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Pathogenicity Studies on Verocytotoxin-producing *Escherichia coli* :
Bacterial adhesion, Toxin Expression and Uptake.

by

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This thesis is presented for the Degree of Doctor of Philosophy, in the Department of Biological Sciences, University of Warwick.

Dedication

For Joan and Peter, for always being there.

Declaration

I declare that this thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself with the exception of immunogold labelling which was carried out by Nicholas Powell at the MRC Clinical Research Centre, Harrow. All work was carried out at the University of Warwick under the supervision of Dr C S Dow apart from studies on the cellular uptake of Verotoxin which were carried out at the MRC Clinical Research Centre, Harrow, under the supervision of Dr S P Borriello.
All sources of information have been specifically acknowledged by means of reference.

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Abbreviations

AE	Attaching-effacing
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Diaminoethane tetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enterinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EVEC	Enterovirulent *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FVT	Free Verotoxin
g	Gramme
GBS	Glycine buffered saline
Gb_3	Globotriaosylceramide
Gb_4	Globotetraosylceramide
HUS	Haemolytic uraemic syndrome
KDa	KiloDalton
LPS	Lipopolysaccharide
LT	Heat-labile toxin (ETEC)
MAb	Monoclonal antibody
MDa	MegaDalton
M	Molar
mg	Microgram
mg	Milligram
mM	Millimolar

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
RME	Receptor -mediated endocytosis
SDS	Sodium dodecyl sulphate
ST	Heat-stable toxin (ETEC)
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris-hydroxymethylaminomethane
TTP	Thrombotic thrombocytopenia purpura
Tween 20	Polyoxyethylenesorbitan monolaurate
VTEC	Verotoxin-producing *Escherichia coli*
VT	Verotoxin (s)
VT1	Verotoxin 1
VT2	Verotoxin 2
VT2vh	Verotoxin 2 human variant
VT2vp	Verotoxin 2 porcine variant
v/v	Volume by volume
w/v	Weight by volume

Aims of This Study

The putative pathogenic mechanisms of VTEC are: cytotoxin production, adherence to epithelial cells in an attaching-effacing manner, and in most cases, possession of a 60-MDa plasmid. The aims of this study were to investigate toxin production and cellular internalization, and, through the production of mutants, to attempt to study the pathogenicity of the organism as a whole. A preliminary step was therefore to develop biological and immunological assays to detect the toxins.

VT1 and VT2 are encoded by lysogenic phages, so it was proposed that a chemical known to induce phages might affect toxin production. The effect of mitomycin C on a VTEC strain, O157:H7 (ATCC 35150), was therefore studied through analysis of extracellular proteins and both intracellular and extracellular toxin levels.

It is likely that *in vivo* it is the cumulative effects of the pathogenic mechanisms described above which bring about the clinical manifestations of a VTEC infection. Many studies have focused on one potential virulence determinant. This study aimed at producing mutants through chemical mutagenesis, in an attempt to investigate the organism as a whole. The mutants were to be characterised with respect to plasmid content, toxin production, and attachment to epithelial cells and mucus.

Although VT1 and VT2 are known to be N-glycosidases, the entry of these toxins into mammalian cells has not been elucidated. Through the use of various conditions and inhibitors, this study aimed to show the mechanism of entry and possible intracellular processing of these toxins. The potential role of the calcium uptake system, in VT internalization was also investigated.

Summary
Verotoxin-producing *Escherichia coli* (VTEC) are a group of medically important bacteria which have been implicated in cases of haemorrhagic colitis and haemolytic uraemic syndrome. They are characterised by the ability to produce Verotoxins (VT), potent exotoxins which kill mammalian cells by inhibiting protein synthesis. Two distinct VT are known, Verotoxin 1 (VT1) and Verotoxin 2 (VT2), and variants of VT2 have been described.

VTEC adhere to intestinal cells via an attaching-effacing mechanism and many strains carry a 60-MDa plasmid which was thought to be involved in adhesion. Attachment of VTEC is thought to be an important pathogenic mechanism since it allows colonisation to occur, and facilitates delivery of toxin. The exact mechanisms of VTEC infection, however, especially the variation in severity of disease have not been established.

This study aimed to investigate the putative virulence attributes of VTEC, namely toxin production and bacterial attachment. In order to study the toxins, it was first necessary to develop a reliable method of assay, and for this, two assays were chosen; a Vero cell assay which measured biological activity, and a colony ELISA, which detected the presence of toxin antigens.

Since VT are produced by lysogenic phages, the effect of a phage inducer, mitomycin C, on VT production, was investigated. Increased levels of VT appeared to be detected and SDS-PAGE analysis of extracellular proteins revealed that the amount of toxin, and other phage proteins increased in the presence of mitomycin C, whilst bacterial protein levels remained constant. This effect was not seen when a non-VTEC strain was tested. The specific activity of VT increased inside the cell, where bacterial protein synthesis had ceased, but decreased extracellularly due to cell lysis. Extracellular phage particles were demonstrated by plaque assay.

Mutants of O157:H7 strain ATCC 35150, were produced by chemical mutagenesis. They did not possess the 60-MDa plasmid, found in the parent strain, and had an altered biochemical profile. Immunological assays revealed that they were altered in the O side chain of the LPS and it was shown by immunoblotting, that only the major outer membrane proteins reacted with anti-O157:H7 although they were not rough mutants. Analysis of the LPS neutral sugars showed that the mutants contained rhamnose, instead of fucose which was present in the parent strain. Despite these changes, however, the hydrophobicity of the mutants, or their ability to attach to colonic mucus or HeLa cells was unaffected, indicating that the 60-MDa plasmid is not significant in attachment. The mutants appeared to produce toxin with little activity, which was also biologically unstable. Secretion of the toxin appeared to be reduced, possibly as a result of the LPS mutations.

The internalisation and intracellular processing of VT1 and VT2 was studied, and it was shown that the toxins were internalised by receptor-mediated endocytosis, but could probably by-pass this pathway. There was no requirement for exposure to an acidic environment, although fusion of endosomes with lysosomes was essential. It was also necessary for cells to possess physiologically functioning calcium channels for intoxication to occur.

Chapter 1 Introduction

1.1 Pathogenic *Escherichia coli*

Escherichia coli is the predominant species among the facultative anaerobic normal flora of the human intestine. Certain members of this species however, are associated with intestinal infections and have been termed enterovirulent *E.coli* (EVEC) (Lupski & Feigin 1988). EVEC fall into four main categories : enterotoxigenic *E.coli* (ETEC), enteroinvasive *E.coli* (EIEC), enteropathogenic *E.coli* (EPEC), and enterohaemorrhagic *E.coli* (EHEC). A fifth group, enteroadherent *E.coli* (EAEC) is less well defined, and will not be discussed further. EVEC are categorised according to the distinct clinical syndromes they induce. Within each grouping, strains tend to fall into characteristic O:H serotypes (Table 1.1). (*E.coli* are serotyped according to the O (somatic) and H (flagellar) antigens they possess.)

1.1.1 Enterotoxigenic *E.coli* (ETEC)

ETEC are a major cause of traveller's diarrhoea and infant diarrhoea in less-developed countries. They cause disease by colonization of the mucosal surface of the small intestine, and by production of heat-labile (LT) and/or heat-stable (ST) enterotoxins, that cause fluid secretion, resulting in diarrhoea. Attachment of the bacteria to the intestinal wall is mediated by fimbriae. The clinical features of ETEC infection are watery diarrhoea, nausea, abdominal cramps and low-grade fever.

1.1.2 **Enteroinvasive *E.coli* (EIEC)**

EIEC cause a dysenteric form of diarrhoeal illness, by invasion and proliferation within epithelial cells, predominantly in the colon, resulting in cell death. The ability of these bacteria to invade epithelial cells is due to possession of a 140-MDa plasmid which codes for production of several outer membrane proteins, which are involved in invasiveness. The resulting illness is characterized by fever, severe abdominal cramps, toxaemia, and watery diarrhoea followed by gross dysentry consisting of scanty stools of blood and mucus.

1.1.3 **Enteropathogenic *E.coli* (EPEC)**

EPEC are an important cause of infantile diarrhoea in many developing countries. They lack specific virulence determinants characteristic of other diarrhoeagenic *E.coli* strains, namely invasiveness or toxin production, and are thought to cause disease by an attaching and effacing adherence to intestinal mucosal epithelial cells. There is no invasion of these cells, but disruption of cytoskeletal elements is followed by loss of the microvillous border, resulting in characteristic attaching and effacing (AE) lesions. EPEC illness tends to be clinically more severe than other diarrhoeal infections in infants, and is characterized by fever, malaise, vomiting, and diarrhoea containing little blood, but large amounts of mucus.

1.1.4 Enterohaemorrhagic *E.coli* (EHEC)

It is the fourth EVEC category, EHEC, which is the subject of this study. This group is defined by the ability to produce exotoxins known as Verotoxins (VT). These bacteria are also known as Verotoxin-producing *E.coli* (VTEC) and this is the preferred terminology in this thesis. In addition to VT production, VTEC adhere to epithelial cells by an attaching and effacing mechanism characteristic of EPEC (Levine 1987), and many strains carry a 60-MDa plasmid thought to be involved in attachment (Karch *et al* 1987). Although several serotypes have been classified as VTEC (Table 1.1), most human strains belong to the O157 serogroup, and of these a high percentage possess the H7 flagellar antigen (O157:H7), although some are non-motile. Two distinct VTs are known, Verotoxin 1 (VT1), which is immunologically identical to Shiga toxin, produced by *Shigella dysenteriae* type 1, and Verotoxin 2 (VT2) which is antigenically distinct from Shiga toxin and VT1. Variants of VT2, VT2vp and VT2vh, from animal (Marques *et al* 1987) and human (Oku *et al* 1989) origin respectively have also been described. VTEC cause disease ranging from mild diarrhoea, which may be bloody, through to haemorrhagic colitis or the haemolytic uraemic syndrome (HUS). Haemorrhagic colitis consists of bloody diarrhoea, abdominal pain, and low grade or no fever. These symptoms also describe the prodromal phase of HUS, where acute renal failure, thrombocytopenia, haemolytic anaemia and even death may result.

The relationship between VT and Shiga toxin led to an alternative nomenclature system of Shiga-like toxins (SLT) (Strockbine *et al* 1986). It is common therefore to find reference to SLT1 and SLT11 instead of VT1 and

Table 1.1 Enterovirulent *E.coli*

Pathogenic *E.coli* can be divided into four main groups according to the clinical symptoms they induce. Within each grouping, strains tend to fall into characteristic O:H serotypes.

Group		**Most common serotypes**
Enterotoxigenic *E.coli*	ETEC	O6,O8,O15,O20,O25, O27,O63,O78,O80, O85,O115,O128ac, O139,O148,O153, O159,O167.
Enteroinvasive *E.coli*	EIEC	O28ac,O29,O124, O136,O143,O144, O152,O164.
Enteropathogenic *E.coli*	EPEC	O55,O111,O119,O127O1 42.
Enterohaemorrhagic *E.coli*	EHEC	O157, (O111,O26)

VT2. In this report however, the original nomenclature of VT1 and VT2 will be adhered to.

1.2 History of Isolation of VTEC

Verotoxins (VT) were first described in 1977 by Konowalchuk *et al* (1977), after an observation that culture filtrates of certain strains of *E.coli*, from various sources, contained a heat-labile toxic substance which was lethal to Vero (African Green Monkey kidney) cells. This cytopathic activity contrasted with the cytotonic effect on Vero cells of the heat-labile toxin (LT) produced by ETEC, and in addition, antisera to LT did not neutralize the effect of VT. Further investigation of one of these *E.coli* strains, O26:H30, showed the presence of two toxins, with isoelectric points of 7.2 and 6.8 (Konowalchuk *et al* 1978). Since seven of the ten strains producing VT were isolated from infants with diarrhoea, it seemed possible that VT were involved in diarrhoeal disease. A clinical study by Wade *et al* (1979), in which VT-producing *E.coli* (serogroup O26) was isolated from three children with bloody diarrhoea, supported this hypothesis.

It was in the early 1980's that the link between VTEC and diarrhoeal disease became established, with reports of VTEC isolation from cases of haemorrhagic colitis (Karmali *et al* 1983), and HUS (Riley *et al* 1983), conditions of previously unknown aetiology. *E.coli* O157:H7, until then thought of as a 'rare' *E.coli* serotype was isolated more frequently (Wells *et al* 1983). This serotype is now acknowledged as the most common VTEC isolate. In one survey, *E.coli* O157:H7 was found to be the third most frequently isolated enteric bacterial pathogen following *Campylobacter jejuni* and *Salmonella* species (Cahoon & Thompson 1987). Prior to 1982 this serotype was rarely isolated from humans or animals, for example

only one strain was detected at the Center for Disease Control in Atlanta, in >3000 isolates serotyped between 1973-1982 (Riley *et al* 1983). Since 1982, O157:H7 strains have frequently been recovered from patients with haemorrhagic colitis and HUS (Table 1.2). Studies indicate that *E.coli* isolates of serotype O157:H7 are members of a single clone widely distributed throughout North America (Whittam *et al* 1988). This study also showed that O157:H7 strains are no more closely related to other VTEC serotypes than are randomly selected clonal genotypes from the *E.coli* population as a whole.

It is important to note that other VTEC serotypes are becoming increasingly isolated, and in a recent study in Thailand, none of the strains possessing VT genes were O157:H7 (Seriwatana *et al* 1988). It is becoming apparent that a great variety of *E.coli* serotypes can obtain the genes coding for VT, although it is the level of expression of these genes which is ultimately important in the role of these strains in clinical disease.

1.3 **Animal Reservoir for VTEC**

The main reservoir of VTEC is the intestinal tract of animals, predominantly cattle. *E.coli* O157:H7 has been isolated from cattle after cases of both haemorrhagic colitis and HUS were associated with the ingestion of raw milk (Borczyk *et al* 1987). This serotype was also isolated when contaminated ground beef was implicated in an outbreak of haemorrhagic colitis and HUS (Wells *et al* 1983). Hence, it has been deduced that the route of transmission of these pathogens is primarily zoonotic in nature. Person-to-person spread has been reported (Karmali *et al* 1988), indicating that a relatively small dose of the infectious agent may

Table 1.2 Number of isolates of *E.coli* O157:H7 in Canada from 1978-1987

Year	Number of isolates
1978-81	5
1982	25
1983	59
1984	163
1985	294
1986	750
1987	1342

(From Hockin *et al* 1988)

be sufficient to initiate disease, but this is a less common mode of transmission.

There is only one reported case of isolation of O157:H7 from a sick cow (Orskov *et al* 1987), however, in a survey in North America this serotype was isolated from >3% of retail fresh meats and poultry, indicating that it is not a rare contaminant of these products (Doyle & Schoeni 1987). Cattle have also been implicated as a source of VTEC in England, approximately 1% of cattle at an abattoir were found to carry this serogroup in their faeces (Chapman *et al* 1989). As cattle appear to be the main reservoir for O157:H7, there is some speculation that this VTEC serotype has evolved from the normal flora of these animals (Whittam *et al* 1988).

VTEC serogroups other than O157 are frequently isolated from cattle with diarrhoea (Mohammed *et al* 1986) and weaned pigs with diarrhoea and oedema disease (Dobrescu 1983). The VTEC serogroups associated with diarrhoea and oedema disease in pigs are O45, O138, O139, and O141, but there are no reports of these strains being implicated in human disease. Around 10% of healthy cattle were found to carry VTEC (Montenegro *et al* 1990) including serogroups implicated in human infections, but they appear to form part of the normal flora of these animals. This figure correlates with that of Read *et al* (1990), who detected VTEC in 10.4% of beef samples from meat processing plants, although it must be stressed that not all of these VTEC serogroups have been implicated in human disease.

1.4 Clinical Manifestations of VTEC Infections

The disease produced as a result of VTEC infection ranges from mild uncomplicated diarrhoea, through haemorrhagic colitis to HUS. Infection usually results in a self-limiting diarrhoeal illness, and asymptomatic infection has been reported (Cleary 1988). In some patient groups, however, there is a progression to more serious symptoms, and one of the predisposing factors towards these groups is age, with the very young and the elderly being most at risk. The reason for this is not clear, but in any one outbreak of VTEC infection a wide spectrum of illness may be reported. Diarrhoea due to VTEC, which may or may not be bloody, develops 3-4 days after infection as a result of reduced absorption in the gut, and loss of fluid, electrolytes and plasma proteins is secondary to severe mucosal damage in the large bowel. The more severe manifestations of human VTEC infection, haemorrhagic colitis and HUS, as well as oedema disease of swine, will be described in more detail to emphasize the importance of studying VTEC as pathogens.

1.4.1 Haemorrhagic colitis

Haemorrhagic colitis is usually a self-limited, acute, afebrile illness that is characterized by abdominal cramps and watery diarrhoea which becomes bloody, resembling lower gastrointestinal bleeding. The young and elderly are prone to complications and in one severe outbreak a 31% fatality rate was reported among nursing home residents (Krishnan *et al* 1987).

Since the syndrome was first linked to VTEC (Riley *et al* 1983), both sporadic cases (Pai *et al* 1984) and outbreaks of VTEC-associated haemorrhagic colitis have been reported (Johnson *et al* 1983, Wells *et al*

1983). Most cases have been associated with contaminated meat products, and haemorrhagic colitis was at one stage referred to as the "hamburger disease". Whilst O157:H7 is the most common VTEC isolate from cases of haemorrhagic colitis (Kleanthous *et al* 1988) other serotypes have been reported, as well as atypical O157 strains (Bopp *et al* 1987). The use of antibiotics to treat VT-associated haemorrhagic colitis is not beneficial, and the early use of antimotility agents to control the diarrhoea may increase the duration of the colitis (Robson *et al* 1990).

1.4.2 Haemolytic Uraemic Syndrome

HUS is the leading cause of acute renal failure in children, with the highest incidence occuring in late summer/early autumn, either in small outbreaks or as sporadic cases. Around 8% of children with O157:H7 infections will develop HUS (Rowe *et al* 1991) and in North America the mortality rate is about 5% (Stavric & Spears 1989). HUS is not a common disease of adults, although a few cases have been reported (Neill *et al* 1985, Crosse & Naylor 1990). As mentioned previously the elderly are particularly susceptible to VTEC infections, and in one nursing home outbreak 30% of infected residents died of HUS (Karmali 1987).

HUS patients characteristically display a triad of features : acute renal failure, microangiopathic anaemia and thrombocytopenia. The most common variety of HUS, the idiopathic form, is typically preceded by a prodromal phase which resembles haemorrhagic colitis. The pathogenesis of HUS has not been established although an observation that most sera from acute HUS cases contained antibodies that lysed human umbilical vein endothelial cells suggests that autoimmunity may play a role in the disease (Leung *et al* 1988).

It is possible to link the incidence of HUS to the frequency of isolation of VTEC. The highest reported frequency of HUS is in Argentina (250 cases/year) where VTEC levels are high, although O157:H7 is not the most common isolate (Lopez *et al* 1989). Conversely a low incidence of both HUS and VT-associated diarrhoea is seen in Thailand (Seriwatana *et al* 1988). It should be noted that although the link between HUS and VTEC is clear, HUS is still a relatively uncommon complication of VTEC infection.

Thrombotic thrombocytopenia purpura (TTP) has also been linked to O157:H7 where symptoms resemble those of HUS, but with neurological manifestations which are only rarely linked to HUS (Ramsey & Neill 1986). Studies have been carried out to investigate a link between genotype and disease produced by VTEC, and in patients with HUS or TTP, O157:H7 strains producing VT2 only, were the most likely isolates (Ostroff *et al* 1989). VTEC containing more than one copy of VT2 and VT2vh genes have been isolated (Schmitt *et al* 1991, Ito *et al* 1990), although multiple copies of VT1 genes have not been reported. Since VT2 has been associated with the more serious manifestations of VTEC infections, there is speculation that multiple gene copies could play a role in the pathogenesis of these infections.

1.4.3 **Oedema Disease of Swine**

VT2vp is associated with oedema disease of swine which affects mainly weaning pigs, and can result in serious economic losses. It is a neurological disease which begins with staggering gait and limb paralysis and progresses to convulsions, coma, and finally death. The disorder is characterized by oedema which is restricted mainly to the stomach wall and colonic mesentery, and profound vascular damage. The pathology of

oedema disease can be induced in weaning pigs by administration of <10ng purified VT2vp per kg of body weight, so it appears that the toxin alone is responsible for the majority of the symptoms of this infection (Gordon *et al* 1992). This means that a vaccine currently under development to protect against the action of VT2vp, based on a toxoid preparation should protect against oedema disease(MacLeod & Gyles 1991).

1.5 **Diagnosis of VTEC infections**

VTEC infections can be diagnosed on the basis of isolation of the organism, detection of VT, or the presence of neutralising antibodies in the patient's serum. VT can be detected as free toxin (FVT), usually in stool filtrates, or it can be released from cells by use of compounds which alter the permeability of the cell envelope, such as polymyxin B, and subsequently assayed. It is well recognized however that the most important and sensitive investigation in diagnosis of VTEC infection is the detection of FVT (Karmali *et al* 1985).

1.5.1 **Detection of Verotoxins**

The detection of FVT is the most important factor in diagnosis of VTEC infections, especially as many VTEC-negative patients are positive for FVT as organisms are often present in numbers too low to detect. FVT is usually assayed from stool filtrates in tissue culture assays where the toxin is titrated across monolayers of Vero (African Green Monkey kidney) cells, although the use of Vero cell suspensions may be a more rapid method of detecting VT (Maniar *et al* 1990). The presence of VT can be confirmed by a neutralization test with appropriate antiserum. A less expensive and more

rapid method of detecting VT from supernatants or cell extracts is an enzyme linked immunosorbent assay (ELISA), which can detect ng toxin ml^{-1}, although this is far less sensitive than tissue culture assays where pg toxin ml^{-1} can be measured (Kongmuang *et al* 1987). Colony ELISA using monoclonal antibodies against VT1 (Strockbine *et al* 1988) and VT2 (Perera *et al* 1988) detect moderate- to high-level producers of VT and have proved useful for epidemiological screening.

Although tissue culture techniques are expensive and time consuming, most other methods fail to detect such low levels of toxin.

1.5.2 **Isolation of VTEC**

The initial diagnostic procedure in most laboratories is the isolation of the causative organism, and O157:H7 is routinely investigated by use of selective medium. The selection is based on the fact that this serotype, unlike 95% of *E.coli* strains, does not ferment sorbitol in 24 hours. A modified MacConkey agar containing sorbitol instead of lactose (SMAC agar) is used to select for non-sorbitol fermenters such as *E.coli* O157:H7. However, many other bacteria do not ferment sorbitol, and even when present, O157:H7 may constitute only a small percentage of non-sorbitol fermenters, so further tests are necessary to confirm the presence of the organism. This is usually done by serotyping the isolate with O157 antiserum (Kleanthous *et al* 1988) although for identification, it is preferable to demonstrate that organisms are able to decarboxylate lysine and ornithine, as cross-reaction of other serotypes with O157 antiserum has been reported (Haldane *et al* 1986).

Several groups have incorporated a further selective step to identify O157:H7. Farmer & Davis (1985) added H7 antiserum to a sorbitol medium, which reacted with the flagella of O157:H7 resulting in loss of

motility. It is also possible to differentiate between O157:H7 and other non-sorbitol fermenters, which are mostly *Proteus* species and other *E.coli* serotypes, by addition of rhamnose and cefixime to SMAC agar. *Proteus* species are inhibited by cefixime, and the *E.coli* strains will ferment rhamnose, while O157:H7 is unable to do this (Chapman *et al* 1991).

These routine screening procedures are clearly limited in that VTEC other than O157:H7, all of which are able to ferment sorbitol, will not be detected. It has been estimated, for example, that in HUS infections 30% VTEC can be attributed to non-O157 serogroups (Kleanthous *et al* 1988). Thus, although O157 is the most commonly isolated VTEC serogroup, this is in part due to the fact that other serogroups are not looked for. More VTEC infections would therefore be diagnosed if the production of VT was investigated. One solution to this problem is to screen colony sweeps for VT production by releasing the toxin with polymyxin B. This method allows VT detection even if VTEC numbers are low, and although less sensitive than demonstration of FVT, it ensures that all VTEC can be detected not just O157:H7. VT released in this manner has been assayed by standard Vero cell assays (Karmali et al 1985), or, more rapidly by an ELISA based on the VT cell receptor, globotriosylceramide (Ashkenazi & Cleary 1990).

In addition to the problem of detecting relatively low numbers of VTEC in a mixed flora, it is necessary to test faeces within four days of onset of symptoms, as VTEC are rapidly cleared from stools of patients. Often, by the time HUS develops, characteristically one week after onset of symptoms, the organism is no longer present in faeces.

1.5.3 Detection of Neutralizing Antibodies

Chart *et al* (1991a) have suggested that serological testing of patients with HUS for antibodies to the lipopolysaccharide (LPS) of O157 provides evidence of infection with O157 when faecal bacteria or VT cannot be detected, since healthy controls failed to demonstrate these antibodies. 73% of children with HUS were serum-positive in an ELISA compared to 23% which gave positive results for bacteriology or toxin testing alone. Similar findings were reported by Bitzan & Karch (1992) using an indirect haemagglutination assay. A diagnosis based on seropositivity alone should be interpreted cautiously, however, as cross-reaction has been reported between the LPS of O157:H7 and *Brucella abortus*, *B.melitensis*, *E.hermannii* (Perry & Bundle 1990) and *Yersinia enterocolitica* O9 (Chart *et al* 1991b). This is because the LPS of these organisms share common sequences.

Rather than detect antibodies to O157:H7, it is possible to look for neutralizing antibodies to VT using a modification of the receptor enzyme linked immunosorbent assay (the NeutRELISA). This method measures the ability of antibodies in patients' sera to inhibit the binding of a known concentration of VT to the VT receptor, globotrioasylceramide, Gb3 (Boulanger *et al* 1990). A positive result would confirm a VT infection, and would allow detection of VTEC other than O157:H7.

1.5.4 The Use of DNA Probes in Diagnosis

DNA probes have been used to detect the presence of genes coding for VT, and for the lysogenic phages which carry these genes (Seriwatana *et al* 1988, Bettelheim *et al* 1990), and whilst this is an effective method of screening for epidemiological studies, it is not a routine diagnostic

procedure due to expense and the need for specialized equipment. Screening for the presence of VT genes gives no indication of expression of the toxins, although it does indicate the incidence of the genes in the *E.coli* population.

1.6 **Adherence of VTEC**

Attachment of VTEC is an important pathogenic mechanism, allowing colonization to occur, which in turn facilitates toxin delivery. VTEC adhere to the intestine of infected animals, in the attaching and effacing (AE) manner which is characteristic of EPEC isolates (Moon *et al* 1983, Sherman *et al* 1988b). EPEC and VTEC can be distinguished, however, because VTEC colonize and adhere to the caecum and colon, whereas EPEC primarily colonize the duodenum and upper small bowel (Karmali 1989). This mode of adherence is characterized by the close attachment of the organism to the enterocyte, effacement of the microvilli, and disruption of the cytoskeleton at the site of attachment. The cell membrane is commonly seen "cupping" around the bacterium, and there is an accumulation of actin beneath the attachment site. This characteristic has been exploited in the detection of bacteria which attach in the AE manner (Knutton *et al* 1989).

Early studies indicated that VTEC adhered to epithelial cells via fimbriae which were encoded by a 60-MDa plasmid (Karch *et al* 1987). However, Ashkenazi *et al* (1992) have since demonstrated that some non-fimbriate VTEC strains hybridise with a probe derived from the 60-MDa plasmid (Levine *et al* 1987). In addition, some non-fimbriate *E.coli* O157:H7 have been shown to adhere to epithelial cells *in vitro* (Sherman *et al* 1987), therefore there is much controversy over the role of the 60-MDa plasmid in VTEC adherence.

The results of the present study support the hypothesis that possession of the 60-MDa plasmid is not a major factor in VTEC attachment. O157:H7 (ATCC 35150) was investigated and found to be non-fimbriated (personal communication, I. Urabi) although it did possess a large plasmid of ~60-MDa. Mutants of this strain, which had lost the plasmid, were able to adhere to HeLa cells to the same extent as the parent strain. Tzipori *et al* (1987) failed to find an association between possession of a 60-MDa plasmid, and virulence of O157:H7 strains in gnotobiotic piglets. Thus there are strong indications that the 60-MDa plasmid is not in itself responsible for adherence of VTEC.

Analysis of genes which hybridised with the plasmid-derived probe indicated that the ability to produce AE lesions was not plasmid-encoded (Hall *et al* 1990). Studies using plasmid-cured strains suggest that the plasmid is involved in initial adherence to the brush border, but is not required for AE activity (Tzipori *et al* 1989). Adherence of VTEC *in vivo* and the formation of AE lesions is probably mediated by a 94-kDa membrane protein. In EPEC strains AE activity is dependent upon a 94-kDa membrane protein encoded by the chromosomal *eae* gene (Jerse & Kaper 1991). Homologous sequences found in VTEC strains indicate that a chromosomal gene could be responsible for VTEC AE adherence (Jerse *et al* 1991). This evidence is supported by the finding that a 94-kDa outer membrane protein competitively inhibits adherence of O157:H7 (Sherman *et al* 1991). Antiserum to the Eae protein of EPEC recognized a corresponding protein in O26:H11 strains, but not in O157:H7 strains (Jerse & Kaper 1991). Thus, it is proposed that these proteins serve similar functions, but are antigenically distinct, emphasizing that O26:H11 is more closely related to EPEC strains than it is to O157:H7 (Whittam *et al* 1988). Regulation of AE attachment in EPEC strains is positively regulated by a 60-MDa plasmid (which unlike the VTEC plasmid does not encode

fimbriae), possession of which is related to increased virulence (Jerse & Kaper 1991). It is speculated that the 60-MDa plasmid in VTEC may play a similar role in VTEC pathogenicity, since at least in some cases, there is correlation between adherence and possession of fimbriae (Winsor *et al* 1992). In addition, the plasmid-encoded adhesins may be involved in tissue specificity of VTEC.

