THE REGULATION AND BIOLOGICAL ACTIVITY OF CELL SURFACE VIRULENCE DETERMINANTS IN MODEL OPPORTUNIST AEROBIC AND ANAEROBIC BACTERIAL PATHOGENS

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CONTENTS

ABBREVIATIONS

Abbreviations used in this thesis

2,4,5,-T	2,4,5,-trichlorophenoxyacetic acid
APS	ammonium persulphate
AQ	aqueous layer
ATCC	American Type Culture Collection
BA	blood agar
Bc	*Burkholderia cepacia*
Bg	*Burkholderia gladioli*
BPI	bactericidal permeability increasing protein
CEP	*Pseudomonas cepacia* medium
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cfu	colony forming units
CGD	chronic granulomatous disease
CHO	carbohydrate
CIE	crossed immunoelectrophoresis
DMSO	dimethylsulphoxide
EDDA	ethylene-N,N'-diacetic acid
EPS	exopolysaccharide
ETOH	ethanol
FCS	foetal calf serum
GI	gastrointestinal
GPI	glycosylphosphatidylinositol
HBP	haem binding protein
HIC	hydrophobic interaction chromatography
HLT	heart-lung transplant
HRP	horse radish peroxidase
IBD	inflammatory bowel disease
Ig	immunoglobulin
IL-8	interleukin-8
Kdo	3-deoxy-D-*manno*-2-octulosonic acid
Ko	D-*glycero*-α-D-*talo*-2-octulosonic acid
LAL	limulus ameobocyte lysate
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MA	malka A medium
MAb	monoclonal antibody
MA+E	malka A + EDDA
MAN	carbon-limited medium
MA+S	malka A + 50% heat inactivated sheep serum
MB	nitrogen-limiting medium
MD	high osmolarity med ium
MH	nitrogen-limiting/high osmolarity medium
MIC	minimum inhibitory concentration

MNL	mononuclear leucocytes
MODS	multiple organ dysfunction syndrome
MOF	multiple organ failure
NB+YE	nutrient yeast broth
NCTC	National Collection of Type Cultures
OA	onion agar
O-Ag	O-antigen
OD	optical density
OM	outer membrane
OMP	outer membrane proteins
P	phosphorus
Pa	*Pseudomonas aeruginosa*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCP	phenol/chloroform/petroleum spirit
per	periodate
PFGE	pulse field gel electrophoresis
PH	phenol layer
PHA	phytohaemagglutanin
PIA	*Pseudomonas* isolation agar
plt	pellet
PPY	proteose peptone yeast extract medium
Prt	protein
PW	phenol water
RIE	rocket immunoelectrophoresis
R-LPS	rough-lipopolysaccharide
S-LPS	smooth-lipopolysaccharide
SDS	sodium dodecylsulphate
SIRS	systemic inflammatory response syndrome
sn	supernate
TBA	thiobarbituric acid
TCE	trichloroethylene
TEMED	NNN'N'-tetramethyl-1,2-diaminoethane
TNF	tumour necrosis factor
VT&B	VT&W + bile salts
VT&E	VT&W + EDDA
VT&S	VT&W + 50% heat inactivated sheep serum
VT&W	Van Tassell and Wilkin's medium

ABSTRACT

Burkholderia cepacia is an aerobic Gram-negative bacterium originally described as the cause of soft rot in onions but now recognised as a serious human pathogen most notably in patients with cystic fibrosis (CF), the most common, fatal inherited disease affecting Caucasian populations. *Bacteroides fragilis*, a Gram-negative commensal associated with the mucosal surface of the human colon, is the most frequently isolated anaerobic bacterium from clinical specimens and is increasingly implicated as an important source of endotoxin in gut-derived sepsis. Previously, both organisms were considered to pose little hazard to human health and consequently, their pathogenesis and virulence factors are poorly understood. The cell surface components of a bacterium are classic virulence determinants and the influence of growth environment on the plasticity of the bacterial surface is well-established. This thesis considers the environmental regulation and biological activity of putative virulence factors for both organisms.

Initial studies focused on the expression and antigenicity of the cell surface components; lipopolysaccharide (LPS), exopolysaccharide (EPS) and outer membrane from several representative strains. To determine the influence of growth environment, these components were investigated under different culture conditions and extraction methods. Results demonstrated that as with other Gram-negative organisms, the composition of outer membrane proteins of both *B. cepacia* and *B. fragilis* were influenced by cultural conditions with bacteria inducing or repressing protein structures presumably to influence overall permeability and survival. The EPS and LPS of *B. fragilis* also varied with environmental growth condition. Interestingly, extraction method was found to influence the LPS structure of *B. cepacia* including the loss of the distinctive rough LPS phenotype of an 'epidemic' strain.

B. cepacia and *B. fragilis* exhibited a greater biological activity than previously recognised both in terms of endotoxicity and cytokine induction. For *B. cepacia* the capacity to induce the proinflammatory cytokines TNF-α and IL-8 from several cell types was significantly, and unexpectedly, higher compared to the other major CF pathogen, *Pseudomonas aeruginosa.* This enhanced inflammatory potential of *B. cepacia* was not due to a more efficient LPS signalling pathway. As both CF pathogens appeared to induce TNF-α in a similar manner, the combined effect of both species was examined. Surprisingly, when *P. aeruginosa* was present in increasing amounts compared to *B. cepacia,* cytokine levels were down-regulated. These results indicate that *B. cepacia* has a major potential to cause immune-mediated damage and concurrent colonisation with *P. aeruginosa* may modulate this effect. For *B. fragilis* cytokine levels were compared to *Escherichia coli*, a facultative anaerobic commensal considered of great importance in gut-derived sepsis due to its extremely active LPS. TNF-α levels induced by *B. fragilis* were 20-fold lower than *E. coli.* However, considering the predominance of *Bacteroides* species in the gut, outnumbering facultative organisms by 20-300 fold, results imply that as a population *B. fragilis* may possess as much biological potential as *E. coli.* Thus, *B. fragilis* may play a vital role in gut-derived sepsis. The relevance of these findings to the understanding of *B. cepacia* in CF and of *B. fragilis* in sepsis is discussed.

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DECLARATION

All of the experiments and procedures presented in this thesis were performed by the author unless otherwise indicated in the text.

CHAPTER 1

INTRODUCTION

1.1 THE BACTERIAL CELL SURFACE IN PATHOGENESIS

1.1.1 PATHOGENESIS

Bacterial pathogenesis can be defined simply as the mechanisms involved in the development of a disease. In a small number of disease states caused by microorganisms, pathogenesis can be attributed to a single element, but in the majority of cases damage usually occurs as a result of a complex mixture of interactions involving both microbial and host factors.

1.1.2 BACTERIAL VIRULENCE DETERMINANTS

For a bacterium to be able to cause disease it must be able to gain entry into or onto a suitable host site, survive and multiply, resist the host defence mechanisms and damage the host tissue (Mims *et al*, 1995). Components of a bacterium that have the potential to harm a host are described as bacterial virulence determinants and can be divided into two main types; cell surface determinants (structural components of the bacterial cell envelope) or extracellular determinants (products made and secreted by a bacterial cell).

Figure 1.1 shows the structure of a typical Gram-negative cell. The cell surface structures relevant to this thesis- lipopolysaccharide, outer membrane and exopolysaccharide- will be discussed in some detail; other cell components are only briefly described.

1.1.3 LIPOPOLYSACCHARIDE

Structure

Lipopolysaccharide (LPS) consists of three covalently linked, biochemically and antigenically distinct regions; lipid A, core oligosaccharide and O-antigen. Lipid A is a hydrophobic compound embedded in the outer leaflet of the outer membrane and acts to anchor the entire LPS molecule to the cell surface. The core oligosaccharide

links the lipid A to the hydrophilic O-antigen, which extends outwards from the bacterial cell into the surrounding environment. This description of LPS structure represents a typical smooth-type LPS (S-LPS). Bacteria can also possess rough-type LPS (R-LPS) which is characterised by the absence of O-antigen and, in some cases, the outer region of the core oligosaccharide.

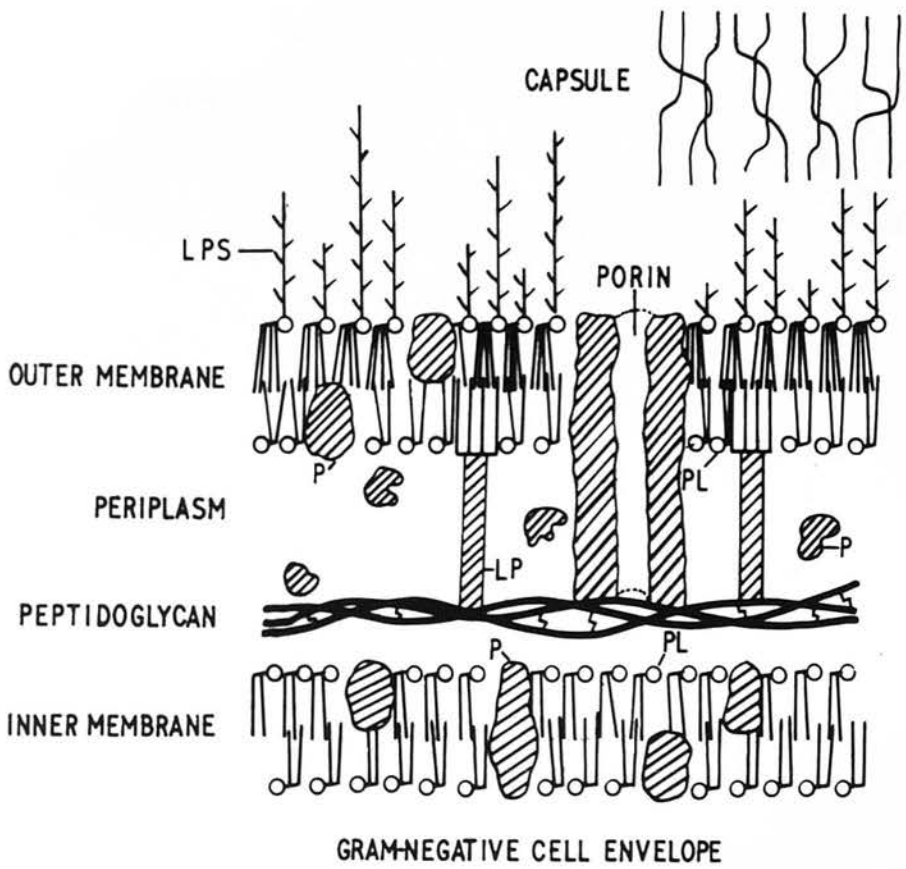


Figure 1.1 The Cell Surface of a Gram-negative Bacterium.
LP = lipoprotein; LPS = lipopolysaccharide; P = protein; PL = phospholipid
(Adapted from Hancock & Poxton, 1988)

Lipid A

The lipid A moiety is an essential structural component of the LPS molecule as lipid A minus mutants are not viable. Indeed, natural free lipid A can only be acquired by chemical degradation, as enzymes capable of cleaving the polysaccharide-lipid bond

have yet to be isolated (Rietschel *et al*, 1993). The structure of lipid A has been elucidated using mainly R-LPS from the Enterobacteriaceae. In *Escherichia coli*, the lipid A structure comprises a β-1,6-linked glucosaminyl-glucosamine disaccharide backbone, substituted with a phosphoryl group at position 4 of the non-reducing sugar and at position 1 of the reducing glucosaminyl residue. Both sugar residues are also substituted at positions 2 and 3 with primary acyl groups joined through amide and ester linkages (Galanos & Lüderitz, 1984; Rietschel *et al*, 1993). This general structure forms the basis of most lipid A moieties. Variations that occur generally involve the length of substituted fatty acid residues, the type and distribution of fatty acid substituents and the presence of additional substituents of the phosphoryl residues (Poxton & Arbuthnott, 1990). Variations in acylation patterns can further differentiate the lipid A region into one possessing either an asymmetrical or a symmetrical fatty acid distribution pattern (Rietschel *et al*, 1993). Despite these potential variations, lipid A remains the most structurally conserved of all LPS regions.

Core oligosaccharide

The core oligosaccharide of LPS consists of an inner and outer region. The inner core region is characterised by the presence of two unusual sugars L-*glycero*-D-*manno*- or D-*glycero*-D-*manno*-heptose and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) as well as phosphate and ethanolamine (Poxton & Arbuthnott, 1990). The inner region is linked to the lipid A component via an α-linked Kdo residue which itself may have substituents of extra Kdo or heptose residues (Rietschel *et al*, 1993). The structure of the inner core region is generally conserved between species. The outer core region consists of the common hexoses, D-glucose, D-galactose and N-acetyl-D-glucosamine and is positioned closest to the O-antigen region.

O-Antigen

The O-antigen (O-Ag) region is the most variable component of LPS and comprises long chains of repeating oligosaccharide units, interlinked by glycosidic bonds. The nature, linkage, sequence, and substitution of individual residues within a repeating unit can vary, giving rise to an enormous range of possible structures (Doran, 1992). The O-Ag region is characteristic of a bacterial strain and forms the basis of bacterial serotype specificity, which can be used as a means of identification. The potential for antigenic variation in the O-antigen region is exemplified by the fact that over 160 serotypes have been described in *E. coli*, compared to only 5 different core types (Rietschel *et al*, 1993).

Biological activity

Approximately 100 years ago, the word 'endotoxin' was introduced to describe a poisonous material, bound to the bacterial body and differing from the exotoxins, that induced severe pathophysiological reactions in experimental animals (Rietschel *et al*, 1993). Today, endotoxin is used almost synonymously with LPS. LPS may be defined in terms of a specified molecular structure whereas the word endotoxin often incorporates a broader functional role. For the purpose of this thesis, LPS and endotoxin will be interchangeable unless otherwise stated.

LPS is well-established as the most virulent of all bacterial cell surface determinants and is responsible for many diverse biological effects, including pyrogenicity, shock and mitogenicity (Table 1.1).

The toxicity of LPS is attributed to the lipid A portion as this region is capable of reproducing all the biological effects observed when LPS is administered to experimental animals. However, the lethality associated with lipid A is highly dependent on structure. Indeed, certain bacterial species (e.g. *Bacteroides*) are said

Table 1.1 Biological effects of lipopolysaccharide

Pyrogenicity	Activation of complement
Lethal toxicity	Activation of clotting factors
Circulatory and vascular damage	B-cell mitogen
Hypotension	Induction of cytokines
Shwartzman reaction	Induction of prostaglandins

(Poxton & Arbuthnott, 1990)

to possess LPS of low endotoxicity whilst other species (e.g. *E. coli*) have LPS of high endotoxicity, reflecting the fatty acid composition and phosphate substitution of the lipid A moiety (Poxton & Arbuthnott, 1990). To determine the specific parts of lipid A responsible for endotoxicity, experiments were performed on lipid A partial structures and lipid A analogues. From these studies it was deduced that full endotoxic activity required a lipid A region containing two β-1,6-linked D-glucosamine residues, two phosphoryl groups and six fatty acids in a defined arrangement as found in *E. coli*. Different structures such as monophosphoryl lipid A or deacylated lipid A demonstrated a reduced toxicity (Doran, 1992).

Although the lethality associated with LPS is ascribed to lipid A, its hydrophobic nature means that the complex is insoluble. It is therefore worth stressing that full biological activity is dependent on sugars present in the inner core, which allow dispersal in aqueous solutions. Indeed, in recent years the inner core region has been recognised as being of great significance for the biological activity and function of LPS. The basis for this is not yet understood although it may be due to a Kdo-dependent conformational change of lipid A (Rietschel *et al*, 1993).

The O-antigen region also plays an important role in the biological function of LPS. O-Ag deficient rough mutants can be 1000 times less virulent than their smooth counterparts, implying that the O-Ag can influence the virulence potential of the LPS (Poxton & Arbuthnott, 1990). The O-Ag may also be involved in inhibiting complement activity and providing protection against phagocytosis (Rietschel *et al*, 1993).

LPS is an amphipathic molecule, containing both a hydrophobic (lipid A) and hydrophilic (O-Ag) region. Other amphiphiles are present in bacterial cell envelopes, for example, lipoprotein and enterobacterial common antigen (ECA) and they share some common properties with LPS. Structurally, ECA consists of a glycerol phosphate backbone with attached fatty acids but the lipid portion is much less complex and significantly different from LPS (Rietschel *et al*, 1993). These structural diversities may explain why LPS is capable of causing lethal toxicity whereas other amphiphiles present in the bacterial cell surface appear to lack such potential.

1.1.4 OUTER MEMBRANE

Structure

Gram-negative bacteria possess a complex cell envelope, distinct from Gram-positive bacterial envelopes by possession of both an inner and outer membrane. The inner membrane of Gram-negative bacteria is similar in structure to the cytoplasmic membrane found in Gram-positive bacteria (Poxton, 1993). However, the outer membrane is characteristic of Gram-negative species and is constructed from phospholipids, proteins (OMPs) and LPS. In enteric bacteria approximately 40% of the outer surface is LPS and 60% is protein (Benz, 1988). The LPS is closely associated with the outer membrane and the packing of the lipid A component into the bilayer results in a more viscous, rigid membrane structure compared to the inner membrane. Proteins may be present as major or minor proteins present in the inner or

outer leaflet of the bilayer, lipoproteins or forming transmembrane channels termed porins. Porins are usually found as trimers, bound non-covalently to the peptidoglycan layer and allow passage of essential hydrophilic nutrients into the cell (DiRienzo *et al*, 1978).

LPS and porins function together as a selective barrier, separating the environment from the delicate workings of the bacterial cell; hydrophobic molecules are kept out whilst hydrolytic enzymes and binding proteins found in the periplasm are retained. However, the pharmaceutical industry has exploited this selective barrier by designing antimicrobials that compete with low molecular weight solutes for transfer through a porin, thus gaining access to the inside of the cell (Benz, 1988).

Biological activity

The bacterial outer membrane is a functioning component of normal cell physiology and is essential for viability, by allowing transfer of ions, vitamins and carbohydrates. Furthermore, in conditions of nutrient stress, bacteria can alter the biosynthesis and expression of OMPs to aid survival. Indeed, the ability to vary OMP antigens is a powerful method to escape the host's antibody-dependent defence mechanisms.

The outer membrane also possesses a degree of pathogenic potential by acting as an adhesin and as a receptor site for siderophores. Iron is an essential requirement for bacterial survival. *In vivo* most extracellular iron is bound to host proteins such as transferrin and lactoferrin leaving only minute amounts free in the host tissue. Bacterial siderophores act as iron-chelators and compete for essential iron with the host (Guerinot, 1994). By possessing OMPs functioning as siderophore receptors, the bacteria can effectively compete for the iron needed to survive in an otherwise iron-depleted environment.

Adherence to host tissue is an important step in the initiation of pathogenesis by allowing bacteria to gain a stable hold before colonisation. For example, some *Neisseria gonorrhoeae* species that do not possess adhesive pili are still able to bind cells by virtue of an OMP adhesin, termed OM protein II or P II. Gonococcal species expressing P II are associated with infection localised to urogenital mucosa (Schwalbe *et al*, 1985) and have several other important virulence attributes, including improved attachment to buccal epithelial cells, increased serum resistance, increased resistance to penicillin and fusidic acid and increased attachment to leucocytes (Lambden *et al*, 1979).

Some bacterial species, for example *Yersinia* and *Shigella*, are invasive pathogens, capable of entering a cell, multiplying and then destroying it. OMPs have been proposed as important virulence determinants in this invasive process, although how the OMPs contribute is not clearly understood. One theory is that some OMPs manipulate the host cytoskeleton to provoke uptake, whilst others are required for transport of essential invasion plasmid antigens (Maurelli, 1992).

Peptidoglycan

Sandwiched between the inner and outer membrane is a layer of peptidoglycan which confers rigidity and shape on the bacterial cell. The peptidoglycan layer is associated with porins from the outer membrane and a small lipoprotein which binds the outer membrane to the peptidoglycan. The structure, attachment and biological functions of peptidoglycan are well-documented and the reader is referred to the following articles (Schleifer & Kandler, 1972; Clarke & Dupont, 1991; Johannsen, 1993).

1.1.5 SURFACE APPENDAGES

Inserted into the outer membrane of many Gram-negative bacteria are two further structures or appendages; fimbriae (pili) and flagella. Both fimbriae and flagella are

composed of subunits of a single protein (pilin and flagellin respectively), that are aggregated to form tight helical chains. Bacteria may possess several types of fimbriae that differ in thickness, length and antigenic specificity. They are shorter and thinner than flagella and function as adhesins in aqueous environments, to particulate material and to host cells (Krogfelt, 1990). Flagella may be present on the bacterial surface as a single polar appendage (monotrichous) or as numerous fibres (peritrichous). Furthermore, flagellin proteins from different species vary in amino acid composition and are highly immunogenic, forming the basis of another serotyping scheme (Poxton & Arbuthnott, 1990). Flagella are responsible for motility, particularly in response to chemoattractants or repellants, a characteristic which is of extreme importance to bacterial survival.

1.1.6 EXOPOLYSACCHARIDE

Structure

Extracellular polysaccharides or exopolysaccharides (EPS) are secreted by many bacterial species to form either loosely associated slime or a discrete layer of mucilaginous material termed a capsule (Whitfield & Valvano, 1993). EPS may be divided into homopolysaccharides or heteropolysaccharides. Homopolysaccharides are composed of a single repeating sugar unit, for example, the slime produced from *Streptococcus mutans* consists entirely of D-glucose. Heteropolysaccharides are more commonly found and contain repeating units of two or more sugar monomers. A large number of heteropolysaccharides have been described, with many different sugar components identified. Monomers may include neutral sugars such as D-glucose and D-galactose, amino sugars, for example N-acetyl-D-glucosamine and uronic acids such as D-glucuronic acid. The latter group confers an acidic property on the EPS as do some non-sugar substituents such as acetate, formate and pyruvate (Whitfield & Valvano, 1993; Sutherland, 1985).

Biological activity

The role of EPS in adhesion has been described for many bacteria including oral streptococci (Loesche, 1986), staphylococci (Christensen *et al*, 1982), common environmental strains (Costerton *et al*, 1987) and freshwater and marine bacteria (Allison & Sutherland, 1987). Initially, the bacterial cell binds to a surface through non-specific adhesion. Cell division produces new daughter cells that also bind so ultimately an adherent microcolony embedded within an EPS biofilm develops (Costerton *et al*, 1987).

In may instances, establishment of a biofilm represents the main role for EPS: protection. EPS is, by its physical nature, highly hydrated and thus protects against desiccation. For pathogens, EPS also affords protection against phagocytosis and the action of phage, antibodies and antibiotics, by the simple fact that the biofilm is too extensive to engulf or penetrate. In large biofilms, insoluble salts produced by bacteria may become trapped in the EPS matrices and form crystallised 'stones' within the colonised host organ (Costerton *et al*, 1987).

The classic example of EPS as a virulence determinant is alginate, an unusual acetylated exopolysaccharide produced by mucoid *Pseudomonas aeruginosa* (section 1.2). EPS has also been reported to induce abscesses, as observed with the capsule of *Bacteroides fragilis* (section 1.5) and to act as a camouflage mechanism against host immune defences, such as occurs with the K1 antigens of *E. coli* (Poxton & Arbuthnott, 1990).

1.1.7 CELL SURFACE HYDROPHOBICITY

The combination of structures associated with the cell envelope bestows on the bacterial surface a relative degree of hydrophobicity. Cell surface hydrophobicity plays an important part in three aspects of bacterial infection; adhesion, phagocytosis

and opsonization (Absolom, 1988). For initial adhesion and orientation of bacteria to a substrate, such as host tissue, the more hydrophobic the cell surface the more readily the bacterium can bind (Dalhback *et al*, 1981). For example, in enteropathogenic *E. coli*, masking of EPS and LPS with hydrophobic fimbriae promotes adhesion to intestinal epithelial cells (Smyth *et al*, 1978). However, a high level of cell surface hydrophobicity also promotes phagocytosis. This could be explained in part by non-specific adsorption of antibody onto the bacterial surface; the more hydrophobic the bacteria, the more IgG is adsorbed, thus augmenting cell surface hydrophobicity and increasing the chances of phagocytosis (Stjernström *et al*, 1977, Absolom, 1988).

1.1.8 EXTRACELLULAR VIRULENCE DETERMINANTS

Extracellular virulence determinants comprise extracellular enzymes and exotoxins. Exoenzymes, such as mucinases, collagenases, hyaluronidases, lipases and haemolysins, to name but a few, are capable of causing host tissue damage by virtue of their degradative properties and can promote the spread of bacteria into deeper tissues (Talaro & Talaro, 1993). Exotoxins are the classic extracellular virulence factors. In some cases they may be almost singularly responsible for the development of disease, for example botulism. Toxins exert their action by damaging an essential metabolic cell function and act on a variety of systems including mucosal epithelial cells, subepithelial tissues and major internal organs (Poxton & Arbuthnott, 1990).

The cell surface of a gram-negative bacterium comprises several closely associated elements, each with a discrete structure, function and level of pathological potential. Together with extracellular virulence determinants, the cell envelope imparts on the bacterium the ability to cause disease and thus plays an important role in pathogenesis.

1.2 ENVIRONMENTAL REGULATION OF THE BACTERIAL CELL SURFACE.

The microenvironment surrounding a bacterium can be highly variable in terms of chemical and physical factors, such as oxygen concentration, pH, osmolarity and temperature. Many bacteria will encounter several different environmental conditions during their life cycle and must be able to respond and adapt to any change if they are to survive. Adaptive responses in bacteria are central to many cellular systems and are tightly controlled by complex pathways. The best-characterised regulatory process is signal transduction, where bacteria 'sense' environmental stimuli through membrane-bound sensory proteins. The sensor proteins, in turn, phosphorylate and activate a regulatory response protein to mediate an appropriate adaptive reaction, usually by control of gene transcription. (Gross, 1993).

The bacterial cell surface plays a major role in pathogenesis and, as such, is flexible to allow survival. Indeed, the bacterial surface is far from a rigid structure but is constantly adapting to new environmental parameters. LPS, OMPs, EPS and thus their corresponding functions, complement resistance, adhesion, invasion, biofilm formation, protection etc., are all well-documented cases of environmentally-regulated bacterial virulence systems. Many fascinating examples are covered in depth in the following reviews; Stock *et al*, 1989; Gross, 1993; Maurelli, 1989; Miller *et al,* 1989). Examples are provided here simply to emphasise the plasticity of the bacterial cell surface structures relevant to this thesis.

Exopolysaccharides

One of the classic examples highlighting sensory regulation of bacterial virulence is the control of the exopolysaccharide alginate, produced by mucoid *Pseudomonas aeruginosa.* Alginate is a β,1-4-linked mannuronic-guluronic acid heteropolysaccharide, the production and secretion of which leads to the distinctive

mucoid phenotype almost exclusively associated with chronic pulmonary infections of Cystic Fibrosis (CF) patients (Pedersen, 1992). The use of phenotypic and genotypic typing systems has shown that initial colonisation in CF patients is due to typical non-mucoid strains and that mucoid variants gradually come to predominate as a result of *in vivo* conversion (Govan & Nelson, 1992). These observations suggest that the production of alginate by *P. aeruginosa* is an adaptation to the specialised conditions in the CF lung, allowing the bacteria to survive inside the host and conferring an advantage to the bacterium in the host-pathogen fight. Indeed, alginate is a key factor in the pathogenesis of CF due to both its adhesive properties and in the formation of microcolonies and biofilms that block phagocytosis and ultimately lead to immune-mediated damage and bronchial obstruction. It has been speculated that many *P. aeruginosa* strains, under conditions of increased osmolarity, nitrogen limitation or dehydration (conditions thought to be prevalent in the CF lung), increase transcription of three key alginate biosynthesis genes, *algD, algC* and *algR* (Deretic *et al,* 1990; Zielinski *et al*, 1992). Thus, alginate production is up-regulated only under certain environmental conditions, which is of obvious benefit, as constitutive expression would place a considerable energy drain on the pathogen.

Environmental regulation of alginate biosynthesis is a complex genetic process involving many regulatory proteins and processes, such as, AlgR, an orthodox response regulator and one component of a bacterial signal transduction system, AlgP, a histone-like protein controlling nucleoid structure, DNA supercoiling and *muc* mutations which are conducive to the mucoid phenotype (Deretic & Konyecsni, 1990; Drlica & Rouviere-Yaniv, 1987; Dorman, 1991; Govan *et al,* 1992). Indeed, such is the intricacy of alginate regulation that control of mucoidy has become a model system for analysing environmental modulation of bacterial virulence determinants.

Outer membrane proteins

When grown in environmentally-stressed conditions, Gram-negative bacteria alter the porin composition of their outer membrane, to change their overall permeability and aid survival. OMPs are regulated in response to a number of environmental stimuli including temperature, carbon source, phosphate and iron starvation (Mekalanos, 1992). Not all cases of OMP regulation involve porin function, in some instances the OMPs induced are invasive proteins (Maurelli, 1989) or iron-binding proteins (Miller *et al*, 1989) vital to pathogenesis.

Lipopolysaccharides

Several bacterial species including *E. coli, P. aeruginosa* and *Serratia marcescens* alter O-Ag chain length in different growth media. Temperature, osmolarity, pH, carbon source, and ion concentration have all been reported to cause appreciable changes in O-Ag length as determined by SDS-PAGE (McGroarty & Rivera, 1990, Weiss *et al*, 1986). These changes obviously alter the bacterium's ability to resist host defences including in particular the action of complement.

Environmental sensors and regulators from different bacterial signal transduction systems share a high degree of sequence homology, thus, the components of a single pathway are not exclusive to each other but can interact with other systems to allow a greater level of flexibility and control (Stock *et al*, 1989). For example, an orthodox histidine-kinase osmosensor, OmpR, may substitute for AlgR in activating the *algD* promoter, under conditions of high osmolarity (Berry *et al*, 1989). Furthermore, the bifunctional enzyme AlgC plays a pivotal role in regulating the loss of LPS O-Ag in mucoid *P. aeruginosa* CF isolates, by switching carbon metabolism from LPS biosynthesis to alginate production (Ye *et al*, 1994). This 'cross-talking' between systems can allow interpretation of multiple signals and subsequent co-ordinated regulation of several genes.

1.3 THE HOST IMMUNE SYSTEM IN PATHOGENESIS

Bacterial pathogenesis involves an ongoing two-way fight for survival between microbe and host. One of the major 'battles' involves the host's immune response, a defensive system which has evolved and adapted to protect the host against infection but which the microbe must resist if it is to survive.

1.3.1 THE IMMUNE RESPONSE AGAINST BACTERIAL PATHOGENS

In humans, the immune response to an invading microbe can be separated into two functional divisions; innate immunity and adaptive immunity.

Innate immunity

Innate immunity is characterised by non-specific defences which individuals possess all their life. Innate immunity can be divided further into those mechanisms that act immediately and non-specific mechanisms that have to be induced and appear early in the infection process.

The first stage of innate immunity, the immediate response, consists of pre-existing defence mechanisms that have no need for induction. For example, our body surfaces, which are defended by epithelial cells that provide a barrier by means of chemical (low pH in stomach, fatty acids on skin, degradative enzymes e.g. lysozyme), microbiological (competition with normal flora) and mechanical (tight junctions between cells) mechanisms. The alternative pathway of complement is also considered an immediate response mechanism as the complement proteins required for the complement cascade are continually present in our plasma (Janeway & Travers, 1994).

If a potential pathogen successfully survives the immediate response and penetrates the epithelial barrier, the host's early defences are induced, although these mechanisms

remain non-specific. The most important response in the early induced phase is the secretion of cytokines. Cytokines are proteins or glycoproteins that can act on numerous cell types both locally and systemically, to initiate a variety of inflammatory reactions (Rees, 1991). The local and systemic effects of cytokine induction shall be discussed in detail in section 1.3.6-7. A group of proteins collectively termed acute phase proteins are also induced in the early phase of infection. Produced in the liver, acute phase proteins can bind to the surface of a wide range of bacteria, facilitating the activation of complement and promoting bacterial uptake by phagocytic cells (Janeway & Travers, 1994).

Adaptive immunity

Most of the bacteria that humans encounter are successfully destroyed by our innate immune mechanisms. However, if after approximately four days an infection remains, components of the host's adaptive immunity can be detected. Adaptive immunity differs from innate immunity by consisting of specific defences that are acquired during the lifetime of an individual against specific pathogens. In other words, adaptive immunity involves immunological memory and generates a lasting, protective response. This type of immunological defence is highly important as it allows the host to respond much more rapidly, intensely and specifically on subsequent encounters with the pathogen. Antibodies are the crux of adaptive immunity, able to initiate the classical pathway of complement and, more importantly, provide a mechanism through which the host can recognise all pathogens. Antibodies *per se* shall not be discussed here although reference to them and, in particular, to their specific binding of cell surface structures shall be made in the following sections.

1.3.2 INTERACTION OF BACTERIA AND THE HOST IMMUNE SYSTEM

Many of the defences used by bacteria to resist the host immune mechanisms involve cell surface structures referred to in section 1.1. For example, EPS can inhibit

phagocytosis and OMP profiles may change to alter the microbe's adhesive properties or permeability to antibiotics.

The interaction between the bacterial cell surface and the host's immune system is very complex, as exemplified by the host's multiple responses to endotoxin. Low dose exposure to LPS released from gut-associated bacteria or in low-level infection may, ironically, be important in the development of lymphoid organs and innate immunity, thus priming the host to resist bacterial infection (Manthey & Vogel, 1992). LPS is also a potent stimulator of cytokines which are essential for the development, maintenance and regulation of immunity. However, the pathological damage resulting from overproduction of cytokines following systemic or high dose endotoxin exposure is extremely severe and potentially fatal.

The following sections shall describe in more detail the steps involved in the LPS-induced stimulation of cytokines and the clinical consequences of cytokine overproduction. It should be noted, however, that other bacterial cell surface structures may play a significant role in cytokine release. EPS has already been implicated in the induction of tumour necrosis factor (Otterlei *et al,* 1993) and the outer membrane will undoubtedly be important in contributing to LPS presentation and thus, indirectly, LPS binding. It should also be emphasised that cytokines can be induced from nearly every cell type in the mammalian body.

1.3.3 LPS-INDUCED RELEASE OF CYTOKINES

LPS receptor binding

The first step in LPS-induced cytokine secretion is the binding of endotoxin to host cells. Initially, three main LPS-receptors were proposed; CD18 antigens, a scavenger receptor and CD14.

The CD18 antigens or leucocyte integrins, comprise a family of three cell-surface glycoproteins; lymphocyte function associated antigen (LFA-1), complement receptor CR-3 and p150,95. Each member consists of two non-covalently bound chains, a β-chain that is identical in all three receptors and an α-chain unique to each molecule (Wright, 1991). The ability of CD18 antigens to bind LPS has been determined by examining macrophages from individuals with a genetic deficiency in CD18. The $CD18^-$ cells were found to be unable to recognise free LPS or unopsonized *E. coli* cells (Wright *et al,* 1989). In addition, the use of R-LPS mutants demonstrated that the CD18 receptor recognised the lipid A region (Wright & Jong, 1986).

The scavenger receptor is a trimeric, transmembrane glycoprotein able to recognise a wide variety of ligands including analogues of lipid A. Found in large quantities in the liver, it was proposed that the scavenger receptor would be well positioned to bind endotoxin entering the circulation from the gut, as might occur following intestinal injury (Wright, 1991).

Although the scavenger receptor and CD18 antigens are all capable of binding LPS and are, therefore, endotoxin receptors, studies in which these molecules were blocked or absent found that cytokine secretion was not affected, showing these receptors to be unnecessary for secretory responses (Wright *et al*, 1990a). It is now thought that the CD18 family and the scavenger receptor play a vital role in the clearance and detoxification of LPS (and thus Gram-negative bacteria) by stimulating phagocytic engulfment (Wright, 1991).

CD14, a 55kD glycoprotein expressed on the surface of monocytes, macrophages and neutrophils, has also been proposed as an endotoxin receptor. Experiments using monoclonal antibodies (MAbs) to deplete macrophage cell surface proteins identified CD14 as an essential component for the binding of an LPS-plasma protein (LBP)

complex (Wright *et al,* 1990b). Lipopolysaccharide binding protein (LBP) is a 60kD glycoprotein present in normal serum at levels less than 0.5μg/ml which increase over one hundred fold after the induction of the acute phase response (Schummann *et al,* 1990). LBP can bind efficiently to lipid A from both R- and S-LPS and to the lipid A precursor IVA. Indeed, LBP can bind lipid A when LPS is in a variety of physical forms including immobilised on microtiter plates, electrophoresed to nitrocellulose and contained in defined buffers and serum (Tobias *et al,* 1989). In contrast, partial lipid A structures such as the diacyl glucosamine phosphate, lipid X, are bound very poorly and no binding is observed for Kdo, DNA, RNA, heparin or phospholipids, indicating that LBP requires an intact lipid A structure for optimal binding. The concentration of LBP present in normal serum is sufficient to increase significantly LPS-induced cytokine secretion, whereas LBP alone does not induce detectable increases in cytokine mRNA or protein. In the absence of LBP, LPS can still bind to CD14 albeit weakly and much more slowly. Expression of CD14 is essential for the function of LBP as neutralising MAbs to CD14 inhibit the cellular responses of LPS-LBP complexes (Dentener *et al*, 1993).

CD14 is also found as a soluble form present in serum (sCD14) and accounts for approximately 99% of the total CD14 content (Hailman *et al*, 1994). sCD14 is capable of binding LPS and inducing cellular responses and it is speculated that sCD14 enables cells that do not express the membrane form of CD14 (for example endothelial cells) to be LPS-responsive. The role of LBP in LPS binding to sCD14 is thought to be primarily as a catalyst, as LBP dissociates from the LPS-sCD14 complex.

Figure 1.2, shows the best-characterised model of endotoxin receptor binding to date. In high concentrations, LPS molecules clump together due to their amphipathic nature in the host's aqueous environment (Schands & Chun, 1980). The ability of LBP to

bind strongly to lipid A (dissociation constant 10^{-9}M) forces the disaggregation of the lipopolysaccharide clump into monomers to form LPS-LBP complexes (Tobias *et al*, 1989). CD14 can recognise and bind the LPS-LBP complexes and initiate secretory responses. Recently, CD14 mutants have been used to demonstrate that amino acids 57-64 are required for LPS binding (McGinley *et al*, 1995; Juan *et al*, 1995). In addition, residues 91-108 in LBP have been identified as forming at least part of the LPS-binding site (Taylor, 1995). Interestingly, both regions can, independently, form part of a high amphipathic domain similar to the amphipathic loops of the antibiotic polymyxin B and Limulus anti-LPS factor, molecules also capable of LPS binding (Juan *et al*, 1995). Further information is awaited to confirm whether amphipathic domains are a prerequisite for LPS binding.

LPS can bind to a variety of other molecules, including the enzyme lysozyme, polymyxin B, bactericidal/permeability increasing protein (a trace plasma protein sharing 44% homology with LBP) and an 80kD protein tentatively suggested as another potential endotoxin receptor (Takada *et al*, 1994). The 80kD protein has been shown to be membrane localised and is present on all LPS-responsive cells (Lei & Morrison, 1988). Further characterisation and binding studies with this 80kD protein are required to assess its importance as an endotoxin receptor.

Intracellular signalling

The second stage in endotoxin-induced cytokine secretion is the transformation of the LPS binding stimulus into an intracellular signal stimulating cytokine release. The mechanism by which the LPS-LBP/CD14 pathway functions in intracellular signalling is not yet understood. Two models have been proposed; LPS-LBP binding to CD14 directly stimulates the cell or CD14 acts as a holding or transfer protein, facilitating the interaction of LPS with another signalling molecule (Figure 1.2).

Evidence has been obtained to support both models. For the former theory, studies have shown that certain anti-CD14 MAbs can mimic the effects of LPS (Schütt *et al*, 1988). However, at present, biochemical and structural evidence favours the latter model. The CD14 receptor does not transverse the membrane but is instead anchored by a glycosylphosphatidylinositol (GPI) tail (Gegner *et al*, 1995). GPI-anchored proteins are unlike the G-linked proteins normally required for eukaryote signalling. The mechanism by which a GPI-protein initiates an intracellular signal is not known, as experiments replacing the GPI anchor with transmembrane domains have shown the GPI tail to be unnecessary for signal transduction, suggesting that CD14 interacts with another, as yet unknown, receptor to initiate the secretory response (Lee *et al*, 1993). Furthermore, as soluble CD14 must bind to a cell surface protein in order to initiate a response, it is probable that membrane CD14 transduces the signal in the same way (Frey *et al*, 1992).

It seems probable that LPS binding to CD14 alters the protein conformation, allowing the complex to interact with a transmembrane receptor. The transmembrane receptor could then transform LPS binding into an intracellular signal by activating a complex interactive network of kinases, similar to signal transduction in bacteria. Indeed, LPS binding is known to induce rapid tyrosine phosphorylation in several proteins including a serine/threonine protein (MAP kinase) known to be important in cell proliferation (Manthey & Vogel, 1992). However, the identification of the tyrosine kinase that phosphorylates the MAP protein remains unknown.

An additional complicating factor in the LPS signalling scenario is that in the absence of LBP and CD14, LPS-induced secretory responses still occur. CD14-independent signalling pathways have been suggested by several workers although, as yet, no conclusive candidates have been found (Lynn *et al*, 1993). It is likely that future studies on eukaryotic cell surface proteins will identify further LPS receptors and

intracellular signalling pathways. It is therefore worth emphasising that although CD14/LBP is the best characterised LPS signalling pathway to date, it is not unique.

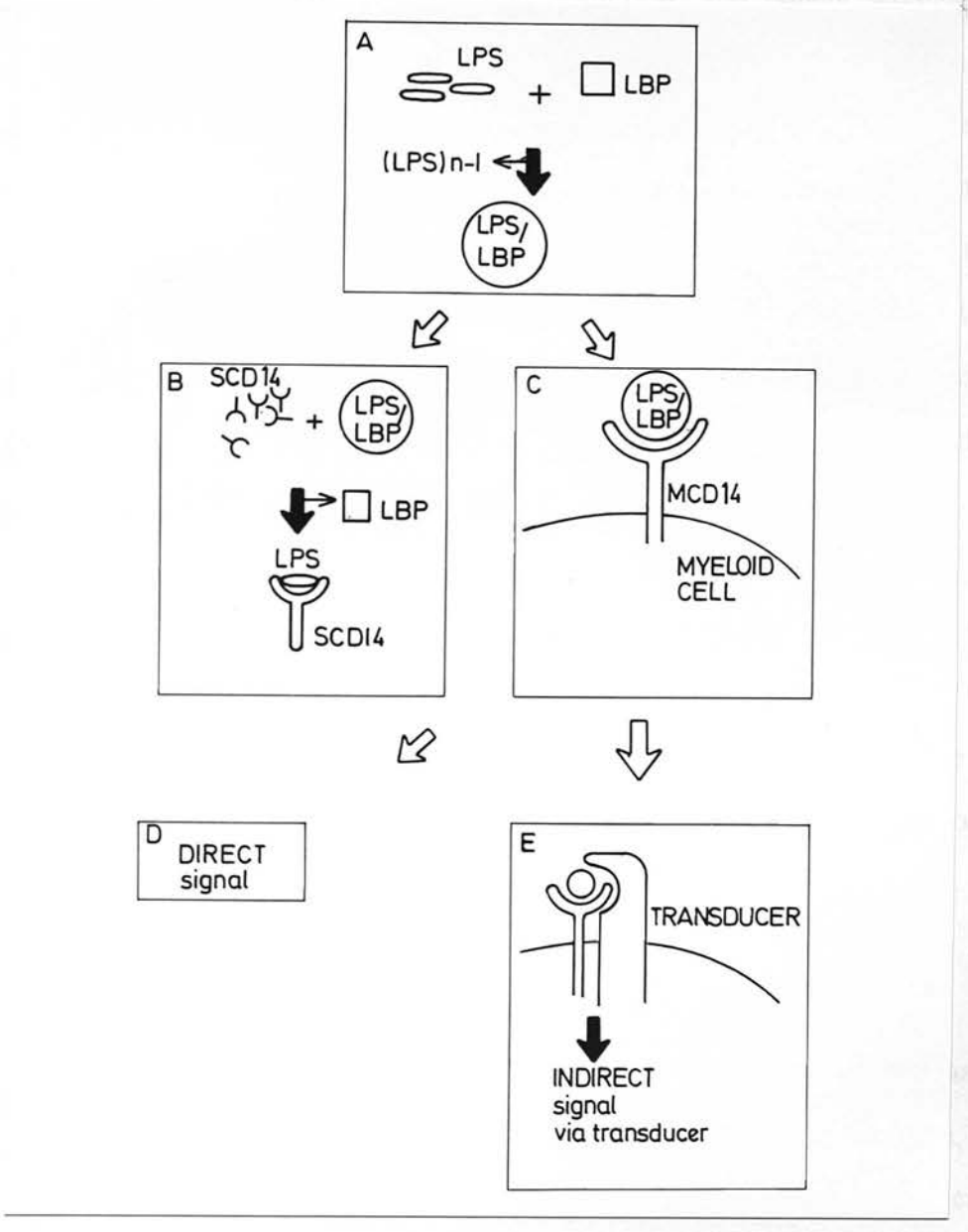


Figure 1.2 LPS receptor binding and intracellular signalling. **(A)** LPS and LBP bind to form an LPS-LBP complex. **(B)** sCD14 recognises the LPS-LBP complex and binds LPS. LBP acts as a catalyst and dissociates. **(C)** The membrane form of CD14 recognises and binds the whole LPS-LBP complex. **(D)** CD14 may transduce the LPS binding stimuli direct. **(E)** CD14 acts as a holding protein for LPS and transduces the binding stimuli via a transmembrane receptor.
LPS = lipopolysaccharide; LBP = lipopolysaccharide binding protein;
sCD14 = soluble CD14; mCD14 = membrane-bound CD14

Cytokine release

The final step after an intracellular signal has been produced is the release of cytokines. Two major cytokines, tumour necrosis factor α (TNF-α) and interleukin 1 (IL-1) are preformed, held within the cell membrane, and rapidly secreted. In contrast, the majority of cytokines require the derepression of regulatory genes prior to cytokine synthesis (Rees, 1991). LPS-inducible genes possess enhancer regions that can be bound by proteins such as NF-κB. In the normal state NF-κB is repressed by another protein, IκB. Upon activation due to LPS stimulation, IκB is phosphorylated and the NF-κB heterodimer dissociates and translocates to the nucleus where it binds upstream of genes possessing the appropriate enhancer region, thus initiating transcription. The protein kinase involved in the phosphorylation of IκB is unknown (Manthey & Vogel, 1992).

The following sections will concentrate on the two major cytokines relevant to this thesis, TNF-α and IL-8, and emphasise their role in both the local and systemic effects of cytokine release. It is important to stress, however, that any one cytokine is just part of a complex biological network, interacting with many other immunological mediators to prevent microbial infection.

1.3.4 TUMOUR NECROSIS FACTOR-α

Tumour necrosis factor alpha (TNF-α) is a 17kD polypeptide existing as three identical subunits and secreted from a variety of cells (Smith & Baglioni, 1987) (Figure 1.3). Initially, TNF-α was named cachectin due to its role in inducing the cachectic or wasted state which may accompany chronic parasitic and viral infection or cancer. As the name suggests, TNF-α has also been shown to cause necrosis in experimental tumours and had seemed promising as an effective treatment for cancer (Rees, 1991). However, in clinical trials, TNF-α inhibited tumour growth in only a few patients and was associated with a variety of toxic effects, including fever,

hypotension, and leukopenia; symptoms also associated with endotoxic shock (Creagan *et al*, 1988). Indeed, TNF-α is now considered to be a primary mediator of endotoxic shock and is one of the first cytokines induced in serum following LPS administration (Manthey & Vogel, 1992). The actions of TNF-α are extremely varied, affecting numerous cell types as shown in Figure 1.3. Due to the extensive literature on the subject, only the most important points are summarised.

Release of TNF-α from macrophages does not require the presence of other cytokines, although an important property of TNF-α is the ability to induce other cytokines, and in some cases, upregulate its own production (Manthey & Vogel, 1992). TNF-α induces cytokine production by binding to specific, high affinity cell surface receptors; a 55kD and a 75kD protein named TNFR1 and TNFR2 respectively. The majority of host cell types and tissues express both receptors although TNFR1 is preferentially expressed on epithelial cells whereas TNFR2 is more abundant on cells of myeloid origin (Ding & Porteu, 1992). The extracellular domains of the two TNF receptors share 28% homology and can be divided into four cysteine-rich repeats. In comparison, the intracellular regions show no apparent similarity, suggesting that the two receptors signal using different pathways (Tartaglia & Goeddel, 1992).

It is proposed that at low concentrations, TNF preferentially binds to the high affinity receptor TNFR2. At higher levels, TNF can bind to TNFR1, due to either increased local concentration (as a result of binding to TNFR2 at the surface) or due to increased release of TNF-α from cells (Tartaglia & Goeddel, 1992). Using receptor antagonists, TNFR1 has been shown to signal a range of biological activities, including cytotoxicity, induction of NF-κB and antiviral activity. Thymocyte and cytotoxic T-lymphocyte proliferation have been attributed to TNFR2. Crosslinking of the receptors and, after binding, internalisation and degradation of the cytokine have

also been suggested as important components of the activation process. Experiments using anti-TNFR MAb fragments (Engelmann *et al*, 1990) and agents inhibiting receptor internalisation and lysosomal processing (Kull & Cuatrecasas, 1981) have been shown to block the effects of TNF-α.

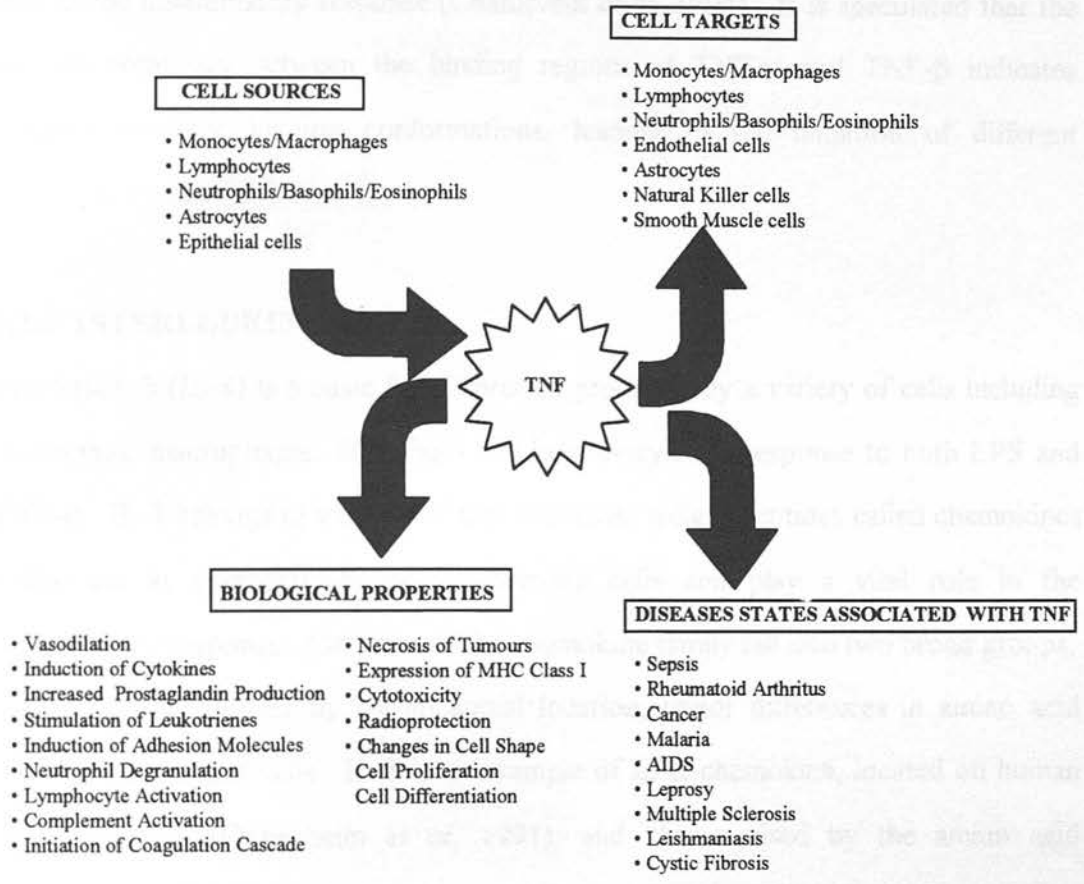


Figure 1.3 Biological properties of TNF-α

Similar to LPS stimulation of cytokines, once TNF-α has bound to its receptors a signal is transduced across the membrane to activate intracellular biochemical pathways. Messenger proteins within the cell including the transcription factor NF-κB associate with promoter sequences upstream of TNF-inducible genes to initiate transcription (Vassalli, 1992).

TNF-β or lymphotoxin shares 28% homology with TNF-α, and shows similar properties associated with inflammation and anti-tumour activity. Furthermore, both cytokines compete for the same receptors and can displace each other. TNF-β, however, is a 25kD glycoprotein, produced only by lymphocytes and is induced much later in the inflammatory response (Chaturvedi *et al*, 1994). It is speculated that the lack of homology between the binding regions of TNF-α and TNF-β indicates different receptor binding conformations, leading to the initiation of different biological events.

1.3.5 INTERLEUKIN-8 (IL-8)

Interleukin-8 (IL-8) is a basic 8.4kD protein produced by a variety of cells including monocytes, macrophages, fibroblasts and keratinocytes in response to both LPS and TNF-α. IL-8 belongs to a family of low molecular weight peptides called chemokines which act as chemoattractants for immune cells and play a vital role in the inflammatory response. Members of the chemokine family fall into two broad groups, α and β, distinguished by chromosomal location, minor differences in amino acid sequence and target cells. IL-8 is an example of an α-chemokine, located on human chromosome 4 (Oppenheim *et al*, 1991), and characterised by the amino acid arrangement -C-X-C-, where C corresponds to a cysteine residue and X any other amino acid (Leibler *et al*, 1994). β-chemokines are found on human chromosome 17 and have the cysteine residue structure -C-C-. α-chemokines possess chemotactic activity for neutrophils, lymphocytes and basophils, whereas β-cytokines attract monocytes (Leibler *et al*, 1994). The cellular effects of IL-8 are shown in Figure 1.4.

Approximately one hour after TNF-α stimulation, IL-8 mRNA levels increase, reaching a maximum after three hours. Site mutagenesis has established the region of the IL-8 gene from -94 to -71bp to be the minimum sufficient for conferring responsiveness to TNF-α. This region resembles a potential binding site for

transcription factors such as NF-κB. In addition, studies using cycloheximide indicated that the induction of IL-8 mRNA occurs despite the absence of new protein synthesis, suggesting that a repressor may be involved in negative regulation of IL-8 (Oppenheim *et al*, 1991).

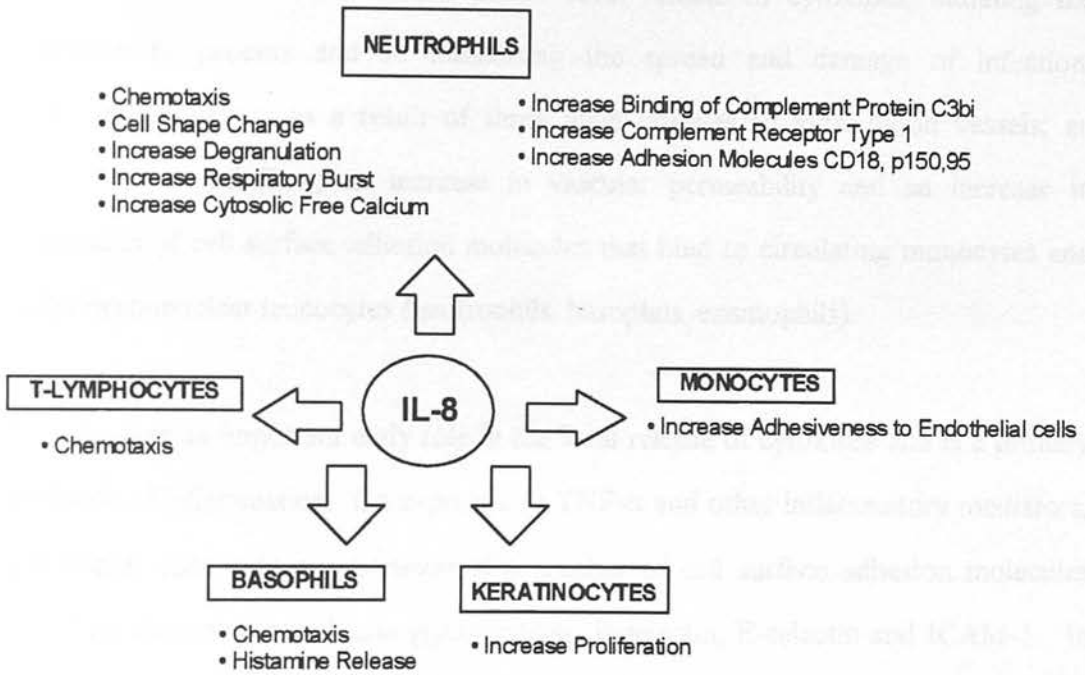


Figure 1.4 Biological properties of IL-8.

Once induced, IL-8 can interact with its own high-affinity receptor, an integral membrane protein present on the surface of neutrophils and T-lymphocytes and comprising seven membrane-spanning helices, capable of activating G proteins (Janeway & Travers, 1994). IL-8 binding to its receptor rapidly phosphorylates two cytosolic proteins of 48kD and 65kD, known previously to be phosphorylated by other chemoattractants. The 48kD and 65kD proteins are therefore thought to play a vital role in the signal transduction of leucocyte migration (Oppenheim *et al*, 1991). Rapid recycling of the IL-8 receptor is also thought to be essential for the chemotactic response of neutrophils, as internalisation of the IL-8-receptor complex transmits

intracellular signals leading to alteration of the actin cytoskeleton, which is known to be involved in the motile response (Downey *et al*, 1994).

1.3.6 LOCAL RESPONSE TO CYTOKINE SECRETION

A local bacterial infection results in the local release of cytokines, initiating the inflammatory process and so minimising the spread and damage of infection. Inflammation occurs as a result of three main changes to local blood vessels; an increase in blood flow, an increase in vascular permeability and an increase in expression of cell surface adhesion molecules that bind to circulating monocytes and polymorphonuclear leucocytes (neutrophils, basophils, eosinophils).

TNF-α plays an important early role in the local release of cytokines and is a primary mediator of inflammation. On exposure to TNF-α and other inflammatory mediators, endothelial cells induce expression of a number of cell surface adhesion molecules including three transmembrane glycoproteins; P-selectin, E-selectin and ICAM-1. In response to mediators such as histamine and thrombin, P-selectin is very rapidly mobilised onto the endothelial cell surface. E-selectin is induced in response to TNF-α but as it requires *de novo* mRNA and protein synthesis, expression is delayed (Springer, 1994). Interaction of the two selectin molecules with the carbohydrate moiety, sialylated Lewis x, present on circulating neutrophils, monocytes and T-lymphocytes, allows the leucocytes to adhere weakly to the blood vessel wall. This initial labile adhesion lets leucocytes 'roll' in the direction of blood flow and permits a stronger binding, dependent on the interaction between ICAM-1 and the leucocyte integrin LFA-1 (previously mentioned in section 1.3.3 as a member of the CD18 family) present on the phagocytic cell surface (Lawrence & Springer, 1991; Springer, 1994). In the resting state, LFA-1 has a relatively low affinity for ICAM-1, however, the presence of IL-8 triggers a conformational change in LFA-1 to increase greatly its

ability to bind to ICAM-1 (Janeway & Travers, 1994). Thus, in the presence of IL-8, leucocytes are held firmly attached to the endothelium (Figure 1.5).

The next stage in inflammation is diapedesis, where leucocytes move out of the blood vessels into infected tissues, a process requiring changes to the blood vessel walls. TNF-α increases vascular permeability and vascular diameter, which allows the attached leucocyte to cross the endothelial wall. The process of diapedesis involves another cell surface adhesion molecule, CD31, a member of the same family as ICAM-1. CD31 is constitutively expressed on all endothelial junctions and on leucocytes. It is thought that mediators such as histamine stimulate phosphorylation of CD31 to modulate cytoskeletal interactions and so permit leucocyte movement (Bevilacqua, 1993). Chemokine binding to proteoglycan molecules in the extracellular matrix directs leucocytes to invading bacteria where immune cells can phagocytose and remove the microbes. The whole process is shown in Figure 1.5.

IL-8 therefore plays a major part in the inflammatory process. Firstly, IL-8 arrests the circulation of the cell. Secondly, release of IL-8 and other chemokines forms a concentration gradient directing the leucocyte cell migration to the infection focus. Thirdly, chemokines activate their respective target cells; thus, cells are not only brought to infection sites but are simultaneously armed to deal with any pathogens they encounter (Janeway & Travers, 1994).

Endothelial cells also express molecules that trigger local blood vessel occlusion, with excess fluid drained to the regional lymph nodes, thereby preventing the spread of infection. Thus, local cytokine release helps the host to fight bacterial infection.

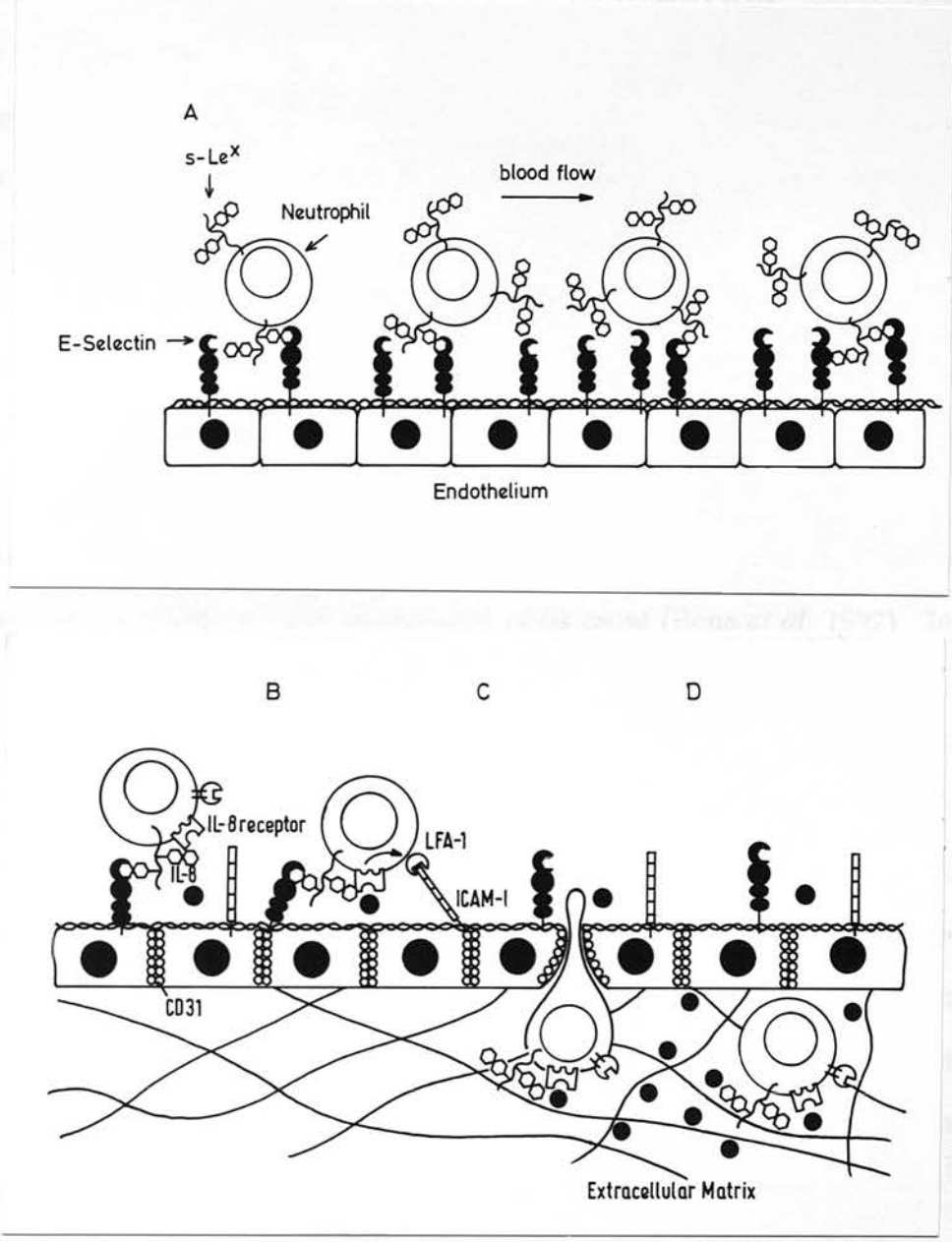


Figure 1.5 Mechanisms of the inflammatory process. **(A)** Weak adhesion of leucocytes to the vascular endothelial surface is mediated via E-selectin and s-Le^X, allowing neutrophils to roll in the direction of the blood flow **(B)** The presence of IL-8 mediates a strong binding between ICAM-1 and LFA-1 holding the leucocytes in place. **(C)** Leucocytes move out of the blood vessels. **(D)** Immune cells migrate to the site of infection along the chemokine concentration gradient.
s-Le^X = sialylated Lewis X; LFA-1 = lymphocyte function associated antigen;
ICAM-1 = intracellular adhesion molecule.
(Adapted from Janeway & Travers, 1994)

1.3.7 SYSTEMIC RESPONSE TO CYTOKINE SECRETION

Once invading bacteria spread from the initial infection site into the circulation cells throughout the body may respond and release cytokines. The mechanisms governing systemic release of cytokines are the same as those that function locally. However, the consequences of systemic cytokine release are catastrophic and often fatal. The term 'sepsis' has traditionally been used to define the systemic response to infection. However, it is worth stressing that a systemic inflammatory response can arise in the absence of detectable infection; non-infectious causes include pancreatitis, ischaemia and trauma. The term 'systemic inflammatory response syndrome' (SIRS), was proposed at a consensus conference of chest physicians in 1991 to describe the systemic inflammatory process, independent of its cause (Bone *et al*, 1992). In this thesis, the terms sepsis and SIRS shall be used interchangeably for this systemic disease.

As described for the local release of cytokines, TNF-α causes an increase in vascular permeability and diameter. If this occurs systemically, the endothelium cannot repair itself and the systemic loss of plasma volume from the circulation leads to sustained hypotension, characteristic of septic shock (Bone, 1993). If Gram-negative bacterial infection is confirmed, this condition may sometimes be referred to as 'endotoxic shock' because of the key role endotoxin plays in initiating this process.

TNF-α also has a central role in endotoxin-induced coagulation activation and ultimately disseminated intravascular coagulation (DIC), another key characteristic of septic shock. TNF-α induces the expression of a membrane glycoprotein, called tissue factor, on monocytes and endothelial cells; involved in the initiation of the extrinsic pathway of coagulation (deBoer *et al*, 1992). Increased levels of cytokines also leads to a marked rise in activated factor XII, which results in fibrinogen to fibrin conversion, and in circulating markers for the generation of thrombin (Levi *et al*,

1993). As a result of systemic stimulation of blood coagulation, fibrin is generated and deposited, leading to the formation of microthrombi in various organs, platelets and the coagulation factors II, V and VII are consumed in excess, and the complement system is activated by both the classical and alternative pathway (Bone, 1993). As with the cytokine network, overstimulation of the complement cascade can have deleterious effects on the host. For example, complement activation products such as C5a induce vasodilation, cooperate with cytokines in the stimulation of tissue factor expression on endothelial cells and activate neutrophils (Carson & Johnson, 1990; deBoer *et al*, 1992). Increased chemotaxis of neutrophils can lead to pulmonary leukostasis, a lesion important in the development of the adult respiratory distress syndrome (ARDS), a further complication of sepsis appearing in approximately 80% of cases (Callery *et al*, 1991).

