immune responses to *C. jejuni* infection. Signaling pathways leading to NF- $\kappa$ B by pathogens and/or their products include transmembrane Toll-like receptors (TLRs) and intracellular receptors nucleotide-binding oligomerization domain proteins (NODs). This study was carried out to: 1) investigate NF- $\kappa$ B activation by *C. jejuni*, 2) provide structural details regarding the NF- $\kappa$ B activating component(s) in *C. jejuni* boiled cell extract (BCE) and 3) investigate the role of TLRs (TLR2 and TLR4) and NODs (NOD1 and NOD2) in mediating NF- $\kappa$ B activation by *C. jejuni*. By means of measuring reporter gene activity, NF- $\kappa$ B activation and subsequent cytokine production by live or heat-killed *C. jejuni*, or BCE were observed in a range of tissue cultures cell lines. Structural characterisation of the NF- $\kappa$ B activating component in BCE indicated that the bioactive structure is an alpha-linked linear oligosaccharide composed of glucose where the activation by the oligosaccharide is suppressed upon pre-treatment of BCE with amyloglucosidase. NF- $\kappa$ B activation was observed to be augmented in cell lines transfected with TLR2 but not with TLR4. This activation is reduced upon transfection of cells with the dominant-negative versions (DNV) of TLR-adaptor molecules MYD88 or IRAK1. Additionally, NF- $\kappa$ B activation by *C. jejuni* was observed to be independent of NOD1 and NOD2 in cells transfected with DNV of these receptors.

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## Abbreviations

AIDS	Acquired immunodeficiency syndrome
AP	Activation protein
BCE	Boiled-cell extract
CDC	Centre for disease control
CD14	Cluster differentiation protein 14
CFU	Colony forming unit
Cadf	<i>Campylobacter</i> adhesion to fibronectin protein
CARD	Caspase recruitment domain
Cia	<i>Campylobacter</i> invasion antigens
COX2	Cyclooxygenase 2
CspA	Cold-shock protein A
DMSO	Dimethyl sulfoxide
ERK	Extracellular signal-regulated kinase
GBS	Guillain-Barre syndrome
GI	Gastrointestinal tract
GRO	Growth-related oncogene
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GLnNAc	N-acetylglucosamine
HD	Human defencin
HBD	Human $\beta$ -defecin
ICAM-1	Intercellular adhesion molecule
IEC	Intestinal epithelial cell
iE-DAP	$\gamma$ -D-glutamyl-meso-diaminopimelic acid
IFN	Interferon
I $\kappa$ B	I-kappa B
IKK	I-kappa B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
kDa	Kilo-Dalton
kGy	Kilo-Gray
LRR	Leucine-rich repeats

MAL	MYD88-adaptor like protein
MAPK	Mitogen-activated protein kinase
MEKK	Mitogen-activated protein kinase kinase
MD-2	MD-2 protein (synonym for Lymphocyte antigen 96)
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MK2	Mitogen-activated protein kinase activated protein kinase 2
MurNAc	N-Acetylmuramic acid
MYD88	Myeloid differentiation factor 88
NF- $\kappa$ B	Nuclear transcription factor kappa B
NEMO	NF- $\kappa$ B essential modulator
NIK	NF- $\kappa$ B-inducing kinase
NOD	Nucleotide oligomerisation domain
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-kinase
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RICK	RIP-like interacting capsase-like apoptosis regulatory protein kinase
RIP2	Receptor-interacting protein 2 (synonym for RICK)
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
SIGIRR	Single immunoglobulin IL-1R-related molecule
SodB	Superoxide dismutase B
SOCS1	Suppressor of Cytokine Signalling 1
ST2L	ST2L protein (synonym for Interleukin-1 receptor-like 1 precursor)
TAB1	TAK1-binding protein 1
TAK1	Transforming growth factor-beta-activated kinase 1
TCR	T cell receptor
TGF	Transforming-growth factor
TIR	Toll/interleukin-1 receptor like
TLR	Toll-like receptor
TNF	Tumor-necrosis factor
TNFR	Tumor-necrosis factor receptor
TOLLIP	Toll-interacting protein
Tpl2	Tumor-progression locus 2

TRAF	TNF-receptor associated factor
TRAM	Toll-like receptor adaptor molecule 2 (synonym for TICAM2)
TRAILR	TNF-related apoptosis-inducing ligand receptor 1
TRIAD3A	TLR-ubiquitinating enzyme
TRIF	TIR domain containing adaptor inducing interferon-beta
VCAM	Vascular cell adhesion molecule

## **CHAPTER 1 Literature review**

# CHAPTER 1

## 1.1 Introduction

*Campylobacter* infections are now recognised as a worldwide health problem (Brown *et al.*, 2004). The infection's prognosis is usually self-limiting diarrhoea (Blaser *et al.*, 1983) but occasionally chronic complications such as Guillain-Barré Syndrome (GBS) can occur (Smith, 1995). Although several virulence factors/mechanisms have been identified in *Campylobacter* (Ketley, 1997), how the bacterium elicits the host immune responses is yet to be understood. The transcription factor NF- $\kappa$ B is one of the central regulators of host-inflammatory genes. *Campylobacter jejuni* has been reported to activate NF- $\kappa$ B and produce various cytokines and chemokines (Mellits *et al.*, 2002; Jin *et al.*, 2003; Jones *et al.*, 2003; Hu and Hickey, 2005; Hu and Hickey, 2006; Johanesen *et al.*, 2006). Transmembrane receptors (TLRs) and the cytoplasmic receptors (NODs) have been shown to mediate the signaling pathways leading to NF- $\kappa$ B activation (Takada and Akira, 2004; Strober *et al.*, 2005). The roles of these receptors in *Campylobacter* pathogenesis, and the NF- $\kappa$ B-activating *Campylobacter* component or components are not known. The aims of the research are to;

- 1- Investigate the receptor-mediated signalling pathways involved in NF- $\kappa$ B activation by *C. jejuni*.
- 2- Characterise the bioactive component or components of *C. jejuni* that activate NF- $\kappa$ B.

## 1.2 History

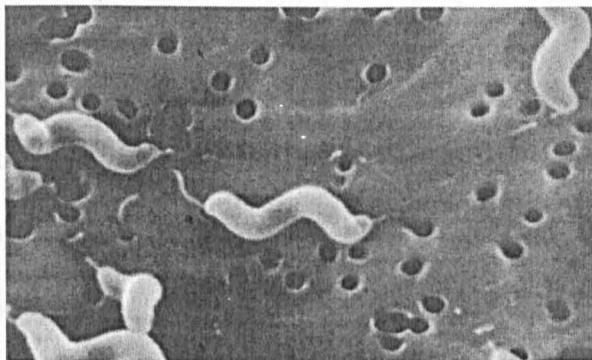
In 1963, Sebald and Veron proposed the genus *Campylobacter* after finding by means of fermentative metabolism and DNA base composition comparisons, that the vibrio-like bacterium was different from *Vibrio fetus*. Ten years later, the mis-placed

members of *Vibrio* species (*V. jejuni*, *V. sputorum*, *V. faecalis*, and *V. coli*) were transferred to the genus *Campylobacter* (Veron and Chatelain, 1973). The recognition of *C. fetus* as the cause of abortion in some farm animals and diarrhoea in others drew more scientific interest to the *Campylobacter* genus (Butzler *et al.*, 1973). This attention led to the discovery of 12 new *Campylobacter* species by the 1990s (Vandamme and Goossens, 1992). Currently, there are 16 species and 6 subspecies in the genus *Campylobacter*. The genetically related *Campylobacter* species *C. jejuni*, *C. doylei*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus* are those most frequently associated with human and animal diarrhoea (On *et al.*, 1998; Vandamme *et al.*, 1997).

### 1.3 Microbiology

Campylobacters are Gram-negative spirally curved small rods, 5-8  $\mu\text{m}$  in length and 0.2-0.8  $\mu\text{m}$  in width. They exhibit cork-screw motility by a polar flagellum at one or both ends of the cell.

**Figure 1.1:** Scanning electron microscope image of *Campylobacter jejuni*, illustrating its corkscrew appearance and bipolar flagella (Altekruse *et al.*, 1999).



Campylobacters are microaerophilic, requiring 3-15%  $\text{O}_2$ , and 3-5%  $\text{CO}_2$  concentrations depending on the species. Some species are thermophilic with an optimal growth temperature of 42  $^{\circ}\text{C}$ . Campylobacters are catalase and oxidase positive, and most are urease negative. All the species have small genome sizes of

approximately 1700 kilobases. The human related pathogens *C. jejuni* and *C. coli* have a similar G+C DNA content of 30-35% (Ketley, 1997; van Vliet *et al.*, 2001).

#### 1.4 Sources of campylobacters

The gut environment of many avian species, including those grown commercially, provides optimum conditions for *Campylobacter* (Newell and Wagenaar 2000). Colonisation of most broiler chicken flocks by *Campylobacter* is not detectable until 10 days following hatching but then persists for many weeks. The introduction of infected birds to hatching chicks will increase the risk of spreading *Campylobacter* in the population (Shanker *et al.*, 1990). Once one bird in a flock is colonised, the infection spreads very quickly through horizontal transfer (Beery *et al.*, 1988). Complete colonisation within a flock has been reported by the 49<sup>th</sup> day, which is the end of the growing period (Gregory *et al.*, 1997). The change of colonisation rate from 0 at hatching to 100% at slaughter has been suggested to be due to the fact that campylobacters can be spread from chick to chick via the communal source of drinking water (Montrose *et al.*, 1985). However, once one bird is infected the spread will be rapid because chickens are coprophagic. Litter, contaminated water, birds, insects, rodents, and farm workers are reported sources for horizontal transmission of campylobacters in poultry (Aarts *et al.*, 1995; Evans and Sayers, 2000; Line, 2000). On entering the slaughterhouse, poultry have large numbers of *Campylobacter* spp. on their skin, feathers and in their intestinal counts of up to  $\log_{10}$  9 CFU/g of caecal content have been reported from birds slaughtered and sampled at the farm. The levels of *Campylobacter* spp. on birds transported from the farm were found to increase from  $\log_{10}$  3 CFU/carcass to  $\log_{10}$  7 CFU/carcass after transport to the processing plant (Stern *et al.*, 1995). In broiler flocks, a survey of faeces found 94% of samples were positive for *Campylobacter* with an average

count of  $\log_{10}$  5.17 CFU/g (Stern and Robach, 2003). Approximately 80% of raw chickens sold in the UK are contaminated with thermophilic campylobacters at levels as high as several thousand per  $\text{cm}^2$  of skin (Corry and Atabay, 2001). It has been estimated that the frequency of human campylobacteriosis can be reduced (up to 30-fold) if a  $\log_{10}$  2.0 reduction in *Campylobacter* numbers on retail chicken carcasses is achieved. Bacteriophage therapy is thought to be an effective means by which this colonisation could be controlled, thus limiting the entry of campylobacters into the human food chain (Loc-Carrillo *et al.*, 2005).

In cattle, experimental infection of ruminating and milk-fed calves with *C. jejuni* and *C. fetus* subsp. *fetus* caused febrile enteritis, and the intestinal lesions were similar to those found in humans (Al-Mashat *et al.*, 1983). Unpasteurised milk consumption was the suspected cause of the largest outbreak of campylobacteriosis that affected 2500 school children in the UK (Jones *et al.*, 1981). *Campylobacter* has been reported to be rarely present in bulk milk tanks (Doyle and Roman, 1982), but a study recorded the presence of 16 organisms per 100 ml in sampled bulk milk tanks (Humphrey and Beckett, 1987). The source of *Campylobacter* in bulk milk was thought to originate from subclinical mastitis of cows. Contamination of milk at the collection point with faecal matter could be another source (Orr *et al.*, 1995). In beef cattle, carriage rates have been reported to be as high as 89% of 306 animals surveyed with an average of 610 organisms/g of faecal matter (Stanley *et al.*, 1998). During the pig fattening period at farms, it was found that pigs could harbour up to eight species of *Campylobacter* where *C. coli* was the most frequently isolated species (Munroe *et al.*, 1983). A study on the prevalence of thermo-tolerant *Campylobacter* spp. in lamb carcasses found that 92% of 360 small intestine samples were positive with an average concentration of  $\log_{10}$  4 CFU/g of intestinal content

(Stanley *et al.*, 1998). In another study on cases of sheep abortion, *C. fetus* subsp. *fetus* caused most abortions while *C. jejuni* was reported as the putative cause of 22% of the cases (Varga *et al.*, 1990).

*Campylobacter jejuni* can cause disease in rabbits and domestic ferrets. The carriage rates of *C. jejuni* in rats, mice, guinea-pigs, and rabbits are generally low. Healthy puppies experimentally infected with *C. jejuni* were colonised without illness or only mild enteric infection (Varga *et al.*, 1990; Skirrow, 1994). The carriage rates in dogs and cats were found to be 25% and 18%, respectively (Sandberg *et al.*, 2002).

Sources of water contamination with *Campylobacter spp.* include wild birds (Sacks *et al.*, 1986), sewage effluent (Jones, 2001), and domestic animals (Blaser *et al.*, 1983). Water contaminated by faecal matter from farm animals has been considered to be the source of some campylobacteriosis outbreaks (Clark *et al.*, 2003). Raw mussels, raw clams, and oysters contaminated with *Campylobacter* were reported to cause disease in humans. Contaminated water may have been the source of shellfish-borne infection (Trezeiva *et al.*, 1991; Stlezer *et al.*, 1989; Blaser *et al.*, 1986).

### **1.5 Environmental survival mechanisms**

Bacteria are considered to enter a viable-but-nonculturable (VBNC) state when subjected to environmental stress. In the VBNC state, bacteria do not grow on laboratory media, but are alive and demonstrate very low levels of metabolic activity (Oliver, 2000). Upon entry into stationary phase or limiting nutrients, *Campylobacter spp.* were shown to enter the VBNC state transforming from a motile spiral form to a coccoid form (Rollins and Colwell, 1986). Although resuscitation of VBNC *Campylobacter* cells has been reported in laboratory animals, isolation of

*Campylobacter* from chicks and detection of specific antibodies from humans were not reported when subjects of these experiments were infected with VBNC *Campylobacter* (Beumer *et al.*, 1992). VBNC remains a source of conjecture with respect to *Campylobacter* research.

*Campylobacter* spp are generally heat-sensitive bacteria, with a decimal reduction time of 1 minute at 55 °C. Foods receiving mild heat treatment may represent a health risk of *Campylobacter* infections. This is supported by the finding that *C. coli* isolated from pork livers subjected to heat treatment in PBS solution at 48-63 °C in water-bath showed a non-logarithmic reduction resulting in a tailing effect at temperatures above 56 °C (Moore and Madden, 2000). Of heat-shock proteins expressed by *Campylobacter*, DnaJ was reported to play a role *in vivo*. A *Campylobacter jejuni* mutation deficient in the DnaJ gene was unable to colonise chickens (Konkel *et al.*, 1998). The major cold-shock protein CspA present in many enteric bacteria is not present in the genome sequence of *C. jejuni* 11168 (Parkhill *et al.*, 2000). A sudden decrease in growth rate was observed when *C. jejuni* was subjected to temperatures a few degrees below the minimum-growth temperature. However, motility, oxygen consumption, protein synthesis and survival of *C. jejuni* at 4°C have been reported (Wilma *et al.*, 1998). *C. jejuni* grow optimally at pH of 6.5-7.5. No surviving *C. jejuni* were detected after one day or 3 days at a pH 5 and 9, respectively (Christopher *et al.*, 1982). The mechanisms of oxygen tolerance and oxygen metabolism that remove reactive oxygen species (ROS) are not clearly understood in *Campylobacter* spp. (van Vliet *et al.* 2001). However, the iron-containing SodB, *katA*-encoded catalase, and alkyl hydroxide reductase are thought to protect *Campylobacter* against oxidative stress (Purdy *et al.*, 1999; Grant and Park, 1995; Baillon *et al.*, 1999). *Campylobacter jejuni* is very sensitive to dry

conditions and storage at room temperature where it will survive less well than in the cold and humid conditions of a refrigerator (Doyle and Roman, 1982).

## 1.6 Epidemiology

Between 1991 and 2001, the number of reported cases of *Campylobacter* infections in the UK per year rose from 32,000 to approximately 56,500 (Brown *et al.*, 2004). It is now estimated that there are about 500,000 cases of *Campylobacter* infections every year in England and Wales (Food Standards Agency, UK, 2000). *Campylobacter jejuni* infections account for 90% of the reported cases (Gillespie *et al.*, 2002). At a cost of four million pounds, and an estimated 25,000 cases of infections per year, *C. coli* are also increasingly becoming recognised as an important pathogen (Tam *et al.*, 2003). Table 1.1 shows the reported incidences of *Campylobacter* enteritis in selected countries.

In the early 1980s, *Campylobacter* infections became reportable illnesses in the United States. However, the impact of *Campylobacter* infections was underestimated because the reporting systems did not culture stool samples for *Campylobacter* when attempting to diagnose other enteric pathogens. *Campylobacter* was identified 2-7 times more frequently than *Salmonella* or *Shigella* when stool samples were cultured for *Campylobacter*. Currently, it is estimated that only 1 in 38 *Campylobacter* cases are reported in the USA. In the United States, the Center for Disease Control estimates the yearly number of *Campylobacter* cases to be around 2.4 million (Mead *et al.*, 1999). For unknown reasons, *Campylobacter* infections have age and sex distributions unique among bacterial pathogens. Two age-peaks of infection occur: The first is at 1 year of age, and the second is at 15-44 years of age. The majority of infections occur among male patients. The seasonal

distribution shows a surge of infections in May and peaks in August (Friedman *et al.*, 2000)

**Table 1.1. Incidences of *Campylobacter* enteritis in selected countries.**

Country	Increasing reported incidence* (Cases per 100,000)			Decreasing reported incidence** (Cases per 100,000)			Isolation rate (%) *** (<5-yr old)
	1986	1992	1998	1997	2000	2003	
England and Wales	34	61	94				
Finland	10	23	37				
Denmark	13	14	42				
Scotland	34	86	118				
Sweden	24	41	58				
USA				25	16	12	
Algeria							17.7
Tanzania							18
Egypt							9
Nigeria							16.5
Cameron							7.7
Brazil							9.9
Guatemala							12.1
Thailand							13
Bangladesh							17.4

\*: Advances in identification methods facilitated better epidemiological reporting (Schlundt *et al.*, 2004). \*\*: Decrease is due to better prevention measures (CDC, 2005). \*\*\*: Rates from laboratory-based statistics since population surveys are not conducted in developing countries (Coker *et al.*, 2002).

In industrialised nations, the single most important route of *Campylobacter* infections is thought to be the consumption of chicken. Studies have reported that 50%-70% of all *Campylobacter* infections investigated were linked to the consumption of chicken (Harris *et al.*, 1986; Adak *et al.*, 1995). Although most *Campylobacter* infections are sporadic, consumption of unpasteurised milk is the most frequent cause of infection outbreaks (Friedman *et al.*, 2000). Consumption of sausages or red meat, contaminated water and contact with pets, are other possible sources of sporadic infections (Kapperud *et al.*, 1992; Nielsen *et al.*, 1998).

Direct transmission is mainly occupational among farmers, butchers, abattoir workers and poultry processors. Human-to-human transmission has been described

infrequently in young children (Allos, 2001). The presence of an underlying disease predisposes patients for acquiring *Campylobacter* and increases the severity of the infection (Pigrau et al., 1997). In one study on patients having bacteraemia caused by *Campylobacter*, most patients also had liver cirrhosis, vascular diseases, or AIDS (Tee and Mijch, 1998). Raising the gastric pH by an antacid medicine or using antibiotics are considered as risks for acquiring infection (Neal *et al.*, 1996). Another risk factor is international travel. Travelling to Mediterranean countries and Asia is a common campylobacteriosis risk factor for European citizens (Kapperaud, 1994; Neimann *et al.*, 2003).

In developing countries, *Campylobacter* infections are hyper-endemic among young children. Unlike infections in industrialised countries, asymptomatic infections occur commonly in both children and adults in tropical developing countries. Additionally, no seasonal peaks are observed and infection outbreaks are uncommon (Allos, 2000).

### **1.7 Prevention**

On the farm *Campylobacter* infection control may assist in reducing contamination of the retail carcasses, poultry and red meat products (Kapperud *et al.*, 1992). Strict hygiene reduces intestinal carriage in food animals (Kazwalla *et al.*, 1990). This is supported by the finding that chlorinating poultry flock drinking water reduces intestinal colonisation rates (Pearson *et al.*, 1993). Other reported ways of reducing colonisation are treatment of chicks with commensal bacteria, bacteriophage and by immunization of older birds (Stern, 1994; Loc-Carrillo *et al.*, 2005; Widder *et al.*, 1996). Reducing *C. jejuni* counts on food animal carcasses at the slaughter and processing facilities is also important for effective control of *Campylobacter* infections (Izat *et al.*, 1988). *Campylobacter jejuni* counts on chicken

and turkeys at slaughter increased by up to 100-fold during the defeathering and eviscerating stages but the bacterial count decreased during subsequent processing steps (Oosterom et al., 1983). Scalding of turkey carcasses reduced the counts to near undetectable levels. *Campylobacter jejuni* contamination of the chiller water can be reduced by the addition of sodium chloride or trisodium phosphate to the chiller water in the presence of an electrical current (Acuff et al., 1986). Up to a 100-fold decrease of carcasse contamination can be achieved by the use of chlorinated water and clean working surfaces at the slaughter house (Mead et al., 1995). The use of lactic acid on swine carcasses reduced *C. jejuni* counts to undetectable levels (Epling et al., 1993). A dose of 0.25 kGy radiation levels has been reported to be sufficient for eliminating *Campylobacter* from poultry carcasses although there is little enthusiasm from consumers for this practice (Patterson 1995).

Natural or artificial *Campylobacter* contamination can be reduced during different processing steps (Table 1.2). However, *Campylobacter* can still be detected in retail foods and in experimental test foods that have been subjected to different storage conditions (Table 1.2). In order to minimise the incidence of campylobacteriosis incidences, safe food preparation and handling procedures need to be followed (CDC, 2005). In most countries, not following safe-food handling procedures (Table 1.3) in kitchens is considered to be the risk factor associated with most cases of food-borne disease (Zhao et al., 1998). In the UK it is estimated that insufficient heat treatment and the cross-contamination of foods account for 35 and 28 %, respectively, of food-borne disease outbreaks investigated (Ryan et al., 1996). In the USA, over a 14 year period, 21% of the reported cases of food-borne diseases were related to private households (Williamson et al., 1992). After handling poultry

and raw meat, careful hand washing is very important to reduce the risk of cross-contamination (Brown *et al.*, 1988).

**Table 1.2. Incidence of *Campylobacter* in tested items.**

Item	Prevalence (%)	Population (CFU/g)	Total decrease (CFU/day)	Reference
Poultry at farm	96.4			Stern and Robach, 2001
Pre-scald skin	52.5	6.6 log <sub>10</sub>		Kotula and Pandya, 1995
Poultry carcass pre-wash	87	4.78 log <sub>10</sub>		Bashors <i>et al.</i> , 2004
Poultry carcass post-wash	80	4.3 log <sub>10</sub>		Bashors <i>et al.</i> , 2004
Poultry carcass pre-chill	99	4.75 log <sub>10</sub>		Northcutt <i>et al.</i> , 2003
Poultry carcass post-chill	83	3 log <sub>10</sub>		Northcutt <i>et al.</i> , 2003
Retail-packaged broiler	98			Stern and Line, 1992
Raw broiler	69			Willis and Murray, 1997
Chicken carcass at retail	63			Bongkot, 1997
Chicken, lamb, pig, and ox liver at retail	83, 72.9, 71.7, and 54.2, respectively.			Kramer <i>et al.</i> , 2000
Chicken, duck, turkey, beef, pork, lamb, bulk milk, and shellfish	49.9, 45.8, 37.5, 3.2, 5.1, 11.8, 1.6, and 2.3, respectively.			Whyte <i>et al.</i> , 200
Chicken carcass stored at -20 °C			1.4 log <sub>10</sub> /64	Oosterom <i>et al.</i> , 1983
Ground beef stored at -15 °C			3 log <sub>10</sub> /14	Stern and Kotula, 1982
Red meat stored at -18 °C			1.7 log <sub>10</sub> /40	Gill and Linda, 1982
Chicken meat stored at 4 °C			1.1 log <sub>10</sub> /18	Blankenship and Craven, 1982

**Table 1.3. Consumer's responses to food safety-procedures surveys.**

Safe food handling/ Hygienic procedures	Percentage of surveyed people who did agree with statement	Reference
Washing hands not important in relation to food hygiene	18.5%	Jay <i>et al.</i> , 1999
Washing hands not performed after handling raw meat and poultry	34%	Altekruse <i>et al.</i> , 1995
Cutting board not washed after handling raw meat and poultry	33%	Altekruse <i>et al.</i> , 1995
Food items handled in a way that could lead to cross-contamination	76%	Daniels, 1998
Sufficient heat treatment not recognised as a preventive option to food borne disease	33%	Altekruse <i>et al.</i> , 1995
Meals not heated to a core-temperature of 74 °C	24%	Griffith <i>et al.</i> , 1998

The isolation of *Campylobacter* from hands that handled raw poultry was reported in 42 of 58 trials (de Boer *et al.*, 1990). When contaminated hands were washed and dried, *Campylobacter* could not be detected. Drying hands after washing is important for elimination of the bacteria from the fingers (Coates *et al.*, 1987). *Campylobacter* was detected on kitchen utensils exposed to raw poultry and on vegetables (handled with these utensils), in 46 and 9 % of the tested samples, respectively (de Boer *et al.*, 1990). Enforcing stricter food safety measures by government agencies would probably minimize the risk for food-borne diseases. At places where foods are prepared or consumed in large quantities, this enforcement would be more effective. In the USA, there was a decline in the incidence of *Campylobacter* in the period from 1996 to 2003. This decline is considered to be as a result of safe food-control measures and educational campaigns carried out by the specialising government agencies (CDC, 2005).

## 1.8 Identification

Direct microscopic examination of fresh liquid faeces with Gram's stain or a phase-contrast optical system can be used for quick *Campylobacter* enteritis diagnosis. The spiral morphology and rapid spinning motion can aid in distinguishing *Campylobacter* spp. from other bacteria. Latex agglutination antigen-based commercial kits can be used for direct detection of *C. jejuni* and *C. coli* in faecal samples (Bolton, 2001). Primary plating on selective blood-based or blood free charcoal-based plates in a micro aerobic atmosphere at 42 °C for 48-72 h is used for *C. jejuni* and *C. coli* isolation (Karmali *et al.*, 1986; Endtz *et al.*, 1991). The selective agent that is used to inhibit most stool flora other than *Campylobacter* species is cephalothin. An antibiotic-free media for culturing stool samples filtered through <0.65 µm filters can be used if the initial results of culture are negative and *Campylobacter* is suspected to be present. This is because some non-*jejuni* campylobacters are susceptible to cephalothin (Bolton, 2001). To isolate *Campylobacter* from food samples, where its presence can be in low numbers compared to other competing bacteria, it may be necessary to use enrichment culture in a broth medium. This allows the recovery of small numbers of cells prior to plating on selective media. This process may take up to five days to achieve definitive results (Sails *et al.*, 2003). A rapid PCR assay for direct detection of *Campylobacter* from chicken faeces has been developed (Bang *et al.*, 2001). Drawbacks of PCR assays include; presence of inhibitors and the sample volumes needed for mass screening. The development of a DNA microarray method, which overcomes the limitations of PCR, that can be used for the direct detection of *Campylobacter* from chicken faecal swabs in 3 h has been reported. Specific sequences targeting the 16S rRNA and, the 16S-23S rRNA intergenic region were

amplified and fluorescently labelled using PCR. The PCR products were then hybridized to immobilised capture probes on the microarray. Using this method *C. jejuni* and *C. coli* can be detected and differentiated directly from chicken faeces (Keramas *et al.*, 2003).

Because of the similarity of the symptoms and the disease prognosis caused by *Campylobacter* species, no further identification is usually performed in routine laboratory settings. In most laboratories, biochemical testing is limited by cost and duration of the tests. To trace the routes of *Campylobacter* transmission, incidence and patterns, an epidemiologist needs the means for species differentiation (Frost, 2001). Given that the incorrect results of some biochemical tests such as antibiotic resistance and the hippurase test can confound the epidemiological investigation, differentiation among campylobacters should not rely on just biochemical testing (Bolton *et al.*, 1984). The biochemical tests used to aid *Campylobacter* spp differentiation include; hippurate hydrolysis, catalase, alpha-haemolysis, urease, nitrate reduction, selenite reduction, hydrogen sulphide production, indoxyl acetate hydrolysis, and growth (at 25/42 °C; on minimal medium and on MacConkey; in the presence of glycine, NaCl, cefoperazone, cephalothin, or nalidixic acid) profiles (Vandamme, 2000).

### **1.8.1 Serotyping**

The serotyping for *C. jejuni* was described by Penner and Hennessy on the basis of using soluble heat-stable antigens in a haemagglutination test. This test comprises of 47 antisera for *C. jejuni* and 18 for *C. coli* (Penner and Hennessy, 1980; Penner *et al.*, 1983). In 1982, a serotyping method that detects variation in heat-labile antigens was proposed (Lior *et al.*, 1982). This method, which is a slide-agglutination based test, recognises 130 serotypes of *C. jejuni*, *C. coli*, and *C. lari*. The need for high quality

antisera, and the non-typeability (up to 20% of isolates) are the main disadvantages of serotyping (Lior *et al.*, 1982; Nielsen *et al.*, 1997).

### **1.8.2 Phage typing**

In 1985, Grajewski and others developed a phage-typing system for differentiating between strains of *C. jejuni* and *C. coli* (Grajewski *et al.*, 1985). The system has an epidemiological potential, and can be used as adjunct or an alternative to serotyping. In the UK, the isolation of *Campylobacter*-specific bacteriophages from broiler chicken (Connerton *et al.*, 2004) and retail poultry (Atterbury *et al.*, 2003) has been reported. Approximately 80.6% of 754 *Campylobacter* isolates from 17 different countries were typable using this method (Khakhria and Lior, 1992).

### **1.8.3 Genotyping**

#### *1.8.3.1 Fla typing*

*Campylobacter*s have two flagellin genes, *flaA* and *flaB*. In *C. jejuni*, the two genes are in tandem, separated by approximately 0.2 kilobases. The genes are conserved, with 92% identity between the *flaA* and *flaB* DNA sequences from individual isolates (Meinnersmann *et al.*, 1997). However, the intragenic region of the *fla* genes differs between isolates, thereby providing the basis of a typing scheme. Indeed, *fla* typing has proved to be valuable for the majority of *C. coli* strains and some strains of *C. lari*, *C. helveticus* (Owen *et al.*, 1993) and *C. jejuni* subsp. *doylei* (Wassenaar *et al.*, 2000). Although *fla* typing has proven to be a useful, reliable and relatively simple subtyping technique, the variations in the procedure that have been described do not allow results obtained in different laboratories to be compared directly (Wassenaar *et al.*, 2000).

### 1.8.3.2 Ribotyping

In 1992, ribotyping based on the detection of RFLP-containing ribosomal RNA genes was introduced. Utilising the 16S gene of the heat-stable serotypes of *C. jejuni* 100% typeability was obtained (Fitzgerald *et al.*, 1996). This time-consuming typing method can not distinguish between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* since these subspecies contain only three ribosomal copies (Denes *et al.*, 1997). However, ribotyping is most useful for distinguishing between *Campylobacter* species that are difficult to analyse by phenotypic tests (Kiehlbauch *et al.*, 1991).

### 1.8.3.3 Random Amplification of Polymorphic DNA (RAPD)

In the RAPD typing method, the result of an amplified random primer shows banding patterns of PCR products when run on agarose gels that are suitable for comparison of isolates within bacterial species. The discrimination level obtained by RAPD for *Campylobacter* was reported to be as good as that of pulsed field gel electrophoresis (Nielsen *et al.*, 2000). However, the reproducibility of RAPD typing may vary in different laboratories (Hernandez *et al.*, 1991). Up to 14% of strains examined were untypeable due to DNase activity (Hernandez *et al.*, 1991; Fitzgerald *et al.*, 1996).

### 1.10.3.4 Amplified Fragment Length Polymorphism (AFLP)

The AFLP method has been used to subtype *C. jejuni* (Duim *et al.*, 1999) but is a complex and expensive typing method to set up. In this method, the genome is digested with two restriction endonucleases, oligonucleotides are ligated to the fragments, and then amplified. This produces fragments of different size which produces a unique fingerprint when separated by electrophoresis (Lindstedt *et al.*,

2000). The possibility of sampling the whole genome randomly is a major advantage of this method (Wassenaar *et al.*, 2000).

#### 1.8.3.5 Multi-locus Sequence Typing (MLST)

Multi-locus sequence typing uses comparative DNA sequencing of conserved housekeeping genes to characterise bacteria. The level of diversity across the MLST loci is important for the potential identification of alleles or sequence types that may correlate with animal host, geographical location, or other factors related to the source of the strain (Miller *et al.*, 2006). MLST was able to discriminate among *C. jejuni* isolates effectively, and is thought to provide a means for the investigation of disease outbreaks (Dingle *et al.*, 2001). As MLST provides unambiguous profiling and data can be compared on internet databases, MLST is thought to be the choice method for genotyping *C. jejuni* during outbreak investigation (Wassenaar *et al.*, 2000).

#### 1.8.3.6 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is a technique used to separate long strands of DNA by length in order to compare differences among samples. Plasmids which may occur in *Campylobacter* spp. may potentially be detected by PFGE together with the chromosomal DNA (Fayos *et al.*, 1993). DNA fragments obtained by digestion with random cutting restriction endonucleases are generally very large (up to 200 kb). These can be separated on the basis of size by using special electrophoretic conditions. Variations in the presence of relevant restriction sites result in genotypic profiles. PFGE has been used to type *C. jejuni*, *C. coli*, *C. fetus* and *C. upsaliensis* (Yan *et al.*, 1991). However, because of the variations in the restriction enzymes and electrophoretic conditions used, comparisons of the PFGE profiles obtained in

different laboratories require careful consideration (Gibson *et al.*, 1994; Bourke *et al.*, 1996).

### **1.9 Symptoms and complications**

The infectious dose of *Campylobacter* and susceptibility of exposed humans to develop the illness were reported to be variable. It is reported that as few as 500 organisms can cause the disease (Walker *et al.*, 1986). The acute self-limited gastroenteritis caused by *Campylobacter* is characterised by watery or bloody diarrhoea, fever, and abdominal cramps. However, because these symptoms can be associated with other bacterial enteric infections, it is not possible to diagnose *Campylobacter* infection based on the symptoms (Blaser *et al.*, 1983). The incubation period can extend to 10 days but it is commonly between 2 and 5 days. Vomiting is rare and abdominal pain may persist after diarrhoea stops (Butzler, 2004). Stool sample examination shows the presence of leukocytes, fresh blood and mucus. Other complications may include mucosal oedema with or without haemorrhage, jejunum mesenteric adenitis and immunoproliferative small intestinal disease. Bacteraemia may occur in immune compromised patients (Skirrow *et al.*, 1993). Patients may rarely develop extraintestinal infections, including meningitis (Goossens *et al.*, 1986), osteomyelitis and neonatal sepsis (Vandenberg *et al.*, 2003). With an incidence of 1/1000 infections, the paralytic condition, Guillain–Barre’ syndrome (GBS), is the most serious complication of *Campylobacter* infection (Smith, 1995). *Campylobacter jejuni* is the most frequently observed pathogen as the antecedent infection in patients with GBS (Leonard *et al.*, 2004). This peripheral neuron demyelinating acute syndrome is characterised by an ascending paralysis. Oligosaccharides from *C. jejuni*, have a structure that mimics the GM1 ganglioside of the peripheral neuron membrane, which may play a role in GBS pathogenesis

(Yuki *et al.*, 1997). Major neurological sequelae occur in 20% of cases, and the mortality rate of GBS is 2-3% (Brescoe *et al.*, 1987).

### **1.10 Treatment and antibiotic resistance**

Preventing dehydration in patients with *Campylobacter* enteritis is necessary for a good prognosis. In severe and prolonged cases of infection, antibiotics such as erythromycin may be used for treatment (Altekruse *et al.*, 1999). The resistance of *Campylobacter* to fluoroquinolones has been reported (Smith *et al.*, 1999). Coincidentally, the existence of fluoroquinolone resistance in *Campylobacter* was dated to the introduction of these agents for use in veterinary medicine (Endtz *et al.*, 1991). Mutations affecting efflux pumps as well as mutations in the genes encoding DNA gyrase and topoisomerase have been reported as the resistance mechanisms toward fluoroquinolones. Although efflux-mediated resistance has been demonstrated in the laboratory, the existence of clinical resistant isolates has not been reported. Wildtype *C. jejuni* gyrase is more susceptible to inhibition by quinolones than that of purified gyrase from fluoroquinolone resistant *C. jejuni* (Gootz and Martin, 1991; Charvalos *et al.*, 1995). Macrolides such as erythromycin bind to the ribosome and cause inhibition of the association of the peptidyl-tRNA. The resistance of *C. jejuni* and *C. coli* to erythromycin is not mediated by efflux, modification of the antibiotic, or the presence of rRNA methylase. The screening of the 23S rRNA genes from erythromycin-resistant campylobacter identified mutations at the same locations in each genome. Alteration of the ribosome may therefore be the mechanism of resistance to macrolides (Yan and Taylor, 1991; Taylor *et al.*, 1997).

## 1.11 *Campylobacter* Pathogenesis

### 1.11.1 Motility

The corkscrew-like motion of *Campylobacter* is facilitated by the polar flagella (Newell *et al.*, 1985). In *C. jejuni*, the flagellum consists of a basal body, hook and filament. Two proteins (FlaA and FlaB) make up the flagellar filament. The FlaA protein (regulated by  $\sigma_{28}$ ) is more highly expressed than than FlaB protein which is regulated by  $\sigma_{54}$  (Nuijten *et al.*, 1991; Henderixon *et al.*, 2001). Mutants of FlaA but not FlaB produced truncated filaments (Guerry *et al.*, 1991; Wassenaar *et al.*, 1991). Although the motility of *Campylobacter* correlates with the FlaA protein synthesis, fully flagellated and motile campylobacters have been reported in a FlaA mutant (Wassenaar *et al.*, 1994). Chemically induced mutations resulted in *C. jejuni* singularly-flagellated and non-flagellated cells. These mutants were non-motile, and were unable to colonise mice. Bipolar flagella may be necessary for *Campylobacter* to move through the intestinal mucus (Morooka *et al.*, 1985). A hypo-motile isolate of *C. jejuni* was able to colonize the mouse intestine as efficiently as the wild-type strain (Newell, 1986). In day-old chicks, the expression of flagellin was more important than motility for optimal colonisation (Wassenaar *et al.*, 1993). However, *C. jejuni* having FlaA but not FlaB resulted in strains that colonised the chicks at higher levels than the wild-type bacteria (Wassenaar *et al.*, 1993).

In epithelial cells cultured with *C. jejuni*, the expression of FlaA was found necessary for maximal invasion and translocation across polarised cell monolayers (Wassenaar *et al.*, 1991; Yao *et al.*, 1994). Interestingly, *C. jejuni* expressing FlaB but not FlaA exhibited higher invasion ability. The flagellar structure may be more important for *C. jejuni* internalisation than motility (Wassenaar *et al.*, 1991; Grant *et al.*, 1993).