1.7 **Verotoxins- General Introduction**

VT are bipartite toxins, composed of a single A subunit which becomes enzymatically active, and a pentamer of smaller B subunits which bind to specific cell-surface receptors. The association between the A and B subunits is non-covalent. Two antigenically distinct VT are known, Verotoxin 1 (VT1) and Verotoxin 2 (VT2), and variants of VT2; VT2vh and VT2vp, have been described from human (Oku *et al* 1989), and animal origin (Marques *et al* 1987). The relative sizes of the subunits of VT1, VT2 and VT2vp are shown in Table 1.3.

All VT are cytotoxins, and VT1, VT2 and VT2vh are enterotoxins as defined by the ability to cause fluid accumulation in the rabbit ileal loop test. VT2vp does not show enterotoxic activity (Tzipori *et al* 1987). With the exception of VT2vp which appears to be the product of a chromosomal gene (Pollard *et al* 1990), VT are produced by lysogenic phage, which at least in some cases are lambdoid in nature (Huang *et al* 1987). Around half of O157:H7 strains are lysogenized by VT1- and VT2-encoding phages, and produce both toxins (Newland & Neill 1988).

VT1 is closely related to Shiga toxin, which is produced by *Shigella dysenteriae* type 1. Antibodies to Shiga toxin will neutralize the activity of VT1 but not VT2, and at the amino acid level VT1 and Shiga toxin differ by only a single amino acid residue in the A subunit (Strockbine *et al*

1988). Expression of Shiga toxin and VT1 is repressed by high levels of iron (van Heyningen & Gladstone 1953, Calderwood & Mekalanos 1987), although VT2 production is not iron-regulated (Weinstein *et al* 1988a).

VT2 is antigenically distinct from VT1, and the toxins share 58% homology at the amino acid level (Jackson *et al* 1987a). The activity of VT1 and VT2 is identical however, and they both inhibit protein synthesis by inactivation of the 60s ribosomal subunit (Igarashi *et al* 1987). VT2 variants include VT2vp which has been isolated from cases of porcine oedema disease, and VT2vh which more closely resembles VT2. They are classified as variants because although they are neutralized by antibodies to VT2 and not VT1, and share >90% nucleotide sequence homology with VT2, they are less cytotoxic for HeLa cells than VT2. This has been shown to be due to differences in receptor binding in the case of VT2vp, but not VT2vh, which differs only slightly from VT2. VT1, VT2 and VT2vh bind to Gb3 (globotriaosylceramide) while VT2vp binds very weakly to this receptor but strongly to Gb4 (globotetraosylceramide), which is not recognized by the other VT (DeGrandis *et al* 1989).

The toxins are heat-labile to varying degrees, with VT1, which is unaffected by heating to 60°C being the most heat-stable, whereas >75% of VT2 activity is lost at this temperature (Head *et al* 1988). VT1 must be held at 80°C for at least 20 minutes or boiled for 2 minutes to be inactivated (Petric *et al* 1987). VT2vp is more heat-labile than either VT1 or VT2, and activity is completely lost at 65°C after 30 minutes (MacLeod *et al* 1991).

Production of VT by certain EPEC strains suggests a putative role for these toxins in the pathogenesis of EPEC infections. Some O26:H11 strains not only produced VT but appeared to possess the 60-MDa plasmid associated with VTEC adherence, and these strains have been recategorized as VTEC (Scotland *et al* 1990). VT production has not been associated with EIEC strains (Cleary & Murray 1988).

Table 1.3 Physicochemical Properties of Verotoxins

Subunit	Toxin	Mol. weight	No. of Nucleo-tides	No. of Amino acid Residues	pI
A	VT1	32,211	879	293	11.1
	VT2	33,135	888	296	9.8
	VT2vp	33,050	891	297	8.7
B	VT1	7,690	207	69	5.9
	VT2	7,817	210	70	5.4
	VT2vp	7,565	204	68	10.2

1.8 Binding of Verotoxins to Eukaryotic Cells

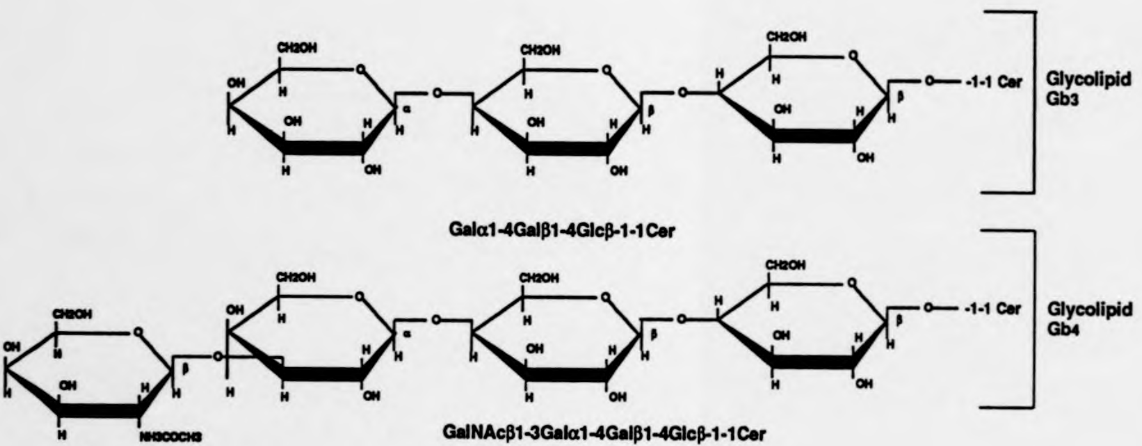
VT exhibit extreme toxicity towards certain cultured cell lines, but most cell types tested are not susceptible to toxin action *in vitro*. The nature of this selectivity is not clear, although it is likely that cells resistant to VT do not contain measurable amounts of toxin receptors. This was found to be the explanation for resistance of certain cells to cytotoxin produced by *Shigella shigae* (Eiklid & Olsnes 1980). Since insertion of the VT receptor into the membrane of resistant cells renders these cells sensitive to VT, this may also explain the cell selectivity of VT (Tyrrell *et al* 1992).

Studies on VT suggest that the holotoxin binds to cells via the B subunits, with the A subunit directed away from the membrane. The cell surface receptor-binding site probably lies in a cleft between adjacent subunits of the B pentamer (Stein *et al* 1992). Cytotoxic activity is lost if the last four amino acids from the carboxy terminus of the 70 amino acid VT2 B subunit are deleted but not if the terminal two amino acids only are lost suggesting that the critical amino acid residues are phenylalanine 67 and asparagine 68 (Perera *et al* 1991b).

The functional receptor for both VT1 and VT2 on HeLa and Vero cells is a neutral glycolipid, globotriaosylceramide (Gb3)(Figure 1.1) (Lingwood *et al* 1987, Waddell *et al* 1987). Shiga toxin binds several Gal α1-4Gal-containing glycolipids (Jacewicz *et al* 1986), but it appears that Gb3 is the high-affinity receptor (Jacewicz *et al* 1989).

The major difference between VT2 and VT2vp is the cytotoxicity towards cultured cell lines. VT2 is cytotoxic towards Vero and HeLa cells, whereas VT2vp has little effect on HeLa cells (Marques *et al* 1987). This is because, despite the fact that VT2 and VT2vp share >90% nucleotide

Fig. 1.1 Structure of Gb_3 and Gb_4



homology (Weinstein *et al* 1988b), they do not bind to the same receptor glycolipid (DeGrandis *et al* 1989). This difference is reflected in the isoelectric points of the B subunits of VT1, VT2 and VT2vp (Table 1.3). VT2vp binds to globotetraosylceramide (Gb4), and binds only weakly to Gb3.

VT1 and VT2 do not recognize Gb4, indicating that a terminal Gal α1-4Gal sequence is essential to binding (Figure 1.1). Vero cells have equal amounts of Gb3 and Gb4 therefore are equally sensitive to VT2 and VT2vp. HeLa cells presumably lack Gb4, and so VT2vp, which binds to Gb3 very weakly, has HeLa cell activity only when the toxin is present in high concentrations. Like VT2, VT2vh binds to terminal Gal α1-4Gal sequences in Gb3 (Samuel *et al* 1990). The difference in receptor binding may result from minor changes in the amino acid sequence since a single change (Asp-18 to Asn) in the B subunit of VT1 changed the binding phenotype to that of VT2vp (Tyrrell *et al* 1992).

The difference in glycolipid binding specificity between VT2 and VT2vp may give some insight into the differences in clinical symptoms seen between VT2 and VT2vp infections. Both purified VT2 and VT2vp are lethal for weaning pigs, but VT2 does not produce the clinical symptoms of oedema disease seen when VT2vp is administered (Samuel *et al* 1990). This suggests that differences in receptor specificity can determine the type of disease seen in animals.

Human Burkitt lymphoma cells (Daudi cells) possess many receptors for VT (Gb3 & Gb4) and so bind large amounts of the toxins. Mutant Daudi cells were produced by Cohen *et al* (1987) which had lost the ability to bind VT and were cross-resistant to inhibition of growth by α-interferon. This suggests that an additional role for these glycolipids *in*

vivo is in the modulation of the affinity of α-interferon for its membrane protein receptors.

1.9 Mechanism of Action of Verotoxins

After binding to the eukaryotic cell, toxin entry is thought to occur via receptor-mediated endocytosis (personal observation, this study). In the process of intracellular translocation, the A subunit undergoes proteolytic cleavage at a trypsin-sensitive site near the amino terminus, to produce the active A' fragment. This inhibits eukaryotic protein synthesis by cleavage of the N-glycosidic bond at adenosine 4324 (A4324) in the 28S rRNA of rat liver 60S ribosomal subunits. This results in the blocking of elongation-factor-1-dependent binding of aminoacyl-tRNA to ribosomes, although non-enzymatic binding of aminoacyl-tRNA, peptide bond formation and translocation are not inhibited (Igarashi *et al* 1987). Shiga toxin, which differs from VT1 in only one amino acid residue, has an identical mode of action, and inactivation has been shown to occur at a rate of at least 40 ribosomes per minute (Reisbig *et al* 1981). This N-glycosidase activity has been demonstrated *in vitro* on isolated ribosomes, but also occurs in living cells since microinjection into Xenopus oocytes has the same effect (Saxena *et al* 1989). The sequence in which A4324 lies, is well-conserved in rat, yeast and *E.coli* ribosomes and is likely to be very important since hydrolysis of a single bond or removal of one base leads to total inactivation of ribosomes (Endo *et al* 1988).

This mode of inactivation may be a general mechanism for ribosome-inactivating cytotoxins since ricin, a higher plant toxin, has the same activity (Endo *et al* 1987). The relatedness of ricin and VT extends not only to the mode of action but also to nucleotide sequence homology. Homology exists between the enzymatically active units of VT and ricin,

namely the A subunit of VT and the A chain of ricin (Calderwood *et al* 1987). The crystal structure of ricin has been determined, and a cleft in the A chain is thought to correspond to the active-site. The conserved residues between ricin and VT were plotted on the ricin A chain crystal structure, and 7 amino acids of VT were shown to lie in the proposed active site. From this data site-directed mutagenesis was used to determine that glutamic acid 167 is an active-site residue in VT1 and is critical for enzymic activity of the toxin (Hovde *et al* 1988). Mutation of the corresponding amino acid residue in VT2vp led to reduction of enzymic activity by approximately 1,500-fold. *In vivo* this mutant toxin failed to affect pathological changes in pigs, but did induce production of neutralising antibodies to VT2vp, therefore this is a possible vaccine candidate (Gordon *et al* 1992). The corresponding glutamic acid residue at position 166 of the A subunit is essential for full toxic activity of VT2 (Jackson *et al* 1990). A second region vital to the cytotoxicity of VT is between amino acid residues 202 and 213 of the A subunit (Jackson *et al* 1990).

1.10 Verotoxin-Converting Phages

VT1 and VT2 are produced by lysogenic phage, which at least in some cases are lambdoid in nature, homology existing with the J gene which codes for the lambda tail fibre, and with the genes which code for DNA replication and integration functions (Huang *et al* 1987). The existence of this relationship is supported by the fact that *E.coli* K12 produces high levels of VT if lysogenized with a VT-converting phage, but this does not occur if the K12 strain is a lambda lysogen (Smith *et al* 1983). VT2vp-encoding phage have not been isolated, and it now appears that the genes encoding this toxin are chromosomally located (Pollard *et al* 1990).

Most of the studies on VT-converting phages, concentrate on the VT-encoding genes, rather than biology of the phages. However, since these are lambdoid phages, it is likely that they share similar characteristics to phage lambda. In a cell lysogenic for lambda, none of the viral genes needed for productive infection are transcribed, and very little viral mRNA is detectable. Six regulatory genes are responsible for ten regulatory proteins which maintain the lysogenic state (Luria *et al* 1978). The repressor protein, coded by the *cI* gene, prevents transcription of the rest of the viral genome, and any conditions which result in breakdown of this protein, cause the phage to enter the lytic cycle. VT-converting phages can be induced by UV light or mitomycin C (O'Brien *et al* 1984, Head *et al* 1988), because these conditions induce the SOS response in the host cells. This involves activation of the RecA protein, which is able to break down the phage repressor causing the phage to enter the lytic cycle (Walker 1987).

VTEC strains which produce both VT1 and VT2 are lysogenized by two distinct phage. O157:H7 strain 933 for example, contains two toxin-converting phages, 933J which produces VT1, and 933W which carries VT2 genes (Strockbine *et al* 1986). The VT1- and VT2-encoding phage of O157:H7 are morphologically indistinguishable, consisting of regular hexagonal heads (74 x 74nm) and short contractile tails (28 x 11nm). This morphology differs from that of VT1-encoding phage H19, isolated from O26:H11, where the hexagonal phage head is more elongated (117 x 67nm) and the longer flexible tail is non-contractile (177 x 12nm). The VT1 region cloned from H19 and 933J is identical however, and was unrelated to the VT2 region from 933W when restriction sites over 2kb were analysed (Willshaw *et al* 1987). VT1-encoding phage isolated from a bovine strain of O26:H11 was similar in morphology to that from a human strain (Rietra *et*

al 1989), so the morphology of the phage seems to be a property of the serotype.

In contrast to other toxin-converting phage eg *Corynebacterium diphtheriae*, the *tox* genes map away from the phage attachment genes. Thus VT-converting phage genes were not picked up by simple imprecise prophage excision. It is interesting that despite the almost identical sequence between VT1 and Shiga toxin, Shiga toxin is the product of a chromosomal gene (Sekizaki *et al* 1987). This difference has been exploited to differentiate between VT1 and Shiga toxin by the polymerase chain reaction (PCR) as oligonucleotide primers based on a common region of VT1 and Shiga toxin will identify both genes, but if a unique sequence in the promoter region of the VT1 gene ie phage DNA is used, Shiga toxin is not recognized (Pollard *et al* 1990).

A study by Brown *et al* (1989) suggested that VT-converting phages in O157:H7 are present in many nontoxigenic *E.coli* isolated from children in Thailand. They proposed that although VTEC are isolated with low frequency this is not due to the absence of phages capable of carrying the genes for VT. This view is not supported by a study in the United States where results suggested that the occurrence of silent or poorly expressed VT genes is low (Newland & Neill 1988).

Phage typing has been postulated as a means, in addition to other studies, to monitor the epidemiology of outbreaks of VT1 and VT2 (Ahmed *et al* 1987).

1.11 Genetics of Verotoxin Production

Nucleotide sequencing has given greater insight into the relatedness between VT, and their relationship with, other toxins. The nucleotide sequence of VT1 is identical to that of Shiga toxin, apart from three

nucleotides in the A subunit which results in a single amino acid difference (Strockbine *et al* 1988). By contrast, VT1 genes share only 57 to 60% nucleotide sequence homology and 55 to 57% deduced amino acid sequence homology with those of VT2 (Jackson *et al* 1987a). The VT2 variants are closely related to VT2, with 91% overall sequence homology between VT2 and VT2vp, and >95% homology between VT2 and VT2vh (Ito *et al* 1990). There is no significant homology between VT and other bacterial toxins with intracellular targets, eg diphtheria toxin, *Pseudomonas* exotoxin A, and cholera toxin, although homology with the plant toxin ricin has been reported (Calderwood *et al* 1987).

1.11.1 **Transcription of Verotoxin Genes**

The genes encoding the A and B subunits of VT constitute an operon with 12 nucleotides separating the coding regions of VT1 (Calderwood *et al* 1987) and 14 nucleotides separating the A and B subunit genes of VT2 (Jackson *et al* 1987a). The intergenic space between the A and B subunit genes of VT2vh and VT2vp is 14 and 15 nucleotides respectively (Ito *et al* 1990, Weinstein *et al* 1988b). A VTEC isolate has been described that contained two VT2vh operons which were almost identical (Ito et al 1990). Similarly Schmitt et al (1991) detected two copies of VT2 genes in an isolate of O157, although there are no reports of multiple gene copies in VT1-producers.

The A and B subunit genes are transcribed into a polycistronic mRNA from a promoter upstream from the A subunit. If this promoter is blocked, VT1 B subunits can be transcribed from a promoter 5' to the B gene, within the C-terminus of the A subunit reading frame (Jackson *et al* 1987b). VT2 B subunits cannot be transcribed independently (Sung *et al* 1990).

VT1 production is repressed at the level of transcription by high levels of iron in the medium. This control requires the product of the chromosomal *fur* gene, a 17-KDa protein which combines with iron to form a repressor which binds to a site within the VT1 promoter, inhibiting transcription (Calderwood & Mekalanos 1987). A 21bp region of dyad symmetry overlapping the proposed promoter region of VT1, is absent from the promoter regions of VT2 and VT2 variants, and these toxins are not iron-regulated (Sung *et al* 1990, Ito *et al* 1990). Unlike the VT1 promoter, the nucleotide sequence of the VT2 promoter is not highly homologous to known *E.coli* promoters and may be a less efficient transcription start signal, which is reflected in lower observed levels of VT2 compared to VT1 (Strockbine *et al* 1986).

1.11.2 **Translation of Verotoxin Subunits**

VT are composed of a single A subunit and 5 B subunits, a stoichiometry which results from independent translation of the A and B subunits. Putative ribosome-binding sites identified upstream of both A and B subunit genes are identical in all VT operons (Ito *et al* 1990). The subunit genes of VT1 and VT2vp are separated by 12 and 15 nucleotides respectively (Calderwood *et al* 1987, Weinstein *et al* 1988b) so they are translated in the same reading frame. In contrast, the A and B subunit genes of VT2 and VT2vh are separated by 14 nucleotides so must be translated in different reading frames (Jackson *et al* 1987a, Ito *et al* 1990). Both the A and B subunit genes are translated with N-terminal signal sequences of around 20 nucleotides, which do not form part of the mature toxin (DeGrandis *et al* 1987, Jackson *et al* 1987a, Gyles *et al* 1988).

The A subunit of VT1 is three amino acids shorter and is more basic than that of VT2, although the B subunits of the two toxins differ by only

one amino acid in length and share a similiar isoelectric point (Table 1.3). VT2 and VT2 variants are closely related at the amino acid level, although within this sub-group of toxins, the A subunits are more highly conserved than the B subunits (Ito *et al* 1990). The A subunits of VT2 and VT2vp for example are 94% homologous, whereas there is only 84% nucleotide homology between the B subunits (Gyles *et al* 1988).

1.12 **Other VT-Producers**

All strains of *S.dysenteriae* type 1 appear to produce VT1 but not VT2, when tested by either genetic or immunological methods (Smith *et al* 1987). This is because Shiga toxin, which these strains actually produce, is identical antigenically to VT1 and differs by only one amino acid. Strains of *S.flexneri*, *S.boydii*, *S.sonnei* and other serotypes of *S.dysenteriae* also produce a cytotoxin, but neutralization by anti-Shiga toxin (equivalent to anti-VT1) is either non-existent, or only partial (Prado *et al* 1986). Thus it appears that toxins of the VT family are not common in other *Shigella* species.

In contrast, cytotoxin produced by some isolates of *Vibrio cholerae* and *V.parahaemolyticus* are neutralizable by anti-Shiga toxin but not by anti-cholera toxin as might be expected. These strains were reported to be Shiga-like toxin producers (O'Brien *et al* 1984a) . However unlike VT, the toxic activity was completely cell-associated, and more significantly was heat-stable. So it appears that this toxin is antigenically cross-reactive with Shiga toxin (and hence to VT1), but is probably not part of this family of toxins. It therefore appears that despite the fact that these toxins are phage-encoded, the host range of these lysogenic phage, as determined by the ability of organisms to produce VT, seems to be very narrow.

1.13 The Role of the Bacterial Cell Envelope in Pathogenicity

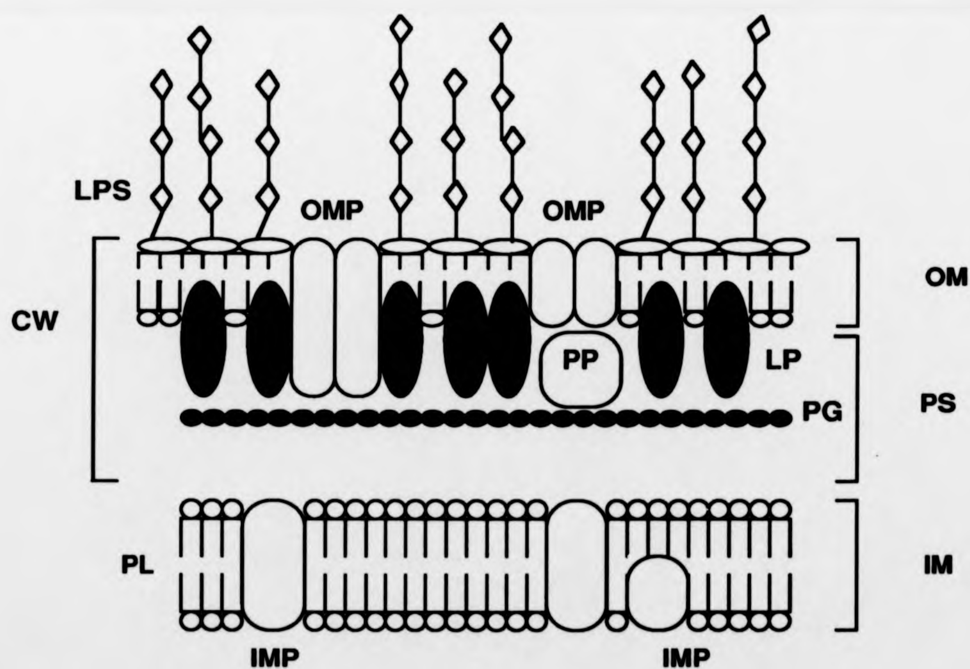
In order to be a successful pathogen, an organism must be able to gain entry into the host, and survive and multiply whilst resisting host defence mechanisms. Most bacteria, except the *Mycoplasma*, possess an additional layer, external to the cytoplasmic membrane, referred to as the cell wall. This is essential to prevent cell lysis, as the cytoplasmic membrane alone could not withstand the osmotic pressure inside the cell caused by the high internal concentration of metabolites. Since the cell wall is the outermost part of the bacterial cell, it is essential that this part of the cell has adapted to aid the survival of the pathogen in the host. In general, bacteria can be classified as either Gram-positive or Gram-negative according to the reaction of the cell wall in the Gram stain. *E.coli* are Gram-negative organisms, so the structure of the Gram-negative cell envelope will be briefly described below.

1.13.1 The Gram-Negative Cell Envelope

The cell envelope of Gram-negative bacteria is much more complex than that of Gram-positive organisms. The cytoplasmic membrane of Gram-positive bacteria is surrounded by a thick cell wall composed of peptidoglycan and teichoic acid. In the case of Gram-negative bacteria however a periplasmic space, containing a narrow layer of peptidoglycan, separates the cytoplasmic membrane from an outer membrane consisting of phospholipids, proteins and lipopolysaccharides (Figure 1.2).

Figure 1.2 Diagrammatic representation of the Gram-Negative Bacterial Cell Envelope

The three main layers of the cell envelope are shown:
outer membrane (OM), inner membrane (IM) and peptidoglycan (PG). Individual components shown are: the cell wall (CW), periplasmic space (PS), lipoprotein (LP), outer membrane protein (OMP), inner membrane protein (IMP), periplasmic protein (PP), lipopolysaccharide (LPS) and phospholipid (PL).



Gram-negative bacteria are more resistant than Gram-positive species to a variety of host defence mechanisms due to the presence of the outer membrane. This protection is physical, biochemical and/or immunological in nature. Physically the outer membrane protects cells from the detergent action of bile salts and degradation by digestive enzymes. This property is especially important in enteric bacteria such as *E.coli*. The outer membrane endows the bacterial surface with strong hydrophilicity which is important in evading phagocytosis, and provides a strong permeability barrier to many antibiotics. The lipopolysaccharide (LPS) component is especially important in pathogenicity, allowing an organism to alter the surface antigen constitution and so evade host defences.

1.13.2 **Bacterial Lipopolysaccharide**

LPS is found exclusively in Gram-negative organisms and is composed of three components: a core polysaccharide common to many Gram-negative bacteria, an O-specific polysaccharide which confers virulence and serological specificity, and a lipid A component mainly responsible for toxicity. The schematic structure of the best characterized LPS, *Salmonella typhimurium*, is shown in Figure 1.3.

The lipid A moiety is hydrophobic and contains six saturated fatty acid chains linked to two glucosamine residues. These fatty acyl chains constitute about half of the outer leaflet of the outer membrane, but are absent from the inner leaflet, which instead contains phospholipids. Lipid A is responsible for the toxic properties and potent biological effects associated with LPS. Mutants incapable of synthesizing lipid A cannot be isolated as loss of this component is lethal to the cell.

Linked to the lipid A is the core oligosaccharide region. This is formed of 10 sugar units which project outward, and join to the O side chain, which is made up of many repeating tetrasaccharide units. Both the core oligosaccharide and the O chain are highly hydrophilic, and contain several sugars rarely found elsewhere in nature. These include an eight-carbon sugar, 2-keto-3-deoxyochonate (KDO); heptose, a seven-carbon sugar; and L-rhamnose and abequose, six carbon sugars. Studies with mutants of *S.typhimurium* defective in LPS have shown that loss of O antigens appear to increase sensitivity to antibodies and complement.

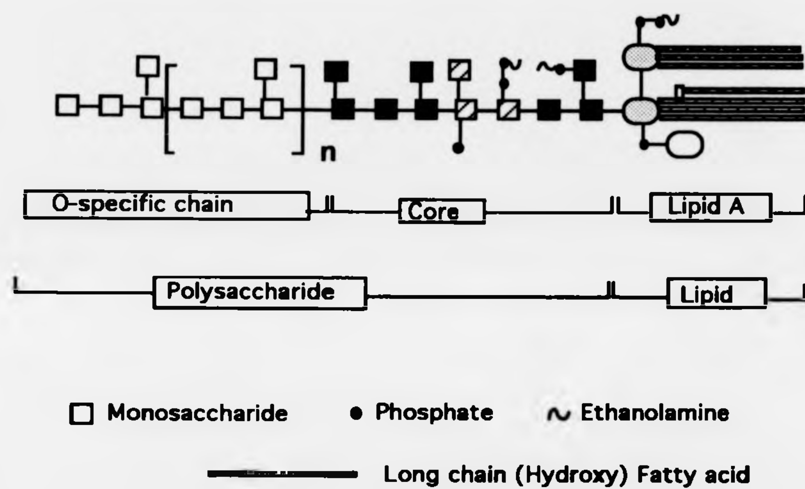
LPSs are synthesized in the plasma membrane and then transferred to the outer membrane. Lipid A is synthesized first and then core oligosaccharide is formed by addition of sugars from activated donors such as UDP-glucose and UDP-galactose. The tetrasaccharide units of the O chain are synthesized on carrier lipids, and assembled by transfer from one carrier to a growing chain on another carrier molecule. The completed O side chain is then added to the core oligosaccharide.

1.13.3 Outer Membrane Proteins

The outer membrane has a characteristic protein composition which differs from that of the cytoplasmic membrane. Outer membrane proteins perform a variety of functions for the cell. Some form diffusion pores or specific transport systems, others are involved in maintaining the structural integrity of the outer membrane, while a few have enzymatic function. A feature of outer membrane proteins is that their profile is dependent on strain,and physiological conditions.

The porins, OmpF, OmpC and PhoE, make up almost 2% of the total cell protein of *E.coli*. Their function is to allow passage of small

Figure 1.3. Schematic structure of LPS from *Salmonella typhimurium*
Adapted from Luderitz *et al* 1982



hydrophilic molecules across the outer membrane. The relative proportions of OmpC and OmpF vary depending on growth conditions, although the sum of their quantities tends to remain constant (Puente *et al* 1991). Under conditions of high osmolarity, synthesis of OmpF is repressed whereas OmpC expression is derepressed. PhoE is produced only under conditions of phosphate starvation. OmpA has a similiar molecular weight to (35-36KDa) , and is almost as abundant as, porins. Although OmpA may be involved with transport across the membrane, it is unlikely by itself to form pores. The function of this protein may be to stabilise the membrane, possibly in conjunction with lipoprotein.

Other proteins may be present in the outer membrane in large copy numbers under certain physiological conditions. The LamB protein for example, which is involved in maltose uptake may be present in comparable amounts to porins when fully induced.