Ultimately the major organs, kidney, liver, heart and lung, are compromised by the failure of normal perfusion. Multiple organ dysfunction syndrome (MODS) or multiple organ failure (MOF) is the net result of a bodily state out of control and unless effective treatment is received, the outcome is likely to be fatal.

In summary, overwhelming Gram-negative bacterial infection can initiate a continuous or systemic release of cytokines leading to a complicated series of severe and often fatal consequences. Ironically, features of the cytokine network that make it so valuable in containing local infection are precisely those that allow it to play a central role in the pathogenesis of sepsis. However, as evolution has deemed to keep cytokines, their value locally must outweigh the consequences of systemic release.

Levels of TNF-α are also increased significantly in other clinical conditions associated with inflammation and autoimmunity, including rheumatoid arthritis, systemic lupus erythematosis, AIDS, leprosy, leishmaniasis and cystic fibrosis. Thus, sepsis is not the

only condition where massive cytokine release is important, although it remains the best documented to date.

1.3.8 TREATMENT OF SEPSIS

Mortality rates of septic patients who develop the complications of shock and MODS can reach up to 90% (Bone, 1993). As a result, several new therapeutic strategies have been proposed to combat this systemic disease. The main target areas for treatment focus on the primary mediators of sepsis; LPS and cytokines, although advances in antimicrobial therapy and medical support aimed at reducing infection in the intensive care setting are also important. The three main strategies are discussed below.

Anti-LPS therapy

The interaction of endotoxin with host cells is a primary event in sepsis. It is therefore logical to prevent the initial binding step. Antibodies against the O-Ag region of LPS are highly protective against infection, increasing opsonisation, phagocytosis and clearance of both LPS and whole bacteria. However, due to the variability and specificity of O-Ag regions between different bacteria, O-Ag antibodies are of limited commercial use as a broad spectrum therapeutic strategy (Baumgartner & Glauser, 1993).

Protective antibodies directed against the lipid A-core region of LPS would, in contrast, be effective against a wide range of Gram-negative bacteria. Two anti-lipid A MAbs have been the subject of large scale clinical trials; a human IgM MAb HA-1A and a murine IgM MAb E5. Unfortunately, neither MAb was found to afford significant protection to sepsis patients, although interestingly, in both cases subgroups of patients showed a trend towards improved survival (Lynn & Cohen, 1995; Baumgartner & Glauser, 1993). For example, the first phase 3 trial using

HA-1A appeared to protect patients with shock, but only when they were bacteraemic (Ziegler *et al,* 1991). Based on current clinical data, neither HA-1A or E5 can be recommended for treatment of sepsis and more studies are required to establish the benefit of anti-lipid A treatment.

Lipid A analogues, due to their altered structure and lower toxicity, have been used as competitive endotoxin antagonists. Lipid X was found to inhibit LPS-induced neutrophil priming (Lynn & Golenbock, 1992) but as a weak LPS antagonist showed no protection in a canine model of sepsis (Danner *et al,* 1993). Lipid IVA is a better antagonist but requires a five to ten fold (w/w) excess to completely block the effects of LPS (Lynn & Golenbock, 1992). Thus, administration of lipid IVA would be difficult due to the large infusions of lipid required. The most promising LPS analogue is monophosphorylated lipid A (MPL), which reduces the haemodynamic effects, cytokine levels and mortality associated with subsequent endotoxin challenge (Astiz *et al,* 1993).

Alternative lipopolysaccharide binding proteins, such as the LBP homologue BPI that can bind and neutralise the biological effects of LPS may be a further anti-LPS measure. BPI has a greater affinity for LPS compared to LBP and can compete effectively with LBP for binding (Lynn & Golenbock, 1992). Preclinical studies with recombinant BPI (rBPI) have shown to protect against endotoxaemia in animal models and a large-scale multicentre trial is now being planned (Kohn *et al,* 1993). Unfortunately, rBPI has a short half life and, therefore, would need to be administered by continuous infusion.

Anti-LPS therapy would be of most benefit when administered early, before the initiation of inflammatory mediators and widespread vascular damage, or prophylactically to high-risk patients to prevent continued cytokine release. The main

disadvantage of anti-LPS therapy, however, is that it is specific to Gram-negative infection. Gram-positive bacteria also cause sepsis (Bone, 1994) and in such cases, any anti-LPS therapy would be redundant.

Anti-Cytokine therapy

The clinical problems associated with sepsis result from cytokine release, thus strategies aimed at preventing secretion or removing excess cytokines from the circulation would be worthwhile. Furthermore, anti-cytokine therapy would aid SIRS patients regardless of the clinical insult; Gram-negative bacteria, Gram-positive bacteria, non-bacterial microbes, trauma, burns and injury. TNF-α and IL-1 are the major cytokines generated during sepsis, playing a pivotal role in the immunological cascade. Consequently, the majority of anti-cytokine therapy has been directed against TNF-α and IL-1. For the purpose and relevance of this thesis only anti-TNF therapy will be detailed.

Both polyclonal and monoclonal antibodies to TNF-α have been shown to be protective against septic shock in animal models, although this effect is seen only when the antibodies are administered before or together with endotoxin challenge (Beutler *et al*, 1985, Tracey *et al*, 1987, van der Poll *et al*, 1994). If the antibodies are received two to three hours after challenge, less protection is observed (Silva *et al*, 1990).

MAbs to TNF-α have, however, been tested in clinical trials. Indeed, the results of a large scale, multi-centre, phase 3 clinical trial were recently published (Abraham, *et al*, 1995). Results showed a reduction in mortality in patients who received the MAb after just three days, and a continued trend at twenty-eight days, but overall, there was no significant difference in mortality between placebo and MAb treated patients. Similar to trials with LPS MAbs, the apparent trend in decreased mortality was

attributed to a subgroup of patients. In this case, improved survival occurred in patients with shock; in non-shock patients there was no increased survival. As this treatment appears to benefit only certain patients, further clinical studies are required to define the role of TNF-α MAbs in sepsis therapy.

TNFR1 and TNFR2 are shed from the surface of immune cells to give soluble, circulating derivatives; sTNFR1 and sTNFR2. The use of sTNF receptors to consume excessive TNF production has been advocated and in earlier studies these proteins appeared to offer protection against sepsis and endotoxaemia (Van Zee *et al,* 1992). Unfortunately, results from a phase 2 and 3 trial suggested that mortality was actually higher in patients receiving the treatment compared to a placebo group (Lynn & Cohen, 1995).

IL-8 is also a potential target for sepsis therapy and indeed, in a rabbit model, a MAb to IL-8, WS-4, inhibited neutrophil-mediated lung injury following reperfusion of pulmonary ischemic tissue (Seikido *et al,* 1993). However, no data is yet available on anti-IL-8 therapy in humans.

Endogenous regulators

The cytokine network is a tightly regulated system and includes 'negative' cytokines, that act to downregulate or counteract the effects of pro-inflammatory mediators such as TNF-α. IL-4 and IL-13 are closely linked genetically and share limited sequence homology (20-25%) (Minty *et al,* 1993). In addition, both are products of activated T-lymphocytes and, more importantly, have anti-inflammatory effects on human endothelial cells and monocytes. In experiments, IL-4 and IL-13 have been shown not only to inhibit cytokine secretion but also LPS- and TNF-α-induced tissue factor expression (Herbert *et al,* 1993). As previously outlined in section 1.3.7, tissue factor initiates blood coagulation and ultimately DIC. IL-10 can block *in vitro* production of

cytokines, including TNF-α, as well as enhance levels of sTNFR (Leeuwenberg *et al*, 1994). In a murine model assessing the use of IL-10 in endotoxaemia, it was found that pretreatment with IL-10 before LPS challenge was protective against sepsis. Currently IL-10 is being evaluated in phase 2 clinical trials (Gérard *et al*, 1993). Thus, all three anti-inflammatory cytokines represent a potentially important therapy in regulating uncontrolled immune responses.

Treatments against different components of the coagulation pathway and MAbs against selectins, integrins and other adhesion molecules have all been used as prospective therapies (Ridings *et al*, 1995). In addition, a more extreme approach, involving the removal of circulating endotoxin and inflammatory mediators by plasma or whole blood exchange, has been tried experimentally. Although only small-scale clinical trials have used this extreme treatment and little data is available, early results are encouraging (Lynn & Cohen, 1995). To date, no suggested treatment for septic shock has conferred an undisputed benefit to patients and indeed, due to the complexity of the disease, a combination of treatments may prove to be the best strategy as was demonstrated in a model of *P. aeruginosa* sepsis (Cross *et al*, 1993).

1.4 *BURKHOLDERIA CEPACIA*

1.4.1 TAXONOMY

Burkholderia cepacia has been described by several different synonyms. Originally termed *Pseudomonas cepacia*, this organism has been called *Pseudomonas kingii*, *Pseudomonas multivorans* and Eugonic Oxidiser 1. In 1992 seven species of the *Pseudomonas* rRNA homology group II were regrouped into the new genus *Burkholderia* on the basis of cellular lipid and fatty acid composition, 16S rRNA and DNA-DNA homology (Yabuuchi *et al*, 1992; List No. 45, 1993). For the purpose of

this thesis, the name *Burkholderia cepacia* will be used. At present, the members of the genus *Burkholderia* are *B. cepacia* (type species), *B. gladioli, B. mallei, B. pseudomallei, B. picketti, B. caryophylli, B. solanacearum,* and recently added to the group, *B. plantarii, B. glumae, B. vandii* (Urakami *et al*, 1994) and *B. cocovenenans* (Zhao *et al*, 1995).

The general characteristics of *B. cepacia* include the following features: Gram-negative, non-spore forming, aerobic rod, motile with a respiratory metabolism and typically catalase and oxidase positive. Various non-fluorescent pigments may be produced and poly-β-hydroxalkanoates can be accumulated as reserve materials. The optimal temperature for growth is 30-35^{o}C (Palleroni, 1984). *B. cepacia* has considerable nutritional versatility with some strains even able to utilise penicillin G as a sole carbon and energy source (Beckman, 1979). *B. cepacia* strains have been shown to possess multiple replicons, plasmids and insertion sequences (Cheng & Lessie, 1994; Rodley *et al*, 1995) that may account for some of this versatility. At first, *B. cepacia* was considered a ubiquitous organism sharing similar environmental niches to *P. aeruginosa.* Indeed the natural habitat of *B. cepacia* has been reported as soil, water and vegetation (Holmes, 1986). However, a recent study has demonstrated that true environmental strains of *B. cepacia* are rather difficult to isolate and surprisingly, quite rare (Butler *et al*, 1995). The following sections describe both the beneficial role and the disease states in which *B. cepacia* is important.

1.4.2 BENEFICIAL ROLE OF *B. CEPACIA*

B. cepacia as a biological control agent

Concern for the environment, public safety and increasing numbers of pesticide-resistant pathogens has raised interest in using alternative methods of plant disease

control. One such method is the use of biological control agents (BCA), a method in which *B. cepacia* has gained considerable attention.

B. cepacia produces the antimicrobial compounds pyrrolnitrin, aminopyrrolnitrin, monochloroaminopyrrolnitrin, altericidins, cepalycins, cepacin A and cepacin B (McLoughlin *et al*, 1992, Jayaswal *et al*, 1993, Abe & Nakazawa, 1994). Studies have shown that these compounds have an antagonistic effect against both bacterial and fungal phytopathogens and are capable of suppressing a wide range of plant diseases including sunflower wilt disease (McLoughlin *et al*, 1992), potato rot (Burkhead *et al*, 1994), tobacco wilt disease (Aoki *et al*, 1991) and blue and grey mould in apples and pears (Janisewicz & Roitman, 1988).

B. cepacia as a decontaminant of soils

In addition to the bactericidal and fungicidal activity of *B. cepacia*, some strains are able to break down herbicides, pesticides and industrial waste material. Trichloroeythlene (TCE) and 2,4,5,-trichlorophenoxyacetic acid (2,4,5,-T; the principle herbicide in 'agent orange') are just two highly volatile, toxic, organic solvents known to disperse through soil, from the original waste site, threatening both soil life and underground water supplies. The documented persistence of these chemicals reflects the fact that many microbes are unable to degrade these compounds, leading to cumulative pollution effects.

By using the chemicals as a sole source of carbon and energy, strains of *B. cepacia* have been shown to degrade both TCE and 2,4,5,-T (Folsom *et al*, 1990). Indeed, *B. cepacia* degraded more than 90% 2,4,5-T from heavily contaminated soil (20,000ppm) within six weeks. This rate is between 200 - 20,000 times greater than other known degrading bacteria (Kilbane *et al*, 1983).

1.4.3 *B. CEPACIA* IN DISEASE

B. cepacia as a phytopathogen

B. cepacia was first reported in 1950 as the causative agent of 'sour skin', a bacterial rot in onion bulbs (Burkholder, 1950). In the report, infected onions were described as slimy, discoloured and producing a sour or vinegar-like odour. Infection was thought to occur through wounds, produced by cutting of the onion tops during harvest time. No evidence was obtained for spread to healthy bulbs, and young onions, potato tubers and narcissus bulbs, inoculated with *B. cepacia* also showed no symptoms.

B. cepacia in nosocomial infections

In humans, *B. cepacia* has been reported in opportunistic infections such as necrotising pneumonitis, macerated wound lesions, urinary tract infections, peritonitis and bacteraemia. In the majority of these cases, *B. cepacia* infection was traced to contamination of hospital water and disinfectant solutions (Table 1.2). For example in one outbreak described by Berkelman *et al* (1982), ten patients had *B. cepacia* recovered from their peritoneal fluid after dialysis treatment, during a three month period. The infective source was found to be the water reservoir in two automatic dialysis machines. Inadequate cleaning and emptying of the water supplies had allowed sustained growth of *B. cepacia.*

B. cepacia has also been associated with foot rot in soldiers training in swamps (Taplin *et al,* 1971). Sampling of the swamp water confirmed the presence of *B. cepacia* and highlights once more the organisms predilection for moist, water-based sources.

Table 1.2 Episodes of *B. cepacia* nosocomial infections

Effect	Source	Reference
Wound infection	Tap water & Chlorhexidine (D)	Basset *et al*, 1970
Pseudobacteremia	Povidone-Iodine (D)	Craven *et al*, 1981
Urinary tract infection	Chlorhexidine (D)	Sobel *et al*, 1982
Infected nasal sprays	Thiomersal preservative	Decicco *et al*, 1982
Infected nebulisers	Water Reservoir	Gelbart *et al*, 1976
Bacteraemia	Blood Gas Analyser	Henderson *et al*, 1988
Septicaemia	Intravenous Infusates	Thong *et al*, 1975

D = disinfectant

B. cepacia in Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is a neutrophil disorder in which cells do not produce oxidative reactive intermediates such as hydrogen peroxide, normally required for effective intracellular killing and degradation. Indeed, although infecting organisms may be readily engulfed by CGD leucocytes, the organisms can survive intracellularly and actually prime the host for infection (Bottone *et al*, 1975). Patients with CGD are thus immunocompromised and appear particularly susceptible to *B. cepacia* infection, normally presenting as pneumonia. The strong association between *B. cepacia* and CGD might be explained by production of catalase, an enzyme known to destroy the reduction products of oxygen (O'Neill *et al*, 1986).

In conclusion, *B. cepacia* may exist as a phytopathogen and, ironically, as a potential biological control agent and decontaminant of soil. Clinically, *B. cepacia* infections are relatively infrequent, requiring a defective immune system and/or arising from contamination. However, in recent years *B. cepacia* has come to prominence as a major threat to patients with cystic fibrosis (CF). Before discussing the role of

B. cepacia in CF the following section will describe the physiology and pathogenesis of this important disease.

1.4.4 CYSTIC FIBROSIS

Cystic fibrosis is the most common lethal inherited disease among the Caucasian population with a frequency of 1 in 2500 live births; 6000 people are affected in the UK alone with 50,000 patients worldwide (Bye *et al*, 1994).

The CF gene

In 1938, Anderson gave the first comprehensive clinical description of CF and within a few years recognised the disease as an autosomal, recessive inherited syndrome (Anderson & Hodges, 1946). However, it was only in 1985 that the genetic locus of CF was mapped, to chromosome 7 (White *et al*, 1985), and in 1989 the CF gene cloned (Riordan *et al*, 1989). The CF gene product, termed the cystic fibrosis transmembrane conductance regulator (CFTR) was identified as a regulated chloride channel when *in vitro* experiments showed that cells, transfected with CFTR cDNA, expressed increased chloride transport (Anderson *et al*, 1991a).

Since the discovery of CFTR over 400 CF-associated mutations have been reported. These mutations can be grouped into four broad classes; defective protein production, defective protein processing, defective regulation and defective conductance. The most common CFTR mutation ΔF508 (so called because of a phenylalanine deletion at residue 508) accounts for around 70% of all CF mutations and results in defective protein processing (Shrimpton *et al*, 1991). It is proposed that the failure to traffic CFTR to the correct cellular location is a result of incorrect folding. Interestingly, experiments delivering CFTR ΔF508 to the plasma membrane show that the protein retains some function (Welsh & Smith, 1993).

The cellular defect

The cellular defect in CF results from dysfunctional chloride channel activity. In healthy epithelial cells sodium ions are actively absorbed into the cell and chloride ions are actively transported out into the luminal area. Water follows the direction of ion transport in order to maintain the osmotic balance. In CF epithelial cells, decreased secretion of chloride ions and hyperabsorption of sodium ions results in the net transport of water out of the lumen and the subsequent production of dehydrated viscous mucus (Boucher *et al*, 1986; Bye *et al*, 1994), which leads to the clinical features of the disease.

Clinical features of CF

Cystic fibrosis is characterised by the accumulation of dehydrated mucus resulting in the blockage of exocrine glands and commonly presenting as pancreatic, gastrointestinal, genitourinary and pulmonary abnormalities.

Pancreatic disease is a common feature of CF with the majority of patients suffering from pancreatic enzyme insufficiency. Pancreatic enzymes normally assist in the digestion and absorption of food in the small intestine, but in CF the enzymes cannot be transported due to blockage of pancreatic ducts with mucus (Bye *et al*, 1994). If uncorrected, the lack of pancreatic enzymes leads to failure to thrive due to fat and protein malabsorption (Boat *et al*, 1989). Pancreatic enzyme supplements have been used by CF patients for many years to relieve the symptoms of maldigestion.

Meconium ileus results from a failure to pass meconium in the first few days of life and occurs in 5-10% of CF newborns. It is believed to be caused by a failure to secrete pancreatic enzymes and therefore incomplete digestion of dehydrated intestinal contents. Obstruction of the bowel also occurs in older children and adults, termed meconium ileus equivalent, and is again associated with sticky, undigested

intestinal contents (Bye *et al*, 1994). Other CF-associated gastrointestinal conditions include rectal prolapses (occurring in around 20% of young CF children) and intussusception (Green, 1993).

Male infertility is a characteristic of CF affecting more than 95% of male patients. Intrauterine obstruction of the genital tract with thickened mucus results in altered Wolffian duct structures, with the vas deferens, epididymis and seminal vesicles atrophic, fibrotic or completely absent (Tussig *et al*, 1972). Fertility in female CF patients is not commonly affected, although the dehydrated cervical mucus may impede normal sperm migration (Kapito *et al*, 1973). CF breast milk has been found to be essentially normal (Alpert & Comier, 1983).

Pulmonary disease in the form of debilitating exacerbations of respiratory infection is the major cause of morbidity and mortality in CF. The lungs of CF patients are histologically normal at birth (Accurso, 1995) although the primary pathogenic events, accumulation of mucus secretions and respiratory infection, have been shown to occur in the first few months of life (Boat *et al*, 1989; Armstrong *et al*, 1995). Mucosal obstruction of the small airways impairs mucociliary clearance, predisposing CF patients to chronic bacterial infection although surprisingly, the spectrum of microorganisms associated with CF is limited. Generally, *Staphylococcus aureus* appears in infancy, *Haemophilus influenzae* in early childhood, followed by *Pseudomonas aeruginosa* in early adolescence. *Burkholderia cepacia* is most common in the late teens (Govan & Nelson, 1992).

Bacterial infection in CF

Prior to 1950, *S. aureus* was the major cause of death in CF but today can be effectively treated by aggressive antibiotic therapy. Indeed, long-term anti-staphylococcal therapy has been implemented in some CF centres, upon diagnosis of

CF, in an attempt to prevent pulmonary damage and subsequent colonisation by *H. influenzae* and *P. aeruginosa.* However, the efficacy of such a strategy has been debated and remains controversial; retrospective studies have shown a correlation between anti-staphylococcal therapy and increased incidence of other CF pathogens, in particular *P. aeruginosa,* and hence a poorer prognosis (Bauemfriend *et al*, 1987). *S. aureus* produces a number of potential virulence determinants including coagulase, catalase, haemolysins, exotoxins, teichoic acid and EPS. However, only the latter two determinants, which play a role in the adherence to pulmonary epithelial cells, have any defined role in CF pathogenesis (Govan & Nelson, 1992).

Less information is available on the non-capsulate *H. influenzae* CF isolates, probably due to the difficulty in cultivating this fastidious organism. However, it is known that *H. influenzae* has increased mucosal adherence, excretes a heat-labile substance to inhibit cilia motion and produces factors to stimulate mucus secretion, possibly including histamine (van Alphen *et al*, 1995).

P. aeruginosa remains the leading CF pathogen colonising up to 90% of patients and once established is very rarely eradicated. *P. aeruginosa* strains produce many virulence factors including exotoxin A and S, haemolysin-phospholipase C, elastase and proteases, all thought to play a role in early colonisation by interfering with host defence mechanisms (Vasil, 1986, Høiby *et al,* 1990). A major virulence determinant of *P. aeruginosa* is alginate (section 1.2). The production of alginate and subsequent biofilm formation protects bacteria from antibiotics, phagocytic cells and antibodies, and leads to the formation of immune complexes causing substantial immune-mediated damage through release of neutrophil elastase, myeloperoxidase and free oxygen radicals (Høiby *et al,* 1990). Longitudinal antibody studies have shown that high and rising anti-*P. aeruginosa* antibodies are associated with a poor prognosis (Høiby *et al,* 1990; Winnie & Cowan, 1991). Current treatment is aimed at

preventing colonisation by non-mucoid strains, using antibiotics such as tobramycin and ciprofloxacin (C. Doherty, personal communication). However, it is unfeasible to totally protect CF patients from such a ubiquitous organism. In the long term, vaccination may be the best solution for inhibiting this organism. Colonisation by *B. cepacia* is discussed in section 1.4.5. Rarer pathogens isolated from CF sputa include *Aspergillus fumigatus, Mycobacterium tuberculosis* and *Stenotrophomonas maltophilia.*

As a consequence of repeated cycles of obstruction, infection and inflammation, irreversible pulmonary tissue damage occurs. Lung function progressively declines with each exacerbation, leading eventually bronchiectasis, hypercapnoea and cor pulmonale. Ultimately, pulmonary failure occurs and is the primary cause of more than 90% of deaths in CF patients (Gilligan, 1991). Several other pulmonary complications may arise, including asthma, pneumothorax and haemoptysis (Green, 1993). At autopsy the air spaces are overinflated, the bronchioles are often obliterated and bronchiectactic cysts are extensive (Boat *et al,* 1989).

Treatment of CF

At present no cure for CF is available although numerous therapies can improve life expectancy including pancreatic supplements, physiotherapy and antibiotics. With the advances of molecular medicine, the discovery and cloning of the CF gene has led to genetic diagnosis and screening of CF and, more excitingly, the prospect of gene therapy. The crux of gene therapy is to deliver CFTR DNA safely and efficiently into affected epithelial cells and thus restore chloride channel activity long term. Currently, there are two gene therapy strategies, one advocating the use of liposomes as DNA carriers and the other using adenoviruses. Both treatments have advantages and problems. The adenoviruses can target and deliver the CFTR DNA package efficiently, as viruses enter cells as part of their natural lifecycle. However, worries

remain about genetic stability and the potential for an immunogenic response. Liposomes do not cause an immune reaction and are safe for use with no indicated side effects. Unfortunately, delivery of DNA by natural uncharged liposomes is inefficient and even using cationic liposomes to aid uptake, targeting the correct cells remains a problem. Both therapeutic strategies have been used in phase 1 clinical trials involving CFTR cDNA delivery to nasal epithelium. Results indicate increased chloride movement and negligible inflammation (Crystal *et al*, 1994; Caplen *et al*, 1995). Further results are eagerly awaited.

Discovery of the CF gene has also led to the development of transgenic CF mice by several groups (Higgins & Trezise, 1992). The Edinburgh CF mouse, produced by targeted insertional mutagenesis, displays several key CF characteristics including pulmonary, gastrointestinal and genitourinary disease and can facilitate pathological, bacteriological and therapeutic studies (Dorin *et al*, 1992).

Using current techniques, gene therapy could truly benefit only very young children who have little or no pulmonary scarred tissue. Once the cycle of inflammation and scarring is established, the use of gene therapy may be of limited use in an inadequate, injured lung. At present the only way to extend the life of CF patients with end stage respiratory failure appears to be through lung transplantation.

Since the first successful heart-lung transplant (HLT) in a CF patient in 1985 over 120 similar operations have been performed in the UK. As with any major surgery risks are considerable but at present 70% of CF patients survive 1 to 2 years post-operatively (Higgenbottam *et al*, 1992). Although not a cure for CF, the majority of patients achieve a better quality of life after transplantation. Unfortunately, infection in the transplanted organs remains a major cause of mortality, with *P. aeruginosa* and *B. cepacia* commonly involved (Noyes *et al*, 1994). In one report (Snell *et al*, 1993)

examining 24 double lung transplant patients, *B. cepacia* was the cause of 14 bouts of pneumonia, 4 empyemas, 1 lung abscess, 1 suppurative pericarditis and 5 cases of sinusitis. Furthermore, ten patients who were *B. cepacia* colonised before transplant grew this bacterium post-operatively, with an additional five patients also colonised. Out of the fifteen *B. cepacia*-positive patients, seven died (five due to sepsis) whereas no patient with only *P. aeruginosa* died.

A few decades ago CF individuals died in infancy; today, through better management and understanding of the disease, patients can expect to live into their twenties and thirties. However, this encouraging picture has been completely devastated by the emergence of *B. cepacia.*

1.4.5 *B. CEPACIA* IN CYSTIC FIBROSIS

In the early 1980's, several North American cystic fibrosis (CF) centres reported a disturbing increase in *B. cepacia* isolates from CF sputa. During the five year period from 1978 to 1983, *B. cepacia* associated CF deaths rose from 9% to 55% (Thomassen *et al,* 1985). At that time, UK centres reported no *B. cepacia* problem but by the early 1990's, several areas were seriously affected. In Edinburgh alone the prevalence of *B. cepacia* had reached over 25% by 1992 and since then *B. cepacia* has been held responsible for the majority of CF deaths. Initially, *P. aeruginosa* colonisation was thought to 'prime' the CF lung for acquisition of *B. cepacia.* However, over 35% of *B. cepacia*-positive CF patients in Edinburgh are not co-colonised with *P. aeruginosa*, highlighting the pathogenicity of *B. cepacia* in its own right. The devastating impact of *B. cepacia* on the CF community is characterised by three unusual features, multiresistance, transmission and 'cepacia syndrome', properties not observed with any other major CF pathogen.

Multiresistance

B. cepacia strains are inherently resistant to numerous antibiotics including aminoglycosides, β-lactams and anti-pseudomonal penicillins. Even when sensitivity has been demonstrated *in vitro*, administration of commonly used CF antimicrobial drugs does not eliminate or reduce *B. cepacia in vivo*, although a decrease in pro-inflammatory markers may occur (Elborn *et al*, 1994).

Transmission

An early feature of *B. cepacia* in CF was the rapid spread and high colonisation rate of this organism, culminating in the realisation that certain *B. cepacia* strains were highly transmissible and responsible for 'epidemic' outbreaks. Indeed, one such 'epidemic' strain, isolated from an Edinburgh CF patient, has colonised several CF individuals both within the same clinic and in other UK CF centres. Recent data from DNA-based fingerprinting and sequence analysis has shown the Edinburgh epidemic strain to be identical to a highly transmissible strain isolated in Toronto (Johnson *et al,* 1994; Sajjan *et al,* 1995).

Tablan *et al* (1987a) identified several factors that increased the risk of acquisition of *B. cepacia* including; increased severity of underlying disease, increasing age, having a sibling colonised with *B. cepacia* and hospitalisation within the previous six months. However, fears arising from the spread of *B. cepacia* led to the introduction of a controversial world-wide segregation policy separating *B. cepacia* colonised from non-colonised patients as the only effective method of reducing transmission rates and cross-infection. It is therefore vital that laboratories remain vigilant in their monitoring of CF sputa and, most importantly, accurately identify this organism, so patients are not wrongly segregated or placed at undue risk. For example, one study demonstrated only 36 out of 115 laboratories investigated, were able to isolate and identify *B. cepacia* from seeded sputum samples (Tablan *et al*, 1987b).

The development of selective media for the isolation and growth of *B. cepacia* together with improved genetic typing systems such as pulsed field gel electrophoresis and ribotyping has helped epidemiological monitoring (Gilligan *et al,* 1985; Anderson *et al,* 1991b; Kostman *et al,* 1992). In particular, the use of DNA-based fingerprinting techniques has provided compelling evidence for person-to-person transmission and nosocomial acquisition of *B. cepacia*. LiPuma *et al* (1990) provided the first scientific evidence for person-to-person transmission, occurring between two CF patients attending a residential summer camp. However, the study did not identify whether transfer occurred directly through droplet spread or indirectly via shared equipment. The isolation of *B. cepacia* from the air of rooms occupied by colonised CF patients supports the proposal of aerosol dissemination (Humphreys *et al,* 1994). With regard to the spread of the Edinburgh/Toronto epidemic strain in the UK, transmission was shown to be due to close social contact such as attendance at meetings, summer camps and at a fitness class (Govan *et al,* 1993). Thus, segregation policies need to encompass both social and hospital contacts, with hygiene practices by patients and carers brought to the highest standards possible to prevent cross-infection.

Epidemiological studies have demonstrated probable transmission routes, although the underlying mechanisms of spread, particularly those of 'epidemic' strains, remain unclear. Recent studies of *B. cepacia* fimbriae (pili) have addressed this issue. Goldstein *et al*, (1995) demonstrated that *B. cepacia* strains produce five distinct classes of pili, mesh, cable, filamentous, spine and spike. Further investigations have revealed a correlation between transmission rates and fimbrial structure, in particular the expression of cable pili by the Edinburgh/Toronto strain, arguably the most transmissible lineage described to date. Non-epidemic CF strains, in general, produce the filamentous fimbriae, non-CF clinical isolates the spine fimbriae and environmental isolates the spike fimbriae. Type I or mesh fimbriae are constitutively co-expressed as

peritrichously arranged dense mats with either cable, filamentous, or spike fimbriae (Goldstein *et al*, 1995). This important new finding leads to exciting possibilities, not only for evaluating the safety of *B. cepacia* strains but also in preventing further transmission with a cable vaccine or neutralising anti-cable antibody. However, it must be stressed that not all transmissible strains possess cable pili, therefore, other transmission factors must exist.

Cepacia syndrome

The third, and perhaps most worrying problem associated with *B. cepacia* acquisition is the inability to predict the clinical outcome. Although some *B. cepacia*-colonised CF patients have asymptomatic carriage and others show a slow and progressive decline in lung function, similar to *P. aeruginosa* colonisation, 20-30% of *B. cepacia*-positive individuals succumb to a rapid, and usually fatal, pulmonary decline associated with necrotising pneumonia and complicated on occasion by septicaemia (Thomassen *et al*, 1985; Rosenstein & Hall, 1980; Isles *et al*, 1984). The latter clinical outcome is unique amongst CF pathogens and has thus been termed 'cepacia syndrome'.

At present, segregation appears to have reduced the spread of *B. cepacia* colonisation (Thomassen *et al*, 1986) but the social and psychological burden on CF communities has been severe. Progress into understanding the pathogenesis of *B. cepacia* has been made but important questions remain; for example, why do different CF patients colonised by the same strain show variable clinical outcome? Identification of transmission factors such as cable pili may explain why some strains are more transmissible, but it must be emphasised that it is not only epidemic strains that correlate with the 'cepacia syndrome'. Indeed, the first reported *B. cepacia*-associated death in a CF patient in the UK, a 9-year old girl, was caused by an apparently non-epidemic isolate (Glass & Govan, 1986). Unfortunately, the patient still showed a

dramatic decline and ultimately died. In contrast, the first patient to acquire the epidemic strain in Edinburgh has shown little deterioration six years after colonisation.

Progress into understanding the epidemiology of *B. cepacia* has been achieved but the pathogenic mechanisms involved in *B. cepacia* infection remain poorly defined and need to be addressed urgently. The following sections describe the mechanisms that may allow *B. cepacia* to prosper in the CF lung: potential virulence factors and host defence mechanisms.

1.4.6 *B. CEPACIA* CELL SURFACE VIRULENCE DETERMINANTS

Lipopolysaccharide

The LPS of *B. cepacia* strains are similar to the established structure described in section 1.1.3. The lipid A region is based on the classic β-1,6-linked D-glucosamine disaccharide, with phosphoryl groups attached at positions 1 and 4' expressed as either two different phosphodiester residues or with one phosphate present in the monoester configuration (Cox & Williamson, 1991).

Initial analytical studies on *B. cepacia* LPS debated the relative absence of Kdo. In 1979, Manniello *et al* found no Kdo present in five *B. cepacia* strains tested. In 1989 Straus *et al* detected Kdo in two out of ten strains and a year later in all six isolates tested although Kdo concentrations were low. A recent finding is that some Kdo can be substituted by Ko (D-*glycero*-α-D-*talo*-2-octulosonic acid) the hydroxylated form of Kdo (Kawahara *et al,* 1994), which is also found in the LPS of *B. pseudomallei* and some *Legionella* species (Wilkinson & Pitt, 1995). This finding points to the presence of only one Kdo residue in *B. cepacia* LPS and may account for the previous difficulty in assaying this compound. Glucose, rhamnose and heptose are the major components present in the core (Manniello *et al,* 1979) but surprisingly isolated core structures show no phosphorus residues present (Cox & Wilkinson, 1991).

The O-Antigen is the best-characterised region of *B. cepacia* LPS with seven serotype groups now identified (Heidt *et al,* 1983). To date all the polymers tested are linear with di- or tri-saccharide repeating units. Some of the O-Ag structures have been found in other bacteria, for example the O3 serotype structure has been reported in *P. aeruginosa* and *Serratia marcescens* (Cox & Wilkinson, 1989a).

B. cepacia LPS may be expressed as either a rough or smooth phenotype (McKevitt & Woods, 1984) and strains do not appear to undergo a smooth to rough transition as occurs with *P. aeruginosa* in CF. A major role for LPS in *B. cepacia* pathogenesis has yet to be defined, although a complex composed of surface carbohydrate, LPS and protein was shown to produce extensive lung pathology in rats, with the toxicity attributed to the LPS component (Straus *et al,* 1989). In addition, the continued presence of LPS may cause inflammatory lung damage as described in section 1.3.

Outer membrane proteins

B. cepacia produces five major outer membrane proteins; A (56kD), B (38kD), C (37kD), D (28kD) and E (21kD). OMP C and D are the most abundant proteins and have recently been shown to form a diffusion porin by non-covalent association (Gotoh *et al,* 1994). As with other Gram-negative bacteria, *B. cepacia* can alter its OMP profile in response to different environmental conditions (section 1.2). Magnesium-depleted cells show a much simpler OMP profile compared to iron-limited or nutrient broth-grown cells in which new OMPs are induced (Anwar *et al,* 1983). These changes undoubtedly contribute to increased resistance to antibiotics and host defence mechanisms, as has been proposed for serum resistance (Butler *et al,* 1994). The outer membrane also contains some unusual polar lipids although how they contribute to the biological properties of the cell surface has yet to be determined (Cox & Wilkinson, 1989b).

The inherent antimicrobial resistance of *B. cepacia* is thought to be due to reduced outer membrane permeability. The small size of *B. cepacia* porins (Parr *et al*, 1987), decreased expression of both OMP C and D (Aronoff, 1988) and inducible β-lactamases are associated with high level β-lactam resistance (Aronoff & Labruzzi, 1986). Similarly, chloramphenicol resistance is believed to be due to decreased outer membrane permeability (Burns *et al*, 1989). In addition, the low concentration of phosphate and Kdo residues in the LPS of *B. cepacia* limits the number of anionic sites required for effective binding by cationic antibiotics (Cox & Williamson, 1991).

B. cepacia produces at least three siderophores, pyochelin, cepabactin and azurechelin, which enable the organism to compete for iron within the host (Sokol *et al*, 1992). Pyochelin associates with a 14kD binding protein in iron-limiting conditions, with the other two siderophores thought to perform a supplementary role when pyochelin-mediated iron translocation is impaired. Pyochelin production has been tentatively associated with mortality in CF patients. Sokol (1986) found that although half of the clinical isolates studied were pyochelin negative, 86% of pyochelin-producing strains were from patients with severe infection.

Exopolysaccharides

Strains of *B. cepacia* deficient in glucose dehydrogenase (Gcd) have been shown to produce large amounts of EPS in the presence of excess glucose (Sage *et al*, 1990). In addition, both Gcd negative and positive strains accumulated EPS when the carbon substrate was changed from glucose to mannitol or glycerol. The EPS produced was not an obvious mucoid EPS such as alginate (*B. cepacia* strains have been shown to be *alg D* negative by PCR analysis; Nelson *et al*, 1994) but consisted of galactose, glucose, mannose, glucuronic acid and rhamnose in the ratio 2:1:1:1:1 (Sage *et al*, 1990). Environmental conditions have been shown to influence EPS production with maximum polymer expressed in conditions of 2% glucose, 0.4M NaCl and a

temperature of 35°C (Allison & Goldsbrough, 1994). However no correlation has been found between the ability of *B. cepacia* to produce EPS and colonisation of the respiratory tract

Extracellular virulence determinants

B. cepacia does not appear to produce a classic exotoxin although several extracellular enzymes have been identified including a protease, haemolysin and gelatinase. None of the exoenzymes are thought to play a major role in pathogenesis, although, the production of a 25kD lipase enzyme has been reported to reduce phagocytosis of *B. cepacia* in rat alveolar macrophages (Straus *et al*, 1992). Several biological properties have been found to be expressed more frequently in *B. cepacia* CF strains compared to non-CF controls such as production of catalase, ornithine decarboxylase, reduction of nitrate to nitrite and hydrolysis of urea (Gessner & Mortensen, 1990) but the role of these factors in CF pathogenesis has yet to be established.

Therefore, with the exception of an apparent role for cable pili in transmission, no potential virulence determinant in *B. cepacia* has yet been conclusively demonstrated. However, as discussed in section 1.2, environmental stimuli may play a part in 'switching on' a more virulent form of cell surface structure or extracellular compound.

1.4.7 THE HOST IMMUNE SYSTEM IN CF

The following section describes the immune response in the CF lung and the major host defence systems as outlined in section 1.3.1.

Innate immunity- immediate protection

In the healthy lung, mucus glands and goblet cells provide a mucus blanket which traps aerosolised bacterial particles and in combination with the upward beating of

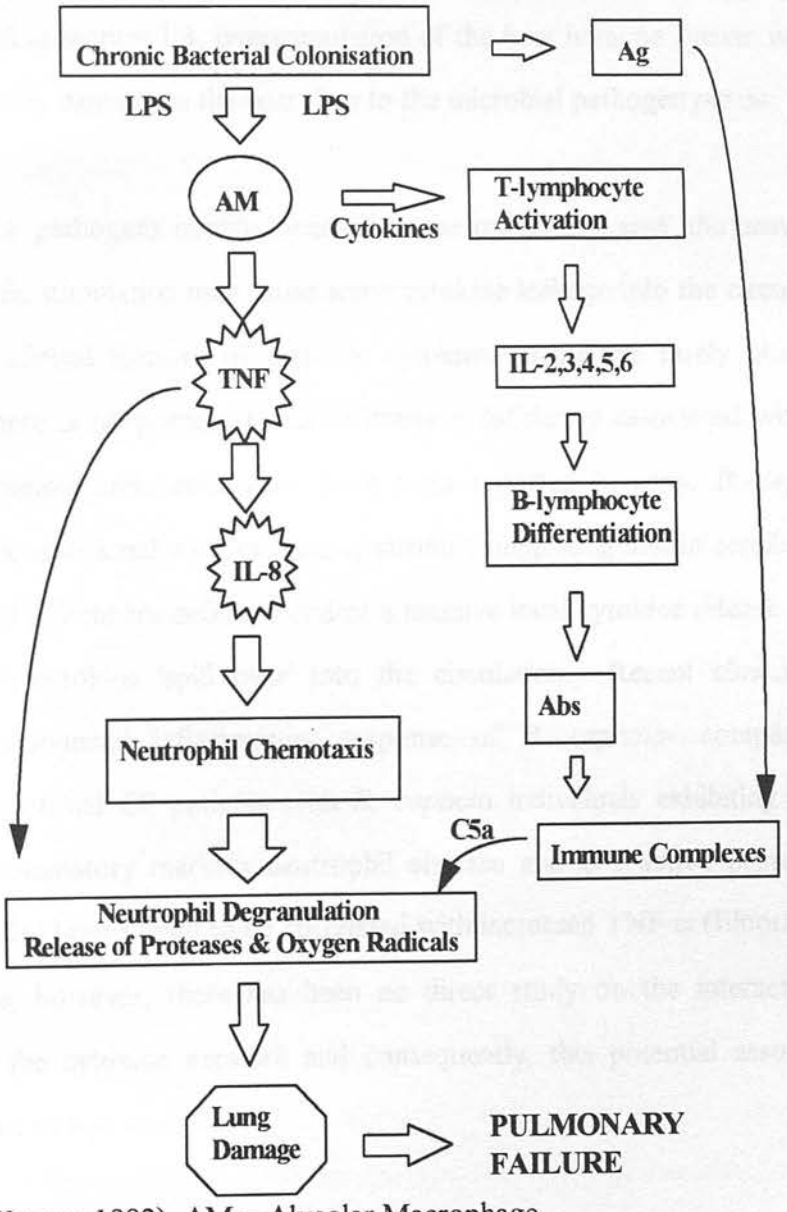
cilia transports and removes inhaled irritants (Piedra & Ogra, 1986). However, in the CF lung, although cilia morphology is normal, accumulation of dehydrated, thickened mucus impairs effective mucociliary clearance allowing colonisation of the lower airways.

At an early stage, invading microbes are exposed to alveolar macrophages which may eliminate the bacteria by non-specific phagocytosis. However, the production of lipase by *B. cepacia* has been reported to impair this process (Straus *et al*, 1992). Alveolar macrophages are primarily located at the interstitium, alveolar epithelium and bronchopulmonary lumen and constitute 80-90% of effector cells. Normally, alveolar macrophages have a half life of around seven days but activation by bacterial stimuli can considerably reduce this (Piedra & Olgra, 1986).

Innate Immunity-early induced protection

In the CF lung, chronic exposure to bacterial products such as LPS results in continued cytokine release and hence a continued inflammatory response, a characteristic feature of CF pulmonary disease. Alveolar macrophages are stimulated by microbial pathogens and other agents to produce pro-inflammatory cytokines, including TNF-α and IL-8, which in turn attract a huge neutrophil influx into the CF lung. Neutrophil release of oxygen radicals, lysosomal enzymes and the granule protease elastase, into the bronchopulmonary microenvironment causes pronounced pulmonary tissue damage (Warner, 1992). TNF-α contributes to the excessive release of enzymes and neutrophil degranulation with a statistically significant correlation between TNF-α levels and granulocyte elastase-α1 proteinase inhibitor complexes (Suter *et al*, 1989). Continuous activation of the complement and clotting cascade by TNF-α amplifies the inflammatory response. In addition, phagocytosis and presentation of bacteria by alveolar macrophages to T-lymphocytes produces cytokines which stimulate antibody production by B-lymphocytes (Warner, 1992).

The inability of the host immune system to eliminate the bacterial infection contributes to the continuous inflammation and vicious cycle of CF that ultimately culminates in pulmonary failure. The whole process is summarised in Figure 1.6 and is described in general terms in section 1.3.



(Adapted from Warner, 1992) AM = Alveolar Macrophage

Figure 1.6 The host immune response in CF

TNF-α and IL-8 levels have been measured in *S. aureus*- and *P. aeruginosa*-colonised CF patients and several studies have reported a strong association between increasing

sputum, plasma and faecal levels of cytokines and pulmonary deterioration (Greally *et al*, 1993; Wilson *et al*, 1993; Dean *et al*, 1993; Briars *et al*, 1995). Raised TNF-α concentrations have also been correlated with nutritional decline in CF, suggesting that continuous secretion of TNF-α acts not only as a major mediator of lung injury but also plays a cachectic role, contributing to the weight loss seen in CF patients. Thus, as described in section 1.3, overstimulation of the host immune system may do as much, if not more, damage to the host than to the microbial pathogen *per se*.

Although most CF pathogens remain localised to the pulmonary area, the heavy and continued antigenic stimulation may cause some cytokine leakage into the circulation. Fortunately, the clinical features of systemic cytokine release are rarely observed, indicating that there is no primary systemic immune deficiency associated with CF. However, bacteraemia and septicaemia have been reported in some *B. cepacia*-colonised patients, associated with 'cepacia syndrome', suggesting that in certain hosts the break down of pulmonary defences and/or a massive local cytokine release causes an uncontrollable cytokine 'spill over' into the circulation. Recent clinical data confirms the pronounced inflammatory response of *B. cepacia*- compared to *P. aeruginosa*-colonised CF patients with *B. cepacia* individuals exhibiting higher levels of the inflammatory markers neutrophil elastase and C reactive protein (an acute phase protein) both shown to be correlated with increased TNF-α (Elborn *et al*, 1994). To date, however, there has been no direct study on the interaction of *B. cepacia* and the cytokine network and consequently, this potential association forms a major part of this thesis.

Adaptive immunity

Studies investigating the antibody response to *B. cepacia* have shown immunoglobulins from all three major classes, IgG, IgM and IgA, to be produced against a range of cell surface antigens; LPS, pili and OMP (Butler, 1994). High

antibody levels against *P. aeruginosa* are often associated with poor clinical condition and are prognostic of a serious decline. In contrast, however, investigations into *B. cepacia* antibodies have shown no protective or prognostic value. For example, some *B. cepacia*-colonised CF patients produce enormous antibody titres and remain clinically unaffected whilst others decline. Similarly, some *B. cepacia* CF individuals deteriorate so rapidly that there is not enough time to mount a significant antibody response (Butler, 1994). However, *B. cepacia* antibodies have been suggested as a form of immunotherapy. A recent report proposes an antibody directed against a conserved 30kD OMP as a protective strategy against further decline (Burnie *et al*, 1995).

Interestingly, when antibiotic therapy has failed, administration of intravenous gammaglobulin has been used successfully to control *B. cepacia* exacerbations (Dr. J. Govan, personal communication). The efficacy of such preparations may be due to increasing the levels of anti-LPS or anti-OMP antibodies although these antibodies are normally present in significant numbers. This leads to speculation that the immunoglobulin mixture contains antibodies capable of neutralising inflammatory mediators such as TNF-α.

In conclusion, the interaction of microbe and host enables *B. cepacia* to become established in the respiratory tract. However, the mechanisms that allow this are poorly understood and once again highlight the need for more information on *B. cepacia* pathogenesis. One unresolved theory is that *B. cepacia* is an intracellular pathogen and therefore escapes the immune system and primes the host for infection. Studies in our laboratory are pursuing this theory.

1.5 *BACTEROIDES FRAGILIS*

1.5.1 TAXONOMY

In 1989, the genus *Bacteroides* was restricted to include only *Bacteroides fragilis* and closely related species based on rRNA and DNA homology (Shah & Collins, 1989). The new classification was an attempt to tighten the membership of the genus and prevent its use as a repository for any anaerobic Gram-negative rod; previous definitions of the *Bacteroides* genus have included 12 (Duerden, 1990), 23 (Holdeman *et al*, 1977) or 40 members (Holdeman *et al*, 1984), with 50 species recognised at the time of reclassification (Moore *et al*, 1985). At present, the genus *Bacteroides* consists of ten species; *B. fragilis* (type species), *B. caccae*, *B. distasonis*, *B. eggerthii*, *B. merdae*, *B. ovatus*, *B. stercoris*, *B. thetaiotaomicron*, *B. uniformis* and *B. vulgatus*. The taxonomic position of *B. variabilis* remains uncertain although on biochemical and physiological data it is likely that this organism also belongs to the *Bacteroides* genus (Shah & Gharbia, 1993).

The general characteristics of *B. fragilis* include the following features; Gram-negative, obligate anaerobe, non-motile, non-spore forming rod. *B. fragilis* is a fermentative, saccharolytic, chemoorganotrophic organism able to grow in the presence of bile. Nutritionally, *B. fragilis* requires sulphates, magnesium, citrate and small amounts of peptone and glucose to grow. Haemin and vitamin K may also stimulate growth. *B. fragilis* is normally found as a commensal in the gastrointestinal tract of man and the following section describes its role as part of the normal colonic flora.

1.5.2 *B. FRAGILIS* AS A COMMENSAL

The human gastrointestinal tract is rapidly colonised after birth with large numbers of bacteria obtained from the mother and surrounding environment. It is postulated that

facultative anaerobes such as *E. coli* are the primary colonisers, creating a reduced atmosphere to permit subsequent colonisation by obligate anaerobes (Hentges, 1993). In healthy adults, although the oesophagus, empty stomach and upper small intestine remain sterile, both the mucosal surface of the large intestine and the intestinal contents are heavily colonised by anaerobes, with the *Bacteroides* species the predominant genus. Indeed, of the 10^{11}-10^{12} bacteria present per gram of colonic contents, anaerobes outnumber aerobes by a factor of 100-1000:1 (Patrick, 1993).

Quantitatively, the *Bacteroides* faecal microflora and epithelium-associated microflora have been reported to differ. Namavar *et al* (1989) in comparing the frequency of bacteria in faeces and colonic tissue found *B. vulgatus* to be the most common *Bacteroides* isolate in faeces at 45%, with *B. fragilis* accounting for only 4%, fewer than for *B. uniformis* (22%), *B. thetaiotaomicron* (15%) and *B. distasonis* (9%). In contrast, sampling of the colonic tissue isolated *B. fragilis* most frequently at 43%, compared to only 25% for *B. vulgatus* and 10% for the other *Bacteroides* identified. These results imply that of the *Bacteroides* species, *B. fragilis* is the most closely associated with the gut mucosa, which may account for the high incidence of this organism in intra-abdominal infections (section 1.5.3.).

Commensal bacteria in the intestine act not only to protect this site against colonisation by non-indigenous organisms but also aid in the digestion and absorption of food. *Bacteroides* species are saccharolytic, capable of hydrolysing a wide range of polysaccharides including, xylans, glucans, pectins, galactomannans and even host-derived mucins (Shah & Gharbia, 1993; Hentges, 1993). The primary end products of polysaccharide fermentation are short chain fatty acids which can be absorbed from the gut to act as potential energy substrates (Drasar & Duerden, 1991). *B. fragilis* also attacks nitrogenous compounds and deconjugates bile acids (Bokkenheuser, 1993), although fermentation products of both processes have been implicated in

cancer (section 1.5.3.). Thus, *Bacteroides fragilis* participates in both colonisation resistance and the degradation of undigested foodstuff as a normal part of the intestinal microflora.

1.5.3 *B. FRAGILIS* IN DISEASE

The indigenous flora comprises virtually the only source of Gram-negative anaerobic bacteria involved in human infection, with *B. fragilis* the species most frequently isolated from clinical samples. The following section focuses on the disease states in which *B. fragilis* is known or thought to be involved.

B. fragilis in Inflammatory Bowel Disease

The immune system in the healthy gut has efficient self-regulatory mechanisms to prevent an uncontrollable inflammatory response in reaction to the huge number of antigens. However, there are a number of GI inflammatory disorders, including ulcerative colitis and Crohn's disease, which together are referred to as Inflammatory Bowel Disease (IBD). Although both diseases are characterised by inflammation of the mucosa due to an infiltrate of cytokines, leukotrienes, macrophages, lymphocytes and neutrophils, in ulcerative colitis only the superficial submucosa is inflammed, whereas in Crohn's disease the inflammation extends into the deeper layers of the bowel wall (Podolsky, 1991a).

The causes of IBD remain unknown although increasing evidence supports the theory that chronic IBD is the result of a dysfunctional immune system manifested by overproduction of mucosal cytokines (Casiniraggi *et al,* 1995). TNF-α, IL-1, IL-6 and interferon-γ have all been implicated as pathologically important in IBD (Woywodt *et al*, 1994). However, the mechanisms of this immune imbalance remain obscure. The improvement of Crohn's disease patients in response to metronidazole administration implies an important role for the anaerobic flora (Borriello, 1991).

Furthermore, as the sera of Crohn's disease patients exhibit increased levels of anti-bacteroides antibodies, an autoimmune reaction to a gut-derived bacterial antigen has also been proposed (Tvede *et al,* 1983). Complications associated with IBD include toxic megacolon, fistulas, abscesses and arthritis. A recent study has proposed a direct link between *Bacteroides* species and IBD-associated arthritis (Lichtman *et al,* 1995). IBD also appears to predispose patients to developing colonic cancer with a six-fold increase in risk association compared to controls (Podolsky, 1991b).

B. fragilis in colonic cancer

Colonic cancer is most common in North America, Australia and Northern and Western Europe, with Asia, Africa and Central and Southern America having relatively few cases (Hill, 1991). From epidemiological studies it is widely believed that environmental factors play the largest part in increasing the risk of developing colonic cancer, with diet one of the most important features. Numerous studies have demonstrated that the risk of colonic cancer is related to the amount of meat or fat in the diet (Armstrong & Doll, 1975; Lui *et al,* 1979) whereas conversely, a strong protective effect is associated with green-leafy vegetables, correlating with the diet of the countries of incidence (Hill, 1991).

B. fragilis, as a commensal, produces metabolic byproducts that are potentially important in human carcinogenesis. Indeed, there is a correlation between the number of *Bacteroides* species per gram of faeces and the risk of colonic cancer (Hill, 1991). The bile acids cholic and chenodeoxycholic acid are degraded by *B. fragilis* into the aromatic compounds deoxycholic and lithocholic acid and the amino acids tyrosine and tryptophan are also metabolised into volatile phenols, all implicated as carcinogens (Bokkenheuser, 1993). Several lines of evidence favour the involvement of bile acid metabolites in particular; 1) bile production is stimulated in the presence of a high fat 'Western' diet, 2) the faecal bile acid concentration is directly related to

adenoma size and 3) higher levels of bile acid byproducts and receptors are present in colonic cancer patients compared to controls (Hill, 1991).

The involvement of *B. fragilis* in colonic cancer and IBD has yet to be conclusively demonstrated, although the circumstantial evidence does postulate a role for this organism as an enteric pathogen. However, *B. fragilis* is most commonly associated with extraintestinal infections that arise from mucosal injury caused by trauma, burns, rupture, a penetrating wound or abdominal surgery.

B. fragilis in peritonitis and abscess formation

The peritoneal cavity is the most common site of contamination from indigenous bacteria with only a few millilitres of spilled intestinal contents, commonly resulting from organ perforation, required to deliver billions of bacteria into an otherwise sterile site. Infection arising from bacterial contamination comprises two phases. Initially, peritonitis, or inflammation of the peritoneal membrane may occur, which if left untreated is usually fatal. In survivors, intra-abdominal abscesses can form secondary to infection (Duerden, 1994). Generally, the purulent material recovered from abscess cavities is polymicrobial, consisting of a facultative anaerobe such as *E. coli* and an obligate anaerobe such as *B. fragilis* (Tally, 1993). Indeed, *B. fragilis* is isolated from the majority of abscess cases including intra-abdominal, vaginal, perianal, and brain abscesses (Patrick, 1993).

Synergism between *E. coli* and *B. fragilis* is thought to function in two ways. Initially, while the peritoneal cavity is well-oxygenated, facultative anaerobes metabolise the oxygen supplies and thus provide conditions for surviving obligate anaerobes to dominate. *B. fragilis* as one of the most oxygen-tolerant of obligate anaerobes is therefore particularly well suited for abscess formation. Secondly, the first line of defence against the infecting bacteria is phagocytic cells and the presence

of *B. fragilis* is thought to impair the phagocytic killing of facultative strains (Tally, 1993). This synergistic relationship was confirmed in initial studies in which bacteria were implanted either alone or in combination in a rat model of intra-abdominal infection. It was found that although mortality was restricted to animals receiving *E. coli* in the inocula, abscess formation did require the presence of both a facultative and obligate anaerobic strain. Abscesses did not form in response to *B. fragilis* alone (Onderdonk *et al*, 1976). These results therefore suggested that facultative organisms caused the peritonitis-associated deaths and together with obligate anaerobes were responsible for the formation of intra-abdominal abscesses. However, these studies used unencapsulated strains of *B. fragilis*.

In contrast, Onderdonk *et al* (1977) found that implanting encapsulated *B. fragilis* strains alone resulted in abscess formation. Furthermore, implantation of purified *B. fragilis* capsular material alone, or in combination with the previously used unencapsulated strains, also caused abscess formation. Abscesses formed in response to the capsule were bacteriologically sterile but histologically identical to abscesses formed in response to intact bacteria. The importance of EPS in the pathogenesis of abscess formation was highlighted by one study in which 75% of *B. fragilis* abscess isolates were found to possess a capsule compared to only 11% of faecal isolates (Brook *et al*, 1992). Capsular material is therefore an abscess-potentiating agent. Other material previously identified as conducive to abscess formation include bran, mucin and autoclaved colonic contents all of which comprise polysaccharides (Finlay-Jones *et al*, 1991).

The mechanisms by which complex polysaccharides enhance abscess formation is not clearly understood although recent work into the structure of *B. fragilis* capsule has provided some clues. The capsule produced by *B. fragilis* comprises two distinct polysaccharide units with oppositely charged amino and carboxyl/phosphate groups.

Alteration of the positive or negative charge on the capsule impairs the ability of encapsulated strains to form abscesses; conversely, the addition of an amino and carboxyl group to a neutral polysaccharide transforms the EPS into an abscess-inducing material (Tzianabos *et al*, 1992). Therefore, bacterial EPS possessing oppositely charged groups is important in the pathogenesis of abscess formation.

B. fragilis in SIRS

In many SIRS patients bacteria cannot be detected in the circulation and it is now becoming increasingly recognised that in these cases the source of endotoxin is likely to be the gut. Following insults such as burns or trauma the onset of shock may reduce the splanchnic circulation leading to intestinal ischaemia and a breakdown of the mucosal barrier. Bacteria and their products, including LPS, can then readily translocate to the systemic circulation via the portal vein to the liver (Runcie & Ramsay, 1990). Consequently, macrophages in the liver (Kupffer cells) may be activated, releasing cytokines and, as a result, inducing the symptoms associated with sepsis (section 1.3). Thus a critically ill patient may be diagnosed as 'septic' but with no obvious source of infection.

B. fragilis is the most common cause of anaerobic bacteraemia, but has lower incidence rates compared to *E. coli, P. aeruginosa* and Gram-negative cocci (Peraino *et al*, 1993). It has generally been assumed that *E. coli*, with its highly biologically active LPS, would play the major role in SIRS. *B. fragilis* possessing a weak endotoxin (10-1000 fold less active than *E. coli*) has been thought to be of little significance. However, as there is 100-1000 fold more *Bacteroides* than *E. coli* in the normal flora, the biological potential of LPS from *Bacteroides* species must be equivalent to that from *E. coli*. Thus *Bacteroides* species may play a vital role in SIRS where there is no obvious foci of infection and should not be disregarded.

1.5.4 *B. FRAGILIS* CELL SURFACE VIRULENCE DETERMINANTS

Lipopolysaccharide

The structure of *B. fragilis* LPS is based on the normal enterobacteriaceae template described in section 1.1.3. but displays many variations when compared to *E. coli* LPS. The lipid A region of *B. fragilis* LPS comprises a typical glucosamine disaccharide backbone but has different fatty acid substitutions. The fatty acids are longer (15-17 carbons compared to C12-C16 for *E. coli*), are branched hydroxylated and non-hydroxylated (not present in *E. coli*) and number only five per lipid A molecule (six in *E. coli*). In addition, in *B. fragilis* lipid A there is only one phosphate substituent; the phosphate group present on position 4 of the non-reducing sugar is missing (Lindberg *et al*, 1990).

Kdo was initially thought to be absent as negative results were obtained in conventional assays. Indeed, Kdo deficiency had been previously defined as a taxonomic criterion for these bacteria (Hofstad, 1974). However, subsequent studies have indicated the presence of a phosphorylated form of Kdo which is unreactive in standard tests (Beckman *et al*, 1989). The sugars D-galactosamine, D-glucose, D-galactose, L-rhamnose and D-glucosamine have been detected in *B. fragilis* LPS with a β,1,6-linked D-galactosyl oligosaccharide chain the immunodominant structure (Lindberg *et al*, 1990).

Despite many studies, there remains conflicting views on the LPS phenotype of *B. fragilis*. Poxton and Brown (1986) showed strains of *B. fragilis* on SDS-PAGE gels to comprise a series of closely spaced bands characteristic of S-LPS. In contrast, Lindberg *et al* (1990) failed to detect the characteristic smooth ladder pattern and proposed *B. fragilis* LPS to comprise lipid A, core and a short O-Ag chain but no long repeating units. However, *B. fragilis* MAb have been produced that react against a ladder pattern confirming the findings of Poxton and Brown (Patrick, 1993).

These discrepancies may be due not only to the extraction method used by the researchers but also the culture conditions, particularly as the closely associated capsular polysaccharide of *B. fragilis* is known to be very dependent on environmental conditions.

It is likely that the structural difference between *B. fragilis* LPS and *E. coli* LPS accounts for their variable toxicity. However, *B. fragilis* LPS has been reported as a potent inducer of DNA replication in B-lymphocytes and hence an inducer of polyclonal antibody production (Hofstad *et al*, 1993). Furthermore, the sheer volume of *B. fragilis* LPS present in the intestine confers a great potential to cause serious disease, as suggested for this organism in SIRS.

Outer membrane proteins

Few studies have investigated the OMP profile of *B. fragilis* but reports have shown the induction of novel OMPs under conditions of iron-limitation, including an immunogenic 44kD protein (Otto *et al*, 1990). Under iron-limited conditions *B. fragilis* uses haem from either haemoglobin or haptoglobin-haemoglobin as the sole source of iron with haem-binding protein complexes (HBP-complexes) involved in haem uptake. Four proteins of approximately 60kD, 58kD, 49kD and 35kD have been identified as haem-binding proteins (Otto *et al*, 1994). The iron-repressible 44kD OMP is required for a functional HBP-complex but the exact role of the protein remains unknown. Siderophore production in *B. fragilis* has not yet been detected although this organism may possess mechanisms for the uptake of siderophores from other intestinal bacteria such as *E. coli* (Patrick, 1993). *B. fragilis* as with all members of the *Bacteroides* genus is resistant to several antibiotics including some cephalosporins and penicillins. The production of β-lactamases by *B. fragilis* is one mechanism of drug resistance, with the role of OMP yet to be defined (Finegold, 1995).

Exopolysaccharide

Recent studies have revealed the *B. fragilis* capsule to comprise two distinct, ionically linked polysaccharide units, termed polysaccharide A and B. Both units are co-expressed on the surface of *B. fragilis* cells in the ratio 1:3.3. Structural analysis of the individual units revealed polysaccharide A to consist of a tetrasaccharide repeating unit with one free amino and one carboxyl group conferring zwitterionic properties on this molecule. Polysaccharide B comprises a hexasaccharide unit with one positive and two negative groups conferring a net positive charge to the polymer (Tzianibos *et al,* 1992).

In concurrent but unrelated studies, the size and antigenicity of the *B. fragilis* capsule has been shown to be heterogeneous in a wild-type population, and found to be extremely sensitive to environmental factors (Patrick & Reid, 1983; Patrick *et al,* 1986). A population of a single *B. fragilis* isolate may possess a large fibrous network (or capsule), a small fibrous network or an electron dense layer, as distinguished by electron microscopy. Furthermore, studies using MAbs raised against the capsular structures, revealed that the different components were not merely varying amounts of the same material but antigenically distinct compounds (Patrick, 1993).

Fimbriae

B. fragilis can successfully attach to host epithelial cells through the possession of fimbriae. Fimbrial structures were observed in 82% of *B. fragilis* strains found in the normal flora, 20% more than any other *Bacteroides* species (Brook *et al,* 1992), and enabling *B. fragilis* to attach more readily to the colonic mucosa. Thus, *B. fragilis* is in a prime position to translocate in response to mucosal injury, which may be one factor for this organisms clinical dominance over the other *Bacteroides* members. Interestingly, of *B. fragilis* abscess isolates, 81% possessed fimbriae compared to only

6% of bacteraemia isolates (Brook *et al*, 1992). It is possible that *B. fragilis* can modulate fimbriae production, and hence virulence, in relation to environmental conditions.

Extracellular factors

B. fragilis produces both an exotoxin and numerous extracellular enzymes including hyaluronidase, DNase, lipase, protease and neuraminidase. Although these hydrolytic enzymes are capable of degrading host tissue, it appears the enzymic activity is somehow localised, thereby preventing rapidly spreading destruction (Duerden, 1994). Recently, the exotoxin has been purified as a 20kD polypeptide containing a zinc-binding epitope, characteristic of a metalloprotease. The exotoxin does show cytotoxic activity but can be easily inhibited by metal chelators (Moncrief *et al*, 1995). The role of this toxin in *B. fragilis* pathogenesis has still to be defined.

The need for specialised equipment and the difficulty in the growth and identification of anaerobic organisms has led to an underestimate of the number of anaerobic infections. *B. fragilis* is primarily considered to be a commensal and has largely been ignored with regards to infection and disease. However, *B. fragilis* along with other members of the genus are becoming increasingly recognised as more than 'innocent bystanders' with recent studies confirming their role as serious opportunistic pathogens. The close association of the various cell surface components, susceptibility to environmental changes and within-strain variation has given rise to confused and contradictory reports on the potential virulence determinants of *B. fragilis*. More work on anaerobic organisms is required if the pathogenesis of *B. fragilis* infection is to be resolved.

AIMS OF THIS THESIS

In the past two decades, increasing scientific and clinical evidence has elevated two apparently harmless Gram-negative bacteria, *Burkholderia cepacia* and *Bacteroides fragilis*, to the status of human pathogens, capable of causing a serious threat to human health. For *B. cepacia* the main concern is the pulmonary colonisation of CF patients and for *B. fragilis* the potential role of the *Bacteroides* population in intra-abdominal sepsis and SIRS. As both organisms were considered previously to be of little significance in human disease, our knowledge of the virulence factors and pathogenesis of *B. cepacia* and *B. fragilis* is limited, particularly with regards to the interaction with the human host. The main aim of this thesis was to extend our understanding of the pathogenesis of *B. cepacia* and *B. fragilis*, by examining possible virulence factors and microbe-host interactions. To achieve the aim of this joint project the following four main areas were investigated for both organisms;

1) To characterise the expression and antigenicity of the cell surface virulence determinants: lipopolysaccharide, exopolysaccharide and outer membrane proteins.