### 1.11.2 Chemotaxis

Chemotaxis, which is the movement of an organism towards or away from chemical substances, of *Campylobacter* has been studied (Hugdahl *et al.*, 1988; Takata *et al.*, 1992). In agar plates containing a variety of different chemicals, *C. jejuni* exhibited a chemotactic response to some amino and organic acids, and L-fucose. A zone of turbidity in the plates indicated that the terminal sugar L-fucose, a component of mucin, acts as chemoattractant for *C. jejuni* (Hugdahl *et al.*, 1988). As with other enteric pathogenic bacteria (Freter *et al.*, 1981), chemotaxis has been reported to be a virulence factor for *C. jejuni* (Takata *et al.*, 1992). Non-chemotactic mutants of *C. jejuni* were not able to colonise mice even at high doses. The flagella of these mutants were similar to those from the wild-type (Takata *et al.*, 1992). A motile non-chemotactic *cheY* *C. jejuni* mutant was capable of adherence and invasion of cultured cells. However, this mutant was not able to colonise mice or cause symptoms in ferrets (a model of infection sometimes used to mimic human infection). Motility without chemotaxis ability may have led to the removal of *C. jejuni* from the host by fluid flow (Yao *et al.*, 1997).

### 1.11.3 Adhesion, invasion, and translocation

Heat-killed or treatment of *C. jejuni* with either sodium azide or chloramphenicol did not affect the ability of the cells to adhere to mammalian tissue culture cell lines. This observation suggests that *C. jejuni* constitutively expresses surface adhesins (Konkel and Ceiplak, 1992). In a non-polarised cell line treated with a chemical mimicking the mucus layer, *C. jejuni* showed increased binding and entry into the cells. The mucus-mimicking chemical treatment prolongs the contact of *C. jejuni* with the cell line resulting in increased adherence and invasion (Szymanski *et al.*, 1995). A protein termed PEB1 has been reported to mediate the

binding of *C. jejuni* to epithelial cells (Pei *et al.*, 1998). Three other outer membrane proteins (OMP) have been proposed to play a role in *C. jejuni* binding to host cells (de Melo and Pechere, 1990). CadF is another OMP that has been identified in the mediation of binding of *C. jejuni* to fibronectin (Konkel *et al.*, 1997). The major outer membrane protein (OmpE) was also reported to act as adhesin (Moser *et al.*, 1997).

The lipoprotein, JlpA was reported to have a role in *C. jejuni* adherence. A JlpA mutant showed reduced ability to bind a HEP-2 cell line (Jin *et al.*, 2001). The flagella contact with host cell, as observed by electron microscopy may indicate that flagella act as adhesins (Konkel *et al.*, 1992). Another deduced role for LPS is supported by the observation that pre-treatment of cell lines with LPS reduced the binding of *C. jejuni* to the cells (McSweegan and Walker, 1986).

Using an antibiotic protection assay, several studies investigated *C. jejuni* entry, survival and replication in tissue culture cell lines (Newell *et al.*, 1985; Konkel *et al.*, 1990; Everest *et al.*, 1992; Doig *et al.*, 1996; Konkel *et al.*, 1999). During incubation with eukaryotic cells, *C. jejuni* secretes invasion antigens, the Cia proteins. A *C. jejuni* mutant in CiaB exhibited reduced invasion but its ability to bind to a eukaryotic cell line was not altered (Konkel *et al.*, 1999). The infection of piglets with a *C. jejuni* CiaB mutant resulted in delayed development of diarrhoea and less severity of histological damage compared to controls. These observations indicate that at least the CiaB protein promotes *C. jejuni* invasion and its uptake by host cells (Konkel *et al.*, 1999). The invasion ability was reported to be strain-dependent (Newell *et al.*, 1985; Konkel and Joens, 1989) with the clinical isolates showing higher invasion level than the environmental isolates (Newell *et al.*, 1985). Clinical isolates from individuals with colitis had higher levels of invasion than those

from asymptomatic individuals (Everest *et al.*, 1992). Invasion has been reported with low (Hu and Kopecko, 1999) and high (Biswas *et al.*, 2000) multiplicities of infection. *Campylobacter jejuni* survives in the invaded cells and induces a cytotoxic response (Konkel *et al.*, 1992). Metabolic inactivation has been reported to prevent endocytosis-mediated internalization of *C. jejuni*, where synthesis of internalization promoting proteins (IPP) is thought to be required to mediate invasion. *Campylobacter jejuni* invasion ability was inhibited by the suppression of IPP synthesis by chemical or heat treatment (Konkel and Ceiplak, 1992). The entry-promoting proteins are reported to be only synthesised by *C. jejuni* when incubated with epithelial cells (Konkel *et al.*, 1993). Animal models of infection revealed that colonic biopsy from *C. jejuni* infected monkeys showed penetration of the epithelial cells by the bacteria (Russell *et al.*, 1993). Other animal model studies on newborn pigs (Babakhani *et al.*, 1993), mice (Sosula *et al.*, 1988), and chicken embryos (Welkos, 1984) reported similar observations indicating that *C. jejuni* is able to invade epithelial cells.

The ability of a bacterial pathogen to translocate across a cell barrier is considered a virulence mechanism. Where a pathogen translocates across a monolayer of polarised cells it causes more cellular damage than a pathogen that minimally translocates (Kops *et al.*, 1996). *Campylobacter* isolates from patients with enteritis were twice as able to translocate across polarised cell lines than non-clinical isolates (Everest *et al.*, 1992). However, non-invasive *C. jejuni* isolates were also reported to translocate across polarized monolayers (Everest *et al.*, 1992). In another experiment, the ability of clinical isolates of *C. jejuni* to translocate across polarised cells and invade polarised and non-polarised cells was investigated. No correlation between invasiveness and the ability of the isolates to translocate was

reported (Harvey *et al.*, 1999). The monolayer integrity was maintained over a 6 h period as measured by trans-epithelial electrical resistance (TEER). However, a loss of TEER was reported after 24 hours of incubating cell monolayers with *C. jejuni* indicating the disruption of the tight junctions (Harvey *et al.*, 1999; Bras and Ketley, 1999). Bacterial translocation, invasion, or toxin production may have led to tight junction disruption (Bras and Ketley, 1999). *Campylobacter jejuni* translocation may be via transcellular or paracellular routes. Internalised bacteria and reducing the host endocytic process by lowering the temperature support the transcellular route (Konkel *et al.* 1992). The use of the paracellular route is supported by the observation that the invasiveness of *C. jejuni* does not correlate quantitatively with translocation efficiency (Harvey *et al.*, 1999).

#### 1.11.4 Cytolethal distending toxin (CDT)

In 1988, Johnson and Lior reported that different *Campylobacter* strains produced CDT. The addition of diluted cell lysates of the whole bacteria to different cell lines caused cell nuclei distension and eventual cell death (Johnson and Lior, 1988). CDT causes eukaryotic cells to arrest in the G2/M transition phase of the cell cycle. Although cell division stops, the cytoplasm continues to grow and distend resulting in morphological changes and cell death (Lara *et al.*, 2000). The production of heat-labile and trypsin-sensitive CDT is dependent on three genes (*cdtA*, *cdtB*, and *cdtC*), the expression of which is not affected by alterations in environmental conditions (Picket *et al.* 1996; Picket, 2000). Three protein subunits (outer-membrane associated) of CDT are necessary for the toxin delivery and activity (Hickey *et al.*, 2000). A role for CDT in *C. jejuni* invasion has been proposed (Purdey *et al.* 2000). In mice challenged with the *C. jejuni* wild-type or a *cdtB* mutant it was shown that there were differences in the presence of the organism in

the various organs examined. At an early time point, the *cdtB* mutant colonisation rate of mice was less than that of wild-type *C. jejuni*. It has been reported that the pathologic effects of CDT are caused by its DNase-like activity. Incubation of human fibroblasts with *C. jejuni* CDT resulted in formation of Rad50 foci (a DNA repair factor) around dsDNA breaks (Hassene *et al.*, 2003). The presence of another toxin was suggested because the *cdtB* mutant lysates caused cytotoxic effects in a HeLa cell line (Purdey *et al.* 2000).

#### *1.11.5 Iron Acquisition*

Pathogenic bacteria have developed mechanisms that allow them to overcome the growth-limiting effects of transferrin and lactoferrin bonded iron (Andrews *et al.*, 2003). Iron uptake by bacteria from outer-membrane receptors into the cytosol is mediated by siderophores (Greek for iron carrier). Genes encoding enterochelin and ferrichrome, which are types of siderophores, have been characterised in *Campylobacter* isolates (Richardson and Park, 1995; Galindo *et al.*, 2001). Purified siderophores were utilised by *C. jejuni* grown in iron-depleted media (Field *et al.*, 1986). The analysis of the *C. jejuni* genome revealed the presence of a periplasmic binding protein dependent system (a siderophore receptor), a hemin uptake operon, a ferrous uptake protein, and genes similar to the *tonB* system. The latter mediates the movement of iron from the siderophore into the periplasm (Parkhill *et al.*, 2000; Van Vliet and Ketley, 2001).

#### *1.11.6 Intracellular survival*

The host and bacterial factors that determine the fate of internalised *Campylobacter* are not understood (Ketley, 1997). In HEP-2 cells, the viability of *C. jejuni* declined 6 hours after internalisation. The invaded cells showed a strong lysosomal response that may have caused the bacterial cell to change to a coccoid

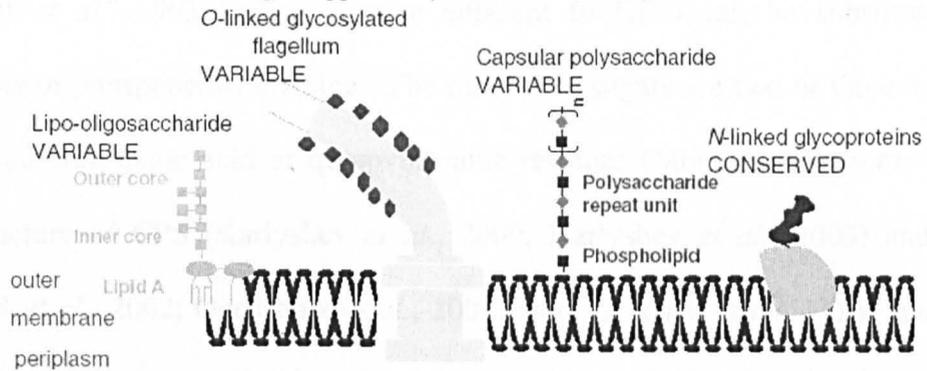
form (de Melo *et al.*, 1989). The bacterial oxidative stress defence system can increase the invasion ability and improve their intracellular survival. A *C. jejuni* mutant in *sodB* (superoxide dismutase gene) exhibited shortened survival times in INT407 cells (Pesci *et al.*, 1994). Catalase has also been reported to be important for *C. jejuni* survival in macrophages. Mutation in *katA* (the gene encoding catalase) made *C. jejuni* more susceptible to killing by the phagocytes. Inhibition of macrophage nitric oxide synthase permitted the recovery of the mutant (Day *et al.*, 2000).

#### *1.11.7 Cell wall components*

##### *1.11.7.1 Lipopolysaccharide (LPS), Capsular Polysaccharide (CPS) and Flagella.*

The synthesis of a high molecular weight glycolipid structure (termed lipopolysaccharide), low molecular glycolipid structure (termed lipooligosaccharide; LOS) and capsular polysaccharide (CPS) have been reported in *C. jejuni* (Karlyshev *et al.*, 2005) (Figure 1.2). The lipid A component of *C. jejuni* LOS has been reported to have endotoxic activity. *C. jejuni* LOS may play a role in pathogenesis through molecular structural mimicry that result in GBS (Moran, 1996). Additionally flagella, LOS and CPS play roles in *C. jejuni* adhesion, invasion, host colonisation, maintenance of cell surface charge and serum resistance (Bacon *et al.*, 2001; Guerry *et al.*, 2002). The part of LOS composed of beta 1-6-linked disaccharides (attached to 10-28 carbon-length fatty acids) linked to sugar residues (by ester or amide linkages) is termed Lipid A. This part, which contains glycosidic and nonglycosidic phosphoryl groups, varies in bacteria with regard to the number and nature of sugars, chain length and location of acyl residues, and the nature of the phosphate group substitution (Henderson *et al.*, 1996).

**Figure 1.2. Outer membrane components of *C. jejuni*** (Karlyshev *et al.*, 2005). LOS is mostly expressed by *C. jejuni* studied strains than LPS. LOS core oligosaccharide is composed of variably-linked glucose, galactose, N-acetyl-D-galactosamine, N-acetylneuraminic acid and L-glycero-D-mannoheptose residues attached to 3-deoxy- $\alpha$ -D-manno-oct-2-ulopyranosonic acid which is linked to lipid-A. CPS is composed of  $\beta$ -d-Ribp,  $\beta$ -d-GalfNAc,  $\alpha$ -d-GlcpA6, a uronic acid amidated with 2-amino-2-deoxyglycerol at, and 6-O-methyl-d-glycero- $\alpha$ -1-gluco-heptopyranose (Michael *et al.*, 2002). The structure of the glycan in the N-linked glycoproteins is GalNAc- $\alpha$ 1,4-GalNAc- $\alpha$ 1,4-[Glc $\beta$ 1,3-]GalNAc- $\alpha$ 1,4-GalNAc- $\alpha$ 1,4-GalNAc- $\alpha$ 1,3-Bac- $\beta$ 1,N-Asn-Xaa, where Bac is bacillosamine, 2,4-diacetamido-2,4,6-trideoxyglucopyranose (Young *et al.*, 2002). The glycosylated flagellum mediates motility and is thought to be an export apparatus (Konkel *et al.*, 2004).



To induce immune responses, lipid-A must have the disaccharide, 2-phosphoryl groups and six fatty acids. The core region of LOS has an inner core (heptose and 2-keto-3-deoxyoctonic acid; KDO), and is reported to exert the lipid-A activity. The biological activities of *S. minnesota* LPS without side-chain polysaccharides were reported to be more potent than that of with side chain polysaccharide (Henderson *et al.*, 1996).

LOS from *C. jejuni* has been reported to be of low molecular weight without detectable O polysaccharide chains (Logan and Trust, 1984). However, the production of high molecular weight LPS by 33% of studied *C. jejuni* isolates was also reported (Preston and Penner, 1987). The high molecular weight polysaccharides were later found to be biochemically similar to group II capsular polysaccharide not O-antigen. Mutation in *kps* genes (similar to capsular polysaccharide genes in *E. coli*) resulted in *C. jejuni* without high molecular weight polysaccharide (Karlyshev *et al.*, 2000). The structural components of *C. jejuni* lipid A are acylated and phosphorylated in a similar fashion to other bacterial species'

Lipid A. The disaccharide backbone of lipid A is composed of diaminoglucose and D-glucosamine of varying molar ratios between strains (Moran, 1997; Moran and Penner, 1999). The inner core of oligosaccharide consists of a trisaccharide (KDO, and two heptoses) in which the heptose is substituted by a beta 1-4 linked D-glucose (Aspinall *et al.*, 1993). The heptose adjacent to KDO can be substituted by phosphate or phosphoethanolamine. The outer core sugars are two or three hexoses substituted with sialic acid or quinovosamine residues (Moran and Penner, 1999). The structure of CPS (Karlyshev *et al.*, 2000; Karlyshev *et al.*, 2005) and LOS (Michael *et al.*, 2002; Godschalk *et al.*, 2007) from *C. jejuni* strain 11168 has been reported.

#### 1.11.8 Peptidoglycan

The polymer of peptidoglycan consists of glycan strands of two alternating sugar derivatives, *N*-acetylglucosamine and *N*-acetylmuramic acid, which form a disaccharide subunit. A peptide subunit of an alternating four or five amino acids, is linked to the carboxy group of *N*-acetylmuramic acid. In most Gram-negative bacteria, diaminopimelic acid is usually the third amino acid of the peptide, whereas it is a lysine in Gram-positive bacteria (Fournier and Philpott, 2005). In *Campylobacter* peptidoglycan is presented as GLcNAc-MurNAc-L-Ala-D-Glu-meso-Dpm-D-Ala (Simbert, 1978) and it has been suggested that its degradation causes the bacterial cell morphology to change to the coccoid form (Amano and Shibata, 1992).

### 1.12 Innate immunity responses to *Campylobacter* infection

The human intestine represents a huge surface area of more than 300 square meters that is inhabited by more than 400 bacterial species of total  $\log_{10}14$  microbial cells. This requires the host defence systems to tolerate commensal bacteria while

being able to discriminate and deal with invading pathogens (Hao and Lee, 2004). The intestinal lumen is mainly occupied by the commensal bacteria, while the mucus covered mucosal surface and the crypts containing intestinal stem cells are not colonised. In non-inflamed mucosa, direct binding to epithelial cells is inhibited (Gusils *et al.*, 2004). Paneth cells (Porter *et al.*, 2002) residing at the base of the crypts play an important role in the innate immune defence in the intestine by producing several types of antimicrobial peptides for example, the pore-forming  $\alpha$ -defensins HD-5, HD-6 (Ouellete, 1999) and enzymes that include bacterial lipid degrading Group IIA phospholipase A2 and peptidoglycan degrading lysozyme (Ouellete, 1999). Intestinal epithelial cells produce pore-forming  $\beta$ -defensins HBD1, HBD2, HBD3, HBD4, and cathelicidin LL-37/hCAP-18 (Ganz, 2003). Additionally, they produce permeability-increasing protein (BPI) which disrupts the outer and inner membranes of Gram-negative bacteria, and serves as a LPS neutralising agent (Ganz, 2003).

Intestinal epithelial innate defence against *C. jejuni* results in the production of the bactericidal  $\beta$ -defensins. The sensitivity of *C. jejuni* to this bactericidal agent may explain why most of *Campylobacter* cases are self-limited (Zilbauer *et al.*, 2005). The resistance of bacteria to defensins can be mediated by lipid-A increased acylation (Guo *et al.*, 1998), or they can be modified with a sugar residue (Gunn *et al.*, 1998), degraded with outer-membrane protease (Guina *et al.*, 2000), or removed with an efflux pump mechanism (Shafer *et al.*, 1998). The successful avoidance of the first innate defences results in invasion of intestinal epithelial cells (IEC) and the subsequent activation of the nuclear transcription factor NF- $\kappa$ B. Invasion of IEC is not a requisite for NF- $\kappa$ B activation as microbial components are reported to induce activation in IEC (Takeda and Akira, 2004).

### 1.12.1 NF- $\kappa$ B activation

In 1986, NF- $\kappa$ B was identified as a regulator for expressing the kappa light-chain gene in murine B-lymphocytes, but has been subsequently found in many different cells (Birbach *et al.*, 2002). Collectively named NF- $\kappa$ B, the inducible dimeric transcription factors are made up of members of the Rel family of DNA binding proteins that recognise a common sequence motif (May and Ghosh, 1998). This group of structurally conserved proteins are regulated via shuttling from the cytoplasm to the nucleus in response to a wide range of stimuli such as inflammatory cytokines (TNF- $\alpha$  or interleukin-1), microbial pathogens, pathogen-derived products, growth factors, and stress inducers (Chen *et al.*, 1999; Baldwin, 2001). Although NF- $\kappa$ B is a ubiquitous transcription factor, most studies of its properties have been performed on cells of the immune system (Birbach *et al.*, 2002). NF- $\kappa$ B plays a central role in immune system responses through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and the inducible enzymes cyclo-oxygenase and nitric oxide synthase (Karin and Ben-Neriah, 2000).

There are five mammalian NF- $\kappa$ B proteins that are assigned to two different groups. Rel-A (p65), c-Rel and Rel-B proteins (C-terminal transactivation domains) constitute the first group. These are synthesised as mature proteins not requiring proteolytic modification. The second group includes the precursor proteins, p105 and p100 (encoded by the NF- $\kappa$ B1 and NF- $\kappa$ B2 genes respectively), that require proteolytic processing to give mature p50 and p52 NF- $\kappa$ B proteins (Ghosh *et al.*, 1998). The I $\kappa$ Bs, (the NF- $\kappa$ B cellular inhibitors), hold NF- $\kappa$ B dimers (Rel-A or c-Rel) in the cytoplasm in the absence of activating stimuli. The I $\kappa$ B family include I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$  and I $\kappa$ B $\gamma$ . Also, the precursor protein p100 may act as an I $\kappa$ B or as

a specific heterodimeric partner for Rel-B with a subsequent release of p50:Rel-B heterodimers. I $\kappa$ Bs have multiple ankyrin repeats that interact with NF- $\kappa$ B via a Rel homology domain (RHD). This N-terminal domain mediates DNA binding, dimerization, nuclear translocation and interaction with the inhibitory I $\kappa$ B proteins (Ghosh *et al.*, 1998; Jacobs and Harrison, 1998). In addition to B cells it is now known that NF- $\kappa$ B is required for the development and function of T cells, dendritic cells, fibroblasts and macrophages (Baeulle and Henkel, 1994; Li and Verma, 2002). In mice, the disruption of the NF- $\kappa$ B p65 subunit was lethal due to developmental defects, whereas the lack of the NF- $\kappa$ B p50 subunit resulted in increased susceptibility to infection (Weih *et al.*, 1997). Mice with transgenic mutated forms of I $\kappa$ B $\alpha$  in T cells were observed to have reduced numbers of CD8<sup>+</sup> T cells and increased apoptosis (Lavon *et al.*, 2000). Additionally, deficiency in B cells and a blocked maturation process were reported in mice with disruption in NF- $\kappa$ B1, NF- $\kappa$ B2 and c-Rel genes (Snapper *et al.*, 1996). In RelB<sup>-/-</sup> and NF- $\kappa$ B2<sup>-/-</sup> mice, lack of thymic dendritic cells (Arron *et al.*, 2001) and follicular dendritic cells (Burkly *et al.*, 1995) were demonstrated. Macrophages from RelB<sup>-/-</sup> mice were deficient in response to TNF- $\alpha$ , whereas those from c-Rel<sup>-/-</sup> were less responsive to TNF- $\alpha$  (Weih *et al.*, 1997).

#### *1.12.1.1 I $\kappa$ B-dependent NF- $\kappa$ B activation*

The activation of the I $\kappa$ B kinase (IKK) complex regulates I $\kappa$ B-mediated NF- $\kappa$ B activation by ultimately phosphorylating I $\kappa$ B $\alpha$  at the serine 32 and 36. Upon phosphorylation, ubiquitin ligase ubiquitin-marks the I $\kappa$ B making them recognised by beta-transducing repeat-containing protein (b-TrCP) such that they are rapidly degraded by the proteasome (Thompson, 1995). The degradation of the inhibitory proteins allows NF- $\kappa$ B to translocate to the nucleus, bind to DNA, and initiate the

transcriptional activities (Thompson, 1995). Although the signalling pathways vary, where Toll-receptors, TNF-receptors and T cell-receptors utilise different mediator molecules, they eventually lead to the activation of the IKK complex and the subsequent degradation of I $\kappa$ B proteins (Hu *et al.*, 1999). The IKK complex has three-core subunits (IKK $\alpha$  kinase, IKK $\beta$  kinase and the regulatory protein, NEMO). The kinases share an N-terminal catalytic domain, a central leucine zipper and a C-terminal motif. The exact mechanism of IKK activation is not understood but gene-disruption experiments have shown that the I $\kappa$ B-dependent NF- $\kappa$ B activation depends on the presence of IKK $\beta$  or NEMO (Barnes and Karin, 1997). Stimulation of gene expression is upregulated when IKK $\alpha$  is recruited from the nucleus to the promoter regions of NF- $\kappa$ B regulated genes where it phosphorylates histone H3 on serine 10 (Hu *et al.*, 1999). IKK $\beta$  plays a major role in the cytoplasm through phosphorylating I $\kappa$ B. In knock-out mice, selective suppression of IKK $\beta$  inhibited NF- $\kappa$ B activation stimulated by TNF- $\alpha$ , LPS and IL-1 (Barkit and Gilmore, 1999; Wang *et al.*, 1998). These defective signalling pathways were not observed in IKK $\alpha$  knock-out mice (Li *et al.*, 1999; Senftleben *et al.*, 2001). Phosphorylation, which can be by the proposed IKK-activating kinases (NIK, MEKK1, MEKK2, MEKK3, and Cot/Tpl-2), is important for the IKK complex activation (Hu *et al.*, 1999). Out of these kinases, only MEKK3 has been reported as essential for TNF- $\alpha$  induced IKK activation in fibroblasts (van Antwerp *et al.*, 1996). The delayed pro-inflammatory induced biphasic NF- $\kappa$ B activation follows MEKK2 complex formation with IKK-I $\kappa$ B-p65 (Beg and Baltimore, 1996). In response to IL-1 stimulation, the conformational change triggered IKK activation may be mediated by the ubiquitination of TRAF6. This is aided by TAB2 or TAB3 which brings TAK1 in proximity with TRAF6 (Wallach *et al.*, 1999, Ishitani *et al.*, 2003). In embryonic

fibroblasts lacking TAK1, TAB1, or TAB2, it has been reported that TNFR1, IL-1R, TLR3, and TLR4-mediated NF- $\kappa$ B and AP-1 activation are severely impaired only in TAK1 lacking cells (Shim *et al.*, 2005).

#### *1.12.1.2 I $\kappa$ B-independent NF- $\kappa$ B activation.*

In B cells, I $\kappa$ B-independent NF- $\kappa$ B activation results in the release of p52-RelB, and p50-RelB dimers in the absence of IKK $\beta$  or NEMO (Li and Verma, 2002). This pathway leads to NIK and IKK $\alpha$  mediated processing of p100, and the subsequent release of p52. Bacterial LPS, lymphotoxin B and Epstein-Barr virus are reported activators of NF- $\kappa$ B independent of I $\kappa$ B (Weih *et al.*, 1997; Arron *et al.*, 2001).

#### *1.12.1.3 NF- $\kappa$ B and intestinal epithelial cells*

The heterodimer composed of the RelA (p65) and NF- $\kappa$ B1 (p50) subunits is the major NF- $\kappa$ B protein found in the nucleus of stimulated IECs (Jobin *et al.*, 1997; Jobin *et al.*, 1999). I $\kappa$ B-dependent NF- $\kappa$ B activation is important for the innate immune responses in IECs. The genes of immune responders are stimulated in IEC by NF- $\kappa$ B, and their induction proceeds immediately after stimulation without new protein synthesis.

Genes regulated by NF- $\kappa$ B in IEC include IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-12, TNF- $\alpha$ , iNOS, COX-2, ICAM-1, VCAM-1, TCR- $\alpha$ , MHC-I and II molecules, GRO- $\alpha$  and GRO- $\beta$ , RANTES and MIP-2 (Baeuerel and Henkel, 1994; Barnes and Karin, 1997; Neurath *et al.*, 1998). Suitably positioned to sample the intestinal antigenic nature and to contact with near by lymphocytes, IECs act as non-professional antigen-presenting cells constitutively expressing MHC-I and II on their surfaces (Shao *et al.*, 2005). Cytokines are important mediators in regulating immune and inflammatory responses. In addition to the immune cells (dendritic, lymphocyte, and

macrophage cells), the IEC can also produce cytokines and chemokines. The cytokines IL-1, IL-10, IL-15, IL-18, and TGF are constitutively expressed by IECs and have roles in epithelial cell growth, homeostasis and regulating the influx of immune cells into the mucosa. Normal epithelial cells also express IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , MCP-1, CCL20 and GM-CSF. The production of these cytokines is highly upregulated in response to pathogens (Jung *et al.*, 1995; Martin *et al.*, 1997; Oswald, 2005). Having chemokine receptors on their apical surface, IECs can respond to chemokines produced by themselves or by other immune cells. The effect of IL-1 is augmented during the infection because IL-1 is synthesised and recognised by IEC (Oswald, 2005). In IECs, stimuli of NF- $\kappa$ B include microbial pathogens, pathogen products, and pro-inflammatory cytokines (Jobin and Sartor, 2000).

In epithelial cells, *C. jejuni* has been reported to activate the transcription factor NF- $\kappa$ B 16 hours post-infection (Mellits *et al.*, 2002). A significant dose-dependent activation was observed 2 hours after incubating epithelial cells with a cell-free boiled extract of *C. jejuni*. The majority of NF- $\kappa$ B activating component(s) were reported to be proteinase K partially insensitive and less than 3 kDa in molecular weight. The activity could not be attributed to secreted molecules as the cell-free filtrate did not activate NF- $\kappa$ B (Mellits *et al.*, 2002). NF- $\kappa$ B activation was subsequently reported to be induced by, but not dependent on, a lipoprotein (JlpA) of *C. jejuni* (Jin *et al.*, 2003). *Campylobacter jejuni* activation of NF- $\kappa$ B was also observed in a THP-1 monocyte cell line (Jones *et al.*, 2003). The influx of neutrophils through the epithelial barrier during infection brings *C. jejuni* in contact with many leukocytes (Jones *et al.*, 2003).

In human intestinal epithelial cells, chemical inhibition of NF- $\kappa$ B activation resulted in suppressed production of many chemokines (Hu and Hickey, 2005). Live

and heat-killed *C. jejuni* activated NF- $\kappa$ B and induced the production of different cytokines in human dendritic cells (Hu *et al.*, 2006). Independent of flagellin, *C. jejuni* could activate NF- $\kappa$ B and induce the production of the dendritic cell chemoattractant, CCL20 (Johanesen *et al.*, 2006).

Composed of transmembrane proteins fibrils, the tight junctions (TJs) encircle the apical region of IEC to restrict diffusion along paracellular borders. Enhancement of TJ permeability after toxin damage by pathogens helps invasive and non-invasive bacteria to colonise IEC or deliver their effectors via type III secretory systems into IEC (Mitic and Anderson, 1998). Histopathological examination of intestinal biopsies from patients suffering *C. jejuni* acute disease revealed the infiltration of neutrophils to the infected mucosa (Skirrow and Blaser, 2000). This may indicate the *C. jejuni* have disrupted TJs, in a similar fashion to that observed in *in vitro* studies (Bras and Ketley, 1999; MacCallum *et al.*, 2005; Chen *et al.*, 2006).

Dendritic cells (DCs) play important roles in innate and adaptive immunity (Rossi and Yong, 2005). In the intestine DCs lie under the surface in Peyer's patches and form an early line of defence against invading pathogens. Upon exposure of DCs to bacterial inflammatory stimuli, these cells are converted from antigen-capturing immature cells to antigen-presenting mature DCs. Moreover, DCs can aid the internalisation of bacterial pathogens following their uptake by M cells or paracellular extensions in the lumen (Rossi and Yong, 2005). A study by Hu and others reported that *C. jejuni* is internalised by DCs at an early time point following infection and killed within 24 hours without cytotoxicity to DCs (Hu *et al.*, 2006). Various cytokines and chemokines are produced by DCs when they interact with pathogenic bacteria (Rossi and Yong, 2005). The production of IL-1, IL-6, IL-8, IL-10, IL-12, IFN- $\gamma$  and TNF $\alpha$ , has been reported in DCs infected with *C. jejuni* (Hu *et*

*al.*, 2006; Johanesen *et al.*, 2006). Intestinal epithelial recruitment of leukocytes plays an important role in the innate mucosal immune response to enteric pathogens in conjunction with DCs and T cells (Hu and Hickey, 2005). *Campylobacter jejuni* induced the production of the DCs chemokine CCL20 and the T-cell chemoattractant CXCL10 from the intestinal epithelial cells. Additionally, infected cells produced the neutrophil chemoattractants CXCL1, CXCL5, and CXCL8 (Priscilla *et al.*, 2006). Human monocytes produced IL-1, IL-6, IL-8 and TNF $\alpha$  when stimulated with *C. jejuni* (Jones *et al.*, 2003). The transcription of GRO (growth-related oncogene), MCP-1 (monocyte chemoattractant protein 1), MIP-1 (macrophage inflammatory protein 1), and IP-10 (interferon-inducible protein) has been reported in epithelial cells exposed to *C. jejuni* (Hu and Hickey, 2005).

#### *1.12.1.4 Activation of NF- $\kappa$ B via Toll-like receptor (TLR)/interleukin-1 receptor (IL-1R) signalling.*

To date, eleven type I transmembrane toll-like receptors have been identified in mammals (Cario, 2005). These receptors feature three common structural motifs: a leucine-rich repeat ligand-binding extracellular domain, a transmembrane region, and a homologous cytoplasmic showing Toll/IL-1 domain (Cario, 2005).

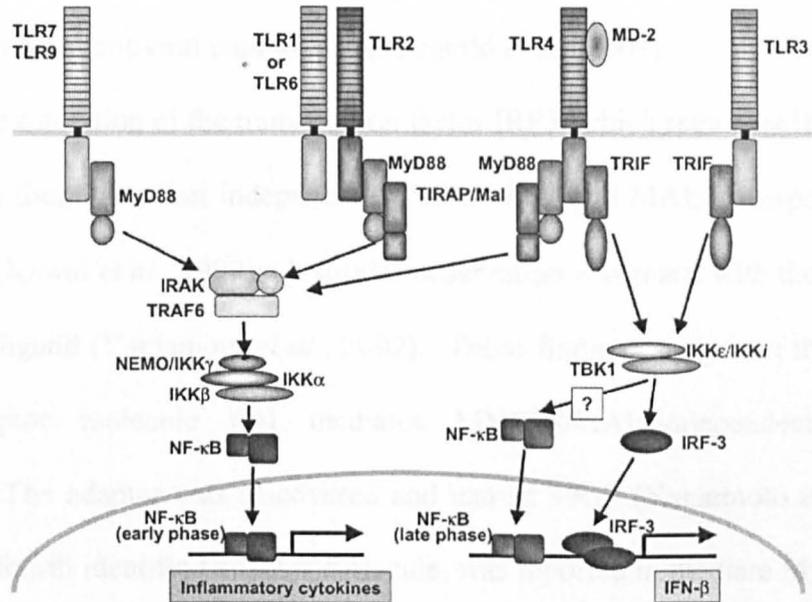
Upon ligand binding, TLRs/IL-Rs dimerise and recruit downstream signalling molecules in a pathway that leads to the activation of NF- $\kappa$ B (Figure 1.3) that results in the expression of a set of defence genes that encode inflammatory cytokines, chemokines and co-stimulatory molecules (Akira and Takada, 2004). TLRs have evolved to detect pathogen-associated molecular patterns (PAMPs) as a front line mechanism to induce innate immune response. In recent years the ligands to which each of the TLRs respond have attracted a good deal of attention.

TLR1 detects triacylated lipoproteins, and can cooperate with TLR2 to detect Pam3CSK4 (Takeuchi *et al.*, 2002). TLR2 recognises bacterial lipoproteins, lipoteichoic acid (LTA) and peptidoglycan (Takeuchi *et al.*, 1999; Aliprantis *et al.*, 1999; Underhill *et al.*, 1999). Viral dsRNA detection is mediated by TLR3 (Alexopoulou *et al.*, 2001). TLR4 senses LPS (Hoshino *et al.*, 1999) and lipoteichoic acid (Takeuchi *et al.*, 1999) and bacterial flagellin is detected by TLR5 (Hiyashi *et al.*, 2001). TLR6 associates with TLR2 and recognises diacylated mycoplasmal lipopeptide (Takeuchi *et al.*, 1999). Human TLR7 and/or TLR8 are known to bind single-stranded RNA (ssRNA) products from viruses, such as human immunodeficiency virus (HIV)-1 and human parechovirus (Diebold *et al.*, 2004; Heil *et al.*, 2004; Triantafilou *et al.*, 2005). DNA containing unmethylated CpG motifs (CpG DNA) also stimulates immune cells via TLR9 (Hemmi *et al.*, 2000). The ligand for TLR10 has yet to be identified (Cario, 2005). TLR11 has been shown to be involved in protection from uropathogenic bacterial infection in mice (Zhang *et al.*, 2004). Endogenous ligands for TLR2 and/or TLR4 include heat shock proteins, fibrinogen, surfactant protein-A, fibronectin extra domain A, heparan sulphate, soluble hyaluronan and  $\beta$ -defensin 2 (Takeda *et al.*, 2003; Sabroe *et al.*, 2003).

The downstream signalling molecules include myeloid differentiation primary response protein 88 (MYD88), IL-1R-associated kinases (IRAKs), transforming growth factor- $\beta$ -activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and tumor-necrosis factor-receptor-associated factor 6 (TRAF6; Takeda *et al.*, 2003). MYD88 was first identified as a gene that is induced upon IL-6 stimulation of M1 myeloleukemic cells. It has a COOH-terminal Toll/IL-1 receptor (TIR) domain and a NH2-terminal death domain (DD). Following pathogen-associated molecular patterns (PAMPs) recognition, MYD88 is recruited to the TLR complex where its

DD engages with with DD of IRAK-4 resulting in the phosphorylation of residues in the kinase-activation loop of IRAK-1. Upon activation of IRAK-1, its affinity with Toll-interacting protein (TOLLIP) is weakened making it accessible for further phosphorylation. The secondary phosphorylated IRAK-1 is released from TOLLIP and interacts with TRAF6. TRAF6 interacts with TAB2 and this complex activates TAK1 that mediates the activation of the I $\kappa$ B kinase complex and NF- $\kappa$ B, p38 and JNK (Akira and Takada, 2004). The signalling pathway activated determines the type of the downstream signalling for the individual TLR. This is dependent on the adaptor molecules that interact with different TLRs (Athman and Philippot, 2004). MYD88 is the adaptor molecule that is utilized by almost all identified TLRs in their signalling cascades (Doyle and O'Neil, 2006). However, in the absence of this molecule, the activation of NF- $\kappa$ B by TLR3 was not affected (Alexopoulou *et al.*, 2003). After ligand binding to its TLR, IRAK4 is recruited to the TLR complex. The MyD88-IRAK4 interaction leads to the phosphorylation of IRAK-1. The dissociation of IRAK-1 from MyD88-IRAK4 complex makes it available to interact with TRAF6 (Cao *et al.*, 1996; Suzuki *et al.*, 2002). The precise role of IRAK-4 has not been clarified. It has been reported that IL-1 signalling is optimized by, but not dependent on, IRAK-4 (Qin *et al.*, 2004; Lye *et al.*, 2004). In IRAK1 deficient macrophage, LPS-induced TNF $\alpha$  production still occurs but at reduced levels. This may indicate the existence of an IRAK-1 activity-mimicking molecule (Swantek *et al.*, 2000). For normal response to LPS, the presence of both of IRAK1 and IRAK2 is required (Zhang *et al.*, 1999). The activation of NF- $\kappa$ B by a MYD88 utilizing ligand requires functional TRAF6 (Cao *et al.*, 1996). A dominant negative form of TRAF6 was found to inhibit NF- $\kappa$ B activation by IL-1 (Cao *et al.*, 1996).

**Figure 1.3. TLR-signalling pathways leading to the activation of NF- $\kappa$ B.** TLR signaling pathways originate from the cytoplasmic TIR domain. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the I $\kappa$ B kinase (IKK) complex consisting of IKK $\alpha$ , IKK $\beta$  and NEMO/IKK $\gamma$ . The IKK complex phosphorylates I $\kappa$ B, resulting in nuclear translocation of NF- $\kappa$ B which induces expression of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN- $\beta$  are observed in a MyD88-independent manner. A third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway. Non-typical IKKs, IKK $\epsilon$ /IKK $\iota$  and TBK1, mediate activation of IRF-3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway. (Akira and Takeda, 2004; Kawai and Akira, 2005).



