PULMONARY COLONISATION OF PATIENTS WITH CYSTIC FIBROSIS BY *BURKHOLDERIA CEPACIA*

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CONTENTS

ABBREVIATIONS

Abbreviations used frequently and/or novel to this thesis:

ACP	alternative complement pathway
ATCC	American type culture collection
Bc^-	CF patient not colonised by *B. cepacia*
Bc^+	CF patient colonised by *B. cepacia*
BcEB	*B. cepacia* enrichment broth
BEC	buccal epithelial cells
BSA	bovine serum albumin
CCP	classical complement pathway
CEP	*Pseudomonas cepacia* medium
CF	cystic fibrosis
CFTB	complement fixation test buffer
cfu	colony forming units
CIC	circulating immune complexes
ELISA	enzyme linked immunosorbant assay
$ESBc^+$	CF patient colonised by the epidemic strain of *B. cepacia*
Gcd^-	glucose dehydrogenase deficient
HBSS	hanks balanced salt solution
HIC	hydrophobic interaction chromatography
HIS	heat inactivated serum
HISS	heat inactivated sheep serum
HRP	horseradish peroxidase
Ig	immunoglobulin
I	intermediate serum resistance
ISTB	isosensitest broth
LPS	lipopolysaccharide
MM	minimal media
NA	nutrient agar
NIC	nitrocellulose
NYB	nutrient yeast broth
OD	optical density
OMP	outer membrane proteins
PAGE	polyacrylamide gel electrophoresis
Pa^-	CF patient not colonised by *P. aeruginosa*
Pa^+	CF patient colonised by *P. aeruginosa*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PIA	*Pseudomonas* isolation agar
PLC	phospholipase C

PNHS	pooled normal human serum
R-LPS	rough-lipopolysaccharide
S-LPS	smooth-lipopolysaccharide
SDS	sodium dodecylsulphate
SR	serum resistant
SS	serum sensitive
TBS	tris buffered saline
TBT	trypan blue/tetracycline agar
TTBS	tween tris buffered saline
URT	upper respiratory tract
WGH	Western General Hospital

ABSTRACT

The spread of *Burkholderia cepacia* within the cystic fibrosis (CF) population has become a major cause of concern for patients and their carers and represents a significant medical and scientific challenge. *B. cepacia* was originally believed to be an environmentally ubiquitous organism and of little importance as a human pathogen and therefore our knowledge of the pathogenic potential of this organism is limited. This thesis considers biological properties and potential virulence factors of *B. cepacia* that may be important in pulmonary colonisation and the immune response in CF patients. The studies have focused on a highly transmissible, epidemic strain of *B. cepacia* isolated in Edinburgh in 1989 and subsequently responsible for colonisation of CF patients in other regional CF centres.

Prior to this study, environmental *B. cepacia* in culture collections often comprised strains that may have originated as contaminants from colonised patients. To obtain 'true' environmental strains for comparative studies and to test for the ubiquity of *B. cepacia* an environmental survey was undertaken. *B. cepacia* was cultured from a minority of sites and further characterisation of the isolates revealed them to be distinct from CF strains suggesting that environmental strains do not pose a major risk to CF patients.

Representative strains of *B. cepacia* were investigated for biological properties, in particular expression of cell-associated and extracellular virulence factors. The ability to survive in unfavourable environments, including antiseptics was also examined. The epidemic strain was distinct from the majority of *B. cepacia* strains in several aspects including the production of melanin, expression of R-LPS and poor motility.

Culture of *B. cepacia* from the oral cavity and saliva of colonised CF patients suggested that the upper respiratory tract may act as a reservoir, both preceding colonisation of the lower respiratory tract and also contributing to the transmissibility of this strain. Comparative studies of the ability of clinical and environmental isolates of *B. cepacia* to adhere to buccal epithelial cells and respiratory mucin performed by fluorescent labelling, flow cytometry and ELISA showed that the most pronounced binding was observed with the epidemic strain.

The humoral immune response in CF patients colonised with *B. cepacia* was investigated by ELISA, incorporating *B. cepacia* R-LPS, and immunoblotting against LPS, flagella and outer membrane antigens. Elevated levels of specific anti-*B. cepacia* IgG, IgA and IgM were observed in serum from CF patients chronically colonised by *B. cepacia*, especially in those patients colonised by the epidemic strain. The detection of anti-*B. cepacia* antibodies prior to laboratory isolation of *B. cepacia* in some patients may aid in the early diagnosis of *B. cepacia* colonisation. Concentrations of one or more IgG subclasses were raised in CF patients, with substantial individual variation. Following chronic colonisation by *B. cepacia*, the specific anti-*B. cepacia*-R-LPS antibody response comprised mainly IgG2 and IgG3. Chemiluminescent opsonophagocytosis assays indicated that CF sera may non-specifically inhibit phagocytosis of *B. cepacia*.

Resistance of *B. cepacia* to the bactericidal activity of normal human serum appeared to be determined by the expression of S-LPS, in particular a full O-side chain. The epidemic strain, which expresses R-LPS was serum sensitive; paradoxically, this strain is associated with bacteraemia in CF patients.

The relevance and significance of these results to the understanding of the pathogenesis, transmissibility and management of *B. cepacia* in CF patients is discussed

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1 **BUTLER, S.L., NELSON, J.W. & GOVAN, J.R.W.** (1992) Cell surface characteristics of *Pseudomonas cepacia* isolates from patients with cystic fibrosis. XIth International Cystic Fibrosis Conference, Dublin. Abstract TP8.

2 **BUTLER, S.L., NELSON, J.W., BROWN, P.H., GREENING, A.P., MADDISON, J., WEBB, A.K. & GOVAN, J.R.W.** (1994) Immunoglobulin G subclasses in patients with cystic fibrosis colonised by *Burkholderia*(*Pseudomonas*) *cepacia.* 19th European Cystic Fibrosis Conference, Paris. Abstract P35.

3 **BUTLER, S.L., NELSON, J.W., POXTON, I.R. & GOVAN, J.R.W.** (1994) Serum sensitivity of *Burkholderia* (*Pseudomonas*) *cepacia* isolates from patients with cystic fibrosis. *FEMS Immunol. Med. Microbiol.* **8**: 285-292.

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9 **SIMPSON, I., FINLAY, J., WINSTANELY, D., HUNTER, R., DEWHURST, N., NELSON, J., BUTLER, S. & GOVAN, J.** (1994) A multi-resistant isolate resembling *Burkholderia* (*Pseudomonas*) *gladioli* and *B. cepacia.* 19th European Cystic Fibrosis Conference, Paris. Abstract 41.

Oral Presentation

BUTLER, S.L., NELSON, J.W. & GOVAN, J.R.W. (1993) *Pseudomonas cepacia*: an emerging pathogen in cystic fibrosis. Scottish Branch Meeting, Society for General Microbiology, St. Andrews.

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DECLARATION

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

CHAPTER 1

INTRODUCTION

1.1. CYSTIC FIBROSIS

1.1.1 General Background

Cystic fibrosis (CF) was first described as a clinical entity in 1938 as "Fibrocystic disease of the Pancreas" (Anderson, 1938), although descriptions of similar clinical syndromes in children can be found in earlier literature. In the 1940's and 1950's, the term mucoviscidosis was applied to CF following descriptions of thick, sticky intestinal secretions (Farber, 1945). At that time few patients survived beyond infancy but advances in the management of CF have increased the present median survival age to 30 years (Shale, 1991). Today, CF is the most common lethal autosomal recessive genetic disease amongst Caucasian populations with an incidence of 1 in 2000 live births and a carrier frequency of around 1 in 22. World-wide there are 50,000 CF patients, in the UK 2 million people are carriers of the CF gene and there are currently approximately 6000 CF patients.

1.1.2 Clinical Features

CF presents with a broad spectrum of symptoms primarily affecting the lungs and gastrointestinal tract (GIT) with variable clinical severity (Boat *et al.*, 1989). Clinical features commonly present in infancy, though detection may be considerably delayed in cases where symptoms are relatively mild (Hunt & Geddes, 1985; Dibble & Savedra, 1988). Defective chloride ion transport across affected epithelia is the hallmark of the disease accompanied by accumulation of dehydrated mucus resulting

in blockage of exocrine glands affecting most of the body's major organ systems. Symptoms related to the GIT include meconium ileus, or obstruction of the bowel in the new-born with inspissated meconium, which occurs in 5-10% of CF cases. In the adult bowel, obstruction (meconium ileus equivalent) may be due to incompletely digested contents. Pancreatic insufficiency is present in most CF patients from birth, although 10-15% of CF patients have some residual pancreatic function (Kubesch *et al.*, 1993). The absence of pancreatic secretions is a consequence of obstruction of the pancreatic ducts with inspissated mucus leading to acinar cell degradation and fibrosis of the pancreas. The resulting lack of pancreatic digestive enzymes and the formation of fibrotic cysts leads to a variety of disorders including malabsorption, steatorrhoea, diabetes mellitus and a general failure to thrive. Similarly, blockage of the hepatic ducts may lead to cirrhosis of the liver. Reproductive potential in male CF patients is greatly reduced; Wolffian duct structure is altered in up to 95% of patients, with vas deferens, epidydimis and seminal vesicles being atrophic, fibrotic or absent (Taussig *et al.*, 1972). In contrast, fertility in CF females is virtually normal although thickened cervical mucus and poor health may delay conception; in one study 75% of CF pregnancies were completed (Cohen *et al.*, 1980). The most important symptom and the principle cause of premature mortality in CF patients is pulmonary failure following chronic lung infection due to a range of opportunistic pathogens. Overproduction of abnormally viscid, dehydrated tracheobronchial mucus results in impaired mucociliary clearance and chronic bacterial colonisation of the major airways. The ensuing cycle of pulmonary inflammation, infection, and mucus hypersecretion causes irreversible lung injury (Boat *et al.*, 1989).

Diagnosis of CF is often indicated by the symptoms and confirmed by the pilocarpine-stimulated sweat test (Gibson & Cooke 1959), which relies on the fact that levels of sodium and chloride in the sweat of children with CF are usually abnormally high (>60 meq/L). In the 1-2% of cases where sweat chloride levels are not elevated, or in

neonates where it is difficult to obtain sufficient quantities of sweat, additional diagnostic approaches are now available, for example the immunoreactive trypsin test and most recently the direct identification of a mutant CF gene.

In the 1940's and 1950's CF patients seldom survived infancy. The increased life expectancy of today's CF patients is attributable to multiple factors; improved and earlier diagnosis, the development of more potent antibiotics and enzyme supplements, advances in surgical procedures and greater attention to the importance of physiotherapy and regular clinical check-ups. Recent advances in our understanding of the basic defect in CF has also led to the development of new therapeutic strategies including amiloride treatment which blocks sodium ion channels resulting in improved mucociliary clearance, steroids to treat inflammation, antiproteinases to combat the destructive potential of both host and bacterial enzymes and DNAse treatment which reduces the viscosity of CF tracheobronchial secretions. Achieving increased longevity is not without a price however and in recent years a range of formidable longer term problems for CF patients has become evident, including profound changes in the homeostasis of the immune response and an array of new immunological syndromes.

1.1.3 The Cystic Fibrosis Gene - Discovery to Gene Therapy

In 1985, localisation of the CF gene on the long arm of chromosome 7 was determined by linkage analysis using the polymorphic markers *met* and *D7S8* (White *et al.*, 1985). Four years of intensive collaborative research followed, utilising novel cloning strategies including chromosome walking and jumping, and complementary DNA hybridisation, and in 1989 the CF gene, comprising 250 kb of DNA, was identified (Riordan *et al.*, 1989; Rommens *et al.*, 1989). Confirmation that this was indeed the CF gene was demonstrated by the fact that the mutant forms of the gene were present in patients with CF but not in normal individuals and that expression of a

6.5 kb mRNA transcript was higher in tissues severely affected in CF including the lungs and pancreas (Riordan *et al.*, 1989). Further genetic analysis indicated that a single mutation, a three base pair (phenylalanine) deletion at position 508 of the proposed protein (the ΔF508 mutation), was present in approximately 70% of CF chromosomes (Kerem *et al.*, 1989). Following the identification of the CF gene, the Cystic Fibrosis Genetic Analysis Consortium consisting of 90 laboratories in 26 countries was formed to accumulate and collate information on the identification and nature of CF mutations. To date, nearly 300 additional mutations have been described and include mis-sense, non-sense, frame-shift and splice-site mutations (Tsui, 1992). The predominance of a particular mutation is population dependant; for example, the frequency of the ΔF508 mutation is 90% in Denmark (CF Genetic Analysis Consortium, 1990) but only 46.2% in Southern Europe (Estivill *et al.*, 1989).

Cloning of the CF gene represented a milestone in molecular genetics and presented the possibility of new approaches to determine the nature of genetic diseases and improve treatment. For CF patients the benefits of characterising the CF gene are already apparent.

Diagnosis and Screening

At the diagnostic level, there is potential to confirm the diagnosis of CF in doubtful cases by identification of the CF mutation within the patient's cells (Scambler, 1989). Pilot population screening programmes, using polymerase chain reaction (PCR) for both prenatal or neonatal diagnosis and heterozygote screening have proved successful and welcome if accompanied by the appropriate counselling. Due to the large number of mutations 100% detection is impossible at present, but it is possible to look for the most common mutations in the local population and to identify up to 85% of the CF mutations (Shrimpton *et al.*, 1991; Burn *et al.*, 1992).

Correlation of Genotype with Phenotype

Attempts to correlate the CF genotype with clinical phenotype are complicated by a number of factors including the large number of mutations, environmental influences and genetic factors outside the CF locus (Kerem *et al.*, 1989; Kerem *et al.*, 1990). However, an association between CF patients homozygous for ΔF508 and earlier diagnosis of CF, meconium ileus, pancreatic insufficiency and even chronic *Pseudomonas aeruginosa* infection is reported (Johansen *et al.*, 1991; Kubesch *et al.*, 1993; Rosenstein, 1994).

Animal Models

The simultaneous development of a transgenic CF mouse in North Carolina, Cambridge, Edinburgh and Texas has facilitated both studies of the clinical pathology associated with CF and the testing of novel therapies (Higgins & Trezise, 1992; Wilson & Collins, 1992). The Edinburgh CF mice, developed by targeted insertional mutagenesis (Porteous & Dorin, 1990; Dorin *et al.*, 1992; Porteous, 1993), differ from the other models in that the mutation is not a null mutation and some residual low level function of native cystic fibrosis transmembrane conductance regulator (CFTR) is present. As a result the Edinburgh mice survive longer and display several key features of CF: male infertility, gut blockage and importantly lung disease. Furthermore these mice have an intrinsic reduction in mucociliary clearance and a decreased ability to clear pathogens (Davidson *et al.*, 1994). In addition the model mice have successfully been used to establish optimum conditions for gene therapy; for example, the influence of liposome properties on the delivery of the gene construct (McLachlan *et al.*, 1994).

Gene Therapy

The aim of gene therapy is to introduce the normal CF gene into the lungs of patients by means of a vector which may be of viral or synthetic origin. At present, researchers in the USA appear to favour the adenovirus approach, however, this may be associated with adverse inflammatory responses. Trials using an adenovirus vector have been performed on six CF patients over the last 12-13 months in the US (Crystal, 1994). Gene transfer was evident in all patients, and no chronic adverse effects have been noted although one patient experienced a mild inflammatory reaction. In late 1993, the first of two separate UK trials using a liposome vector began on CF patient volunteers in the Brompton Hospital in London. Preliminary results of the Phase 1 trials are encouraging; liposome mediated gene transfer was shown to be safe and some correction of chloride and sodium ion transport was observed. It is likely, however, that the efficiency of delivery via liposome vectors will need to be improved (Caplan *et al.*, 1994).

1.1.4 Function of the CF Gene and the Cellular Defect in Cystic Fibrosis

Cloning of the CF gene enabled the primary amino acid sequence to be predicted together with a proposal for a potential structure and function of the gene product. The protein, designated Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), consists of 1480 amino acids with a predicted molecular weight of 168 kDa (Riordan *et al.*, 1989). Comparing amino acid sequences of CFTR with a protein database showed sequence homology and a shared overall structural scheme with the ABC super-family of transporters, which includes the multidrug resistance (MDR) P-glycoprotein and several bacterial periplasmic permeases (Hyde *et al.*, 1990). CFTR is predicted to consist of five domains: two repeats of a membrane spanning domain each composed of 6 transmembrane segments with external carbohydrate regions; and

two nucleotide binding domains separated by a polar regulatory domain (R) which contains multiple phosphorylation sites (Anderson *et al.*, 1991).

Elucidation of the structure of CFTR led to speculation as to the normal function of the protein and how mutations in the CF gene caused dysfunction. Early observations of increased sodium and chloride ion concentrations in sweat (Di' Sant Agnese, 1953), had implied a defect in ion transport in epithelial cells affected by CF. Low permeability of sweat glands to chloride ions resulting in poor sodium and chloride ion reabsorption was reported to account for the saltiness of CF sweat (Quinton, 1990). Studies on chloride channels of the epithelial cells of airway, sweat glands and pancreatic tissue showed that chloride secretion in response to cAMP was impaired in CF (Welsh, 1990). Following cloning of the CF gene, transfection of cDNA into CF airway and pancreatic epithelial cells was shown to correct the defect in cAMP regulated chloride impermeability (Rich *et al.*, 1990). At this time there was some debate as to whether CFTR was a chloride channel or a regulator of chloride channels. Evidence that CFTR was a chloride channel followed expression of CFTR in heterologous cell types: in each case a unique chloride current was generated that was activated by cAMP agonists (Anderson *et al.*, 1991). Further studies in which CFTR was purified and reconstituted into lipid bilayers confirmed that CFTR was the chloride channel (Bear *et al.*, 1992). How mutations in CFTR may alter protein function has recently been addressed by Welsh and Smith (1993). Four mechanisms have been proposed: Class 1 representing defective protein production; Class 2, e.g. the common ΔF508 mutation, is associated with defective processing; Class 3 defective regulation, and Class 4, defective conduction. A relationship between the genotype, biochemical dysfunction and clinical phenotype can be identified in some CF patients (Welsh & Smith, 1993).

Antibody studies and in situ hybridisation have indicated that expression of CFTR on the apical surface of epithelial cells is consistent with clinical pathologies (Gregory *et al.*, 1990; Denning *et al.*, 1992), although the level of expression in lung epithelial cells, the site of most severe symptoms, was unexpectedly low. In spite of this apparent paucity of expression recent reports suggest that the CFTR dysfunction in pulmonary epithelial cells can be correlated with *Pseudomonas aeruginosa* infection (Barasch *et al.*, 1991). CF epithelial cells bind approximately twice as many *P. aeruginosa* as do cells from normal subjects due to an increase in the proportion of glycolipids lacking sialylation resulting from defective acidification in the trans-Golgi caused by mutations in CFTR.

1.1.5 Pulmonary Infection in Patients with Cystic Fibrosis

Pulmonary dysfunction, primarily as a result of chronic airways infection, is responsible for more than 90% of mortality in CF (Shale, 1991). At birth the lungs are structurally normal but pathological changes occur within the first few months of life (Bedrossian *et al.*, 1976). The primary pathogenic event is dilation of the acinar and duct lumens in submucosal glands followed by opportunistic infection and mucus hypersecretion. Hypertrophy of bronchial glands, goblet cell hyperplasia and squamous cell metaplasia further contribute to impaired mucociliary clearance. The outcome is a destructive cycle of obstruction, infection and inflammatory-mediated tissue damage, characterised by recurrent acute exacerbations with extensive bronchiectasis. Pulmonary function progressively declines with each exacerbation and with advancing lung disease, ventilation perfusion imbalance leads to hypoxia, cor pulmonale and ultimately death (Hodson & Warner, 1992).

Microbial infection in CF typically remains localised in the lung, confined to the conducting airways rather than the lung parenchyma. Systemic infection is rare, suggesting that only local immune defences are defective, although the slow,

inexorable progress of lung disease indicates that local defences are able to maintain a quasi-stable homeostasis in the early stages of disease. The various aspects of local respiratory defences, both non-specific defences such as the cough reflex and mucociliary clearance and specific immunity are ineffective once pulmonary infection is established, and eradication of infecting organisms is virtually impossible.

Pulmonary infection in CF patients is characterised by a limited spectrum of pathogens. Chronic bacterial colonisation, notably by *Staphylococcus aureus*, *Haemophilus influenzae* and *P. aeruginosa*, is most commonly associated with episodes of acute pulmonary exacerbation and deterioration in lung function although the role of common respiratory viruses, chlamydia, mycoplasma and fungi in contributing to worsening pulmonary status should not be underestimated (Efthimiou *et al.*, 1984; Friend, 1986; Govan & Nelson, 1992a and b). A negative aspect of the improved life expectancy for CF patients is the evolution of this spectrum to include previously uncommon bacterial opportunists, in particular mycobacterial species and *Burkholderia (aka. Pseudomonas) cepacia.* Susceptibility of CF patients to colonisation by individual pathogens appears to be age-related. Generally *S. aureus* colonisation occurs in early infancy, followed by *H. influenzae* which tends to be replaced by *P. aeruginosa* (Iacocca *et al.*, 1963; Mearns *et al.*, 1972; Govan & Nelson, 1992a and b).

The predominant role of *S. aureus* was first recognised in 1946 in a microbiological study of post-mortem lung tissue from 14 CF infants aged 1-40 months. The fact that in the pre-antibiotic era *S. aureus* was the major cause of mortality in CF infants was emphasised by the culture of the organism from 12 of these lungs (Di' Sant Agnese & Anderson, 1946). *S. aureus* produces a number of virulence factors, comprising adhesins, immuno-evasive mechanisms and toxins although a precise role for any of these putative virulence factors in lung damage has not been defined. There is little

doubt, however, that the organism is an important cause of respiratory distress and progressive lung damage during the life of a CF patient. Effective anti-staphylococcal therapy has been available since the 1950's. The sequential nature of bacterial colonisation in CF led to the hypothesis that *S. aureus* 'primed' the lung for subsequent colonisation by *H. influenzae* and *P. aeruginosa* and in some CF centres, prophylactic therapy was instituted upon diagnosis of CF with the hope of providing prolonged protection. However, this stratagem has been the subject of some controversy and retrospective studies have indicated an association between anti-staphylococcal prophylaxis and increased incidence of *H. influenzae*, *P. aeruginosa* and a poorer prognosis (Bauernfeind *et al.*, 1987; Kulczycki *et al.*, 1988).

The role of non-capsulate, non-typable *H. influenzae* in respiratory exacerbations is probably underestimated, due to difficulties in laboratory culture of this fastidious organism in the presence of other organisms and distinguishing colonisation from infection with this respiratory commensal. In CF children, however, high concentrations of the inflammatory marker C-reactive protein were associated with *H. influenzae* exacerbations (Govan & Glass, 1990).

Both *S. aureus* and *H. influenzae* can be effectively treated by antibiotics. In contrast *P. aeruginosa*, despite significant increases in the potency of anti-pseudomonal antibiotics, is highly resistant to antibiotic therapy and once established is seldom, if ever, eradicated (Govan *et al.*, 1987). *P. aeruginosa* is the major bacterial pathogen in lung disease in CF adolescents and adults and the most common cause of morbidity and mortality. By the 1980's colonisation with *P. aeruginosa* and the subsequent progression to the mucoid alginate-producing phenotype was associated with a poor prognosis and regarded as a 'harbinger of death' (Pier, 1985). *P. aeruginosa* is widely recognised as an opportunist pathogen *par excellence*, and an impressive array of

virulence factors have been well described (Gilligan, 1991; Govan & Nelson, 1992a and b).

The emergence of new opportunistic pathogens during the 1980's in CF centres both in North America and the UK has been a major concern amongst the CF population and their carers, and has prompted clinicians to take unprecedented actions. Current concern is due to three major features associated with colonisation of patients with CF by *B. cepacia*: (1) many clinical strains of *B. cepacia* are highly resistant to currently available antimicrobials and even those showing *in vitro* susceptibility are often clinically intractable; (2) recently some strains of *B. cepacia* have been shown to be readily transmissible (Govan *et al.*, 1993); and (3) approximately 20% of CF patients unexpectedly succumb to an accelerated fatal decline in pulmonary function with necrotising pneumonia and bacteraemia, known as "*Cepacia* Syndrome"(Isles *et al.*, 1984).

1.1.6 Immune Defence in Cystic Fibrosis Patients

There is little evidence to suggest that a generalised primary immune defect exists in CF although certain consistent aberrations, likely to be secondary effects of chronic infection, drug therapy and malnutrition are observed. Infection in CF rarely spreads to extra-pulmonary sites indicating that systemic immunity is intact. In the CF lung a combination of local host and bacterial factors enables opportunistic pathogens to become established.

Anatomical and Mechanical Protection

The aerodynamic design of the upper airways deflects aerosolised particles to impact with the mucosa where they may be removed by effective mucociliary clearance and the cough reflex (Reynolds, 1989). The normal barrier function of the mucus blanket prevents microbial penetration to the underlying epithelia and enhances shedding of

offending particles via mucociliary clearance (Piedra & Ogra, 1986). Mucus retention, however, is characteristic of CF airways and may exert a deleterious role by protecting bacteria from other airway defence mechanisms (Lamblin & Roussel, 1993). Impaired retention and clearance is thought to be due to the inspissated and copious nature of CF mucus as studies to date suggest that cilia morphology and beat frequency are normal (Katz & Holsclaw, 1980; Rutland & Cole, 1981).

Non-Specific Defences

Potential pathogens which manage to bypass mucociliary clearance and reach the lower airways encounter a second line of defence - non-specific phagocytosis by pulmonary macrophages, the primary effector cell in the lung. Multiple roles of the pulmonary macrophage include induction and regulation of cellular and humoral immunity. Colonising microorganisms must also be able to establish themselves in the presence of an array of non-specific protective factors including lysozyme, anti-proteinases, iron chelating proteins (transferrin and lactoferrin) and the anti-bacterial activity of lung surfactant.

Humoral Immunity

Once chronic pulmonary colonisation is established total circulating levels of immunoglobulin G (IgG), IgA and IgM become significantly raised in CF individuals. Paediatric studies have indicated that hypogammaglobulinaemia is associated with less severe disease (Matthews *et al.*, 1980; Wheeler *et al.*, 1984); similarly in adult CF patients high levels of IgG are associated with poor clinical condition and prognosis (Warner, 1992). In the terminal phases of disease, IgG2 becomes the major IgG subclass in serum and lungs. CF patients also mount a specific antibody response against a range of bacterial pathogens; patients with the highest levels of serum precipitins and/or individuals in which the antibody levels rise rapidly have the worst prognosis (Høiby *et al.*, 1977; Baltimore *et al.*, 1986). Phagocytosis in CF patients

was thought to be defective following observations that CF serum inhibited phagocytosis of *P. aeruginosa* by human and rabbit macrophages (Thomassen *et al.*, 1979). More recent observations of the nature of the IgG subclass response imply that the defect is in fact opsonic rather than cellular. Defective opsonisation may be attributed to a number of factors: (1) the predominance of IgG2 which may inhibit opsonic mediated clearance as pulmonary macrophages only express receptors for IgG1 and IgG3 (Fick *et al.*, 1986); (2) proteolytic cleavage of IgG by neutrophil and bacterial elastases (Bainbridge & Fick, 1989); and (3) competitive inhibition for macrophages and neutrophils by circulating immune complexes (CIC). CF sputa are reported to contain a high proportion of free secretory component, suggesting defective secretion or assembly of IgA (Wallwork & McFarlane, 1976), which may explain why levels of IgG are often higher than levels of IgA in respiratory tract secretions (Fick *et al.*, 1984). However, organisms recovered from the lungs of CF patients are often coated with IgA which may further inhibit clearance as IgA does not activate complement and is a poor opsonin (Piedra & Ogra, 1986). A number of studies have identified CIC in CF serum, sputum and also deposited in tissues of patients chronically colonised by *P. aeruginosa*. Although high levels of CIC are associated with an aggressive humoral response to *P. aeruginosa* and decreased survival prospects a precise pathogenic role remains to be established (Lagacé *et al.*, 1989; Warner, 1992).

Cellular Response and Inflammation

In CF patients the numbers of B and T lymphocytes and their ability to proliferate is normal (Warner 1992), although lymphocyte hyporeactivity specifically to *P. aeruginosa* as a result of antigen overload may be observed with increasing severity of pulmonary disease (Sorensen *et al.*, 1979). Polymorphonuclear leukocytes (PMN) and alveolar macrophages are present in huge numbers in CF lungs and sputum. No consistent abnormality in the levels of serum complement components are reported

(Speert, 1985). CF bronchopulmonary secretions are rich in inflammatory exudate and would be expected to contain complement. Several cytokine classes have been measured in CF sputum and serum. Tumour necrosis factor-α (TNFα), produced by macrophages and monocytes in response to lipopolysaccharide (LPS) is considerably increased in CF (Norman *et al.*, 1991), contributing to the inflammatory process by activating neutrophils and causing lung damage and weight loss (Greally *et al.*, 1993). Interleukin 1 (IL-1) levels are raised in CF but there is no correlation with clinical condition, whilst IL-2 levels are raised even prior to the isolation of organisms (Warner, 1992). IL-8, a potent neutrophil chemoattractant produced in response to LPS, TNFα and IL-1 is greatly increased in CF patients and correlates well with disease severity (Assadullahi *et al.*, 1992; Massion *et al.*, 1994).

Continuous antigenic stimulation as a result of persistent microbial infection leads to a chronically over-active immune response and considerable immune-mediated damage to the CF lung as illustrated in Figure 1 (Sordelli *et al.,* 1988; Auerbach, 1988; Elborn & Shale, 1990; Winnie & Cowan, 1991; Warner, 1992;). It is proposed that activated T-helper lymphocytes produce IL-2 and IL-2 receptor which causes T-lymphocyte proliferation and cytokine production. IL-2,3,4,5 and 6 promote B lymphocyte transformation to plasma cells resulting in generation of antibodies. Chemoattractants, IL-3,4,5,6 and 8 in combination with C5a and leukotriene A4 cause massive influx of PMN which release oxygen radicals as well as lysosomal and proteolytic enzymes. Lung damage occurs as PMN products are released by cells ineffectively attempting to engulf *P. aeruginosa* microcolonies (frustrated phagocytosis: Govan & Glass, 1990) and protease inhibitors such as α1-antitrypsin are overwhelmed by sheer volume and are also broken down by bacterial proteases (Suter *et al.*, 1989).

The host - pathogen interaction is a continually evolving process, in CF it would appear that the pathogens have the upper hand; particularly *P. aeruginosa* which also possesses an arsenal of immuno-evasive and modulatory mechanisms (Speert, 1985; Buret & Cripps, 1993).

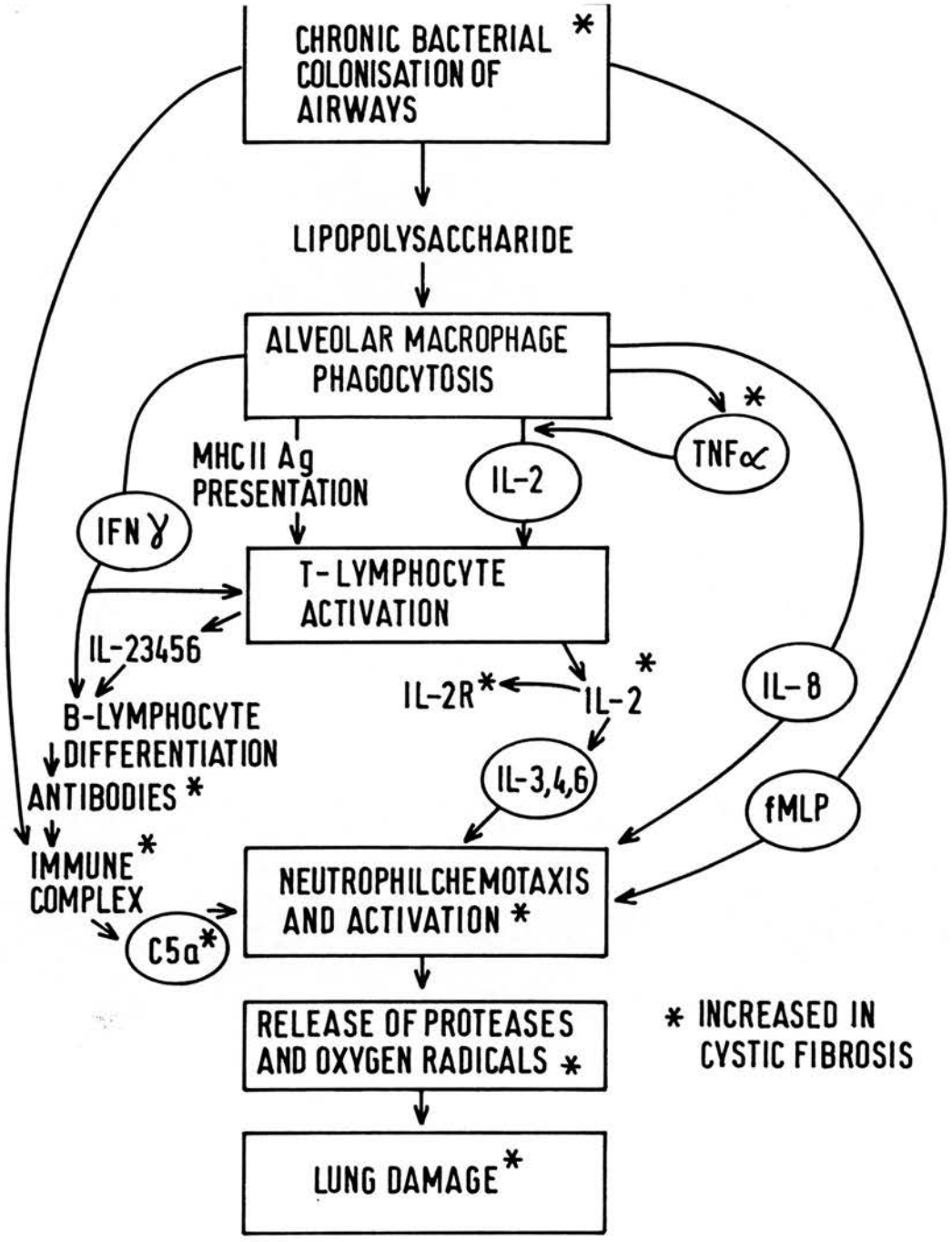


Figure 1. Immune hyper-responsiveness in cystic fibrosis patients (Based on Warner, 1992).

1.2 *BURKHOLDERIA CEPACIA*

1.2.1 TAXONOMY AND NOMENCLATURE

The genus *Pseudomonas* includes a heterologous collection of organisms, some of doubtful taxonomic status, which are widely distributed in nature as saprophytes or as commensals and pathogens for man, animals and plants. In 1992 the transfer of the seven species, *P. cepacia, P. mallei, P. pseudomallei, P. caryophylli, P. gladioli, P. pickettii,* and *P. solanacearum* of the *Pseudomonas* rRNA homology group II, to the new genus *Burkholderia* was proposed with *B. cepacia* as the type species (Yabuuchi *et al.*, 1992; List No. 45, 1993).

B. cepacia (Latin fem. n. *caepa*, onion) was first described in 1950 as a phytopathogen causing soft rot in onions (Burkholder, 1950). The organism was later referred to as Eugonic Oxidiser 1, *P. kingii,* and *P. multivorans* (Stanier *et al.*, 1966; Gilardi, 1970; Jonsson, 1970) before the name *P. cepacia* was revived in 1981 (Palleroni & Holmes, 1981). The previous nomenclature and also the association with CF may imply that *B. cepacia* is closely related to *P. aeruginosa*; however, the two organisms are very different and the renaming emphasises this fact. For the purpose of this thesis the organism will be referred to as *B. cepacia.*

1.2.2 GENERAL CHARACTERISTICS

B. cepacia is an aerobic, Gram-negative, non-fermentative bacillus (Palleroni, 1984). The organism is typically catalase and oxidase positive, although the latter reaction may take some minutes to develop (Mrs C. Doherty, personal communication). Yellow or green pigments, and in some cases the brown pigment melanin, are produced but not fluorescent pigments. Peritrichous fimbriae or pili may be present and the organism is motile by means of multiple polar flagella. *B. cepacia* will grow at 41^{o}C but not at 4^{o}C, and does not survive for sustained periods on agar slopes in

refrigerators (Ballard *et al.*, 1970; Palleroni, 1984). Poly-β-hydroxybutyrate is accumulated as a reserve material (Morris & Roberts, 1959).

B. cepacia is remarkable in its exceptional nutritional versatility, hence the earlier name *P. multivorans* or 'eater of everything'. Most strains are able to utilise a substantially larger number of organic compounds, including penicillin G, as sole carbon and energy sources than any of the fluorescent pseudomonads (Stanier *et al.*, 1966; Beckman & Lessie, 1979). The natural habitat of *B. cepacia* is reported to be soil, water, sewage and vegetation (Ballard *et al.*, 1970; Carson *et al.*, 1973) but, despite being frequently described as ubiquitous, *B. cepacia* is in fact relatively difficult to isolate from the environment (Fisher *et al.*, 1993; J. Govan, unpublished observations). Given that it possesses such extraordinary nutritional capability it is surprising that *B. cepacia* does not predominate in habitats such as soil, where it is often outgrown by fluorescent pseudomonads (Palleroni, 1984). In common with *P. aeruginosa*, *B. cepacia* has successfully adapted to survival within the hospital environment. Reservoirs of *B. cepacia* are commonly moist surfaces or water-based solutions; contamination of disinfectant solutions may occur intrinsically at time of manufacture or extrinsically at the user site (Holmes, 1986; Martone *et al.*, 1987).

1.2.3 Clinical Infections

B. cepacia is an opportunist pathogen and does not generally pose a threat to healthy individuals with an intact immune system, requiring impaired host defences or an unusually large inoculum to initiate infection (Sobel *et al.*, 1982). *B. cepacia* is increasingly recognised as an important cause of nosocomial infection and, given appropriate conditions, can colonise and infect human soft tissue, respiratory tract, urinary tract and cause nosocomial bacteraemia which may lead to endocarditis or septic shock (Basset *et al.*, 1970; Rapkin, 1976; Sobel *et al.*, 1982; Pegues *et al.*, 1993). Generally epidemic infections of *B. cepacia* involve the bloodstream and

urinary tract; in contrast, endemic infection primarily involves the lower respiratory tract (Jarvis *et al.*, 1987). In the majority of cases, this behaviour may be attributed to the ability of *B. cepacia* to contaminate and grow in water supplies and commonly used hospital antiseptics associated with the minimal growth requirements of *B. cepacia*. Several reports indicate that antiseptics such as Povidone-Iodine and Resiguard are bactericidal against *B. cepacia* if used at sufficiently high concentrations or if the contaminating inoculum is small (Berklemman *et al.*, 1981; Craven *et al.*, 1981; Levey & Guiness, 1981).

Children with chronic granulomatous disease (CGD) appear to be particularly susceptible to *B. cepacia* pulmonary infection and to a lesser extent septicaemia (Bottone *et al.*, 1975, O'Neil *et al.*, 1986; Lacy *et al.*, 1993). Phagocytic cells of patients with CGD do not produce peroxide and other reduced oxygen species necessary for efficient intracellular killing and organisms able to resist non-oxidative killing mechanisms, such as *S. aureus* and *B. cepacia* are a particular problem (Speert *et al.*, 1994). *B. cepacia* septicaemia has also been documented in patients with acquired neutrophil dysfunction, a report of a fatal *B. cepacia* pneumonia in a young girl with an acquired bacterial killing defect emphasises that defective neutrophils are an important predisposing factor for infection with *B. cepacia* (McCracken *et al.*, 1972). Recently, there have been reports describing community acquired *B. cepacia* causing pneumonia and septicaemia in apparently previously healthy individuals (Wong *et al.*, 1991; Pujol *et al.*, 1992); however, Cohen *et al.* (1993) questioned the immune status of the case described by Pujol *et al.*.

In conclusion the presence of *B. cepacia* in clinical material may involve contamination rather than infection and the numbers of cases remain relatively low. There is no doubt however that *B. cepacia* poses a significant threat to patients with CF.

1.2.4 BENEFICIAL ROLE OF *BURKHOLDERIA CEPACIA*: BIOLOGICAL CONTROL AGENT

B. cepacia has attracted considerable attention due to its potential for biological control of plant pathogens and may be considered to be a microbial "Jekyll and Hyde". The ability of *B. cepacia* to antagonise and repress soil-borne pathogens makes the organism an interesting proposition from an agricultural point of view (Bevivino *et al.*, 1994). Biological control by *B. cepacia* has already been successfully demonstrated against the aetiologic agents of a number of crop diseases such as southern maize leaf blight, tobacco leaf spot and damping-off of onion seedlings (Sleesman & Leben, 1976; Spurr, 1978; Kawamota & Lorbeer, 1976). More recently antibiotic activity of *B. cepacia* against *Pseudomonas solanacearum* was reported to suppress the incidence of bacterial tobacco wilt disease (Aoki *et al.*, 1991).

Several mechanisms have been proposed for antagonism against fungi: (1) competition for iron by siderophores; (2) production of antibiotics and, (3) competition for nutrients (Homma *et al.*, 1989; Meyer *et al.*, 1989). Jayaswal *et al.* (1993), have isolated a strain of *B. cepacia* with strong antagonistic properties against several plant pathogens due to the production of volatile compounds and, importantly for a biocontrol agent, this strain exhibits this activity over a range of temperatures and pH.

1.3 *BURKHOLDERIA CEPACIA* AND CYSTIC FIBROSIS

1.3.1 EPIDEMIOLOGY

An association between *B. cepacia* and CF was first noted in the 1970's but at that time, was not considered to be of particular concern (Ederer & Matsen, 1972; Laraya-Cuasay, 1977). However, several reports from large North American CF centres in the early 1980's demonstrated a disturbing increase in the prevalence of *B. cepacia* isolated from CF sputa, with prevalence rates doubling in this time (Isles *et al.*, 1984; Thomassen *et al.*, 1985). An increase in patient deaths associated with *B. cepacia* from 9% to 55% during the period 1978 to 1983 also gave rise for concern (Thomassen *et al.*, 1985). Several salient features of *B. cepacia* colonisation in CF were described by Isles *et al.* (1984) following a 10 year study, notably the three distinct clinical sequelae: (1) chronic asymptomatic carriage of *B. cepacia*, alone or in combination with *P. aeruginosa*; (2) progressive deterioration over many months with recurrent fever and frequent hospitalisation; and (3) in approximately 20% of CF patients, a rapid decline in pulmonary function with necrotising pneumonia and bacteraemia with a 67% fatality rate, known as *"Cepacia* Syndrome". Concern over acquisition of *B. cepacia* led to the institution of precautionary infection control measures including segregation of *B. cepacia* colonised CF patients from non-colonised patients in some CF centres. Coincident with these measures the incidence of *B. cepacia* declined from 8.2% in 1983 to 1.7% in 1984 (Thomassen *et al.*, 1986). This data strongly suggested that the increased incidence of *B. cepacia* amongst CF patients resulted from nosocomial infection and person-to-person transmission. In 1984 a collaborative case controlled study by the Centres for Disease Control (CDC) and the Rainbow Babies' and Children's Hospital, Cleveland, investigated the risk factors for acquisition of *B. cepacia*, and evaluated the clinical outcome of colonisation (Tablan *et al.*, 1987). The risk factors associated with *B. cepacia*

colonisation were reported to be:-

(a) increasing severity of underlying pulmonary disease;

(b) increasing age;

(c) having a sibling with cystic fibrosis colonised with *B. cepacia;*

(d) hospitalisation within the previous six months.

In patients with mild CF, no difference in clinical outcome was observed between those colonised with *B. cepacia* and matched controls during the period of study. In contrast, *B. cepacia* colonised CF patients with moderate or advanced disease were hospitalised longer and died sooner after colonisation.

In many respects *B. cepacia* colonisation of patients with CF resembles that of *P. aeruginosa.* Both are opportunist pathogens with a predilection for CF patients with severe pulmonary impairment, causing episodes of acute exacerbation and chronic respiratory tract colonisation that is difficult to eradicate (Tablan *et al.*, 1985; Govan & Glass 1990). Colonisation with either organism can be associated with a poor prognosis due to deterioration in pulmonary function. However, several features of *B. cepacia* infection differ from that of *P. aeruginosa* infection in CF. Firstly a subset of patients with mild or moderate CF succumb to an acute rapid deterioration involving bacteraemia (i.e. *"Cepacia* Syndrome"); a similar clinical course has not been attributed to *P. aeruginosa* infection in CF (Isles *et al.*, 1984; Rosenstein & Hall, 1980). Secondly, the majority of CF isolates of *B. cepacia* are innately resistant to currently available anti-pseudomonal antimicrobials and pose a major therapeutic challenge (Gold *et al.*, 1983). Finally, *P. aeruginosa* is not readily transmitted between patients with CF and epidemic scenarios similar to those currently observed with *B. cepacia* in the CF community are not associated with *P. aeruginosa.*

A number of studies suggest that close and prolonged exposure to *B. cepacia* colonised patients increased the risk of acquisition in non-colonised patients (Thomassen *et al.*, 1986; LiPuma *et al.*, 1990). In addition, early investigations had indicated that hospitalisation was an important factor in acquisition of *B. cepacia* in CF patients (Tablan *et al.*, 1987). There are several routes via which *B. cepacia* may colonise the CF respiratory tract: (1) person-to-person, either directly from a colonised CF patient or indirectly via contact with a contaminated fomite; (2) from an environmental reservoir, either the natural habitat or a contaminated hospital source; and (3) by autoinfection from a site other than the respiratory tract, a route that has not been investigated in CF (Tablan, 1993). The first good evidence for person-to-person transmission of *B. cepacia* between CF patients was provided by the study of LiPuma *et al.* (1991) who used ribotyping to show transfer of *B. cepacia* between two patients attending a residential camp. Transfer of *B. cepacia* occurred within six days of exposure to the initial single colonised patient; however fourteen other camp attendees who remained in contact with the index case showed no evidence of *B. cepacia*. Unfortunately no environmental data was available from this study and therefore indirect transmission cannot be discounted. Recent epidemiological investigations by the CDC and the North American CF Foundation in four CF summer camps have reported a 9-11% sputum conversion from *B. cepacia* negative to positive in CF patients after attending such camps (Pegues & Tablan, 1993). Ribotyping studies indicated that the newly colonised patients had acquired *B. cepacia* strains belonging to the same ribotype as those present in individuals who were colonised prior to attending the camp; strains isolated from the camp environment were found to belong to different ribotypes suggesting that person-to-person transmission was the major mode of acquisition (Pegues & Tablan, 1993). The risk for acquisition of *B. cepacia* by CF patients was found to be proportional to the prevalence of *B. cepacia* colonised individuals attending the camps and to the duration of exposure. Certain activities, such as sharing eating utensils or kissing,

were considered to be 'high-risk' and agree with a contemporary report confirming the importance of social contact in *B. cepacia* transmission (Govan *et al.,* 1993). Evidence that *B. cepacia* may be transmitted by social contact, the unfavourable clinical progression in some patients following acquisition of *B. cepacia* and the threat of litigation has forced clinicians and scientists in North America to recommend that *B. cepacia* colonised CF patients no longer attend summer camps or CF meetings (Speert *et al.*, 1993). As a result of the stigma and anxiety associated with *B. cepacia* colonisation for the CF patient and the risk of transmission to other susceptible patients it has become vital for diagnostic laboratories to correctly identify *B. cepacia.*

The study of CF camps suggests that there is little risk of acquisition of *B. cepacia* from the natural environment (Pegues & Tablan, 1993). Recent studies present strong circumstantial evidence that nosocomial acquisition from contaminated healthcare equipment such as nebulisers and humidifiers does occur (Burdge *et al.*, 1993). Similarly, studies demonstrating that *B. cepacia* colonised patients may contaminate their immediate environment both in the hospital and in the home emphasises the need to maintain good standards of hygiene (Nelson *et al.,* 1991; Fisher *et al.*, 1993).

1.3.2 Isolation and Detection

In the US, the apparent disparity in the prevalence of *B. cepacia* between different CF centres prompted the CDC to investigate the proficiency of microbiology laboratories in culturing and identifying *B. cepacia.* Although 105 out of 111 (95%) of laboratories were able to identify *B. cepacia*, only 36 out of 115 (32%) could detect *B. cepacia* in a simulated sputum specimen. Ability of individual laboratories to detect *B. cepacia* correlated with the use of a selective medium for the organism and when the laboratories were asked to repeat the investigation using selective media 97% were successful (Tablan *et al.*, 1987). *B. cepacia* grows slowly on commonly

used isolation media such as MacConkey agar and is often overgrown by *P. aeruginosa* and other respiratory tract organisms (Gilligan *et al.*, 1985; Welch *et al.*, 1987). The range in prevalence of *B. cepacia* may therefore represent real inter-centre differences or may be an artefact due to lack of laboratory awareness. The CDC has therefore recommended the routine use of *B. cepacia* selective medium in laboratories processing sputa from patients with CF (Tablan *et al.*, 1987). Two selective media are superior for the recovery of *B. cepacia* from respiratory secretions; *B. cepacia* agar (CEP) containing polymyxin B and ticarcillin and OFPBL in which the ticarcillin is replaced by bacitracin (Gilligan *et al.*, 1985; Welch *et al.*, 1987). Additional media for the isolation of *B. cepacia* from soil and environmental samples have also been described, however on this media cultures of *B. cepacia* tend to be overgrown by fungi (Wu & Thompson, 1984; personal observation).

In conclusion laboratory culture and identification of *B. cepacia* may present difficulties; in our experience cultures of *B. cepacia* from sputum may require up to 72 hours incubation, even on CEP, and other species in particular *Xanthomonas maltophilia* and *Comamonas acidovorans*, will grow readily on this medium. It is therefore essential to confirm *B. cepacia* by an appropriate multi-test system such as API 20NE. In the near future it is possible that molecular diagnostic techniques will aid in early diagnosis of respiratory tract infections; recently O'Callaghan *et al.* (1994) have used PCR and DNA hybridisation based on 16S rRNA sequences to detect small numbers of *P. aeruginosa* and *B. cepacia* in respiratory tract secretions.

1.3.3 Typing Systems

Early epidemiological studies of *B. cepacia* were hampered by a lack of typing systems to identify individual strains. Segregation, a policy which is stressful for patients and logistically difficult for clinics, was based on fear and the observation of clusters of cases in centres as there was little scientific evidence to demonstrate

transmission. Initially typing systems for *B. cepacia* were based on phenotypic traits, for example serotyping, biotyping and bacteriocin typing (Heidt *et al.*, 1983; Goldman & Klinger, 1986; Govan & Harris, 1985, McKevitt *et al.*, 1987). Unfortunately serotyping is of little value for CF isolates as 40% of CF *B. cepacia* isolates agglutinate multiple sera or are nonagglutinable (Gilligan, 1991). Typing based on bacteriocin production and sensitivity testing has proved useful in our laboratory; however some strains of *B. cepacia* exhibit instability in bacteriocin type after storage at -70^{o}C (Mrs C. Doherty, personal communication).

Recently a number of molecular typing systems based on detecting genotypic differences have been developed which are able to discriminate between epidemic and non-epidemic strains (Rabkin *et al.*, 1989). Ribotype analysis depends on rRNA probing of restriction endonuclease digests of chromosomal DNA. This technique has been successfully used to confirm patient to patient transmission in a summer camp and has shown that single strains of *B. cepacia* persist in chronically colonised CF patients. More recently acquisition of *B. cepacia* from the 'local' environment has been demonstrated (LiPuma *et al.*, 1990; LiPuma *et al.*, 1991; Fisher *et al.*, 1993). An adaptation of ribotyping, PCR-ribotyping has also been used (Kostman *et al.*, 1992), and similarly Arbitrarily Primed PCR has been used successfully to investigate cross infection in CF patients in a *B. cepacia* outbreak in a French CF clinic (Bingen *et al.*, 1993). Both these techniques are particularly attractive to diagnostic laboratories as they are less time consuming than traditional ribotyping and do not require radioactive probes. Both are, however, less discriminative than Pulsed Field Gel Electrophoresis (PFGE), although this method is somewhat time consuming (Sun *et al.*, 1993).

The choice of typing system for any pathogen depends upon the laboratory facilities available and also the level to which identification is required (Pitt, 1994).

Epidemiological investigations of *B. cepacia* in our laboratory combine a number of approaches. Routinely bacteriocin typing and the API 20NE multi-test system are used, the latter detects a range of biochemical properties allowing diagnosis and preliminary strain identification. PFGE is used in cases to confirm or differentiate clonal relationships; for example, in our own laboratory PFGE has recently confirmed that two new concurrent cases of *B. cepacia* in the Edinburgh paediatric CF clinic were unrelated.

1.3.4 ANTIMICROBIAL THERAPY

B. cepacia is innately resistant to a wide range of currently available antimicrobials, including colistin, aminoglycosides and traditional anti-pseudomonal agents such as penicillins, third generation cephalosporins and quinolones (Gilligan *et al.*, 1985; Prince, 1986). Undoubtedly, the resistance of *B. cepacia* plays an important role in the persistence of this organism and antimicrobial therapy rarely, if ever, leads to eradication of *B. cepacia*. Treatment of patients colonised by *B. cepacia* therefore poses a significant challenge in the management of CF patients; there has been some concern that the aminoglycosides and penicillins used to treat *P. aeruginosa* infection might be selective for *B. cepacia* (Goldman & Klinger, 1986; Govan & Glass, 1990). Historically, trimethoprim-sulfamethoxazole and chloramphenicol were the most effective antibiotics but resistance soon develops during or after therapy (Nord *et al.*, 1974; Gilligan, 1991). More recently developed antimicrobials such as imipenem, aztreonam and ceftazidime exhibit some *in vitro* activity against *B. cepacia* (Aronoff & Klinger, 1984; Klinger & Thomassen, 1985; Tablan *et al.*, 1987). Ceftazidime appeared particularly promising initially as most CF isolates of *B. cepacia* were susceptible *in vitro*, but clinical experience proved variable (Gold *et al.*, 1985). In the most recently reported study, Lewin *et al.* (1993), investigated the activity of three newly developed antimicrobials meropenem and two quinolones, PD 127391 and PD 131628, against 130 isolates of *B. cepacia* from various sources. Although the results

were promising two CF strains of *B. cepacia*, both particular 'problems' in the Edinburgh CF clinic, were highly resistant even to these new agents. A novel approach has been to combine tobramycin with amiloride in an aerosol. Cohn *et al.* (1988) have shown that tobramycin and amiloride act synergistically against *B. cepacia.* As it is unlikely that any current antimicrobial or those under development will have a significant impact on *B. cepacia* infection in CF patients, clinicians and scientists will be well advised to concentrate on preventing the acquisition of *B. cepacia.*

1.3.5 *BURKHOLDERIA CEPACIA* IN THE UK: THE EDINBURGH EXPERIENCE

Experience of *B. cepacia* colonisation in CF patients treated at UK regional centres has provided important new data on *B. cepacia* and confirmed both the threat for colonised patients and the efficacy of precautionary measures. In the UK, surveillance in large CF centres indicated that *B. cepacia* presented little problem amongst CF patients in the early 1980's. By the end of the decade, however, several regional CF centres had reported a situation reflecting that previously described in North America (Simmonds *et al.*, 1990; Lancet Editorial, 1992; Millar-Jones *et al.*, 1992; Govan *et al.*, 1993; Smith *et al.*, 1993; Smyth *et al.*, 1993). In the UK studies, regional differences in the prevalence of *B. cepacia* could, in part, be attributed to the emergence of a particularly transmissible strain of *B. cepacia* with well defined characteristics (Table 1.) and originally known as the Epidemic Strain.

The studies by LiPuma *et al.* (1988; 1991) had previously shown that CF patients within a clinic tended to harbour the same strain of *B. cepacia* and demonstrated transfer of *B. cepacia* from a colonised CF patient to a previously non-colonised patient. The most compelling evidence for person-to-person transmission of *B. cepacia* has been provided by the recent studies on the epidemic strain of *B. cepacia*, the first strain of *B. cepacia* to show transmission between patients attending different

regional centres including Belfast, Birmingham, Cardiff, Edinburgh, Leeds, Liverpool and Manchester. The clonal relationship of epidemic strain isolates from the different centres within the UK and, interestingly strains from Ontario, Canada has recently

Table 1. Phenotypic and genotypic characteristics of the Epidemic Strain of *B. cepacia* (Govan *et al.*, 1993).

Characteristic	Comment
Biochemical profile	API 20NE - 99.9%
Colonial morphology	small, dry colonies (see Figure 2.)
Lipopolysaccharide	rough
Pigment production	melanin
C14-lipase	negative
Bacteriocin type	S3/P0
Multiresistance profile	resistant to: ceftazidime; meropenem; piperacillin; tobramycin; gentamicin; ciprofloxacin; imipenem; azlocillin; aztreonam; colomycin; cotrimoxazole; chloramphenicol.
Ribotype	"A"
PFGE	discrete pattern

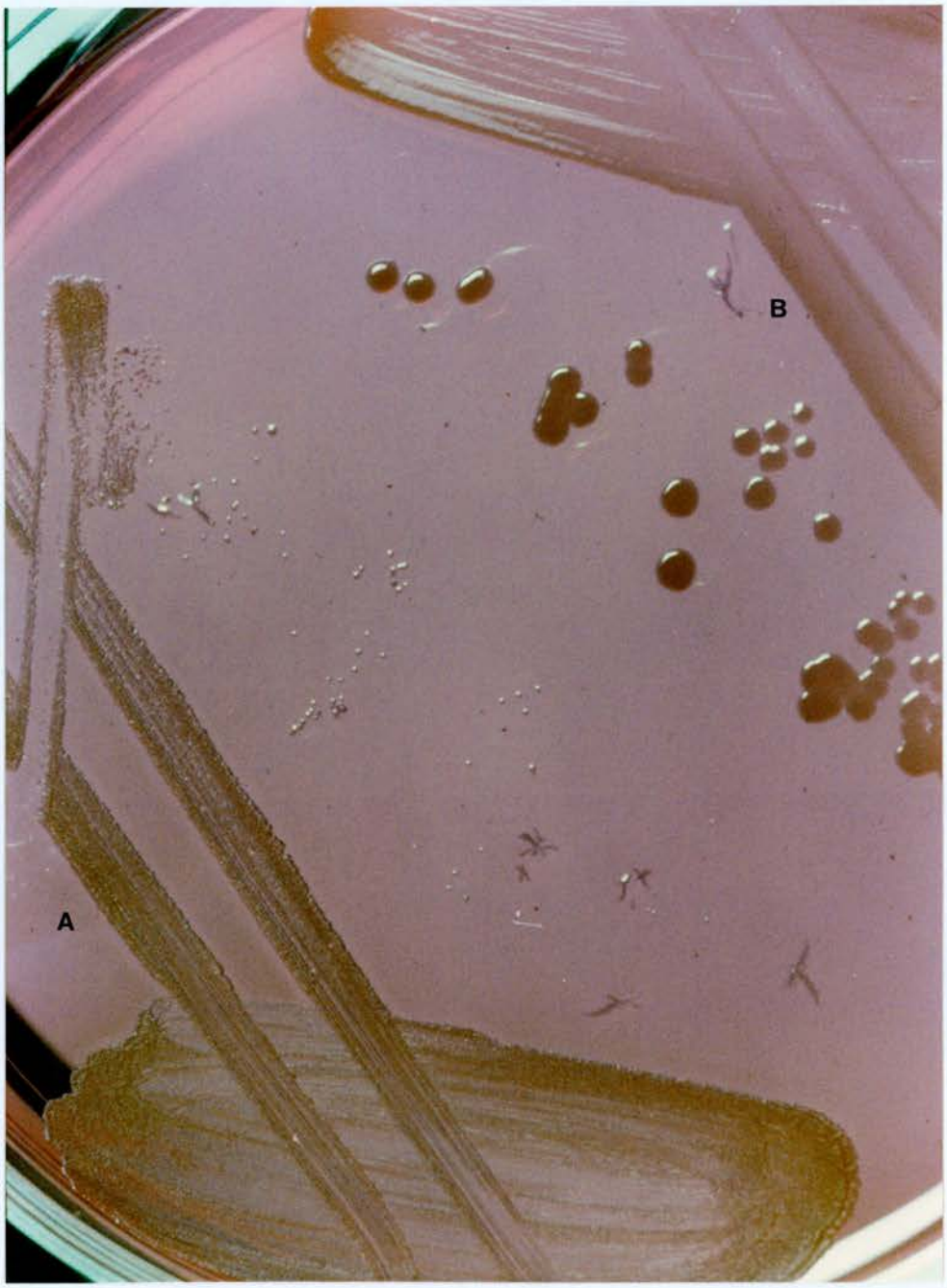


Figure 2. Colony morphology of *B. cepacia*: (a) the epidemic strain with distinctive small, dry colonies; (b) C1409, a CF isolate with 'typical' *B. cepacia* colony morphology.

been confirmed by multilocus enzyme typing (Johnson *et al.*, 1994). Intense epidemiological investigations revealed a considerable degree of inter-regional social contact between adult CF groups, providing strong circumstantial evidence that social contact outwith the hospital environment had been important in the spread of this strain.

The spread of the epidemic strain of *B. cepacia* within and between CF centres is typified by the experience in the Edinburgh Adult and Paediatric CF Clinics (Figure 3.) where surveillance for *B. cepacia* had began in 1982 (Govan *et al.*, 1993). In 1986, Glass & Govan described an isolated case of "*Cepacia* Syndrome" in a nine year old female CF patient, the first report of a CF death due to *B. cepacia* in the UK. In 1989 the first isolate of the epidemic strain was cultured from the index case, a 20 year old CF male (patient 7), and in the subsequent four years a further 12 patients attending the adult CF clinic became colonised with the epidemic strain; in 1992 the prevalence of *B. cepacia* had reached 26.7%. Investigation, in view of the opinion that colonisation with *P. aeruginosa* precedes colonisation with *B. cepacia*, revealed that only eight of the 13 (62%) patients colonised by the epidemic strain in Edinburgh were co-colonised by *P. aeruginosa*. The clinical significance of colonisation by the epidemic strain varied between patients; however, between 1990 and 1992, seven deaths (five female) occurred in *B. cepacia* colonised CF patients of whom six were colonised by the epidemic strain. In the same period no deaths occurred in *B. cepacia* negative patients. The impact of the epidemic strain in Edinburgh can clearly be seen from Figure 3. Analysis of the social contacts of colonised patients revealed that a number of patients attended a weekly CF fitness class, which was disbanded in 1991 following the deaths of two of the attending CF patients within the same week. The relationship between male patient 19 who was colonised by the epidemic strain and an additional unrelated strain of *B. cepacia,* and female patient 22 who was *B. cepacia* negative at the start of the relationship, provides the most convincing evidence of the

transmissibility of this strain. During the course of a short and intimate relationship patient 22, who had severe pulmonary dysfunction became *B. cepacia* positive and died six weeks later. Interestingly, despite the fact that patient 19 was colonised by two distinct strains of *B. cepacia*, patient 22 only acquired the epidemic strain. Following the death of his girlfriend patient 19 experienced considerable emotional trauma and despite having comparatively good lung function died one month later due to pulmonary failure with overwhelming *B. cepacia* pulmonary infection and bacteraemia. Following the segregation of *B. cepacia* colonised CF patients in the Edinburgh clinic in August 1992 there has been no further acquisition of the epidemic strain by Edinburgh CF patients.