2) To investigate the influence of relevant environmental conditions on the induction of new, or production of altered, cell surface components which may play a vital role in survival and pathogenesis.

3) To investigate the biological activity of *B. cepacia* and *B. fragilis* cell surface determinants, focusing on their endotoxicity and cytokine inducing ability from a range of clinically important cell types.

4) In addition, taxonomically and pathogenically related controls were examined to distinguish any variation between strains and species which may provide clues to pathogenic methods *in vivo*.

Results obtained from the above experiments could then be used to ascribe further the role of these organisms in infection.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 BACTERIA

Bacterial strains

Bacterial strains used in this thesis are listed in Table 2.1 and were obtained from the Cystic Fibrosis Laboratory (CFL) collection or Microbial Pathogenicity Research Laboratory (MPRL) collection, both at the Department of Medical Microbiology, University of Edinburgh.

Bacterial identification

Bacterial identification was performed by Mrs C. Doherty for CFL strains and by Mr R. Brown for MPRL strains. All CFL isolates were identified by API 20NE system (bioMerieux, Marcy l'Etoile, France). In addition, strains of *B. cepacia* were analysed by bacteriocin typing as described by Govan & Harris (1985) and by genotypic typing using Pulsed Field Gel Electrophoresis (PFGE· CHEF Bio-Rad Laboratories Inc., CA, USA). *Bacteroides* strains were identified by biochemical and physiological analysis (Brown *et al*, 1995).

Maintenance of bacterial strains

Bacteria were maintained at -70°C in 10%(w/v) skimmed milk (Oxoid, L31 Unipath Ltd., Basingstoke, Hants., UK.). Bacterial suspensions were prepared by emulsifying several colonies in 1ml volumes of skimmed milk in 2ml cryotubes (NUNC, Inter Med, Kamstrup, Denmark). Fresh growth cultures were obtained from -70°C after bacteria had been subcultured a maximum of four times. Fresh maintenance cultures were prepared at approximately five monthly intervals.

Table 2.1 Bacterial strains used in this thesis

Strains obtained from CFL	Origin
Burkholderia cepacia C1359[a]	CF Sputum
Burkholderia cepacia C1409	CF Sputum
Burkholderia cepacia C1504	CF Sputum
Burkholderia cepacia ATCC 17762	Urinary Tract Infection
Burkholderia cepacia J762	Wound Infection
Burkholderia cepacia C1732	CDC Pseudobacteraemia
Burkholderia cepacia C1744	CDC Povidone Iodine Solution
Burkholderia cepacia J2395	Hospital environment
Burkholderia cepacia J2540	Soil
Burkholderia cepacia ATCC 29424	Soil
Burkholderia cepacia J366	Soil
Burkholderia gladioli ATCC 10248	Type Strain
Pseudomonas aeruginosa C1250	CF Sputum
Pseudomonas aeruginosa PAO1	Genetic Type Strain
Pseudomonas aeruginosa J35	Derivative of PAO1
Pseudomonas aeruginosa J36	Derivative of PAO1
Pseudomonas aeruginosa J37	Derivative of PAO1
Pseudomonas aeruginosa J38	Derivative of PAO1

[a] a highly transmissible strain isolated from several CF centres (Govan *et al,* 1993)

Strains obtained from MPRL	Origin
Bacteroides fragilis NCTC 9343	Type strain (Appendix abscess)
Bacteroides fragilis MPRL1504	Wound swab
Bacteroides vulgatus MPRL 1651	Faeces
B. thetaiotaomicron NCTC 10582	Faeces
Bacteroides uniformis MPRL1721	Unknown
Escherichia coli O18K	A.S. Cross, Walter Reed Army Institute for Research, Washington D.C., USA

2.1.2 PREPARATION AND STERILISATION OF CULTURE MEDIA

Chemicals used throughout this thesis were of Analar Grade from BDH (Merck Ltd., Dorset, UK.) unless otherwise stated. All solutions were made with pyrogen-free water obtained from a Milli-Q Reagent Grade Water System (Millipore Corporation, Molshiem, France) and sterilised by autoclaving at 121°C/15psi for 15min unless otherwise stated.

2.1.3 MEDIA USED FOR CFL STRAINS

Nutrient rich medium

a) Nutrient yeast broth (NB+YE) was Oxoid No.2 supplemented with 0.5%(w/v) yeast extract (Difco Laboratories, Michigan, USA).

b) Nutrient agar (NA) was Columbia agar base (Oxoid)

c) *Pseudomonas cepacia* selective medium (CEP; Mast Laboratories, Bootle, UK.).

d) *Pseudomonas* Isolation agar (PIA; Difco).

e) Onion-agar medium (OA) was developed because of the phytopathogenic nature of *B. cepacia* to onions. The medium was prepared as follows; two onions were peeled, diced and homogenised until a thick suspension was obtained. The mixture was filter sterilised and added at a concentration of 5%(v/v).

Minimal medium

a) Malka medium A (MA; Robert-Gero *et al*, 1970) was prepared by the following recipe:-Solution A: Na_2HPO_4 (73.4g/l); KH_2PO_4 (32.4g/l)

Solution B: $MgSO_4.7H_20$ (20.5g/l)

Solution C: 50% (w/v) Glucose

Solution D: $FeSO_4.7H_2O$ (1.83g/l)

Solution E: $(NH_4)_2SO_4$ (50g/l)

All solutions were filter sterilised. Solutions A, B and E were stored over 5%(v/v) chloroform and to solution D one drop of concentrated hydrochloric acid was added. To prepare 1 litre of Malka A medium, 20ml of solutions A, B, C and E and 1ml of solution D were added to 919ml of sterile pyrogen-free water.

b) Malka A + 50%(v/v) serum (MA+S) was used to mimic a physiological medium. Sheep serum (Moredun Animal Diseases Research Institute, Gilmerton Road, Edinburgh) was heat inactivated at 56°C for 30min to destroy any complement that may have interfered with bacterial growth.

c) Nitrogen-limited medium (Malka B) was MA with solution E substituted with 1%(v/v) 1M KNO_3.

d) High osmolarity medium (Malka D) was MA with the addition of 6%(v/v) 5M NaCl.

e) Nitrogen-limited/high osmolarity medium (Malka H) was MA with the changes associated with both Malka B and D.

Malka A medium was used as the basal medium to which ingredients were either added, taken away or substituted in order to produce the desired environmental change as shown below.

f) Iron-limited medium was MA minus solution D and with the addition of 1mM of the iron chelator ethylenediamine-N,N'-diacetic acid; EDDA (Sigma, St. Louis, USA).

g) Magnesium-limited medium was MA with 0.1%(v/v) solution B.

h) Phosphate-limited medium was MA with 0.1%(v/v) solution A.

i) Semi-solid medium was MA with 0.4%(w/v) agar.

j) Alcohol-enriched medium was MA with the addition of 5%(v/v) ethanol.

k) Carbon-limited medium was MA with the substitution of solution C with 1%(w/v) mannose.

To prepare Malka agar plates 2%(w/v) agar was added to pyrogen-free water, autoclaved and the Malka solutions and supplements added as before.

Phosphate buffered saline (PBS) was made by dissolving 1 PBS tablet (Oxoid) in 100ml of pyrogen-free water and sterilised by autoclaving.

Aerobic bacteria were grown at 37°C overnight with broth cultures shaken in an orbital incubator at 180 rev. min^{-1} unless otherwise stated. All cultures were checked for purity by both Gram stain and streaking for single colonies on blood agar plates.

2.1.4 MEDIA USED FOR MPRL STRAINS

Nutrient rich medium

a) Proteose Peptone Yeast extract medium (PPY; Holbrook *et al,* 1977) comprised;

Protease Peptone (Oxoid) 20g/l;

Yeast Extract (Difco) 10g/l;

Sodium chloride 5g/l

3.75%(w/v) L-cysteine hydrochloride 20ml/l

2%(w/v) Sodium carbonate 20ml/l

Haemin (250mg/ml) + Menadione (50mg/ml) 20ml/l

b) Columbia Agar with Horse Blood (BA) was supplied ready-made from Oxoid

Minimal medium

a) Van Tassell & Wilkins' medium (VT&W) (Van Tassell & Wilkins, 1978) was

prepared by the following recipe:-

Solution A: $(NH_4)_2SO_4$ (2g/l); Sodium citrate (0.5g/l); Vitamin B_{12} (5µg/l); KH_2PO_4 (7g/l); K_2HPO_4 (8g/l). The pH was adjusted to pH 6.93

Solution B: $MnCl_2.4H_2O$ (10mg/l); ,$MgCl_2.6H_2O$ (20mg/l); $FeCl_3.6H_2O$ (0.3mg/l); $CaCl_2.2H_2O$ (30mg/l)

Solution C: 10%(w/v) Glucose

Solution D: 5%(w/v) L-cysteine hydrochloride

Solution E: 10%(w/v) $NaHCO_3$

Solution F: Haemin made up in 0.1M NaOH (5mg/l)

Solutions A-E were autoclaved and solution F was filtered sterilised. To prepare 1 litre of VT&W medium, 350ml of solution A, 500ml of solution B, 100ml of solution C, 10ml of solution D and 1ml of solution E were mixed together.

VT&W was used as the basal medium to which ingredients were added, taken away or substituted in order to produce a desired environmental change as shown below.

b) VT&W + 50%(v/v) serum (VT&S). Sheep serum was heat inactivated as described above for MA+S.

c) Iron-deficient media was VT&W minus $FeCl_3.6H_2O$ and the addition of 1mM of the iron chelator EDDA.

d) Bile-enriched media was VT&W with the addition of 0.5%(w/v) Bile salt solution (Oxoid).

e) Cysteine-enriched media was VT&W with the addition of a further 0.5%(w/v) L-cysteine hydrochloride.

Media used for anaerobic growth was prereduced overnight and anaerobic bacteria grown overnight in a Forma Scientific Anaerobic System Cabinet, model 1024 (Forma Scientific, Ohio, USA) in a 10% CO_2, 10% H_2, 80% N_2 atmosphere unless otherwise stated. All cultures were checked for purity by both Gram stain and streaking for

single colonies on blood agar plates.

2.1.5 ANTISERA

Polyclonal antisera to *B. cepacia* was obtained from the CFL serum collection. Initial serum came from either CF patients (sera prepared by Mrs C. Doherty, Department of Medical Microbiology, University of Edinburgh) or from Dutch rabbits inoculated with heat-killed whole bacteria (sera prepared by Dr. S. Butler, formerly Department of Medical Microbiology, University of Edinburgh). When using serum from a CF patient the bacteriology of the patient was taken into account.

Polyclonal antisera to *B. fragilis* was obtained from the MPRL serum collection. Initial serum was from rabbits inoculated with whole bacteria (sera prepared by Mr R. Brown, Department of Medical Microbiology, University of Edinburgh).

2.1.6 EQUIPMENT

Equipment used in this thesis is indicated in the relevant section of text.

2.2 METHODS

2.2.1 INFLUENCE OF ENVIRONMENTAL CONDITIONS ON BACTERIAL SURVIVAL AND GROWTH

Nutrient changes and atmosphere

Bacteria were patch inoculated onto the various minimal media as described in section 2.1.3 for CFL strains. Plates were incubated at 37°C in one of the following four atmospheres;

Aerobic: as normal

Microaerophillic: a 4% O_2/96% CO_2/H_2 or 4% O_2/96% N_2 atmosphere was produced in a gas jar system as described by Collee & Marr (1989). Air was evacuated to a

pressure of 24" Hg and refilled with either a 10% CO_2/90% H_2 background or 80% N_2 background.

Anaerobic: a 10% CO_2/10% H_2/80% N_2 atmosphere was used as described for maintenance of anaerobic cultures.

Survival, growth and changes in colony morphology were monitored every 24h over a four day period.

pH

B. cepacia strains (C1359, C1409, J366, J762, C1732 and C1744) were inoculated onto OA, CEP, and MA agar plates; the pH of the MA media was preadjusted to pH 7.0, 6.3, 5.55, 5.0 and 4.0. pH changes occurring during growth were monitored every day for eight days using a combination pH electrode (Fisons, Loughborough, UK).

Antibiotics

The minimum inhibitory concentration (MIC) of the antibiotics metronidazole and chloramphenicol against the *B. fragilis* strains NCTC 9343 and MPRL 1504 was ascertained by the serial discontinuous concentration tube method as described by Scott (1989). The MIC for the two *B. fragilis* strains on BA plates (kindly supplied by Mr R. Brown, Department of Medical Microbiology, University of Edinburgh) was used as an initial test concentration for the antibiotics. VT&W medium was prepared with 8 times-$^1/_{16}$ times the BA-determined MIC of the antibiotics.

B. fragilis strains grown overnight in 10ml PPY were diluted 1:100 in VT&W medium and incubated for a further 3h. When the bacteria had reached mid-log phase three drops of the bacterial suspension was added to each dilution tube. VT&W media without any added antibiotic or bacterial suspension were used as controls. The tubes were incubated anaerobically overnight and the MIC defined as the lowest

concentration of antibiotic that inhibited visible growth.

2.2.2 PREPARATION OF LPS

Proteinase K method

LPS was prepared by the Proteinase K digestion method as described by (Hitchcock & Brown, 1983). Bacteria grown overnight in 10ml broth culture were harvested by centrifugation and washed twice in PBS at 4000 *g* for 15min. The bacterial suspension was diluted in PBS to give an optical density (OD_{525nm}) of between 0.5-0.6 as measured by a Pye Unicam SP600 spectrophotometer. Aliquots (1.5ml) were placed in eppendorf tubes and centrifuged at 10,000 *g* for 3min in a Microcentaur centrifuge. The pellet was then resuspended in 50µl of double-strength sample buffer and heated at 100°C for 10min in a boiling water bath. Once cooled, 10µl of a 2.5mg/ml solution of proteinase K (protease Type XI, Sigma) in double-strength sample buffer was added and the mixture incubated at 60°C for 1hour. The sample was stored at -20°C until required.

Phenol water (PW) method

This procedure was based on the hot aqueous phenol method of Westphal and Lüderitz (1954) as described by Hancock & Poxton (1988). No plastic material was used during this procedure.

Bacteria grown overnight in five litres of medium were harvested and washed twice in PBS at 10,000 *g* for 15min in a Sorvall RC-5B centrifuge (Dupont UK Ltd., Stevenage, UK). The resulting pellet was frozen at -20°C, lyophilised (Edwards Modylo freeze dryer, Edwards High Vacuum Ltd., Surrey, UK) and weighed.

The lyophilised pellet was resuspended to a 5%(w/v) solution and heated to 67°C in a water bath. An equal volume of 90%(w/v) aqueous phenol was also heated and added to the cell suspension. The mixture was stirred every minute for 15min and cooled in an ice-bath for 30min to allow initial separation of the phenol and water

phase. To complete the separation, the mixture was centrifuged at 10,000 *g* for 15min in a Sorvall centrifuge. The upper aqueous phase was collected, transferred to pre-boiled dialysis tubing (3-20/32"; Medicell International Ltd., London, UK.) and dialysed against running tap water until the smell of phenol was no longer detectable (approximately 24h). Insoluble material was removed by centrifugation at 10,000 *g* for 15min. For crude LPS extracts, the dialysate was then freeze-dried, weighed and stored in a sterile container at -20°C until required. The lower phenol layer from *B. cepacia* C1359, C1409 and *P. aeruginosa* C1250 were also collected, dialysed and lyophilised.

For further purification of the LPS, the dialysed extracts were concentrated by rotary evaporation (Buchi Rotavapor-RE111, Switzerland) to approximately one-fifth the original volume and ultracentrifuged (Sorvall ultracentrifuge-OTD65B, Dupont) at 100,000 *g* for 3h. The resulting pellet was resuspended in 5ml of pyrogen-free water using a syringe fitted with a 23-gauge needle. The final LPS solution was freeze-dried, weighed and stored in a sterile container at -20°C until required.

Phenol chloroform petroleum spirit (PCP) method

This method is based on the procedure described by Galanos *et al* (1969) but incorporates the ether-acetone precipitation of LPS as described by Qureshi *et al* (1982). No plastic material was used during this procedure.

Bacteria were grown, harvested and lyophilised as described for the PW preparations. The extraction solution (PCP) consisted of a 90%(w/v) phenol, chloroform and petroleum spirit (boiling point 40°C-60°C) mixture in the ratio 2:5:8 by volume. Dried bacteria were resuspended in 25%(w/v) PCP, stirred for 2 min at 4°C and centrifuged at 10,000 *g* for 15min. The supernate was filtered through Whatman No.1 filter paper (Whatman International Ltd., Maidstone, UK) into a round bottom flask and the pellet re-extracted using PCP as before. The filtered supernates were pooled and the chloroform and petroleum spirit (volatile solvents) removed by rotary

evaporation. To precipitate the LPS six volumes of diethyl ether-acetone in the ratio 1:5 by volume were added to the remaining one volume of phenol solution and left to stand for at least 30min. The LPS was sedimented by centrifugation at 10,000 *g* for 15min and washed twice in the diethyl ether/acetone mixture and twice in diethyl ether alone. The final pellet was dried by leaving the ether to evaporate for 48h in a fume cupboard. The dried pellet was resuspended in pyrogen-free water, dialysed against running tap water for 48h and finally lyophilised.

Periodate-treated and Proteinase K-treated LPS samples

To 100µl of PW-LPS sample (5mg/ml), 100µl of 0.1M sodium periodate was added and the mixture stored at room temperature in the dark overnight. Excess periodate was removed by adding 50µl of ethylene glycol and the sample stored at -20°C until required.

To 150µl of LPS sample (5mg/ml) 30µl of proteinase K (2.5mg diluted in 1ml 0.125M Tris-HCl) was added and heated at 60°C for 1h.

2.2.3 PREPARATION OF OUTER MEMBRANES

Bacteria were grown in 500ml of medium overnight. Cells were harvested and washed twice in PBS at 8000 *g* for 15min in a Sorvall centrifuge. The pellet was resuspended in 5ml pyrogen-free water and broken by sonication (Microson, Ultrasonic Cell Disrupter, Heat Systems-Ultrasonics Inc., NY, USA) by ten 1min bursts with five 30 second intervals at an amplitude of 8-10µm. Unbroken cells were removed by centrifugation at 4,000 *g* for 10min. Outer membranes were derived by solubilisation by addition of 0.5ml of 7%(v/v) solution of Sarkosyl, 0.1mM of phenylmethylsulphonylfluoride was added as a protease inhibitor. Outer membranes were collected by ultracentrifugation for 1h at 50,000 *g*. Pellets were washed in 5ml of water, ultracentrifuged as before and finally resuspended in 1ml of pyrogen-free water by repeated passage through a 23-gauge needle and stored at -20°C until

required. Protein content in the samples was determined by the Folin assay of Lowry *et al* (1951) and adjusted to give a protein concentration of 800µg/ml.

2.2.4 PREPARATION OF ALGINATE

An overnight culture (300ml) of a mucoid *P. aeruginosa* (C1250) was centrifuged at 20,000 *g* for 1h and the supernate transferred to a sterile beaker. Two volumes of acetone were added, to separate out the alginate, and the mixture left overnight. The resulting precipitate was washed twice in ethanol at 10,000 *g* for 15min before redissolving in pyrogen-free water. The alginate was reprecipitated as before and left overnight. Once redissolved in pyrogen-free water the solution was dialysed against running tap water for 48h and in 2l of pyrogen-free water containing 1mM EDTA overnight. The EDTA solution was changed three times with pyrogen-free water and the final dialysate frozen and lyophilised.

2.2.5 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Lipopolysaccharide samples

LPS samples were added to gels at 20µl per track for silver-staining or 45µl per track for immunoblotting. Proteinase K samples prepared as described in section 2.2.2. and PW and PCP samples (5mg/ml) mixed with an equal volume of double-strength sample buffer, were heated at 100°C for 5min. LPS separation was performed on 14%(w/v) acrylamide gels omitting SDS from both the separating and stacking buffers.

Outer membrane samples

OM samples were added to gels at 40µl per track. Samples were heated to 100°C for 5min prior to addition to a gel. OM separation was performed on 10%(w/v) acrylamide gels with SDS included in both buffers.

LPS and OM preparations were separated on polyacrylamide gels using the buffer system of Laemmli (1970) with the following solutions used:

a) Double strength sample buffer (pH6.8) comprised 0.125M Tris-HCl, 4%(w/v) sodium dodecylsulphate (SDS), 20%(v/v) glycerol, 2%(v/v) 2-mercaptoethanol and 0.002%(w/v) bromophenol blue.

b) Double-strength separating buffer (pH8.8) comprised 0.75M Tris-HCl and 0.2%(w/v) sodium dodecylsulphate SDS.

c) Double strength stacking buffer (pH6.8) comprised 0.25M Tris-HCl and 0.2%(w/v) SDS.

d) 40%(w/v) Acrylamide comprised 100g acrylamide and 2.7g methylene bis acrylamide.

e) Electrode buffer (pH8.3) comprised 0.025M Tris, 0.192M glycine and 0.1%(w/v) SDS.

The volume of reagents required to prepare polyacrylamide gels of various acrylamide concentrations is shown in Table 2.2.

The separating gel (14% or 10%) and stacking gel (4%) were prepared as shown in Table 2.2, and degassed to remove any air bubbles prior to the addition of the TEMED and APS. Once poured, the separating gel was overlaid with water-saturated butan-2-ol and left to set. After removal of the butan-2-ol, the stacking gel was poured on top of the separating gel, a 20 well comb inserted, and once the gel had set, the comb was removed and the gel apparatus placed into an electrophoresis tank. Samples were loaded into the wells of the stacking gel and electrophoresed through the stacking gel at a constant 60V and through the separating gel at a constant 150V until the dye front had run approximately 9cm. After electrophoresis, the samples were visualised by either staining the gel or immunoblotting after transfer to nitrocellulose sheets.

Table 2.2 Preparation of polyacrylamide gels

Reagent	Stacking gel	Separating gel	
	4%	14%	10%
Pyrogen-free water	3.5	3.45	6.95
Separating buffer	-	17.5	17.5
Stacking buffer	5.0	-	-
40%(w/v) acrylamide	1.0	12.25	8.75
TEMED (NNN'N'-tetramethyl-1,2-diaminoethane)	0.02	0.05	0.05
Ammonium persulphate (APS) (15mg/ml)	0.5	1.75	1.75

(Hancock & Poxton, 1988)

2.2.6 SILVER STAINING FOR VISUALISING LIPOPOLYSACCHARIDE

This procedure is based on the method of Tsai & Frasch (1982) as described by Hancock & Poxton (1988). The following solutions were used:

a) Fixative solution comprised 7%(v/v) acetic acid and 25%(v/v) propan-2-ol.

b) Oxidising solution comprised 1.05g periodic acid in 150ml pyrogen-free water containing 4ml of fixative.

c) Ammonical silver nitrate solution comprised 1.4ml ammonia solution and 21ml of 0.36%(w/v) sodium hydroxide to which 4ml of 19.4%(w/v) silver nitrate was slowly added with mixing. The solution was made up to 100ml with pyrogen-free water.

d) Developing solution comprised 200ml formaldehyde solution containing 10mg citric acid.

After overnight soaking in fixative, gels were oxidised for 15min in freshly prepared periodic acid solution. Gels were then washed in four changes of distilled water over a period of 3h and silver-stained for LPS by the addition of freshly-prepared ammonical silver nitrate solution for 15min. Over a period of 40min the gels were washed in at least four changes of distilled water. The LPS was visualised by adding 200ml of freshly prepared developing solution. The above steps were carried out on a

shaking platform. Once the desired staining intensity was reached gels were washed thoroughly in distilled water.

2.2.7 COOMASSIE BLUE STAINING FOR VISUALISING PROTEINS

This procedure is based on the method described by Hancock & Poxton (1988). The following solutions, made up in distilled water, were used:

a) Solution 1 comprised 25%(v/v) propan-2-ol, 10%(v/v) acetic acid and 0.05%(w/v) Coomassie brilliant blue R-250 (Bio-Rad).

b) Solution 2 comprised 10%(v/v) propan-2-ol, 10%(v/v) acetic acid and 0.005%(w/v) Coomassie blue.

c) Solution 3 comprised 10%(v/v) acetic acid and 0.025%(w/v) Coomassie blue.

d) Solution 4 comprised 40%(v/v) methanol and 10%(v/v) acetic acid.

e) Solution 5 comprised 10%(v/v) acetic acid.

After overnight soaking in solution 1 gels were placed sequentially through solutions 2-5 for 60min each. All steps were carried out on a shaking platform.

2.2.8 IMMUNOBLOTTING

This procedure is based on the method of Towbin *et al* (1979) as described by Hancock & Poxton (1988). The following solutions were used:

a) Immunblot transfer buffer (pH8.3) comprised 6g of Tris, 29g of glycine and 500ml of methanol in two litres distilled water.

b) Tris buffered saline (TBS; pH7.5) comprised 4.84g Tris and 58.48g sodium chloride in two litres distilled water.

c) Tween-Tris buffered saline (TTBS) comprised TBS with 0.025%(v/v) Tween-20.

d) Blocking solution comprised TBS with 3%(w/v) gelatin.

e) Antibody diluent comprised TBS with 1%(w/v) gelatin.

f) Polyclonal antisera for the first antibody was obtained from either the CFL serum collection or from the MPRL serum collection. Sources of the antisera are indicated

in section 2.1.5 and in the relevant result section. Peroxidase-labelled anti-rabbit or anti-human antibody conjugate (IgG) was diluted 1:1000 as according to the manufacturers' instructions (Sigma).

f) Horseradish peroxidase (HRP) colour development solution comprised 30mg HRP colour reagent (Bio-Rad EIA Purity Grade) dissolved in 10ml of methanol, added to 50ml TBS containing 30µl hydrogen peroxide.

Samples were separated by PAGE as described above. The gel was placed on a Scotchbrite™ pad of a blotting apparatus and covered by a nitrocellulose membrane (0.2µm pore size, Schleicher & Schuell, Dassel, Germany) presoaked in immunoblot transfer buffer. The immunoblot cassette was closed sandwiching the gel and nitrocellulose sheet between the Scotchbrite™ pads and placed in an immunoblotting tank. Care was taken to expel any air bubbles from the cassette system that may have interfered with transfer. Antigens were transferred from the gel to the nitrocellulose membrane overnight at 10-12V; a constant current of 40mA.

The nitrocellulose membrane was washed in TBS for 10min and placed in blocking solution for 45min, before incubating with a 1:200 dilution of the first antibody for 3h. The nitrocellulose membrane was washed briefly in distilled water before two 10min washes in TTBS. The nitrocellulose membrane was then incubated for 1h with the HRP-conjugated second antibody. After washing as before, the binding of antibody to separated antigenic determinants was visualised by addition of the HRP colour solution until the required intensity was reached. All the above steps took place on a shaking platform. The nitrocellulose membrane was washed thoroughly in distilled water, blotted dry and stored in the dark.

2.2.9 PERCOLL DISCONTINUOUS DENSITY CENTRIFUGATION

This method was described by Patrick & Reid (1983). Percoll (Pharmacia LKB, Uppsala, Sweden) was diluted to 80%, 60%, 40% and 20%(v/v) solutions using

0.15M NaCl. Starting with the 80% solution and working sequentially, 1ml of each solution was carefully layered (80% at the bottom) into 70 x 20mm sterile glass tubes. A 1ml sample of an overnight bacterial culture was carefully applied to the top of the 20% layer and the gradient centrifuged at 3000 *g* for 15min in a bench centrifuge, allowing the bacteria to sediment according to their buoyant density.

2.2.10 CELL SURFACE HYDROPHOBICITY

Cell surface hydrophobicity was determined by Hydrophobic Interaction Chromatography (HIC). This procedure is based on the method described by Smyth *et al* (1978) and uses the following solutions:

a) Octyl sepharose CL-4B (Pharmacia) was used as the non-polar ligand.

b) Sepharose CL-4B (Pharmacia) was used to correct for non-specific adsorption.

c) Binding buffer comprised 50mM phosphate buffer (0.2mol/l NaH_2PO_4 + 0.2mol/l Na_2HPO_4) containing 1.7M $(NH_4)_2SO_4$.

d) The gel slurry referred to throughout this section comprised 75%(v/v) binding buffer and either 25%(w/v) Octyl sepharose or Sepharose.

Preparation of the HIC columns

To a disposable polystyrene chromatography column, 2mm in diameter, (Pierce & Warner Ltd., Chester, UK), 2ml of degassed water was added and a polyethylene disc pushed to the bottom of the column. The water was removed and 2ml of degassed pre-swollen gel slurry added. A polyethylene disc was added to the top of the slurry, the column capped to prevent drying out and the gel slurry left to settle for 30min. Columns were prepared fresh on the day of use or overnight for anaerobic reduction.

Determination of bacterial cell surface hydrophobicity by HIC.

Bacteria grown overnight in 10ml of broth were harvested and washed twice in sterile binding buffer. Bacteria were resuspended in buffer to give 10^4cfu/ml. Aliquots

(2ml) of bacterial suspension were added to the column and the eluate collected in 70 x 20mm sterile glass tubes. To ensure adequate wash-through of cells 2ml of binding buffer was also added and the eluate collected in the same glass tube. Retention of cells by the columns was determined by the difference in viable counts between the initial suspension and the column eluate. All counts were performed in duplicate.

2.2.11 ROCKET IMMUNOELECTROPHORESIS (RIE)

This procedure is described by Hancock & Poxton (1988). The following solutions were used:

a) Electrophoresis buffer comprised two solutions;

Solution 1 comprised 1.3%(w/v) barbitone sodium and 0.2%(w/v) barbitone.

Solution 2 comprised 5.6%(w/v) glycine and 4.5%(w/v) Tris.

Equal volumes of solutions 1 and 2 were mixed and the pH adjusted to 8.8.

b) Agarose gel comprised 1.5%(w/v) agarose, 25%(v/v) electrophoresis buffer and 1%(w/v) Triton X-100.

c) Stain comprised 0.5%(w/v) Coomassie blue R-250, 45%(v/v) ethanol and 10%(v/v) acetic acid made up in distilled water.

d) Destain comprised the stain as in c) above but omitted the Coomassie blue dye.

A 50mm square sheet of Gelbond™ (Pharmacia) was placed hydrophilic side up on to a glass plate and laid on a flat level table. To a 3ml aliquot of molten agarose at 50°C, 0.5ml of polyclonal antiserum, obtained from either the CFL or MPRL serum collection was added, taking care that the agarose had sufficiently cooled so the antibodies were not destroyed. The agarose was cast onto the Gelbond™ square and left to set. Up to six 3mm diameter wells were cut into the middle of the agarose and loaded with 20µl of LPS or OM sample.

Gels were placed in an electrophoresis tank with the wells set perpendicular to the line of current. Presoaked wicks of Whatman No.1 filter paper were used to connect the edges of the gel to the electrophoresis buffer and a current of 100mA applied for

4-5h. Gels were then overlaid with a sheet of filter paper, several sheets of blotting paper, a glass plate and pressed for 15min with a 1kg weight before being washed in two changes of 0.01M sodium chloride and one wash of distilled water for 15min each and pressed as before. The flattened gels were dried with a hair-drier, stained for 10min and then destained to visualise the antigen-antibody reaction.

2.2.12 CROSSED IMMUNOELECTROPHORESIS (CIE)

This procedure is a two-dimensional version of RIE and is described by Hancock & Poxton (1988). The solutions used are the same as described above for RIE.

Preparation of the first-dimensional gel

A 80mm square of Gelbond™ was placed hydrophilic side up onto a glass plate and 15ml of molten agarose poured onto the square. Once set, up to four circular wells, approximately 3mm in diameter were cut into the agarose, spaced 20mm apart and positioned 20mm from one edge. Wells were loaded with 20µl of sample and the gel placed in an electrophoresis tank with the edges of the gel connected to the buffer by Whatman No.1 presoaked filter paper. After applying a current of 100mA for 1h, 50mm x 10mm agarose strips, incorporating the wells, were cut from the gel and placed on the edge of a 50mm square sheet of Gelbond™. Wells were positioned at the bottom right hand corner.

Preparation of the second-dimensional gel

The second-dimensional gel was prepared in a similar way to RIE. A 3ml aliquot of molten agarose containing 0.5ml of polyclonal serum was cast against the first-dimension strip and allowed to set. Gels were placed in the electrophoresis tank with the first-dimensional strips nearer the cathode. The edge of the gels were connected to the buffer by means of pre-soaked filter paper. A current of 100mA was applied overnight at 4°C.

Visualisation of antigen-antibody interactions in CIE gels

Gels were pressed, washed, stained and destained as described above for RIE.

2.2.13 CHEMICAL ANALYSIS OF LPS SAMPLES

LPS preparations were used at a concentration of 5mg/ml. Optical densities were read against a reagent blank, (pyrogen-free water taken through the same procedure as the samples) in a SP-6 Pye-Unicam Spectrophotometer. All readings were performed in duplicate.

Estimation of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) content.

LPS preparations were assayed for Kdo by the standard thiobarbituric acid (TBA) method (Kharkanis *et al*, 1978).

The following solutions were used:

a) 25mmol periodic acid in 62.5mmol sulphuric acid

b) 2%(w/v) sodium arsenite in 0.5M hydrochloric acid

c) 0.6%(w/v) thiobarbituric acid (TBA) adjusted to pH 9 with sodium hydroxide

A 10µl aliquot of a 1mg/ml solution of Kdo was used as the standard.

TBA method

LPS samples (40µl, 100µl, 400µl, made up to 0.5ml with pyrogen-free water) were mixed with 0.5ml of 0.25M H_2SO_4 and heated at 100°C for 8min. Insoluble material was removed from turbid suspensions by centrifugation at 4000 *g* for 10min. Aliquots (0.5ml) of the acid hydrosylate were mixed with 0.25ml of periodic acid reagent and incubated at 37°C for 30min. Once cooled, 0.25ml of sodium arsenite solution was added and the solution mixed until the brown colour disappeared, before addition of 0.25ml of TBA solution. The solution was heated at 100°C for 7.5min and whilst still hot 1ml of dimethylsulphoxide added to preserve the colour. Adsorption was measured at 548nm.

Hydrofluoric acid Dephosphorylation

This method is based on the procedure described by Beckmann *et al* (1989). Hydrofluoric acid dephosphorylation was used to remove any potential phosphate substituent from the Kdo moiety which would have prevented a detectable reaction in the standard TBA assay.

LPS samples (1mg) were added to 0.3ml of aqueous 50% hydrofluoric acid and sealed in polypropylene tubes at 4°C for 48h. Volatile material was removed at room temperature by a stream of nitrogen gas and the residue dissolved in 0.3ml of pyrogen-free water. The sample was again dried by a stream of nitrogen gas and the residue resuspended in 1ml of pyrogen-free water and kept at -20°C until required. The Kdo content of the samples was analysed by the TBA method described above.

Estimation of phosphorus content.

The phosphorus content was estimated by the method of Chen *et al* (1956). The following solutions were used:

a) Digestion mixture comprised concentrated sulphuric acid and 60% perchloric acid mixed 3:2(v/v).

b) Phosphate reagent comprised 10% 3M sulphuric acid, 0.25%(w/v) ammonium molybdate and 1%(w/v) ascorbic acid.

A 100µl aliquot of a 10µg/ml sample of phosphorus (NaH_2PO_4) was used as a standard.

LPS samples (20µl and 40µl) were reduced to dryness by heating on a rack, before 0.1ml of digestion mixture was added and the solutions heated to boiling point. Solutions were then refluxed for 20min or until a clear solution remained. After cooling, 8ml of freshly prepared phosphate reagent was added, mixed, and the solution incubated at 37°C for 90min. Absorbance was read at 820nm.

Estimation of Carbohydrate content

The presence of neutral sugars was estimated by the phenol-sulphuric acid method described by Dubois *et al* (1956).

A 50µl aliquot of a 180µg/ml glucose solution was used as a standard.

LPS samples (20µl and 40µl) were made up to 0.5ml with pyrogen-free water and mixed before addition of 0.5ml of 5%(w/v) phenol. After mixing, 2.5ml of sulphuric acid was pipetted directly onto the samples by aid of a dispensette (Brand, West Germany). After cooling for 30min the absorbance was read at 490nm.

Estimation of protein content

The protein concentration was estimated for both LPS and OM preparations by the Folin assay of Lowry *et al* (1951). The following solutions were used:

a) 12.5%(w/v) sodium carbonate

b) 0.1%(w/v) copper sulphate

A 25µl aliquot of a 2mg/ml solution of bovine serum albumin was used as a standard. Samples (5µl and 25µl) were made up to 0.4ml with pyrogen-free water and 1.2ml of 12.5% Na_2CO_3 and 0.2ml of 0.1% $Cu.SO_4.5H_2O$ added. Solutions were mixed and left to stand for 1h. Folin reagent diluted 1:3 was added (0.2ml) and the mixtures left for 25min. The absorbance was read at 750nm.

2.2.14 LIMULUS AMOEBOCYTE LYSATE (LAL) ASSAY

LPS preparations (5mg/ml) were assessed for endotoxic activity by the 'kinetic method for the determination of endotoxin in water' by the Coatest© Endotoxin Kit (Chromogenix, Mölndal, Sweden). LPS samples were diluted in pyrogen-free water to a concentration of 5ng/ml-0.05ng/ml and 50µl of each sample added to a flat-bottomed microtitre plate. The control endotoxin (*E. coli* 0111:B4) was serially diluted 1 in 5 in pyrogen-free water to provide a standard concentration range of 24-0.0384 endotoxic units (EU)/ml. The outer wells were omitted to avoid

temperature gradients that may have interfered with the result. Freshly prepared chromogenic LAL reagent (20µl) was added to the wells by means of a transfer plate to ensure that each well received the reagent at the same time. Plates were read kinetically every 19 seconds for 90 minutes in a Thermomax plate reader (Molecular Devices, London, UK) at 405nm. All samples were tested in duplicate. All material coming into contact with the sample was purchased as endotoxin-free (Rainin Instrument Co. Inc., CA, USA) or depyrogenated by heating to 250°C for 2.5h.

2.2.15 PREPARATION OF CELLS FOR CYTOKINE ASSAYS

Separation of mononuclear leucocytes (MNL)

Human mononuclear leucocytes were separated from heparin treated human leukocyte concentrate (buffycoat; 30% monocytes; supplied by Blood Transfusion Service, Edinburgh) on lymphocyte separation medium (ICN Flow, CA, USA) following a two-fold dilution in RPMI 1640 (ICN Flow). Cells were harvested at 1000 *g* for 30min with the MNL forming a white interface between the serum and separation medium. This layer was carefully transferred with a sterile pasteur pipette to a sterile tube and washed three times in RPMI 1640 at 4000 *g* for 15mins. At the last wash, cells were resuspended in pre-warmed RPMI 1640 supplemented with 10% Foetal Calf Serum (FCS; Sigma), 1mM L-glutamine (Greiner Labortechnik Ltd., Gloucestershire, UK) and penicillin + streptomycin (100µg/ml; Gibco BRL Life Technologies, Paisley, UK) to a concentration of 8 x 10^6 cells /ml. Cells were counted in a haemocytometer.

Separation of alveolar macrophages

Human alveolar macrophages from broncheoalveolar lavage fluid were separated and kindly gifted by M. Imrie and Dr A. Greening, Western General Hospital, Edinburgh. Cells were suspended in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin and streptomycin to a concentration of 1 x 10^6 cells /ml.

Separation of neutrophils

Neutrophils were separated from heparinised blood from healthy volunteers by Dr J. Hughes, Department of Medical Microbiology, University of Edinburgh. Cells were resuspended in RPMI 1640 supplemented with 10%FCS, L-glutamine, penicillin and streptomycin to a concentration of 2 x 10^6 cells/ml.

Growth and maintenance of cell lines

The cell lines used throughout this thesis are listed in Table 2.3.

Table 2.3 Origin, source and cell type of cell lines used in this thesis

Cell Line	Origin	Cell Type	Source
THP-1	Human Blood	Monocyte	Dr D. Morrison, University of Kansas Medical Centre, USA
A549	Human Lung	Epithelial	ECCC[a]
L929	Mouse Connective Tissue	Fibroblastic	ECCC

a = European cell culture collection

Growth of cell lines from liquid nitrogen

Ampoules were removed from liquid nitrogen and rapidly thawed at 37°C. Contents were transferred to a sterile container with 1ml of pre-warmed media added to the ampoule and the washings transferred to the container. Cells were harvested at 4000 *g* for 10min, resuspended in approximately 25ml of media, transferred to a 50cm^2 tissue culture flask and incubated at 37°C in a 5% CO_2 atmosphere.

Maintenance of cell lines

All cell lines were checked every two days for growth and divided approximately twice a week by one of the following methods;

a) Attached cells. The fibroblastic cell lines A549 and L929 adhere to the plastic of

flask walls and had to be detached once a confluent monolayer was established. For maintenance purposes this was achieved by discarding approximately half the media and dislodging the cells by use of a cell scraper. Contents were split into two flasks and MEM media (Sigma) added to the same volume as before. To prepare the A549 and L929 cells for the bioassays, media was discarded and cells dislodged by 0.05% trypsin/0.02%EDTA (Gibco) digestion, added at a 20% volume of discarded media. Cell detachment was confirmed by viewing under an inverted microscope. Cells were transferred to a sterile container and media containing FCS added, to ensure the digestion of excess trypsin. Cells were centrifuged at 4000 *g* for 15min, washed twice and finally resuspended to the required concentration in MEM media containing 10%FCS, L-glutamine, penicillin and streptomycin.

b) Suspension cells. ThP-1 cells are non adherent and were maintained by pouring half the contents of a flask into a new container and adding RPMI 1640 media to the new flask to the appropriate volume. For use in the bioassays, cells were harvested and washed twice at 4000 *g* for 15min and finally resuspended in fresh media to the required concentration.

Enhancement for CD14 in ThP-1 cells

ThP-1 cells were enhanced for expression of CD14, an LPS binding receptor, by addition of 0.1μM of 1,25-dihydroxyvitamin D_3 (ICN Flow). Cells were grown in 75cm^2 tissue culture flasks for 72h and a half volume of vitamin D_3-containing medium added and the flasks incubated for a further 24h. Cells were prepared as described previously. CD14 IgG MAb (SAPU) was used neat for inhibition assays.

Freezing cell lines

To establish a stock of cell lines, cells were frozen approximately every three months and viability checked accordingly. The following solution was required:

Freezing medium comprised 10% DMSO, 5%FCS in RPMI 1640

Cells were harvested in the same manner described above and resuspended in freezing medium to a concentration of between 8 x 10^6-2 x 10^6 cells/ml. Aliquots (1ml) were transferred into cryogenic ampoules (NUNC) and frozen at -20°C for 30min. Ampoules were then transferred to liquid nitrogen.

2.2.16 STIMULATION OF CELLS FOR CYTOKINE ASSAYS

Bacterial extracts, diluted in pyrogen-free water, were added to 96-well round bottom plates or 24-well plates for time-course experiments (Greiner), in a ratio of 1:10 of the cells to be stimulated. Plates were incubated at 37°C in a 5%CO_2 atmosphere, with 100µl of supernate removed at appropriate intervals, frozen and stored at -20°C until required for the bioassay.

2.2.17 TNF BIOASSAY

The bioassay for TNF used a mouse fibroblastic cell line, L929, that is sensitive to the cytotoxic effects of TNF. Cells were harvested and resuspended to a concentration of 3 x 10^5 cells/ml in MEM media containing 5%FCS, L-glutamine, penicillin and streptomycin as described in 2.2.15. L929 cells (100µl) were added to wells of a 96-well flat-bottom plate and incubated overnight at 37°C in a 5%CO_2 atmosphere to form a confluent monolayer.

Bioassay for TNF

Growth medium from the plate was discarded and replaced with 100µl of fresh MEM containing Actinomycin D (2µg/ml; Sigma) to inhibit further growth of the L929 cells. Supernates from the stimulation assays were diluted 1:5 in MEM and 100µl added to the wells, thus giving a final dilution of 1:10. A human rTNF-α standard (National Institute for Biological Standards and Control) at a stock concentration of 40,000 International Units was serially diluted 1:5 in MEM and 100µl added to provide a standard concentration range of 1000-0.0128 IU/ml. All samples and standards were tested in duplicate. Samples of pyrogen-free water and MEM media were also tested

as controls. Plates were incubated at 37°C in a 5%CO_2 atmosphere overnight. After incubation, the medium was discarded and 100µl of filtered crystal violet solution (0.5%w/v crystal violet in 20%v/v methanol) added. Only living L929 cells take up the stain. After two minutes, plates were washed in running tap water until no further colour was removed. Plates were dried by use of a hairdryer, 100µl of 20%(v/v) acetic acid added to the wells and crystals lysed by shaking on a microtitre plate shaker. Plates were read at 585nm in a Vmax plate reader (Molecular Devices). Optical densities were converted to TNF equivalents using the TNF standard as a reference.

2.2.18 SPECIFICITY OF TNF BIOASSAY

A human anti-TNFα antibody (Genzyme, NBS Biologicals, Herts, UK) was serially diluted 1:10 in MEM down to a 1:10,000 dilution. Pooled supernates from stimulation assays were diluted 1:5 in MEM to which 10µl of each dilution of anti-TNF antibody was added. After a 30min incubation at 37°C, 100µl of dilution was added to a plate of L929 cells and incubated for a further 24h. The plate was then washed, stained and read as before. Supernate alone and antibody alone were used as comparative controls.

2.2.19 INDUCTION AND BIOASSAY FOR IL-8

Stimulation of IL-8 from MNL or alveolar macrophages was carried out by the same method as described for TNF induction.

The bioassay for IL-8 was a radioimmunoassay and was carried out by J. McColm, Department of Child Life and Health, University of Edinburgh.

2.2.20 MEASUREMENT OF LPS MITOGENITY

The following experiment was performed in conjunction with D. Delahooke, Department of Medical Microbiology, University of Edinburgh.

Spleen and lymph nodes from C3H/HeN mice (removed by M. Kerr, Department of Medical Microbiology) were placed in RPMI 1640 and homogenised into a single cell suspension. Filtered cells were harvested at 4000 *g* for 15min, washed twice in RPMI 1640 and resuspended to a concentration of 2 x 10^6 cells/ml in RPMI 1640 supplemented with 5%FCS. Aliquots (100µl) of cell suspension were added to an equal volume of either PHA or LPS in a 96-well plate and incubated for 48h at 37°C, 5% CO_2. Each sample was tested in triplicate. After incubation, 10µCuries of ^{3}H-thymidine (Amersham International, Little Chalfont, Bucks., UK.) diluted in 20µl of RPMI 1640 was added to each well and the plates incubated for a further 24h as before. Each well was washed ten times in distilled water with an Autowash 2000 wash machine (Dynatech Laboratories Ltd., West Sussex, UK) and the washings deposited onto filter paper (Filter Paper for Cell Harvester, ICN Flow) which was left to dry overnight. The filter paper discs were removed and placed into polyvial polyethylene tubes to which 1ml of scintillation fluid was added. The tubes were sealed and beta-emissions registered in a Tricarb Liquid Scintillation Analyzer 1900 CA (Packard Instrumentation BV, Berks, UK).

2.2.21 STATISTICAL ANALYSIS

The Wilcoxon rank sum test for nonparametric data was used for comparison of results in the LAL and TNF-α assays. Advice on statistical analysis of the data was obtained from Dr. H. Brown, Medical Statistics, Edinburgh.

CHAPTER 3

RESULTS

ENVIRONMENTAL MODULATION OF CELL SURFACE VIRULENCE DETERMINANTS OF *BURKHOLDERIA CEPACIA*

The influence of growth environment on both the phenotypic expression of cell surface components and the induction of virulence factors is well documented. The following experiments aimed to investigate potential virulence determinants of *B. cepacia*, focusing in particular on environmental modulation of cell surface factors.

Throughout the present study the isolate referred to as the 'epidemic' strain is known to be highly transmissible between CF patients (Govan *et al*, 1993); isolates referred to as 'non-epidemic' do not appear, to date, to be highly transmissible.

3.1 GROWTH AND SURVIVAL OF *B. CEPACIA* IN DIFFERENT ENVIRONMENTAL CONDITIONS

The influence of nutrient-limitation on growth

The influence of 12 different growth media on the colonial morphology of two strains of *B. cepacia*, C1359 (epidemic) and C1409 (non-epidemic), was investigated. Colonies of the epidemic strain normally appear as dry, rough and much smaller than the larger, smoother colonies of C1409. Four genetically-characterised strains of *P. aeruginosa* with defined environmentally-regulated alginate production were used as controls. Strains were inoculated onto various solid media, passaged once and growth marked at 24h, 48h, 72h and 96h (Table 3.1). All plates were incubated aerobically at 37°C.

From Table 3.1 it can be seen that certain nutrient conditions inhibit growth of *B. cepacia*. For example, both strains tested did not grow well on phosphate-limiting medium. However, no major change in colonial morphology, including the presence of a mucoid phenotype, was observed. In all culture conditions, with the exception of CEP media, growth of C1409 was faster compared to growth of C1359; colonies of C1359 reaching 'full size' only after 48h growth compared to 24h for C1409. The defined *P. aeruginosa* strains grew well in all conditions with the exception of the

carbon-limited medium, where growth was inhibited and much lower than for the *B. cepacia* strains. As expected, alginate biosynthesis was modulated in response to varying nutrient conditions.

Table 3.1 The Influence of Growth Media on Colonial Morphology[a]

Media[b]	C1359	C1409	J35	J36	J37	J38
CEP/ PIA	+++[c]	+++	+++M	+++M	+++M	+++
MA	++	+++	+++	+++M	+++M	+++
MB	++	+++	+++M	+++M	+++M	+++
MD	++	+++	+++M	++(+)	+++M	+++
MH	-	+(+)	+++M	++	++M	++(+)
Mg-limit	+	(+)	+	++M	++M	+
Fe-limit	+	+(+)	++(+)M	+++M	+++M	+++
P-limit	+(+)	+(+)	+(+)M	+(+)M	+/-M	+(+)
ETOH	+	++	+++M	+++M	+++M	+++
Acetone	+(+)	++(+)	+++M	+++M	+++M	+++
Semi-solid	++	+++	+++	+++M	+++M	+++
Carbon-limited	+/-	++(+)	+/-	-	-	-

a = results tabulated are for 96h growth compared to CEP or PIA controls
b = refer to section 2.1.3 for recipe
c = growth scored from +++ (maximum) to - (no growth)
M = mucoid phenotype characteristic of high EPS production

The influence of atmosphere on growth

The experiment above was repeated except that plates were incubated under microaerophilic and anaerobic conditions to investigate the influence of atmosphere

on growth and survival of *B. cepacia*. In microaerophilic conditions, no changes in colonial morphology were observed, although growth of C1359 was much slower; colonies required 72h to reach 'full size'. This observation suggests that a microaerophilic atmosphere inhibits growth of the epidemic strain.

Neither *B. cepacia* strain grew on any medium incubated anaerobically. However, due to the exceptional versatility of this organism, (for example survival in pyrogen-free water), it was considered that *B. cepacia* might adopt a dormant form. To test this theory, C1359 and C1409 were inoculated onto MB plates, (which allow anaerobic growth of *P. aeruginosa*), and incubated in an anaerobic cabinet. Swabs of the anaerobic plate were taken at weekly intervals for four weeks and subsequently inoculated onto CEP media incubated aerobically. Although both strains of *B. cepacia* did not show any visible growth on the anaerobic plates, growth did occur on the CEP plates. Even the swab taken four weeks after initial inoculation produced growth, indicating that *B. cepacia* can survive anaerobic conditions. Strains were confirmed as *B. cepacia* by API 20NE.

The influence of pH on growth

pH may be an important factor for the growth of *B. cepacia* since this organism is pathogenic for onions, one of the few vegetables with a pronounced acidic composition (Geigy, 1962). The influence of pH on the growth of six strains of *B. cepacia* from clinical and environmental sources was investigated. Malka A medium was prepared with predetermined volumes of 1M HCl, to give a range of media with different initial pH levels. In addition, onion-agar medium was investigated as a growth substrate. Growth was marked at 24h, 48h and 72h relative to a CEP control (Table 3.2). With the exception of the epidemic strain which was very acid-sensitive, there was no difference in pH tolerance between clinical and environmental strains.

pH measurements during growth were taken over time to determine any trend in growth metabolism. The pH of each medium was determined using a combination pH electrode specially constructed for agar plates. Initial pH measurements were taken before bacterial inoculation, then every 24h for eight days. Figure 3.1 shows the pH measurements taken with *B. cepacia* C1409, representative of all strains examined. Uninoculated control plates did not exhibit any change, from the initial pH level, during the course of the experiment. Interestingly, the culture medium which supported the best growth of *B. cepacia* CEP, was metabolised to an alkaline pH. Furthermore, of the MA plates, only the MA(pH7) produced substantial growth. As all MA plates were identical with the exception of the initial pH levels, these results indicate that *B. cepacia* does not grow well in low pH conditions with neutral or alkaline levels preferred.

Table 3.2 The Influence of pH on Growth of *B. cepacia* [a]

Media	J366[b]	J762[c]	C1732[c]	C1744[b]	C1359[c]	C1409[c]
CEP	+++	+++	+++	+++	+++	+++
OA	+++	+++	+++	++(+)	++	++(+)
MA(pH7)	+++	+++	+++	+++	++	+++
MA(pH6.3)	++(+)	+++	+++	++(+)	+	+++
MA(pH5.5)	+	++(+)	++	++	+/-	++
MA(pH5)	+/-	++	+	+	-	+
MA(pH4)	+/-	+/-	+/-	+/-	-	+/-

a = results shown are for 72h growth compared to a CEP control
b = environmental strains
c = clinical strains

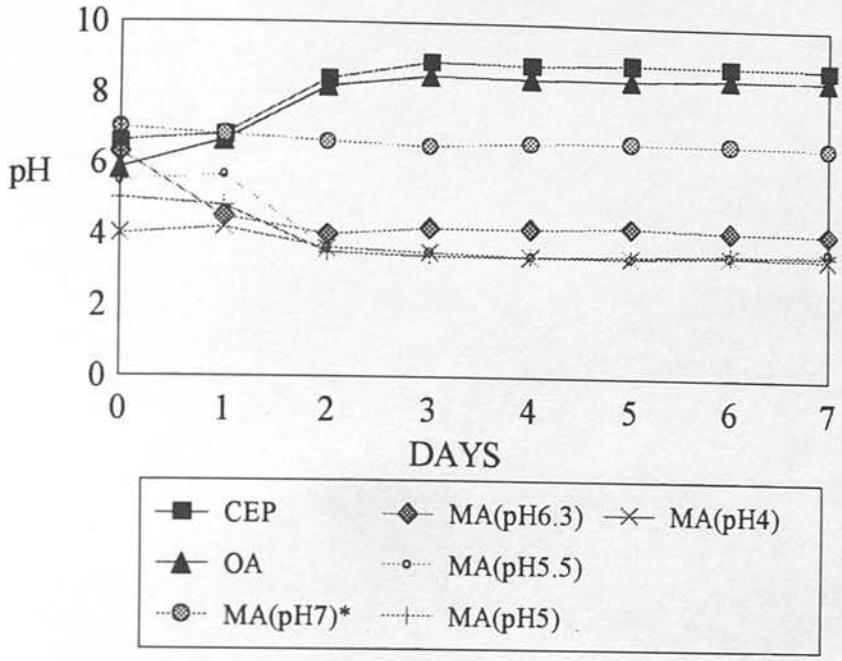


Figure 3.1 pH change during the growth of *B. cepacia* C1409
* = initial pH level

3.2 CHARACTERISTICS AND ENVIRONMENTAL REGULATION OF LPS

The aim of this section was to characterise the lipopolysaccharide phenotype from strains of *B. cepacia*, using *B. gladioli* and *P. aeruginosa* isolates as comparative controls, and in addition, investigate whether growth in different environmental conditions altered the LPS structure. LPS was extracted by either the phenol water (PW), phenol-chloroform-petroleum spirit (PCP) or proteinase K method, separated by PAGE and visualised by silver-staining and immunoblotting.

LPS Phenotype

Five strains of *B. cepacia*; C1359 (epidemic CF strain), C1409 & C1504 (non-epidemic CF strains), ATCC 17762 (non-CF clinical strain) and J2540 (environmental isolate), and one strain of *B. gladioli* (ATCC 10248) were examined for LPS profile by silver-stained PAGE. Figure 3.2 shows the LPS profiles extracted by the PW method and confirms that strains of *B. cepacia* may possess rough or smooth LPS.

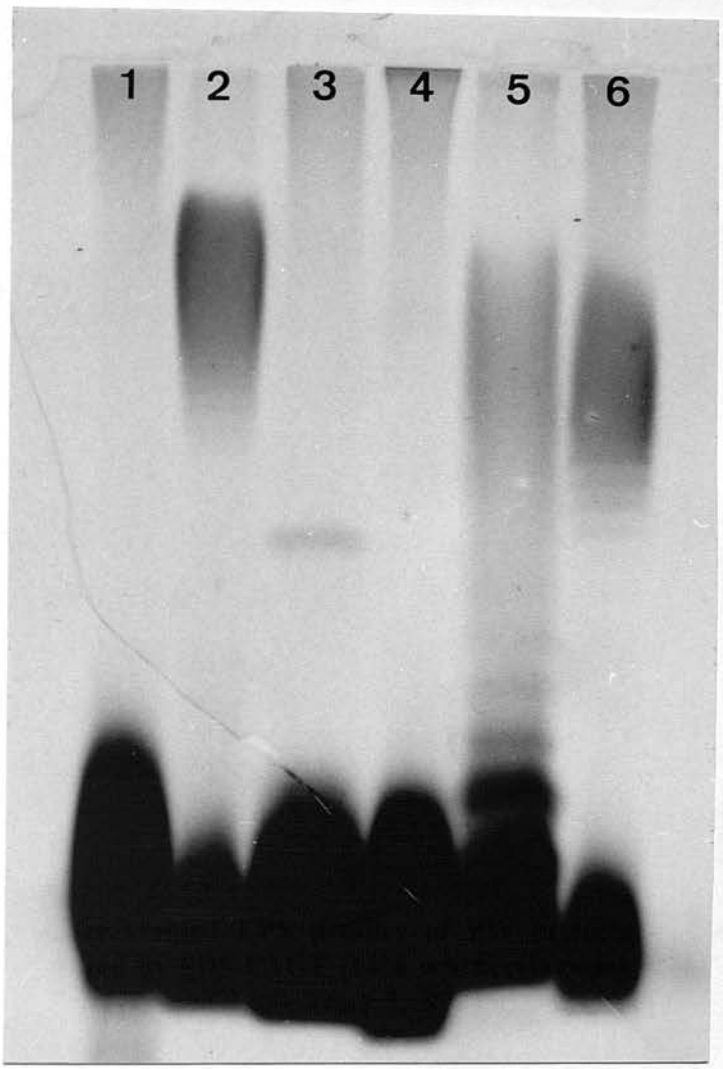


Figure 3.2 Silver stained LPS profiles of PW extracts of *B. cepacia* and *B. gladioli* separated by SDS-PAGE (14% w/v acrylamide). Track 1, *B. cepacia* C1359; Track 2, *B. cepacia* C1409; Track 3, *B. cepacia* C1504; Track 4, *B. cepacia* ATCC 17762; Track 5, *B. cepacia* J2540, Track 6, *B. gladioli* ATCC 10248.

The influence of environmental growth conditions on LPS structure

The same bacterial isolates used in the above experiment were grown in different culture media and their LPS profiles examined. All strains were grown in a nutrient rich medium NB+YE, a defined minimal medium MA and a physiological medium MA+S. In addition, LPS from two strains of *B. cepacia*, C1359 (epidemic) and C1409 (non-epidemic), were extracted from growth in high osmolarity medium MD, iron-limiting medium MA+E and carbon-limiting medium MAN (Figure 3.3 and 3.4). Differences in band staining intensity could be seen but no major structural changes were observed.

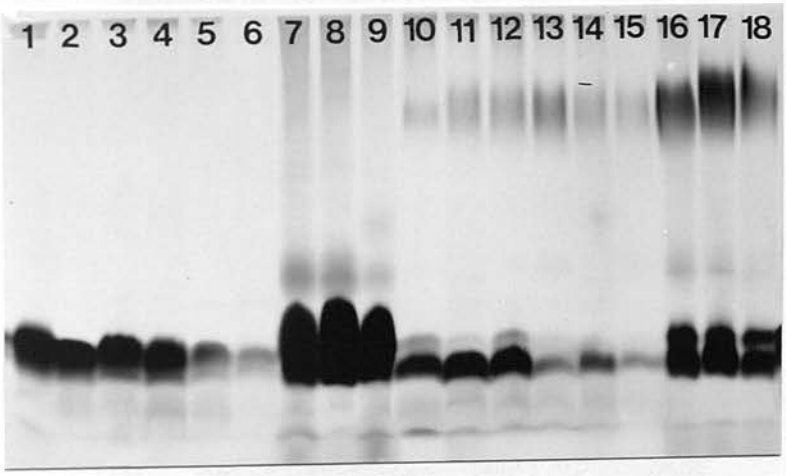


Figure 3.3 Silver stained LPS profiles of *B. cepacia* C1359 and C1409 extracted from different growth conditions. Tracks 1-6, C1359 proteinase K digests from NB+YE, MA, MA+S, MD, MA+E and MAN; Tracks 7-9, C1359 PW extracts from NB+YE, MA, MA+S; Tracks 10-15 C1409 proteinase K digests and Tracks 16-18 C1409 PW extracts from the same growth media.

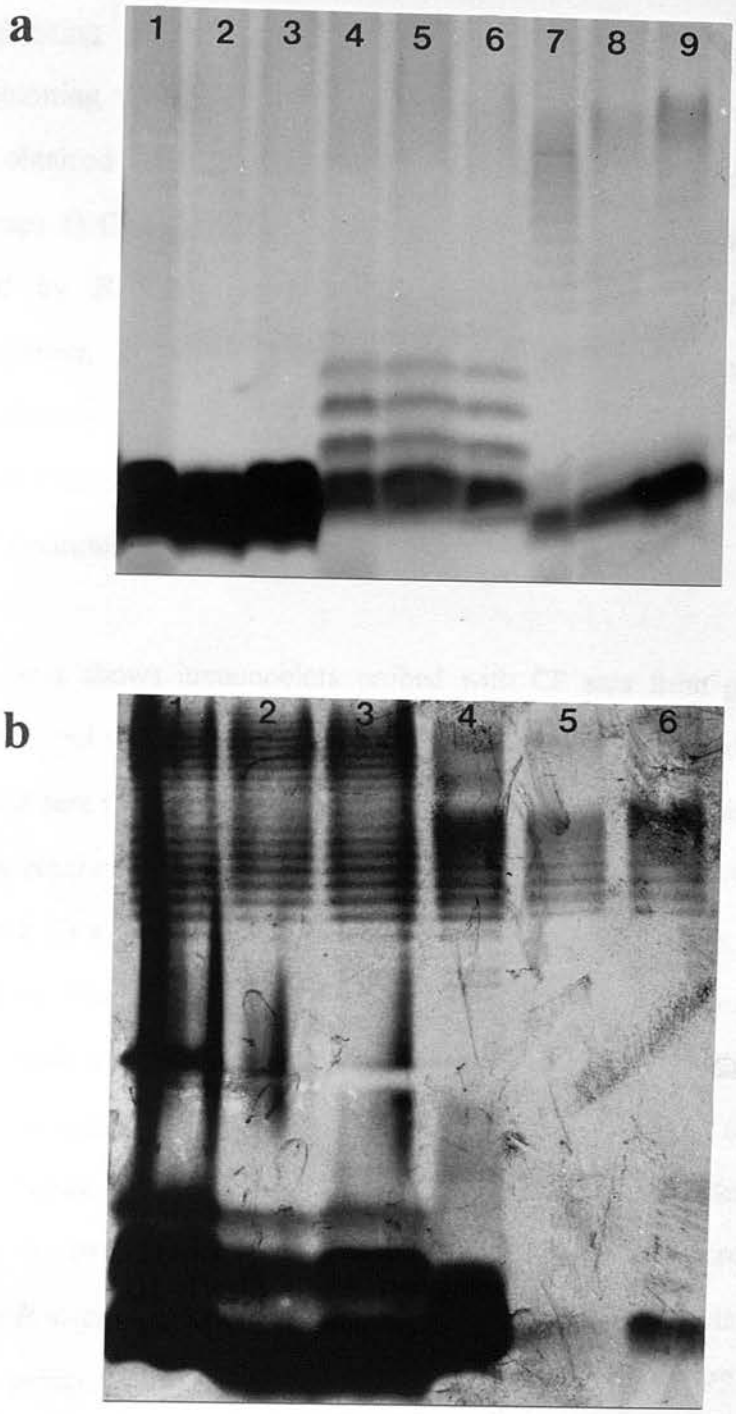


Figure 3.4a-b. Silver stained LPS profiles of proteinase K whole cell digests of *B. cepacia, B. gladioli* and *P. aeruginosa* strains extracted from different growth environments. Figure 3.4a Tracks 1-3, *B. cepacia* ATCC 17762; Tracks 4-6, *B. cepacia* J2540; Tracks 7-9 *B. gladioli* ATCC 10248; **Figure 3.4b** Tracks 1-3, *P. aeruginosa* PAO1; Tracks 4-6, *P. aeruginosa* C1250; all LPSs were extracted from NB+YE, MA and MA+S respectively.

Immunoblotting

Immunoblotting was performed on the same strains as above using a variety of antisera obtained from both CF patients and rabbits. CF sera were categorised into four groups 1) CF patients colonised by *B. cepacia* epidemic strain, 2) CF patients colonised by *B. cepacia* non-epidemic strain, 3) CF patients colonised by *P. aeruginosa*, and 4) CF patients not colonised by either *B. cepacia* or *P. aeruginosa*. Rabbit sera was raised against heat-killed whole cells of either *B. cepacia* C1359 (epidemic), *B. cepacia* C1409 (non-epidemic) or *B. cepacia* J2395 (hospital environment).

Figure 3.5a-c shows immunoblots probed with CF sera from group 1, 2 and 3 respectively and reveals a difference in immunogenic structure between the bacterial strains. CF sera from group 1 (patients colonised only with the epidemic strain) gave a positive reaction with the low molecular mass core LPS from this strain (Figure 3.5a; Track 1) and one band from two other *B. cepacia* strains, C1504 & J2540, (Figure 3.5a; Track 3 & 5). The reaction to the CF sera from group 2 (patients colonised with a non-epidemic strain; C1409) was markedly different. The high molecular weight moiety of the LPS from C1409 and surprisingly *B. gladioli* reacted strongly (Figure 3.5b; Track 2 & 6), with no reaction from the epidemic strain observed. *P. aeruginosa* LPS from strain C1250 did not exhibit cross-reactivity with any of the *B. cepacia* strains at the serum concentration used. Furthermore, using CF sera from group 3 (patients colonised with *P. aeruginosa*), only LPS samples from the two *P. aeruginosa* strains tested gave a reaction (Figure 3.5c; Track 2 & 4). No reaction was observed for any LPS sample probed with group 4 CF sera, (patients *B. cepacia* and *P. aeruginosa* negative).

Figure 3.6a-c shows the immunoblots obtained using rabbit sera raised against C1359, C1409 and J2395 respectively. Immunoblots using C1359 and C1409 rabbit sera

(Figure 3.6a & 3.6b) were similar to the reactions observed with CF serum, except C1409 rabbit serum also reacted against a high molecular weight structure of C1504, ATCC 17762 and J2540 (Figure 3.6b; Tracks 3-5). This greater cross-reactivity could be due to the higher level of antibody present in the rabbit sera. The reaction of the antisera from the rabbit inoculated with J2395 (an environmental isolate) showed, rather surprisingly, a similar response to that observed from CF sera group 1 (Figure 3.5a). The strongest reaction was against the environmental isolate (Figure 3.6c; Track 5), with C1504 also giving a reaction (Track 3).