The IRAK1-TRAF6 complex interacts with transforming growth factor- $\beta$  activated kinase (TAK1) and TAK1-binding proteins (TAB1 and TAB2). Both TRAF6 and TAK1 are then ubiquitinated. Subsequently, TAK1 and TAB2 are phosphorylated. This phosphorylation leads to dissociation of the TRAF6-TAK1 protein complex from the membrane and IRAK1 degradation (Wang *et al.*, 2001). Phosphorylation of the downstream IKKs is mediated by the activated TAK1. The IKK complex mediated phosphorylation and degradation of I $\kappa$ B allows the translocation of NF- $\kappa$ B to the nucleus leading to the upregulation of NF- $\kappa$ B-controlled pro-inflammatory genes (Qian *et al.*, 2001). In mice deficient in MYD88, NF- $\kappa$ B activation and cytokine production were found to be inhibited in response to

TLR2, TLR7, and TLR9 ligands. However, LPS induced NF- $\kappa$ B activation but with delayed kinetics and without inflammatory cytokine production (Kawai *et al.*, 1999). MAL is a molecule that shares structural similarity with MYD88. The MAL dominant negative form has been found to suppress NF- $\kappa$ B activation by LPS but not by IL-1R. Mice lacking MAL show an impaired response to TLR2 and TLR4 ligands but responded normally to IL-1, flagella, unmethylated DNA, dsRNA, and low molecular weight antiviral compounds (Fitzgerld *et al.*, 2001).

The activation of the transcription factor IRF3, which regulates IFN inducible genes, was found to occur independently of MYD88 and MAL in response to LPS, via TLR4 (Kawai *et al.*, 1999). A similar observation was made with the response to the TLR3 ligand (Yamamoto *et al.*, 2002). These findings suggested that there is a third adaptor molecule that mediates MYD88/MAL-independent signalling pathways. The adaptor was discovered and named TRIF (Yamamoto *et al.*, 2002). TRAM, a fourth identified adaptor molecule, was reported to mediate MyD88/MAL-independent signalling in response to TLR4 but not TLR3 ligand (Fitzgerald *et al.*, 2003). In a similar way to MYD88 and MAL, NF- $\kappa$ B activation was mediated by the overexpression of TRIF. The IFN- $\beta$  promoter was activated by TRIF but not by MYD88 or MAL (Yamamoto *et al.*, 2002).

To activate NF- $\kappa$ B, TRIF needs to associate with the receptor interacting protein 1 (RIP1; Meylan *et al.*, 2004). The cytokine production in response to LPS was reported to be dependent on the TRIF pathway (Gohda *et al.*, 2004). In mice deficient in TRIF and its related adaptor molecule TRAM, cytokine production was inhibited in response to LPS but normal for TLR2, TLR7 and TLR9 ligands (Yamamoto *et al.*, 2003). The expression of high levels of TLR antagonists (Zhong and Ghosh 2002; Fukao *et al.*, 2002; Nakajawa *et al.*, 2002; Wald *et al.* 2003; Brint

*et al.*, 2004; Diehl *et al.*, 2004; Boone *et al.*, 2004; Chuang *et al.*, 2004) is one strategy used to regulate TLR activities in IEC. This expression suppresses the activation of TLRs present at the cell surface. Intracellular negative regulators of TLRs that are expressed in most tissues include: PI3K (affects TLR-2,4,9; inhibits p38, JNK and NF- $\kappa$ B functions), TOLLIP (affects TLR-2,4; autophosphorylates IRAK1), SIGIRR (affects TLR-4,9; interacts with TRAF6 and IRAK), TRAILR (affects TLR-2,3,4; stabilizes I $\kappa$ B $\alpha$ ), and TRAF3A (affects TLR-4,9; ubiquitinates TLRs). Other TLR antagonists that are induced by LPS and expressed by macrophages include SOCS1 (affects TLR-4,9; suppresses IRAK), A20 (affects TLR-2,3,4,9; de-ubiquitinates TRAF6), and ST2L (affect TLR-2,4,9; sequesters MYD88 and MAL). Another TLR2 antagonist, which is constitutively expressed in breast-milk and in plasma, is the soluble form of TLR2 (LeBouder *et al.*, 2003). The soluble forms of TLR4 and MYD88 down-regulate TLR4 by blocking TLR4 interaction with MD2 and acting as antagonist for MYD88, respectively (Iwami *et al.* 2000; Janssens *et al.*, 2002).

Although most of immune responses to bacterial pathogens and/or their components are mediated through TLR2 and TLR4, it is not surprising given their exposure that IECs are poorly responsive to these receptor ligands (Melmed *et al.*, 2003; Abrue *et al.*, 2005). IEC may not be able to discriminate among PAMPs of commensal and pathogenic bacteria without other pathogen-associated signs such as invasion or toxin production (Otte *et al.* 2004). *In vitro* prolonged exposure of IEC to TLR2/TLR4 ligands resulted in tolerance to these ligands (Otte *et al.* 2004). A decrease in the surface-expression of these receptors with decreased IRAK activity and increased TOLLIP expression are the suggested mechanisms for the observed tolerance (Otte *et al.* 2004). Receptors relocate to the basolateral membrane of the

cell or to cellular compartments such as the Golgi apparatus as a consequence of the continuous stimulation by components of commensal microflora (Hornef *et al.*, 2002). A study of human intestinal epithelial cell line T84 has shown that peptidoglycan or LPS stimulation relocates the surface expression of TLR2 (Tohno *et al.*, 2005) and TLR4 (Hornef *et al.*, 2003) to cytoplasmic compartments near the basolateral membrane. Of importance for the host to detect pathogens, intracellular TLR must retain their full signalling ability (Otte *et al.* 2004). It was noted that intracellular TLR4 was able to detect internalised bacteria and LPS (Hornef *et al.*, 2003).

The precise cellular localisation of TLR5, the flagellin receptor in IEC is open to debate. Following TLR5 stimulation with *E. coli* flagellin, both basolateral and apical TLR5 expression were reported in HT29-19A and Caco-2 IEC lines (Gweritz *et al.*, 2001; Bambou *et al.* 2004). Only basolateral membrane expression of TLR5 was reported in the T84 IEC (Lyons *et al.*, 2004).

Since flagellin from commensal bacteria is not translocated to the basolateral membrane and therefore is not considered bioactive, the basolateral expression of TLR5 assists in the maintenance and homeostasis of the GI. Again, specific TLR expression and distribution are necessary for the IEC to avoid unwanted TLR activation while allowing them at the same time to initiate an efficient response against pathogen invasion (Lyons *et al.*, 2004).

In the sub-epithelial compartment, intestinal myofibroblasts upregulate the expression of TLRs; 2, 3, 4, 6, and 7 after stimulation with LPS or LT (Otte *et al.*, 2003). The continued expression of TLR3 only was reported in mature crypt epithelial cells where the weak expression of TLR2 and TLR4 was lost as the cells matured and moved toward the gut lumen (Furrie *et al.*, 2005). The continued

expression of TLR3 in progression to the lumen was not considered detrimental as TLR3 responds to dsRNA of viruses and is not a PAMP of gut bacteria (Furrie *et al.*, 2005). IECs constitutively express TLR9 but are unresponsive to its ligand (Pedersen *et al.*, 2005). After adhesion to IEC, *C. jejuni* causes cellular damage, excessive inflammation, and CDT production. *Campylobacter jejuni* infection of the IEC results in an enhanced IL-8 production that is dependent on adhesion (Ketley, 1997). The precise role of TLR and the exact nature of the bioactive components involved in this response are currently unknown. Flagellin from *C. jejuni* was reported not to activate TLR5. In CHO K1 cell line transfected with TLR5, *C. jejuni* flagellin did not activate NF-Kb. Avoidance of *C. jejuni* flagellin detection by TLR5 (Andersen-Nissen *et al.*, 2005) is mediated by the lack of the TLR5-site specific residues (amino acids 89-96) that have been shown to be essential for the agonistic activity of *S. typhimurium* *fliC* (Mizel *et al.*, 2003).

The interaction of *S. typhimurium* with TLRs is the most studied among the enteric bacterial pathogens. It has been shown that TLR2, TLR4, and TLR5 are important in the host defence against *S. typhimurium* infection. Invasion of IEC by *S. typhimurium* results in the sequestering and relocation of the bacteria to intracellular vacuoles in addition to localised inflammation and the lysis of infected cells (Lembo *et al.*, 2003). TLR4 is important for immediate detection of *S. typhimurium* and early responses in infected macrophage and for later responses after invasion. Infection of TLR4-deficient mice demonstrates the role of TLR4 in limiting infection, and the induction of TNF and chemokines (Lembo *et al.*, 2003; Weiss *et al.*, 2004). In IEC, a strong inflammatory response induced by *S. typhimurium* flagellin is mediated by TLR5. Given that IEC do not express TLR5 on the apical membrane, the immune response to *S. typhimurium* flagellin suggests that a

translocation of flagellin to the basolateral membrane where TLR5 is expressed, is necessary (Gewritz *et al.*, 2001). A study reported that *S. typhimurium* strains lacking flagellin expression induced only a weak inflammatory response indicating that flagellin may be the primary pathogenesis factor in *S. typhimurium* infection. The expression of capsular antigen, a virulence factor of *S. Typhi*, inhibits the TLR4 and TLR5 response, allowing dissemination of the infection. Infection with *S. Typhi* does not result in IL-8 induction or neutrophil recruitment to the infected IEC (Sebastiani *et al.*, 2000; Zeng *et al.*, 2003; Raffatellu *et al.*, 2005).

Because of the conserved structure of Lipid A (the bioactive TLR4-stimulating structure of LPS) among pathogenic and non-pathogenic commensal strains of *E. coli*, it is unlikely that LPS recognition by TLR4 plays a major role in diarrhoeagenic *E. coli* pathogenesis (Beckhed *et al.*, 2003). LPS from apically adhered *E. coli* does not upregulate TLR4 in IEC. Moreover, variant O antigens among *E. coli* LPS do not activate TLR4 (Cario *et al.*, 2002). TLR5-dependent activation of NF- $\kappa$ B in IEC has been reported using flagellin from several strains of pathogenic *E. coli*. Enteroaggregative *Escherichia coli* infect IEC to produce IL-8 that was reported to be caused by aggregative adherence fimbriae. The involvement of TLR in mediating this action is not understood (Berin *et al.*, 2002; Khan *et al.*, 2004; Harrington *et al.*, 2005). TLR2 and TLR4 were reported to be activated by the uropathogenic *E. coli* fimbriae (Frendeus *et al.*, 2001; Ogawa *et al.*, 2002). The B subunit of the ETEC *E. coli* type II heat-labile enterotoxin activates TLR2 (Hajishengallis *et al.*, 2005). Independent of TLR and MYD88, *Shigella flexneri* has been reported to invade the IEC, activate NF- $\kappa$ B, and induce IL-8 production via NOD1 (Girardin *et al.*, 2001).

In the hemopoietic cells (macrophages, dendritic cells, B cells, and T cells) of the intestinal lamina propria, TLR2 and TLR4 are expressed at low levels in a non-inflammatory state. The macrophages are unresponsive to LPS whereas the dendritic cells are responsive to LPS and peptidoglycan (Rossi and Yong, 2005).

Appropriate stimulation of TLRs on DCs by their respective ligands can thus initiate the entire spectrum of innate and, in turn, acquired immunity. Ligand binding to TLRs up-regulates CD83, co-stimulatory molecules, and CCR7, which drives DC migration to T cell areas of draining lymph nodes (Jarrossay *et al.*, 2001; Neiss and Reineker, 2006). These early activated DCs thus play an important role in the activation of other DCs and the recruitment of specialising lymphocytes that support the adaptive immune response (Rossi and Yong, 2005). As noted above, *C. jejuni* stimulates the production of NF- $\kappa$ B- regulated cytokines in DCs. C-type lectin receptors are also expressed by DCs. These receptors sense the carbohydrate present on pathogens (Rossi and Yong, 2005).

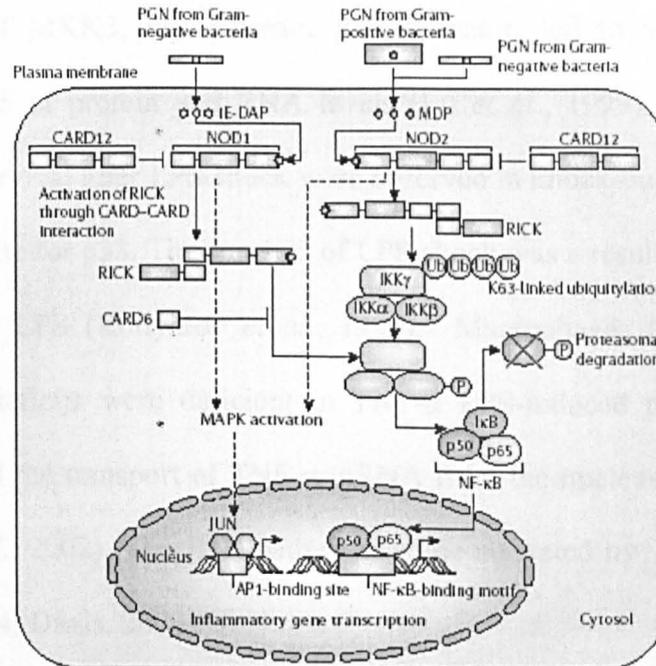
#### *1.12.1.5 NOD signalling*

NOD1 and NOD2 are intracellular receptors that are generally expressed by antigen-presenting cells (Inohara *et al.*, 2003) and most epithelial cells (Kim *et al.*, 2004; Hisamatsu *et al.*, 2003). Unlike macrophages and dendritic cells, T cells and B cells do not express NOD1 or NOD2 (Gutierrez *et al.*, 2002; Ogura *et al.*, 2001). In most IEC, and particularly primary cells, NOD1 is expressed while the expression of NOD2 is undetectable at the protein level (Hisamatsu *et al.*, 2003). The expression of NOD2 is limited to Paneth cells, at the base of intestinal crypts (Ogura *et al.*, 2003). Baseline expression of NOD1 in the primary epithelial cells can be upregulated by IFN- $\beta$  but not by TNF $\alpha$ , via the interaction with IRF1 at the CARD4 promoter. IFN- $\beta$  increases the baseline TNF-induced upregulation of NOD2 (Hisamatsu *et al.*,

2003). In IFN- $\beta$ -deficient mice, no upregulation of NOD1 or NOD2 was observed in response to challenge with *Listeria monocytogenes* (Stockinger *et al.*, 2003).

The synthetic peptidoglycan-derived structures iE-DAP and MDP are sensed by NOD1 (Chamillard *et al.*, 2003) and NOD2 (Inohara *et al.*, 2003), respectively. NOD2 is considered a general sensor of most bacteria because its ligand is present in both Gram negative and Gram positive bacteria (Inohara *et al.*, 2004). Macrophages from NOD1 or NOD2-deficient mice were unresponsive to the ligands or these receptors (Chamillard *et al.*, 2003; Pauleau *et al.*, 2003). As they are intracellular receptors, NOD1 or NOD2 ligands must reach the NOD-LRR (leucine rich repeat) domains for activation. This may be mediated through the process of phagocytosis, delivery of peptidoglycan into the cells through a secretion system, or via an intestinal peptide transporter (Viala *et al.*, 2004; Vavricka *et al.*, 2004). Following NOD1 or NOD2 activation by their ligands, NF- $\kappa$ B is activated (Figure 1.5) through a RICK-mediated pathway (Inohara *et al.*, 2003). NOD1 and NOD2 are comprised of a C-terminal series of LRR, central nucleotide binding domains (NBs) and a caspase-activating and recruitment domain (CARD). Overexpression of NODs results in the exposure of CARD, and the subsequent interaction with the CARD domain of a serine/threonine kinase called RIP2 (Kobayashi *et al.*, 2002). The oligomerization of RIP2 allows it to interact with the regulatory subunit of the IKK complex. Such interaction leads to the phosphorylation of I $\kappa$ B $\alpha$  and the release of NF- $\kappa$ B. NF- $\kappa$ B was activated in epithelial cells transfected with constructs of NOD1 or NOD2. The activation was highly inhibited in fibroblasts expressing NOD1 or NOD2 but deficient in RICK (Kobayashi *et al.*, 2002).

**Figure 1.5. NF- $\kappa$ B and MAPK NOD-mediated activation by peptidoglycan (PGN).** Recognition of muramyl dipeptide(MDP) and  $\gamma$ -d-glutamyl-meso-diaminopimelic acid (iE-DAP) through leucine-rich repeat(LRR) domains activates the NOD (nucleotide-binding oligomerization domain) proteins NOD2 and NOD1, respectively, which then recruit receptor-interacting serine/threonine kinase (RICK) through caspase-recruitment domain (CARD)–CARD interactions. In the case of NOD2, activation of RICK leads to K63 (Lys63)-linked polyubiquitylation of IKK $\gamma$ , the scaffold of the inhibitor of NF- $\kappa$ B (I $\kappa$ B)-kinase complex (the IKK complex), which also consists of IKK $\alpha$  and IKK $\beta$ . This is followed by the phosphorylation of IKK $\beta$ , as well as the phosphorylation of I $\kappa$ B and the release of nuclear factor- $\kappa$ B (NF- $\kappa$ B) for translocation to the nucleus. CARD12 negatively regulates RICKmediated NF- $\kappa$ B activation by both NOD1 and NOD2, whereas CARD6 negatively regulates only RICK-mediated NF- $\kappa$ B activation by NOD1. In addition to NF- $\kappa$ B activation, NOD1 and NOD2 signalling gives rise to the activation of mitogen-activated protein kinases (MAPKs) such as JUN amino-terminal kinase (JNK), extracellular-signal regulated kinase (ERK) and p38MAPK by as-yet unknown mechanisms (denoted by dashed arrows). (Strober *et al.*, 2006; Fritz *et al.*, 2006).



MAPK pathway activation was reported following NOD activation. The activation of p38 MAPK and ERK were observed in NOD2-expressing macrophages in response to MDP (Pauleau *et al.*, 2003; Kobayashi *et al.*, 2005). The c-Jun NH2-terminal kinase (JNK) was activated upon NOD1 activation by its ligand (Girardin *et al.*, 2001).

### 1.12.2 Activation of MAPKs

The main mammalian MAPK families are the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases and the c-Jun NH2-terminal kinases (JNK) (Schaefer and Weber, 1999; Davis, 2000; Chang and Karin, 2001). The activation loop of these families has a Thr-X-Tyr motif. Phosphorylation of tyrosine

and threonine is essential for the activation. Upon phosphorylation, a core MKKK-MKK-MAPK cascade is initiated in all MAPK pathway activation processes (Cobb and Goldsmith, 1995; Dong and Davis, 2002).

The role of MAP kinases in the innate immune responses has been demonstrated using specific inhibitor studies. The activity of p38 MAPK in macrophages was reduced by 70% using the p38 inhibitor SB203580. Additionally, the disruption of MKK3, an upstream p38 activator, led to a defect in IL-12 induction by LPS at protein and RNA levels (Lu *et al.*, 1999). Increased stress resistance and survival after LPS shock were observed in knock-out mice deficient in MK2, the substrate for p38. The survival of LPS shock was a result of reduced TNF- $\alpha$  production by LPS (Kotlyarov *et al.*, 1999). Macrophages from mice having selective ERK deficits were deficient in TNF- $\alpha$  LPS-induced production. ERK inhibitor blocked the transport of TNF- $\alpha$  mRNA from the nucleus to the cytoplasm (Eliopoulos *et al.*, 2002). The JNK pathway can be activated by TNF, IL-1 or LPS (Sluss *et al.*, 1994; Davis, 2000).

In the IEC line T84, *C. jejuni* activated ERK strongly but weakly activated p38 MAP kinase. Inhibition of ERK but not p38 suppressed the ability of *C. jejuni* to stimulate IL-8 production (MacCallum *et al.*, 2005). A *C. jejuni flaA* mutant was defective in stimulating these responses. However, purified flagellin from *C. jejuni* did not stimulate ERK, p38 or cytokine production, and was found to be defective in activating TLR5 (Priscilla *et al.*, 2006). The activation of MAPKs by *Campylobacter* is thought to be mediated by adhesion and invasion or an interaction of a cell-surface receptor with a bacterial component. The cell surface lipoprotein JlpA expressed by *C. jejuni* was reported to interact with Hsp 90 and activate p38 MAPK (Jin *et al.*, 2003).

### 1.13 Reporter gene studies of pathogenesis

The lack of a suitable animal model has slowed the progress towards a clear understanding of *Campylobacter* pathogenesis. An alternative is to use *in vitro* manipulation of the genes to study microbial-host interactions at the molecular level. The genes under control of transcriptional regulation at the end points of signalling pathways induced by stimulating factors maybe studied using transfection of a reporter gene such as luciferase under control of the response promoter (Gould and Subramani, 1988). Reporter constructs carry specific pieces of the promoter under investigation or promoter sequences with specified mutations linked to a reporter gene are transfected into the cells to identify pathways leading to these responses. Subsequently, the reporter activity is measured from the cell lysate, and is considered as an indicator for the promoter activity. The activities of different constructs are compared to each other to obtain information about the specific sequence functions in determining the promoter activity (Huzar *et al.*, 2001). The measured reporter activity is variable and it depends on both the activity of the promoter and on the transfection efficiency (which depends on the plasmid DNAs and number of cells transfected and is determined by response to stimulating ligands). To correct for variation in measured reporter activity, a second reporter, such as beta-galactosidase should be co-transfected. This reporter gene is under the control of minimal promoter or a viral promoter of constitutive activity. Its expression is presumed to be proportional to the number of transfected cells. The activity of the beta-galactosidase reporter is used to normalize the primary promoter activity for subsequent comparisons (Gould and Subramani, 1988).

In experiments where the signalling pathways induced by a specific agonist are to be investigated, then co-transfection of interfering expression constructs or the use

of specific inhibitors may be included before the stimulation with the specific agonist (Huzar *et al.*, 2001). The luciferase-reporter activity and  $\beta$ -galactosidase reporter activity of stimulated cells and unstimulated cells are measured, corrected and expressed as a multiple of the basal stimulation (Gould and Subramani, 1988).

#### **1.14. Current work**

This thesis has adapted the reporter gene approach to study the activation of the central immune response regulator NF- $\kappa$ B in response to *C. jejuni* and *C. jejuni* products. This is mainly due to the lack of a suitable animal model for *C. jejuni* infection, and the possibilities to manipulate signalling pathways leading to NF- $\kappa$ B activation. The reporter gene approach study of pathogenesis has been widely used in literature also to avoid issues of ethical concerns in using animal subjects.

The transcription factor NF- $\kappa$ B activation by *C. jejuni* has been previously reported. However, there is a lack in the knowledge of the pathways of activation. In this study, tissue culture cell lines were transfected with reporter gene (NF- $\kappa$ B-dependent luciferase and NF- $\kappa$ B-independent  $\beta$ -galactosidase) along with receptors/adaptor molecules (e.g TLRs, NODs) to investigate the signalling pathways involvement of these receptors in NF- $\kappa$ B activation by *C. jejuni*.

## **CHAPTER 2 Materials and methods**

## CHAPTER 2

### 2.1 MATERIALS AND METHODS

#### 2.1.1 Bacterial strains and growth conditions

The *Campylobacter jejuni* type strain NCTC 11168, *C. jejuni*, NCTC 11168 lipooligosaccharide mutant *waaF* (Oldfield *et al.*, 2002) and *C. jejuni* NCTC 11168 capsular polysaccharide mutant *kpsM* (Karlyshev *et al.*, 2000) were used in these studies for which the genome sequence is available (Parkhill *et al.*, 2000). They were propagated on blood agar plates (Blood-agar base CM271, Oxoid, with 5% v/v defibrinated horse blood, TCS) under microaerobic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85 % N<sub>2</sub>) for 24 h at 42 °C. When growing mutants the plates were supplemented with kanamycin at 25 µg/ml.

#### 2.1.2 Plasmid constructs

Reporter plasmids pRc\RSVlacZ and p3EnCONALuc used to monitor the specific induction of NF- $\kappa$ B were a gift from Professor R. Hay, University of St Andrews, UK (Rodriguez *et al.*, 1999). The expression plasmids pcDNA3 TLR2, pcDNA3 TLR4, pEF-BOS MD-2 and pcDNA3 CD14 (Carl *et al.*, 2002); and the dominant negative expression construct pEF-BOS DN-MYD88 (Muzio *et al.*, 1997) were gifts from Professor D. Golenbock, University of Massachusetts Medical School, USA. The plasmid expression constructs pcDNA3 NOD1, pcDNA3 DN-NOD1 (Girardin *et al.*, 2001), pcDNA3 NOD2, pcDNA3 DN-NOD2, and pcDNA3 DN-MAL (Fritz *et al.*, 2005) were gifts from Professor D. Philpott, Institute Pasteur, France. The pcDNA3 DN-IRAK1 and pcDNA3 DN-p38 were gifts from Dr. W. Li, University of Southern California, USA. Plasmid constructs used in this study and their description are shown in table 2.1. *Escherichia coli* containing these constructs were grown on Luria Bertani agar plates (LB broth , Difco, with 1.5% w/v bacteriological agar No.

1, Oxoid) cultured over night at 37 °C. They were used to inoculate 400 ml of LB broths and incubated with shaking 200 rpm at 37 °C overnight. The extraction of DNA was performed using the Plasmid Maxi Prep kit according to the manufacturer's instructions (Qiagen).

**Table 2.1. Plasmid constructs used in this study.**

<b>Plasmid construct</b>	<b>Description</b>
pRc\RSVlacZ	Reporter gene; non-specific activation measurement*
p3EnCONALuc	Reporter gene; specific $\kappa$ B-dependent activation measurement*
pcDNA3 TLR2	Transmembrane receptor**
pcDNA3 CD14	Accessory molecule for TLR2 mediated signalling**
pcDNA3 TLR4	Transmembrane receptor**
pEF-BOS MD-2	Accessory molecule for TLR4 mediated signalling**
pEF-BOS DN-MYD88	Adaptor molecule for TLRs signalling (NR)**
DN-IRAK1	Downstream signalling molecule for MYD88 pathway (NR)^
pcDNA3 DN-MAL	Adaptor molecule for TLR2 and TLR4 signalling (NR)***
pcDNA3 NOD1	Intracellular receptor; detection of PGN***
pcDNA3 NOD2	Intracellular receptor; detection of PGN ***
pcDNA3 DN-NOD1	Intracellular receptor; detection of PGN (NR)***
pcDNA3 DN-NOD2	Intracellular receptor; detection of PGN (NR)***
pcDNA3 DN-p38	p38 MAPK signalling (NR)^

NR= negative regulation. \*: (Rodrigues *et al.*, 1999). \*\*: (Muzio *et al.*, 1997; Carl *et al.*, 2002). \*\*\*: (Yamamoto *et al.*, 2002; Girardin *et al.*, 2003). ^: (Yeo *et al.*, 2003).

### 2.1.3 Preparation of *Campylobacter* boiled cell extracts

A 24 h plate culture of *C. jejuni* NCTC 11168, *C. jejuni* NCTC 11168 LOS mutant, or *C. jejuni* NCTC 11168 KpsM mutant were used to inoculate 400 ml of Nutrient Broth No 2 (CM 67, Oxoid) in 1 L conical flasks and then shaken under microaerobic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85 % N<sub>2</sub>) for 24 h at 42 °C. The bacteria were collected by centrifugation at 10,000 g for 15 min. The bacterial cell pellet was then re-suspended in 1 ml of phosphate buffered saline (PBS; BR14a, Oxoid) and washed by centrifugation for a total of 3 times. The cell pellet was weighed then re-suspended in PBS to 10% w/v. This suspension was then boiled for 10 min and cooled on ice. The suspension was then centrifuged at 13,000 g and the supernatant

collected. This extract was then filtered through a 0.2  $\mu\text{m}$  filter (Minisart, Sartorius) to remove any residual bacteria, freeze-dried and stored at  $-20\text{ }^{\circ}\text{C}$  until required.

#### **2.1.4 Ethanol precipitation**

To precipitate proteinaceous contaminants (Oss, 1989), dried BCE was mixed with ice-cold ethanol, kept at  $-20\text{ }^{\circ}\text{C}$  for 2 h, and then centrifuged at 15,000 g for 20 min. The supernatant was concentrated by evaporation under a stream of nitrogen.

#### **2.1.5 Enzymatic treatments**

To remove nucleic acids BCE samples were incubated at  $37\text{ }^{\circ}\text{C}$  for 4 h with either RNase 1  $\text{ng } \mu\text{l}^{-1}$  or DNase 1  $\mu\text{g } \mu\text{l}^{-1}$  (Sigma Aldrich). Proteins were removed by Proteinase K (Sigma Aldrich) treatment at a final concentration of 100  $\mu\text{g ml}^{-1}$  for 1 h at  $55\text{ }^{\circ}\text{C}$ . All BCE samples were heated at  $100\text{ }^{\circ}\text{C}$  for 10 min subsequent to enzymatic treatment and stored at  $-20\text{ }^{\circ}\text{C}$  until use. TNF- $\alpha$  and flagellin samples were used directly to prevent denaturation.

#### **2.1.6 Centrifugal filtration**

Three kDa and 1 kDa molecular cut-off filters (Pall) were used to remove high molecular weight components. BCE (3 ml) was centrifuged using these filters at 7000 g for 3 h at room temperature.

#### **2.1.7 Gel filtration**

After passing through 3 kDa filters, BCE was freeze dried, re-suspended in 50  $\mu\text{l}$   $\text{H}_2\text{O}$ , and injected into Superdex PC 3.2/30 (100 to 7000 Da separation range; 2.4 ml bed volume) column (Amersham Bioscience) which was run using an FPLC system (Pharmacia). The running (gradient 0-60% in 60 min) buffer (A) was 0.1 M ammonium acetate in  $\text{H}_2\text{O}$  where the elution buffer (B) was  $\text{H}_2\text{O}$ . A total of 24 fractions (100  $\mu\text{l}$  each/ min) were collected, dried, re-suspended in sterile  $\text{H}_2\text{O}$ , and tested for bioactivity.

### **2.1.8 RP- chromatography**

The BCE active fractions obtained from gel filtration were combined and applied (50  $\mu$ l injection volume) to the reverse-phase  $\mu$ RPC C2/C18 column (Amersham Bioscience) run by the FPLC system (Pharmacia) with eluent A, 0.1% trifluoroacetic acid (TFA); eluent B, 70% acetonitrile containing 0.1% TFA; gradient, 0–60% in 60 min; flow rate 100  $\mu$ l/min. A total of 40 fractions were collected, dried, re-suspended in sterile H<sub>2</sub>O, and tested for bioactivity.

### **2.1.9 Solid phase extraction (SPE)**

The BCE fraction containing material <1000 Da was further purified using disposable reverse-phase, then normal-phase cartridges as the following:

A reverse-phase (Sep-pak C18, Waters) cartridge was conditioned with 10 ml of HPLC-grade methanol (Sigma), and washed with 10 ml of H<sub>2</sub>O. Then, the BCE fraction containing material <1000 Da suspended in 10 ml H<sub>2</sub>O was applied to the cartridge and allowed to enter the column by gravity. The cartridge was then washed with 5 ml of H<sub>2</sub>O. Products of this step were concentrated, and tested for bioactivity.

A normal-phase (CN-500 mg, Phenomenex) cartridge was conditioned with 3 ml of HPLC-grade acetonitrile (Pierce), the active fraction from reverse-phase separation was applied (10% H<sub>2</sub>O in acetonitrile) to the cartridge which was then washed with 10% H<sub>2</sub>O in acetonitrile. The elution step was performed using 20, 30, and 40 % H<sub>2</sub>O in acetonitrile. Products of this step were concentrated, and tested for bioactivity. Additional normal-phase purification for the active fraction was performed as described above using a Hypercarb (porous graphitized carbon) 200 mg cartridge (Thermoelectric).

### **2.1.10 Normal phase high pressure liquid chromatography (NP-HPLC)**

The active fraction from the third SPE step (Hypercab purification) was loaded (in 10% acetonitrile) on a NP-HPLC (Luna CN) column (Phenomenex). A gradient 0-40% over 50 min was run (on PU980 pumps, Jasco) using mobile phase: A; HPLC grade acetonitrile and B; H<sub>2</sub>O. The UV absorbance was monitored using Spectro-Monitor 3000 (Spectra Physics, LDC) and recorded using Picolog computer software (Picotech). Forty fractions (1 ml in volume) were collected, concentrated and tested for activity. This step was repeated with combined active fractions from the first passage.

### **2.1.11 Permethylation of BCE**

For permethylation, a modification of the Ciucanu and Kerek method was used (Weiskopf *et al.*, 1997). Permethylated is thought to enhance MS sensitivity for carbohydrate containing samples (Weiskopf *et al.*, 1997). Purified BCE (1 mg) was dried down in a Teflon-capped reaction vial, to which DMSO–NaOH slurry (1 ml) was added. After thorough mixing, the sample was allowed to incubate at room temperature for 60 min. Methyl iodide (1 ml) was added to the vial, mixed vigorously, and then incubated at room temperature for 60 min. With the reaction vial placed in a small ice bath for cooling, the reaction was quenched by slow, dropwise addition of water (2 ml). Samples were then extracted with chloroform (4 ml), and the extracted phase was rinsed with 4 ml aliquots of water until the organic phase was no longer cloudy. The extract was then dried under a dry nitrogen stream, redissolved in 1:1 methanol–water containing 0.02 % formic acid for ESI-MS analysis.

### **2.1.12 Mass Spectrometry (MS)**

The active BCE fractions from the second NP-HPLC run were analysed by ESI-MS in positive mode using Q-ToF2 tandem mass spectrometer (Waters Co). Samples (10 $\mu$ l) were delivered to the MS in 50% acetonitrile and 0.2% formic acid. The capillary voltage was 900-1200 V and the collision gas was Argon. The mass spectra were acquired using MaxEnt3 software (Waters Co).

### **2.1.13 Treatment of BCE with glucosidases**

The initial structural characterization of BCE by MS indicated the presence of carbohydrate component(s). BCE was therefore treated with an alpha-exo-glucosidase (amyloglucosidase, Sigma) or an alpha-endoglucosidase (dextranase; Sigma) or an endo-glucosidase (glucanase, Sigma) at concentration of 1 mg BCE: 0.5mg/ml of enzyme in H<sub>2</sub>O with incubation for 24 h at 37 °C. The mixture was then boiled for 5 min to inactivate the enzyme, centrifuged at 10,000 g for 10 min, and the supernatant tested for bioactivity.

### **2.1.14 Gas chromatography (GC)**

The monosaccharide composition of BCE (1 mg) was analysed by GC as their alditol acetate derivatives (Sawardeker *et al.*, 1967; Papp-Szabo *et al.*, 2005). Hydrolysis (in screw cap tube) of the glycosyl was performed in 4 M trifluoroacetic acid (300  $\mu$ l) at 100 °C for 4 h after which the solution was dried under stream of compressed air. Reduction of the sample was performed in 300  $\mu$ l of H<sub>2</sub>O with NaBD<sub>4</sub> (Sigma) at room temperature for 30 min. After quenching with 500  $\mu$ l 10% HOAc in MeOH and evaporation to dryness, subsequent acetylation was accomplished by Ac<sub>2</sub>O treatment with residual sodium acetate as the catalyst at 100 °C for 1 h. Then, alditol acetate derivatives were analyzed (helium was the carrier gas; mobile phase) by GC using a Varian 3400 gas chromatograph equipped with a DB-17 capillary (fused silica;

stationary phase) column (Agilent Technologies) heated from 210 °C to 240 °C over 30 min (2 °C increment /min).

#### **2.1.15 Nuclear magnetic resonance (NMR)**

<sup>1</sup>H P-NMR analysis was performed on the combined active BCE fractions from 1<sup>st</sup> NP-HPLC step. BCE was exchanged (3 times) with D<sub>2</sub>O (99.8%), lyophilized, and dissolved in D<sub>2</sub>O (99.9%). <sup>1</sup>H NMR spectra were recorded by using a 5-mm probe at 25 °C, and chemical shifts were measured relative to internal acetone (2.25). Spectra were recorded on DRX500 (500MHz) spectrometer (Bruker) using BrukerWinNMR software.

#### **2.1.16 Preparation of flagellin extracts**

*Campylobacter jejuni* were cultured in Nutrient Broth No. 2 (Oxoid) as described in section 2.1.1, harvested by centrifugation at 10,000 g for 10 min and re-suspended in 1 ml of PBS. *Escherichia coli* were cultured in Nutrient Broth No. 2 but under atmospheric conditions. Cells suspensions were passed through narrow bore tubing a total of 20 times to shear flagella. The flagella were isolated in the supernatant following centrifugation at 10,000 g for 10 minutes at 20 °C. The supernatant was then re-centrifuged at 50,000 g for 60 min at 4 °C and the resulting flagella pellet re-suspended in a 5 ml volume of PBS. Flagella preparations were monitored by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Enteropathogenic *E. coli* E2348/69 was used as source of flagellin to stimulate an innate immune response (Zhou *et al.*, 2003).

To precipitate Lipid-A (Schromm *et al.*, 2000), boiled cell extract (BCE) was treated with acetate buffer (0.1M pH 4.4; at 100 °C for 1h) and centrifuged (10,000 g at 4 °C for 10 min) after cooling. The supernatant was desalted using a Sephadex-10

(Amersham Biosciences) column with H<sub>2</sub>O as the eluting buffer. The precipitated pellet was washed 3 times with H<sub>2</sub>O.

To inactivate LPS (Paik *et al.*, 2003), a fraction of BCE was incubated with Polymyxin B (10 µg/ml; Sigma) before the induction assay. To hydrolyze lipopeptide (Shibata *et al.*, 2000) that may be present in BCE, a BCE fraction was treated with lipopeptide lipase (8 µg/ml; Sigma) before the induction assay.

#### **2.1.17 Cell culture and transfection**

HELA 57A cervical epithelial cells (Rodriguez *et al.*, 1999), HCA-7 colonic epithelial cells (Kirkland, 1985), Caco-2 colonic epithelial cells (Jung *et al.*, 1995) and HEK293 kidney derived cells (Graham *et al.*, 1977) were grown in monolayer cultures of approximately  $5 \times 10^6$  in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with penicillin at 100 µg ml<sup>-1</sup>, streptomycin at 100 µg ml<sup>-1</sup> and foetal calf serum (FCS) at 10% (v/v) (Invitrogen Ltd). Cell viability was monitored by performing microscopic examination of 0.4% Trypan Blue stained cells. To provide selection for the transcriptional markers present in the HeLa 57A cell line, such cultures were supplemented with G418 at 0.5 µg ml<sup>-1</sup> (Invitrogen). All cell lines were grown under 5% CO<sub>2</sub> conditions at 37 °C. The calcium phosphate procedure (Jordan and Wurm, 2004) was used to transfect plasmid DNAs into Caco-2, HEK293 and HCA-7 cells. Cultured cells were seeded in D-MEM supplemented with 10% FCS and allowed to grow until 80% confluence. Three hours before transfection the growth medium was replaced fresh. Transfection mixes (150 µl of 2X HBS {HEPES 50 mmol, KCl 10 mmol, glucose 12 mmol, NaCl 280 mmol, and Na<sub>2</sub>HPO<sub>4</sub> 1.5 mmol; final pH 7.05}, 37 µl of 2M CaCl<sub>2</sub>, and H<sub>2</sub>O to final volume of 300 µl) containing plasmid DNAs at total mass 4 µg (where the balance in all experiments was made up to the target DNA mass with pcDNA3 empty vector), were incubated

for 40 min at room temperature, remixed and added drop-wise onto the cells (300  $\mu$ l/well) that were incubated at 5% CO<sub>2</sub> for 8 h at 37 °C. Cells were then washed with pre-warmed PBS, supplemented with growth media and incubated 36 h as before.