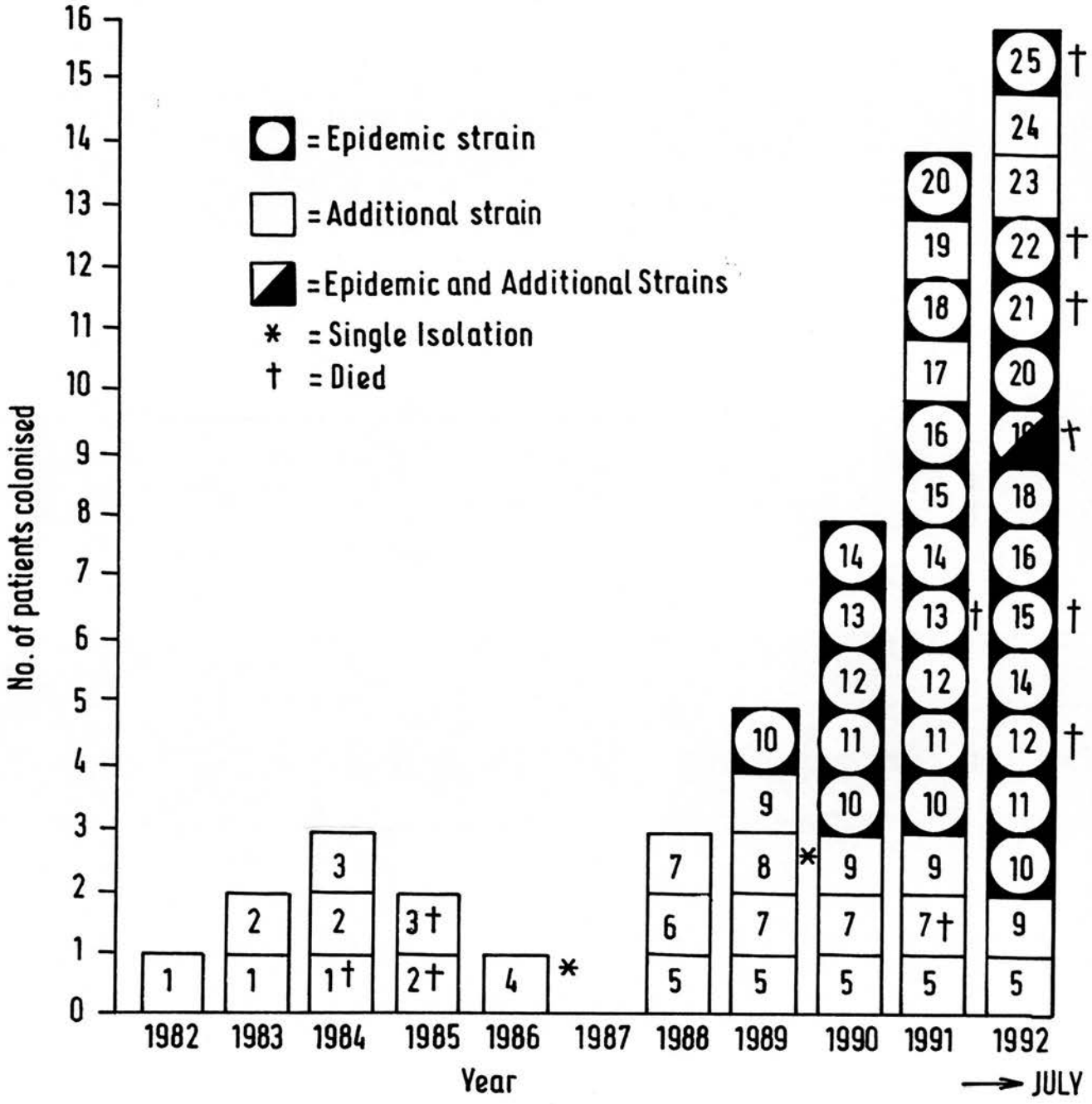


Figure 3. Consecutive cases of *B. cepacia* colonisation in CF patients attending the Edinburgh adult and paediatric CF clinics.

1.4 PATHOGENICITY AND VIRULENCE OF *BURKHOLDERIA CEPACIA*

1.4.1 VIRULENCE FACTORS AND PATHOGENICITY

Virulence factors may be defined as components and products of the bacterial cell which bestow on the bacterium the potential to cause disease (pathogenicity). Instances in which pathogenicity is the result of a single bacterial factor, for example the neurotoxin of *Clostridium tetani*, are rare. More often, pathogenesis is a multifactorial interaction involving both bacterial and host factors. Many potential virulence factors have primary functions in the normal anatomy and physiology of the bacterium - any association with disease may be purely coincidental. While some pathogens regularly cause disease in individuals with intact host defences, other potential pathogens, the microbial opportunists, are only able to take advantage of immunocompromised hosts (Finlay & Falkow, 1989).

A sequence of potentially pathogenic interactions between a bacterium and a susceptible host begins when the bacterium has reached a target or colonisation site on or in the host (Finlay & Falkow, 1989; Poxton & Arbuthnott, 1990). Bacterial adherence to host mucosal or epithelial surfaces via specific receptor-ligand interactions is generally considered to be prerequisite for initial colonisation. Subsequently, the bacterium multiplies and may need to adapt in order to persist within the host; for example, by regulating the production of factors which enable it to compete against commensal flora for nutrients and avoid non-specific and specific host immune responses. Damage to the host tissues may occur directly via toxins or invasion, or indirectly via the immune response. Finally, to complete the process the pathogen may disseminate to other susceptible hosts.

Bacterial virulence factors are generally classified as being cell surface factors such as fimbriae, or extracellular such as the toxins or exo-enzymes (Poxton & Arbuthnott,

1990). Studies to improve our understanding of the pathogenicity and virulence of individual pathogens are challenging for several reasons. Firstly, virulence may be primarily manifested *in vivo*; *in vitro* grown bacteria may not express all their virulence factors, thus relevant model systems are required which mimic the disease situation. Another problem is that virulence factors relevant to one type of infection may be of no importance in another. Thirdly, when the organism involved is an opportunistic pathogen, such as *P. aeruginosa* or *B. cepacia*, it is difficult to decipher which aspects of the disease are mediated by the bacterium and which are due to pre-existing host factors and the host immune response.

1.4.2 The Bacterial Cell Surface

The cell envelope of Gram-negative bacteria (Figure 4.) is a complex structure conferring shape and rigidity on the bacterial cell and functioning as a barrier through which the bacterium interacts with the environment. Three principal layers make up the cell envelope; 1) the outer membrane; 2) the periplasmic space; and 3) the cytoplasmic membrane. Additionally an extracellular polysaccharide material may also be present, as a capsule or slime layer (Stanier *et al.*, 1986). The outer membrane is an asymmetric bilayer containing phospholipids, proteins and the unique lipid, lipopolysaccharide (LPS). Three major classes of protein are found in the outer membrane including lipoprotein, major outer membrane proteins and minor outer membrane proteins, each being induced or repressed depending on the environment. Many of the major outer membrane proteins are pore proteins or porins which form relatively non-specific, water-filled pores spanning the outer membrane for the passive entry of low molecular weight, hydrophilic solutes (Nakae, 1986). Other minor outer membrane proteins act as specific transport systems for solutes which are too large or present at low concentrations. The tubular protein polymers of the flagella and fimbriae that are involved in motility and adherence are associated with both the cytoplasmic and outer membrane of the bacterial cell surface. LPS largely replaces

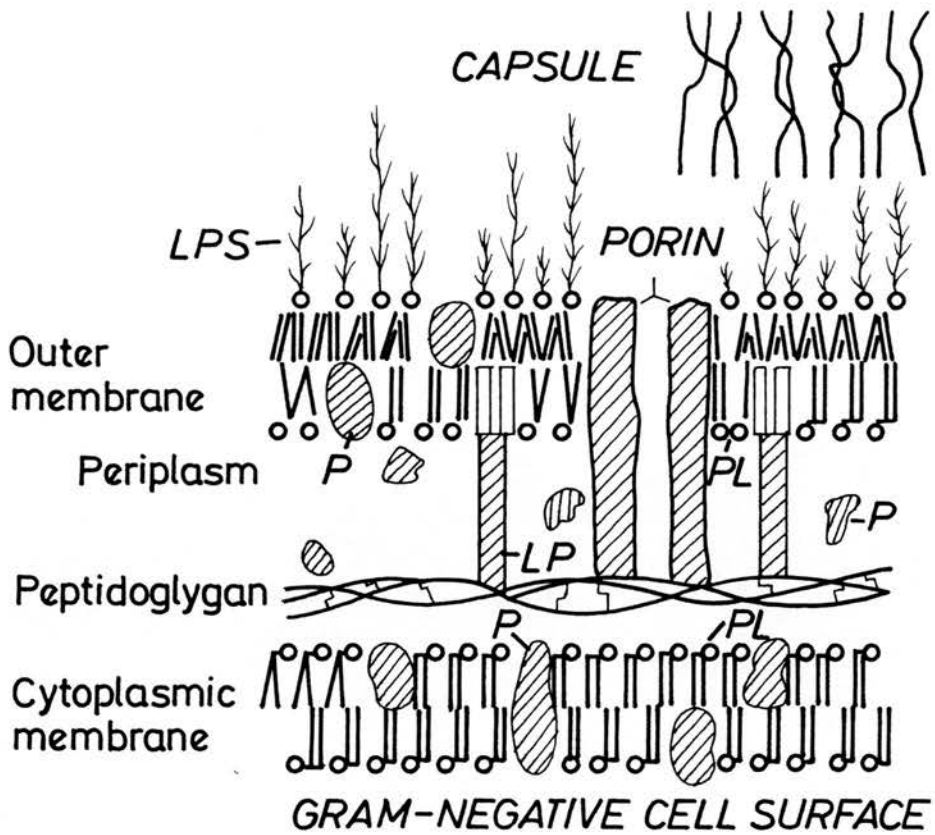


Figure 4. The cell surface of a Gram-negative bacterium.
LP=lipoprotein; LPS=lipopolysaccharide; P=protein; PL=phospholipid;
(From Hancock & Poxton, 1988).

phospholipids in the outer leaflet; the hydrophobic terminal is inserted into the hydrophobic core of the membrane whilst the hydrophilic terminal extends outward from the cell surface. The varied normal physiological functions of the cell surface components include acquisition and transport of nutrients, receptors for bacteriophage and bacteriocins and bacterial adhesins; several of these cell surface components have been shown to be potential virulence factors for *B. cepacia.*

1.4.3 PATHOGENICITY OF *B. CEPACIA*

Our knowledge and understanding of the pathogenesis of *B. cepacia* infection in the lungs of patients with cystic fibrosis and the bacterial factors responsible for colonisation and virulence is minimal, particularly when compared to the wealth of literature concerning the pathogenesis of *P. aeruginosa* in CF. There is also debate as to whether *B. cepacia* is a true pathogen or merely a marker of severe lung disease; despite the fact that a number of potential virulence factors have been described for *B. cepacia* there is little evidence to demonstrate that primary pathology in the CF lung results directly from any of these products. The aim of this chapter is to review the literature describing potential virulence factors of *B. cepacia*; most of the available information concerns the role of this organism in CF as it is in this area that interest and concern are focused.

Several theories have been postulated in attempts to explain the spectrum of disease associated with *B. cepacia* colonisation in CF: (1) different strains of *B. cepacia* may vary significantly in virulence; (2) synergistic interaction with other CF pathogens such as *P. aeruginosa* (Sokol, 1986; Saiman *et al.*, 1990): and (3) the severity of *B. cepacia* infection may be dependant on the degree of pre-existing tissue damage (Gilligan, 1991). Postulates (2) and (3) may be questioned as not all patients colonised by *B. cepacia* are co-colonised by *P. aeruginosa* (Govan *et al.*, 1993) and a

recent report describes CF patients with new lung transplants succumbing to overwhelming pulmonary infection with *B. cepacia* (Snell *et al.*, 1993).

Data pointing to a purely commensal role for *B. cepacia* comes from several studies with animal models which show that *B. cepacia* is avirulent compared to *P. aeruginosa*. For example, in a guinea pig model of pneumonia, *B. cepacia* caused only mild disease whilst equivalent concentrations of *P. aeruginosa* caused fulminant fatal pneumonia (Goldman & Klinger, 1986). Similarly, *P. aeruginosa* was highly virulent in a burned mouse model but *B. cepacia* was avirulent although it did show tenacious persistence in the wound (Stover *et al.*, 1983). The apparent avirulence of *B. cepacia* may largely be explained by the fact that *B. cepacia* does not appear to express any of the virulence factors associated with *P. aeruginosa* such as exotoxin A and alginate and, as implied by several studies, *B. cepacia* strains may vary in virulence with certain isolates being highly virulent and others essentially avirulent (McKevitt & Woods, 1984; Goldman & Klinger, 1986; Gilligan, 1991).

1.4.4 Colonisation and Adherence

A dogma of bacterial pathogenesis states that the first interaction between a potential pathogenic organism and its host entails attachment to a eukaryotic cell surface and that this is a prerequisite for local bacterial proliferation in the host and therefore for tissue damage, bacterial invasion and dissemination (Finlay & Falkow, 1989; Krogfelt, 1991). Attachment to host cell surfaces occurs via receptor-ligand interactions; the bacterial ligand or adhesin is located on the bacterial cell surface and recognises specific binding sites on the host cell surface. Fimbriae are amongst the best described of bacterial adhesins; for example the PAP fimbriae of uropathogenic *Escherichia coli* (Pere *et al.*, 1986). Correlation between the presence of fimbriae and pathogenicity of *B. cepacia* in CF has been addressed by several groups investigating the adhesive properties of *B. cepacia* fimbriae.

B. cepacia has been reported to express polar and peritrichous fimbriae (Saiman *et al.*, 1990; Sajjan & Forstner, 1992; Kuehn *et al.*, 1992). Kuehn and colleagues (1992) showed that outer membrane protein preparations of *B. cepacia* were enriched with 3 proteins at 16, 20 and 40 kDa which were not present in non fimbriated strains. The fimbrial subunit (16 kDa) appeared similar to those observed with other bacteria and showed homology with PAK fimbriae of *P. aeruginosa*. In contrast, Saiman *et al.* (1990a & b) demonstrated minimal cross-reactivity of *B. cepacia* fimbriae with monoclonal antibodies to *P. aeruginosa* fimbriae, and no homology between *P. aeruginosa* fimbrial probes and *B. cepacia* genomic DNA. This apparent discrepancy may be due to sequence variation of fimbrial genes between different strains of *B. cepacia* (Kuehn *et al.*, 1992).

Krivan and co-workers (1988) demonstrated in *in vitro* binding experiments that both *P. aeruginosa* and *B. cepacia* adhere to GalNAcβ1-4Gal sequences in the glycolipids Asialo GM1 and Asialo GM2. No competition for epithelial receptors between these two organisms has been observed indicating that different receptors may be used preferentially or that the bacteria may bind to each other (Saiman *et al.*, 1990). In the same report, the authors described an 8-fold increase in the binding of two strains of *B. cepacia* in the presence of *P. aeruginosa* or *P. aeruginosa* products. The authors postulated that enhanced binding occurred through a synergistic relationship between *P. aeruginosa* and *B. cepacia* whereby *P. aeruginosa* exoproducts modified the epithelial cell surface exposing receptors and facilitating *B. cepacia* adherence (Saiman *et al.*, 1990).

Several studies describe specific binding of *B. cepacia* isolates from CF patients to CF and non-CF mucins (Sajjan *et al.*, 1992; Sajjan & Forstner, 1992; Sajjan & Forstner, 1993). Isolates exhibiting the highest degrees of mucin binding tended to be

associated with CF patients with the most severe illness leading to speculation that variability in binding of different *B. cepacia* isolates to respiratory mucin may contribute to morbidity and mortality or that tissue damage results in increased adhesion. Variable strain-specific binding potentially may explain why some *B. cepacia* strains colonise CF patients transiently and other strains, once acquired, are never lost. Unfortunately in the study by Sajjan *et al.* (1992) typing data was not provided; *B. cepacia* is known to be highly transmissible and the possibility of a clonal relationship between *B. cepacia* strains could not be excluded. Deglycosylation procedures indicated that mucin receptors for *B. cepacia* include N-acetylglucosamine and N-acetylgalactosamine (Sajjan *et al.*, 1992). *B. cepacia* isolates also bind to buccal epithelial cells (BEC) via two different adhesin-receptor systems one of which is independent of bacterial fimbriation. The other binding mechanism is mediated by a 22 kDa adhesin located on the fimbriae and a 55 kDa epithelial cell receptor. The 22 kDa adhesin is also involved in binding to mucin and may therefore be an important virulence factor in the early stages of colonisation. The presence of fimbriae also increased the binding of *B. cepacia* to pneumocytes *in vitro* (Kuehn *et al.*, 1992).

1.4.5 Outer Membrane Proteins

B. cepacia expresses five major OMP's; A (56 kDa), B (38 kDa), C (36 kDa), D (27 kDa) and E (21 kDa). The C and D proteins have been identified as porins and are antigenic in most patients chronically colonised by *B. cepacia* (Parr *et al.*, 1987; Aronoff & Stern, 1988). Synthesis of the outer membrane constituents is influenced by the environment; for example Anwar *et al.* (1983) showed that the simplest OMP profile of *B. cepacia* was obtained following growth in magnesium deficient conditions and that a 66 kDa OMP was induced in iron deficient conditions. Similarly the variable micro-environment of the CF lung, in particular ionic content and concentrations of essential elements, may explain the intra-isolate variability observed in the OMP profiles of single strains of *B. cepacia* from patients with CF (Larsen *et*

al., 1993). Major roles for OMP's in *B. cepacia* colonisation in CF include enabling the organism to persist by promoting nutrient uptake and by preventing antibiotic agents from reaching their target sites.

Siderophores

One of the most important roles of OMP's in terms of virulence are to act as receptors for iron-chelating siderophores, which are induced in iron deficient conditions. Siderophore-mediated iron acquisition is an important prerequisite in establishing and maintaining infection in many bacterial pathogens (Payne & Finklestein, 1978). Production of siderophores enables the bacteria to compete against host iron binding proteins such as transferrin and against other bacteria for the limited amount of free iron. The fact that many bacteria express more than one iron transport mechanism and may be able to utilise systems from other species, thus gaining a competitive advantage, emphasises the importance of iron.

B. cepacia expresses at least three siderophore-mediated iron transport mechanisms including pyochelin, cepabactin and azurechelin (Sokol, 1986; Sokol & Woods, 1988; Meyer *et al.*, 1989; Sokol *et al.*, 1992). In the study by Sokol (1986), pyochelin production was observed in only 49% of *B. cepacia* strains examined. The fact that the majority of pyochelin producing strains (86%) were from patients with severe infection suggested that pyochelin may contribute to virulence. Later studies using a chronic pulmonary infection model in rats demonstrated that exogenously supplied pyochelin increased the area of pathology observed in the rat lungs, suggesting a role for pyochelin in dissemination of *B. cepacia* in the lung and also in the induction of a greater inflammatory response (Sokol & Woods, 1988). Both pyochelin producing strains of *B. cepacia* and those which do not produce pyochelin can take up ferripyochelin. This may be important in CF infections where *P. aeruginosa* is also involved as the pyochelin of *P. aeruginosa* and *B. cepacia* appear identical suggesting

that, in some cases, a synergistic relationship may occur between these opportunistic pathogens (Sokol & Woods, 1988).

Two additional iron transport systems have been described in *B. cepacia*; cepabactin which strongly chelates Fe III and azurechelin which is a salicylic acid and has been identified in 88% of *B. cepacia* strains isolated from the respiratory tract (Meyer *et al.*, 1989; Sokol *et al.*, 1992; Visca *et al.*, 1993). No potential role in virulence has yet been described for either of these siderophores; they may simply perform a supplementary role as pyochelin is reported to be inefficient when competing for iron against host serum proteins (Meyer *et al.*, 1989).

1.4.6 LIPOPOLYSACCHARIDE

LPS is considered to be one of the main virulence factors of Gram-negative bacteria and is a major surface antigen (Poxton & Arbuthnott, 1990). Numerous and varied biological activities are associated with LPS including antigenic and endotoxic properties, as a receptor for bacteriophage and bacteriocins and possibly a role in recognition and adhesion via lectins (Wilkinson, 1983). Immunomodulatory functions of LPS include influencing polymorphonuclear leukocyte function (Kharazmi *et al.*, 1991), and smooth-LPS (S-LPS) serves to protect the organism from opsonisation and phagocytosis as well as the bactericidal activity of serum (Taylor, 1983; Crokaert *et al.*, 1992).

Structure

The architectural norm for LPS is an amphipathic tripartate macromolecule with an O-specific polymer side chain attached to a hydrophobic lipid via a common core (Wilkinson, 1983). The O-side chain is highly variable, consisting of repeating oligosaccharide units and is the basis for O-serotyping. Lipid A, based on a 1,6'-β-linked disaccharide of glucosamine with O and N fatty acid substituents and the

proximal region of the core typically containing L-glycero-D-*manno*-heptose and 3-deoxy-D-*manno*-octulosonic acid (KDO) are relatively conserved. Both Lipid A and the core are usually phosphorylated. The LPS of *B. cepacia* has a number of unusual features. Initial chemical analysis indicated an absence of KDO (Manniello *et al.*, 1979; Anwar *et al.*, 1983); however, Straus *et al.* (1989; 1990) reported the isolation of KDO from the culture supernate of 2 out of 10 strains of *B. cepacia* examined and from six clinical isolates of *B. cepacia* and more recently Cox & Wilkinson (1991) detected low levels of KDO for one strain of *B. cepacia*. Compared to LPS of *P. aeruginosa* that of *B. cepacia* has a low phosphorus content but more heptose (Manniello *et al.*, 1979). Extensive studies on the O-side chain of *B. cepacia* serogroups 01, 03, 05 and 07 have indicated that in serogroups 01, 03 and 05 the metabolic versatility of *B. cepacia* extends to the biosynthesis of unusual sugars including D-rhamnose and D-fucose (Cox & Wilkinson, 1989; 1990a & b).

B. cepacia LPS and Cystic Fibrosis

An unusual feature of chronic *P. aeruginosa* colonisation in CF is the emergence of mucoid, serum-sensitive, polyagglutinating phenotypes lacking the O-side chain of LPS (Hancock *et al.*, 1983; Govan & Harris, 1986). Although in one study the majority of CF isolates of *B. cepacia* (54%) expressed R-LPS (McKevitt & Woods, 1984), there is no evidence to date to confirm that *B. cepacia* undergoes a similar transition in chronically colonised CF patients.

The investigations of Straus *et al.* (1988; 1990) indicated that an extracellular toxic complex (ETC) produced by various strains of *B. cepacia* may be responsible for the lethality and lung tissue destruction associated with *B. cepacia* pneumonia. The ETC consisted of a surface carbohydrate antigen, LPS and protein and enhanced the virulence of *B. cepacia* when co-injected into mice (Straus *et al.*, 1990). The most toxic ETC preparation was that which contained the most LPS. Immunisation of

rabbits with ETC resulted in the production of antibodies that were protective in 90% of mice inoculated with the homologous strain.

1.4.7 ANTIMICROBIAL RESISTANCE

The high intrinsic resistance to antibiotics in Gram-negative bacteria is often associated with the barrier properties of the outer membrane. Various studies indicate that in *B. cepacia* reduced permeability is a major contributing factor in resistance to β-lactam antibiotics as well as to aminoglycosides and other hydrophobic compounds. The small size of the hydrophobic channels formed by *B. cepacia* porins retards diffusion of β-lactams (Parr *et al.*, 1987). In addition decreased expression of the major C and D porin proteins is associated with high level β-lactam resistance in some CF isolates of *B. cepacia* (Aronoff, 1988). Similarly resistance to aminoglycosides and chloramphenicol is due to the inability of the antibiotic to disrupt and permeabilise the outer membrane (Moore & Hancock, 1986, Burns *et al.*, 1989).

Resistance to cationic antimicrobial agents is, in part, attributable to the unusual structure of *B. cepacia* LPS with its relative dearth of anionic sites and the presence of fixed counter-cationic sites (Moore & Hancock, 1986; Cox & Wilkinson, 1991). In *P. aeruginosa* the loss of the O-side chains, independent of diminished porin expression, has also been associated with β-lactam resistance (Godfrey *et al.*, 1984). Recent studies on *B. cepacia* have also demonstrated that a high level of β-lactam resistance correlates with the expression of rough-LPS (R-LPS) (Simpson *et al.*, 1994).

In addition to an impermeable outer membrane, all pseudomonads produce an inducible chromosomal β-lactamase (Prince, 1986). At least three β-lactamases have been described for *B. cepacia*; one with the unique ability to utilise β-lactam compounds as a source of carbon (Beckman & Lessie, 1979; Aronoff & Labrazzi,

1986). Activity of *B. cepacia* β-lactamases has been shown to increase in a CO_2 enriched atmosphere (Corkill *et al.*, 1994), which may contribute to the clinical intractability of *B. cepacia* in CF. Recently carbapenemase activity has been described as widespread in *B. cepacia* (Simpson *et al.*, 1993).

1.4.8 EXOPOLYSACCHARIDE

The role of exopolysaccharide is another key example of the differences between *P. aeruginosa* and *B. cepacia* colonisation in CF. Many bacterial species synthesize organic polymers which are deposited outside the cell wall as a capsule or as a slime layer (Sutherland, 1985). The roles of these exopolymers are varied including receptors for bacteriophage, mediating cell-to-cell interactions and also providing a protective barrier as typified by bacterial microcolonies (Costerton *et al.*, 1990). A prime example of a bacterial exopolysaccharide is the alginate polymer produced by mucoid strains of *P. aeruginosa*, which is considered to be the major virulence factor in pulmonary colonisation and immune-mediated damage in CF lungs (Govan & Harris, 1986). There is little evidence for alginate biosynthesis in *B. cepacia* with the exception of an alginic acid-like compound, containing 72% guluronic acid with 1.75% acetylation, described in one strain of *B. cepacia* (Straus *et al.*, 1990). Recent PCR studies with primers of the *P. aeruginosa alg* D gene, which encodes the essential enzyme GDP mannose dehydrogenase, indicate that it is unlikely that *B. cepacia* could produce an alginate like compound as this gene was absent in 10 *B. cepacia* strains studied (Nelson *et al.*, 1994). Sage *et al.* (1990) demonstrated that *B. cepacia* strains which were glucose dehydrogenase deficient (Gcd^-) formed large amounts of an exopolysaccharide comprising galactose, glucose, mannose, guluronic acid and rhamnose. Surveys of clinical isolates from patients with CF indicated that there was no correlation between the ability of *B. cepacia* to colonise the respiratory tract and the capacity of Gcd^- strains to produce exopolysaccharide.

1.4.9 EXTRACELLULAR VIRULENCE FACTORS

P. aeruginosa colonisation in CF is associated with the production of several classic extracellular virulence factors such as exotoxin A, an ADP-ribosyl transferase similar to diphtheria and cholera toxin, and the potent proteolytic enzyme elastase in addition to a multitude of other factors (Vasil, 1986; Bainbridge & Fick, 1989). *B. cepacia* does not appear to produce the factors associated with *P. aeruginosa* virulence and pathogenicity in CF lung infections, namely exotoxin A or elastase. *B. cepacia* does however, produce a number of extracellular products including protease, gelatinase, haemolysin and lipase although a role for any of these products in the pathogenesis of CF lung infection has not been shown (McKevitt & Woods, 1984; Nakazawa *et al.*, 1987; Gessner & Mortensen, 1990). *B. cepacia* isolates from plants produce high levels of pectolytic enzyme; however this enzyme has only been found in low levels in strains of clinical origin (Gonzalez & Vidaver, 1979). In a study of potentially pathogenic factors, Gessner & Mortensen (1990) demonstrated that a number of characteristics are more frequently observed in isolates of *B. cepacia* from CF patients than from other clinical sources or the environment. These factors include the production of catalase, ornithine decarboxylase, valine aminopeptidase, C14 lipase, alginase, trypsin, the reduction of nitrate to nitrite, hydrolysis of urea and xanthine and complete haemolysis of bovine erythrocytes. The significance of these observations in relation to pathogenesis in CF is not clear. Indeed, the epidemic strain of *B. cepacia* which is associated with a fatal clinical outcome in CF does not produce C14 lipase (Govan *et al.*, 1993).

B. cepacia Protease

Bacterial proteases have been shown to damage host tissues directly (Marks, 1981). Several studies describe protease production by CF and non-CF isolates of *B. cepacia* suggesting that protease production is probably a characteristic feature of most strains (Burkholder, 1950; McKevitt & Woods, 1984; Montie *et al.*, 1985; Nakazawa *et al.*,

1987; McKevitt *et al.*, 1989). Further characterisation of the *B. cepacia* protease showed that it was a 34 kDa metalloprotein capable of cleaving hide powder and collagen but, unlike *P. aeruginosa* elastase, the *B. cepacia* protease did not degrade IgG, IgM, IgA or secretory-IgA. Immunologic cross-reactivity of *B. cepacia* protease with *P. aeruginosa* elastase was observed, although the amino acid composition of the two proteases is dissimilar (McKevitt *et al.*, 1989). Intratracheal installation of *B. cepacia* purified protease into rat lungs produced bronchopulmonary pneumonia characterised by polymorphonuclear cell infiltration and proteinaceous exudation into large airways. Active immunisation of rats with *B. cepacia* protease elicited an immunological response, although this was not protective against subsequent lung infection with *B. cepacia* (McKevitt *et al.*, 1989). Montie *et al.* (1985) reported that a highly proteolytic isolate of *B. cepacia* from a patient with CF was found in cultures of liver cells five days after inoculation into the wound in a burned-mouse model, suggesting that bacteraemia had occurred. A clinical corollary to this was observed when two gelatinolytic strains of *B. cepacia* were isolated from two cases of *B. cepacia* bacteraemia subsequent to burn wound colonisation (Brauner *et al.*, 1985).

B. cepacia Lipases

There is some evidence that lipases, especially phospholipase C (PLC), play an important role in virulence. PLC is a lecithinase which cleaves phosphatidylcholine, a phospholipid found in mammalian cell membranes, into phosphorylcholine and diacylglycerol (Esselmann & Liu, 1961). The normal physiological role of lipases is probably nutritional - liberating fatty acids for use as an energy source, for example PLC of *P. aeruginosa* has been implicated in the scavenging of phosphates (Lonon *et al.*, 1988). PLC is associated with the cytopathology of lung disease and by acting directly on pulmonary tissue the enzyme may be responsible for the extensive

destruction of lung parenchyma observed in post-mortem lungs from CF patients with "*Cepacia* Syndrome" (Thomassen, *et al.*, 1985).

B. cepacia has often been described as lipolytic (Starr & Burkholder, 1941; Morris & Roberts, 1959; Carson *et al.*, 1973; McKevitt & Woods, 1984; Nakazawa *et al.*, 1987; Lonon *et al.*, 1988). Lipase activity has been detected on a number of different substrates including egg-yolk agar, polyoxyethylene sorbitans (Tweens) and *p*-nitrophenylphosphorylcholine (Lonon, *et al.*, 1988). *B. cepacia* lipase activity has been detected in culture supernatants of exponentially growing and stationary phase cells in the absence of exogenously supplied substrate, suggesting that lipase is produced constitutively. The purified enzyme was found to have a molecular weight of approximately 24 kDa and was not cytotoxic for HeLa cells or for mice injected intravenously with the purified lipase. A recent study by Straus *et al.* (1992) demonstrated that *B. cepacia* lipase acts indirectly to exert an antiphagocytic effect on rat pulmonary macrophages. Phagocytosis of *B. cepacia* by rat pulmonary macrophages was significantly reduced when the cells were preincubated with lipase or when phagocytosis occurred in the presence of lipase. Scanning electron microscopy showed that macrophages exposed to *B. cepacia* lipase had fewer pseudopodia and microvilli when compared to untreated macrophages (Straus, *et al.*, 1992). Thus *B. cepacia* lipase may be an important virulence factor allowing the bacteria to evade the mammalian host defences.

B. cepacia produces more than one haemolysin, including a heat-labile haemolysin that has both PLC and sphingomyelinase activities (Vasil *et al.*, 1990). The frequency of haemolysin expression amongst *B. cepacia* strains is inconsistent, ranging from 4-40% dependent on the type of erythrocytes used (Nakazawa *et al.*, 1987; Gessner & Mortensen, 1990; Vasil *et al.*, 1990). Unlike PLC activity in *P. aeruginosa*, the activity of PLC in *B. cepacia* does not correlate with haemolytic activity, although all

strains of *B. cepacia* do produce detectable amounts of extracellular PLC activity (Nakazawa *et al.*, 1987; Vasil *et al.*, 1990). The gene that encodes the PLC of *B. cepacia* is largely homologous to the *P. aeruginosa* PLC gene, particularly at the amino terminus with the exception that there is considerable hypervariability in the genetic organisation of the *B. cepacia* gene (Vasil *et al.*, 1990).

1.4.10 Regulation of Virulence Factors in *Burkholderia cepacia*

The ability of a bacterial pathogen to sense a changing or new environment and to modify expression of surface related and extracellular features accordingly confers survival advantage and may contribute to pathogenicity. Several possible mechanisms for genetic control of the regulation and expression of the potential virulence factors of *B. cepacia* exist. Plasmids which may contain antibiotic resistance or virulence genes have been described in *B. cepacia* although their distribution among *B. cepacia* strains appears to be erratic (Beckman & Lessie, 1979; Gonzalez & Vidaver, 1979; Williams *et al.*, 1979; McKevitt & Woods, 1984; Gaffney & Lessie, 1987; Larsen *et al.*, 1993). Interestingly, *B. cepacia* genomic DNA contains numerous insertion sequences (IS) (Gaffney & Lessie, 1987). IS are transposable elements which have the ability to insertionally regulate chromosomal or plasmid-encoded determinants and to mediate incorporation of foreign genes. *B. cepacia* is unique in the large numbers of IS identified and, in contrast, these elements have yet to be discovered in *P. aeruginosa* (Scordilis *et al.*, 1987). The ability to control gene expression by transposable gene-activating elements may explain the phenotypic variability, including the apparent difference in virulence and virulence factors, that is observed in isolates of *B. cepacia*.

1.4.11 THE HOST RESPONSE

B. cepacia has several potential virulence factors which may be involved in colonisation and pathogenesis in CF. In this section I have attempted to summarise the accumulating data available on the pathogenicity and virulence factors of *B. cepacia.* A striking feature of this literature is the dearth of information on the host immune response to this opportunist pathogen. Observations of patients attending the Edinburgh adult CF clinic, particularly those patients colonised with the epidemic strain, lead us to the speculation that undetermined host factors are a major influence on the clinical course of *B. cepacia* colonisation.

Aronoff and colleagues have shown that colonisation with *B. cepacia* occurs in CF patients already colonised by *P. aeruginosa* despite the presence of serum IgG antibodies against shared outer membrane proteins (Aronoff & Stern, 1988; Aronoff *et al.*, 1991). Similarly McKevitt *et al.* (1989) observed immunogenic cross-reactivity between *B. cepacia* protease and *P. aeruginosa* elastase in CF patients colonised with *P. aeruginosa* who subsequently acquired *B. cepacia.* Failure to prevent subsequent *B. cepacia* colonisation suggests that these antibodies are not protective. More recently, Jensen *et al.* (1993) have shown considerable homology between the GroEl stress proteins of *P. aeruginosa* and *B. cepacia.* The authors speculate that colonisation of CF patients by either or both of these organisms may be associated with the autoimmune diseases seen in CF as there is extensive homology between bacterial stress proteins and the homologous host protein. Immunological studies on *B. cepacia* colonisation of patients with CF indicate that the organism persists in the presence of a considerable antibody response and suggest the possibility of immune mediated damage.

In the single study describing killing by human polymorphonuclear leukocytes and normal human serum of one strain of *B. cepacia* (Anwar *et al.*, 1983) the degree of

sensitivity observed varied according to the conditions in which *B. cepacia* was cultured.

Our knowledge of the behaviour and the significance of *B. cepacia* as a pathogen in non-CF patients is limited. In chronic granulomatous disease where oxidative killing mechanisms are absent, patients are at particular risk as *B. cepacia* is resistant to non-oxidative neutrophil-mediated killing. However such a deficiency has not been described in patients with CF.

Recently a strain of *B. cepacia* associated with rapidly fatal illness in a CF patient has been shown to invade respiratory epithelial cells *in vitro* (Burns *et al.*, 1992). The ability to invade respiratory epithelial cells might not only be important in the context of virulence, but also has implications for antibiotic therapy as bacteria in an intracellular location would be protected from the activity of antimicrobial agents such as β-lactams and aminoglycosides which are often used in the management of infection in CF patients.

In summary, the few studies that have been carried out to date indicate that *B. cepacia* is able to persist in the presence of a pronounced humoral immune response and evade other immunological defences. *B. cepacia* may be able to evade eradication by inactivating defence mechanisms; for example the *B. cepacia* lipase activity which appears to affect phagocytosis (Straus *et al.*, 1992). The possibility that *B. cepacia* may be intracellular is a concept which is gaining increasing support, particularly since *B. cepacia* is closely related to *Burkholderia pseudomallei* which has recently been shown to be an intracellular pathogen (Pruksachartvuthi *et al.*, 1990).

AIMS OF THIS THESIS

Pulmonary colonisation of patients with cystic fibrosis by *B. cepacia* has become a cause of considerable concern for CF patients, their relatives and their carers and represents a significant medical and scientific challenge. Our knowledge and understanding of the virulence and pathogenesis of *B. cepacia*, particularly with respect to the interaction with the CF host, is inadequate to answer the multitude of questions posed by those involved with CF patients. The objective of this thesis was to evaluate the basic characteristics of *B. cepacia*, in particular those which may be relevant to pulmonary infection in the CF lung, and to study the interaction of *B. cepacia* with the CF host by investigating aspects of the humoral immune response. The following four major topics of research were covered:-

1. A study of the general characteristics and biological properties of *B. cepacia*, with particular emphasis on the properties of the highly transmissible epidemic strain.

2. Investigation of the adherent properties of *B. cepacia* to mucin and epithelial cells and the possible contribution of adherence to the initial stages of pulmonary colonisation.

3. In view of the association of *B. cepacia* with bacteraemia in cystic fibrosis, the ability of *B. cepacia* to resist the bactericidal activity of normal human serum was examined.

4. Detection and characterisation of anti-*B. cepacia* antibodies in serum and sputum from patients with CF. The nature of the antibody response was investigated to determine specificity, the potential use for diagnosis and as a prognostic indicator

and finally the antibodies were examined for deficiencies which may explain why *B. cepacia* is not eradicated.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PATIENTS AND SPECIMENS

Patients

Sputum and serum were obtained from CF patients attending the adult CF clinic (age range 19 - 29 years) at the Western General Hospital (WGH), Edinburgh. In addition saliva, nasal and buccal scrapes were also obtained from selected patients as required. Sputum from CF patients attending the Edinburgh paediatric unit and serum from adult CF patients attending the Manchester CF clinic were also examined. For the purpose of this thesis, CF patients were classified on the basis of colonisation with *B. cepacia*: a) non-colonised patients, from whom *B. cepacia* had never been isolated; b) transiently colonised patients from whom *B. cepacia* had been isolated on one or two occasions only; c) chronically colonised patients who were persistently colonised by *B. cepacia*; and d) those patients who were chronically colonised with the epidemic strain of *B. cepacia*. The bacteriology of each patient, particularly co-colonisation with *P. aeruginosa*, was taken into account. Controls were chronic bronchitics attending the Respiratory Unit at the WGH matched for degree of bronchiectasis, and normal human volunteers from the laboratory.

Specimen Processing

Sera to be used in antibody studies were aliquoted into micronic tubes (Alpha Laboratories, Eastleigh, Hants.) and stored at -20^{o}C until analysis. Serum required in

serum sensitivity assays was prepared by placing fresh venous blood in a water bath at 37°C for 30 min to allow clotting and then separated by centrifuging at 3980 *g* for 20 min (Bactifuge, Heraeus-Christ GMBH, Osterodf. W, Germany). Normal human sera were pooled (PNHS) and half was aliquoted in 2 ml volumes in cryotubes (Nunc, Inter Med, Kamstrup, Roskilde, Denmark) and stored at -70°C whilst the remaining sera was heat inactivated (HIS) for 30 min at 56°C prior to storage. CF sera were treated in the same manner but not pooled.

Bacteriology of sputum specimens was routinely performed by Mrs C. Doherty (Department of Medical Microbiology, University Medical School, Edinburgh). Sputa were homogenised in sputalysin (Calbiochem, La Jolla, CA, USA) and following dilution were cultured quantitatively on blood agar, Hartley broth agar (incorporating bacitracin), *Pseudomonas* isolation agar (PIA) and *Pseudomonas cepacia* Medium (CEP). Any remaining sputum was aliquoted into 1.5 ml Eppendorf tubes (Elkay Products Inc., Shrewsbury, MA, USA) and centrifuged (Micro Centaur, MSE, Crawley, Surrey) at 11,600 *g* for 15 min. The sol phase was aliquoted in 200 µl volumes and stored at -70°C until analysed.

2.1.2 BACTERIA

Bacterial cultures were identified as *B. cepacia* by growth on CEP, colonial morphology and by biochemical profile using API 20NE multi-tests.

B. cepacia Typing

B. cepacia typing, performed by Mrs C. Doherty, included bacteriocin typing of *B. cepacia* strains by the spotting method described by Govan & Harris (1985) and genotypic typing by Pulsed Field Gel Electrophoresis (PFGE: CHEF Bio-Rad Laboratories Inc., Hercules, CA, USA).

Table 2. Bacterial Strains used in this Thesis.

Bacterial Strains	Description	Source
B. cepacia ATCC: 10856; 17762; 17765; 25416; 25608; 29414.	ATCC Catalogue (1989)	Dr I.N. Simpson Smithkline Beecham Pharmaceuticals Brockham Park Surrey
B. gladioli ATCC 10248	ATCC Catalogue (1989)	Dr I.N. Simpson
B. cepacia ATCC 17616 (and derivatives)	Sage *et al.* (1990)	Dr. T.G. Lessie Dept. of Microbiology University of Massachusetts Amherst, Massachusetts.
B. cepacia Pc 5, Pc 24.	Sajjan *et al.* (1992)	Dr J. Forstner Hospital For Sick Children University of Toronto Toronto, Ontario.
B. cepacia: J2534; 2535; 2536; 2537; 2538; 2539; 2540; 2441; 2542; 2543.	Environmental survey, Royal Botanic Gardens, Edinburgh	Dr J.R.W Govan Dept. of Medical Microbiology University Medical School Edinburgh
P. aeruginosa PAO1	Holloway (1955)	
P. aeruginosa J1385	Jacuzzi isolate	
E. coli J2319	UTI isolate	
S. aureus C1705	CF isolate	
E. coli R3	core R-LPS mutant	Dr I.R. Poxton Dept. of Medical Microbiology University Medical School, Edinburgh.
E. coli 018: K5	S-LPS	
P. aeruginosa PAC608	Rowe & Meadow (1983)	
Neisseria meningitidis A14	clinical isolate	Dr John Stewart Dept. of Medical Microbiology University Medical School Edinburgh.

Strains used in this thesis

The bacterial strains used in this thesis are shown in Table 2. All other strains, including environmental, clinical and CF isolates of *B. cepacia* and *P. aeruginosa*, are indicated in the relevant section of the Results, prefixed with SBC, J , JN or C.

Maintenance of bacterial strains

Bacterial strains were maintained at -70^{o}C in 10% w/v skim milk (Oxoid L31, Oxoid Ltd., Basingstoke, Hants.). Bacterial suspensions were prepared by emulsifying several single colonies in 1.0 ml volumes of skim milk in 2.0 ml cryotubes. Fresh maintenance cultures were prepared at approximately four monthly intervals.

2.1.3 CHEMICALS AND MEDIA

Chemicals

Unless otherwise stated all chemicals were Analar grade (BDH, Merck Ltd., Poole, Dorset) and were prepared in distilled water.

Complex Media

a) Nutrient yeast broth (NYB) was Oxoid No.2 supplemented with 0.5% w/v yeast extract (Oxoid).

b) Isosensitest broth (ISTB) was Oxoid.

c) Nutrient agar (NA) was Columbia agar base (Oxoid).

d) *Pseudomonas cepacia* selective Medium (CEP) (Mast Laboratories, Bootle, Merseyside).

e) *Pseudomonas* Isolation agar (PIA) (Difco Laboratories, Detroit, Michigan, USA).

f) Furunculosis agar (Ogunnariwo & Hamilton-Miller, 1975) for detection of melanin pigment production consisted of 1.0% w/v tryptone, 0.5% w/v yeast extract, 0.1% w/v L-tryptone, 0.25% w/v sodium chloride and 1.5% w/v Bacteriological agar (Oxoid).

g) Elastin-TSA plates for determination of elastase production consisted of 4.5% w/v tryptone soy agar (TSA, Oxoid) with 0.1% w/v UV sterilised elastin-orcein (Sigma Chemical Co., Poole, Dorset).

h) Tryptose minimal broth (TMB) for PLC production consisted of 0.2% w/v tryptose (Sigma), 120 mM Tris HCl pH 7.2, 50 mM glucose, 16 mM $CaCl_2$, 10 mM KCl and 20 mM $(NH_4)_2SO_4$ (Based on Berka *et al.*, 1981).

i) Semi-solid agar for motility studies (Luzar *et al.*, 1985) consisted of 0.1% w/v tryptone (Difco), 0.3% w/v yeast extract (Oxoid), 0.5% w/v sodium chloride and 0.4% w/v Bacteriological agar (Oxoid).

j) EMB/Glucose Indicator Media (Sage *et al.*, 1990) for detection of glucose dehydrogenase deficient (Gcd^-) strains of *B. cepacia* consisted of 0.2% w/v yeast extract, 1.0% w/v glucose, 1.5% w/v Bacteriological agar, 200 µg/ml eosin Y (Sigma) and 33 µg/ml methylene blue (Sigma).

k) TBT media for recovery of *B. cepacia* from soil (Hagedorn *et al.*, 1987) consisted of 2% w/v Bacteriological agar (Oxoid), 0.1% w/v L-asparagine, 0.1% w/v $NaHCO_3$, 0.05% w/v KH_2PO_4, 0.01% w/v $MgSO_4.7H_2O$, 0.005% w/v Trypan Blue (Sigma) and 0.002% w/v tetracycline. The pH was adjusted to 5.5 with 10% phosphoric acid and the filter sterilised tetracycline was added to the autoclaved

medium. To reduce fungal contamination crystal violet (5 mg/litre) and 0.005% w/v filter sterilised nystatin were added.

All commercial media were prepared by the departmental Media Kitchen according to manufacturers' instructions. Media were made up in distilled water and sterilised by autoclaving at 15 psi for 15 min.

l) Malka minimal media (MM) was prepared as follows:-

Solution A: Na_2HPO_4 (73.4 mg/ml); KH_2PO_4 (32.4 mg/ml)

Solution B: $MgSO_4.7H_2O$ (20.5 mg/ml)

Solution C: 1% w/v glucose

Solution D: $FeSO_4.7H_2O$ (1.83 mg/ml) in sterile distilled water to which one drop of concentrated hydrochloric acid (HCl) was added.

Solution E: $(NH_4)_2SO_4$ (50.0 mg/ml)

Solutions were prepared with sterile distilled water and were filter sterilised. All solutions except C were stored over chloroform. To prepare 1 litre of Malka, 20 ml A, 20 ml B, 20 ml C, 1 ml D and 20 ml E were added to 919 ml sterile distilled water.

m) *B. cepacia* enrichment broth (BcEB) was Malka minimal media with 300 U filter sterilised polymyxin B (Sigma).

2.1.4 MISCELLANEOUS

Animal Blood and Serum

Citrated animal blood for use in haemagglutination assays was obtained from Moredun Animal Diseases Research Institute, Gilmerton Road, Edinburgh; the Transgenic Unit, University Medical School, Edinburgh and Becton Dickinson Laboratories. For serum sensitivity assays heat inactivated sheep serum was also obtained from Moredun and sheep erythrocytes for determination of complement

activity were obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire.

Lipopolysaccharide

Rough- and smooth-LPS from *P. aeruginosa* were prepared by Dr J. Nelson (Dept. of Medical Microbiology, University Medical School, Edinburgh). Phenol water extracts of *B. gladioli* ATCC 10248 were prepared by Mrs D. Shaw (Dept. Medical of Microbiology, University Medical School, Edinburgh).

Mucins

Purified human mucins for adherence studies were obtained from Dr J.K. Sheehan and Dr D.J. Thornton (Department of Biochemistry and Molecular Biology, University of Manchester) from asthmatic and CF patients. Mucin was supplied and stored as a solution in 4 M guanidinium chloride at 4^{o}C. Prior to use in adherence assays mucins were diluted in coating buffer (100 µg/ml), added to pre-boiled dialysis tubing with a 2000 molecular weight cut-off (Spectrum Medical Industries Inc., Los Angeles, CA, USA) and dialysed overnight with stirring against 1 litre of coating buffer at 4^{o}C.

Buffy Coat

Monocytes for chemiluminescent opsonophagocytosis assays were prepared from Buffy Coat obtained from the Blood Transfusion Service (BTS, RIE, Lauriston Place, Edinburgh, Lothian).

Antisera

Polyclonal antisera to *B. cepacia* was prepared in Dutch rabbits provided by Dr I.R. Poxton (Department of Medical Microbiology, University Medical School, Edinburgh). *B. cepacia* (J2395, C1359 and C1409) were heat killed in a water bath at 62^{o}C for 15 min (C1359) or 30 min (J2395 and C1409). Rabbits were immunised

with 1.0 ml doses into the marginal vein of the ear on days 1,2,3,8,9,10 and 22 by Mr J. Verth (Transgenic unit, University Medical School, Edinburgh) and a test bleed performed on day 29 according to the method of Hancock & Poxton (1988). The rabbit anti-*P. aeruginosa* flagella type-a and -b antisera were provided by Dr G.H. Shand (Statens Seruminstitut, Copenhagen, Denmark).

2.1.5 EQUIPMENT

Equipment and apparatus used in the experimental studies is indicated in the relevant section of the text. Manufacturers' addresses are only cited once.

2.2 EXPERIMENTAL METHODS

2.2.1 CULTURE OF BACTERIA

Unless stated otherwise bacteria were all cultured at 37°C. Bacteria were streaked onto NA or CEP as appropriate from the maintenance culture and were subcultured onto fresh plate media within seven days. A maximum of four subcultures were performed after which time fresh cultures were prepared from maintenance cultures.

Bacteria were inoculated into 10 ml of liquid medium which when required could be inoculated into larger volumes for bulk growth. Liquid cultures were incubated in an orbital incubator (Gallenkamp, Loughborough, Leics.) at 100-120 rpm unless otherwise indicated.

2.2.2 MEASUREMENT OF BACTERIAL CONCENTRATION

Viable counts

Serial ten-fold dilutions of bacteria were prepared in sterile phosphate buffered saline (PBS; 50 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl) and volumes of 100 µl were spread onto NA plates. Plates were incubated at 37°C for 24-48 h, depending on the *B. cepacia* strain, and colonies were counted using a colony counter (Gallenkamp).

Measurement of Optical Density

Measurements of absorbance were made at 525 nm (spectrophotometer, Spectronic 20, Bausch & Lomb). The suspending medium was used as a blank and as a diluent for optical density (OD) measurements. A standard curve of log bacterial numbers (as determined by viable count) against OD 525 was drawn. An optical density of 0.15 at 525 nm is equivalent to a concentration of approximately $1x10^8$ colony forming units (cfu) of *B. cepacia* /ml.

2.2.3 Measurement of Bacterial Growth

Effect of Temperature

A 250 ml flask containing 150 ml NYB was inoculated with 150 µl of an overnight preculture grown in NYB. The flasks were incubated at 25°, 37° or 43°C in an orbital incubator. At 2 h intervals (up to 10 h) and at 24 h, samples were removed for measurement of optical density and viable counts.

Effect of Culture Conditions

An overnight preculture of *B. cepacia* grown in NYB was harvested by centrifugation (3250 *g* for 10 min) and washed twice in PBS. The bacteria were resuspended in PBS and retained at room temperature for 2-3 h prior to adding 150 µl of this suspension to a 250 ml flask containing 150 ml NYB or MM. Flasks were incubated statically or with aeration at 37°C. Measurement of optical density and viable counts were performed as above.

2.2.4 Survival of *B. cepacia*

Water

Several single colonies of *B. cepacia* were emulsified in 10 ml of tap or pyrogen-free water which were retained at room temperature. At weekly intervals, for a period of 4 months, 100 µl was removed and spread onto CEP. After incubation at 37°C for 24-48 h the plates were examined for growth of *B. cepacia*.

Antiseptics

The triple challenge capacitance test of Kelsey & Sykes (1969) was used to determine the efficacy of antiseptics against *B. cepacia*. The antiseptics were used neat (as directed by manufacturer) or diluted in sterile distilled water to three concentrations: (1) the manufacturer's recommended concentration; (2) 25% less concentrated; and (3) 25% more concentrated than the recommended concentration. Bacteria (1.0 ml of

an overnight culture) in NYB were added to 3 ml of the antiseptic, and the mixture was shaken. After 8 min at room temperature, five 50 µl volumes were removed and spotted onto NA. After 2 min the antiseptic was challenged with a further 1 ml of the bacterial culture, and 8 min later five 50 µl volumes were removed as before. The process was repeated once more. After allowing the spots to dry the plates were incubated for 24-48 h. The antiseptics and recommended concentrations were Povidone Iodine (neat; Betadine, Napp Laboratories Ltd., Cambridge, Norfolk), Listerine (neat; Warner Lambert Healthcare, Hampshire), 70% ethanol, Cetavlon (1:20; ICI Pharmaceuticals Division, Macclesfield, Cheshire), Hibitane (1:100; ICI) and Roccal (1:10; Winthrop Pharmaceuticals, Surbiton-upon-Thames, Surrey)

2.2.5 Screening *B. cepacia* for Melanin Production

B. cepacia was subcultured from CEP to furunculosis agar and incubated for 48 h at 37°C. Plates were then examined for the production of melanin, a dark brown diffusable pigment.

2.2.6 Determination of Elastase Production

An elastase assay, based on that of Baker (1982) was used to examine strains of *B. cepacia* for elastase production. Elastase-TSA plates were inoculated with bacterial suspensions (1 colony emulsified in 1 ml sterile saline) using a multi-point inoculator (Denley Instruments Ltd., Billingshurst, Sussex). After incubation for 24-48 h plates were examined and the diameter of any zone of clearing was measured.

2.2.7 Determination of Phospholipase C Activity

The assay to determine PLC activity was based on the method of Berka *et al.* (1981). Single colonies from CEP and PIA were used to inoculate 2 ml TMB in capped test tubes (16 x 150 mm). The tubes were shaken at 120 rpm for 48 h at 37°C, and the optical density of each culture was measured at 540 nm. Cells were removed from 1

ml of culture by centrifugation at 11,600 *g* for 5 min, and approximately 10 µg of activated charcoal was added to the supernatant to remove pigment. After centrifuging to remove the charcoal 10 µl of the clear supernatant was added to 90 µl NPPC reagent in a 96 well flat-bottomed microtitre plate (Nunc). The NPPC reagent contained 250 mM Tris HCl pH 7.2; 60% w/w glycerol; 1.0 µM $ZnCl_2$ and 10 mM *p*-nitrophenylphosphorylcholine (NPPC; Sigma). The plates were incubated for 1 h in a plate incubator (Dynatech Laboratories Ltd., Billingshurst, West Sussex) shaking at 37°C before the absorbance at 405 nm was measured in an automated plate reader (Titertek Multiskan/MC, Flow Laboratories Ltd., Irvine, Strathclyde).

2.2.8 SCREENING OF *B. CEPACIA* FOR EXOPOLYMER PRODUCTION

B. cepacia strains were patch inoculated on to the following plate media: CEP; NA; PIA; EMB/glucose indicator medium; MM and MM with solution E replaced by 10 mM KNO_3. The plates were incubated at 37°C for 72 h in a moist atmosphere and then examined for mucoid colonial phenotype. Those strains which appeared to produce an exopolymer were mixed with India ink and examined under phase contrast microscopy for capsule production.

2.2.9 MOTILITY ASSAYS

Motility in Semi-solid Agar

Bacterial strains were cultured on CEP and five colonies for each strain were stab inoculated into the centre of the motility medium. For each assay triplicate plates per strain were incubated at 37°C and examined for colony spreading. Colony diameter was measured after 24-48 h.

Motility in Liquid Culture

Bacterial strains were cultured in NYB for 16 h and examined for motility under phase contrast microscopy.

2.2.10 Electron Microscopy of Whole Cells

Simple negative staining was performed using the method described by Hancock & Poxton (1988). Overnight cultures of *B. cepacia* grown in NYB were harvested by centrifugation, washed once in sterile PBS and the pellet was resuspended to milky turbidity in 1.0% w/v ammonium acetate. Formvar-coated 400 mesh copper grids were floated on a drop of this suspension for approximately 1 min then touched against filter paper (Whatman No. 1) to remove excess moisture. The grid was then placed on a drop of 2.0% w/v phosphotungstic acid (PTA) (neutralised to pH7.0 with sodium hydroxide) for 1 min. Grids were dried in a desiccator prior to viewing in a Hitachi 12A transmission electron microscope at 75 KV.

2.2.11 Measurement of Cell Surface Hydrophobicity

Cell surface hydrophobicity was determined by hydrophobic interaction chromatography (HIC) based on the method of Smyth *et al.* (1978) using octyl sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) as the non-polar ligand and Sepharose CL-4B (Pharmacia) to correct for non-specific adsorption.

Disposable polystyrene chromatography columns (Pierce & Warriner (UK) Ltd., Chester) were prepared fresh according to the manufacturing instructions. Columns were packed with pre-swollen gel slurries which had been washed to remove preservative and then prepared with 50 mM phosphate binding buffer (pH 7.0) containing 1.7M $(NH_4)_2\ SO_4$ (gel constitutes approximately 70% of the final volume). The gel slurries were degassed prior to adding to columns to give a 2 ml packed bed volume. Columns were then washed with binding buffer and capped until required to prevent drying.

Bacteria cultured overnight in 10 ml NYB were harvested and washed twice in sterile binding buffer. The bacteria were resuspended in binding buffer to give 10^4 cfu/ml before application to the columns. Bacterial suspensions (2 ml) were added to the column followed by 2 ml 4M NaCl to ensure adequate wash-through of cells. Retention of cells by the columns was assessed by viable counts of the initial suspension and of the column eluates.

2.2.12 Proteinase K digestion of Bacteria for the Preparation of Lipopolysaccharides

The Proteinase K digestion of bacteria (Hitchcock & Brown, 1983), suitable for both R- and S-LPS types, was used to prepare LPS for analysis by polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Bacteria cultured overnight in 10 ml NYB were harvested by centrifugation and washed twice in PBS. The bacteria were resuspended in PBS to give an absorbance between 0.5 and 0.6 at 525 nm as measured on the spectrophotometer. Bacterial suspension (1.5 ml) was transferred to an Eppendorf tube and the bacteria sedimented by centrifugation at 11,600 *g* for 3 min. The pellet was resuspended in 50 µl single strength PAGE sample buffer (see page 71) and boiled (100°C) for 10 min. Once cooled 10 µl sample buffer containing 25 µg Proteinase K (protease Type XI, Sigma) was added and samples were incubated at 60 °C for 60 min in a water bath. Samples were stored at -20°C.

2.2.13 Preparation of Rough Lipopolysaccharide

The aqueous phenol, chloroform, petroleum ether (PCP) method of Galanos *et al.* (1969) incorporating the diethyl ether precipitation of LPS described by Qureshi *et al.* (1982), was used to prepare LPS from the epidemic strain of *B. cepacia* which expresses the R-LPS phenotype.

Two litre flasks (ten) containing 1 litre of NYB were inoculated with 2.0 ml of an overnight preculture grown in NYB. After incubation in an orbital incubator (120 rpm) at 37°C for 48 h bacterial cells were harvested by centrifugation at 15,000 *g* using a KSB continuous flow system (Dupont UK Ltd., Stevenage, Herts.). Cells were then washed twice in PBS with centrifugation (Sorval RC-5B refrigerated superspeed centrifuge, Dupont) at 10,000 *g* for 10 min. The bacterial pellet was frozen at -20°C, freeze dried (Edwards Modylo freeze dryer, Edwards High Vacuum Ltd., Crawley, Surrey) and weighed.

The extraction solvent (PCP) consisted of 90% w/v aqueous phenol (90 g phenol were dissolved in 10 ml water at 45°C and then made up to a total of 100 ml with distilled water), chloroform and petroleum spirit (boiling point 40°-60°C) in the proportions 2:5:8 by volume. Dried bacteria were solubilised in PCP at 25% w/v, stirred for 2 min at room temperature and centrifuged at 10,000 *g* for 15 min. The supernatant was filtered through Whatman No. 1 filter paper into a round bottomed flask. The centrifuged pellet was re-extracted by the same procedure and the filtered supernatants were pooled and then rotary evaporated (Buchi Rotavapor-RE111, Switzerland) to remove petroleum spirit and chloroform.

In order to precipitate the LPS six volumes of diethyl ether/acetone in the ratio of 1:5 by volume were added to one volume of the phenol solution (Qureshi *et al.*, 1982). After standing at room temperature for at least one hour the LPS was sedimented by centrifugation at 5000 *g* for 10 min. The centrifuge tubes were drained and the pellet washed three times with diethyl ether/acetone, the pellet being recovered by centrifugation each time. The final pellet was dried under vacuum until the smell of ether/acetone was no longer detectable. The dried pellet of LPS was resuspended in 5 ml pyrogen-free water with the aid of a syringe and a 23-gauge needle and recovered by ultracentrifugation (Sorval ultracentrifuge - OTD65B, Dupont) at 100,000 *g* for 4

h. The LPS was then taken up in a minimal amount pyrogen-free water, lyophilised and stored at -20°C.

2.2.14 Preparation of Smooth Lipopolysaccharide

Extraction of S-LPS from *B. cepacia* strains was based on the aqueous phenol method of Westphal & Luderitz (1954). Bacteria were cultured and lyophilised as described for R-LPS preparations.