Outer membrane proteins are important in disease as antibodies are commonly raised against them. Antibodies against *Salmonella typhimurium* outer membrane proteins for example, confer protection against infection by this organism (Udhayakumari & Muthukkaruppan 1987). Attachment of VTEC is also thought to be mediated by outer membrane proteins (Sherman *et al* 1991).

1.13.4 **Flagella**

Many bacteria possess appendages, such as flagella, which increase motility of the organism. Colonization and penetration of the surface mucous layer which overlies the lining of the gastrointestinal tract is an important aspect of the pathogenesis of enteric bacteria, and flagella may aid this process. Flagella may also function as bacterial attachment factors to promote binding to receptors on enterocytes or colonocytes. Expression of

flagella appears to be an important virulence factor for *Vibrio cholerae* and *Campylobacter jejuni* (Yancy *et al* 1978, Morooka *et al* 1985), and as most clinically isolated strains of O157 possess H7 flagella, they may be important in pathogenesis. Attachment of O157:H7 is not competitively inhibited by H7 flagella, so clearly any role flagella have in pathogenesis is not at the actual level of attachment (Sherman *et al* 1988a).

1.14 Cellular Internalization of Toxins

In general, bacterial toxins can be classified according to whether they damage the cell membrane, or act intracellularly. VT inhibit protein synthesis by acting on the 60S ribosomal subunit (Endo *et al* 1988), therefore they fall into the category of intracellularly acting toxins. Several other toxins have been shown to act on intracellular targets, including diphtheria toxin, *Pseudomonas* exotoxin A, Shiga toxin, and *E.coli* enterotoxins (Middlebrook & Dorland 1984). They all appear to share a common mechanism of action whereby binding to specific receptors on the target cell, is followed by internalization and subsequent interaction with the intracellular target.

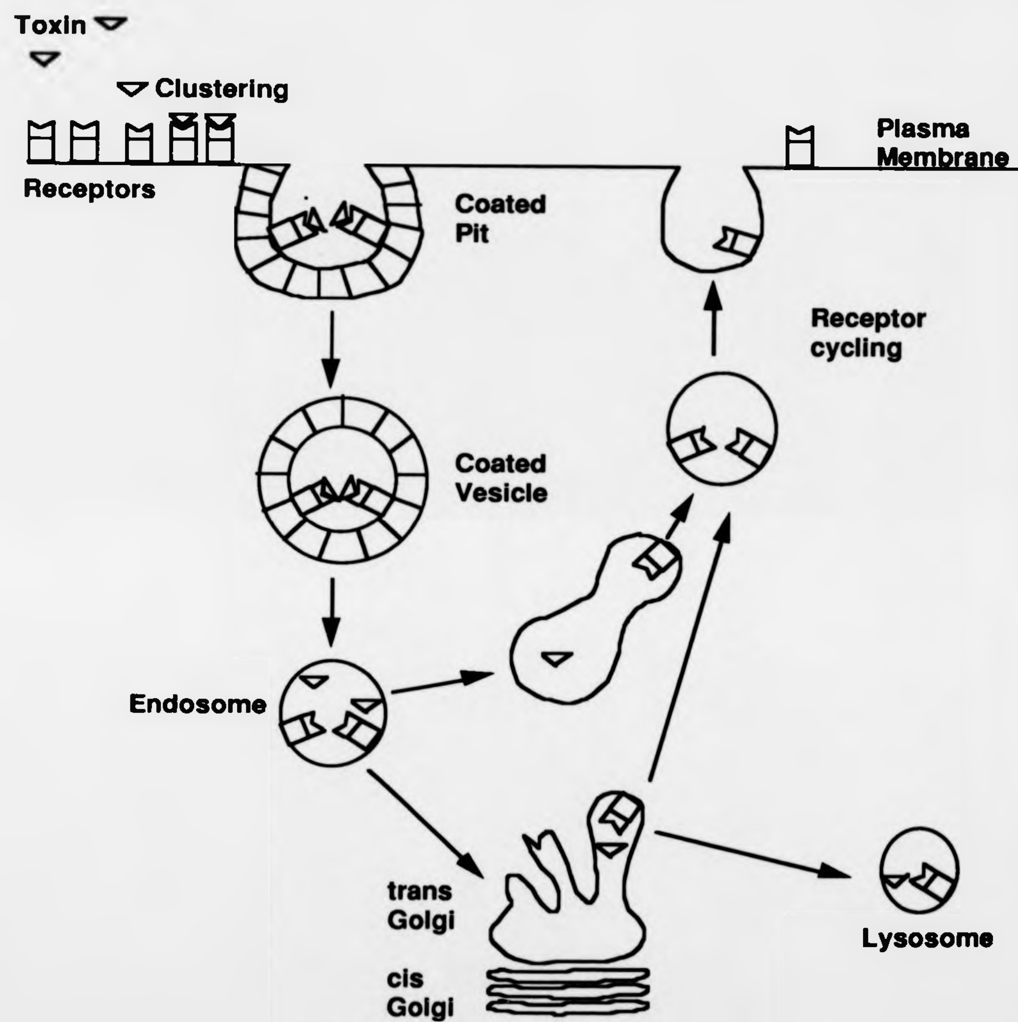
It is known that several toxins with intracellular targets are able to enter cells via receptor-mediated endocytosis (RME) (Figure 1.4) (Willingham & Pastan 1984). This process begins with the binding of the toxin to a specific receptor on the target cell. The toxin-receptor complexes rapidly migrate to specialized regions of the cell membrane known as coated pits. Most animal cells possess these areas (500-1500 per cell) which are rich in the protein clathrin. This clustering, which is thought to be mediated by the enzyme transglutaminase (Middlebrook & Dorland 1984), seems to be temperature-dependent as, in the case of Shiga toxin, it occurs at 37°C but at 0°C the binding sites are randomly distributed (Sandvig *et al*

1989). The toxin is internalized by invagination of the plasma membrane to form coated vesicles, which, upon loss of the clathrin coat are known as endosomes. Some of the toxin is then transported to the trans-Golgi region, and 30-60 minutes after entry, fuse with primary lysosomes or the trans-reticular Golgi apparatus. During this transit the intravesicular pH falls to about pH5, approaching that of lysosomes, and some toxins require this acidic environment to maintain their activity. The remainder of the endocytosed toxin is routed back to the cell surface and released in an intact form, as only a few toxin molecules are actually transported to the cytoplasm (Olsnes & Sandvig 1983).

In the case of diphtheria toxin, once the endosomal pH has fallen to 5.3, a hydrophobic domain is inserted into the endosomal membrane, allowing the active fragment of the toxin to enter the cytosol via transmembrane channels. Other toxins must pass from the endosome to the cytosol via lysosomes, which may indicate a requirement for further processing by lysosomal enzymes which are not present in endosomes. The mechanism of translocation to the cytosol is not fully understood, although diphtheria toxin, which normally passes to the cytosol directly from the endosome, can enter cells directly across the plasma membrane, if the external pH is low. Translocation is clearly in this case triggered by acidic conditions, and, as lysosomal pH is also low, it is assumed that this is one of the requirements for entry of toxins to the cytosol.

Figure 1.4

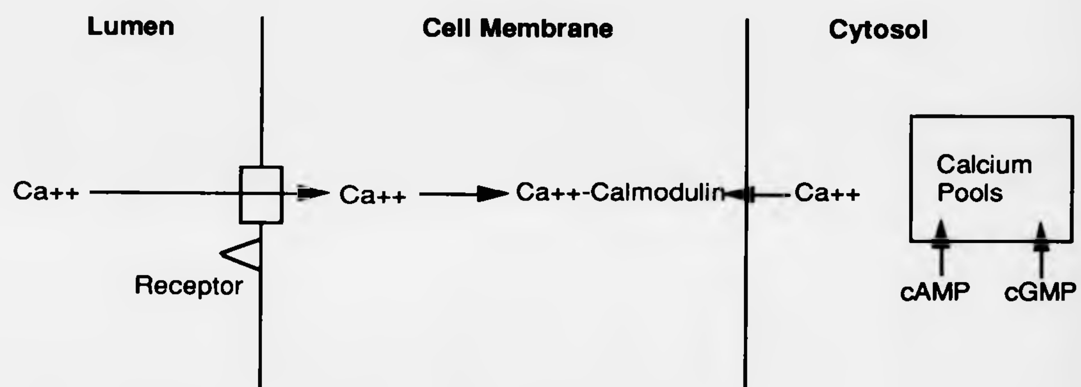
Schematic Representation of Receptor-Mediated Endocytosis



1.15 The Role of Calcium Uptake in Toxin Internalization

Since the outcome of toxin internalization is cell death, it is unlikely that mammalian cells have a specific uptake mechanism for toxins. It is, however, probable that existing transport pathways are involved in the uptake of these proteins. Calcium plays a regulatory role in all living organisms, and it is known that the activity of bacterial toxins such as Shiga toxin (Sandvig & Brown 1987) and *C.difficile* toxin B (Caspar *et al* 1987), and the plant toxins abrin and modeccin (Sandvig & Olsnes 1982a) are affected in the presence of drugs which block calcium transport across membranes. Calmodulin is a calcium-binding protein required to facilitate calcium transport in mammalian cells, and this pathway is represented in Figure 1.5. It appears that a calcium flux through naturally occurring channels is necessary for toxin uptake, but must be controlled, since calcium ionophores, which cause a strong influx of calcium ions, and thus upset the calcium gradient across the membrane, protect rather than sensitise cells against Shiga toxin (Sandvig & Brown 1987), abrin and modeccin (Sandvig & Olsnes 1982a). The protection afforded, is at the level of toxin entry since addition of calcium channel blockers such as verapamil is only effective before internalization of the toxins.

Fig 1.5 The Calcium Pathway in Mammalian cells



Chapter 2 Materials and Methods

2.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1.

2.2 Monoclonal Antibodies

Monoclonal antibodies were provided by N Strockbine as detailed in Table 2.2.

Table 2.2 Monoclonal Antibodies

MAb	Source	Reference
MAb 13C4 (ascites)	N Strockbine	Strockbine *et al* 1985
MAb BC5 BB12 (ascites)	N Strockbine	Downes *et al* 1988

Table 2.1 Bacterial Strains

Strain	Genotype	Source	Reference
E.coli O157:H7	-	ATCC 35150	Wells (1983)
E.coli O26:H11	-	NCTC 8781	PHLS Manchester
E.coli O157 Clinical isolate	-	Warwick Hospital	-
E.coli K12	-	University of Warwick	-
E.coli O26:H11 (E3787/H19)	-	S. Scotland	Willshaw *et al* (1987)
E.coli O157:H- (E32511)	-	S. Scotland	Willshaw *et al* (1987)
E.coli C600 (933J)	-	T. Meyer	Meyer *et al* (1989)
E.coli C600 (933W)	-	T. Meyer	Meyer *et al* (1989)
E.coli DH1	F^-, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, ($r_k M_k$), *sup*E44, l^-	P. Reeves	Hanahan (1983)
E.coli Econ1	-	Northwick Park Hospital	-
S.dysenteriae type 1	-	NCTC 4837	

2.3 Cytotoxicity Assay

2.3.1 Tissue Culture

Vero cells were grown in 75ml flasks in growth medium consisting of Medium 199 (Flow) supplemented with 10% foetal calf serum (FCS), 10mM L-glutamine, penicillin ($50Uml^{-1}$) and streptomycin ($50mgml^{-1}$). Confluent monolayers were removed with trypsin-EDTA and resuspended at $4x10^5$ cells ml^{-1} in growth medium. A 100 µl volume of diluted cells was added to each well of a 96-well microtitre plate. The cells were allowed to reach confluency by overnight incubation at 37°C in an atmosphere of 5% CO_2. The growth medium was then replaced with maintenance medium, which was the same as growth medium except that it contained only 2% FCS. The monolayers were then ready to be used in the Vero cell assay, although were stable in maintenance medium for up to two days.

2.3.2 Vero Cell Assay

Bacterial cultures were grown, with shaking, in 10ml of Penassay broth at 37°C for 18 hours. Cells were harvested by centrifugation at 10,000g for 10 minutes and the supernatant was passed through a 0.22µm membrane filter. The filtrate was then diluted two-fold across a microtitre plate and 100µl aliquots of each dilution were transferred to the Vero cell monolayers using a multichannel pipette. The plates were incubated at 37°C for 24-48 hours in 5% CO_2. Toxin titres were assessed as the dilution of the sample that killed 50% (CD_{50}) of the Vero cell monolayer. Negative controls, containing uninoculated broth, were included in each experiment. Where it was necessary to confirm the presence of VT, antitoxin (at 1:1000) was added to each well with the toxin.

2.4 Latex Agglutination Assay

2.4.1 Coating Latex Particles

The method of Bernard & Lauwerys (1983) was used to coat latex particles as follows.

50µl antibody (MAb 13C4 or MAb BC5 BB12) and 4ml freshly prepared, 0.22µm-filtered, GBS (10X stock contained: 1M glycine, 1.7M NaCl & 76mM NaN_3 adjusted to pH9 with NaOH) were mixed in a plastic universal tube. 0.5ml latex particles (0.73µm, 2.5% solids) were added and incubated for 60 minutes at room temperature with continuous agitation. After centrifugation at 25,000g for 10 minutes at 25°C, the particles were washed twice in 10ml of a solution containing 0.1M NaCl and 15mM NaN_3. Particles were resuspended in 10ml of this solution and stored at 4°C for up to 6 months.

2.4.2 Stabilising Coated Latex Particles

Before use of coated latex particles in an agglutination assay, stabilization was necessary to prevent non-specific aggregation between particles. The antibody-coated particles from stock were resuspended by vortexing, and 0.5ml of 1.1% bovine serum albumin (BSA) was added to 3.6ml of latex particle suspension. The BSA was necessary to overcome non-specific protein-protein interactions. After vigorous vortexing for 5 minutes, 0.4ml GBS pH 10.1, was added and the suspension was homogenized by further vortexing.

2.4.3 Preparation of the Antigen Filtrate

A modification of the method of Karmali *et al* (1985) was used to extract VT.

100μl of an overnight Penassay (Difco Antibiotic medium no.3) broth culture was inoculated into 20ml of fresh broth, and incubated with shaking at 37°C for 5 hours. Cells were harvested by centrifugation at 10,000g for 10 minutes in glass centrifuge tubes. The cell pellets were washed twice in PBS and suspended in 1ml of a polymyxin B solution ($2mgml^{-1}$ in PBS) which alters the permeability of the cell envelope and releases intracellular toxin. After 10 minutes incubation at 37°C, the suspension was centrifuged at 10,000g for 10 minutes. The pellet was discarded, and the supernatant (antigen) was passed through a 0.22μm membrane filter.

2.4.4 Latex Agglutination Test

The polymyxin B-treated culture filtrate (antigen) was diluted 1:100 in GBS pH9 containing 0.1% BSA. 50μl of stabilized coated latex particles were added to 20μl of diluted sample and vortexed rapidly. This mixture was incubated for 30 minutes at 37°C in a shaking water bath, and the reaction was stopped by adding 5ml of GBS containing 0.1% Tween 20 (0.45μm filtered).

Latex agglutination was stable for up to 12 hours at 25°C or 24-48 hours at 4°C. To detect latex agglutination, 50μl of sample was added to 20ml Isoton (Coulter) in an accuvette. The particles were counted according to size using a Coulter Counter II fitted with a 30/80 orifice.

2.5 Colony ELISA

Bacterial colonies were inoculated onto Penassay agar plates and incubated at 37°C for 16-20 hours. The surface of the agar was then overlaid with dry nitrocellulose membrane (Hybond-C, Amersham plc) ensuring that no air bubbles were trapped. 2ml polymyxin B (2mgml^{-1} in PBS) was added and then incubated at 37°C for 60 minutes. This allowed the polymyxin B to permeate the cell envelope and release intracellular toxin. After incubation the membrane was gently peeled off the agar and thoroughly washed in PBS to remove bacterial debris. To prevent non-specific binding of the antibody, the membrane, in a glass Petri dish, was blocked by addition of 10ml TBS (150mM sodium chloride, 50mM Tris-HCl pH 7.5) containing 2% Marvel. After shaking at room temperature for 10 minutes, the TBS-2% Marvel was poured off and replaced by an identical fresh solution, to which 10µl 1° antibody (MAb 13C4 or MAb BC5 BB12) was added (to give a 1:1000 dilution). This was left shaking at room temperature overnight. Unbound 1° antibody was then removed by three 10 minute washes in TBS containing 0.1% Tween 20. 10ml of fresh TBS-0.1% Tween 20 was then added containing 10µl 2° antibody (Anti-mouse peroxidase conjugate, Sigma). After 1-2 hours incubation with shaking, at room temperature, the membrane was washed twice in TBS-0.1% Tween 20 (10 minutes per wash) and twice more in TBS only. The membrane was then developed using the DAB/nickel method.

2.6 DAB/Nickel Development System

6mg DAB (3' 3' diaminobenzidene tetrachloride) was dissolved in 9ml of 0.05M Tris pH 7.6. To this, 1ml of a 0.3% w/v stock solution of nickel chloride in water, was added, followed by 0.1ml of a 3% solution of hydrogen peroxide in water. This solution was applied to the membrane,

which developed in 1-20 minutes. The membrane was washed in water before the reaction was complete as development continued for 10 minutes after washing.

2.7 Phage Induction

Plaque assays were carried out to demonstrate the presence of phages in culture filtrate from *E.coli* O157:H7 which had been grown in the presence of 1.0μgml^{-1} mitomycin C. Ten-fold dilutions of the filtrate were made in modified LB broth (10g tryptone, 5g yeast extract, 5g sodium chloride per litre, + 10mM calcium chloride & 0.001% thiamine). Each dilution was allowed to adsorb onto indicator cells, *E.coli* DH1 (grown to an OD_{600} of 0.5), for 20 minutes at 37°C. 100µl samples from the absorption mixture were mixed with 3ml modified LB soft agar (0.7%). This was immediately poured into Petri dishes containing modified LB agar (1.5%). Plates were examined for the presence of plaques in the bacterial lawn after overnight incubation at 37°C.

2.8 Preparation of crude toxin for SDS-PAGE analysis.

2.8.1 Mitomycin C treatment

A single bacterial colony (O157:H7 ATCC 35150) was inoculated into 10ml Penassay broth (Difco Antibiotic medium No. 3) and incubated at 37°C overnight. 2.5ml of this culture was inoculated into 1litre of fresh broth and incubated at 37°C until the bacterial suspension reached an OD_{600} ~0.5. At this stage, 0.1μgml^{-1} of mitomycin C was added to each flask. Seven flasks were set up in this way, so that one flask could be harvested every hour after addition of the drug. After harvesting the cells at 10,000g for 10 minutes, the supernatant was reduced in volume by differential ammonium sulphate precipitation.

2.8.2 Ammonium sulphate precipitation

Solid ammonium sulphate was added to the supernatant at 4°C to 40% saturation, and after 30 minutes incubation at 4°C, with agitation, the precipitate was removed by centrifugation at 10,000g for 10 minutes, and discarded. Further ammonium sulphate was added to the supernatant to 60% saturation. The second precipitate containing the toxin was recovered by centrifugation as before. The pellet was resuspended in 10ml of 0.01M phosphate buffer and dialysed overnight against 2 litres of this buffer, with 3 changes of buffer. The crude toxin was then analysed by SDS-PAGE or further purified by hydroxylapatite column chromatography.

2.8.3 Hydroxylapatite column chromatography

The dialysed crude toxin preparation was applied to a 15 x 150mm hydroxylapatite column (Biorad) equilibrated with 0.01M sodium phosphate buffer, pH 7.2. After washing with two column volumes of this buffer, the crude toxin was eluted with 0.2M sodium phosphate buffer pH 7.2. Protein levels were monitored at 280nm during elution, using a Pharmacia UV detector. 1ml fractions were collected, and fractions containing the protein peak were pooled and dialysed against 0.01M sodium phosphate buffer overnight, with 3 changes of buffer. The dialysate was analysed by SDS-PAGE analysis.

2.9 Polyacrylamide Gel Electrophoresis (PAGE)

2.9.1 SDS-PAGE

The following stock solutions were prepared:

Buffer A
0.75M Tris-HCl pH 8.8
0.2% SDS

Buffer B
0.25M Tris-HCl pH 6.8
0.2% SDS

Acrylamide stock (100ml)
44g acrylamide
0.8g bisacrylamide

Samples were analysed on 11% resolving gels with 4.5% stacking gels. Gel plates were assembled according to the manufacturers instructions (LKB Biotechnology Inc.). The resolving gel was made up as shown below and poured using a 10ml pipette immediately after addition of the ammonium persulphate and TEMED, so that the top of the gel was approximately 40mm below the top of the plate. After pouring, the gel was overlaid with water-saturated butanol and allowed to set for 30 minutes. The butanol was poured off, and 10ml Buffer B was poured onto the top of the gel, then thoroughly removed along with traces of butanol. The stacking gel was poured to the top of the gel plate and allowed to set around a comb, which on removal produced wells for sample loading. The gel was placed into the electrophoresis tank and running buffer (0.025M Tris, 0.129M glycine, 0.1% SDS) was added to the upper and lower reservoirs.

Resolving gel (11%)		Stacking gel (4.5%)	
Buffer A	27ml	Buffer B	10ml
Acrylamide stock	13.6ml	Acrylamide stock	3ml
Distilled water	12ml	Distilled water	7ml
Ammonium persulphate (10%) 1.9ml		Ammonium persulphate (10%) 0.5ml	
TEMED	130µl	TEMED	40µl

Prior to loading, samples were boiled for 5 minutes in an equal volume of sample buffer which contained, per 40ml, 10ml 0.025M Tris-HCl pH 6.8, 0.8g SDS, 4ml glycerol, 2ml 2-mercaptoethanol and 0.025g bromophenol blue. Sample buffer was made up in advance and stored at 4°C. Denatured samples were then loaded onto the gel using a Hamilton syringe. Molecular weight standards (Pharmacia) were included on each gel.

Samples were run at 20mA through the stacking gel and 40mA through the resolving gel. When the dye front reached the lower edge of the gel, the power was switched off, and the gel removed from the apparatus. The samples were then stained by the silver nitrate method or transferred to nitrocellulose prior to immunoblotting.

2.9.2 **4-15% Non-Denaturing PAGE**

The following stock solutions were prepared:

25% (w/v) acrylamide (100ml)	**30% (w/v) acrylamide (100ml)**
acrylamide 25g	acrylamide 30g
bisacrylamide 1.25g	bisacrylamide 0.15g

Lower gel buffer (100ml)

Tris 36.6g (pH 8.8 with HCl)

From these solutions, 4% and 15% gel solutions were made up.

4% (w/v) acrylamide solution		**15% (w/v) acrylamide solution**	
25% (w/v) acrylamide	9.2ml	30% (w/v) acrylamide	10ml
lower gel buffer	7.5ml	lower gel buffer	2.6ml
distilled water	40.8ml	30% (v/v) glycerol	7.4ml
TEMED	12.2µl	TEMED	2.6µl
* ammonium persulphate	104.25µl	* ammonium persulphate	26µl

* 10% ammonium persulphate was made up immediately before use.

The 4% and 15% acrylamide solutions were placed into separate compartments of a gradient maker. The gel was then poured so that the acrylamide concentration ranged from 15% at the bottom of the gel to 4% at the top. The gel was placed in the electrophoresis tank containing running buffer (0.025M Tris, 0.129M glycine), and prerun for 1 hour at 18mA. Samples were then loaded in a buffer containing 75% (v/v) glycerol

and 0.1% (w/v) bromophenol blue. Gels were run at 18mA until the dye front reached the bottom of the gel.

After removal from the gel tank the samples were transferred to nitrocellulose.

2.10 Silver nitrate staining of polyacrylamide gels.

Samples which had been separated by PAGE were stained by the silver nitrate method of Wray *et al* (1981). A modification of this method was used to stain LPS.

2.10.1 Protein staining

After PAGE the gel was soaked in 50% methanol, in a sandwich box, for at least 8 hours, with three changes of wash. 2.5ml ammonia was added to 42ml 0.36% (w/v) sodium hydroxide in a measuring cylinder. To this, silver nitrate (1.6g dissolved in 8ml distilled water) was added with shaking, until the solution was transparent. Distilled water was then added to a total volume of 200ml. The gel was stained in this solution for 15 minutes, with shaking. After thoroughly washing out the box, the gel was washed twice for 5 minutes in distilled water. The stain was developed in 500ml of a solution containing 2.5ml of 1% (w/v) citric acid and 0.4ml formaldehyde solution.

To stop the stain, 500ml of a solution containing 225ml methanol and 50ml acetic acid was added. The stained gel was then photographed on a light box.

2.10.2 LPS staining

After PAGE, the gel was fixed overnight in 200ml of a solution containing 25% (v/v) isopropanol in 7% (v/v) acetic acid. The gel was then soaked for 5 minutes in an oxidising solution (150ml distilled water containing 1.05g

periodic acid and 4ml 25% (v/v) isopropanol in 7% (v/v) acetic acid) which was made up just before use.

After eight 20 minute washes in distilled water, the gel was stained for 10 minutes in : 0.1M sodium hydroxide (28ml), concentrated ammonia (1ml), 20% (w/v) silver nitrate (5ml), and water 115ml. This solution was used immediately after preparation. Excess stain was removed by four 10 minute washes in water.

A developing solution was made up containing 50mg citric acid and 0.5ml of 37% formaldehyde per litre. It was essential to keep this solution at 25°C as at higher temperatures, staining of protein as well as LPS occurs. The stain was developed in this solution for 10-20 minutes until bands appeared. To stop the developer, the gel was placed in 200ml distilled water containing 10ml of 7% (v/v) acetic acid for 1 hour. The stained gel was then washed and stored in distilled water prior to photography.

2.11 Protein Assay

Protein concentrations were determined by the method of Lowry *et al* (1951).

Reagents: **A** 5% sodium carbonate

B (i) 1% $CuSO_4.5H_2O$

(ii) 2% sodium potassium tartrate

C 50ml reagent **A** + 1ml **B** (i) + 1ml **B** (ii)

- used immediately.

Each sample was diluted as appropriate, and 0.5ml were added to two boiling tubes. To each tube, 0.5ml 1M sodium hydroxide was added, followed by 2.5ml of reagent C. After 10 minutes incubation at room temperature, 0.5ml of 50% (v/v) Folin-Ciocalteu reagent in water, was added to each tube, and thoroughly vortexed. Tubes were then incubated

at room temperature for 30 minutes to allow the development of a colour reaction. The optical density of each tube was read at 650nm. To assess protein levels, a series of protein solutions (bovine serum albumin) of known concentrations (0-1mg protein) were tested in parallel. The optical densities of the standard solutions were used to draw a calibration curve from which the protein concentrations of unknown solutions were extrapolated.

2.12 Plate ELISA

100µl MAb to either VT1 or VT2 diluted 1:1000 in coupling buffer (sodium carbonate 0.795g, sodium hydrogen carbonate 1.465g in 500ml distilled water, pH 9.6) was added to each well of a 96-well ELISA plate. The plate was sealed with plastic film and incubated overnight at 4°C. The MAb acted as capture antibodies to concentrate the antigen.

Unattached antibodies were removed by washing the plate 3 times in ELISA wash, a saline solution containing 0.1% (v/v) Tween 20. To prevent non-specific binding of antigen, each well was blocked for 1 hour at 37°C with 100µl ELISA diluent (0.6g Tris, 0.1g bovine serum albumin in 100ml ELISA wash, adjusted to pH 7.4 with glacial acetic acid). The plate was washed 3 times in ELISA wash.

100µl of a 1:10 dilution of antigen (culture filtrate) was added to each well and incubated at 37°C for 1 hour. 100µl polyclonal rabbit anti-VT (1:100, antiserum was a gift from S. Scotland) was added to each well and incubated at 37°C for 1-2 hours, then washed 3 times to remove unattached antibodies. Anti-rabbit peroxidase conjugate (Sigma) diluted 1:1000 in ELISA diluent was then added. After incubation at 37°C for 1 hour, the plate was washed 3 times with ELISA wash.

TMB substrate was made up from 10ml substrate buffer (0.1M sodium acetate adjusted to pH 5.2 with glacial acetic acid), 100µl TMB

(58mg tetramethylbenzidine dissolved in 10ml DMSO) and 1µl hydrogen peroxide. 100µl of this solution was added to each well and incubated at 37°C for 10 minutes. The reaction was stopped by addition of 50µl 2M sulphuric acid, and the absorbance of each well was read at 450nm using a Biorad ELISA plate reader.

The following controls were included in each ELISA: 1. The antigen was omitted, and replaced by broth instead of culture supernatant, or PBS in place of sonicate. This ensured that the polyclonal antiserum did not bind non-specifically. 2. The primary antibody (anti-VT polyclonal) was omitted to ensure that the anti-rabbit serum was binding only to rabbit antigens (the MAb were of mouse origin).

No antigen was added to the first row of wells in each plate, as a blank for the plate reader.

2.13 Chemical mutagenesis

E.coli O157:H7 was grown overnight at 37°C in Penassay broth (Difco Antibiotic medium no.3). 500µl was then harvested in a microfuge and resuspended in 10ml of fresh Penassay broth. After incubation at 37°C for 1 hour, 0.1μgml^{-1} mitomycin C was added, before further incubation at 37°C for 2 hours. The bacterial suspension was then serially diluted and plated onto Penassay agar plates to obtain single colonies.

After overnight incubation, six of these clones were subcultured onto fresh agar. Plasmids were isolated from these clones and analysed by agarose gel electrophoresis.

2.14 Small-scale preparation of plasmid DNA

2.14.1 Isolation of Plasmids

Plasmid DNA was extracted using an alkaline lysis method.