HEK293 cells were transfected with; the reporter genes plasmid DNAs (500 ng of NF- $\kappa$ B dependent luciferase, and 250 ng NF- $\kappa$ B-independent  $\beta$ -galactosidase gene) plus; A- TLR2 (300 ng)-CD14 (200 ng), or A+DN-MYD88 (600 ng), or B-TLR4 (300 ng)-MD2 (200 ng) or B+ DN-MYD88 (600 ng), or 600 ng of DN-MYD88 or 50 ng of DN-NOD1, or 50 ng of NOD1, or 1 ng of NOD2, or 1 ng of DN-NOD2, or A+NOD1, or A+DN-NOD1, or B+NOD2, or B+DN-NOD2. The DN-p38 plasmid DNA (200 ng; Yeo *et al.*, 2003), or DN-IRAK1 (50 ng), or DN-MAL (100 ng; Yamamoto *et al.*, 2003) was transfected in HEK-293 cells along with the reporter genes plasmid DNA. Hela 57A cells were transfected with MD-2 or TLR2-CD14. The plasmid DNA concentrations (Table 2.2) used were selected to minimize basal NF- $\kappa$ B activation.

**Table 2.2. Plasmid constructs and transfection concentration used in this study.**

Plasmid construct	Transfected concentration (ng)
NF- $\kappa$ B dependent luciferase	250
NF- $\kappa$ B-independent $\beta$ -galactosidase gene	250
TLR2 and CD14	300, 200
TLR2, CD14 and DNMYD88	300,200, 600
TLR4 and MD2	300, 200
TLR4, MD2 and DNMYD88	300,200, 600
DNMYD88	600
DN-NOD1	50
NOD1	50
DN-NOD2	1
NOD2	1
TLR2, CD14 and NOD1	300, 200, 50
TLR2, CD14 and DN-NOD1	300, 200, 50
TLR4, MD2 and NOD2	300, 200, 1
TLR4, MD2 and DN-NOD2	300, 200, 1
DN-p38	200
DN-IRAK1	50
DN-MAL	100

### 2.1.18 Induction with BCE and live *C. jejuni*

For induction with BCE, cultured cells were starved of serum for 16 h and inductions carried out by adding BCE or by adding tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ; EU Programme EVA/MRC Centralized Facility for AIDS Reagents, NIBSC, UK; Grant numbers QLK2-CT-1999-00609 and GP828102) to a final concentration of 50 ng ml<sup>-1</sup>. The TLR2 agonist N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-Cys-[S]-Ser1-[S]-Lys (4) trihydrochloride (Pam3SK4) was used at 100 ng ml<sup>-1</sup> and purchased from EMC microcollections, Tübingen, Germany. Ultrapure grade *E. coli* K12 LPS (Invivogen) was used to stimulate TLR4 at 10  $\mu$ g ml<sup>-1</sup>. The synthetic peptidoglycan derivative GlcNAc-MurNAc-L-Ala- $\gamma$ -D-Glu-meso-diamino-pimelic acid (M-triDAP) was a gift from Dr Dominique Mengin-Lecreulx Institute Pasteur, and used to stimulate NOD1 at 10  $\mu$ g ml<sup>-1</sup>. The muramyldipeptide MurNAc-L-Ala-D-isoGln was purchased from Invivogen and used at 10  $\mu$ g ml<sup>-1</sup> to stimulate NOD2.

For induction with live bacteria the eukaryotic cells were removed from antibiotic-containing medium and the serum reduced to 1% v/v for 16 h prior to the addition of *C. jejuni* at the multiplicity of infections indicated or by adding TNF- $\alpha$  to a final concentration of 50 ng ml<sup>-1</sup>. *Campylobacter jejuni* were cultured in Nutrient Broth No. 2 as described above but harvested by centrifugation at 10000 g for 5 min at 22 °C. The bacterial cell pellet was re-suspended in D-MEM plus 1% v/v FCS and incubated at 42 °C for 1h shaking at 140 rpm under microaerobic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85 % N<sub>2</sub>). Viable counts were performed on these cultures using blood-agar plates to determine the true inocula applied to cultured host cells, to monitor the viability of the bacteria in the presence of tissue culture cells and to ensure the absence of contaminating bacteria. Heat killed bacteria were prepared as above but were heated for 1h at 75 °C and then cooled to 22 °C prior to addition to eukaryotic cells.

#### **2.1.19 Polarised colonic epithelial cells**

Caco-2 and HCA-7 cells were cultured on semi-permeable polycarbonate filter inserts of 0.4  $\mu$ m pore size in Transwell units (Corning-Costar). Transepithelial electrical resistance (TEER) was measured on a daily basis (by Prof. Ian Connerton) using a voltmeter (World Precision Instruments). Confluent monolayers reached TEER values between 800 and 1,500  $\Omega$  cm<sup>2</sup> (from 25 days after seeding).

#### **2.1.20 Reporter cell assays**

HELA 57A cells are stably transfected with an NF- $\kappa$ B-dependant promoter driving *luc* transcription, and an independent Rous Sarcoma Virus promoter driving *lacZ* expression (Rodriguez *et al.*, 1999). Other cell lines were transiently transfected with reporter plasmids pRc\RSVlacZ and p3EnhCONALuc to perform these functions. After the specified induction time (6 h for BCE and heat-killed bacteria, or 16 h for

live bacteria), cultured cells were washed with PBS and lysed by incubating them with 250  $\mu$ l/well of 1X lysis buffer (Promega) for 10 min at room temperature. The cells were then harvested using cell scraper (Nunc) centrifuged at 10,000 g for 1 min and the supernatants were stored at -70 °C. Replicate luciferase and  $\beta$ -galactosidase reporter assays were performed. Luciferase activity (40  $\mu$ l of luciferin added to cell lysate supernatant containing 10  $\mu$ g of protein concentration) was measured using a Turner Bioluminometer as recommended by the manufacturer (Promega Ltd). To measure  $\beta$ -galactosidase activity, 400  $\mu$ l of lacZ buffer (60 mmol  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 40 mmol  $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ , 10 mmol KCl, 1 mmol  $\text{MgSO}_4$ ; and 1.35  $\mu$ l beta-2-mercaptoethanol; pH 6.95) were added to 100  $\mu$ l of cells lysate supernatant in eppendorf tube. Then, 100  $\mu$ l of O-nitrophenyl- $\beta$ -D-galactopyranoside (4 mg/1 ml  $\text{H}_2\text{O}$ ) were added to the tube and the mixture incubated at 37 °C until yellow colour development. The reaction was stopped by adding 250  $\mu$ l of 1M  $\text{Na}_2\text{CO}_3$  and the activity measured (after centrifugation at 10,000 g for 5 min) at absorbance of 420 nm using the Cecil spectrophotometer (Cecil Instruments).

To determine the degree of NF- $\kappa$ B induction, all luciferase activity values were normalised against internal  $\beta$ -galactosidase activities in order to provide a constitutive control of basal expression levels, and further expressed as a multiple of the mock-induced control (fold-induction).

Fold of induction= SV/MV

SV= stimulated cell luciferase activity value/ stimulated cell  $\beta$ -galactosidase value, where MV= mock treated cell luciferase value/ mock treated cell  $\beta$ -galactosidase value. Experimental data represent the means and standard deviations of three independent determinations. The data were analysed using either t-tests or ANOVA, where  $p < 0.05$  was considered significant.

### **2.1.21 Cytokine analysis**

Cultured cells were prepared and induced as described above. After 6 h incubation the external media was removed and stored at  $-20^{\circ}\text{C}$  until examined using a Coulter-Alter Flow Cytometer in conjunction with a BD cytometric bead array human inflammation kit according to manufacturer's instructions (BD Biosciences). IL-8 was specifically measured (by Prof. Ian Connerton) using a sandwich ELISA, by capture with a murine anti-human IL-8 and detected using biotinylated goat anti-human IL-8 using streptavidin-coupled horseradish-peroxidase according to the manufacturer's instructions (R&D Systems).

### **2.1.22 Determination of TLR and Nod expression in tissue culture cells**

Eukaryotic cell-lines were cultured as detailed above and harvested at 80% confluence using a cell scraper. RNAs were extracted from cells using the Qiagen RNeasy kit as per the manufacturer's instructions (Qiagen). RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies) and  $0.5\ \mu\text{g}$  was used to generate cDNA using Oligo(dT) primers with Superscript III reverse transcriptase (Invitrogen). The primers used to PCR-amplify TLRs, NODs, and TOLLIP are given in Table 2.2 using Taq DNA polymerase (Qbiogene) typically for 30 cycles at  $90^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 1 min. PCR products (191 to 622 bp) were examined on 4% polyacrylamide gels electrophoresed in TBE (100 mM Tris-Borate, 1mM EDTA) and stained with ethidium bromide (Sigma Aldrich) following electrophoresis.

**Table 2.3. PCR primers used in this study.**

Gene	Forward (5'-3')	Reverse (5'-3')	Product (bp)	Reference
TLR1	CTATACACCAAGTTGTCAGC	GTCTCCAACCTCAGTAAGGTG	220	Young <i>et al.</i> , 2004
TLR2	GTACCTGTGGGGCTCATTGT	CTGCCCTTGCAGATACCATT	191	Young <i>et al.</i> , 2004
TLR3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	319	Saikh <i>et al.</i> , 2003
TLR4	CTGCAATGGATCAAGGACCA	CCACCACCATGATGAGAGCA	622	Saikh <i>et al.</i> , 2003
TLR5	CTAGCTCCTAATCCTGATG	CCATGTGAAGTCTTTGCTGC	438	Young <i>et al.</i> , 2004
TLR6	AGGTGCCTCCATTATCCTCA	GAATCCATTTGGGAAAGCAG	211	Young <i>et al.</i> , 2004
TLR7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG	542	Saikh <i>et al.</i> , 2003
TLR8	GCCAGCGAGTCTCACTGAACT	GCCAGGGCAGCCAACATA	558	Young <i>et al.</i> , 2004
TLR9	TTCCCTGTAGCTGCTGTCC	ACAGCCAGTTGCAGTTCACC	207	Young <i>et al.</i> , 2004
TLR10	GGCCAGAAACTGTGGTCAAT	CTGCATCCAGGGAGATCAGT	199	Young <i>et al.</i> , 2004
NOD1	TCCAAAGCCAAACAGAAACTC	CAGCATCCAGATGAACGTG	180	Kim <i>et al.</i> , 2004
NOD2	GAAGTACATCCGCACCGAG	GACACCATCCATGAGAAGACAG	174	Kim <i>et al.</i> , 2004
TOLLIP	TGGAATAAGGTCACTCACTG	AGAAGGCTCTCTCATCGAA	78	Melmed <i>et al.</i> , 2003

## **CHAPTER 3 Activation of NF- $\kappa$ B by *Campylobacter jejuni***

## CHAPTER 3

### 3.1 Introduction

The inducible transcription factor NF- $\kappa$ B is ubiquitously expressed in different tissues where it regulates the cellular immune responses to inflammatory stimuli such as foodborne pathogens. *Campylobacter jejuni* causes enteritis by mechanisms that are not clearly understood (Ketley, 1997, Mellits *et al.*, 2002, Hu *et al.*, 2006). However, the ability of *C. jejuni* to activate NF- $\kappa$ B is thought to be responsible for the pathological and clinical symptoms during the infection (Mellits *et al.*, 2002; Hu *et al.*, 2006; MacCallum *et al.*, 2006). *In vitro* studies indicated that when *C. jejuni* is incubated with epithelial cell lines NF- $\kappa$ B is induced resulting in the production of pro-inflammatory cytokines and chemokines (Mellits *et al.*, 2002; Jin *et al.*, 2003; Jones *et al.*, 2003; Hu and Hickey, 2005; Hu and Hickey, 2006; Priscilla *et al.*, 2006; MacCallum *et al.*, 2006). Interleukin-8 (IL-8) is a potent immune cell chemo-attractant and activator that is thought to be important for host immune response to *C. jejuni in vivo*.

The activation of NF- $\kappa$ B and the subsequent production of the pro-inflammatory molecules by live enteric bacterial pathogens and their products have been reported (Jobin and Sartor, 2000; Akara and Takeda, 2004; Strober, 2006). It has been previously demonstrated that *C. jejuni* and heat-stable cell free extract (BCE) of *C. jejuni* induce NF- $\kappa$ B and IL-8 production in HeLa 57A cells stably transfected for NF- $\kappa$ B-dependant (*luc*) and independent (*LacZ*) reporter genes (Mellits *et al.*, 2002). This observation was extended by transfecting HEK293, Caco-2 and HCA-7 cells with the corresponding reporter plasmids and treating these cells with live *C. jejuni* 11168 or heat-killed bacteria or BCE.

Additionally, the effect of specific mutations in *C. jejuni* or pre-treatments of *C. jejuni* boiled cell extract (BCE) on NF- $\kappa$ B activation has been investigated.

### 3.2 Results

#### 3.2.1 Live *C. jejuni*, or heat-killed *C. jejuni*, or BCE can activate NF- $\kappa$ B and induce IL-8 production in tissue culture cell lines.

HeLa 57A, HEK293, Caco-2 and HCA-7 cells stably or transiently transfected with the reporter plasmids  $\kappa$ B-luc and pRSV-lac were treated for 6 h with *C. jejuni* 11168 (m.o.i of 100) or alternatively for 6 h with either heat-killed bacteria (at an equivalent m.o.i.) or BCE. Activation of NF- $\kappa$ B together with the secretion of the pro-inflammatory cytokine IL-8 were observed in the cell lines following the various treatments (Table 3.1).

**Table 3.1.** NF- $\kappa$ B and IL-8 induction levels by *C. jejuni* in the tested cells. The positive control was TNF- $\alpha$  (50 ng/ml<sup>-1</sup>). Data represent the means of three independent measurements  $\pm$  standard deviation.

Cell line	Live bacteria NFI	Heat-killed bacteria NFI	BCE NFI	TNF- $\alpha$ NFI	BCE IFI (initial pg ml <sup>-1</sup> )
Hela 57 A	130 $\pm$ 27	90 $\pm$ 15	275 $\pm$ 25	225 $\pm$ 20	48 (50)
HEK293	5.2 $\pm$ 0.8	2.1 $\pm$ 0.2	14.5 $\pm$ 27	2.1 $\pm$ 15	66 (80)
Caco-2	2.5 $\pm$ 0.2	2 $\pm$ 0.2	5.4 $\pm$ 1.2	12 $\pm$ 3.3	4 (50)
HCA-7	9.3 $\pm$ 3	6 $\pm$ 2	26 $\pm$ 4	16 $\pm$ 2	460 (40)

NFI: NF- $\kappa$ B fold of induction. IFI: IL-8 fold of induction (data provided by Prof. Ian Connerton). HeLa 57A cells stably expressing the reporter genes are more responsive.

All cells induced NF- $\kappa$ B and secreted IL-8 in response to live/or heat killed *C. jejuni* or *C. jejuni* BCE. However, it is evident that there are inherent differences in the sensitivities of these cells to *C. jejuni* products based on the IL-8 response levels. Most notably the HCA-7 and Caco-2 cell lines are both derived from human colon carcinomas, and are capable of polarisation and tight junction formation in culture but HCA-7 cells secrete relatively high levels of IL-8 in response to a 6 h *C. jejuni* BCE treatment compared to Caco-2 cells, which show

a significantly weaker response from similar pre-induction levels of IL-8 (Table 3.1). Treatment of the colonic epithelial cells (Caco-2 or HCA-7) with *C. jejuni* NCTC 11168 (m.o.i. 100) did not result in cell death over a 30 h time course, since trypan blue staining of parallel infections showed no significant differences between the viability of mock infected cells and those infected with bacteria. Viable counts of *C. jejuni* present in the tissue culture medium of these cells revealed that the bacteria were not adversely affected by either the incubation conditions or the cellular response, as the counts remained static over the 30 h time course.

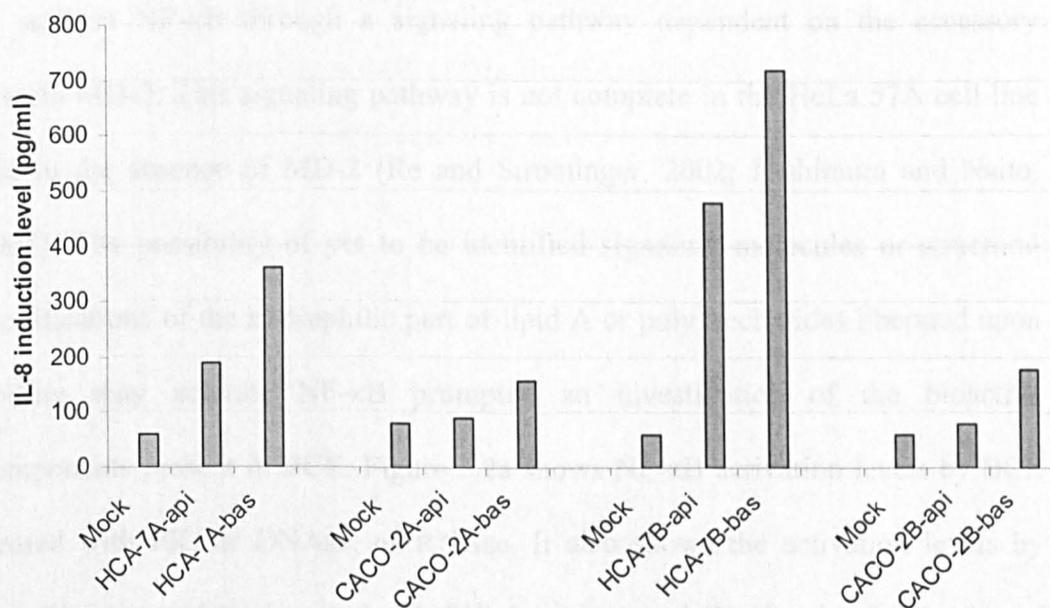
### **3.2.2 *Campylobacter jejuni* and BCE induce polarised colonic epithelial cells to produce IL-8.**

IL-8 is an important chemokine that is regulated by NF- $\kappa$ B, and has been reported to increase NF- $\kappa$ B activation. It is produced mainly by macrophages, and IEC to attract neutrophils to the site of infection. Production of IL-8 from the basolateral gut epithelial cell will initiate signalling to underlying cells types.

Polarised cell monolayers (TEER  $>800 \Omega\text{cm}^2$ ) of HCA-7 or Caco-2 exposed to either live cells (m.o.i 100) or BCE for 6 h on their apical face also show differences in IL-8 production measurable from their basolateral side (Figure 3.1). *Campylobacter jejuni* treatment of HCA-7 polarised cells produced a six-fold increase in IL-8 in the basolateral chamber after 16 h compared to a mock treated control (corresponding to  $360\pm40 \text{ pg ml}^{-1}$  and  $190\pm30 \text{ pg ml}^{-1}$  of IL-8 respectively in the basolateral and apical chambers), whereas Caco-2 cells produced a two-fold increase in IL-8 over a mock treated control (corresponding to  $160\pm30 \text{ pg ml}^{-1}$  and  $90\pm30 \text{ pg ml}^{-1}$  of IL-8 respectively in the basolateral and apical chambers). At 16 h post-infection the tight junctions of these monolayers

remained largely intact as judged by modest falls in the TEER of 50-100  $\Omega\text{cm}^2$ , around 5% of the total. Although, 12 h later the TEER values began to fall with bacteria present in the basolateral chamber. BCE treatments of HCA-7 or Caco-2 resulted in temporary falls in TEER values up to 100  $\Omega\text{cm}^2$  that showed recovery 24 h later. BCE treatment of HCA-7 polarised cells produced a twelve-fold increase in IL-8 in the basolateral chamber after 6 h compared to a mock treated control (corresponding to  $720\pm 60$  pg ml<sup>-1</sup> and  $480\pm 40$  pg ml<sup>-1</sup> of IL-8 respectively in the basolateral and apical chambers). In contrast at 6 h post BCE treatment polarised CACO-2 cells produced a three-fold increase in IL-8 over a mock treated control (corresponding to  $180\pm 40$  pg ml<sup>-1</sup> and  $80\pm 20$  pg ml<sup>-1</sup> IL-8 respectively in the basolateral and apical chambers).

**Figure 3.1. IL-8 induction levels by *C. jejuni*.** Live *C. jejuni* induced IL-8 in the apical (HCA-7A-api, CACO-2A-api) and basolateral (HCA-7A-bas, CACO-2A-bas) chambers. Induction levels by BCE in the apical (HCA-7B-api, CACO-2B-api) and basolateral (HCA-7B-bas, and CACO-2B-bas) chambers.

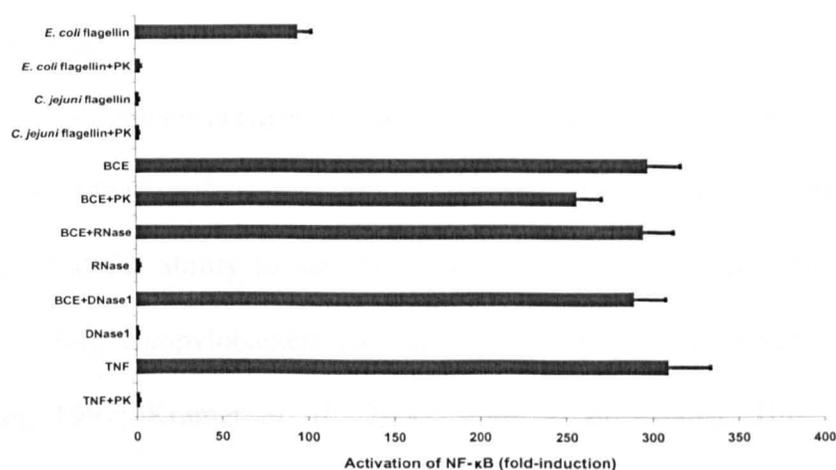


### **3.2.3 NF- $\kappa$ B activation by BCE is independent of peptide, nucleic acids, capsular polysaccharide, and lipid containing components.**

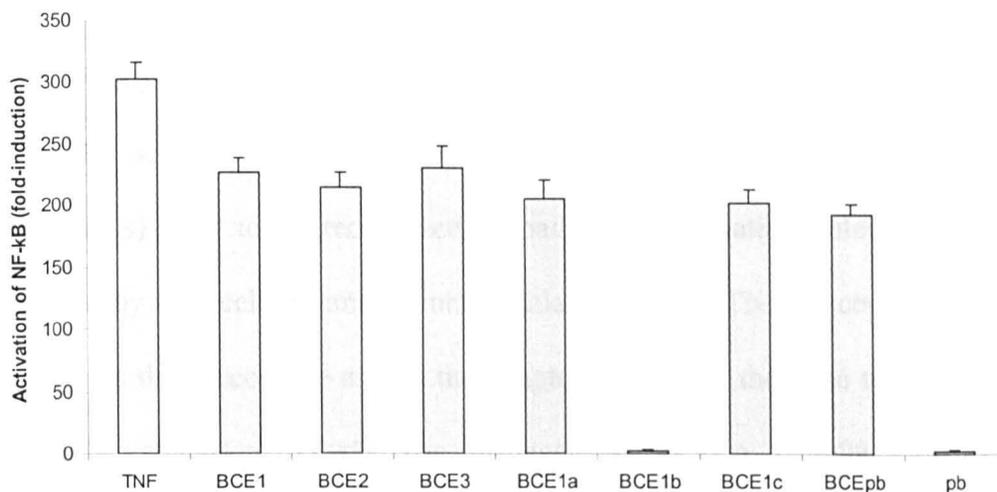
Subsequent to the finding above, BCE was prepared from *C. jejuni* mutants affected in LOS or CPS production, treated with proteinase K (PK), DNase, RNase, lipopeptide lipase, sodium acetate buffer (to precipitate lipid A), or pre-incubated with polymyxin B ( binds to LPS) before the induction assay on the HeLa 57A cell line. Since proteinaceous structures (e.g. flagellin), CpG DNA, RNA, LPS, CPS, and lipoproteins have been reported to induce host immune responses (Akira and Takeda, 2004), the treatments above were performed to investigate the possibility of these structures contributing to NF- $\kappa$ B activation as components of BCE, and gain insight into what factors might be responsible for the inflammatory responses characteristic of *C. jejuni* caused enteritis. Moreover, the lipid-A component of Gram-negative lipopoly/lipooligosaccharides are known to activate NF- $\kappa$ B through a signaling pathway dependent on the accessory protein MD-2. This signaling pathway is not complete in the HeLa 57A cell line due to the absence of MD-2 (Re and Strominger, 2002; Nishimura and Naito, 2005). The possibility of yet to be identified signaling molecules or structural modifications of the hydrophilic part of lipid A or polysaccharides liberated upon boiling may activate NF- $\kappa$ B prompted an investigation of the bioactive components present in BCE. Figure 3.2a shows NF- $\kappa$ B activation levels by BCE treated with PK, or DNase, or RNase. It also shows the activation levels by flagellin prepared (and treated with PK) from *C. jejuni*. The levels of activation of NF- $\kappa$ B by precipitated crude BCE (the pellet presumably contains lipid A), the supernatant, BCE treated with lipopeptide lipase, BCE from a *C. jejuni waaF* mutant, and from a *C. jejuni kpsM* mutant are shown in figure 3.2b.

The data presented in figure 3.2 indicate that the bioactive component(s) in BCE are not flagellin, DNA, RNA, lipopolysaccharide, CPS, or lipoprotein. Previously, the bioactive component in BCE was reported to be a proteinase K resistant molecule less than 3 kDa (Mellits *et al.*, 2002). Here in this study, the result of the extended treatments on BCE has not been reported for *C. jejuni* or other foodborne bacterial pathogens.

**Figure 3.2a. Induction-fold of NF- $\kappa$ B in HeLa 57A cells incubated with *C. jejuni* BCE and flagellin with or without enzymatic treatment; *E. coli* flagellin (50 ng/ml<sup>-1</sup>), *E. coli* flagellin treated with proteinase K(PK; at 100  $\mu$ g/ml<sup>-1</sup>), *C. jejuni* flagellin (50 ng/ml<sup>-1</sup>), *C. jejuni* flagellin treated with proteinase K(PK; at 100  $\mu$ g/ml<sup>-1</sup>), *C. jejuni* boiled-cell extract (BCE), BCE treated with DNase (1 $\mu$ g/ml<sup>-1</sup>), or BCE treated with RNase (1ng/ml<sup>-1</sup>). TNF- $\alpha$  was used as positive control of activation and PK activity at 50 ng/ml<sup>-1</sup>. The data are recorded as the means of 3 independent determinations.**



**Figure 3.2b. Induction-fold of NF- $\kappa$ B in HeLa 57A cells incubated with *C. jejuni* BCE (prepared from wild-type, mutants, or subjected to pre-treatments); wt- *C. jejuni* boiled cell extract (BCE1), *kpsM*-*C. jejuni* boiled-cell extract (BCE2), *waaF*- *C. jejuni* boiled-cell extract (BCE3), supernatant of BCE1 after lipid-A removal (BCE1a), precipitated lipid-A from BCE1 (BCE1b), BCE1 treated with lipopeptide lipase (BCE1c), BCE pre-incubated with polymyxin B (BCEpb), polymyxin B (pb). TNF- $\alpha$  was the control activation (50 ng/ml<sup>-1</sup>). The data are recorded as the means of three independent determinations.**



### 3.3 Discussion

*Campylobacter* is currently the most reported cause of foodborne mediated enteritis worldwide (Brown *et al.*, 2004). Having a range of environmental reservoirs and the ability to survive outside the host organisms (Ketley, 1997; Butzler, 2004), campylobacters can still be detected in processed retail foods (Bongkot, 1997; Kramer *et al.*, 2000; Wyte *et al.*, 2000). This along with unhygienic practices during food preparation by households may contribute to the high incidences of infection by this bacterium (Altekruse *et al.*, 1995; Griffith *et al.*, 1998; Jay *et al.*, 1999).

The infection prognoses of *C. jejuni* can be self-limiting. The disease is characterized by watery or bloody diarrhoea accompanied by abdominal cramps, or chronic condition affecting the peripheral nerves (Smith, 1995; Ketley, 1997). In intestinal epithelial cells (IECs), where the interactions between bacterial foodborne pathogens and the host take place, the innate immune responses, which are mainly responsible for the disease pathology, are mainly controlled by the

transcription factor NF- $\kappa$ B (Jobin and Sartor, 2000). The production of cytokines, chemokines, inflammatory enzymes, and immunoregulatory molecules are regulated by the heterodimer composed of the RelA (p65) and NF- $\kappa$ B1 (p50) subunits whose activation proceeds via the I $\kappa$ B dependent pathway (Jobin and Sartor, 2000).

To activate NF- $\kappa$ B, the inflammatory stimuli (e.g. a pathogen or its components) need to be recognized as pathogen associated molecular patterns (PAMPs) by extracellular and/or intracellular receptors. These receptors differ in the ligands they recognize and in the adaptor molecules they use to transfer the stimulating signals to NF- $\kappa$ B. The virulence factors (e.g. flagella, CDT, LOS, CPS, peptidoglycan, lipoproteins, and OMP) and mechanisms (e.g. motility, chemotaxis, adhesion, invasion, translocation, iron acquisition, and intracellular survival) may contribute to the pathogenesis of *Campylobacter* and the induction of the host immune responses. In *in vitro* studies, the activation of NF- $\kappa$ B by live bacteria, flagella, nucleic acids, LPS/LOS, PGN, CPS, and lipoproteins have been reported. Apart from the nucleic acids, these structures are bound to Gram-negative bacteria' cell-surfaces and are thought to be extracted with minimal changes by repeated centrifugations followed by boiling in a saline buffer (Mellits *et al.*, 2002).

Live *C. jejuni* or its cell-free boiled extract (BCE) have been reported to activate NF- $\kappa$ B in epithelial cells (HeLa 57A and HCA-7) via an I $\kappa$ B-dependent pathway, and induce the production of TNF- $\alpha$  and IL-8 (Mellits *et al.*, 2002). The activation by BCE, which was dose-dependent and immediate, may imply that *C. jejuni* can cause inflammatory responses independent of its adherence to or invasion of the host cell (Mellits *et al.*, 2002). In this study, the activation of NF- $\kappa$ B and IL-8 production were observed in HeLa 57A, HEK293, HCA-7, and

Caco-2 cell lines. The higher levels of activation in all cell lines by BCE may suggest that the bioactive component is liberated by heat to be more readily available for recognition by cellular receptor(s). Additionally, the NF- $\kappa$ B activation by BCE occurred independently of the major components of *C. jejuni* cell surface and its nucleic acids. The host immune responses to these components in IECs are regulated by means that accommodate the commensal bacteria and detect the presence of pathogens. At the apical membrane of IEC, which is continuously exposed to the gut flora, the adherence of bacterial pathogens coupled with their ability to invade and reach the basolateral membrane is thought to be responsible for immune stimulation and a precursor to systemic infection. IL-8, a potent chemokine produced at the basolateral surface and controlled by NF- $\kappa$ B, attracts the neutrophils to the site to limit the infection. Here, during co-incubation of polarized HCA-7 and CACO-2 cell lines with *C. jejuni* or BCE, IL-8 was produced at higher levels in basolateral chambers by both cell lines compared to the apical chambers. This may reflect that *C. jejuni* interaction with host cells does not have to involve invasion and intracellular survival to cause inflammatory response *in vivo*. Although inflammatory responses may be insufficient to characterise *Campylobacter* enteritis, they will contribute to the disease symptoms. Moreover, inappropriate pro-inflammatory responses to bacterial components in the absence of live bacteria may contribute to temporary bouts of discomfort. IL-8 has been reported to be produced by a variety of cell lines exhibiting differences in the induction levels in response to *C. jejuni*. The differences in production levels have been reported to be due to the contamination of cell lines by another cell line, or the requirement of a cell-to-cell cross talk for immune response in the gut mucosa (MacCallum *et al.*, 2006).

The data presented in this chapter report NF- $\kappa$ B activation by live *C. jejuni* in different cell lines, and provided initial structural details about the NF- $\kappa$ B activating component in BCE. In chapter 4, different fractionation techniques, mass spectrometry, gas chromatography, and nuclear magnetic resonance were applied to identify the bioactive component in BCE. The signalling pathways leading to NF- $\kappa$ B activation by *C. jejuni* are investigated in chapter 5.

**CHAPTER 4 Structural characterisation of NF- $\kappa$ B  
activating components in *Campylobacter jejuni* BCE**

## CHAPTER 4

### 4.1 Introduction

One of the measures taken to minimize the risk for contracting a foodborne disease is sufficient heat-treatment. However, the reported activation of NF- $\kappa$ B by BCE from *C. jejuni* may invalidate this measure. Unlike other methods for extracting bacterial immune-response provoking components, boiling-mediated liberation of the bioactive component(s) from *C. jejuni* will not introduce any further components that could add to the response. Using the HeLa 57A cell line as a model for studying NF- $\kappa$ B activation by BCE, Mellits *et al.* (2002) reported that BCE is heat-stable and treatment of BCE with proteinase K did not suppress its activation of NF- $\kappa$ B. Moreover, the cell line does not express the full complement of receptors known to lead to NF- $\kappa$ B activation (Re and Strominger, 2002; Nishimura and Naito, 2005). This along with the results presented in chapter 3, which indicate that BCE is not affected by various treatments, may imply that BCE of *C. jejuni* represents a novel component responsible for NF- $\kappa$ B activation.

The aim of this chapter is to characterize the structure of the NF- $\kappa$ B activating component(s) in *C. jejuni* BCE. This was carried out using several different purification steps and the product analysed using mass spectrometry, gas chromatography, enzymatic treatments and by NMR.

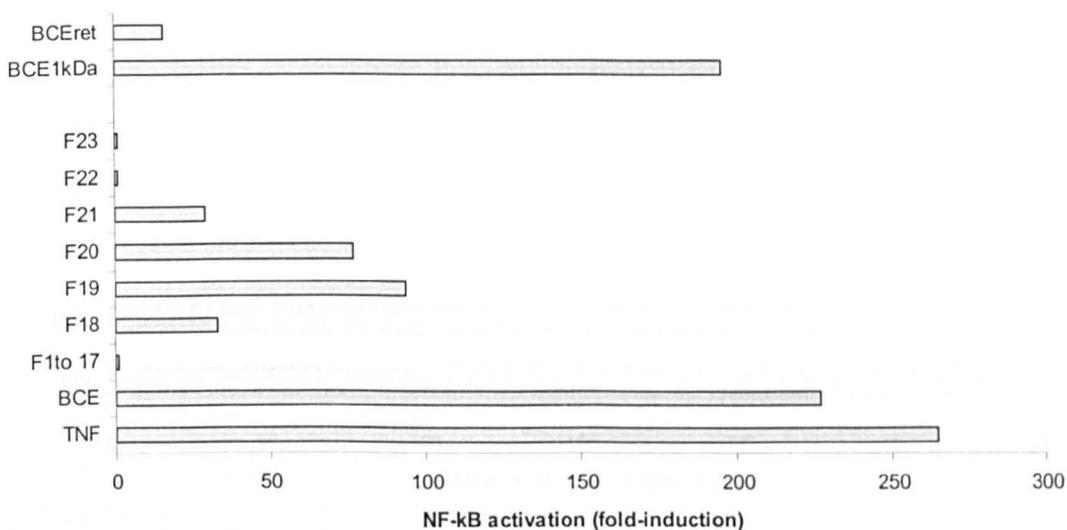
### 4.2. Results

#### 4.2.1 NF- $\kappa$ B is activated mainly by a fraction less than 1000 Da

The majority of the components capable of BCE activation of NF- $\kappa$ B were reported to be less than 3000 Da (Mellits *et al.*, 2002). Molecular size-based fractionation was the first step in purifying BCE for further structural

characterization methods. This was performed using a size-exclusion column (SEC) and 1000 Da molecular cut-off filters. The separation of BCE on a SEC gave 4 active fractions that were eluted from a column fractionating between 100 to 7000 Da. The broad peak of activity could be due to eluting efficiency rather than structural difference. However, since BCE preparation involves a boiling step that may break the physiological structure of a parent molecule, the observed size may not reflect the true molecular weight of the activating component sizes. NF- $\kappa$ B activation by BCE applied to the SEC and 1K filter are shown in figure 4.1.

**Figure 4.1. Induction-fold of NF- $\kappa$ B in HeLa 57A cells incubated with *C. jejuni* BCE fractionated by a size-exclusion column and a 1 kDa filter; *C. jejuni* boiled-cell extract (BCE), fractions of BCE obtained from separation on the size-exclusion column (numbered F1 to F23), BCE 1kDa fraction (BCE1kDa), and the BCE retained fraction on the 1kDa filter (BCEret). TNF- $\alpha$  was the control activation (50 ng/ml<sup>-1</sup>).**

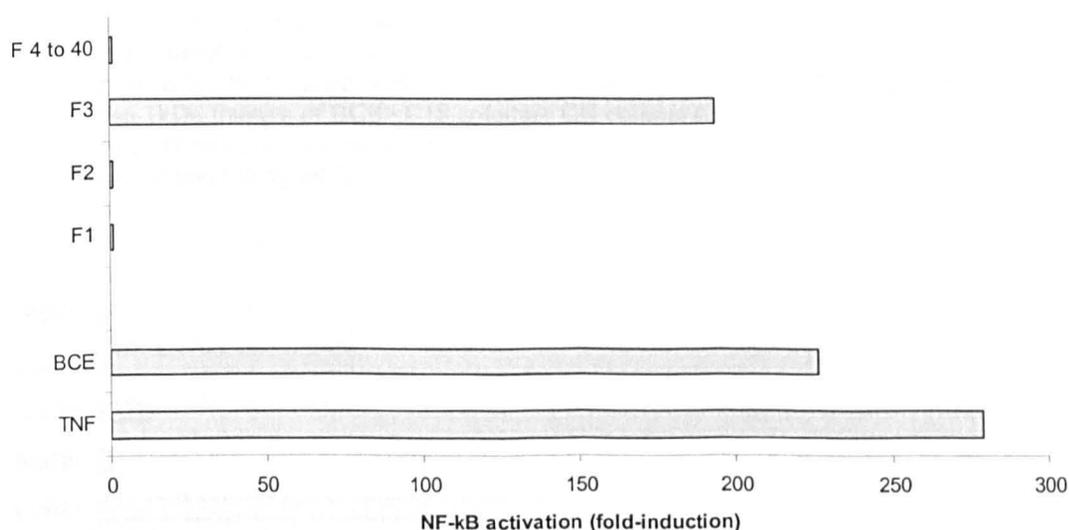


#### **4.2.2 The NF- $\kappa$ B activating component in BCE does not adsorb to the reverse-phase column under hydrophobic conditions.**

NF- $\kappa$ B can be activated by water-soluble and water-insoluble bacterial-derived components (Yoshihiko *et al.*, 2005). Thus, knowing the polarity of the active component in BCE will indicate if the active component belongs to specific

polarity-categorized ligands. The resultant active fractions from the SEC column were combined and applied to a reverse-phase column following the operational conditions recommended by the manufacturer for characterising unknown samples. As shown in figure 4.2, the result indicates that the NF- $\kappa$ B activating component did not adsorb to the column and was eluted immediately in the void volume. Thus, the active component is a hydrophilic structure. An advantage of using an RP-column here would be to filter-out hydrophobic contaminants that are often present in biological samples as high-molecular weight structures.