The lyophilised pellet was finely divided, resuspended to a concentration of 5% w/v in pyrogen-free water and heated to 67°C in a water bath. An equal volume of 90% w/v aqueous phenol was added to the bacterial suspension in the water bath and the mixture stirred at 67°C for 15 min. The mixture was transferred to 50 ml centrifuge tubes, cooled in an ice bath to allow initial separation of the aqueous and phenol phases and centrifuged at 10,000 *g* for 15 min to complete the separation. The upper (aqueous) phase containing the LPS was removed and the extraction procedure repeated on the lower phase. The aqueous phases were pooled, transferred to pre-boiled dialysis tubing and dialysed against running tap water overnight until the smell of phenol was no longer detectable. Any insoluble deposit was removed by centrifuging twice for 15 min at 10,000 *g*. The dialysed extract was then concentrated by rotary evaporation to approximately one-fifth of the original volume and then ultracentrifuged at 100,000 *g* for 3 h. The gelatinous pellet obtained was resuspended in pyrogen-free water with the aid of a syringe and a 23-gauge needle and recentrifuged. The final LPS pellet was suspended in approximately 0.5 ml pyrogen-free water, freeze dried, weighed and stored at -20°C.

2.2.15 Crude Preparation of Outer Membrane Proteins

Overnight cultures of *B. cepacia* strains grown in one litre flasks containing 500 ml ISTB at 37°C with aeration were harvested and washed twice in PBS at 6000 *g* for

10 min at 4°C. The pellet was resuspended in 20 ml pyrogen-free water and sonicated at an amplitude of 8 μm (Microson, Ultrasonic Cell Disruptor, Heat Systems-Ultrasonics Inc., NY, USA) to break cells. Unbroken cells were removed by centrifugation at 7000 *g* for 10 min. Outer membranes were derived by solubilisation in 2% w/v Sarkosyl (N-lauroyl sarcosinate; Sigma) for 45 min at room temperature. Outer membranes were collected and washed by centrifugation at 38,000 *g* for 1 h at 4°C. The pellet was resuspended in 0.5 ml pyrogen-free water and frozen at -20°C. Protein concentration in the samples was approximated by comparing density of bands on a polyacrylamide gel to known standards.

2.2.16 Preparation of Flagella

For preparation of *B. cepacia* flagella, 2 litre flasks containing 1 litre NYB (two flasks per strain) were inoculated with 2.0 ml of an overnight preculture of *B. cepacia* grown in NYB. The flasks were incubated for 48 h at 37°C in an orbital incubator (120 rpm). Bacterial cultures were then harvested by centrifugation at 10,000 *g* for 15 min at 4°C and washed twice in PBS. Each bacterial pellet was suspended in 2 ml PBS and homogenised (T1500, Ystral GmbH, W. Germany) for approximately 2 min. Bacterial cells were removed by up to ten cycles of centrifugation (10,000 *g* for 10 min at 4°C). Removal of cells was monitored at intervals by microscopy and culture of supernatant. The cell free supernatant was ultracentrifuged at 100,000 *g* for 2 h at 4°C, and the resultant pellet washed twice in pyrogen-free water. The pellet was then resuspended in 0.5 ml pyrogen-free water, dialysed overnight with stirring at 4°C against pyrogen-free water containing 0.05% w/v sodium azide (Sigma) and stored at -20°C. The amount of protein in each sample was determined using a protein assay (Bradford reagent, Bio-Rad).

Negative Staining of Flagella with PTA

The flagella preparations were viewed by electron microscopy to confirm absence of whole cells. Formvar-coated 400 mesh copper grids were floated onto a 10 µl drop of flagella sample diluted 1:10 in pyrogen-free water. Grids were then processed and viewed as previously described.

2.2.17 Preparation of Samples for Polyacrylamide Gel Electrophoresis

Sample buffer (pH 6.8) contained 0.0625 M Tris with 2.0% w/v sodium dodecyl sulphate (SDS) (BDH Specially Pure), 10% v/v glycerol, 1% v/v 2-mercaptoethanol and 0.001% bromophenol blue. Double strength sample buffer (pH 6.8) was prepared in the same manner except that concentrations of constituents were doubled.

Protein Samples

The protein concentration of flagella preparations, as determined by the Bradford reagent, was adjusted to 1.0 mg/ml. The outer membrane preparations were used directly. Samples were mixed with an equal volume double strength sample buffer and denatured by boiling at 100°C for 10 min prior to adding to a gel.

Lipopolysaccharide

Proteinase K samples prepared as described, were added to gels at 15 µl per track. Samples of extracted LPS (1.0 mg/ml), were mixed with an equal volume of single strength sample buffer and boiled at 100°C for 10 min. Samples were added at 10 µg per track.

2.2.18 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was performed with the buffer system of Laemmli (1970). The following buffers and solutions were used in PAGE:

a) Electrode buffer (pH 8.3) consisted of 0.025 M Tris, 0.192 M glycine (BDH Chromatographically Homogeneous) and 0.1% w/v SDS. For the Mini-Gel system 10x Tris/glycine/SDS buffer (Bio-Rad, Electrophoresis Purity Reagent) was used, made up in pyrogen-free water.

b) Separating gel buffer (double strength, pH 8.8) consisted of 0.75 M Tris-HCl to which 0.2% w/v SDS was added, (SDS was omitted in buffer intended for use in preparing immunoblots).

c) Stacking gel buffer (double strength, pH 6.8) consisted of 0.25 M Tris-HCl to which 0.2% w/v SDS was added, (SDS omitted as in (b) above).

d) Acrylamide stock solution (40% w/v) contained 100 g acrylamide (BDH Electrophoresis Grade) and 2.7 g methylene bis acrylamide (BDH Electrophoresis Grade) and was made up to 250 ml with distilled water.

The separating gel was prepared as described in Table 3 and poured between cleaned (with methanol) glass plates (160 mm x 125 mm x 1.5 mm) with the aid of a 50 ml syringe and a 19-gauge needle. The gel was overlaid with water-saturated butan-2-ol and allowed to set. After removal of the butan-2-ol, the stacking gel (see Table 3) was poured onto the separating gel and a 20 well comb was inserted before the gel began to set. On removing the comb the gel was fitted into an electrophoresis tank (Jencons Scientific Ltd., Leighton Buzzard, Beds.) and the electrode buffer added.

Samples, prepared as described, were loaded into the wells of the stacking gel. Samples were electrophoresed through the stacking gel at a constant 60 V and through the separating gel at a constant 150 V until the dye front had run 80-100 mm.

After electrophoresis, samples were analysed by staining or by immunoblotting after transfer to nitrocellulose.

Table 3. Preparation of Polyacrylamide Gels

Reagent	Volume (ml) to give acrylamide concentration of:		
	Separating gel		Stacking gel
	12%	14%	4%
Distilled water	5.2	3.45	3.5
Separating buffer	17.5	17.5	-
Stacking buffer	-	-	5.0
Acrylamide solution (40% w/v)	10.5	12.25	1.0
TEMED (NNN'N'-tetramethyl-1,2-diaminoethane)	0.05	0.05	0.02
Ammonium persulphate (15 mg/ml)	1.75	1.75	0.5

2.2.19 Silver Staining of Polyacrylamide Gels for Lipopolysaccharide

The methods of Tsai & Frasch (1982) and Hitchcock & Brown (1983) were adapted to visualise LPS in polyacrylamide gels. The following reagents and solutions were used in the silver staining procedure:

a) Fixative consisted of 7% v/v acetic acid and 25% v/v propan-2-ol.

b) Oxidising solution contained 1.05 g periodic acid in 150 ml distilled water to which 4.0 ml of fixative was added.

c) Ammoniacal silver nitrate solution was made up by mixing 1.4 ml ammonia solution with 21.0 ml of 0.36% w/v sodium hydroxide and the slow addition of 4.0 ml 19.4% w/v silver nitrate accompanied by vigorous agitation. The volume was made up to 100 ml with distilled water.

d) Developing solution consisted of 0.019% v/v formaldehyde solution containing 0.005% w/v citric acid.

Following electrophoresis the gel was placed in fixative and left overnight at room temperature. Fixative was poured off and freshly prepared oxidising solution added to the gel for 5 min. The gel was washed in at least four changes of 200 ml distilled water over a period of 4 h. Silver staining was carried out by addition of fresh ammoniacal silver nitrate solution. After staining for 15 min the gel was washed in four changes of distilled water (200 ml) over 40 min. LPS was visualised by the addition of 200 ml of fresh developing solution. Once the desired staining intensity was attained (approximately 15 min) the gel was washed in large volumes of distilled water. All the above reaction steps were carried out on a shaking platform.

2.2.20 Silver Staining of Polyacrylamide Gels for Protein

The method of Oakley *et al.* (1980) was adapted for the staining of polyacrylamide gels to visualise protein. The following solutions and reagents were used in addition to those described for silver staining LPS:

a) Prefix 1 consisted of 50% v/v methanol and 10% v/v acetic acid made up to 200 ml in distilled water. Prefix 2 consisted of 5% v/v methanol and 7% v/v acetic acid also made up to 200 ml with distilled water.

b) 200 ml of 10% v/v glutaraldehyde (unbuffered) in distilled water.

c) Dithiothreitol, 5µg/ml in distilled water.

The gel was placed in prefix 1 for 30 min, followed by prefix 2 also for 30 min. Prefix 2 was poured off and 10% glutaraldehyde solution added for a further 30 min. The gel was then washed twice in 250 ml distilled water and then soaked overnight in fresh distilled water. Freshly prepared dithiothreitol solution was added for 30 min, poured off and replaced by fresh ammoniacal silver nitrate. The remaining steps were as described for silver staining of LPS.

2.2.21 Coomassie Blue Staining of Polyacrylamide Gels for Protein

The Coomassie blue stain described by Hancock & Poxton (1988) was used. The solutions were made up in distilled water and were:

a) Solution 1 - 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 - 10% v/v propan-2-ol, 10% v/v acetic acid and 0.005% w/v Coomassie blue.

c) Solution 3 - 10% v/v acetic acid and 0.0025% w/v Coomassie blue.

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

e) Solution 5 - 10% v/v acetic acid.

The gel was placed in solution 1 overnight and then sequentially through solutions 2-5, each for 45-60 min at room temperature with gentle shaking throughout.

2.2.22 IMMUNOBLOTTING

A modification of the method of Towbin *et al.* (1979) was used in the immunochemical analysis of bacterial cell components separated by PAGE and transferred to nitrocellulose (NIC) paper (pore size 0.3 μm) (Schleicher & Schuell, Dassel, Germany). The following reagents and solutions were used:

a) Electrode transfer buffer (pH 8.3) contained 12 g Tris (Bio-Rad, Electrophoresis Grade), 57.68 g glycine (BDH Chromatographically Homogeneous) in 4 litres distilled water and 1 litre methanol.

b) Tris buffered saline (pH 7.5) (TBS) contained 4.84 g Tris and 58.48 g sodium chloride in 2 litres distilled water.

c) Tween-Tris buffered saline (pH 7.5) (TTBS) was as for TBS with 0.025% v/v Tween-20 (Sigma).

d) Blocking solution was as for TBS with 3% w/v gelatin (Bio-Rad, EIA Purity Grade)

e) Antibody diluent was as for TBS with 1% w/v gelatin.

f) Horseradish peroxidase (HRP) colour development solution was made up directly before use and contained 30 mg HRP colour reagent (Bio-Rad EIA Purity Grade) dissolved in 10 ml methanol and added to 50 ml TBS containing 70 μl hydrogen peroxide.

Electrophoretic Transfer of Antigens from Polyacrylamide Gels to Nitrocellulose

The gel was removed from the PAGE apparatus and placed in a Scotchbrite^{TM} pad cassette of the blotting apparatus (made by Mr J. Duffus, Dept. of Medical Microbiology, University Medical School, Edinburgh). The gel was then covered with a sheet of NIC pre-soaked for 10 min in transfer buffer. The cassette was then closed sandwiching the gel and NIC between the Scotchbrite pads and placed into the immunoblotting tank containing transfer buffer, ensuring that the gel was placed towards the cathode and the NIC towards the anode. A constant current of 40 mA was applied overnight at 4^{o}C.

Visualisation of the Antibody-antigen Reactions

After transfer the NIC was removed and washed in TBS for 10 min before placing in blocking solution for 30-45 min. The blocking solution was poured off and the antibody diluent, containing an appropriate dilution of sample (serum or sputum), added and incubated for 3 h at room temperature. The NIC was rinsed briefly in distilled water and then washed twice (each for 10 min) in TTBS, following which it was incubated for 60 min with HRP-conjugated secondary antibody diluted 1:500 (for anti-human conjugates) or 1:1000 (for anti-rabbit conjugate) in antibody diluent. The type of conjugate used depended upon the particular investigation (see Results) and included: anti-human IgG; anti-human-IgA and anti-rabbit IgG (ICN Biomedicals Ltd, high Wycombe, Bucks.). After rinsing in distilled water and washing in TTBS (as above), the binding of antibody to separated antigenic determinants was visualised by addition of the HRP colour solution until colour development was sufficient. The reaction was stopped by rinsing with copious quantities of distilled water, the NIC was then dried and stored in the dark. (The above steps involved gentle shaking throughout).

2.2.23 MULTI-BLOTTING

For analysis of multiple sera against a single antigen Mini-Gel and Multi-Screen apparatus (Bio-Rad) were used. The procedures for running mini PAGE, electrophoretic transfer and visualisation of the antibody-antigen reaction were essentially the same as for the larger system, with the following exceptions.

Following electrophoretic transfer and blocking the NIC was placed in the multi-screen apparatus which was then firmly secured. Samples (sera or sputa) were diluted 1:200 in TTBS containing 1.0% v/v Bovine serum albumin (BSA; Boseral, Organon Teknika B.V., Boxtel, Holland) and added to each lane carefully to avoid formation of air bubbles. Following overnight incubation in the multi-screen apparatus, the apparatus was shaken vigorously over a sink to empty-out the TTBS-BSA/sample, the NIC was removed and placed in a staining tank, rinsed twice in distilled water and then washed twice in TTBS. The remaining stages, from the addition of HRP-conjugated secondary antibody were as described above.

2.2.24 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Polystyrene microwell strips (Greiner Labotechnik, Dursley, Glos.) fixed in to a frame to form 'plates' were used in ELISA experiments. Mini-sorb tubes (Nunc) were used for making antigen dilutions. The following diluents and buffers were used in the various ELISA procedures:

a) Coating buffer (pH 9.6) consisted of 0.05 M carbonate/bicarbonate buffer (6.2 g/litre $Na_2CO_3.H_2O$ and 4.2 g/litre $NaHCO_3$) with 0.05% w/v sodium azide.

b) Post-coat buffer consisted of PBS (pH 7.2) containing 5% v/v BSA and 0.05% w/v sodium azide.

c) Wash buffer consisted of PBS (pH 7.2) containing 0.05% v/v Tween-20 and 0.05% w/v sodium azide.

d) Dilution buffer consisted of PBS (pH 7.2) containing 0.05% v/v Tween-20, 4.0% w/v polyethylene glycol 6000 (Sigma) and 0.05% w/v sodium azide.

e) Alkaline phosphatase substrate buffer consisted of 0.05 M carbonate buffer (pH 9.8) (21.2 g/litre Na_2CO_3 and 16.8 g/litre $NaHCO_3$) and 1.0 mM $MgCl_2.6H_20$. Immediately prior to use, one alkaline phosphatase 5 mg tablet (Sigma 104 phosphatase substrate tablets) per 5 ml buffer was added. The alkaline phosphatase substrate buffer was also used as a blank to calibrate the plate reader.

ELISA Coating Procedure

Coating with LPS-polymyxin complex (Scott & Barclay, 1987)

Stock solutions of LPS and polymyxin were made up in distilled water and sonicated for 8x30 s prior to mixing. Complexes were formed by adding polymyxin B sulphate (Sigma) at a concentration of 0.2 mM to LPS at a concentration of 0.1 mM and then sonicating for another 8x30 s. The mixture was then dialysed (in 2000 molecular weight cut-off dialysis membrane) overnight at 4°C against pyrogen-free water containing 0.05% w/v sodium azide to remove unbound polymyxin. Complexes were stored in mini-sorb tubes at -20°C. To coat microwell strips, the complex was diluted 1:50 in coating buffer and added at 100 µl per well. After coating overnight at room temperature plates were washed four times with wash buffer (Microwash 2, Skatron, Traby, Lier, Norway). Wells were post-coated with post-coat buffer at 100 µl per well overnight at room temperature. (Wells coated with post-coat only were background controls). After washing four times plates were rinsed with distilled water and stored at -20°C. Plates were also coated with *P. aeruginosa* LPS in the same manner.

Coating with Whole Bacteria

Bacteria were grown overnight in NYB, harvested and washed twice with PBS. Cells were resuspended to a density of 10^7 cfu/ml (determined spectrophotometrically) in coating buffer and added at 100 µl per well. Plates were then centrifuged at 1365 *g* for 5 min to sediment bacteria to the wells. Following overnight coating at room temperature the procedures were as described for LPS-polymyxin coating.

Coating with Mucin

Mucins were diluted in coating buffer and added at 100 µl per well. A coating concentration of 10 µg/ml gave optimal sensitivity, showing the maximal difference in OD between background controls and mucin-coated wells. Wells were coated overnight at room temperature, washed and post-coated as described above.

ELISA Experimental Procedures

The coated microwell strips were used in the following ELISA studies:

Detection of serum IgA, IgG and IgM, anti-*B. cepacia* LPS antibodies

Serially diluted sera (1:100 - 1:12800 for IgA and IgM and 1:400 - 1:52000 for IgG) in dilution buffer were added to the R-LPS/polymyxin B microwell strips at 100 µl per well in triplicate. After incubation at 37°C for 90 min in a plate incubator, plates were washed four times with wash buffer. Conjugates, alkaline phosphatase conjugated to anti-human IgA, IgG or IgM (Sigma) were diluted 1:1000 in dilution buffer and added at 100 µl per well. Plates were incubated for a further 90 min at 37°C, washed as above and then rinsed five times in distilled water before addition of alkaline phosphatase substrate at 100 µl per well. After incubation at room temperature for 30 min, the OD of wells was read at 405 nm in an automated plate

reader. Final results were expressed as the titre giving an OD >0.1 after subtraction of the OD of negative control wells.

Detection of Serum IgG Subclass Anti-*B. cepacia* Antibodies

Serially diluted serum was added to R-LPS/polymyxin coated plates as above. After washing, mouse anti-human IgG (clone HP-6017, Sigma), IgG1 (clone HP-6069, ICN), IgG2 (clone HP-6002, ICN), IgG3 (clone HP-6047, ICN) and IgG4 (clone HP-6025, Sigma) monoclonal antibodies conjugated to biotin, diluted 1:1000 in dilution buffer were added and incubated at 37°C for 90 min. Plates were washed and streptavidin alkaline phosphatase (Sera-Lab Ltd., Crawley Down, Sussex) diluted 1:500 in dilution buffer was added and plates re-incubated. After washing, substrate was added the plates treated as described above.

Detection of Sputum IgA and IgG

The assay was as for detection of antibodies in serum with the exception that sputum was diluted 1:25 - 1:3200.

Anti-*B. cepacia* LPS Antibody Specificity

To investigate the specificity of the anti-*B. cepacia* LPS antibodies serum was serially absorbed with preparations of purified R-LPS of the epidemic strain of *B. cepacia* (C1359) and *P. aeruginosa* PAC608. Sera were mixed with an equal volume of R-LPS at 1 mg/ml and incubated for 60 min at 37°C followed by overnight incubation at 4°C. Samples were suitably diluted prior to use in ELISA studies.

2.2.25 Measurement of Serum IgG

The concentration of serum IgG subclasses was determined by Radial Immunodiffusion Assay (BindArid, the Binding Site, Birmingham). The assay was performed as described in the manufacturing instructions; for determination of IgG1

and IgG2 concentration, test and control serum (the latter provided by manufacturers) were diluted 1:10 in BSA, whilst serum was used neat for determination of IgG3 and IgG4 concentration. Volumes of serum (5 µl) and calibrators (also provided) were added to the radial immunodiffusion plates, which were then incubated at 22°C in a moist box for 72 h. Diffusion was read, to the nearest point-one of a millimetre, with the aid of an eye-piece. Concentration of each IgG subclass was calculated from a calibration curve drawn using the diffusion results of the calibrators and control sera.

2.2.26 Chemiluminescent Opsonophagocytosis Assay

The potential of the antibodies in CF sera to inhibit opsonophagocytosis was investigated by chemiluminescent opsonophagocytosis assay.

Preparation of Mononuclear Cells from Buffy Coat

Buffy coat was diluted 1:1 with RPMI-1640 (Gibco BRL, Paisley, Strathclyde) and carefully layered onto 25 ml lymphocyte separation medium (ICN Flow) in 50 ml centrifuge tubes. The diluted buffy coat was centrifuged at 1000 *g* for 30 min without the brake. The white layer (mononuclear cells) at the interface of the plasma and lymphocyte separation medium was carefully removed. After centrifuging at 180 *g* for 15 min the cell pellet was resuspended in RPMI-1640 and centrifuged again. The final pellet was then resuspended in 8-10 ml HBSS (Sigma with Ca^{2+}, Mg^{2+}, 1 mg/ml filter sterilised glucose but no phenol red). The cells were counted using a haemocytometer (Thoma, Hawksley, England), adjusted to approximately $4x10^7$ cells/ml and placed on a continuous roller until used.

Preparation of Bacteria

Overnight cultures of bacteria grown in NYB were harvested and washed twice in HBSS and then resuspended in HBSS to a concentration of $1x10^9$ cells/ml (determined spectrophotometrically). Bacterial suspension (0.5 ml) was added to 0.5

ml heat inactivated serum (HIS) in a sterile Eppendorf tube and incubated at room temperature for 90 min. The mixture was centrifuged at 5800 *g* for 5 min and the supernatant carefully pipetted off. After washing twice in HBSS the pellet was resuspended in 0.5 ml HBSS. Controls were bacteria treated in the same way without addition of serum.

Chemiluminescence Assay

The order and timing of sample addition to the wells of luminostrips (Labsystems, Basingstoke, Hants) is important and where possible a multi-channel pipette was used. After addition of HBSS (100 µl per well) the bacteria/antibody suspension or bacteria alone were added in duplicate at 50 µl per well and 50 µl of 6 mg/ml Zymosan A (Sigma) in HBSS was also added in two control wells (as a control stimulant of phagocytosis to indicate the activity of the mononuclear cells). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma: 10^{-3} M) diluted in HBSS was added at 100 µl per well and finally 50 µl per well of mononuclear cell suspension. The plate was shaken and immediately placed in a Luminoskan (Labsystems) pre-warmed to 37°C where readings of chemiluminescence were taken every 5 min for 90 min.

2.2.27 Examination of Upper Respiratory Tract Specimens for *B. cepacia*

Mouth and peri-nasal swabs, buccal scrapings and saliva from CF patients on two separate occasions were examined for *B. cepacia*. All samples were taken prior to sputum collection. Swabs and scrapings were transported from the WGH in BcEB. The BcEB containing peri-nasal and mouth swabs were incubated at 37°C for up to 3 weeks prior to subculturing onto CEP. The BcEB containing buccal scrapings was filtered through a 2.2 µm pore filter, which allows passage of *B. cepacia* but not buccal epithelial cells (BEC). The filter paper was then placed onto CEP, and the filtrate cultured as above. Saliva was cultured quantitatively onto CEP.

2.2.28 HAEMAGGLUTINATION ASSAY

The capacity of *B. cepacia* to agglutinate a variety of defibrinated or citrated erythrocytes was investigated by a haemagglutination assay based on that of Duguid *et al.* (1979). Overnight cultures of *B. cepacia* grown in NYB at 37°C were centrifuged and the pellet resuspended in 0.5 ml fresh NYB. Erythrocytes were separated by centrifuging at 2545 *g* for 10 min and washed in sterile PBS four times prior to resuspending in PBS to give a cell concentration of 3% v/v. One drop of sterile PBS or 2.0% w/v α methyl mannoside was added to a porcelain tile, followed by one drop of the erythrocyte suspension and finally by one drop of bacterial suspension. The tile was rocked gently at room temperature for up to 30 min and the wells examined for haemagglutination. Variations on the assay were culture of bacteria in MM broth or growth at 30°C.

2.2.29 BACTERIAL ADHESION TO BUCCAL EPITHELIAL CELLS

The binding of *B. cepacia* to BEC was investigated using fluorescent staining and flow cytometry.

Preparation of Bacteria

Bacteria grown overnight in NYB, (the *N. meningitidis* control was grown on New York City Agar (Oxoid) in 5% CO_2), were washed three times in PBS at 1341 *g* for 25 min. The supernatant was poured off and the pellet 'loosened' with a 1 ml pastette prior to addition of 1.0 ml FITC (fluorescein isothiocyanate, Sigma) in FITC buffer (0.05 M $NaCO_3$, 5.84 g/litre; 0.1 M NaCl, 5.3 g/litre; pH 9.2). After washing twice as above the bacteria were adjusted to $3x10^7$ cfu/ml (determined spectrophotometrically).

Preparation of BEC

BEC from normal volunteers and CF patients were scraped, (six cotton wool swabs per individual), suspended in PBS and then washed twice at 200 *g* for 10 min. After washing, BEC were resuspended in 5 ml PBS, counted using a haemocytometer and the cell number adjusted to $3x10^5$ BEC/ml. The BEC were then mixed with the bacterial suspension (ratio 1:100) and incubated at 37°C for 30-45 min. The mixture was washed three times at 200 *g* for 10 min, the supernatant poured off with care each time so as not to lose cells. Cells were fixed in 3.0 ml buffered paraformaldehyde and then placed at 4°C overnight prior to reading fluorescence on an EPICS 'C' flow cytometer (Coulter Electronics, operated by Dr J. Stewart, Dept. of Medical Microbiology, University Medical School, Edinburgh).

2.2.30 Bacterial Adhesion to Mucin

Bacteria, cultured overnight in NYB were washed twice in PBS and resuspended to a concentration of $1x10^9$ cells/ml (determined spectrophotometrically) in freshly made PBS containing 0.05% v/v Tween-20 and 1.0% v/v BSA. The bacterial suspensions were added in triplicate to microtitre wells coated with mucin at 100 µl per well and incubated at 37°C for 60 min. After washing, hyperimmune CF sera (as determined in LPS-ELISA) diluted 1:1000 in dilution buffer was added at 100 µl per well. The remaining steps in the adherence assay ELISA were as described for detection of serum IgG anti-*B. cepacia* LPS antibodies. Binding to mucin was determined by calculating the mean of the triplicates and dividing the mean binding to mucin by the mean binding to the control wells. The assay conditions were varied by changing the growth environment, using MM or static incubation, and varying the incubation time of the first step of the assay (bacteria binding to mucin).

2.2.31 SERUM SENSITIVITY ASSAY

The method for the serum sensitivity assay was based on a method obtained from Miss Elizabeth Allen (Dept. of Medical Microbiology, University Medical School, Edinburgh).

Determination of Complement Activity

Complement activity (CH_{50}) was determined by sheep erythrocyte lysis assay based on that of Hudson & Hays (1980). Sheep erythrocytes (1.0 ml) were washed twice in PBS at 200 *g* for 10 min and resuspended in 5.0 ml PBS. Erythrocyte suspension (0.2 ml) was added to 2.8 ml distilled water to determine the required proportion of PBS to erythrocytes to give a concentration of 1 $x10^9$ rbc/ml (an OD 540 nm of 0.35 is equivalent to 1 $x10^9$ rbc/ml). Equal volumes of the erythrocyte suspension and rabbit anti-sheep antibody diluted 1:1000 in PBS were mixed and incubated at 37°C for 30 min prior to use. Test serum, stored at -70°C for a maximum of four weeks was maintained on ice during this assay. Serum was diluted 1:2 in PBS and increasing volumes (0.1 ml to 0.4 ml) were added to 0.3 ml of sensitised sheep erythrocytes in individual tubes and incubated at 37°C for 45 min. Ice-cold PBS (3.0 ml) was then added to each tube and tubes were centrifuged at 200 *g* for 5 min. The OD at 540 nm for the supernatant in each tube was then measured and complement activity determined.

Serum Bactericidal Assay

Overnight cultures of *B. cepacia* grown in ISTB were harvested and washed twice in complement fixation test buffer (CFTB, pH 7.4, Oxoid). Bacterial pellets were resuspended in CFTB to a density of $1x10^8$ cfu/ml (determined spectrophotometrically) and further diluted 100-fold to give a final cell density of $1x10^6$ cfu/ml. The assay tube consisted of 1.0 or 1.6 ml CFTB with 0.8 or 0.2 ml test serum respectively. Controls were 1.0 ml CFTB plus 0.8 ml HIS and 1.8 ml CFTB

alone. To each tube 0.2 ml bacterial suspension was added. Assay tubes were incubated in a shaking incubator at 37°C for 180 min. Samples (100 µl) were removed at T_0, T_{30}, T_{90} and T_{180} min, diluted 1:50 in CFTB and 100 µl was either added directly to NA and spread or diluted 10-fold in CFTB before adding to NA. NA plates were incubated at 37°C for 24-48 h before scoring.

Investigation of Serum Components

The roles of the classical complement pathway (CCP) and the alternative complement pathway (ACP) were investigated by treating serum with 10 mM $MgCl_2$-EGTA (200 mM ethyleneglycol-bis (beta-amino-ethyl- ether) N,N'-tetraacetic acid, Sigma) which specifically inactivates the CCP by chelating Ca^{2+} ions and not Mg^{2+} ions as described by Fine *et al.* (1979). EGTA was dissolved in sterile normal saline by heating at 60°C in a water bath and adding 5.0 M NaOH until EGTA went into solution. The pH was titrated back to 7.45 with 1.0 M HCl and the volume made up to 200 ml with saline. The EGTA stock was added to PNHS to give a final concentration of 10 mM.

PNHS was replaced by hyperimmune CF serum in order to investigate the role of specific anti-*B. cepacia* antibodies in serum killing. The CF sera were subjected to a series of absorptions with *B. cepacia* whole cells to remove the specific antibody. Overnight cultures of bacteria grown in NYB were harvested by centrifugation, washed twice in PBS and resuspended to a cell density of 10^8 cfu/ml. Bacterial suspension (1.0 ml) was added to a 1.5 ml Eppendorf tube and bacteria were pelleted by centrifugation at 1365 *g* for 5 min. After removal of supernatant, bacteria were resuspended in serum diluted 1:200 in CFTB, incubated for 15 min at room temperature and recentrifuged. The supernatant was then added to another pellet of cells and the process repeated. This step was repeated at least three times.

Influence of Culture Medium on Serum-sensitivity

To determine the influence of the culture medium on serum sensitivity, *B. cepacia* strains were cultured in MM and 50% heat inactivated sheep serum.

2.2.32 Environmental Isolation of *B. cepacia*

As part of a study to determine the environmental habitat of *B. cepacia* in the environment, a laboratory 'field trip' to the Royal Botanic Gardens, Edinburgh, was undertaken. The Botanic Gardens were thought to be appropriate as they provided a variety of environmental sites and microclimates. Several different 'micro-environments' were investigated including pools and vegetation of tropical and temperate greenhouses, the local Edinburgh soil, plants and ponds. Specimens including 100 ml volumes of pond water; 10 g soil samples collected in sterile universals; leaf debris which was transported in BcEB; and finally swabs taken of moist surfaces (for example rock pools) and transported in BcEB. *B. cepacia* enrichment broth was added to any dry specimens. In the laboratory, specimens were incubated at 30^{o}C for seven days after which time the broths were subcultured onto CEP and TBT media and re-incubated. Water specimens were filtered, and the filter paper incubated on CEP as previously described. Any bacterial colonies were subcultured onto fresh CEP and TBT. Individual colonies were then subcultured onto MacConkey agar to enable elimination of lactose producers. Non-lactose fermenting colonies were transferred to King's B medium (King *et al.*, 1954) and fluorescent colonies discarded whilst non-fluorescing colonies were inoculated onto Stewarts AG medium (Stewart, 1972) (performed by Mrs C. Doherty). The colonies which were identified as *B. cepacia* on AG medium were then screened by API 20NE. The clonal relationships of colonies positively identified as *B. cepacia* were investigated by bacteriocin typing and PFGE by Mrs Doherty. Individual isolates were then further investigated to determine the nature of the LPS (R or S).

CHAPTER 3

BIOLOGICAL PROPERTIES AND CHARACTERISTICS OF *BURKHOLDERIA CEPACIA*

Biological properties and characteristics of *B. cepacia* strains, in particular the epidemic strain, which may be of importance in contributing to the ability of *B. cepacia* strains to colonise and infect the CF host were investigated.

3.1 ISOLATION OF *B. CEPACIA* FROM THE ENVIRONMENT

The aim of this study was to estimate the prevalence of *B. cepacia* in the environment and determine the risk of environmental acquisition of *B. cepacia* by CF patients. To investigate the general view that *B. cepacia* is ubiquitous in the environment, 55 samples from the different micro-environments; e.g. soil, water and plant debris from the tropical, temperate and external areas within the Botanic Gardens were screened for *B. cepacia.* Environmental samples were processed as illustrated in Figure 5. Bacterial colonies obtained on *B. cepacia* selection media were subcultured onto MacConkey agar and King's B media in order to eliminate those colonies which were lactose fermenters or fluorescent. The remaining colonies were confirmed as *B. cepacia* by use of the API 20NE multi-test system. Table 4 identifies the sites from which 12 *B. cepacia* were isolated.

Further characterisation of the 12 *B. cepacia* strains was performed by phenotypic typing (Bacteriocin typing) and genotypic typing (PFGE). Although several isolates were shown to share the same bacteriocin type and five isolates were untypable (Table 4), PFGE

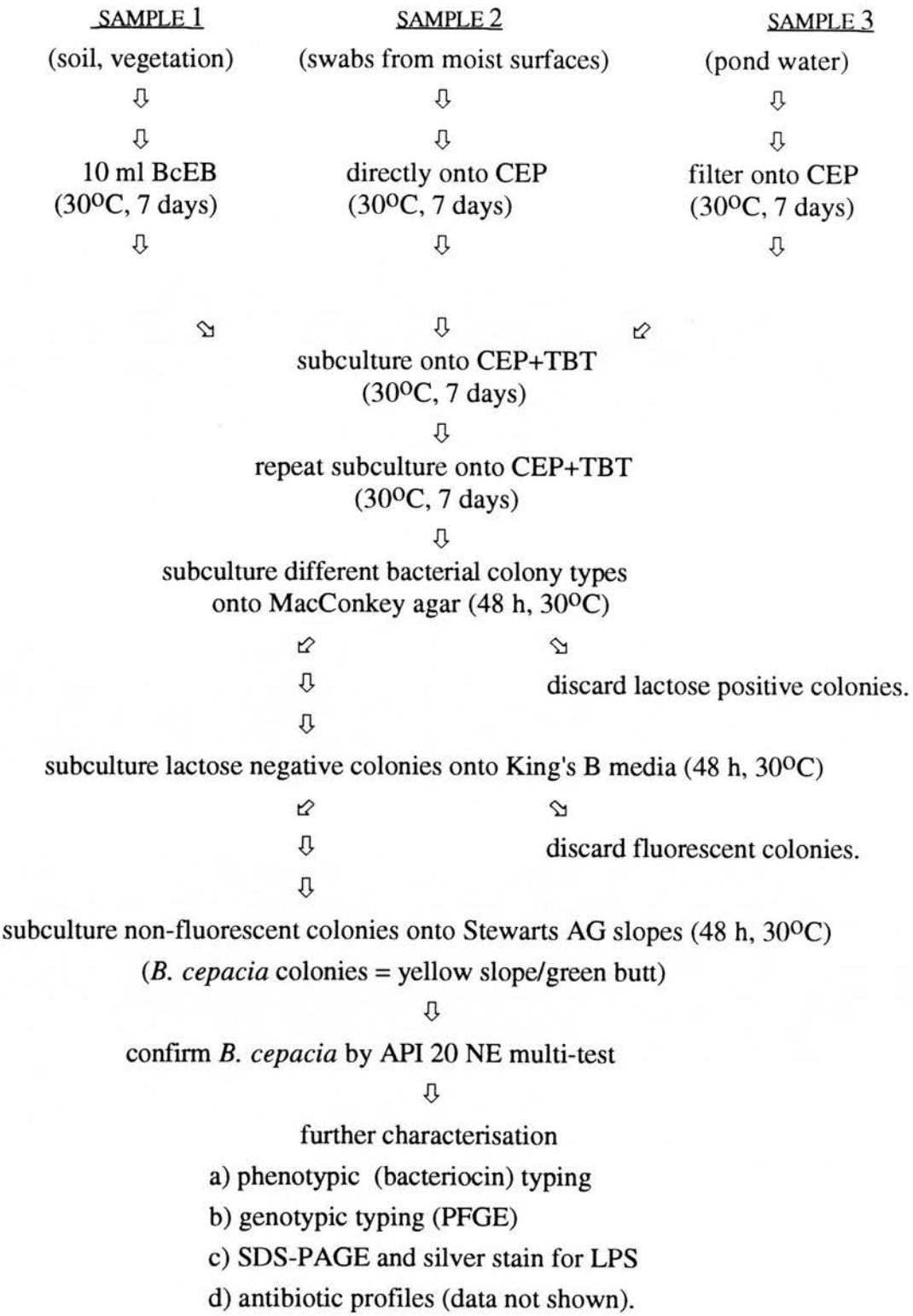


Figure 5. Processing of environmental samples obtained from the Royal Botanic Gardens, Edinburgh

Table 4. Environmental sites in the Royal Botanic Gardens, Edinburgh from which *B. cepacia* strains were cultured.

Isolate	Site	Area	Bacteriocin type
J2534	rotting bark	orchid and cycad house	S16/P3
J2535	rotting bark	orchid and cycad house	S16/P3
J2536	soil	gardens	S1/P0
J2537	soil	rhododendron house	S1/P0
J2538	soil	rhododendron house	S1/P0
J2539	soil (near *Hohenbergia stalata*)	temperate aquatic house	S3/P0
J2540	soil (near banana plant)	tropical aquatic house	UT
J2541	soil (near *Epidendrum o'brieniamum*)	orchid and cycad house	UT
J2542	soil (near *Nautilocalyx lynchii*)	orchid and cycad house	S1/P0
J2543	pond water	tropical aquatic house	UT
J2552	soil (near *Carludoucas palmata*)	tropical palm house	UT
J2553	*sanseveira* leaf	tropical palm house	UT

UT - untypable by bacteriocin typing

confirmed that the *B. cepacia* strains isolated were clonally distinct with the exception of isolates J2541 and J2543 which shared a PFGE profile. The study suggests that a preferred habitat for *B. cepacia* includes warm moist soil environments.

3.2 GROWTH OF *B. CEPACIA*

The aim of this study was to determine the optimal growth conditions for *B. cepacia* in terms of temperature, aeration, incubation time and nutrient availability. Three representative strains of *B. cepacia* were used in this study; the epidemic strain (C1359); a CF isolate (C1409) and an environmental isolate (J2395). Optimal growth of each *B. cepacia* strain was observed following aerated culture for 24 h at 37°C in NYB as shown in Table 5.

Table 5. Growth of *B. cepacia* in NYB and MM following static or aerated culture.

Strain	Growth of *B. cepacia* (mean cfu/ml)			
	NYB		MM	
	static	aerated	static	aerated
C1359	$1.0x10^8$	$1.0x10^9$	$3.4x10^7$	$7.5x10^7$
C1409	$5.0x10^7$	$2.1x10^9$	$1.6x10^7$	$3.5x10^8$
J2395	$4.5x10^7$	$1.7x10^9$	$5.1x10^7$	$1.6x10^9$

The growth of each *B. cepacia* strain in NYB at 25°, 37° and 43°C over 24 h was measured by optical density and by viable count (Figure 6). Under all conditions higher growth rate and a greater final cell density was observed for J2395. The epidemic strain (C1359) had the slowest growth at each temperature, with the longest initial lag phase preceding logarithmic growth and also the smallest cell density at 24 h. Final counts for

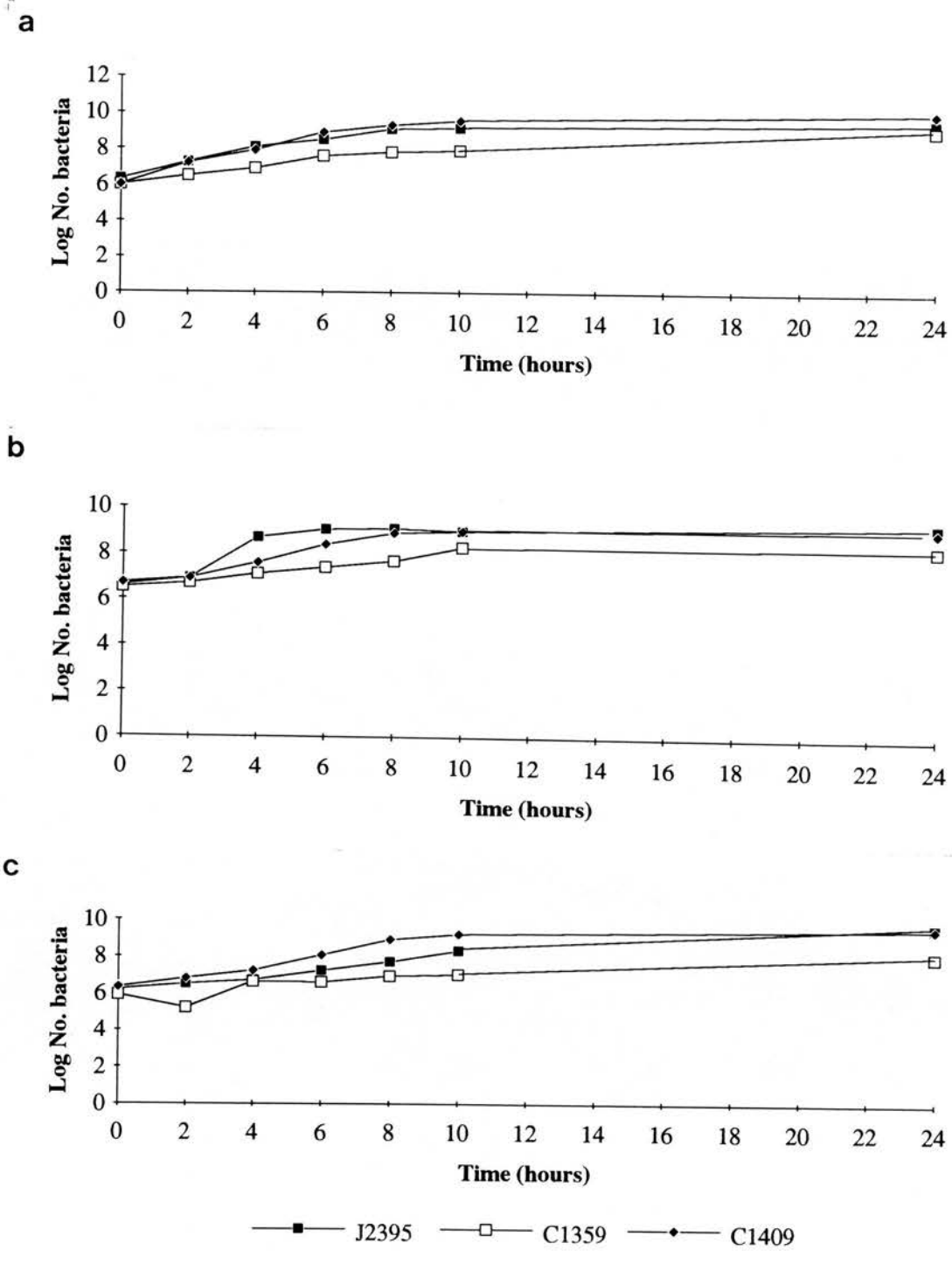


Figure 6. Growth curves of *B. cepacia* strains J2395, C1409 and C1359 in NYB for 24 h at: a) 37°C; b) 43°C; and c) 25°C.

J2395 were similar in NYB and MM; in contrast both C1359 and C1409 showed lower final counts in MM.

3.3 SURVIVAL OF *B. CEPACIA*

Water

Single colonies (approximately 10^5 cfu/ml) of *B. cepacia* strains C1359, C1409 and J2395 were inoculated into 10 ml volumes of tap and pyrogen-free water and examined at weekly intervals by visual observation of turbidity and by subculture onto CEP for survival and growth. In no instance did the water become turbid, suggesting that cell numbers were no greater than approximately 10^7 cfu/ml. All three *B. cepacia* strains survived in both tap and pyrogen-free water up to 1 month, thereafter *B. cepacia* was recovered only from pyrogen-free water. No difference was observed in the survival of the individual *B. cepacia* strains, with the exception that when subcultured onto CEP colonies of C1409 and J2395 were visible at 24 h, but 48-72 h incubation were required before any colonies were visible for the epidemic strain and even at that time the colonies remained very small.

Antiseptics

The antimicrobial efficacy of six antiseptics was examined according to the Kelsey-Sykes Capacitance test procedure against the three *B. cepacia* strains (C1359, C1409 and J2395), and also against a non-mucoid clinical isolate of *P. aeruginosa* (SBC 88) and *S. aureus* for comparison. An antiseptic was considered to be effective when not more than five colonies were observed after the second incremental addition of bacterial suspension. The efficacy of the antiseptics at the manufacturers recommended concentration against bacteria grown in NYB is shown in Table 6. The data indicate that the strains of *B.*

Table 6. Efficacy of Antiseptics against *B. cepacia* as determined by the Kelsey-Sykes Capacitance Test.

Strain	Antiseptic					
	Povidone Iodine *	Listerine *	70% Ethanol	Cetavlon	Hibitane	Roccal
B. cepacia (epidemic strain)	+++	++	+++	+++	-	+++
B. cepacia (C1409)	+++	++	+++	+++	-	++
B. cepacia (J2395)	+++	++	+++	+++	-	++
P. aeruginosa (SBC 88)	+++	++	+	-	++	-
S. aureus	+++	++	+	++	+++	++

* : Povidone iodine and Listerine used neat and at 75% v/v only
+++ : antiseptic effective at all concentrations
++ : antiseptic effective at greater than or equal to recommended concentration
\+ : antiseptic effective at greater than recommended concentration only
\- : antiseptic ineffective

cepacia investigated do not show particular resistance to the antiseptics in the test conditions used in the assay. To investigate whether resistance might be increased following growth in a nutrient depleted environment and also to try to replicate more accurately what may be occurring *in situ*, the experiment was repeated following culture of *B. cepacia* C1359 and J2395 and *P. aeruginosa* SBC 88 in MM instead of NYB. No difference in the effect of the antiseptics on *P. aeruginosa* or *B. cepacia* J2395 was observed following culture in MM. In contrast, against the epidemic strain of *B. cepacia* C1359, Cetavlon was not effective at recommended concentration and Roccal was not effective at the lowest concentration, suggesting that culture of the epidemic strain in a MM promoted resistance to some antiseptics.

3.4 EXTRACELLULAR PRODUCTS OF *B. CEPACIA*

Melanin

One hundred isolates of *B. cepacia* were screened for melanin production by culture on furunculosis agar. The only strain of *B. cepacia* associated with melanin production was the epidemic strain (Figure 7). Figure 7 illustrates melanin production by the epidemic strain of *B. cepacia* (C1359) grown on furunculosis agar and growth of a non-melanin producing CF strain of *B. cepacia* (C1409).

Elastase

Fifty three isolates of *B. cepacia*, including the epidemic strain, and three strains of *P. aeruginosa* PAO1, J1385 and SBC 88 were screened for elastase production (the former two *P. aeruginosa* strains were known elastase producers). Elastase production was determined by a zone of clearing of the elastin-TSA medium around bacterial growth. Elastase production was observed for each of the *P. aeruginosa* controls. In contrast, no

Figure 7. Melanin production by the epidemic strain of *B. cepacia* grown on Furunculosis agar (Ogonariwo & Hamilton-Miller, 1975) at 37°C for 48 h. a) The epidemic strain, represented by C1359; b) CF isolate of *B. cepacia* (C1409).

evidence for elastase production was found for any of the *B. cepacia* isolates investigated.

Phospholipase C

Phospholipase (PLC) production by *B. cepacia* has been described previously (Nakazawa *et al.*, 1987; Vasil *et al.*, 1990). The aim of this study was to compare production of PLC in *B. cepacia* strains from different sources and in particular to determine whether there was any difference in production of PLC between the epidemic strain and other strains of *B. cepacia*. Table 7 shows PLC production by 32 isolates of *B. cepacia* and a *P. aeruginosa* PAO1 control. PLC production was evaluated in a microtitre assay by measuring the release of the yellow chromogen *p*-nitrophenol after hydrolysis of the substrate NPPC by PLC. Activity was calculated as the absorbance at 405 nm with NPPC divided by the optical density of the culture at 540 nm.

Table 7. Phospholipase C production by *B. cepacia*

Source	No. of strains tested	Range of relative activity [a]	Mean +/- SEM[b]
CF: epidemic strain[c]	6	0.20-0.88	0.42+/-0.034
CF: other strains	10	0.03-0.94	0.34+/-0.019
Environmental	7	0.07-0.40	0.23+/-0.008
Clinical (non-CF)	4	0.06-0.62	0.30+/-0.031

[a] - Relative activity was defined as the ratio of activity of the *B. cepacia* to the activity of PAO1.
[b] - SEM, Standard error of the mean.
[c] - Epidemic strain isolates from six individual CF patients attending the Edinburgh adult CF clinic.

PLC production by all the *B. cepacia* isolates investigated was less than that for PAO1. The difference in PLC activity between the epidemic strain and other strains of *B. cepacia* was not significant ($p<0.1$ Students *t*-test).

Exopolymer Production

Following culture on nitrogen-deficient MM or glucose/EMB medium exopolymer production was observed in a minority of the 53 *B. cepacia* strains investigated. Exopolymer production following culture on the glucose/EMB media was observed for two *B. cepacia* strains, CDC 8 and a glucose dehydrogenase-negative (gcd^-) derivative of *B. cepacia* ATCC 17616 (obtained from Dr T. Lessie). An alternative exopolymer was produced by five gcd^+ strains: two CF strains (J415 and C1409), two clinical strains (J761 and J762) and one environmental strain (J2395) cultured on nitrogen-deficient MM. Mucoid colonial morphology was observed for the environmental strains of *B. cepacia* isolated from the Botanic Gardens when cultured on TBT media and also on PIA, but not on either of the previously described media. Examination of India ink stained preparations under phase contrast microscopy confirmed that the exopolymer was not of a capsular nature.

3.5 MOTILITY OF *B. CEPACIA*

Motility was investigated in 39 *B. cepacia* isolates by measuring migration in semi-solid motility agar (Table 8) and also by observing the motility of *B. cepacia* in liquid culture under phase contrast microscopy . The 17 isolates of the epidemic strain showed a mean diameter of spreading colony of 9.5 mm (range 5.0 - 14.0 mm), approximately half the mean spreading diameter observed for the non-epidemic strains of *B. cepacia* investigated

(mean spreading diameter 18.0 mm; range 4.0 - 46.0 mm). Statistical analysis showed that this difference was significant ($p<0.005$, Students *t*-test).

A number of selected strains from each group of *B. cepacia* strains were additionally examined for motility in liquid culture. Observations of motility under phase contrast microscopy were consistent with those on semi-solid agar.

Table 8. Motility of *B. cepacia* strains in semi-solid agar as determined by diameter (mm) of the spreading colony, 48 h.

Source	No. of isolates tested	Mean spreading diameter (mm)+/-sd	Range of spreading diameter (mm)
Environmental	5 (1nm)	30+/-13.8	18 - 46
Clinical/CF	17 (2nm)	16+/-8.7	4 - 41
Epidemic strain [a]	17 (1nm)	9.5+/-2.8	5 - 14
P. aeruginosa [b]	2	37	35 - 39

[a] - Epidemic strain isolates from 9 individual CF patients from the Edinburgh and Manchester CF clinics, plus paired strains from four CF patients.
[b] - *P. aeruginosa* spreading motility measured at 24 h.
nm - non-motile

3.6 Cell Surface Features of *B. cepacia*

Cell surface features of *B. cepacia* which could be involved in colonisation and virulence were investigated by comparing surface features of the epidemic strain to other CF and non-CF strains of *B. cepacia*.

Electron Microscopy of Whole Cells

The aim of this study was to investigate the general cell morphology of *B. cepacia* including expression of fimbriae and flagella. Twenty of the 21 strains of *B. cepacia* examined were coccobacilli in shape, one strain (NCTC 10744) was bacilli; all fimbriate strains possessed peritrichous fimbriae and multitrichous polar flagella. Fimbrial expression differed between strains of *B. cepacia* and even between individual cells of a single culture. Sixteen (70%) of the *B. cepacia* strains examined expressed fimbriae, however expression was rare; for example on a typical specimen grid approximately 10% of cells were observed to express fimbriae. The range of fimbriae observed is shown in Figures 8, 9 and 10. Two distinct fimbrial types were observed as shown in the electron micrograph of a CF isolate of *B. cepacia* C1394 (Figure 8). The larger fimbriae were regular and thick with no visible channels and typical of enterobacterial Type 1 fimbriae, the smaller fimbriae were 'crinkly' in appearance. Long flagella can also be clearly observed. Figure 9 is an electron micrograph of *B. cepacia* J415, a CF isolate associated with a fatal case of '*Cepacia* syndrome' (Glass & Govan, 1986). In this dividing cell only one fimbrial type (Type 1) was visible. Figure 10 is an electron micrograph of the epidemic strain, showing the extremes of fimbriation of cells from a single culture.

Characterisation of Isolated Flagella

Flagellar antigens were prepared from three *B. cepacia* strains: the epidemic strain (C1359); CF isolate C1524 and environmental isolate J2395. In addition, flagellar samples of *P. aeruginosa* JN62 (*P. aeruginosa* flagella type 'a') and PAO1 (*P. aeruginosa* flagella type 'b'), prepared by Dr J. Nelson, were included in SDS-PAGE as standards and

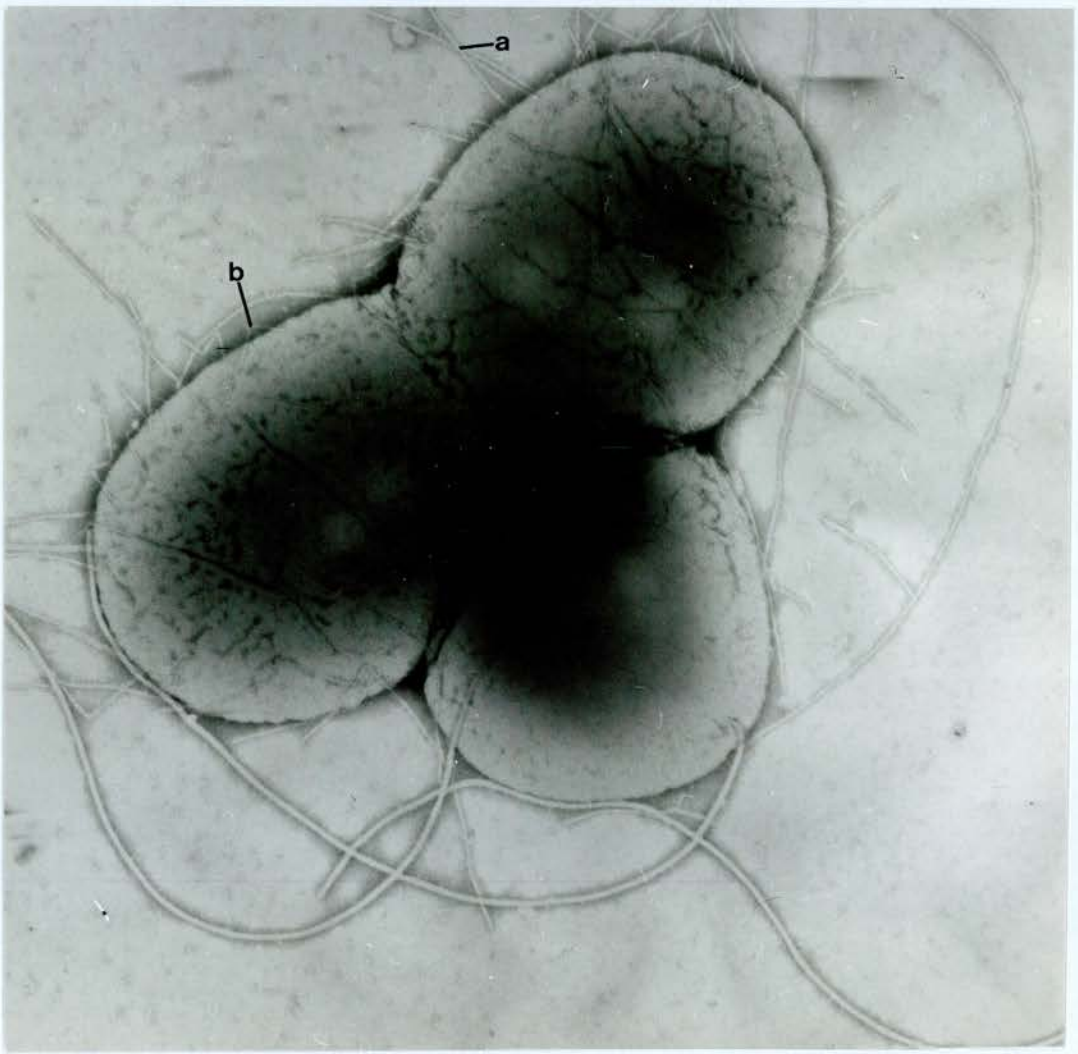


Figure 8. Electron micrograph of *B. cepacia* C1394 stained with 2% w/v PTA, showing both fimbriae and flagella. a) large, Type 1 fimbriae, b) small 'crinkly' fimbriae. Magnification x 40,000.

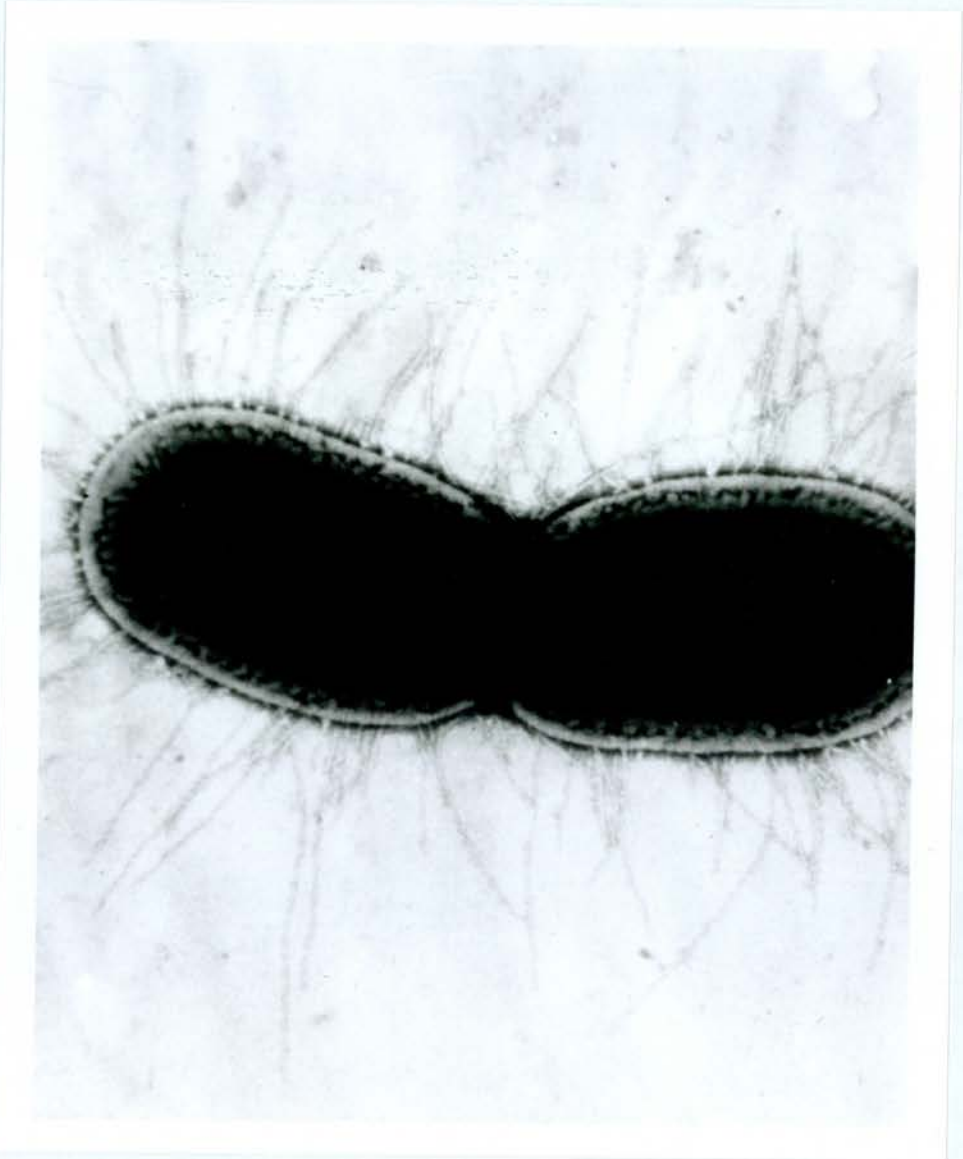


Figure 9. Electron micrograph of *B. cepacia* J415 stained with 2% w/v PTA, expressing peritrichous fimbriae. Magnification x 25,000.

Figure 10. Electron micrograph of the epidemic strain of *B. cepacia* (SBC 9) stained with 2% w/v PTA, showing a highly fimbriated and a less fimbriated cell. Magnification x 23,000.

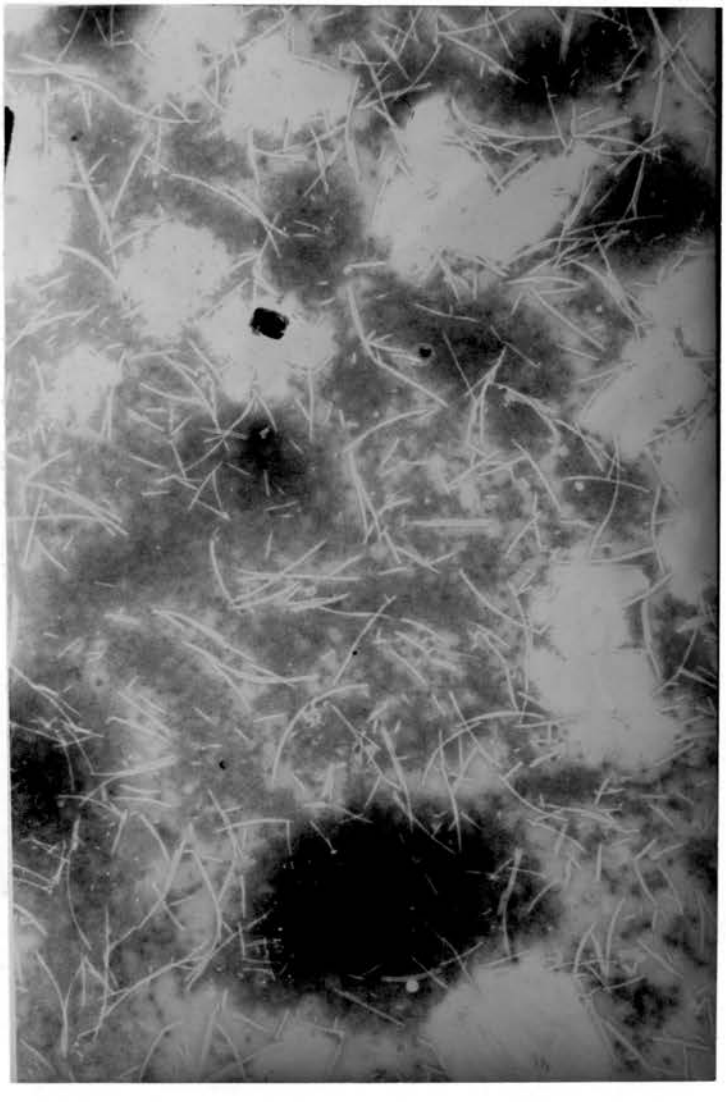


Figure 11. Electron micrograph of flagellar antigen preparation from the epidemic strain of *B. cepacia* stained with 2% w/v PTA. Magnification x 15,000.