1.5ml of an overnight culture, in an Eppendorf tube, was centrifuged in a microfuge for 1 minute. The medium was removed by aspiration and the pellet was resuspended by vortexing in 100µl of cell resuspension solution. After 5 minutes at room temperature, 200µl of a freshly prepared cell lysis solution was added, and mixed gently by inverting the tube 2 or 3 times. The tube was stored on ice for 5 minutes after which 150µl of ice-cold neutralisation solution was added and mixed by inversion of the tube several times. After 5 minutes on ice the preparation was centrifuged in a microfuge at 4°C. The supernatant was transferred to a fresh tube and an equal volume of phenol/chloroform was added and mixed by vortexing. After centrifugation for 2 minutes the supernatant was transferred to a fresh tube. 2 volumes of ice-cold 100% ethanol were added and mixed by vortexing. After 2 minutes at room temperature, the tube was centrifuged for 5 minutes in a microfuge. The supernatant was removed by aspiration and the pellet, containing the plasmid DNA, was dried under vacuum for 5-10 minutes. The dried pellet was resuspended in 50µl TE (10mM Tris-HCl pH 8.0, 1mM EDTA) containing DNAse-free pancreatic RNAse (20µgml^{-1}).

Plasmid DNA was analysed by agarose gel electrophoresis on 0.7% agarose gels.

The following solutions were used in the preparation of plasmid DNA:

Cell resuspension solution	Cell lysis solution	Neutralization solution
50mM Glucose	0.2M sodium hydroxide	5M potassium acetate 60ml
10mM EDTA	1% SDS	Glacial acetic acid 11.5ml
25mM Tris-Cl pH 8.0		Distilled water 28.5ml
4mgml-1 lysozyme *		

* Powdered lysozyme was added to the solution just before use.

2.14.2 Agarose gel electrophoresis

Agarose gels were used to separate plasmid bands. 0.7g agarose was dissolved in 100ml 1xTBE (10x TBE contains 1) by boiling for several minutes. After cooling to ~50°C, 10µl ethidium bromide (10mgml^{-1}) was added to the gel before pouring into a horizontal gel tank (Hybaid) containing a well-forming comb. After setting, the comb was removed, and buffer (1xTBE) was added to the tank so that the gel was just covered. DNA samples were added to 6X loading buffer (40% sucrose + 0.25% bromophenol blue) before loading. Gels were run for 3-4 hours at 100V.

2.15 Api 20E Tests

Api 20E tests were carried out according to the manufacturers instructions. A single bacterial colony was resuspended in distilled water, and dispensed into the cupules of an assay strip. Reactions which required an anaerobic environment were overlaid with mineral oil. The assay strips were

incubated for 16-20 hours at 37°C, then developed using reagents supplied by the manufacturer, where necessary. The results were translated into a 7-digit figure and from this, using the Apilab software, the identity of the organism was deduced.

2.16 Saline Extraction of Heat-Stable Antigens

Confluent bacterial growth from four nutrient agar plates was transferred to 4ml of 0.9% (w/v) sodium chloride, and heated at 100°C for 60 minutes. Cells were pelleted by centrifugation at 10,000 rpm for 10 minutes and the supernatant, containing soluble antigens, was filtered through a 0.22μm membrane filter, and stored until use, at 4°C.

2.17 Passive Haemagglutination Assay

Saline-extracted antigens were diluted 1:10 in PBS (pH 7.0) and incubated for 60 minutes at 37°C with an equal volume of a 1% suspension of sheep red blood cells (RBC), previously washed in PBS. The sensitized RBC were centrifuged, washed three times in PBS, and resuspended in PBS to give a 0.5% RBC suspension.

Two-fold dilutions of antibody to O157:H7 (previously diluted to 1:40) were made in a 96-V-shaped well microtitre tray (25μl per well). 25μl sensitized RBC were added to each well, and the plates were shaken gently to mix the contents of each well. After incubation for 60 minutes at 37°C, haemagglutination was observed by eye. The titre was recorded as the highest dilution of antiserum to give agglutination of sensitized RBC. Pre-immune serum was used in negative control assays.

2.18 Oxoid O157 Latex Agglutination Test

This test was carried out according to the manufacturers instructions. Briefly, bacterial cells were mixed on a glass slide, with latex particles coated with O157. Any bacteria able to agglutinate the latex particles were further tested with negative control latex particles. Agglutination of these particles was indicative of non-specific agglutination.

2.19 Western Blotting

2.19.1 Preparation of Whole Cell Antisera

Bacteria were grown up overnight at 37°C in 10ml Penassay broth (Difco Antibiotic Medium no.3), harvested by centrifugation, and washed twice in PBS (pH 7.0). Cells were further incubated at 37°C, overnight, in 1% Formalin (v/v). After centrifugation, the cells were washed six times in PBS to remove Formalin, then resuspended in 2ml PBS. 100µl of this suspension was plated onto nutrient agar and incubated overnight at 37°C, to ensure that all bacteria were dead.

0.5ml of formalinised bacteria were injected intravenously into a New Zealand White rabbit. The procedure was repeated after two weeks and again after four weeks. After 10 weeks the rabbit was bled from an ear vein. 10ml of blood was collected at each bleeding. Blood was stored at 4°C for 24 hours before serum was removed using a Pasteur pipette and centrifuged at 1500g for 20 minutes to remove red blood cells. Serum was stored in 1ml aliquots at -20°C.

Preimmune serum was obtained by bleeding the rabbit in the same way, before immunization with formalinized bacteria.

2.19.2 **Immunoblotting**

A modification of the method described by Towbin *et al* (1979) was used for the transfer of saline-extracted antigens from SDS-PAGE gels to nitrocellulose membranes.

A nitrocellulose membrane filter (Amersham plc) was cut to the size of the polyacrylamide gel, and soaked, with the gel, in Western transfer buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 20% (v/v) methanol). The membrane was then placed on top of the gel, with the exclusion of air bubbles, and sandwiched between soaked Whatman no. 1 paper cut to the same size. This was placed in the holder of a Biorad Transblot tank, and transfer of the proteins from the gel to the nitrocellulose was carried out according to the manufacturers instructions, at 300mA for 3 hours.

After transfer, the nitrocellulose filter was rinsed in distilled water to remove salt, from the buffer, and soaked in 100ml Ponceau S (0.5% (w/v) in 5% trichloroacetec acid). After rinsing briefly to remove excess dye, the positions of the molecular weight standards were marked on the membrane.

The nitrocellulose membrane was soaked briefly in 20ml TBS (150mM sodium chloride, 50mM Tris-HCl pH 8.0), in a sandwich box, until all traces of the dye were removed. Fresh TBS containing 2% dried skimmed mik (Marvel), which acted as a blocking agent, was added and incubated, with shaking, at room temperature. After 30 minutes this solution was poured off and a fresh TBS-Marvel solution was added, containing primary antibody (anti-O157:H7 or anti-O26:H11) at a final dilution of 1:500. This was incubated, with shaking, overnight at room temperature.

The solution containing primary antibody was discarded, and the nitrocellulose membrane was washed three times (10 minutes /wash) in

TBS containing 0.1% Tween 20. A fresh solution of TBS-Tween 20 was added, containing a 1:1000 dilution of secondary antibody (anti-rabbit peroxidase conjugate, Sigma). This was incubated on a shaker at room temperature for 1-2 hours, after which the filter was washed twice (10 minutes /wash) in TBS-Tween 20 and twice in TBS only. The membrane was then developed using the chloronaphthol method.

2.19.3 Chloronaphthol development system

Solution A (1.5g sodium chloride, 1ml 1M Tris-HCl pH 7.5 made up to 50ml with water) and solution B (50mg 4-chloronaphthol, 10ml methanol made up to 50ml with water) were prepared less than 1 hour before use. 50μl of hydrogen peroxide were added to solution A, before solutions A and B were mixed. The mixture was added to the membrane, and shaken gently at room temperature until a colour reaction developed (5-30 minutes). The membrane was removed from the solution before the reaction went to completion, and placed into distilled water. The water was changed 3 times over a 30 minute period, after which the nitrocellulose membrane was dried on filter paper and stored in the dark.

2.20 Outer Membrane Preparation

100ml LB broth (10g tryptone, 5g yeast extract, 10g sodium chloride per litre pH 7.0) was inoculated with 1ml of an overnight bacterial culture and incubated with shaking at 37 °C for several hours to allow cells to reach mid-exponential phase (4×10^8 cells ml^{-1}). After centrifugation at 10,000 rpm for 10 minutes at 4°C, cells were resuspended in 10ml of 10mM Tris-Cl pH 8 and sonicated (3 x 30 second bursts). Unbroken cells were removed by centrifugation at 3000 rpm for 20 minutes at 4°C. The resulting supernatant was centrifuged at 20,000 rpm for 60 minutes at 4°C. The pellet

containing the outer membrane was resuspended in 150ml distilled water and stored at -20°C.

The crude outer membrane preparation was thawed, and 50µl samples were extracted with 8 volumes of a detergent solution containing 1.67% (w/v) Sarkosyl and 11.1mM Tris-Cl pH 7.6 for 20 minutes at 20°C. The insoluble outer membranes were pelleted by centrifugation at 20,000 rpm for 90 minutes at 20°C. The pellet was suspended in 50µl of SDS-PAGE sample buffer prior to analysis by SDS-polyacrylamide gel electrophoresis.

2.21 Neutral Sugar Analysis

2.21.1 Phenol Extraction of Lipopolysaccharide

2g of freeze-dried *E.coli* O157:H7 cells, or mutant cells of this serotype, were resuspended in 100ml of distilled water at 67°C. 100ml of 91% phenol (w/v), preheated to 67°C, were added, and the mixture was stirred at 67°C for 20 minutes. The homogenate was cooled in iced water and was continuously stirred for 5 minutes before centrifugation at 3000g for 20 minutes at 20°C. The upper aqueous phase was carefully removed and kept at room temperature, before the lower phenolic phase was reheated to 67°C. 75ml of distilled water, preheated to 67°C were added to the phenolic phase, and after mixing and centrifugation as before, the phases were separated. The aqueous phases were pooled, and alongside the remaining phenol phase, were dialysed against running water to remove the phenol. The combined aqueous phases were dialysed for 2-3 days whereas the phenol phase required 7 days. Cell debris was removed from the dialysed phenol phase by centrifugation at 2000g for 20 minutes. The volume of each phase was reduced to approximately 30ml by rotary evaporation at room temperature. The concentrated extracts were

centrifuged at 105,000g for 4 hours at 4°C in a 60 Ti Beckmann rotor. The LPS pellet from each phase was resuspended in water with a glass homogeniser and recentrifuged twice as above. After the final centrifugation the pellets were freeze-dried.

2.21.2 Thin Layer Chromatography

The neutral LPS sugars were analysed by thin layer chromatography (TLC).

To 1mg of freeze-dried LPS, 100µl of 0.5M H_2SO_4 was added. After hydrolysis for 4 hours at 100°C, the mixture was neutralised with a saturated solution of barium hydroxide. Small samples were spotted onto indicator paper to test for neutralisation. The hydrolysis tubes were then spun at 3000g for 15 minutes to pellet barium sulphate formed during neutralisation. The supernatants were removed and then freeze-dried.

For analysis by TLC, hydrolysates were resuspended in 10µl water. 2µl samples were then spotted onto cellulose-coated glass plates (Merck). Further samples were applied, with drying between each application. 3µl of sugar standards were also loaded, containing 10mgml^{-1} of glucose, rhamnose, mannose, galactose, ribose and fucose.

Plates were placed in a tank containing a solvent system of n-butanol:pyridine:water in a ratio of 6:4:3 volumes. The solvent had been stored for 24 hours in the tank to allow the atmosphere to become saturated. The plates were left in the tank for several hours until the solvent front was approximately 1cm from the top. After removal from the tank the plates were allowed to air-dry. They were then returned to the tank and rerun in the same direction, as before. This allowed better separation of the sugars. After drying, the plates were developed by spraying with anilinium hydrogen phthalate (0.7ml aniline, 1.6g O-phthalic acid in 100ml n-butanol saturated with water). Sugars were visualised after baking at 100°C for 10-15 minutes.

2.22 **Hydrophobicity studies**

2.22.1 **Microbial Adhesion to Hydrocarbons (MATH)**

This method was described by Rosenberg *et al* (1980).

Bacteria were grown in 10ml volumes, overnight at 37°C. Cells were harvested by centrifugation, washed twice, and resuspended in 1.2ml of PUM buffer (22.2g $K_2HPO_4.3H_2O$, 7.26g KH_2PO_4, 1.8g urea, 0.2g $MgSO_4.7H_2O$, per litre distilled water, pH 7.1). The optical density at 400nm was determined spectrophotometrically and adjusted, if necessary, to give a standard opacity for each bacterial suspension. A volume of n-hexadecane (0.1-0.4ml) was added, and after 10 minutes of incubation at 30°C the contents of each tube were mixed uniformly in a vortex mixer for 2 minutes. After allowing 15 minutes for the phases to separate, the OD_{400} of the lower phase was measured, and compared to the initial reading. The fraction of adherent cells was taken as the percentage decrease in OD_{400} of the aqueous phase after mixing and phase separation, as compared with that of the original separation.

2.22.2 **Salt Aggregation Test (SAT)**

SAT was carried out as described by Lindahl *et al* (1981).

Ammonium sulphate concentrations in sodium phosphate buffer (pH 6.8, 0.02M) ranging from 4M to 0.02M, in 0.2 increments, were prepared. 10µl aliquots of bacterial suspensions were spotted onto an equal volume of each of the salt concentrations, on glass slides. The slides were rocked gently for 1-2 minutes until aggregation was observed. The last dilution at which aggregation occurred was recorded. Bacterial suspensions used were of standard opacity so that equivalent cell concentrations were used in each test.

2.22.3 Hydrophobic Interaction Chromatography (HIC)

The method of Mozes & Rouxhet (1987) was used.

Glass Pasteur pipettes were plugged with glass wool and packed to a height of 20mm with octyl-Sepharose CL-4B (Sigma). The column was washed with 20 bed-volumes of sterile PBS. Bacterial strains of the same optical density, were suspended in 2ml of the same buffer and the optical density at 580nm was measured before loading onto the column. The absorbance reading of the eluent was measured at 580nm (the first 2ml from the column was discarded as void volume). The percentage hydrophobicity was calculated by the formula;

$$\frac{ODi - ODe}{ODe} \times 100 = \% \text{ hydrophobicity}$$

where ODi was the absorbance of the suspension before loading onto the column and ODe was the absorbance of the eluent.

2.23 Attachment Studies

2.23.1 Attachment of Bacteria to Mucus

Mouse colonic mucus, which had been isolated by colonic washing, was provided (M Krishna). Mucus was run out on a 4-15% native gel (Section 2.9.2), and transferred to nitrocellulose overnight, using a Biorad Transblot apparatus. The membrane was then blocked in 3% Marvel in TBS (150mM sodium chloride, 50mM Tris-HCl pH8.0). After washing in TBS, the membrane was probed with 10ml of an overnight bacterial culture ($>10^9 ml^{-1}$) for 2 hours. Unattached bacteria were removed by washing twice in TBS, and antiserum (anti-*E.coli*, provided by M Krishna) was added in TBS at a dilution of 1:1000, for 1.5 hours. The membrane was washed thoroughly in TBS to remove unbound antibodies, after which

secondary antibody (anti-rabbit peroxidase conjugate, Sigma) at 1:1000 in TBS was added for 1 hour. After washing several times with TBS, the membrane was developed using the chloronaphthol method.

A control was carried out, using nutrient broth instead of a bacterial culture, to check that the antibodies used did not bind directly to the mucus.

2.23.2 Attachment of Bacteria to HeLa cells

HeLa cells were inoculated into 12-well tissue culture dishes ($4x10^5 ml^{-1}$) in 199 medium (Flow), and allowed to form monolayers overnight in 5% CO_2 at 37°C. Cells were then washed 3 times in PBS (pH 7.0).

Bacteria (10^8) were resuspended in 2ml 199 medium and added to the HeLa cells. The tissue culture dishes were incubated at 37°C for 3 hours to allow the bacteria to attach. The culture medium was then removed and the HeLa cells were washed 6 times in PBS to remove loosely attached organisms. The HeLa cells with adherent bacteria were removed from the plate with 0.5ml 0.25% trypsin, and HeLa cells were lysed with 0.5ml BSA (0.1% in water). After vortexing, lysates were serially diluted tenfold in sterile PBS, and plated out in duplicate to calculate the number of viable bacteria adherent to the HeLa cells.

Inhibition studies were also carried out, where whole cell antiserum (100µl), raised against O157:H7 ATCC 35130, was added with the bacteria. Pre-immune serum controls were included and results were expressed as a percentage of bacteria adherent to cells in the presence of pre-immune serum.

HeLa cells were cultured as for Vero cells (section 2.3.1)

2.23.3 Staining Tissue Culture Cells

The attachment of bacteria was viewed microscopically by staining the cells after the washing step which removed unattached bacteria.

Tissue culture cells which were to be stained were grown on 13mm round coverslips in 24-well plates. To stain cells, the coverslip was air-dried and then fixed for 5-10 minutes in a solution composed of 3 parts methanol to 1 part glacial acetic acid, which was made fresh and kept on ice. It was necessary to then wash the coverslip 3 times in 95-100% methanol to remove the acid which would prevent staining. Each wash lasted 5 minutes. After the final wash the coverslip was air-dried. The cells were then stained in 5% Gurr Giemsa stain in phosphate buffer for 5-10 minutes. Excess stain was removed by washing with tap water. The slide was then air-dried, mounted and viewed by light microscope.

2.24 Immunogold labelling

Immunogold labelling was carried out by Nicholas Powell at the Clinical Research Centre, Northwick Park Hospital, Harrow.

Chapter 3 The Development of Toxin Assays and the Effect of Mitomycin C on Verotoxin Production.

3.1 The Development of Assay Systems

In any study on toxins, it is essential to have a reliable assay system. The assay of choice will depend upon the aspect of toxin production which is of importance to the study. If toxin activity is investigated for example, an assay which detects this property would be chosen, such as a cytotoxicity assay using a susceptible cell line. Immunoassays using antibodies raised against the toxin will monitor the production of toxin, but will not confirm that the toxin is biologically active. Alternatively, it may be only the presence of the toxin gene which is of interest, in which case DNA hybridisation studies would suffice as an assay system. Such studies would probably be followed up by assaying for the toxin itself, to confirm that the gene was expressed.

In this study, both a cytotoxicity assay and an immunoassay were required. A Vero cell assay was chosen as a method of assessing toxin activity, and two immunoassays were compared to find a rapid method of detecting toxin production, and differentiating between VT1 and VT2. Through a combination of these assays it was possible to characterise the production of toxin by mutants of O157:H7 ATCC 35150. The Vero cell assay was further used in a study of the internalisation mechanisms of VT1 and VT2.

3.1.1 Cytotoxicity Assay

3.1.1.1 Introduction

VT were originally described after the cytotoxic effect of an *E.coli* culture filtrate on Vero cells was observed (Konowalchuk *et al* 1977); and a Vero cell cytotoxicity assay has remained an important method of identifying VT. When unknown samples are tested, it is important to incorporate a neutralization step with anti-VT serum to confirm that the cytotoxic action results from VT and is not due to an unrelated toxin or a result of non-specific cell death. Kongmuang *et al* (1988) have produced mutant Vero cells which have lost the VT receptor, Gb_3. VT are cytotoxic towards parent but not mutant cells, whereas other toxins, which attach to different receptors on the Vero cells, are either cytotoxic towards both cell types, or do not affect either cell type. Such a modification of Vero cells improves the specificity of the tissue culture assay, since although it is time-consuming and requires specialized equipment it remains one of the most sensitive assays for VT.

In the present study, the strains used were known to produce VT, and this was confirmed by cytotoxicity assays and neutralization tests. Two other *E.coli* strains, which do not produce VT, had no effect on the Vero cells.

3.1.1.2 Results and Discussion

Culture filtrates of several *E.coli* strains were titrated across 96-well microtitre plates. Filtrates containing VT were cytotoxic towards the cells as shown in Figure 3.1. Toxin titres (TC_{50}) were calculated as the reciprocal dilution of culture filtrate to kill 50% of cells, as assessed microscopically.

Four VTEC strains were tested: O26:H11 E3787 (a VT1-producer), O157:H- E32511 (a VT2-producer), O157:H7 (reference strain ATCC 35150) and O157 (clinical isolate). All strains were cytotoxic towards Vero cells (Table 3.1). To confirm that the CPE were caused by VT, neutralisation assays were carried out, whereby neutralising monoclonal antibodies against VT1 and VT2 were incorporated into the cytotoxicity assays. The antibodies reduced the TC_{50} of all strains confirming that the CPE on Vero cells were due to VT (Table 3.1). The O157:H7 and O157 strains produced both VT1 and VT2, so MAb against both toxins were used to produce the neutralisation titre. Neither of the non-VTEC *E.coli* strains tested, K12 and O26:H11 (an EPEC strain) had any effect on Vero cells.

Strains E3787 and E32511 were used as positive controls to ensure that the Vero cells used were susceptible to both VT1 and VT2. The fact that the titre of VT1 was considerably lower than that of VT2 can be explained by the fact that VT1 is thought to be almost completely cell-associated whereas VT2 is more equally distributed between the interior of the cell and the surrounding medium (Strockbine *et al* 1986). Thus in a situation where no agents such as mitomycin C or polymyxin B are present to extract intracellular toxin, lower levels of VT1 compared to VT2, would be expected.

3.1.2 Latex Agglutination Assay

3.1.2.1 Introduction

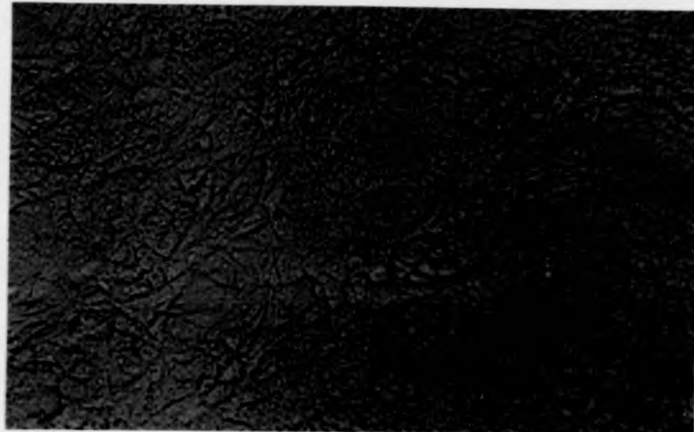
Agglutination of antibody-coated latex particles has frequently been used to detect homologous antigens. Commercial latex agglutination tests are available to detect many medically important bacteria including *E.coli* O157. These tests generally depend upon assessment of agglutination by

Figure 3.1 Vero Cell Assay. The effect of Verotoxins on Vero cells.

a) A healthy cell monolayer.

b) Cytotoxic effect of VT on a Vero cell monolayer.

a)



b)

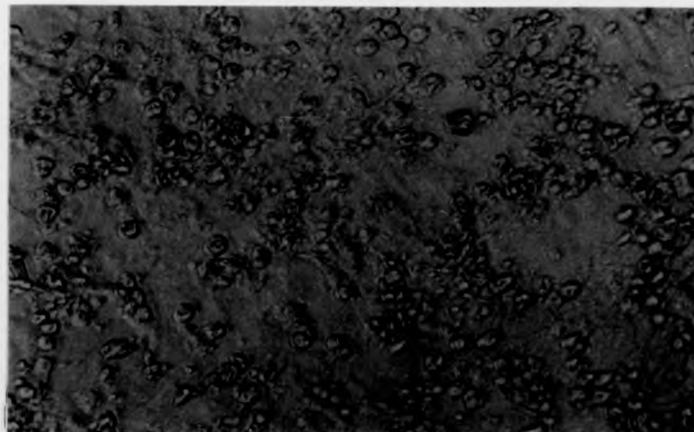


Figure 3.1 Vero Cell Assay. The effect of Verotoxins on Vero cells.

a) A healthy cell monolayer.

b) Cytotoxic effect of VT on a Vero cell monolayer.

a)

b)

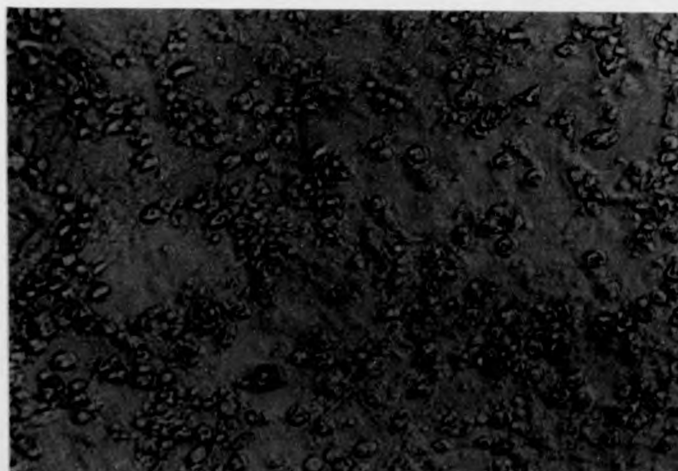


Table 3.1 Cytotoxicity Assay

E.coli strain	TC_{50}	Neut. titre
E3787 (VT1 producer)	128	32
E32511 (VT2 producer)	131,072	4096
O157:H7 (reference strain)	32	4
O157 (clinical isolate)	32	4
O26:H11 (EPEC)	No CPE	
K12	No CPE	

Culture filtrates were titrated across Vero cells grown in 96-well microtitre plates. TC_{50} represents the reciprocal filtrate dilution to kill 50% cells assessed microscopically after 20-24 hours incubation at 37°C in 5% CO_2.

Incorporation of neutralising anti-VT MAb confirmed the identity of the toxins by a reduction in TC_{50} (Neut titre).

Results shown were reproducible in triplicate, and when carried out on different days.

eye, compared to a negative, and sometimes a positive, control. With some of these kits, including the O157 test, false positive identification has been reported (Borczyk *et al* 1990).

In this study, an alternative method of detecting agglutination of latex particles was employed, that of particle size counting using a Coulter counter. The latex particles were coated with monoclonal antibodies against either VT1 or VT2. Specific and non-specific agglutination was easily differentiated although attempts to distinguish between VT1 and VT2 production were unsuccessful.

3.1.2.2 **Results and Discussion**

Agglutination of latex particles coated with anti-VT1 in the presence of an O157:H7 culture filtrate is demonstrated in Figure 3.2. The first peak represents single latex particles (volume 0.141mm^3 = diameter 0.73µm) and the smaller peak (volume 0.226mm^3= diameter 1.46µm), results from agglutination of these particles due to the formation of complexes between antigen in the culture filtrate and antibody adsorbed onto the latex particle. It was possible to convert the volume reading to a diameter using the Coulter counter. When a culture filtrate of O26:H11, a non-VT-producer, was used as a source of antigen no agglutination was seen, and the small peak corresponding to agglutinated latex particles results from non-specific agglutination only (Figure 3.2.). The ratio of the height of the peak representing single latex particles to that representing agglutinated particles is significantly smaller when the antigen is homologous to the antibody coating the particles. O157:H7, which produces VT1, gave a ratio of 3:1 for single: multiple latex particles . This ratio was 10:1 when a non-VT-producer was used (Figure 3.2), therefore this method could be used to detect at least high-level VT-producers. Ratios of around 3:1 were

obtained when known VT1- and VT2 -producers (E3787 and E32511) were reacted with latex particles coated with the homologous antibody, whilst a non VT-producer, *E.coli* K12, gave a ratio greater than 10:1 with both anti-VT1 and anti-VT2 coated particles.

This method was not suitable for differentiating between VT1 and VT2 as some cross-reaction occurred using these monoclonal antibodies. Working with the same MAb Downes *et al* (1988) reported similar cross-reaction. Latex particles were coated with either anti-VT1 or anti-VT2, and reacted with culture filtrate from E3787, a VTEC strain known to produce only VT1. The ratio of peak sizes was 2:1 for the homologous antigen-antibody system, but was 3:1 when anti-VT2 was used, indicating that significant agglutination had occurred. From the earlier experiment, it was assessed that a ratio of around 10:1 corresponded to a negative result. It was clear therefore that cross-reaction had occurred between VT2 and anti-VT1.

It would be appropriate to use this method for detection of VT, although with these MAb it would not be possible to assess the type of toxin produced. For maximum detection it may be appropriate to use a mixture of VT1 and VT2 antibodies in a latex agglutination assay.