These results suggest that there is marked intra-species variation between the LPS types of *B. cepacia*. Furthermore, two *B. cepacia* strains, C1504 and ATCC 17762, although observed to have R-LPS by silver-staining (Figure 3.2), showed high molecular weight bands upon immunoblotting (Figure 3.6b; Track 3 & 4) suggesting that cross-reactive antigenic epitopes were present. However, whether these bands are part of an LPS structure is not known. The lack of cross-reaction between *P. aeruginosa* LPS and *B. cepacia* sera highlights the difference between these two CF pathogens, whereas the positive reaction of *B. gladioli* confirms the close taxonomic relationship of these species within the new genus *Burkholderia.*

All the bacterial LPS samples used in the above immunoblots were PW extracts from growth in NB+YE. Figure 3.7 shows the immunoblot reaction using PW-LPS samples from *B. cepacia* C1359 and C1409 grown in NB+YE, MA and MA+S. The sera used to probe the blots was the same rabbit sera as described above. No difference in LPS structure from the different culture media was observed and thus LPS antigenicity does not appear to be dependent on environmental conditions.

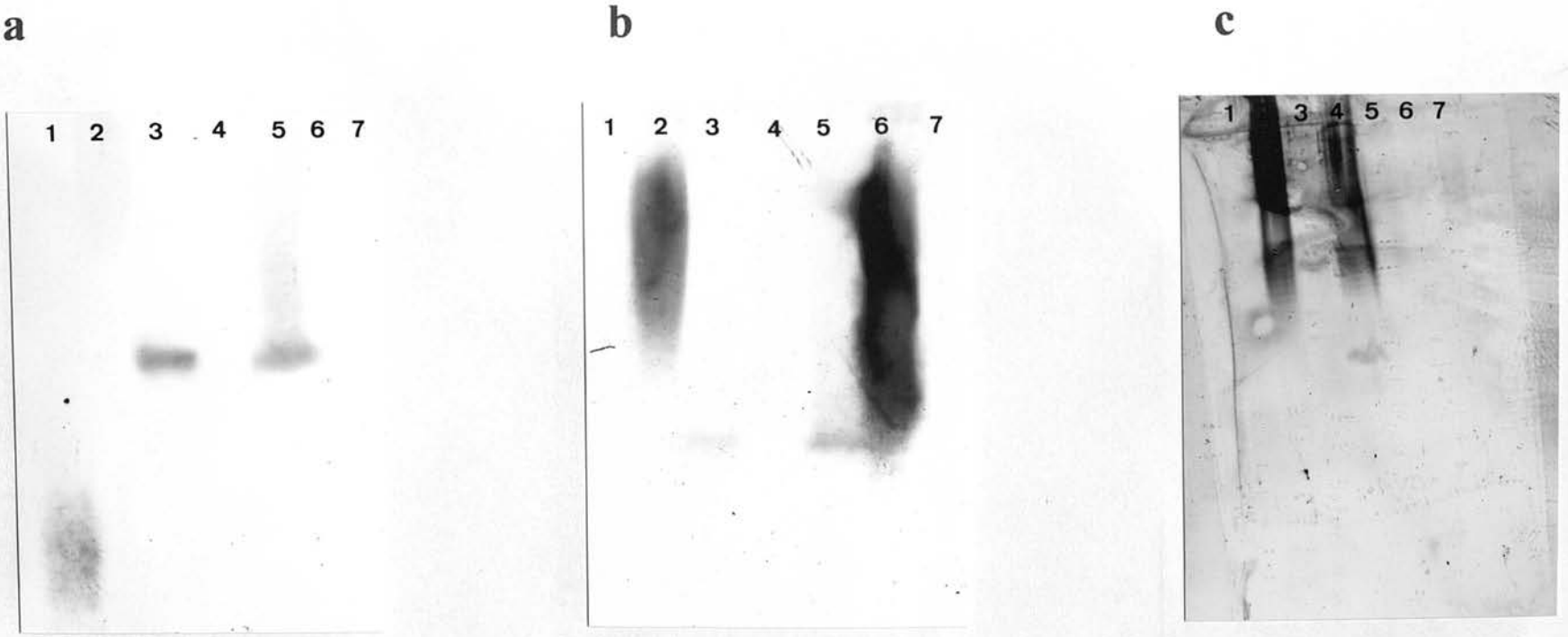


Figure 3.5 a-c Immunoblots of LPS antigens extracted by the PW method and probed with sera from CF patients colonised by (a) an epidemic *B. cepacia* strain, (b) a non-epidemic *B. cepacia* strain and (c) a *P. aeruginosa* strain. Figure 3.5a and 3.5b Track 1, *B. cepacia* C1359; Track 2, *B. cepacia* C1409; Track 3, *B. cepacia* C1504; Track 4, *B. cepacia* ATCC 17762; Track 5, *B. cepacia* J2540, Track 6, *B. gladioli* ATCC 10248; Track 7 *P. aeruginosa* C1250. **Figure 3.5c** Track 1 *B. gladioli* ATCC 10248; Track 2, *P. aeruginosa* PAO1; Track 3, *B. cepacia* ATCC 17762; Track 4, *P. aeruginosa* C1250; Track 5, *B. cepacia* C1504; Track 6, *B. cepacia* C1409; Track 7, *B. cepacia* C1359.

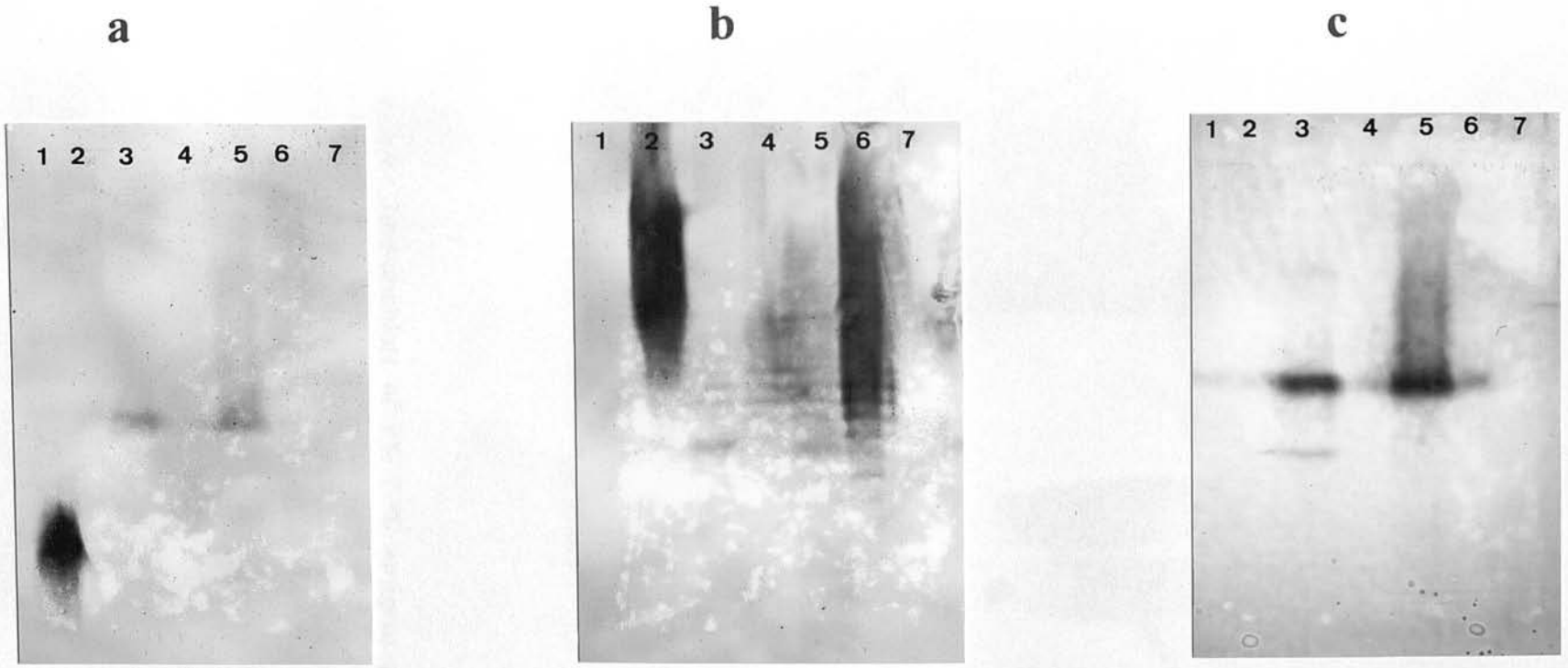


Figure 3.6 a-c Immunoblots of LPS antigens extracted by the PW method and probed with rabbit sera raised against heat-killed whole cells of (a) *B. cepacia* C1359, (b) *B. cepacia* C1409 and (c) *B. cepacia* J2395. Track 1, *B. cepacia* C1359; Track 2, *B. cepacia* C1409; Track 3, *B. cepacia* C1504; Track 4, *B. cepacia* ATCC 17762; Track 5, *B. cepacia* J2540, Track 6, *B. gladioli* ATCC 10248; Track 7 *P. aeruginosa* C1250.

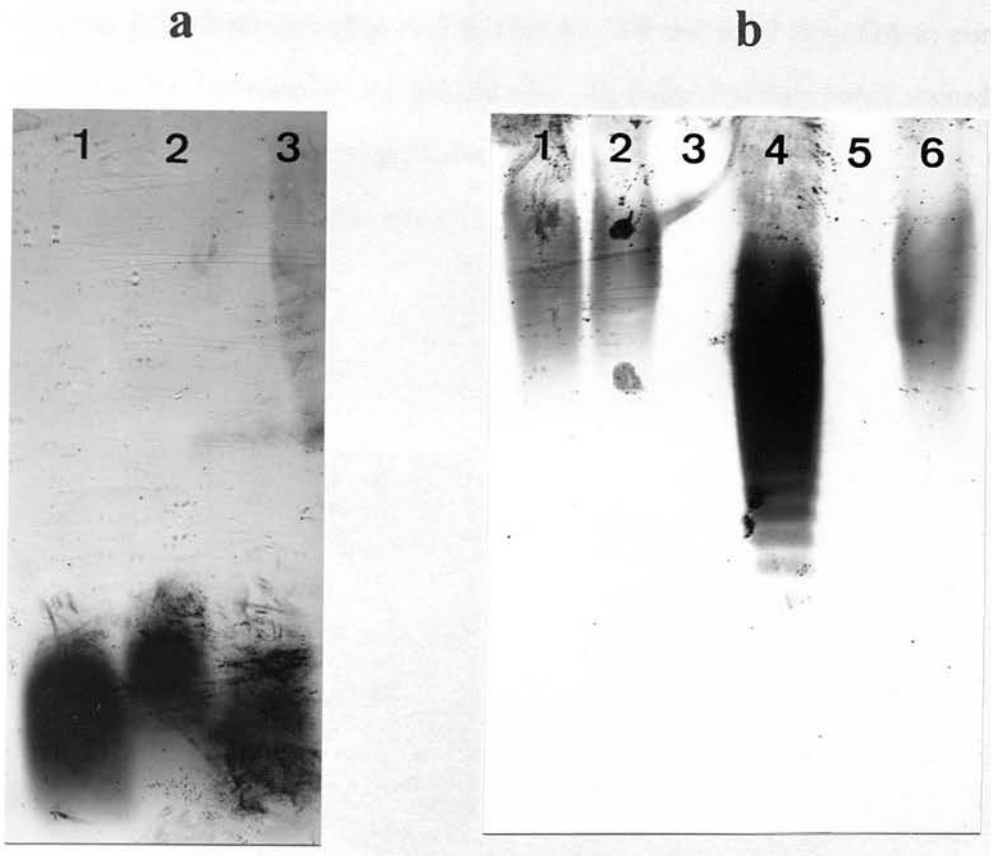


Figure 3.7a-b Immunoblots of PW-LPS antigens extracted from different growth environments and probed with rabbit sera raised against heat-killed whole cells of (a) *B. cepacia* C1359, and (b) *B. cepacia* C1409. Figure 3.7a Tracks 1-3, *B. cepacia* C1359 LPS extracted from NB+YE, MA and MA+S. **Figure 3.7b** Track 1 *B. cepacia* C1409 LPS extracted from NB+YE; Track 2, *B. cepacia* C1409 LPS extracted from MA; Track 3, Blank; Track 4, *B. gladioli* ATCC 10248; Track 5, Blank; Track 6, *B. cepacia* C1409 LPS from MA+S.

The influence of pH on *B. cepacia* LPS structure

The three most acid-tolerant *B. cepacia* strains, as determined by the pH growth experiment described in section 3.1, were selected, J762, C1744 and C1409, and their LPS extracted from growth in MA media with an initial pH level of 7.0, 6.3, 5.5 and 5.0. LPS from growth in NB+YE and OA was also extracted. Analysis of the proteinase K LPS profiles (Figure 3.8) showed LPS extracted from OA to comprise more core-LPS compared to the MA samples. In addition certain bands stained more intensely suggesting increased production of O-antigen of this chain length. However, no major structural differences were observed with regards to the different MA media.

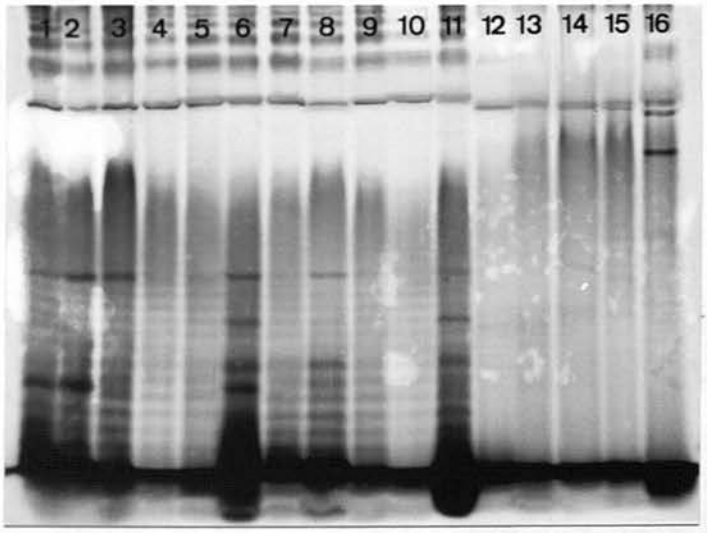


Figure 3.8 Silver-stained LPS profiles of proteinase K whole cell digests of *B. cepacia* strains grown under a range of pH conditions. Tracks 1-6, *B. cepacia* C1744 (environmental isolate); Tracks 7-11, *B. cepacia* J762 (non-CF clinical isolate) and Tracks 12-16 *B. cepacia* C1409 (CF isolate), all LPSs extracted from NB+YE(pH6.8), MA(pH7), MA(pH6.3), MA(pH5.5), MA(pH5; for C1744 only) and OA(pH6.3).

The influence of different extraction methods on LPS structure

It has been reported that the endotoxicity of a bacterial strain is dependent on the extraction method employed (Delahooke *et al*, 1995a). Therefore, as well as the actual growth environment, the LPS extraction method will be important in affecting LPS profile. To determine whether LPS extraction method influenced LPS profile, LPS from *B. cepacia* C1359 and C1409 were obtained by both the PW and PCP method and compared to the proteinase K profile from Figure 3.3.

A silver-stained gel and immunoblot of the LPS of *B. cepacia* C1409 extracted by the PW and PCP methods are shown in Figure 3.9a and 3.9b respectively. Using the PW method the LPS of C1409 appears mainly as high molecular weight material with some core antigen visible by silver-staining. No difference in LPS profile compared to a proteinase K sample was observed. However, using the PCP method, although the LPS again appeared the same by silver staining (Figure 3.9a; Track 2), immunoblots revealed two very dense bands (Figure 3.9b; Track 2), lower in molecular weight than normally observed with either the PW or proteinase K extracts.

The LPS profile of C1359 obtained by PW extraction was the same as a proteinase K sample, low molecular weight core LPS, as observed by silver-staining and immunoblotting (Figure 3.9c; Track 1 & Figure 3.9d; Track 1 respectively). However, LPS obtained by the PCP method differed in several respects. Firstly, the LPS extract was extremely hydrophobic and is best described as a piece of rubber/hard chewing gum! The extract would not form an aqueous solution easily even when heated, sonicated and repeatedly freeze-thawed but did dissolve rapidly in 90%(w/v) phenol. Secondly, when the sample was examined by silver-stain the track appeared blank (Figure 3.9c; Track 2). Thirdly, and quite surprisingly, immunoblotting of the PCP sample probed with rabbit sera raised against C1359 did show a reaction. However, in contrast to the normal low molecular weight core LPS,

three high molecular weight dark bands and two very faint bands were observed (Figure 3.9d; Track 2). These bands are unlikely to be part of an LPS ladder pattern as they were not regularly spaced and unlikely to be an artifact of the PCP procedure as they reacted with specific antisera but not upon silver-staining. Whether these bands are present *in vivo* and are involved in pathogenicity requires further studies.

In the PW extraction method, LPS is normally obtained from the upper aqueous layer and the bottom phenol layer is discarded. Due to the extreme hydrophobic nature of the C1359 PCP extract and its ability to dissolve easily in phenol, further experiments examined the LPS from both the aqueous phase and the phenol phase of a PW extract. This process was performed to determine whether any LPS was present in the phenol layer and if so, whether it differed from that obtained from the aqueous layer. *B. cepacia* C1409 and *P. aeruginosa* C1250 were also re-examined as a comparison. In addition, both phases extracted from C1409 were ultracentrifuged and the pellet and supernate retained to compare both the LPS from the pellet and supernate and from the ultracentrifuged and uncentrifuged samples.

Figure 3.10a shows the immunoblot of LPS from C1359 obtained from the aqueous and phenol layer of a PW extraction. The sample obtained from the phenol layer was not observed on silver-staining but was observed by blotting. Samples from the two layers reacted differently; the aqueous layer appeared as a low molecular weight core (Figure 3.10a; Track 1), but the phenol layer in contrast, showed a large high molecular weight moiety not observed previously (Figure 3.10a; Track 2). Similarly, the samples of *P. aeruginosa* C1250 obtained from the two PW phases also varied. The phenol phase extract was not observed on silver-staining even though there was a reaction by blotting (Figure 3.10b; Track 2); indeed, more reactive bands were observed in the phenol extract compared to the aqueous sample.

Figure 3.11a and 3.11b show the results obtained with C1409 LPS by silver-staining and immunoblotting. Firstly, there was no visual difference between LPS obtained from the ultracentrifuged pellet, the supernate or the crude extract (pellet + supernate) that is normally used (Figure 3.11a; Tracks 1, 2 & 4). However, there was a variation between the samples obtained from the aqueous and phenol layers, with the phenol layer LPS being of a lower molecular weight. Furthermore, the supernate from the ultracentrifuged phenol layer gave a different reaction to the other samples upon both silver-staining and immunoblotting. As all three organisms show variations in LPS structure between the PW phases this may be important in terms of LPS expression *in vivo* and hence pathogenicity.

In order to establish the purity of the LPS extracts, proteinase K (40µg/mg LPS sample) and 0.1M sodium periodate (200µl/mg LPS sample) were added to the PW extracts from C1359. As the sample did not show any reaction after treatment with periodate (Figure 3.12; Track 3) it may be concluded that the majority of the extract is periodate-labile LPS. However, upon treatment with proteinase K, some of the core LPS mass is also lost (Figure 3.12; Track 2) indicating that a small amount of protein may be present.

B. cepacia strains exhibit a variety of LPS profiles ranging from the rough-LPS core structure to the characteristic ladder pattern of smooth LPS. Minor variations in LPS profile were observed from samples extracted from different growth environments although the variations were mainly differences in band intensity. However, extraction method appears to have a considerable influence on LPS structure with PCP extracts and the aqueous and phenol phase PW extracts exhibiting different structures when probed with specific sera. Unfortunately *B. cepacia* isolates, in general, do not resolve well on SDS-PAGE which makes observing structural changes

difficult. It is possible that LPS changes may be occurring but cannot be detected due to the limited resolution of the techniques employed.

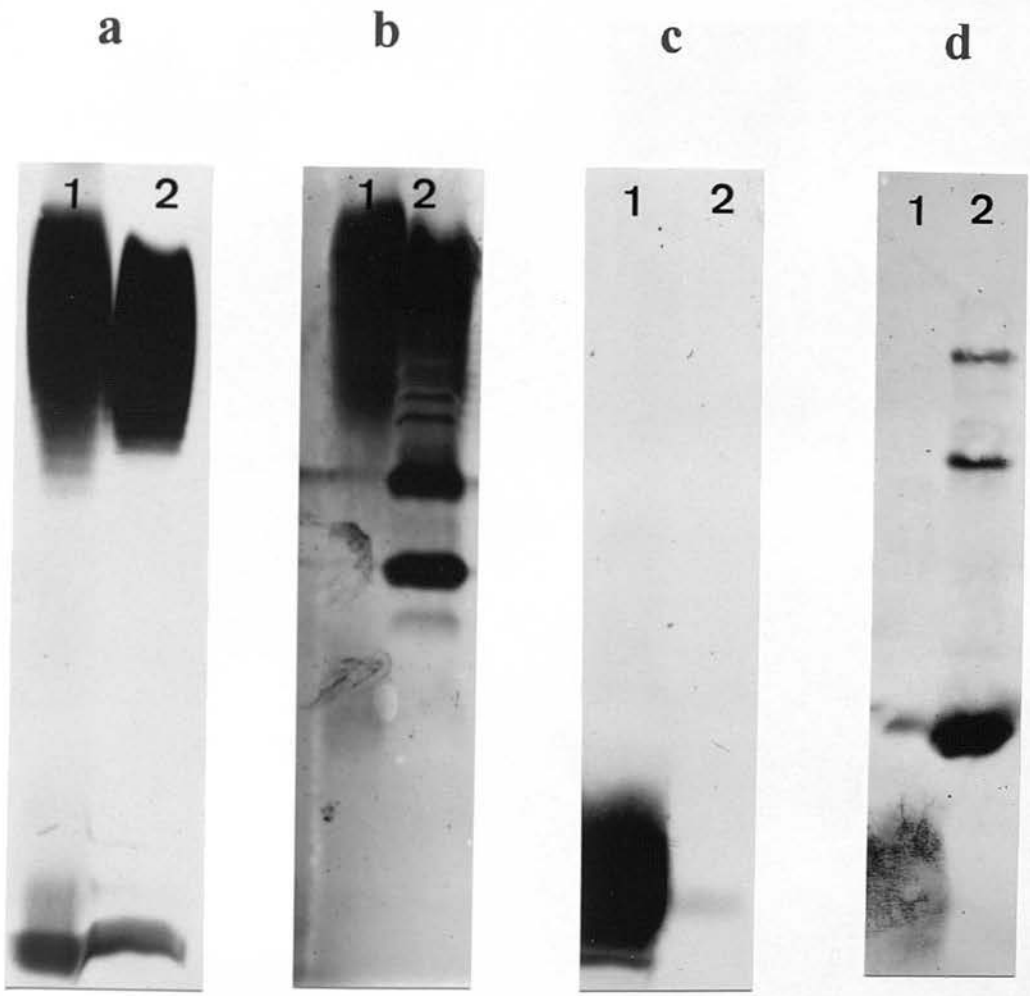


Figure 3.9 Profile of LPS samples from different extraction methods. (a & b) *B. cepacia* C1409 LPS samples and **(c & d)** *B. cepacia* C1359 LPS samples as observed by **(a &c)** Silver-stained LPS PAGE and **(b & d)** Immunoblot probed with rabbit sera raised against the homologous strain. Track 1, PW method; Track 2, PCP method.

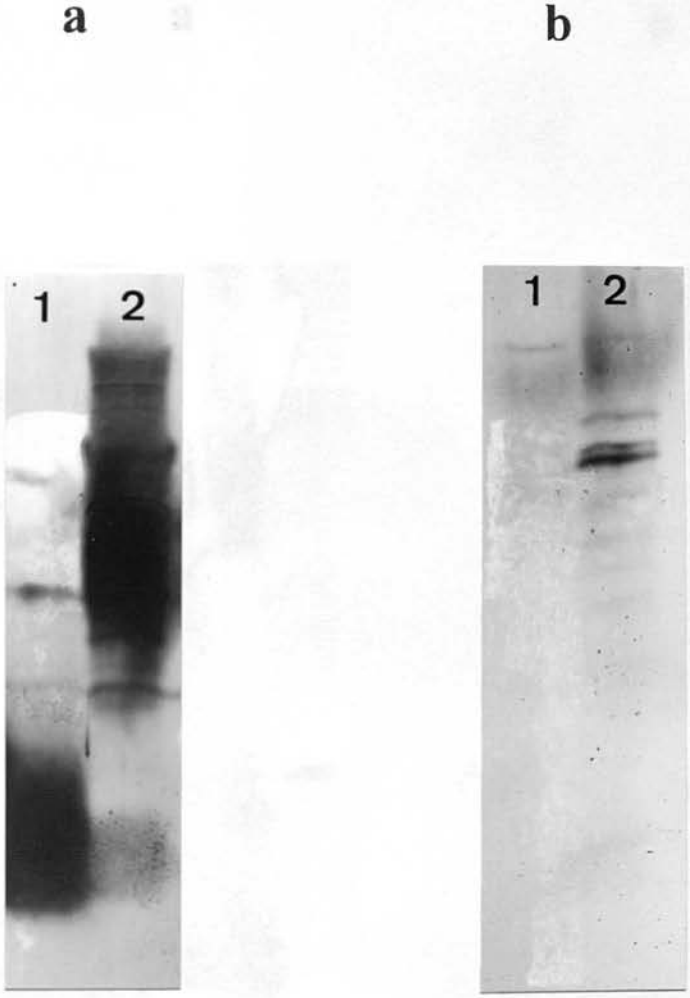


Figure 3.10 Immunoblots of LPS samples obtained from different phases of a PW extract. **(a)** *B. cepacia* C1359 LPS samples and **(b)** *P. aeruginosa* C1250 LPS samples as observed by immunoblots probed with **(a)** rabbit serum raised against C1359 and **(b)** serum from a *P. aeruginosa* colonised CF patient. Track 1, Aqueous layer from a PW extract; Track 2, Phenol layer from a PW extract.

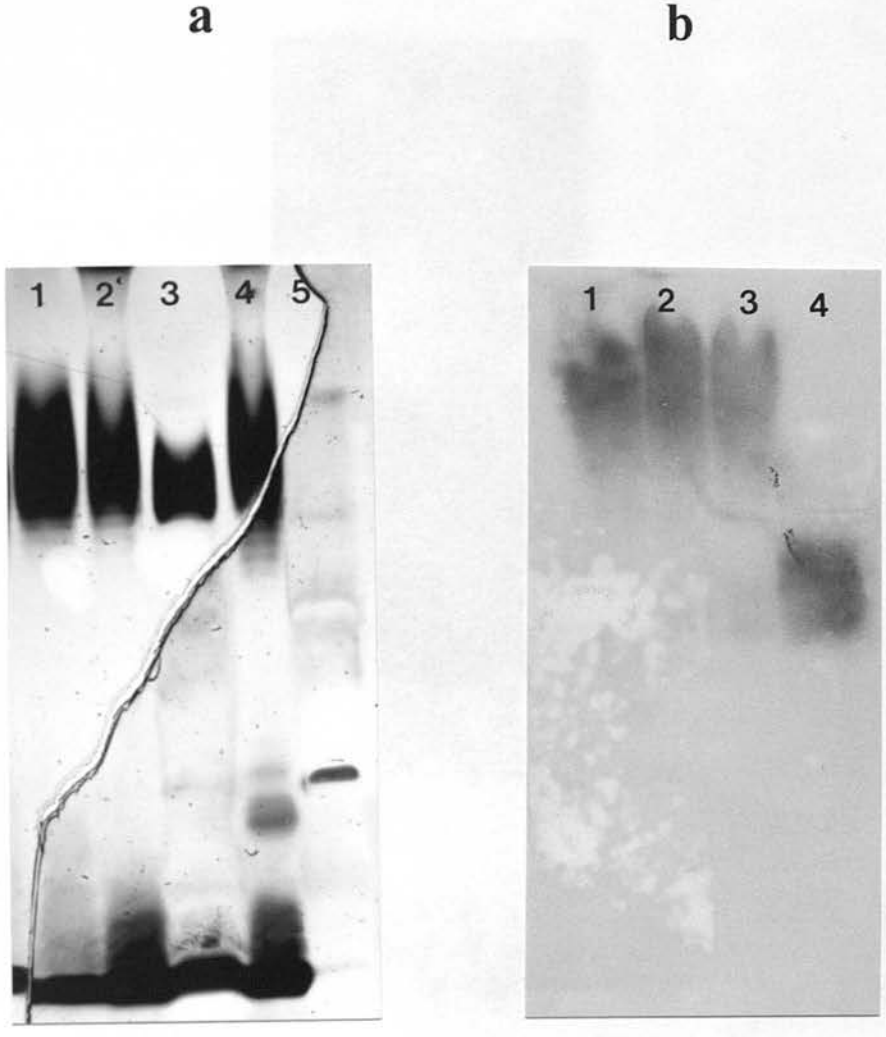


Figure 3.11a-b. Profile of LPS samples from *B. cepacia* C1409 obtained from different phases of a PW extract as observed by (a) silver-stained LPS PAGE and (b) immunoblot probed with rabbit serum raised against the homologous strain. **Figure 3.11a** Track 1, uncentrifuged aqueous layer extract; Track 2, ultracentrifuged aqueous layer pellet; Track 3, ultracentrifuged phenol layer pellet; Track 4, ultracentrifuged aqueous layer supernate; Track 5, ultracentrifuged phenol layer supernate. **Figure 3.11b** Track 1, uncentrifuged aqueous layer extract; Track 2, ultracentrifuged aqueous layer pellet; Track 3, ultracentrifuged aqueous layer supernate; Track 4, ultracentrifuged phenol layer supernate.

Figure 3.12. PW-LPS samples of *B. cepacia* C1359 after treatment with proteinase K and periodate as observed by silver-stained LPS PAGE. Track 1, C1359 PW-LPS sample; Track 2, after treatment with proteinase K; Track 3, after treatment with periodate.

Chemical analysis of LPS

Simple chemical analysis was carried out on the LPS samples to characterise further the LPS structure and to investigate whether the chemical composition was altered due to environmental growth conditions.

Table 3.3 shows the yield of lyophilised cells and PW-LPS extracts obtained from strains of *B. cepacia, B. gladioli* and *P. aeruginosa* grown in NB+YE, and in addition *B. cepacia* C1359 and C1409 grown in MA and MA+S. The yield of cells extracted

from the different bacterial species did not correlate with the final amount of lyophilised PW extract. Interestingly, growth in minimal media produced the lowest yield of cells although the lowest yield of LPS was obtained from growth in serum supplemented media. This discrepancy may be due to the varying hydrophobicity of the LPS extracts as the PW method selects preferentially for hydrophilic material (see results in section 3.5).

Table 3.3 Yield of cells and PW-LPS extracts obtained from different bacterial isolates and growth media.

Strain	Growth media	Yield of lyophilised	
		cells (g/l)	PW extract (% of cell dry wt.)
B. cepacia C1359	NB+YE	0.658	5.6
B. cepacia C1359	MA	0.446	6.7
B. cepacia C1359	MA+S	0.564	2.3
B. cepacia C1409	NB+YE	0.667	12.0
B. cepacia C1409	MA	0.525	16.8
B. cepacia C1409	MA+S	0.533	3.7
B. cepacia C1504	NB+YE	0.320	1.9
B. cepacia ATCC 17762	NB+YE	0.890	7.5
B. cepacia J2540	NB+YE	0.470	14.6
B. gladioli ATCC 10248	NB+YE	0.958	7.1
P. aeruginosa PAO1	NB+YE	0.900	8.1
P. aeruginosa C1250	NB+YE	0.270	0.4

Chemical analysis of the PW-LPS preparations is summarised in Table 3.4 and provides several interesting observations. The Kdo content of the various PW-LPS samples differed slightly when assayed by the standard TBA method or after prior

dephosphorylation with hydrogen fluoride; the latter method generally selecting for less Kdo content. This finding would imply that the dephosphorylation process for *B. cepacia* actually destroys the reactive components rather than releasing more, unlike the findings described for other Gram-negative organisms (Beckmann *et al,* 1989). Furthermore, the amount of Kdo differed dramatically between the *B. cepacia*/*B. gladioli* preparations and those from *P. aeruginosa* with the latter samples containing on average five-fold more Kdo. The average Kdo content of the *B. cepacia* extracts is 0.4% which agrees with previous reports (Cox & Wilkinson, 1991) and reinforces the theory that *B. cepacia* contains only one Kdo unit. Very little protein was detected in the samples, the largest proportion amounting to only 0.37%, confirming the observations made by silver-stained PAGE in Figure 3.12. The carbohydrate content of the PW-LPS samples is greater than the other chemical components as may be expected, although, it must be remembered that the assay used in the present study measures only neutral sugars and therefore more unreactive carbohydrate may be present. The phosphorus content of the *B. cepacia* samples is higher than previously reported but is unlikely to be due to phosphorus in the reagents as the assay was repeated four times with different PW extracts and freshly prepared reagents each time.

There was no obvious distinction between the chemical composition of samples from clinical and environmental *B. cepacia* isolates, or between *B. cepacia* and *B. gladioli* samples and no correlation to LPS phenotype was detected. Furthermore, there was no difference between epidemic and non-epidemic *B. cepacia* isolates, with the exception of the epidemic strain (C1359) possessing less carbohydrate.

Chemical variations were also observed between PW-LPS samples extracted from different growth environments. MA+S extracts possessed more Kdo but less carbohydrate compared to the other samples. In addition, both MA and MA+S

extracts possessed a greater amount of protein contamination compared to the nutrient rich media NB+YE.

Table 3.4 Chemical analysis of PW-LPS preparations

Strain	Growth media	Kdo[d]		Prt	CHO	P
		TBA	HF			
C1359[a]	NB+YE	3.4±0.3[e]	3.3±0.2	1.9±0.1	210±18	40.7±3.5
C1359	MA	2.0±0.2	1.8±0.1	3.0±0.2	200±10	38.1±2.4
C1359	MA+S	5.6±0.4	5.3±0.3	2.8±0.1	180±9	40.2±2.4
C1409[a]	NB+YE	2.5±0.2	2.7±0.2	1.9±0.1	344±12	25.0±2.2
C1409	MA	2.6±0.2	1.9±0.2	3.0±0.2	370±15	27.9±2.1
C1409	MA+S	2.6±0.2	4.0±0.3	2.9±0.2	259±15	45.7±2.5
C1504[a]	NB+YE	3.4±0.4	NT	2.1±0.1	288±9	30.1±0.6
ATCC17762[a]	NB+YE	4.9±0.4	2.9±0.2	3.7±0.2	282±15	49.2±2.1
J2540[a]	NB+YE	6.5±0.2	NT	2.1±0.1	290±27	31.5±2.0
ATCC10248[b]	NB+YE	3.6±0.2	2.8±0.4	1.1±0.1	243±24	24.6±2.3
PAO1[c]	NB+YE	23.4±1.5	17.5±1.3	2.9±0.2	239±11	57.3±4.2
C1250[c]	NB+YE	19.9±0.5	NT	3.7±0.1	203±16	37.0±0.9

a = *B. cepacia* isolates; *b* = *B. gladioli* isolates; *c* = *P. aeruginosa* isolates
d = Kdo measured by the standard thiobarbituric acid method (TBA) or after prior dephosphorylation with hydrogen fluoride (HF)
e = All results are shown as μg/mg dry weight sample
All results are given as the mean ± standard error from at least three experiments
prt = protein; CHO = carbohydrate; P = phosphorus; NT = not tested

The chemical composition and yield of lyophilised LPS obtained by different extraction methods is shown in Table 3.5. For *B. cepacia* C1359 and C1409, the yield of sample obtained from the phenol layer of a PW extract was less than the amount obtained from the aqueous layer. In contrast, the yield of sample obtained for

P. aeruginosa C1250 from the two PW layers was the same. This finding correlates with a previous report in which only 25% of LPS from a rough *P. aeruginosa* isolate was recovered from the aqueous phase (McGroarty & Rivera, 1990). The PW aqueous layer gave the highest yield of LPS for C1409 whereas the PCP method gave the highest yield for C1359. This may have been expected as the PCP method preferentially selects for rough LPS. However, whether C1359 expresses rough LPS *in vivo* is debatable considering the immunoblot results in Figure 3.9 and 3.10.

Table 3.5 Chemical analysis and yield of LPS from different extraction methods

Strain	Sample	Yield of lyophilised sample (% of cell dry wt.)	Kdo[c]	prt	CHO	P
C1359[a]	PW-aq	4.8	3.2	1.9	226	56.4
C1359	PW-ph	1.1	1.4	587	144	12.6
C1359	PCP	10.0	1.0	92	102	0.5
C1409[a]	PW-aq(plt)	2.5	3.2	1.1	292	23.4
C1409	PW-aq(sn)	11.5	2.5	1.3	360	19.9
C1409	PW-ph(plt)	0.7	0.6	4.4	160	9.66
C1409	PW-ph(sn)	1.0	0.8	665	154	5.3
C1409	PCP	3.6	2.0	55	239	9.2
C1250[b]	PW-aq	1.1	20.1	3.5	196	40.0
C1250	PW-ph	1.1	11.4	754	117	6.4

a = *B. cepacia* isolates; *b* = *P. aeruginosa* isolate
c = results are µg/mg dry weight sample
prt = protein CHO = carbohydrate P = phosphorus
PW-aq = aqueous layer of a phenol water extract; PW-ph = phenol layer of a phenol water extract; plt = pellet from an ultracentrifuged PW extract; sn = supernate from an ultracentrifuged PW extract; PCP = phenol chloroform petroleum extract.

Different LPS extraction methods select for samples of varying chemical composition; reflecting the varying structures observed in Figures 3.9-3.11. As expected for a PW extract, LPS samples obtained from the aqueous layer possessed the highest levels of Kdo and phosphorus, whereas samples from the phenol layer, in particular the supernate, possessed a high level of protein. Similarly, the PCP extracts also contained more protein contamination compared to the PW aqueous extracts. Carbohydrate content was more uniform between the samples, although levels were, in general, higher for the aqueous PW extracts. The variation in chemical composition between the samples once again lends speculation to the LPS structure expressed in the host.

3.3 CHARACTERISTICS AND ENVIRONMENTAL REGULATION OF OMP

Outer membrane profile

Outer membranes were extracted from eight strains of *B. cepacia*, two strains of *P. aeruginosa* and one strain of *B. gladioli*. For two of the *B. cepacia* strains, isolates C1359 (epidemic) and C1409 (non-epidemic), outer membranes were extracted from growth in five different media; NB+YE, MA, MD, MA+E and MAN. Samples were stained by the Coomassie blue method. In addition, immunoblots of the OMP antigens were performed using both human and rabbit sera as described for the LPS samples.

On analysis of the OMP Coomassie blue stained gel (Figure 3.13), it is immediately obvious that no two strains share the same profile. However, several bands are common to all the *B. cepacia* strains studied, including a major band at approximately 30kD, recently proposed as a potential immunotherapeutic target on account of its conserved nature (Burnie *et al*, 1995). All the *B. cepacia* strains expressed high molecular weight bands with the exception of the non-epidemic CF isolate C1409 and

the environmental isolate J366.

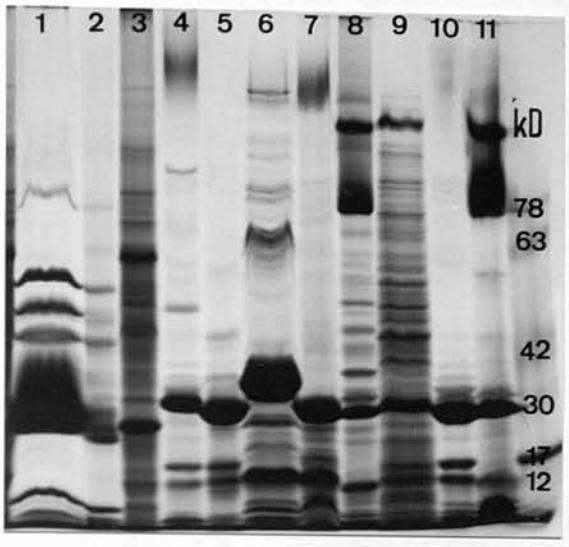


Figure 3.13 Coomassie blue stained outer membrane profiles of *B. cepacia*, *B. gladioli* and *P. aeruginosa* separated by SDS-PAGE (10% w/v acrylamide). Track 1, *P. aeruginosa* C1250; Track 2, *P. aeruginosa* PAO1; Track 3, *B. gladioli* ATCC 10248; Track 4, *B. cepacia* J2395; Track 5, *B. cepacia* J366; Track 6, *B. cepacia* J2540; Track 7, *B. cepacia* ATCC 29424; Track 8, *B. cepacia* ATCC 17762; Track 9, *B. cepacia* C1504; Track 10, *B. cepacia* C1409; Track 11, *B. cepacia* C1359. Molecular weights of protein standards are indicated.

The influence of growth environment on OM profile

Figure 3.14 shows the OMP profile of *B. cepacia* C1409 and C1359 grown under different environmental conditions and again highlights the dissimilar profiles between these two isolates. As with other Gram-negative bacteria, *B. cepacia* strains change their protein composition in response to growth environment. This adaptation will affect the overall permeability of the outer membrane and allow the organism to survive in unfavourable conditions. C1409 grown in NB+YE (Figure 3.13; Track 10) expresses fewer high molecular weight bands compared to the other growth conditions studied which may be expected in a nutrient-rich environment. OMP profiles from C1409 grown in MA, MA+E and MAN (Tracks 2, 4 & 5) show two new high molecular weight bands at approximately 65kD and 78kD respectively, the OMP extracted from MD (Track 3) shows a unique band at 44kD and from MAN an

extra intense band at just under 30kD. The function of these induced bands is unknown. OM preparations from C1359 grown in MA and MD (Tracks 7 & 8) produce the largest number of bands, although a major protein over 78kD, approximately 110kD, is not expressed when grown in conditions of high osmolarity (MD). Again the purpose of the proteins that are induced or repressed remains to be determined. Band intensity also varied between the growth conditions, (for example the 12kD band common to all the samples), and highlights the influence of the environment on levels of protein expression. It is interesting to note, that when grown under the same condition the two strains did not induce or repress proteins of the same molecular weight, implying that different *B. cepacia* isolates adapt to stressful environments in a variable manner.

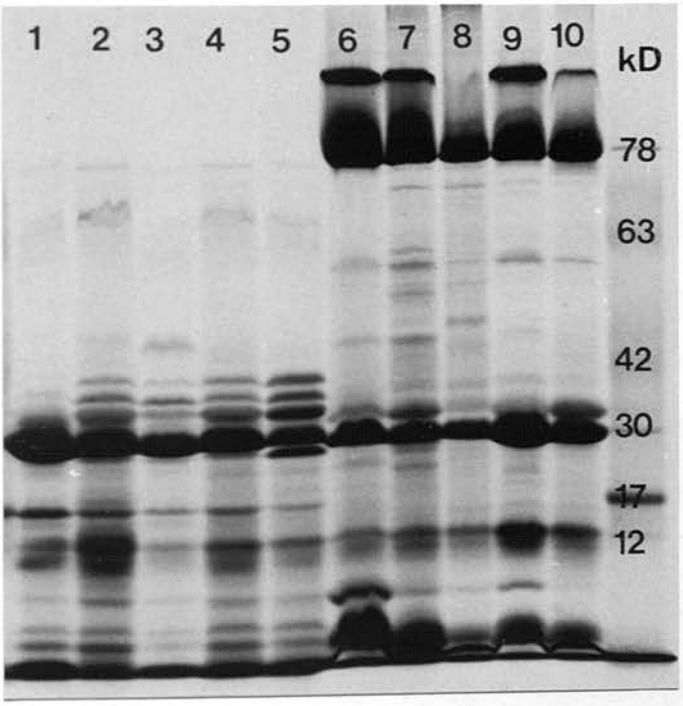


Figure 3.14 Coomassie blue stained outer membrane profiles of *B. cepacia* C1409 and C1359 extracted from different growth environments. Tracks 1-5 C1409 OM samples extracted from NB+YE, MA, MD, MA+E and MAN; Tracks 6-10 C1359 OM samples extracted from the same growth media. Molecular weights of protein standards are indicated.

Immunoblot analysis of the OM profile

Immunoblot analysis of the OM samples revealed differences in antigenic structure between the strains. Figure 3.15a and 13.15b show the immunoblots of OM preparations probed with sera from a CF patient colonised with an epidemic and non-epidemic *B. cepacia* strain respectively. All the OM preparations gave reaction with serum from an epidemic-colonised CF patient, except the environmental *B. cepacia* isolate J366 (Figure 3.15a; Track 10). The strongest reaction was against C1359 grown in NB+YE (Figure 3.15a; Track 1), although *B. cepacia* ATCC 17762, J2395 and *B. gladioli* ATCC 10248 also reacted well (Figure 3.15a; Track 7, 8 & 9). With regards to the antigenicity of the OMs extracted from different growth environments, C1359 grown in MD gave reaction with only two bands compared to the numerous bands reacting from the MA and NB+YE samples. Similarly, the OM sample from C1409 grown in MD also showed fewer bands reacting.

Serum from a CF patient colonised with an non-epidemic strain (C1583) gave a very different response to that observed in Figure 3.15a described above. The *P. aeruginosa* OM sample and the high molecular weight bands of *B. cepacia* ATCC 17762 gave the strongest reaction (Figure 3.15b; Track 1 & 9). However, all other OM preparations gave a poor response. Thus, the two CF sera used in the present study gave a very different reaction to the OM samples with regards to strength of response and cross-reaction.

The response of the OM samples to rabbit sera raised against C1359 (epidemic strain), C1409 (non-epidemic strain) and J2395 (environmental strain) are shown in Figure 3.16a-c respectively. The reaction observed using rabbit sera against C1359 (Figure 3.16a) differs from that seen using serum from a CF patient colonised with the homologous strain (Figure 3.16a); less reactive bands are observed with the rabbit serum, for example there is no reaction with the *P. aeruginosa* sample. It seems unlikely that the greater reaction against the CF serum is due to cross reaction with

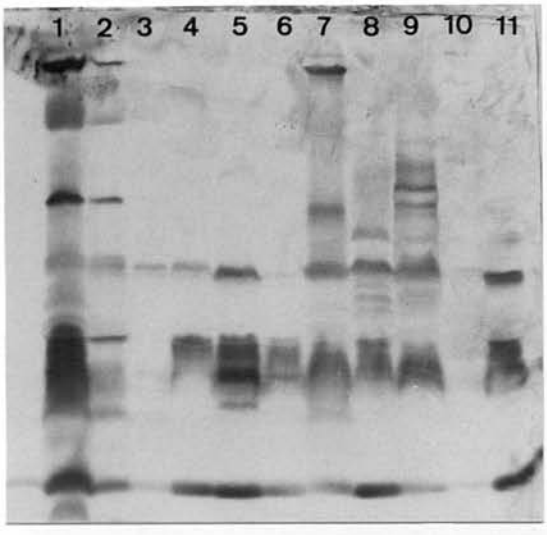
other bacteria as the CF patient was colonised only by the epidemic strain of *B. cepacia* and no other *Burkholderia* or *Pseudomonas* isolate.

The immunoblot using rabbit sera raised against C1409 (Figure 3.16b) cannot be compared with the blot shown in Figure 3.15b as the CF patient, from whom the serum was obtained, was not colonised with the same non-epidemic *B. cepacia* isolate as used to inoculate the rabbit. The immunoblot in Figure 3.16b does however show several interesting results. Firstly, *P. aeruginosa* was the only OM sample not to show any reaction. Secondly, for C1359, NB+YE (Figure 3.16b; Track 3) gave the strongest reaction of all growth conditions although two bands from the OM extracted from MA+E (Track 6) also reacted well. Thirdly, although all OM samples from C1409 reacted strongly there was obvious differences between the preparations from different growth conditions. For example, growth in MA and MA+E (Tracks 9 & 11) produced four highly reactive bands above the common 30kD band, compared to only three from growth in MD (Track 10) and two from growth in NB+YE and MAN (Track 8 & 12).

Rabbit sera raised against J2395 showed a different and weaker reaction against the OM preparations. All the samples reacted but only the homologous strain and *B. cepacia* ATCC 29424 showed a strong response. The 30kD band was highly reactive, as was observed using other sera, confirming the antigenicity and conserved nature of this protein.

Unlike the LPS samples, the OMs of *B. cepacia* and *P. aeruginosa* cross-reacted, although this depended highly on the source of serum. Furthermore, the influence of different growth environments on the antigenicity of the outer membranes can readily be seen.

a



b

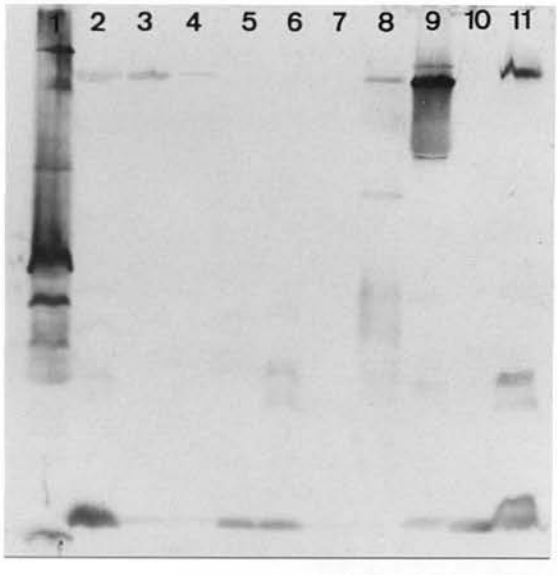
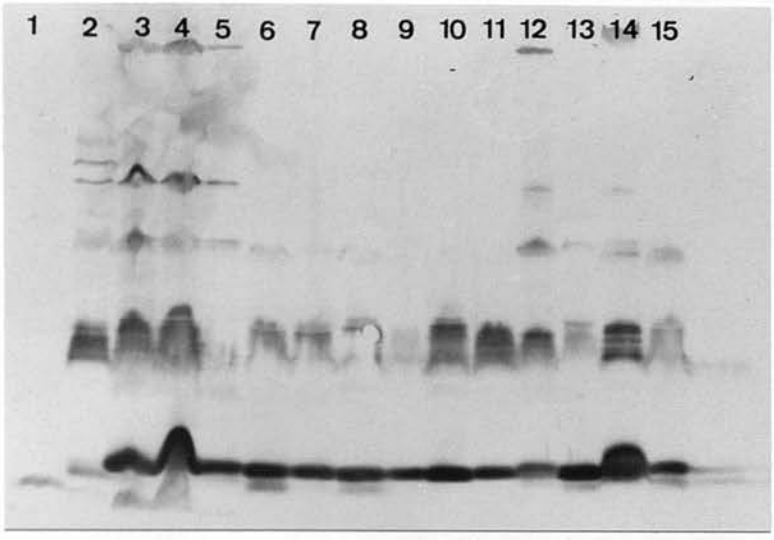


Figure 3.15 Immunoblots of outer membrane antigens of *B. cepacia, B. gladioli* and *P. aeruginosa* probed with sera from a CF patient colonised with (a) the epidemic strain and (b) a non-epidemic strain. Figure 3.15a Track 1, *B. cepacia* C1359 from NB+YE; Track 2, MA; Track 3, MD; Track 4, *B. cepacia* C1409 from NB+YE; Track 5, MA; Track 6, MD; Track 7, *B. cepacia* ATCC 17762; Track 8, *B. cepacia* J2395; Track 9, *B. gladioli* ATCC 10248; Track 10, *B. cepacia* J366; Track 11, *P. aeruginosa* PAO1. **Figure 3.15b** Track 1, *P. aeruginosa* PAO1; Track 2, *B. cepacia* C1359 from NB+YE; Track 3, MA; Track 4, MD; Track 5, *B. cepacia* C1409 from NB+YE; Track 6, MA; Track 7, MD; Track 8, *B. gladioli* ATCC 10248; Track 9, *B. cepacia* ATCC 17762; Track 10, *B. cepacia* J2395; Track 11, *B. cepacia* ATCC 29424.

a



b

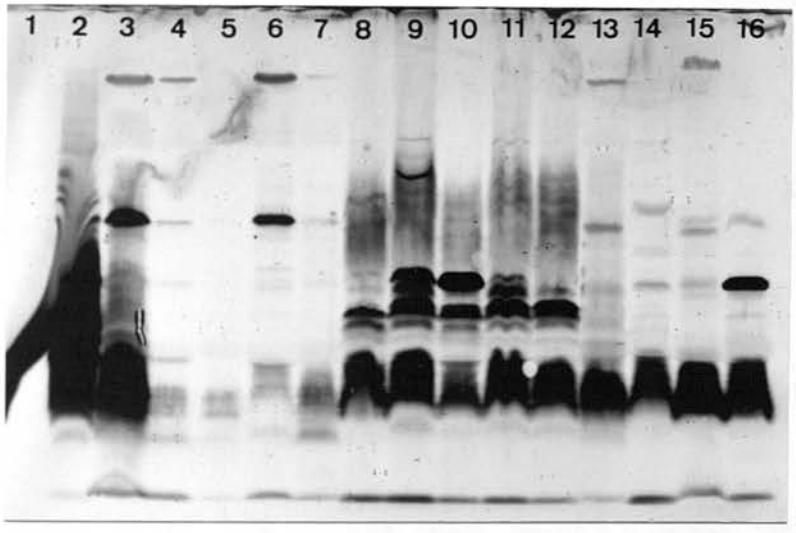


Figure 3.16 Immunoblots of outer membrane antigens of *B. cepacia, B. gladioli* and *P. aeruginosa* probed with rabbit sera raised against (a) *B. cepacia* C1359, (b) *B. cepacia* C1409 and (c) *B. cepacia* J2395. Figure 3.16a Track 1, *P. aeruginosa* PAO1; Track 2, *B. gladioli* ATCC 10248; Track 3, *B. cepacia* C1359 from NB+YE; Track 4, NB+YE; Track 5, MA; Track 6, MD; Track 7, MA+E; Track 8, MAN; Track 9, *B. cepacia* C1409 from NB+YE; Track 10, MA; Track 11, MD; Track 12, *B. cepacia* ATCC 17762; Track 13, *B. cepacia* J2395; Track 14, *B. cepacia* ATCC 29424; Track 15, *B. cepacia* J366. **Figure 3.16b** Track 1, *P. aeruginosa* PAO1; Track 2, *B. gladioli* ATCC 10248; Track 3, *B. cepacia* C1359 from NB+YE; Track 4, MA; Track 5, MD; Track 6, MA+E; Track 7, MAN; Track 8, *B. cepacia* C1409 from NB+YE; Track 9, MA; Track 10, MD; Track 11, MA+E; Track 12, MAN; Track 13, *B. cepacia* ATCC 17762; Track 14, *B. cepacia* J2395; Track 15, *B. cepacia* ATCC 29424, Track 16, *B. cepacia* J366.

c

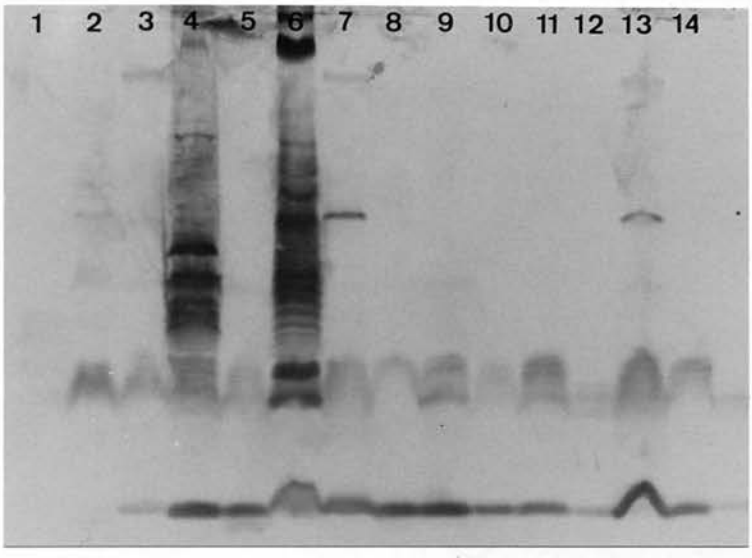


Figure 3.16c Track 1, *P. aeruginosa* PAO1; Track 2, *B. gladioli* ATCC 10248; Track 3, *B. cepacia* ATCC 17762; Track 4, *B. cepacia* J2395; Track 5, *B. cepacia* J366; Track 6, *B. cepacia* ATCC 29424; Track 7, *B. cepacia* C1359 from NB+YE; Track 8, *B. cepacia* C1409 from NB+YE; Track 9, MA; Track 10, MD; Track 11, MA+E; Track 12, MAN; Track 13, C1359 from NB+YE; Track 14, C1409 from NB+YE.

3.4 CHARACTERISTICS AND ENVIRONMENTAL REGULATION OF EPS

EPS profile

The *B. cepacia* strains used in this study do not produce high levels of exopolysaccharide as observed by simple colonial morphology. However, to investigate further the possibility of EPS production Percoll discontinuous density centrifugation was carried out on 11 strains of *B. cepacia,* one strain of *B. gladioli* and two strains of *P. aeruginosa* with results shown in Figure 3.17. (Refer to Figure 6.7 for a photograph of Percoll profiles).

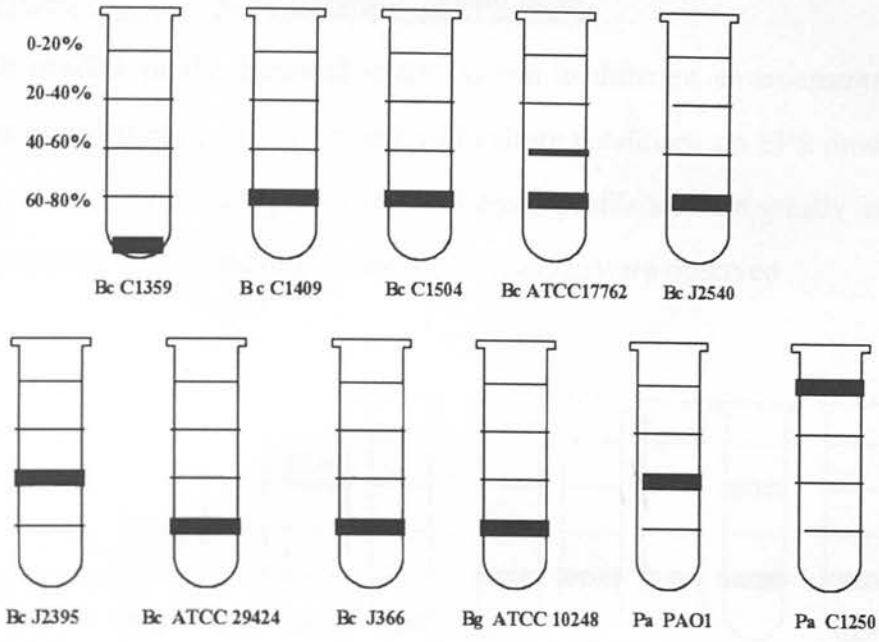


Figure 3.17 Percoll profiles of *B. cepacia, B. gladioli* and *P. aeruginosa* strains grown in NB+YE. The main areas of bacterial concentration are marked by the dark bands.

The Percoll profiles show that the majority of strains centrifuged to the 60-80% interface indicating that strains of *B. cepacia* produce little EPS. Investigation of the Percoll populations using an India ink stain confirmed the lack of capsular material. However, *B. cepacia* ATCC 17762 and J2395 gave some precipitation at the 40-60% interface, although again no EPS could be observed by India ink staining. In contrast, *P. aeruginosa* C1250 produced a mucoid phenotype that was easily identified on agar plates and precipitated to the 0-20% interface. All Percoll centrifugations were carried out on 24h old cultures, although no difference in profiles was observed after 48h growth.

The influence of growth environment on EPS profile

Percoll profiles of the bacterial strains grown in different environments were also carried out to investigate the influence of culture conditions on EPS production. As seen from the results in Figure 3.18, the Percoll profile was not greatly influenced by environmental factors although some slight changes were observed.

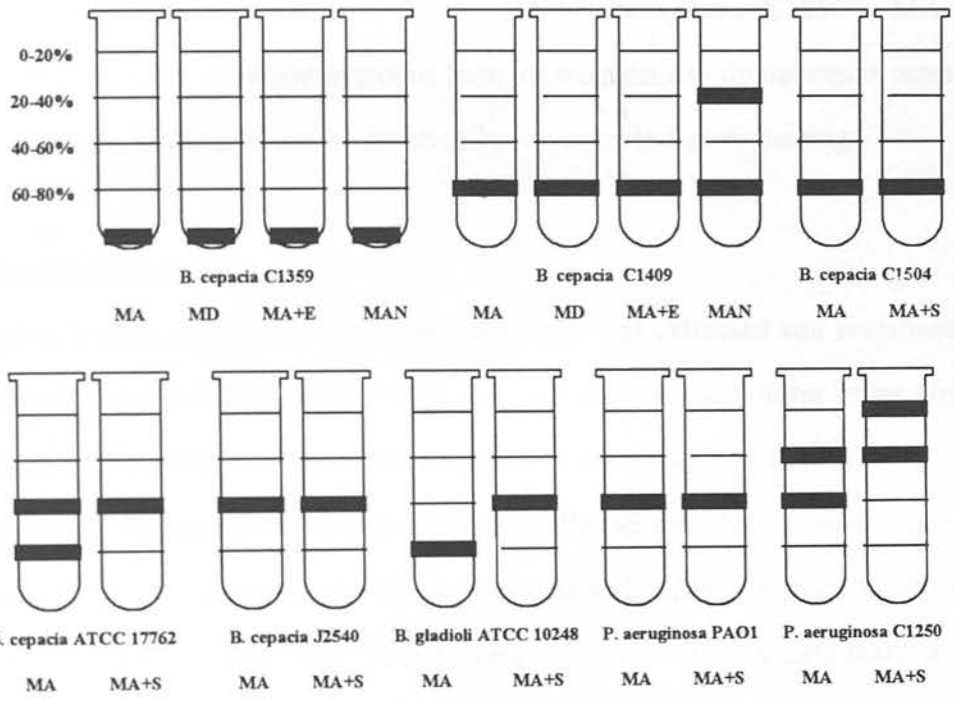


Figure 3.18 The influence of growth environment on the Percoll profile of *B. cepacia, B. gladioli* and *P. aeruginosa* strains. The main areas of bacterial concentration are marked by the dark bands.

No difference in Percoll profile was seen for C1359, C1504, ATCC 17762 and PAO1. Changes were observed in C1409 grown in MAN which produced an additional small band at the 20-40% interface, in J2540 which precipitated to the 40-60% interface in MA and MA+S, and in *B. gladioli* which centrifuged to the 40-60% layer in MA+S, indicating increased production of EPS in those growth conditions. Surprisingly, the

mucoid *P. aeruginosa* strain C1250 grown in MA sedimented out to both the 40-60% and the 20-40% interface, suggesting that less alginate is produced in minimal medium. Furthermore growth in MA+S also produced two bands, present at the 0-20% and 20-40% interfaces. Thus, the majority of strains appeared as homogeneous populations with respect to EPS production. In other words, the bacterial sample centrifuged to only one interface. However, this was not observed with *B. cepacia* C1409 in MAN, ATCC 17762 and *P. aeruginosa* C1250 in MA and MA+S, which suggests these three strains form heterogeneous populations in terms of EPS production. This could not be detected however by India ink staining.

EPS extraction and analysis

The alginate polysaccharide of *P. aeruginosa* C1250 was extracted and visualised on silver-stained 12% and 10% SDS-PAGE gels and by immunoblotting using human serum from a CF patient colonised with a mucoid *P. aeruginosa* as a probe.

The alginate did not separate out well on the SDS-PAGE gel which is what would be expected for such a high molecular weight material and, more importantly, no other polysaccharide material was observed indicating the absence of any LPS material. In addition no reaction was observed by immunoblotting.

3.5 CELL SURFACE HYDROPHOBICITY

The cell surface hydrophobicity of five *B. cepacia* isolates, two *P. aeruginosa* isolates and one *B. gladioli* strain was determined by HIC. Specific hydrophobic interactions were measured by octyl Sepharose CL-4B with Sepharose CL-4B used as a control for non-specific absorption. Results (Figure 3.19) are expressed as the percentage of cells retained by the octyl Sepharose relative to retention by Sepharose. In addition, the hydrophobicity of *B. cepacia* C1359 (epidemic CF strain), C1409 (non-epidemic CF strain) and J2540 (environmental strain) grown in different culture media was measured (Figure 3.20).

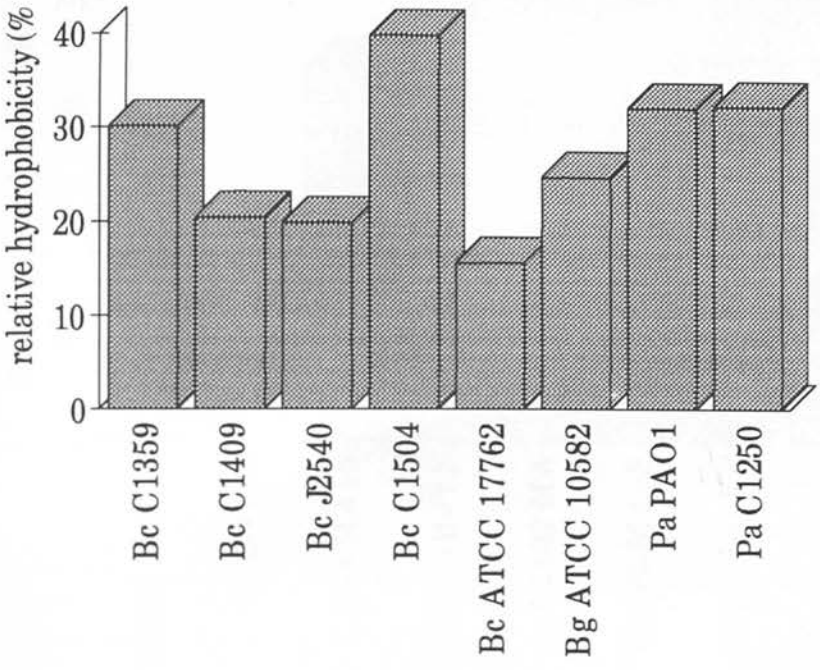


Figure 3.19 Cell Surface Hydrophobicity of *B. cepacia, B. gladioli* and *P. aeruginosa* strains.

The cell surface hydrophobicity of *B. cepacia* averaged at 25%, the same as for *B. gladioli,* but slightly less than the average for *P. aeruginosa*, measured at 32%. However, no correlation could be determined between cell surface hydrophobicity and bacterial source or LPS phenotype.

For the three *B. cepacia* strains tested, C1359, C1409 and J2540, growth in MA+S increased hydrophobic interactions (Figure 3.20). This increase was particularly pronounced in the epidemic strain, with relative hydrophobicity reaching levels of 70%. For the two CF strains, growth in MA produced bacteria of low hydrophobicity, whereas for the environmental strain J2540, NB+YE gave the least hydrophobic interactions. However, the difference in cell surface hydrophobicity between the growth conditions varied for each strain tested.