**Figure 4.2. Induction-fold of NF- $\kappa$ B in HeLa 57A cells incubated with *C. jejuni* BCE fractionated by reverse-phase HPLC column: *C. jejuni* boiled-cell extract (BCE), combined BCE active fractions from the size exclusion column run on the reverse-phase column (F1 to F40). TNF- $\alpha$  was the control activation (50 ng/ml<sup>-1</sup>).**

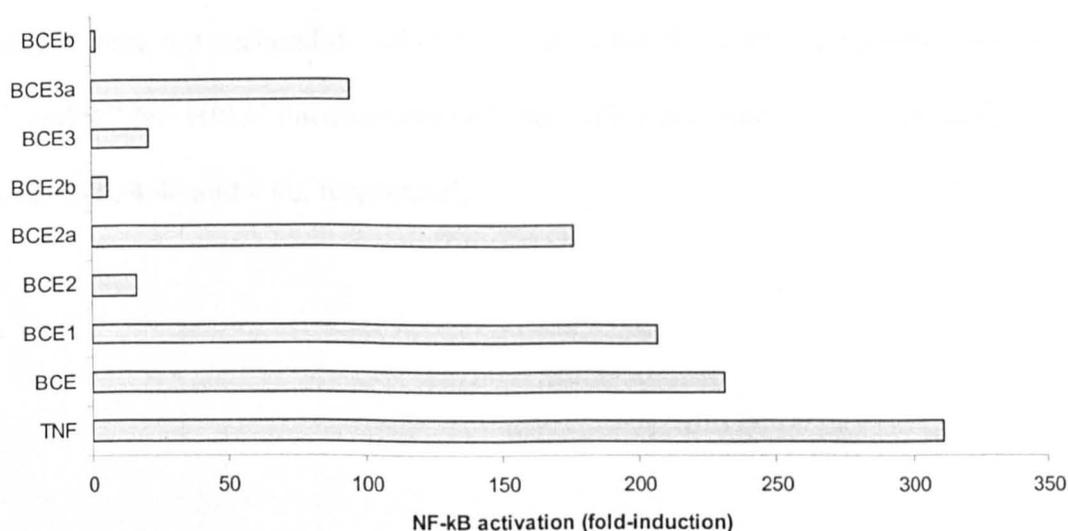


#### **4.2.3 Solid Phase Extraction (SPE) purification of BCE indicates that the active component is optimally eluted using a specific solvent composition.**

Subsequent to the finding above, BCE-1kDa from wild-type *C. jejuni* was purified using SPE disposable (C18, CN, and PGC) columns before further fractionation on the normal-phase- HPLC column. SPE (reviewed in Hennion, 1999) is a simple chromatographic process that provides pre-HPLC purification for higher

sample volumes in a convenient time frame. In SPE, the sorbent is the stationary phase while the mobile-phase is the solvent of the sample. BCE-1kDa was purified by the RP-SPE column (C18; traps hydrophobic contaminants), by the NP-SPE column (CN; traps polar components), then by another NP-SPE column (PGC). During the purification steps using the NP-SPE column, the NF- $\kappa$ B activating component was optimally-eluted using a 70/30 acetonitrile-water mixture, and this behaviour is typically observed when separating oligosaccharides. Recorded NF- $\kappa$ B activation fold-induction of BCE purified by SPE columns are presented in figure 4.3.

**Figure 4.3. Induction-fold of NF- $\kappa$ B in HeLa 57A cells incubated with *C. jejuni* BCE fractionated by solid-phase extraction columns:** *C. jejuni* boiled-cell extract (<1kDa fraction of BCE), <1kDa fraction of BCE, BCE purified by the reverse-phase C18 column (BCE1), BCE purified by normal-phase CN column and eluted with; 20% H<sub>2</sub>O in acetonitrile (BCE2), BCE eluted with 30% H<sub>2</sub>O in acetonitrile (BCE2a), BCE eluted with 40% H<sub>2</sub>O in acetonitrile (BCE2b). BCE purified with the second normal-phase porous-graphitized carbon (hybercarb) column (BCE3), BCE eluted with; 20% H<sub>2</sub>O in acetonitrile (BCE3), BCE eluted with 30% H<sub>2</sub>O in acetonitrile (BCE3a), BCE eluted with 40% H<sub>2</sub>O in acetonitrile (BCE3b). The purification order was; less than 1kDa fraction of BCE> C18 column> CN column (20, 30, and 40% elutions from the same column)> Hypercarb column (20, 30, and 40% elutions from the same column). TNF- $\alpha$  was the control activation (50 ng/ml<sup>-1</sup>).



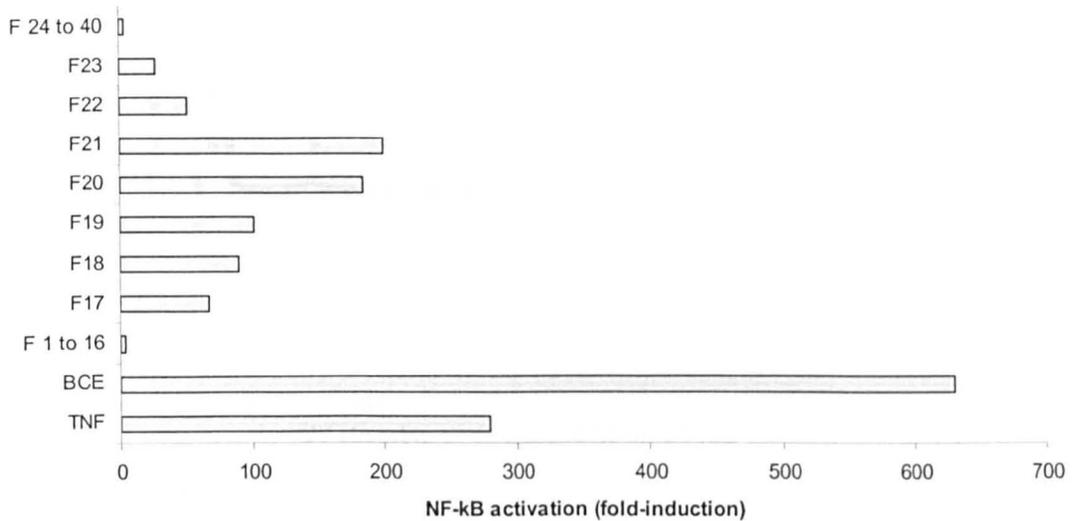
The retention of an oligosaccharide on a normal-phase SPE or HPLC column is controlled by the concentration of the organic modifier in the mobile phase. At high acetonitrile concentration, the bonding formation between the hydroxyl

groups of the oligosaccharide with the column's stationary phase is increased, whereas higher water content and less acetonitrile weaken the bond formation and permits the oligosaccharide elution from the column (Lo-Guidice and Lhermitte, 1996; Hennion, 1999).

#### **4.2.4 Normal-phase HPLC of BCE gave active fractions that have very low UV-absorbance.**

To further fractionate BCE for MS and NMR studies, purified BCE-1kDa by SPE was applied to a NP-HPLC column. This cyanopropyl-bonded silica column is recommended for fractionating polar samples. The first NP-HPLC run resulted in broad-range NF- $\kappa$ B activating fractions (Figure 4.4a) where the second NP-HPLC run (on the active fraction from the first NP-HPLC) gave two NF- $\kappa$ B active fractions (Figure 4.4b). This could be due to the column efficiency regarding its sample-loading limit. Of interest during NP-HPLC fractionation was the demonstration of UV absorbance at 195nm which may indicate the presence of carbohydrate in the sample. Attempts to record UV absorbance at higher wavelengths were not successful. NF- $\kappa$ B activation levels by BCE fractions from the 1<sup>st</sup> and 2<sup>nd</sup> NP-HPLC purifications and their UV absorbance are shown in figure 4.4a, 4.4b, 4.4c and 4.4d, respectively.

**Figure. 4.4a. Induction-fold of NF- $\kappa$ B in HeLa 57A cells incubated with *C. jejuni* BCE fractionated by normal-phase HPLC column (1<sup>st</sup> run):** *C. jejuni* boiled-cell extract from 5-litres culture (BCE: <1kDa fraction of BCE purified by SPE), fractions (F1 to 40) obtained by purifying BCE by NP-HPLC. TNF- $\alpha$  was the control activation (50 ng/ml<sup>-1</sup>).



**Figure 4.4b. Induction-fold of NF- $\kappa$ B in HeLa 57A cells incubated with *C. jejuni* BCE fractionated by normal-phase HPLC column (2<sup>nd</sup> run):** *C. jejuni* boiled-cell extract (BCE; combined active fractions from the 1<sup>st</sup> NP-HPLC purification), fractions (F1 to 40) of BCE from 2<sup>nd</sup> NP-HPLC purification. TNF- $\alpha$  was the control activation (50 ng/ml<sup>-1</sup>).

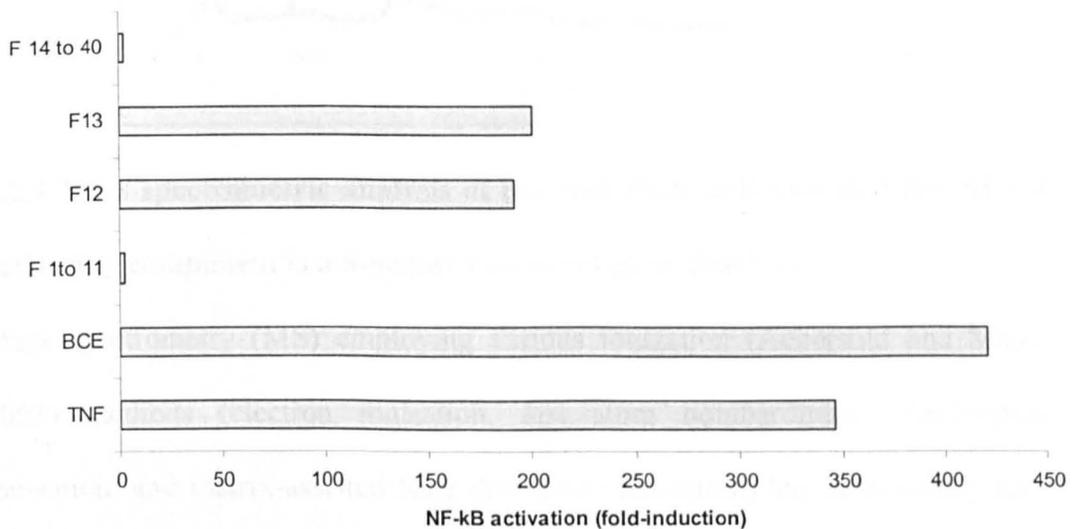


Figure 4.4c.

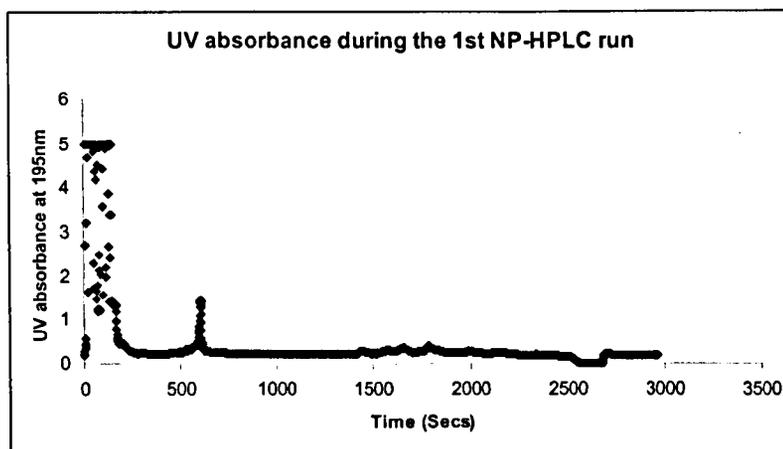
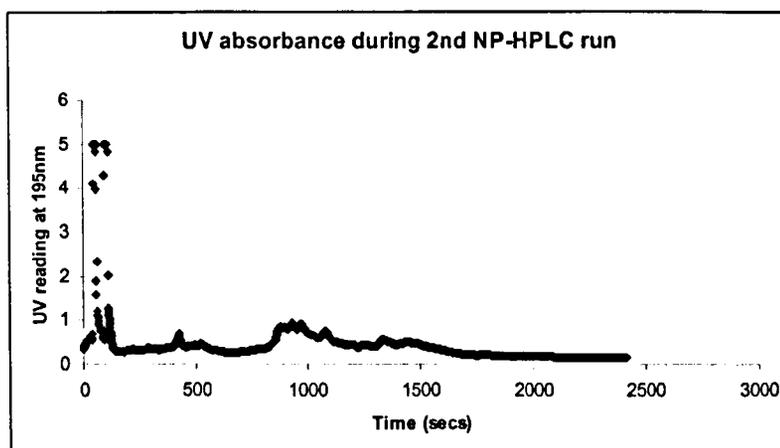


Figure 4.4d.



#### 4.2.5 Mass spectrometric analysis of purified BCE indicates that the NF- $\kappa$ B activating component is a 5-hexose residue oligosaccharide.

Mass spectrometry (MS) employing various ionization (Aebersold and Mann, 2003) methods (electron ionisation, fast atom bombardment, electrospray ionisation, and matrix-assisted laser desorption ionisation) has been widely used in protein profiling, metabolic analysis, and structural characterization of bacterial-derived components such as LPS (Risberg *et al.*, 1999), peptidoglycan (Xu *et al.*, 1997), lipopeptide (Hashimoto *et al.*, 2004), and carbohydrate (Harvey, 1999). A mass spectrometer separates and detects ionised forms of the analyte on the basis of their mass to charge ratio. Therefore, MS is a valuable technique for

molecular-weight determination (Aebersold and Mann, 2003). Boiled-cell extract (BCE) from *C. jejuni* wild-type, *C. jejuni* waaF mutant and *C. jejuni* kpsM mutant were analyzed by MS using the electrospray method for ionisation (Table 4.1 and Figure 4.5).

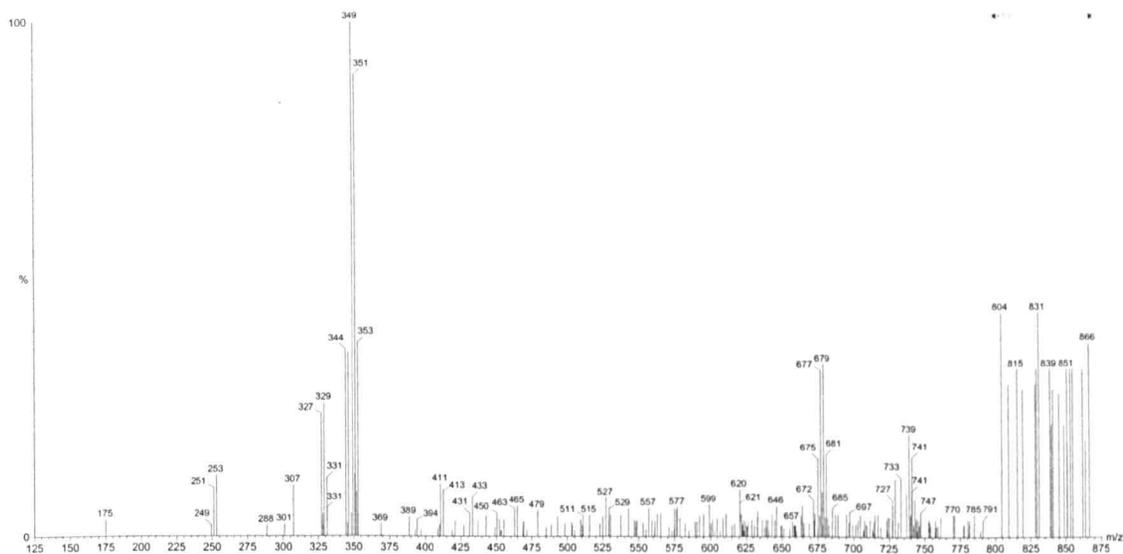
**Table 4.1. Electrospray mass spectrometry analysis of the combined BCE active fractions obtained from the 2<sup>nd</sup> NP-HPLC purification.** The analysis was performed in positive mode, negative mode and MS/MS positive mode for ion 851m/z. The proposed moiety is based on matching the ions of BCE to that of theoretical 5-hexose residue oligosaccharide. Interpretation of the analysis results was performed using the Glycofragment online tool\*.

Ion (+mode)	Ion type	Moiety
851	[M+Na]H	5-hexose residue
689	[M+Na]H	4-hexose residue
527	[M+Na]H	3-hexose residue
365	[M+Na+H <sub>2</sub> O]H	2-hexose residue
203	[M+Na+H <sub>2</sub> O]H	1-hexose residue
185	[M+Na]H	1-hexose residue
Ion (-mode)		
827	[M-H]	5-hexose residue
665	[M-H]	4-hexose residue
503	[M-H]	3-hexose residue
341	[M-H]+H <sub>2</sub> O	2-hexose residue
322	[M-H]-H <sub>2</sub> O	2-hexose residue
179	[M-H]+H <sub>2</sub> O	1-hexose residue
161	[M-H]	1-hexose residue
MS/MS (851)		
851	[M+Na]H	5-hexose residue
689	[M+Na]H	851-162 (1 hex)
671	[M+Na]H-H <sub>2</sub> O	851-180 (1 hex+H <sub>2</sub> O)
585	<sup>2,5</sup> X + Na+	
527	[M+Na]H	851-324 (2 hexoses)
509	[M+Na]H	851-342 (2 hexoses+H <sub>2</sub> O)
407	<sup>0,2</sup> X + Na+, <sup>2,4</sup> A + Na+	
393	<sup>1,5</sup> X + Na+	
365	[M+Na+H <sub>2</sub> O]H	851-486 (3 hexoses)
347	[M+Na]H-H <sub>2</sub> O	851-504 (3 hexoses+H <sub>2</sub> O)
265	<sup>2,5</sup> A + H+	
225	<sup>0,2</sup> X + 2H+, <sup>2,4</sup> A + 2 H+	
185	[M+Na]H	851-666 (4 hexoses+ H <sub>2</sub> O)

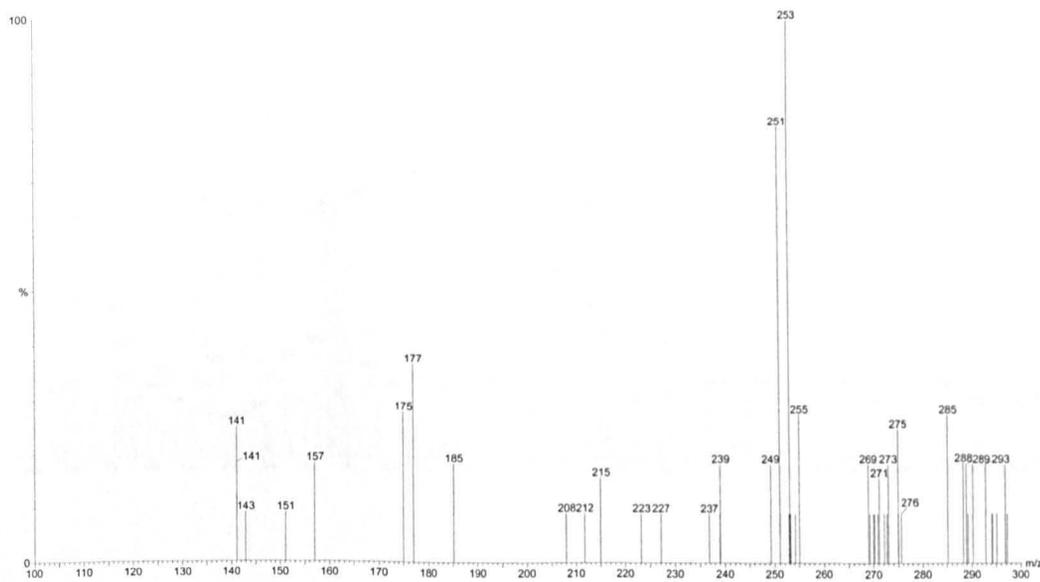
\*: <http://www.dkftz.de/spec/glycosciences.de/tools/glycofragments/fragment.php4>

**Figure 4.5. Mass spectra of BCE of wt-*C. jejuni* (a-d), *C. jejuni waaF*-mutant (a1-d1), *C. jejuni kpsM* mutant (a2-d2), negative-mode MS spectra of wt-BCE (a3-d3). Figures show whole spectra and focused region m/z spectra. MS/MS of ion 851 from BCE mass spectrum of wt-BCE (a4)**

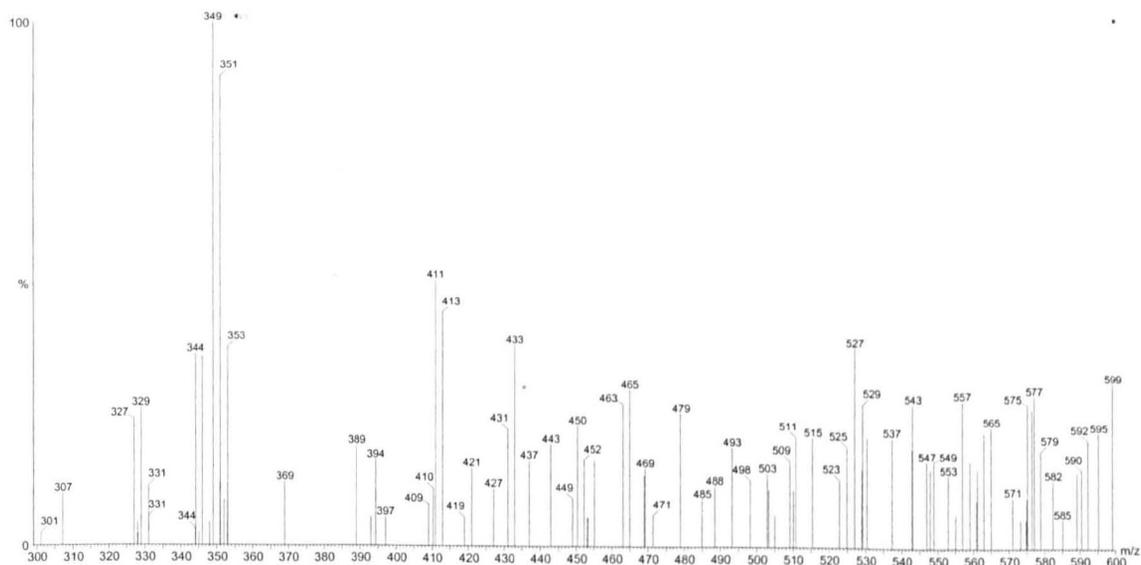
(a); whole spectrum



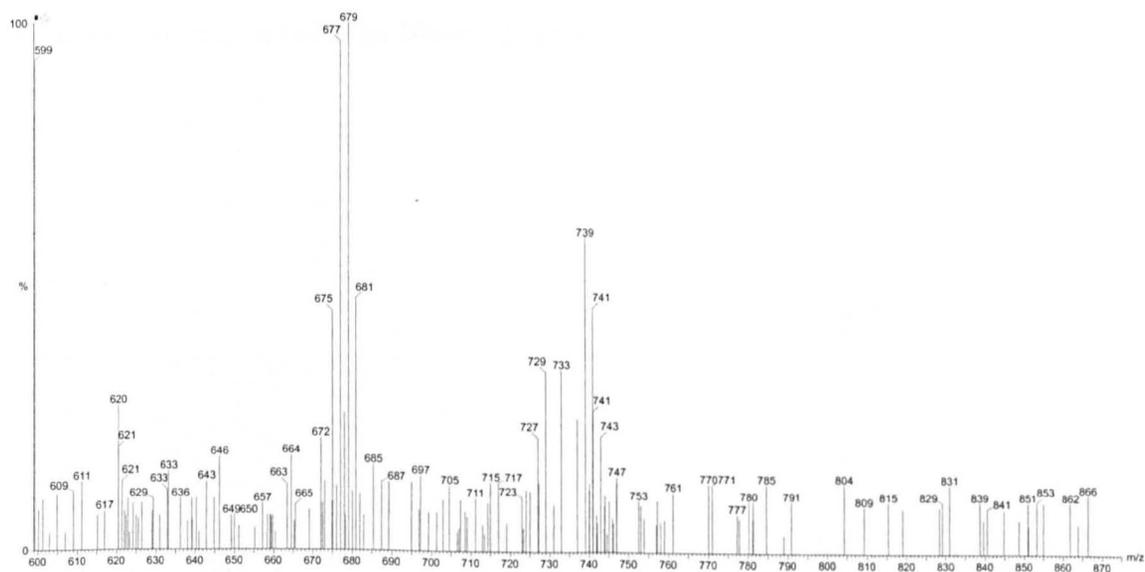
(b); focused region (100 to 300 m/z) spectrum



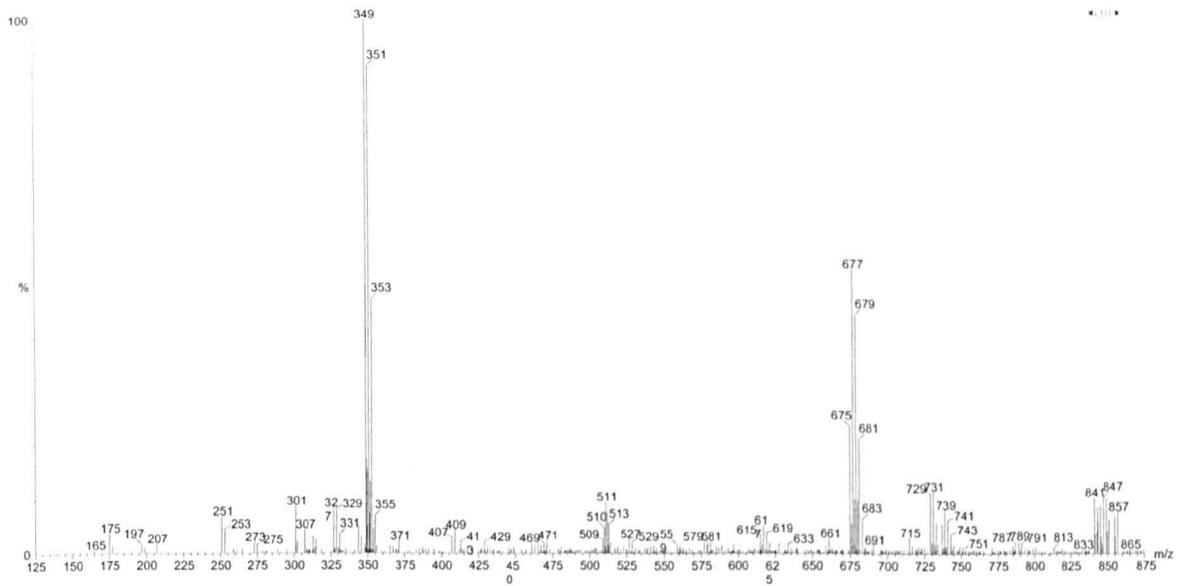
(c); focused region (300 to 600 m/z) spectrum



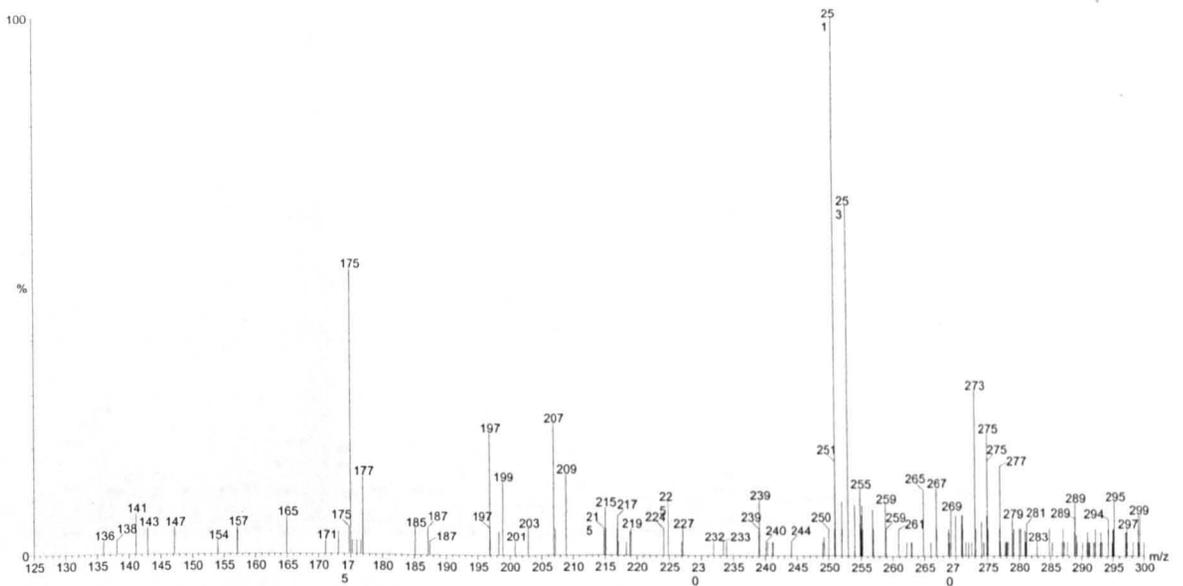
(d); focused region (600 to 870 m/z) spectrum



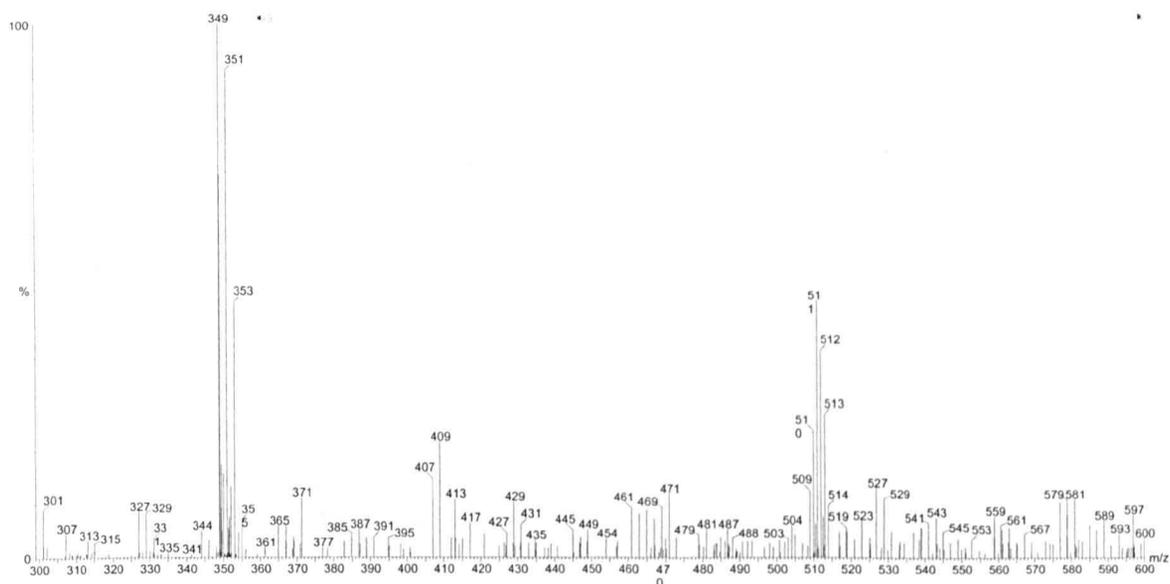
(a1); whole spectrum



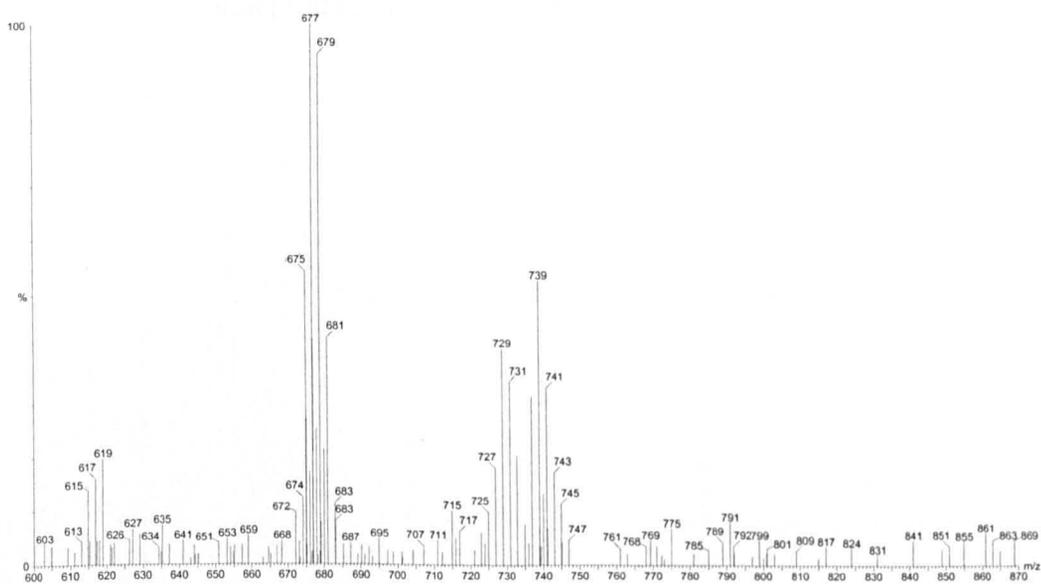
(b1); focused region (1250 to 300 m/z) spectrum



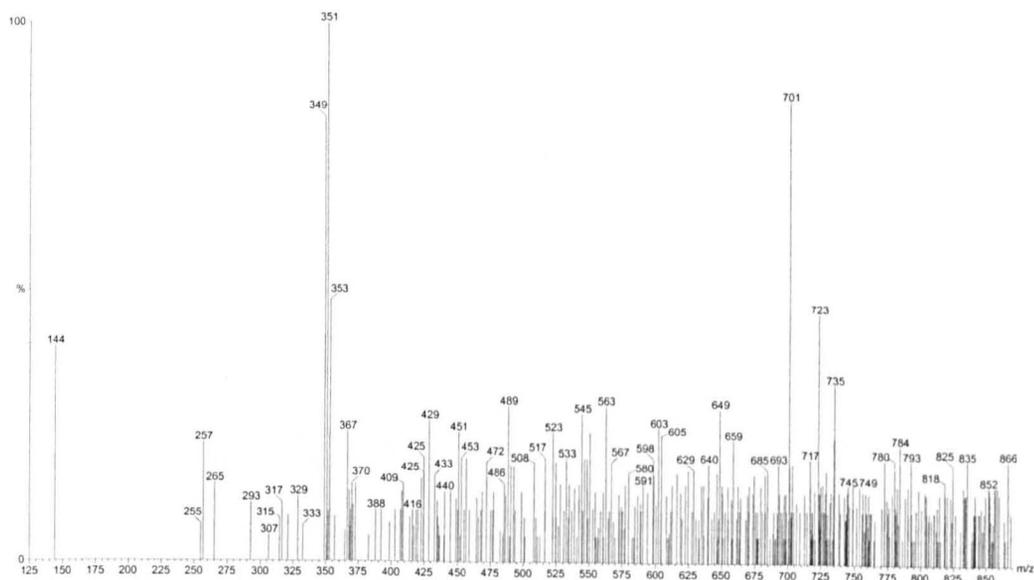
(c1); focused region (300 to 600 m/z) spectrum



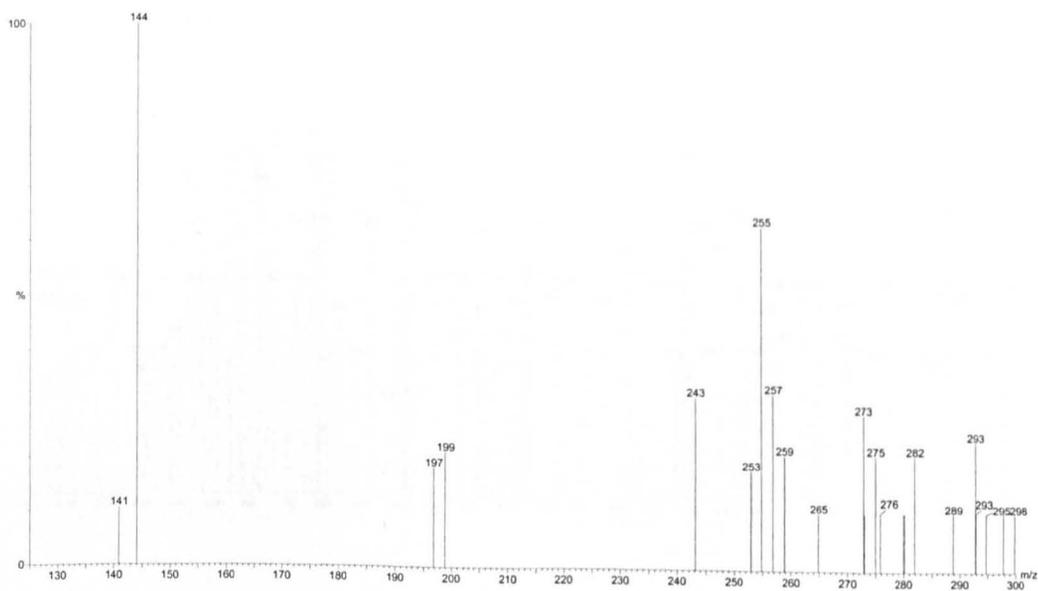
(d1); focused region (600 to 870 m/z) spectrum



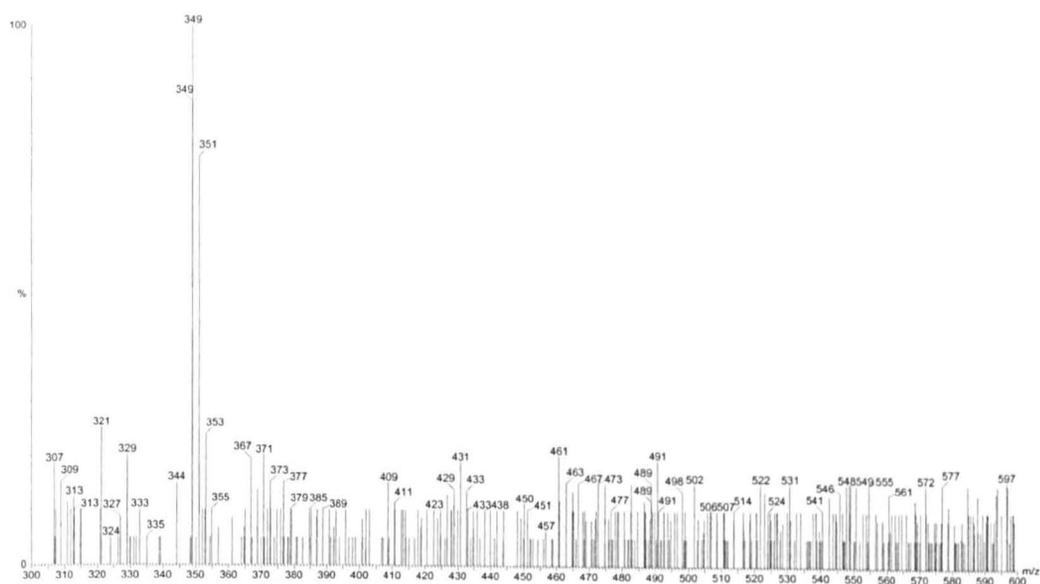
(a2); whole spectrum



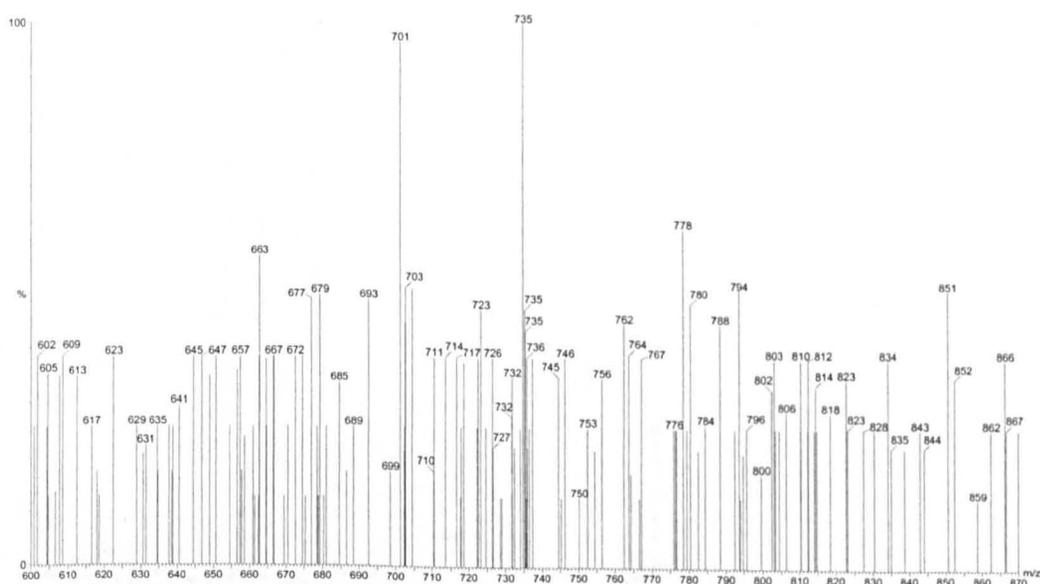
(b2); focused region (100 to 300 m/z) spectrum