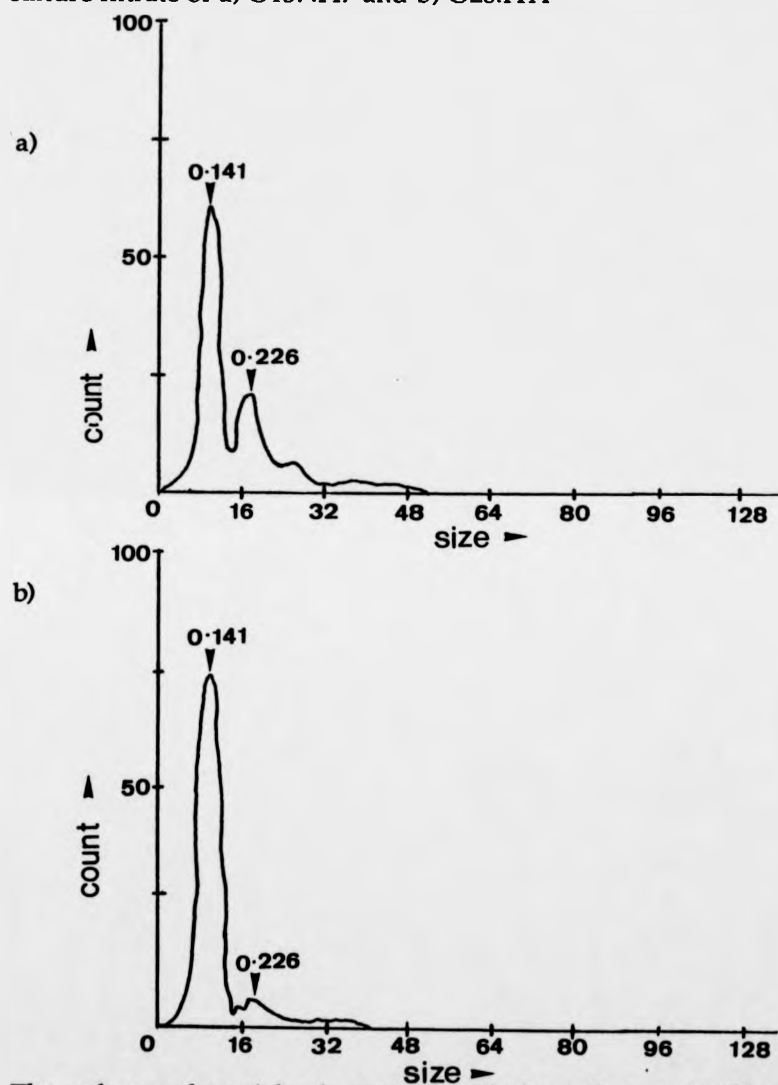
3.1.3 Colony ELISA

3.1.3.1 Introduction

Since VT are produced during the late log growth phase of VTEC (MacLeod & Gyles 1989) , it is necessary to grow bacteria, usually overnight, to obtain detectable levels of toxin. In order to screen several strains comparatively, it is advantageous to grow all

Figure 3.2. Latex agglutination assay.

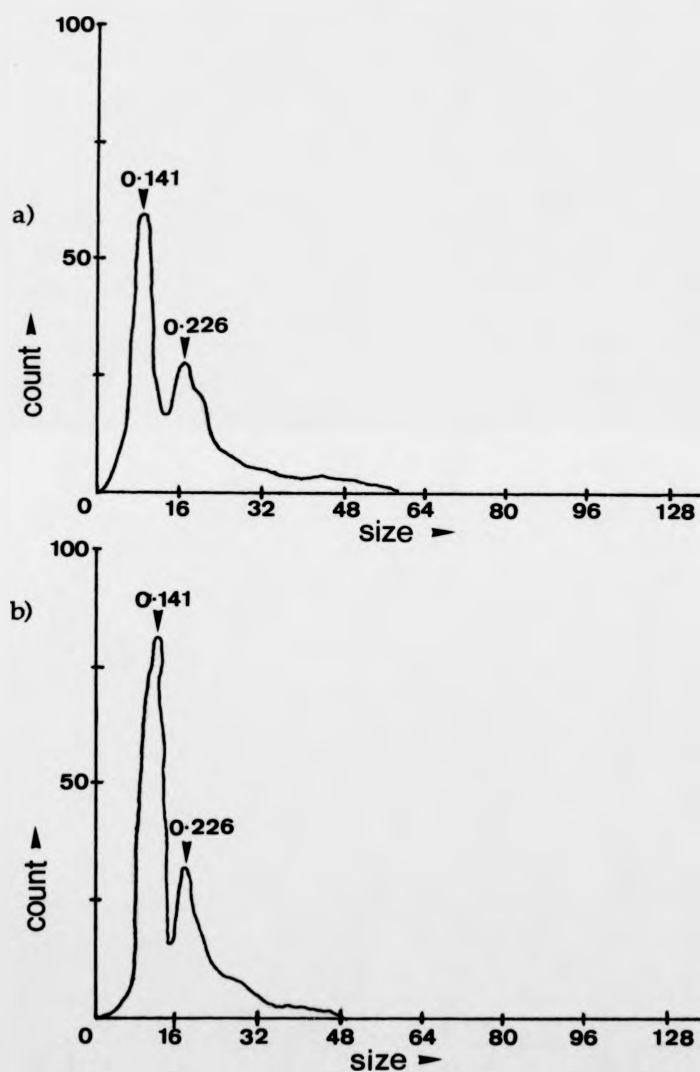
Latex particles coated with MAb against VT1 were reacted with a culture filtrate of a) O157:H7 and b) O26:H11



The volume of particles is represented along the x-axis. The peaks at $0.141\mu m^3$ and $0.226\mu m^3$ correspond to single latex particles and agglutinated latex particles respectively. The count and size are expressed in arbitrary units.

Figure 3.3 Latex agglutination assay

Culture filtrate from a VT1-producer (E3787) was reacted with latex particles coated with MAb against a) VT1 and b) VT2.



The peaks at 0.141μm^3 and 0.226μm^3 correspond to single latex particles and agglutinated latex particles respectively. The count and size are expressed in arbitrary units.

organisms on a single agar plate. This ensures that all strains have been subjected to the same conditions, and allows direct comparison of toxin levels. To satisfy these criteria, a colony ELISA was developed using monoclonal antibodies, which allowed detection of VT1 and VT2 within the cell. Colonies were transferred onto nitrocellulose membrane and then incubated in the presence of polymyxin B. This drug permeabilises the cell membrane allowing release of intracellular toxin without affecting normal levels of toxin production.

3.1.3.2 **Results and Discussion**

Figure 3.3 shows that the VT1 positive control (C600933J) reacted more strongly with the MAb against VT1 than did any of the test organisms apart from *S.dysenteriae*. type 1.The strong reaction of *S.dysenteriae* type 1 with anti-VT1 emphasizes the antigenic relatedness of Shiga toxin to VT1. Of the test organisms only O157:H7 (ATCC 35150) and the clinical isolate of O157 gave a positive reaction. This shows that the two *E.coli* strains O26:H11 and K12 did not produce VT1. The VT2 positive control (C600933W) did not react with the anti-VT1 MAb, so at least in this assay, cross-reaction does not occur and the two toxin types can be differentiated.

The colony ELISA using MAb to VT2 gave similar results, in that only the positive control and the two O157 strains were positive. These reactions were not as strong as those for VT1, which apparently contradicts the results of the cytotoxicity assays where VT2 titres were consistently higher than those of VT1. Since VT1 is predominantly cell-associated however, polymyxin B treatment allows detection of intracellular toxin not released during Vero cell assays. Thus VT1 levels were considerably higher in the colony ELISA than in the cytotoxicity assay. It is possible that total VT2 levels are lower than those of VT1, as observed by Strockbine *et*

al (1986), although using higher concentrations of antibody for the colony blots using anti-VT2 may have improved the intensity of the positive reactions. Significantly *S.dysenteriae*, type 1 which reacted most strongly with anti-VT1, did not react at all with anti-VT2, further proof that there is no cross-reaction between the MAbs in this assay. In a similar immunoassay, Perera *et al* (1988) found that low-level VT producers could not be detected, and these results indicate that this applies to this assay, even though a more sensitive developing system was used. From a clinical viewpoint, however, this is unlikely to be a problem since *E.coli* producing low levels of VT are not implicated in disease (Marques *et al* 1986).

These results indicated that the strain of O26:H11 did not produce either VT1 or VT2. As some strains of this serotype are VT-producers, this strain is an ideal organism to use as a negative control. Further studies by S.Fox (personal communication) using DNA probes, confirmed that this O26:H11 strain does not carry the genes for VT production.

The colony ELISA seemed to be a reliable assay for VT and there did not appear to be cross-reaction between VT1 and VT2, which was a problem with the latex agglutination assay (section 3.1.2). This may in part be due to the repeated washing involved in the colony ELISA which would remove cross-reacting antibodies, however, this discrepancy between the latex and colony blot tests cannot fully be explained. An immuno-dot-blot assay evaluated by Panigrahi *et al* (1987) for detection of *S.typhimurium* toxin was also found to be more sensitive than latex agglutination tested in parallel. This assay was similar to the colony ELISA in the present study, except that instead of testing cell-bound toxin, extracted toxin was spotted onto membranes. This technique was also successfully tested in the present study using culture filtrates as the toxin

Figure 3.4 Colony ELISA

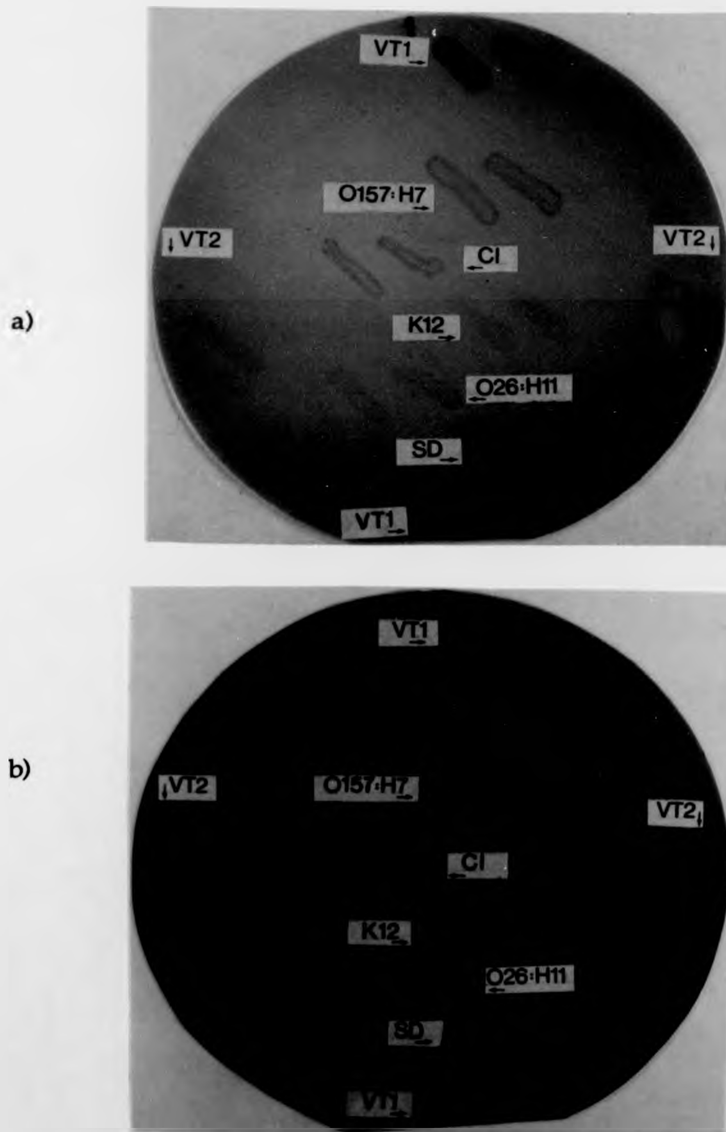
Colonies were transferred to nitrocellulose and treated with polymyxin B.

Toxin was detected using MAb against a) VT1 and b) VT2.

The DAB/nickel chloride development method was used.

Control organisms were: **VT1** - C600933J , **VT2** - C600933W

Test organisms were : **O157:H7** (ATCC 35150), O157 -clinical isolate **(CI)**, *E.coli* **K12** , *E.coli* **O26:H11** (EPEC strain), *S.dysenteriae* type 1 **(SD)**,



source. However, using colony ELISA it is possible to test many strains on a single agar plate rather than growing up each strain individually in liquid broth, therefore this was the assay of choice for subsequent characterisation of mutants of O157:H7. Colony ELISA results were compared to Vero cell assays, to assess whether toxin was biologically active.

3.2 The Effect of Mitomycin C on Verotoxin Production

3.2.1 Introduction

Mitomycin C, an antibiotic produced by *Streptomyces* species, inhibits bacterial DNA synthesis. Its action as a bifunctional alkylating agent leads to the formation of covalent crosslinks between complimentary strands of DNA, so preventing the two DNA strands from separating during replication. Inhibition of RNA synthesis and protein synthesis seem to be non-specific manifestations of cell toxicity (Verweij & Pinedo 1990). The drug is also thought to be involved in the induction of lysogenic phage. This is because when *E.coli* is exposed to agents or conditions which damage DNA or interfere with DNA replication, a diverse set of physiological responses known as SOS responses, are induced. More than 17 *din* (damage-inducible) genes are induced in response to such conditions (Walker 1987). The SOS regulatory system is controlled in part by the interplay of two proteins, LexA and RecA. During normal growth, the LexA protein represses the *din* genes, but when DNA is damaged or DNA replication is inhibited, an inducing signal is generated, which activates the RecA protein. The activated RecA protein interacts with, and causes proteolytic cleavage of, LexA. Activated RecA is also capable of mediating the cleavage of phage λ repressor, not necessarily by direct

proteolytic activity, but possibly by altering the conformation of the protein and increasing the susceptibility of, for example, a specifically labile bond to hydrolysis (Walker 1987), or facilitating autodigestion (Little 1984). That the RecA protein is involved in phage induction is clearly indicated by the evidence that RecA (def) (RecA-) mutants, which are defective in all activities of RecA protein, are defective in recombination, SOS induction and lambda induction (Walker 1987). Viral DNA replication is not affected by mitomycin C, although the mechanism of this resistance has not been elucidated (Gale *et al* 1972). It is possible that the viral DNA is modified and is thus resistant to the action of the drug. Mitomycin C is thus a valuable tool in the study of viral DNA in the absence of host cell DNA synthesis

VT1 and VT2 are produced by lysogenic phages (Strockbine *et al* 1986), and *E.coli* K12 produce high levels of VT if lysogenised with VT-converting phages (Smith *et al* 1983). Mitomycin C should result in cessation of bacterial DNA synthesis and induction of the VT-converting phage(s), which may result in increased production of VT, since this is a phage product. It is already known that mitomycin C treatment of *E.coli* O157:H- strain E32511 results in elevated levels of extracellular VT2 (Head *et al* 1988), although the mechanism of this enhancement has not been established. It could simply be due to the fact that mitomycin C causes cell lysis which releases pre-formed toxin, or in addition to this, induction of the toxin-converting phage may result in *de novo* synthesis of toxin.

This study was carried out to demonstrate the effect of mitomycin C on synthesis and release of VT, and has shown that this drug is able to increase toxin production and the level of extracellular viral proteins while bacterial protein synthesis is inhibited. The specific activity of toxin inside the cells also increased in the presence of mitomycin C, although

extracellular specific activity levels were lower in the mitomycin C-treated cells as a result of bacterial proteins released during cell lysis.

3.2.2 Results and Discussion

3.2.2.1 Lysis of Cells

To confirm whether mitomycin C caused cell lysis, the optical density (600nm) of an exponentially growing culture of O157:H7, after addition of mitomycin C, was monitored (Figure 3.5).

5ml of an overnight culture of ATCC 35150 was added to a 250ml flask containing 100ml Difco Antibiotic Medium no.3. The culture was allowed to reach the exponential growth phase (3 hours), at which time the mitomycin C was added. The effect of the drug is very clear, in that a decrease in the optical density corresponding to cell lysis was apparent in the cultures containing the drug, after 5-6 hours, which was 2-3 hours after the drug was added. The control culture continued to increase in optical density throughout this experiment. Cell lysis in the mitomycin C-treated culture was confirmed by light microscopy.

After 24 hours, phage (2.5×10^3 pfu ml^{-1}) could be demonstrated in the supernatant of the culture containing $1.0 \mu g ml^{-1}$ mitomycin C, by the formation of clear plaques in a lawn of *E.coli* DH1 (Figure 3.6). It was necessary to use a lambda-free strain, as VT-converting phages are lambdoid in nature, and will not infect a lambda lysogen. No plaques were seen when supernatant from a culture grown in the absence of mitomycin C, was used. VT-encoding phages from O157:H7 consist of regular, hexagonal heads and short contractile tails, but unfortunately electron microscopy of the phage-containing supernatants in this study, was unsuccessful. However production of viral plaques confirms the lysogenic

Figure 3.5 The effect of mitomycin C on growth of *E.coli* O157:H7. The drug, which was added after 3 hours, was tested at two concentrations, 0.4μgml^{-1} & 1.0μgml^{-1}

No mitomycin C was added to the control culture.

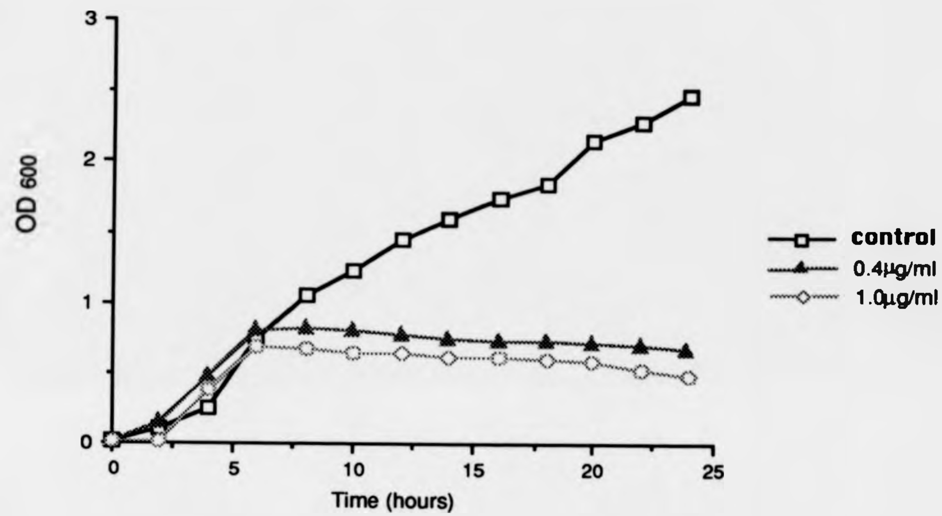
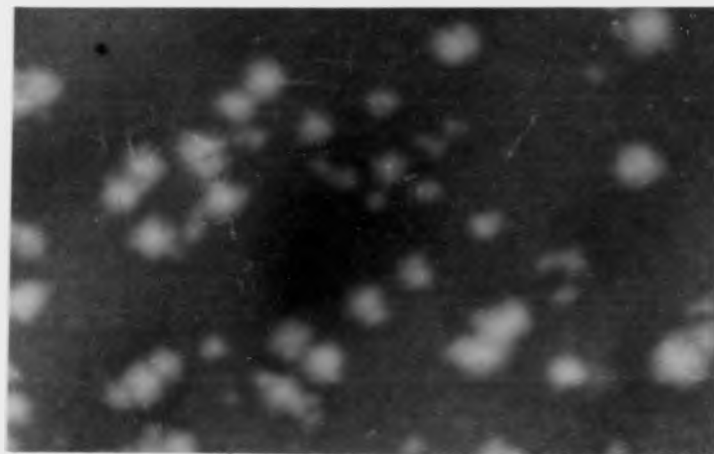


Figure 3.6 Induction of bacteriophage in *E.coli* O157:H7



A culture filtrate from cells grown in the presence of mitomycin C ($1.0\mu g ml^{-1}$) was added to *E.coli* DH1. Clear plaques in the lawn of DH1, corresponding to induced phage, were observed after 16 hours at 37°C.

nature of VTEC, and also that viral DNA synthesis is not inhibited by this concentration of mitomycin C.

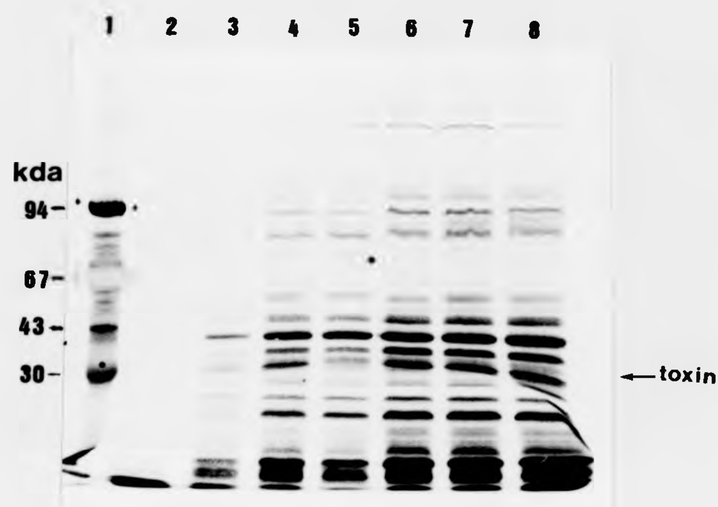
These observations tie in with published studies on the effect of mitomycin C on levels of *Salmonella* toxin, where a drastic decrease in viable cells was seen, and thought to be caused by the selective inhibition of non-toxin protein synthesis with concomitant lytic release of toxin (Houston *et al* 1981).

3.2.2.2 **Analysis of Extracellular Proteins**

In order to assess whether the inhibition of protein synthesis was selective, extracellular proteins were analysed by SDS-PAGE. Cells were harvested at hourly intervals, between 1 and 7 hours after addition of mitomycin C ($0.1\mu gml^{-1}$) to exponentially growing cells. The cell-free supernatants were then concentrated by ammonium sulphate precipitation and subsequently analysed by SDS-PAGE (Figure 3.7). It was shown by SDS-PAGE that some proteins were removed in the 40% ammonium sulphate precipitate, however, these were mainly non-toxin proteins. Petric *et al* (1987), reported that at 40% saturation, 5% toxin activity was precipitated, while at 60% saturation, >90% of toxin was precipitated.

The appearance of protein bands after 2 hours, confirmed the cell lysis seen in Figure 3.5 where the optical density of cultures to which mitomycin C had been added, began to decrease 2-3 hours after addition of the drug. The concentration of toxin, and of some low molecular weight proteins, increased proportionally with the length of exposure to mitomycin C. The toxin band corresponds to the A subunit, as the samples had been denatured by treatment with SDS. The smaller B subunits are

Figure 3.7 SDS-PAGE analysis of extracellular proteins from a culture of mitomycin C-treated O157:H7.

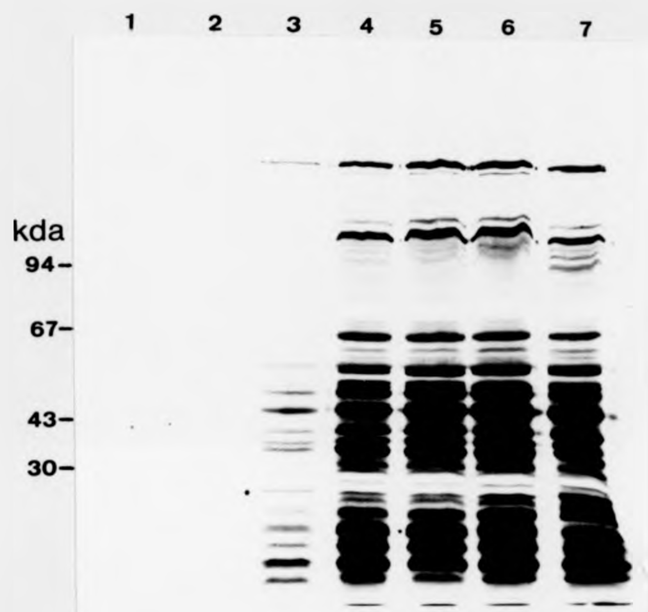


Proteins were concentrated by ammonium sulphate precipitation.

Lane 1 contains molecular weight markers. Lanes 2-8 contain extracellular proteins from cells harvested between 1 and 7 hours after addition of mitomycin C to exponentially growing cells.

Proteins were visualised by silver nitrate staining.

Figure 3.8 SDS-PAGE analysis of extracellular proteins from a culture of mitomycin C-treated O26:H11.



Lanes 1-7 contain ammonium sulphate concentrated supernatants from cells harvested at hourly intervals between 1 and 7 hours after addition of mitomycin C ($0.1 \mu g ml^{-1}$) to exponentially growing cells.

Proteins were visualised by silver nitrate staining.

not shown, as for unknown reasons the B polypeptides of VT migrate as diffuse and heterogeneous bands during SDS-PAGE (Newland *et al* 1985).The concentration of other non-toxin proteins increased only slightly throughout the experiment, corresponding to a gradual cessation of transcription and hence bacterial protein synthesis. The non-toxin proteins which significantly increased in concentration were thought to be other phage proteins, since it is known that mitomycin C inhibits bacterial protein synthesis (Verweij & Pinedo 1990). One of the most prominent of these proteins has a molecular weight of ~43kDa, and this corresponds in size to a VT-phage structural protein reported by O'Brien *et al* (1984b).

This experiment was repeated with an enteropathogenic *E.coli* (O26:H11) which does not produce VT, and in which a lysogenic phage could not be demonstrated. With this organism, the concentration of all proteins appeared to increase at a similar rate, due to the release of intracellular proteins by cell lysis (Figure 3.8). There was no selective inhibition of protein synthesis, as these bacteria were not lysogens. This observation reinforces the hypothesis that the extracellular proteins of O157:H7, which increase in concentration with increasing exposure time to mitomycin C, including toxin, are phage products.

The ammonium sulphate precipitates were further analysed by hydroxylapatite column chromatography (Figure 3.9). The column eluate was monitored spectrophotometrically, and the first four samples (1-4 hours incubation with mitomycin C) resulted in a single protein peak. This was analysed by SDS-PAGE, and the results of the samples taken at 1-3 hours, can be seen in Figure 3.9, lanes 1-3. After 5 hours incubation however, a second, smaller protein peak appeared, which at subsequent time intervals, became larger than the original peak. Lanes 4 and 5 of Figure 3.9, show analysis of the original peak, and the second protein peak,

respectively. The two peaks which resulted from hydroxylapatite chromatography of the 6 hour sample, are similarly shown in, in lanes 6 and 7. It is thought that the second peak represented assembly of the phage proteins seen in Figure 3.7.

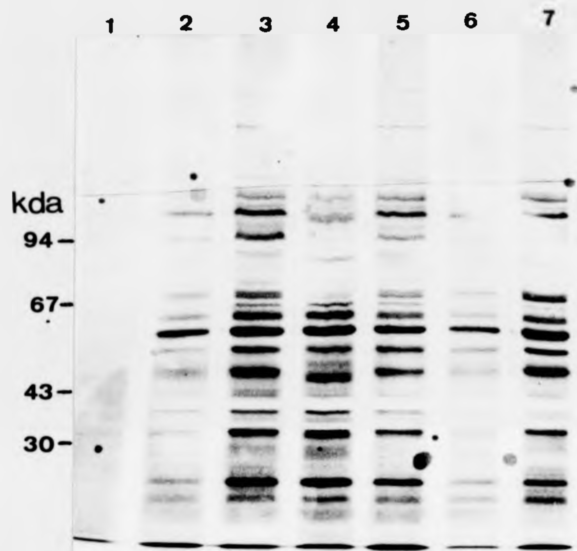
For the purposes of purification, it is desirable to achieve a situation where there is the greatest ratio of toxin to non-toxin proteins, ie before other phage proteins begin to assemble. From this data an incubation period of 3-3.5 hours appeared to be optimal.

3.2.2.3 Total Protein Concentration

As cell lysis will result in release of toxin and many non-toxin proteins into the supernatant, the levels of intracellular (sonicate) and extracellular (supernatant) protein were compared in the presence and absence of mitomycin C.

The effect of mitomycin C was immediately obvious with respect to the increase of extracellular protein from 6% to 40% of the total protein (Figure 3.10). However these values give no indication of the amount of toxin involved, or whether any of the proteins released into the supernatant were synthesized as a result of phage induction.

Figure 3.9 SDS-PAGE analysis of ammonium sulphate-precipitated O157:H7 extracellular proteins after hydroxylapatite column chromatography.



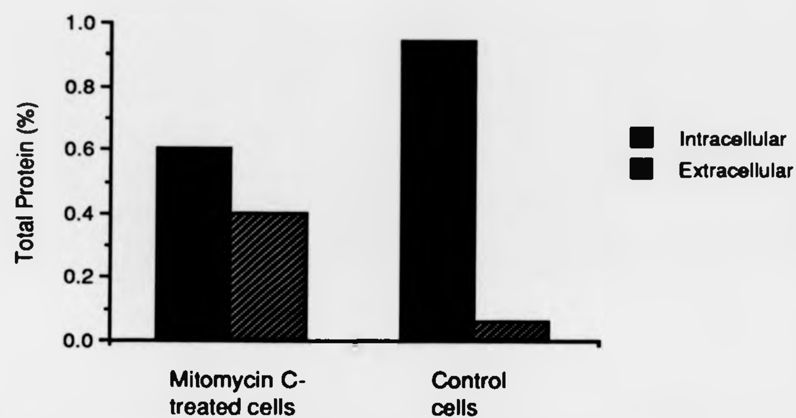
Samples were taken between 1 and 6 hours after addition of mitomycin C to exponentially growing cells. Samples were subjected to ammonium sulphate precipitation followed by hydroxylapatite column chromatography. A single peak was eluted from the column for samples exposed to the drug for 1-3 hours. Two protein peaks were eluted from subsequent samples.

Lanes 1-3 contain peak 1 from samples taken at 1-3 hours.

Lanes 4 and 5 contain peaks 1 & 2 from the 5 hour sample.

Lanes 6 and 7 contain peaks 1 & 2 from the 6 hour sample.

Figure 3.10 The effect of mitomycin C (0.1μgml^{-1}) on distribution of cell protein between the inside of the cell and its environment



Figures are expressed as a percentage of the total cell protein.