Flagella of the epidemic strain (C1359) and J2395 were characterised by molecular mass determination in SDS-PAGE. Molecular mass of flagella separated by SDS-PAGE (Figure 12) were estimated from a calibration curve of log molecular mass of protein standards against Rf, where Rf is the distance moved by a standard protein through the separating gel divided by the distance moved by the bromophenol blue marker. Flagella from strain J2395 (Track 3) appeared to have mobility in SDS-PAGE intermediate between JN62 (Track 1) and PAO1 (Track 2) and had a calculated *Mr* of 51,000. Flagella from the epidemic strain C1359 migrated further than either PAO1 or JN62 flagella, and had a calculated *Mr* of 44,000.

Immunoblot analysis of flagella with *P. aeruginosa* flagellar type-a and -b antisera showed that there was no cross-reactivity between either antisera and flagellar antigens of *B. cepacia* C1359 and J2395 (Figure 13a & b).

Lipopolysaccharide Analysis

Lipopolysaccharide prepared from 72 isolates of *B. cepacia* (including 16 isolates of the epidemic strain) was examined after separation by SDS-PAGE and visualisation by silver staining and immunoblotting (Table 9).

The majority of *B. cepacia* isolates examined (68%) expressed S-LPS. All isolates of the epidemic strain express R-LPS, which may suggest that the majority of CF isolates express R-LPS as the epidemic strain is the most prevalent strain of *B. cepacia* in several UK CF centres. Figure 14a shows the S-LPS profiles for the ten genotypically distinct environmental isolates of *B. cepacia* (isolated from the Royal Botanic Gardens, Edinburgh) confirming the observation that a S-LPS profile is the more common form for

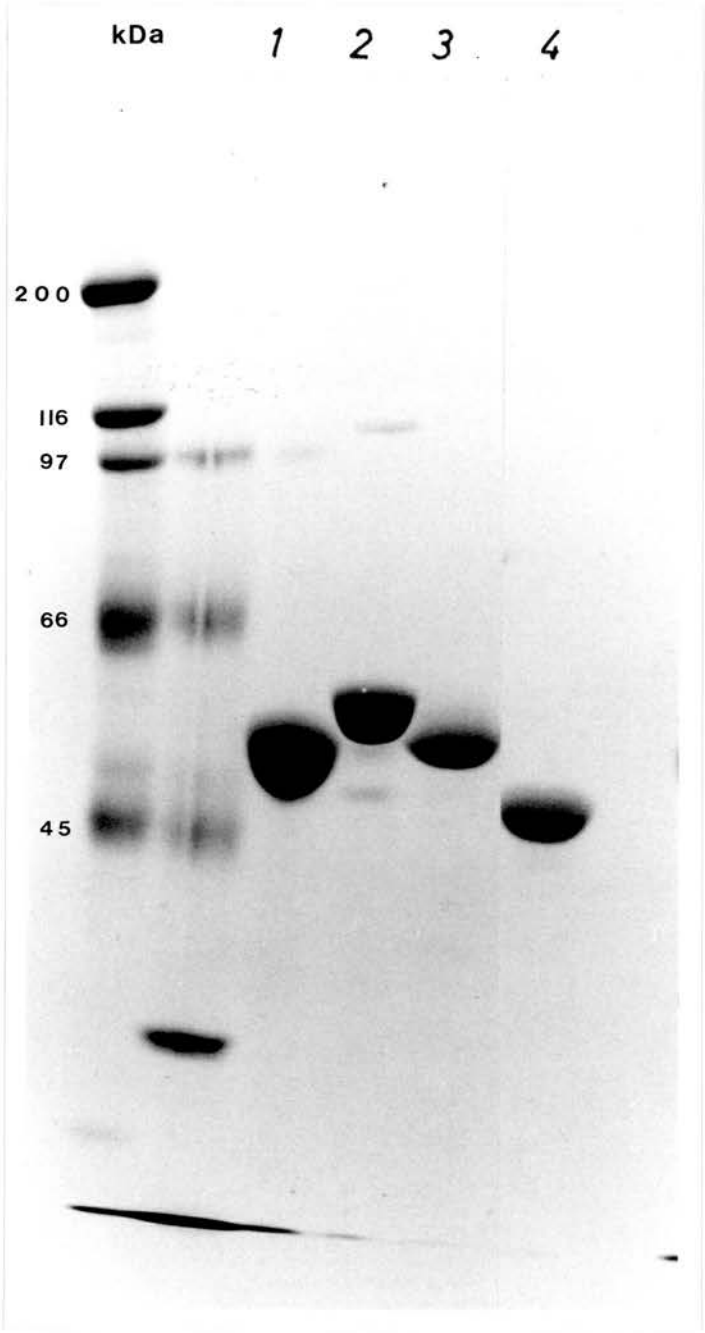


Figure 12. SDS-PAGE of flagella preparations from *P. aeruginosa* strains JN 62 (Track 1), PAO1 (Track 2) and *B. cepacia* strains J2395 (Track 3) and the epidemic strain (Track 4). Flagella were separated using 12% w/v acrylamide gels and stained with Coomassie blue. Molecular weights of protein standards are indicated.

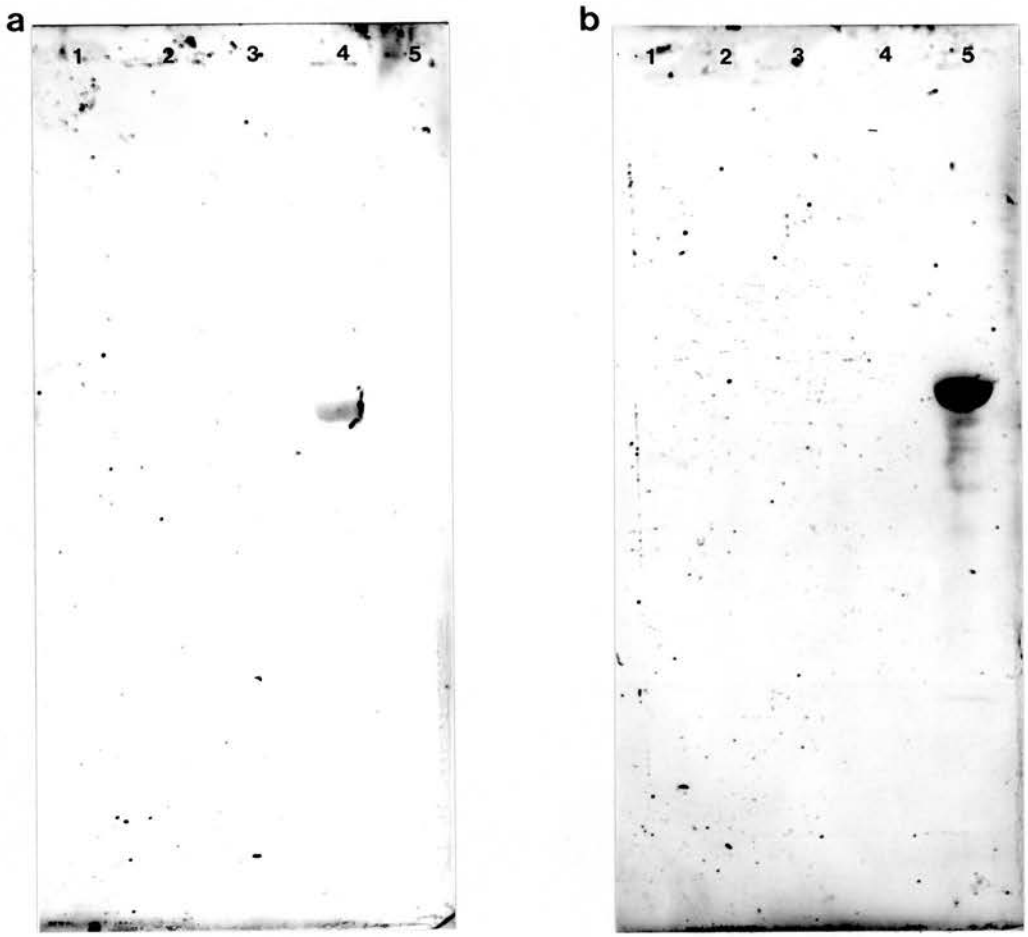


Figure 13. Immunoblots of flagellar antigens separated by SDS-PAGE followed by electrophoretic transfer to NIC paper and probed with anti-*P. aeruginosa* flagellar type-a antisera (a) and anti-*P. aeruginosa* flagellar type-b antisera (b): flagellar antigens from J2395 (Track 1); C 1524 (Track 2); the epidemic strain (Track 3); JN 62 (Track 4) and PAO1 (Track 5).

B. cepacia. Figure 14b shows the range of S-LPS profiles observed in clinical, environmental and CF isolates of *B. cepacia* and also the typical R-LPS profile for the epidemic strain.

Table 9. Lipopolysaccharide Analysis of *B. cepacia*

Source	No. of isolates tested	LPS	
		R-LPS	S-LPS
CF: epidemic strain	16	16	0
other strains	17	5	12
Environmental	23	1	22
Clinical	16	1	15

Outer Membrane Protein Profile

Outer membrane preparations were prepared from five *B. cepacia* strains, the epidemic strain (C1359), two other CF strains (C1409 & C1559) and the environmental strains (ATCC 17616 & J2395). The outer membranes were separated by SDS-PAGE and visualised by silver or Coomassie staining or by immunoblotting. Coomassie staining for proteins was superior to the protein silver stain which was too sensitive and stained LPS present in the crude outer membrane preparations.

Molecular masses of the major protein bands were determined as described above in SDS-PAGE. Coomassie staining of the SDS-PAGE showed that the five *B. cepacia* strains had dissimilar OMP profiles, with the exception of a major band with an *M*r of approximately 29,000 (Figure 15), possibly equivalent to the D porin described by

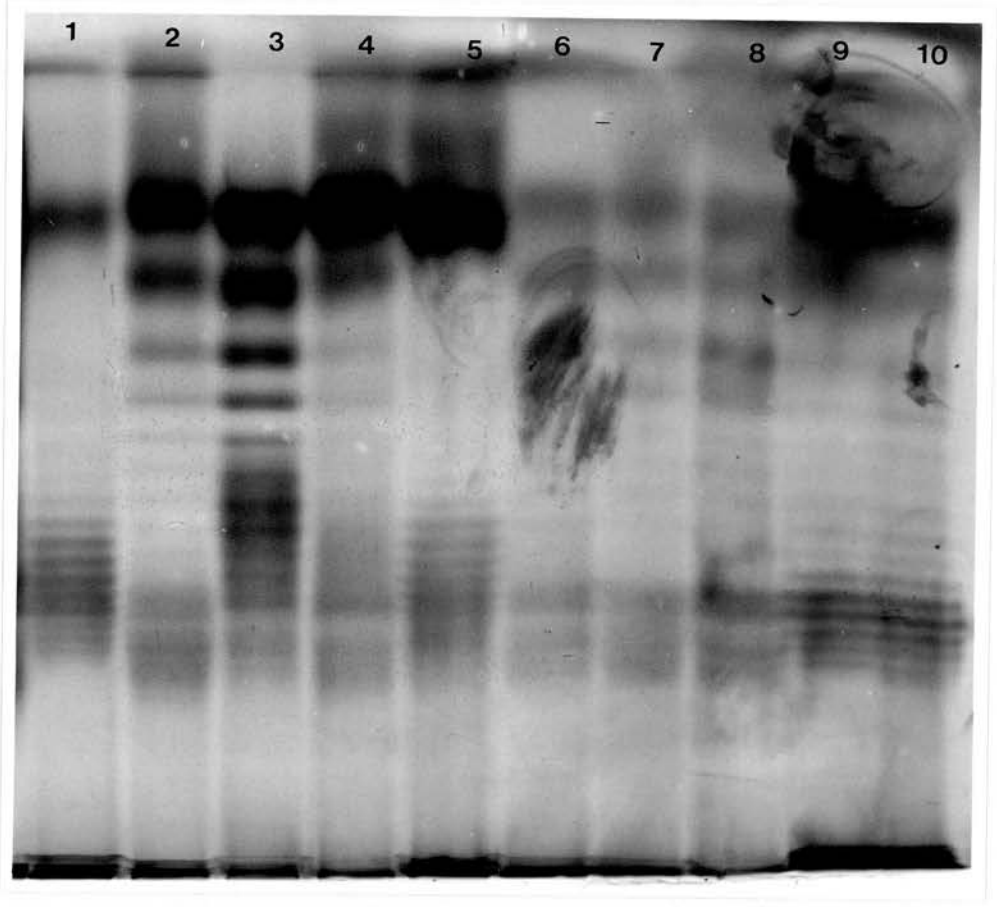


Figure 14a. Silver stained LPS profiles of proteinase K whole cell digests of 10 environmental isolates of *B. cepacia* separated by SDS-PAGE (14% w/v acrylamide). Track 1 to Track 10 are J2534 through J2543 sequentially.

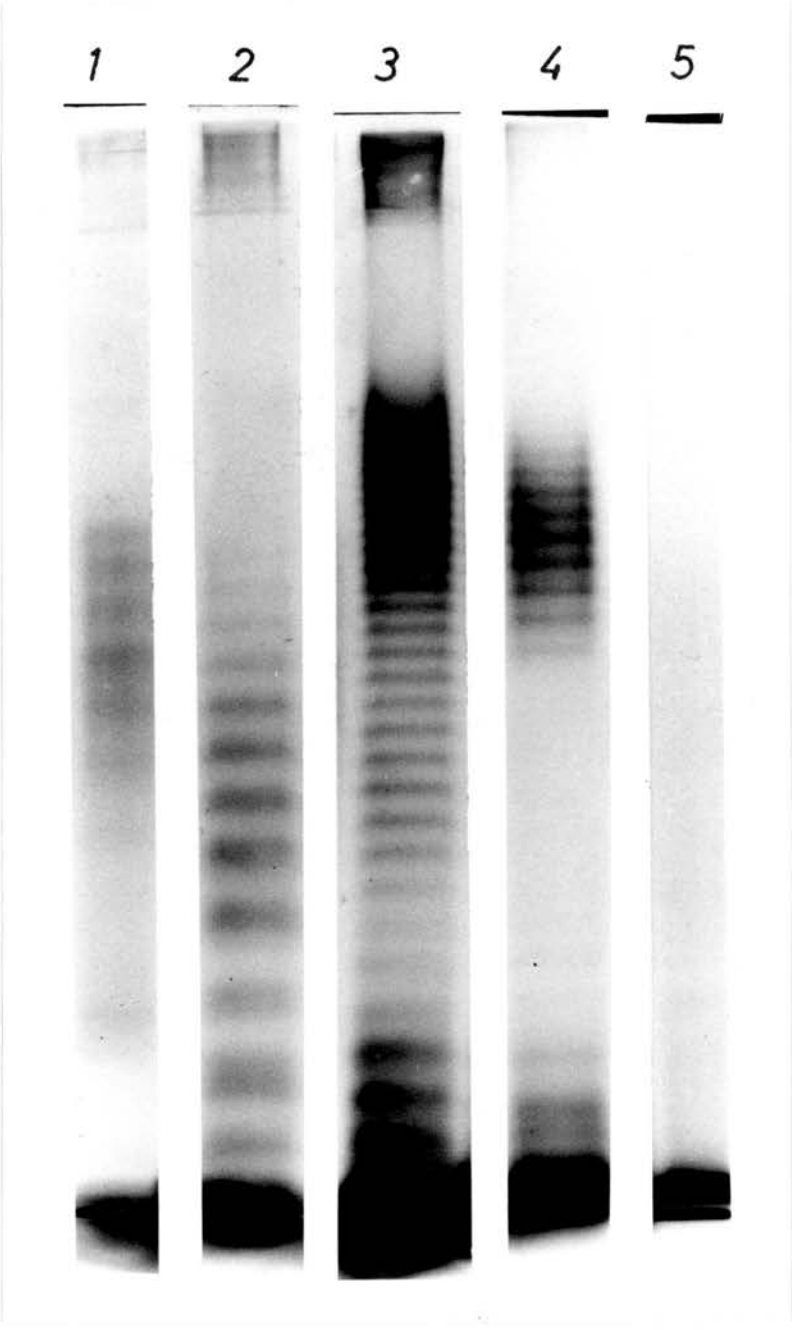


Figure 14b. Silver stained LPS profiles of proteinase K whole cell digests of five *B. cepacia* strains separated by SDS-PAGE (14% w/v acrylamide). Track 1, J716 (UTI); Track 2, J2395 (environment); Track 3, J1745 (environment); Track 4, C1409 (CF) and Track 5, the epidemic strain (CF).

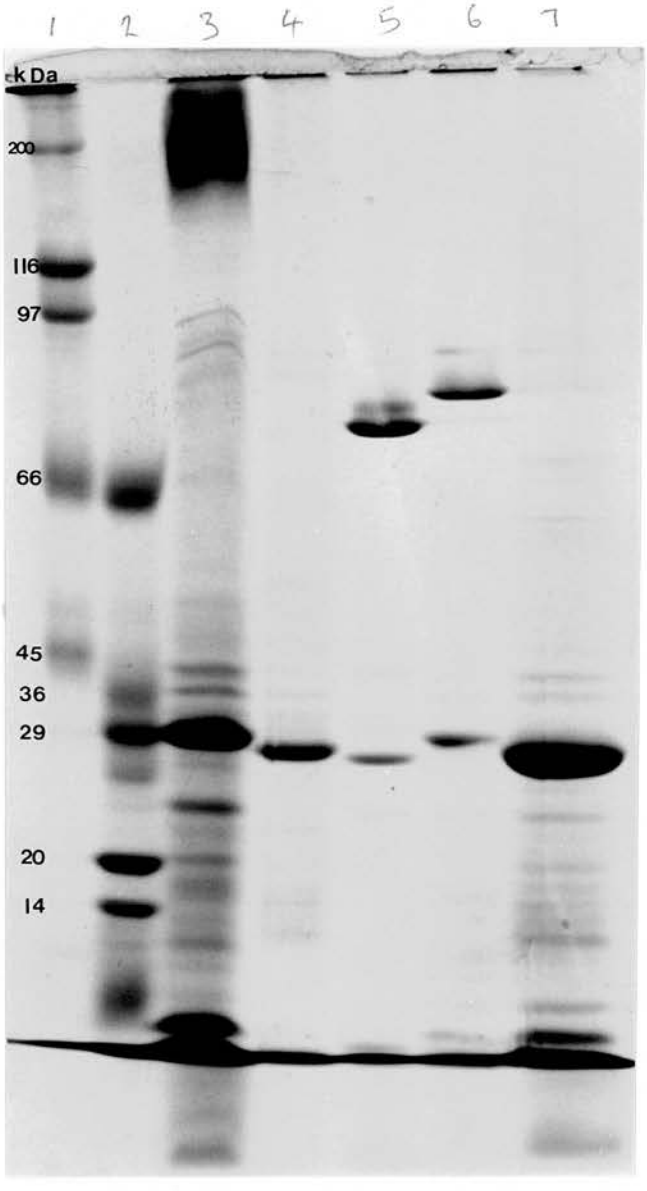


Figure 15. SDS-PAGE of outer membrane preparations from *B. cepacia* strains J2395 (Track 1), C1409 (Track 2), the epidemic strain (Track 3), C1559 (Track 4) and ATCC 17616 (Track 5). Outer membranes were separated using a 12% w/v acrylamide gel and stained with Coomassie blue. Molecular weights of protein standards are indicated.

Aronoff (1988). The OMP profile of the environmental isolates, J2395 and ATCC 17616 (Tracks 1 and 5 respectively) showed greatest similarity, with the exception of a broad high *Mr* band expressed by J2395. The CF isolates C1359 and C1559 (Tracks 3 and 6) express major high *Mr* bands of approximately 79,000 and 80,000 respectively, but other bands are very faint suggesting a low level of expression. The remaining CF isolate, C1409 (Track 2) expresses only the one major band.

Immunoblot analysis of the outer membrane preparations, probing with antisera raised in rabbits against heat-killed whole cells of J2395, C1409 and the epidemic strain, C1359 showed that there is variable cross-reactivity between the OMP antigens (Figure 16a, b & c). The sera from the rabbit inoculated with J2395 reacted strongly with the outer membrane preparation from the autologous strain, particularly the O-side chain moiety of the LPS (Figure 16a, Track 5), and also with two bands of ATCC 17616, C1559 and C1409 (Track 1, 3 and 4) and less strongly with the same bands of C1359 (Track 2). A weak reaction was also observed with various high molecular bands of each *B. cepacia*. Similarly, the response observed in the immunoblots probed with sera from the rabbit inoculated with C1409 (Figure 16b) showed a strong response against the autologous *B. cepacia*, particularly the O-side chain of the LPS (Track 4). The rabbit antisera reacted with the same two bands on each of the other strains of *B. cepacia*, and also faintly with some high *Mr* bands. The reaction of the antisera from the rabbit inoculated with the epidemic strain C1359 was slightly different (Figure 16c). There was no response to any O-side chain LPS and additional bands were observed for C1409 (Track 4) and J2395 (Track 5). The antisera reacted strongly with the same pair of bands as the previous antisera and with additional bands. The presence of the very high *Mr* band of J2395 was confirmed. This antisera also reacted strongly with a low molecular weight band,

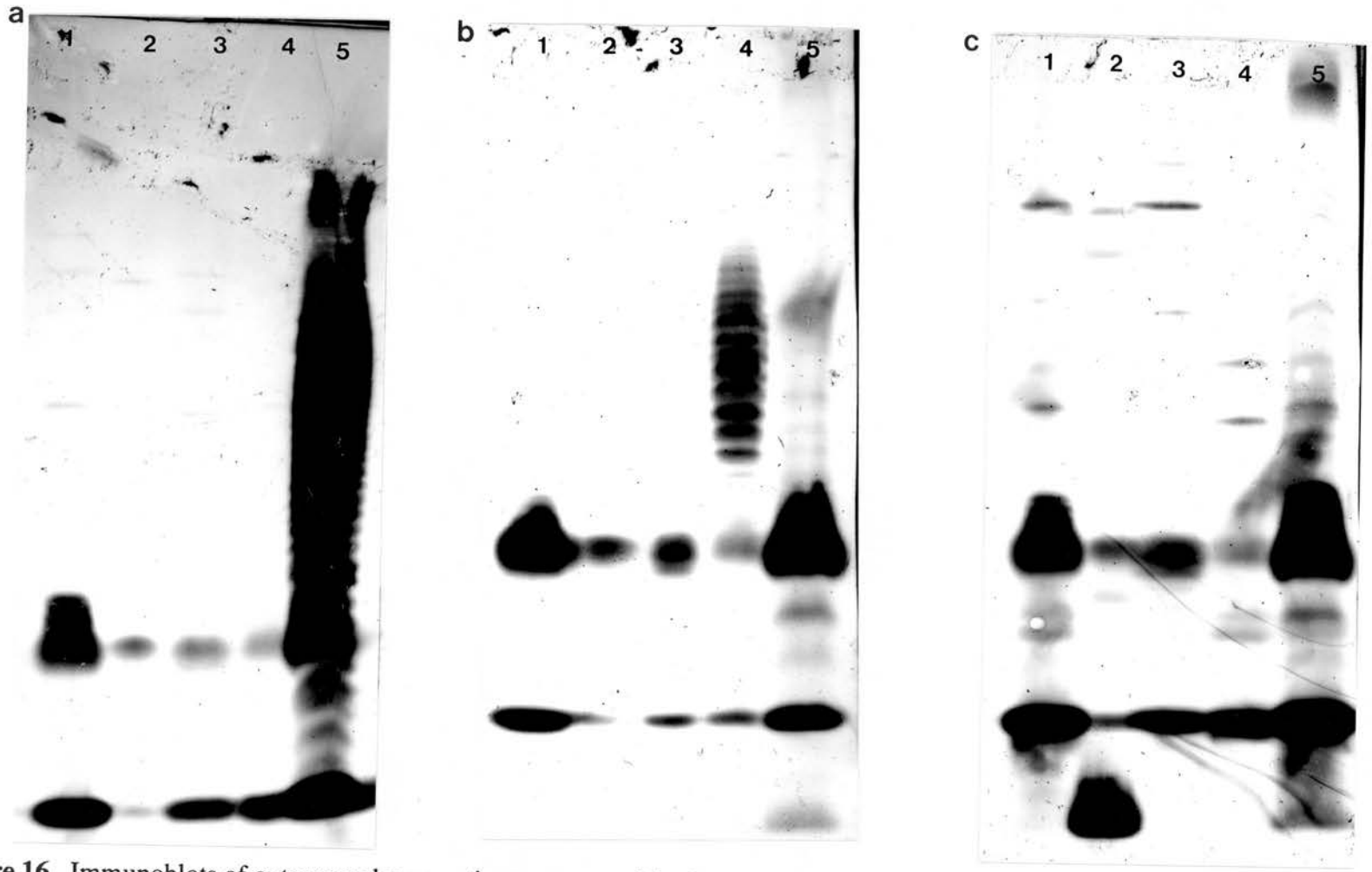


Figure 16. Immunoblots of outer membrane antigens separated by SDS-PAGE followed by electrophoretic transfer to NIC paper and probed with anti-*B. cepacia* antisera raised in rabbits to heat-killed whole cells of *B. cepacia* (a) J2395, (b) C1409 and (c) the epidemic strain: outer membrane antigens from ATCC 17616 (Track 1); the epidemic strain (Track 2); C1559 (Track 3); C1409 (Track 4) and J2395 (Track 5).

possibly core-LPS, of the epidemic strain. The immunoblot results emphasised that OMPs were heterogeneous in this group of *B. cepacia* isolates; however the immunoblots also verify the existence of common antigens, demonstrated by the paired bands apparent in each blot. The three CF isolates, C1359, C1559 and C1409 (Track 2, 3 and 4) appear to express fewer OMP than the two environmental isolates ATCC 17616 and J2395 (Track 1 and 5), which may be consistent with increased antibiotic resistance.

The immunoblot analysis (Figure 16c) confirms the earlier observation that the epidemic strain of *B. cepacia* expresses R-LPS.

Cell Surface Hydrophobicity

Surface hydrophobicity of eight *B. cepacia* isolates, *B. gladioli* ATCC 10248 and *P. aeruginosa* PAO1 was determined by HIC (Figure 17). Bacterial suspensions were chromatographed on octyl sepharose in the presence of binding buffer and relative hydrophobicity was expressed as the percentage of cells retained on octyl sepharose relative to the percentage retention by sepharose. A greater degree of retention was considered to be due to higher cell surface hydrophobicity.

In general the cell surface hydrophobicity for the bacterial strains investigated was low, less than 15% relative hydrophobicity, with the exception of one of the epidemic strain isolates (C1359). The per cent relative hydrophobicity tended to be slightly greater for *B. cepacia* isolates expressing R-LPS (C1359, C1430, C1559, C1394 and ATCC 17616).

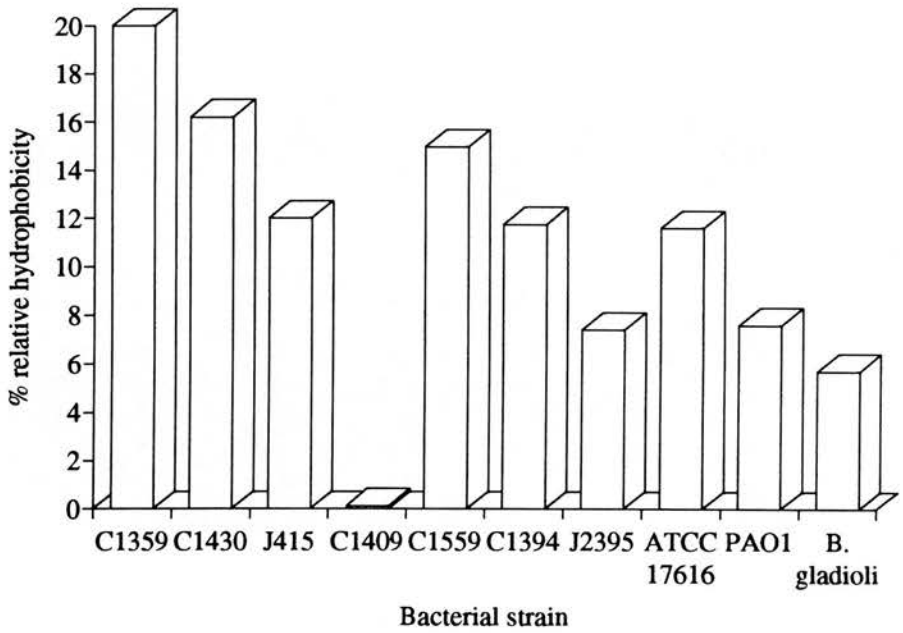


Figure 17. Cell surface hydrophobicity of eight *B. cepacia* strains, *P. aeruginosa* PAO1 and *B. gladioli* ATCC 10248.

CHAPTER 4

ADHERENCE AND COLONISATION OF *BURKHOLDERIA CEPACIA* IN CYSTIC FIBROSIS

In order to assess the possible role of the upper respiratory tract (URT) as a human reservoir for *B. cepacia* and in particular for the epidemic strain, the URTs of CF patients were examined for carriage of *B. cepacia.* The potential colonising ability of *B. cepacia* isolates was then assessed by investigating haemagglutination and adherence to buccal epithelial cells (BEC) or purified respiratory mucin

4.1 Determination of an Upper Respiratory Tract Reservoir for *B. cepacia*

In order to determine whether the URT acts as a reservoir for *B. cepacia* in colonised CF patients, samples from three URT sites (nasal swab, mouth swab and buccal scrape) were investigated on two separate occasions in CF patients colonised or not colonised by *B. cepacia* as determined by sputum culture of the organism. Quantitative counts for *B. cepacia* and *P. aeruginosa* were also performed with saliva samples (Table 10). The corresponding strain of *B. cepacia* was isolated from the mouth swab only in CF patients I, II and IV, colonised with two different non-epidemic strains of *B. cepacia* and the epidemic strain respectively. CF patients III to IX were colonised by the epidemic strain, which was isolated from all sites in patient III and from two sites in patients V - IX. With the exception of patient III, all the *B. cepacia* colonised patients studied were co-colonised by *P. aeruginosa.* *B. cepacia* was not isolated from the URT sites in 17

Table 10. Isolation of *B. cepacia* from Upper Respiratory Tract Sites in *B. cepacia* colonised CF patients on two separate occaisions.

		B. cepacia isolated from				
Patient	*B. cepacia*	nasal swab	mouth swab	buccal scrape	saliva (mean cfu/ml)	sputum (mean cfu/ml)
I	C1524	ng	+	ng	ng	$4.0x10^5$
II	C1705	ng	+	ng	ng	$2.0x10^6$
III	ES	+*	+	+	$7.8x10^5$	$3.0x10^7$
IV	ES	ng	+	ng	$1.1x10^5$	$4.5x10^7$
V	ES	ng	+	+	$9.0x10^5$	$5.0x10^6$
VI	ES	ng	+	+	NA	$7.0x10^7$
VII	ES	ng	+	+	$2.0x10^6$	$6.0x10^7$
VIII	ES	ng	+	+	NA	$6.0x10^7$
IX	ES	ng	+	+	$3.1x10^5$	$1.1x10^8$

+ - growth of autologous strain of *B. cepacia*
* - on one occasion
NA - not available
ng - no growth

control CF patients who were sputum culture negative for *B. cepacia* (11 of these were colonised by *P. aeruginosa*). Mean counts for *P. aeruginosa* in saliva of CF patients colonised by *P. aeruginosa* alone and in those co-colonised by the epidemic strain of *B. cepacia* were 10-100 fold less than counts for *B. cepacia*, although sputum counts of both organisms were similar. On the basis of these results it would appear that the URT may be a reservoir for the epidemic strain of *B. cepacia* in CF patients colonised by this strain.

4.2 HAEMAGGLUTINATION BY *B. CEPACIA*

Fimbriation in some Gram-negative bacteria may be associated with the ability to agglutinate various red blood cell types, and may therefore be detected by haemagglutination assay (Tweedy *et al.*, 1968). Furthermore, fimbrial haemaggluination by Type 1 fimbriae is typically mannose sensitive, whereas non-fimbrial haemagglutination is not inhibited by mannose. The capacity of 37 *B. cepacia* isolates from environmental, CF and other clinical sources to agglutinate erythrocytes from seven different species was investigated. Haemagglutination by *B. cepacia* was compared to that of a control strain of *E. coli* expressing mannose-sensitive Type 1 fimbriae. Table 11 shows the number of *B. cepacia* isolates agglutinating each red blood cell type and the effect of α methyl-mannoside.

Mannose sensitive haemagglutination was observed with the *E. coli* control strain and each of the haemagglutinating *B. cepacia* isolates. Addition of α methyl-mannoside inhibited haemagglutination regardless of erythrocyte type although the degree of inhibition was dependent on the *B. cepacia* isolate. Four patterns of haemagglutination were observed: (1) no agglutination with any cell type; (2) very weak agglutination,

observed with guinea pig and horse red blood cells only; (3) weak agglutination with all cell types; and (4) strong agglutination with guinea pig and horse erythrocytes but weak agglutination with the remaining cell types. The epidemic strain isolate examined in this study (C1359) was not observed to cause any of the red blood cell types to agglutinate. In order to pursue this observation a further 23 isolates of the epidemic strain from individual CF patients were investigated. None of the epidemic strain isolates were capable of haemagglutination.

Table 11. Number of *B. cepacia* isolates haemagglutinating each of seven erythrocyte types with and without addition of α methyl-mannoside.

Red blood cell type	No. of agglutinating isolates out of 37 tested	No. of mannose sensitive isolates* out of 37 tested
Human	17	17
Guinea-pig	27	27
Horse	27	27
Sheep	8	8
Goose	11	11
Ox	3	3
Goat	8	8

* - determined by addition of 2.0 w/v % α methyl-mannoside

The haemagglutination patterns of *B. cepacia* with guinea pig and horse erythrocytes and the effect of α methyl-mannoside on agglutination of each cell type was analysed with respect to the origin of the *B. cepacia* isolate. The data presented in Table 12 indicate that there is no association between the source of *B. cepacia* and capacity to agglutinate red blood cells, as agglutination patterns are similar for isolates of *B. cepacia* from different sources.

Table 12. Haemagglutination of guinea-pig and horse red blood cells by *B. cepacia* isolates and effect of α methyl-mannoside on haemagglutination of both cell types.

	Haemagglutination		
B. cepacia	Guinea pig erythrocytes	Horse erythrocytes	α methyl-mannoside
J365[a]	+++	+++	-
J759[a]	++	+	-
J760[a]	+++	++	-
J761[b]	+++	+++	++
J762[b]	++	++	-
J1743[b]	+	+	-
J1744[a]	++	++	-
J2395[a]	++	++	-
C133[c]	+++	++	+
C1394[c]	+++	+++	++
C1402[c]	++	++	-/+
C1406[c]	++	+	-
J1745[a]	+++	+++	++
J1749[b]	+++	+++	++
J415[c]	+++	+++	++
C1409[c]	+++	+++	-
J1875[c]	+++	+++	++
J752[c]	+++	+++	++
J772[c]	+++	+++	++
J763[b]	+++	+++	++
CDC 1[b]	+	-	-
CDC 26[b]	+	-	-
CDC 96*	+	-	-
CDC 94*	++	++	+
CDC 11[a]	+++	+++	++
CDC 8[a]	++	++	+
CDC 80[b]	+++	++	+
E. coli J2319	+++	+++	-

+++ strong agglutination
++ moderate agglutination
\+ weak agglutination
\- no agglutination

[a] - environmental isolate of *B. cepacia*
[b] - clinical isolate of *B. cepacia*
[c] - CF isolate of *B. cepacia*
* - source not known

To investigate whether haemagglutination was associated with fimbriation, transmission electron microscopy was performed on representative isolates of *B. cepacia* from Table 12. The electron microscopy showed that both haemagglutinating and non-haemagglutinating isolates of *B. cepacia* expressed fimbriae; for example the CF isolate J415 (Chapter 3, Figure 9) has previously been shown to express peritrichous fimbriae and strongly agglutinated all the red blood cell types whereas the epidemic strain of *B. cepacia* also expressed peritrichous fimbriae (Chapter 3, Figure 10) yet did not agglutinate any of the erythrocytes types. The *B. cepacia* isolates which were not observed to express fimbriae by electron microscopy did not haemagglutinate. The inability of *B. cepacia* isolates to haemagglutinate may be explained by the absence or low frequency of fimbrial expression observed or alternatively the different fimbrial types expressed by *B. cepacia* may not have equal capacity to haemagglutinate.

Overnight culture of *B. cepacia* isolates in NYB at 30°C instead of 37°C, did not affect the degree of haemagglutination observed, however the capacity of ten of the agglutinating strains of *B. cepacia* to agglutinate guinea pig and horse erythrocytes was eliminated following culture in MM.

4.3 Adherence of *B. cepacia* to Buccal Epithelial Cells

Adhesion of three representative isolates of *B. cepacia* (the epidemic strain C1359, CF isolate C1409 and environmental isolate NCTC 10744), *P. aeruginosa* SBC88 and *N. meningitidis* A14 to BEC obtained from three CF patients and five normal controls was analysed by flow cytometry (Table 13). The binding index is a guide as to the degree of binding of the bacteria to the BECs. Table 13 shows clearly that there is considerable variation in the degree of binding for each of the bacterial strains to the different BEC.

Table 13. Flow cytometric analysis of binding of 3 *B. cepacia* strains, *P. aeruginosa* and *N. meningitidis* to BEC from CF patients and normal subjects. The binding index is the mean level of fluorescence per cell divided by the percentage of the cell sample population with fluorescence greater than the background controls.

Source of BEC	Binding Index				
	C1409	epidemic strain	NTCC 10744	*P. aeruginosa*	*N. meningitidis*
I [a]	1.22	40.3	0.17	22.9	7.7
II [b]	0.47	24.0	0.83	3.8	2.3
III [b]	0.08	15.3	0.43	2.3	1.0
IV [c]	0.11	12.4	0.07	4.2	2.8
V [c]	0.29	17.7	0.70	4.5	2.1
VI [c]	0.28	8.9	0.17	1.9	1.6
VII [c]	0.25	0.77	0.79	0.70	0.47
VIII [c]	0.12	1.2	0.55	0.38	0.31

[a] - CF patient colonised by the epidemic strain only
[b] - CF patients colonised by *P. aeruginosa* only
[c] - normal controls.

With the exception of the BEC from subject VII, the greatest binding of *B. cepacia* to BEC was observed with the epidemic strain. Maximum binding was demonstrated to the BEC from subject I (a CF patient colonised by the epidemic strain) by the epidemic strain, C1409, *P. aeruginosa* and *N. meningitidis*. Interestingly the binding index for the epidemic strain and BEC I was almost twice the binding index for *P. aeruginosa* and more than five times that for the control strain of *N. meningitidis*, which is considered to demonstrate relatively high levels of binding to BEC (Dr J. Stewart, personal communication). The second highest binding index was also observed with the epidemic strain and BEC II from a CF patient not colonised by *B. cepacia*. The binding of *B. cepacia* strains C1409 and NCTC 10744 to all the BEC samples is significantly less ($p<0.01$, Students *t*-test) than the binding of the epidemic strain to the BEC. The binding indices of the control organisms, *P. aeruginosa* and *N. meningitidis,* and all of the BEC samples were greater than those for the *B. cepacia* strains C1409 and NCTC 10744 but less than those for the epidemic strain. There is no significant difference ($p<0.1$) between the binding of the CF isolate of *B. cepacia* and the environmental isolate of *B. cepacia.*

4.4 Adherence of *B. cepacia* to Purified Respiratory Mucin

Several methods were investigated to examine the adherence of *B. cepacia* to purified respiratory mucin. The most successful method was a microtitre ELISA incorporating hyperimmune sera obtained from CF patients colonised by the epidemic strain of *B. cepacia* to measure adhesion of thirty *B. cepacia* strains to purified respiratory mucin bound to the wells of polystyrene microtitre plates. In all mucin adherence experiments adherence was considered to be significant when R value > 2.0 (Dr J Forstner, personal communication).

In preliminary experiments, hyperimmune sera from *B. cepacia* colonised CF patients were screened against whole cells of several strains of *B. cepacia* bound to polystyrene microtitre plates in a chequerboard ELISA to determine the optimum serum concentration required for detecting multiple strains of *B. cepacia*. Sera from several CF patients colonised by the epidemic strain or other strains of *B. cepacia* were screened individually and also as a pool. Serum from CF patients colonised by the epidemic strain diluted 1:1000 was observed to be superior in detecting *B. cepacia* bound to the microtitre plates. In addition the concentration of mucin required to coat wells optimally and the optimal concentration of bacteria added to wells were determined. A mucin coating concentration of 10 μg/ml was shown to give optimal sensitivity, yielding a maximal difference in OD between coated wells and background controls. A bacterial concentration of 10^9 cfu/ml gave maximal measurable binding to mucin coated wells with minimal non-specific to background coated wells. In control experiments performed with strains of *P. aeruginosa* previously shown to exhibit high binding to mucin (Nelson, 1990) the optimal bacterial concentration was 10^7 cfu/ml, as at concentrations higher than this significant binding to uncoated wells was evident for *P. aeruginosa*.

Significant adherence to mucin, following overnight static culture in NYB at 37°C, was observed in seven of the 30 *B. cepacia* isolates investigated (Table 14); all of these seven isolates were obtained from CF patients. Of these adherent strains of *B. cepacia*, the greatest adherence to the respiratory mucin was observed with the epidemic strain (R value 20.0). To further investigate whether the enhanced capacity to adhere to respiratory mucin was typical in isolates of the epidemic strain of *B. cepacia*, the adherence of a further sixteen isolates of the epidemic strain from different CF patients was examined. Significant adherence (R value 3.3 - 55.0) to respiratory mucin was

observed in ten (62.5%) of the epidemic strain isolates. In control assays, adherent strains of *P. aeruginosa* had mean adherence to mucin at least ten times greater than that observed for *B. cepacia*. However, the background binding to non-coated wells was also significantly higher than that for *B. cepacia* suggesting that adherence in *P. aeruginosa* is non-specific.

Table 14. Adherence of *B. cepacia* strains to purified respiratory mucin following overnight static culture in NYB at 37°C. Significance of adherence to mucin determined by calculation of R value.

	Mucin binding
B. cepacia strain number	R value[a]
C1359[b]	20.0
C1409	3.90
Pc 5[c]	4.75
Pc 24[c]	7.20
SBC74	5.00
SBC78	2.30
SBC67	2.70

[a] R value - mean binding to mucin / mean background (BSA)
An R value >2 is considered to show significant binding to mucin
[b] epidemic strain
[c] CF isolates from Toronto

The effect of altering the culture conditions; (1) growing bacteria in aerated culture, and (2) culture in nutrient rich or nutrient poor media on the ability of the bacteria to adhere to mucin was examined and found to be strain dependant. The epidemic strain and SBC67 demonstrated greater adherence following static culture whereas J762, J2395 and C1409 demonstrated significant binding only after aerated culture (Table 15). Following

culture in nutrient deficient media the binding of two non-epidemic strain CF isolates (C1406 and C1409) and two epidemic strain isolates (C1359 and SBC28), to mucin appeared to increase. Non-specific binding to the control wells had also increased proportionally however, suggesting that culture in nutrient-deficient conditions induced a general increase in adhesion in some *B. cepacia* strains (data not shown).

Table 15. Adherence of *B. cepacia* strains to purified respiratory mucin following static or aerated culture in NYB at 37°C.

	Mucin Binding (R value [a])	
B. cepacia strain number	Static culture	Aerated culture
SBC28[b]	24	14.5
C1359[b]	20	9.0
C1409[c]	3.9	4.5
SBC67[c]	2.7	0.6
J762[d]	0.42	3.9
J2395[e]	1.3	3.0

a R value - mean binding to mucin / mean background (BSA)
An R value >2 is considered to show significant binding to mucin
b - epidemic strain
c - CF isolate
d - clinical non-CF isolate
e - environmental isolate

To determine whether binding of *B. cepacia* to the mucin coated wells reached a saturation point the binding of *B. cepacia* isolates was measured at 30 min intervals over a period of 2 h (Figure 18). Figure 18 confirms the variable nature of mucin adherence in *B. cepacia* strains for example; for the two epidemic strain isolates SBC58 and SBC75 maximum adherence is achieved by 60 min and 90 min respectively. The maximum

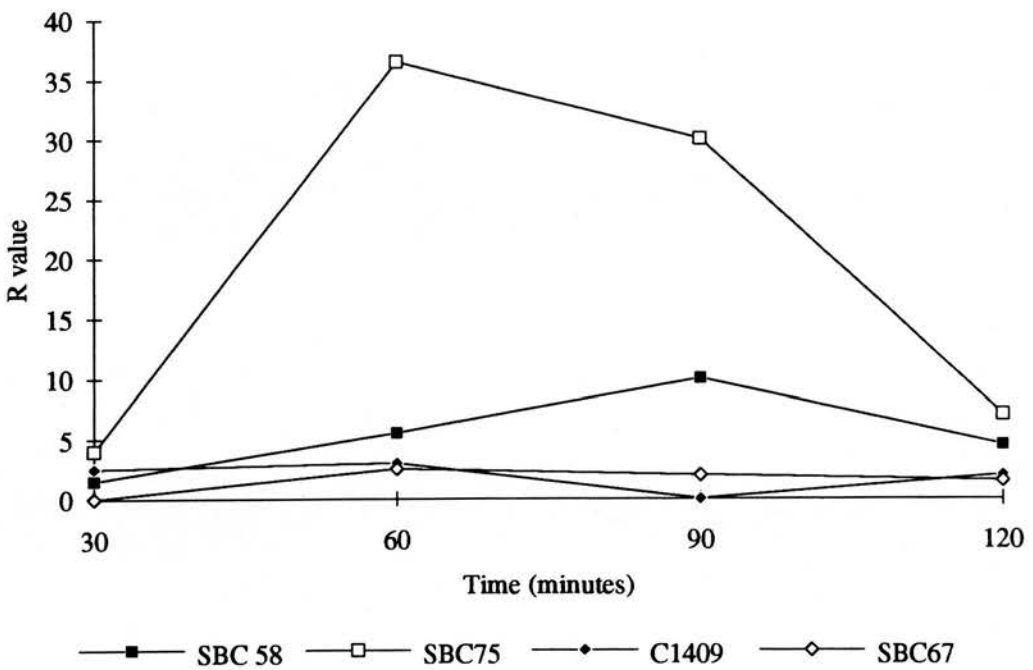


Figure 18. Binding of four *B. cepacia* isolates to purified respiratory mucin measured at 30 min intervals over 2 h. Degree of binding determined by R value.

adherence for C1409 and SBC67 is also achieved at 60 min and 90 min but the R value is much lower. Two additional strains of *B. cepacia* were examined: for one strain (Pc 5) the R value was still increasing at 2 h and the remaining isolate (J415) did not show significant binding at any time (data not shown).

The purified respiratory mucin used in the above experiments was obtained from a chronic asthmatic patient. Purified CF mucin from several patients became avilable at a later date and the assay was repeated to compare the binding of *B. cepacia* to the different purified respiratory mucins. In the first instance it appeared that the *B. cepacia* strains bound with greater affinity to some of the CF mucins. However, when the assay was repeated without bacteria, it was observed that the OD obtained depended on the CF serum used to detect binding and that the reaction between CF serum and CF mucin was variable. The OD obtained with asthmatic mucin was constant with different CF sera, therefore this was used in all assays.

In this study, the epidemic strain of *B. cepacia*, which is associated with poor pulmonary status and marked transmissibility exhibited the greatest binding to BEC and purified respiratory mucin yet did not agglutinate any red blood cell type. In contrast, *B. cepacia* J415, also associated with fatality in CF but not transmissibility (Glass & Govan, 1986), strongly agglutinated red blood cells yet did not adhere significantly to mucin. On the basis of the results obtained, there appears to be no correlation between the capacity of *B. cepacia* strains to agglutinate erythrocytes and that to adhere to BEC or mucin, indicating that different *B. cepacia* isolates may express different adhesins. Furthermore any relation of the above with colonisation of the CF patient and clinical outcome would be extremely difficult to determine from these results.

CHAPTER 5

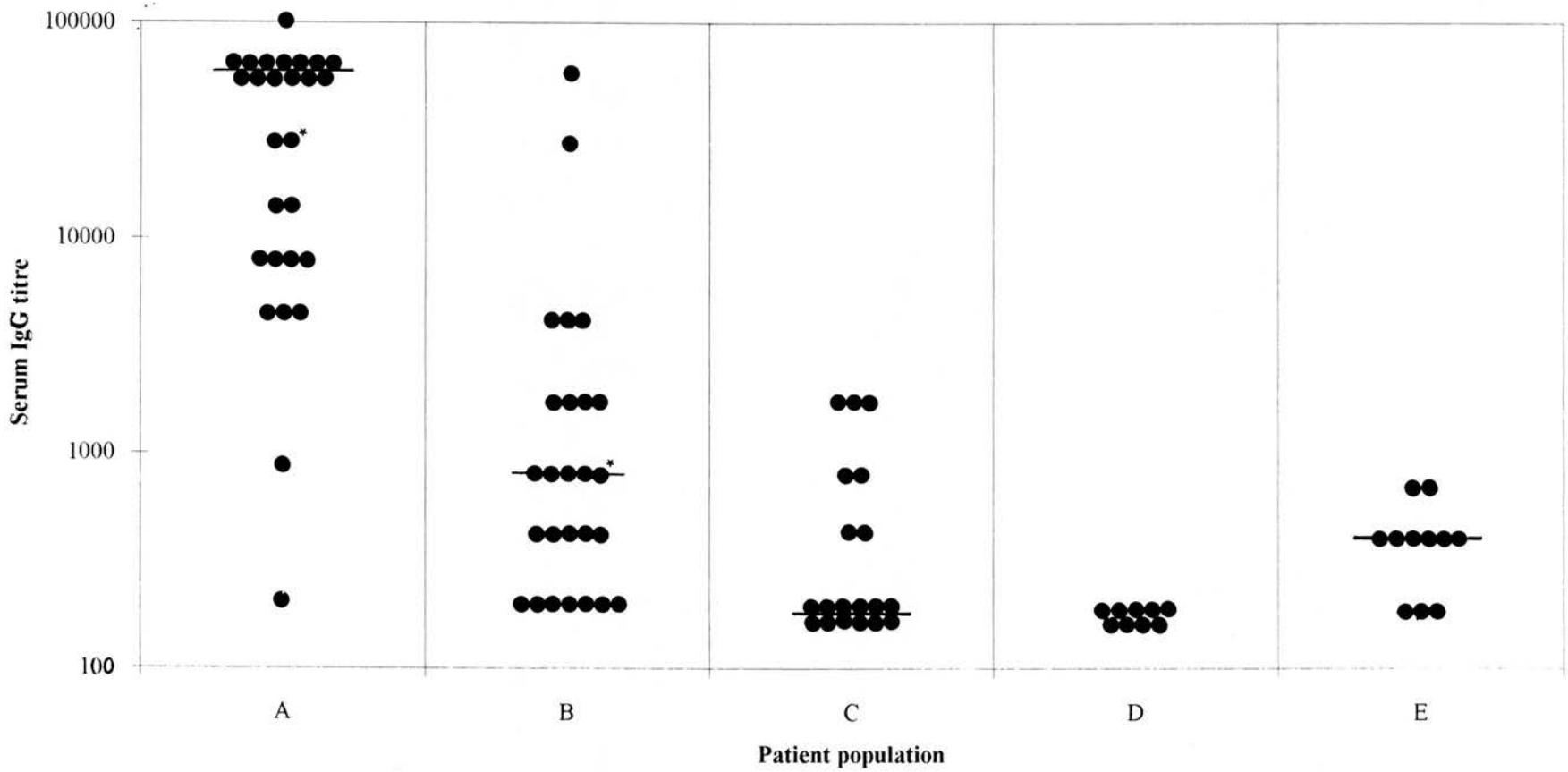
INVESTIGATION OF THE ANTIBODY RESPONSE TO *BURKHOLDERIA CEPACIA* IN COLONISED CYSTIC FIBROSIS PATIENTS

The aim of this study was to investigate the anti-*B. cepacia* antibody response in CF patients using an ELISA system based on defined antigenic preparations and immunoblotting. The potential role of such assays in the early diagnosis of *B. cepacia* colonisation in CF patients and also as an indication of prognosis was evaluated. Finally, explanations were sought to account for the failure of the anti-*B. cepacia* antibodies to eliminate *B. cepacia* from colonised CF patients. In this chapter, CF patients colonised by *B. cepacia* will be referred to as Bc^+, those colonised by the epidemic strain as $ESBc^+$ and CF patients not colonised by *B. cepacia* as Bc^-.

5.1 Serum Anti-LPS Antibodies Detected by ELISA

LPS prepared from the epidemic strain of *B. cepacia*, shown in Chapter 3 to express rough-form LPS, was used in the ELISA system at a coating concentration of 20 μg/ml. Results for all the ELISAs are expressed as final serum titres, i.e. the reciprocal of the serum dilution which gives an OD >0.1 after subtraction of background controls. Analytical variation of the ELISA, including intra-plate and day-to-day (inter-plate) variation, was determined using six serum samples (two each with low, medium and high ELISA titres) to calculate the coefficient of variation. The intra-plate and inter-plate variations of the ELISA were 5.5% and 13.9% respectively.

Serum anti-*B. cepacia* LPS IgG titres were investigated in 74 Bc^+CF patients who represented a cross section of the CF patient population from UK CF centres in Cardiff; Edinburgh, Leeds, Manchester and Newcastle. Controls comprised CF patients not colonised by *B. cepacia* matched for age, sex and clinical status and chronic bronchitic patients not colonised by either *B. cepacia* or *P. aeruginosa* but matched for clinical status and normal healthy individuals (Figure 19). The duration of *B. cepacia* colonisation in individual patients at the time of sampling ranged from six weeks to 4.5 years; the IgG titres shown in Figure 19 were obtained using the most recent serum available for each CF patient. Elevated titres of anti-*B. cepacia* LPS IgG were observed in Bc^+CF patients (Bc^+ CF IgG titre range 200 - 51200; titre median 800) and in particular in $ESBc^+$ CF patients ($ESBc^+$ CF patients: IgG titre range 200 - 102400; titre median 51200). Low serum IgG titres (<1600) were measured in patients recently colonised by the epidemic strain, whilst titres in chronically colonised patients were >6400. The serum IgG titres for Bc^- CF patients (IgG titre range 200 - 1600; titre median 200), and the two non-CF control groups (titre range 200 - 800; titre median 200) were lower than in the *B. cepacia* colonised patients. Statistical analysis of transformed data (converted to logarithms) revealed a highly significant difference between IgG anti-*B. cepacia* antibody titres in CF patients colonised by the epidemic strain and patients colonised by other strains of *B. cepacia*, Bc^- patients and non CF controls ($p<0.0001$ in each case). The anti-*B. cepacia* antibody titres in CF patients colonised by other strains was significantly higher than titres in Bc^- CF patients ($p<0.01$). There were no significant difference in the titres between the controls and Bc^- CF patients.



* patients colonised by two strains of *B. cepacia*

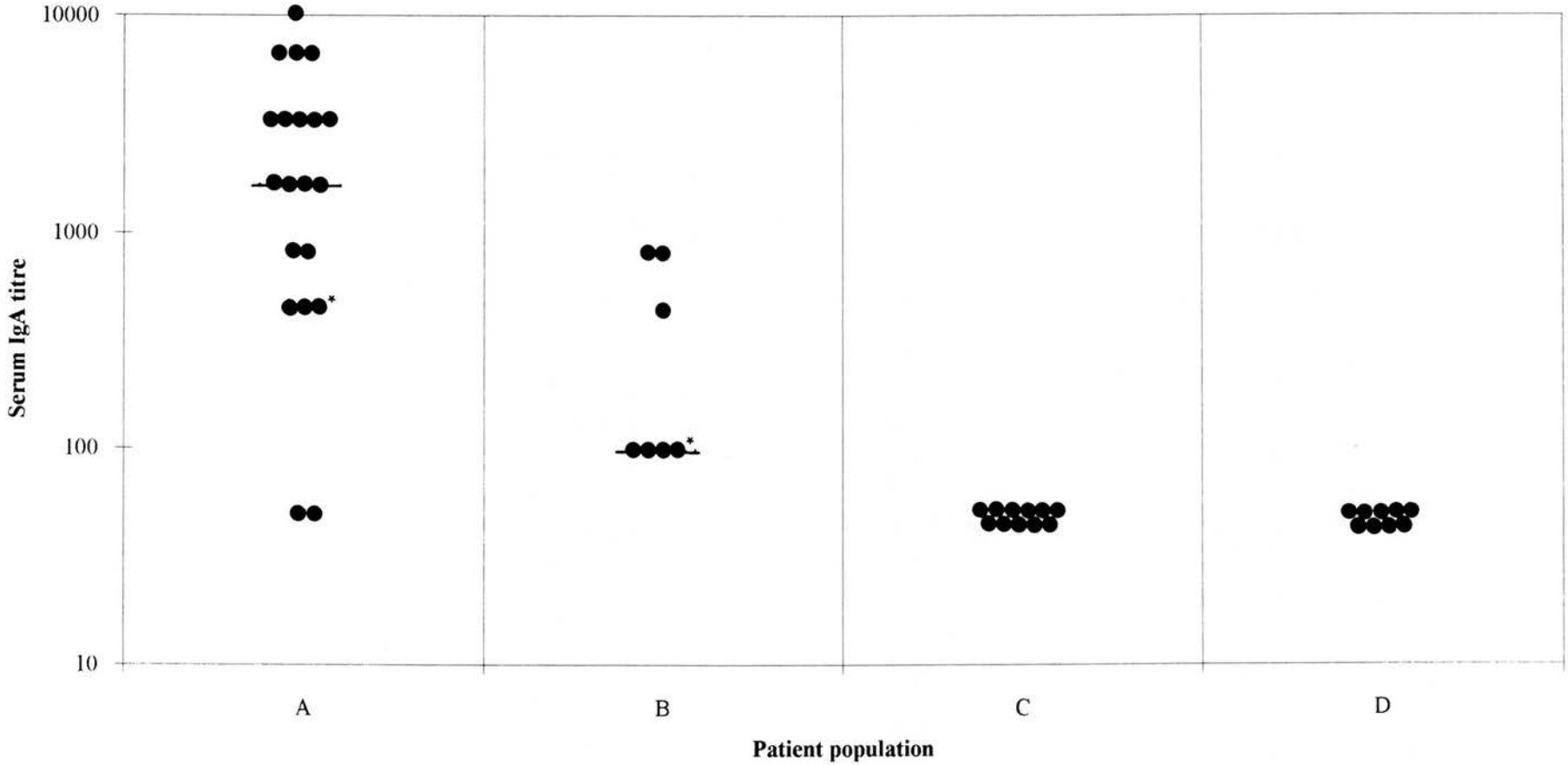
Figure 19. Serum IgG anti-*B. cepacia* antibody titre measured by ELISA with *B. cepacia* R-LPS as coating antigen in five populations: (A) $ESBc^+$ CF patients, n=29; (B) Bc^+ CF patients, n=26; (C) Bc^- CF patients, n=19; (D) chronic bronchitic patients, n=9; and (E) normal healthy controls, n=11. Bars represent median values.

Serum IgA and IgM antibodies to *B. cepacia* LPS were also investigated in CF patients and controls (Figure 20 and 21). The results for serum IgA (Figure 20) agreed with those for serum IgG in that the IgA titres in patients colonised by *B. cepacia*, in particular by the epidemic strain, are greater than the titres in Bc^- patients and controls. Similarly, serum IgM anti-*B. cepacia* LPS antibodies were raised in CF patients colonised by the epidemic strain ($p<0.0001$); interestingly, however there was no significant difference between the titres in patients colonised by other strains of *B. cepacia* and Bc^- CF patients or the non-CF patients (Figure 21).

To confirm that the increased antibody titre observed in CF patients colonised by the epidemic strain of *B. cepacia* was a result of colonisation, paired sera from eight CF patients was investigated by ELISA. Figure 22 shows the serum IgG, IgA and IgM anti-*B. cepacia* antibody titres in patients prior to colonisation and after the establishment of chronic colonisation with the epidemic strain. A rise in antibody titre, for each class of immunoglobulin was observed for each CF patient. Serum from 10 Bc^- CF was investigated for anti-*B. cepacia* LPS antibodies at similar time intervals. No increase in antibody titre was noted for any of these control patients.