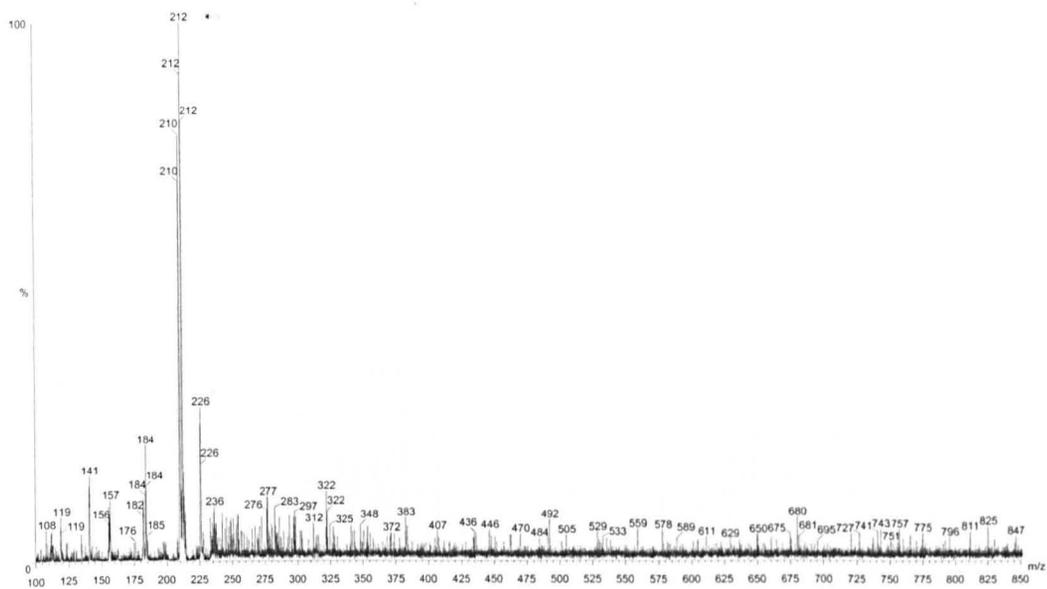
(c2); focused region (100 to 300 m/z) spectrum



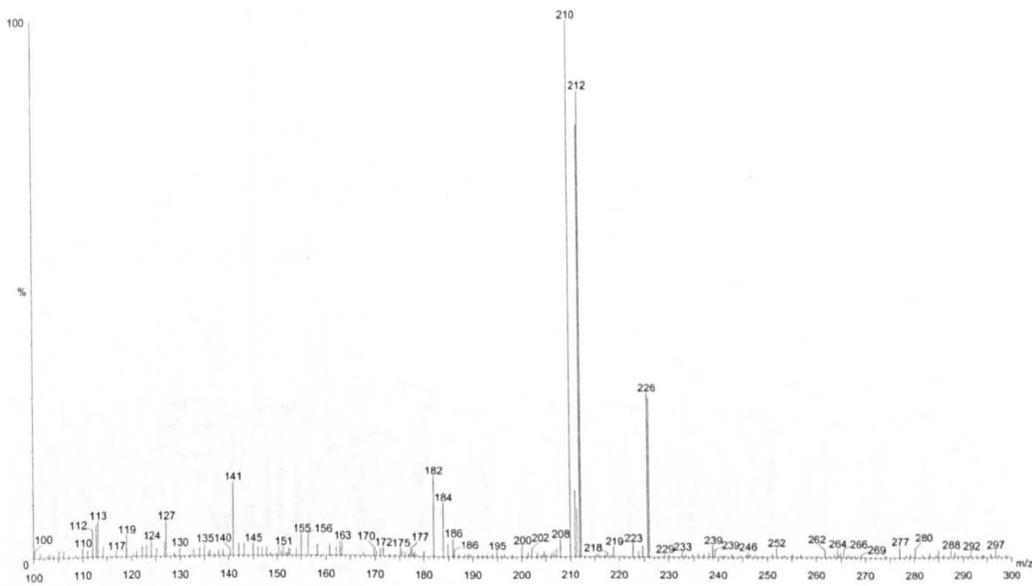
(d2); focused region (600 to 870 m/z) spectrum



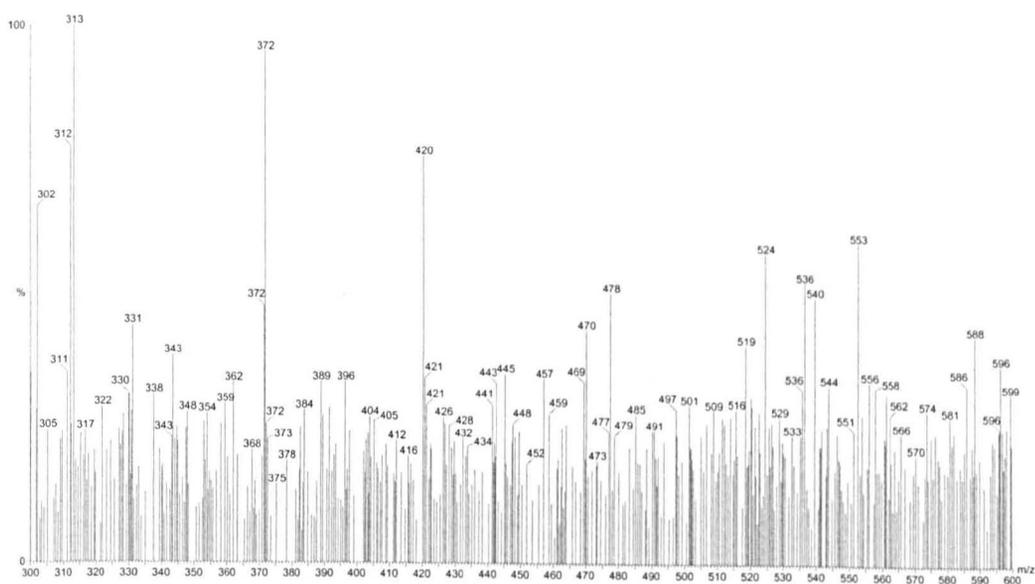
(a3); whole spectrum



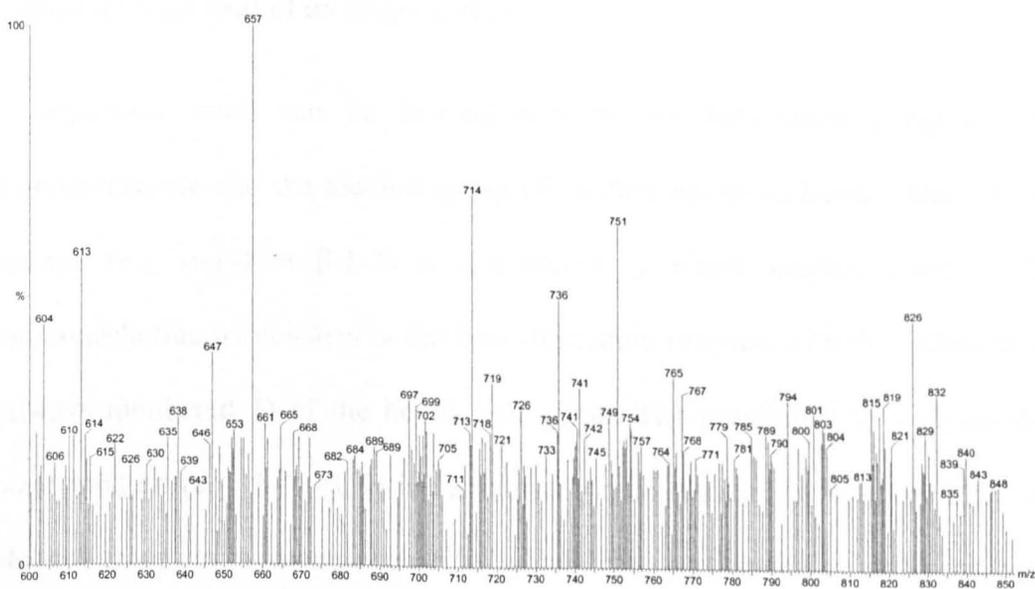
(b3); focused region (100 to 300 m/z) spectrum



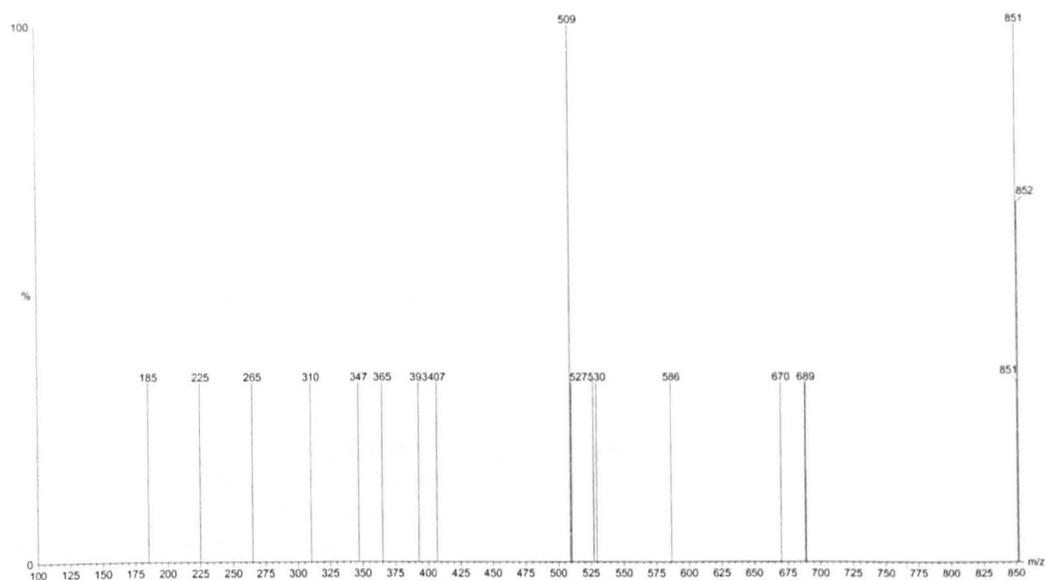
(c3); focused region (300 to 600 m/z) spectrum



(d3); focused region (600 to 870 m/z) spectrum



(a4); MS/MS spectrum of ion 851 m/z.

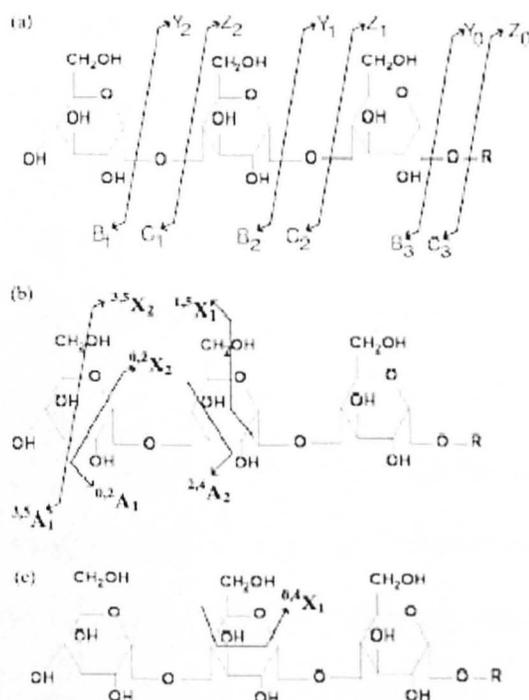


#### 4.2.6 Fragmentation pattern in mass spectrometry analysis of BCE is consistent with that of an oligosaccharide.

A glycosidic bond can be formed between the hemiacetal group of one monosaccharide and the alcohol group of another monosaccharide. The type of linkage (e.g.  $\alpha$ -1-4 or  $\beta$ -1-3) is determined by which alcohol group of the monosaccharide participates in the bond-formation reaction with the carbon atom (always numbered 1) of the hemiacetal group. The possibility of a glycosidic-bond mixture (e.g. 1-4 >> 1-6) is facilitated by the availability of more than one alcohol-group in a monosaccharide which can participate in different bond formations. Due to these various linkage possibilities and anomeric configurations, oligosaccharides have greater complex structural possibilities than either amino acids or nucleotides. However, despite the plethora of possible structures the fragmentation pattern produced upon mass spectrometry of a sample can provide useful structural details about the component. Based on the proposed

fragmentation pattern of oligosaccharides (Domon and Costello, 1988), the oxonium ions ( $B_n$ -type ions) result from the direct cleavage of the glycosidic bonds and contain the non-reducing terminus of the oligosaccharides.  $Y_n$ -type ions result from the transfer of a proton from the non-reducing part of the oligosaccharide to the reducing-terminus. The glycosidic bond cleavage ions on the reducing terminal side of the glycosidic oxygen are assigned  $C_n$  and  $Z_n$ -type ions.

**Figure 4.6. Fragmentation patterns of oligosaccharides during mass spectrometry analysis** (Domon and Costello, 1988).  $Y_n$  and  $B_n$  ion types (A; glycosidic bonds cleavage),  $Z_n$  and  $C_n$  ion types (B; glycosidic bonds cleavage), and  ${}^{n,m}X$  and  ${}^{n,m}A$  ion types (C; cross-ring cleavage). Figure adapted from (Tang *et al.*, 2005).



Due to the facile nature of the glycosidic bond, double or multiple  $B_n Y_n$  ions can occur in the cleavage of one molecule (Domon and Costello, 1988). The nature and the monosaccharide sequence in an oligosaccharide can be deduced from the mass differences (corresponding to the monosaccharide residue masses) between the observed  $B_n$  and  $Y_n$  ions. Cross-ring fragmentation ions ( ${}^{n,m}X$  and  ${}^{n,m}A$ ) result

from the cleavage of carbon-carbon or carbon-oxygen bonds in the monosaccharide rings (Domon and Costello, 1998). The type of linkage in the oligosaccharide can be deduced by observing which cross-ring ions are present and absent (Lemoine *et al.*, 1993; Mulroney *et al.*, 1995). The fragmentation pattern of BCE generates numerous ions as can be observed in the BCE MS spectra (Figure 4.5). Of the observed ions, only those matched in the database search are listed in table 4.1. For underivatized oligosaccharide, attempts to get information on the linkage types have been reported (Yoon and Laine, 1992; Yamagaki *et al.*, 1998). However, the samples analyzed in these reports were pure low molecular weight compounds and were not applied to unknown samples. The fragmentation pattern resulting in  $^{02}A_n$  ion (by the loss of 60 amu from  $M+Na$ ) is indicative of 1-4-linkage where the formation of  $^{02}A_n$  (-60 amu),  $^{03}A_n$  (-90 amu), and  $^{04}A_n$  (-120 amu) are indicative of 1-6-linkage (Reinhold *et al.*, 1995). The fragment ions observed for BCE indicate the presence of a mixture of 1-4 and 1-6-type linkages. The 1-3-linkage type is not indicated by a specific fragment ion formation (Reinhold *et al.*, 1995). Relative-intensity of MS ions provides limited information on the linkage type because of the need for a wide-range of standards that would resemble the unknown sample (Yamagaki *et al.*, 1998). Fragmentation ions of BCE are shown in table 4.2.

**Table 4.2. Fragmentation pattern of BCE as interpreted by Glycofragment tool. Many of the ions observed in BCE show fragmentation pattern consistent with that of oligosaccharide (glycosidic bond and cross-ring cleavage).**

Mass*	Fragmentation ions	Mass**	Mass*	Fragmentation ions	Mass**
38	$^{0.5}A_n + Na^+$		319	$^{1.5}A_n + Na^+$	317
53	$^{1.2}X_n + Na^+, ^{2.3}A_n + Na^+$		328	$Z_n - H_2O + Na^+, B_n - H_2O + Na^+$	328
67	$^{4.5}A_n + Na^+$		335	$^{3.4}A_n + Na^+, ^{0.1}A_n + Na^+$	335
83	$^{1.3}X_n + Na^+, ^{0.4}A_n + Na^+$		346	$Y_n - H_2O + Na^+, C_n - H_2O + Na^+$	346
97	$^{3.5}A_n + Na^+$		347	$Z_n + Na^+, B_n + Na^+$	347
113	$^{1.4}X_n + Na^+, ^{0.3}A_n + Na^+$	113	365	$Y_n + Na^+, C_n + Na^+$	365
127	$^{2.5}A_n + Na^+$	127	377	$^{3.4}X_n + Na^+, ^{0.1}X_n + Na^+$	377
143	$^{0.2}A_n + Na^+$	143	393	$^{1.5}X_n + Na^+$	394
157	$^{1.5}A_n + Na^+$	157	407	$^{0.2}X_n + Na^+, ^{2.4}A_n + Na^+$	407
166	$Z_n - H_2O + Na^+, B_n - H_2O + Na^+$	166	421	$^{3.5}A_n + Na^+$	421
173	$^{0.1}A_n + Na^+$	173	423	$^{2.5}X_n + Na^+$	423
184	$Y_n - H_2O + Na^+, C_n - H_2O + Na^+$	184	437	$^{1.4}X_n + Na^+, ^{0.3}X_n + Na^+$	437
185	$Z_n + Na^+, B_n + Na^+$	185	451	$^{2.5}A_n + Na^+$	451
203	$Y_n + Na^+, C_n + Na^+$	203	453	$^{3.5}X_n + Na^+$	453
215	$^{0.1}X_n + Na^+, ^{3.4}X_n + Na^+$	215	467	$^{2.4}X_n + Na^+, ^{0.2}A_n + Na^+$	467
231	$^{1.5}X_n + Na^+$	231	481	$^{1.5}A_n + Na^+$	481
245	$^{0.2}X_{4n} + Na^+, ^{2.4}A_n + Na^+$	244	490	$Z_n - H_2O + Na^+, B_n - H_2O + Na^+$	490
259	$^{3.5}A_n + Na^+$	259	497	$^{0.1}A_n + Na^+, ^{3.4}A_n + Na^+$	498
261	$^{2.5}X_n + Na^+$	261	508	$Y_n - H_2O + Na^+, C_n - H_2O + Na^+$	508
275	$^{0.3}X_n + Na^+, ^{1.4}X_n + Na^+, ^{0.3}A_n + Na^+$	275	509	$Z_n + Na^+, B_n + Na^+$	509
289	$^{2.5}A_n + Na^+$	289	527	$Y_n + Na^+, C_n + Na^+$	527
291	$^{3.5}X_n + Na^+$	291	539	$^{3.4}X_n + Na^+, ^{0.1}X_n + Na^+$	539
305	$^{2.4}X_n + Na^+, ^{0.2}A_n + Na^+$	307	555	$^{1.5}X_n + Na^+$	555
Mass*	Fragmentation ions	Mass**			
569	$^{0.2}X_n + Na^+, ^{2.4}A_n + Na^+$	569			
583	$^{3.5}A_n + Na^+$	583			
585	$^{2.5}X_n + Na^+$	585			
599	$^{1.4}X_n + Na^+, ^{0.3}X_n + Na^+, ^{0.3}A_n + Na^+, ^{1.4}A_n + Na^+$	599			
613	$^{2.5}A_n + Na^+$	611			
615	$^{3.5}X_n + Na^+$	613			
629	$^{2.4}X_n + Na^+, ^{0.2}A_n + Na^+$	629			
643	$^{1.5}A_n + Na^+$	643			
653	$Z_n - H_2O + Na^+, B_n - H_2O + Na^+$	653			
659	$^{0.1}A_{4n} + Na^+, ^{3.4}A_n + Na^+$	659			
671	$Y_n - H_2O + Na^+, C_n - H_2O + Na^+, Z_n + Na^+, B_n + Na^+$	671			
689	$Y_n + Na^+, C_n + Na^+$	689			
701	$^{0.1}X_n + Na^+$	701			
717	$^{1.5}X_n + Na^+$	717			
731	$^{0.2}X_n + Na^+$	731			
747	$^{2.5}X_n + Na^+$	747			
761	$^{0.3}X_n + Na^+, ^{1.4}A_n + Na^+$	761			
777	$^{3.5}X_n + Na^+$	777			
791	$^{0.4}X_n + Na^+, ^{2.4}X_n + Na^+, ^{1.3}A_n + Na^+$	791			
807	$^{4.5}X_n + Na^+$	809			
821	$^{2.3}X_n + Na^+, ^{1.2}A_n + Na^+, ^{3.4}A_n + Na^+$	823			
835	$^{0.5}X_n + Na^+$	835			

\*: All theoretical ions for a sodiated 5-hexose residue oligosaccharide. \*\*: BCE ions.

#### 4.2.7 Mass spectrometric analysis of permethylated BCE indicates that the active component is a linear oligosaccharide.

In methylated oligosaccharides, the free hydroxyl groups are completely methylated. In this method, the glycosidic linkages are not broken because of their insensitivity to the basic conditions required for alkylation (Ciucanu and Kerek, 1984). Mass spectrometry of methylated oligosaccharide benefits from better sensitivity, and can provide information about the linkage type (Visuex *et al.*, 1999).

In the mass spectrum of a methylated oligosaccharide three ion-products can be observed (Reinhold *et al.*, 1995). They are non-reducing, reducing, and internal ions (noted; Me (Hex)<sub>n</sub>-H, H(Hex)<sub>n</sub>OMe, and H(Hex)<sub>n</sub>-H, respectively). By adding or subtracting the symbol's masses, one can determine the ions masses, where 'n' indicates the number of residues in the ion fragment, H and O are the usual elemental symbols, the masses of Me (methyl group) and methylated-hexose (Hex) are 15 and 204 Da, respectively. In a linear methylated oligosaccharide, the three fragment ions will be present, where the absence of the reducing ion H(Hex)<sub>n</sub>OMe and the observation of H(Hex)-OMe(14 Da) would indicate a branching at the reducing-terminus (Reinhold *et al.*, 1995).

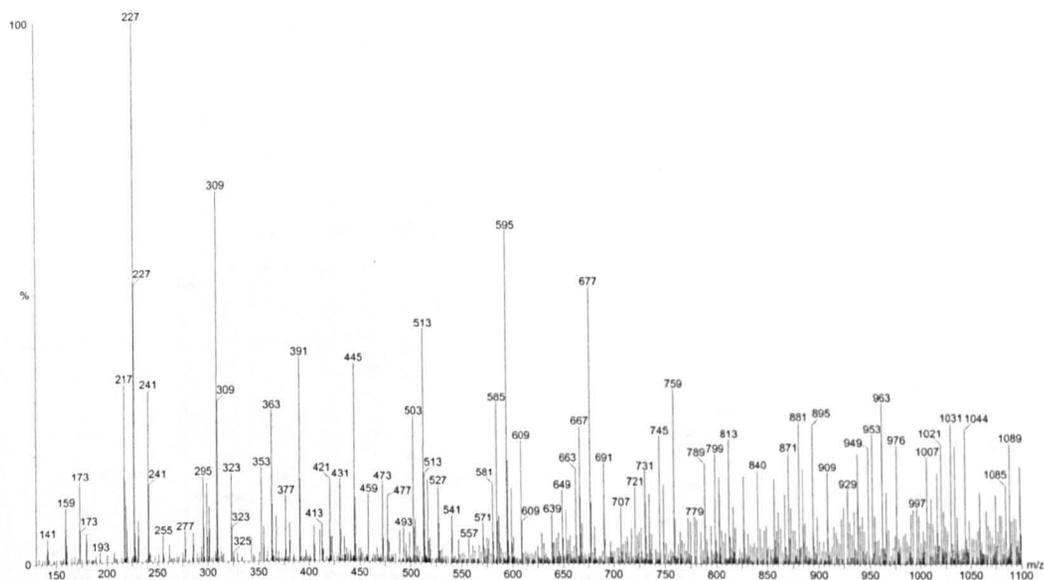
In the MS spectrum of permethylated BCE, the three fragment ions can be observed in their sodiated forms; Me(Hex)<sub>n</sub>-H.Na, H(Hex)<sub>n</sub>OMe.Na, and H(Hex)<sub>n</sub>.Na. Table 4.3 shows ion types observed in the mass spectra of permethylated BCE. Figure 4.7 shows mass spectra of permethylated BCE.

**Table 4.3. Ion types of permethylated BCE.** Masses and ion types observed in BCE that correspond to that observed in MS analysis of permethylated oligosaccharide are listed. Interpretation is based on that recommended by Reinhold (Reinhold *et al.*, 1995)

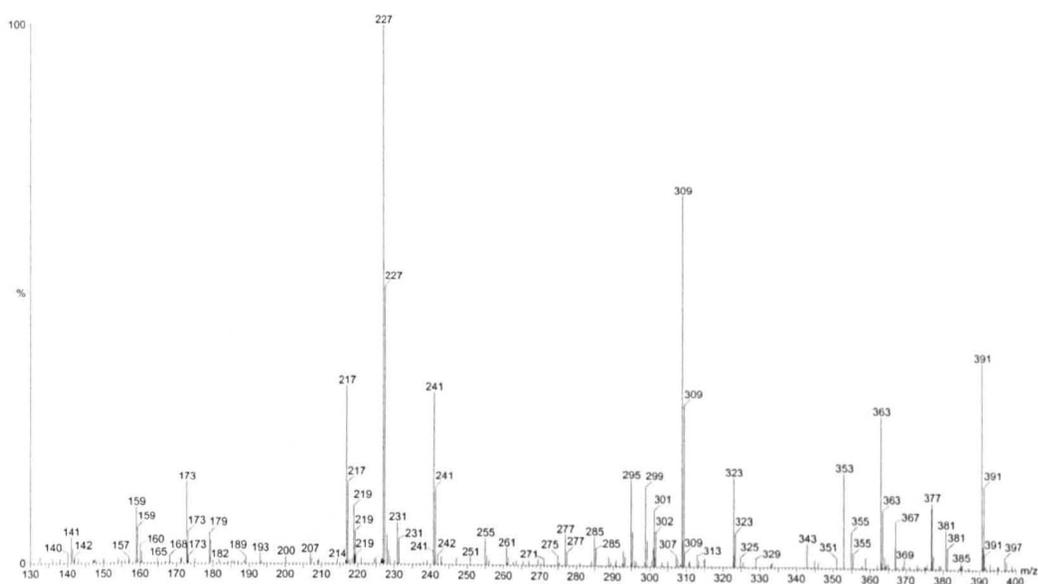
Mass	Ion type	Comment
1075	H(Hex)5OMe	5-hexose residue
1058	Me(Hex)5-H	
1044	H(Hex)5-H	
871, 667, 463, and 259		Losses of 204 from 1075
854, 650, 446, and 242		Losses of 204 from 1058
840, 636, 432, and 228		Losses of 204 from 1044

**Figure 4.7. Mass spectra of permethylated BCE (a-d).** Whole spectrum (a), focused region (b to d)

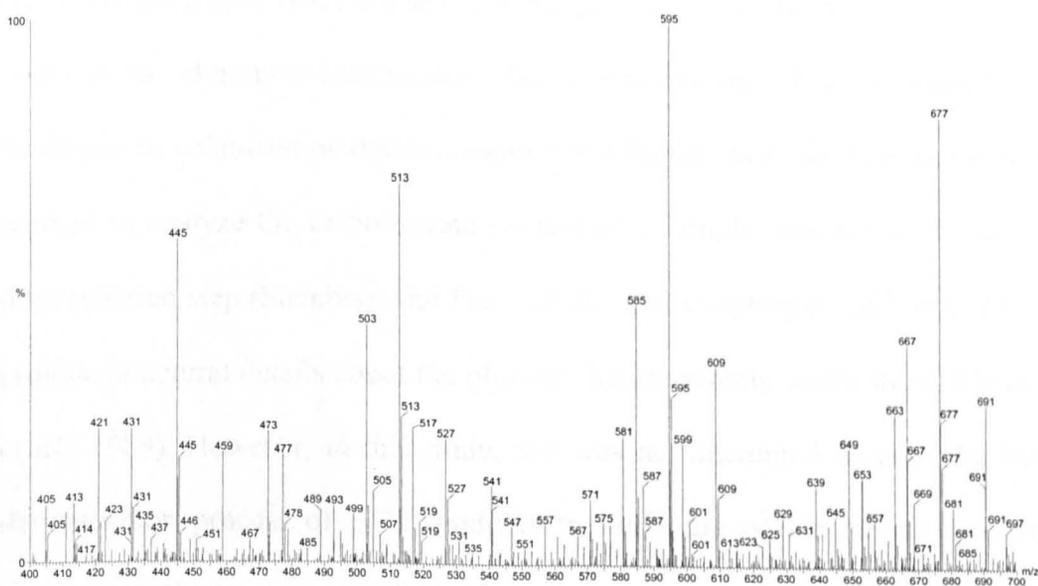
(a); whole spectrum



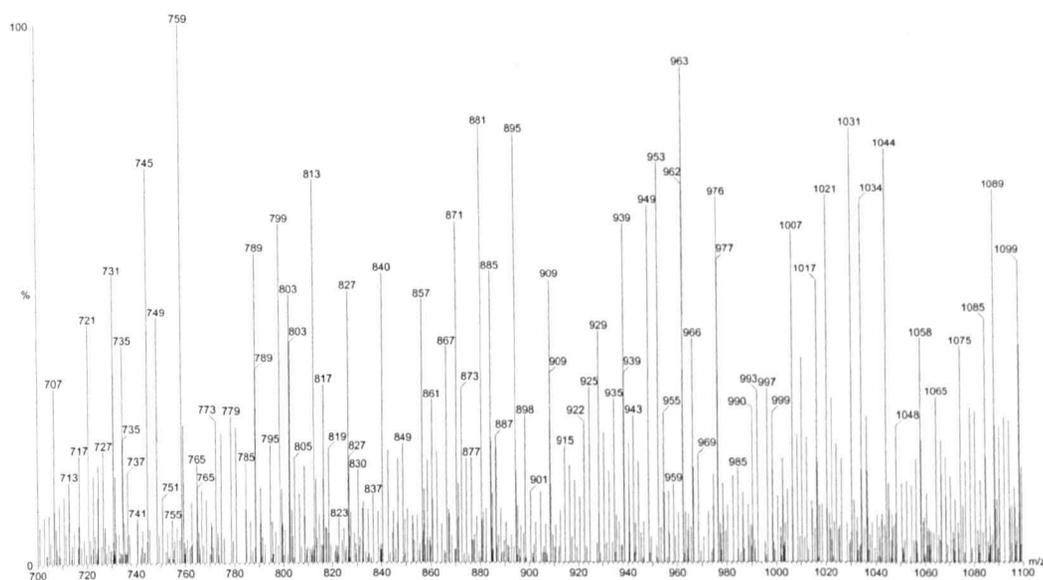
(b); focused region (130 to 400 m/z) spectrum



(c); focused region (400 to 700 m/z) spectrum



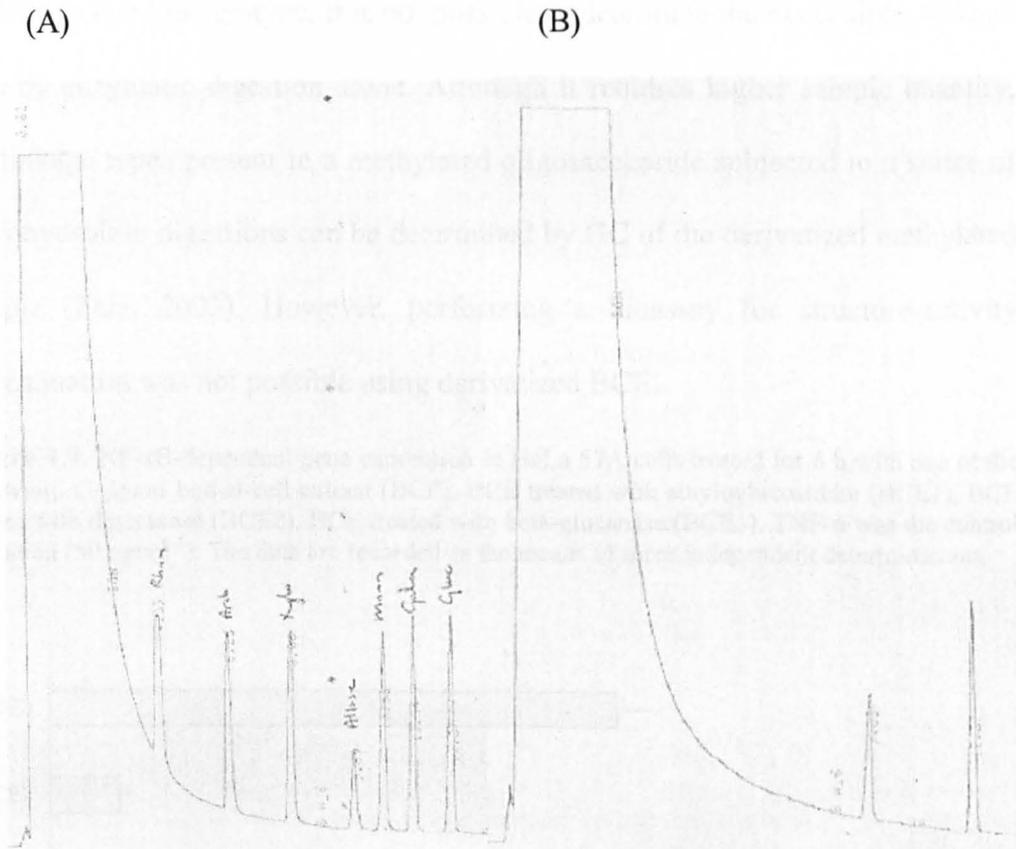
(d); focused region (700 to 1100 m/z) spectrum



#### 4.2.8 Gas-chromatography analysis indicates that glucose is the sole component in fractionated BCE.

Gas chromatography (GC) is a sensitive method for analysing the monosaccharide contents of oligo/poly-saccharides. The derivatization of a monosaccharide facilitates its volatilization that is a requirement for GC analysis. The common GC method to analyze the carbohydrate content of a sample uses the alditol-acetate derivatization step (Steinberg and Fox, 1999). The coupling of GC with MS can provide structural details about the oligosaccharide sample under study (Hansson *et al.*, 1989). However, in this study, this was not attempted because the harsh derivatization process of BCE modifies the structure of the NF- $\kappa$ B-activating component. Hence, corresponding structural-activity to data from GC-MS was not possible. In figure 4.8b, glucose was observed as the only monosaccharide present in purified BCE. The glucose-peak was identified by comparing its retention time (rt) to that of a series of standards (figure 4.8a).

**Figure 4.8. Gas-chromatography (GC) analysis of fractionated BCE.** GC of external monosaccharide standards (A; from left to right: rhamnose (rt; 4.39 min), arabinose (rt; 6.26 min), xylose (rt; 8 min), allose (6.69), mannose (rt; 10.53 min), galactose (rt; 11.34 min) and glucose (rt; 12.37 min). GC of BCE (B) where the internal standard was allose (rt; 9.64 min).

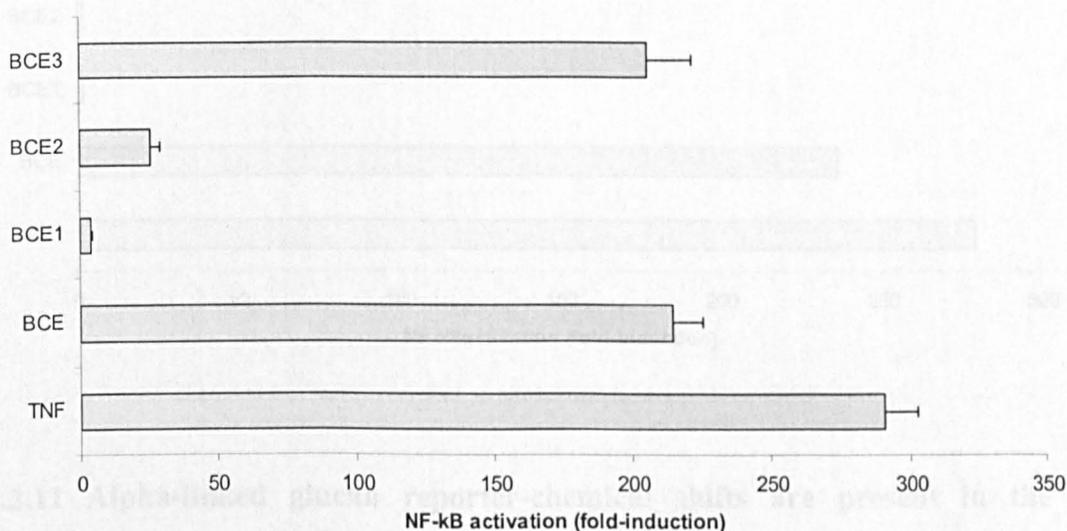


#### **4.2.9 NF- $\kappa$ B activation by BCE is inhibited by treatment of BCE with alpha-glucosidases.**

To investigate whether the NF- $\kappa$ B activating structure in BCE is sensitive to glucosidase-hydrolysing enzymes (GHE), BCE was incubated with the exo-alpha-GHE (Sun and Henson, 1990) amyloglucosidase (has hydrolysing activity on 1-4, 1-3, and 1-6 linkages), endo-alpha-GHE (Wheatley and Moo-Young, 1977) dextranase (acts on endo-1-6 linkage), or endo-beta-GHE (Malet *et al.*, 1993) beta-glucanase; (acts on beta-endo-1-3/1-4 linkages). The NF- $\kappa$ B activation levels by BCE treated with these enzymes are shown in the figure 4.9.

The results of the enzymes treatment indicate that the bioactive-structure in BCE is an alpha-type linked oligosaccharide. However, because these enzymes are not specific to one linkage-type, it is not possible to determine the exact alpha linkage type by enzymatic digestion alone. Although it requires higher sample quantity, the linkage types present in a methylated oligosaccharide subjected to a series of glucohydrolase digestions can be determined by GC of the derivatized methylated sample (Zaia, 2003). However, performing a bioassay for structure-activity determination was not possible using derivatized BCE.

**Figure 4.9.** NF- $\kappa$ B-dependent gene expression in HeLa 57A cells treated for 6 h with one of the following: *C. jejuni* boiled-cell extract (BCE), BCE treated with amyloglucosidase (BCE1), BCE treated with dextranase (BCE2), BCE treated with beta-glucanase (BCE3). TNF- $\alpha$  was the control activation (50 ng/ml<sup>-1</sup>). The data are recorded as the means of three independent determinations.