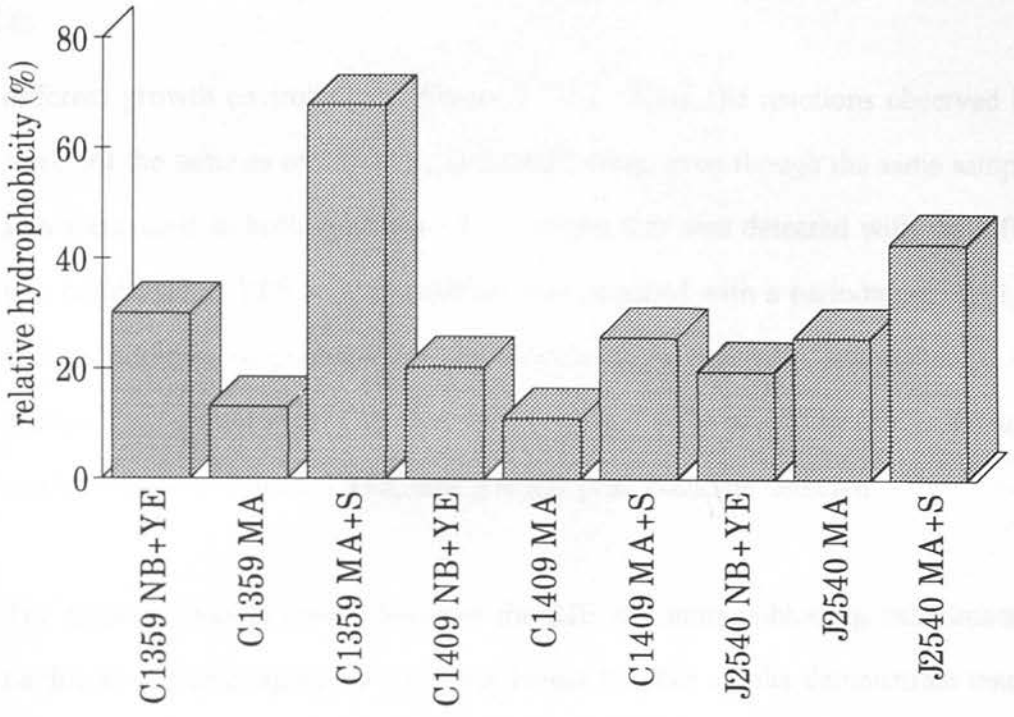


Figure 3.20 The Cell Surface Hydrophobicity of *B. cepacia* C1359, C1409 and J2540 grown in NB+YE, MA and MA+S.

3.6 ANTIGEN DETECTION BY RIE

Further investigations of the surface antigenic structures were performed with both rocket (RIE) and crossed immunoelectrophoresis (CIE). Samples of LPS extracted by different methods and from various growth conditions and OMs from different growth environments were loaded onto agarose gels containing either human or rabbit antisera.

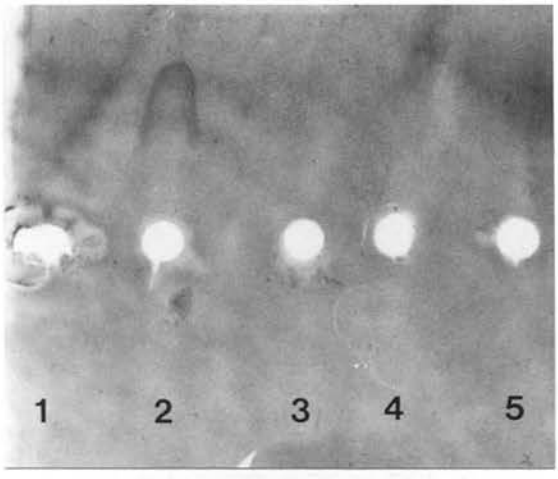
Figure 3.21a-f shows the RIE gels of several LPS and OM samples, with some interesting results emerging. Firstly, and most surprisingly, no sample gave reaction with any of the rabbit sera. Secondly, only one major reaction was ever observed using the CF sera; between the PW-LPS sample of the epidemic strain and serum from a patient colonised by the homologous strain (Figure 3.21a). Furthermore, this unique reaction of the epidemic strain was observed using PW-LPS extracts from

different growth environments (Figure 3.21b). Thus, the reactions observed by RIE were not the same as observed by immunoblotting, even though the same samples and sera were used in both methods. The antigen that was detected with the CF serum was confirmed as LPS as little reaction was obtained with a periodate-treated sample and on addition of proteinase K or autoclaving a precipitin line remained (Figure 3.21c). Two-dimensional CIE was used to observe the precipitin further, although as can be seen from Figure 3.21d, only a single peak could be detected.

The discrepancies in results between the RIE and immunoblotting experiments must be due to the techniques *per se*; nevertheless the RIE results demonstrate that out of all the reactions studied, the interaction between the epidemic strain and CF patients is the strongest. Furthermore as there was a difference between human and rabbit sera, this leads to speculation that the core-LPS antigens were somehow masked on the whole cells used to raise the rabbit antisera. Masking would most likely be due to EPS, although none could be detected for the epidemic strain. Therefore, why the rabbit serum gave no reaction against the PW-LPS sample of C1359 is not clearly understood, although the presence and, hence, masking effect of a high molecular weight compound in the epidemic strain should not be discounted.

OM samples also reacted against the CF serum, although a 'streak' rather than a precipitin arc was produced (Figure 3.21e). It is possible that these streaks are actually salt present in the samples precipitating out. Interestingly, using serum from a CF patient colonised by both C1359 (epidemic) and C1409 (non-epidemic), OM samples from both strains gave a reaction (Figure 3.21f), although only C1359 LPS precipitated out. However, whether this reaction is again due to salt precipitate remains to be clarified.

a



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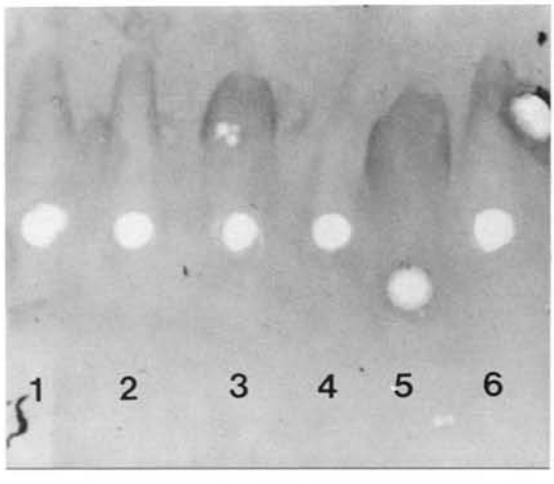
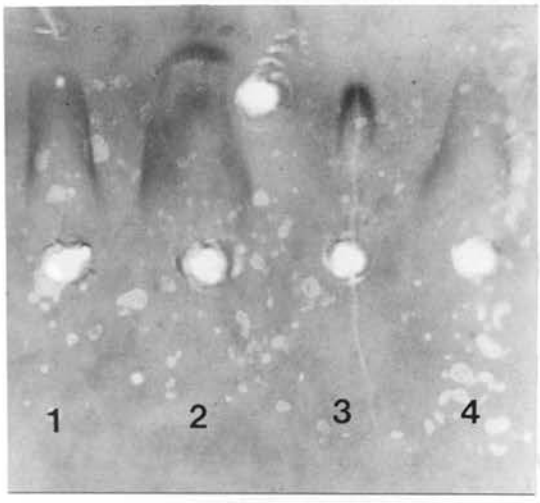


Figure 3.21a. Rocket immunoelectrophoresis of LPS samples from *B. cepacia, B. gladioli* and *P. aeruginosa* strains. Track 1, *P. aeruginosa* PAO1 PW-LPS; Track 2, *B. cepacia* C1359 PW-LPS; Track 3, *B. cepacia* C1409 PW-LPS; Track 4, *B. cepacia* ATCC 17762 PW-LPS; Track 5, *B. gladioli* ATCC 10248 PW-LPS. **Figure 3.21b RIE of *B. cepacia* PW-LPS samples from different growth environments.** Track 1, *B. cepacia* C1359 PW-LPS from NB+YE; Track 2, PW-LPS from MA; Track 3, PW-LPS from MA+S; Track 4, *B. cepacia* C1409 PW-LPS from NB+YE, Track 5, *B. cepacia* C1359 PW-LPS from MA+S; Track 6, C1359 PW-LPS from MA. Serum used in both **a** and **b** was obtained from a CF patient colonised by the epidemic strain.

c



d

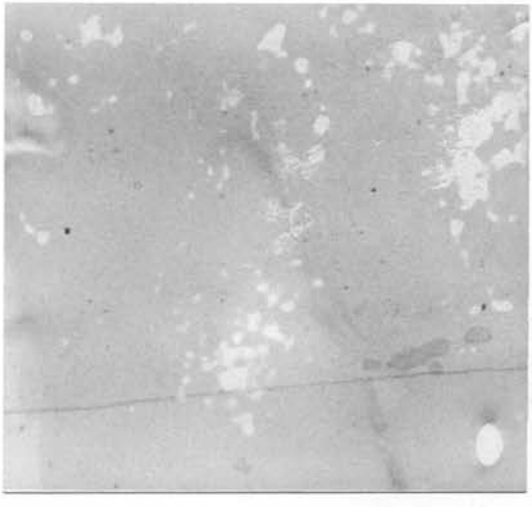
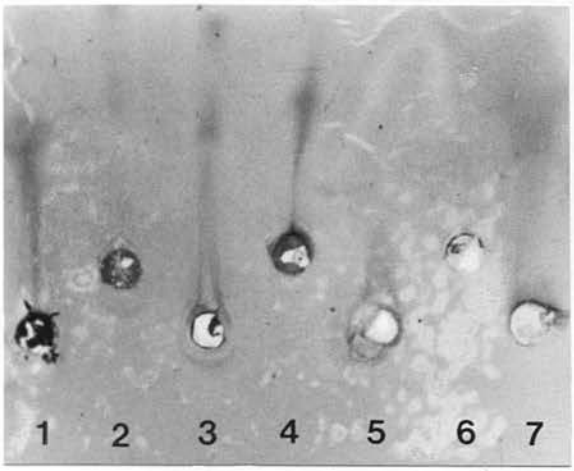


Figure 3.21c RIE of *B. cepacia* C1359 PW-LPS samples after treatment with proteinase K and periodate. Track 1, PW-LPS; Track 2, PW-LPS after periodate treatment; Track 3, PW-LPS after proteinase K treatment; Track 4, PW-LPS after autoclaving. **Figure 3.21d Crossed immunoelectrophoresis of *B. cepacia* C1359 PW-LPS extract.** Serum used in both **c** and **d** was obtained from a CF patient colonised by the epidemic strain.

e



f

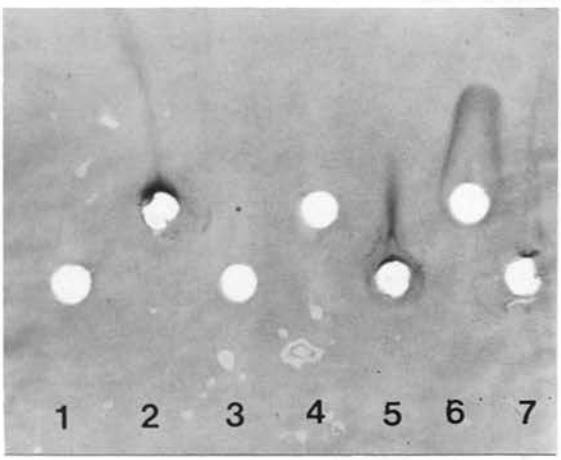


Figure 3.21e RIE of OM samples from *B. cepacia, B. gladioli* and *P. aeruginosa* strains. Track 1, *B. gladioli* ATCC 10248 OM from NB+YE; Track 2, *B. cepacia* C1409 OM from MA; Track 3, *B. cepacia* C1409 OM from NB+YE; Track 4 *B. cepacia* ATCC 17762 OM from NB+YE; Track 5, *B. cepacia* C1359 OM from NB+YE; Track 6, *B. cepacia* C1359 OM from MA; Track 7, *P. aeruginosa* PAO1 OM from NB+YE. **Figure 3.21f RIE of LPS and OM samples from *B. cepacia* strains.** Track 1, C1409 PW-LPS; Track 2, C1409 OM sample from NB+YE; Track 3, C1409 PCP; Track 4, C1359 PCP; Track 5, C1359 OM sample from NB+YE; Track 6, C1359 PW-LPS; Track 7, C1359 OM sample from MA. Serum used for **3.21e** was from a CF patient colonised by the epidemic strain, serum used for **3.21f** was from a CF patient colonised by both the epidemic strain, C1359, and a non-epidemic strain, C1409.

CHAPTER 4

RESULTS

BIOLOGICAL ACTIVITY OF THE CELL SURFACE COMPONENTS OF *BURKHOLDERIA CEPACIA*

The interaction between the bacterial cell surface and the host immune system plays a major part in pathogenesis. Experiments described in this section aimed to determine the biological activity, and in particular the inflammatory potential, of two bacterial cell surface components, LPS and OM, focusing on their endotoxicity and capacity to induce TNF-α and IL-8, two major proinflammatory cytokines involved in CF.

Throughout the following experiments the same eight bacterial strains were used, *B. cepacia* C1359 (CF, epidemic), C1409 (CF, non-epidemic), C1504 (CF, non-epidemic), ATCC 17762 (non-CF), J2540 (environmental), *B. gladioli* ATCC 10248 (environmental, type strain) and *P. aeruginosa* C1250 (CF, mucoid) and PAO1 (genetic type strain).

4.1 THE ENDOTOXICITY OF LPS SAMPLES

PW-LPS samples from the bacterial strains described above were assayed for their endotoxic potential *in vitro* using the Limulus Amoebocyte Lysate (LAL) assay.

Dose response

To determine the optimal concentration of LPS extract to use in the LAL assay, PW-LPS samples were serially diluted ten fold in pyrogen-free water to provide a concentration range from 5ng/ml-0.05pg/ml. Figure 4.1 shows the dose response curve for three representative strains. An LPS concentration lying between 5ng/ml-0.5ng/ml was chosen as the optimal concentration of PW sample to use in the LAL assay, as the optical density values produced from this concentration range corresponded to the middle of the standard curve and would, therefore, provide more accurate results in future experiments.

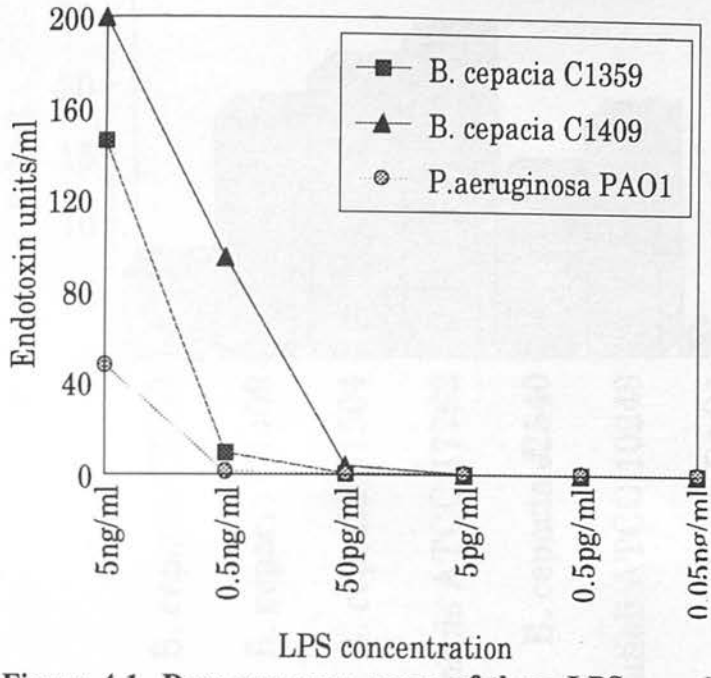


Figure 4.1 Dose response curve of three LPS samples as measured by the LAL assay. Results shown are the mean of duplicates.

The endotoxicity of PW-LPS samples from different bacterial strains

PW-LPS samples from *B. cepacia, B. gladioli* and *P. aeruginosa*, at a concentration of 1ng/ml, were assayed for their endotoxic potential (Figure 4.2). In the LAL assay, *B. cepacia* LPS was significantly more endotoxic ($P<0.01$) than LPS preparations from *P. aeruginosa*. No correlation was observed between LPS phenotype and LAL activity although, interestingly, the LPS from the epidemic strain gave the lowest endotoxic readings from all *B. cepacia* strains tested. Further experiments carried out using LPS samples of 5ng/ml and 0.5ng/ml confirmed the difference between *B. cepacia* and *P. aeruginosa*, indicating the trend observed was not dependent on LPS concentration.

The influence of growth environment on LPS endotoxicity.

The PW-LPS extracts of *B. cepacia* C1359 and C1409 grown in NB+YE, MA and MA+S were assayed to determine whether growth environment influenced the endotoxicity of the LPS samples (Figure 4.3).

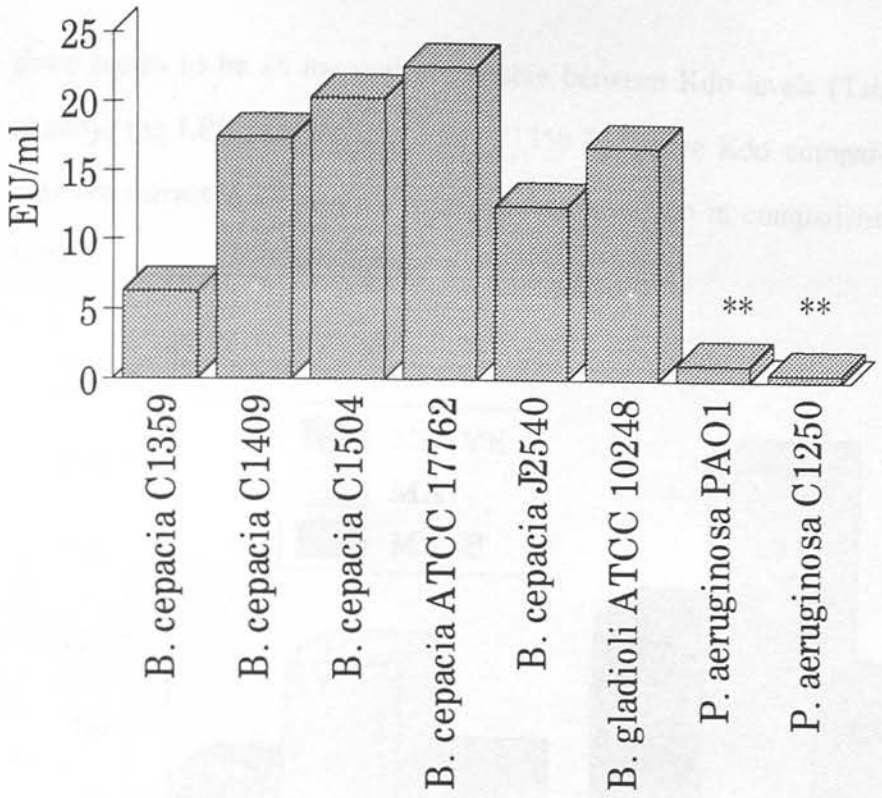


Figure 4.2 The endotoxic activity of PW-LPS preparations as measured by the LAL assay. LPS samples were used at a concentration of 1ng/ml. The results shown represent the mean of three experiments. The endotoxic activity of *B. cepacia* was significantly different from *P. aeruginosa* ($P<0.01$) which is highlighted by the ** symbol

For both strains, LPS preparations extracted from growth in MA gave the highest endotoxin values. For the epidemic strain, no difference in endotoxicity between LPS extracted from growth in NB+YE and MA+S could be detected, whereas for C1409, the LPS sample extracted from NB+YE gave a markedly higher endotoxic response compared to the sample from MA+S. Furthermore, for all three growth conditions, LPS preparations from C1409 had a greater endotoxic potential than C1359.

Thus, although no structural difference between the LPS samples from different growth media could be detected by silver-stained PAGE and immunoblotting, endotoxicity does appear to be dependent on environmental conditions. Interestingly,

there seems to be an inverse relationship between Kdo levels (Table 3.4) and LAL activity; the LPS preparations from C1359 had more Kdo compared to C1409 and samples extracted from growth in MA had less Kdo in comparison to NB+YE and MA+S.

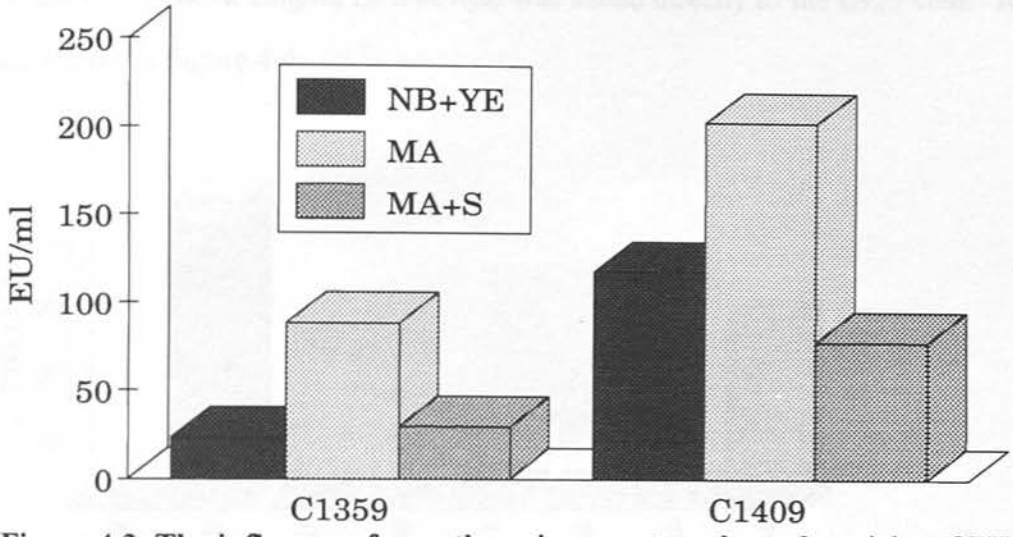


Figure 4.3 The influence of growth environment on the endotoxicity of PW-LPS samples. LPS was used at a concentration of 1ng/ml. Each value represents the mean of two experiments.

4.2 INFLAMMATORY POTENTIAL OF LPS

Increasing levels of TNF-α have been reported to be associated with pulmonary deterioration in CF patients (for example Wilson *et al,* 1993). LPS is well-established as a potent stimulator of TNF-α, therefore, the following experiments were performed to investigate the potential of *B. cepacia* LPS to induce TNF-α, using both *B. gladioli* and *P. aeruginosa* LPS extracts for comparison.

TNF-α levels were measured in a two-stage bioassay. LPS samples were used to stimulate cells, and hence induce cytokine production, and the supernate collected. Subsequently, diluted supernates were added to L929 cells, which are sensitive to the cytotoxic effects of TNF-α, and the levels of L929 cell killing used to calculate the amount of TNF-α present.

Specificity of TNF-α bioassay.

To ensure that the L929 bioassay was measuring TNF-α specifically, an anti-TNF-α MAb was mixed with supernate from a stimulation experiment and added to the L929 cells. In addition, to ensure that the PW-LPS samples themselves had no direct effect on the L929 cells a 1mg/ml LPS sample was added directly to the L929 cells. Results are shown in Figure 4.4.

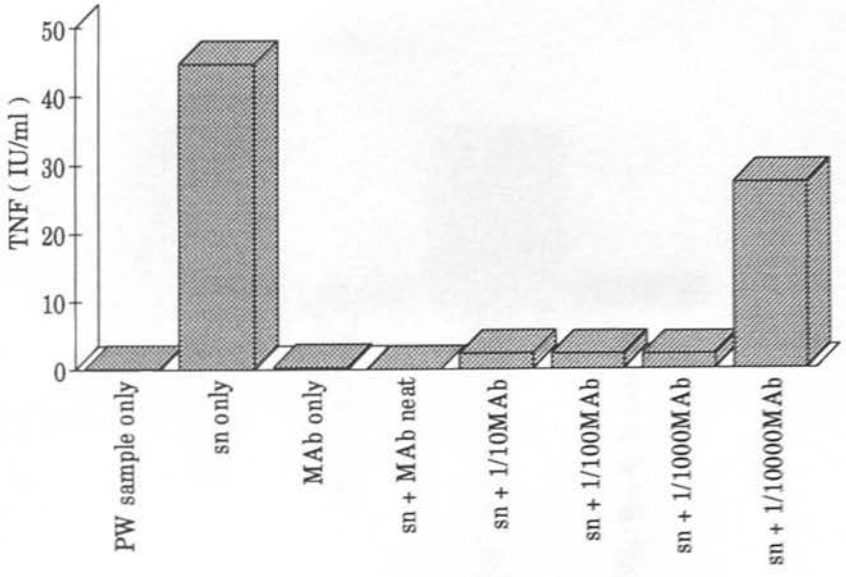


Figure 4.4 Specificity of TNF-α Bioassay. Supernate was collected at 4h. Results shown are the mean of duplicates. MAb = monoclonal antibody; sn = supernate.

Figure 4.4 shows that the anti-TNF-α MAb neutralises TNF-α in the supernate up to a dilution of 1:10,000. In addition, the PW-LPS samples alone had no effect on the L929 cells. Thus, it can be concluded that the bioassay used in the present study measures only TNF-α in the culture supernate and it is solely responsible for lysis of the L929 cells.

PW-LPS samples were also treated with either periodate, proteinase K or both, to confirm that TNF-α activity was indeed being induced by the LPS molecule (Figure 4.5). It is interesting to note, however, that there is a small reduction in TNF-α levels

on addition of proteinase K. This observation implies that either the protein present in the sample is inducing a small cytokine response *per se* or the protein, in combination with the LPS molecule, presents a more active form of compound compared to pure LPS. Alternatively, the proteinase K may have been contaminated with an enzyme which degraded some of the LPS sample. Proteinase K and periodate alone did not stimulate any detectable TNF-α.

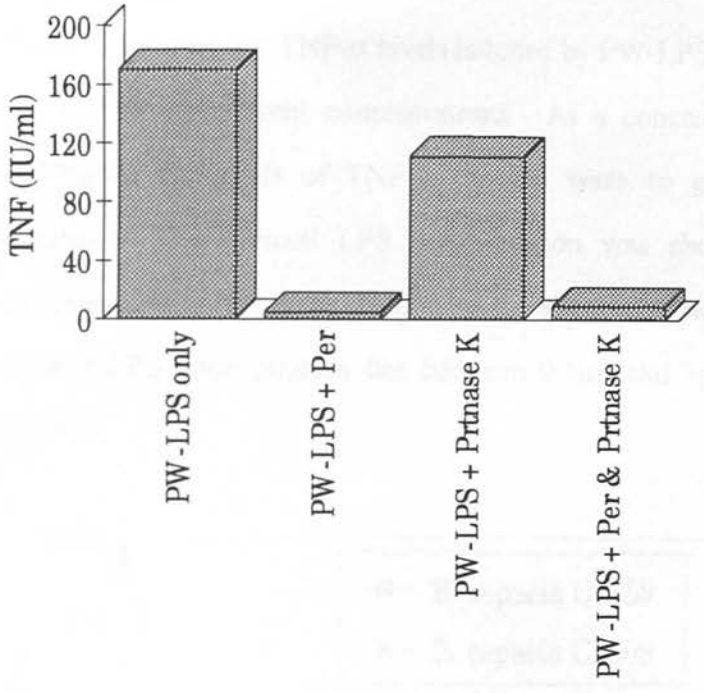


Figure 4.5 The influence of periodate and proteinase K treated PW-LPS samples on induction of TNF-α. Results are the mean of four wells. Supernate was collected at 4h. Per = periodate; Prtnase K = Proteinase K.

Further controls in each experiment consisted of assaying the supernate from wells containing medium only, MNL cells only and water used to dilute the LPS samples. Assays in which TNF-α levels could not be detected above the background ODs (determined as the OD produced from a well containing L929 cells only) were rejected. Fresh samples were diluted every time from 5mg/ml stock solutions kept at -20°C, with fresh stock solutions prepared approximately every two months.

4.3 TNF-α INDUCTION FROM MONONUCLEAR LEUCOCYTES

Mononuclear leucocytes (MNL) were separated from healthy blood donors and used as a source of immune cells. As with the LAL assay, the optimal parameters of the TNF assay were assessed in order to obtain the most accurate and reliable results for future experiments.

Dose response

Figure 4.6 shows the TNF-α levels induced by PW-LPS samples of *B. cepacia* C1359 and C1409 at different concentrations. At a concentration equal or greater than 1000ng/ml the levels of TNF-α induced were so great that no value could be assigned. The optimal LPS concentration was chosen so that the OD values corresponded to the linear section of the sigmoid standard curve. In this study, the optimal LPS concentration lies between 0.1μg and 1pg/ml and was determined as 1ng/ml.

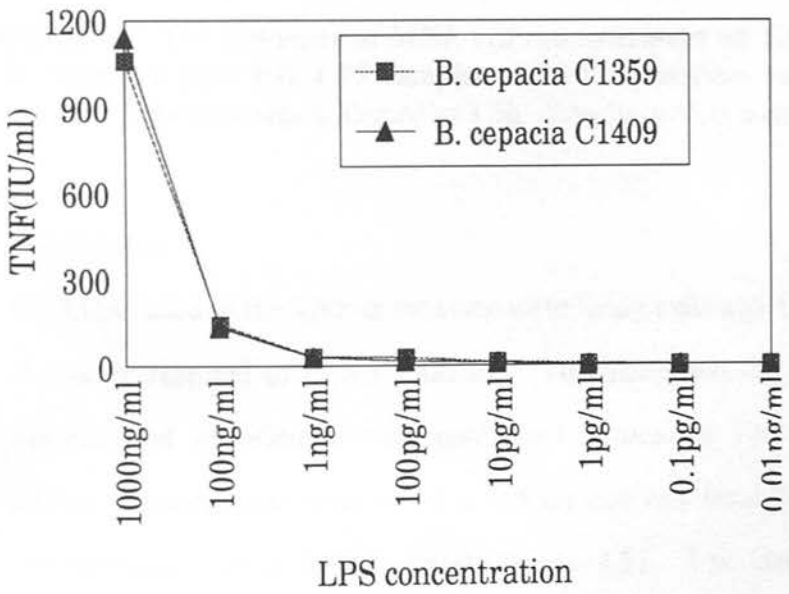


Figure 4.6 Dose response of PW-LPS samples from MNL cells. Supernate was collected at 3.5h Results are the mean of four readings.

MNL cell concentration

In the present study, MNL cells were used at a concentration of 8 x 10^6 cells/ml; chosen as the optimum cell number by preliminary experiments in another study. However, as shown in Figure 4.7, MNL cell concentrations ranging from 9 x 10^6-1 x 10^6 cells/ml gave detectable levels in response to a PW-LPS sample of 1ng/ml.

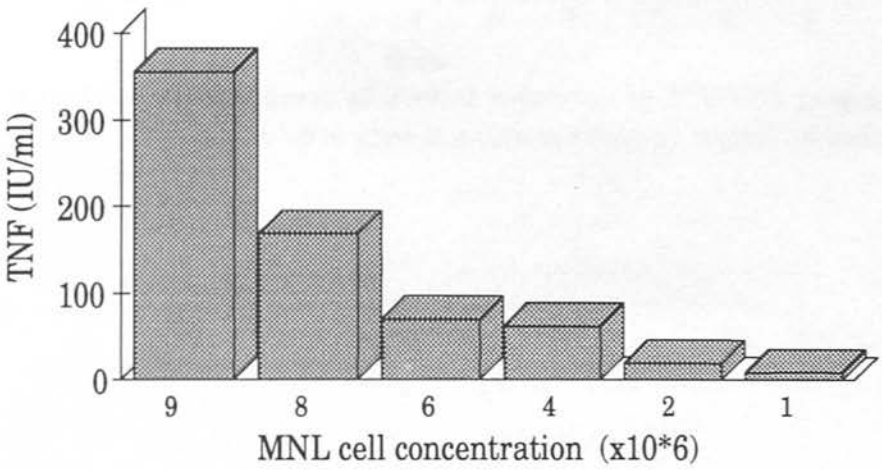


Figure 4.7 The influence of MNL cell concentration on TNF-α levels induced by *B. cepacia* C1409 PW-LPS samples. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 3.5h. Results are the mean of duplicates.

Time course

The MNL used in the TNF-α bioassay were living cells and as such required a period of time to respond to LPS stimulation. To ensure that the peak cytokine response was assessed, experiments were performed to measure TNF-α induction over time. TNF-α measurements were made at 30min intervals from 0-9h (Figure 4.8) and at 24h intervals over a 6-day period (Figure 4.9). For clarity, results from three representative strains only are shown.

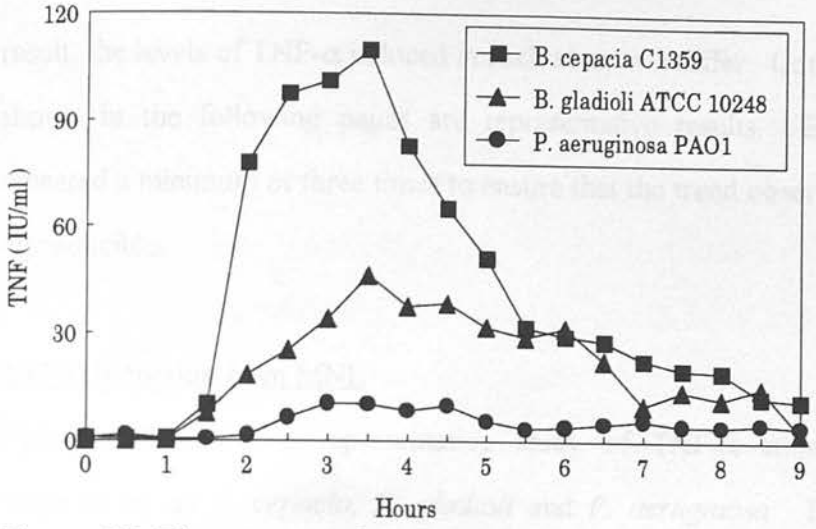


Figure 4.8 Time-course of TNF-α induction by PW-LPS preparations from 0-9 hours. LPS samples were used at a concentration of 1ng/ml. Results are the mean of duplicates.

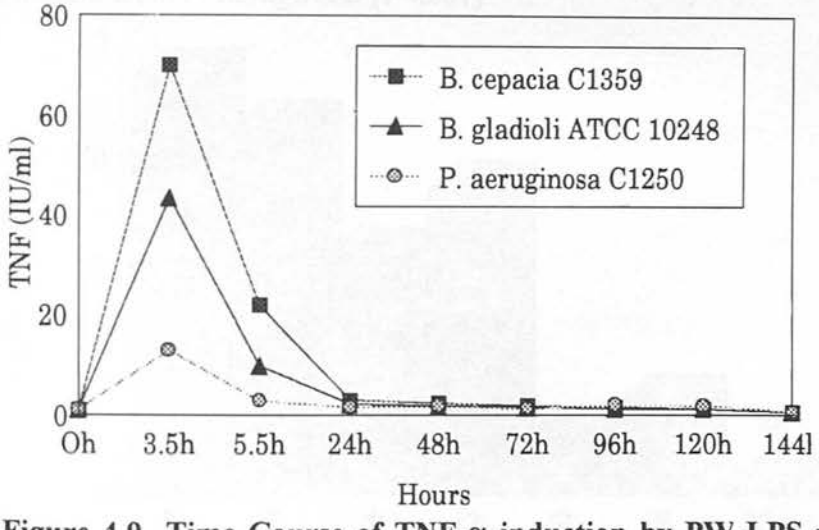


Figure 4.9 Time Course of TNF-α induction by PW-LPS preparations over a six-day period. LPS samples were used at a concentration of 1ng/ml. Results shown are the mean of duplicates.

For all PW-LPS preparations the peak TNF-α response was observed between approximately 2.5h-4.5h (Figure 4.8). Furthermore, after 24 hours negligible TNF-α was detected. For all future experiments LPS preparations at 1ng/ml were used and supernatant collected at 3.5h.

It must be stressed that MNL cells from different blood donors vary slightly, and as a

result, the levels of TNF-α induced in each assay will differ. Consequently, the graphs shown in the following pages are representative results. Each experiment was repeated a minimum of three times to ensure that the trend observed was accurate and reproducible.

TNF-α induction from MNL

Figure 4.10 shows a representative study of TNF-α stimulation by PW-LPS preparations of *B. cepacia, B. gladioli* and *P. aeruginosa*. Both *B. cepacia* and *B. gladioli* samples induced at least a nine-fold higher activity compared to either of the *P. aeruginosa* preparations and as a population, *B. cepacia* was significantly different from *P. aeruginosa* (P<0.01).

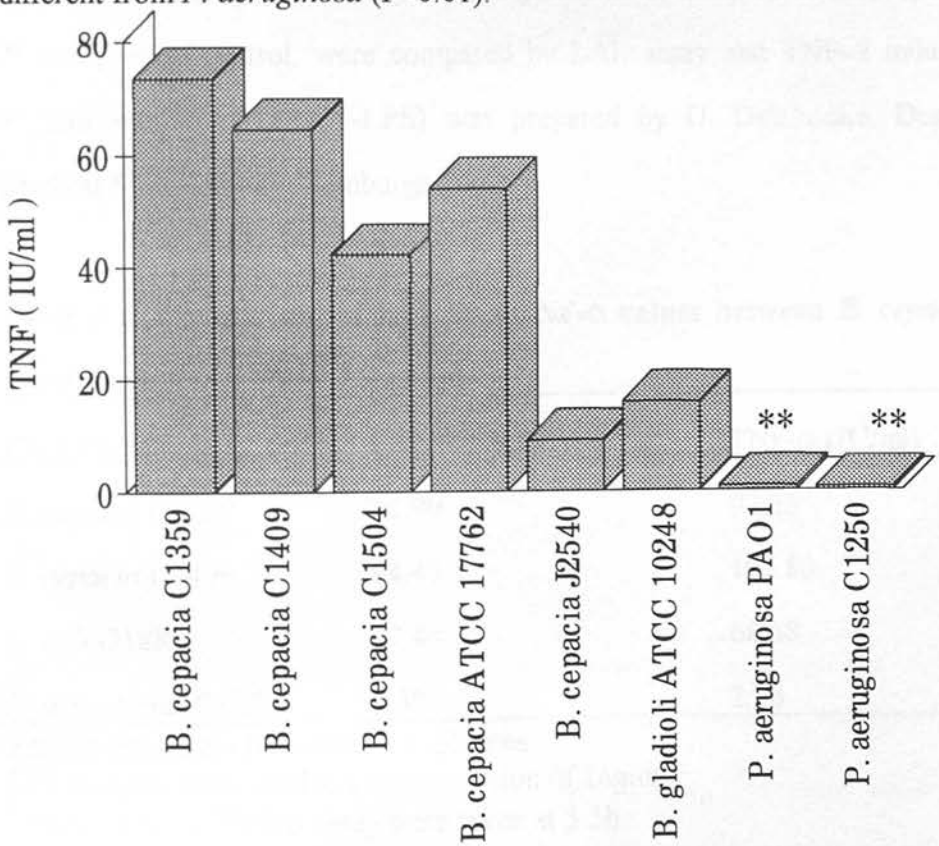


Figure 4.10 TNF-α levels produced from MNL in response to PW-LPS preparations of *B. cepacia, B. gladioli* and *P. aeruginosa*. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 3.5h. Results are the mean of duplicates. The levels of TNF-α induced by *B. cepacia* were significantly different from *P. aeruginosa* (P<0.01) which is highlighted by the ** symbol.

In contrast to the LAL assay results, in which the epidemic strain had the lowest endotoxicity of the *B. cepacia* strains tested, the epidemic strain produced one of the highest levels of TNF-α. Furthermore, there was a significant difference in TNF-α levels induced from *B. cepacia* clinical and environmental strains ($P<0.05$) which would apparently confirm the view that a sub-group of *B. cepacia* may be 'safe' for environmental release as a biocontrol agent or decontaminant of soil. However, it must be stressed that all the *B. cepacia* samples tested possessed a greater biological activity compared to the *P. aeruginosa* extracts.

E. coli is considered to have one of the most potent LPS structures, therefore, to define more clearly the activity of *B. cepacia* LPS, samples of both species, plus a *P. aeruginosa* control, were compared by LAL assay and TNF-α induction. The *E. coli* sample (O18K; S-LPS) was prepared by D. Delahooke, Department of Medical Microbiology, Edinburgh.

Table 4.1 Comparison of LAL and TNF-α values between *B. cepacia, E. coli* and *P. aeruginosa* PW-LPS samples.

PW-LPS sample	LAL (EU/ml)	TNF-α (IU/ml)
B. cepacia C1359	60.99	97.23
B. cepacia C1409	94.40	106.80
E. coli O18K	17.44	68.38
P. aeruginosa C1250	2.32	2.36

Results shown are the mean of duplicates.
LPS samples were used at a concentration of 1ng/ml.
Samples for the TNF-α assay were taken at 3.5h.

As expected, both *B. cepacia* and *E. coli* samples were more active than the *P. aeruginosa* sample. Unexpectedly however, *B. cepacia* PW-LPS samples gave a greater response compared to the *E. coli* PW-LPS sample in both the LAL and

TNF-α assays. Therefore, it appears that *B. cepacia* produces an LPS with extremely high inflammatory potential, which may be a major factor in *B. cepacia* pathogenesis.

The influence of growth environment on TNF-α induction

The PW-LPS samples from *B. cepacia* C1359 and C1409 grown in NB+YE, MA and MA+S were used to determine the influence of growth environment on TNF-α induction.

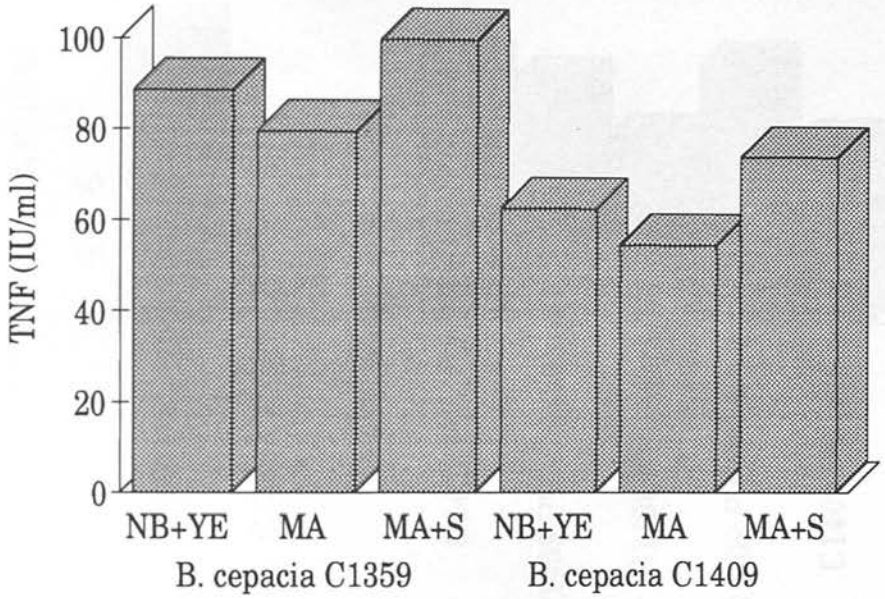


Figure 4.11 The influence of growth environment on TNF-α induction from MNL by PW-LPS samples. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 3.5h. Each value represents the mean of duplicates.

For both strains tested, PW-LPS preparations extracted from growth in MA+S were more stimulatory than samples extracted from NB+YE, which in turn produced more TNF-α than the MA preparations. However, although this was a trend observed in four different assays, there was no significant difference between growth conditions. Furthermore, these results do not correlate with the LAL assay, where LPS preparations from MA were the most endotoxic.

The influence of LPS extraction method on TNF-α induction.

Results in chapter three showed that the LPS extraction method affected LPS profile, antigenicity and chemical composition. To determine the influence of LPS extraction method on TNF-α induction, LPS samples extracted by the PCP method and preparations from both the aqueous and phenol layer of a PW extract of *B. cepacia* C1409 and C1359 were assayed (Figure 4.12).

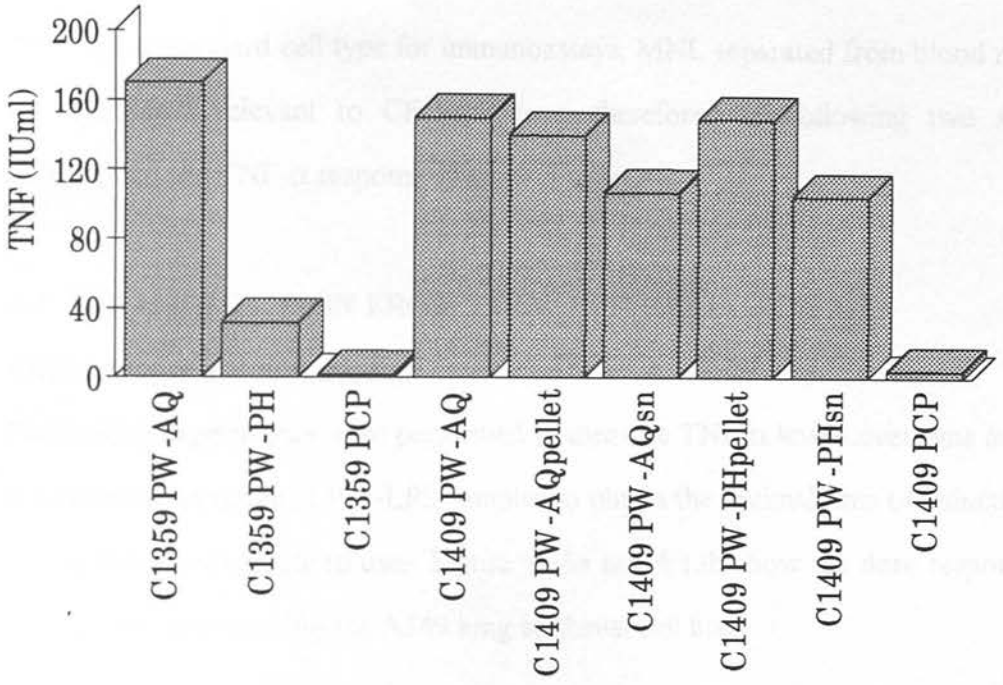


Figure 4.12 The influence of LPS extraction method on TNF-α induction. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 3.5h Results are the mean of four readings. PW = phenol water extract; AQ = aqueous layer; PH = phenol layer; PCP = phenol-chloroform-petroleum spirit extract; sn = supernate.

The aqueous layer and phenol layer extracts of *B. cepacia* C1409 showed no major difference in induction of TNF-α. In contrast, the biological activity of the phenol layer extract of C1359 was markedly reduced compared to the aqueous sample. Furthermore, the PCP samples of both *B. cepacia* strains tested, induced very little detectable TNF-α activity. Thus, it can be concluded that LPS extraction method not

only alters LPS structure but also biological activity, a factor which should be remembered when relating results to bacterial pathogenic potential. PW-LPS samples were used in all future LPS experiments due to the higher biological potential. In addition, the ultracentrifuged pure LPS pellet, supernate and the uncentrifuged preparation from C1409 did not differ significantly, showing that the uncentrifuged LPS samples used in these experiments are good representatives of the pure LPS.

Although a standard cell type for immunoassays, MNL separated from blood may not be considered relevant to CF pathogens; therefore, the following two sections investigated the TNF-α response from lung cells.

4.4 TNF-α INDUCTION FROM LUNG EPITHELIAL CELLS

Dose response and time course

Preliminary experiments were performed to measure TNF-α levels over time and with a concentration range of PW-LPS samples to obtain the optimal time of induction and concentration of sample to use. Figure 4.13a and 4.13b show the dose response and time course graphs using the A549 lung epithelial cell line.

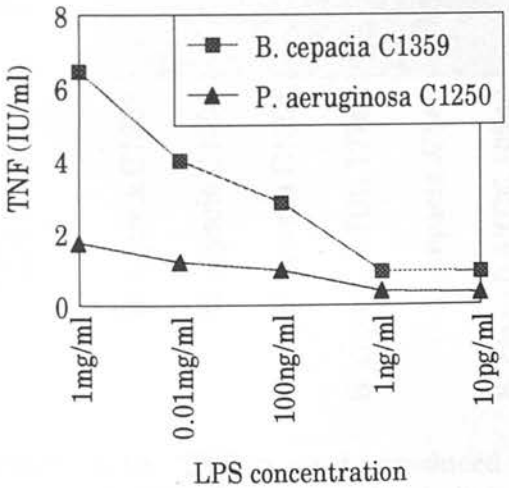


Figure 4.13a Dose response of PW-LPS samples from A549 lung epithelial cells. Results are the mean of duplicates. Supernate was collected at 4h.

From the concentration range tested, the optimum TNF-α response was obtained with the LPS sample of 1mg/ml. The peak time of TNF-α production from the A549 cells was approximately 4h (Figure 4.13b).

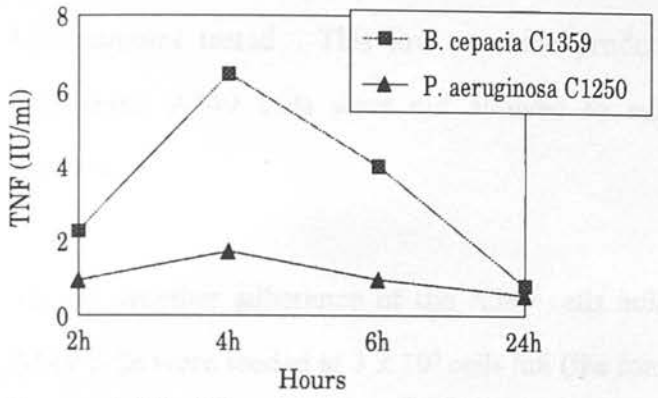


Figure 4.13b Time-course of TNF-α induction by PW-LPS preparations from A549 cells. LPS samples were used at a concentration of 1mg/ml. Results are the mean of duplicates.

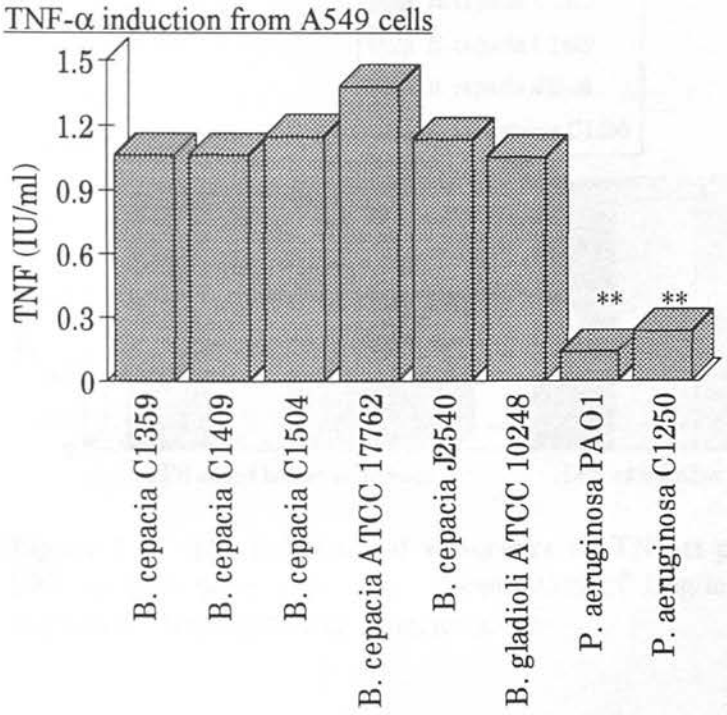


Figure 4.14 TNF-α levels produced from A549 cells in response to PW-LPS preparations of *B. cepacia, B. gladioli* and *P. aeruginosa*. LPS samples were used at a concentration of 1mg/ml. Supernate was collected at 4h. Results are the mean of duplicates. The levels of TNF-α induced by *B. cepacia* were significantly different from *P. aeruginosa* ($P<0.01$) which is highlighted by the ** symbol.

The *B. cepacia* PW-LPS samples induced a significantly higher level of TNF-α ($P<0.01$) compared to the *P. aeruginosa* samples, suggesting once more that *B. cepacia* plays a vital role in the immune-mediated damage observed in the CF lung. However, the A549 cells did not produce large quantities of TNF-α in response to the LPS samples tested. This low cytokine production may have been because the fibroblastic A549 cells were not allowed to adhere before addition of the LPS samples.

To test whether adherence of the A549 cells influenced production of TNF-α, the A549 cells were seeded at 3 x 10^5 cells /ml (the same as used for the fibroblastic L929 cells) and left for 24h to form a confluent monolayer. LPS samples were subsequently added and supernate collected at 4h (Figure 4.15).

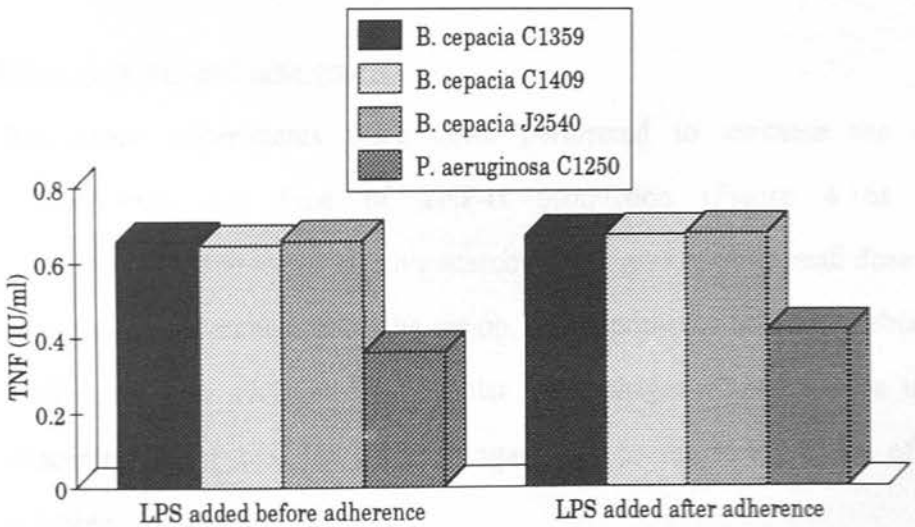


Figure 4.15 The influence of adherence on TNF-α production from A549 cells. LPS samples were used at a concentration of 1mg/ml. Results are the mean of duplicates. Supernate was collected at 4h.

No difference in TNF-α levels was observed between adhered and non-adhered A549 cells with very little TNF-α induced overall (Figure 4.14). Thus, although *B. cepacia* LPS samples stimulated more TNF-α with respect to *P. aeruginosa* LPS, the levels

induced are very small compared to the levels from MNL cells, implying that lung epithelial cells do not contribute substantially to the clinically relevant TNF-α levels observed in CF patients.

4.5 TNF-α INDUCTION FROM ALVEOLAR MACROPHAGES

Alveolar macrophages are the major inflammatory cells of the lung, releasing large quantities of immune mediators. Therefore, it was essential not only to investigate the cytokine response from these important lung cells but also to determine if the trend observed previously with blood-derived monocytes (Figure 4.10) was representative of alveolar macrophages. Samples of purified human alveolar macrophages, prepared from broncheoalveolar lavage fluid, were gifted by Dr. A. Greening and M. Imrie, Western General Hospital, Edinburgh.

Dose response and time course

Preliminary experiments were again performed to estimate the optimal LPS concentration and time of TNF-α production (Figure 4.16a and 4.16b). Unfortunately, due to the relative scarcity of samples, only a small dose response and time course experiment could be set up, based primarily on results obtained with the MNL cells. In addition, the alveolar macrophages could only be used at a cell concentration of 1 x 10^6 cells/ml, again due to the low number of cell samples available.

All three concentrations of PW-LPS sample tested, 1000ng/ml, 1ng/ml and 1pg/ml, produced a good TNF-α response with optimal OD values, thus an LPS concentration of 1ng/ml was chosen to conform with the samples used in the MNL assays.

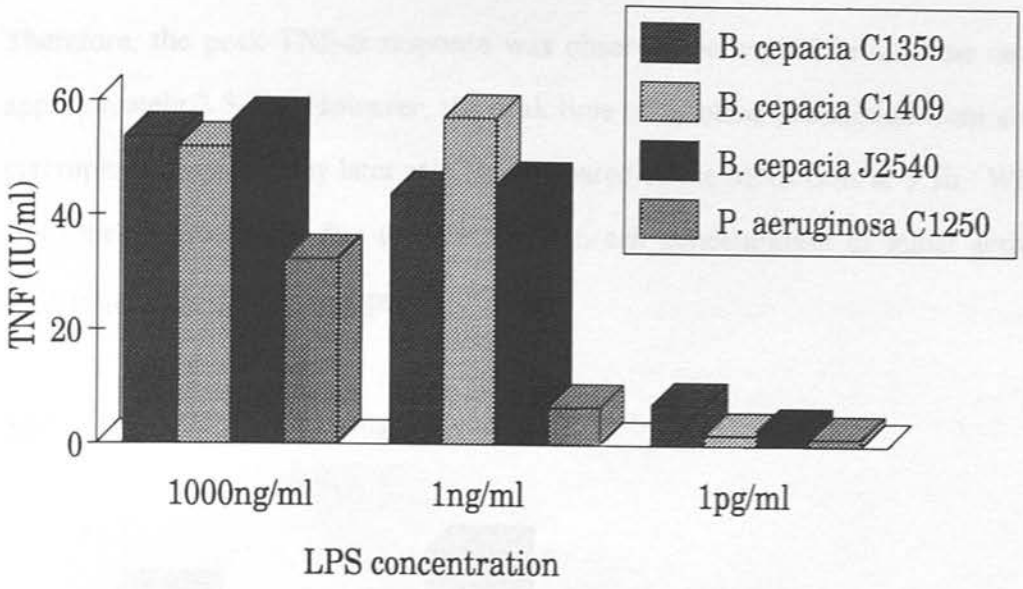


Figure 4.16a Dose response of PW-LPS samples from alveolar macrophages. Results are the mean of duplicates. Supernate was collected at 4h.

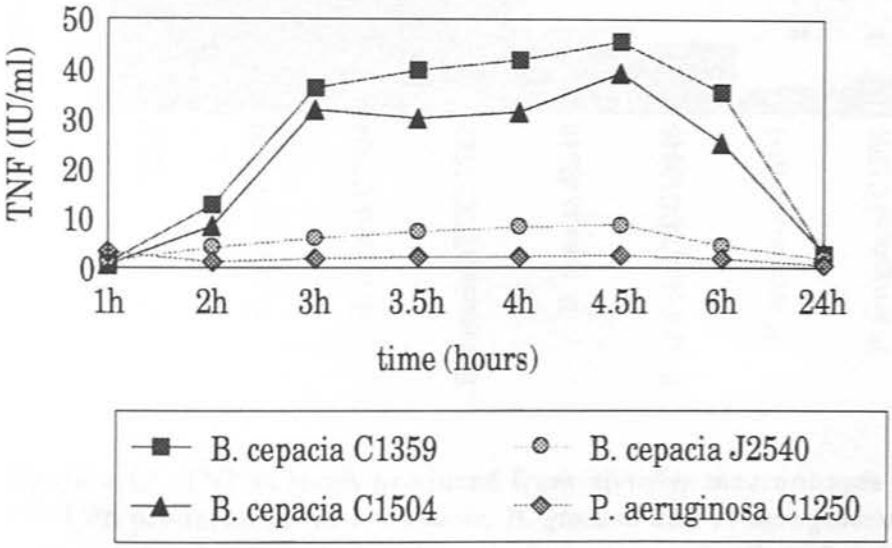


Figure 4.16b Time course of TNF-α induction from alveolar macrophages in response to PW-LPS samples. LPS samples were used at a concentration of 1ng/ml. Results are the mean of duplicates.

The time-course in Figure 4.16b shows the levels of TNF-α produced from alveolar macrophages over a 24h period. The TNF-α levels rise dramatically after 2h, reach a peak at 4.5h, before decreasing at 6h and reaching negligible levels after 24h.

Therefore, the peak TNF-α response was observed between a broad time range of approximately 2.5-5h. However, the peak time of cytokine production from alveolar macrophages was slightly later at 4.5h, compared to the MNL cells at 3.5h. Whether this time variation was due to differences in cell concentration or initial activation state requires further investigation.

TNF-α induction from alveolar macrophages

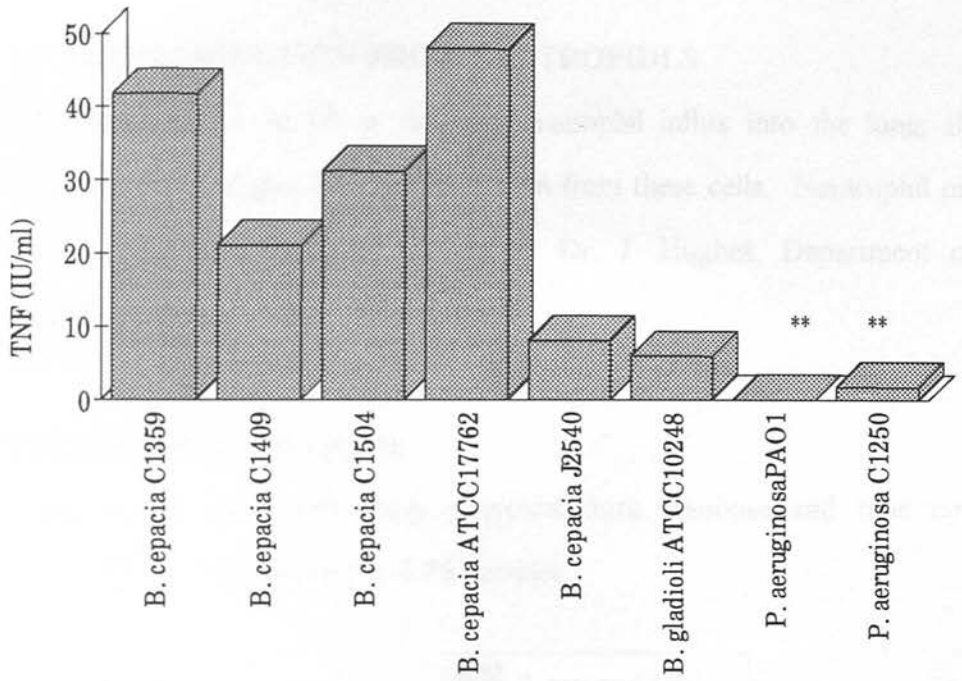


Figure 4.17 TNF-α levels produced from alveolar macrophages in response to PW-LPS preparations of *B. cepacia, B. gladioli* and *P. aeruginosa.* LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 4.5h Results are the mean of duplicates. The levels of TNF-α induced by *B. cepacia* was significantly different from *P. aeruginosa* ($P<0.01$) which is highlighted by the ** symbol.

Figure 4.17 shows TNF-α production from alveolar macrophages in response to PW-LPS preparations at a concentration of 1ng/ml and from supernate collected at 4.5h. Most importantly, the general trend in TNF-α production from alveolar macrophages was similar to that observed with MNL. The levels of TNF-α produced from

B. cepacia PW-LPS preparations were at least five times greater than either of the two *P. aeruginosa* samples and as a population *B. cepacia* produced a significantly different TNF-α response compared to *P. aeruginosa* ($P<0.01$). Similarly, there was a difference in TNF-α levels between *B. cepacia* clinical isolates and the environmental strain J2540 and *B. gladioli.* Therefore, from the similarity in responses it can be concluded that MNL cells act as a suitable model for alveolar macrophages.

4.6 TNF-α INDUCTION FROM NEUTROPHILS

One characteristic of CF is the large neutophil influx into the lung, thus it was essential to investigate TNF-α production from these cells. Neutrophil preparations were separated from blood donors by Dr. J. Hughes, Department of Medical Microbiology, University of Edinburgh.

Dose response and time course

Figure 4.18a and 4.18b show a typical dose response and time course from neutrophils in response to PW-LPS samples.

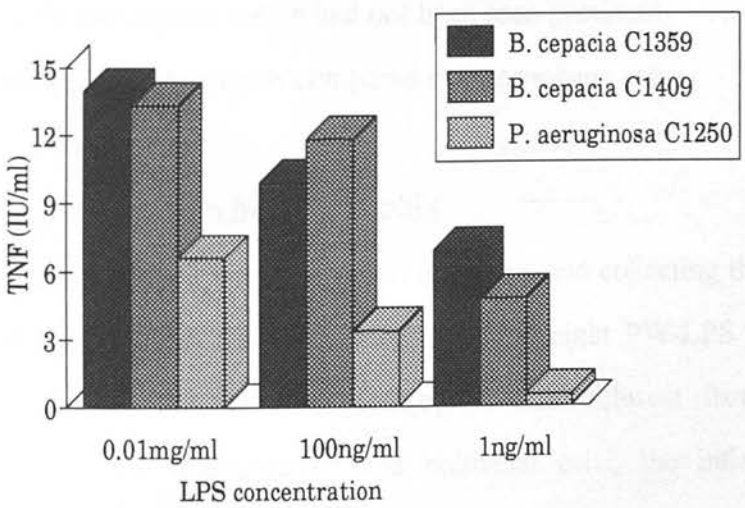


Figure 4.18a Dose response of neutrophils in response to PW-LPS preparations. Supernate was collected at 3.5h. Results are the mean of duplicates.

Two *B. cepacia* strains and one *P. aeruginosa* strain were used for the dose response measurements, with three concentrations of LPS used: 0.01mg/ml, 100ng/ml and 1ng/ml. All three PW-LPS concentrations tested induced detectable TNF-α which corresponded with optimal OD values, thus, to conform with previous experiments it was decided to use a PW-LPS concentration of 1ng/ml.

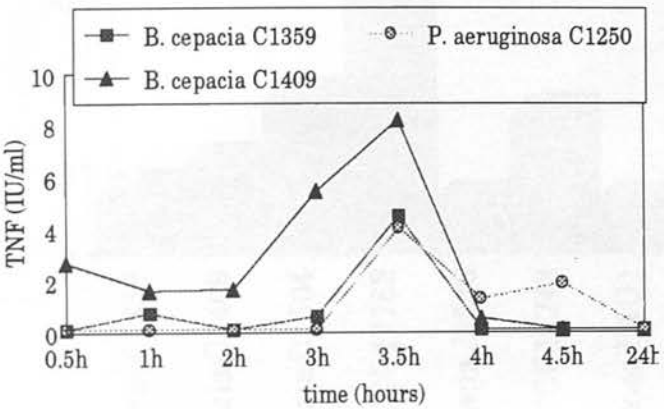


Figure 4.18b Time course of TNF-α induction from neutrophils in response to PW-LPS preparations. Results are the mean of duplicates.

The peak of TNF-α production by the neutrophil cells was observed at 3.5h, similar to other cell types investigated. However, there was a rapid decline in TNF-α levels at 4h and beyond which had not been seen previously. This may be due to the shorter lifespan of neutrophils compared to macrophage cells.

TNF-α induction from neutrophils

Using a PW-LPS concentration of 1ng/ml and collecting the supernate at 3.5h, Figure 4.19 shows the TNF-α induced from all eight PW-LPS preparations. The TNF-α response observed from neutrophils was different from the reaction seen with monocytes, macrophages and epithelial cells; the inflammatory capacity of the *B. cepacia* samples did not differ significantly from the *P. aeruginosa* PW-LPS preparations. Similarly, there was no distinct variation between *B. cepacia* clinical

and environmental strains. However, the *B. cepacia* extracts still induced an equal, if not greater, amount of TNF-α compared to the *P. aeruginosa* samples, reiterating the importance and huge inflammatory potential of *B. cepacia*.