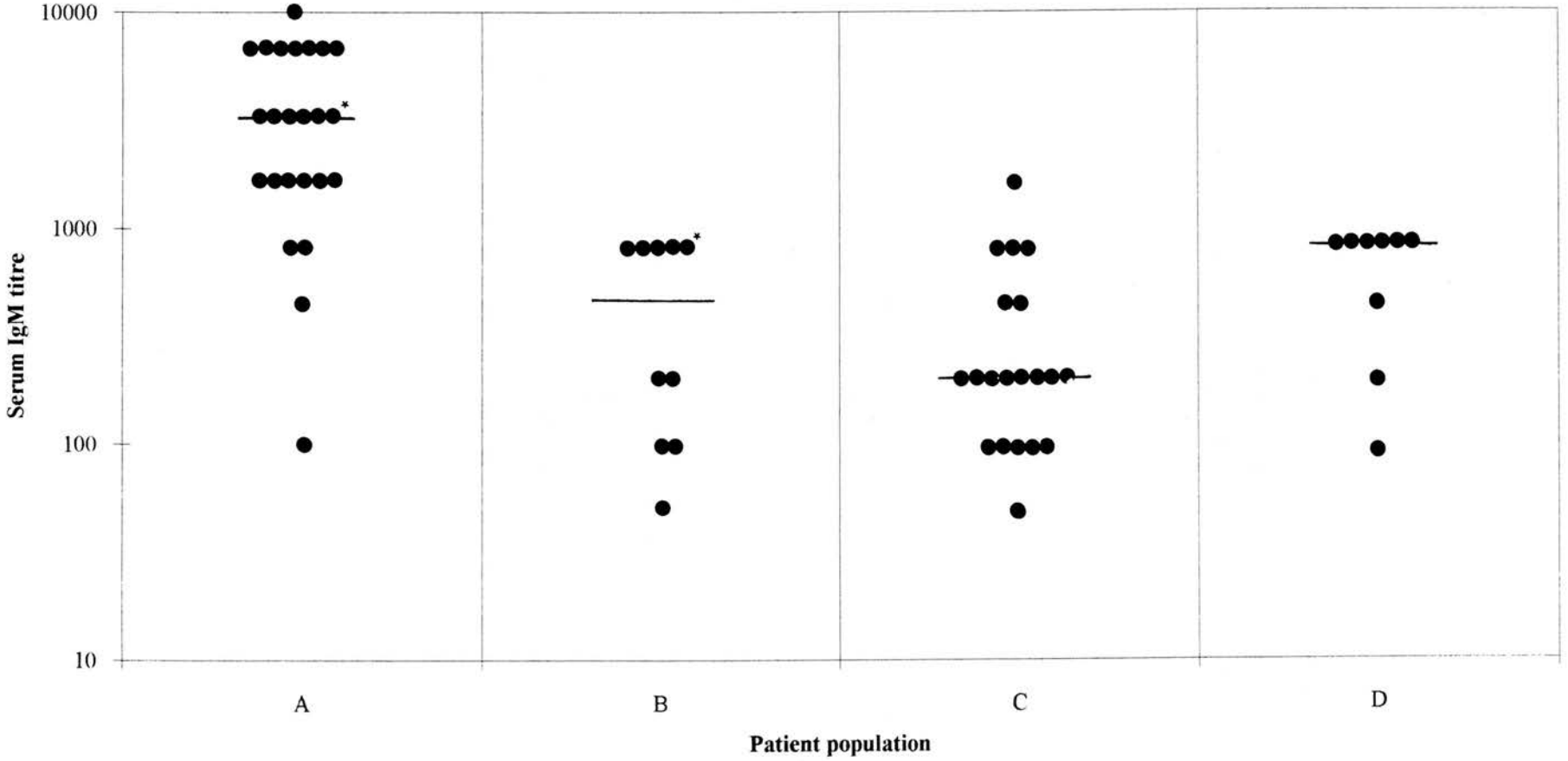
5.2 Absorption of Sera with *B. cepacia* and *P. aeruginosa* LPS

The possibility that there may be cross reactivity between the anti-*B. cepacia* IgG, anti-*P. aeruginosa* IgG and antigens from either organism was investigated. Serum anti-*P. aeruginosa* LPS IgG antibody titres were measured in 20 Bc^+ and Bc^- CF patients, the majority of which were colonised by *P. aeruginosa*. Regardless of *B. cepacia* colonisation status, a specific anti-*P. aeruginosa* response was observed in those patients colonised by *P. aeruginosa* (titre range 400 - 12800), whilst the anti-*P. aeruginosa* titre



* patients colonised by two strains of *B. cepacia*

Figure 20. Serum IgA anti-*B. cepacia* antibody titre measured by ELISA with *B. cepacia* R-LPS as coating antigen in four populations: (A) ESBc$^+$ CF, n=20; (B) Bc$^+$ CF patients, n=6; (C) Bc$^-$ CF, n=11 and (D) chronic bronchitic patients, n=9. Bars represent median values.



* patients colonised by two strains of *B. cepacia*

Figure 21. Serum IgM anti-*B. cepacia* antibody titre measured by ELISA with *B. cepacia* R-LPS as coating antigen in four populations: (A) $ESBc^+$ CF patients, n=23; (B) Bc^+ CF patients, n=10; (C) Bc^- CF patients, n= 20; (D) chronic bronchitic patients, n= 9. Bars represent median values.

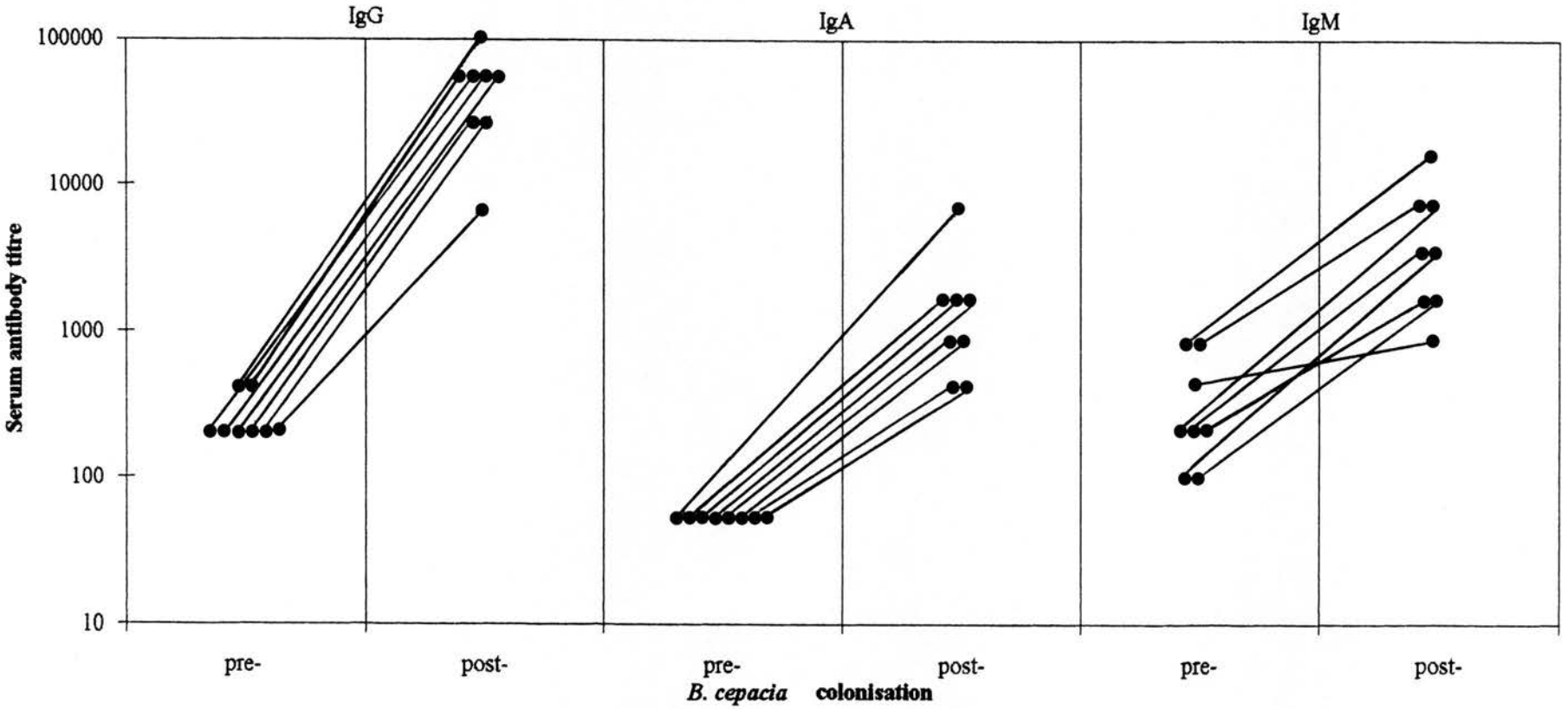


Figure 22. Serum IgG, IgA and IgM anti-*B. cepacia* antibody titre measured by ELISA with *B. cepacia* R-LPS as coating antigen in eight CF patients pre- and post-colonisation with the epidemic strain of *B. cepacia*.

(titre range 200 - 800) observed in non-*P. aeruginosa* colonised CF patients equivalent in magnitude to the anti-*B. cepacia* titre in Bc^- CF patients.

Sera from CF patients colonised with *B. cepacia* and/or *P. aeruginosa* or neither were absorbed with purified R-LPS of the epidemic strain and the *P. aeruginosa* strain PAC 608 (expressing R-LPS) and analysed by ELISA (Figure 23a & b) and multiblotting. The anti-*B. cepacia* IgG antibody titre in CF patients colonised by the epidemic strain (patients I-VII), was considerably reduced following absorption of serum from with the autologous strain but not after absorption with the *P. aeruginosa* LPS. Similarly, in those CF patients colonised by *P. aeruginosa* (patients II, VI, VII and VIII) the IgG antibody titre measured against the *P. aeruginosa* core LPS coating antigen was reduced following absorption with *P. aeruginosa* but not after absorption with *B. cepacia* LPS. These immunoblotting results confirmed the observation that specific serum antibodies were only removed by *B. cepacia* or *P. aeruginosa* LPS as appropriate (data not shown) and indicate that the anti-*B. cepacia* R-LPS antibody response was specific for *B. cepacia* and not cross-reactive with *P. aeruginosa* R-LPS.

5.3 Longitudinal Studies of Serum IgG and IgM Anti-LPS Antibodies

The next experimental approach was to follow the serum IgG anti-*B. cepacia* LPS antibody response at approximately six monthly intervals in 10 $ESBc^+$ CF patients attending the Edinburgh adult CF clinic (Figure 24a & b). Prior to colonisation with the epidemic strain each patient had low anti-*B. cepacia* antibody titres in the range observed for titres measured in Bc^- CF patients. Following colonisation by *B. cepacia* the profile of the serum IgG response was patient dependent with a variable rate of increase in titre. Interestingly, a 2-4 fold increase in serum IgG titre preceded laboratory culture of *B.*

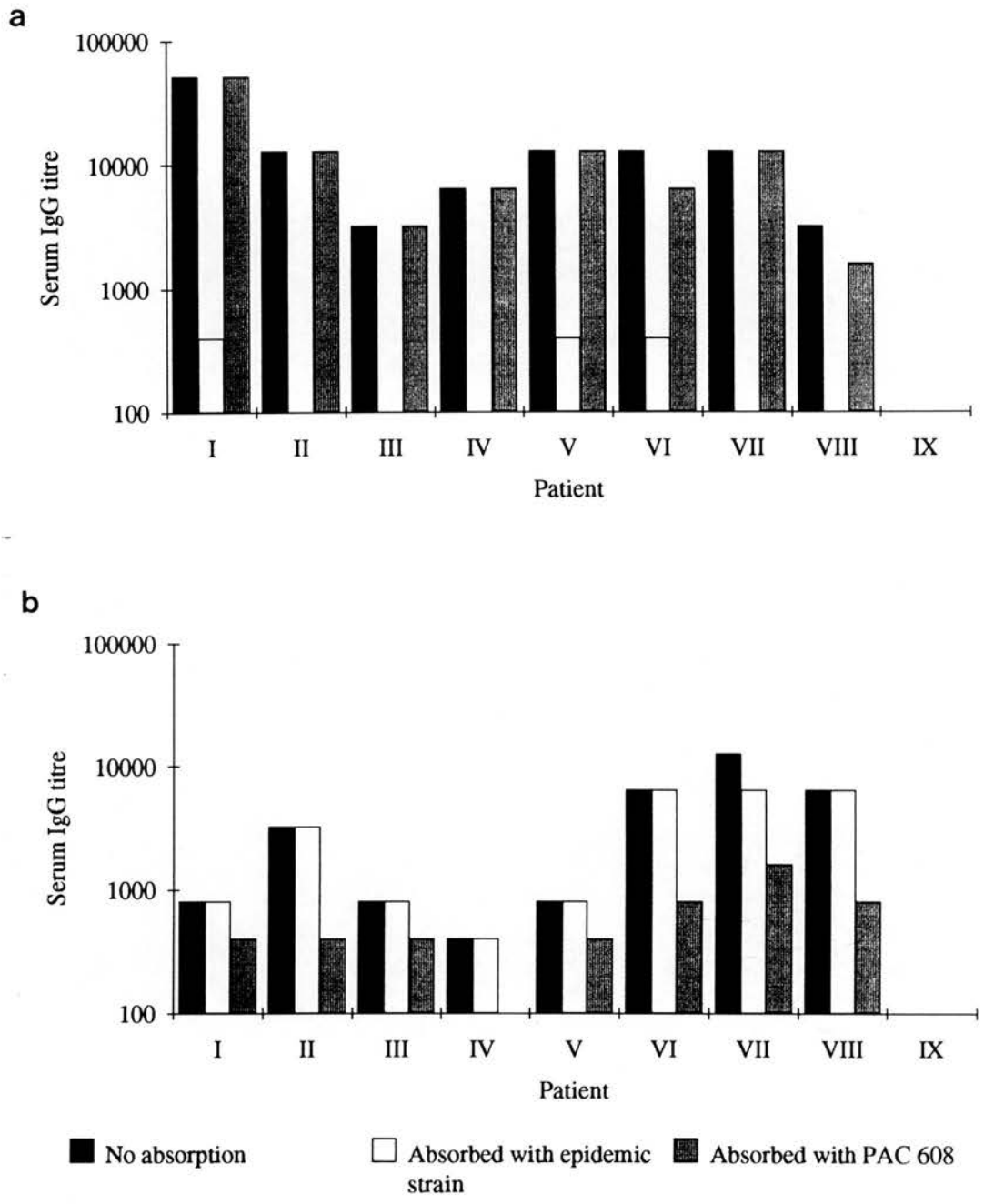


Figure 23. Serum IgG anti-*B. cepacia* and anti-*P. aeruginosa* core LPS antibodies in CF patients measured by ELISA with (a) core R-LPS from the epidemic strain of *B. cepacia* and (b) core R-LPS from *P. aeruginosa* PAC608 as coating antigens. Patients I, III, IV, and V were $ESBc^+$; patients II, VI, and VII were $ESBc^+/Pa^+$; patient VIII was colonised by *P. aeruginosa* only and patient IX was not colonised by either organism.

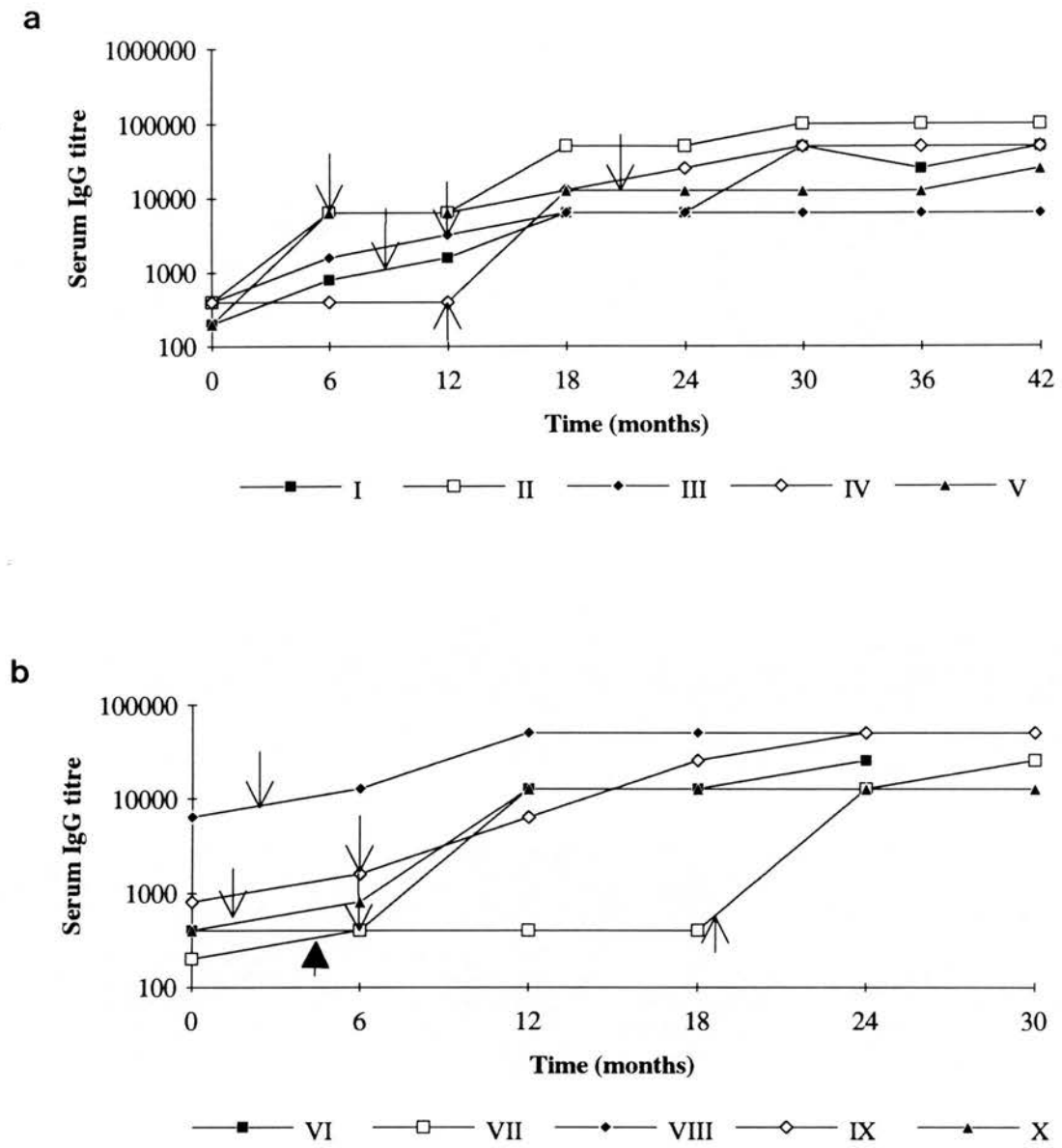


Figure 24. Longitudinal study of serum IgG anti-*B. cepacia* LPS antibodies in 10 CF patients colonised by the epidemic strain measured by ELISA with R-LPS from the epidemic strain as coating antigen. Arrows indicate when the epidemic strain (↑) or other strains of *B. cepacia* (♠) were first isolated from sputum.

cepacia in all patients with the exception of patient IV who was initially transiently colonised by the epidemic strain and patient VII who was initially colonised by a different strain of *B. cepacia.* Following the onset of chronic colonisation by the epidemic strain, the serum IgG antibody titre in both patients IV and VII increased. The serum IgG titre in each patient increased following chronic colonisation until a maximum level was attained. None of the CF patients in this study lost *B. cepacia* once chronic colonisation was established.

Longitudinal measurement of IgM anti-*B. cepacia* LPS antibody titres in serum obtained from clinic visits immediately prior to the first isolation of *B. cepacia* and also from subsequent visits were performed (data not shown). The aim was to investigate any increase in specific IgM antibodies to *B. cepacia* LPS around the time of colonisation, in order to relate laboratory isolation with colonisation. Clinic visits were not sufficiently frequent (time in between visits varied from two weeks to two months) to enable any correlation to be made. In three out of 10 $ESBc^+$ CF patients investigated, a peak anti-*B. cepacia* LPS IgM titre coincided with laboratory isolation of *B. cepacia.*

5.4 Sputum Anti-LPS Antibodies Detected by ELISA

Anti-*B. cepacia* LPS IgA and IgG antibodies in sputum were also measured by ELISA with epidemic strain core-LPS as coating antigen in nine $ESBc^+$ CF patients, nine Bc^- CF patients and nine chronic bronchitic patients. Longitudinal measurements of sputum IgA antibody indicated that there was considerable variation in titre between samples, as shown in 3 CF patients (Figure 25). Similar variation was not observed in either Bc^- CF patients or in the chronic bronchitic control patients. Figure 26 shows anti-*B. cepacia* sputum IgA antibody titres in three patient populations. The sputum IgA titres in $ESBc^+$

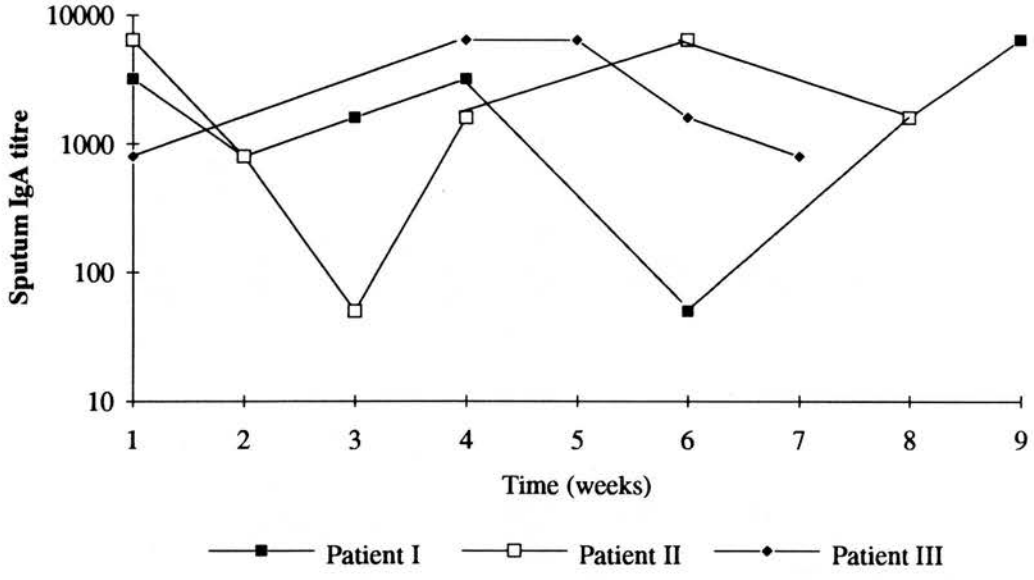


Figure 25. Longitudinal study of sputum IgA anti-*B. cepacia* LPS antibodies in 3 CF patients colonised by the epidemic strain of *B. cepacia* measured by ELISA with R-LPS from the epidemic strain as coating antigen.

Figure 26. Sputum IgA anti-*B. cepacia* LPS antibody titre measured by ELISA with R-LPS from the epidemic strain of *B. cepacia* as coating antigen in three patient populations: (A) ESBc$^+$ CF patients, n=9; (B) Bc$^+$ CF patients, n=9; and (C) chronic bronchitic patients, n=9. Bar represents median values.

CF patients (sputum IgA titre range 200 - 6400; titre median 1600) were significantly higher ($p<0.001$) than for non-*B. cepacia* colonised CF patients (titre range 25 -100; titre median 50) and chronic bronchitics (titre range 25 - 400; titre median 50).

In contrast to the sputum IgA antibodies, IgG antibodies in sputum were difficult to detect; in 10 BC^- CF patients titre were <25, whilst 10 Bc^+ CF patients showed titres of <200 with the exception of one CF patient colonised by the epidemic strain who had a sputum IgG titre of 3200.

5.5 Immunoblot Analysis of Serum Against *B. cepacia* Antigens

The serum IgG response in Bc^+ CF patients was further investigated by immunoblotting and multiblotting. Antigens were separated by SDS-PAGE and electrophoretically transferred to NIC paper for subsequent probing with sera from CF patients or controls. The antibody response to the following *B. cepacia* preparations was investigated: (1) purified R-LPS from the epidemic strain; (2) purified S-LPS from the environmental isolate J2395; (3) Proteinase K digests of whole cells of six different *B. cepacia* isolates; (4) outer membrane preparations of five *B. cepacia* isolates; and (5) flagellar preparations from three *B. cepacia* isolates. Sera from CF patients colonised by the epidemic and other strains of *B. cepacia* and also Bc^- CF patients were analysed.

Serum IgG Response to Purified LPS

Multiblots of purified R- and S-LPS from the epidemic strain and J2395 respectively, probed with serum from CF patients pre- and post-colonisation with *B. cepacia* are shown in Figure 27a & b (serum as for Figure 22). Patients I - IX (Tracks 1-18) were colonised by the epidemic strain. Prior to colonisation serum from patient V only showed

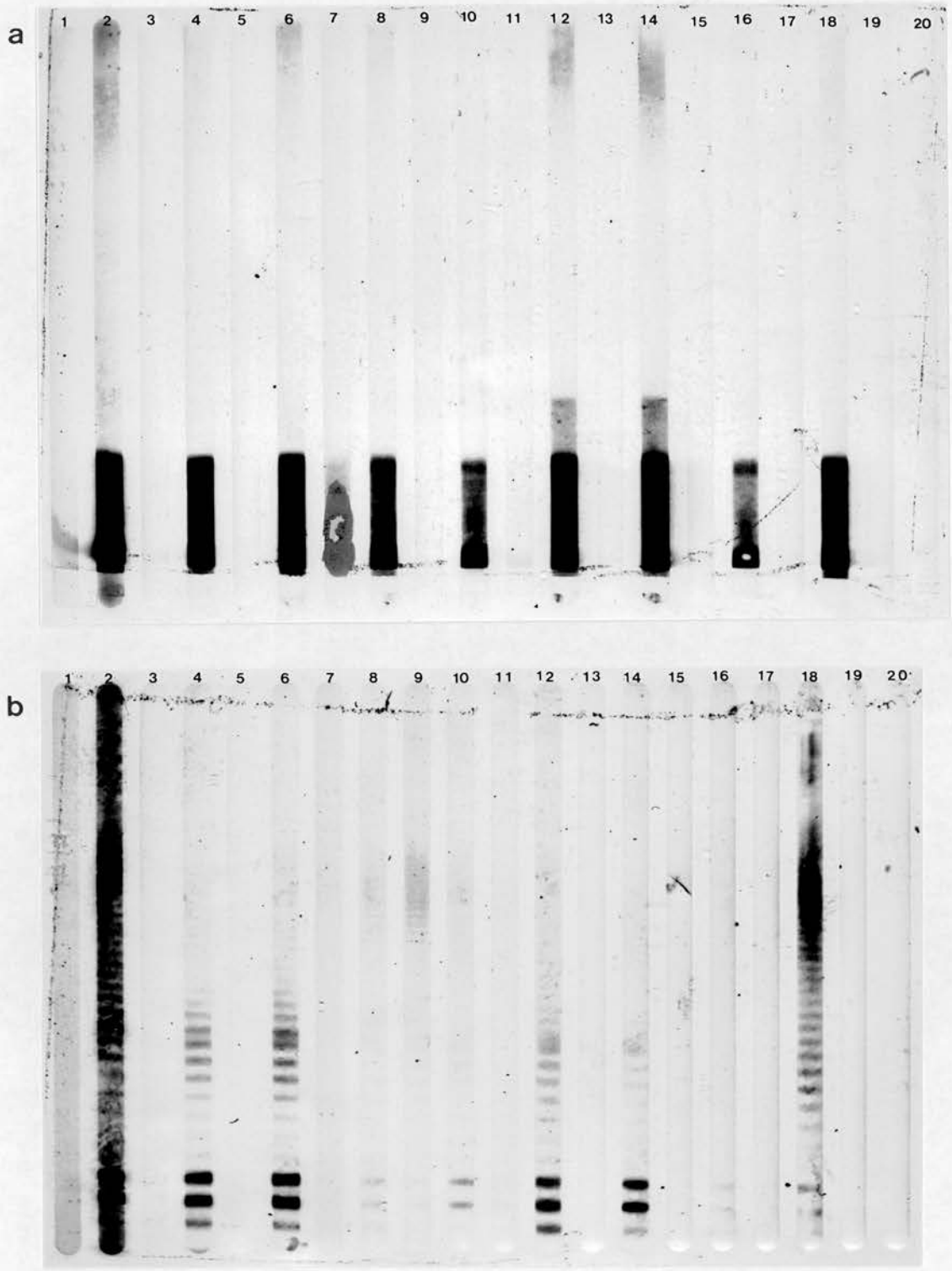


Figure 27. Multiblots of purified R- and S-LPS from (a) the epidemic strain and (b) J2395 separated by SDS-PAGE (12% w/v acrylamide, for the mini-gel) followed by transfer to NIC paper and probed with paired serum (1:200) from 9 ESBc$^+$ CF patients (Tracks 1-18: 'odd' numbers are serum pre-colonisation, 'even' numbers are serum post-colonisation). No pre-colonisation was available for patient IV (Track 7). Patient X (Tracks 19 and 20) was colonised by a non-epidemic strain of *B. cepacia*, C1524.

low level reactivity with the O-side chain portion of the J2395 S-LPS. No reaction was observed with pre-colonisation sera from the other CF patients with either of the LPS preparations indicating that no detectable level of specific IgG anti-*B. cepacia* antibodies were present. An anti-LPS antibody response to the core-LPS of the epidemic strain and to the ladder of the O-side chain of J2395 following onset of chronic colonisation was detected by immunoblot for patients I - IX. Patient X was colonised by a non-epidemic strain of *B. cepacia* C1524; a positive reaction between serum antibodies from this patient and LPS preparations from the epidemic strain and J2395 was not detected. Interestingly, sera from all the ESBc$^+$ CF reacted with LPS from *B. cepacia* J2395, an isolate from a flower vase in a hospital ward near to patient VIII (Tracks 15 and 16; Nelson *et al.*, 1991). Based on phenotypic and genomic fingerprinting, this strain has never been isolated from the sputum of any of the CF patients.

Figure 28 shows the LPS preparations from the epidemic strain and J2395 and also a preparation of S-LPS from a representative strain of the closely related species *B. gladioli* probed with antisera raised in rabbits and with CF sera. Rabbits inoculated with the epidemic strain or J2395 only reacted with LPS antigens of the autologous *B. cepacia* strain. In contrast, serum from CF patient I, colonised only by the epidemic strain, reacted with the core-LPS of the epidemic strain, the O-side chain of J2395 and also with a single band of the *B. gladioli* preparation. All of the ESBc$^+$ CF patients attending the Edinburgh adult CF clinic, and some of those attending the Manchester CF clinic react to the O-side chain of J2395. The reactivity of CF patients' sera with J2395, and the lack of reactivity of the rabbit antisera from the rabbit inoculated with the epidemic strain suggests that 'silent' colonisation rather than the possession of shared antigens is responsible for cross-reactivity. It is possible that these CF patients may have been

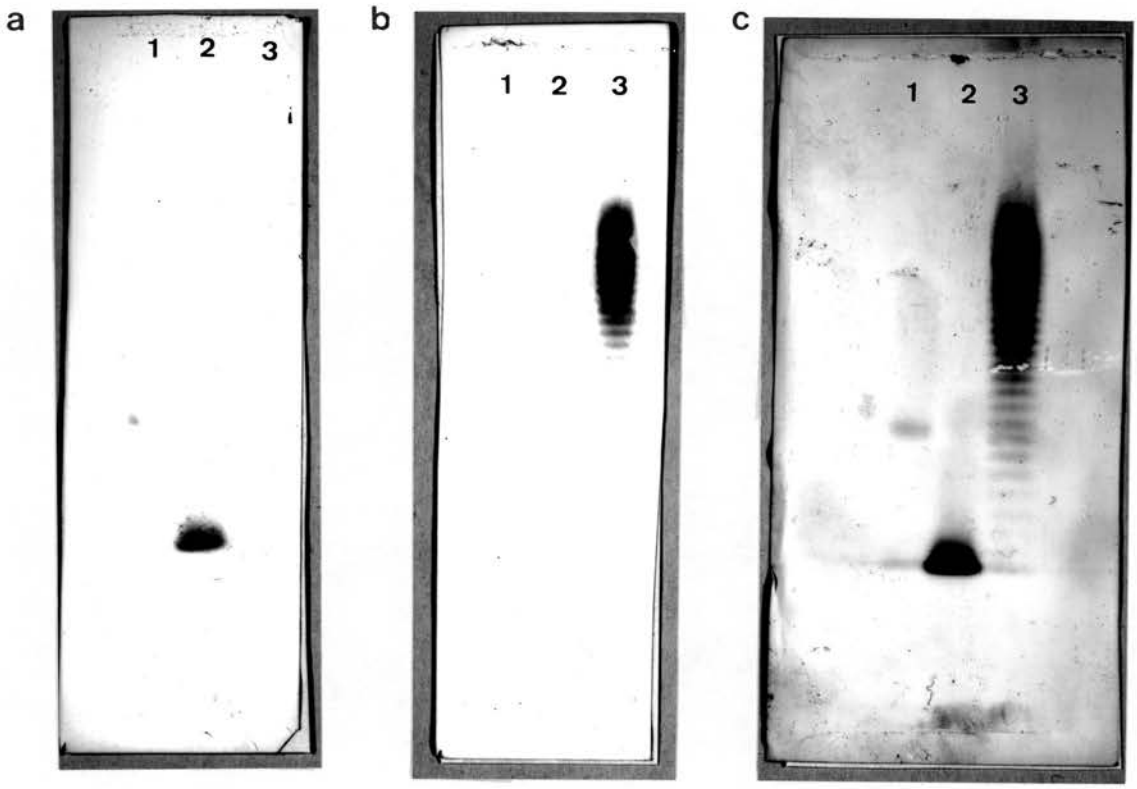


Figure 28. Immunblots of phenol water extracts of *B. gladioli* ATCC 10248 (Track 1); purified R-LPS from the epidemic strain (Track 2) and purified S-LPS from J2395 (Track 3) and separated by SDS-PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with rabbit antisera or CF sera (1:200) from: (a) rabbit inoculated with epidemic strain (C1359); (b) rabbit inoculated with J2395; and (c) $ESBc^{+}$ CF patient I.

transiently colonised by additional strains of *B. cepacia* or that *in vitro* the epidemic strain may not express the same antigens that are expressed *in vivo*.

Serum IgG Response to Proteinase K Digests of Whole Cells

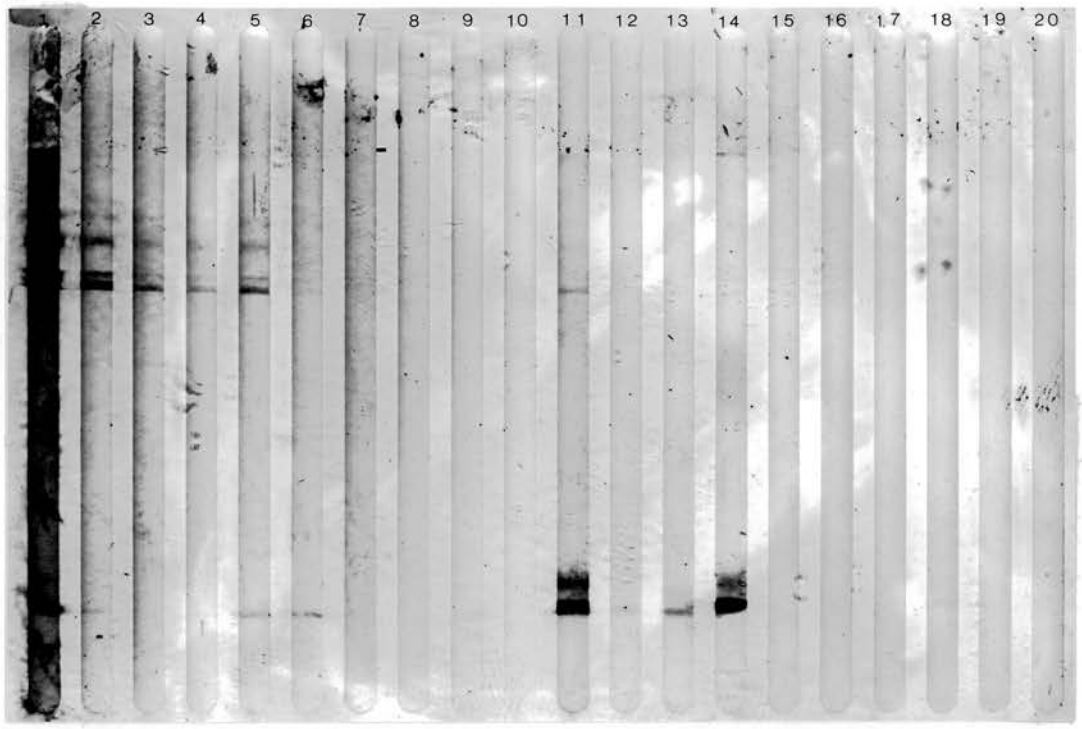
Proteinase K digests of six non-epidemic strains of *B. cepacia* strains were probed with sera from 20 CF patients attending the Edinburgh and Manchester CF clinics, (nine $ESBc^+$,10 Bc^+ and one Bc^-CF patient). Multiblots of the reaction of the CF sera with each *B. cepacia* strain are shown in Figure 29 (a-f). Patients 1-7, 9 and 10 were also analysed in the previous LPS study. Serum from $ESBc^+$ CF patients contained IgG directed against some bands of each Proteinase K digest (Figure 29 a-f; Tracks 1-7, 11 and 18). Sera from CF patients colonised with the non-epidemic *B. cepacia* strains C1495 (Track 12), C1548 (Track 13), and C1454+C1499 (Track 14) reacted similarly. No reaction was observed with sera from CF patients colonised by *B. cepacia* SBC 74 (Track 8), C1524 (Tracks 10), C1559 (Track 15), C1454 (Track 16) and C1449 (Track 19) or from the Bc^- CF patient (Track 20), even when the digest was of the autologous colonising strain. The absence of any reactive bands suggests a lack of serum IgG directed against *B. cepacia* antigens in these patients.

Serum IgG Response to Outer Membrane Preparations

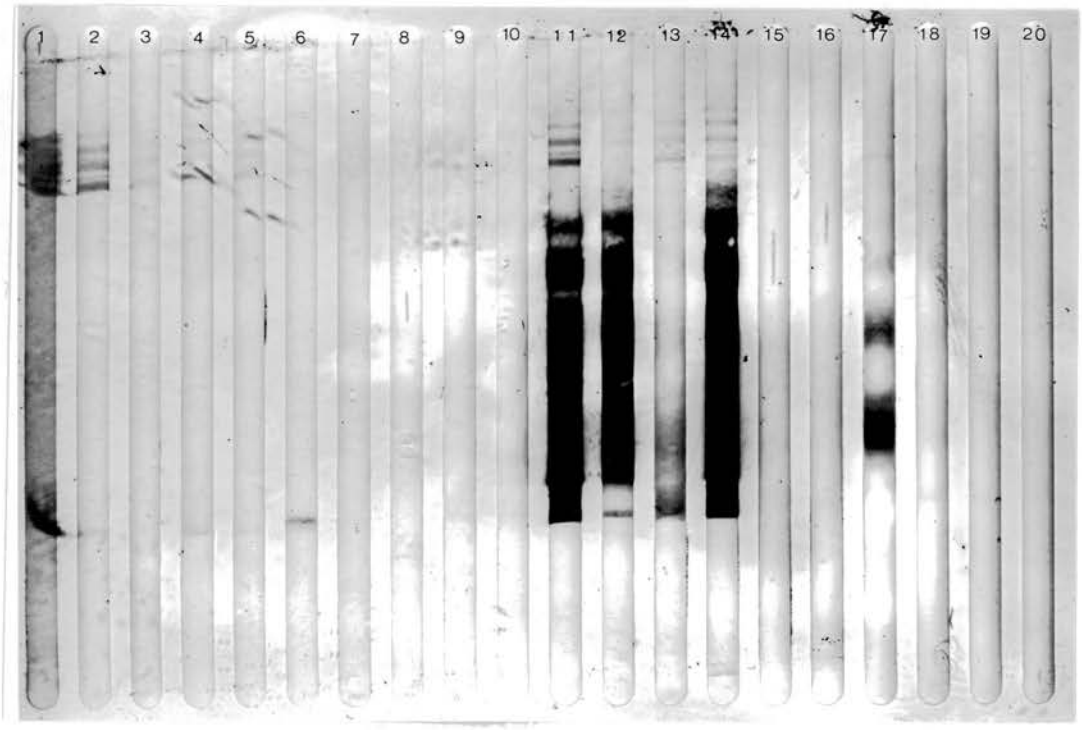
The serum IgG anti-*B. cepacia* antibody response in 13 *B. cepacia* colonised CF patients, five non-colonised CF patients and two non-CF patients to the outer membrane preparations (see Figure 15 for Coomassie stained OMP profile) of five *B. cepacia* strains was analysed by multiblotting (Figure 30a-e). The observations agree with those in the previous Proteinase K study in that sera from CF patients colonised by the epidemic strain (Tracks 1-3, 5-8 and 20) generally reacted more strongly with the outer membrane

Figure 29 (a-f). Multiblots of proteinase K digests of *cepacia* strains: (a) C1409; (b) C1524; (c) C1449; (d) C1499; (e) SBC74; (f) C1454, separated by SDS-PAGE (12% w/v acrylamide) followed by transfer to NIC paper and probed with CF sera (1:200). CF patients were colonised by the following *B. cepacia* strains: Tracks 1-7, 11 and 18 the epidemic strain; Tracks 10,12, 13, 15 and 17 *B. cepacia* strains not used in this study; Track 8, SBC74; Track 9, $ESBc^{+}$+C1409; Track 14, C1454+C1499; Track 16, C1454; Track 19, C1449; and Track 20, Bc^{-}.

c


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

d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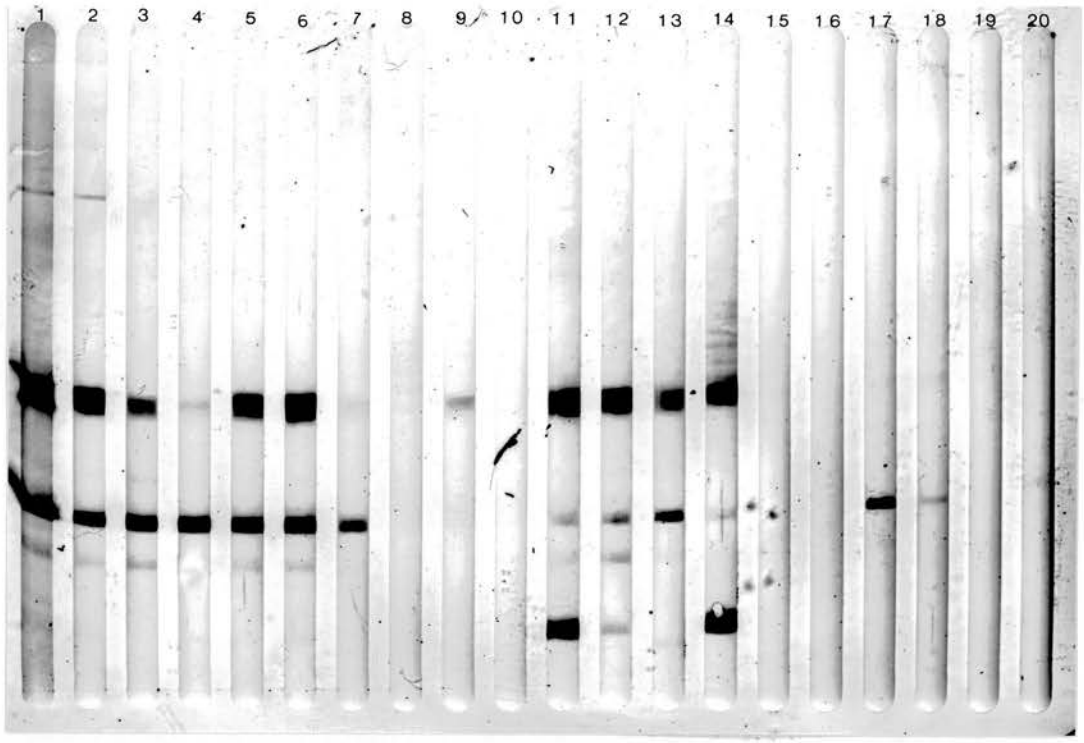
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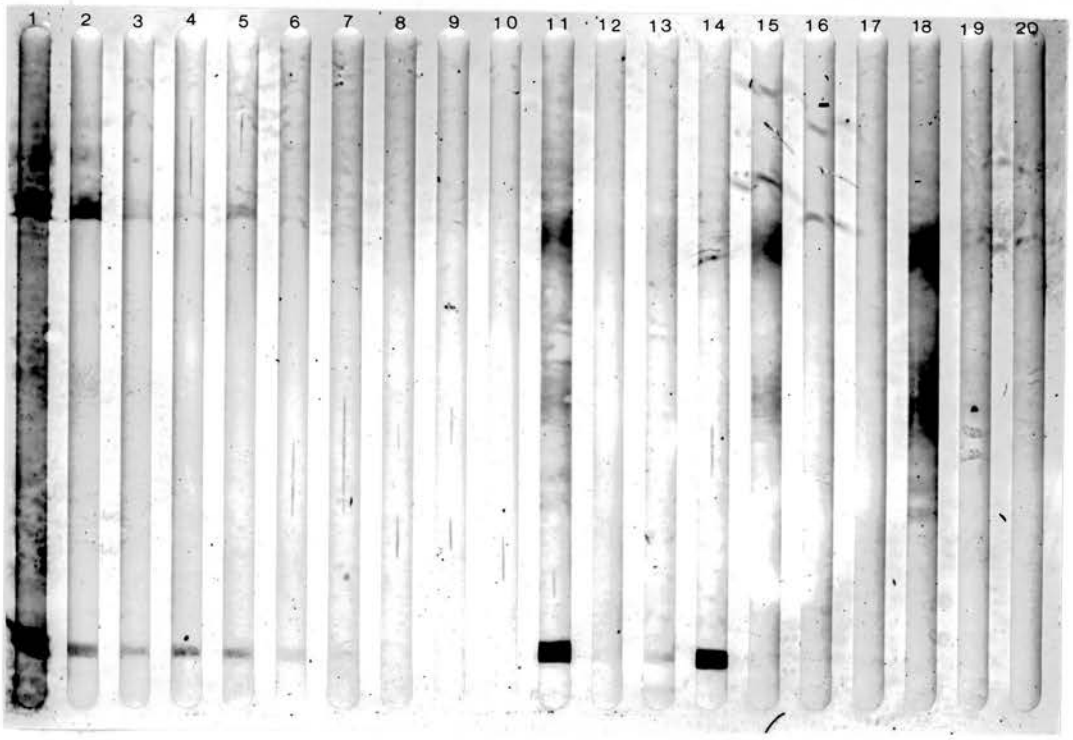
f


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

a

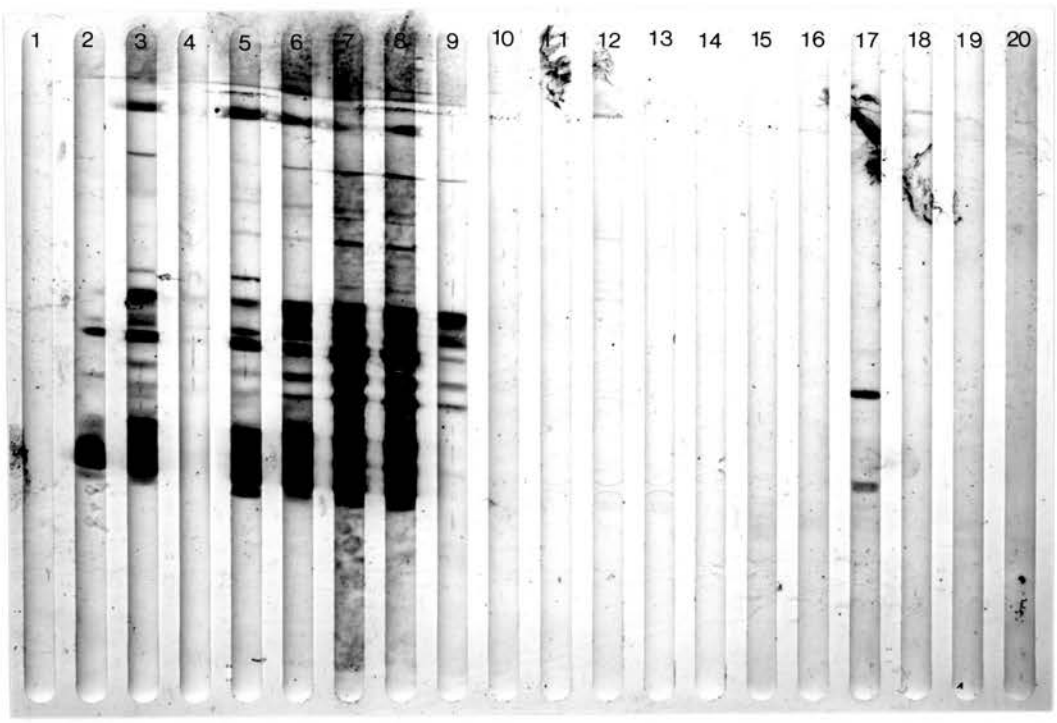
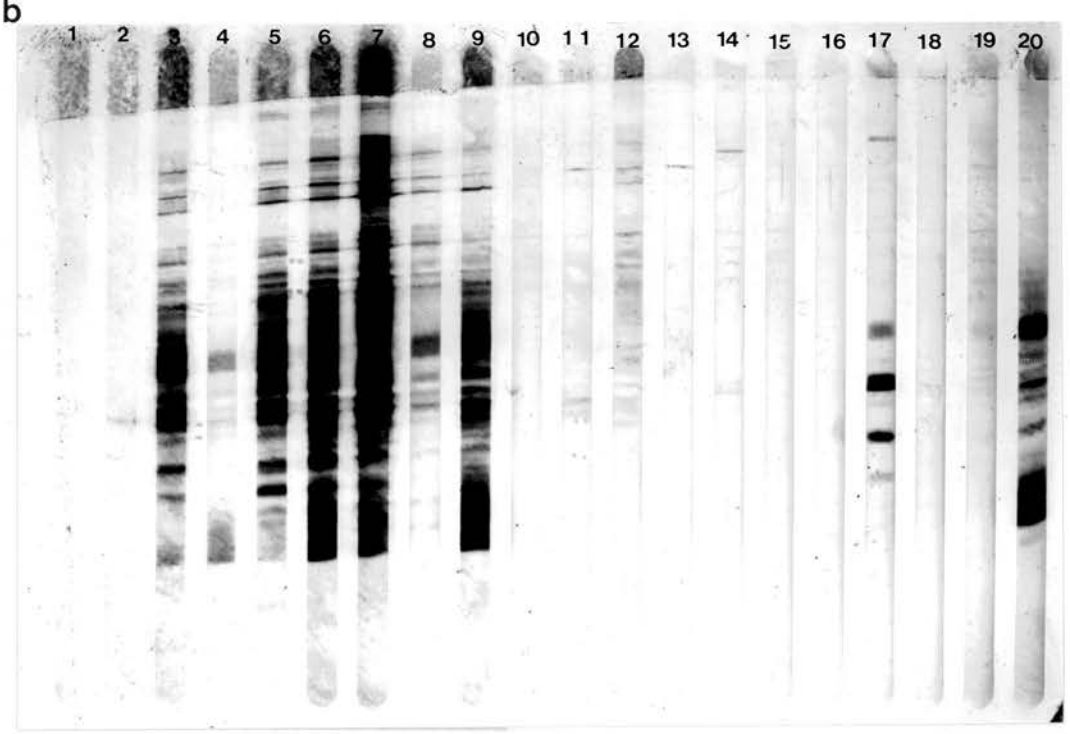


Figure 30 (a-e). Multiblots of outer membrane preparations of *B. cepacia* strains: (a) the epidemic strain; (b) J2395; (c) ATCC 17616; (d) C1409 and (e) C1559, separated by SDS-PAGE (12% w/v acrylamide) followed by tranfer to NIC paper and probed with CF sera (1:200). CF patients were colonised by the following strains of *B. cepacia*: epidemic strain, Tracks 1,2,3,5-8,20; other strains of *B. cepacia*, Tracks 4,9,17,18; C1559, Track 19; Bc^- CF patients, Tracks 1, 10-14 ;and non-CF patients, Tracks 15 &16. Serum from patient 1 not available in Figure 30a, b and d.

b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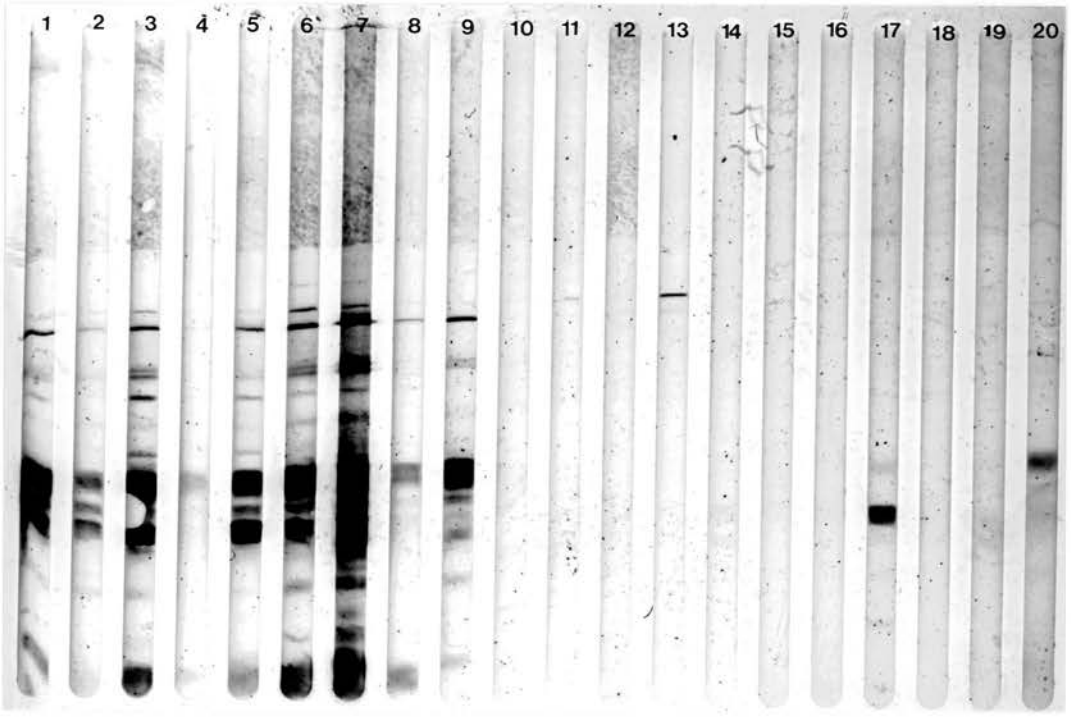
c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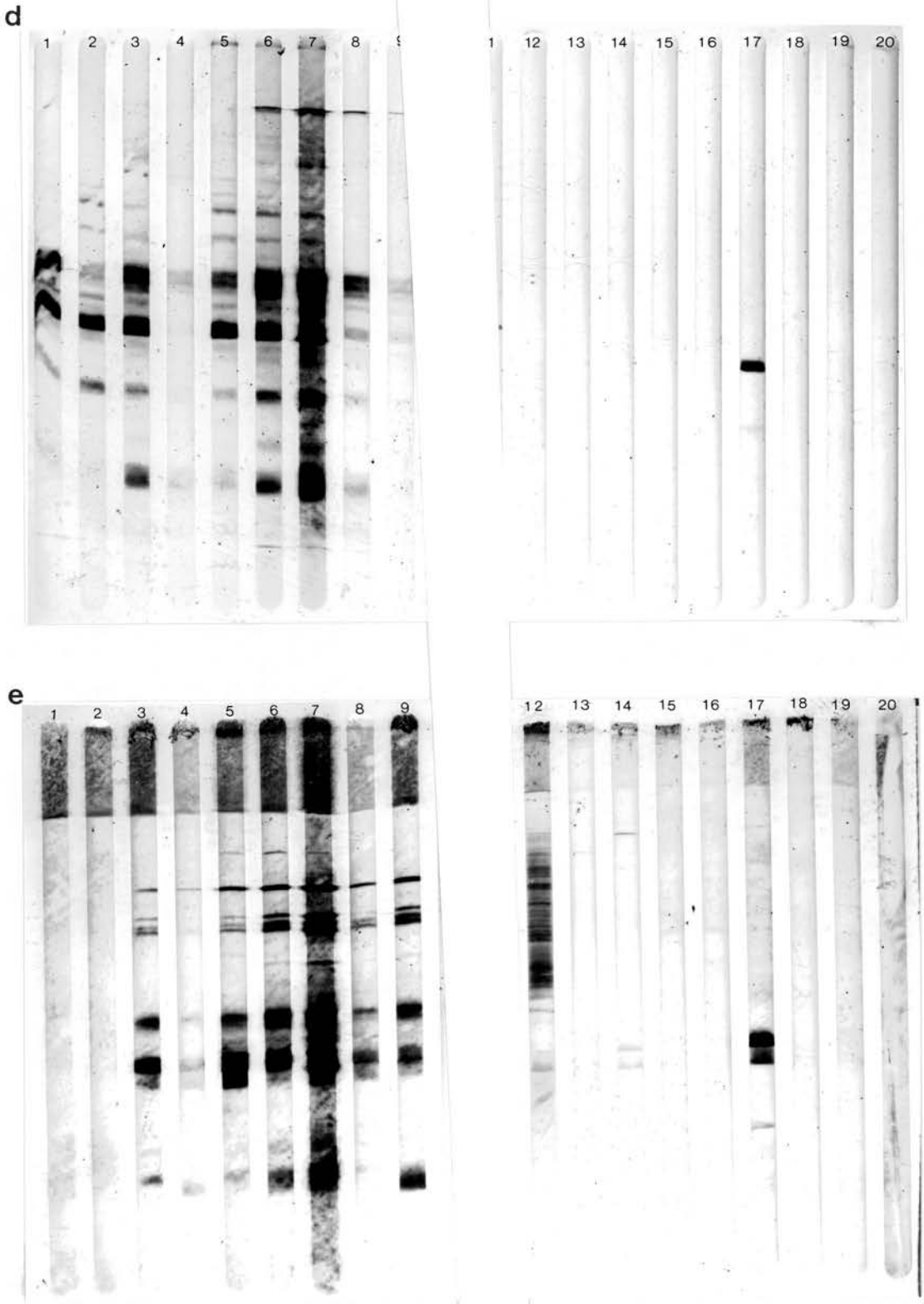
d
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e
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antigens from each strain than sera from CF patients colonised by other strains of *B. cepacia* (Tracks 4, 9, 17-19). The serum from Bc^- CF patients colonised by *P. aeruginosa* (Tracks 10-14) contained antibodies directed against some OMPs of J2395 and C1409 which was in agreement with the earlier studies of Aronoff *et al.* (1991). CF patients colonised by C1559 and C1454 do not react with OMP bands of any of the *B. cepacia* strains studied, even the homologous strain.

Serum IgG Response to *B. cepacia* Flagella Preparations

Serum IgG responses to flagella preparations of three *B. cepacia* strains were analysed by multiblotting (Figure 31a & b) in 17 $ESBc^+$ CF patients, including two patients colonised by additional *B. cepacia* strains, and three Bc^+ CF patients. Although the multiblots demonstrated that the flagella preparations were crude, the heterogeneity of the serum IgG response between *B. cepacia* colonised CF patients was striking, even in those patients colonised by the 'identical' strain, may also be observed. Figures 31a & b show the serum IgG response to flagella preparations of J2395 and C1524. Serum from patients colonised by the epidemic strain (Tracks 1-9,11,13,15-20) with the exception of patients 13 and 19, reacted with both flagellar preparations, although the strength of the reaction and the bands against which the serum reacted varied. Serum from patient 10 (Track 10), colonised by C1524, responded to antigens of the autologous colonising strain but to different bands than sera from the other CF patients. Serum from patient 12 (Track 12) responded strongly to low molecular weight bands of J2395 and weakly to two bands of C1524. In the previous investigations, serum from patients 10 and 12 failed to react with antigenic preparations from the epidemic strain. No antibody response was detected by immunoblotting against any of the flagellar antigens in this study for Patient 14 (Track 14). Multiblots against flagella preparations of the epidemic strain showed an

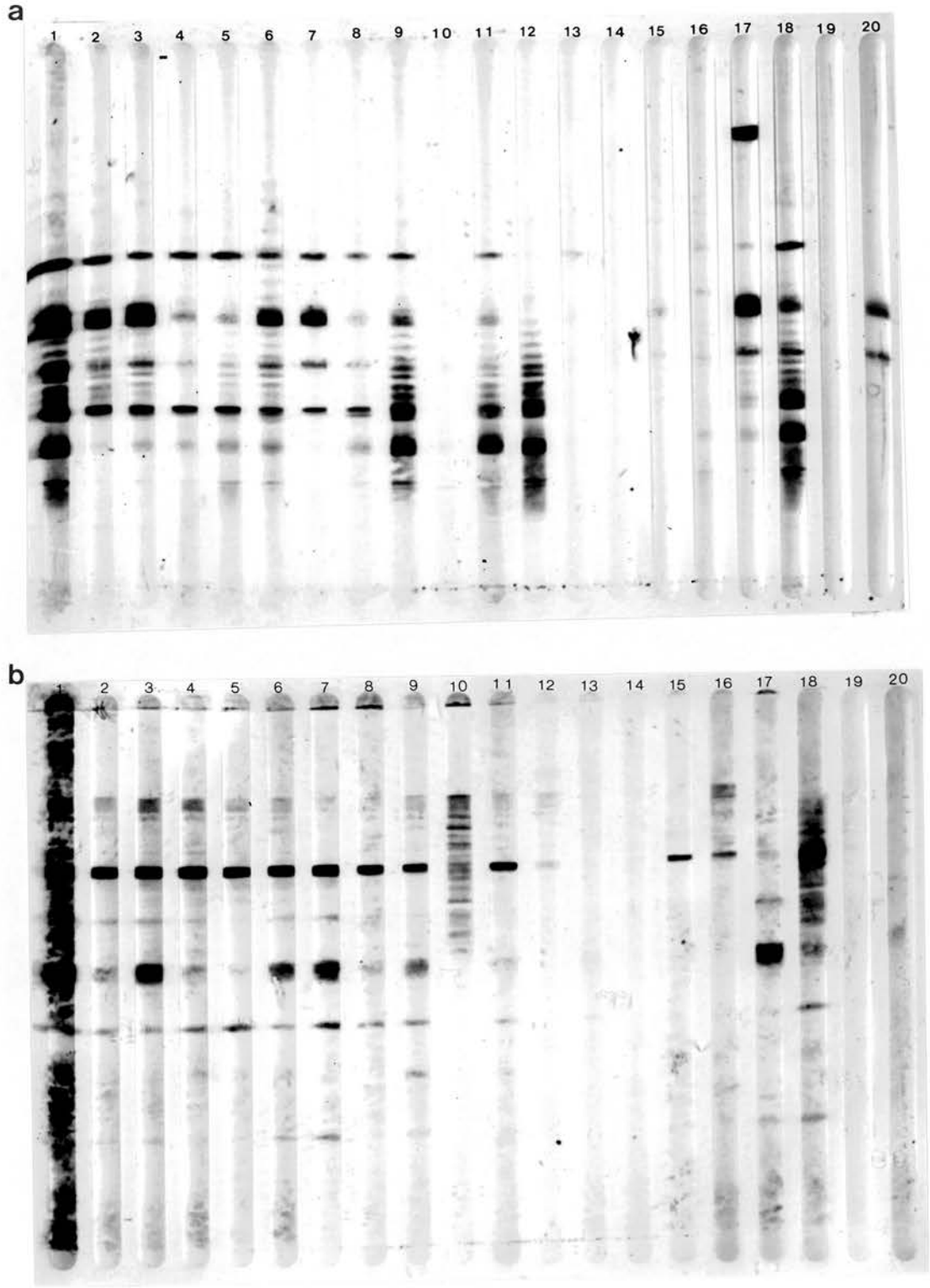


Figure 31. Multiblots of flagellar preparation of *B. cepacia* strains: (a) J2395 and (b) C1524, separated by SDS-PAGE (12% w/v acrylamide) followed by transfer to NIC paper and probed with CF sera (1:200). CF patients were colonised with the following strains of *B. cepacia*: the epidemic strain, Tracks 1-8, 11, 15-20; the epidemic strain+C1409, Track 9; C1524 (Track10); C1449 (Track 12); the epidemic strain+C1506; Track 13; and C1454 (Track 14).

antibody response only in sera from patients colonised by the epidemic strain (data not shown), suggesting that the epidemic strain flagella may be antigenically distinct from other *B. cepacia* flagella.