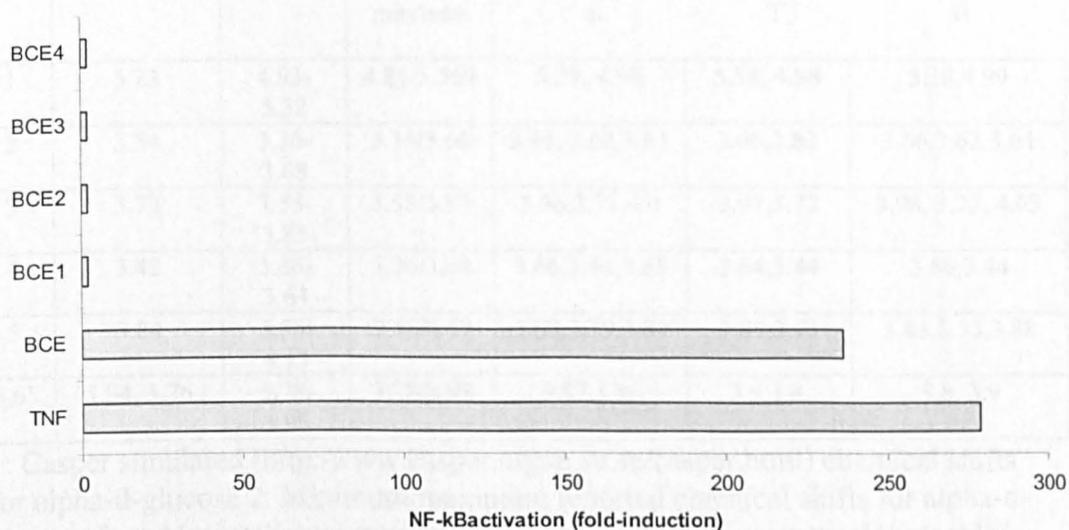


#### 4.2.10 NF- $\kappa$ B is not activated by BCE applied to the aminopropyl column or by permethylated BCE.

Aminopropyl columns can be used for separation of polar compounds (Hennion, 1999). However, in this study, NF- $\kappa$ B was not activated by BCE once applied to this column (Figure 4.10). The active component in BCE could not be detected in the pass-through, wash, or elute of this column. This could be explained by the

glycosylamine formation between the reducing-terminus of the oligosaccharide and the amino group of the column's stationary phase (Robinson *et al.*, 2007). Permethylation of BCE replaces the hydroxyl groups of the oligosaccharide with methyl groups. This makes BCE less polar and can be retained by a reverse-phase column under hydrophobic conditions. NF- $\kappa$ B was not activated by permethylated BCE (Figure 4.10) implies the importance of its polarity for the activation.

**Figure 4.10.** NF- $\kappa$ B-dependent gene expression in HeLa 57A cells treated for 6 h with one of the following: *C. jejuni* boiled-cell extract (BCE), the pass-through of BCE applied to aminopropyl column (BCE1), the wash of BCE applied to aminopropyl column (BCE2), the elute of BCE applied to aminopropyl column (BCE3), permethylated BCE (BCE4). TNF- $\alpha$  was the control activation ( $50 \text{ ng/ml}^{-1}$ ).



#### 4.2.11 Alpha-linked glucan reporter-chemical shifts are present in the spectrum of BCE analysed by nuclear magnetic resonance (NMR)

NMR is a non-destructive technique that has been used for carbohydrate structural determination (Duus *et al.*, 2000). Data from  $^1\text{H}$  NMR, which show the anomeric proton resonances (chemical shift range; 4.40 to 5.5 ppm) and the ring resonances (chemical shifts range; 3 to 4.2 ppm), can provide useful structural details when combined with data from other techniques such as MS, GC, and GC-MS. Requiring purified samples, the application of NMR to BCE in this study was

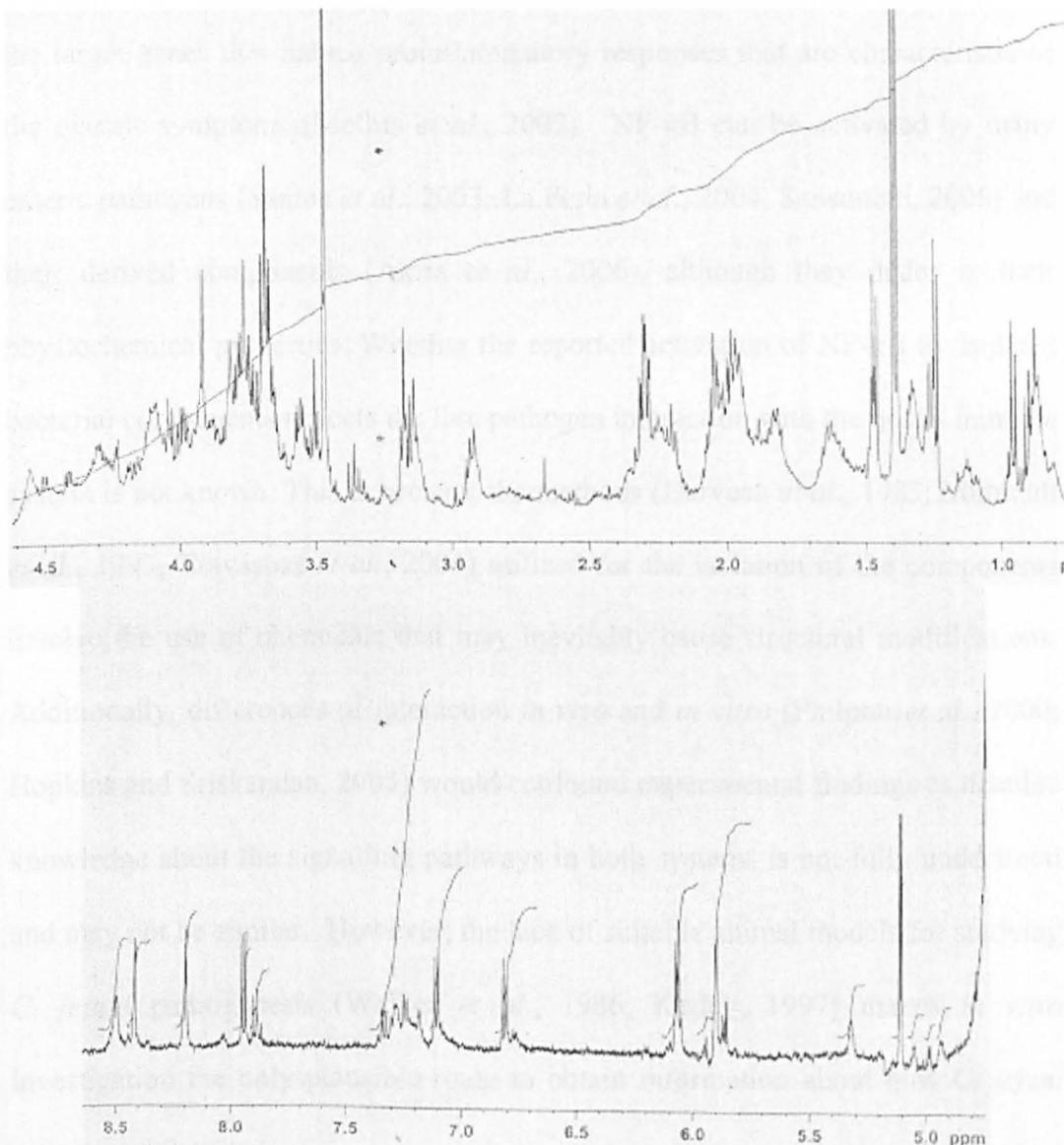
limited to  $^1\text{H}$  NMR because of the quantity of sample needed. Additionally, the highly polar nature of carbohydrate means the purification steps before NMR is of limited performance. This would result in polar but not active component being co-eluted during the purification. The  $^1\text{H}$  NMR spectrum of purified BCE is shown in figure 4.11. Interpretation of the chemical shifts (Table 4.4) was performed using online CASPER computer software.

**Table 4.4.** Interpretation of BCE NMR chemical shifts. The online CASPER tool was used to interpret the NMR data. Chemical shifts of alpha-glucose simulated by Casper software, BCE, found in database and reported in literature are listed.

H	Casper <sup>1</sup> ppm	BCE	Database <sup>2</sup> min/max	Literature <sup>3</sup>		
				L	T	B
1	5.23	4.93- 5.32	4.81/5.569	5.39, 4.98	5.38, 4.98	5.38, 4.99
2	3.54	3.36- 3.68	3.36/3.68	3.66, 3.63, 3.61	3.66, 3.62	3.66, 3.62, 3.64
3	3.72	3.58- 3.85	3.58/3.85	3.96, 3.71, 4.0	3.97, 3.72	3.98, 3.72, 4.03
4	3.42	3.46- 3.64	3.36/3.64	3.66, 3.44, 3.65	3.64, 3.44	3.66, 3.44
5	3.84	3.56- 4.13	3.56/4.13	3.88, 3.79, 3.89	3.85, 3.73	3.85, 3.73, 3.88
6,6'	3.84, 3.76	3.78- 3.98	3.78/3.98	3.87, 3.86	3.9, 3.8,	3.8, 3.9

1: Casper simulated (<http://www.casper.organ.su.se/casper.html>) chemical shifts for alpha-d-glucose 2: Minimum/maximum reported chemical shifts for alpha-d-glucose found in database: <http://www.dkfz.de/spec/glycosciences.de/sweetdb/>  
3- Reported chemical shifts for linear (L), terminal (T), and branched (B) alpha-glucan (Zhang *et al.*, 1991; Dinadayala *et al.*, 2004; Bittencourt *et al.*, 2006).

**Figure 4.11.**  $^1\text{H}$  proton NMR spectrum of BCE. The combined active fractions from the 1<sup>st</sup> NP-HPLC purification of BCE (from wild-type *C. jejuni*) were analyzed by NMR. The chemical shift of acetone (2.25 ppm) was used as internal reference. The other chemical shifts found in 1 to 3 ppm range might be that of co-eluting components. BCE analysis show chemical shifts that fall in the range of anomeric chemical shifts (4.40-5.50 ppm) and the ring chemical shifts (3- 4.2 ppm)



#### 4.3 Discussion

*Campylobacter* is one of the most reported causative agents of foodborne diseases worldwide where most of *Campylobacteriosis* cases are largely caused by *C. jejuni* in developed countries with active surveillance. During infection, *C. jejuni* interacts with the host by poorly understood mechanisms (Ketley, 1997). These

interactions ultimately result in the up-regulation of the inflammatory- response genes that are generally controlled by the transcription factor NF- $\kappa$ B. Upon cellular stimulation by live *C. jejuni* or its extracted component(s), NF- $\kappa$ B translocates from the cytoplasm to the nucleus where it binds to DNA turning on the target genes that induce proinflammatory responses that are characteristic of the disease symptoms (Mellits *et al.*, 2002). NF- $\kappa$ B can be activated by many enteric pathogens (Santos *et al.*, 2003; La Ferla *et al.*, 2004; Sansonetti, 2006) and their derived components (Akira *et al.*, 2006), although they differ in their physiochemical properties. Whether the reported activation of NF- $\kappa$ B by isolated bacterial components reflects the live pathogen interaction with the host's immune system is not known. This is because the methods (Darveau *et al.*, 1983; Muhlradt *et al.*, 1997; Travasoss *et al.*, 2004) utilized for the isolation of the components involve the use of chemicals that may inevitably cause structural modifications. Additionally, differences of interaction *in vivo* and *in vitro* (Philpott *et al.*, 2000; Hopkins and Sriskandan, 2005) would confound experimental findings as detailed knowledge about the signalling pathways in both systems is not fully understood and may not be similar. However, the lack of suitable animal models for studying *C. jejuni* pathogenesis (Walker *et al.*, 1986; Ketley, 1997) makes *in vitro* investigation the only plausible route to obtain information about how *C. jejuni* may cause disease.

In a dose-dependent manner, BCE from *C. jejuni* has been reported to activate NF- $\kappa$ B maximally 6 hours after stimulation of the HeLa 57A cell line (Mellits *et al.*, 2002). Given the insensitivity of BCE to boiling and proteinase K digestion, one may rule out a proteinaceous structure as the activating component in BCE. Additionally, a lipid-moiety containing structure does not participate in the

activation since BCE is also resistant to lipopeptide lipase treatment. Unlike BCE, cell-filtrate did not activate NF- $\kappa$ B indicating the bioactive structure is liberated or modified during BCE preparation that involves a boiling step (Mellits *et al.*, 2002).

The cell wall of *C. jejuni*, from which BCE components derive, like other Gram-negative bacteria, mainly contains LPS, PGN, membrane proteins, and lipoproteins. The lipid components of LPS (Dixon and Darveau, 2005) and lipoproteins (Okusawa *et al.*, 2004) are the most important for NF- $\kappa$ B activation by these two structures. Peptidoglycan NF- $\kappa$ B activation is induced by the beta 1-4 linked NAG-NAM moiety of PGN (Chamaillard *et al.*, 2003). The bioassays used to evaluate NF- $\kappa$ B activation by these structures have been performed in cell lines that express cellular receptors needed for the signalling pathways leading to the activation.

The cell line HeLa 57A was used in this study to determine NF- $\kappa$ B activation by BCE during different purification steps. This cell line does not express TLR2 (lipoprotein and PGN receptor), MD2 (necessary for LPS signalling pathway), and NOD2 (recognizes the minimal activating moiety of PGN). NOD1, which also recognizes PGN, is expressed in this cell line. However, the stimulatory component of NOD1 has been reported to be found in the culture supernatant but not in cell extracts from different bacteria (Hasegawa *et al.*, 2006). Culture supernatant and LOS of *C. jejuni* did not activate NF- $\kappa$ B (Mellits *et al.*, 2002).

BCE prepared from *C. jejuni* LOS and capsule polysaccharide mutants or BCE subjected to treatment that precipitates lipid-A (if any) are able activate NF- $\kappa$ B. Generally capsular polysaccharide is thought to be a weak immune-stimulant

(Henderson *et al.*, 1996). Given the information above, the active component in BCE are not LOS, PGN, CPS, or lipo-proteins.

As supported by evidence from structural analysis (MS, GC, and NMR) and inactivation by alpha-glucohydrolases, NF- $\kappa$ B is activated by a short-alpha linked oligosaccharide in BCE. Activation of NF- $\kappa$ B by similar structures has not been reported for *Campylobacter* or any other Gram-negative bacteria.

Recently, NF- $\kappa$ B activation and production of pro-inflammatory cytokines by alpha-linked glucan has been reported for other organisms such as *Pseudallescheria boydii* (Bittencourt *et al.*, 2006), *Tinospora cordifolia* (Nair *et al.*, 2006) and *Streptococcus mutans* (Choi *et al.*, 2005; Okamoto *et al.*, 2007). However, macrophages have been used in these reports. Many receptors are expressed by macrophages (Taylor *et al.*, 2005) that may lead to NF- $\kappa$ B activation in a different manner than possible in the HELA 57A cell line.

A cell-surface expressed alpha 1-4 linked glucan has been recently detected in *C. jejuni* (Papp-Szabo *et al.*, 2005). In the *C. jejuni* CPS mutant (*kpsM*), sugar linkage analysis showed that this glucan is made up of 25-glucose residues that have infrequent branching points. Glucose was found as the sole sugar present in the identified glucan. <sup>1</sup>H NMR spectra showed chemical shifts of 5.42, 3.64, 3.98, and 3.66 for H1, H2, H3, H4 resonances, respectively. In *C. jejuni*, the genetic basis for alpha-glucan synthesis has not been determined (Papp-Szabo *et al.*, 2005). Substituted with phosphoglycerol, phosphoethanolamine, and succinyl residues, osmoregulated periplasmic glucans (OPGs) are synthesized by members of the *Enterobacteriaceae* (Bohin, 2000). Made of glucose only, linear and branched substituted OPGs of 5-13 residues have been reported in Gram-negative bacteria (Page *et al.*, 2001). The role of OPGs mostly  $\beta$ -linked glucan in *C. jejuni*

pathogenesis is yet to be determined. OPGs can exist as mixture of substituted/unsubstituted  $\alpha/\beta$ -glucans and are thought to be important for interaction with the host's cells in addition to their osmoprotectant function. In *E. coli*, synthesis of OPGs is regulated by the mdoGH operon (Bohin, 2000).

Different types of chromatography columns have been used for fractionating carbohydrate containing samples (Davies and Hounsell, 1996). However, there is no single system that is capable of purifying all possible carbohydrate isomers utilizing volatile buffers. The performance of size-exclusion column (SEC) chromatography is limited because separation of the carbohydrate mixture is not based on the possible linkage types (Deery *et al.*, 2001). Since carbohydrates are of polar nature, separation using a reverse-phase chromatographic method is limited to only removing hydrophobic contaminants. Unless non-volatile ion-pairing agents are used, carbohydrate will elute in the void volume when applied to a RP-HPLC column (Davies and Hounsell, 1996).

Oligosaccharides lack UV absorbing groups such as nitriles and double bonds making their detection only available below 210nm. At such low wavelength, NH and C=O UV absorbance is poor, and solvents usually cause observed drifting in baseline absorbance (Paulus and Klockow, 1996). Detection of carbohydrate during an HPLC separation using reflective index (RI) values is limited because the sensitivity of the detector to the mobile-phase composition changes (Davies and Hounsell, 1996).

Derivatization and chromophoric labelling of oligosaccharides will increase their detection during HPLC purification. However, in this study, chemical labelling of BCE interfered with the bioassay for NF- $\kappa$ B activation and was avoided. This made identifying the active fractions only possible after performing the cell

culture based bioassays. Determination of the proposed structure of the NF- $\kappa$ B activating component was only possible by combining information obtained from different experiments. The use of MS, GC, enzymatic inactivation, and NMR for the structural characterization of BCE were limited by the inability of MS to differentiate carbohydrate isomers, the hydrolysing nature of GC derivatization step, the non-one-linkage type specific action of glucohydrolases, and the sample quantity and purity requirements for NMR analysis, respectively.

**CHAPTER 5 The role of TLR and NOD receptors in NF- $\kappa$ B  
activation by *Campylobacter jejuni***

## CHAPTER 5

### 5.1 Introduction

The host's immune responses during the course of foodborne infection are mainly regulated by NF- $\kappa$ B (Gewirtz *et al.*, 2002). Depending on the immune system status of the patient, campylobacteriosis can be a self-limiting infection, or accompanied by systematic and chronic complications (Coker *et al.*, 2002). In intestinal epithelial cells, where the first interaction with the host's immune system takes place, the signalling cascade by pathogens or their products proceeds generally via an I $\kappa$ B-dependent pathway resulting in NF- $\kappa$ B activation and up-regulation of the inflammatory response genes (Schottelius and Baldwin, 1999; Jobin *et al.*, 2000). This pathway utilizes trans-membrane receptors (e.g. TLRs) and/or cytoplasmic receptors (e.g. NODs) via adaptor molecules (e.g. MYD88, IRAKs, and MAL for TLRs and RIP2 for NODs) leading eventually to the translocation of NF- $\kappa$ B from the cytoplasm to the nucleus where it binds target gene DNA sequences (Akira and Takada, 2004; Strober *et al.*, 2005). Signalling via other pathways such as MAPK has been reported to mediate NF- $\kappa$ B regulation of the immune responses (Zhang and Dong, 2005).

How these receptors/adaptor molecules contribute to NF- $\kappa$ B activation by *C. jejuni* would be of significant help in understanding the pathogenesis of *C. jejuni*. In this chapter, the roles of these molecules in NF- $\kappa$ B activation are investigated by means of adding (transfection of wild type genes), or masking (transfection of dominant negative alleles) of the receptor/adaptor in question.

## 5.2 Results

### 5.2.1 *Campylobacter jejuni* activates NF- $\kappa$ B through TLR/MYD88

#### independent and dependent pathways

##### 5.2.1.1 TLR/MYD88 independent pathway

Lipopolysaccharide, lipoproteins/peptidoglycan, flagella, and CpG DNA are major PAMPs that are recognized by TLR4, TLR2, TLR5, and TLR9. The expression of TLRs in tissue culture cell lines has been reported to vary among laboratories (Nishimura and Naito, 2005). Therefore, reverse-transcriptase PCR was used to verify the expression of TLR1-10 mRNAs in HeLa 57A, HEK-293, HCA-7, and Caco-2, the cell lines used in this study (Table 5.1).

Table 5.1. Expression pattern\* of TLRs and NODs receptors in cell lines used in the study.

Product	Caco-2	HeLa 57A	HCA-7	HEK293	Ligands
TLR1	-	-	-	-	triacylated lipoproteins
TLR2	-	-	-	-	lipoproteins
TLR3	-	+	+	-	dsRNA
TLR4	-	+	-	-	lipopolysaccharide
TLR5	-	+	+	+	flagellin
TLR6	-	-	-	-	diacylated lipoprotein
TLR7	-	+	-	-	ssRNA
TLR8	-	-	-	-	ssRNA
TLR9	-	-	-	-	unmethylated CpG DNA
TLR10	-	-	-	-	
NOD1	+	+	+	+	iE-DAP
NOD2	-	-	+	-	Muramyl dipeptide
TOLLIP	+	+	+	-	Inhibitor of TLRs

\*: Data provided courtesy of the technical assistance of Mrs. E. Dillon.

The HeLa 57A cell line that is stably transfected for the reporter plasmids and produces robust responses to NF- $\kappa$ B activators (Rodriguez *et al.*, 1999), was shown to express mRNAs to TLRs 3, 4, 5 and 7, but does not express MD-2, an accessory molecule necessary for TLR4 signalling (Re and Strominger, 2002; Nishimura and Naito, 2005). However, Caco-2 cells cultured under these conditions produced no detectable TLR expression and the alternative colonic cell

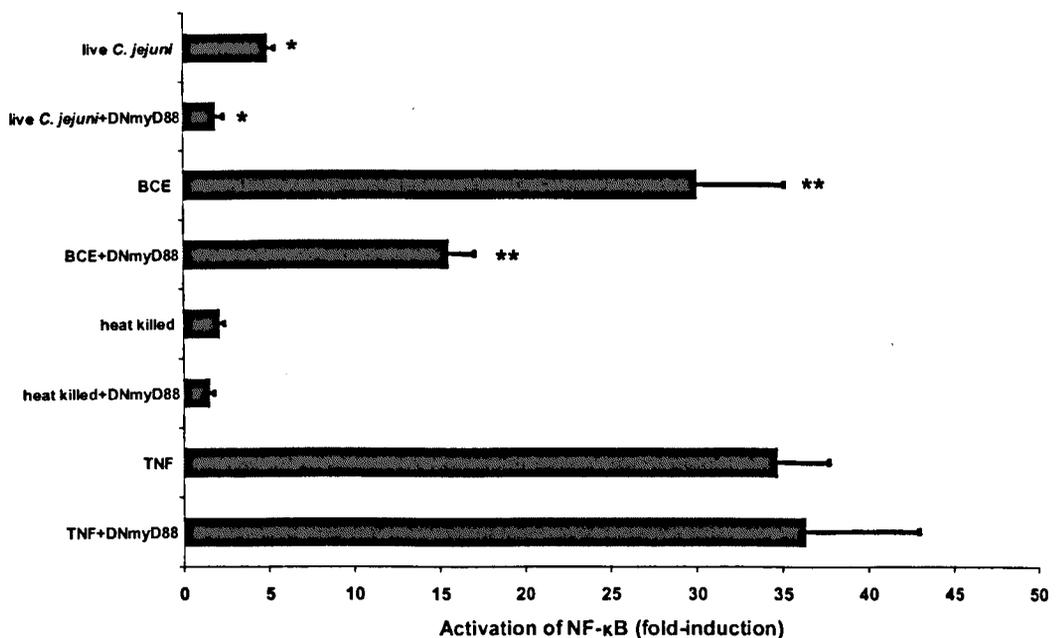
line HCA-7 exhibited only TLR3 expression. HEK293 cells exhibited mRNA expression of TLR5 and no other TLR. The influence of the TLR inhibitor protein TOLLIP was considered as it has been reported to suppress PAMP recognition. TOLLIP was found to be expressed in Caco-2, HeLa 57A and HCA-7 cells (Table 5.1), and would therefore have to overcome if these cells were to respond to *C. jejuni* via their TLRs. Overall, the cell lines did not express a common TLR that could account for recognition of a discrete *C. jejuni* pro-inflammatory product or known NF- $\kappa$ B activator. It is possible that *C. jejuni* products may be recognized by alternative yet to be identified TLRs depending on the cell context but the possibility exists that *C. jejuni* can activate NF- $\kappa$ B independent of TLRs.

The data presented in chapter 3 indicated that *C. jejuni* flagellin present in BCE does not activate NF- $\kappa$ B through TLR5 present in either HEK293 or HELA 57A cells since HELA 57A cells were not responsive to purified *C. jejuni* flagellin but that they could respond to flagellin of *E. coli* (Zhou *et al.*, 2003). These data are consistent with recent reports that *C. jejuni* flagellin along with other  $\alpha$  and  $\epsilon$  proteobacterial flagellins can evade innate immune recognition by TLR5 (Watson, & Galan, 2005; Andersen-Nissen *et al.*, 2005). The negative response to flagellin is also consistent with the observation that the majority of the *C. jejuni* BCE activity is proteinase K insensitive.

We further disrupted the classical TLR responsive pathway by the expression of a dominant negative form of the adaptor molecule MYD88. HEK293 cells were co-transfected with the reporter plasmids and either a plasmid bearing a dominant negative form of MYD88 or empty vector as a control. The transfected cells were treated with live *C. jejuni* 11168 (m.o.i. 100) for 6 h or alternatively heat killed *C. jejuni* or BCE for 6 h (Figure 5.1). A significant difference was detected between

the dominant negative MYD88-expressing cells and the control cells for treatments with live *C. jejuni* ( $P=0.019$ ) and BCE ( $P=0.035$ ) that was not evident in the TNF- $\alpha$  control, which activates NF- $\kappa$ B independent of MYD88. However, we note that HEK293 cells retain their ability to respond to *C. jejuni* and its products, and are able activate NF- $\kappa$ B in the presence of transfected dominant negative MYD88.

**Figure 5.1. Induction-fold of NF- $\kappa$ B in HEK293-DNMYD88 cells by *C. jejuni*.** HEK293 cells were transfected with reporter plasmids DNA ( $\kappa$ B-luc and RSV-lacZ) and DN-MYD88. Transfected cells were treated with either live (for 16 h) or heat-killed *C. jejuni* 11168 or *C. jejuni* boiled cell extract (BCE) for 6 h. Significant differences between the DN-MYD88-expressing cells and the control cells for live *C. jejuni*\* ( $P=0.019$ ) and BCE\*\* ( $P=0.035$ ) corresponds with the asterisks as indicated. The data are recorded as the means of 3 independent determinations.



### 5.2.1.2 Activation of NF- $\kappa$ B by *C. jejuni* and BCE is augmented through TLR2

We further sought to establish if *C. jejuni* 11168 and its heat-stable products could activate NF- $\kappa$ B through TLRs 2 and 4 if present. We therefore specifically expressed these receptors in HEK293 cells that are naive for TLRs 2 and 4 and do

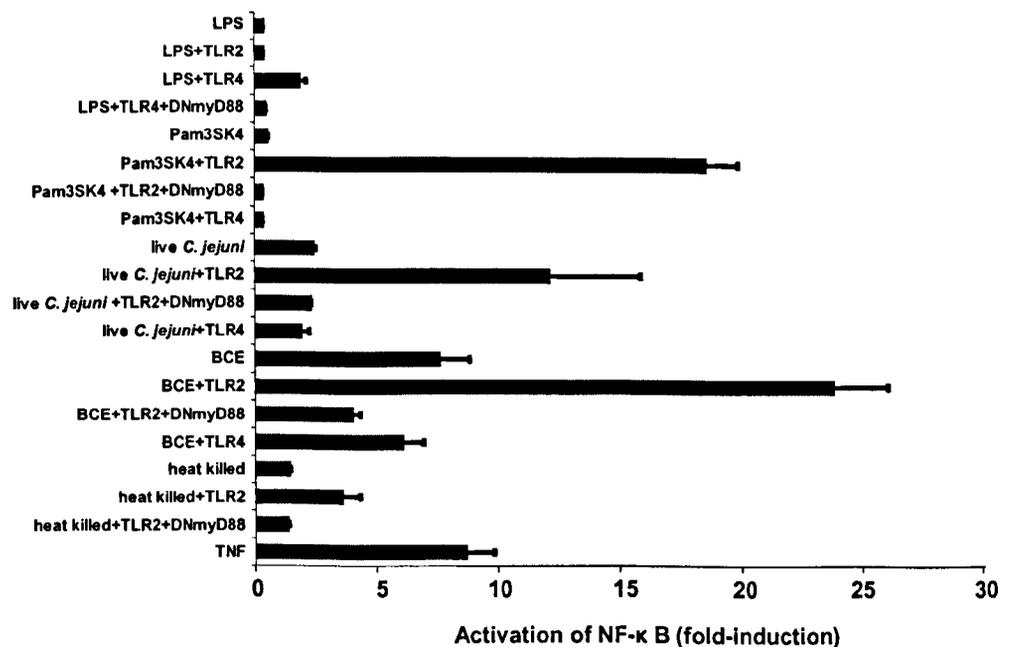
not respond to the microbial ligands associated with these receptors. Co-transfection of HEK293 cells with plasmid DNAs directing the expression of human TLRs and the accessory proteins MD-2 and CD-14 confer the ability to respond to previously unrecognised microbial products. Figure 5.2a shows that transient expression of functional TLR2 results in a nine-fold increase in the NF- $\kappa$ B-dependent reporter gene activity in the presence of live *C. jejuni* (m.o.i. 100) compared to HEK293 cells transfected with MD-2, CD14 and empty vector control plasmid DNAs. Heat-killed *C. jejuni* and *C. jejuni* BCE also exhibited similar trends with respective four- and three-fold increases in NF- $\kappa$ B-dependent reporter gene activity when transfected with the TLR2 expression plasmid. By way of controls, HEK293 cells were transfected with TLR2 and shown to gain the ability to respond to the synthetic TLR2 ligand Pam3CSK4, but not pure *E. coli* LPS that is dependent on activation of NF- $\kappa$ B through TLR4. Conversely expression of TLR4 in HEK293 cells results in sensitivity to *E. coli* LPS but does not result in sensitivity to Pam3CSK4. However, TLR4 expression in HEK293 cells did not result in any significant increase in their NF- $\kappa$ B-dependent response to *C. jejuni* or BCE (Figure 5.2a). To demonstrate the increase in activity was dependent on the TLR2/MYD88 pathway, HEK293 cells were co-transfected with TLR2-CD14 and a dominant negative MYD88-expressing plasmid. Expression of dominant negative MYD88 prevented NF- $\kappa$ B induction via Pam3CSK4 with TLR2 and reduced the response to live *C. jejuni*, heat-killed *C. jejuni* and BCE to either similar or lower levels than those observed in the absence of TLR2 (Figure 5.2a).

TLR2-CD14 or TLR4-MD-2 were further introduced into CACO-2 cells to examine if these colonic epithelial cells could also demonstrate an enhanced NF-

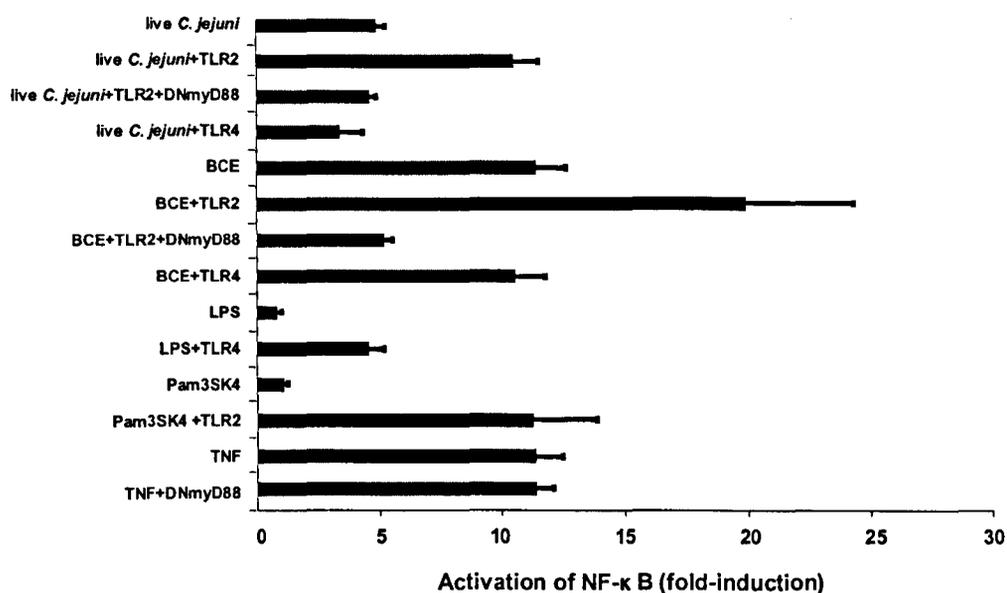
$\kappa$ B response to *C. jejuni* via TLRs. Figure 5.2b shows CACO-2 cells transfected with TLR2 show a significant increase in their response to *C. jejuni* (m.o.i. 100), and that the increased response may be abrogated by co-expression of dominant negative MYD88. However, TLR4 expression in CACO-2 cells did not enhance their response to live *C. jejuni* or BCE to complete a similar trend to that observed with HEK293 cells.

**Figure 5.2. Induction-fold of NF- $\kappa$ B in HEK293/Caco2-TLR2/4 cells by *C. jejuni*.** HEK293 (a) and Caco-2 (b) cells were transfected with the reporter plasmids DNA, plasmid DNA of TLR4 and MD-2 or TLR2 and CD14, and DN-MYD88 as indicated. Transfected cells were treated for 6 h with one of the following: ultrapure *E. coli* LPS (TLR4 agonist; at 10  $\mu$ g/ml<sup>-1</sup>); PAM3SK4 (TLR2 agonist; at 100 ng/ml<sup>-1</sup>); heat-killed *C. jejuni*, or *C. jejuni* boiled cell extract (BCE). Incubation with live *C. jejuni* was at m.o.i of 100 for 16 h. The activation control TNF- $\alpha$  was added at 50 ng/ml<sup>-1</sup>. The data are recorded as the means of 3 independent determinations.

(a): HEK293 cells



(b): Caco-2 cells

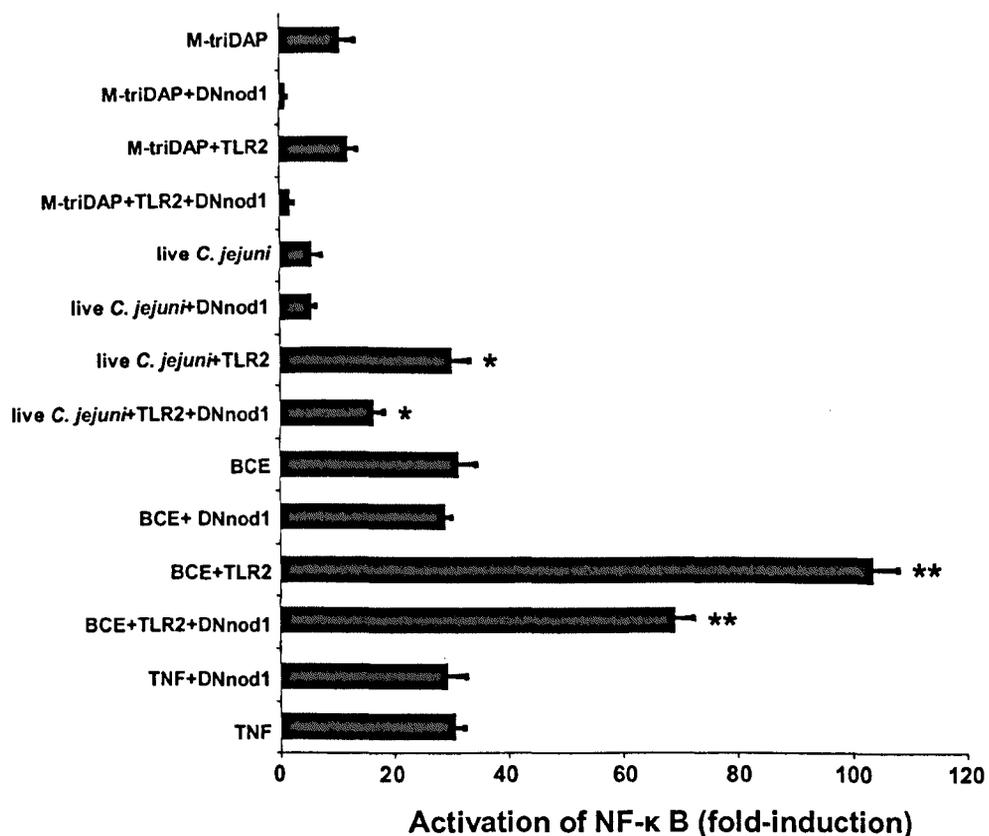


### 5.2.2 *Campylobacter jejuni* exhibits synergy between TLR2 and NOD1 to activate NF-κB

Consistent with reports that NOD1 is expressed in many tissues (Girardin and Philpott, 2004), we found NOD1 expression in all the cell lines examined including intestinal epithelial cells. NOD1 has been shown to respond to a Gram-negative peptidoglycan product M-triDAP, and is a candidate mechanism by which *C. jejuni* could activate NF-κB in the absence of TLR/MYD88 pathways. HEK293 cells were transfected with a plasmid expressing a dominant negative form of NOD1 and NF-κB was induced in these cells with either live *C. jejuni* (m.o.i. 100) or BCE. Figure 5.3 shows that no significant falls in NF-κB activity were recorded in response to either live *C. jejuni* or BCE upon expression of dominant negative NOD1. However, when dominant negative NOD1 was

expressed with the TLR2-CD14 constructs required to sensitise HEK293 cells to TLR2 ligands and enhance the activation of NF- $\kappa$ B, the comparative activation levels exhibited significant reductions for live *C. jejuni* ( $P=0.047$ ) and BCE ( $P=0.021$ ). It appears that although *C. jejuni* does not appear to require NOD1 within the 6 h period of this experiment to activate NF- $\kappa$ B, the presence of functional NOD1 appears to act synergistically to enhance the NF- $\kappa$ B response to BCE through the TLR2/MYD88 pathway.

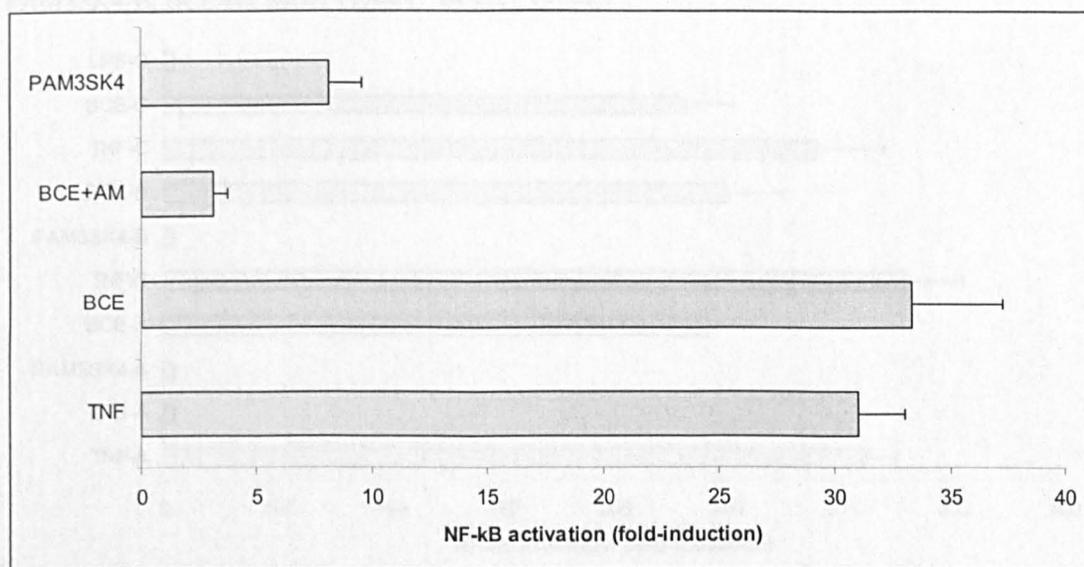
**Figure 5.3. Synergized induction of NF- $\kappa$ B in HEK293-TLR2-NOD1 cells by *C. jejuni*.** HEK293 cells were transfected with the reporter plasmid DNA, TLR2-CD14 and with NOD1 or DN-NOD1 as indicated. Transfected cells were treated for 6 h with one of the following; NOD1 agonist M-triDAP (10  $\mu$ g/ml<sup>-1</sup>), live *C. jejuni* 11168; *C. jejuni* boiled-cell extract (BCE). The activation control was TNF- $\alpha$  (50 ng/ml<sup>-1</sup>). Significant differences between TLR2-expressing cells and those co-expressing DN-NOD1 are indicated with asterisks for live *C. jejuni*\* ( $P=0.047$ ) and BCE\*\* ( $P=0.021$ ). The data are recorded as the mean of 3 independent determinations.