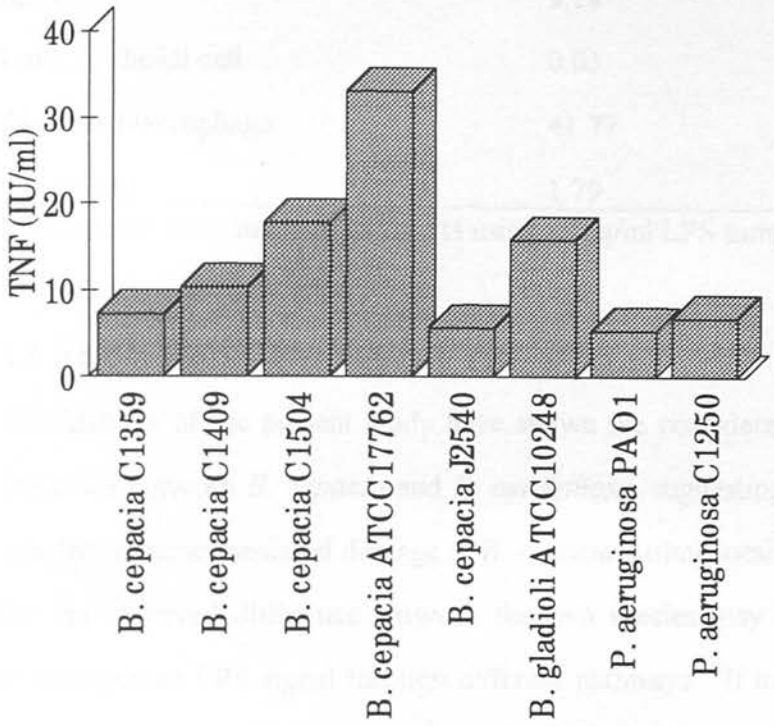


Figure 4.19 TNF-α levels produced from neutrophils in response to PW-LPS preparations. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 3.5h. Results are the mean of duplicates.

4.7 TNF-α INDUCTION FROM DIFFERENT CELL TYPES

Table 4.2 shows the levels of TNF-α induced, per cell, from the different cell types tested in this study in response to *B. cepacia* C1359 LPS.

Results from Table 4.2 show that alveolar macrophages produce the highest level of TNF-α per cell. This observation supports the major role of alveolar macrophages in contributing to the immune-mediated damage observed in the CF lung.

Table 4.2 Levels of TNF-α produced per cell in response to *B. cepacia* C1359 LPS.

Cell type	TNF-α (IU/ml x10^{-6})
MNL	9.18
Lung epithelial cell	0.03
Alveolar macrophage	41.77
Neutrophil	1.79

Calculations were made from results using a 1ng/ml LPS sample.

4.8 TNF-α INDUCTION FROM THP-1 CELLS

The findings of the present study have shown the considerable difference in TNF-α induction between *B. cepacia* and *P. aeruginosa*, suggesting a potentially important role for immune-mediated damage in *B. cepacia* pathogenesis. However, one reason for the observed difference between the two species may be that *B. cepacia* and *P. aeruginosa* LPS signal through different pathways. If indeed this were the case then one pathway may be more or less efficient and hence produce more or less TNF-α. To test this theory a monocyte/macrophage cell line ThP-1 was used, which can be enhanced for the expression of CD14, the best characterised LPS receptor. It was postulated that if CD14 was required for *B. cepacia* or *P. aeruginosa* LPS signalling then levels of TNF-α should be increased when CD14 expression is enhanced, as has been observed for *E. coli* (Delahooke *et al*, 1995a).

Dose response and time course

Figure 4.20a and 4.20b show the dose response and time course for the ThP-1 cell line in response to PW-LPS samples.

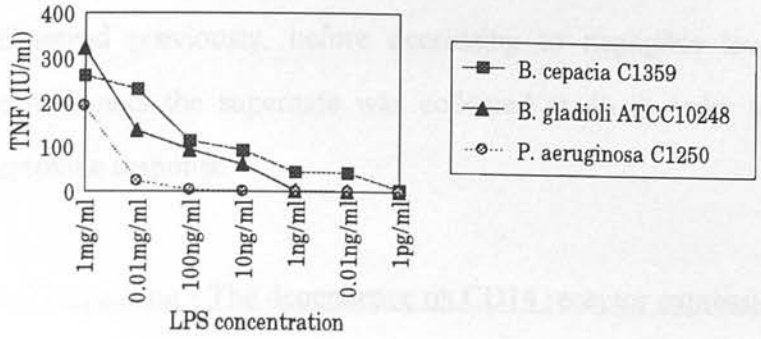


Figure 4.20a Dose response of ThP-1 cells in response to PW-LPS extracts. Supernate was collected at 4h. Results are the mean of duplicates.

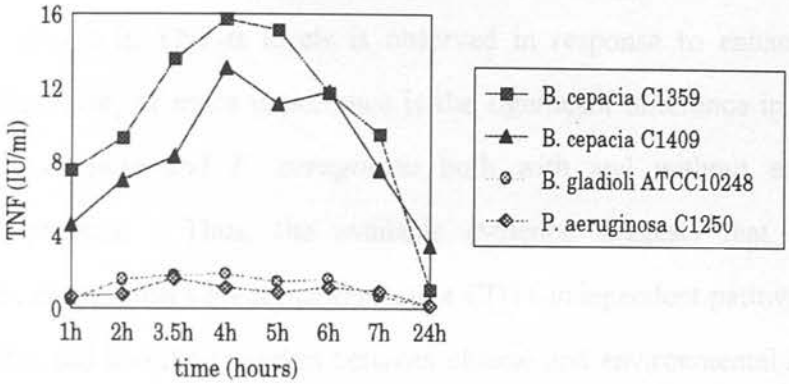


Figure 4.20b Time course of TNF-α induction from ThP-1 cells by PW-LPS samples. LPS samples were used at a concentration of 1ng/ml. Results are the mean of duplicates.

The levels of TNF-α induced from ThP-1 cells decreased in a dose-dependent manner as expected. TNF-α levels induced by LPS at a concentration of 1mg/ml and 0.01mg/ml were calculable, but outwith the linear range of the TNF standard curve. Thus, as with the MNL, alveolar macrophages and neutrophil cells, an LPS concentration of 1ng/ml was chosen as the optimum concentration to produce OD values corresponding to the linear section of the standard curve and providing the most accurate measurements of TNF-α. The time course experiment using ThP-1 cells, shows a familiar trend which correlates with the response observed with the other monocytes and macrophage cells tested. Increasing TNF-α levels were detected in a broad time frame between approximately 2h and 7h, longer than

observed previously, before decreasing to negligible levels at 24h. For future experiments the supernate was collected at 4h in order to measure the maximum cytokine response.

LPS signalling - The dependence on CD14 receptor expression

Figure 4.21 shows TNF-α levels induced from ThP-1 cells both with and without enhancement for CD14 in response to PW-LPS extracts from *B. cepacia, B. gladioli* and *P. aeruginosa*. Results show that for some PW-LPS samples a non-significant increase in TNF-α levels is observed in response to enhanced CD14 expression. However, of more importance is the significant difference in TNF-α levels between *B. cepacia* and *P. aeruginosa* both with and without enhancement for CD14 expression. Thus, the available evidence suggests that both *B. cepacia* and *P. aeruginosa* LPS signal through a CD14-independent pathway. Interestingly, using this cell line the variation between clinical and environmental strains of *B. cepacia* is not observed. Furthermore, *B. gladioli* gives a comparable reaction to the *P. aeruginosa* samples.

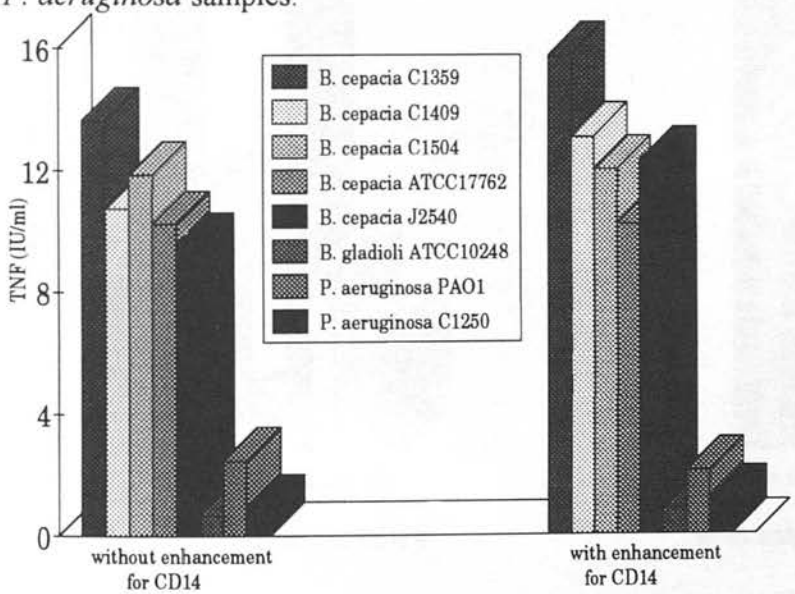


Figure 4.21 TNF-α stimulation from ThP-1 cells with and without enhancement for CD14 in response to PW-LPS samples from *B. cepacia, B. gladioli* and *P. aeruginosa*. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 4h. Results are the mean of four readings.

To confirm the redundancy of the CD14 receptor in *B. cepacia* and *P. aeruginosa* LPS signalling, a further experiment was performed. A CD14 MAb was incubated with ThP-1 cells 30min prior to addition of the PW-LPS extracts. It was speculated that if *B. cepacia* or *P. aeruginosa* LPS operated through a CD14-dependent pathway then the MAb would block LPS binding and TNF-α levels should fall.

No significant decrease in TNF-α levels occurred on addition of an anti-CD14 MAb. Some PW-LPS samples do show a fall in TNF-α induction levels, although in contrast, other samples show a rise in TNF-α, reiterating the redundancy of CD14 in *B. cepacia* and *P. aeruginosa* LPS signalling. Furthermore, there is no correlation between rising or falling TNF-α levels and the enhanced expression of CD14.

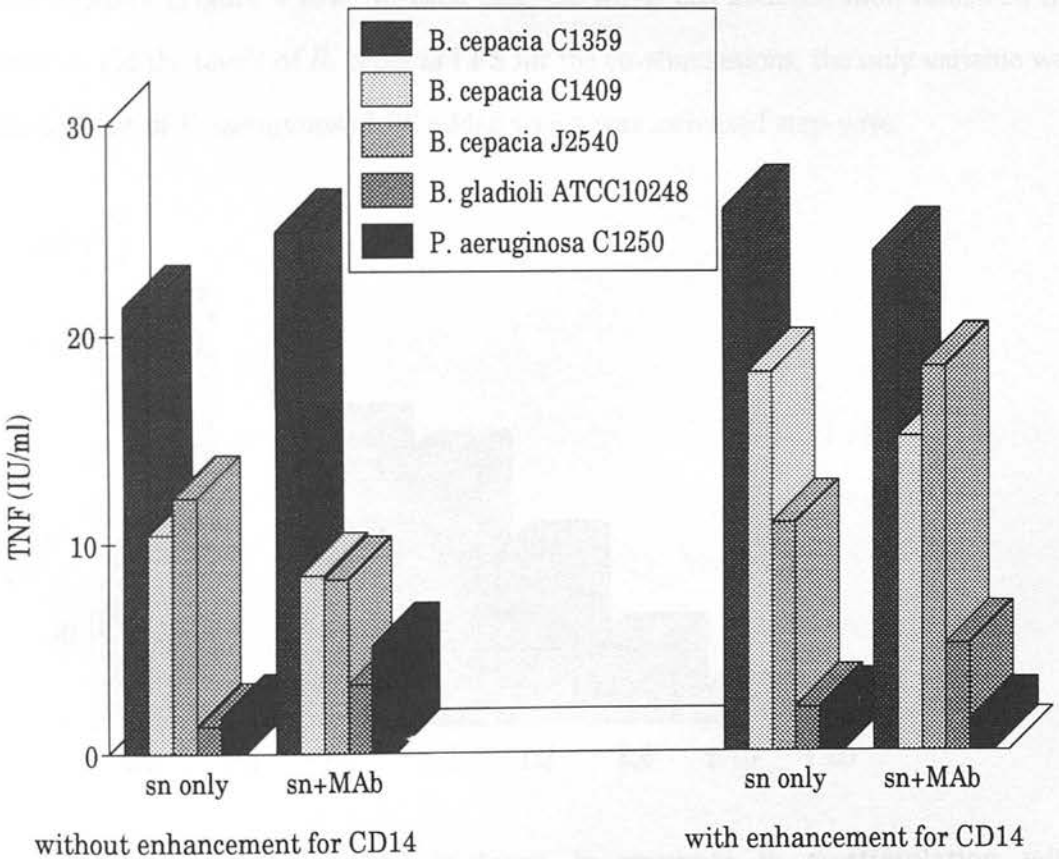


Figure 4.22 The influence of an anti-CD14 MAb on TNF-α induction from ThP-1 cells with and without enhancement for CD14. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 4h. Results are the mean of duplicates.

4.9 THE INFLUENCE OF CO-STIMULATION BY *B. CEPACIA* AND *P. AERUGINOSA* LPS ON TNF-α INDUCTION.

Evidence from the ThP-1 experiments suggests that both *B. cepacia* and *P. aeruginosa* initiate LPS signalling through a pathway independent of CD14. This poses the question that if both LPS types signal through the same pathway, what would happen when both LPSs are presented together, as is often the case in the CF lung? The following experiments were set up to try to answer this question and to investigate whether there was a synergistic or competitive effect on co-stimulation.

B. cepacia C1409 and *P. aeruginosa* C1250 were used to stimulate MNL both alone and together (Figure 4.23). In each case the MNL cell concentration remained the same as did the levels of *B. cepacia* LPS for the co-stimulations, the only variable was the amount of *P. aeruginosa* LPS added which was increased step-wise.

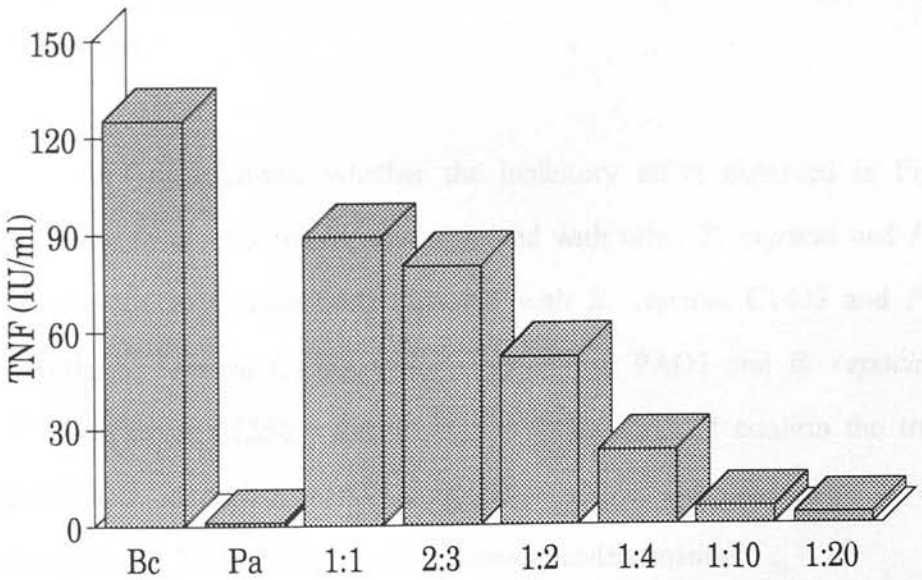


Figure 4.23 TNF-α levels produced in response to co-stimulation with *B. cepacia* C1409 and *P. aeruginosa* C1250 PW-LPS together. The numbers indicate the ratio of *B. cepacia* to *P. aeruginosa* LPS. Supernate was collected at 3.5h. Results are the mean of duplicates.

The results of the co-stimulation assay gave several interesting findings. Firstly, as expected, the levels of TNF-α induced in response to *B. cepacia* LPS alone were markedly higher compared to *P. aeruginosa* LPS alone. Secondly, when equal amounts of *B. cepacia* and *P. aeruginosa* LPS were added together the TNF-α levels decrease. Thirdly, as *P. aeruginosa* LPS becomes more in excess of *B. cepacia*, so the levels of TNF-α drop in a dose-dependent manner. These results are extremely important as it implies colonisation by *B. cepacia* alone may cause more immune-mediated damage than co-colonisation with both organisms. Fourthly, even when *P. aeruginosa* LPS is in a twenty-fold excess of *B. cepacia* LPS the levels of TNF-α are still greater than *P. aeruginosa* LPS alone. This latter finding re-emphasises the importance of *B. cepacia* as an opportunistic pathogen in its own right and highlights the major inflammatory potential of this organism in CF pathogenesis. Therefore, this study suggests that both organisms are competing for the same LPS receptors and surprisingly, that *P. aeruginosa* can modulate the inflammatory response of *B. cepacia.*

In order to determine whether the inhibitory effect observed in Figure 4.23 in response to co-stimulation was observed with other *B. cepacia* and *P. aeruginosa* strains, the experiment was repeated with *B. cepacia* C1409 and *P. aeruginosa* PAO1, *B. cepacia* C1359 and *P. aeruginosa* PAO1 and *B. cepacia* C1359 and *P. aeruginosa* C1250. Results shown in Figure 4.24 confirm the trend observed previously, namely excess *P. aeruginosa* LPS in the presence of *B. cepacia* LPS, dampens the TNF-α response in a dose-dependent manner.

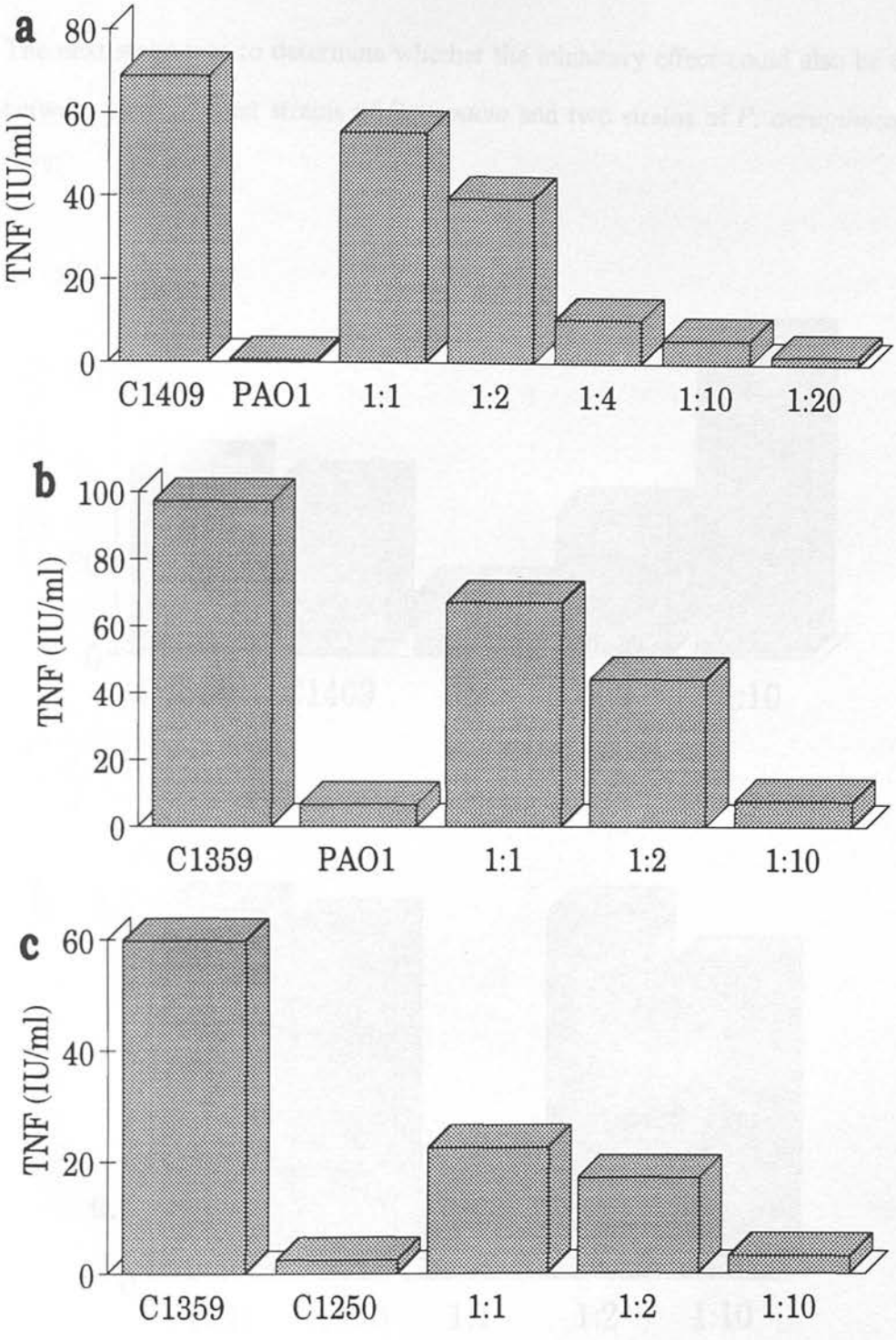


Figure 4.24 TNF-α levels produced in response to co-stimulation with *B. cepacia* and *P. aeruginosa* PW-LPS together. The numbers indicate the ratio of *B. cepacia* to *P. aeruginosa* LPS. Supernate was collected at 3.5h. Results shown are the mean of duplicates. **(a)** Co-stimulation with *B. cepacia* C1409 and *P. aeruginosa* PAO1; **(b)** Co-stimulation with *B. cepacia* C1359 and *P. aeruginosa* PAO1 and; **(c)** Co-stimulation with *B. cepacia* C1359 and *P. aeruginosa* C1250. *P. aeruginosa* LPS was in excess in each experiment.

The next stage was to determine whether the inhibitory effect could also be observed between two different strains of *B. cepacia* and two strains of *P. aeruginosa* (Figure 4.25).

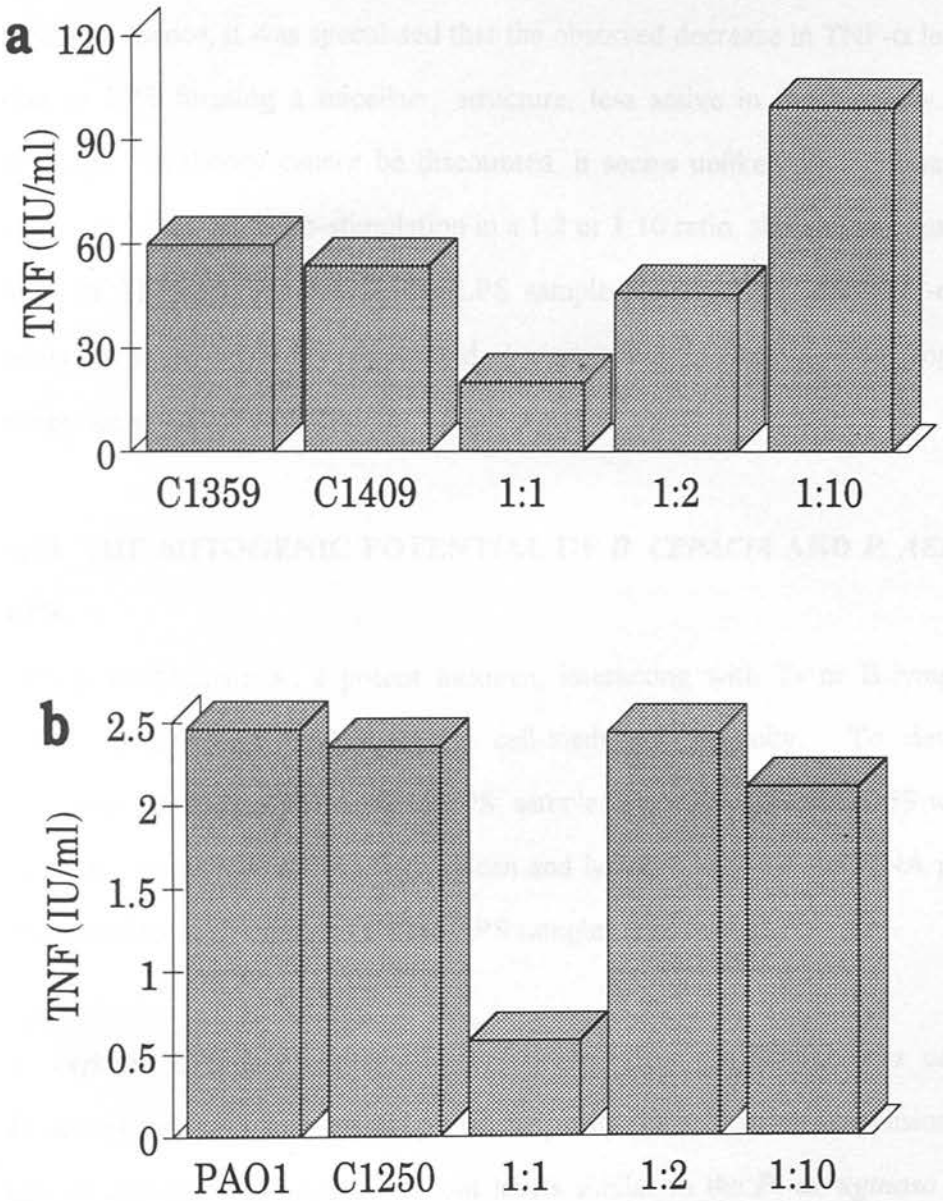


Figure 4.25 TNF-α levels produced in response to co-stimulation with two *B. cepacia* PW-LPS samples or two *P. aeruginosa* PW-LPS samples. The numbers indicate the ratio of *B. cepacia* to *P. aeruginosa* LPS. Supernate was collected at 3.5h. Results shown are the mean of duplicates. **(a)** Co-stimulation with *B. cepacia* C1359 and C1409; **(b)** Co-stimulation with *P. aeruginosa* PAO1 and C1250. C1409 and C1250 were randomly chosen as the LPS samples to be in excess.

The results of co-stimulation by two *B. cepacia* strains or two *P. aeruginosa* strains differed from the results observed upon co-stimulation with both species together. As observed previously, TNF-α levels drop when equal amounts of the two LPS samples were added together. The biological activity of LPS is thought to be dependent on its solubility, hence, it was speculated that the observed decrease in TNF-α levels may be due to LPS forming a micellar structure, less active in the bioassay. However although this theory cannot be discounted, it seems unlikely as the assays using an excess of LPS, that is co-stimulation in a 1:2 or 1:10 ratio, showed an equal or greater level of TNF-α compared to the LPS sample alone. Thus, the TNF-α reduction observed when both *B. cepacia* and *P. aeruginosa* LPS were added together must reflect competitive inhibition for LPS receptors.

4.10 THE MITOGENIC POTENTIAL OF *B. CEPACIA* AND *P. AERUGINOSA* LPS.

LPS is well-known as a potent mitogen, interacting with T- or B-lymphocytes to initiate proliferation and stimulate cell-mediated immunity. To determine the mitogenic potential of *B. cepacia* LPS, samples from *B. cepacia* C1359 were used to stimulate lymphocytes from both spleen and lymph nodes and the DNA proliferation compared to *P. aeruginosa* C1250 LPS samples (Figure 4.26).

B. cepacia LPS has a greater proliferative effect on lymphocytes compared to *P. aeruginosa* LPS, emphasised particularly with the spleen cell suspension. PHA is a known mitogen and produced count levels similar to the *P. aeruginosa* sample and also the *B. cepacia* sample acting on the lymph node suspension. Both organisms produced greater levels than the control as expected. The difference in count results between the spleen and lymph node samples may be due to the greater number of lymphocytes obtained from the spleen suspension. These results indicate that *B. cepacia* may stimulate a greater cell-mediated response than *P. aeruginosa*.

However, further work is required to investigate if the proliferative response was mainly T-or B- lymphocyte-dependent, as that finding would have implications for antibody production.

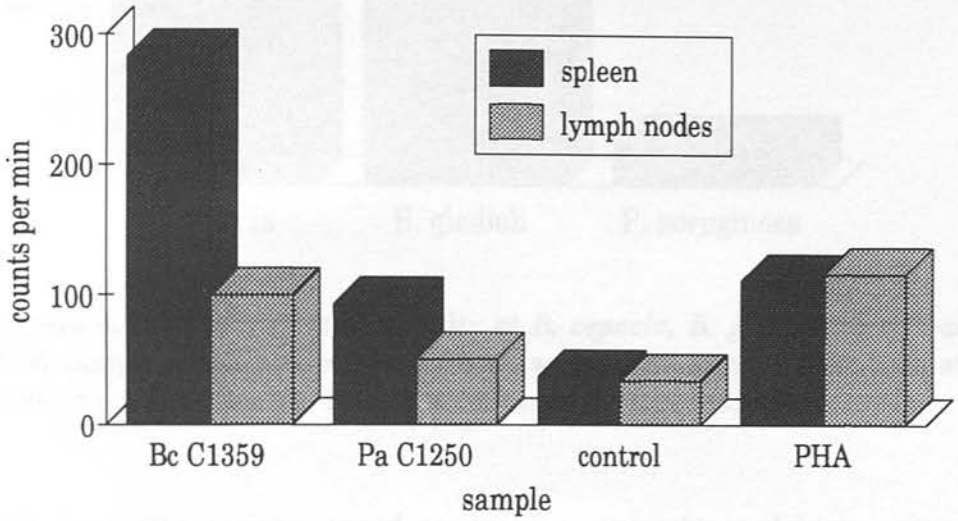


Figure 4.26 The mitogenic potential of *B. cepacia* and *P. aeruginosa* PW-LPS samples on lymphocytes from spleen and lymph nodes. PW-LPS samples were used at a concentration of 25µg/ml, results are the mean of triplicates.
control = no stimulant; PHA = phytohaemagglutanin

4.11 THE BIOLOGICAL ACTIVITY OF OM SAMPLES

The next section assessed the biological activity of *B. cepacia*, *B. gladioli* and *P. aeruginosa* OM samples, as in the host LPS would be unlikely to be found pure but rather complexed to protein or other components of the bacterial cell surface. Thus, these experiments aimed to determine the influence of the outer membrane on LPS endotoxicity and inflammatory potential.

4.11.1 THE ENDOTOXICITY OF OM SAMPLES

To confirm the presence of LPS in the OM samples, the OM preparations were tested in the LAL assay (Figure 4.27).

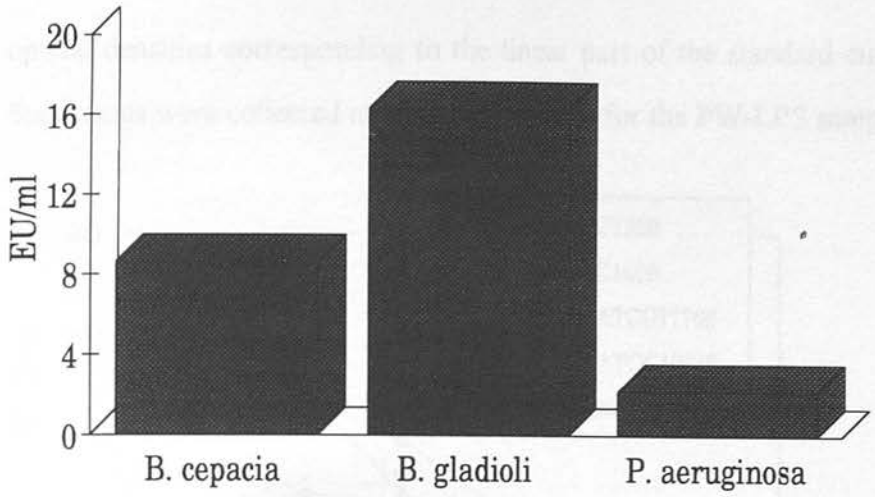


Figure 4.27 The endotoxic activity of *B. cepacia, B. gladioli* and *P. aeruginosa* OM samples as measured by the LAL assay. OM samples were used at a protein concentration of 1ng/ml. Results are the mean of four readings.

All three OM samples tested produced a detectable endotoxic response. The *B. gladioli* OM sample exhibited the greatest endotoxicity followed by *B. cepacia* C1359 and *P. aeruginosa* C1250, similar to the observation made with the PW-LPS samples (Figure 4.2).

4.11.2 THE INFLAMMATORY POTENTIAL OF OM SAMPLES.

The OM samples were tested in the TNF-α assay to determine their inflammatory potential. OM preparations of *B. cepacia*, *B. gladioli* and *P. aeruginosa* were diluted in pyrogen-free water to provide a protein concentration range from 1.6μg/ml-0.16pg/ml and were used to induce TNF-α from MNL cells (Figure 4.28).

Surprisingly, the TNF-α levels did not follow a dose-dependent response. The most concentrated OM sample at 1.6μg/ml, produced lower levels of TNF-α compared to less concentrated OM preparations. However, this may be partly explained by the high viscosity of the OM samples at this concentration, particularly those of *B. gladioli* and *P. aeruginosa*. Thus, the optimal OM protein concentration to obtain

optical densities corresponding to the linear part of the standard curve was 16ng/ml. Supernates were collected at 3.5h, the same as for the PW-LPS samples.

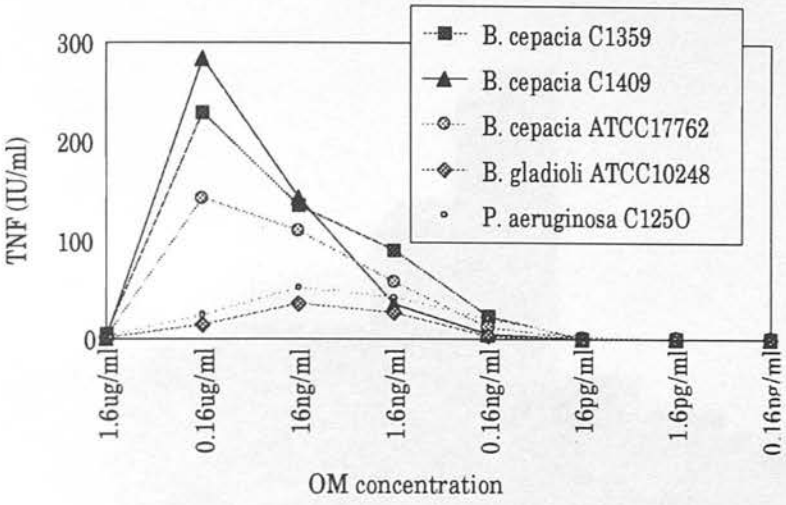


Figure 4.28 Dose response of OM samples from MNL cells. The OM concentration corresponds to the concentration of protein present in the sample. Supernate was collected at 3.5h. Results are the mean of duplicates.

Figure 4.29 shows the TNF-α levels produced from MNL in response to OM samples prepared from the same eight bacterial strains used to obtain the PW-LPS samples. The trend in TNF-α induction observed in response to the OM samples was different from the response observed by the PW-LPS samples. There were two major variations. Firstly, although all the *B. cepacia* OM samples induced more TNF-α than the *P. aeruginosa* preparations, there was no significant difference between the two bacterial populations. Thus, it may be concluded that for the outer membrane there is a comparable TNF-α response between *B. cepacia* and *P. aeruginosa.* Secondly, the OM sample inducing the greatest response was J2540, the environmental strain. Using PW-LPS samples, this strain induced less TNF-α compared to the clinical isolates. This finding suggests that all *B. cepacia* strains regardless of origin exhibit a degree of inflammatory potential. However, it is unclear whether these variations between LPS and OM results are due to protein present in the OM samples, the

influence of the outer membrane on the presentation of LPS or whether the varying levels of LPS within the OM samples are solely responsible.

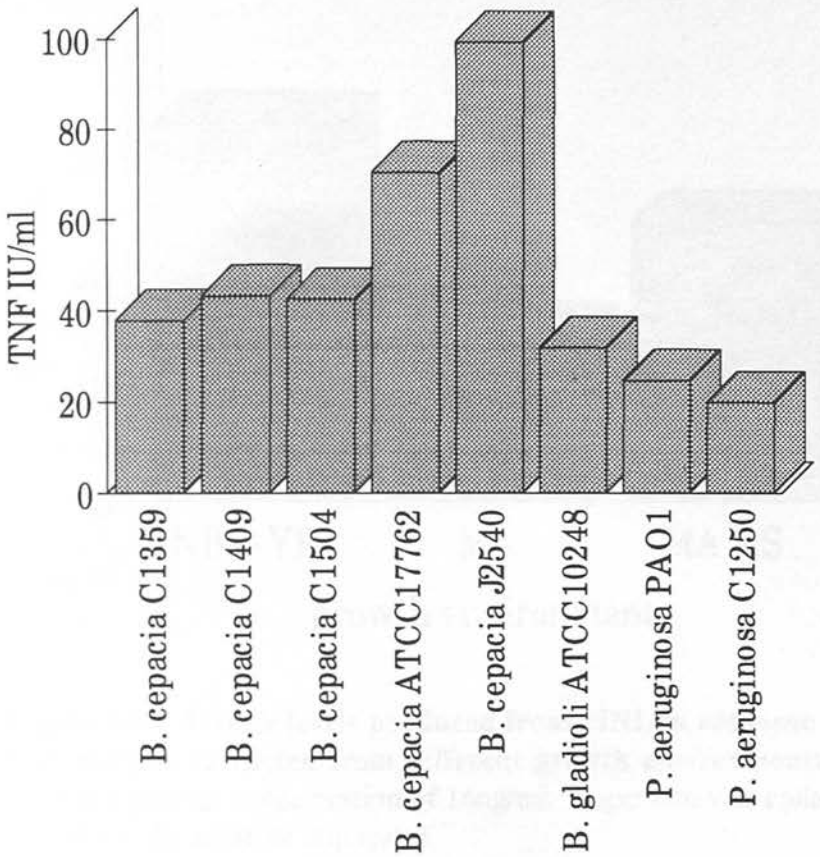


Figure 4.29 TNF-α levels produced from MNL in response to *B. cepacia*, *B. gladioli* and *P. aeruginosa* OM samples. OM samples were used at a protein concentration of 16ng/ml. Supernate was collected at 3.5h. Results are the mean of duplicates.

The influence of growth environment on TNF-α induction by OM samples

OM preparations from *B. cepacia* C1409 grown in NB+YE, MA and MA+S were used to assess the influence of growth environment on the stimulation of TNF-α. As seen in Figure 4.30, growth environment affects the induction of TNF-α in response to stimulation by OM extracts. The OM preparation extracted from NB+YE

produced the greatest amount of TNF-α followed by MA+S and finally MA. Although these results are from only one strain they do correspond to the trend observed with the PW-LPS samples.

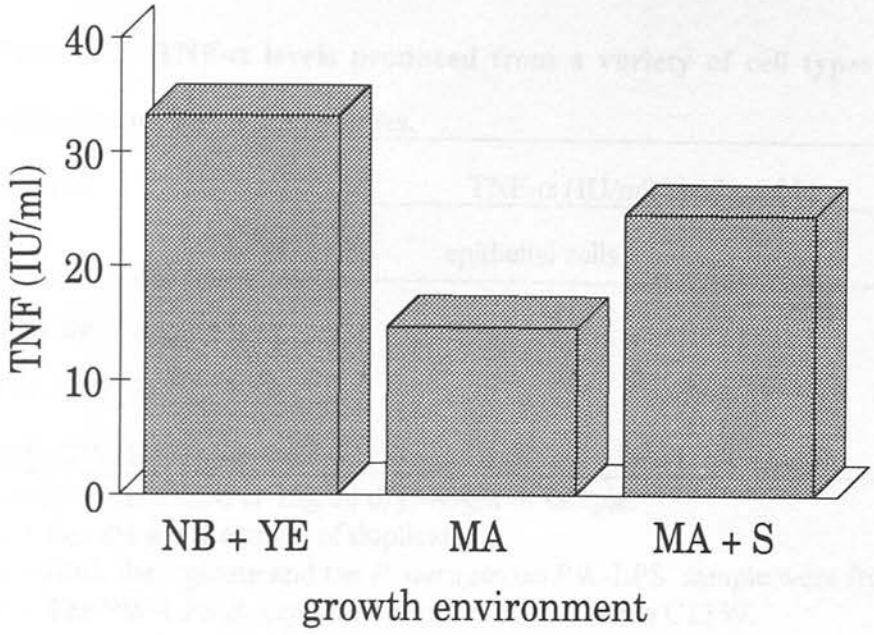


Figure 4.30 TNF-α levels produced from MNL in response to *B. cepacia* C1409 OM samples extracted from different growth environments. OM samples were used at a protein concentration of 16ng/ml. Supernate was collected at 3.5h. Results shown are the mean of duplicates.

4.12 THE BIOLOGICAL ACTIVITY OF EPS SAMPLES

Previous reports investigating the inflammatory potential of EPS found a high biological activity for alginate (Ottlerlei *et al*, 1993). Therefore, although the LPS from the mucoid *P. aeruginosa* strain C1250 has been shown to be of low activity, it was thought prudent to investigate the inflammatory potential of its EPS, as this may partly account for the discrepancy observed between the two species.

Alginate samples at 1ng/ml dry weight were initially tested in the LAL assay for the presence of LPS. No endotoxicity was detected and indeed the onset time for the

start of the LAL reaction was beyond the standard range, confirming both the absence of LPS and the purity of the alginate sample. Alginate samples were also used to stimulate TNF-α from a variety of cells (Table 4.3).

Table 4.3 TNF-α levels produced from a variety of cell types in response to alginate and PW-LPS samples.

Sample	TNF-α (IU/ml) produced by			
	MNL	epithelial cells	alveolar macrophages	neutrophils
alginate	1.65[a]	1.621	3.07	1.96
PW-LPS (Pa)[b]	1.18	2.34	1.81	6.82
PW-LPS (Bc)[c]	49.04	9.02	41.77	10.27

samples were used at 1ng/ml dry weight of sample.
a = Results are the mean of duplicates.
b = Both the alginate and the *P. aeruginosa* PW-LPS sample were from strain C1250
c = The PW-LPS *B. cepacia* sample was from strain C1359.

As shown in table 4.3, alginate produced a detectable TNF-α response but only at levels comparable to the PW-LPS extracts. Indeed, even if both the alginate and *P. aeruginosa* PW-LPS response were taken together, the levels of TNF-α induced would still be below the levels observed for *B. cepacia.* Thus, the presence of alginate does not appear to increase substantially the biological activity of the *P. aeruginosa* strain tested in this study.

4.13 IL-8 INDUCTION BY PW-LPS SAMPLES

Interleukin-8 (IL-8) levels are substantially increased in CF patients and thought to be largely responsible for the observed neutrophil influx. Assays were performed to determine the induction of IL-8 by PW-LPS samples. The stimulation was performed by myself although the assay to measure IL-8 concentration was performed by J. McColm, Department of Child Life and Health, Edinburgh.

Using the PW-LPS samples from *B. cepacia* C1359 and *P. aeruginosa* C1250 at the same concentration used for the TNF-α assays, 1ng/ml, a time course was initially performed to obtain the peak time of IL-8 production from MNL (Figure 4.31).

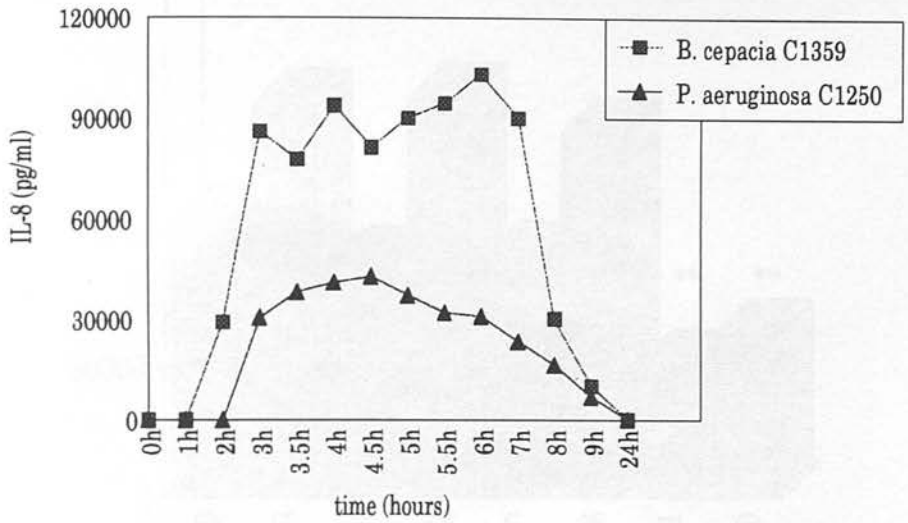


Figure 4.31 Time course of IL-8 induction from MNL in response to PW-LPS preparations. LPS samples were used at a concentration of 1ng/ml. Results are the mean of duplicates.

A broad peak of IL-8 production was observed between approximately 3h-7h, longer lasting than for the TNF-α response. Although any sampling would be satisfactory in that broad time frame an optimal sampling time of 5h was chosen for future experiments.

Induction of IL-8 from MNL in response to PW-LPS samples

Figure 4.32 shows IL-8 stimulation from MNL in response to PW-LPS preparations of *B. cepacia, B. gladioli* and *P. aeruginosa*. Similar to the findings of the TNF-α assays, *B. cepacia* strains induce more IL-8 compared to the *P. aeruginosa* strains. If *B. cepacia* and *P. aeruginosa* are treated as two distinct populations, there is a significant difference in IL-8 levels ($P < 0.05$). However, the statistical analysis must be treated with some caution due to the smaller number of IL-8 assays performed

Further experiments examining IL-8 production from more PW-LPS samples would be required before any satisfactory conclusion could be reached.

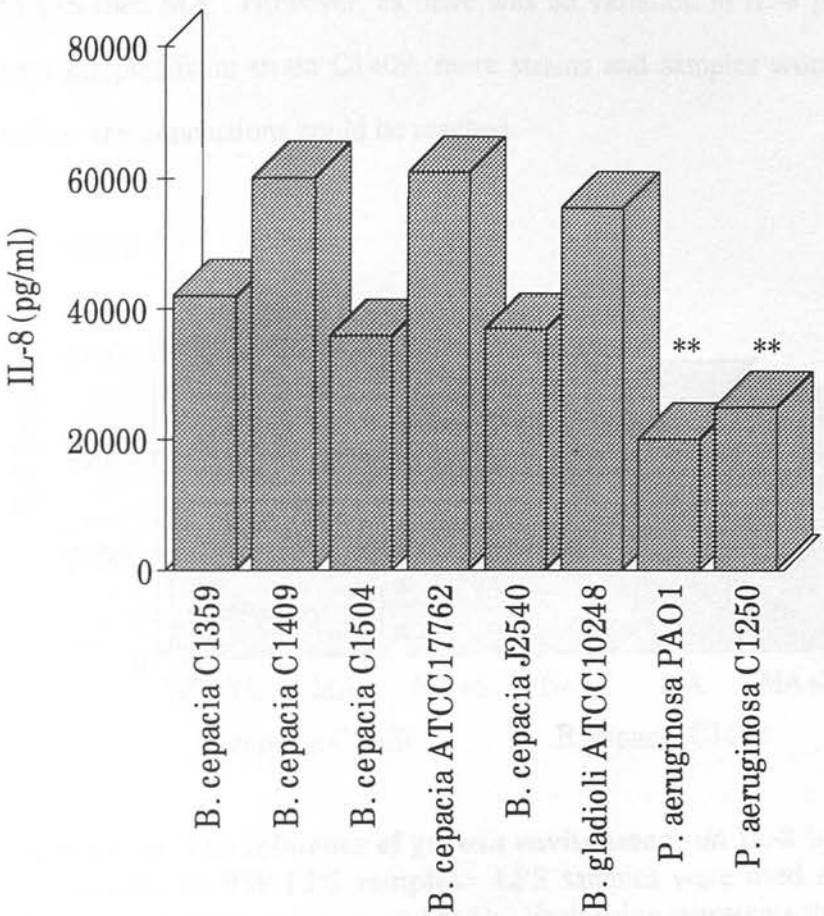


Figure 4.32 IL-8 levels produced from MNL in response to *B. cepacia*, *B. gladioli* and *P. aeruginosa* PW-LPS extracts. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 5h. Results shown are the mean of duplicates. The levels of IL-8 induced by *B. cepacia* was significantly different from *P. aeruginosa* ($P<0.05$) which is highlighted by the ** symbol

The influence of growth environment on IL-8 production

PW-LPS samples of *B. cepacia* C1359 and C1409 extracted from growth in NB+YE, MA and MA+S, were used to investigate the influence of growth environment on IL-8 production (Figure 4.33).

For *B. cepacia* C1359 the influence of culture condition on IL-8 production follows the same pattern as for TNF-α, namely NB+YE produces most IL-8, followed by MA+S then MA. However, as there was no variation in IL-8 production with PW-LPS samples from strain C1409, more strains and samples would have to be tested before any conclusions could be reached.

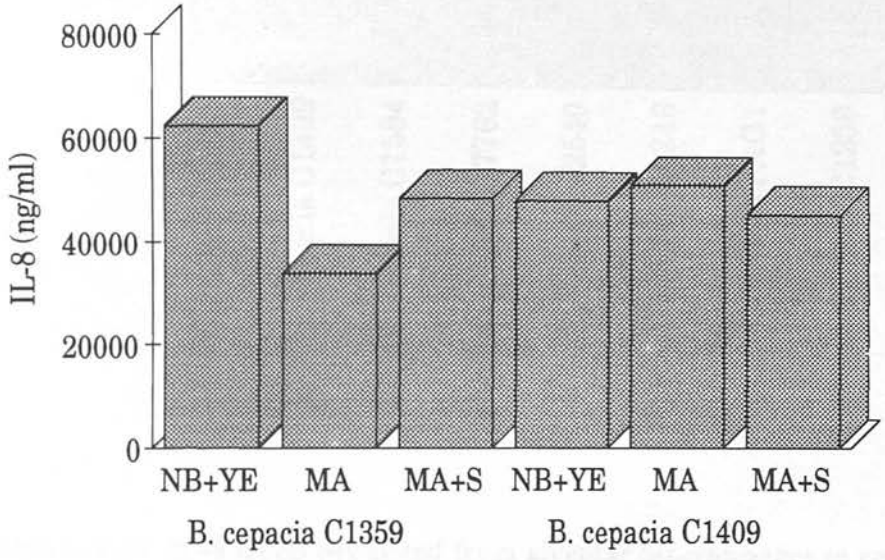


Figure 4.33 The influence of growth environment on IL-8 induction from MNL in response to PW-LPS samples. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 5h. Each value represents the mean of duplicates.

IL-8 induction from alveolar macrophages

IL-8 induction from alveolar macrophages in response to PW-LPS samples is shown in Figure 4.34. The *B. cepacia* samples again induced a greater response compared to the *P. aeruginosa* samples. In addition, less variation between *B. cepacia* isolates was noted compared to the IL-8-MNL assay (Figure 4.32). However, due to the scarcity of the alveolar macrophage extracts, this assay was only able to be performed on one occasion and further experiments would be required to confirm these observations.

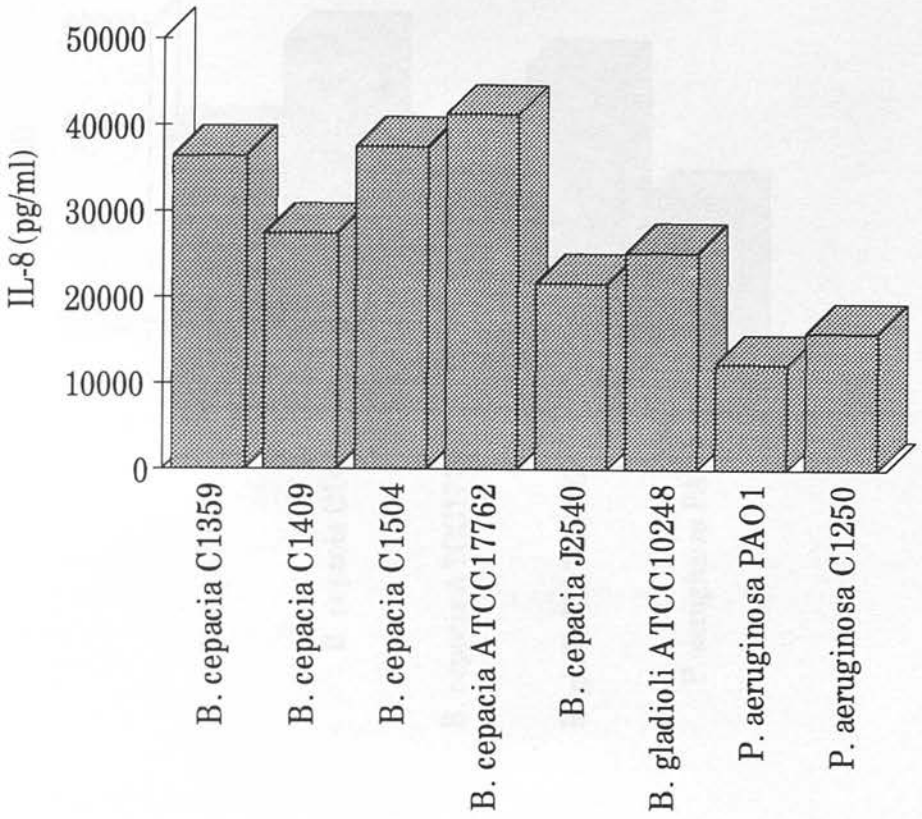


Figure 4.34 IL-8 levels produced from alveolar macrophages in response to PW-LPS samples. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 5h. Results shown are the mean of duplicates.

4.14 IL-8 INDUCTION FROM OM SAMPLES

Five OM samples from *B. cepacia* C1359, C1409, ATCC 17762, *B. gladioli* ATCC 10248 and *P. aeruginosa* PAO1 were tested for IL-8 production from MNL cells (Figure 4.35). IL-8 production in response to the OM samples varied from the response to the PW-LPS samples. For the OM preparations, it is concluded that only comparable levels of IL-8 are produced from *B. cepacia* and *P. aeruginosa.*

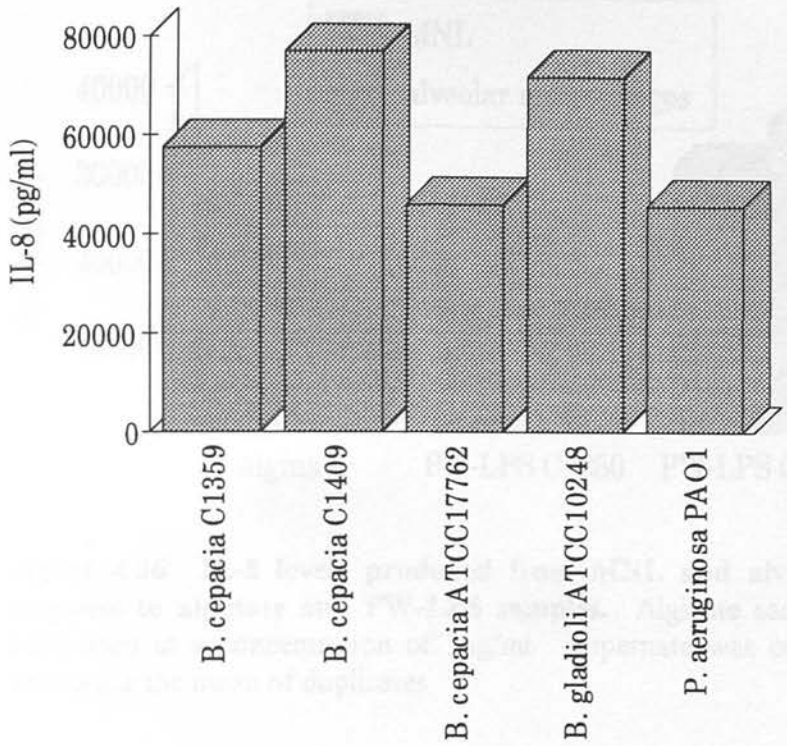


Figure 4.35 IL-8 levels produced from MNL in response to OM samples. OM samples were used at a protein concentration of 16ng/ml. Supernate was collected at 5h. Results shown are the mean of duplicates.

4.15 IL-8 INDUCTION BY EPS SAMPLES

Alginate samples were tested for their capacity to stimulate IL-8 from both MNL and alveolar macrophages.

Alginate samples induced comparable levels of IL-8 to the PW-LPS extract of the homologous strain C1250, and in addition, the levels of IL-8 produced were only slightly less than for *B. cepacia* C1359.

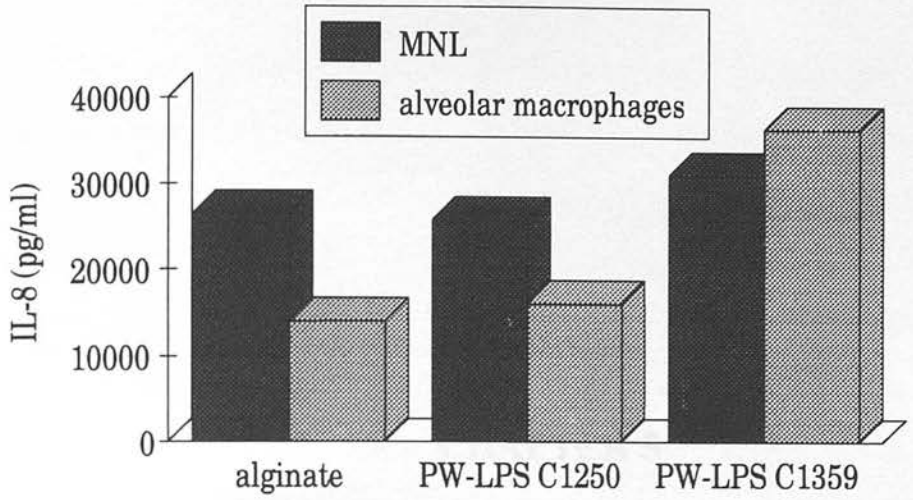


Figure 4.36 IL-8 levels produced from MNL and alveolar macrophages in response to alginate and PW-LPS samples. Alginate samples and LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 5h. Results shown are the mean of duplicates.

Overall, the results of the present study highlight the significant inflammatory capacity of *B. cepacia,* confirming the pathogenic potential of this species and challenging the hypothesis that *B. cepacia* acts merely as a marker of existing lung disease. A significant difference in TNF-α levels between *B. cepacia* and *P. aeruginosa* PW-LPS samples was observed from MNL, alveolar macrophages and lung epithelial cells. Most importantly, however, LPS co-stimulation experiments demonstrated that *P. aeruginosa* can modulate the inflammatory response of *B. cepacia.* It seems reasonable to conclude that immune-mediated damage is an important factor in *B. cepacia* pathogenesis.

CHAPTER 5

DISCUSSION

(*Burkholderia cepacia*)

Burkholderia (formerly *Pseudomonas*) *cepacia* was first described in 1950 as a causative agent of bacterial rot in onion bulbs, but has in the last decade, emerged as a serious human pathogen, most notably in patients with cystic fibrosis (CF), the most common, fatal inherited disease affecting Caucasian populations. The clinical impact of *B. cepacia* is based on its inherent multiresistance to antibiotics, direct transmissibility between CF patients and the occurrence of 'cepacia syndrome', a rapidly fatal necrotising pneumonia, observed in 20% of colonised patients and rarely associated with other CF pathogens. These three factors have had devastating consequences for the CF population leading, ultimately, to the introduction of a world-wide segregation policy separating *B. cepacia* colonised from non-colonised CF patients in an attempt to reduce cross-infection.

The impact of *B. cepacia* on the CF community, and the recent reports of serious community-acquired *B. cepacia* infection in non-CF patients (Hobson *et al*, 1995), has prompted urgent clarification on the pathogenic potential of this organism. Considerable progress has been made in elucidating the transmission and epidemiology of *B. cepacia* (Goldstein *et al*, 1995; Govan *et al*, 1993); in contrast virulence determinants and pathogenic mechanisms remain poorly understood. Part of this problem is highlighted by suggestions that *B. cepacia* isolates vary considerably in virulence; some strains are highly virulent whilst others are essentially avirulent (Gilligan, 1991). This leads to the question of whether the highly-transmissible 'epidemic' strains, or indeed CF isolates as a whole, represent a distinct, virulent subpopulation of *B. cepacia*; a question which requires resolving for two important reasons. Firstly, the health hazards associated with the release of *B. cepacia* into the environment as a biocontrol agent or soil decontaminant must be addressed; is there a group of *B. cepacia* strains which are 'safe' to use? Secondly, and more importantly, there are calls for the abolition of the controversial segregation policy in areas where a *B. cepacia* problem has not yet arisen. However, before any relaxation of the

segregation policy is introduced, it is essential that the virulence factors of *B. cepacia* are identified so that virulent strains can be distinguished from avirulent isolates.

Therefore, the aims of this thesis were to identify putative virulence factors, identify possible pathogenic mechanisms *in vivo* and to distinguish any variation in virulence between *B. cepacia* isolates from a variety of sources.

5.1 ENVIRONMENTAL MODULATION OF CELL SURFACE VIRULENCE DETERMINANTS OF *B. CEPACIA*

Progress into the understanding of *B. cepacia* pathogenesis has been hampered by the finding that this organism produces few recognised virulence factors such as exotoxin A or alginate; associated with *P. aeruginosa* infection in CF (McKevitt & Woods, 1984). The surface components of Gram-negative bacteria are well-established as classic virulence factors. Furthermore, these surface structures can be heavily influenced by environmental conditions. Indeed, in many cases of infection, growth environment induces the expression of a more virulent form of cell surface structure, for example the production of alginate in the CF lung (section 1.2). Thus, the first section of this study characterised the expression and antigenicity of cell surface factors from a variety of *B. cepacia* strains. The influence of the environment on these components, and on growth and survival mechanisms, was also investigated.

Growth and survival

Preliminary studies investigated changes in growth and colonial morphology in several different nutrient-limiting media, mimicking conditions thought to be encountered *in situ* or previously reported to induce cell surface alterations. No obvious change in morphology was observed on any of the media, although certain culture conditions, for example phosphate-limiting medium, did restrict growth.

In addition, low pH minimal medium was found to be highly inhibitory to all *B. cepacia* strains tested, with growth decreasing in a pH-dependent manner. This observation is in contrast to a previous report in which one *B. cepacia* strain grew well at pH 4.5 and 7 but was inhibited at pH 9.5 (Dejsirilert *et al*, 1991). Interestingly, however, in the study of Dejsirilert *et al,* the complex media used (heart infusion broth), was rapidly metabolised to produce an alkaline pH, regardless of initial pH level; indeed, the media supporting the best growth of *B. cepacia* in this study, the commercially prepared laboratory medium CEP, although initially prepared to pH 6.2, was also rapidly metabolised to produce an alkaline pH. Therefore, the discrepancy between these two studies is likely to be due to the different metabolic by-products and buffering capacity of the various media rather than a true pH response. Taken together, however, the results from the two studies show that growth of *B. cepacia* can be restricted in an environment maintained at a low or high pH.

It must be stressed, however, that in this study, all the *B. cepacia* strains grew to some extent at low pH levels, with the exception of the epidemic strain. This observation is slightly surprising, as previous studies have opted to use a low growth pH in selective media for *B. cepacia* environmental isolates (Hagedorn *et al,* 1987). In contrast, media used to isolate clinical *B. cepacia* strains is generally prepared around pH 6.7 (Wu & Thompson, 1984; Gilligan *et al*, 1985; Welch *et al*, 1987), implying that clinical and environmental isolates vary in their pH tolerance. In the present study however, no difference in pH tolerance was observed between clinical and environmental isolates.

It was initially speculated that the ability to grow at a low pH would be an important property of *B. cepacia*, not only because onions, a natural host, are highly acidic (Geigy, 1962) but also because phagocytes kill ingested bacteria in an acidic

phagolysosome (Janeway & Travers, 1994). Indeed, the prevalence of *B. cepacia* in patients with CGD and the theory that *B. cepacia* may survive intracellularly in CF patients would suggest that *B. cepacia* must tolerate low pH conditions. However, many Gram-negative organisms, including *P. aeruginosa,* are killed in phagocytic cells by cationic proteins, active during a transient increase in pH before acidification of the phagolysosome (Roitt *et al*, 1989). Thus, to survive inside phagocytes an organism must be able to tolerate both high and low pH conditions.

Oxygen tension is an important environmental factor, as any bacterium growing as part of a colony, or close to a surface, will experience a gradual decrease in oxygen levels as the bacterial colony becomes larger or the organism is surrounded by debris. Indeed, alginate biosynthesis in *P. aeruginosa* has been reported to be dependent on oxygen levels (Leitão & Sá-Correia, 1993). The ability of *B. cepacia* to exist in soil depths where oxygen tension is low would suggest that tolerance of low oxygen levels may play a role in survival and virulence. In this project, both *B. cepacia* strains tested grew in conditions of reduced oxygen levels, but, neither strain grew (to an observable level) under anaerobic conditions. Interestingly, however, both strains survived, at least four weeks in the oxygen-free atmosphere, despite the fact that *B. cepacia* does not possess the metabolic machinery required for anaerobic growth (Zannoni, 1989). This implies that *B. cepacia* has the ability for dormancy, an important biological property which could contribute to the success of an opportunistic pathogen; allowing an organism to remain viable in otherwise lethal situations. For example, to survive intracellularly a pathogen must be able to tolerate low oxygen levels. Indeed, in anaerobic conditions, *Shigella* species increase their invasiveness (Kanai & Kondo, 1994). Thus, the ability of *B. cepacia* to survive in conditions of low and high pH, nutrient limitation and oxygen-free conditions lends further credence to the proposal that intracellular survival may be a factor in *B. cepacia* pathogenesis.

Of all the *B. cepacia* strains tested in this study, the epidemic strain was the slowest growing and the most susceptible to adverse conditions, as has been observed by laboratory colleagues (Butler, 1994). However, as the anaerobic studies demonstrate this observation may only be a reflection of growth and not an indicator of survival.

Cell surface factors - Lipopolysaccharide

Lipopolysaccharide is widely regarded as the most potent of all bacterial cell surface factors, with a diverse range of biological properties important in the infection process (section 1.1). Characterisation of the LPS from several *B. cepacia* strains, originating from different sources and clonally distinct (based on observations made by PFGE, C. Doherty personal communication), confirmed previous reports that *B. cepacia* strains may possess rough or smooth LPS (McKevitt & Woods, 1984; Simpson *et al*, 1994). Surprisingly, however, two of the strains previously reported to possess R-LPS exhibited reactive high molecular weight bands on immunoblot analysis. A distinct ladder pattern was observed using serum raised against a smooth LPS CF isolate. The reason why these reactive bands are only observed with immunoblotting is not understood. However, the positive reaction of the high molecular weight structures suggests that these components are expressed *in vivo*, and leads to speculation on the morphological heterogeneity of *B. cepacia*; a single strain varying in amount and structure of LPS.

Results from the immunoblots revealed a lack of homology between LPS of different *B. cepacia* strains and other species including *B. gladioli* and *P. aeruginosa*. Serum raised against the epidemic strain reacted only with the core LPS of the homologous strain and a high molecular weight band of two other isolates. Conversely, serum raised against a S-LPS non-epidemic *B. cepacia* strain reacted very strongly against the homologous strain and with *B. gladioli*, and to a lesser degree with three other *B. cepacia* strains. A similar response was obtained using sera from CF patients. No

cross reaction with any of the *P. aeruginosa* isolates was observed, confirming the difference between these two species. The findings of the present study agree with previous reports (Nelson *et al*, 1993; Burnie *et al*, 1995), showing both LPS heterogeneity between *B. cepacia* isolates and the specificity of LPS antigens. LPS preparations were also extracted from different growth media and examined by SDS-PAGE and immunoblotting but no major variation in LPS structure was observed.