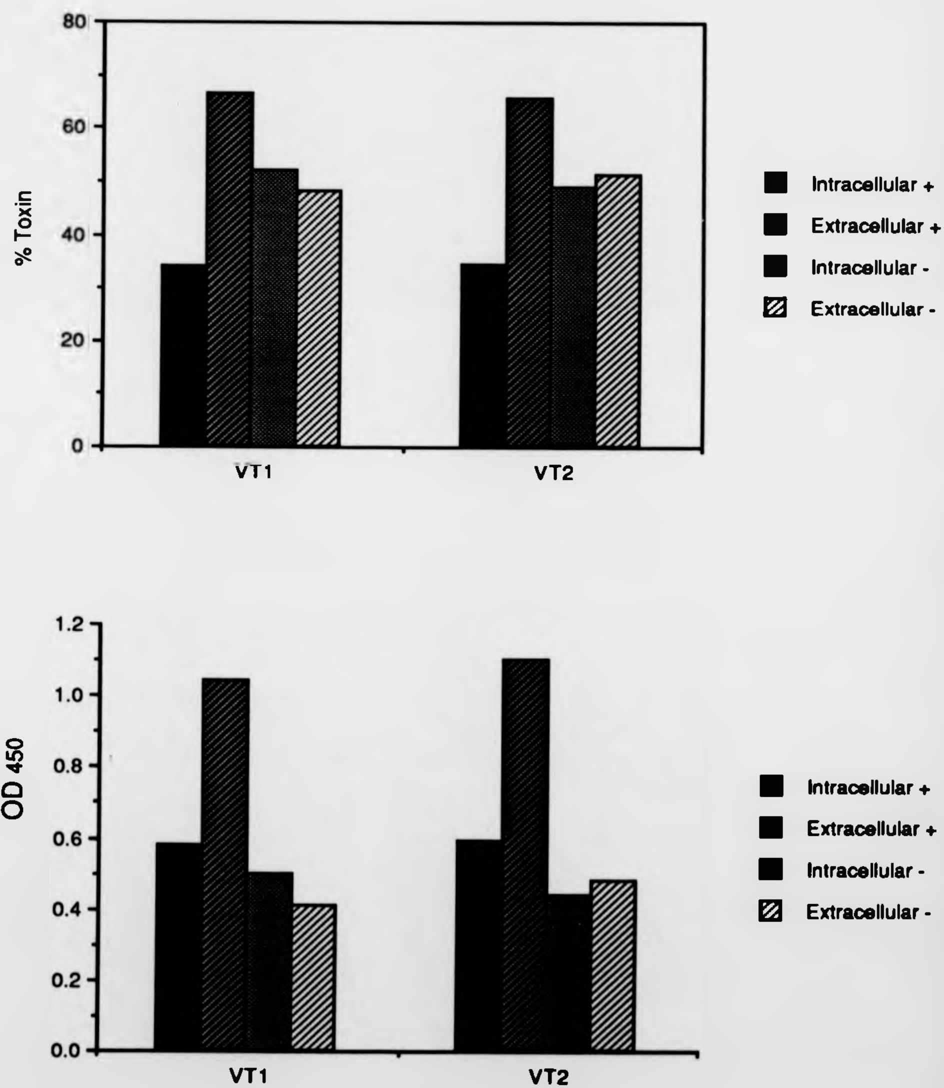
3.2.2.4 Toxin Production

To assess the levels of toxin in the supernatant, an enzyme-linked immunosorbent assay (ELISA) was used, and toxin was detected using monoclonal antibodies to VT1 and VT2. The results were expressed as a percentage of the total toxin detected in each culture (Figure 3.11).

The most obvious effect of mitomycin C was in the distribution of toxin within the culture. In both the VT1 and VT2 experiments, the amount of toxin in the supernatant, increased. This observation in itself, does not show that mitomycin C treatment results in synthesis of increased levels of toxin due to phage induction, since it has been shown that cell lysis occurs. The increased extracellular toxin levels could simply result from lytic release of pre-formed toxin. By analysing the actual figures from the ELISA experiment rather than considering percentage distributions for both VT1 and VT2, there is an increase in both intracellular and extracellular toxin levels in the cells treated with mitomycin C (Figure 3.11). If the increase in extracellular toxin was due to lytic release of pre-formed toxin, there would be a corresponding decrease in toxin inside the cell, in the presence of mitomycin C. Since this clearly was not the case it is proposed that mitomycin C causes an increase in VT levels by induction of toxin-converting phages and *de novo* synthesis of toxin.

Control experiments showed that non-specific binding of either antigen or primary antibody was not significant. Attempts to purify VT had been unsuccessful, so it was not possible to standardise the assay with serial dilutions of toxin. Despite the fact that the ELISA was only semi-quantitative, there is clear indication that the amount of intracellular and extracellular toxin increased due to mitomycin C action.

Figure 3.11 The effect of mitomycin C ($0.1\mu gml^{-1}$) on % distribution of VT1 and VT2. Bacteria were grown with (+) and without (-) the antibiotic, and intracellular and extracellular toxin levels were determined by an ELISA.



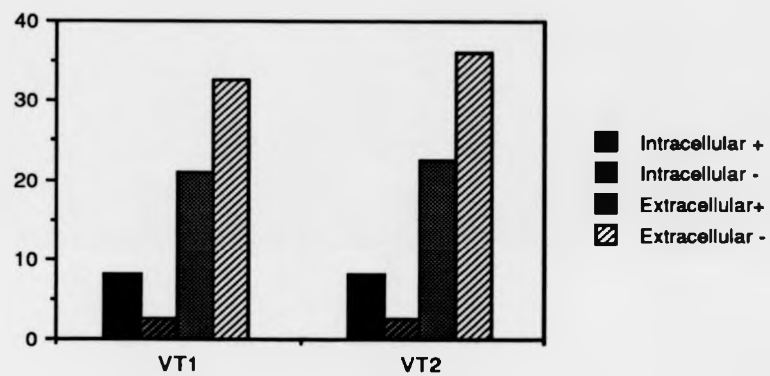
3.2.2.5 **Specific Activity**

The specific activity was calculated as optical density units per mg protein. From Figure 3.12 it can be seen that despite the fact that extracellular toxin levels were much higher in mitomycin C-treated cultures, because of lytic release of many non-toxin proteins, the specific activity was lower than in control cultures. The reverse was true for intracellular values, where phage induction resulted in increased toxin production, but the synthesis of bacterial proteins was gradually inhibited. The net result of this is an increase in the specific activity of VT.

3.2.2.7 **Tissue Culture Assays**

It has been shown that incubation of O157:H7 ATCC 35150 in the presence of mitomycin C results in increased VT production compared to control cells. This strain produces both VT1 and VT2, as shown by the ELISA results, where monoclonal antibodies to VT1 and VT2 both gave positive results (Figure 3.11). It is known however that there is some cross-reactivity between these monoclonal antibodies (Downes *et al* 1988), therefore to determine whether production of both of these toxins is genuinely increased by mitomycin C, two other strains were examined , E3787 which produces VT1 only, and E32511, a VT2 producer.

Figure 3.12 Intracellular and extracellular specific activity of VT1 and VT2.



Specific activity is expressed as OD units per mg protein.

Table 3.2 Vero cell assay

	-mitomycin C	+ mitomycin C	Increase in titre
VT1	32	512	16-fold
VT2	2,048	65,536	32-fold

Culture filtrate of E3787 (VT1) and E32511 (VT2) were titrated across Vero cell monolayers. Titre is a reciprocal of the dilution to cause 50% cell death.

Bacteria were grown in the presence (+) or absence (-) of mitomycin C. Results were reproducible on three separate days.

Table 3.2 shows that levels of both VT1 and VT2 were increased by incubating VTEC in the presence of mitomycin C. This supports the results of the ELISA (Figure 3.11), where it is clear that the amount of extracellular VT antigen increased in the presence of the drug, The tissue culture assay showed an increase in the titre of VT1 of 16-fold, and that of VT2, 32-fold, although since a 2-fold dilution series was used, these differences were probably not significant. Head *et al* (1988) noted a 100-fold increase in VT2 production in the presence of mitomycin C, although this figure corresponded to polymyxin B-extracted toxin from the cell pellets. However, the increase in extracellular levels appears to be similar to that found in this study.

It is concluded from these studies that addition of mitomycin C to an exponentially growing culture results in a large increase of extracellular toxin due to induction of toxin-converting phage. This contrasts with the effect of this drug on production of heat-labile toxin (LT) by enterotoxigenic *E.coli* (Gemski *et al* 1978). The increase in extracellular levels of LT caused by the mitomycin C treatment reflected lytic release of cell-bound toxin only. The cells lysed due to phage induction, but LT is plasmid- rather than phage-encoded so synthesis of this toxin will cease as bacterial protein synthesis is eventually inhibited by the mitomycin C. Other studies on the effect of mitomycin C on toxin production, including that of Houston *et al* (1981) on *Salmonella* toxin found, as in this study, that addition of mitomycin C increased the amount of toxin and total protein in culture supernatants, and that, as a result of cell lysis, there was a decreased amount of protein in mitomycin C-treated sonic extracts. As with LT however, the increase in biological activity of *Salmonella* toxin in the supernatant was almost equal to that lost from the sonic extracts. This

contrasts to the findings in this study, where increase in both extracellular and intracellular toxin levels in the presence of mitomycin C further confirms the phage-encoded nature of VT.

Chapter 4 The Production and Characterization of Mutants of *E.coli* O157:H7

4.1 Introduction

In order to determine the importance of various characteristics of *E.coli* O157:H7 in pathogenesis, a general mutagenesis method was chosen. Exponentially growing bacteria (O157:H7 ATCC 35150) were incubated with mitomycin C, an antibiotic produced by *Streptomyces* spp., which is also used as a plasmid curing agent. The activity of mitomycin C is dependent upon its *in vivo* reduction to the highly reactive hydroquinone derivative, a process which involves an $NADPH_2$-dependent enzyme. Reduced mitomycin C behaves as a bifunctional alkylating agent which forms covalent cross-links between complementary strands of DNA, usually involving guanine residues (Verweij & Pinedo 1990). As a mutagen, it is thought that mitomycin C induces mispairing during DNA replication by alkylating certain bases at certain positions. Alkylation-induced mispairing specifically generates transitions (A-T→C-G) *in vivo* (Drake & Baltz 1976). The drug also acts as a curing agent by inhibiting replication of the plasmid, resulting in loss of the plasmid after one or two generations. Mitomycin C as used in this study, could therefore cause alterations in both the chromosomal and plasmid DNA. Since it is not clear whether chromosomal or plasmid elements are involved in pathogenesis, mutations in both forms of DNA could lead to greater understanding of disease caused by O157:H7. Preliminary identification of mutants was made, based on alterations in plasmid profiles and biochemical reactions.

Two putative virulence attributes were considered in the assessment of the mutants: attachment of the organisms, and toxin production. With respect to attachment, the outer membrane, and especially the LPS was investigated. Two mutants, clones 5 and 6, with altered LPS components, were identified using antibodies raised against O157:H7 and were further characterised by thin layer chromatography (TLC) of LPS neutral sugars. Studies on the hydrophobicity of mutants in which the LPS was altered, and their ability to attach to human epithelial cells and to intestinal mucus *in vitro*, were carried out. Immunological methods were used to screen toxin production by mutants. The amount of biologically active toxin produced, and the distribution of toxin between the inside and outside of the cell was then measured, and comparisons were made between the mutants and the parent strain. Clones 5 and 6 were reduced in toxin activity, and secretion appeared to be impaired compared to the wild-type. It is likely that at least some of the virulence factors of O157:H7 are common to all VTEC.

4.2 Results & Discussion

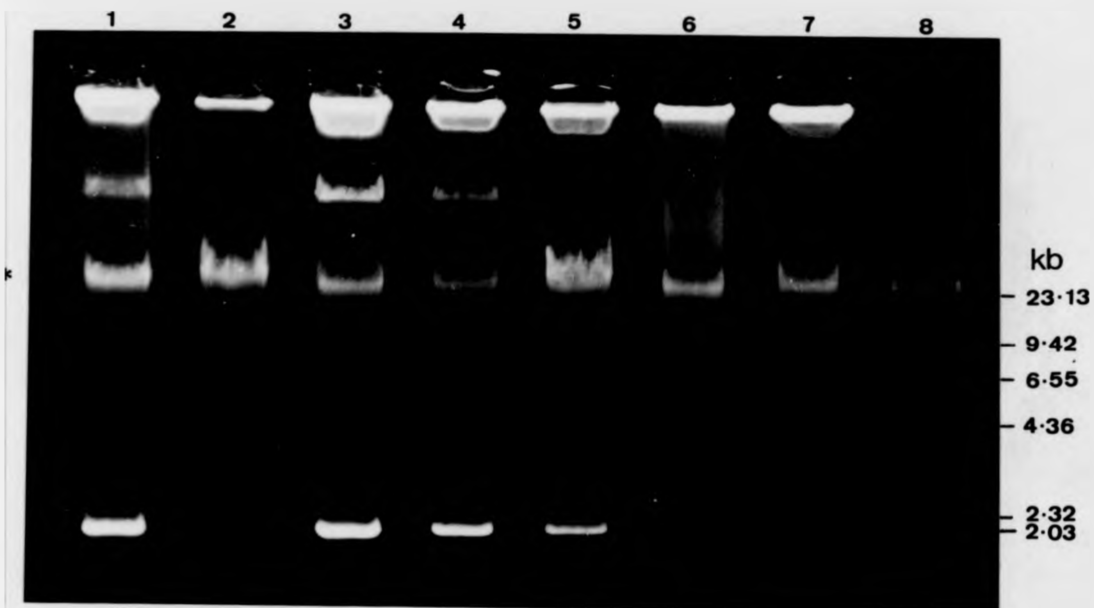
4.2.1 Plasmid Profiles

After incubating O157:H7 ATCC 35150 in the presence of mitomycin C, cells were plated onto Penassay plates to obtain single colonies. Six clones were chosen for further investigation. Since mitomycin C is a plasmid curing agent, the plasmid profiles of the clones were investigated to give an indication that the drug had been effective.

The plasmid content of the parent strain was identical to that of clones 2 and 3, and consisted of three plasmids of ~2kb, ~4kb and >23kb . Clone 4 contained in addition, a plasmid of ~4.2kb (Figure 4.1). Clones 1, 5

Figure 4.1 Plasmid Profile

Plasmid preparations from O157:H7 and clones 1-6 were separated on a 0.7% agarose gel, and visualised by ethidium bromide staining.



Lanes : **1** - O157:H7 (parent strain). **2** - clone 1. **3** - clone 2. **4** - clone 3. **5** - clone 4. **6** - clone 5. **7** - clone 6. **8** - λ HindIII markers.

* Chromosomal DNA

and 6 did not possess any of these plasmid bands, and only the chromosomal DNA band was visualised on the agarose gel (Figure 4.1).

Since the largest marker used was 23kb, it was not possible to estimate the size of the large plasmid, however, it is thought that this was the ~60-MDa plasmid (equivalent to ~94kb) which is carried by most O157:H7 strains (Karch *et al* 1987). The identity of this plasmid could have been confirmed by hybridization studies using a 3.4kb probe, known as CVD419, which was derived from the ~60-MDa plasmid (Levine *et al* 1987). It is also possible to detect proteins which are associated with possession of the ~60-MDa plasmid using a direct ELISA (Toth *et al* 1991). A plasmid of similar size (~98kb) was found in all isolates of O157:H7 by Ostroff *et al* (1989), and 85% of these strains contained in addition, between one and four other plasmids, ranging from 2-87kb. It appeared therefore that the plasmid profile of the parent strain was not untypical of O157:H7 strains.

The nature of the smaller O157:H7 plasmids is not known, although they are not essential, as some strains do not carry these plasmids. The ~60-MDa plasmid appears to code for a novel fimbrial antigen (Karch *et al* 1987), although the significance of this in attachment and hence pathogenesis is unclear. Since clones 1, 5 and 6 did not possess the plasmids present in the parent strain, they appeared to be most suitable for further studies.

4.2.2 Biochemical Profiles

Since it was likely that chromosomal mutations had also occurred, biochemical profiles of all six mutants and the wild-type were investigated. An EPEC strain, O26:H11 was included for comparison.

Table 4.1 Api 20E Biochemical Profiles

Strain	Numerical Profile	Identification	Atypical Reactions
O157:H7 (35150)	5144172	*E.coli* 90.5%	Sorbitol 90%
Clone 2	5144372	*E.coli* 94.5%	Sorbitol 90%
Clone 3	5144172	*E.coli* 90.5%	Sorbitol 90%
Clone 4	5144172	*E.coli* 90.5%	Sorbitol 90%
Clone 5	5154562	*E.coli* 97.4%	Rhamnose 87%
Clone 6	5154562	*E.coli* 97.4%	Rhamnose 87%
O26:H11	5144562	*E.coli* 99.3%	Rhamnose 87%

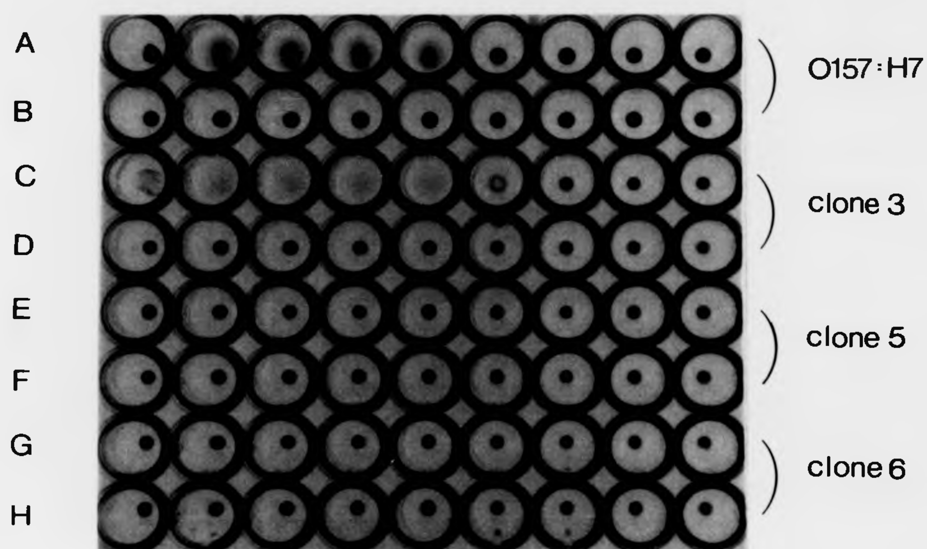
Api 20E- Results of 21 biochemical tests were processed to give a numerical profile. Through computer analysis, the percentage probability of an organism belonging to a particular species was calculated. The percentage values under 'Identification' refer to the probable identity of the organism as *E.coli* , and those under 'Atypical Reactions' refer to the percentage of *E.coli* which are able to utilise a certain sugar which the organism tested is unable to.

To analyse biochemical reactions, an Api 20E test was chosen. This is a commercial test to identify members of the Enterobacteriaceae family. The results of 21 biochemical reactions are processed to give a 7-digit numerical profile which identifies the probability of an organism belonging to a particular species. Any reactions which are atypical of the probable organism are highlighted. The results of the Api 20E tests are shown in Table 4.1. Of interest, were the atypical reactions of these organisms. Unlike 90% of *E.coli*, O157:H7 is unable to ferment sorbitol. This is a characteristic which is exploited in the isolation of this serogroup (Kleanthous *et al* 1988). The metabolism of clones 5 and 6 had clearly been altered as they were able to ferment sorbitol, but, in common with O26:H11, were unable to ferment rhamnose. Clones 3 and 4 have identical profiles to the wild-type, and although clone 2 has an altered biochemical profile, further studies were concentrated on clones 5 and 6. Attempts to produce a biochemical profile for clone 1 were unsuccessful as the organism failed to grow in the test kit. The implication of this is that major changes in the metabolism of this organism had occurred as a result of mutagenesis, which meant that the organism required a richer, more complex medium than is available in the Api test strip.

4.2.3 Passive Haemagglutination Assay

Red blood cells (RBC) coated with protein or polysaccharide antigens will agglutinate in the presence of the appropriate antibody. In this study, RBC were coated with heat-stable saline-extracted antigens from the reference strain of O157:H7 and clones 2, 3, 4, 5 and 6. The sensitised RBC were then reacted with antibodies raised against the reference strain of O157:H7. The aim was to demonstrate any changes in the antigenic

Figure 4.2 Passive haemagglutination assay



Antiserum raised against O157:H7 was titrated across rows A, C, E & G. Preimmune serum was titrated across rows B, D, F &H. Sheep RBC coated with heat-stable antigens from O157:H7 and clones 3, 5 & 6 were added to the titrated antisera as indicated. Titres are represented as reciprocals of the last antiserum dilution at which agglutination was seen.

determinants by comparing the passive haemagglutination titre with that of the parent strain. The antigen was extracted by heating bacteria resuspended in saline, at 100°C for 60 minutes. Although the exact composition of the antigen was not known, in a similar study, Penner & Hennessy (1980) observed that antigens extracted in this way from *Campylobacter* species possessed characteristics of O antigens. These included stability at 100°C and the ability to readily adsorb to RBC. In addition, the antigens elicited production of specific antibodies in rabbits.

The results of the passive haemagglutination assay of clones 3, 5 and 6, are shown in Figure 4.2 alongside the parent strain. The wild-type and clone 3 had passive HA titres of 1280. This was the highest dilution of antiserum at which there was clear agglutination. The antigenic determinants of clone 3 clearly had not been altered significantly. Clones 2 and 4 were also tested, and gave a passive HA titre of 1280 (not shown). Clones 5 and 6, however, failed to react with anti-O157:H7, indicating major changes in the antigenicity of these strains compared to the wild type. No haemagglutination was observed when sensitised RBC were reacted with pre-immune serum. This confirmed that aggregation of RBC resulted from specific antigen-antibody interactions. Clone 1 could not be tested because attempts to extract heat-stable antigens by saline treatment were unsuccessful. There was a possibility at this stage that the putative mutants were in fact contaminants, but subsequent colony ELISA and tissue culture assays showed that they were still able to produce toxin, so the possibility of these clones not being mutants was very low.

It was noted that in the first well, containing the highest concentration of antiserum, there was often no agglutination. This is known as the 'prozone phenomenon' and is thought to be due to the effect of high protein concentrations causing an increase in the net charge of the coated RBC. This increases the electrostatic repulsion between

individual particles, which opposes the action of the antibody molecules to link the erythrocytes together (Cruickshank *et al* 1975). Once the protein concentration was reduced by dilution, the antibodies were able to aggregate the RBC and bring about agglutination.

4.2.4 Oxoid Latex Agglutination Assay

The Oxoid latex agglutination assay is a commercially available test, which detects the presence of O157 antigens. The saline-extracted antigens of the O157:H7 clones 5 and 6 had failed to react with antiserum raised against whole cells of O157:H7 in a passive haemagglutination assay. These antigens were thought to consist predominantly of LPS, part of which is the O side chain, which is responsible for the O antigenicity of Gram-negative organisms. The Oxoid latex agglutination test, which consists of latex particles coated with antibodies to O157, would give a negative result with clones 5 and 6 if the O side chain was significantly altered.

O157:H7 clones 3, 5 and 6 were tested alongside the parent strain, the O157 clinical isolate and, as a negative control, *E.coli* O26:H11. The results of the assays are shown in Table 4.2. Clones 5 and 6 failed to agglutinate latex particles coated with anti-O157, confirming that these strains were antigenically distinct from the wild-type strain. Both the O157:H7 parent strain and the O157 clinical isolate gave a positive reaction in this assay. To confirm that this agglutination was specific, these strains were further reacted with control latex particles, with which they gave a negative reaction. As expected, O26:H11 failed to agglutinate the test particles in this assay (Table 4.2).

This latex agglutination assay confirmed that the O side chains of clones 5 and 6 had been altered by the mutagenesis procedure.

Table 4.2 The Oxoid latex agglutination assay

Organism	Test latex particles	Control latex particles
Clone 3	Positive	Negative
Clone 5	Negative	nt
Clone 6	Negative	nt
O157:H7 ref strain	Positive	Negative
O157 clinical isolate	Positive	Negative
O26:H11	Negative	nt

Bacterial cells were mixed with test latex particles coated with anti-O157. Organisms able to agglutinate these particles were reacted with control latex particles to confirm that the initial agglutination was specific.

nt - not tested

4.2.5 SDS-PAGE Analysis of Saline-extracted Antigens

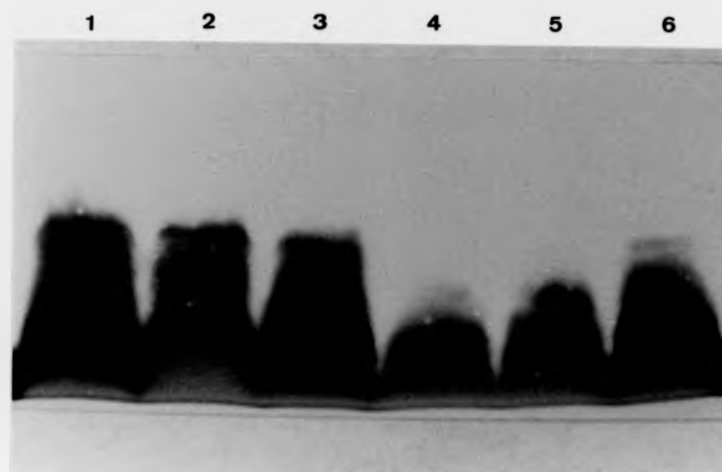
It was clear from the results of the passive haemagglutination and latex agglutination assays that the O side chains of clones 5 and 6 had been altered as a result of mutagenesis. The saline-extracted antigens were therefore separated by SDS-PAGE, and visualised by silver nitrate staining under conditions which visualise LPS rather than proteins.

The results of the SDS-PAGE analysis are shown in Figure 4.3 . This shows an apparent reduction in the amount of LPS in clones 5 and 6 compared to the parent strain. The LPS of clones 2, 3 and 4 is comparable to that of the wild-type. Clone 1 could not be investigated since all attempts to extract heat-stable antigens were unsuccessful.

It was expected that the distinctive ladder-like pattern of LPS would be visualised by this staining method. This did not occur however, and only those portions of the LPS which did not comigrate with proteins were stained, giving all strains the appearance of rough LPS which consists of only lipid A and core oligosaccharides. It was subsequently discovered that if the samples had been treated with proteinase K the entire LPS would have been visualised (Hitchcock & Brown 1983), however, due to time limitations, this was not attempted. Compression of LPS by the proteins probably explains why the banding is well-defined. Hitchcock & Brown (1983) found that when samples had been proteased, banding of LPS was much more diffuse, especially along the upper edges.

Thus although it appeared that clones 5 and 6 possessed a reduced amount of LPS compared to the parent strain, this was only demonstrated amongst the low molecular weight fractions. Since the full LPS profile was not visualised, conclusions about the extent of mutation could not be drawn from these SDS-PAGE profiles.

Figure 4.3 SDS-PAGE analysis of saline extracted antigens.



Antigens were separated on a 11% resolving gel and visualised by LPS-silver nitrate staining.

Lanes 1-5 contains clones 2-6. Lane 6 contains O157:H7 (35150)

4.2.6 Immunoblotting of Saline-extracted Antigens

It was known that the LPS, notably the O side chains, of clones 5 and 6 no longer reacted with either anti-O157 or with antiserum raised against the whole organism (O157:H7 ATCC 35150). To investigate this further, the saline-extracted antigens of clones 2, 3, 4, 5 and 6 were separated by SDS-PAGE as before, alongside the wild-type. The antigens were then transferred from the unstained gel to nitrocellulose and immunoblotted with antiserum raised against O157:H7.

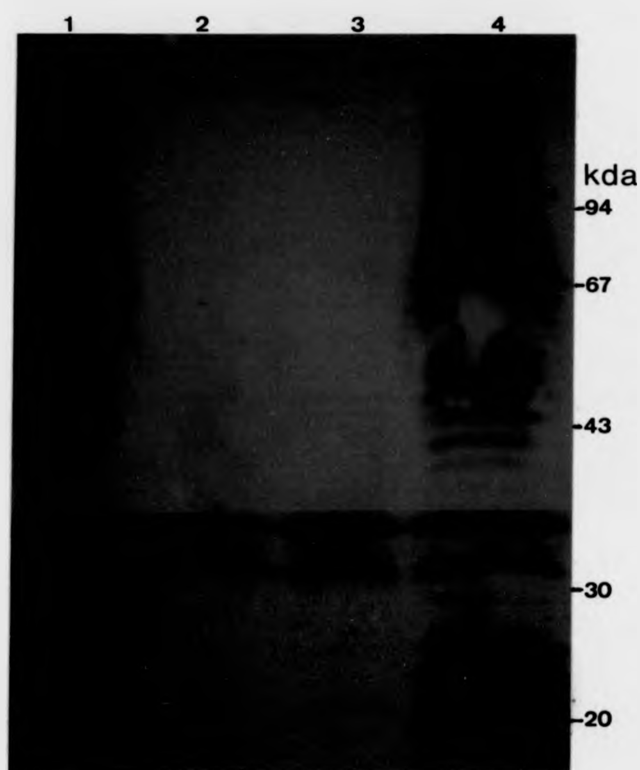
The saline-extracted antigens of O157:H7 and clones 4, 5 and 6 reacted with anti-O157:H7 as shown in Figure 4.4. The distinctive LPS ladder-like pattern was seen in the lanes containing clone 4 and the parent strain. It appeared that clone 4 was not altered with respect to antigenicity, neither were clones 2 and 3 which were also tested (not shown). Due to difficulties in preparing the antigen extract of clone 1, it was not possible to test this mutant. The ladder-like pattern was not seen in the lanes containing clones 5 and 6 however, where only two bands of 32 and 35 kDa which were probably proteins, were visible. Overbeeke & Lugtenberg (1980) analysed many human *E.coli* strains from different sources and found that in all cases the fastest moving band in the 30-42kDa region was OmpA. All other major outer membrane proteins were related to porins OmpC and OmpF. Thus it was thought that the 32kDa band was OmpA and the larger band corresponded to the porins, which are closely related in size (36-37kDa, Smyth 1985). The identity of OmpA could have been confirmed as it is the only outer membrane protein which is degraded during incubation of cell envelopes with trypsin. This yields a large degradation product with a molecular weight of approximately 18kDa associated with the cell envelope. It appeared that

the major outer membrane proteins of clones 5 and 6 were not significantly altered, so further studies were confined to the LPS, which did not react with the O157 antiserum. Saline-extracted heat-stable antigens were prepared several times, and the results as descibed above, were reproducible each time.