5.6 Serum IgG Subclasses in *B. cepacia* Colonised CF Patients

The serum IgG subclass response to *B. cepacia* core R-LPS was investigated in CF patients to clarify the nature of the immunoglobulin response in *B. cepacia* colonised CF patients.

Total Serum IgG Subclass Concentration

Preliminary analysis was performed by RID with selected sera to confirm that the total serum IgG subclass concentrations in $ESBc^+$ and Bc^+ CF patients were 'normal' and that there were no previously undetected subclass deficiencies which might predispose this group of CF patients to *B. cepacia* colonisation. Sera from 19 CF patients (nine $ESBc^+$, seven Bc^+, three Bc^-/Pa^+) and one Bc^-/Pa^- chronic bronchitic patient were analysed. The serum concentrations for the IgG subclasses and normal ranges are shown in Figure 32. No subclass deficiency was observed for any of the patients investigated, however a wide range of concentration was obtained for each of the IgG subclasses. The serum IgG subclass concentrations from the majority of the patients were in excess of the normal ranges indicated. Serum concentrations of IgG1 and IgG3 were significantly higher in $ESBc^+$ CF patients than in those Bc^+ CF patients ($p<0.1$ and $p<0.05$ respectively, Students *t*-test).

Increased concentrations of total serum IgG appeared, in some patients to correlate with poor pulmonary status: for example, two $ESBc^+$ CF patients (patients 1 and 2, Figure

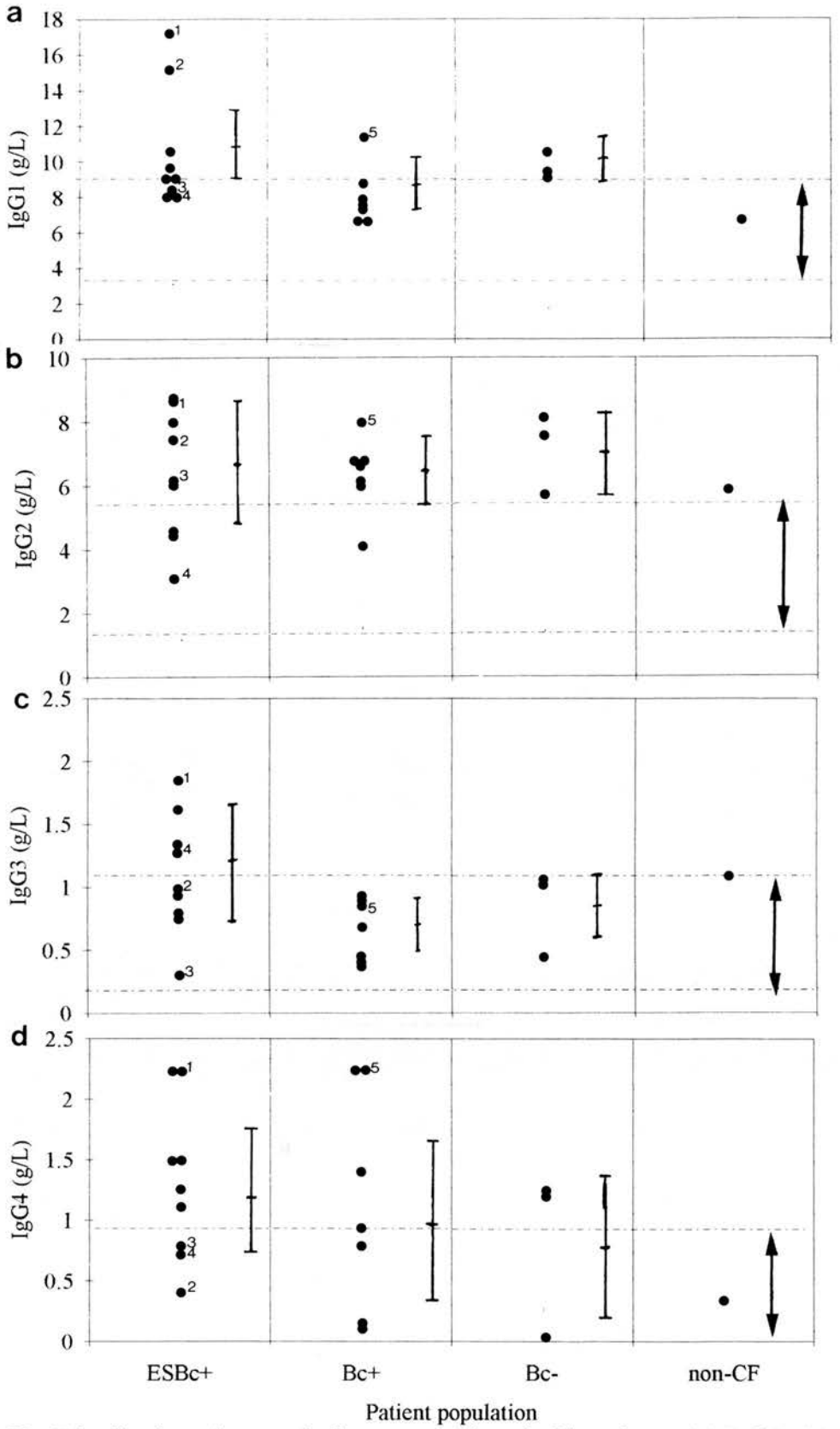


Figure 32. Distribution of serum IgG concentrations in 20 patients: (a) IgG1; (b) IgG2; (c) IgG3; (d) IgG4. Vertical bar represents mean +/- 1SD. Normal values are indicated. Patient populations are as follows: $ESBc^+$, CF patients colonised by the epidemic strain of *B. cepacia*; BC^+, CF patients colonised by other strains of *B. cepacia*; Bc^-, CF patients colonised by *P. aeruginosa* only; the non-CF patient was not colonised by either *B. cepacia* or *P. aeruginosa*.

32) had increased concentrations of all (patient 1) or some (patient 2) of the subclasses. Patient I is chronically unwell and is awaiting heart-lung transplantation; patient II died as a result of overwhelming *B. cepacia* infection shortly after this serum was obtained. In contrast, patients 3 and 4, also $ESBc^+$, have serum concentrations of all the IgG subclasses near or within normal ranges and are relatively well. Patient 5 had increased concentrations of serum IgG1, 2 and 4, however in previous antibody studies no antibody response against any of the *B. cepacia* antigens was observed and in the following study no specific anti-*B. cepacia* LPS IgG subclass antibodies were detected.

Detection of Serum IgG Subclass Anti-*B. cepacia* R-LPS Antibodies

A modified ELISA, incorporating a streptavidin-biotin conjugate system, was used to measure the serum IgG and IgG subclass anti-*B. cepacia* R-LPS antibodies in 13 $ESBc^+$ CF patients (including two patients colonised by additional strains of *B. cepacia*), eight Bc^+ CF patient and 20 Bc^- CF patients.

Figure 33 shows the total anti-*B. cepacia* core LPS IgG antibody titres and the titres for IgG1-3 in $ESBc^+$ and Bc^+ CF patients. The patients numbered 1-5 correspond to the patients numbered 1-5 in Figure 32. The range in antibody titres for the $ESBc^+$ CF patients was: total IgG, 200-25600, median 6400; IgG1, 25-6400, median 400; IgG2, 50-12800, median 3200 and IgG3, 50-6400, median 1600. The range in antibody titres for Bc^+ CF patients was: total IgG, 200-6400, median 400; IgG1, 50-800, median 50; IgG2, 50-800, median 50 and IgG3, 25-800, median 50. No anti-*B. cepacia* core-LPS specific IgG4 was detected in any of the patients; although serum concentrations of IgG4 were elevated in many of these patients. In $ESBc^+$ CF patients and two of the Bc^+ CF patients colonised by two different *B. cepacia* strains, the highest antibody titres were observed

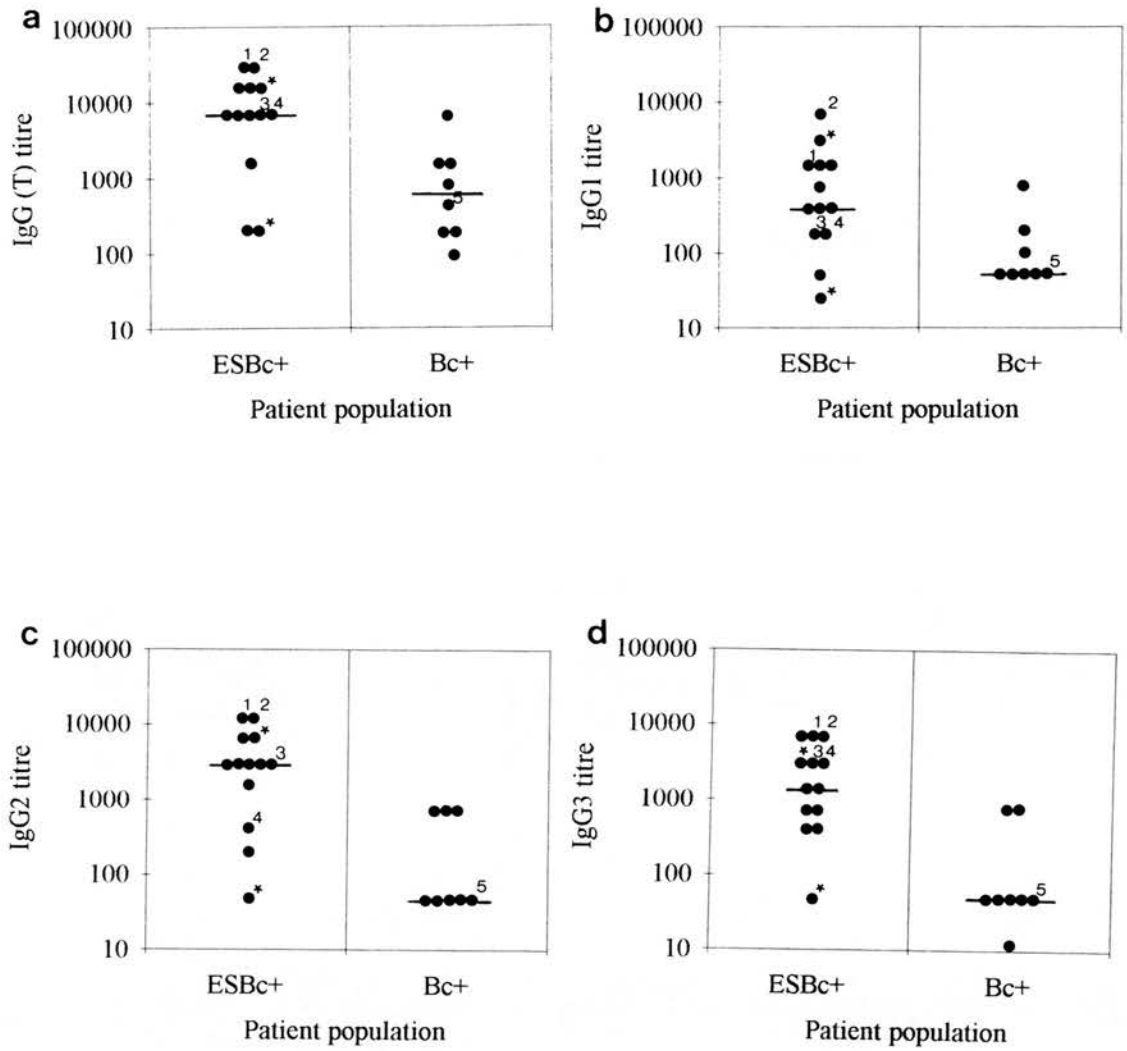


Figure 33. Anti-*B. cepacia* LPS IgG subclass antibody titres in ESBc$^+$ and Bc+ CF patients detected by ELISA with R-LPS of the epidemic strain as coating antigen. (a) anti-*B. cepacia* IgG; (b) anti-*B. cepacia* IgG1; (c) anti-*B. cepacia* IgG2; (d) anti-*B. cepacia* IgG3. Data for IgG4 not shown as all anti-*B. cepacia* LPS IgG4 antibody titres were <25. Bar represents median value. * indicates CF patients colonised by two strains of *B. cepacia*.

for IgG2 and IgG3 (median titres for ESBc+ patients 3200 and 1600 respectively). For most of the CF patients colonised by non-epidemic strains of *B. cepacia* and non-colonised controls (data not shown) there was no detectable levels of the IgG subclasses.

These studies are not conclusive but provide an interesting insight into pathogenicity of *B. cepacia* and CF for example; a high level of specific IgG2 directed against *B. cepacia* antigens may be important in preventing clearance of *B. cepacia* by pulmonary macrophages as this subclass of IgG is a poor opsonin.

5.7 Opsonic Potential of Serum from CF Patients Colonised by *B. cepacia*

Following the observation of the pronounced immunoglobulin response to *B. cepacia* colonisation the next step was to investigate the ability of serum from CF patients colonised by *B. cepacia* to promote or inhibit opsonic phagocytosis of *B. cepacia*. A chemiluminescent opsonophagocytosis assay was performed in 96 well microtitre plate which enabled large numbers of samples to be investigated simultaneously whilst requiring minimal numbers of phagocytic cells. Phagocytic activity was analysed by measuring the luminol (which is linked to the myeloperoxidase-H_2O_2 system) enhanced chemiluminescence which results from the oxidative burst induced by the phagocytosis of bacteria.

In the first instance, preliminary experiments were performed to determine the optimum concentrations of phagocytic cells, bacteria and serum. Phagocytic cells were obtained from human buffy coat and were predominately monocytes. A monocytic cell concentration of 2.0×10^7 cells/ml (1.0×10^6 cells/well) was found to give optimal chemiluminescence without giving a double peak or interfering with chemiluminescence.

The optimal bacterial concentration was $1.0x10^9$ cfu/ml ($5.0x10^7$ cfu/well), resulting in a ratio of phagocytic cells to bacteria in each well of 1:50. Undiluted serum was found to give the most reproducible results.

The characteristics and magnitude of the oxidative burst were strain and serum dependent. Figure 34 shows the chemiluminescent output of phagocytes incubated with Zymosan A or with the five bacterial strains (nonopsonic phagocytosis) measured at 5 min intervals over a period of 90 min. Results are the mean of four experiments. The greatest peak was observed with *P. aeruginosa* PAO1 (mean peak CL=117.2; sd+/-20.8), which was larger than the peak chemiluminescence observed for the Zymosan positive control (mean peak CL=78.0; sd+/-22.8). The magnitude of the oxidative burst induced by the three *B. cepacia* strains and *S. aureus* was much lower than that for PAO1 or Zymosan: C1359 mean peak CL= 30.6 sd+/-10.4; C1559 mean peak CL=7.0 sd+/-3.5; J2395 mean peak CL=18.4 sd+/-5.9; *S. aureus* mean peak CL=13.0 sd+/-7.0. The time at which peak chemiluminescence occurred ranged from 15 min to 35 min, chemiluminescent output was then observed to diminish at a varying rate until baseline levels were reached.

The assay was repeated following preincubation of bacteria for 90 min at room temperature with sera from 10 CF patients; four colonised by the epidemic strain; four colonised by other strains of *B. cepacia* and two colonised by *P. aeruginosa* only. Figures 35a - e show the chemiluminescence measured in phagocytic cells incubated with bacteria and serum as a percentage of the chemiluminescence resulting from nonopsonic phagocytosis of bacteria. The oxidative burst was clearly reduced in the presence of any serum for both PAO1 and C1359 (epidemic strain) (Figures 35a & b). In contrast,

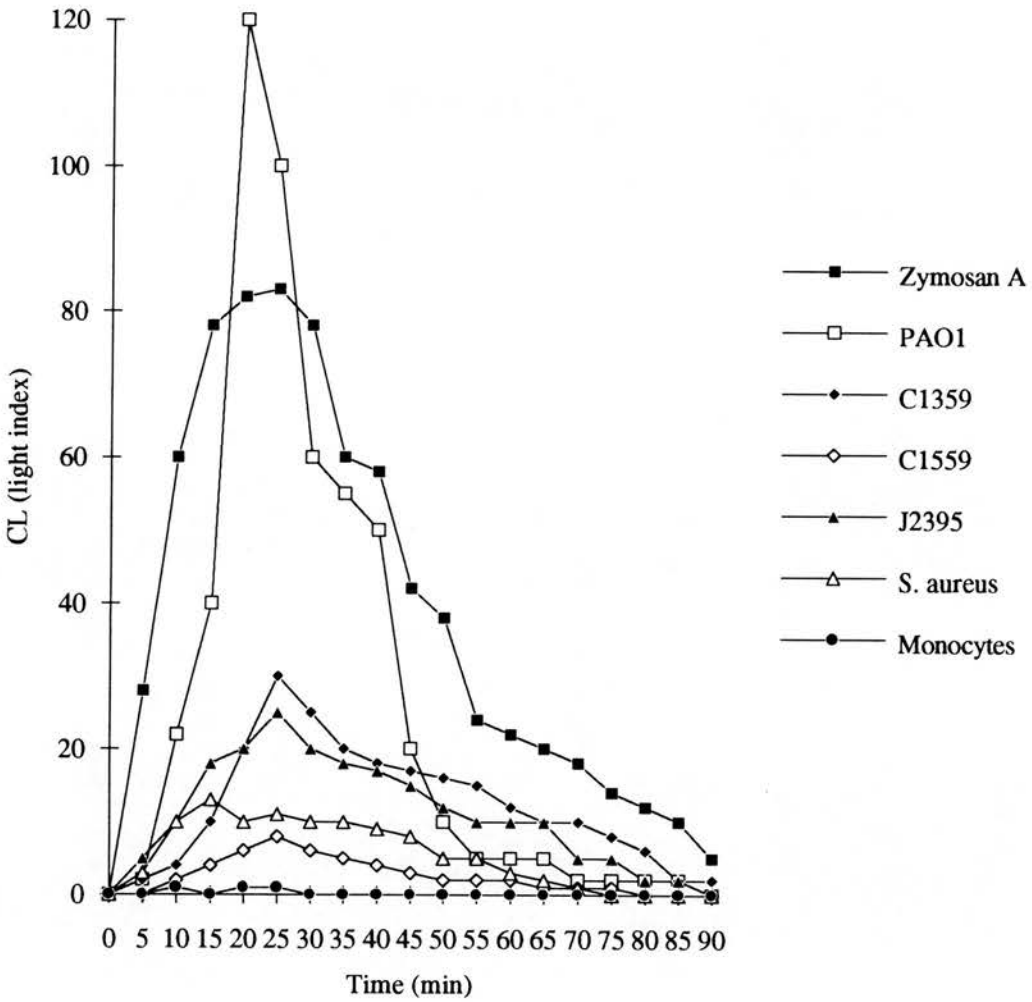


Figure 34. Nonopsonic phagocytosis of bacterial cells by monocytes measured by luminol enhanced chemiluminescence.

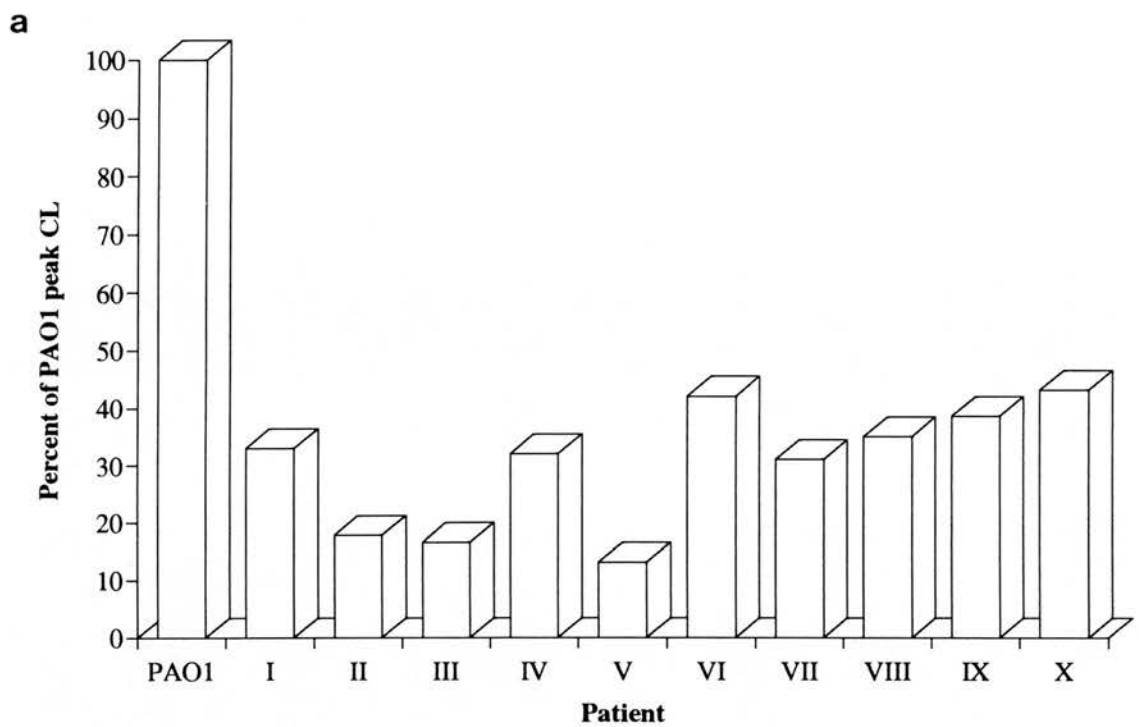
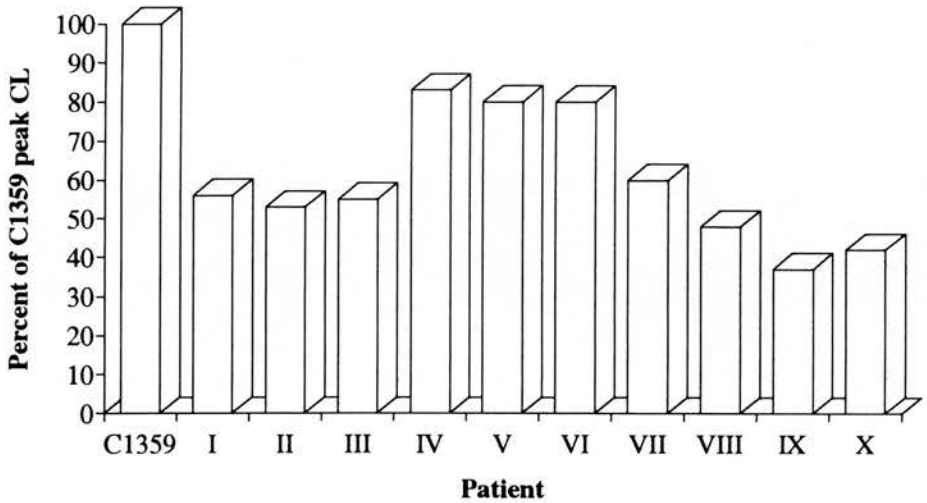
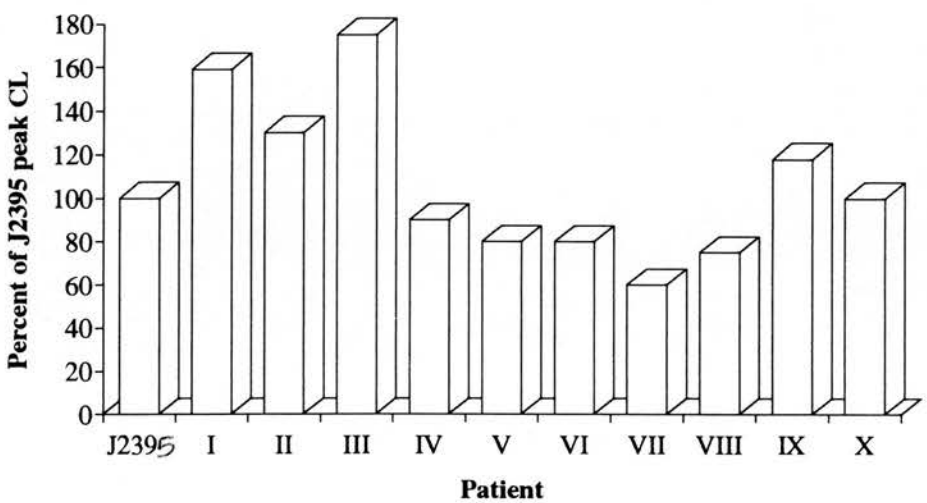


Figure 35 (a-e). Effect of preincubation of bacteria with serum on oxidative burst in monocytes. Results are peak CL expressed as a percentage of bacterial strain alone: (a) PAO1; (b) epidemic strain; (c) J2395; (d) C1559 and (e) *S. aureus*. CF patients colonised by the following: Patients I - IV, the epidemic strain; patients V-VII and X non-epidemic strains; patients VIII & IX, Bc^{-}.

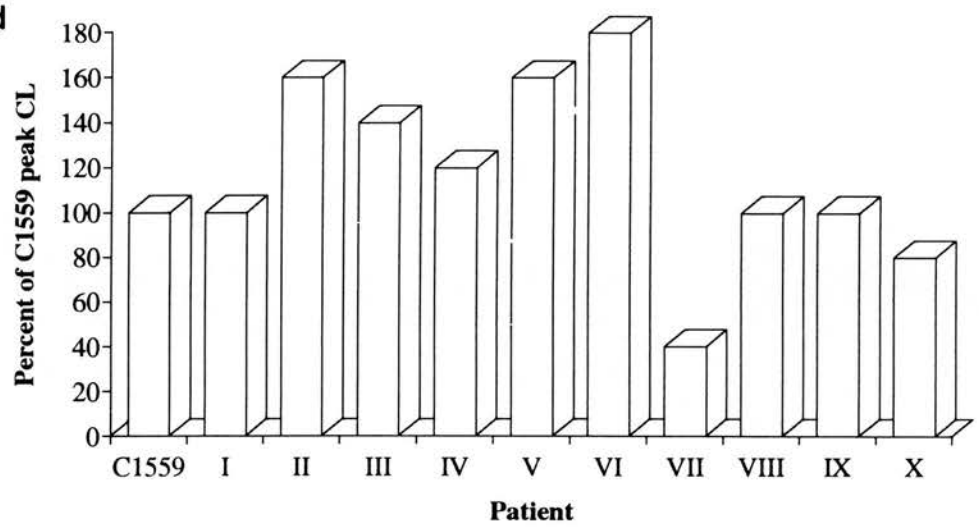
b


Percent of C1359 peak CL
100
90
80
70
60
50
40
30
20
10
0
C1359
I
II
III
IV
V
VI
VII
VIII
IX
X
Patient

c


Percent of J2395 peak CL
180
160
140
120
100
80
60
40
20
0
J2395
I
II
III
IV
V
VI
VII
VIII
IX
X
Patient

d


Percent of C1559 peak CL
180
160
140
120
100
80
60
40
20
0
C1559
I
II
III
IV
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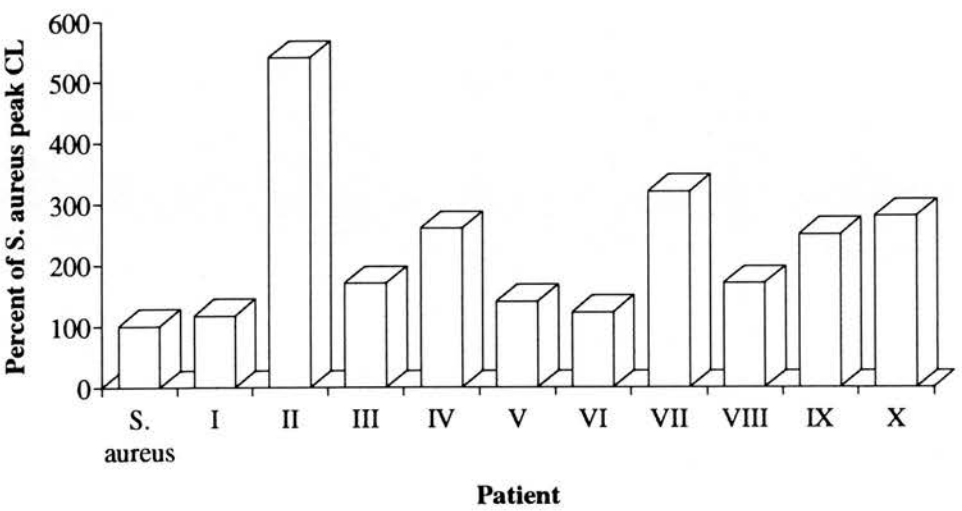
VI
VII
VIII
IX
X
Patient

e


Percent of S. aureus peak CL
600
500
400
300
200
100
0
S. aureus
I
II
III
IV
V
VI
VII
VIII
IX
X
Patient

chemiluminescence was increased for the remaining *B. cepacia* strains (C1559 and J2395) and also for *S. aureus* (Figures 35c-e). The degree of inhibition or promotion of the oxidative burst was highly variable. There did not appear to be any correlation between the bacterial status of the CF patient (with respect to colonisation by *B. cepacia*) and the effect of the serum on the opsonophagocytosis. However the fact that opsonophagocytosis of the epidemic strain was reduced in the presence of CF sera may be of importance when considering the persistence of this organism.

CHAPTER 6

BACTERICIDAL ACTIVITY OF NORMAL HUMAN SERUM AGAINST *BURKHOLDERIA CEPACIA*

Traditionally, bacteria expressing R-LPS are considered to be serum-sensitive and are not associated with bacteraemia. Approximately 20% of CF patients colonised by *B. cepacia* succumb to '*Cepacia* Syndrome', the rapid fatal decline in pulmonary function which may be accompanied by *B. cepacia* bacteraemia. The aim of this study was to investigate the bactericidal activity of normal human serum against strains of *B. cepacia*, particularly the epidemic strain which has been shown to express R-LPS yet has been associated with bacteraemia in CF patients.

6.1 Determination of Complement Activity

Serum obtained from two normal healthy volunteers and three CF patients colonised by the epidemic strain of *B. cepacia* was examined by sheep erythrocyte lysis assay to determine the activity of serum complement. Each of the sera tested had levels of haemolytic complement within the acceptable range (20 - 50 CH_{50} units).

6.2 Serum Killing of *B. cepacia*

The sensitivity of 19 *B. cepacia* isolates from different sources, expressing both R- and S-LPS phenotypes, to the bactericidal activity of normal human serum was investigated. PFGE and bacteriocin typing demonstrated that there was no clonal relationship amongst the *B. cepacia* isolates used in this study with the exception of C1359 and C1602 which

are representative isolates of the epidemic strain. For each experiment the percent killing was calculated from the number of organisms, as determined by viable count, at T_0 min and those still viable at the end of the experiment. Results were compared with those for the controls (CFTB only and heat inactivated serum: HIS) and expressed as: serum-resistant (SR) = <10% killing of bacteria; intermediate (I) = 10-90% killing; and serum-sensitive (SS) = >90% killing.

Serum killing of *B. cepacia* by pooled normal human serum (PNHS: pooled from two healthy adults) was measured at 90 min and also at 180 min to detect delayed killing (Table 16). After 90 min exposure to serum, 7 (58.3%) of the 12 isolates expressing S-LPS were resistant to serum bactericidal activity, 3 (25%) showed intermediate sensitivity and 2 (16.7%) were serum-sensitive. In contrast, none of the seven *B. cepacia* isolates expressing R-LPS were serum-resistant at 90 min, 4 (57.2%) showed intermediate sensitivity and 3 (42.8%) were serum-sensitive. Following 180 min exposure to PNHS there was a marked increase in the number of serum-sensitive isolates. All of the *B. cepacia* isolates expressing R-LPS were serum-sensitive at 180 min. Of the *B. cepacia* isolates expressing S-LPS seven (58.3%) were serum-sensitive at 180 min, one showed intermediate sensitivity and four (33.3%) remained serum resistant. The two control *E. coli* strains, R3 and 018, included in this study expressed R- and S-LPS respectively. The rough strain was serum-sensitive at 90 min whilst the smooth strain of *E. coli* remained serum-resistant at 180 min.

After 180 min exposure to PNHS, 18 of the *B. cepacia* strains examined fell within the boundaries of the sensitivity classifications described above. Although serum sensitivity was classified as a >90% reduction in the number of colony forming units when

Table 16. Serum killing of *B. cepacia* by 40% PNHS at 90 min and 180 min.

B. cepacia	Source	LPS (R or S)	90 min	180 min
C1518	CF (sputum)	S	SR	SR
C1394	"	R	SS	SS
C1524	"	S	I	SS
C1548	"	R	SS	SS
C1559	"	S	I	SS
C1454	"	R	I	SS
C1409	"	S	I	SS
C1704	"	S	SR	SR
C1359*	"	R	I	SS
C1602*	CF (blood)	R	I	I
ATCC25609	Bronchial wash	S	SS	SS
ATCC25608	Wound	S	SR	SS
ATCC27515	Infected tissue	S	SR	I
ATCC17762	Urine	R	SS	SS
J162	Wound	S	SR	SS
J2395	Flower vase	S	SR	SR
SBC100	Potato	S	SS	SS
ATCC17616	Soil	R	I	SS
J159	Onion	S	SR	SR
E. coli R3	-	R	SS	SS
E. coli 018	-	S	SR	SR

*epidemic strain
SR - serum resistant
SS - serum sensitive

I - intermediate

compared to the viable counts at 0 min, greater than 96% killing or reduction was measured for the majority of serum-sensitive *B. cepacia* isolates with the exception of the epidemic strain isolate C1602 (90% killing). At the other extreme 0-3% killing was measured for the four serum-resistant *B. cepacia* isolates. Finally, a 58% reduction in the viable count was observed for the intermediately sensitive isolate ATCC 27515.

The observation that all the isolates which remained serum-resistant after 180 min exposure to PNHS expressed S-LPS, suggests that S-LPS may contribute to the ability of *B. cepacia* isolates to resist the bactericidal activity of PNHS. Interestingly, the two representative isolates of the epidemic strain expressimg R-LPS, C1359 and C1602 from sputum and blood culture respectively, were serum-sensitive.

The killing curves for three *B. cepacia* isolates J2395 (serum-resistant), C1409 (delayed killing) and the epidemic strain (C1359; serum-sensitive) over 180 min are shown in Figure 36. The figure clearly illustrates the contrast in killing profiles: no decrease in cell numbers was observed with J2395; for C1409 an initial gradual decline in cell numbers up to 90 min was then followed by a steeper decline until 180 min; the epidemic strain was rapidly serum-sensitive by 90 min.

B. cepacia strains expressing S-LPS exhibit a range of responses to the bactericidal activity of PNHS; for example, the distinct killing curves for J2395 and C1409 (Figure 36). The O-side chain portion of the LPS of J2395 showed a greater degree of polysaccharide substitution than the LPS of C1409 (Chapter 3, Figure 14b) and this may contribute to increased the serum resistance observed for J2395.

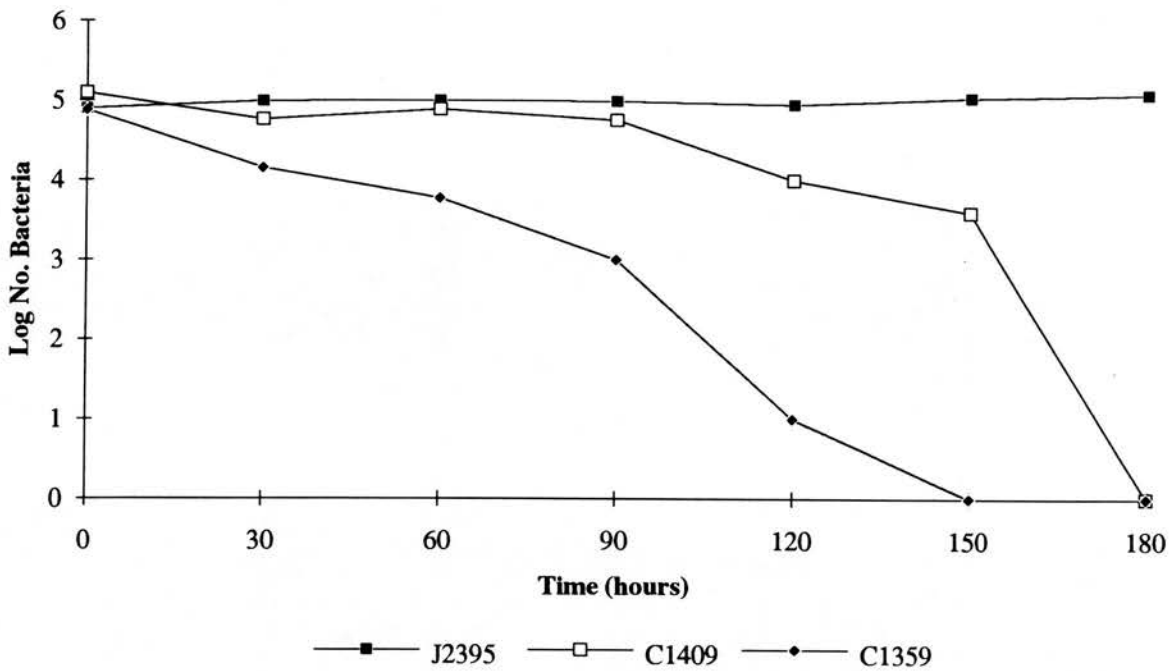


Figure 36. Serum killing of *B. cepacia* strains J2395 (serum-resistant, S-LPS), C1409 (intermediate, S-LPS) and C1359 (epidemic strain; serum-sensitive, R-LPS) by 40% PNHS over 180 min. Each point is the mean of triplicates.

Investigations with other bacteria, for example *Aeromonas* species, indicate that LPS is not the sole determinant of serum resistance in Gram negative bacteria and that other cell surface components such as OMPs and capsules may also be involved (Taylor, 1983). A possible role of OMP in contributing to serum resistance in *B. cepacia* was investigated by examining crude Sarkosyl preparations of serum-resistant, intermediate and serum-sensitive *B. cepacia* strains by PAGE and Coomassie staining (Chapter 3, Figure 15). The three CF isolates, C1359, C1559 and C1409, expressed fewer OMP than the environmental isolates J2395 and ATCC 17616, however as the latter strain was not serum resistant the bands observed presumably have no role in resistance. J2395 expressed a broad band of high molecular weight (150 - 200 kDa) not observed in any of the other *B. cepacia* strains which may contribute to serum resistance in this strain.

6.3 Serum Requirement for Bacterial Killing

The aim of this next series of experiments was to investigate several of the major serum components; for example the two complement pathways and specific serum antibodies, to determine their role in the serum killing of *B. cepacia*.

The majority of experiments were performed with 40% serum, although killing was observed with 10% serum. Serum killing by 10% or 40 % PNHS was proportional to the serum concentration; killing of serum-sensitive and intermediate strains of *B. cepacia* was slower in the presence of 10% serum and complete killing was not always achieved. Two controls were included in each serum sensitivity assay; PNHS was replaced by CFTB or HIS. No bactericidal activity was associated with either of these controls indicating that a serum factor was involved in serum killing and that the factor was heat labile.

Role of Antibody in Serum Killing

To investigate the role of specific anti-*B. cepacia* antibodies in serum killing, sera were obtained from three CF patients of variable clinical status chronically colonised by the epidemic strain of *B. cepacia*. In contrast to PNHS which contained negligible amounts of anti-*B. cepacia* antibodies (mean antibody titre, 400), the hyperimmune CF sera contained significant amounts of specific anti-*B. cepacia* antibodies. At the time serum was obtained the general clinical status and antibody titre of each CF patient was: (a) Patient I was chronically unwell with an anti-*B. cepacia* LPS IgG antibody titre of 102400; (b) Patient II was 'well' with an anti-*B. cepacia* LPS IgG antibody titre 51200; and (c) Patient III was experiencing acute pulmonary exacerbation also with an anti-*B. cepacia* LPS IgG antibody titre 51200.

The bactericidal activity of PNHS and the CF serum against four strains of *B. cepacia* (J2395, C1548, the epidemic strain (C1359) and ATCC 17616) is shown in Figure 37. The serum from two of the CF patients (Patients I and II) were comparable in their killing activity against the three heterologous strains of *B. cepacia* (C1548, J2395 and ATCC 17616) and also the epidemic strain. Serum from the third CF patient, however, whilst equally active against heterologous strains of *B. cepacia*, was much less active against the epidemic strain of *B. cepacia*.

Each CF sera was then subjected to a series of four absorptions against whole cells of each of the *B. cepacia* strains in order to specifically remove anti-*B. cepacia* antibodies and the serum sensitivity assay was repeated with the absorbed serum. No difference was observed in the bactericidal potential of any of the CF sera. A belated ELISA with the absorbed sera and whole cells as coating antigen showed unexpectedly that the

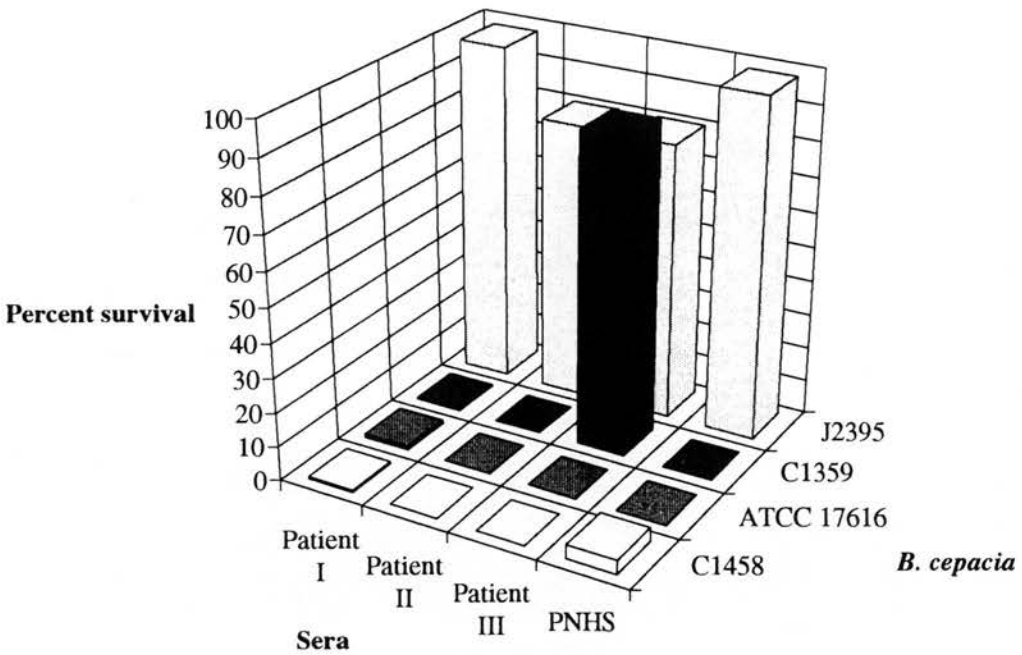


Figure 37. Serum killing of 4 *B. cepacia* isolates the epidemic strain, C1548, J2395 and ATCC 17616 by 40% PNHS and 40% CF serum. CF Patients I-III were chronically colonised by the epidemic strain of *B. cepacia*, represented by C1359. Serum from patient III was obtained during an acute pulmonary exacerbation.

absorption process had not resulted in a significant decrease in the anti-*B. cepacia* antibody titre. It was not possible to repeat the absorptions as the serum sensitivity assays had used all the CF sera available from patients I - III. Attempts to obtain more serum from the same patients or others in similar clinical condition were unsuccessful.

Role of the Complement Pathways

To determine whether the classical complement pathway (CCP) or the alternative complement pathway (ACP), was more important in the complement mediated killing of serum-sensitive *B. cepacia,* serum killing was investigated in eight isolates which were incubated with 40% PNHS containing magnesium EGTA (in which only the ACP is functional). Table 17 shows serum killing of these strains in the presence of 40% HIS, 40% PNHS and 40% PNHS+10mM $MgCl_2$ EGTA.

Decreased cell numbers in the presence of HIS was observed to a minor extent with C1548 and more so for ATCC 17616 but not at all for the remaining six *B. cepacia* isolates. With the exception of the serum-resistant *B. cepacia* isolate J2395, serum killing of all of the *B. cepacia* strains was reduced in the presence of $MgCl_2$ EGTA, although the extent of this was strain dependent. The most marked reductions in serum killing were observed with the two representative isolates of the epidemic strain, C1602 and C1359, and for environmental isolate ATCC 17616. Each of these *B. cepacia* isolates express R-LPS. The results obtained in this study suggest that the CCP is the complement pathway primarily involved in serum killing of *B. cepacia.*

Table 17. Serum killing of eight *B. cepacia* strains by 40% PNHS, 40% PNHS+10mM $MgCl_2$ EGTA and HIS at 180 min. Serum killing was calculated as the percent decline in cell numbers between 0 and 180 min.

Strain	Killing of *B. cepacia* strains (%)		
	HIS	40% PNHS	40% PNHS+ 10mM $MgCl_2$ EGTA
C1602	0	91	32
C1359	0	96	32
C1548	5	97	87
C1409	0	72	37
ATCC 17762	0	100	47
ATCC 17616	17	98	18
C1518	0	4	0
J2395	0	0	0

HIS - heat inactivated serum
PNHS - pooled normal human serum

6.4 Effect of Culture Media on Serum Sensitivity of *B. cepacia*

To investigate the effect of the culture media on the serum sensitivity of *B. cepacia* isolates were cultured overnight at 37°C in either 50% v/v heat inactivated sheep serum (HISS) or minimal media (MM). *B. cepacia* isolates were then exposed to PNHS in the same manner as previously described and the results compared with those obtained following culture in isosensitest broth (ISTB). Figure 38 shows the percent survival observed for nine *B. cepacia* isolates following culture in ISTB, HISS or MM.

The effect of culture media on serum sensitivity of *B. cepacia* was observed to be strain dependent. Five of the nine *B. cepacia* strains investigated were more resistant to the bactericidal activity of PNHS following culture in HISS whilst culture in ISTB or HISS appeared to make no difference to the serum resistance or sensitivity of three of the

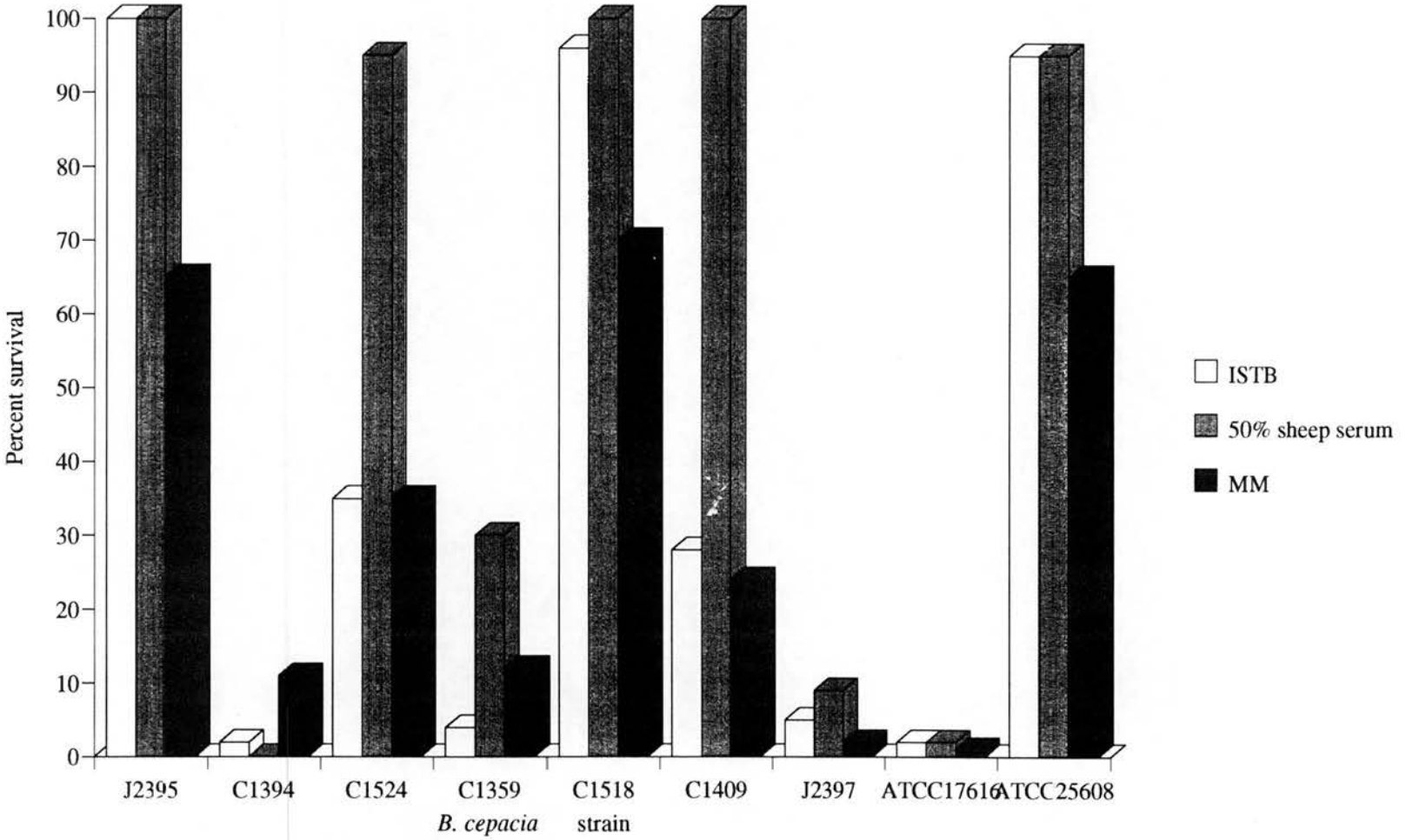


Figure 38. Effect of culture medium on the serum sensitivity of nine *B. cepacia* isolates. Viable counts measured at 180 min.

isolates (J2395, ATCC 17616, ATCC 25608). Following culture in MM all nine *B. cepacia* isolates were less resistant to the bactericidal activity of PNHS than following culture in HISS or ISTB, with the exceptions of C1394 which was serum-sensitive (100% ki'ling) in HISS and C1359 which exhibited the greatest serum sensitivity following culture in ISTB. The most marked increases in serum resistance were observed for C1524 and C1409, the observed differences with the other isolates were not as marked. The results show that culture medium does have an effect on the serum sensitivity of *B. cepacia* isolates. Of interest, the epidemic strain was not observed to be serum resistant following culture in any of the above conditions examined.

In conclusion, the association of *B. cepacia* with bacteraemia in CF patients, including the epidemic strain which expresses R-LPS, cannot be explained by serum resistance and *B. cepacia* may have different *in vivo* characteristics or resist serum killing by an undetermined mechanism.

CHAPTER 7

DISCUSSION

Three important features of *B. cepacia* colonisation in CF patients account for current concern: (1) the organism's characteristic multiresistance to available antimicrobial agents, which limits possible therapeutic options; (2) the high risk of transmission of *B. cepacia* from either person to person or contaminated environmental fomites; and (3) the life threatening clinical sequelae in which approximately 20% of *B. cepacia* colonised CF patients succumb to '*Cepacia* syndrome', a rapidly fatal decline in lung function accompanied by bacteraemia. These features cause major problems for the management of colonised and non-colonised CF patients in CF clinics and in general hospital wards; in addition recent reports of social acquisition pose a threat to the continuation of the adult CF groups in the community which provide great benefit to both patients and their families (Walters & Smith, 1993). In response to concern and requests for information guidelines have been issued by national organisations, including the CF Trust, with the aim of reducing the risk of cross-infection. Such guidelines will require to be updated as further information becomes available in order that any recommendations are justified.

Until recently *B. cepacia* was not considered to be particularly important as a human pathogen; this is reflected by the fact that literature concerning *B. cepacia* pathogenesis is scarce in comparison to the copious literature associated with the pathogenesis of *P. aeruginosa*. The recent creation of a new genus, *Burkholderia*, with *B. cepacia* as the type species (Yabuuchi *et al.*, 1992) emphasises that *B. cepacia* is distinct taxonomically from *P. aeruginosa* a fact which is supported by the

differences in epidemiology and pathogenic potential between the two organisms. In contrast to *B. cepacia*, *P. aeruginosa* in CF is not associated with a rapid decline in pulmonary function and bacteraemia and, in general, is not readily transmitted between patients (Tablan, 1993). Furthermore, *B. cepacia* has not been shown to produce alginate or express any of the virulence factors classically associated with *P. aeruginosa* infection in CF. Nor has *B. cepacia* been observed to undergo the phenotypic adaptations, including a transition from S- to R-LPS expression, serum-sensitivity, development of hypersensitivity to antimicrobial agents and the over production of alginate, which are observed in *P. aeruginosa* isolates from chronically colonised CF patients (Hancock *et al.*, 1983; Penketh *et al.*, 1983; Burke *et al.*, 1990).

The aim of this thesis was to investigate *B. cepacia* pulmonary colonisation in CF patients, particularly the biological properties which may be important in colonisation by the multiresistant, highly transmissible strain of *B. cepacia* which was identified during a survey of antibiotic resistance in *B. cepacia* strains (Lewin *et al.*, 1993) and also by improved typing facilities. These studies indicated that the multiresistant strain of *B. cepacia* accounted for 75% of the *B. cepacia* isolates from adult CF patients in Edinburgh and that it was prevalent amongst CF patients across the UK (see Introduction). As a result of this outbreak the majority of investigations focused on the epidemic strain of *B. cepacia* and the CF patients colonised by this strain.

7.1 The Natural Habitat of *B. cepacia* and Risk of Environmental Acquisition

B. cepacia is frequently described in the literature as a ubiquitous saprophyte found in soil, water and vegetation of diverse geographic distribution (Berkelman *et al.*, 1981). The use of the word "ubiquitous" may be an overstatement of the organism's distribution in nature and to clarify this issue an environmental survey was carried out

using improved selective *B. cepacia* culture techniques (Nelson *et al.*, 1991) of the Royal Botanic Gardens in Edinburgh, which provided a range of soil and water microenvironments. The results of the Botanic Gardens survey demonstrated that *B. cepacia* could be isolated from soil, water and plant material from the varied microenvironments ranging from tropical houses to the outdoor pond. The fact that *B. cepacia* was isolated from a relatively small percentage of the sites examined (12 of 55) supported the suggestion that 'ubiquitous' is an exaggeration. Interestingly, it also confirmed the observation of Palleroni (1984) that despite extraordinary nutritional and survival capabilities *B. cepacia* does not appear to predominate in the natural environment.

Biochemical and genetic analysis of the strains isolated during the microbiological survey of the Botanic Gardens suggested that *B. cepacia* strains isolated from the 'natural' environment are distinct from their clinical counterparts confirming the observations of Gonzalez & Vidaver (1979) and more recently those of Bevivino *et al.* (1994). Genotypic characterisation of the Botanic isolates by PFGE demonstrated that the majority of environmental isolates were clonally distinct from each other and also from the clinical and CF isolates. With the exception of *B. cepacia* type strain ATCC 17616, all the environmental *B. cepacia* isolates, including those from the Botanics, expressed S-LPS with a full O-side chain; in addition, environmental isolates exhibited a lower degree of antimicrobial resistance than strains from CF patients or *B. cepacia* from the hospital environment (Mrs C. Doherty, personal communication).

Increased interest in environmental strains of *B. cepacia* has emerged because the risk which such strains present to CF patients is unknown. In the past, the literature has not clearly differentiated between true environmental strains and contaminating environmental strains (e.g. strains contaminating hospital equipment after use by

colonised patients). The improved selection procedures which have been successfully developed should provide sufficient material to further clarify this issue. Previous studies, which examined the hospital and home environments of CF patients (Nelson *et al.*, 1991; Fisher *et al.*, 1993) have shown that *B. cepacia* colonised CF patients may contaminate their immediate environment. In contrast, in the report of Pegues & Tablan (1993) isolates of *B. cepacia* from the 'natural' environment were distinct from the *B. cepacia* isolated from CF patients.

The ability of *B. cepacia* to survive in conditions of low nutrient availability undoubtedly contributes to the organism's success as a nosocomial pathogen. Carson *et al.* (1973) demonstrated that distilled water may contain high numbers of *B. cepacia* (10^7 cfu/ml) which are not detected as the small cell size does not result in turbidity. Commercial products, such as disinfectants, may become contaminated following dilution with the 'sterile' water resulting in outbreaks of *B. cepacia* pseudobacteraemia or tissue infection (Bassett *et al.*, 1970; Berkelman *et al.*, 1981; Craven *et al.*, 1981; Decicco *et al.*, 1981). Various factors influence bacterial resistance to disinfectant agents: Vess *et al.* (1993) proposed that the ability of *B. cepacia* strains to attach to surfaces and form cellular masses or biofilms provided protection from agents active against planktonic populations; alternatively Pyle *et al.* (1994) suggested that the mechanism of resistance to iodophor agents involved physiological mechanisms such as catabolite repression. In contrast to the *in situ* resistance of *B. cepacia* strains to disinfectant agents, a comparable degree of resistance *in vitro* could not be demonstrated (Bassett *et al.*, 1970; Craven *et al.*, 1981); indeed, the six commonly used disinfectants were effective against two strains of *B. cepacia* investigated in this study. However, conditions likely to be encountered *in situ*, in particular nutrient deprivation which may induce adaptations in *B. cepacia* to promote survival, were not accurately reproduced in the assay. The influence of culture conditions in experimental investigations cannot be ignored; the above

experiments will be repeated following culture in appropriate conditions as part of a future project.

In conclusion, the risk environmental strains of *B. cepacia* present to CF patients remains unclear. Based on present evidence patients are probably at greatest risk from person-to-person transmission or indirect acquisition from contaminated fomites in the hospital or home where the ability of *B. cepacia* to survive in conditions of low nutrient availability allows a contaminated reservoir to be a source of infection (Fisher *et al.*, 1993; Govan *et al.*, 1993; Pegues & Tablan, 1993; Burdge *et al.*, 1993).

7.2 *B. cepacia* Colonisation of Patients with Cystic Fibrosis

Several studies have proposed that *B. cepacia* strains may vary in virulence, with some strains being highly virulent and others essentially avirulent (McKevitt & Woods, 1984; Goldman & Klinger, 1986; Gilligan, 1991). This issue will be addressed in the following section.

Extracellular Virulence Factors

In previous studies the virulence factors associated with *P. aeruginosa* colonisation in CF (elastase, phospholipase C (PLC), alginate and exotoxin A) have not been demonstrated in *B. cepacia* isolates. However, because of the clearly documented virulence and transmissibility of the epidemic strain (Govan *et al.*, 1993) attempts were made to investigate putative virulence factors in epidemic strain isolates. In the present study, investigation of the epidemic strain of *B. cepacia* confirmed the previous reports that *B. cepacia* lacks the classic *P. aeruginosa* virulence factors (McKevitt & Woods, 1984; Montie *et al.*, 1985; Nakazawa *et al.*, 1987; McKevitt *et al.*, 1989; Vasil *et al.*, 1990) and also indicated that the epidemic strain was no different from other *B. cepacia* strains. All strains of *B. cepacia* showed some PLC activity but no elastase production was observed for any of the *B. cepacia* strains

investigated. Melanin production was characteristically associated with the epidemic strain and has not previously been described in *B. cepacia*. A pathogenic role for melanin is unlikely as non-melanin producers have also been associated with fatalities in CF. However, identifying melanin production is a useful marker for the epidemic strain. Although exopolysaccharide production was observed in several *B. cepacia* strains following culture on the appropriate media, it would seem unlikely that exopolysaccharide production contributes to virulence as many CF isolates including the epidemic strain did not produce exopolysaccharide under any growth conditions. This result agreed with the survey of polysaccharide biosynthesis by Sage *et al.* (1990) which indicated that there was no correlation between the ability of *B. cepacia* to colonise the respiratory tract and the production of exopolysaccharide. Finally, Southern hybridisation studies with an exotoxin A gene probe performed by Dr J. Nelson showed a positive band with the epidemic strain although subsequent experiments failed to demonstrate biosynthesis of a 66 kDa protein equivalent to *P. aeruginosa* exotoxin A. In contrast, Vasil *et al.* (1986) demonstrated that the gene encoding exotoxin A was absent in eight *B. cepacia* strains.

B. cepacia Cell Surface

Bacteria interact with their environment via their cell envelope and therefore the components of the envelope are important in enabling an organism to adapt and survive in a susceptible host. The next section of this thesis concerned the investigation of the major cell surface components of *B. cepacia* namely fimbriae, flagella, LPS and OMPs.

Tweedy *et al.* (1968) first described the fimbriae of *B. cepacia* and showed them to be similar to Type 1 fimbriae of *E. coli*. Investigation of the epidemic strain and other CF isolates of *B. cepacia* confirmed this and agree with the majority of subsequent observations (Fuerst & Hayward, 1969; Sajjan & Forstner, 1992; Kuehn *et al.*, 1992)

that *B. cepacia* fimbriae are peritrichous and morphologically similar to Type 1 fimbriae. A further comparison with Type 1 fimbriae is that haemagglutination of guinea pig red blood cells by some *B. cepacia* strains was also inhibited by D-mannose. Only one study has described *B. cepacia* strains expressing polar fimbriae, similar to the fimbriae of *P. aeruginosa* (Saiman *et al.*, 1990). The present study and that of Tweedy *et al.* (1968) noted that fimbriation was rare in *B. cepacia* strains with only 10% of organisms from solid or liquid culture observed to express fimbriae. In the present study, electron microscopy (Figures 8-10) indicated that *B. cepacia* expressed two different types of fimbriae; one (Figure 9) comparable to Type 1 fimbriae which was associated with mannose sensitive haemagglutination by strains of *B. cepacia*; and a second 'crinkly' type (Figure 10) observed on non-haemagglutinating *B. cepacia* strains. It is possible that expression of a particular fimbrial type in any strain of *B. cepacia* might be associated with the colonising potential of that *B. cepacia* strain; for example from available evidence it is not clear whether the 22 kDa adhesin found on fimbriae of adherent *B. cepacia* strains (Sajjan & Forstner, 1992 and 1993) is associated with more than one fimbrial type. Different fimbrial morphological types have not previously been described for *B. cepacia*; interestingly a recent paper presented by Forstner *et al.* (1994) described seven different structural classes of *B. cepacia* fimbriae. Co-expression of two different fimbrial classes was common, particularly amongst CF isolates. The Toronto and Edinburgh epidemic strain isolates expressed a 'cable' pilus, so called because of its rather unusual morphology, assembled from a 17 kDa pilin subunit.

In some Gram-negative bacteria fimbrial-mediated haemagglutination has been reported to be associated with adherence and colonisation for example *Helicobacter* species and *Haemophilus influenzae* type b (Gilsdorf *et al.*, 1992; Taylor *et al.*, 1992). *B. cepacia* strains investigated in this study did not show such an association. *B. cepacia* strains which were capable of haemagglutination (those with Type 1 type

fimbriae) were not particularly adherent to mucin, whilst mucin adherent strains which were also observed to express fimbriae did not haemagglutinate. As the 22 kDa adhesin involved in adherence to mucin and BEC has been shown to be located on *B. cepacia* fimbriae (Sajjan & Forstner, 1992), it is possible that strains of *B. cepacia* which are capable of haemagglutination do not express this adhesin and therefore that the different adhesins are associated with different fimbriae.