The results of the LPS studies emphasise the distinct nature of the epidemic strain, not only by the lack of cross-reactivity to other *B. cepacia* isolates but also because it is the only *B. cepacia* strain consistently expressing R-LPS. Indeed, R-LPS has even been considered as one of the physiological markers in distinguishing these highly-transmissible strains (Simpson *et al*, 1994).

Using different LPS extraction methods, however, it was shown that the epidemic strain may not always possess R-LPS. Two very important results were noted. Firstly, LPS extracted by the PCP method, produced a highly hydrophobic compound, which was not revealed by silver-staining but showed five high molecular weight bands on immunoblotting. The positive antigenic reaction suggests these bands are expressed *in vivo* although the role of the corresponding structure in pathogenesis can only be speculated. The irregularity of the band spacing implies the structure may not be smooth-type LPS but another amphipathic molecule, perhaps similar to the enterobacterial common antigen, or protein, which may account for the lack of reaction on silver-staining. Further work is required to determine whether the structure and function of these high molecular weight bands are similar to the bands cross-reactive with the epidemic strain serum as discussed previously.

Secondly, the extreme hydrophobic nature of the PCP extract led to investigations of the phenol hydrophobic phase of a PW-LPS preparation. Very little LPS sample was

obtained in the phenol layer, compared to the normally used aqueous layer and no observation of the phenol sample was made by silver-staining. However, again, a large high molecular weight moiety reacted upon immunoblotting with a similar result obtained using another *B. cepacia* strain.

It is well-established that different extraction procedures select for LPS of different structures and composition. However, the PW method usually selects for high molecular weight material, whilst the PCP selects for low molecular weight material; the opposite of the results found for the epidemic strain. Furthermore, the fact that the LPS from the epidemic strain can vary depending on the extraction procedure casts doubt on the possession of R-LPS by the epidemic strain *in vivo* and emphasises the importance of extraction procedures in studying bacterial components.

A mystery therefore exists about the nature of the LPS of the epidemic strain, with several questions remaining unanswered; for example what LPS structure is expressed *in vivo* R-LPS, S-LPS or a mixture of both? Furthermore, if the LPS of the epidemic strain is indeed rough and highly hydrophobic why does the majority dissolve into the aqueous layer of a PW preparation? To answer these questions experiments were carried out to determine the cell surface hydrophobicity of whole cells. High hydrophobicity may play an important role in *B. cepacia* pathogenesis as it would allow bacteria to bind more efficiently to cells. This property may also render the bacteria more susceptible to phagocytic uptake, although this could be construed as an advantage for intracellular growth. Unfortunately, the epidemic strain did not show any major difference in cell surface hydrophobicity compared to other *B. cepacia* isolates. Interestingly, however, the hydrophobicity of the epidemic strain increased to approximately 70% when grown in minimal media supplemented with 50% serum, far greater than any other strain or growth condition. Although no difference in LPS structure was seen with this growth condition, further extraction

procedures from serum supplemented media may elucidate the nature of the LPS of the epidemic strain.

The distinctive nature of the epidemic strain is further highlighted by the observations made by rocket immunoelectrophoresis (RIE). Initially, RIE was performed as an additional method to investigate the antigenicity of cell surface factors. Although all the samples and sera used were the same as in the immunoblot experiments, only one positive reaction was ever observed; between the PW-LPS sample from C1359 (epidemic) and serum from a CF patient colonised by this strain. No other sera or LPS sample gave a positive response. The discrepancies between the RIE and immunoblot assays can only be explained by an intrinsic difference in the two techniques (Shand *et al*, 1988). The results do however suggest that the epidemic strain is far more immunogenic than any other *B. cepacia* strain studied in this project. OM samples showed a different response; a streak was observed rather than a precipitin arc. Whether this streak is a true antigen-antibody reaction or an artifact produced by salt precipitation, moving by force of the electric current, is not clear.

Chemical analysis was also performed on LPS samples from different strains, growth conditions and extraction procedures. The only major difference observed between the strains was the levels of Kdo in *B. cepacia* and *P. aeruginosa* LPS, with the latter species possessing over five fold more Kdo. Straus *et al* (1989) found a correlation between lethality in a mouse model and the presence of Kdo in an extracellular toxic complex of *B. cepacia* isolates. However, the toxicity observed in that study is more likely to be attributed to the overall composition of the complex rather than the presence of Kdo alone. The average Kdo content of the *B. cepacia* extracts used in the present study is 0.4% which agrees with other reports (Cox & Wilkinson, 1991). This level of Kdo is generally considered to be low and may account for the previous debate on the apparent absence of this compound. However, it is now known that

B. cepacia also possesses D-*glycero*-α-D-*talo*-2-octulosonic acid (Ko), an unusual hydroxylated form of Kdo. Ko is also found in the LPS of *B. pseudomallei*, and some *Legionella* species (Wilkinson & Pitt, 1995), both intracellular bacteria, which adds further support to the hypothesis that *B. cepacia* strains are able to invade cells and survive intracellularly as part of their pathogenic strategy.

Cell surface factors - Outer membrane

All the *B. cepacia* strains studied possessed a distinct OM profile, highlighting the heterogeneity between isolates. Immunoblot analysis however, revealed that OMs from all the *B. cepacia* strains tested, regardless of origin, cross-reacted, suggesting that although the outer membrane profiles from different strains appear distinct, the strains express common epitopes *in vivo*.

P. aeruginosa and *B. cepacia* OMs also cross-reacted, albeit to varying degrees depending on the sera used. Previous studies have concluded that some *B. cepacia* OM components may be antigenically related to *P. aeruginosa* (Aronoff, 1988; Nelson *et al*, 1993). In contrast, a recent report (Lacy *et al*, 1995) found that despite extensive preabsorption with *P. aeruginosa* whole cells, the IgG antibody response against the OMs of *B. cepacia* was not reduced, implying that the OM antigens were *B. cepacia*-specific and not cross-reactive with *P. aeruginosa*. In the present study, the cross-reactivity between *B. cepacia* and *P. aeruginosa* depended largely on the serum used. Rabbit sera raised against whole cells of three different *B. cepacia* strains showed no response against a *P. aeruginosa* OM sample. In contrast, sera from CF patients colonised with *B. cepacia* but not *P. aeruginosa* exhibited a positive reaction against the *P. aeruginosa* sample. In all of the previous reports only sera from a CF patient was used.

The rabbit sera used came from animals kept in relatively sterile conditions with a low

risk of exposure to bacteria; in contrast, CF patients stand a high risk of exposure to the ubiquitous *P. aeruginosa.* Thus, the results of the immunoblots using rabbit sera suggest that anti-*B. cepacia* OM antibodies recognise distinct epitopes and do not cross-react with *P. aeruginosa* OMs. Therefore, the immunoblot reactions using sera from *P. aeruginosa* negative CF patients suggest prior exposure to *P. aeruginosa*, rather than a true cross reaction between *B. cepacia* and *P. aeruginosa* OM antigens. However, the rabbit sera used was raised against heat-killed whole cells and the cross reactive OM antigens may simply have been denatured or masked by other cell components, thereby preventing the production of antibodies against these epitopes.

Variation in OM profile within a single strain was observed when isolates were grown in different conditions. Growth in the nutrient-rich media produced fewer proteins compared to conditions of environmental stress. This observation may correlate with nutrient requirements, as bacteria growing in nutrient-depleted environments would possess larger, more numerous porins to allow entry of sufficient nutrient supplies. Proteins induced in the nutritionally depleted environments may be important in the transfer of these limited nutrients across the cell membrane although, surprisingly, no growth condition induced specifically the same protein in both *B. cepacia* strains tested. Variation in OM profile either between growth environments or between strains may be correlated not only to nutrient requirements but also to survival, including increased resistance to antimicrobial agents (Parr *et al,* 1987) and serum killing (Butler *et al,* 1995). Indeed, resistance to β-lactams, tetracycline, quinolones and chloramphenicol have all been related to a decrease in porin number or porin size (Burns, 1995; Aronoff, 1988; Moore & Hancock, 1986; Burns *et al,* 1989).

Immunoblot analysis of the OM samples from different growth conditions revealed some changes in antigenicity, although a number of proteins were conserved both between strains and between growth conditions. It is interesting to note that one of

these conserved epitopes, a major protein of approximately 30kD, described previously as the porin D antigen of *B. cepacia*, has recently been suggested as a potential immunotherapeutic agent (Burnie *et al*, 1995). However, Aronoff *et al* (1991), showed that anti-porin D antibody did not protect CF patients against *B. cepacia* colonisation. Therefore, the prospective use of this therapy would be limited.

The *B. cepacia* strains tested in this study possessed varied OM profiles that could be influenced by growth condition. The function of many of these proteins, and their potential role in *B. cepacia* pathogenesis remains to be defined.

Cell surface factors - Exopolysaccharide

In the present study, the relative absence of EPS production by *B. cepacia*, as compared to a mucoid *P. aeruginosa* control, was confirmed by Percoll profiles of washed whole cells from a number of isolates. Only two of the *B. cepacia* strains tested, one clinical and one environmental isolate, exhibited a profile consistent with moderate EPS production. More importantly, the epidemic strain was unique in possessing a Percoll profile characteristic of no exopolysaccharide production. This observed absence or low level EPS production from a number of *B. cepacia* strains would suggest that, in contrast to *P. aeruginosa* infection, EPS does not play a major role in *B. cepacia* pathogeneis. Indeed, Sage *et al* (1990) found no correlation between production of EPS by *B. cepacia* and the ability to colonise the respiratory tract. However, *B. cepacia* strains used in the study of Sage *et al*, and in more recent investigations (Allison & Goldsborough, 1994), have been found to produce copious amounts of EPS in the presence of high osmolarity and excess glucose. Further experiments to study the influence of such growth parameters on EPS production induced alterations in Percoll profiles for three of the strains tested, although the majority of isolates did not vary. Thus, for the *B. cepacia* strains tested in this study, EPS does not appear to be a major virulence factor in pathogenesis.

The studies in this section examined the cell surface components of representative *B. cepacia* strains, not only to investigate the expression of such components *per se* but also to characterise the extracts which were to be used in the future studies of inflammatory potential, detailed in the next section. Intra- and inter-species OM cross reaction was observed, depending on the source of sera used. As expected, OM profiles were adaptable to changing environmental conditions. LPS samples, in general, were more heterogeneous. Of importance was the loss of the characteristic R-LPS phenotype of the epidemic strain when different LPS extraction methods were employed. This finding leads to new speculation on virulence factor expression *in vivo* and the loss of important cell surface factors when grown *in vitro*. For example can the epidemic strain produce both smooth and rough LPS? Although no S- to R-LPS transition has been observed in this present study, previous reports have suggested this mechanism on the basis of experimental findings (Lacy *et al*, 1995).

The adaptation of the outer membrane to changing surroundings is an important intrinsic survival mechanism. In addition, this study has shown the ability of *B. cepacia* to survive in adverse conditions such as low pH, high pH, oxygen limitation and nutrient limitation which is an important property for the success of an opportunistic pathogen. No major virulence determinant was specifically induced, although the OM and LPS samples were shown to be adaptable and both surface components have been reported as important factors in bacterial infection. Thus the role of the OM and LPS in *B. cepacia* pathogenesis was investigated further in the next section.

5.2 BIOLOGICAL ACTIVITY OF CELL SURFACE COMPONENTS OF *B. CEPACIA*

CF patients colonised by exactly the same strain of *B. cepacia*, as distinguished by genetic typing methods, present variable clinical outcomes. This observation, plus the knowledge that *B. cepacia* produces few possible virulence determinants, leads to the

suggestion that the host, and in particular the immune response, plays the major role in *B. cepacia* pathogenesis. Unfortunately, studies have shown anti-*B. cepacia* antibodies to be neither protective nor prognostic for CF patients (Nelson *et al*, 1993; Butler, 1994).

Cytokine production is an essential component of the host defence system, required for development and maintenance of the immune response. However, excess cytokine production is now widely accepted as a primary cause of several diseases (section 1.3). Furthermore, previous reports have found a correlation between increasing cytokine levels and pulmonary deterioration in CF patients. Wilson *et al*, (1993) reported a strong association between increasing plasma and mRNA levels of TNF-α and IL-1β and pulmonary and nutritional decline in *P. aeruginosa*-colonised CF patients. Similarly, Greally *et al* (1993) and Norman *et al* (1989) suggested that the observed cytokine response to *P. aeruginosa* infection in CF patients was contributing significantly to the airway inflammation and airflow obstruction associated with acute lung injury.

To date, no specific study on cytokine induction by *B. cepacia* has been performed. Thus, the induction of two major proinflammatory cytokines were studied and their potential role in *B. cepacia* pathogenesis in the CF lung assessed. TNF-α is one of the first cytokines produced during infection and is a key factor in inducing the cytokine cascade observed during inflammation. IL-8 is a chemotactic cytokine responsible for attracting neutrophils to the site of infection, a major characteristic of CF lung disease. Both cytokines play an important role in the immune-mediated damage observed in CF patients. The next section therefore focused on the inflammatory capacity of *B. cepacia* cell surface factors compared to the activity of *B. gladioli* and *P. aeruginosa* controls.

Endotoxicity

LPS, or endotoxin, has long been recognised as possessing many biological properties harmful to a host and remains one of the most potent inducers of cytokines. One of the methods to determine the toxic potential of LPS is the LAL assay, often used to detect LPS in products such as blood, saline and dialysate materials. Initial investigations determined the endotoxicity of LPS samples from a variety of strains and growth conditions. Surprisingly, and very unexpectedly, LPS samples from *B. cepacia* were found to possess significantly higher endotoxic potential compared to *P. aeruginosa* LPS. Furthermore, growth condition influenced the endotoxicity of the samples, implying that although no structural changes could be observed by SDS-PAGE or immunoblotting, the LPS composition is somehow varied and virulence altered correspondingly.

Induction of TNF-α by LPS

Initial experiments determined the capacity of several PW-LPS samples to induce TNF-α from human mononuclear leucocytes (MNL). Cytokine levels induced by *B. cepacia* LPS were significantly higher compared to *P. aeruginosa* PW-LPS samples. This important result indicates that *B. cepacia* has a greater potential to cause immune-mediated damage in the CF lung compared to *P. aeruginosa* and may be a vital pathogenic mechanism of *B. cepacia in vivo.*

MNL are generally used as the standard leucocyte cell source for *in vitro* cytokine studies (for example Fomsgaard *et al,* 1990) and are obtained from healthy blood donors. To confirm the relevance of the above results to CF lung disease, experiments were performed with lung epithelial cells, alveolar macrophages and neutrophils. Lung epithelial cells play a vital role in the initial stages of bacterial adherence and colonisation and are extensively damaged during periods of exacerbation (Stadnyk, 1994). Alveolar macrophages are the pivotal effector cell

against invading bacteria in CF, able to secrete a variety of proteases, complement components and cytokines (Buret & Cripps, 1993). Neutrophils are the predominant cells of the alveolar space in CF patients, representing a substantial systemic circulatory component, and producing products such as elastase and superoxide which can also cause extensive tissue injury (Elborn & Shale, 1990). Therefore, all three cell types play a fundamental role in the host-pathogen interaction of cystic fibrosis.

As with the MNL experiments, results obtained with the alveolar macrophages and lung epithelial cells showed that *B. cepacia* possessed a significantly higher inflammatory potential than *P. aeruginosa*. However, the same trend was not observed with the neutrophil preparations; *B. cepacia* producing only comparable levels of TNF-α to *P. aeruginosa*. It must be emphasised however, that *P. aeruginosa* LPS can cause extensive damage. Indeed, previous studies have found *P. aeruginosa* LPS to be highly active in both the LAL and the TNF assay (Fomsgaard, 1990) and clinical studies, focusing on *P. aeruginosa*-colonised CF patients, have detected clinically relevant levels of cytokine both locally in sputum and systemically in serum (Norman *et al*, 1989; Greally *et al*, 1993). Thus, although the result of the neutrophil stimulation was different from the other cell types studied, it still signifies that *B. cepacia* has the potential to cause serious tissue damage and inflammation and is not merely a marker of pulmonary disease. This conclusion is further substantiated by comparison of *B. cepacia* LPS to an *E. coli* control. Although only tested with MNL cells, the *B. cepacia* LPS was more active in both the LAL and TNF assay.

LPS samples from different growth conditions were also tested, and showed some variation in their capacity to induce TNF. Although the difference was not significant this observation shows that the components of LPS required for receptor binding and cytokine induction can be sensitive to environmental change.

The above cytokine results are highly important in terms of understanding further the mechanisms of *B. cepacia* pathogenesis in the CF lung. A positive correlation has been found previously between increasing cytokine levels and pulmonary decline in CF patients. This study has shown *B. cepacia* to possess the capacity to stimulate a massive cytokine release that could lead to an uncontrollable response, causing rapid acceleration of pulmonary inflammation, damage and deterioration. Furthermore, these results firmly resolve the controversy; *B. cepacia* is not simply a marker of pre-existing lung disease but an organism with a high degree of pathogenic potential.

The influence of LPS extraction method on TNF-α induction.

For both *B. cepacia* strains tested, the LPS samples extracted by the PCP method induced very little detectable TNF-α compared to a PW control. The higher biological activity of PW-LPS samples compared to PCP-LPS has been described previously for other species of bacteria (Delahooke *et al*, 1995) and may be a general distinguishing property of the two procedures. PCP extraction produces a more hydrophobic material (as was observed), suggesting that the LPS could bind with greater efficiency to leucocytes. As this does not appear to occur, the epitopes required for efficient LPS receptor-binding (and correspondingly cytokine induction) must be either extensively different compared to PW-LPS samples, or masked in the high molecular weight moiety obtained by the PCP method. Which LPS structure and, hence, biological activity reflects that produced *in vivo* remains to be defined.

For the epidemic strain, the phenol-layer extract of a PW-LPS preparation was also found to be a poor cytokine inducer, in comparison to the aqueous-layer sample. However, for C1409 (non-epidemic) both phenol and aqueous layers have a comparable TNF response. These variations in biological activity between the samples must be due to the structural differences observed by immunoblotting. Further investigation of the different structures may lead to important new

information on the LPS binding regions of *B. cepacia*.

Induction of TNF-α by OM

LPS is unlikely to be present in a pure form *in vivo*, but rather attached to parts of the outer membrane. Investigations were therefore performed to establish the inflammatory potential of the outer membrane samples, which were shown in this study to contain levels of endotoxin as detected by the LAL assay. TNF-α was induced by all the outer membrane samples, although there was no significant difference between the levels stimulated by the *B. cepacia* OM extracts and the *P. aeruginosa* OM extracts. Furthermore, the influence of growth environment was found to exert an effect on OM inflammatory potential. These results indicate that TNF-α induction is dependent on the presentation, composition and surrounding environment of the LPS and confirm the conclusions made with the PW and PCP extracts. However, the contribution of the protein present in the OM samples is unknown and cannot be dismissed, as a proteinase K treated LPS sample showed a reduction in TNF-α levels compared to a crude extract.

Induction of TNF-α by EPS

The results in this section have established that the biological activity of *B. cepacia* LPS is equal to, if not greater than, *P. aeruginosa* LPS. A major characteristic of *P. aeruginosa* infection in CF is the production of alginate, which may effectively shield the LPS from leucocytes *in vivo*. Otterlei *et al* (1993), reported that alginate blocks possessed a high biological activity and were able to stimulate a greater level of cytokine compared to *P. aeruginosa* LPS. This finding may account for the variation in inflammatory capacities between *B. cepacia* and *P. aeruginosa*. To test this theory, alginate was extracted from the *P. aeruginosa* mucoid strain used throughout this study C1250, and assayed for TNF-α induction. However, alginate samples induced only a similar level of cytokine as the *P. aeruginosa* LPS samples;

thus alginate production cannot account for the discrepancy between the two species.

Induction of IL-8

One characteristic of CF lung disease is the migration of neutrophils into the airway lumen, where neutrophil proteases, elastase and other products are important mediators of local tissue destruction. IL-8 is a major neutrophil chemoattractant and CF patients have been found to exhibit increased IL-8 levels compared to non-CF controls (Richman-Eisenstat *et al*, 1993; Dean *et al*, 1993). Therefore, this study investigated the ability of *B. cepacia* cell surface factors to produce IL-8.

IL-8 assays were performed with MNL and alveolar macrophages using the same PW-LPS preparations as before. Results of the studies showed that *B. cepacia* LPS produced a higher level of IL-8 compared to *P. aeruginosa* samples, confirming once more the inflammatory potential of *B. cepacia*. However, the difference between the two species was not so great as observed with the TNF assays. In addition, there was a good correlation between high TNF-inducing strains and high IL-8-inducing strains (from alveolar macrophages) which would support the suggestion that TNF-α derived from alveolar macrophages is a major stimulator of IL-8 in the lung (Standiford *et al*, 1990). Both OM and EPS preparations were assayed for IL-8 production, but as with the TNF-α results, no significant difference between any of the samples was found.

Intraspecies variation - are distinct subgroups of *B. cepacia* evident?

With the exception of the epidemic strain, which has been shown to possess a distinct biological character, no major difference between the *B. cepacia* clinical strains was observed. However, an important result emerging from this study was the finding that *B. cepacia* clinical isolates induced a far greater level of TNF-α from MNL and alveolar macrophages than the environmental strain of both *B. cepacia* and *B. gladioli*. This observation confirms previous reports that *B. cepacia* strains vary in

virulence and a sub-group of avirulent *B. cepacia* may be safe for environmental release (Bevivino *et al*, 1994). Results from chapter three have demonstrated the structural heterogeneity among *B. cepacia* isolates and recent taxonomic studies (Vandamme, 1995) have shown that three or more new species may emerge on taxonomic properties alone. Furthermore, studies continuing in our laboratory suggest that there may indeed be certain clones of *B. cepacia* with a predilection for colonisation of the lungs of CF patients. However, as a word of caution, the environmental strains tested in the present study still induced equal, if not greater TNF-α and IL-8 levels compared to *P. aeruginosa* samples. This shows that environmental strains themselves possess considerable inflammatory potential, and until more is understood about how this potential may function *in vivo*, release of any strain seems premature. For example, we do not know whether a subgroup of environmental isolates can cause infections in humans *de novo* or whether all *B. cepacia* isolates can adapt *in vivo*, although results from this study would suggest the former. Future studies continuing in our laboratory and involving the CF mouse may help to determine the virulence of environmental strains in an *in vivo* situation.

Interspecies variation - *B. cepacia* and *B. gladioli*

The similarity between *B. cepacia* and *B. gladioli* throughout this study supports the taxonomic regrouping of the *Pseudomonas* rRNA homology group II. Information on *B. gladioli* remains scarce although it is known to act primarily as a plant pathogen. Due to the similarities of the two species, *B. gladioli* was considered more of a hindrance to the unequivocal identification of *B. cepacia*, rather than a human pathogen in its own right. Indeed, one of the very few reports on the pathogenic potential of *B. gladioli* found no harmful consequences of pulmonary colonisation in CF patients (Christenson *et al* 1989). However, the findings of Simpson *et al* (1994) showed that some clinical isolates of *B. cepacia* exhibited characteristics of both species, leading to the theory that a bacterial hybrid may have emerged with a

composite of virulence determinants required for life as a successful human pathogen. The results in the present study would certainly support the cautionary comments of Simpson *et al* as the *B. gladioli* LPS sample used in these investigations possessed comparable levels of biological activity to *P. aeruginosa* and *B. cepacia* LPS. Interestingly, another member of the same taxonomic group, *B. pseudomallei,* has been shown to possess the same level of endotoxicity as *B. cepacia*, and an equal level of TNF-α induction to *Salmonella abortus* (Kanai & Kondo,1994). As these three members all show a similar degree of endotoxicity and, more importantly, as *B. cepacia* strains appear to possess characteristics of both *B. gladioli* and *B. pseudomallei*, further taxonomic and phylogenetic studies are urgently required to assess the possibility and pathogenic potential of 'hybrid' organisms.

B. cepacia and *P. aeruginosa* - LPS signalling pathway and co-stimulation

This study has highlighted the differences between *B. cepacia* and *P. aeruginosa.* The structure and composition of the LPSs are very different, as established by the chemical analysis and lack of homology in the immunoblot experiments (section 3.2). These variations are one explanation for the observed difference in biological activity between the two species. However, the different antigenic epitopes may mean that the LPSs bind to different receptors, which may signal a higher or lower level of cytokine production.

To test this theory LPS samples from both species were assayed in a monocyte/macrophage cell line able to be enhanced for expression of CD14, the best characterised LPS receptor to date. In the presence of both enhanced CD14 production and a CD14 monoclonal antibody, used to inhibit receptor binding, no difference in cytokine production was detected, indicating, as far as possible, that both LPS types signalled in a similar manner. This is confirmed further by the observation that both LPS types induce only a single peak of TNF-α, whereas the CD14-

dependent *E. coli* induces multiple peaks (Delahooke *et al*, 1995). It is suggested that the periodic response of *E. coli* may be related to the requirement for CD14. In addition, a recent report by Merten *et al* (1995), showed CD14 was not involved in the LPS-induced increase in IL-6 production by a *P. aeruginosa* CF isolate.

CD14 has been shown to be unessential for the induction of cytokines by bacterial LPS (Lynn *et al*, 1993). In their report, Lynn *et al* suggested that a CD14-independent pathway may be of more importance in sites of local infection, where LPS concentration is high. This would be the scenario in the CF lung where high levels of LPS would be situated. If both *B. cepacia* and *P. aeruginosa* LPS function in a similar manner then this suggests there may be competition for LPS receptors when both types are present, as is often the case in the CF lung. Both LPS types presenting together may produce a more exaggerated response or an inhibitory effect.

Studies to investigate this possibility found that when *P. aeruginosa* was present in increasing amounts compared to *B. cepacia,* TNF-α levels fell correspondingly. Furthermore, even when *P. aeruginosa* was in a twenty-fold excess of *B. cepacia* more TNF-α was still secreted compared to *P. aeruginosa* alone, reiterating the major inflammatory potential of *B. cepacia.* The inhibitory effect of co-stimulation was repeatable with different *B. cepacia* and *P. aeruginosa* strains but was not observed with two different *B. cepacia* strains together or two *P. aeruginosa* strains together. This observation leads to the important suggestion that the inhibitory effect of co-stimulation is not merely a solubility phenomenon, due to the presence of a critical LPS concentration, but rather that *P. aeruginosa* LPS actually modulates the inflammatory response of *B. cepacia*. If reflected *in vivo*, this exciting finding could open up numerous opportunities for new therapies, for example antibodies directed against the LPS receptor.

Preliminary experiments with the PW-LPS samples showed *B. cepacia* LPS to be more mitogenic than *P. aeruginosa* LPS. Ko, a component of *B. cepacia* LPS, has been proposed as a strong mitogen which may account for the observed difference (Wilkinson & Pitt, 1995). Further work is required to establish the independent T-cell and B-cell response and thus the impact of *B. cepacia* on antibody production and cell mediated immunity compared to *P. aeruginosa.*

In summary, this study has established that *B. cepacia* does not produce a predominant virulence determinant, even when grown under conditions of environmental stress. Furthermore, *B. cepacia* strains have been shown to share similar properties with both *B. gladioli* and *B. pseudomallei*, re-emphasising the desperate need for a re-evaluation of the *Burkholderia* genus. Crude groupings of *B. cepacia* strains can be achieved, as determined by LPS homology on immunoblotting or capacity to induce TNF-α, although the distinct character of the 'epidemic' strain was clearly seen. The recent recognition of transmissibility factors will be a useful tool for the rapid identification of these highly transmissible strains and may lead to new prospects of vaccine development against colonisation. However, due to the variation within the *B. cepacia* species, the antigen to which a vaccine would be directed would have to be chosen carefully. A vaccine directed against a cable pilus would stop only some highly transmissible strains and would be no use against 'non-epidemic' strains, some of which have been found to cause a rapid clinical deterioration. Therefore, work must continue in discovering the pathogenic mechanisms of *B. cepacia* in the hope of developing novel therapeutic strategies against all *B. cepacia* and *B. cepacia*-like organisms.

To this end this study has provided some very important clues. This study firmly established that *B. cepacia* possessed a significantly higher inflammatory potential than *P. aeruginosa*, and may explain the ability of *B. cepacia* to cause severe immune-

mediated damage in the CF lung. It is speculated that the potentially huge level of cytokine induction by *B. cepacia* may contribute not only to a destructive local inflammation of the airways, but also a systemic inflammatory response, manifested by increased cytokine levels in the plasma and, in certain patients, contributing to rapidly fatal decline. Indeed, this hypothesis is confirmed by recent clinical data in which levels of the immune markers, C-reactive protein and neutrophil elastase (shown to be directly correlated to circulating TNF), were increased during periods of *B. cepacia*-associated exacerbations compared to *P. aeruginosa*-associated exacerbations (Elborn *et al*, 1994). Interestingly, the present study also showed that the *B. cepacia* inflammatory response may be modulated in the presence of *P. aeruginosa*, giving an important clue to why there are variable clinical outcomes in *B. cepacia* colonised CF patients. The clinical course of infection may be related not only to pre-existing lung disease, activated leucocytes or cytokine production *per se,* but also to co-colonisation with other organisms, such as *P. aeruginosa.* Of course, administration of *P. aeruginosa* or *P. aeruginosa* cell surface components to CF patients is not being suggested as any form of treatment. However, antibodies directed against the shared LPS receptor may be one possible therapy. In addition, recent unpublished clinical evidence indicates that patients who do not respond well to antibiotics but continue to exhibit rapid pulmonary decline, respond to treatment with commercial preparations of immunoglobulins (Dr J. Govan personal communication). *B. cepacia* antibodies are unlikely to be present in these preparations in significant numbers and, as the work of Nelson *et al* (1993) and Butler (1994) show, many CF patients already mount an enormous anti-*B. cepacia* response. Thus, the benefit of additional specific *B. cepacia* antibodies is doubtful. However, pooled immunoglobulin preparations may act as anti-inflammatory agents, perhaps containing anti-cytokine antibodies or anti-cytokine receptors.

If indeed commercial immunoglobulin preparations are acting in an anti-inflammatory capacity then this may lead to the much needed development of a positive therapeutic

strategy. However, better understanding of *B. cepacia* pathogenesis in CF is still urgently required, as only then will *B. cepacia* infection truly be brought under control without the need for such controversial and draconian measures that are currently in place.

CHAPTER 6

RESULTS

ENVIRONMENTAL MODULATION OF CELL SURFACE VIRULENCE DETERMINANTS OF *BACTEROIDES FRAGILIS*

The cell surface components of *Bacteroides fragilis* are considered to be the major virulence factors. However, the expression and role of these structures in *B. fragilis* pathogenesis is still debated. The following experiments aimed to characterise the expression and antigenicity of potential virulence determinants of *B. fragilis* NCTC 9343 and *B. fragilis* MPRL 1504 and of the related strains; *B. vulgatus* MPRL 1651, *B. thetaiotaomicron* NCTC 10582 and *B. uniformis* MPRL 1721.

6.1 CHARACTERISTICS AND ENVIRONMENTAL REGULATION OF LPS

Controversy still remains as to the exact phenotype of *B. fragilis* LPS and its potential role in disease. Part of this problem is due to the particularly close association of the *Bacteroides* cell surface components and the influence of culture conditions; factors not taken into account by previous workers.

The influence of growth environment on LPS phenotype

The LPS from the above five *Bacteroides* strains were extracted by proteinase K digestion from growth in a nutrient-rich proteose peptone-yeast extract medium PPY; a chemically defined minimal medium VT&W; and a physiological medium VT&S. In addition, the LPS of *B. fragilis* NCTC 9343 and MPRL 1504 was extracted from growth in 2%(w/v) bile salts, 0.5%(w/v) bile salts, 0.5%(w/v) cysteine hydrochloride and low pH medium (pH5.5).

From results obtained by SDS-PAGE and silver-staining, the following observations were made about the different *Bacteroides* species; the two *B. fragilis* strains possessed a typical core LPS structure, a series of closely spaced bands running approximately half-way down the gel area and particularly in strain NCTC 9343, a darkly staining high molecular mass antigen (speculated to be capsular polysaccharide; Figure 6.1a). *B. vulgatus* was the only *Bacteroides* strain to show a ladder pattern characteristic of smooth LPS (Figure 6.1d), with both *B. thetaiotaomicron* and

B. uniformis exhibiting core LPS and a single dark staining band but no obvious high molecular weight moiety (Figure 6.1e-f).

Due to problems of poor separation and resolution, the LPS phenotype of *B. fragilis* has, in the past, been proposed as rough, semi-rough with common antigen or smooth. For the *B. fragilis* strains tested, the observed LPS phenotype was similar to that reported by Poxton & Brown (1986), although the distinctive common antigen, defined by these authors was not clearly seen in the LPS samples.

Growth environment appears to influence *B. fragilis* LPS structure. LPS preparations of *B. fragilis* extracted from bile supplemented media showed the loss of the high molecular weight moiety, (Figure 6.1b-c; Track 3 & 4). For *B. fragilis* NCTC 9343, a decrease in production of core LPS was also observed and for MPRL 1504 production of two extra high molecular weight bands was noted. As the gut contains bile salts and *B. fragilis* is noted for its bile-tolerance, these observed structural changes may be significant to the virulence of this organism. In addition, LPS samples of *B. fragilis* and *B. vulgatus* extracted from growth in PPY showed variation in banding pattern and core production compared to LPS profiles from VT&W and VT&S, with *B. vulgatus* exhibiting a pronounced decrease in O-antigen chain length. No structural changes were observed with *B. thetaiotaomicron* or *B. uniformis.*

Immunoblotting

To investigate the structures contributing to LPS antigenicity, immunoblots of the five *Bacteroides* strains were performed. Rabbit sera raised against the homologous strains were used as probes, except for *B. fragilis* NCTC 9343, where serum raised against the similar strain *B. fragilis* NCTC 9344 was used (Figure 6.2a-c). For the two *B. fragilis* strains the banding pattern gave the strongest reaction (Figure 6.2a-b),

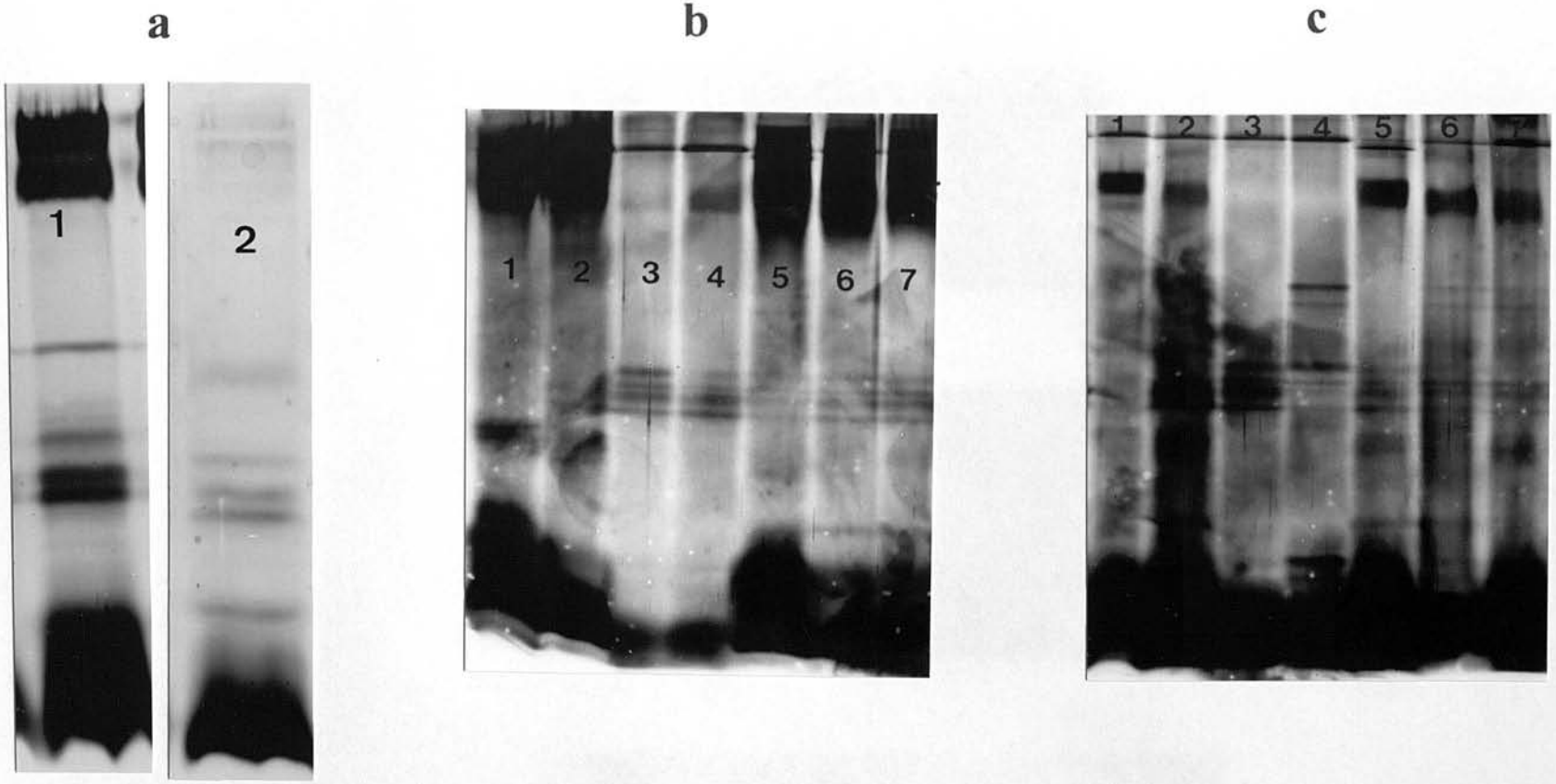


Figure 6.1a-f Silver stained LPS profiles of proteinase K whole cell digests of *B. fragilis, B. vulgatus, B. thetaiotaomicron* and *B. uniformis* extracted from different growth conditions. Figure 6.1a *B. fragilis* LPS profile, Track 1 NCTC 9343; Track 2, MPRL 1504. **Figure 6.1b** *B. fragilis* NCTC 9343 LPS extracted from growth in; Track 1, 0.5% cysteine hydrochloride; Track 2, low pH media; Track 3, 2% bile salts; Track 4, 0.5% bile salts; Track 5, PPY; Track 6, VT&W; Track 7, VT&S. **Figure 6.1c** *B. fragilis* MPRL 1504 LPS extracted from the same growth conditions.

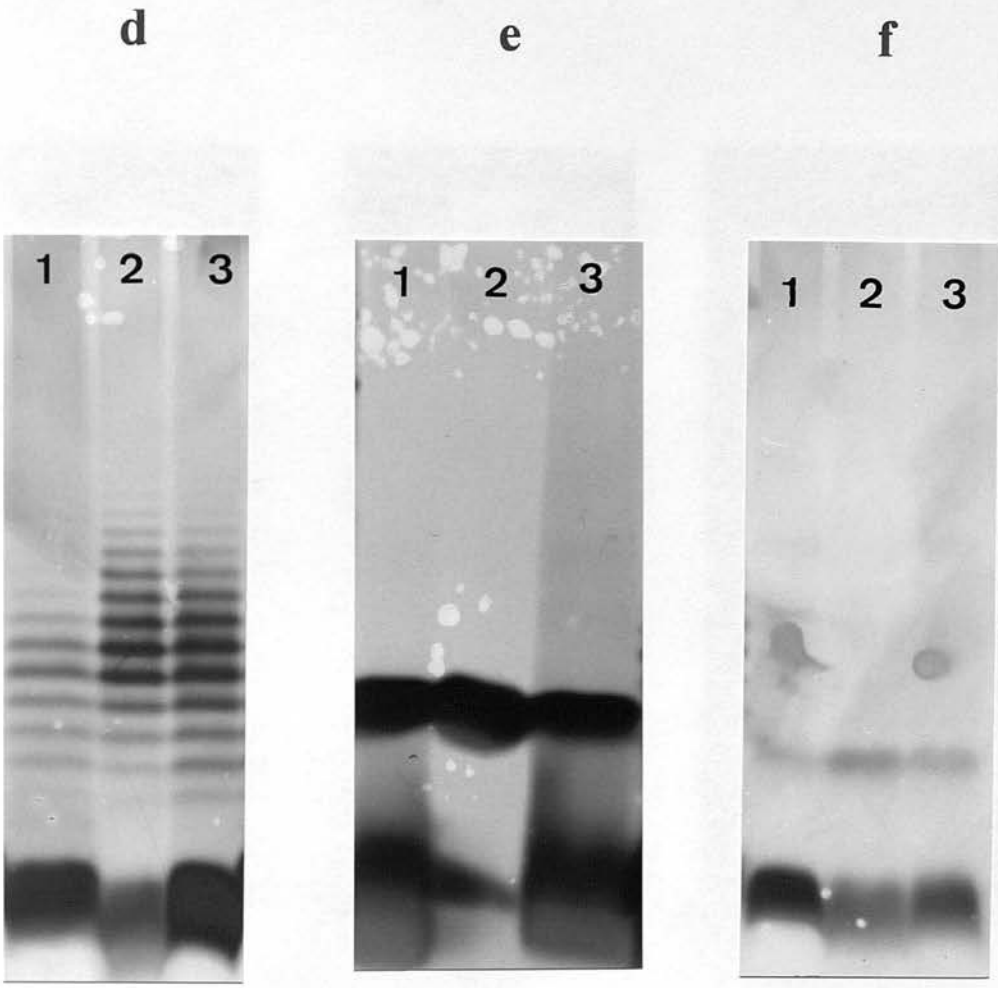


Figure 6.1d LPS profile of *B. vulgatus* MPRL 1651 extracted from growth in PPY, VT&W and VT&S; **Figure 6.1e** LPS profile of *B. thetaiotaomicron* NCTC 10582 and **Figure 6.1f** LPS profile of *B. uniformis* MPRL 1721 extracted from the same growth conditions.

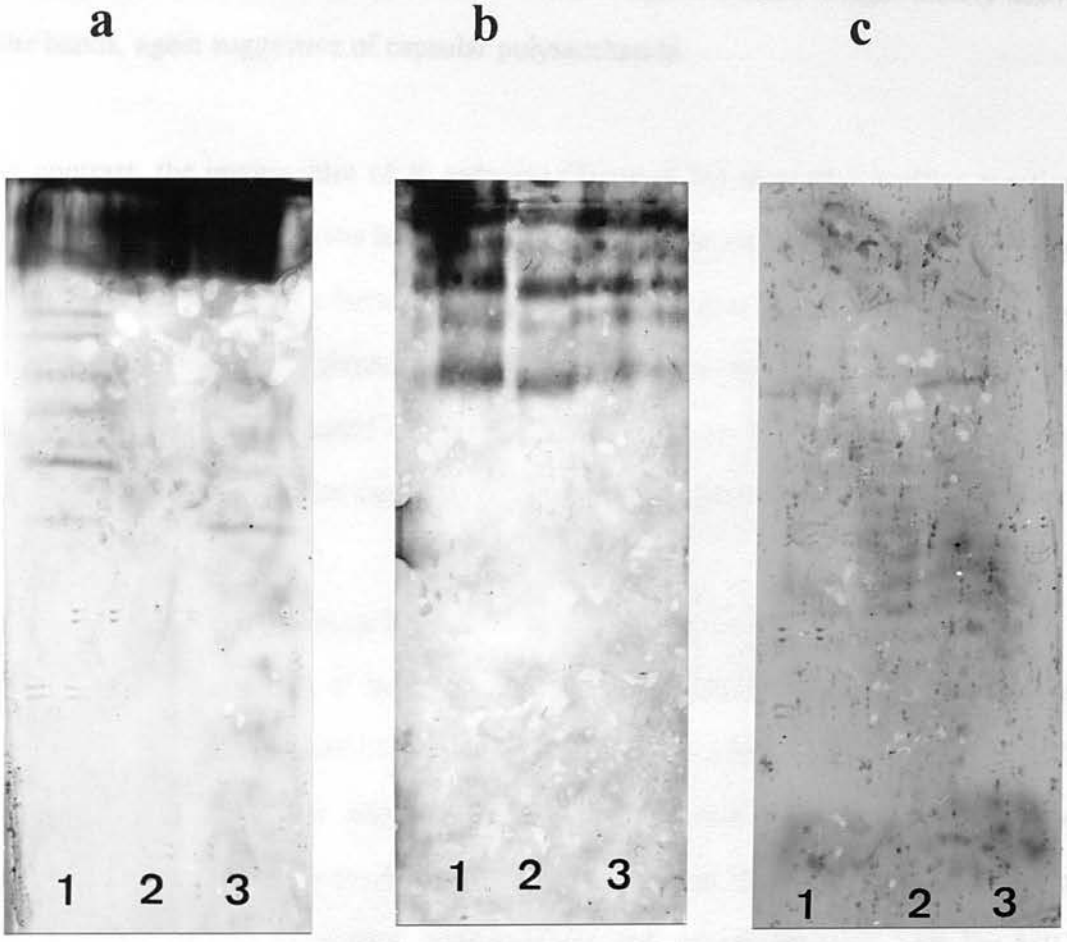


Figure 6.2a-c Immunoblots of proteinase K LPS digests of *B. fragilis*, and *B. vulgatus* extracted from different growth conditions. Figure 6.2a *B. fragilis* NCTC 9343 LPS extracted from growth in; Track 1, PPY; Track 2, VT&W; Track 3, VT&S; **Figure 6.2b** *B. fragilis* MPRL 1504 LPS; and **Figure 6.2c** *B. vulgatus* MPRL 1651 LPS extracted from the same growth conditions. Immunoblots were probed with rabbit sera raised against NCTC 9344, MPRL 1504 and MPRL 1651 respectively.

although the number and spacing of the bands varied between the two isolates. *B. fragilis* NCTC 9343 also exhibited a reactive high molecular weight moiety above the bands, again suggestive of capsular polysaccharide.

In contrast, the immunoblot of *B. vulgatus* (Figure 6.2c) showed a positive reaction against the core LPS and the ladder pattern but, the response was much weaker. The differences in antigenicity between the *Bacteroides* species may be important for the increased virulence of *B. fragilis*. No reaction was seen with *B. thetaiotaomicron* or *B. uniformis* despite repeated attempts. Furthermore, no change in antigenicity was observed with LPS samples extracted from different growth conditions.

The influence of antibiotics on LPS structure.

Growth in the presence of antibiotics has been reported to influence cell surface structure. As an inhabitant of the intestinal tract, it is probable that *B. fragilis* will encounter oral antibiotics which may influence LPS structure and hence virulence potential. To test this theory, *B. fragilis* NCTC 9343 and MPRL 1504 were grown in the presence of two antibiotics, metronidazole and chloramphenicol, with the MIC level determined by the standard discontinuous tube dilution assay (Table 6.1).

Table 6.1 MIC levels of *B. fragilis* NCTC 9343 and MPRL 1504 grown in the presence of metronidazole and chloramphenicol.

Strain	MIC (mg/l)	
	Metronidazole	Chloramphenicol
B. fragilis NCTC 9343	2	16
B. fragilis MPRL 1504	8	8

B. fragilis NCTC 9343 and MPRL 1504 were grown in minimal medium supplemented with ½, ¼ and $^1/_8$-MIC level of metronidazole and chloramphenicol. LPS was extracted by proteinase K digests including an unsupplemented minimal medium control.

a

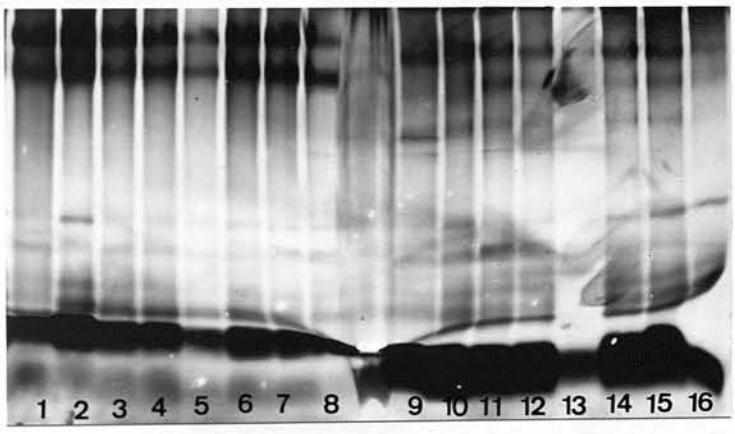
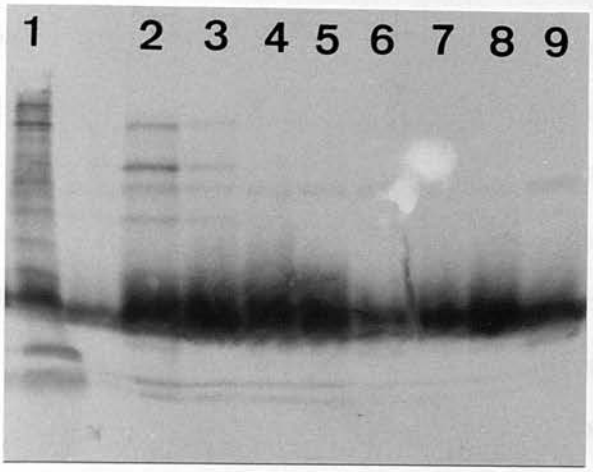


Figure 6.3a-c LPS profile of *B. fragilis* NCTC 9343 and MPRL 1504 grown in sub-MIC conditions. Figure 6.3a Silver-stained SDS-PAGE, Tracks 1-8 NCTC 9343 LPS extracted from growth in PPY; VT&W; $^1/_8$-MIC metronidazole; $^1/_4$-MIC metronidazole; $^1/_2$-MIC metronidazole; $^1/_8$-MIC chloramphenicol; $^1/_4$-MIC chloramphenicol; $^1/_2$-MIC chloramphenicol; Tracks 9-16 MPRL 1504 LPS extracted from the same growth conditions.

b



c

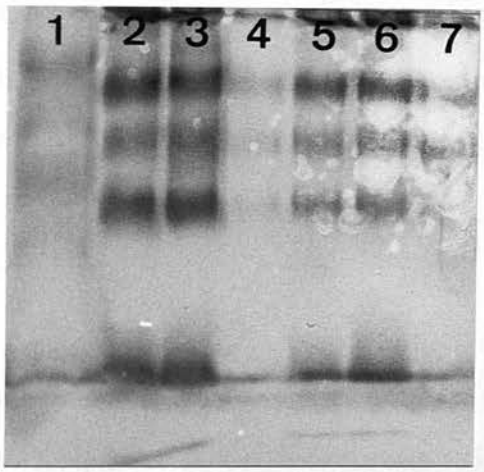


Figure 6.3b Immunoblot of NCTC 9343 LPS extracted from growth in; Track 1, VT&W; Track 2, $^1/_8$-MIC metronidazole; Track 3, $^1/_8$-MIC metronidazole; Track 4, $^1/_4$-MIC metronidazole; Track 5, $^1/_4$-MIC metronidazole; Track 6, $^1/_2$-MIC metronidazole; Track 7, $^1/_8$-MIC chloramphenicol; Track 8, $^1/_4$-MIC chloramphenicol; Track 9, $^1/_2$-MIC chloramphenicol; and **Figure 6.3c** Immunoblot of MPRL 1504 LPS extracted from growth in; Track 1, VT&W; Track 2, $^1/_8$-MIC metronidazole; Track 3, $^1/_4$-MIC metronidazole; Track 4, $^1/_2$-MIC metronidazole; Track 5, $^1/_8$-MIC chloramphenicol; Track 6, $^1/_4$-MIC chloramphenicol; Track 7; $^1/_2$-MIC chloramphenicol. Immunoblots were probed with rabbit sera raised against NCTC 9344 and MPRL 1504 respectively.

Figures 6.3a-c show the silver-stained PAGE and immunoblots of LPS preparations from NCTC 9343 and MPRL 1504 grown in sub-MIC conditions. For the silver-stained PAGE (Figure 6.3a), the characteristic core LPS, closely spaced bands and high molecular weight antigen is seen; with no obvious structural differences observed between growth in the sub-MIC antibiotic medium compared to the control medium. Immunoblots probed with rabbit antiserum did, however, show slight changes in antigenicity (Figure 6.3b-c). For *B. fragilis* NCTC 9343 (Figure 6.3b) the rabbit sera reacted with the core LPS and the banding pattern but not with the high molecular weight moiety, unlike the immunoblot in Figure 6.2a. In addition, LPS samples extracted from sub-MIC antibiotic media exhibited a reduction in band production, compared to LPS from minimal medium alone. For both *B. fragilis* isolates, LPS from the sub-MIC media showed an increase in core antigen production compared to the control, with the exception of MPRL 1504 LPS samples extracted from ½-MIC media which reacted very weakly and gave little reaction with core antigen (Figure 6.3c; Tracks 4 & 7). Thus, growth in antibiotic-supplemented medium induces slight antigenic changes which highlights the plasticity of the cell surface of *B. fragilis*.

6.2 CHARACTERISTICS AND ENVIRONMENTAL REGULATION OF OM

OM phenotype

Outer membranes were extracted from *B. fragilis* NCTC 9343 and MPRL 1504, grown in PPY. OM samples of *B. vulgatus* MPRL 1651 and *B. thetaiotaomicron* NCTC 10582 were gifted by E. Allan, Department of Medical Microbiology, Edinburgh. Samples separated by SDS-PAGE were stained with Coomassie blue (Figure 6.4) and as expected each *Bacteroides* species showed a distinct OM profile.

The influence of growth environment on OM structure

Outer membranes from two *B. fragilis* strains were extracted from growth in VT&W, VT&S, iron-limiting medium VT&E and bile-containing medium VT&B. As

observed with other Gram-negative bacteria, *B. fragilis* was responsive to different growth conditions with outer membrane protein production increasing or decreasing to change overall permeability and aid survival (Figure 6.5). Production of a major band of 41kD is induced for *B. fragilis* NCTC 9343 when grown in PPY (Track 1). Similarly, growth in VT&W appears to increase production of a 58kD OMP (Track 2). OM preparations from VT&B and VT&E gave a weaker response. For *B. fragilis* MPRL 1504, only the OM samples extracted from PPY, VT&S and VT&E gave a reaction; VT&W and VT&B samples did not react due to the low protein concentration in these extracts. However, differences in OMP profile between growth conditions were still observed, for example a 12kD band was seen only in the sample from VT&S (Track 8). Variation between the two *B. fragilis* samples was also highlighted, for example the band at 41kD was present in NCTC 9343 grown in PPY (Track 3) but not in MPRL 1504 (Track 6).

Immunoblotting

The outer membranes from *B. fragilis* NCTC 9343 and MPRL 1504 were extracted from growth in PPY, VT&W and VT&S and probed using rabbit sera raised against NCTC 9344 and MPRL 1504 (Figure 6.6a-b). A large number of bands gave a strong reaction, particularly the 41kD band from NCTC 9343 grown in PPY (Figure 6.6a, Track 3). Interestingly, there was a large cross-reaction between the OM samples of the two *B. fragilis* strains tested, implying that OM antigenic epitopes are conserved within the *B. fragilis* species.

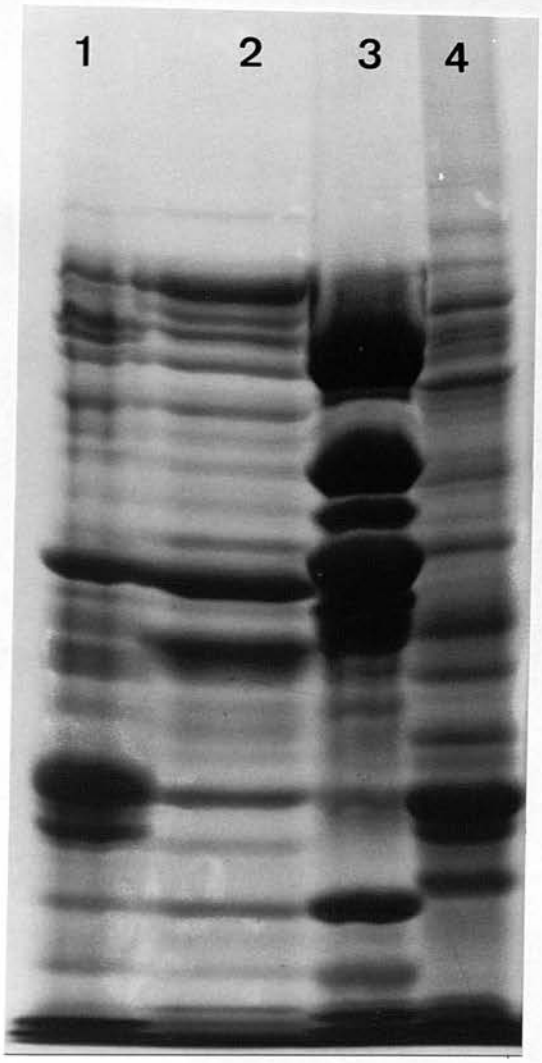


Figure 6.4 Coomassie blue stained outer membrane profiles of *B. fragilis*, *B. vulgatus* and *B. thetaiotaomicron* separated by SDS-PAGE (10% w/v acrylamide). Track 1 *B. fragilis* NCTC 9343; Track 2, *B. fragilis* MPRL 1504; Track 3, *B. vulgatus* MPRL 1651 and Track 4, *B. thetaiotaomicron* NCTC 10582.

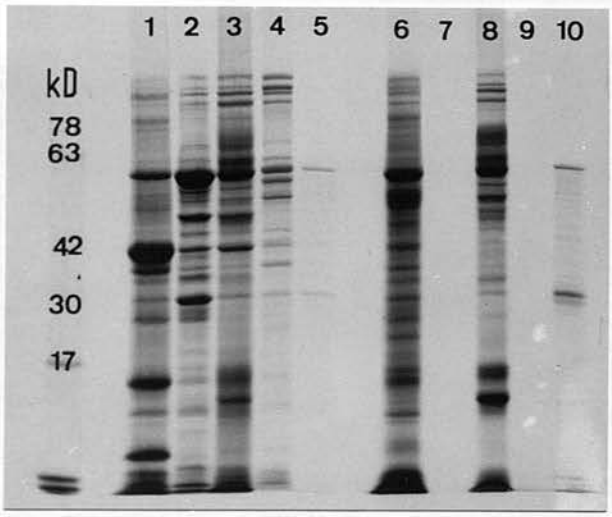


Figure 6.5 Outer membrane profiles of *B. fragilis* NCTC 9343 and MPRL 1504 extracted from different growth conditions and stained with Coomassie blue. Tracks 1-5 *B. fragilis* NCTC 9343 OM extracted from growth in PPY, VT&W, VT&S, VT&B, VT&E; Tracks 6-10 *B. fragilis* MPRL 1504 OM extracted from the same growth conditions.

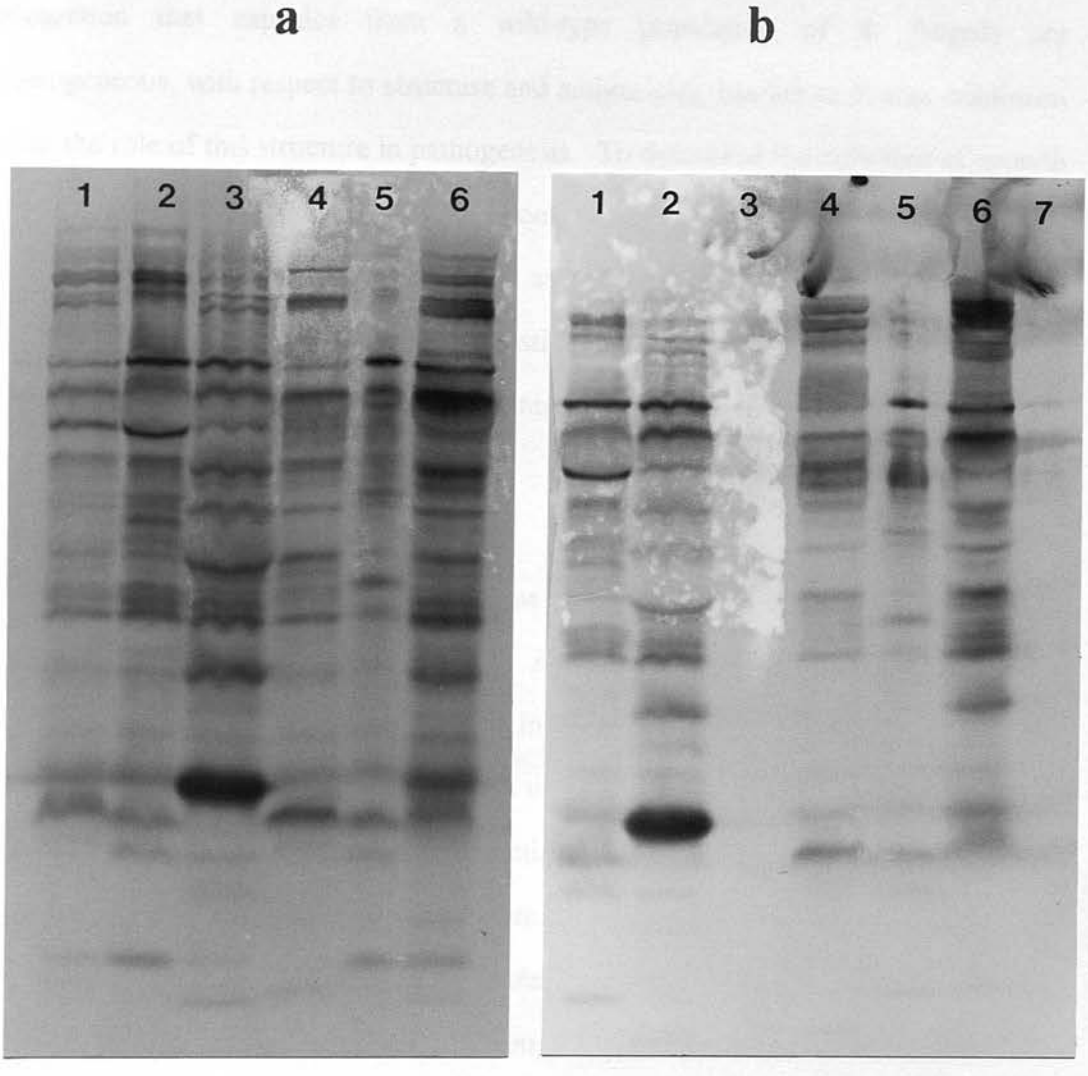


Figure 6.6a-b Immunoblots of outer membrane antigens of *B. fragilis* extracted from different growth conditions and probed with rabbit sera raised against (a) NCTC 9344 and (b) MPRL 1504. Figure 6.6a Tracks 1-3, NCTC 9343 OM samples from growth in PPY, VT&W, VT&S; Tracks 4-6 MPRL 1504 OM samples extracted from the same growth conditions. **Figure 6.6b** Tracks 1-2, NCTC 9343 OM samples from growth in PPY, VT&W; Tracks 3-7, MPRL 1504 OM samples from growth in VT&E, PPY, VT&W, VT&S and VT&B.

6.3 CHARACTERISTICS AND ENVIRONMENTAL REGULATION OF EPS

The influence of growth condition on EPS profile.

The capsular polysaccharide of *B. fragilis* is regarded as a major virulence determinant. However, the close association of the capsule with LPS and the recognition that capsules from a wild-type population of *B. fragilis* are heterogeneous, with respect to structure and antigenicity, has led to further confusion about the role of this structure in pathogenesis. To determine the influence of growth environment on EPS production, the Percoll profile of two *B. fragilis* strains, one *B. vulgatus* strain, one *B. uniformis* strain and one *B. thetaiotaomicron* strain grown in PPY, VT&W and VT&S, were investigated. In addition, the profile of the *B. fragilis* isolates grown in bile-supplemented medium VT&B, and iron-limited medium VT&E, was also examined.

EPS production from *Bacteroides* strains was found to be highly sensitive to environmental conditions. For example, *B. fragilis* MPRL 1504 grown in PPY produced mainly small capsules, growth in VT&W mainly large capsules and for growth in VT&S no capsular production was observed (Figure 6.7a). This result implies *B. fragilis* increases EPS production in conditions of nutrient limitation. However, the Percoll profile of other *Bacteroides* strains differed from MPRL 1504 (Figure 6.7b) and therefore no overall correlation between culture conditions and EPS production could be reached. Thus, although capsule production was shown to be dependent on environmental conditions, the pathological basis of these changes requires further investigation.

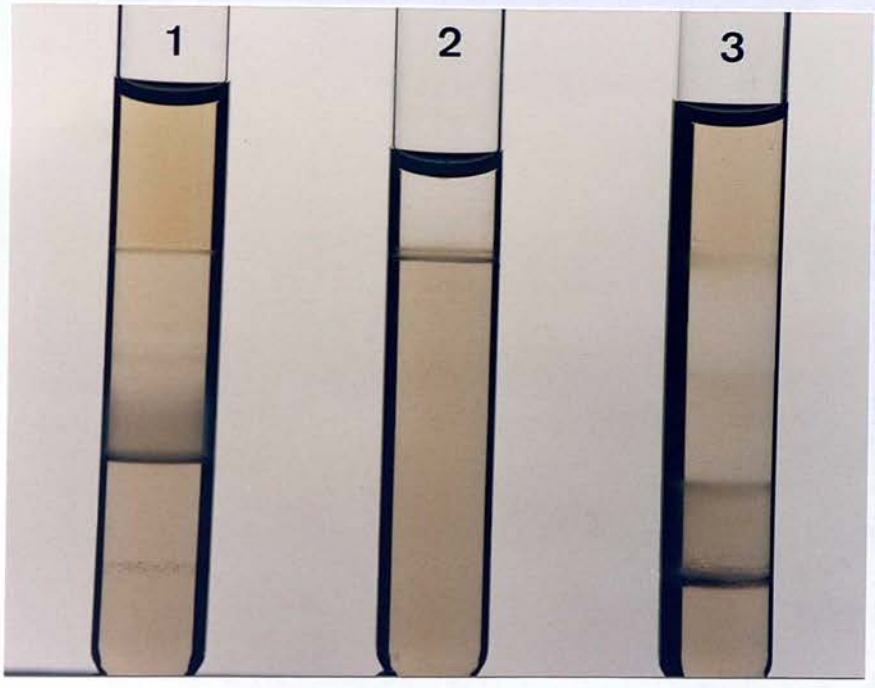


Figure 6.7a The influence of growth environment on the Percoll profile of *B. fragilis* MPRL 1504. Track 1, PPY; Track 2, VT&W; Track 3, VT&S.