There are two explanations for the lack of reaction between the LPS of clones 5 and 6, and anti-O157:H7. One is that the O antigens had been altered to the extent that they no longer reacted with anti-O157, but would react with antibodies raised against another O antigen. The second explanation is that these mutants no longer possessed an O side chain, and would therefore be described as rough mutants. Rough LPS consists of lipid A and core oligosaccharide, but does not give rise to the ladder-like pattern in SDS-PAGE gels which results from the separation of a mixture of molecules of increasing molecular weight, representing additional O-antigen units added to the basic lipid A-core oligosaccharide structure.

To assess which explanation was most feasible, two identical Western blots were prepared of clones 2, 3, 4, 5 and 6 alongside the parent strain. The clinical isolate of O157 was also included, as well as O26:H11 and *S.dysenteriae* type 1. One of the blots was then reacted with antiserum against O157:H7, and the other with anti-O26:H11. The results of these blots are shown in Figure 4.5. When antiserum to O157:H7 was used, the characteristic ladder-like banding of LPS was seen in the lanes containing clones 2, 3 and 4, the parent strain, and the clinical isolate of O157. This confirmed that these clones were not significantly altered with respect to antigenic determinants, and that the antiserum was specific to O157. These bands were not visualised by silver staining, as the stain was specific for LPS, and was not effective in the presence of proteins. Only two bands from clones 5 and 6, corresponding to the outer membrane

Figure 4.4 Immunoblot of SDS-PAGE gel (11% acrylamide) of saline-extracted antigens with anti-O157:H7.



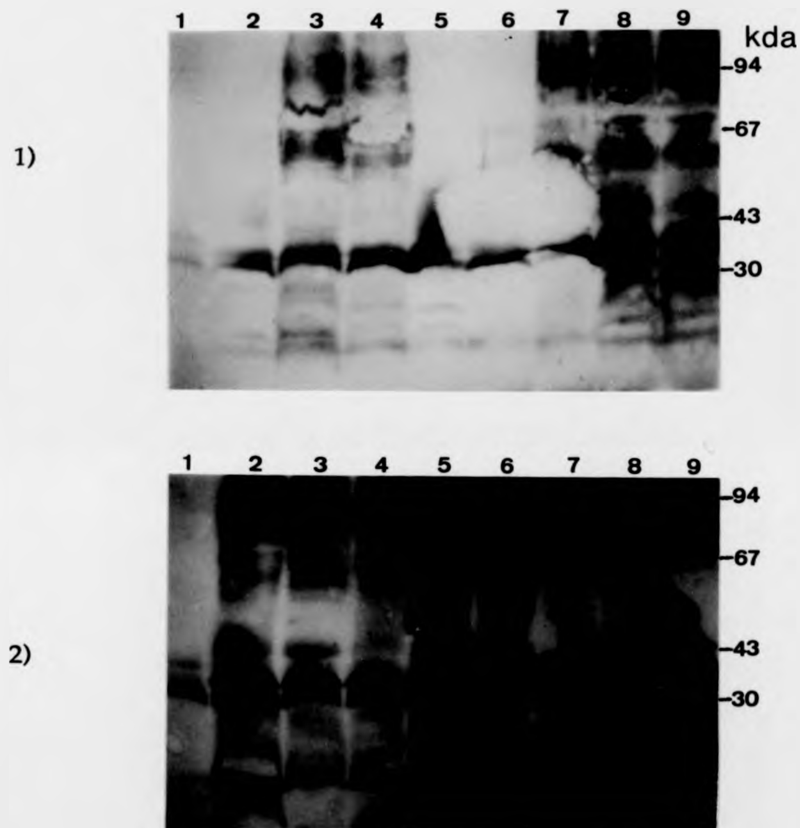
The following strains were tested : wild-type O157:H7 (lane 1), clone 6 (lane 2), clone 5 (lane 3), clone 4 (lane 4)

The 4-chloronaphthol development system was used

Figure 4.5 Immunoblots of SDS-PAGE gels (11% acrylamide) of saline-extracted antigens.

1)

2)



Identical gels were immunoblotted with 1) anti-O157:H7 or 2) anti-O26:H11. Lanes: **1**- *S.dysenteriae* type 1, **2**- O26:H11, **3**- O157 clinical isolate, **4**- O157:H7 parent strain, **5**- clone 6, **6**- clone 5, **7**- clone 4, **8**- clone 3

proteins seen in Figure 4.4, reacted strongly with this antiserum. This pattern was similar to that of O26:H11 and *S.dysenteriae* type 1 and was due to antigenic cross-reactivity. This phenomenon has been reported by other workers between outer membrane proteins of several serotypes of *E.coli* and other Enterobactericeae (Hofstra & Dankert 1979, Nicolle *et al* 1989). When anti-O26:H11 was used however, there was a strong reaction throughout the LPS profile of clones 5 and 6 (Figure 4.5). Similar reactions occurred in the lanes containing the clinical isolate of O157, clones 2, 3 and 4, and the O157:H7 parent strain. The LPS of *S.dysenteriae* type 1 did not bind to anti-O26:H11, however, so this appears to be cross-reactivity rather than non-specific binding. Only the outer membrane proteins of *S.dysenteriae* type 1, which also cross-reacted with anti-O157:H7, reacted with this antiserum.

Taken together, these results showed that clones 5 and 6 were antigenically distinct from O157:H7, but the fact that the LPS reacted with anti-O26:H11 means that they are not rough mutants. It was not thought that these clones possessed the O26 antigen, however, since the other O157 strains also cross-reacted with anti-O26:H11. This antiserum is clearly less specific than that raised against O157:H7. Cross-reaction between serotypes and even different species is not uncommon when sugar groups and molecular structure are similar. For example, a common sugar group is responsible for the antigenic cross-reactivity observed between *E.coli* O157:H7, *Brucella abortus* and *B.melitensis* (Perry & Bundle 1990). It is more difficult to explain why the anti-O157:H7 did not react with the O26:H11 antigens. Clearly the O157:H7 antigenic determinants are more specific. In support of this hypothesis, the presence of unique epitopes for O157:H7 which could be used to produce specific immunological reagents, has been reported (Rice *et al* 1992). It is not known whether O157:H7 and O26:H11 share similar LPS structures,

but it seems likely, since in addition to the cross-reaction seen here, anti-O157:H7 was found to inhibit attachment of O26:H11 to HeLa cells by >50% (this study).

The immunoblotting indicated that the LPS, but probably not the outer membrane proteins of clones 5 and 6 differed from the parent strain. Further studies were therefore concentrated on the LPS and not the protein moiety of the outer membrane.

4.2.7 **Neutral Sugar Analysis**

Studies on the heat-extracted antigens of O157:H7 clones 5 and 6 had shown that the LPS was significantly altered in the O side chain and no longer reacted with antiserum raised against the parent strain. The neutral sugars were therefore extracted from purified LPS and analysed by TLC.

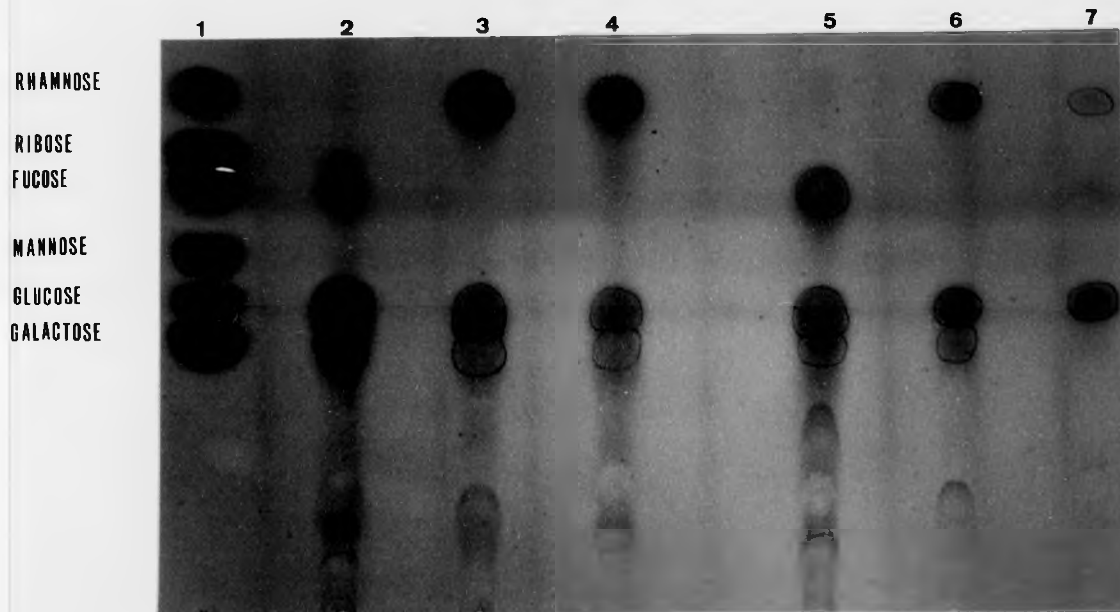
The results of TLC analysis of mutant and wild-type LPS is shown in Figure 4.6. LPS was isolated using a phenol/water procedure which results in a product containing only around 1% contaminating proteins. Most LPS separates into the aqueous phase but occasionally some lipophilic LPS remains in the phenol phase. This also occurs with rough LPS. To ensure maximum yield of LPS, in this study both phases were kept and analysed separately. Both the wild-type and the mutants contained glucose and galactose, but fucose in the wild-type was absent in clones 5 and 6, which instead contained rhamnose. This pattern was seen in both the aqueous and phenol phases, although more of the LPS had separated into the aqueous phase.

It is hypothesised that the altered sugar profile results from a mutation in the LPS synthetic pathway. The O side chain of LPS is made up of many repeating tetrasaccharide units which are synthesised on carrier lipids and assembled by transfer from one carrier, to a growing

chain on another carrier molecule. During synthesis, carbohydrates are added to the repeat unit from sugar nucleotide intermediates. In the case of *E.coli* O157:H7, these sugars are mannose, galactose, glucose and fucose (Perry *et al* 1986). These are incorporated into the O chain by membrane-bound transferases, in the form of GDP-D-mannose, UDP-D-galactose, UDP-D-glucose and GDP-L-fucose. In the mutants however, it appeared that rhamnose was incorporated into the repeat units in place of fucose. In order to understand this, it is necessary to look at the biosynthetic pathway of sugar residues (Figure 4.7). In the wild-type O157:H7, GDP-L-fucose is synthesised from glucose-6-phosphate via a series of intermediates including GDP-D-mannose. Glucose, galactose and rhamnose nucleotides are synthesised along a different pathway. If the pathway leading to GDP-L-fucose was blocked as a result of the mutagenesis procedure, it is speculated that the synthesis of dTDP-L-rhamnose is favoured. This would also prevent synthesis of GDP-mannose. In this study, mannose was not detected in the mutants or the wild-type O157:H7. This is due to the method of analysis, using mineral acid hydrolysis which destroys the mannose component in the O chain. It would be necessary to extract the mannose using anhydrous hydrofluoric acid to see whether it was present in the mutants. Since this was not possible, it can only be speculated that mannose would be absent from the mutants. Alternatively the pathway could have been blocked after GDP-mannose, that is, at the level of production of GDP-4-keto-6-deoxymannose or GDP-L-fucose. Since antigenic changes in the LPS were major, and rhamnose was present in the mutants, it is hypothesised that the pathway was blocked before GDP-mannose, and intermediates were redirected along the rhamnose fork of the pathway.

Figure 4.6 Neutral sugar analysis.

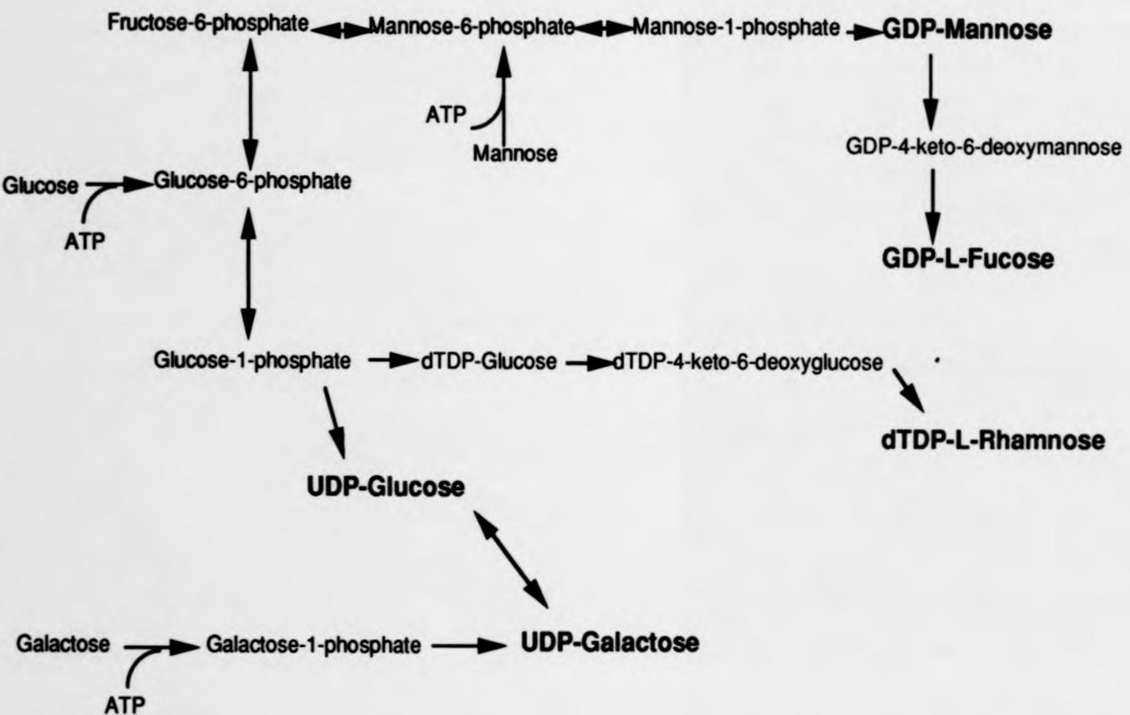
Neutral sugars were extracted from purified LPS by mineral acid hydrolysis and separated by thin layer chromatography.



The LPS extraction yielded an aqueous phase and a phenolic phase.

Lanes : **1**- Sugar standards. **2** - wild-type (aqueous phase). **3** - clone 5 (aqueous phase). **4** - clone 6 (aqueous phase). **5** - wild-type (phenolic phase). **6** - clone 5 (phenolic phase). **7** - clone 6 (phenolic phase).

Figure 4.7 Biosynthesis of Sugar Residues.
Major metabolic steps involved in the biosynthesis of LPS carbohydrate precursors



Adapted from Gabriel 1987

4.2.8 Hydrophobicity Studies

Hydrophobic interactions are thought to be important in pathogenesis with respect to adhesion of bacterial cells, and in overcoming repulsive forces between the bacterial and host cells. Since the LPS of clones 5 and 6 had been altered, it was possible that the hydrophobicity of these bacteria was modified. The hydrophobicity of clone 1 was of interest since when grown on solid media this strain produced more mucoid colonies than the other O157:H7 strains. A human *E.coli* strain, Econ1, was included as a hydrophobic control. This organism had been isolated from a urine sample, found to be hydrophobic in nature, and was routinely used as a hydrophobic control at Northwick Park Hospital.

Three methods were chosen to assess hydrophobicity: microbial adhesion to hydrocarbons (MATH), hydrophobic interaction chromatography (HIC) and a salt aggregation test (SAT). All three methods gave an indication of the net hydrophobicity in terms of adhesion. Bacteria can have a net hydrophobicity through a small hydrophobic area which enables the cell to adhere even though the majority of the cell is more hydrophilic (Rosenberg & Doyle 1990). In addition, SAT gives an indication of the hydrophobic properties of the cell as a whole. MATH is a simple quantitative test, which relies on the fact that hydrophobic organisms adhere to liquid hydrocarbons whereas hydrophilic strains do not. HIC measures microbial adhesion to octyl- sepharose beads in small chromatography columns. The higher the percentage retention in the column, the more hydrophobic the organism. SAT works on the principle that increasingly hydrophobic bacteria will aggregate at correspondingly lower salt concentrations. These three methods were used since a single assay does not necessarily indicate the extent of hydrophobicity of the

microbial cell surface. It should be noted that hydrophobicity is a relative term since even organisms described as being hydrophobic disperse readily in the bulk aqueous phase.

Each of the three methods used to assess hydrophobicity indicated that all the VTEC tested ie O157:H7 ATCC 35150, clones 1, 5 and 6, and the clinical isolate of O157, were hydrophilic compared to the hydrophobic control. Whilst SAT (Table 4.4) and HIC (Table 4.5) were very reproducible, MATH (Table 4.3), proved less reliable. One reason for this is that the vigorous vortexing of the bacteria in the presence of hydrocarbon often leads to cell damage, and hence spurious data. N-hexadecane which was used in this study, is less damaging in this respect however, than the aromatic hydrocarbons such as xylene which were used in the original MATH tests (Rosenberg & Doyle 1990).

Clone 1 appeared slightly more hydrophobic than the wild type, although not significantly when compared to the hydrophobic control. This could be a consequence of the fact that this mutant was more mucoid than the other VTEC tested, which may have affected hydrophobicity, or simply meant that the cells had a tendency to clump together. Another disadvantage of the MATH assay is that bacteria which clump, will give low turbidity readings in the aqueous layer, falsely indicating that the organisms have adhered to the hydrocarbon. In a similar way, these organisms may have become physically entrapped in the HIC column, rather than adhering due to their hydrophobic properties. Non-specific clumping would not explain the SAT result however, as no aggregation occurred in the control test. It has been reported, however, that SAT is only satisfactory for the detection of hydrophobic organisms and HIC is the best method for hydrophilic bacteria (Mozes & Rouxhet 1987). Thus, although clone 1 appeared to have slightly altered hydrophobicity compared to the wild type, it may not be a true reflection of its

hydrophobic properties. Clones 5 and 6 were not significantly altered compared to the parent strain.

The MATH and HIC tests (Tables 4.3 and 4.5) showed that the clinical isolate of O157 was slightly more hydrophobic than the reference strain. This may be more significant, since sequential subculturing of clinical isolates can result in gradual loss of hydrophobic properties. It may be the case therefore, that this strain was originally more hydrophobic than it now is. There is evidence that other non-invasive *E.coli*, EPEC, are more hydrophobic than non-pathogenic strains (Smyth *et al* 1978), so it seems possible that prior to sub-culturing, the clinical isolate of O157 was more hydrophobic than shown here.

The overall conclusion is that none of the VTEC tested were strongly hydrophobic. This supports the findings of Sherman *et al* (1987) who using HIC, tested several O157:H7 strains and found that all had a high anionic surface charge, but low surface hydrophobicity. The LPS mutations in clones 5 and 6 had not affected the hydrophobicity of these organisms. It also seemed unlikely that the hydrophobic properties of clone 1 had been significantly changed, as slight differences are probably explained by the mucoid nature of these bacteria.

Table 4.3 Microbial Adhesion to Hydrocarbons (MATH)

Strain	**Volume of Hexadecane added**		
	0.1ml	0.2ml	0.4ml
O157:H7 Parent strain	98.4 ± 3.2	97.8 ± 4.3	90.1 ± 1.2
Clone 1	100.3 ± 4.1	101.5 ± 7.2	87.2 ± 2.3
Clone 5	99.1 ± 0.9	96.6 ± 3.4	93.5 ± 3.1
Clone 6	97.4 ± 2.6	98.7 ± 0.8	93.5 ± 5.7
O157 Clinical isolate	98.0 ± 2.2	93.7 ± 6.2	87.6 ± 4.3
Econ1	93.3 ± 3.5	85.4 ± 8.4	64.9 ± 3.8

Bacteria were suspended in saline, and a volume of hexadecane ranging from 0.1ml to 0.4ml was added and thoroughly mixed. Results are expressed as mean percentage retention of organisms in the aqueous layer for triplicate tests (± = range of values).

Table 4.4 Salt Aggregation Test (SAT)

Strain	Ammonium Sulphate Conc.
O157:H7 Parent strain	2.4M
Clone 1	1.7M
Clone 5	2.3M
Clone 6	2.3M
O157 Clinical isolate	2.3M
Econ1	0.6M

A bacterial suspension was mixed with a range of ammonium sulphate solutions ranging from 0-4.0M. Figures refer to the highest concentration of ammonium sulphate, at which aggregation of organisms was observed. This value was reproducible even when performed on separate days.

Table 4.5 Hydrophobic Interaction Chromatography (HIC)

Strain	% Retention by HIC column
O157:H7 Parent strain	48.8 ± 0.5
Clone 1	53.0 ± 3.1
Clone 5	45.5 ± 4.2
Clone 6	45.5 ± 1.0
O157 Clinical isolate	50.5 ± 2.6
Econ1	75.3 ± 0.3

A suspension of bacteria was applied to an octyl-sepharose chromatography column. The pre- and post-column optical densities were then compared, and the percentage of organisms retained by the column was calculated. Mean values of three tests are shown (± = range of results).

4.2.9. Attachment of Bacteria to Mucus and HeLa cells.

The successful colonization of host mucosal surfaces by pathogenic bacteria results from a complex interaction between the bacterial and host cells. The initial association involves interaction of the pathogen with extracellular host products such as mucus which is secreted by goblet cells of the epithelium. Adhesion then involves highly specific interactions between bacterial adhesins and receptors on the epithelial surface.

Since the cell envelope of clones 5 and 6 differed from the parent strain, the attachment properties of these bacteria were investigated and compared. Their ability to attach to mucus and to a human epithelial (HeLa) cell line was studied.

4.2.9.1 Attachment to Mucus

Mouse colonic mucus was separated by SDS-PAGE and transferred to nitrocellulose. Bacteria which attached to the mucus fractions were detected immunologically using mouse antiserum to *E.coli*, followed by goat anti-mouse horseradish peroxidase conjugate. There was no apparent difference in the binding capacity of the wild-type and clones 5 and 6, which all bound to a ~70 kDa protein of mouse mucus (Figure 4.8). An *E.coli* strain expressing K88 fimbrial antigens which are associated with attachment to mucus (Laux *et al* 1986), was included as a positive control. Thus although a blot was not done with whole cells to see how reactive the serum was, the K88 antigens acted as an indicator that the expected reaction had worked. As a negative control, culture medium was added in place of bacteria and no band was visualised in this test. This ensured that the antibodies themselves did not react with the mucus. In an identical experiment none of the strains bound to any component of hamster

mucus (data not shown), but this may be because hamsters do not appear to be colonized by *E.coli* (S P Borriello, personal communication). A parallel study on *C.difficile* revealed that the bacteria were probably binding to glycoprotein (Krishna & Borriello 1992).

Attachment of *E.coli* to mucus has been studied by other workers who concluded that the bacteria bind to fractions in the 50-70-kDa range (Cohen *et al* 1986, Laux *et al* 1986). Mucin consists of high-molecular-weight polymeric glycoproteins and a smaller link glycopeptide. It appears that O157:H7 bind to the 118-kDa link glycopeptide (Sajjan & Forstner 1990b). In an earlier study, however, it was suggested that mucin samples which were prepared from secretions in which there was prolonged exposure of the mucus to proteolytic enzymes, may become partially degraded during the harvesting procedure. Ferret mucus was found to contain a 70-kDa band which was thought to represent partial degradation of the 118-kDa component (Roberton *et al* 1989). Thus it is possible that the observed binding of the O157:H7 strains to a 70-kDa band in this study, is a consequence of the mucus preparation, whereby some proteolytic activity occurred, and the bacteria attached to a product of degradation of the link glycopeptide thought to bind to O157:H7 (Sajjan & Forstner 1990b).

Binding of *E.coli* to mucin has in some cases been shown to be dependent upon the possession of adhesins such as fimbriae (pili). *E.coli* K12, for example, does not bind to mucus, but if it carries the K88ab plasmid which encodes K88 fimbrial antigens, the cells will attach to mucus via two glycoproteins of 57- and 64-kDa (Laux *et al* 1986). Adherence by some fimbriae can be blocked by D-mannose, and such adhesins, also known as type-1 pili are found on 50-70% of *E.coli* (Karch *et al* 1985). Sajjan & Forstner (1990a) showed that only a type-1 piliated O157:H7 strain was capable of binding to mucus, although this strain was more hydrophobic than any of the other type-1 piliated strains

investigated. Hydrophobicity studies (Section 4.2.8) show that the O157:H7 strain used in the present study was less hydrophobic than any of the type-1 piliated strains studied by Sajjan & Forstner (1990a), who found it necessary to induce the pili by repeated subculturing. It is unlikely therefore that the bacteria used in the present study were type-1 piliated. Possession of these pili therefore, appears not to be essential to mucus binding.

In conclusion it appears that *E.coli* O157:H7 bind to a ~70-kDa fraction, of mouse colonic mucus which is possibly a degradation product of the 118-kDa link glycopeptide which has been shown to bind O157:H7 (Sajjan & Forstner 1990b). Lack of attachment to hamster colonic mucus is attributed to lack of receptors, because hamsters are not natural hosts of *E.coli*. The binding capacity of clones 5 and 6 was not affected by the alterations to these bacteria, indicating that this binding is not O-type specific since clones 5 and 6 no longer possess O157 antigens.

4.2.9.2 **Attachment to HeLa Cells**

It is known that VTEC attach to the surface of certain eukaryotic cell lines but are non-invasive. O157:H7 and clones 5 and 6 were allowed to attach to HeLa cells to see whether the alterations to the outer membrane of the mutants had affected their ability to attach to epithelial cells. The results of these assays are shown in Table 4.6. Attachment assays using clone 1 were unsuccessful as the bacteria were unable to divide in tissue culture medium (even over a shorter time period) so any results would not be comparable to the other strains tested. A comparable number of bacterial cells (~ 5×10^{7} ml^{-1}) had been added in each case, and since the growth rate of clones 5 and 6 was the same as the parent strain, these results indicated

Figure 4.8 Attachment to mucus

Mouse mucus was separated by PAGE and transferred to nitrocellulose. Bacterial cells were allowed to attach and were detected with mouse antiserum to *E.coli* followed by goat anti-mouse peroxidase conjugate.
Lanes: **1**- E.coli K88 (positive control). **2**- O157 (clinical isolate).
3- O157:H7 ATCC 35150 (parent strain). **4**- clone 5. **5**- clone 6
No band was visualised in the negative control (culture medium, no bacteria) - not shown.

that the alterations in the LPS of the mutants had not significantly affected their ability to attach to HeLa cells. It is likely that during the 3 hour incubation period of the assay (at 37°C) the bacteria reached the exponential growth phase, therefore the observation that twice as many wild-type cells had attached compared to clone 5, is probably not significant. Incubation periods of less than 3 hours were not tested.

In separate assays, O157:H7 whole cell antiserum was added with the bacterial cells to compare the degree of inhibition of attachment of the wild-type and mutants. The results of these assays are shown in Table 4.7.

When pre-immune serum was used, there was less than 50% inhibition of attachment in each case. The % inhibition of clones 5 and 6 (31.1% and 35.2% respectively) was slightly less than that of the wild-type (45.9%) but this difference is not probably significant since this inhibition, by components of the serum (which had not been heated to destroy complement), is non-specific. In the presence of O157:H7 antiserum, however, almost all of the wild-type (95.6%) was unable to attach to the HeLa cells (Figure 4.9). In contrast, around 50% of clones 5 and 6 were still able to attach to the HeLa cells in the presence of anti-O157:H7. The antiserum had some inhibitory effects on attachment of the mutants, but this was not comparative to the inhibition of wild-type attachment.

Anti-O157:H7 did not inhibit the attachment of the mutants to the HeLa cells to the same extent as the wild-type. Immunoblotting had shown that the LPS of clones 5 and 6 did not react with this antiserum so the implication was that LPS was involved in attachment. However, other studies have shown that LPS is unlikely to be involved in attachment (Sherman & Soni 1988). VTEC attach to epithelial cells in an attaching-effacing (AE) manner (Sherman *et al* 1988b), also characteristic of EPEC,

Table 4.6 Attachment of O157:H7 and clones 5 and 6 to HeLa cells.

Strain	**Number of cells attached ml^{-1}**
Wild-type O157:H7	3.45×10^6
Clone 5	5.71×10^6
Clone 6	7.03×10^6

Bacterial cells (5×10^7 ml^{-1}) were allowed to attach to HeLa cells for 3 hours at 37°C. After washing, the HeLa cells were lysed and attached bacteria were serially diluted and cultured. The assays were carried out in triplicate, and a mean value is shown in the above table.

Table 4.7 Attachment of O157:H7 and clones 5 and 6 to HeLa cells in the presence of O157:H7 antiserum.

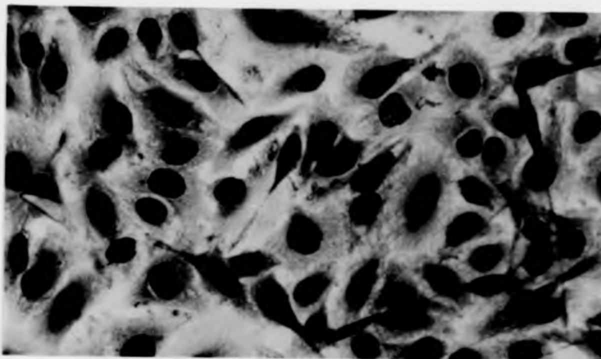
Strain	**% inhibition by pre-immune serum**	**% inhibition by O157:H7 antiserum**
Wild-type O157:H7	45.9± 3.6	95.6± 2.9
Clone 5	31.1± 1.5	51.7± 3.0
Clone 6	35.2± 1.4	45± 1.9

Bacterial cells (5 x 10^7 ml-1) were allowed to attach to HeLa cells in the presence of either O157:H7 antiserum, or pre-immune serum from the same rabbit. Assays without serum additions were also carried out. Attached cells were enumerated as described in Table 4.6.