### 5.2.3 TLR2 only senses the oligosaccharide present in BCE

Since BCE activates NF- $\kappa$ B independent of TLR2, but the levels of activation increase upon transfection of the cell line with this receptor, the effect of BCE treatment with amyloglucosidase on this increase was investigated to know whether the sensing is due to the alpha-oligosaccharide in BCE or another component. In HEK-293 cell line transfected with TLR2-CD14, the activation of NF- $\kappa$ B by BCE treated with amyloglucosidase was significantly reduced (Figure 5.4). This is consistent with the recent reporting that TLR2 is the receptor mediating an immune response to alpha-glucan (Bittencourt *et al.*, 2006).

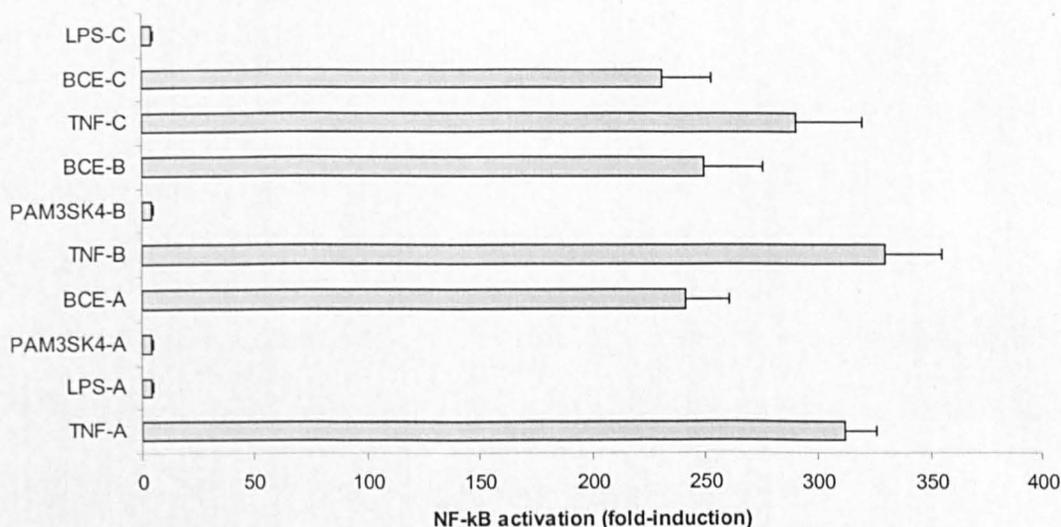
**Figure 5.4. Induction-fold of NF- $\kappa$ B in HEK293-TLR2 cells by *C. jejuni* BCE pre-treated with amyloglucosidase.** *C. jejuni* BCE treated with amyloglucosidase (BCE+AM) minimally induced NF- $\kappa$ B- dependent gene expression in HEK293 cells transfected with the reporter plasmid DNA and TLR2-CD14. The transfected cells were incubated for 6 h with one of the following: *C. jejuni* boiled cell extract (BCE), BCE treated with amyloglucosidase, TLR2 agonist PAM3SK4 (100 ng/ml<sup>-1</sup>), and TLR2-independent activation control TNF- $\alpha$  (50 ng/ml<sup>-1</sup>). The data are recorded as the means of three independent determinations.



### 5.2.4 Expression of TLR2 or MD-2 in HELA 57A has no increasing effect on NF- $\kappa$ B activation by BCE

HELA 57A cells were transfected with TLR2-CD14 or MD-2 to see the effect on NF- $\kappa$ B activation levels. Although TLR2 increases the activation in HEK293 cells, this increase was not observed in HeLa 57A. Additionally, the transfection of the TLR4 accessory protein (MD-2) did not result in BCE sensing by this receptor (Figure 5.5). In transfected HeLa57A, TLR2 or TLR4 did not sense their ligands. This may be due to low transfection efficiency, very low expression of MYD88, and high levels of TOLLIP present in this cells line (Nishimura and Naito, 2005).

**Figure 5.5. Induction-fold of NF- $\kappa$ B in HeLa 57A-TLR2/TLR4 cells by *C. jejuni* BCE.** NF- $\kappa$ B-dependent gene expression by BCE is not increased by transfecting HeLa 57A cells with TLR2 or MD-2. HeLa 57A stably transfected with the reporter plasmid DNA were transiently transfected with empty plasmid DNA, or TLR2-CD14, or MD-2. Transfected cells were incubated for 6 h with one of the following: BCE, TLR2 agonist PAM3SK4 (100 ng/ml<sup>-1</sup>), TLR4 agonist LPS (10  $\mu$ g/ml<sup>-1</sup>). The data are recorded as the means of three independent determinations. Transfection with empty plasmid DNA (TNF-A, LPS-A, PAM3SK4-A, BCE-A), TLR2-CD14 (TNF-B, PAM3SK4-B, BCE-B), MD-2 (TNF-C, BCE-C, LPS-C)

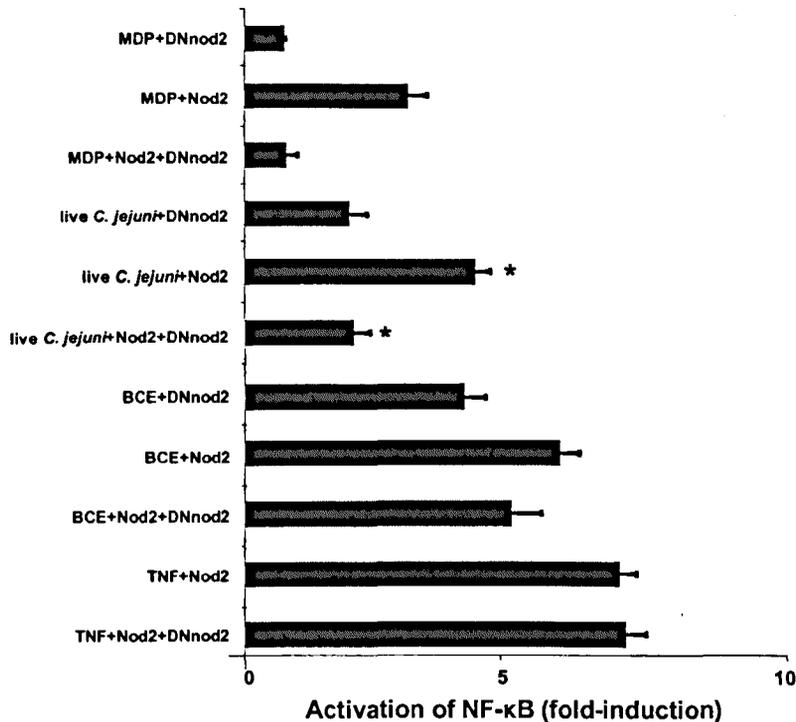


### 5.2.5 *Campylobacter jejuni* can activate NF- $\kappa$ B via NOD2

NOD2 was only found to be expressed in the HCA-7 cell line of those examined (Table 5.1). We therefore elected to transfect HEK293 cells with a plasmid expressing NOD2 to examine its effect on NF- $\kappa$ B induction levels. Figure 5.6

shows NOD2 expression conferred sensitivity to the NOD2 agonist MDP. However, consistent with previous reports the introduction of NOD2 resulted in an increase in the background levels of NF- $\kappa$ B-driven luciferase activity, making comparison between cells transfected with NOD2 encoding plasmids and those with reporter plasmids/empty vector controls difficult to interpret (Ogura *et al.*, 2001). Co-transfection of a dominant-negative form of NOD2 also resulted in a similar increase in non-induced NF- $\kappa$ B-driven luciferase levels but these could not be induced by MDP. Therefore to assess if the introduction of NOD2 affected the ability of live *C. jejuni* (m.o.i. 100) or BCE to activate NF- $\kappa$ B, we compared the induction factors in the presence and absence of DN-NOD2. Using this criterion the expression of NOD2 increases the activation of NF- $\kappa$ B to live *C. jejuni*, in that the co-expression of DN-NOD2 significantly reduces the response to live *C. jejuni* ( $P=0.04$ ). The reduction in the activation of NF- $\kappa$ B by BCE in the presence of DN-NOD2 was not significant.

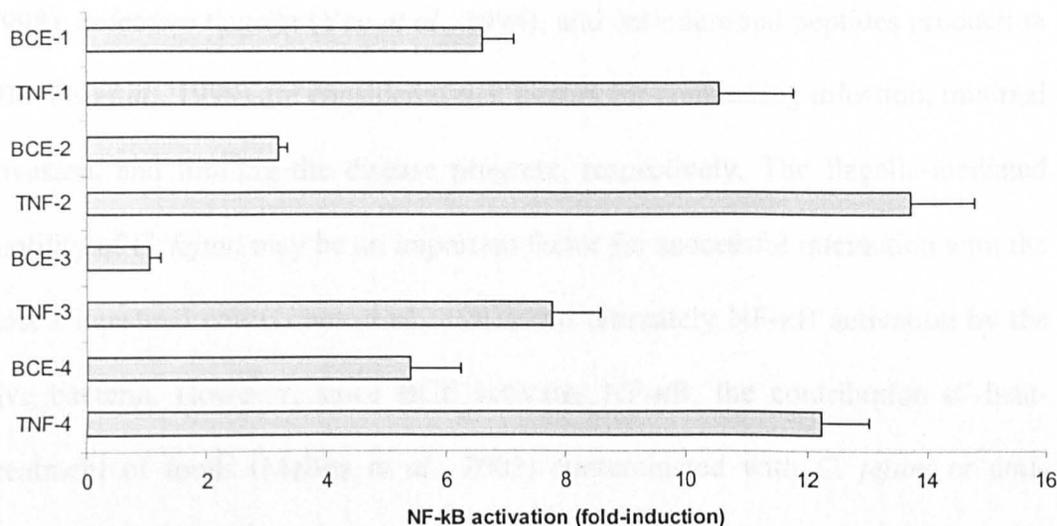
**Figure 5.6. Induction-fold of NF- $\kappa$ B in HEK293-NOD2 cells by *C. jejuni*.** HEK293 cells were transfected with the reporter plasmids DNA, along with NOD2 or DN-NOD2 as indicated. Transfected cells were treated for 6 h with one of the following: NOD-2 agonist MDP (100  $\mu$ g/ml<sup>-1</sup>); live *C. jejuni* 11168, *C. jejuni* boiled-cell extract (BCE). The activation control was TNF- $\alpha$  (50 ng/ml<sup>-1</sup>). Significant differences between the NOD2-expressing cells and those co-expressing DN-NOD2 ( $P=0.04$ ) for live *C. jejuni* are indicated by the asterisk. The data are recorded as the means of 3 independent determinations.



### 5.2.6 NF- $\kappa$ B activation by BCE is severely inhibited by DN-IRAK1 but not by DN-MAL or DN-p38

IRAK1 is a downstream molecule of the MYD88-dependent TLR signalling pathway. It acts to transfer signalling from MYD88 to TRAF6 leading to NF- $\kappa$ B activation (Alexopoulou and Kontoyannis, 2005). Although BCE can activate NF- $\kappa$ B in cells transfected with DN-MYD88, the activation is suppressed (Figure 5.7) by disrupting the signalling pathway in cells transfected with DN-IRAK1 ( $P<0.001$ ). What molecule participates of NF- $\kappa$ B activation in the absence of MYD88 and may interact with IRAK1 is not known.

**Figure 5.7. Induction-fold of NF- $\kappa$ B in HEK293-DNp38/DNIRAK1/DNMAL cells by *C. jejuni*.** NF- $\kappa$ B-dependent gene expression in HEK293 cells transfected with reporter plasmid DNA (BCE-1, TNF-1), along with DN-p38 (BCE-2, TNF-2), DN-IRAK1 (BCE-3, TNF-3), DN-MAL (BCE-4, TNF-4). Transfected cells were incubated for 6 h with *C. jejuni* boiled-cell extract (BCE) or control activation TNF- $\alpha$  (50 ng/ml<sup>-1</sup>). The data are recorded as the means of three independent determinations.



MAL (also known as TIRAP) is an adaptor molecule reportedly important for MYD88-dependent TLR2/TLR4 signalling (Takada and Akira, 2004). However, HEK-293 cells transfected with DN-MAL did not show impaired response to BCE ( $P=0.14$ ) (Figure 5.7). Signalling cross-talks between p38 and NF- $\kappa$ B have been reported to regulate inflammatory responses (Olson *et al.*, 2006). Therefore, the effect of DN-p38 on NF- $\kappa$ B activation was investigated in HEK-293 cells. Cells transfected with DN-p38 showed a decrease in NF- $\kappa$ B activation levels in response to stimulation with BCE ( $P=0.0015$ ) (figure 5.7).

### 5.3 Discussion

The mechanisms used by *C. jejuni* to cause disease in humans are not fully understood. However, the ability of this pathogen to activate NF- $\kappa$ B and other immune response regulators has been reported (Mellits *et al.*, 2002; McCallcum *et al.*, 2005). To cause an enteric illness, a pathogen must first overcome the first

responses of the host's innate immune defence. The highly acidic environment of the stomach, the constant physical movement in the gut, and production of antimicrobial peptides in the intestine are examples of these defence responses (Muller *et al.*, 2005). The reduction of stomach acidity (Waterman and Small, 1998), defective flagella (Yao *et al.*, 1994), and antimicrobial peptides production (Bevins *et al.*, 1999) are considered risk factors for contracting infection, minimal invasion, and limiting the disease progress, respectively. The flagella-mediated motility of *C. jejuni* may be an important factor for successful interaction with the host's intestinal cell (Grant *et al.*, 1993) and ultimately NF- $\kappa$ B activation by the live bacteria. However, since BCE activates NF- $\kappa$ B, the contribution of heat-treatment of foods (Mellits *et al.*, 2002) contaminated with *C. jejuni* or antimicrobial-killed (Zilbauer *et al.*, 2005) bacteria in liberating the bioactive components needs to be considered.

The disruption of the IECs tight junction and the subsequent infiltration of professional phagocytes by *C. jejuni* have been reported (Chen *et al.*, 2006). This is aided by the finding that *C. jejuni* induces the production of IL-8 that attracts neutrophils to the site of infection. Depending of the host's immune system status, this may limit the infection or being the early defences are overcome by *C. jejuni* resulting in more complications.

To activate NF- $\kappa$ B in IECs, *C. jejuni* or its products must be recognized by cellular receptor(s) that initiates the signalling cascade leading to NF- $\kappa$ B activation. In attempt to illustrate *C. jejuni in vivo* interactions with the host, *C. jejuni* and its BCE activation of NF- $\kappa$ B were investigated in different cell lines varying in the pattern expression of the receptors under study.

To date 11 transmembrane receptors (TLRs) and 2 cytoplasmic receptors (NODs) have been identified (Akira and Takada, 2004; Strober *et al.*, 2006). Regarding the sensing of foodborne bacterial pathogens, TLR2, TLR4, TLR5, and TLR9 are well characterized to recognize specific ligand(s). Bacterial ligands delivered into the host cell by invasion, phagocytosis, or injection are recognized by NOD1 or NOD2 (Strober *et al.*, 2006).

Because flagella are sensitive to boiling and proteinase K treatment, TLR5 was not investigated in this study. As BCE treated with DNAase still activates NF- $\kappa$ B, TLR9 was also not included in this study. The results obtained here indicate the live/heat-killed *C. jejuni* or BCE can activate NF- $\kappa$ B independent of TLR2 and TLR4. The activation was also independent of NOD1 and NOD2. These findings have not been previously reported for *C. jejuni*.

MYD88, the central adaptor used by most TLRs, was found have a role but not necessary for observed NF- $\kappa$ B activation. However, disruption of the signalling molecule IRAK1, a downstream molecule of MYD88, severely suppressed NF- $\kappa$ B indicating that signalling by *C. jejuni* is initiated by an IL-1/Toll like receptor which is not TLR2/TLR4 and yet to be identified. It might be possible that *C. jejuni* does not activate NF- $\kappa$ B by an IL-1/Toll like-independent receptor. The other adaptor molecule is MAL, which is reported to be important for MYD88-TLR2/TLR4 -dependent signalling was found not to be necessary for the activation. Studies about MYD88-independent TLR signalling have reported the importance of the TRIF adaptor in this pathway for TLR3 and TLR4 (Akira and Takada, 2004). Because BCE was found not to be sensed by TLR4, and TLR3 recognizes viral dsRNA, the role of this pathway in NF- $\kappa$ B activation by *C. jejuni* was not investigated.

NODs, the cytoplasmic receptors that sense peptidoglycan fragments, are not necessary for NF- $\kappa$ B activation by *C. jejuni*. The activation occurs in HELA 57A cells, where NOD2 is not expressed. Additionally, the presence of NOD1, which mainly senses a secreted peptidoglycan fragment (Hasegawa *et al.*, 2006), may not be functional as its commercial ligand does not activate NF- $\kappa$ B in these cells. Previously, *C. jejuni* culture supernatant was reported not to induce NF- $\kappa$ B activation (Mellits *et al.*, 2002).

Since structural characterization of BCE showed that the proposed NF- $\kappa$ B activating component is an alpha-linked oligosaccharide, the sensing of BCE treated with amyloglucosidase by TLR2 was investigated. As recently reported, TLR2 sensing of alpha-glucan was lost upon treatment with this enzyme (Bittencourt *et al.*, 2006).

Although GC analysis showed glucose as the sole component in BCE, one must take into consideration that not all carbohydrates can be analysed by GC as alditol-acetate derivatives. Additionally, the observed inactivation of BCE by amyloglucosidase might not exactly reflect the component's nature since this enzyme exhibits low foreign activity (1%) toward other sugars.

The MAPK p38 was reported to partially regulate the production of inflammatory cytokines by *C. jejuni* (Watson and Galan, 2005; McCallum *et al.*, 2005). In this study, HEK-293 cells transfected with DN-p38 showed decreased levels of NF- $\kappa$ B activation by BCE. The role of p38 in regulating NF- $\kappa$ B function is thought to occur in the nucleus independently of its translocation or binding to specific DNA target sequences, and it is a consequence of phosphorylating the transcriptionally active subunit of NF- $\kappa$ B, RelA (Olson *et al.*, 2006).

In IECs, the expression of TLRs is highly regulated to discriminate among normal gut microflora and pathogens. This is mediated by very low levels of receptor expression, site of expression and high levels of TLR negative-regulating molecules (Abreu *et al.*, 2005). The results obtained here may suggest that *C. jejuni* interacts with the host's IECs through yet-to be understood mechanism/receptor(s). However, it is clear that the unknown receptor utilizes the adaptor molecules (MYD88, IRAK1) that mediate the classical NF- $\kappa$ B activation by IL-1/Toll like receptors family.

*Campylobacter* has been reported to cause enteritis at low infectious doses (Robinson, 1981). One risk factor for contracting the infection is the consumption of foods such as raw milk (Peterson, 2003). The subsequent disease onset after contaminated milk ingestion indicates that *Campylobacter* has strategies to avoid the host initial defence responses. Enteric pathogenic bacteria have evolved mechanisms for overcoming these defences. The mechanisms include evasion of PAMPs detection at the mucosal surface, resistance to anti-bacterial peptides, invading and crossing the epithelial cells, escaping the phagocyte responses, and interfering with humoral immune responses (Hornef *et al.*, 2002). Since TLR2, TLR4, and TLR5 were found not necessary for NF- $\kappa$ B activation by *C. jejuni*, the PAMPs detection evading mechanism is expected to be important for *C. jejuni* pathogenesis. In IECs, which are characterised by their unresponsiveness to TLRs signal, NOD1 has been reported to be the general sensor for enteroinvasive *E. coli* and *S. flexneri* that mediates NF- $\kappa$ B activation (Strober *et al.*, 2006). Unlike these pathogens, *C. jejuni* activation of NF- $\kappa$ B was found independent of NOD1. The production of antimicrobial peptides (Zilbauer *et al.*, 2005), regulated by NOD2 in

IECs (Hisamatsu *et al.*, 2003; Strober *et al.*, 2006) may indicate a role for NOD2 in limiting *C. jejuni* infection.

## **CHAPTER 6 Discussion**

## CHAPTER 6

### 6. Discussion

*Campylobacter* species, a genus with a range of reservoirs and the ability to survive in the environment, are the major cause of food-borne bacterial gastroenteritis worldwide. Principally caused by *C. jejuni*, the incidence of campylobacteriosis has followed an increasing pattern since the 1990s. Given that campylobacters are found in foods in retail stores, educational campaigns for consumers are thought to be an important factor to minimise the risk of contracting infections. It has been reported that most of the food-borne infections that occur in private households can be reduced if the consumers follow basic steps in handling and processing of food items. To better design control/prevention measures for *Campylobacter* enteritis, understanding how this pathogen causes disease is important. This can be partially investigated by studying the pathogen interaction with the host's cells at the molecular levels.

Live *C. jejuni* (NCTC 11168) can activate the transcription factor NF- $\kappa$ B in epithelial cells but this response requires around 16 h before a sustained induction of the transcription factor takes place (Mellits *et al.*, 2002). In contrast a boiled cell extract could activate NF- $\kappa$ B dependent gene transcription within two hours but that this response was transient peaking at 6 h and demonstrating the re-synthesis of I $\kappa$ B $\alpha$  after 90 min (Mellits *et al.*, 2002). Consistent with these data we show here that BCE and heat-killed bacteria can activate NF- $\kappa$ B-dependent reporter genes transfected into a range of cell types at 6 h (HELA 57A, HEK-293, CACO-2 and HCA-7), and that the result of this activation is correlated with the elaboration of the cytokine IL-8. The presentation of live *C. jejuni* leading to the invasion of tissue cultured cells has the potential to stimulate internal receptors

differentially to cell surface receptors. This demonstrated for the first time that *C. jejuni* can elicit a pro-inflammatory response through the surface receptor TLR2 in a MYD88 dependent manner, and that this response can be increased by the presence of the internal receptor NOD1. However, in the absence of TLR2 or when a dominant negative form of MYD88 is co-expressed in cultured cells, *C. jejuni* and BCE can still activate NF- $\kappa$ B leading to IL-8 production. How this MYD88 independent activity occurs is not known at this time but the general consequence is that all the cell lines studied here, and likely a wider array of cell types, will inherently produce a pro-inflammatory response when challenged by *C. jejuni* or its products. This basal response may be enhanced in certain cell types through the expression of established receptors leading to robust activation of NF- $\kappa$ B. *Campylobacter* enterocolitis is predominantly an inflammatory disease and the ability of the bacteria to activate NF- $\kappa$ B in a range of cell types likely contributes to the disease process.

The responses of intestinal epithelial cells need to be tightly controlled because they are continually exposed to bacteria and bacterial products. However, these cells must retain the ability to respond to pathogen associated damage (Abreu *et al.*, 2005). The activation of NF- $\kappa$ B by *C. jejuni* in intestinal epithelial cells leads to the secretion of a series important cytokines and chemokines (IL-8, growth related oncogene  $\alpha$  or GRO $\alpha$ , GRO $\gamma$ , macrophage inflammatory protein 1, monocyte chemoattractant protein 1 and IFN $\gamma$ -inducible protein 10) that contribute to the induction of inflammatory and adaptive immune responses (Hickey *et al.*, 1999; Mellits *et al.*, 2002; Bakhiet *et al.*, 2004; Watson & Galan, 2005; Hu & Hickey, 2005). It is the activation of the innate immune system by *C. jejuni* to produce pro-inflammatory cytokines that leads to localised inflammation and to

the development of symptoms associated with the disease. Cells responsible for mobilising adaptive immunity also respond to *C. jejuni*. Human monocytes have been reported to respond to *C. jejuni* by induction of IL-1, IL-6, IL-8, and TNF- $\alpha$  via NF- $\kappa$ B (Jones *et al.*, 2003; Hickey *et al.*, 2005), and similarly *C. jejuni* induces immature dendritic cells to produce IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, IFN $\gamma$ , and TNF- $\alpha$  (Hu *et al.*, 2006). Dendritic cells are of key importance to the intestinal immune system as they not only release cytokines but upon antigen capture mature to become the major antigen presentation cells capable of initiating proliferation in naive T-cells to induce a primary immune response and enable immunological memory. In addition to the expression of many TLRs, dendritic cells express C-type carbohydrate-recognizing receptor (Rossi and Young, 2005). The importance of this C-type receptor in sensing *C. jejuni*- derived carbohydrate is not currently known.

In addition to the proposed alpha-glucan in BCE, TLR2 recognises a wide variety of ligands that include bacterial lipopeptides/lipoproteins, lipoteichoic acid, lipoarabinomannan as well as trypanosome glycosylphosphatidylinositol-anchored protein and yeast zymosan (Takeda *et al.*, 2003). Lipoproteins and lipopeptides are candidate ligands for the TLR2 dependent activation of NF- $\kappa$ B by *C. jejuni* and BCE. Genome sequences of *C. jejuni* encode a number of putative lipoproteins that include the *C. jejuni* surface lipoprotein JlpA, which has been reported to interact with cellular heat shock protein Hsp90 to activate NF- $\kappa$ B (Jin *et al.* 2003). The expression of TLR2 increases the ability of HEK293 and CACO-2 cells to respond to BCE in a similar way to live *C. jejuni*. BCE contains a heat-stable TLR2 agonist that is insensitive to proteinase K digestion. It is possible that residual lipopeptides could survive these processes and therefore are candidates

for the activation of NF- $\kappa$ B via TLR2. However, treatment of purified 1kDa fraction of BCE with amyloglucosidase abolished NF- $\kappa$ B activation even in the presence of TLR2 indicating that this receptor only sensed an oligosaccharide structure in BCE. In contrast the expression of TLR4 in HEK293 cells did not result in enhanced activation of NF- $\kappa$ B by *C. jejuni* or BCE. The natural agonist of TLR4 is bacterial lipopolysaccharide (LPS), however, *C. jejuni* synthesises lipooligosaccharide (LOS) as a component of its cell surface. LOS differs from LPS common to other Gram-negative species in that the O-chain polysaccharide is absent leaving core polysaccharide attached to lipidA. There is considerable variation in the LOS structures of *C. jejuni* and structural variants arise within cultures of the same strain due to phase variation in the reading frames of LOS biosynthetic genes (reviewed by Karlyshev *et al.*, 2005). The core polysaccharide structure of *C. jejuni* NCTC 11168 has been determined and exhibits structural variants, most notably in the terminal structures that are analogous to human gangliosides GM1a and GM2 and subject to phase variation (Oldfield *et al.*, 2002; Michael *et al.*, 2002). The lipid-A backbone of *C. jejuni* NCTC 11168 also shows variation with combinations of the disaccharides 2,3-diamino-2,3-dideoxy-D-glucose and D-glucosamine exhibiting different patterns of pyrophosphorylethanolamine attachment. All this structure variation could mean that only selective LOS types have the possibility of acting as agonists of TLR4 or possibly they could contribute to TLR2 dependent activation. Regardless of these possibilities the expression of TLR4 with MD2 and CD14 in HEK293 cells did not significantly increase activation of NF- $\kappa$ B by *C. jejuni* NCTC 11168 or BCE, and signalling by this pathway appears limited compared to the strong pro-inflammatory responses recorded for the alternative pathways investigated here.

NOD1 and NOD2 are internal cellular sensors of muropeptides derived from the breakdown of bacterial peptidoglycan. NOD1 responds to the naturally occurring product GlcNAc-MurNAc-L-Ala- $\gamma$ -D-Glu-*meso*-diamino-pimelic acid formed upon the breakdown of peptidoglycan from Gram-negative bacteria (Girardin *et al.*, 2003), whereas the minimal structure to which NOD2 will respond is the muramyl dipeptide MurNAc-L-Ala-D-isoGln that is a common component of Gram-negative and Gram-positive bacteria (Inohara *et al.*, 2003; Girardin *et al.*, 2003). *C. jejuni* is clearly a source of these molecules or related structures, which could be delivered as breakdown products in BCE or through invasion by live bacteria. However, NOD1 has only been conclusively shown to affect NF- $\kappa$ B activation in cells co-expressing TLR2-CD-14. This effect appears to be a feature of the response to *C. jejuni* because the NOD1 agonist M-triDAP did not show any significant increase in the activation of NF- $\kappa$ B following co-expression of TLR2. The expression of NOD2 in HEK293 cells leads to an increase in the background levels of the luciferase reporter of NF- $\kappa$ B but clearly further sensitises the cells to *C. jejuni*.

The presentation of heat killed or dead bacteria to cultured cells may lead to internalisation but these particles will not be replaced unlike the situation with a growing bacterial culture. Consistent with this view heat-killed *C. jejuni* produce a transient activation of NF- $\kappa$ B compared to the delayed but sustained stimulation by live bacteria (Mellits *et al.*, 2002) *C. jejuni* BCE represents a soluble diffusible activator that may be rapidly recognised by cell surface receptors and has the possibility of being transported within the cell to stimulate internal receptors. Stimulation by BCE is by nature transient but represents a stable material that gives rise to a strong reproducible response that can be easily quantified to test the

effects of chemical or enzyme treatments on BCE, or alternatively changes in the responses of host cells to manipulation of the signal transduction pathways available to them. Response to BCE was not confined to sub-confluent tissue culture cells but was also evident when applied to polarised monolayers of colonic epithelial cells (Caco-2 and HCA-7) by the production of IL-8. Application of BCE to the apical face of polarised monolayers resulted in a greater accumulation of IL-8 in the physiologically relevant basolateral chamber. The application of live *C. jejuni* to the apical face of polarised Caco-2 and HCA-7 monolayers induced a slower accumulation of IL-8 over 16 h but with a similar result in that the IL-8 levels recorded in the basolateral chambers were greater than those of the apical chamber. Viable counts of *C. jejuni* applied at an m.o.i. of 100 in D-MEM to cell cultures under these conditions remain static, likely due to the bacteria being in stationary phase, as it is evident from microscopic examination that a proportion of the extracellular bacteria continue to divide. At 16 h the effective dose of live and dead bacteria will be greater than the initial input m.o.i. of 100. However, despite the increase in microbial load at this time the tight junctions of these monolayers remain largely intact as judged by modest falls in the TEER of around 5%. Although, 12 hours later against a greater microbial load the TEER values were observed to fall implying the loss of tight junctions to provide access to the basolateral chamber for *C. jejuni* via a paracellular route, as reported by previous authors (Bras & Ketley, 1999; MacCallum *et al.*, 2005).

All the cell types investigated responded to treatment with *C. jejuni* or BCE by induction of NF- $\kappa$ B but the levels of IL-8 elaborated by the cells were markedly different. Of particular note the HCA-7 and Caco-2 cell lines are similarly derived from human colon carcinomas and capable of forming polarised monolayers in

culture as discussed above, but HCA-7 cells secrete almost 100-fold the quantity of IL-8 in response to BCE treatment compared to Caco-2 cells. Polarised cells also show a five-fold difference in the basolateral levels of IL-8 between the two cell lines. A possible explanation for this difference could be in the expression of TLR3 or NOD2, which were detectable by RT-PCR from HCA-7 cells but absent in Caco-2 cells. Treatment with nucleases that would remove the dsRNA ligand for TLR3 did not reduce the ability of BCE to activate NF- $\kappa$ B. However, we demonstrate that NOD2 expression in HEK293 cells results in a general up-regulation of the NF- $\kappa$ B reporter response, and that inhibition of NOD2 function by co-expression of DN-NOD2 results in a reduction in the ability of live *C. jejuni* to activate NF- $\kappa$ B. The presence of NOD2 in HCA-7 could at least in part account for the ability of *C. jejuni* to induce increased levels of IL-8. This with another reported function of NOD2, that is regulating antimicrobial peptides in IECs, suggests that NOD2 assists in early recognition and limiting *C. jejuni* infection. However, it is not certain this explanation could extend to BCE, where co-expression with DN-NOD2 did not produce a significant fall in the activation of NF- $\kappa$ B. NOD2 has been reported to be expressed at low levels in colonic epithelial cells but to be up-regulated by pro-inflammatory cytokines, TNF- $\alpha$  and IFN $\gamma$  (Rosenstiel *et al.*, 2003). Indeed the NOD2 gene promoter contains a  $\kappa$ B response element that will ensure a response to internalised pathogens following initial surface receptor stimulation even if NOD2 levels are initially low (Gutierrez *et al.*, 2002). It is possible that the rapid and robust response to diffusible BCE will lead to increased NOD2 expression that may in part overcome the dominant negative phenotype of the plasmid borne DN-NOD2 allele.

That heat inactivated *C. jejuni* can activate NF- $\kappa$ B has been reported for human monocyte derived THP-1 and dendritic cells (Jones *et al.*, 2003; Hu *et al.*, 2006), although a recent report has suggested that heat inactivated *C. jejuni* 81-176 does not activate NF- $\kappa$ B in intestinal derived T84 cells (Johanesen & Dwinell, 2006). In these experiments the chemokine CCL20 (synonymous with macrophage inflammatory protein 3 $\alpha$ ) was monitored over a 24 h period where the infection model was reported to consist of a 2 h incubation of *C. jejuni* or dead bacteria with the T84 cells before treatment with gentamycin and removal of the bacteria. Continued stimulus could only be possible through internalised bacteria. Our data clearly demonstrate for that stimulation through the internal receptor NOD1 is only evident when the cells co-express and respond to TLR2, and that this response requires MYD88 and the accessory protein CD-14. However, the expression of NOD2 does lead to an enhanced activation of NF- $\kappa$ B that is not reliant on the co-expression of TLR2. It would seem that NOD2 is likely responsible for the NF- $\kappa$ B activation by internalised bacteria but the response may be weak if the combination of *C. jejuni* 81-176 and T84 cells do not exhibit the basal response to heat-stable products we note here in several cell lines and previously with other *C. jejuni* strains (Mellits *et al.*, 2002). However, it has been recently reported that with the notable exception of the anti-inflammatory cytokine IL-10, cytokine production in dendritic cells did not show any significant differences between live and heat-killed *C. jejuni* 81-76 (Hu *et al.* 2006). Taken together these latter findings would suggest that the signals required for dendritic cell trafficking and response to *C. jejuni* are not confined to live cells, and that invasion can contribute to cytokine induction but is certainly not a prerequisite.

To summarise we have demonstrated that *C. jejuni* and its heat-stable products can activate NF- $\kappa$ B in a range of cell types that are independent of the expression of Toll-like receptors, and the internal receptors NOD1 and NOD2. However, the presence of TLR2 with accessory protein CD-14 will further sensitise cells to *C. jejuni* and its heat-stable products in a MYD88 dependent manner to enhance the activation of NF- $\kappa$ B, and that the activation conferred by TLR2 shows synergy with NOD1. The expression of NOD2 also increases the sensitivity of cells to *C. jejuni*. The response of humans to the food borne pathogen *C. jejuni* is likely to have several facets that include recognition of several PAMPs by a variety of surface and internal receptors that stimulate pathways that converge on the transcription factor NF- $\kappa$ B to elicit a potent pro-inflammatory response that is the hallmark of the pathogen.

Because BCE of *C. jejuni* is a serious concern for food safety structural characterization was limited to understand the nature of the NF- $\kappa$ B activating component in BCE. The structures of other *C. jejuni* derived products, which are thought not to contribute to NF- $\kappa$ B activation have been reported previously (Michael *et al.*, 2002; Karlyshev *et al.*, 2005). The understanding that BCE is heat, proteinase K, and lipopeptide lipase resistant, was the initial consideration towards the development of purification methods. Size-exclusion chromatography based separation is usually the first choice for estimating the molecular weight for the component of interest. Followed by SPE and the suitable phase of HPLC, the structure under study can be identified by MS, GC (if sample is derivatizable), MS-GC, and/or NMR. However, the combination of structural characterization with tracking of the bioactive components can limit methods of purification and make structural details about the molecules difficult to obtain or indeed confound

any conclusion. In this study, the use of strong buffers or chemicals that would require the sample to be desalted before MS/NMR was avoided to eliminate affecting the bioassay results or loss of sample during desalting. Therefore, the purification methods development were limited by the suitability of available buffer systems.

The activation of the calcium-dependent transcription factor NF-AT (reviewed by Macian, 2005) and its contribution to innate immune responses have not been clearly demonstrated (Goodridge *et al.*, 2007). Zymosan, a yeast-derived product mostly composed of  $\beta$ -glucan, was reported to activate NF-AT and induce the production of early growth response 2 and early growth response 3 transcription factors, cyclooxygenase 2, IL-2, IL-10 and IL-12 p70 by Dectin-1 expressing stimulated dendritic cells (Goodridge *et al.*, 2007).

The nature of the NF- $\kappa$ B-activating structure in BCE was found to be an alpha-glucan (5-glucose residue). Only until recently, the activation of NF- $\kappa$ B by alpha-glucan has been reported (Bittencourt *et al.*, 2005). Given that beta-glucan is of beneficial health effect, that is by shifting of pro-inflammatory responses to anti-inflammatory responses through the regulation of the interleukin-1 receptor antagonist gene (Luhm *et al.*, 2006), what makes alpha-glucan induce inflammatory responses is not known. This is assuming that NF- $\kappa$ B activation as observed in cell line bioassays reflects the host's inflammatory responses to infection. How alpha-glucan can be recognised by trans-membrane or cytoplasmic receptors is not understood. However, for BCE it seems that the hydroxyl groups in the structure play a role in its recognition by the host's receptors because replacing these groups with methyl groups inhibits BCE ability to activate NF- $\kappa$ B. If BCE is recognized by an IL-1/Toll like receptor, then its hydrophilic active

component must interact with the extracellular domain (ED) of the receptor. The ED, which has a conserved LRR motif, varies in structure for different TLRs to enable binding to wide range of PAMPs (Strober *et al.*, 2006). After binding, the signal is initiated at the conserved 160 amino acids-motif (TIR domain) that is found at the cytosolic domain of TLRs. Binding of BCE to ED could be facilitated by a hydroxyl (of BCE) –hydroxyl (of amino acids in ED) interaction. Recognition of PAMPs by NODs is also mediated by their LRR motif (Strober *et al.*, 2006). Although alpha-glucan has been recently described for *C. jejuni*, its synthesis and contribution to the interaction with host cells are not known. The OPGs, mostly beta-linked but reported to contain alpha-linked glucan, could be a possible source for the bioactive component in *C. jejuni*.

The knowledge obtained here has highlighted the possible interactions of *C. jejuni* products with the host, and will assist in developing better prevention/control methods of *Campylobacter* mediated infections. Additional investigations to identify the cellular receptors used by *C. jejuni* in the host's IECs to induce inflammatory responses and characterization of the bioactive structure(s) responsible for the responses in IECs are needed.

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