Motility, in association with chemotaxis, enables a bacterium to move towards a more favourable environment and may promote localisation to the host surface (Finlay & Falkow, 1989). Motility in *P. aeruginosa* has been associated with increased virulence; for example non-flagellate or non-motile mutants of *P. aeruginosa* show decreased virulence in a burned mouse model (Montie *et al.*, 1982; Drake & Montie, 1988). Furthermore reduced motility and impaired virulence were observed in strains expressing R-LPS isolated from chronically colonised CF patients with poor pulmonary status (Luzar *et al.*, 1985). In this study the *B. cepacia* strains investigated exhibited a range of motility. Interestingly, the motility of the epidemic strain isolates of *B. cepacia* (which express R-LPS) was significantly less than the motility of the environmental, clinical and other CF isolates. The lack of motility observed in a strain associated with transmission and considered to be 'virulent' indicates that being highly motile may not be as important for *B. cepacia* as for *P. aeruginosa*.

McKevitt & Woods (1984) reported that the majority (54%) of CF isolates of *B. cepacia* expressed R-LPS. In their study no typing data was available to confirm that the CF isolates studied were clonally distinct. In contrast, the results from the present study showed that the majority of *B. cepacia* strains investigated (regardless of source) expressed S-LPS. An explanation for this apparent contradiction may lie in the transmissibility of *B. cepacia* and the lack of typing data available for the

McKevitt & Woods study. For example, 75% of the *B. cepacia* isolates from CF patients in the Edinburgh clinic express R-LPS, but these isolates are clonally related. As discussed earlier, *B. cepacia* isolated from the 'natural' environment expresses S-LPS; the epidemic strain would appear to be rather unusual in the consistent expression of R-LPS. It is interesting to speculate what advantage the expression of R-LPS could confer on to the epidemic strain as a potential pathogen, as the organism would have increased susceptibility to the bactericidal activity of serum and would be more susceptible to phagocytosis due to increased hydrophobicity. In the context of intracellular survival however, increased uptake by phagocytes might be an advantage.

Increased resistance to antimicrobial agents is associated with reduced expression of OMPs in *B. cepacia* (Aronoff , 1988). Godfrey *et al.* (1984) also observed that in *P. aeruginosa* the loss of the LPS O-side chain contributed to resistance to β-lactam antibiotics. In this study the CF isolates of *B. cepacia* were observed to express fewer OMPs than the isolates from the hospital environment or the soil (Figure 15). The CF isolates were also found to be less sensitive to a range of antimicrobial agents (Mrs C. Doherty, personal communication). Expression of R-LPS by the epidemic strain may contribute to the high levels of antimicrobial resistance observed with this strain (Simpson *et al.*, 1994). A recent report demonstrated that the expression of extracellular virulence factors and cell surface components of *B. cepacia* was influenced by sub-MIC levels of antibiotics (McKenney *et al.*, 1994). Although *B. cepacia* is seldom eradicated by antimicrobial agents, altering the expression of potential virulence factors may reduce the pathogenic potential of the organism and enable the host's immune response to control the infection.

B. cepacia Adherence

Adherence to an appropriate host mucosal or epithelial cell surface, mediated via specific interactions between bacterial adhesins and host receptors, is often a pivotal

event in bacterial colonisation (Krogfelt, 1992). *B. cepacia* adherence has recently been the focus of research for several groups and has been reviewed in the Introduction.

Evidence suggests that oral colonisation by both Gram-positive and Gram-negative pulmonary pathogens is a prerequisite for subsequent colonisation of the respiratory tract (Johanson *et al.*, 1979; Woods *et al.*, 1980; Komiyama *et al.*, 1987). After colonisation of the oral cavity, aspiration of the contents may lead to colonisation of the lower respiratory tract. The role of adherence in the pathogenesis of Gram-negative respiratory tract infection has been investigated largely in association with BEC; the ability of *P. aeruginosa* and *S. aureus* to adhere to BEC *in vitro* has been reported to be directly related to the severity of lung infection (Woods *et al.*, 1980; Schwab *et al.*, 1993). In contrast Paranchych *et al.* (1986) reported that *P. aeruginosa* bound less well *in vitro* to BEC from CF patients and a prospective *in vivo* study showed little evidence of buccal cell adhesion by *P. aeruginosa* (Dr J. Govan, personal communication). In the present study *B. cepacia* was successfully cultured from various sites within the oral cavity and also from saliva of *B. cepacia* colonised CF patients. It seems reasonable to postulate, that these sites could act as a reservoir for *B. cepacia* in preceding colonisation of the lower airways as described for *P. aeruginosa* (Woods *et al.*, 1980). In addition the relatively high concentrations of the epidemic strain cultured from saliva may contribute to the transmissibility of this strain. Since only a small proportion of the patients attending the Edinburgh adult CF clinic are colonised by other strains of *B. cepacia* (2 out of 9 in the present study) it is not clear whether oral carriage of *B. cepacia* is a general phenomenon or limited to the epidemic strain.

Binding of *B. cepacia* to BEC was demonstrated in a small number of unmatched subjects and clearly showed that the epidemic strain binds to BEC with more affinity

than other strains of *B. cepacia*, *P. aeruginosa* or *N. meningitidis*. Woods *et al.* (1981) proposed that in *P. aeruginosa* colonisation, serious illness was accompanied by a marked increase in patient susceptibility to respiratory tract colonisation associated with increased protease activity of secretions and alterations in epithelial cell surfaces such as loss of fibronectin. The greater degree of adherence observed with all the organisms to the BEC of patient I (Table 13), a chronically unwell patient colonised by the epidemic strain might be due to such alterations in epithelial cell surfaces.

Reports have suggested that adherence to BEC and respiratory mucin may be mediated via fimbrial adhesins, for example the *B. cepacia* 22 kDa fimbrial adhesin described by Sajjan *et al.* (1992) or the PAK pilin of *P. aeruginosa* which mediated adhesions to respiratory epithelial cells (Doig *et al.*, 1988). Alternatively, Ramphal *et al.* (1991) demonstrated that adhesion may be mediated by several classes of non-fimbrial adhesins including alginate. The study of Sajjan *et al.* (1992) indicated that the ability to adhere to mucin may contribute to the virulence of a strain; adherent isolates were obtained from CF patients with more advanced disease, suggesting that increased mucin adherence may contribute to morbidity and mortality. An alternative hypothesis, emphasising the role of the host in colonisation is emerging from the work of Barasch *et al.* (1991) who showed that decreased sialylation of epithelial cells in patients homozygous for ΔF508 is associated with increased *P. aeruginosa* binding *in vitro*.

In this study mucin adherence of *B. cepacia* was measured in a microtitre assay incorporating hyperimmune serum from CF patients colonised with the epidemic strain to measure binding. The assay had several drawbacks, principally concerning the use of CF serum: (1) serum obtained from patients colonised by the epidemic strain might have been less efficient at detecting other strains of *B. cepacia* and hence

bias the results; (2) serum from three different patients had to be used, as the first and second choices unfortunately died during the course of the studies and the experiments had to be repeated with serum from the next patient.

The trend in results obtained for the adherence of each *B. cepacia* strain to purified mucin were consistent for the different sera. Specific adherence to purified respiratory mucin was observed in only seven of 30 strains investigated, all of these were CF isolates. In normal healthy lungs mucin serves to protect the underlying respiratory epithelia from bacterial colonisation as adherent bacteria are removed by mucociliary clearance (Reynolds, 1989). In CF airways impaired clearance and viscid stagnant mucus mean that adherent organisms are not removed; thus an enhanced ability to adhere may be an important virulence factor in some CF isolates of *B. cepacia*. Binding of *B. cepacia* to mucin was approximately ten-fold less than that observed for *P. aeruginosa*. *B. cepacia* binding to the control (no mucin) was negligible but that for *P. aeruginosa* was almost as high as binding to mucin which agrees with previous observations by Sajjan *et al.* (1992) who reported that *P. aeruginosa* adherence to mucin was non-specific. In contrast to the results obtained for this study, Sajjan *et al.* (1992) reported that the majority of *B. cepacia* strains (28 of 30) were adherent to purified mucin. However no typing data was available in this study and a clonal relationship between strains could not therefore be discounted, an important oversight considering the potential transmissibility of some *B. cepacia* strains. The need for typing was emphasised following collaboration between our laboratory and that of Dr J. Forstner in Toronto in which strains were exchanged to be analysed in our respective mucin adherence assays involving ELISA or radiolabelling. The results for the adherence assays performed in each centre were consistent for each *B. cepacia* strain analysed. The collaboration also revealed important information on the epidemiological background and origin of the Edinburgh epidemic strain. Genomic fingerprinting of the epidemic strain and also the Toronto

high binding strain showed similar PFGE profiles. The Toronto strain was a melanin producer, had R-LPS and was bacteriocin type S3/P0. Although not conclusive, the data suggest a clonal and epidemiological link between the Edinburgh and Toronto isolates. This link has recently been confirmed by the multilocus enzyme typing study of Johnson *et al.* (1994).

7.3 The Humoral Immune Response to *B. cepacia* in Colonised Cystic Fibrosis Patients

Studies of the humoral immune response in *P. aeruginosa* colonised CF patients have provided important evidence on the nature of the interaction between the CF host and the colonising organism and may explain, at least in part, why *P. aeruginosa* is able to persist in the CF lung. The occurrence of a specific anti-*P. aeruginosa* antibody response has been shown to be useful in diagnosing infection at an early stage and also in differentiating between infection and harmless colonisation (Brett *et al.*, 1988; Cordon *et al.*, 1992). Furthermore, measurement of an antibody response against *P. aeruginosa* has been found useful in monitoring the progress of infection, as a guide to therapeutic intervention and also as a prognostic indicator. The present study identified several similarities between the humoral immune response of CF patients colonised by *P. aeruginosa* and the humoral immune response of CF patients colonised by *B. cepacia* and therefore it is reasonable to propose that the explanations may be extrapolated to *B. cepacia* colonisation and persistence in the lungs of CF patients.

In chronically colonised CF patients the anti-*P. aeruginosa* antibodies belong to all the major immunoglobulin classes and are directed against all the major *P. aeruginosa* antigens (OMP, flagella, LPS and alginate) and toxins (exotoxin A, elastase and PLC: Doring & Høiby, 1983; Hancock *et al.*, 1984; Pier *et al.*, 1987; Albus *et al.*, 1989; Pedersen *et al.*, 1990, Fomsgaard, 1990; Nelson *et al.*, 1990; Kronborg *et al.*, 1992;

Fournier *et al.*, 1993). These studies have demonstrated a strong correlation between the development of an anti-*P. aeruginosa* antibody response and the onset of chronic colonisation. By monitoring the immunological responses of the CF host it is possible to identify stages in chronic infection: for example, serum antibodies to exotoxin A, phospholipase C and flagella appear during the initial stages of colonisation indicating the role of these virulence factors in colonisation. A beneficial role for anti-*P. aeruginosa* antibodies has not been established. The antibody response is ineffective in eliminating *P. aeruginosa* and high and rising levels of antibodies paradoxically correlate with a poor prognosis (Wheeler *et al.*, 1984; Høiby *et al.*, 1990). In contrast, it has been suggested that the large amount of anti-LPS antibodies protect against endotoxaemia in CF patients (Fomsgaard, 1990, Kronborg *et al.*, 1992) and recently Morrin & Reen (1993a and b) demonstrated that specific anti-*P. aeruginosa* antibodies could inhibit adherence of some *P. aeruginosa* strains to BEC. Furthermore, Pier *et al.* (1987) and Cryz *et al.* (1988) reported that opsonophagocytic antibodies to LPS and alginate are protective, conferring resistance to colonisation by *P. aeruginosa*, and forming the rationale behind initiations to develop vaccine strategies, either actively using a conjugate vaccine (Cryz *et al.*, 1988) or by passive administration of hyperimmune globulin (Genzyme - personal communication with Dr J. Govan).

The failure of the antibody response to eliminate *P. aeruginosa* from CF lungs may be a consequence of the inappropriate nature of the antibody response with respect to IgG subclass. Several groups have speculated that the functional capacity of the anti-*P. aeruginosa* antibodies is "blocking" rather than opsonising or lysis-promoting (Fick *et al.*, 1981; Pedersen *et al.*, 1989; Moss *et al.*, 1986). The relative amounts of the different isotypes present in serum have been correlated with pulmonary function, degree of opsonophagocytosis and the efficacy of immunoprotection: for example, in *P. aeruginosa* colonised cystic fibrosis patients increased levels of IgG2 and IgG3 are

associated with decreased lung function whilst high concentrations of IgG4 correlate directly with disease severity (Pressler *et al.*, 1988; Pedersen *et al.*, 1989; Likavconova & Lagace, 1992). Marked shifts in the IgG1-4 responses to specific *P. aeruginosa* antigens are also observed, notably an increase in specific IgG2 coupled with a lack of IgG1 in response to chronic stimulation by LPS (Fick *et al.*, 1986; Shryock *et al.*, 1986); recently Kronborg *et al.* (1993) showed that IgG2 anti-lipid A, IgG3 anti-lipid A and IgG2 anti-polysaccharide correlated with lung deterioration before the onset of chronic infection was diagnosed. A high concentration of specific anti-*P. aeruginosa* IgG2 may inhibit clearance of *P. aeruginosa* from the CF lung as pulmonary macrophages are restricted in FcγR expression, with receptors for IgG1 and IgG3 only. Formation of immune complexes may also indirectly inhibit phagocytosis as IgG2 is the major constituent of immune complexes (Hornick & Fick, 1990). It has been suggested that terminal CF is an immune complex disease (Fomsgaard, 1990); the high concentration of anti-LPS antibodies observed in the later stages of infection supports this hypothesis.

The anti-*B. cepacia* antibody responses to several *B. cepacia* envelope components was investigated by ELISA and immunoblot systems; both of which have been shown to be sensitive and specific for determining antibodies to *P. aeruginosa* (Brett *et al.*, 1986; Shand *et al.*, 1988; Fomsgaard, 1990). The ELISA based system incorporated purified core R-LPS from the epidemic strain of *B. cepacia* and was able to differentiate between non-colonised patients and CF patients colonised by the epidemic strain or other strains of *B. cepacia*. The R-LPS preparation was chosen as a coating antigen for the following reasons:

1. LPS represents a more defined antigenic preparation than a system based on whole cells and was better able to discriminate CF patients colonised with *B. cepacia* from patients colonised with *P. aeruginosa*;

2. Available evidence suggested antigenic cross-reactivity between some outer membrane proteins of *B. cepacia* and *P. aeruginosa* (Aronoff & Stern, 1988; Aronoff *et al.*, 1991);
3. Our own absorption studies demonstrated that the anti-*B. cepacia* LPS IgG antibodies are specific and do not react with *P. aeruginosa* core LPS and vice versa confirming previous findings that the composition and structure of core-LPS between *P. aeruginosa* and *B. cepacia* are different (Palleroni, 1984; Nelson *et al.*, 1992);
4. Core LPS is believed to be a relatively conserved component of LPS (Wilkinson, 1983), and therefore is a suitable antigen for the detection of an antibody response against all serotypes of *B. cepacia*;
5. The majority of *B. cepacia* isolates from CF patients were reported to express R-LPS (McKevitt & Woods, 1984).

The ELISA data obtained in both the cross-sectional study and the study of paired sera clearly show elevated anti-*B. cepacia* antibody titres, for the three major immunoglobulin classes IgG, IgA and IgM, in sera from CF patients chronically colonised by the epidemic strain of *B. cepacia*. For patients colonised by other strains of *B. cepacia* and non-colonised patients the difference was less marked. Similar observations were made for sputum IgA anti-*B. cepacia* LPS antibody titres.

Several theories may explain the absence of significant serum anti-*B. cepacia* IgG (i.e. a titre < 1600); firstly the CF patient has never been colonised with or exposed to *B. cepacia*, secondly the colonising strain of *B. cepacia* may be non-immunogenic and finally in patients 'intermittently' colonised by the epidemic strain lack of detection in sputum and low antibody titres may result from a low concentration of *B. cepacia* in the lower respiratory tract rather than an actual absence of the organism (Figure 21; Dr S. Elborn, personal communication). The detection of a rising antibody titre as

shown in the longitudinal study (Figure 21), is therefore of more value in confirming colonisation with *B. cepacia* than a single measurement. Indeed, in the longitudinal studies a rise in the level of anti-*B. cepacia* LPS IgG antibodies was demonstrated in some of the patients prior to or accompanying the first positive sputum culture of *B. cepacia*.

The prognostic value of measuring anti-*B. cepacia* IgG antibody titres is less obvious from this limited study. Hopefully, analysis of data from a large ongoing multicentre study will provide further information. At present, available evidence does not show a correlation between increased antibody titres and pulmonary decline (Brown *et al.*, 1993). For example, the index case, who became colonised with *B. cepacia* in 1989 (see patient 10, Figure 3, Introduction), has had relatively high anti-*B. cepacia* LPS IgG titres (12800) for the last four years. In contrast, the last CF patient to contract the epidemic strain (patient 25, Figure 3) who was severely ill and awaiting heart-lung transplant at the time of acquisition of *B. cepacia*, died so rapidly that there was no time for an anti-*B. cepacia* antibody response to develop.

Examination of the immunoblots of paired serum from CF patients revealed a strong response to the R-LPS of the epidemic strain following colonisation with *B. cepacia* (Figure 24a) and confirmed the ELISA results. The presence of a serum IgG response to the O-side chain of the environmental isolate of *B. cepacia* J2395 in patients not known to be colonised by this strain was interesting (Figure 24b). There is no satisfactory explanation for the presence of these antibodies. One possibility is that the CF patients involved had been co-colonised with an additional strain of *B. cepacia* which was never cultured from sputum. However, it is unlikely that so many patients would be colonised by a strain which was never detected in an efficient diagnostic laboratory. Alternatively it is possible that the epidemic strain may express S-LPS *in vivo*. There is no evidence to indicate that the epidemic strain expresses S-

LPS *in vivo* or *in vitro*, despite investigation of LPS from newly acquired and chronic isolates of the epidemic strain and also following culture of the organism in a range of conditions.

Immunoblots obtained with serum from CF patients colonised by various strains of *B. cepacia* against the Proteinase K digests of six non-epidemic strains of *B. cepacia*, OMPs and also flagella preparations emphasise that the immune response in *B. cepacia* colonised CF patients is heterogeneous; for example, CF patients colonised by the 'same' strain exhibit different degrees of response to antigens of homologous and heterologous *B. cepacia* strains. The lack of serum IgG in some of the patients against antigens from a homologous strain of *B. cepacia* suggests that strains of *B. cepacia* vary in their capacity to stimulate the immune response. The results from this study indicate that the epidemic strain may be particularly immunogenic. It is interesting to speculate whether increased immunogenicity of a strain correlates with increased virulence, and whether the excessive immune response observed in some CF patients colonised with the epidemic strain contributes to the pathology observed in these patients.

Respiratory tract infections have been reported to be associated with an IgG2 subclass deficiency (Bradwell, 1993). In this study however, raised concentrations of at least one of the IgG subclasses, including IgG2, were observed. Analysis of the IgG subclasses in CF patients colonised with *B. cepacia* were consistent with subclass data obtained for CF patients colonised with *P. aeruginosa*. The most marked increases in total IgG concentration were observed for IgG1 and IgG4, whilst the greatest percentage increase in specific antibody against *B. cepacia* R-LPS was observed for IgG2 and IgG3 (Shryock *et al.*, 1986; Pressler *et al.*, 1988; Fomsgaard, 1990). A correlation between the antibody subclass response and clinical status in the *B. cepacia* colonised CF patients was observed for some of the CF patients studied (for

example Patient 1, Figure 32 and 33). Patient 1, colonised by *B. cepacia* only, had raised concentrations of all four IgG subclasses; the anti-*B. cepacia* antibody response comprised largely IgG2, which as described earlier has poor opsonic potential and may inhibit clearance of *B. cepacia* from the lung. The titre of specific anti-*B. cepacia* LPS IgG1 was low and for IgG4 not detectable, but total serum concentrations were markedly increased. Based on the immunoglobulin data obtained it is tempting to speculate that a combination of hypergammaglobulinaemia and non-opsonic specific antibody may contribute to the persistence of *B. cepacia* and immune mediated damage.

The chemiluminescent opsonophagocytosis assay provides a simple method of assessing phagocyte function *in vitro* by measuring oxygen radicals produced by phagocytes during the respiratory burst following phagocytosis of particles (Blair *et al.*, 1988). In the absence of serum, *P. aeruginosa* PAO1 was more susceptible to non-opsonic phagocytosis than *B. cepacia* or *S. aureus*. Non-opsonic phagocytosis of *P. aeruginosa* has been described by Speert *et al.* (1984, 1986) who showed that susceptible strains expressed more fimbriae and were more hydrophobic and concluded that non-opsonic phagocytosis was facilitated by hydrophobic interactions mediated by fimbriae. In the present study the epidemic strain, which has been shown to express peritrichous fimbriae (Figure 10) and to be slightly more hydrophobic than either J2395 or C1559 (Figure 17), was more susceptible to non-opsonic phagocytosis then either of the latter two *B. cepacia* strains. The results following incubation with serum are more difficult to interpret. Chemiluminescence was reduced for the epidemic strain and also *P. aeruginosa* PAO1 in the presence of serum yet increased for the remaining *B. cepacia* strains and for *S. aureus*. Inhibition of chemiluminescence appeared to be non-specific as all sera, regardless of the bacterial status of the patient, were capable of inhibiting phagocytosis.

In summary, analysis of the anti-*B. cepacia* antibody response in CF patients shows some parallels with the well documented anti-*P. aeruginosa* antibody response. CF patients colonised by *B. cepacia* may mount a significant and specific antibody response against the colonising strain. In contrast to *P. aeruginosa*, the ability of *B. cepacia* to stimulate an antibody response was observed to be strain dependent and some strains, notably the epidemic strain, appeared to be particularly immunogenic. Serum from patients colonised by the immunogenic strains of *B. cepacia* contained antibodies which were cross-reactive with epitopes of other strains of *B. cepacia*, whilst serum from patients colonised with some other strains of *B. cepacia* contained few antibodies directed even against the homologous strain. Detection of an anti-*B. cepacia* antibody response in CF patients may be a useful aid in conjunction with other available methods for diagnosing colonisation with *B. cepacia*, and indeed has been used to confirm colonisation by the epidemic strain in our laboratory. However the variation in immunogenicity between *B. cepacia* strains means detecting an antibody response cannot be relied upon to confirm colonisation with *B. cepacia*.

In CF patients, a beneficial role for anti-*B. cepacia* antibodies is not obvious. Previous studies have shown that specific anti-*B. cepacia* antibodies are not protective (Aronoff & Stern, 1988; Aronoff *et al.*, 1991), and the results of this study indicate that the antibodies are not effective in eliminating *B. cepacia* once it is established in the CF lung. The role of other host defences in controlling *B. cepacia* colonisation is not clear. The results from the ongoing multicentre study may indicate the role of cytokines and some inflammatory markers *in vivo*. Unrelated studies by colleagues in the department are currently underway investigating the *in vitro* interaction of *B. cepacia* with cytokines. If *B. cepacia* is shown to be able to survive intracellularly, the role of the cell mediated immune response against *B. cepacia* would also be important. There is no information available to date on the interaction of *B. cepacia* with the cell mediated immune response.

7.4 SERUM BACTERICIDAL ACTIVITY AGAINST *B. CEPACIA*

The ability of Gram-negative bacteria to invade and survive in the bloodstream correlates strongly with resistance to the complement-mediated bactericidal activity of serum; serum-resistant bacteria have been shown to be more virulent and less immunogenic than their serum-sensitive counterparts (Clumeck *et al.*, 1982; Taylor, 1983). Studies with animal models have demonstrated that serum-resistant strains of *E. coli* consistently caused infective endocarditis in rabbits whilst serum-sensitive strains did not (Durack & Beeson, 1977). *N. gonorrhoeae* associated with localised infections are serum-sensitive whilst strains associated with disseminated systemic infection are serum-resistant (Rice, 1989). Similarly, a higher proportion of *E. coli* isolated from the blood of bacteraemic patients are serum-resistant compared with isolates from faeces or urinary tract infections (Taylor, 1983). *P. aeruginosa* isolates from the lungs of chronically colonised CF patients are typically serum-sensitive; the infection remains localised and bacteraemia does not occur (Høiby & Olling, 1977).

A number of cell envelope polymers are considered to be important in protecting the bacterium from complement-mediated serum killing: for example, the acidic polysaccharides or capsules such as the K antigens of *E. coli* and *K. aerogenes* (Williams *et al.*, 1983), outer membrane proteins such as the *Aeromonas* A protein (Munn *et al.*, 1982) and most significantly the role of LPS, in particular the O-side chain which presents a steric barrier to the stable insertion of the C5b-9 membrane attack complex and has been well studied in *E. coli*, *K. pneumoniae* and *P. aeruginosa* (Taylor, 1983; Schiller & Joiner, 1986; Ciurana & Tomas, 1987; Williams & Tomas, 1990). Additional mechanisms of serum resistance are exhibited by *N. gonorrhoeae* and include phenotypic shifting of lipooligosaccharide antigen expression and addition of sialic acid residues and stimulation of blocking antibodies directed at outer membrane protein antigens which prevent binding of bactericidal antibodies to lytic epitopes (Rice, 1989; Smith *et al.*, 1992).

The lack of standardised methods and definitions of serum resistance and sensitivity makes accurate assessment of the impact of serum activity *in vivo* difficult (Crokeart *et al.*, 1992). DeMatteo *et al.* (1981) showed that survival of *P. aeruginosa* isolates in human serum was highly dependent on *in vitro* conditions. Expression of cell envelope components is influenced by both the growth conditions and growth phase and *in vitro* susceptibility may not reflect the *in vivo* situation. Anwar *et al.* (1983) reported that nutrient conditions had a profound effect on the susceptibility of *B. cepacia* to serum killing. The classification of serum resistance and sensitivity in this study was initially based on that of Thomassen & Demko for *P. aeruginosa* (1981) but was subsequently influenced by the nature of the results obtained; for example after a 3 h exposure to PNHS, a 90% or greater decline in cell numbers was measured for all the strains classified as sensitive, whilst a 10% or less decline was typical for those strains classified as serum-resistant. In the present study only one *B. cepacia* strain produced results outwith these limits and was classified as intermediate.

Serum sensitivity was influenced by the time of exposure of the *B. cepacia* strains to PNHS. A higher proportion of *B. cepacia* strains were serum-sensitive after 3 h than after 90 min, suggesting that serum killing of some of the *B. cepacia* isolates was slow or delayed and indicating that stopping the reaction after 1 or 2 h as in some studies would not identify all serum-sensitive strains (Crokeart *et al.*, 1992). The major determinant of serum resistance in *B. cepacia* was expression of S-LPS as the four serum-resistant isolates of *B. cepacia* expressed S-LPS with a full O-side chain. Delayed serum killing of *B. cepacia* isolates was observed where the O-side chain was lacking or where the chain length was shorter. The length of the O-side chain has also been shown to be important in evasion of serum killing by *K. aerogenes* (Williams *et al.*, 1983).

A role for the other envelope components in protecting *B. cepacia* from serum bactericidal activity was less clear. As described in Results (Chapter 3), some *B. cepacia* strains produce an exopolymer when cultured under appropriate conditions. This material was observed in both serum-resistant and serum-sensitive *B. cepacia* isolates expressing S-LPS, but not with strains expressing R-LPS including the epidemic strain. Analysis of the outer membrane proteins of the serum-sensitive *B. cepacia* isolates C1359 (epidemic strain), C1559, ATCC 17616, C1409 (which exhibited delayed killing as shown in Figure 32) and the serum-resistant isolate J2395 revealed that only the latter expressed an OMP of between 150-200 kDa (Figure 15). It is possible that this protein may complement the barrier effect of the O-side chain.

The nutrient conditions under which *B. cepacia* was grown affected the susceptibility to serum killing in a strain-dependent manner. Serum sensitivity was increased for eight of the nine *B. cepacia* isolates investigated following culture in a minimal medium compared to that following culture in ISTB or HISS. Five *B. cepacia* isolates were more resistant to serum bactericidal activity following culture in HISS compared to ISTB, and three of the strains were equally susceptible under all culture conditions. These results agree with observations on the serum susceptibility of *Bacteroides* following culture in the same nutrient conditions (Miss E. Allan, personal communication). The epidemic strain was not serum-resistant following culture in nutrient rich or deficient media, suggesting that serum sensitivity in this strain is genuine and not an artefact of the assay or culture conditions.

The bactericidal activity of serum against *B. cepacia* was observed to be heat-labile and, as observed with *P. aeruginosa*, appears to result principally from activation of the classical complement pathway (CCP) but with some killing resulting from activity of the alternative complement pathway (ACP: Thomassen & Demko, 1981; Schiller, 1988). Serum treated with 10 mM $MgCl_2$ EGTA had a reduced ability to kill six *B.*

cepacia isolates which were serum sensitive in the presence of PNHS. EGTA binds calcium ions strongly and magnesium ions poorly thus inhibiting the CCP which requires both ions whilst the ACP requires only magnesium ions (Fine *et al.*, 1979). The fact that the CCP is of major importance in serum killing of *B. cepacia* suggests that antibodies, which initiate the activity of the CCP, may also play a role in the process. The requirement for specific antibody in the serum killing of *B. cepacia* is unclear. PNHS contained negligible amounts of anti-*B. cepacia* antibodies as shown by ELISA incorporating both the R-LPS of the epidemic strain or *B. cepacia* whole cells as coating antigen and yet was as efficient at killing *B. cepacia* as the three CF sera from patients chronically colonised by the epidemic strain. An attempt to remove the anti-*B. cepacia* antibodies from the CF sera by serial absorption with *B. cepacia* whole cells was unsuccessful, possibly because of the extremely high concentration of antibodies present. This problem was also encountered by Thomassen & Demko (1981) who proposed that in CF patients chronically colonised by *P. aeruginosa* with advanced lung disease, autologous strains are protected from serum bactericidal activity by the presence of blocking antibodies. A similar mechanism may explain the inability of the sera from patient III (Figure 37) to effectively kill *B. cepacia.*

The results from this study do not explain why strains of *B. cepacia*, in particular the epidemic strain which expresses R-LPS and was serum sensitive in all investigations, are associated with bacteraemia in CF patients. A number of possibilities exist to explain the ability of *B. cepacia* to invade and survive in the bloodstream circumventing serum bactericidal activity: (1) the *in vivo* characteristics of the epidemic strain are not reflected in the *in vitro* assay; (2) serum from bacteraemic patients has been reported to be ineffective in killing the homologous organism *in vivo* (Taylor, 1983); (3) bacteria with a slower growth rate may be less susceptible than rapidly dividing cells (the epidemic strain does grow slowly in conditions of poor

nutrient availability, Results Chapter 3); and (4) bacteria may adopt an intracellular location in the blood or reticuloendothelial system (Taylor, 1983).

The proposal that *B. cepacia* may be able to survive in an intracellular location not only avoiding serum bactericidal activity but also the humoral immune response is particularly appealing. Taxonomically, the idea is supported by the fact that *B. cepacia* is closely related to *B. pseudomallei*, the causative agent of melioidosis and a highly virulent intracellular pathogen (Pruksachartvuthi *et al.*, 1990). More recently intracellular survival has been described for other well known respiratory tract pathogens, *Bordetella pertussis* and even *P. aeruginosa* (Chi *et al.*, 1991; Freidman *et al.*, 1992) which, in addition to the well known intracellular pathogens, indicates that such an ability is widespread. Research to determine whether *B. cepacia* is able to survive intracellularly is currently underway in several laboratories in the UK and also in the USA. If *B. cepacia* is found to be an intracellular pathogen the inability of the immune response to eliminate the organism and the ability of a serum-sensitive organism to survive in the blood might be explained. In addition, such an observation might have important implications for the future treatment of *B. cepacia* infections both in terms of antibiotic therapy and in a rational approach to vaccination.

CONCLUSIONS

Anxiety concerning the transmission of *B. cepacia* amongst CF patients attending different regional CF centres continued to grow throughout the duration of this project. On conclusion of this project, transmission *per se* appears to have been brought under control but only at the social and logistical cost of patient segregation. In the late 1980's, there was some debate as to whether *B. cepacia* was truly a pathogen or simply a marker of advanced lung disease. As a result of growing evidence from CF clinics it is now generally accepted that some strains of *B. cepacia* are pathogenic. '*Cepacia* syndrome' has been observed in CF patients previously considered to be in relatively good health and this study has demonstrated that patients mount a significant humoral immune response against some colonising strains. Major problems remain with regard to the modes of pathogenesis of *B. cepacia* in CF, as the organism has few demonstrable virulence determinants. Previous studies have clearly demonstrated that environmental and clinical or CF isolates of *B. cepacia* are different (Bevivino *et al.*, 1994). This study has elaborated on these differences and has shown that CF strains differ significantly from each other with respect to colonising potential, transmissibility and the ability to stimulate the host immune response and it is proposed that these differences are accompanied by variable virulence. The evidence suggests that there are clones of *B. cepacia* which are particularly virulent and transmissible, for example the Edinburgh 'epidemic strain'. It will soon be possible to investigate the virulence of the different *B. cepacia* strains and verify (or otherwise) this proposal using the CF mouse developed by Drs David Porteous and Julia Dorin. Preliminary studies with the epidemic strain have confirmed the pathogenic potential of that strain; pneumonia developed in six out of nine CF homozygous mice inoculated with the epidemic strain but not in the control mice. The observation that there are differences between CF isolates of *B. cepacia* raises the question that blanket segregation of all *B. cepacia* colonised CF patients might not be

necessary. If the different *B. cepacia* strains are distinct the nomenclature of this species must be questioned. Should a strain such as the epidemic strain be classified with the seemingly harmless strains isolated from the Botanic Gardens? This idea is further supported by the recent studies of Simpson *et al.* (1994) which showed that the epidemic strain has a surface fatty acid composition more closely resembling *B. gladioli*, an organism previously thought to be harmless in CF (Christenson, *et al.*, 1989) than *B. cepacia*. On the other hand, biochemical data clearly identify the epidemic strain as *B. cepacia*. This study shows that the epidemic strain is distinct from other *B. cepacia* strains and may even be a hybrid!

Finally, the association of *B. cepacia* with CF is far from understood. This study reiterates the heterogeneity of both *B. cepacia* strains and the nature of their interaction with CF patients and emphasises that each new case should be considered individually.

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Serum sensitivity of *Burkholderia* (*Pseudomonas*) *cepacia* isolates from patients with cystic fibrosis

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Abstract: Bacterial strains which are sensitive to the bactericidal activity of serum are generally considered to be less virulent than serum-resistant strains and are seldom associated with bacteraemia. *Burkholderia* (*Pseudomonas*) *cepacia* is an important pathogen in cystic fibrosis and is associated with rapid fatal pulmonary decline and bacteraemia in 20% of colonised patients. In this study 19 isolates of *B. cepacia* expressing either rough or smooth LPS were investigated to determine the degree of serum sensitivity. Strains expressing rough-LPS were serum-sensitive: these included a highly transmissible strain of *B. cepacia* isolated from approximately 50 cystic fibrosis patients attending various U.K. regional centres and associated with cases of bacteraemia.

Key words: *Burkholderia cepacia*; *Pseudomonas cepacia*; Cystic fibrosis; Bacteraemia; Serum susceptibility; Lipopolysaccharide

Introduction

Burkholderia cepacia, formerly *Pseudomonas cepacia*, [1] is an important pulmonary pathogen in patients with cystic fibrosis (CF). Clinical sequelae of *B. cepacia* colonisation are variable and include asymptomatic carriage or a slow decline in lung function. The third outcome of *B. cepacia* colonisation is observed in approximately 20% of patients and involves a rapidly fatal decline in pulmonary function accompanied by necrotising pneumonia and, in some patients, bacteraemia [2]. *B. cepacia* is inherently resistant to most antibiotics [3,4] and is able to persist despite a specific anti-*B. cepacia* antibody response [5,6]. The role of the bactericidal activity of human serum against *B. cepacia* has not been extensively investigated but is of particular importance due to the association of *B. cepacia* with bacteraemia. In a study of a single *B. cepacia* strain (NCTC 10661) Anwar et al. [7] described killing by serum factors and polymorphonuclear leukocytes (PMN).

It is widely accepted that *Pseudomonas aeruginosa* isolates from chronically infected CF patients exhibit phenotypic changes rarely observed in isolates of *P. aeruginosa* from other sources. This phenotype includes loss of the O-side chain portion (B-band) of lipopolysaccharide (LPS), alginate biosynthesis, and susceptibility to serum killing [8–12]. Longitudinal studies on the phenotypic characteristics of *B. cepacia* cultured from

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the CF lung have not been carried out and it is unclear whether *B. cepacia* undergoes phenotypic alterations similar to those observed with *P. aeruginosa*. Although a mucoid phenotype of *B. cepacia* may be observed under appropriate cultural conditions [13] biochemical characterisation of the exopolysaccharide indicate that it is not alginate (S. Butler, unpublished observations). The absence of alginate biosynthesis in *B. cepacia* is supported by PCR studies which showed no evidence of the *alg*D gene, encoding the key enzyme mannose dehydrogenase, in ten strains of *B. cepacia* [14]. In addition, there is no evidence to date to suggest that a change from smooth-LPS (S-LPS) to rough-LPS (R-LPS), due to loss of O-antigen, occurs in *B. cepacia* strains following colonisation of the CF lung. Indeed, a highly transmissible strain of *B. cepacia* cultured in a number of CF Centres in the U.K. [15] invariably expresses R-LPS and has never been associated with a S-LPS phenotype even in newly colonised patients (S. Butler, unpublished observations).

The aim of this study was to investigate and compare the susceptibility of clinical and environmental isolates of *B. cepacia* to normal human serum (NHS) and in particular to investigate the degree of serum sensitivity in isolates from CF patients.

Table 1

Serum killing of *B. cepacia* by 40% PNHS at 90 min and 180 min

Strain	Source	LPS (R or S)	90 min	180 min
C1518	CF (sputum)	S	SR	SR
C1394	CF (sputum)	R	SS	SS
C1524	CF (sputum)	S	I	SS
C1548	CF (sputum)	R	SS	SS
C1559	CF (sputum)	S	I	SS
C1454	CF (sputum)	R	I	SS
C1409	CF (sputum)	S	I	SS
C1704	CF (sputum)	S	SR	SR
C1359 *	CF (sputum)	R	I	SS
C1602 *	CF (blood)	R	I	I
ATCC 25609	Bronchial wash	S	SS	SS
ATCC 25608	Wound	S	SR	SS
ATCC 27515	Infected tissue	S	SR	I
ATCC 17762	Urine	R	SS	SS
J162	Wound	S	SR	SS
J2394	Plant	S	SR	SR
SBC 100	Potato	S	SS	SS
ATCC 17616	Soil	R	I	SS
J159	Onion	S	SR	SR
E. coli R3		R	SS	SS
E. coli 018		S	SR	SR

* Epidemic strain [15].
R, rough LPS.
S, smooth LPS.
SR, serum-resistant.
SS, serum-sensitve.
I, intermediate.

Materials and Methods

Bacterial cultures

Nineteen isolates of *B. cepacia* were studied (Table 1). Nine isolates cultured from sputum and one from the blood of a colonised CF patient attending the Edinburgh adult CF clinic were characterised phenotypically and genotypically by bacteriocin typing [16] and pulsed field gel electrophoresis (PFGE: CHEF, BioRad Laboratories Inc.) respectively. The remaining 9 non-CF isolates were either of clinical or environmental origin. All strains were identified as *B. cepacia* by growth and colonial morphology on *Pseudomonas cepacia* Medium (CEP) (Mast Laboratories Ltd., Bootle, U.K.) and by API 20NE (bioMerieux, 69280 Marcy l'Etoile, France). Control strains were *Escherichia coli* O18 and *E. coli* R3 expressing smooth and rough LPS phenotypes respectively.

Lipopolysaccharide analysis

LPS was prepared for polyacrylamide gel electrophoresis (PAGE) analysis by the proteinase K digestion method [17] and separated on 14% w/v polyacrylamide gels incorporating the buffer system of Laemmli [18]. The gels were stained with silver by the modified method of Tsai and Frasch [19] as described by Hancock and Poxton [20].

Outer membrane protein analysis

Overnight cultures of *B. cepacia* C1359, C1409 and J2394 grown at 37°C with aeration, were harvested from Isosensitest broth (IST) (Oxoid) and washed twice in sterile phosphate buffered

saline (PBS; pH 7.2) at $6000 \times g$ for 10 min at 4°C (Sorvall, RC5B). The pellet was resuspended in 20 ml pyrogen-free water and sonicated at 8 μm (Microson, Ultrasonic Cell Disruptor, Heat Systems-Ultrasonics Inc., NY, U.S.A.) to break cells. Unbroken cells were removed by centrifugation at $7000 \times g$ for 10 min. Outer membranes were derived by solubilisation in 2% w/v Sarkosyl (N-lauroyl sarcosinate: Sigma) for 45 min at room temperature. Outer membranes were collected and washed by centrifugation at $38000 \times g$ for 1 h at 4°C. The crude outer membrane preparation was separated on a 12% w/v polyacrylamide gel, and OMP's were stained with Coomassie blue [20].

Serum

Blood was obtained from 2 normal human volunteers and 3 CF patients colonised by the same strain of *B. cepacia* [15]. After incubation at 37°C for 30 min to allow clotting, serum was separated by centrifugation at 5000 rpm (Heraeus, Bactifuge) for 20 min. The normal sera were pooled (pooled normal human serum; PNHS) and half of the serum pool was immediately aliquoted in 2-ml volumes and stored at −70°C whilst the remaining sera was heat inactivated (HIS) for 30 min at 56°C prior to storage. Each CF serum was treated in the same manner but not pooled. Both PNHS and CF sera were screened for the presence of anti-*B. cepacia* antibodies by ELISA as described by Nelson et al. [5]. Complement activity (CH_{50}) was determined by the sheep erythrocyte lysis assay [21]. The roles of the classical complement pathway (CCP) and the alternative complement pathway (ACP) were investigated by treating serum with 10 mM MgCl EGTA (200 mM ethyleneglycol-bis (beta-amino-ethyl ether) *N,N'*-tetraacetic acid; Sigma) which specifically inactivates the CCP by chelating Ca^{++} ions and not Mg^{++} ions as described by Fine et al. [22].

Serum bactericidal assay

Overnight cultures of *B. cepacia* grown in IST were harvested by centrifugation, and washed twice in complement fixation test buffer (CFTB, pH 7.4: Oxoid). Bacterial pellets were resuspended in CFTB to a density of 1×10^8 cfu ml^{-1}, and diluted 100-fold in CFTB to give a final suspension of 1×10^6 cfu ml^{-1}. The assay tube consisted of 1.0 or 1.6 ml CFTB with 0.8 ml or 0.2 ml PNHS, PNHS + EGTA, or CF sera respectively. Controls were 1 ml CFTB + 0.8 ml HIS and 1.8 ml CFTB. To each tube 0.2 ml of bacterial suspension was added. Assay tubes were incubated in a shaking incubator at 37°C for 180 min. Samples (100 μl) were removed at T_0, T_{30}, T_{90} and T_{180} min, diluted 1 in 50 in CFTB and 100 μl was either plated directly onto a nutrient agar (NA) plate and spread or further diluted tenfold in CFTB before being added to NA. NA plates were incubated at 37°C for 24–48 h before scoring.

For each experiment the percent killing was calculated from the number of organisms counted at T_0 and those still viable at the end of the experiment, taking into account any background decline in cell numbers as observed in the controls. The results were classified as: resistant (SR) = < 10% killing of bacteria; intermediate (I) = 10–95 killing; and sensitive (SS) = > 95% killing.

Results

With the exception of C1359 and C1602, which represent isolates of the epidemic strain of *B. cepacia*, PFGE and bacteriocin typing demonstrated no clonal relationship amongst the *B. cepacia* strains selected for this study.

Serum sensitivity of B. cepacia

The sensitivity of *B. cepacia* strains expressing both smooth or rough LPS phenotypes from different sources to the bactericidal activity of normal human serum was investigated.

Sensitivity to PNHS

Serum killing was measured at 90 min and 180 min to detect delayed killing. After 90 min, 7 (58.3%) of the isolates expressing S-LPS were resistant to serum bactericidal activity, 3 (25%) showed intermediate serum sensitivity and the remaining 2 (16.7%) were serum-sensitive. In contrast, none of the isolates expressing R-LPS were serum-resistant, 3 (42.8%) were serum-sen-

sitive and 4 (57.2%) of rough strains showed intermediate serum sensitivity. There was a marked increase in the number of *B. cepacia* strains which were serum-sensitive at 180 min (Table 1). All strains which were resistant to serum killing at 180 min and the majority of strains in which delayed killing was observed expressed S-LPS. After 180 min, 6 (85.7%) of the rough *B. cepacia* isolates were serum-sensitive, 7 (58.3%) of the *B. cepacia* strains expressing S-LPS were serum-sensitive, only 4 (33.3%) remained serum-resistant. Typical killing curves for three *B. cepacia* strains, serum-resistant (J2394), serum-sensitive (C1359) and intermediate (C1409) over 180 min are shown in Fig. 1.

Involvement of OMP in serum resistance

B. cepacia strains expressing S-LPS exhibit a range of responses to the bactericidal activity of PNHS suggesting that the presence of a full O-antigen is not the sole determinant of serum resistance. To examine the role of OMP, crude Sarkosyl OMP preparations of serum-resistant and serum-sensitive strains were examined by PAGE and Coomassie staining (Fig. 2). The two CF isolates C1359 (R-LPS) and C1409 (S-LPS) expressed fewer OMPs than the environmental strain J2394 (S-LPS), which expressed a broad band of high molecular mass (150–200 kDa) not observed in C1409 CF smooth strain, which exhibited intermediate serum sensitivity.

Serum requirement for bacterial killing

The next series of experiments were performed to confirm that the serum components required for killing of *B. cepacia* were consistent with those described for other bacterial species [11,27].

All sera used had levels of haemolytic complement within normal ranges (20–50 CH_{50} units). Serum killing of *B. cepacia* was similar using both 10% and 40% PNHS, and was proportional to the serum concentration as killing of serum-sensitive and intermediate strains was slower and complete killing was not always achieved with 10% PNHS.

Role of antibody in serum killing

In contrast to PNHS which contained negligible amounts of anti-*B. cepacia* antibodies (mean antibody titre, 400), the hyperimmune CF sera had a mean anti-*B. cepacia* antibody titre of 25 600. The bactericidal potential of PNHS and individual serum from two CF patients were comparable in their killing activity against 3 heterologous strains of *B. cepacia* and the autologous *B. cepacia* strain from each patient. However, serum from a third CF patient, taken during an acute

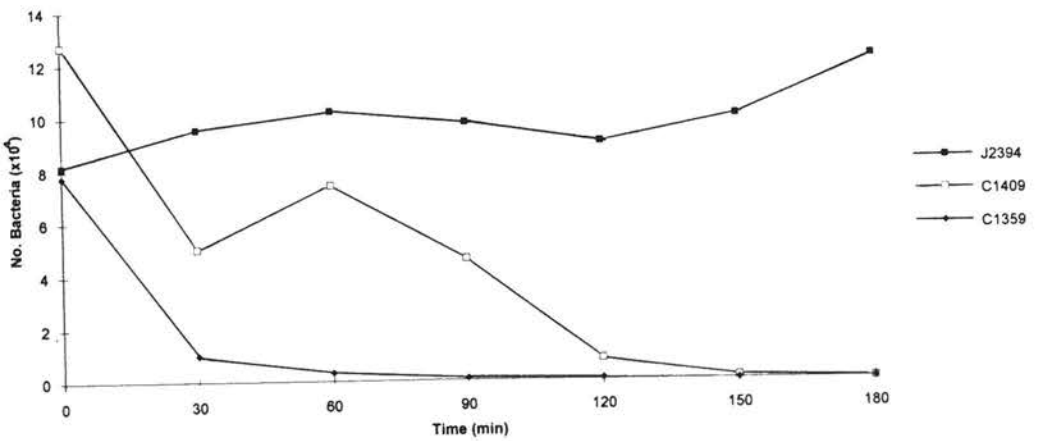

Fig. 1. Serum killing of *B. cepacia* strains J2394 (serum-resistant, S-LPS), C1409 (intermediate, S-LPS) and C1359 (serum-sensitive, R-LPS – 'Epidemic strain') by 40% PNHS over 180 min. Each point is the mean of triplicates.

pulmonary exacerbation, whilst equally active against heterologous strains of *B. cepacia* was inactive against the autologous *B. cepacia* strain. (Fig. 3).

Little or no reduction of serum killing was observed in the presence of HIS indicating that

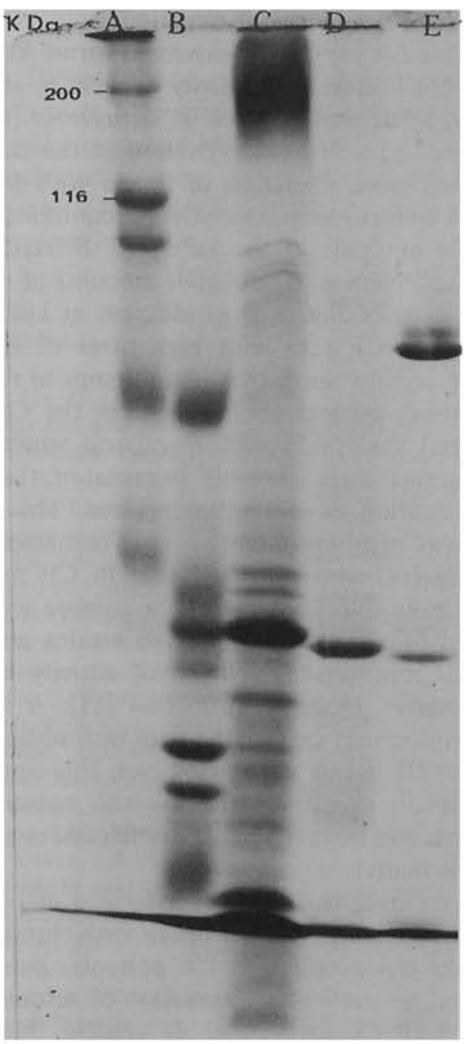

Fig. 2. SDS-PAGE of crude Sarkosyl outer membrane extracts of *B. cepacia* stained with Coomassie Blue. (A) and (B) molecular mass standards, (C) J2394 (S-LPS, serum-resistant strain) (D) C1409 (S-LPS, serum-sensitive strain) (E) C1359 (R-LPS, serum-sensitive strain).

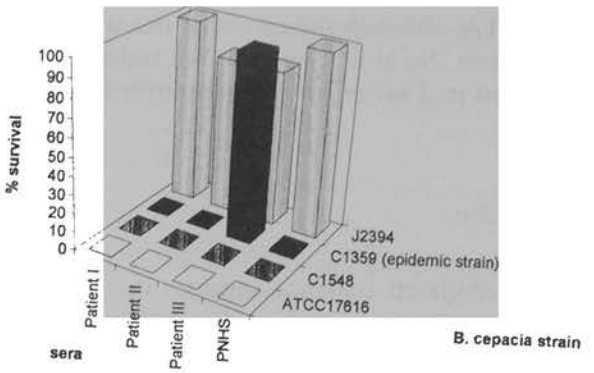

Fig. 3. Serum killing of 4 *B. cepacia* isolates by PNHS and CF serum. Patients I–III were chronically colonised by the same epidemic strain of *B. cepacia*, represented by C1359. Serum from patient III was obtained during an acute pulmonary exacerbation.

the bactericidal activity of both PNHS and CF sera was heat-labile (Table 2). To determine whether the classical complement pathway (CCP) or the alternative complement pathway (ACP) is more important in the complement mediated killing of serum-sensitive *B. cepacia*, serum killing was investigated in 10 strains which were incubated with 40% PNHS containing magnesium EGTA (in which only the ACP is functional). Serum killing of 7 of these strains representing either sensitive or intermediate killing in 40% PNHS was decreased in the 40% PNHS +

Table 2

Serum requirement for killing of *B. cepacia*

HIS	40% PNHS	Killing of *B. cepacia* strains (%)	
		Strain	40% PNHS + $MgCl_2$ EGTA
0	91	C1602	32
0	91	C1359	32
5	97	C1548	87
0	72	C1409	37
0	100	ATCC 17762	47
17	90	ATCC 17616	18
0	4	C1518	0
0	0	J2394	0

HIS, heat inactivated serum.
PNHS, pooled normal human serum.

MgEGTA, although the extent of this was strain-dependent. No alteration in serum resistance was observed in 2 serum resistant controls (Table 2).

Discussion

Accumulated evidence suggests that bacterial resistance to serum bactericidal activity is an important virulence determinant in Gram-negative bacteria [23–25]. In 19 *B. cepacia* isolates, 13 (68.5%) were sensitive to the bactericidal activity of PNHS after a 3 h period of exposure, 2 (10.5%) were intermediately sensitive and 4 (21%) were resistant. After 90 min exposure to PNHS only 5 (26.3%) of *B. cepacia* strains were serum-sensitive. The major determinant of serum resistance in *B. cepacia* appeared to be possession of a complete O-side chain. In other Gram-negative bacilli including *E. coli* and *Salmonella* species [25] the O-side chain is thought to interfere with serum killing by steric hindrance, preventing stable insertion of the membrane attack complex, and it seems reasonable to speculate that a similar mechanism operates in *B. cepacia*.

There is evidence that resistance to serum killing does not rely solely on a single factor, for example LPS, and other cell surface components including OMPs and capsular polysaccharide have been implicated [24,25]. Some *B. cepacia* strains may produce an extracellular polysaccharide when grown in appropriate nutrient-deficient conditions. This material has been observed in both serum-resistant and serum-sensitive strains expressing S-LPS, but has not been observed in strains expressing R-LPS (S. Butler, unpublished observations). Analysis of the outer membrane proteins of three strains of *B. cepacia* revealed that neither the rough strain *B. cepacia* C1359 nor the smooth strain C1409 expressed an OMP of between 150–200 kDa which was expressed by the highly serum-resistant strain J2394. It is possible that the presence of this protein may complement the protective action of the O-side chain in inhibiting stable insertion of the membrane attack complex into the outer membrane. Expression of cell surface components is influenced by both growth conditions and growth phase and the in vitro serum susceptibility observed may not accurately reflect the in vivo situation [6,26]. Anwar et al. [7] reported that the nutrient conditions under which a single strain of *B. cepacia* was grown had a profound effect on the killing by serum factors and polymorphonuclear leukocytes. It was proposed that variation in outer membrane protein and phospholipid concentration resulting from growth in nutrient depleted media was responsible for varying sensitivity to serum killing.

Serum bactericidal activity against *B. cepacia* is heat-labile and, as with *P. aeruginosa* [11,27], appears to result from activation of the classical pathway, since chelation of serum with $MgCl_2$-EGTA reduces killing activity. A requirement for specific antibody in the killing of *B. cepacia* is unclear. PNHS with negligible amounts of anti-*B. cepacia* antibodies, was as efficient at killing *B. cepacia* as CF sera with high titres of specific anti-*B. cepacia* antibodies. An attempt to remove the anti-*B. cepacia* antibodies from the CF sera by serial absorption with *B. cepacia* whole cells was unsuccessful, possibly because of the high concentration of antibodies present. This problem was also encountered by Thomassen and Demko [11] who proposed that in CF patients chronically colonized with *P. aeruginosa* with advanced lung disease, autologous strains are protected from serum bactericidal activity by the presence of blocking antibodies [11]. A similar mechanism may explain the data with serum from patient III in this study. However, this could not be proven experimentally for the reasons described, and further studies are necessary to clarify this matter.

In contrast to the well-recognised association of serum-sensitive *P. aeruginosa* with chronic pulmonary colonization of CF patients, our study showed no particular association of serum sensitivity with CF isolates of *B. cepacia*. McKevitt and Woods reported that the majority of CF isolates of *B. cepacia* express R-LPS [28]; it seemed reasonable to assume therefore that a majority of CF isolates would also be serum-sensitive. In other infections, for example *P. aeruginosa* colonisation of CF lungs [11,26] and *E. coli* urinary tract infections [26] strains are typically

serum-sensitive strains, express R-LPS and are not associated with bacteraemia. Our finding that rough forms of *B. cepacia* are associated with bacteraemia in CF suggests that the in vivo characteristics of the organism are different from those observed in vitro, or that *B. cepacia* resists serum killing by an as yet unknown mechanism.

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MiniReview

Virulence factors of *Burkholderia cepacia*

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Introduction

Originally named following its identification as a cause of soft rot in onions, *Burkholderia cepacia* has also been known as *Pseudomonas multivorans* and *Pseudomonas kingae* [1,2], and until most recently, as *Pseudomonas cepacia* [3]. A proposal for the transfer of 7 species of the genus *Pseudomonas* RNA Homology group II to a new genus *Burkholderia* with the type-species *Burkholderia cepacia* has been validated [3]. For the purpose of this review the name *B. cepacia* will be used.

Once considered solely as a phytopathogen, *B. cepacia* is now recognized as an important pathogen in nosocomial infection and in patients with chronic granulomatous disease and particularly in those with cystic fibrosis (CF). CF is the most common autosomal recessive lethal disease in Caucasian populations with an incidence of approximately 1 in 2500 live births and a carrier frequency of 1 in 20. The basic cause of the pathophysiological symptoms of CF is a defect in epithelial ion transport which results in viscous dehydrated bronchopulmonary and gastrointestinal secretions. Build-up of viscid mucus is associated with impaired mucociliary clearance and susceptibility to bacterial colonization which in turn initiates a vicious cycle of chronic inflammatory reaction. The susceptibilty of CF patients to pulmonary colonization has been recognised since the earliest descriptions of the disease when patients seldom survived infancy. Advances in management of CF have meant that today most patients survive to early adulthood. However, this increased longevity has in part created its own problems including the emergence of new opportunistic pathogens, including and most notably, *B. cepacia*.

The last decade has seen *B. cepacia* emerge as a particular problem amongst patients with CF, where colonization may be symptomless or associated with a slow decline in lung function. A more serious clinical outcome, not observed with other CF pathogens, in which some colonized CF patients experience *B. cepacia* bacteraemia and/

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or succumb to an accelerated and fatal deterioration in pulmonary function [4–7], is central to the current concern over *B. cepacia* in the CF community.

Based on nucleic acid homology, *B. cepacia* is more closely related to *B. pseudomallei*, *B. mallei* and *B. gladioli* than to *P. aeruginosa* and other fluorescent pseudomonads, and was placed in the separate subgroup, *Pseudomonas* RNA homology group II. *B. cepacia* is nutritionally versatile, with minimal growth requirements and the ability to survive in unfavourable environments: it has been isolated from disinfectants and antiseptics and can even use penicillin G as a nutrient [8–11]. The organism is intrinsically resistant to most antibiotics, and even if individual strains show in vitro susceptibility to an antibiotic, there is little clinical response [10,12–15].

In contrast to the large amount of information on *P. aeruginosa* virulence factors, knowledge of the virulence factors and pathogenesis of *B. cepacia* is scanty. Animal models have indicated that *B. cepacia* is less virulent than *P. aeruginosa* [16]. The aim of this review is to discuss the main features and properties of *B. cepacia* and, in particular, to focus on those which may contribute to its ability to colonize patients with CF.

Colonization and adherence

The ability of a potential pathogen to adhere to the host mucosal or epithelial cell surfaces is often pivotal in the subsequent establishment of infection. Few potential adhesins have been described for *B. cepacia*, and most attention to date has been focused on the adhesive properties of fimbriae.

Electron microscope studies have shown that approximately 60% of *B. cepacia* strains express peritrichous fimbriae (see Fig. 1) [17,58]. Other *B. cepacia* strains possess polar fimbriae, similar to those expressed by *B. aeruginosa* [18]. Kuehn et al. [17] showed that outer membrane protein preparations of *B. cepacia* were enriched with 3 proteins (16, 20 and 40 kDa) which were not present in a non-fimbriated strain. The fimbrial subunit was identified as the 16 kDa protein; the protein appeared similar to those seen in other bacteria and showed homology with PAK fimbriae of *P. aeruginosa* [17]. This data contrasts with that of Saiman et al. who found minimal cross-reactivity with anti-*P. aeruginosa* anti-pilin monoclonal antibodies and no homology between *P. aeruginosa* pilin gene probes and *B. cepacia* genomic DNA [18,19]. It is possible that sequence variation exists among the pilin genes of different *B. cepacia* strains and that any individual pilin gene probe from *P. aeruginosa* may not reveal a specific *B. cepacia* gene [17].

The presence of fimbriae increases the ability of *B. cepacia* to adhere to pneumocytes in vitro [17]. In vitro binding experiments by Kirvan et al. [20] demonstrated that both *B. cepacia* and *P. aeruginosa* adhere to the same Galβ1–4GalNAc sequence present in many asialoglycolipids. The experiments of Saiman et al. [19] did not demonstrate competition for epithelial receptors, indicating that different epithelial receptors may be used preferentially by each of the *Pseudomonas* species or that the bacteria may bind to each other. Binding of 2 *B. cepacia* strains to epithelial monolayers increased in the presence of *P. aeruginosa* indicating a possible synergistic relationship whereby *P. aeruginosa* exo-products modify epithelial cell surfaces, exposing receptors and facilitating increased *B. cepacia* attachment [19]. It must be stressed, however, that not all CF patients are colonized with *P. aeruginosa* prior to acquisition of *B. cepacia*: in the Edinburgh CF clinic 38% of patients with *B. cepacia* are not co-colonized with *P. aeruginosa* [6].

Sajjan et al. [21] were able to demonstrate specific binding of *B. cepacia* isolates from patients with CF to both CF and non-CF mucins as well as to buccal epithelial cells. Unfortunately no typing data was available to exclude the possibility of clonal relationships between the strains [21]. The degree of binding observed with *B. cepacia* is considerably less than that observed with *P. aeruginosa* [58]. Deglycosylation of mucin indicated that the mucin receptors for *B. cepacia* include N-acetylglucosamine and N-acetylgalactosamine. Isolates exhibiting the highest mucin binding values tended to correlate with those patients with severe illness leading to speculation

that variability in the binding of different *B. cepacia* isolates to respiratory mucin may contribute to morbidity and mortality, and may explain why some *B. cepacia* strains colonize patients transiently whereas other strains, once acquired, are never lost. A sparsely distributed 22 kDa pilin-associated protein was identified as a mucin binding adhesin specific to piliated strains of *B. cepacia* [22].