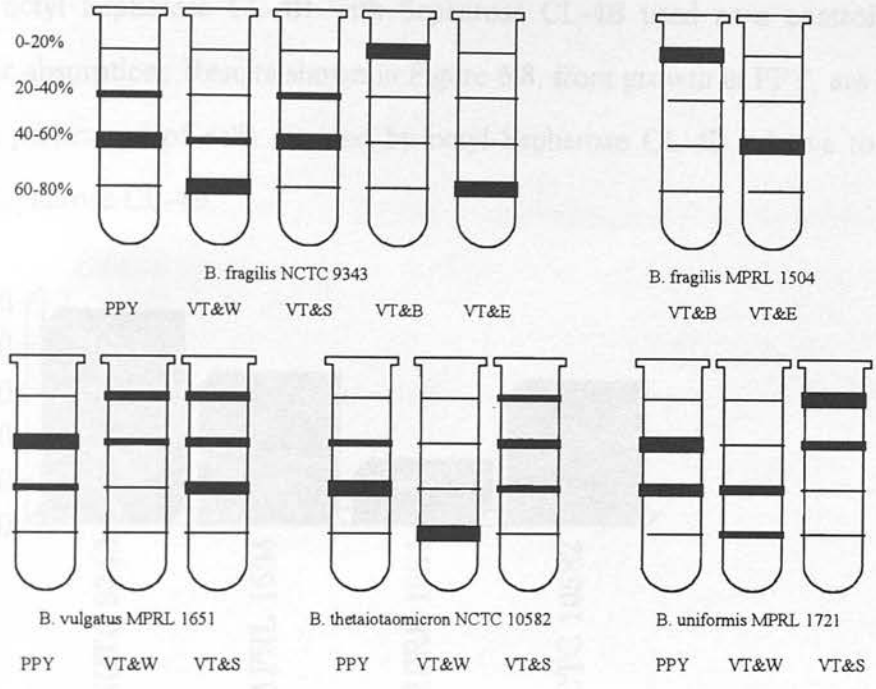


Figure 6.7b The influence of growth environment on the Percoll profile of *B. fragilis, B. vulgatus, B. thetaiotaomicron* and *B. uniformis* strains. The main areas of precipitation are marked with dark bands.

6.4 CELL SURFACE HYDROPHOBICITY

The cell surface hydrophobicity of four *Bacteroides* strains grown in PPY, VT&W and VT&S was determined by HIC. Specific hydrophobic interaction was measured using octyl Sepharose CL-4B with Sepharose CL-4B used as a control for non-specific absorption. Results shown in Figure 6.8, from growth in PPY, are expressed as the percentage of cells retained by octyl-Sepharose CL-4B relative to retention with Sepharose CL-4B.

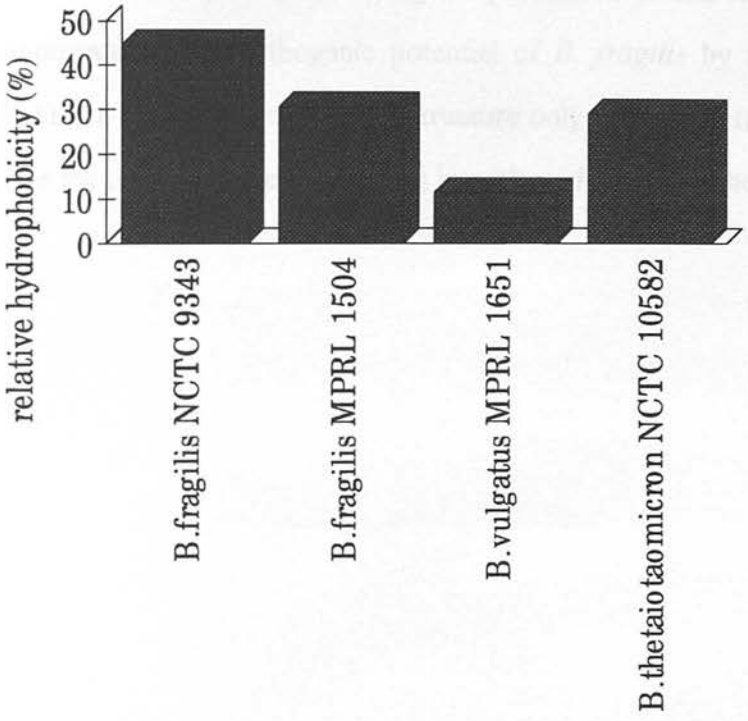


Figure 6.8 Cell surface hydrophobicity of *B. fragilis, B. vulgatus* and *B. thetaiotaomicron* grown in PPY.

Differences in cell surface hydrophobicity between the *Bacteroides* species are apparent; *B. fragilis* NCTC 9343 possessed the highest degree of hydrophobicity whereas *B. vulgatus* MPRL 1651 was found to be the least hydrophobic. The greater hydrophobicity of the *B. fragilis* isolates, and thus in theory the greater adherence, suggests a close association of *B. fragilis* with mucosal surfaces, confirming previous clinical observations. Unfortunately, due to the oxygen sensitivity of the bacteria

grown in VT&W and VT&S, no measurement of cell surface hydrophobicity could be determined for these culture conditions and thus the influence of growth environment could not be assessed.

In summary, members of the *Bacteroides* genus produce distinct LPS, OM and EPS profiles which vary with environmental growth conditions. This plasticity is a vital biological property, allowing organisms to adapt and survive in otherwise lethal situations. Furthermore, varying the production of cell surface components may be important to the pathogenic potential of *B. fragilis* by allowing this organism to induce a more virulent form of structure only when required. A possible pathogenic role for these observed changes is investigated further in the next chapter.

CHAPTER 7

RESULTS

BIOLOGICAL ACTIVITY OF THE CELL SURFACE COMPONENTS OF *BACTEROIDES FRAGILIS*

Experiments in this chapter were designed to investigate the potential role of *B. fragilis* cell surface components in SIRS, examining in particular biological endotoxicity and capacity to induce TNF-α. The influence of growth environment on these pathogenic properties was also determined.

PW-LPS samples were used throughout this chapter. *Bacteroides* extracts were prepared by E. Allan and the *E. coli* sample (O18K) by D. Delahooke, both of the Department of Medical Microbiology, Edinburgh.

7.1 THE ENDOTOXICITY OF *B. FRAGILIS* LPS SAMPLES

Dose response of LPS for LAL assay.

The LPS stock solution of *B. fragilis* NCTC 9343 was diluted in pyrogen-free water to a concentration of 10ng/ml, 1ng/ml and 0.1ng/ml to determine the optimum LPS concentration to use in the LAL assay.

Figure 7.1 shows the results obtained and as expected endotoxin values correlated with LPS concentration. The 10ng/ml sample gave the highest endotoxin reading but, unfortunately, produced optical density values outwith the normal standard range. The 1ng/ml sample, although lower in endotoxicity, produced OD values corresponding to the middle of the standard range and was thus chosen as the optimal LPS concentration to use.

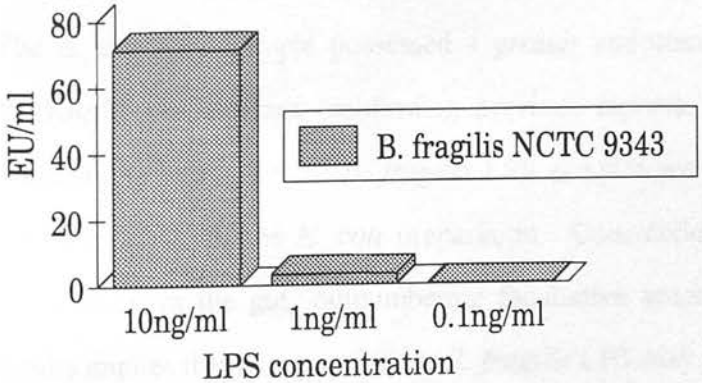


Figure 7.1 Dose response curve of *B. fragilis* NCTC 9343 LPS as measured by the LAL assay. Results shown are the mean of duplicates.

Comparison of LPS endotoxicity between *B. fragilis* and *E. coli*

The endotoxicity of LPS from *B. fragilis* NCTC 9343 and MPRL 1504 was compared to *E. coli* O18K (Figure 7.2).

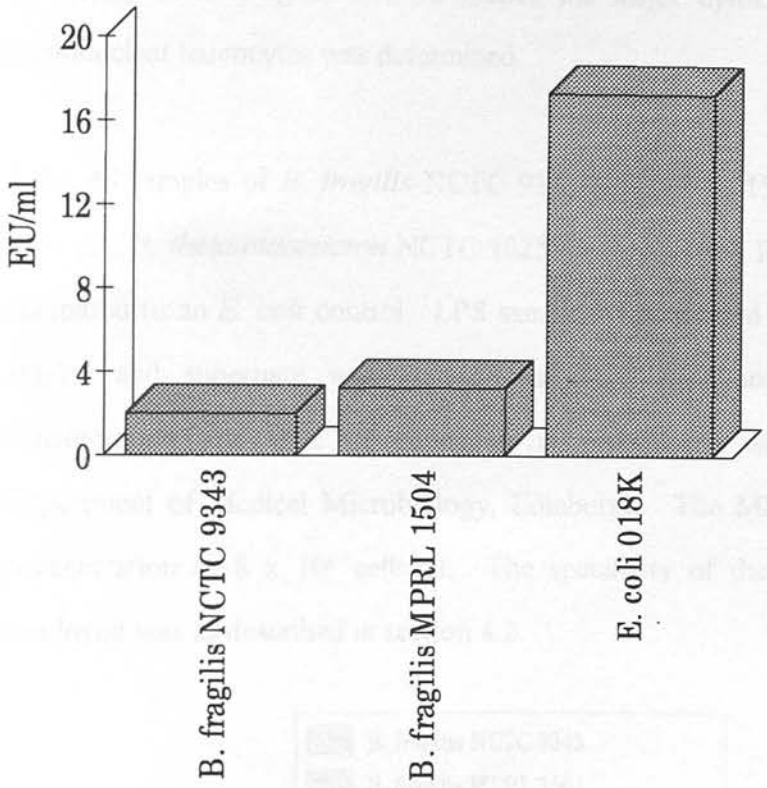


Figure 7.2 The endotoxic activity of *B. fragilis* and *E. coli* LPS preparations as measured by the LAL assay. LPS samples were used at a concentration of 1ng/ml. Results are the mean of duplicates.

The *E. coli* LPS sample possessed a greater endotoxic potential compared to the *B. fragilis* preparations, confirming previous reports. Surprisingly, however, the endotoxicity levels for the *B. fragilis* LPS samples were only five- to nine-fold less than the levels for the *E. coli* preparation. Considering the huge predominance of *Bacteroides* in the gut, outnumbering facultative anaerobes by 100-1000 fold, this results implies that as a population, *B. fragilis* LPS may possess as much, if not more, biological potential than *E. coli* LPS.

7.2 INFLAMMATORY POTENTIAL OF *B. FRAGILIS* LPS

A primary characteristic of SIRS is excessive and uncontrollable cytokine production. To test the hypothesis that *B. fragilis*, as a population, may play a vital role in SIRS, the ability of *B. fragilis* LPS to induce the major cytokine TNF-α from human mononuclear leucocytes was determined.

PW-LPS samples of *B. fragilis* NCTC 9343 and MPRL 1504, *B. uniformis* MPRL 1721 and *B. thetaiotaomicron* NCTC 10258 were assessed for inflammatory potential compared to an *E. coli* control. LPS samples were diluted in pyrogen free water to 1ng/ml and supernate was collected at 4h; determined as the optimal LPS concentration and time of collection in preliminary work by D. Delahooke, Department of Medical Microbiology, Edinburgh. The MNL cells were used at a concentration of 8 x 10^6 cells/ml. The specificity of the assay and the controls employed was as described in section 4.2.

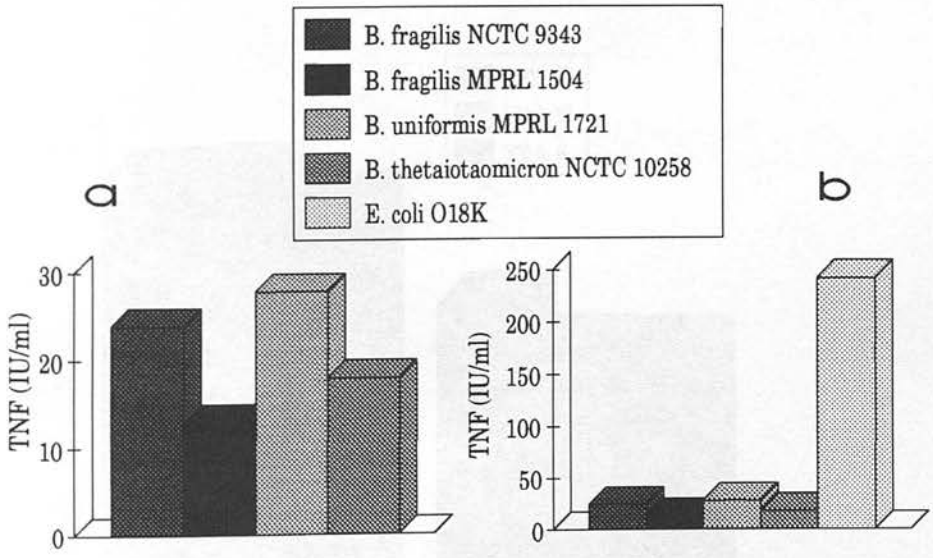


Figure 7.3 TNF-α levels produced from MNL in response to LPS preparations of *B. fragilis, B. uniformis, B. thetaiotaomicron* and *E. coli*. **(a)** The TNF-α response from *Bacteroides* strains only. **(b)** The TNF-α response from both *Bacteroides* strains and *E. coli*. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 4h. Results are mean of duplicates.

TNF-α induction levels varied between the *Bacteroides* species although no significant difference was measured, implying *B. fragilis, B. uniformis* and *B. thetaiotaomicron* possess the same degree of inflammatory potential (Figure 7.3). *E. coli* LPS induced a significantly higher level of TNF-α ($P<0.01$) than LPS preparations from *Bacteroides* species. However, more importantly, *E. coli* induced on average only twenty-fold more cytokine compared to *B. fragilis,* considerably less activity than the 100-1000 fold required to match, population for population, the potential of *Bacteroides* in the gut. This result contrasts with previous reports where *B. fragilis* was found to be 100-1000 fold less active than *E. coli.* Thus, the findings of the present study confirm the hypothesis that as a population, *Bacteroides* hold as much if not more biological activity compared to *E. coli.*

The influence of growth environment on TNF-α induction

PW-LPS samples from two strains of *B. fragilis* grown in PPY, VT&W and VT&S were used to assess the influence of growth environment on TNF-α induction.

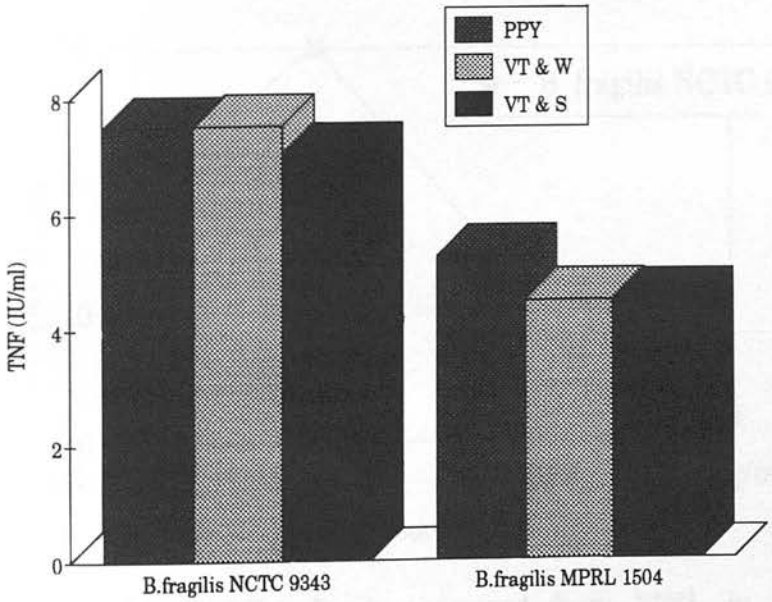


Figure 7.4 TNF-α levels induced by LPS samples of *B. fragilis* extracted from different growth conditions. LPS samples were used at a concentration of 1ng/ml. Supernate was collected at 4h. Results are the mean of duplicates.

No significant difference in TNF-α induction between the LPS samples extracted from different growth conditions was observed. Therefore, although growth condition influenced the structure of *B. fragilis* LPS, inflammatory potential does not appear to be affected by the growth media tested in this study.

7.3 INFLAMMATORY POTENTIAL OF *B. FRAGILIS* OM

Initial experiments assessed the inflammatory potential of OM preparations from *B. fragilis* NCTC 9343 and MPRL 1504, *B. vulgatus* MPRL 1651 and *B. thetaiotaomicron* NCTC 10258.

Dose response

The OM preparation from *B. fragilis* NCTC 9343 was diluted in pyrogen-free water to a protein concentration of 800μg/ml, 8μg/ml, 80ng/ml and 0.8ng/ml. Supernates were collected at 4h.

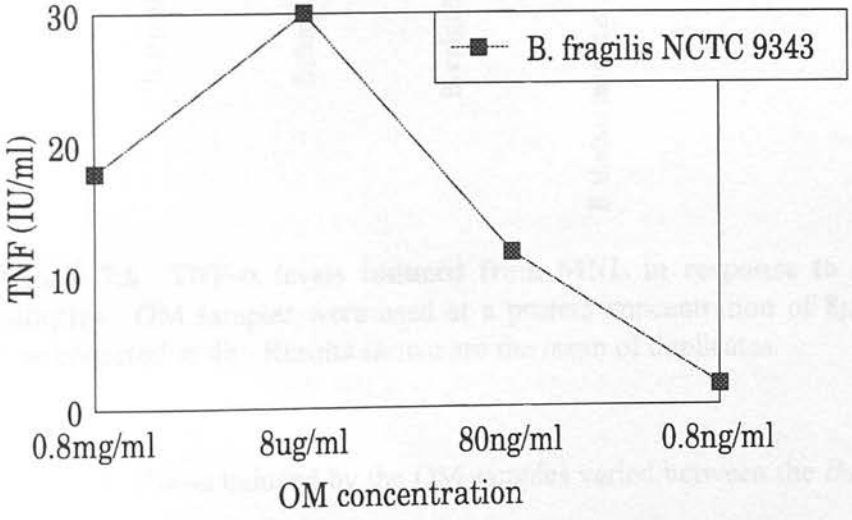


Figure 7.5 TNF-α levels produced from MNL in response to a protein concentration range of *B. fragilis* NCTC 9343 OM sample. Supernate was collected at 4h. Results are the mean of duplicates.

The OM concentration corresponding to optimal OD values was 8μg/ml. OM samples, diluted to a protein concentration of 8μg/ml, were assayed for their ability to induce TNF-α (Figure 7.6).

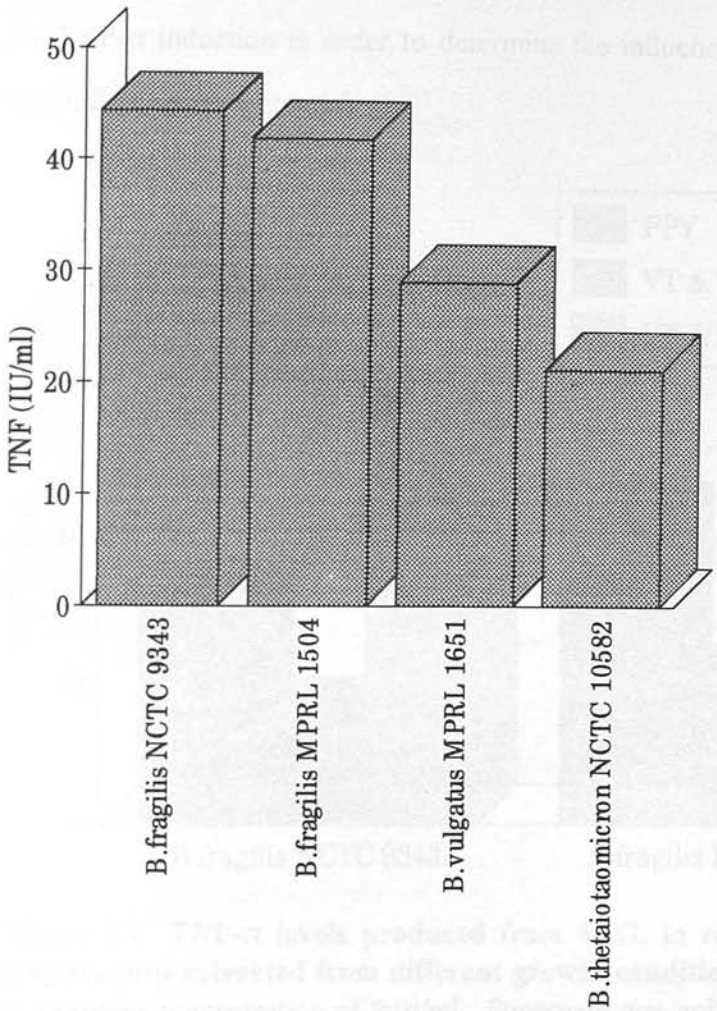


Figure 7.6 TNF-α levels induced from MNL in response to *Bacteroides* OM samples. OM samples were used at a protein concentration of 8μg/ml. Supernate was collected at 4h. Results shown are the mean of duplicates.

Levels of TNF-α induced by the OM samples varied between the *Bacteroides* strains. OMs from the *B. fragilis* strains induced more TNF-α compared to *B. vulgatus* MPRL 1651 and *B. thetaiotaomicron* NCTC 10582, which may be important for the greater virulence recognised with *B. fragilis*. However, further work is required to establish whether these differences are significant.

The influence of growth environment on TNF-α induction by OM samples

The structure of the Gram-negative outer membrane is more susceptible to environmental changes than LPS. Thus, outer membrane preparations from two *B. fragilis* strains, extracted from growth in PPY, VT&W and VT&S, were assessed for TNF-α induction in order to determine the influence of growth environment on OM inflammatory potential.

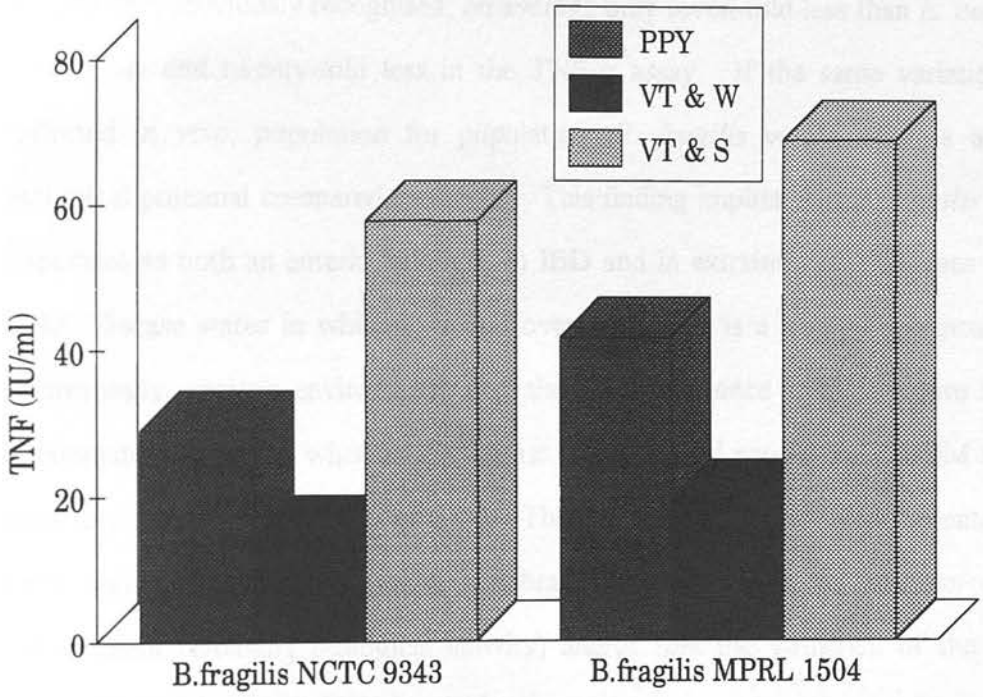


Figure 7.7 TNF-α levels produced from MNL in response to *B. fragilis* OM preparations extracted from different growth conditions. OM samples were used at a protein concentration of 8μg/ml. Supernate was collected at 4h. Results shown are the mean of duplicates.

The growth environments tested in this study influenced the inflammatory potential of the OM samples (Figure 7.7). OMs extracted from both *B. fragilis* strains grown in VT&S induced the highest levels of TNF-α whereas OMs extracted from growth in VT&W produced the lowest amounts. As the greatest activity was observed in serum-supplemented media this suggests an increased pathogenic potential for *B. fragilis in vivo,* both in the gut and in the systemic circulation. Furthermore, this

finding leads to two suggestions. Firstly, TNF-α induction is dependent on the presentation of LPS within the outer membrane, highlighting the influence of extraction method on biological activity. Secondly, the protein present in the OM plays an important role in the virulence of Gram-negative bacteria.

Thus, results gained in this section show that *B. fragilis* exhibits more biological activity than previously recognised; on average only seven-fold less than *E. coli* in the LAL assay and twenty-fold less in the TNF-α assay. If the same variation were reflected *in vivo*, population for population, *B. fragilis* would possess as much biological potential compared to *E. coli*. This finding implies that *B. fragilis* may be important as both an enteric pathogen in IBD and in extraintestinal diseases such as SIRS; disease states in which cytokine overproduction is a key pathological event. Interestingly, growth environment was shown to influence LPS structure but not inflammatory capacity, whereas in contrast the biological activity of the OM samples were dependent on growth conditions. This result suggests that the presentation of LPS within the Gram-negative membrane is susceptible to the surrounding environment (effecting biological activity) and/or that the virulence of the protein component of the OM is highly variable.

CHAPTER 8

DISCUSSION

(*Bacteroides fragilis*)

Historically, *Bacteroides fragilis* has been regarded primarily as a commensal of the human colon, despite being the most commonly isolated anaerobic bacterium from clinical specimens. Part of this paradoxical thinking is based on previous reports of the relative avirulence of *B. fragilis* in a variety of *in vivo* and *in vitro* models. For example Sveen *et al* (1977) found the doseage of *B. fragilis* LPS required to induce a local Shwartzman reaction in rabbits was over 1000-fold greater than for a *Salmonella* control. However, in recent years *B. fragilis* has gained increasing prominance as a serious human pathogen implicated in disease states such as abscess formation, colonic cancer, inflammatory bowel disease and, most importantly, SIRS.

In a large number of critically ill patients presenting with a septic clinical state there may be no positive bacterial culture or obvious foci of infection (Pape *et al*, 1994). It is now accepted that the probable source of bacteria for these patients is the gut microflora. Following episodes of shock-induced gut ischaemia, bacteria and their products may translocate from the intestine into the circulation via the portal vein. As a consequence, leucocytes, and in particular the liver macrophages or Kupffer cells, may become activated, stimulating production of proinflammatory cytokines, which may ultimately lead to the pathological changes associated with sepsis (Runcie & Ramsay, 1990). *Escherichia coli* has been considered previously the most important bacterium in SIRS by virtue of its extremely active LPS. However, the huge predominance of *Bacteroides* species in the intestine, outnumbering organisms such as *E. coli* by 100-1000 fold, suggests that as a population *Bacteroides* may possess as much biological potential as *E. coli*.

Progress into defining the potential pathogenic role of *B. fragilis* in disease has been hampered by the confusion and continuing controversy on the cell surface factors of this organism. For example, there remains debate on whether the LPS is rough or smooth. These problems are further emphasised by the discovery of Patrick *et al*

(1986) that a wild-type laboratory culture of *B. fragilis* is morphologically heterogeneous with growth conditions having a marked influence on cell surface structures.

Thus, this section of work aimed to determine the influence of environmental growth conditions on the expression of *B. fragilis* cell surface factors and the possible role of these structures in SIRS.

8.1 THE ENVIRONMENTAL MODULATION OF THE CELL SURFACE FACTORS OF *BACTEROIDES FRAGILIS*

Lipopolysaccharide

LPS preparations from *B. fragilis* and other representatives of the *Bacteroides* genus; *B. uniformis, B. vulgatus* and *B. thetaiotaomicron*, were analysed by SDS-PAGE and immunoblotting. Previous studies on *B. fragilis* have resulted in conflicting views on the LPS profile of this organism. Weintraub *et al* (1985) concluded that most strains of *B. fragilis* possessed a chemically and antigenically similar, if not identical, R-LPS type molecule. In contrast, Poxton & Brown (1986) found strains of *B. fragilis* to possess predominantly smooth, strain-specific LPS. In the present study the strains of *B. fragilis* tested exhibited core LPS, a series of high molecular weight bands and a high molecular weight moiety believed to be capsular antigen, highlighting the close association of *B. fragilis* cell surface factors. This finding agrees with the previous observations of Poxton & Brown (1986), although the common antigen defined by these authors was not always detected. *B. uniformis* and *B. thetaiotaomicron* possessed predominantly rough-LPS whereas *B. vulgatus* exhibited a ladder pattern characteristic of smooth LPS, emphasising the LPS heterogeneity between the different *Bacteroides* species. Whether the observed differences in LPS structure can be related to the reported variation in pathogenicity between *Bacteroides* species is discussed later.

In addition, growth environment was found to influence LPS structure. Two strains of *B. fragilis* showed variation in LPS structure between growth in rich and minimal media, furthermore, *B. vulgatus* exhibited a reduction in O-antigen when grown in the nutrient rich medium. However, no obvious difference in antigenicity between LPS samples from different growth conditions was observed. These findings highlight the complexity involved in defining the profile of *B. fragilis* LPS expression *in vivo*. This problem is further emphasised by the poor resolution of *B. fragilis* LPS by conventional PAGE methods, although attempts have been made to improve this situation (Maskell, 1994).

The most noticeable structural change in *B. fragilis* LPS was in minimal media supplemented with bile; a reduction in core LPS antigen and loss of the high molecular weight moiety, thought to be capsule, was observed. As bile salts are an important environmental factor in the gut, this finding leads to speculation on the co-ordinated expression of cell surface factors *in vivo*. For example, as capsular polysaccharide biosynthesis is increased in the presence of bile (section 6.3), the drain on energy resources may be compensated for by reducing LPS production. The genetic basis of these changes requires further investigation.

LPS was also extracted from *B. fragilis* strains grown in a variety of minimal media supplemented with a range of sub-MIC concentrations of metronidazole and chloramphenicol. Chloramphenicol is an effective antimicrobial agent affecting protein synthesis. Metronidazole is one of the most active non-β-lactam antibiotics used against anaerobes, effective when intracellular reduction of its nitro group produces cytotoxic compounds such as hydroxylamine, which inhibit nucleic acid synthesis. Metronidazole has been reported previously to increase both cell surface hydrophobicity and aniongenicity of *B. fragilis* strains, changing the *B. fragilis*-host interactions (Cavalcanti *et al*, 1991). In their study Cavalcanti *et al* concluded that

metronidazole may induce these alterations by interacting with negatively charged surface groups such as LPS.

Silver-stained SDS-PAGE of the sub-MIC LPS extracts failed to reveal any variation in LPS structure although immunoblots did show some slight changes in antigenicity compared to an unsupplemented control. *B. fragilis* NCTC 9343 grown in sub-MIC media produced fewer bands compared to the control. In addition, both *B. fragilis* strains exhibited a greater expression of core-LPS antigen when extracted from $^1/_8$- and ¼-MIC compared to the control. These findings suggest that in the presence of sub-MIC concentrations of metronidazole and chloramphenicol, LPS O-antigen is reduced and production of core LPS increased, implying that a more hydrophobic LPS is indeed expressed which may increase both binding efficiency to host cells and phagocytic engulfment. How the antibiotics regulate the LPS biosynthetic enzymes remains to be defined.

To establish whether other growth environments shown in this study to influence LPS structure also altered cell surface hydrophobicity, strains of *B. fragilis, B. vulgatus, B. thetaiotaomicron* and *B. uniformis* grown in nutrient rich medium, minimal medium and serum-supplemented medium were assessed by HIC. Unfortunately, although all material was prereduced and kept as anaerobic as possible, the HIC procedure lasted approximately three hours and none of the strains tested gave viable counts from the VT&W and VT&S media, highlighting the oxygen-sensitivity of *Bacteroides* strains in these growth environments. Therefore, the influence of growth environment on cell surface hydrophobicity could not be ascertained. However, cell surface hydrophobicity measurements were made from the strains grown in PPY.

Variations between different *Bacteroides* species and different *B. fragilis* isolates were noted. *B. fragilis* NCTC 9343 was found to be the most hydrophobic and

therefore, in theory, the most efficient at binding, whilst *B. vulgatus* MPRL 1651 was the least hydrophobic. Further experiments with more strains would be required to establish whether the cell surface hydrophobicity of these species correlated with their predominant association with either faecal or colonic wall material. However, the influence of oxygen-levels on these results is undetermined and should not be disregarded, as this may account for the observed differences.

Outer membrane

The OMPs of *B. fragilis* are not considered major virulence determinants although a 44kD protein has been postulated as an iron-receptor and may play an important role *in vivo* (Otto *et al*, 1990). The OMP profile of four *Bacteroides* strains were shown to be distinct, although as expected, the two *B. fragilis* strains showed the most similarity. *B. fragilis* produced an unusually large number of bands, detectable both by Coomassie blue staining and by immunoblotting, implying that *B. fragilis* has a large number of transport mechanisms.

The OMs of *B. fragilis* extracted from growth conditions likely to be encountered *in vivo*, influenced both OM structure and antigenicity. The function of many of the OM proteins that were induced or repressed have yet to be characterised, although a similar study in our laboratory showed such changes are unlikely to be related to serum-sensitivity (E. Allan, personnal communication). However, the observed variations in OM profile may be influencing the presentation and biological activity of LPS in the outer membrane, as discussed in section 8.2. The co-ordinated regulation of *B. fragilis* cell surface structures is possible as a signal transduction system in this organism, thought to be responsible for regulating outer membrane proteins, was initially identified because of increased antibiotic resistance (Rasmussen & Kovacs, 1993), which, in this study, has been shown to alter LPS structure.

Exopolysaccharide

The capsular polysaccharide of *B. fragilis* is well-known as a potentiating agent of abscess formation and is regarded as a major virulence determinant (Tzianabos *et al*, 1992). The capsule of *B. fragilis* is heterogeneous, distinguished under the electron microscope, as large, small or an electron dense layer. Percoll discontinuous density centrifugation has been used to separate out these heterogeneous populations for further investigations and was used in this study to determine the influence of growth environment on EPS production. The particularly close association of the surface polysaccharides of *B. fragilis* and the flexibility of the LPS under different growth conditions suggests EPS may behave in a similar manner. Indeed, the results in this study show EPS to be highly influenced by growth condition, with different subpopulations apparent. However, no correlation with growth condition and capsule production between different strains was determined. For example, one *B. fragilis* isolate possessed a large capsule when grown in minimal media, whereas another *B. fragilis* isolate produced a small capsule. However, in bile-supplemented minimal media, the EPS profile of both *B. fragilis* isolates showed production of a large capsule. This may account for the absence of the high molecular weight moiety (speculated to be capsular antigen) in the LPS SDS-PAGE gels, as the larger capsule may simply have been too large to enter the gel.

Once again these findings lend speculation to the co-ordinated environmental regulation of *B. fragilis* cell surface factors. It is interesting to consider that if indeed co-ordinated regulation occurs *in vivo*, then a relatively avirulent *B. fragilis* strain may express a more virulent form of surface structure when exposed to a suitable environmental stimulus, as may occur when bacteria move from inside to outside the gut. Co-ordinated environmental regulation of the cell surface would undoubtedly be an important intrinsic property contributing to *B. fragilis* pathogenesis.

In summary, all the cell surface factors of *B. fragilis* were shown to be flexible to growth conditions, reiterating the complexity of the surface of this organism which has not always been taken into account by previous workers investigating the biological potential. Thus, the following section reassessed the biological activity of *B. fragilis* cell surface factors from different growth environments and the potential role of *B. fragilis* in SIRS was determined.

8.2 THE BIOLOGICAL ACTIVITY OF CELL SURFACE FACTORS OF *BACTEROIDES FRAGILIS*.

In the present study the capacity to stimulate TNF-α from mononuclear leucocytes was determined. TNF-α is a major cytokine induced from monocytes and macrophages and is a prime mediator of the pathological changes observed in septic patients (section 1.3).

LPS extracts from *B. fragilis, B. uniformis* and *B. thetaiotaomicron* were shown to induce variable TNF-α levels, although the variation could not account for the previously observed pathogenicity differences between these species. For example, *B. fragilis* has been reported to have a greater pathogenic potential compared to *B. uniformis* which was not reflected here by inflammatory capacity. Furthermore, TNF-α induction did not depend on LPS profile. Surprisingly, growth environment did not appear to influence the biological activity of the LPS samples.

However, regardless of growth media, *B. fragilis,* and indeed all the *Bacteroides* isolates, induced on average only a 20-fold lower TNF-α level compared to an *E. coli* control, far less than the 100-1000 fold level that *Bacteroides* outnumber facultative anaerobes in the gut. This surprising result was further confirmed by endotoxicity measurements, as determined by the LAL assay, where *B. fragilis* and *E. coli* LPS differed by less than nine-fold. These findings are particularly important as they

suggest that in cases of intra-abdominal infection, and particularly in SIRS, where cytokine production is a major feature, *B. fragilis,* as a population, possesses more biological potential compared to *E. coli.* This theory is further confirmed by complementary studies in our laboratory investigating *Bacteroides* antibody levels in septic patients (Allan *et al*, 1995). In that study, Allan *et al*, found that in the majority of non-survivors tested, IgG levels against *B. fragilis* LPS were significantly higher than for a control mixture of enterobacteria and *P. aeruginosa* LPS. This finding implies that some septic patients, do indeed, have a high exposure to *B. fragilis* LPS.

Furthermore, anaerobic bacteraemia comprises 5% to 25% of all bloodstream cases with *B. fragilis* stated as the most common cause, accounting for up to 65% of anaerobic isolates and with an attributable mortality of 19% (Redondo *et al*, 1995). However, *B. fragilis* still remains behind *E. coli, Klebsiella, Serratia* and *P. aeruginosa* in terms of overall blood culture isolates. The results of the present study show the importance of *B. fragilis* as a human pathogen and suggest that the lower culture frequency of this organism in bacteraemia may simply be due to the high redox potential of blood, especially considering the oxygen-sensitivity of this organism when grown in serum-supplemented media as was observed in this study.

OM samples extracted from different growth conditions were also tested for inflammatory capacity. Different *Bacteroides* species induced variable TNF-α levels, highlighting once more the heterogeneity of this genus. In contrast to the observations made with the LPS samples growth environment influenced the inflammatory capacity of the OM samples. OMs extracted from both *B. fragilis* strains grown in VT&S induced the highest levels of TNF-α whereas OMs from growth in VT&W gave the lowest amounts. These findings suggest that the biological activity of *B. fragilis* and other *Bacteroides* species depends on the presentation of the LPS within the outer membrane.

It is important to stress that these studies show *B. fragilis* to be more biologically active than previously recognised. The discrepancy between the present study and previous reports may, in part, be due to extraction methods and culture conditions. However, the present study confirms that *B. fragilis* can play a vital role in gut-derived sepsis and any new anti-sepsis therapy should consider this organism. Studies on the pathogenic potential of *B. fragilis* have been extended in concurrent investigations by fellow laboratory colleagues and similar observations have been made. Furthermore, the presentation and surrounding environment of *B. fragilis* LPS is important to its biological activity, particularly the influence of closely-associated surface structures. To what extent, or if at all, co-ordinated regulation of *B. fragilis* cell surface factors occurs *in vivo*, remains to be defined.

CONCLUSIONS

To the reader who has reached the end of this thesis it will be apparent that this study was a joint project, investigating two apparently diverse Gram-negative bacteria, *Burkholderia cepacia* and *Bacteroides fragilis*. As with most studies, results obtained from preliminary investigations and advances made by laboratory colleagues and other researchers within the field, has meant more emphasis has been placed on one particular area, in this case investigating *B. cepacia*, which accounts for the difference in volume of thesis dedicated to each organism.

However, significant results were obtained for both *B. cepacia* and *B. fragilis*. The cell surface of both organisms was shown to be influenced by environmental conditions and the biological activity of constituent molecules much greater than was previously recognised. For *B. cepacia*, the ability to induce two major proinflammatory cytokines was greater than the other major CF pathogen *Pseudomonas aeruginosa*. In addition, this study has established the LPS of both *B. cepacia* and *P. aeruginosa* to signal via a CD14-independent pathway and, surprisingly, the ability of *P. aeruginosa* to modulate the inflammatory response of *B. cepacia*. Therefore, the inflammatory potential of *B. cepacia* may contribute to the destructive immune-mediated damage observed in CF patients. For *B. fragilis*, the ability to induce TNF-α was only twenty-fold lower than *Escherichia coli*, much less than the 100-1000 fold excess required for *E. coli* to outnumber, population for population, *B. fragilis*. Thus, *B. fragilis* may play a vital role in disease states with an intestinal origin, including IBD and SIRS. Future studies should include investigations into the influence of anti-inflammatory cytokines and neutralising antibodies on the biological potential and hence pathogenesis of *B. cepacia* and *B. fragilis*.

PUBLICATIONS

SHAW, D. AND POXTON, I.R. (1994) The endotoxic activity of *Burkholderia cepacia* lipopolysaccharide. *J. Endotoxin Res.* 1 S1: 40

SHAW, D., POXTON, I.R. AND GOVAN, J.R.W. (1995) Biological activity of *Burkholderia* (*Pseudomonas*) *cepacia* lipopolysaccharide. *FEMS Immunol. Med. Microbiol.* 11: 99-106.

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ORAL PRESENTATION

SHAW, D., POXTON, I.R. AND GOVAN, J.R.W. (1995) The induction of TNF by *B. cepacia* and *P. aeruginosa* lipopolysaccharide. 20th European cystic fibrosis conference, Brussels: O9

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Biological activity of *Burkholderia (Pseudomonas) cepacia* lipopolysaccharide

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Abstract

Burkholderia cepacia has emerged as an important multiresistant pathogen in cystic fibrosis (CF), associated in 20% of colonised patients with a rapid and fatal decline in lung function. Although knowledge of *B. cepacia* epidemiology has improved, the mechanisms involved in pathogenesis remain obscure. In this study, *B. cepacia* lipopolysaccharide (LPS) was assessed for endotoxic potential and the capacity to induce tumour necrosis factor (TNF). LPS preparations from clinical and environmental isolates of *B. cepacia* and from the closely related species *Burkholderia gladioli* exhibited a higher endotoxic activity and more pronounced cytokine response in vitro compared to preparations from the major CF pathogen *Pseudomonas aeruginosa*. This study may help to explain the vicious host immune response observed during pulmonary exacerbations in CF patients colonised by *B. cepacia* and lead to therapeutic advances in clinical management.

Keywords: Burkholderia cepacia; *Pseudomonas cepacia*; Cystic fibrosis; Lipopolysaccharide; Tumour necrosis factor

1. Introduction

Chronic pulmonary infection leading to an intense host immune response and recurring episodes of exacerbation continue to be the major causes of lung disease in patients with cystic fibrosis (CF) [1]. Although *Pseudomonas aeruginosa* remains the leading CF pathogen affecting up to 90% of patients, *Burkholderia (Pseudomonas) cepacia* has recently emerged as a major cause for concern based on its multiresistance [2,3], transmissibility [4] and association with the 'cepacia syndrome'; a rapid fatal decline in lung function seen in 20% of colonised patients, complicated on occasion by septicaemia [5,6]. *B. cepacia* produces few recognised virulence factors and the mechanisms of pathogenesis in the CF lung associated with this organism remain obscure [7].

Lipopolysaccharide (LPS; endotoxin) is a classic bacterial virulence factor the biological properties of which include potent immunostimulatory effects on mononuclear cells, granulocytes and B lymphocytes [8]. Activation of the immune cells results in the synthesis and secretion of inflammatory mediators or cytokines that are required for the development, maintenance and regulation of the host immune response [9]. However, the pathophysiological consequences of overproduction of cytokines can be severe as observed by the numerous clinical sequelae associated with endotoxic shock. Greally et al. [10]

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suggested that the cytokine response they observed to *P. aeruginosa* infection in CF patients contributed to the airways inflammation and airflow obstruction associated with acute lung injury. Similarly, Wilson et al. [11] found a strong association between increasing plasma levels of TNF-α and IL-1β (two pivotal cytokines produced early in infection) and pulmonary deterioration.

The aim of our study was to assess the capacity of *B. cepacia* LPS to induce TNF from human mononuclear cells and to compare this activity with LPS from the related phytopathogen, *Burkholderia gladioli* and from *P. aeruginosa*. *B. gladioli* was included because of controversy concerning its clinical significance in CF patients, including speculation that multiresistant, epidemic *B. cepacia* strains may be hybrids of both *B. cepacia* and *B. gladioli* [12,13]. The Limulus amoebocyte lysate (LAL) assay was used as an additional indicator of endotoxic activity.

2. Materials and methods

2.1. Bacteria and media

The bacterial strains used in this study are described in Table 1. All isolates were identified as *B. cepacia* by the API 20NE system (bioMerieux, Marcy l'Etoile, France) and individual strains characterised further by bacteriocin typing [14] and pulse field gel electrophoresis (CHEF, BioRad Laboratories Inc.) [15] to ensure a lack of clonal relationship. Bacteria were grown in 5l batches of nutrient broth (Oxoid) + 0.5% yeast extract (Difco) (NB + YE) at 37° C, in an orbital incubator at 200 rev min^{-1} overnight.

Table 1
Bacterial strains, origin and LPS phenotype

Strain	Origin	LPS Phenotype
B. cepacia C1359 [a]	CF sputum	R
B. cepacia C1409	CF sputum	S
B. cepacia C1504	CF sputum	R/S [b]
B. cepacia ATCC 17762	Urine	R/S [b]
B. cepacia J2540	Soil	S
B. gladioli ATCC 10248	Type strain	S
P. aeruginosa PAO1	Type strain	S
P. aeruginosa C1250	CF sputum	S

[a] An epidemic strain isolated from several CF centres [4].
[b] R-LPS observed by silver-stained PAGE, S-LPS observed by immunoblot.
R = rough-type LPS.
S = smooth-type LPS.

2.2. LPS extraction and analysis

Bacterial LPS preparations were obtained by a version of the phenol-water (PW) method described by Westphal and Luderitz [16] which omitted ultracentrifugation. LPS PW samples were resuspended to a concentration of 5 mg ml^{-1} in pyrogen-free water. PAGE analysis was performed on 14% w/v acrylamide gels using the buffer system of Laemmli [17] with SDS omitted from the stacking and separating gel buffers. Samples (15 μl for silver staining or 45 μl for immunoblotting) were loaded onto gels. Gels were oxidised with periodic acid and silver-stained by the modified method of Tsai and Frasch [18] as described by Hancock and Poxton [19].

2.3. Immunoblotting

Antigens separated by PAGE gels as described above were transferred to nitrocellulose membranes (0.2 μm pore size, Schleicher and Schuell, Dassel) overnight at 10–12 V using the Tris, glycine, methanol buffer of Towbin et al. [20]. Blots were developed as described by Hancock and Poxton [19] using either rabbit sera raised against whole bacteria or human sera from colonised CF patients as the first antibody. Serum was diluted 1 in 200 in 1% gelatin Tris buffered saline. Anti-rabbit (ICN Flow) and anti-human (Sigma) IgG-horseradish peroxidase antibodies were used as the anti-first antibody conjugate.

2.4. Limulus amoebocyte lysate (LAL) assay

The endotoxicity of the LPS preparations was measured by the LAL assay using the kinetic method of the Coatest© Endotoxin Kit (Chromogenix). LPS samples (50 μl) were diluted in pyrogen-free water (Blood Transfusion Service) to 5 ng ml^{-1}–0.05 ng ml^{-1} and added to a flat-bottomed microtitre plate. The control endotoxin (*E. coli* O111:B4) was serially diluted 1 in 5 to provide a standard range of 24–0.0384 EU ml^{-1}. Chromogenic LAL reagent (20 μl) was added to the wells by means of a transfer

plate to ensure that each well received the reagent at the same time. Plates were read kinetically every 19 s for 90 min in a thermomax plate reader (Molecular Devices) at 405 nm. All material coming in contact with the sample was purchased as endotoxin-free or depyrogenated by heating to 250° C for 2.5 h.

2.5. Induction and estimation of TNF from human leucocytes by LPS

Human mononuclear leucocytes (MNL; approximately 30% monocytes) from freshly collected buffycoats (supplied by the Blood Transfusion Service, Edinburgh, UK) were separated on lymphocyte separation medium (ICN Flow) following a two-fold dilution in RPMI 1640. Cells were harvested at 1000 × g for 30 min and the monocyte layer washed three times in RPMI for 15 min. Leucocytes were counted in a haemocytometer and diluted in RPMI supplemented with 10% foetal calf serum (FCS), penicillin (100 μg ml^{-1}; Sigma), streptomycin (100 μg ml^{-1}; Sigma) and 1 mM L-glutamine to a concentration of 8×10^6 cells ml^{-1}. Cells (180 μl) and LPS samples (20 μl) diluted to the ng ml^{-1} range were added to a round-bottom microtitre plate and incubated at 37° C in a 5% CO_2 atmosphere. Culture supernates (100 μl) were collected 3.5 h after incubation, except for the time-course experiments, and stored at −20° C until required.

The bioassay for TNF used a mouse fibroblastic cell line, L929, which is sensitive to the cytotoxic effects of TNF. Cells were grown in minimum essential medium eagle (MEM; Sigma) supplemented with 5% FCS, penicillin, streptomycin and L-glutamine as before. Cells were dislodged by 0.05% trypsin/0.02% EDTA digestion and resuspended to a concentration of 3×10^5 cells ml^{-1}. L929 cells (100 μl) were added to the wells of a flat-bottom

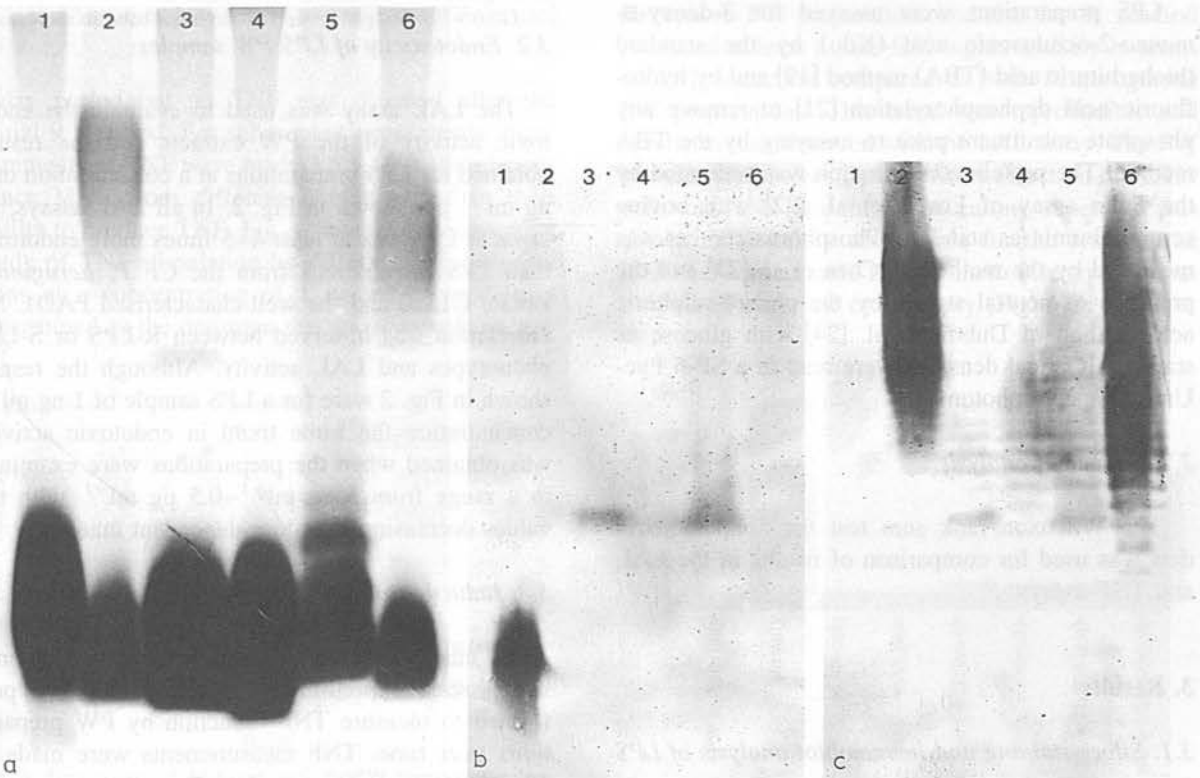

Fig. 1. (A) Silver-stained PAGE (14% acrylamide) of PW preparations. Track 1, C1359; 2, C1409; 3, C1504; 4, ATCC 17762; 5, J2540; 6, ATCC 10248. (B) Immunoblot of PW preparations probed with rabbit serum raised against *B. cepacia* C1359. Track 1, C1359; 2, C1409; 3, C1504; 4, ATCC 17762; 5, J2540; 6, ATCC 10248. (C) Immunoblot of PW preparations probed with rabbit serum raised against *B. cepacia* C1409. Track 1, C1359; 2, C1409; 3, C1504; 4, ATCC 17762; 5, J2540; 6, ATCC 10248.

microtitre plate and incubated overnight at 37° C, 5% CO_2. After the growth medium was discarded and replaced by MEM containing actinomycin D (2 μg ml^{-1}; Sigma), to stop growth of the L929 cells, 100 μl of test supernate diluted 1 in 5 in MEM was added. Human recombinant TNF-α standard (National Institute for Biological Standards and Control) was serially diluted 1 in 5 in MEM to give a standard range of 1000–0.0128 IU ml^{-1} TNF. After overnight incubation at 37° C, 5% CO_2, filtered crystal violet (0.5% CV in 20% methanol) was added at 100 μl per well to stain surviving cells. Plates were washed under running tap water, dried and 100 μl of 20% (v/v) acetic acid added to dissolve the CV crystals. Plates were read at 585 nm in a V_{max} plate reader (Molecular Devices) and converted to TNF equivalents.

2.6. Chemical analysis of LPS

LPS preparations were assayed for 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) by the standard thiobarbituric acid (TBA) method [19] and by hydrofluoric acid dephosphorylation [21] to remove any phosphate substituent prior to assaying by the TBA method. The protein concentration was estimated by the Folin assay of Lowry et al. [22] with bovine serum albumin as standard. Phosphorus content was measured by the method of Chen et al. [23] and the presence of neutral sugars by the phenol-sulphuric acid method of Dubois et al. [24] with glucose as standard. Optical densities were read in a SP-6 Pye-Unicam spectrophotometer.

2.7. Statistical analysis

The Wilcoxon rank sum test for nonparametric data was used for comparison of results in the LAL and TNF assays.

3. Results

3.1. Silver-staining and immunoblot analysis of LPS

Silver-stained polyacrylamide gels employed to visualise the content of the PW extracts used in the bioassays confirmed that clinical isolates of *B. cepacia* could possess either rough or smooth LPS (Fig. 1a). Immunoblots of the PW extracts showed some cross-reactivity between the LPS of different *B. cepacia* strains and other species including *B. gladioli*. Serum from a rabbit immunized with *B. cepacia* C1359 demonstrated a positive reaction with the low molecular mass core LPS of this strain (Fig. 1b, track 1). Two other *B. cepacia* strains gave reaction with one band at the lower end of the high molecular mass ladder (Fig. 1b, tracks 3 and 5). However, with serum from a rabbit immunized with *B. cepacia* C1409, the high molecular weight moiety of LPS from C1409, C1504, ATCC 17762, J2540 and *B. gladioli* showed a positive reaction (Fig. 1c, tracks 2–6). Identical reactions were produced using serum from CF patients colonised with the C1359 or C1409 strain (data not shown). *P. aeruginosa* LPS from strains PAO1 and C1250 did not exhibit cross-reactivity with any of the *B. cepacia* strains at the serum concentration used (data not shown).

3.2. Endotoxicity of LPS PW samples

The LAL assay was used to evaluate the endotoxic activity of the PW extracts and the results obtained for LPS preparations at a concentration of 1 ng ml^{-1} are shown in Fig. 2. In all LAL assays, *B. cepacia* LPS was at least 4–5 times more endotoxic than LPS preparations from the CF *P. aeruginosa* isolate C1250 and the well-characterised PAO1. No correlation was observed between R-LPS or S-LPS phenotypes and LAL activity. Although the results shown in Fig. 2 were for a LPS sample of 1 ng ml^{-1} concentration the same trend in endotoxic activity was obtained when the preparations were examined in a range from 5 ng ml^{-1}–0.5 pg ml^{-1} with the values decreasing in a dose-dependent manner.

3.3. Induction of TNF by LPS PW preparations

To ensure that the maximum cytokine response was assessed, preliminary experiments were performed to measure TNF induction by PW preparations over time. TNF measurements were made at 30-min intervals from 0–9 h. For all preparations, the peak TNF response was observed between approximately 2.5–4.5 h (Fig. 3). Further measurements of TNF were taken at 24 h intervals over a

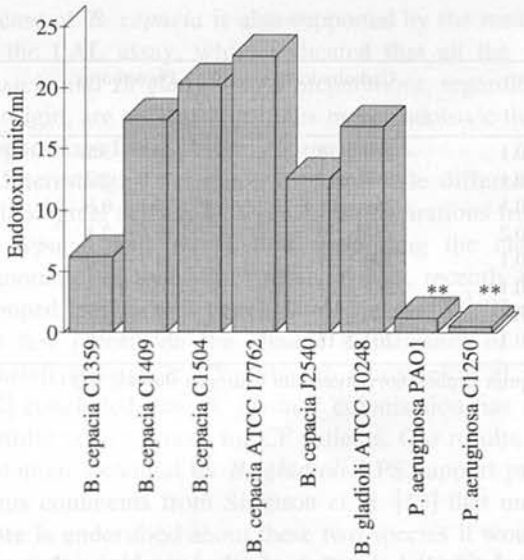

Fig. 2. The endotoxic activity of PW preparations diluted to 1 ng ml^{-1} as measured by the LAL assay. Each value is represented as the mean of three experiments. ** The endotoxic activity of *P. aeruginosa* differed significantly from *B. cepacia* ($P < 0.01$).

7-day period but no TNF was detected after the initial 9 h period. For subsequent experiments, measurements of TNF were made 3.5 h after stimulation. Since MNL from different donors varied in their ability to produce TNF, Fig. 4 shows a representative study of TNF stimulation by different LPS preparations at a concentration of 1 ng ml^{-1}, previously determined as the optimum dosage of sample to use.

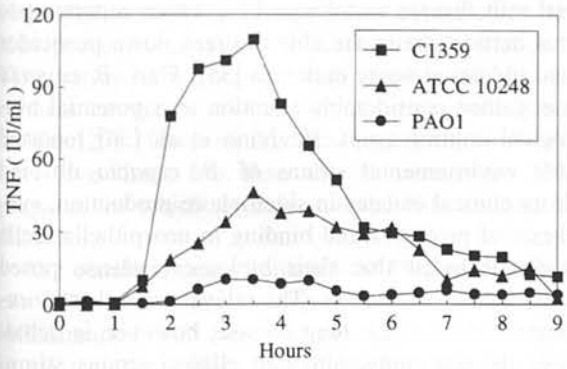

Fig. 3. Time-course of TNF induction by PW preparations. TNF released from MNL by PW samples (1 ng ml^{-1}) was measured from 0–9 h at 30 min intervals. For clarity only *B. cepacia* C1359 (■), *B. gladioli* ATCC 10248 (▲) and *P. aeruginosa* PAO1 (●) are shown.

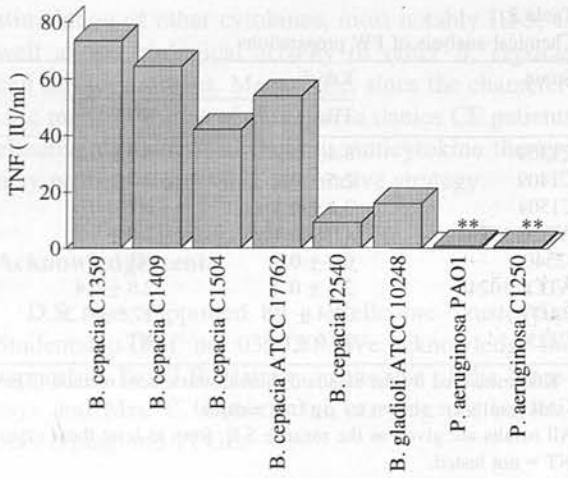

Fig. 4. TNF levels produced from MNL in response to PW preparations (1 ng ml^{-1}) after a 3.5 h stimulation. Each value is represented as the mean of duplicates. ** TNF production from *P. aeruginosa* differed significantly from *B. cepacia* ($P < 0.01$).

B. cepacia PW preparations and that for *B. gladioli* produced at least a nine-fold higher activity in terms of TNF induction compared to both *P. aeruginosa* PW preparations. This trend was reproducible and similar to that observed with the LAL assay. The PW samples themselves had no direct effect on the L929 cells and TNF present in the culture supernate was shown to be solely responsible for the lysis of the L929 cells as an anti-TNF-α mAb (Genzyme) neutralised all observed cytotoxicity (Fig. 5).

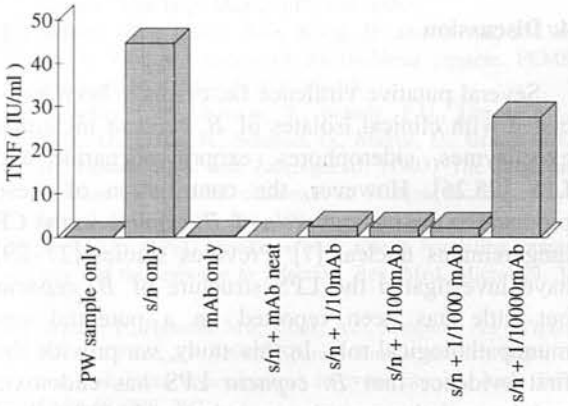

Fig. 5. Anti-TNF-α mAb neutralisation of lysis of L929 cells. The effect of the PW preparations alone is also shown for comparison. Each value represents the mean of duplicates. s/n = culture supernate.

Table 2
Chemical analysis of PW preparations

Strain	Kdo [a]		Protein	Carbohydrate	Phosphorus
	TBA	HF			
C1359	3.4 ± 0.3 [b]	3.3 ± 0.2	1.9 ± 0.1	210 ± 18	40.7 ± 3.5
C1409	2.5 ± 0.2	2.7 ± 0.2	1.9 ± 0.1	344 ± 12	25.0 ± 2.2
C1504	3.4 ± 0.4	NT	2.1 ± 0.1	288 ± 9	30.1 ± 0.6
ATCC 17762	4.9 ± 0.4	2.9 ± 0.2	3.7 ± 0.2	282 ± 15	49.2 ± 2.1
J2540	6.5 ± 0.2	NT	2.1 ± 0.1	290 ± 27	31.5 ± 2.0
ATCC 10248	3.6 ± 0.2	2.8 ± 0.4	1.1 ± 0.1	243 ± 24	24.6 ± 2.3
PAO1	23.4 ± 1.5	17.5 ± 1.3	2.9 ± 0.2	239 ± 11	57.3 ± 4.2
C1250	19.9 ± 0.5	NT	3.7 ± 0.1	203 ± 16	37.0 ± 0.9

[a] Kdo measured by the standard thiobarbituric acid method (TBA) or after prior dephosphorylation with hydrogen fluoride (HF).
[b] All results are shown as μg (mg sample)$^{-1}$.
All results are given as the mean ± S.E. from at least three experiments.
NT = not tested.

3.4. Chemical analysis

Chemical analysis of the PW LPS preparations is summarised in Table 2 and provides several interesting observations. Firstly, although the LAL data showed a greater endotoxic activity in the *B. cepacia* and *B. gladioli* preparations than in those from *P. aeruginosa*, the latter contained on average a five-fold greater concentration of Kdo. Secondly, there was no difference in Kdo content between *B. cepacia* and *B. gladioli*. Thirdly, the phosphorus, carbohydrate and protein content of the LPS preparations did not differ greatly between strains.

4. Discussion

Several putative virulence factors have been associated with clinical isolates of *B. cepacia* including exoenzymes, siderophores, exopolysaccharide and LPS [25,26]. However, the contribution of these products to the pathogenesis of *B. cepacia* in the CF lung remains unclear [7]. Previous studies [27–29] have investigated the LPS structure of *B. cepacia* but little has been reported on a potential immunopathological role. In this study, we provide the first evidence that *B. cepacia* LPS has endotoxic activity and the capacity to induce a high level of TNF.

TNF-α is one of the major cytokines produced in response to LPS stimulation and plays a key role in regulating the secretion of other cytokines, thus amplifying and diversifying the immune response [30]. The biological activity of LPS from *P. aeruginosa* has been studied previously and found to be highly active in both LAL and TNF assays [31]. In our study, we have established that *B. cepacia* and *B. gladioli* LPS, on a weight-for-weight basis, induces approximately nine times as much TNF compared to *P. aeruginosa* LPS indicating that *B. cepacia* has a greater potential than *P. aeruginosa* to cause and sustain immune-mediated damage in the lung.

Several reports have described strains of *B. cepacia* to be an antagonist of fungal and bacterial plant pathogens capable of suppressing dry rot of potatoes [32], blue and grey mould of apples [33] and bacterial wilt disease in tobacco [34]. Other reports state that certain strains are able to break down pesticides and industrial waste materials [35]. Thus, *B. cepacia* has gained considerable attention as a potential biological control agent. Bevivino et al. [36] reported that environmental strains of *B. cepacia* differed from clinical isolates in siderophore production, synthesis of proteases and binding to uroepithelial cells and concluded that their biological release posed little hazard to humans. The relevance of these virulence factors in CF lung disease, however, is debatable. In our study, although clinical strains stimulated more TNF activity compared to the environmental isolate, all the *B. cepacia* and *B. gladioli* PW preparations showed a high potential to stimulate TNF. Caution with respect to environmental

release of *B. cepacia* is also supported by the results of the LAL assay, which indicated that all the *B. cepacia* and *B. gladioli* LPS preparations, regardless of origin, are at least four times more endotoxic than preparations from *P. aeruginosa* strains.

Interestingly, our study detected little difference in biological activity between LPS preparations from *B. cepacia* and *B. gladioli* supporting the close taxonomic relationship of these species, recently regrouped into the new genus *Burkholderia* [37]. There are few reports on the clinical significance of *B. gladioli* isolates in CF although Christenson et al. [12] concluded that *B. gladioli* colonisation has no harmful consequences for CF patients. Our results of endotoxic potential by *B. gladioli* LPS support previous comments from Simpson et al. [13] that until more is understood about these two species it would be prudent to treat isolates of *B. gladioli* from CF patients with the same caution reserved for *B. cepacia*.

PW preparations from *B. cepacia* and *P. aeruginosa* differed not only in biological activity but also in structure and composition as observed by the lack of cross-reactivity in the immunoblots and the differences in Kdo content. Although the structure of several *B. cepacia* LPS serotypes has now been investigated [27–29] initial chemical analysis debated the relative absence of Kdo [38–40]. In our study, Kdo was detectable in all the bacterial preparations based on both the TBA assay and following dephosphorylation by hydrofluoric (HF) acid prior to assaying with TBA. HF acid dephosphorylation was employed in case any phosphorus substitution of the Kdo prevented a detectable reaction in the standard TBA assay. The average Kdo content of the *B. cepacia* extracts was 0.4% which agrees with other studies [29]. However, this Kdo concentration is still over five times less than the *P. aeruginosa* PW preparations which gave an average Kdo content of 2.2%.

We conclude that stimulation of TNF by *B. cepacia* LPS may contribute to destructive pulmonary inflammation. This hypothesis is supported by recent in vivo evidence that *B. cepacia* stimulates a pronounced inflammatory response in mutant CF mice [41] and in CF patients as measured by levels of neutrophil elastase and C-reactive protein [42]. Further studies in our laboratory are investigating the stimulation of other cytokines, most notably IL-8, as well as the biological activity of other *B. cepacia* cell surface antigens. Meanwhile, since the characteristic multiresistance of *B. cepacia* denies CF patients effective antimicrobial therapy, anticytokine therapy may prove a worthwhile alternative strategy.

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