Siderophores

Production of siderophores enables bacteria to compete for iron with host iron-binding proteins including transferrin and lactoferrin and has been correlated with the ability of various bacteria to establish and maintain infection. *B. cepacia* strains express at least 3 siderophore-mediated iron transport systems, including pyochelin, cepabactin and azurechelin [23–26]. Pyochelin produced by *B. cepacia* is chemically unrelated to the pyochelin siderophore of *P. aeruginosa* [23,24]. A 14 kDa ferripyochelin binding protein, present in increased amounts in the outer membrane of iron-starved *B. cepacia* cells, has also been described [23]. Morbidity and mortality in infected CF patients has been correlated to the production of pyochelin. Although such evidence may point to a role in pathogenicity, half of the clinical isolates investigated by Sokol [23] were pyochelin-negative. Pyochelin may increase the ability of *B. cepacia* to disseminate throughout the lungs and perhaps induce a greater inflammatory

Fig. 1. *Pseudomonas cepacia* J1359 in the process of dividing into 2 separate cells which exhibit peritrichous pili. Staining is 2% w/v phosphotungstic acid. Magnification ×25000.

response due to the increased area of infection in the lung [27]. Exogenously supplied pyochelin enhanced the virulence of non-pyochelin producing *B. cepacia* strains in a chronic pulmonary model in rats [27]. Meyer et al. [26] showed that *B. cepacia* ATCC25416 excreted both pyochelin and a lower molecular mass compound, cepabactin, which strongly chelated Fe III and facilitated iron translocation. Azurechelin, another distinct iron-binding compound, has been identified in 88% of *B. cepacia* strains isolated from the respiratory tract [25].

Extracellular virulence factors

B. cepacia produces a number of extracellular products including protease, gelatinase, haemolysin and lipase, although no pathogenic role for these factors has been demonstrated [28,29]. Unlike *P. aeruginosa*, *B. cepacia* does not appear to produce toxin A, exoenzyme S, or other detectable extracellular factors capable of producing a cytotoxic effect in vitro [28]. In a study of putative pathogenic factors of *B. cepacia* [30] a number of characteristics were demonstrated more frequently in isolates from CF patients than control isolates. These factors included production of catalase, ornithine decarboxylase, valine aminopeptidase, C14 lipase, alginase, and trypsin; reduction of nitrate to nitrite; hydrolysis of urea and xanthine and complete haemolysis on bovine red blood cells. The role of any of these factors in respiratory colonization or infection in CF patients is not clear [30]. Indeed, an epidemic strain of *B. cepacia* CF5610 associated with fatal clinical outcome in CF does not produce C14 lipase or haemolysis [6].

Molecular studies of the *Pseudomonas* exotoxin A gene by Vasil et al. [31] concluded that the production of exotoxin A and the presence of the exotoxin A gene are probably limited to *P. aeruginosa* and is not found in other *Pseudomonas* spp. Southern hybridization experiments under low, medium and high stringency conditions with an exotoxin A gene probe failed to produce a positive signal with any of 8 *B. cepacia* strains tested. Similar experiments conducted by ourselves also failed to demonstrate the presence of the exotoxin A gene in 3 environmental strains of *B. cepacia* although a positive band was obtained with *B. cepacia* CF5610 strain isolated from a patient with CF. However, growth of this strain in both iron replete and depleted medium and subsequent analysis of the cell free culture supernate by polyacrylamide gel electrophoresis and immunoblot analysis with anti-*P. aeruginosa* exotoxin A antisera, failed to confirm production of a 66 kDa protein equivalent to *P. aeruginosa* toxin A. (J.W. Nelson, unpublished results).

The extracellular proteinase of *B. cepacia*, a 34 kDa protein, has antigenic similarities to *P. aeruginosa* elastase and cleaves gelatin, hide powder, collagen but not human immunoglobulin IgG, IgM, secretory IgA, or IgA [32]. Intratracheal instillation of purified proteinase into rat lungs produces a bronchopneumonia characterized by polymorphonuclear cell infiltration and proteinaceous exudation into large airways. Active immunization of rats with *B. cepacia* proteinase elicits an immunological response although this is not protective against subsequent lung infection with *B. cepacia* [32].

There is also evidence that lipases, particularly phospholipases, may play an important role in bacterial virulence [33,34]. Phosphoplipase C is an enzyme that cleaves phosphatidylcholine, a major lung surfactant, to yield phosphorylcholine and diacylglycerol, and has been associated with cytopathology of lung tissue. *B. cepacia* has frequently been described as being lipolytic [28,33–35]. McKevitt and Woods [28] reported that 32 of 48 strains of *B. cepacia* isolated from CF patients demonstrated lipase activity on egg-yolk agar whilst Carson et al. [35] showed that *B. cepacia* could hydrolyse Tween 20, 40, and 80. In another study [34] 6 out of 10 clinical strains of *B. cepacia* from the sputum of CF patients produced lecithinase by the egg-yolk reaction, whilst lipase activity on 4 different Tweens was strain-dependent. Purified enzyme had a molecular weight of 25 000 and was not cytotoxic for Hela cells or for mice injected intravenously with purified lipase. It has been reported, however, that lipase adversely affected the phagocytic function of rat pulmonary

alveolar macrophages in a dose-dependent manner [36]. Phagocytosis of *B. cepacia* by rat pulmonary alveolar macrophages was significantly reduced when the cells were either preincubated with lipase or when phagocytosis occurred in the presence of the lipase [36]. Scanning electron microscopy showed that the macrophages exposed to *B. cepacia* lipase had fewer pseudopodia, microvilli and other projections compared to untreated macrophages. Thus *B. cepacia* lipase may be an important virulence factor which allows the bacteria to evade the mammalian host defence system.

B. cepacia produces a heat-labile haemolysin which has both phospholipase C and sphingomyelinase activities [37]. Haemolytic and phospholipase C (lecithinase) expression in *B. cepacia* appears to be a complex phenomena. The study of Nakazawa et al. [29] found that only 4% of clinical isolates were β-haemolytic, whilst 67% of isolates produced lecithinase. Others have found higher percentages of haemolytic isolates of *B. cepacia* if a variety of erythrocyte types were tested, including a study of clinical isolates of *B. cepacia* from CF patients which found that 40% were haemolytic when erythrocytes from various animals were tested [37]. Unlike the PLC activity of *P. aeruginosa* the PLC activity in *B. cepacia* does not correlate with haemolytic activity [29,37]. However, all haemolytic strains produce detectable lecithinase activity, and strains of *B. cepacia*, whether haemolytic or non-haemolytic, appear to produce detectable amounts of extracellular PLC activity. In contrast to the consistent patterns observed in the PLC gene of *P. aeruginosa* there is hypervariability in genetic organization of the PLC gene of *B. cepacia* [37]. The variable manner in which a *B. cepacia* PLC specific gene probe hybridizes with restricted *B. cepacia* DNA, the variability in expression of haemolytic and PLC activities of different strains, and the association of DNA arrangements with conversion of an Hly + to an Hly − variant may be related to the relatively large number of distinct insertion sequences (IS) reported for *B. cepacia* (> 25) [37,38]: in contrast these elements have yet to be discovered in *P. aeruginosa*. Some of these IS elements of *B. cepacia*, can be found in multiple copies and have been shown to both activate or inactivate gene expression.

Cell surface antigens

Lipopolysaccharide

B. cepacia strains isolated from patients with CF may express either the rough (R) or smooth (S) lipopolysaccharide (LPS) phenotype, whereas the majority of *B. cepacia* strains isolated from other clinical conditions or from the environment express S-LPS (S.L. Butler, unpublished results). This is in agreement with the study of McKevitt and Woods [28] where 22 strains examined possessed S-LPS and 26 strains possessed R-LPS. The epidemic strain of *B. cepacia* isolated from a number of CF patients in the UK invariably has a R-LPS phenotype and is associated with the appearance of dry colonies [6]. There is no evidence to date to confirm that *B. cepacia* strains undergo a phenotypic change from S to R LPS within the CF lung as is observed with *P. aeruginosa*.

Western blotting and absorption studies demonstrated that a significant proportion of serum antibodies from *B. cepacia*-infected CF patients which reacted with the core LPS of *B. cepacia* did not react with the core LPS of *P. aeruginosa* [39]. These observations indicate differences in the structure and composition of core LPS between *B. cepacia* and *P. aeruginosa* confirming previous findings, including the lack of phosphorus in the core of *B. cepacia* LPS [40] and the inability of a monoclonal antibody reactive with *P. aeruginosa* and *P. fluorescens* core LPS to react with *B. cepacia* [41]. Core heterogeneity between different isolates of *B. cepacia* may also exist because immunoblotting demonstrated that serum from patients colonized with *B. cepacia* produced a band reactive with some but not all core LPS preparations [39].

Initial chemical analysis of *B. cepacia* LPS indicated the absence of detectable 3-deoxy-D-manno-2-octulosonic acid (KDO) in LPS from *B. cepacia* [42,43]. However, Straus et al. [44] reported the isolation of KDO from the culture supernate of 2 out of 10 strains of *B. cepacia* and

in a further study KDO was demonstrated in 6 clinical isolates of *B. cepacia* and all 6 LPS preparations were equally toxic for mice when injected intraperitoneally [45]. Compared with LPS from *P. aeruginosa* that from strains of *B. cepacia* has less phosphorus and more heptose. Glucose and rhamnose were the major saccharide components of LPS from the organisms tested [42]. An extracellular material isolated from a clinical *B. cepacia* consisted of a surface carbohydrate antigen, LPS and protein, the toxicity of which appeared to be associated with the LPS portion of the complex [46]. It has been proposed that this extracellular toxic complex produced by *B. cepacia* is responsible for the lethality and extensive pulmonary tissue necrosis associated with pneumonia produced by this organism.

Outer membrane proteins

B. cepacia produces 5 major outer membrane proteins A (56 kDa), B (38 kDa), C (37 kDa), D (28 kDa) and E (21 kDa). The C and D proteins have been identified as porin proteins [47,48], and appear to be antigenic in most patients with CF who are chronically colonized with *B. cepacia* [48,49]. In the study of Anwar et al. [55], outer membrane protein profiles of magnesium-depleted cells were much simpler than that of iron-depleted cells and nutrient broth grown cells. Synthesis of a 66 kDa outer membrane protein was induced when *B. cepacia* was grown under iron depletion. *B. cepacia* isolates from individual CF patients may exhibit marked phenotypic variability, including manifestation of different patterns of outer membrane proteins separated on a polyacrylamide gel: up to 5 OMP patterns have been identified from *B. cepacia* isolates derived from a single strain [50].

Various studies indicate that the outer membrane of *B. cepacia* is a major contributing factor in the β-lactam resistance of this species, retarding the diffusion of β-lactams to their penicillin-binding protein targets [47,51,52]. Resistance to aminoglycosides and hydrophobic compounds in *B. cepacia* is largely due to the low outer membrane permeability [51]. Loss of the major porin protein D and decreased expression of protein C may also be associated with high level β-lactam resistance in some CF isolates of *B. cepacia* [52]. Production of β-lactamases, including carbapenases capable of hydrolyzing the most potent and broad spectrum of the β-lactam antibiotics, imipenem and meropenem, also contribute significantly to the resistance of *B. cepacia* [53].

Exopolysaccharide

Production of alginate by mucoid strains of *P. aeruginosa* is the major virulence determinant associated with strains which colonize the lungs of patients with CF. In contrast, *B. cepacia* does not appear to produce alginate. PCR studies with primers of the *P. aeruginosa alg* D gene, encoding the essential enzyme GDP mannose dehydrogenase, indicate that this gene was absent in 10 *B. cepacia* strains studied and therefore that *B. cepacia* is unlikely to produce an alginate-like polymer (J.W. Nelson, unpublished results). Additional studies in our laboratory and by Sage et al. [54] showed that some *B. cepacia* strains do produce an exopolysaccharide comprising galactose, glucose, mannose, glucuronic acid and rhamnose, with lesser amounts of uronic acid: no mannuronic or guluronic acid was detected. Surveys of clinical isolates from patients with CF indicate that there is no correlation between the ability of *B. cepacia* to colonize the respiratory tract and capacity to form exopolysaccharide [6,54]. In contrast, Straus et al. [46] observed that 1 strain of *B. cepacia* produced an alginate-like compound containing 72% guluronic acid with 1.75% acetylation.

Evasion of the immune system

Immunological studies on *B. cepacia* colonization of patients with CF indicate that the organism persists despite a considerable antibody response and suggest the possibility of immune-mediated damage. Aronoff et al. [48,49] demonstrated the presence of IgG antibodies to outer membrane antigens of *B. cepacia* in serum from patients with CF colonized with *B. cepacia* and/

or *P. aeruginosa*. These authors concluded that some *B. cepacia* outer membrane components may be antigenically related to those of *P. aeruginosa* and that colonization with *B. cepacia* occurs in the presence of antibodies specific for the outer membrane of the organism. Serum IgG and sputum IgA antibodies directed towards the core LPS of *B. cepacia* have also been described [39].

Investigations into the bactericidal effect of human serum have shown a large variation in the responses of the *B. cepacia* strains investigated. All strains expressing R-LPS were serum-sensitive under a variety of test conditions whilst strains expressing S-LPS exhibit a range of responses (S.L. Butler, unpublished results). Anwar et al. [55] showed that a *B. cepacia* strain grown in different nutrient depletions in batch culture showed varying degrees of sensitivity to engulfment and killing by human polymorphonuclear leucocytes (PMN) and to killing by human serum. The wide range of sensitivity shown by the organism may reflect the phenotypic variation in cell envelope composition caused by specific nutrient depletions. Patients with chronic granulomatous disease (GCD) are at particular risk of infection with *B. cepacia*, which is able to resist neutrophil-mediated non-oxidative bactericidal killing (D.P. Speert, personal communication). The ability of *B. cepacia* to survive a pronounced humoral response and other immunological defences is intriguing and requires further investigation. Indeed there is a suggestion that *B. cepacia* may invade and survive within respiratory epithelial cells, enabling the organism to persist within the CF lung [56].

Concluding remarks

Acquisition of *B. cepacia* is a major concern among patients with CF although the exact pathophysiological role of the organism remains controversial and unsolved. Epidemiological data and the use of phenotypic and genotypic typing systems for *B. cepacia* suggest that certain strains are particularly transmissible, although there is no evidence at present that some strains are more virulent. The role of any of the described virulence factors of *B. cepacia* relating to its pathogenesis in patients with CF remains unclear. Enhanced adhesion to mucin of certain *B. cepacia* strains may aid initial colonization whilst multiresistance to antibiotics and possible intracellular localization may contribute to persistence of the organism. Production of anti-*B. cepacia* antibodies by the host and subsequent immune complex mediated damage, is probably responsible for pulmonary decline. The development of a CF mouse model carrying precise and clinically relevant mutations [57] will allow in vivo investigation of *B. cepacia* colonisation and virulence factors. Current concern over *B. cepacia* colonisation amongst CF patients has highlighted the urgent need to identify *B. cepacia* colonising factors and the pathophysiological and/or immunological factors which account for the rapid clinical deterioration in some CF patients.

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Serum IgG and sputum IgA antibody to core lipopolysaccharide antigen from *Pseudomonas cepacia* in patients with cystic fibrosis

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Summary. The immunological response of cystic fibrosis (CF) patients to lipopolysaccharide (LPS) antigens of *Pseudomonas cepacia* was investigated. Enzyme-linked immunosorbent assays (ELISA) with either *P. cepacia* whole cells or extracted core LPS from a clinical isolate of *P. cepacia* as antigen were used to measure serum IgG and sputum IgA anti-*P. cepacia* antibodies. The ELISA with core LPS distinguished nine CF patients colonised by *P. cepacia* from nine age- and sex-matched non-colonised CF patients. The rate of increase of anti-*P. cepacia* IgG antibodies after bacteriologically proven *P. cepacia* colonisation varied in individual patients: in some patients the first isolation of *P. cepacia* was preceded or accompanied by a two-to-four-fold rise in anti-*P. cepacia* LPS IgG titres. Absorption studies and immunoblot analysis of serum from patients colonised with *P. cepacia* demonstrated that a significant component of the anti-*P. cepacia* core LPS antibodies was specific for *P. cepacia* and did not react with the core LPS of *P. aeruginosa*. Immunoblotting also illustrated that there may be a degree of core heterogeneity between different isolates of *P. cepacia*. Detection of *P. cepacia* LPS specific antibodies in serum (IgG) and sputum (IgA) from CF patients is recommended to assist the identification of *P. cepacia* colonisation in CF patients.

Introduction

Pseudomonas cepacia is a major pulmonary pathogen in patients with cystic fibrosis (CF).[1–3] Originally considered a phytopathogen, the organism has been isolated with increasing frequency from patients with CF[3–6] and is also recognised as an important aetiological agent in nosocomial infection.[7,8] The possession of innate and inducible resistance to many anti-pseudomonal antibiotics[9,10] and the ability to survive under conditions of minimal nutrition or in the presence of certain disinfectants contribute to the role of *P. cepacia* as a formidable nosocomial pathogen.[8,9] Prevention and treatment of pulmonary infections due to *P. cepacia* present a major challenge. The association of *P. cepacia* with the CF lung is complex, and clinical sequelae include rapidly fatal deterioration of pulmonary function, long term colonisation accompanied by a slow decline in lung function, and chronic asymptomatic carriage.[11]

Although the humoral response of CF patients to *P. aeruginosa* antigens has been investigated extensively,[12] there are relatively few reports defining the response to *P. cepacia* antigens.[13–14] The aim of this study was to investigate the antibody response of CF patients to *P. cepacia* whole-cell and extracted lipopolysaccharide (LPS) antigens. The extent to which antibodies cross-react with *P. cepacia* and *P. aeruginosa* core LPS was also analysed.

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Materials and methods

Patients

Nine patients (five female and four male, mean age 21·6 years, range 16–27 years) attending the Edinburgh adult CF clinic, were identified as being persistently colonised by *P. cepacia* (PC+) by serial bacteriological sputum cultures (at least three consecutive samples positive). For each patient an age- and sex-matched CF control was selected from whom *P. cepacia* had never been isolated (PC−).

Serum and sputum samples

Serum samples were obtained from CF patients and from healthy blood donors at the Blood Transfusion Centre, Edinburgh; all were stored at −20°C. Sputum used for antibody analysis was centrifuged at 10000 *g* for 15 min and the supernate was stored at −70°C. Control sputum samples obtained from patients with chronic bronchitis were processed as for CF samples. Whenever possible, sputum and serum were obtained

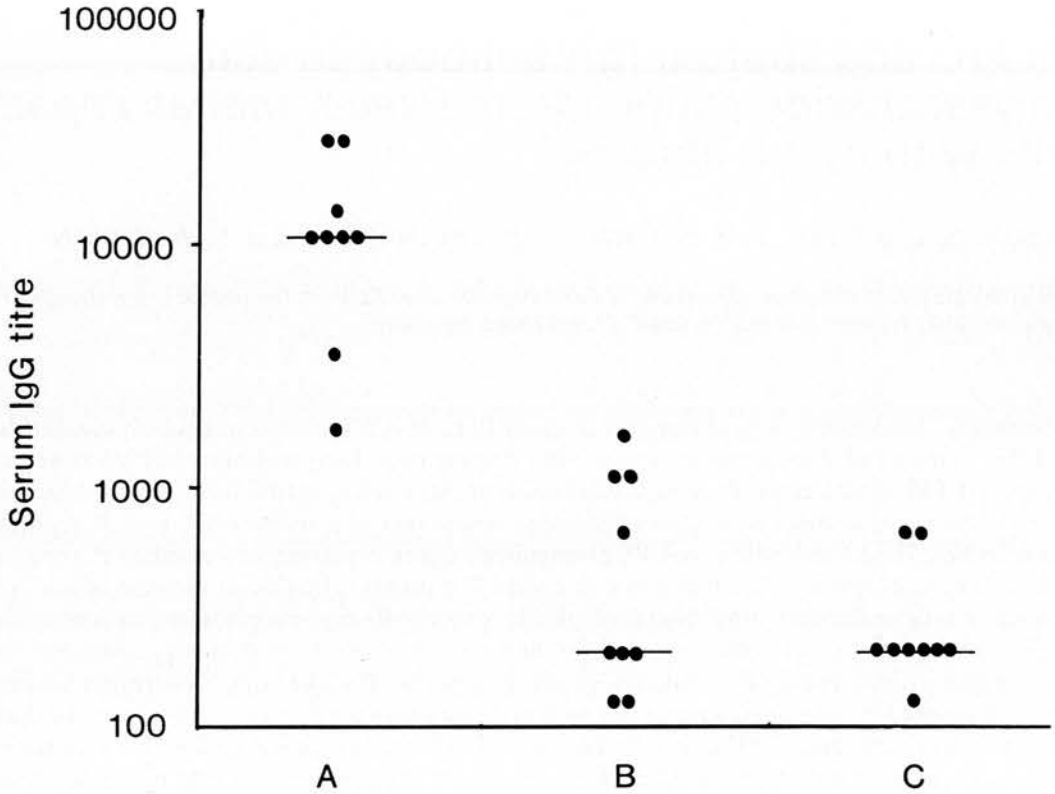

Fig. 1. Serum IgG anti-*P. cepacia* core R-LPS antibodies in (**A**) nine CF patients persistently colonised with *P. cepacia* (titre range 3200–51200), (**B**) nine non-colonised CF patients (titre range 200–3200) and (**C**) nine healthy controls (titre range 200–800) measured by ELISA with core R-LPS as coating antigen; bars represent median values. Antibody titres for the PC+ CF patients were from serum samples obtained after colonisation with *P. cepacia* was confirmed by bacteriological culture.

from CF patients at the same time. Specimens from PC+ and PC− CF patients, including those with and without *P. aeruginosa* colonisation, were subjected to detailed immunological analysis; these patients were designated I–IX (see *Results*).

Bacteriological analysis of sputum

Bacteriological examination was performed on sputum which had been homogenised in sputalysin (Calbiochem, La Jolla, CA, USA). After appropriate dilutions, sputum was cultured quantitatively on blood agar, horse digest agar, Pseudomonas Isolation Agar (Difco), and *P. cepacia* Selective Medium (Mast Laboratories, Bootle). Bacteria cultured on *P. cepacia* selective medium were identified biochemically by the API 20 NE system (API System, La Balme les Grottes, France).

Bacterial strains

P. cepacia reference strains J1780 (serotype 1), J1680 (serotype 2), J1705 (serotype 3), J1774 (serotype 4), J1479 (serotype 5), J1772 (serotype 6), J1690 (serotype 7), J1687 (serotype 8), J1758 (serotype 9) and J1745 (non-typable) were obtained from the Centers for Communicable Disease Control (Atlanta, GA, USA). The serotype was based on the serotyping scheme proposed by Heidt *et al.*[15] *P. cepacia* strains J1359 (non-typable), SBC21 (non-typable), SBC42 and SBC29 were isolated from the sputum of CF patients. *P. cepacia* SBC8 was an environmental strain isolated from a plant. Serotyping sera were unavailable for the typing of strains SBC42, SBC29 and SBC8, but these were confirmed as distinct strains by ribotyping.[16] *P. aeruginosa* R-mutant PAC608, defective in the production of LPS, was obtained from Professor P. M. Meadow (University College, London).

Preparation of LPS

The aqueous phenol, chloroform, petroleum ether method of Galanos *et al.*[17] incorporating the diethyl ether precipitation of LPS described by Qureshi *et al.*[18] (as described by Hancock and Poxton[19]) was used to prepare LPS from *P. cepacia* strains expressing R-form LPS. Briefly, LPS was extracted from washed, freeze-dried bacteria from an overnight culture, washed and purified by centrifugation at 100000 *g* for 4 h and freeze-dried. The proteinase K digestion method[20] was also used to prepare LPS for analysis by polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

SDS-PAGE and immunoblotting

LPS was separated on polyacrylamide 14% gels with the buffer system of Laemmli[21] (except that

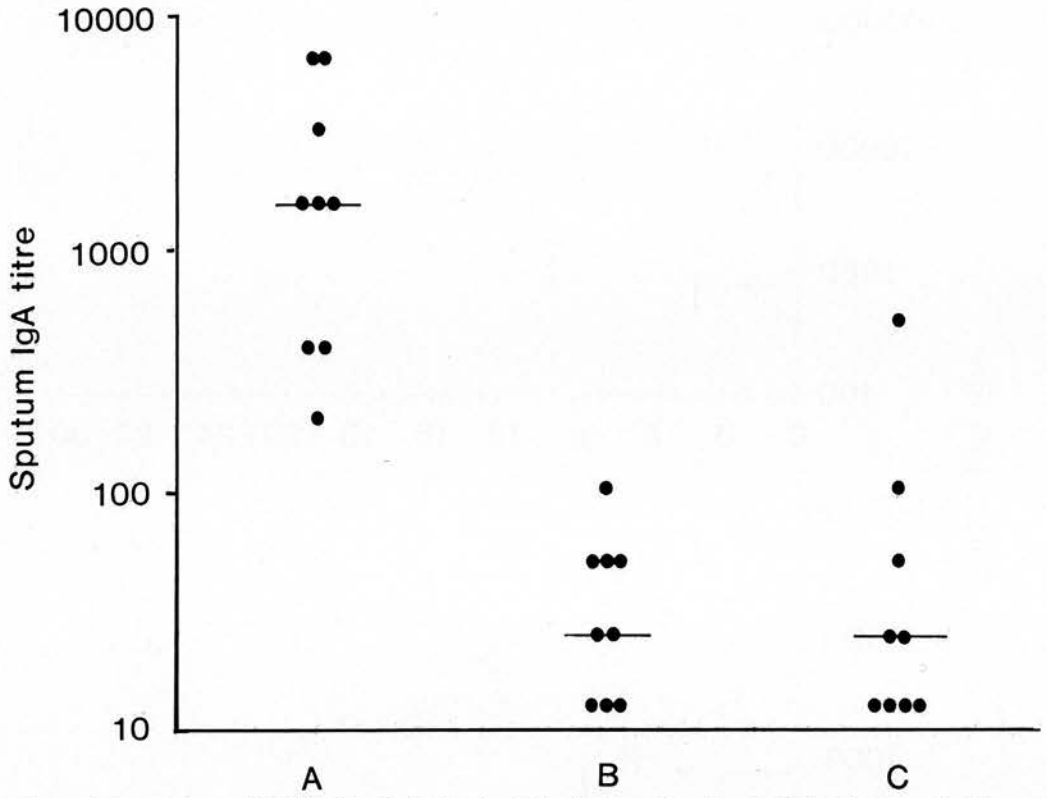

Fig. 2. Sputum IgA anti-*P. cepacia* core R-LPS antibodies in (**A**) nine CF patients persistently colonised with *P. cepacia* (titre range 200–6400), (**B**) nine non-colonised CF patients (titre range 25–100) and (**C**) nine chronic bronchitics (titre range 25–400) measured by ELISA with core R-LPS as coating antigen; bars represent median values. Antibody titres for the PC+ CF patients were from sputum samples obtained after colonisation with *P. cepacia* was confirmed by bacteriological culture.

sodium dodecyl sulphate was omitted from the stacking and separating gel buffers in gels used for immunoblotting). The LPS separating gels were stained with silver by the method of Tsai and Frasch.[22] For immunoblotting, separated antigens were transferred to nitrocellulose membranes (pore size 0·2 μm, Schleicher and Schuell, Dassel, Germany) by the method of Towbin *et al.*[23] Antigens were probed with serum diluted 1 in 200 and sputum diluted 1 in 100 for 3 h at room temperature, and the immune complexes were detected with anti-human IgG and IgA horseradish peroxidase conjugates (ICN Biomedicals Ltd, High Wycombe) and HRP colour development reagent (BioRad Laboratories, Richmond, USA).

Enzyme-linked immunosorbent assay (ELISA)

Antigens used for coating microtitration plates were: (a) *P. cepacia* whole cells, serotypes 1–9 and a non-typable strain; and (b) extracted LPS from a rough isolate, *P. cepacia* J1359. For the whole-cell assay, bacteria were grown overnight in nutrient broth containing yeast extract 0·5% w/v (NYB), harvested and washed twice with PBS. Cells were resuspended to a density of 10^7 cells/ml in carbonate-bicarbonate coating buffer (pH 9·6) and 100 μl was added to the wells of polystyrene "Polysorb" microtitration plates (Nunc, Roskilde, Denmark). Plates were centrifuged at 1365 *g* for 5 min to sediment bacteria on to the wells. In the case of the LPS antigen, extracted LPS was complexed with polymyxin as described by Scott and Barclay[24] and used at a final concentration of 10 ng/ml. LPS-polymyxin complexes were diluted in coating buffer and added to microtitration plates at 100 μl/well. All plates were coated overnight at room temperature and washed four times with wash buffer (PBS, pH 7·2, containing Tween 20 0·05% w/v and sodium azide 0·02% w/v). All plates were then post-coated with post-coat buffer consisting of PBS containing bovine serum albumin (BSA) 5% w/v, at 100 μl/well. After being washed four times with wash buffer, plates were stored at −20°C until used.

Serum and sputum samples were serially diluted in dilution buffer and added to coated microtitration plates at 100 μl/well in triplicate. Antibody diluent consisted of PBS (pH 7·2) containing Tween 20 0·05% v/v, BSA 0·5% w/v, polyethylene glycol 6000 4% w/v and sodium azide 0·002% w/v. After incubation at 37°C for 90 min, plates were washed four times with wash buffer. Conjugates, including alkaline phosphatase conjugated to anti-human IgG or anti-human IgA (Sigma) were diluted 1 in 1000 in dilution buffer and added at 100 μl/well. Plates were incubated for a further 90 min at 37°C and then washed and rinsed before addition of alkaline phosphatase substrate (Sigma) at 100 μl/well. After incubation for 30 min at room temperature, the optical density (OD) of wells was read at 405 nm in a Titertek Multiscan plate reader (Flow Laboratories Ltd, Irvine). Final results were expressed as the titre giving an OD > 0·1 after

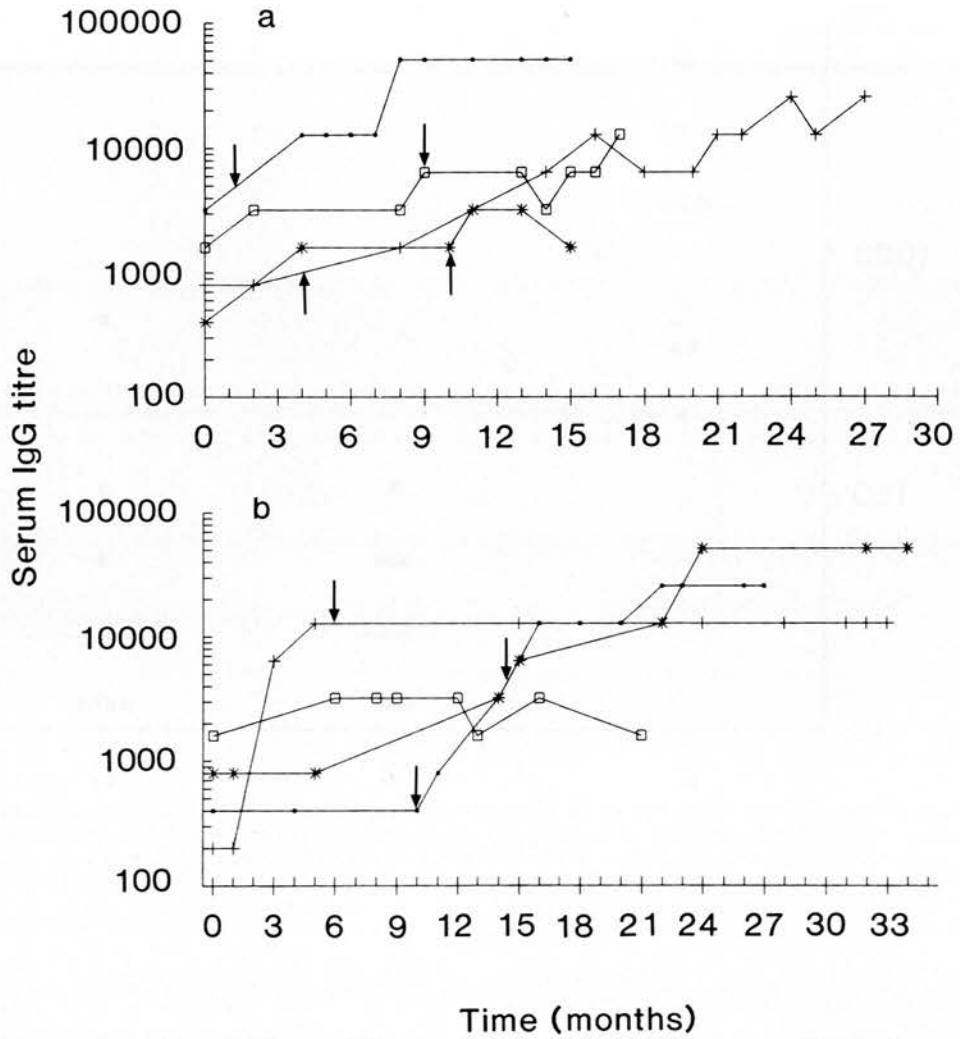

Fig. 3. Longitudinal study of serum IgG anti-*P. cepacia* LPS antibodies in eight CF patients measured by ELISA with core R-LPS as coating antigen. Patients I (**a**, —●—) III (**a**, —✳—) IV (**a**, —□—) and V (**b**, —●—) were colonised by *P. cepacia* only; patients II (**a**, —+—), VI (**b**, —+—) and VII (**b**, —✳—) were colonised by both *P. cepacia* and *P. aeruginosa*; patient VIII (**b**, —□—) was colonised by *P. aeruginosa* only. The arrows indicate the time when *P. cepacia* was first isolated from sputum from each patient.

subtraction of the OD of negative controls (wells coated only with post-coat buffer) for each sample. Analytical variation of the ELISA, including intra-plate and day-to-day (inter-plate) variations, was performed with six serum samples (three determinations for each), two each with low, medium and high ELISA titres, and the coefficient of variation was calculated. The intra- and inter-plate variations of the ELISA were 5·5% and 13·9%, respectively.

Absorption studies

Serum from CF patients was serially absorbed with whole cells of *P. cepacia* J1359 and *P. aeruginosa* PAC608. Overnight cultures of bacteria grown in NYB were harvested by centrifugation, washed twice in PBS and resuspended to a density of 10^8 cells/ml. Bacterial suspensions (1 ml) were placed in Eppendorf tubes and cells were harvested by centrifugation with a microcentrifuge. Bacteria were resuspended in serum diluted 1 in 200 in dilution buffer, incubated for 15 min at room temperature and re-centrifuged. The supernate was then added to another pellet of cells and the process was repeated. This step was repeated three times for each absorbing bacterial strain. Serum anti-*P. cepacia* and anti-*P. aeruginosa* core LPS antibodies were assayed by ELISA and immunoblotting as described above.

Statistical analysis

Data were not normally distributed and statistical analysis was by Wilcoxon signed rank tests.

Results

Whole cell ELISA

IgG antibodies directed to *P. cepacia* whole-cell antigens were detected in serum from both PC+ and PC− CF patients. A response to each of the serotype strains was evident although the difference in antibody titres between PC+ and PC− patients was significant

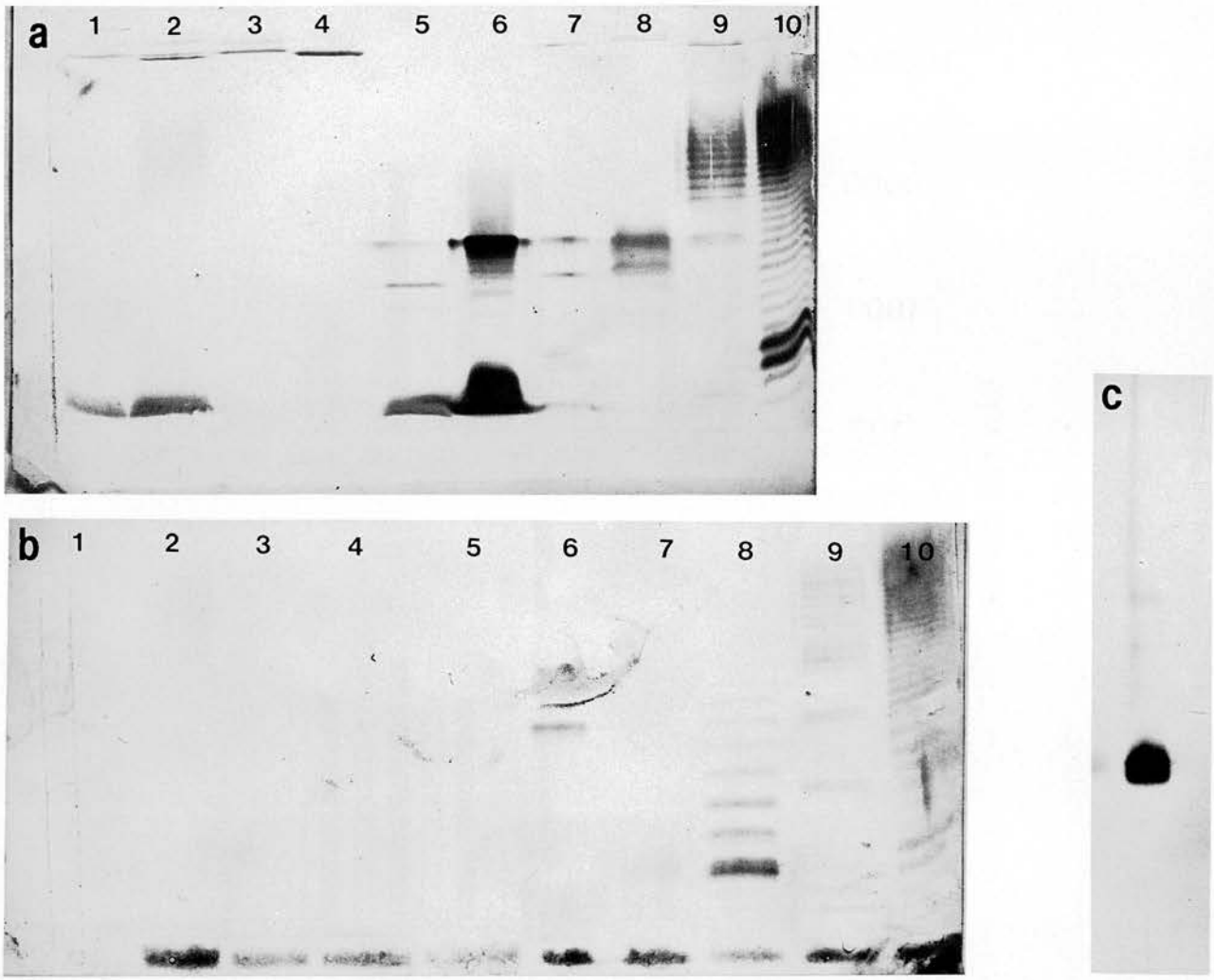

Fig. 4. Immunoblots of *P. cepacia* LPS antigens with serum from two CF patients colonised with *P. cepacia* and analysed for IgG anti-LPS antibodies; (**a**) serum from patient III, (**b**) serum from patient VII. The *P. cepacia* LPS antigens preparations were: lane **1**, J1780 (serotype 1); **2**, J1680 (serotype 2); **3**, J1774 (serotype 4); **4**, J1690 (serotype 7); **5**, J1758 (serotype 9); **6**, J1359 (non-typable); **7**, SBC21 (non-typable); **8**, SBC42; **9**, SBC29; **10**, SBC8. (**c**) Silver stained PAGE of isolated core LPS from *P. cepacia* J1359.

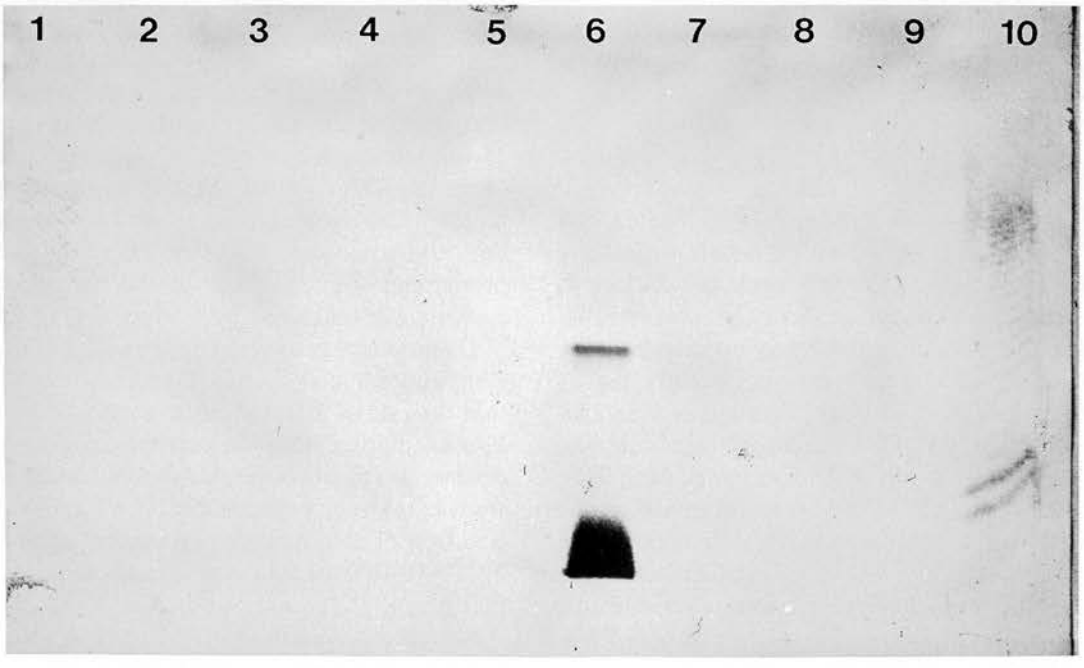

Fig. 5. Immunoblot of *P. cepacia* LPS antigens with sputum from CF patient III colonised with *P. cepacia* and analysed for IgA anti-LPS antibodies. *P. cepacia* LPS antigens were as in fig. 4.

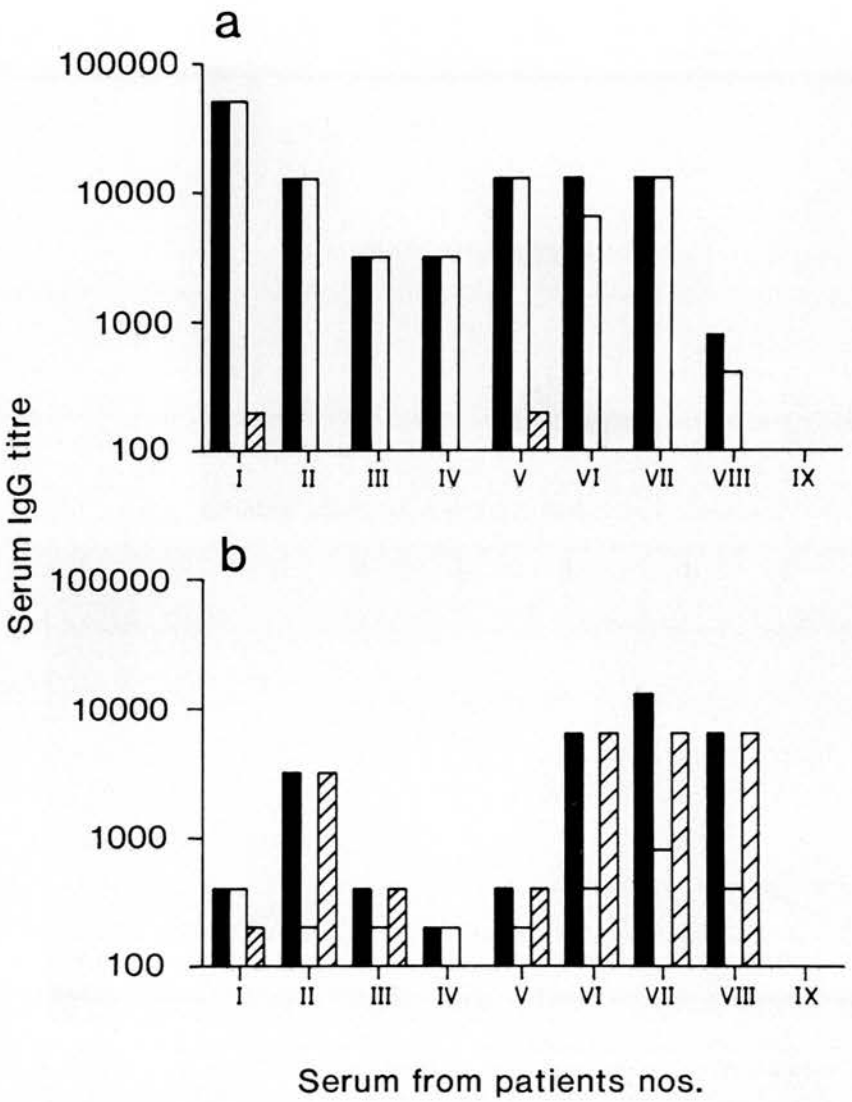

Fig. 6. Serum IgG anti-*P. cepacia* and anti-*P. aeruginosa* LPS antibodies in CF patients measured by ELISA with (**a**) core R-LPS from *P. cepacia* J1359 and (**b**) core R-LPS from *P. aeruginosa* PAC608 as coating antigens before (■) and after absorption with *P. cepacia* J1359 (▨) and *P. aeruginosa* (□) whole cells. Patients I, III, IV and V were colonised by *P. cepacia* only; patients II, VI and VII were colonised by both *P. cepacia* and *P. aeruginosa*; patient VIII was colonised by *P. aeruginosa* only; patient IX was not colonised by either organism.

only in the case of serotype 5 and the non-typable whole cells ($p < 0{\cdot}05$).

Core R-LPS ELISA

Serum IgG and sputum IgA antibodies. The ELISA employing extracted LPS from an isolate expressing core R-LPS was better than the whole-cell ELISA in discriminating between PC+ and PC− patients (fig. 1). In nine CF patients persistently sputum-positive for *P. cepacia*, IgG titres were significantly higher (median 12800, range 3200–51200) than in nine age- and sex-matched CF PC− controls (median 400, range 200–3200) ($p < 0{\cdot}01$ Wilcoxon signed rank test), and in nine healthy blood donors (median 400, range 200–800). There was no significant difference in the IgG titres between the PC− CF patients and the healthy blood donors. Seven of nine PC− CF controls were colonised by *P. aeruginosa* and six of nine PC+ CF patients were colonised by *P. aeruginosa*.

The nine PC+ CF patients also had significantly higher titres of sputum IgA antibodies reactive with core LPS of *P. cepacia* (median 1600, range 200–6400) than did the nine PC− patients (median 50, range 25–100) and nine chronic bronchitics (median 50, range 25–400) ($p < 0{\cdot}01$ Wilcoxon signed rank test); there was no significant difference between the titres of the non-colonised CF patients and the chronic bronchitics (fig. 2). None of the chronic bronchitic patients was colonised by *P. cepacia*.

Longitudinal analysis of serum IgG and sputum IgA. Longitudinal analysis of CF serum samples indicated that the rate of increase in the levels of anti-*P. cepacia* IgG antibodies after bacteriological diagnosis of *P. cepacia* colonisation varied from patient to patient; in some cases (e.g., patients III, IV, VI and VII) the first isolation of *P. cepacia* was preceded or accompanied by a two-to-four-fold rise in anti-*P. cepacia* R-LPS IgG titre (fig. 3).

Longitudinal analysis of sputum IgA titres from five CF patients, four of whom were colonised by *P. cepacia*, demonstrated the variable IgA antibody

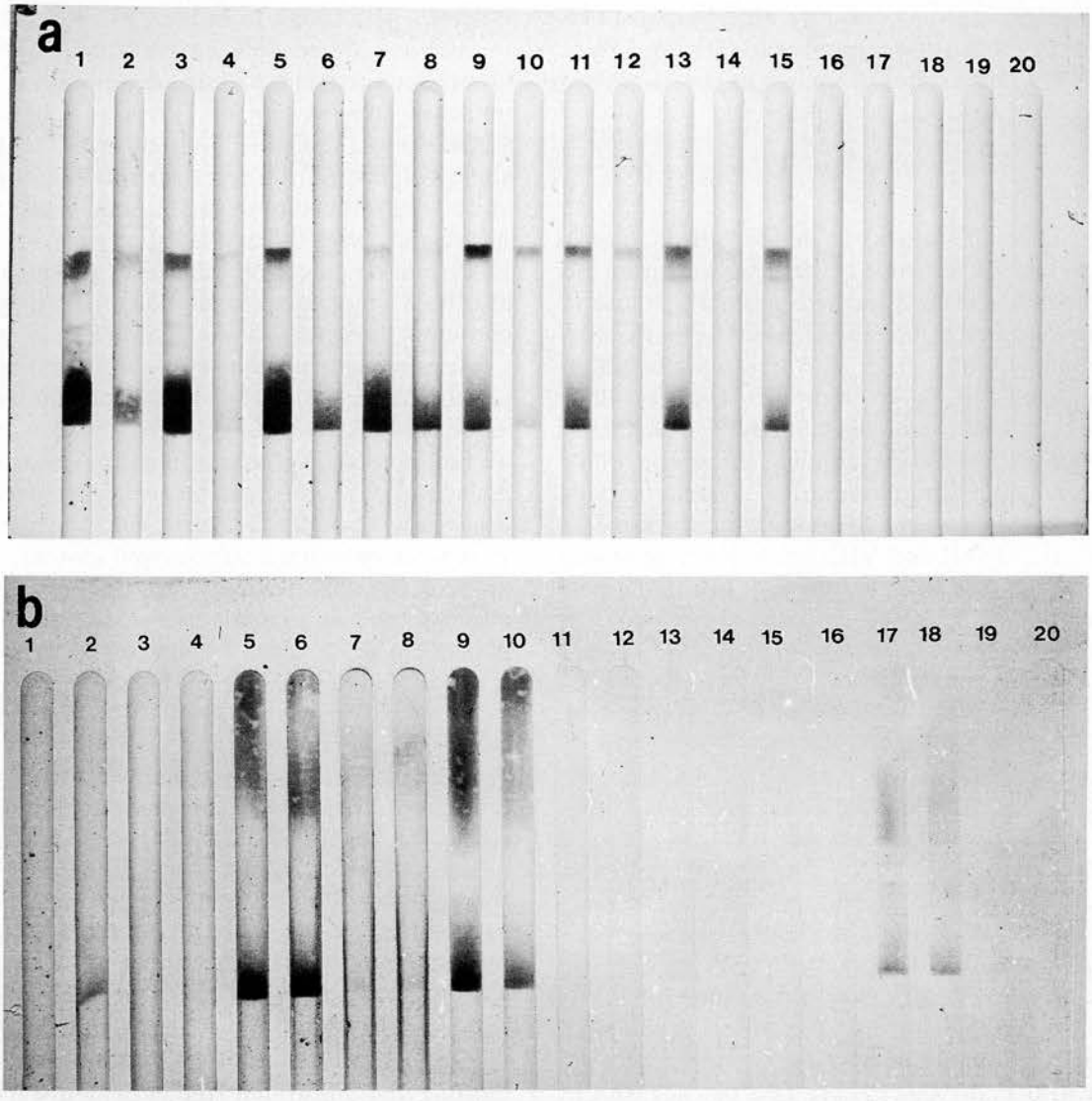

Fig. 7. Immunoblot of (**a**) anti-*P. cepacia* and (**b**) anti-*P. aeruginosa* core LPS IgG antibodies in serum from five CF patients before and after absorption with *P. cepacia* J1359 or *P. aeruginosa* PAC608, or both, whole cells. Lanes 1, 5, 9, 13 and 17, unabsorbed serum samples; lanes 2, 6, 10, 14 and 18; serum samples absorbed with *P. cepacia* J1359 whole cells; lanes 3, 7, 11 and 19 serum samples; absorbed with *P. aeruginosa* PAC608 whole cells; lanes 4, 8, 12, 16 and 20, serum samples absorbed with both *P. cepacia* J1359 and *P. aeruginosa* PAC608 whole cells. Patients I (lanes 1–4) and IV (13–16) were colonised by *P. cepacia* only; patients II (lanes 5–8) and III (9–12) were colonised by *P. cepacia* and *P. aeruginosa*; and patient VIII (lanes 17–20) was colonised by *P. aeruginosa* only.

response to core R-LPS of *P. cepacia*. During a 10-week study period, the sputum IgA antibody titre ranged from 400 to 6400 for two of the PC+ CF patients, and from 800 to 3200 for a third PC+ patient. In a fourth PC+ patient, the IgA antibody titre was maintained at 100–200, which was similar to that of the PC− patient included in the study. There was no correlation between the sputum IgA antibody titre and the number of *P. cepacia* isolated by bacteriological culture from sputum, but there was a tendency for IgA levels to rise during hospitalisation for pulmonary exacerbations.

Immunoblot analyses

Isolated core LPS from *P. cepacia* J1359 is shown in the silver-stained PAGE gel (fig. 4c). Reactivity of serum from two PC+ CF patients against *P. cepacia* LPS antigens is shown in the Western blots (fig. 4a and b). In both cases, a positive reactive band corresponding to the low mol. wt core LPS of most but not all of the *P. cepacia* isolates was observed: reactivity with nine and six of the 10 core LPS antigens was observed with serum from patients III and VII, respectively (fig. 4a and b). In addition, a response corresponding to higher mol. wt O-antigen subunits of LPS from strains SBC42, SBC29 and SBC8 was noted. The serum used for the preparation shown in fig. 4a was obtained from a CF patient colonised with strain JI359 (ribotype A); the serum for the preparation shown in fig. 4b was obtained from a CF patient colonised with strains J1359 (ribotype A) and SBC42 (ribotype C). Immunoblot analysis of serum from two PC− CF patients, one of whom was colonised with *P. aeruginosa*, did not produce a visible response to any of the *P. cepacia* LPS antigens used.

Sputum from the same PC+ CF patient whose serum was used for fig. 4a was used for immunoblot

analysis of IgA antibodies reactive with *P. cepacia* LPS antigens (fig. 5). A strong response to the core LPS of strain J1359 and a weak response to the O-antigen subunits of strain SBC8 was observed.

Absorption studies

Serum from CF patients colonised with *P. cepacia* or *P. aeruginosa*, or both or neither, was absorbed with whole cells from *P. cepacia* strain J1359 and *P. aeruginosa* strain PAC608 and analysed by ELISA (fig. 6) and immunoblotting (fig. 7). With serum from PC+ CF (patients I–VII, fig. 6) antibodies reactive with *P. cepacia* core LPS were substantially removed after absorption with the corresponding *P. cepacia* whole cells, but not after absorption with *P. aeruginosa* whole cells. Serum from patients colonised with *P. aeruginosa* (patients II, VI, VII and VIII, fig. 6) had a relatively high antibody titre to *P. aeruginosa* core LPS; these antibodies were removed by absorption with *P. aeruginosa* whole cells but not by absorption with *P. cepacia* whole cells. These absorption studies demonstrated that a significant component of the anti-*P. cepacia* core LPS antibodies was specific for *P. cepacia* and not reactive with core LPS from *P. aeruginosa*; e.g., patient I, colonised by *P. cepacia* but not by *P. aeruginosa*, had a serum IgG titre of 51 200 against *P. cepacia* core LPS and a titre of 200 against *P. aeruginosa* core LPS. The antibodies against *P. cepacia* core LPS were removed by absorption with *P. cepacia* whole cells but not by the *P. aeruginosa* whole cells as shown by ELISA (fig. 6) and immunoblotting (fig. 7). Conversely, serum from a CF patient colonised by *P. aeruginosa* but not by *P. cepacia* (patient VIII) reacted with *P. aeruginosa* core LPS but not with *P. cepacia* core LPS.

Discussion

Antibodies reactive with *P. cepacia* whole cells and extracted core R-LPS were demonstrated in serum and sputum from patients with CF. A core LPS preparation was used as a coating antigen because (a) LPS represents a more defined antigenic preparation than a system based on whole cells; (b) available evidence suggests antigenic cross-reactivity between some outer-membrane protein antigens of *P. cepacia* and *P. aeruginosa*;[13,14] (c) core LPS is believed to be a relatively conserved component of LPS[25] and, therefore, provides a suitable antigen for the detection of an antibody response against all serotypes of *P. cepacia*; and (d) many strains of *P. cepacia* isolated from CF patients produce rough LPS (i.e., lacking O-specific side-chains)[26] (S. Butler, unpublished observations). The ELISA system based on core R-LPS from a CF clinical isolate of *P. cepacia* clearly differentiated between serum samples from PC+ and PC− CF patients. However, figs. 1 and 2 showed that a range of serum IgG and sputum IgA antibody titres were obtained with serum from both PC+ and PC− CF patients and it is possible that overlap between the two groups could occur. Thus, detection of a rising antibody titre between two or more serum samples would appear desirable. Indeed, in the longitudinal studies a rise in the level of anti-*P. cepacia* IgG antibodies was demonstrated in some patients before or accompanying the first isolation of *P. cepacia*. An ELISA based on core R-LPS from *P. aeruginosa* has also been reported to be useful for the diagnosis of chronic *P. aeruginosa* infection in CF.[27]

Measurement of anti-*P. cepacia* antibodies to identify *P. cepacia* colonisation may prove useful. Although the value of selective media for *P. cepacia* is well recognised,[1,6] problems may still be encountered, including growth of *Xanthomonas maltophilia* and *P. acidovorans*[4], and the fact that some clinical isolates of *P. cepacia*, on initial isolation from sputum, may take up to 7 days to grow. In view of the considerable concern relating to pulmonary colonisation of CF patients by *P. cepacia*,[4,5,28] the ability to identify *P. cepacia* colonisation early and accurately is important, especially in clinics where segregation of colonised and non-colonised patients is practised.

Recent studies[13,14] have demonstrated the presence of IgG antibodies to outer-membrane antigens of *P. cepacia* in serum from CF patients colonised with *P. cepacia* or *P. aeruginosa* or both. These authors concluded that some *P. cepacia* outer-membrane components may be related antigenically to those of *P. aeruginosa*. Similarly, in our study, serum antibodies reactive with *P. cepacia* whole cells were found in CF patients colonised with *P. cepacia* or *P. aeruginosa* or both. In terms of LPS antigens however, the Western blotting and absorption studies demonstrated that a significant proportion of the antibodies that reacted with core LPS of *P. cepacia* did not react with core LPS of *P. aeruginosa*. These observations indicate differences in the structure and composition of core LPS between *P. aeruginosa* and *P. cepacia*, confirming previous findings, including structural differences[29] (e.g., the lack of phosphorus in the core LPS of *P. cepacia* and the inability of a monoclonal antibody reactive with *P. aeruginosa* core LPS to react with *P. cepacia*[30]). Furthermore, there is unlikely to be cross-reactivity with core LPS of *X. maltophilia* because of the structural differences reported by Neal and Wilkinson.[30] There may be some core heterogeneity between different isolates of *P. cepacia* because Western blotting demonstrated that serum from patients colonised with *P. cepacia* produced a band reactive with some but not all *P. cepacia* core LPS preparations.

Although anti-core IgA antibodies were demonstrated in sputum from chronically colonised patients, the levels of these antibodies may have been underestimated because of the possibility of immune-complex formation and fragmentation of antibody by elastases derived from neutrophils or *P. aeruginosa*.[32,33]

Our data suggest that, in CF patients colonised by

P. cepacia, antibodies reactive with *P. cepacia* are unable to eliminate the organism from the lungs. Indeed, this study and those of Aronoff *et al.*[13,14] have shown that *P. cepacia* colonisation occurs in the presence of antibodies specific for outer-membrane components of the organism. However, a relatively high titre of IgG antibodies against *P. cepacia* was observed in one of the PC− patients from whom *P. cepacia* has never been isolated despite contact with members of the PC+ group. It is interesting to speculate on the possible protective effect of antibodies in a subset of PC− CF patients.

Measurement of anti-*P. cepacia* antibodies currently forms part of a prospective longitudinal study on a larger number of CF patients from different regional centres. Such investigation will provide important information regarding the diagnostic applicability and usefulness of the antibody assay for detection of *P. cepacia* in patients with CF.

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Human hydatid disease: evaluation of an ELISA for diagnosis, population screening and monitoring of control programmes

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Summary. The routine use of ELISA and complement fixation tests in the diagnosis of suspected clinical cases of hydatid disease was evaluated. In the ELISA test, dialysed and filtered sheep cyst fluid was used as antigen and two positive cut-off points—+3SD and +2SD of the mean absorbance values of the control sera—were evaluated. The predictive values of ELISA tests were 82% and 90% for positive tests, and 86% and 82% for negative tests, respectively with the two cut-off points. In a population survey of blood donors and veterinary workers in Powys, 4% and 8%, respectively, had ELISA values above the lower cut-off point. However, it would not be appropriate to use the same test for diagnostic population screening in Wales since the predictive value of the test is likely to be very low in this setting. Serological surveys with the ELISA may be of use in monitoring the progress of the South Powys Hydatid Control Programme. The use of cumulative percentages was found to be a useful method of comparing whole distributions of results in different populations.

Introduction

Hydatid disease in man is caused principally by infection with the larval stage of the dog tapeworm *Echinococcus granulosus*. It is acquired by ingestion of the eggs of the tapeworm which are excreted in the faeces of infected dogs. In the UK, the intermediate host for *Echinococcus granulosus* is sheep, and the sheep farming areas of Mid-Wales and Herefordshire have the highest incidence of hydatid disease in the UK.[1] Serological tests developed for routine use in the diagnosis of human infection include an enzyme-linked immunosorbent assay (ELISA) and complement fixation tests (CFT).[2–4] In this study, the use of these methods for the diagnosis of clinically suspected cases was evaluated. The value of the ELISA test for monitoring the population prevalence of infection in Wales as part of a long-term control programme[5] was also assessed.

Materials and methods

Laboratory methods

Hydatid antigen was obtained from hydatid cyst fluid aspirated from fertile sheep cysts, dialysed and filtered through a 0·45-μm acetate membrane.[6,7] The filtered dialysate was stored in small volumes at −20°C for use in both ELISA and CFT tests.

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ELISA. An indirect ELISA was performed by a modification of the method described by McLaren *et al.*[8] The antigen was diluted to its optimum concentration (1 in 500) in carbonate buffer, pH 9·6, and 150 μl was placed in each well of a microtitration plate. After overnight incubation in a moist chamber at room temperature, the plates were washed four times in phosphate-buffered saline (PBS), pH 7·2, containing Tween 20 0·05% to remove excess antigen before use.

Test serum samples and control sera (150 μl of each) diluted 1 in 200 in PBS-Tween were added to duplicate wells. The standard positive control, similarly diluted, was added to four wells on each plate. The plates were incubated for 2 h in a moist chamber at room temperature and washed with PBS-Tween.

Rabbit anti-human IgG peroxidase conjugate (Dako) was diluted to its optimum concentration (1 in 7000) in PBS-Tween and 150 μl was added to all wells of the tray. The tray was incubated for 3 h at room temperature in a moist chamber, then washed in PBS-Tween.

The reporter substrate—orthophenylene diamine (OPD 0·01%; 150 μl)—was added to each well immediately after preparation and the plates were incubated in a moist chamber at room temperature. The reaction was stopped in one of the standard control wells at intervals to enable the rate of reaction to be monitored. The reaction was stopped by the addition of 25 μl of 2·5 M H_2SO_4 to all wells when the standard positive control had attained an absorbance value of