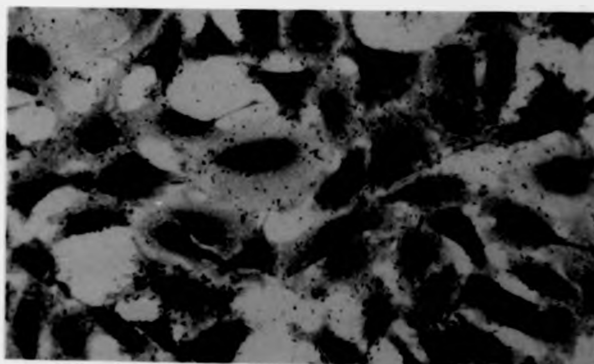
The number of cells attaching in the presence of serum is expressed as a percentage of the number which attached in the absence of serum. Assays were carried out in triplicate and the figure in the above table is the mean value (± = range of values)

Figure 4.9 Attachment of O157:H7 to HeLa cells in the presence and absence of O157:H7 antiserum.

1.



2.

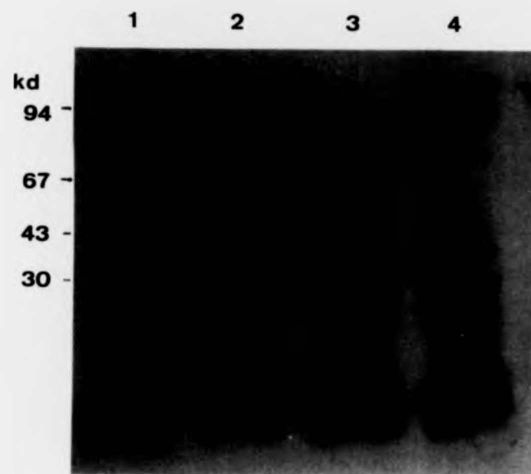


Bacteria were allowed to attach to HeLa cell monolayers which had been cultured on 13mm round glass coverslips. After several washes to remove unattached bacteria, the HeLa cells were stained with Giemsa stain and photographed. Assays were carried out in the presence (1) and absence (2) of anti-O157:H7.

which are thought to attach via a 94-kDa membrane protein encoded by the chromosomal *eae* gene (Jerse & Kaper 1991). Homologous sequences in VTEC strains indicate that a chromosomal gene may be responsible for VTEC adherence (Jerse *et al* 1991). A possible adhesin is therefore a 94-kDa outer membrane protein which competitively inhibits adherence of VTEC (Sherman *et al* 1991). It is speculated that to inhibit attachment of the wild-type, the antiserum either attached directly to the adhesin(s), or antibody-antigen complexes masked the adhesin(s), blocking attachment. It follows therefore that the antiserum was less effective at inhibiting attachment of the mutants either because the adhesin itself was altered antigenically, or because the adhesin was not masked by antibodies attaching to the LPS. To investigate this further, outer membrane preparations were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-O157:H7. This showed that unlike the parent strain and a clinical isolate of O157, outer membrane components from clones 5 and 6, in the 94-kDa region, did not react with the antiserum (Figure 4.10). This could indicate that a protein of 94-kDa is involved in attachment of VTEC to epithelial cells, and that in these mutants this protein is antigenically distinct from the parent strain. Such an alteration would not necessarily affect the function of the protein, since it appears that the Eae protein of EPEC, which is involved in attachment, is antigenically distinct from the putative homologue in VTEC (Jerse *et al* 1991). In the absence of anti-O157:H7 the adhesion of the mutants was not different to that of the wild-type, therefore it is concluded that the components(s) of the outer membrane responsible for attachment had probably been altered antigenically but not functionally.

In a study on the adhesion of *E.coli*, from various sources, to mammalian cells, it was concluded that binding was associated with the presence of fimbriae (Jann *et al* 1981). The role of fimbriae in attachment of

Figure 4.10 Immunoblotting of outer membrane preparations with anti-O157:H7.



Outer membranes were separated on 11% SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with anti-O157:H7.

Lanes: **1**- O157 (clinical isolate). **2**- clone 5. **3**- clone 6. **4**- O157:H7 ATCC 35150 (parent strain).

O157:H7 to mammalian cells is controversial. Most strains possess a 60-MDa plasmid which encodes fimbriae which were thought to be required for adhesion to epithelial cells (Karch *et al* 1987). Subsequent studies have shown that possession of this plasmid is not essential for either adherence to epithelial cells or virulence (Junkins & Doyle 1989, Tzipori *et al* 1987, Hall *et al* 1990). A recent study indicated that the plasmid is not necessarily associated with possession of fimbriae (Ashkenazi *et al* 1992). The present study supports the theory that the 60-MDa plasmid is not essential for adherence of O157:H7, since both the wild-type, and the mutants, which had lost the plasmid, attached to HeLa cells to the same degree.

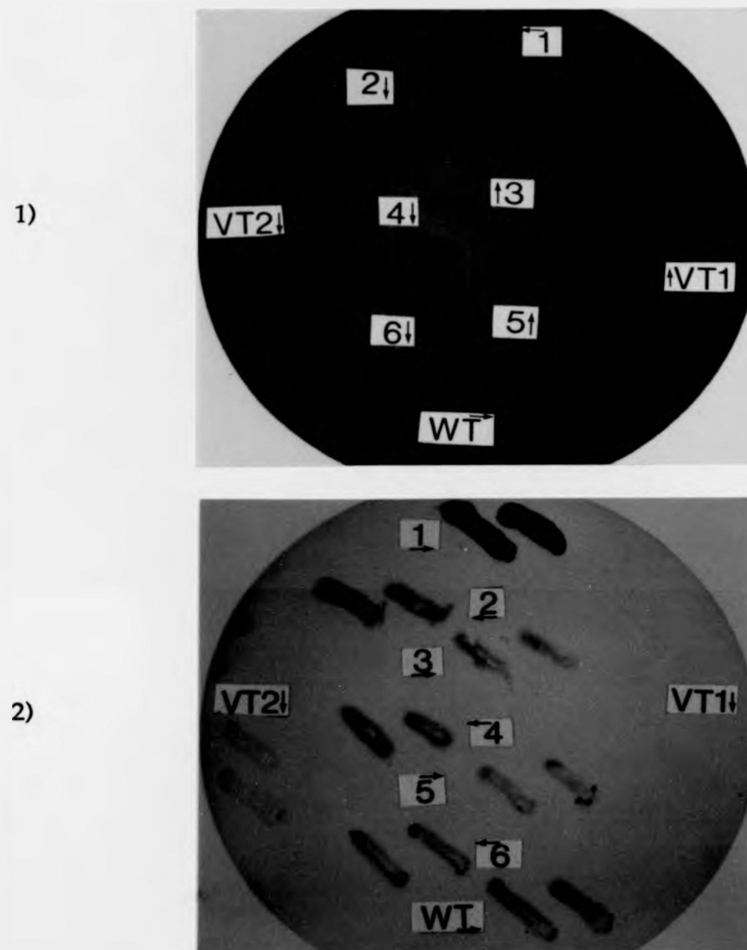
4.2.10 Toxin Production

All six clones were screened for toxin production using immunological and biological techniques. The initial screen was a colony ELISA which detected the presence of the toxin antigens. Following this, Vero cell assays were carried out to see whether the toxin detected antigenically was biologically active. The distribution of toxin between the inside and outside of the cell was then determined by a standard plate ELISA.

4.2.10.1 Colony ELISA

The results of the colony ELISA are shown in Figure 4.11. It had been established that the wild-type produced both VT1 and VT2 (Section 3.1.3), so assays were carried out in duplicate to assess the relative amount of each toxin produced by the clones. Clones 2, 3, 4, 5 and 6 seemed to produce comparable amounts of VT1 and VT2 to the wild-type, but clone 1 appeared to produce more of each toxin than any of the other strains. This clone however had been more severely altered by the mutagenesis

Figure 4.11 Colony ELISA of O157:H7 (WT) and clones 1-6.



Strains were grown overnight on Penassay agar plates then transferred to nitrocellulose membrane in the presence of polymyxin B, and immunoblotted with MAb against 1) VT1 and 2) VT2.

The DAB/nickel horseradish peroxidase development system was used.

Controls :VT1- C600 933J. VT2- C600 933W.

procedure than the others. Evidence of this included the inability of these organisms to grow in the Api 20E biochemical assay or to survive in the tissue culture attachment assays. In addition, all attempts to extract heat-stable antigens from this mutant were unsuccessful. It appeared that this strain, which when grown on solid media had more mucoid colonies than the wild-type or the other clones, had an altered cell wall. Thus the apparent increase in toxin production seen in the colony ELISA may have been due to a more permeable cell wall which allowed more efficient extraction of toxin by polymyxin B treatment.

4.2.10.2 **Vero Cell Assays**

A preliminary Vero cell assay was carried out to determine whether the toxin detected by colony ELISA was biologically active. Undiluted supernatant filtrate from an overnight culture of clones 1, 5 and 6 and the parent strain, was added to Vero cell monolayers. Clones 5 and 6 were of interest since it had already been determined that the LPS of these strains differed from the wild-type. Clone 1 was tested to see whether this strain produced more toxin than the wild-type, or whether the apparent increased toxin levels detected by colony ELISA reflected differences in the permeability of the cell wall which increased the efficiency of toxin extraction by polymyxin B.

The assay revealed that none of the mutants produced active extracellular toxin even if toxin production was induced by mitomycin C (Table 4.8). Intracellular toxin levels were therefore examined, by breaking open cells through sonication and adding filtered sonicate to Vero cells. This showed that clones 1, 5 and 6 were able to produce some biologically active toxin, although intracellular levels were much lower than the parent strain and in some cases induction by mitomycin C was essential.

This indicated that most of the toxin detected by colony ELISA was not biologically active.

If these crude toxin preparations from the mutants were kept at 4°C overnight, all toxic activity was lost. This could either be due to increased protease activity in these preparations or an increased susceptibility of mutant toxin to proteolytic activity. To assess whether there was increased protease activity in the mutant filtrates, wild-type toxin was added to an equal volume of the non-active mutant toxin preparation. After 48 hours at 4°C the activity of the normal toxin was compared to a control sample which had been incubated in PBS. Both toxin samples were still active, indicating that the loss of activity of the mutant toxin was not due to increased protease activity, but resulted from the instability of the protein.

The quantity of toxin required to elicit detectable immunoreactivity in a colony ELISA is much greater than the amount which can be detected by a cytotoxicity assay. The lack of detectable extracellular active toxin from clones 1, 5 and 6 is therefore probably due to attenuation of cytotoxic activity rather than the instability of the mutant polypeptides.

4.2.10.3 **Plate ELISA**

The results of the colony ELISA indicated that the clones of most interest, 5 and 6, produced comparable levels of VT1 and VT2 to the wild-type (Figure 4.11). Also of interest was the fact that clone 1 appeared to over-produce both toxins. Vero cell assays detected very little active toxin in any of these mutants however, and no activity was detected extracellularly. This raised the possibility that in addition to a reduction in toxin activity in these mutants, the secretion of the toxin was affected.

Table 4.8 Vero cell assay.

	Intracellular		**Extracellular**	
	Induced	Normal	Induced	Normal
Wild-type	+++ (+++)	+++ (+++)	+++ (+++)	+++ (+++)
Clone 1	+/- (-)	- (-)	- (-)	- (-)
Clone 5	+ (-)	+/- (-)	- (-)	- (-)
Clone 6	+ (-)	+/- (-)	- (-)	- (-)

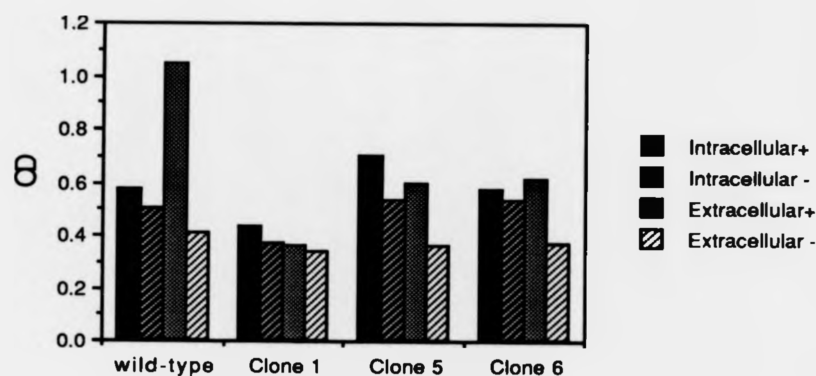
+++ = 100%. + =25-50%. +/- =10-25%. - = 0%.

Figures correspond to % cell death

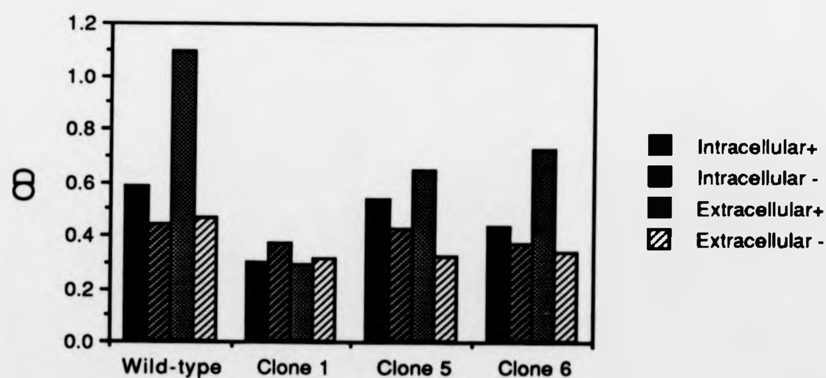
The cytotoxicity of culture supernatant (extracellular) and sonicated cells (intracellular) of O157:H7 and clones 1, 5 and 6 was investigated. Cultures had been grown in the presence (induced) or absence (normal) of $0.1\mu gml^{-1}$ mitomycin C. Results were recorded after 3 days (day 1 results in parentheses).

Figure 4.12 Plate ELISA to demonstrate distribution of VT1 and VT2 of O157:H7 (wild-type) & clones 1, 5 and 6 .

1) Distribution of VT1



2) Distribution of VT2



MAb against 1)VT1 and 2)VT2 were used to detect toxin in intracellular (sonicate) and extracellular (supernatant) fractions of overnight cultures grown with (+) and without (-) mitomycin C.

Cultures were standardised spectrophotometrically before fractionating.

The distribution of toxin between the inside and outside of the cell was therefore examined using a standard plate ELISA. MAb against VT1 or VT2 were used as capture antibodies to coat an ELISA plate. Toxin which bound to these antibodies was detected by rabbit polyclonal VT antibodies followed by anti-rabbit peroxidase conjugate.

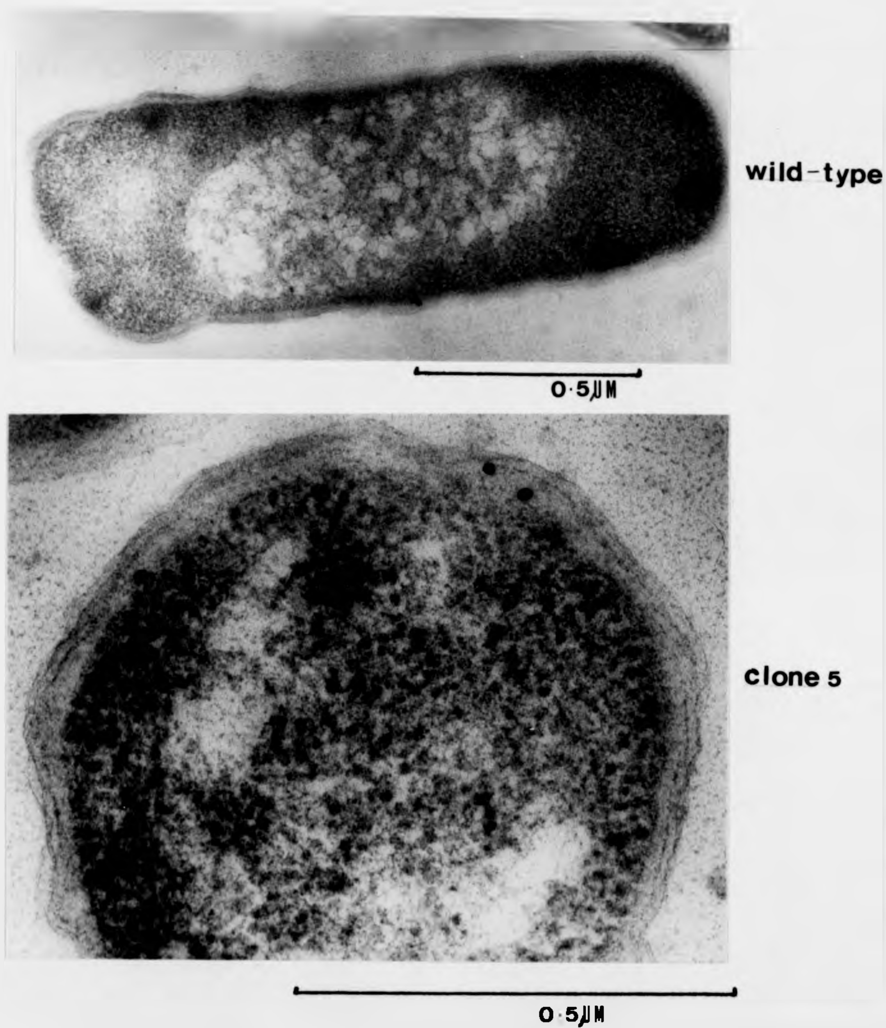
The results of the ELISA are shown in Figure 4.12. It is immediately obvious that not all toxin is located intracellularly, which combined with the results of the Vero cell assay indicates that the extracellular toxin and most of the intracellular toxin must be biologically inactive. In general, levels of VT1 and VT2 detected in all three mutants were lower than wild-type levels and extracellular levels were more greatly reduced. The toxin-converting phages did not appear to be affected as mitomycin C still caused an increase in VT production by phage induction. The increase in intracellular toxin levels of clones 5 and 6 was comparable to the wild-type, but extracellular increases were significantly lower. This implies that secretion is impaired.

4.2.10.4 **Immunogold Labelling**

Immunogold labelling studies were carried out in an attempt to explain the reduced level of secreted toxin by the mutants. MAb tagged with colloidal gold were used to visualise toxin in thin sections of bacterial cells, which were viewed by transmission electron microscopy.

The results of the immunogold labelling are shown in Figure 4.13. The technique was only partially successful as only 2-6 gold particles were seen per cell, and most cells were unlabelled. This is thought to be a reflection of the fixation regime used. There was no discernible difference between cells which had been grown in the presence or absence of mitomycin C. The labelling of anti-VT2 was not successful even when the

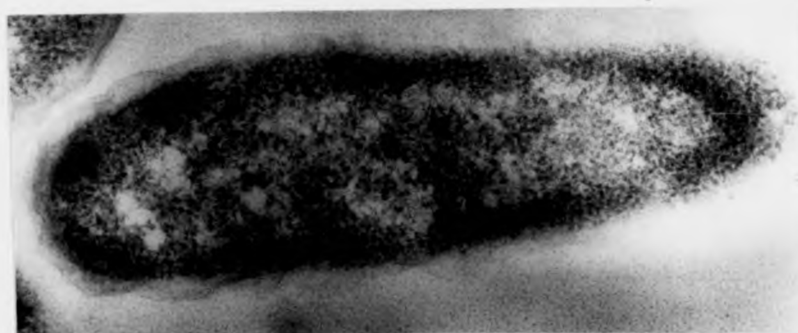
Figure 4.13 Immunogold labelling of VT1

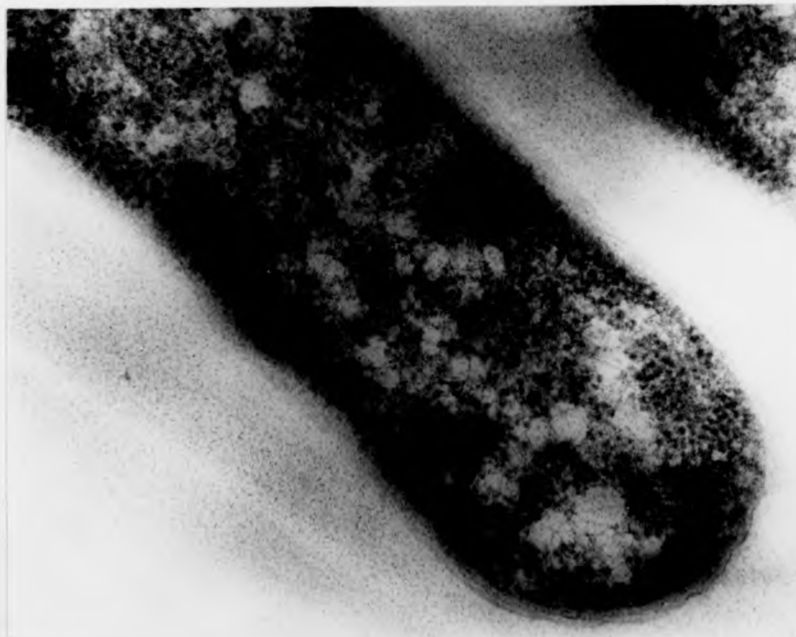


Bacterial thin sections were treated with MAb to VT1 followed by rabbit anti-mouse then Protein A-gold. Sections were washed before each addition. After the final step the sections were allowed to dry before post-staining, and viewed by transmission electron microscopy.

continued overleaf

Figure 4.13 continued from previous page.





concentration of MAb was increased from 1:1000 to 1:100, so the following results refer to detection of VT1 only. Unfortunately it was not possible to repeat this work.

In the wild-type, the toxin was located either outside of the cell, in the periplasmic space, or in the cell periphery. Similar observations were made of VT1 in clone 5. In clone 6 however the toxin tended to be located more towards the centre of the cell, and in several cells a clumping of 5-6 gold particles was observed. This observation was made in >20 cells studied. It is not known whether this reflects a discrete localization of VT1 or passive clumping of the gold. No toxin was observed in the periplasm. Naturally these observations are limited and may not be significant, however they support the hypothesis that secretion is affected, possibly because the release of toxin from the cytoplasm is impeded.

It appears from these results that clones 5 and 6 produce slightly less toxin than the wild-type and activity is greatly reduced. No toxin activity was detected outside of the cell and this may reflect the fact that the mutant toxin was unstable. It is hypothesized therefore, that when the mutant toxin is synthesized it possesses low activity, but, during passage across the outer membrane this activity is lost, so no active toxin is detected outside the cell. The toxin is thus affected biologically but still cross-reacts antigenically with the wild-type.

VT are composed of a single A subunit non-covalently associated with a pentamer of B subunits. To be functionally active, the B subunit must possess the ability to bind to Gb3 and to oligomerize correctly with the A subunit. The A subunit, in addition to assembling correctly with B subunits must possess RNA N-glycosidase activity. If any of these characteristics are altered, toxin activity will be affected. Several workers have reported that changes to a few amino acid residues can reduce toxin activity. A critical site for enzymatic activity of the A subunit of VT1 is

glutamic acid residue 167. If this residue is replaced with aspartic acid, specific activity is decreased 1000-fold, although the change does not affect the polypeptide conformation, and full-length proteins are produced which are correctly processed by signal peptidase (Hovde *et al* 1988). The corresponding amino acid residue, glutamic acid 166, is essential for VT2 activity (Jackson *et al* 1990). Deletion of amino acids 3-18 of the VT2 A subunit results in loss of cytotoxicity and enzymatic activity (Perera *et al* 1991b). Mutations in the B subunit have also been shown to be essential to toxin activity. Perera *et al* (1991a) identified 3 amino acid residues in Shiga toxin and VT2 B subunits which were essential for holotoxin activity. These mutants were immunogenic but not cytotoxic, much like the mutant toxins produced in the present study. It would have been of great interest to sequence the toxin genes from clones 5 and 6 to investigate the reduced activity.

It is possible that toxin activity was affected at the level of toxin binding. This could have been investigated using a receptor analogue ELISA such as that described by Weinstein *et al* (1988a). The receptor analogue used was Gal-Gal-BSA, which is commercially available. This was attached to an ELISA plate and toxin allowed to bind. The extent of binding was detected by specific MAb to the toxins. Reduced binding, or the inability of the mutant toxins to bind to the VT receptor would explain the observed loss of cytotoxicity.

As mentioned above, it has been established that minor changes in the amino acid sequence of VT can dramatically affect their activity. It would have been of great interest therefore to sequence the toxins from the mutants and possibly relate alterations at the nucleotide level to reduced activity. Since the nucleotide sequences of VT1 and VT2 are known (Calderwood *et al* 1987, Newland *et al* 1987) it would be possible to utilise the polymerase chain reaction (PCR) in sequencing the mutant

toxins. Primers could be synthesized to amplify the entire toxin gene, or shorter sequences could be amplified, and readily cloned into suitable vectors and sequenced. Perera *et al* (1991a,1991b) have demonstrated that both 5' and 3' terminal coding sequences are essential for functional VT operons, so these areas could be concentrated on initially. Although glutamic acid residue 167 (VT1) or 166 (VT2) has been shown to be essential for toxin activity, it is probably not affected in these mutants since there is some activity, albeit greatly reduced.

In addition to reduced biological activity, there is some evidence that toxin secretion is also impaired. VT are periplasmic proteins which are secreted from the cell via a 'leaky membrane', that is, there is no active secretion. Proteins destined for an extracytoplasmic location are synthesized with an additional hydrophobic amino-terminal extension (approximately 20 residues) known as the signal sequence. Single amino acid substitutions in the signal sequence can affect the export of a protein. Maltose binding protein (MBP), for example, accumulates in a precursor form in the cytoplasm if a single hydrophobic or uncharged amino acid is replaced with a charged residue (Bedouelle *et al* 1980). This suggests that the hydrophobicity of the signal sequence is important in localisation of an exported protein. In the present study the results of the plate ELISA indicated that secretion of VT by the mutants was not as efficient as the wild-type. It may be the case that toxin secretion is reduced due to an altered signal sequence. This could be confirmed by sequencing the signal sequence.

An alternative explanation to reduced secretion of toxin is that other molecules which are essential to secretion are affected. In order to translocate across biological membranes, proteins assume a loosely folded structure, the 'pre-folded confirmation' (Kumamoto 1991). This is thought to be maintained by soluble protein factors known as molecular

chaperones. One of the better characterised of these agents is SecB. Mutants in SecB exhibit severe defects in some proteins for example MBP. In $SecB^-$ mutants 70% of intracellular pre-MBP was found to be in an export-competent conformation but was exported extremely slowly, showing that SecB also accelerates the export process (Kumamoto 1991). It may be possible to test this theory because only a subset of exported proteins are affected by a mutation in SecB. Mutants in SecB are inhibited in MBP secretion but export normal levels of alkaline phosphatase (Kumamoto & Beckwith 1985). These proteins can be assayed, and reduced levels of extracellular MBP but not alkaline phosphatase, compared to wild-type levels, could be indicative of SecB mutations.

The observed reduction in secreted toxin could be a function of the toxin itself, either by alterations in the signal sequence or the tertiary structure of the molecule. The latter may be less likely since antigenic determinants seemed to be unaffected in the mutants. Molecular chaperones may have been altered by the mutagenesis, in which case it is likely that other secreted proteins would also be affected. Alternatively, the export of toxin across the membrane may have been affected by the changes in the outer membrane itself. Since secretion is not active, it could simply be that the mutated membrane is less 'leaky' than the wild-type membrane. It should be possible to test this hypothesis by inducing the phages from the mutants and the wild-type, and lysogenising another *E.coli* strain such as K12. The amount of extracellular toxin produced by the K12 lysogens could then be compared, and reduction in mutant levels would be a function of the toxin or phage genes and not a property of the cell membrane.

In summary, mutants of O157:H7 ATCC 35150 have been created by chemical mutagenesis. Six clones were analysed, and clones 1, 5 and 6 were

found to be altered with respect to plasmid content. Clones 5 and 6 were altered with respect to outer membrane components, and all three mutants showed a reduced capacity to produce toxin, compared to the wild-type. The O chain of the LPS of mutants 5 and 6, failed to react with anti-O157:H7 although the major outer membrane proteins, Omp F, C, and A, appeared to be unaffected antigenically. Neutral sugar profiles of clones 5 and 6 revealed that the LPS contained rhamnose instead of fucose, which was present in the wild-type. The hydrophobicity of these mutants, and their ability to attach to colonic mucus and epithelial cells was not affected. However, it appeared that a protein responsible for binding of the bacteria to epithelial cells may have been altered antigenically since it no longer reacted with anti-O157:H7, and this antiserum was not effective at blocking attachment. Clones 5 and 6 produced very little active toxin, and secretion may have been impaired. Immunological toxin screening revealed that the mutants produced only slightly less toxin than the wild-type.

Attempts to extract outer membrane components from clone 1 were unsuccessful, so LPS analysis and attachment studies were not carried out on this mutant. However, toxin secretion and activity were affected. Clones 2, 3 and 4 were not significantly altered by the mutagenesis, and were included in studies only for comparative purposes.

Chapter 5 Mechanism of Internalisation and Intracellular Processing of Verotoxins

5.1 Receptor-Mediated Endocytosis

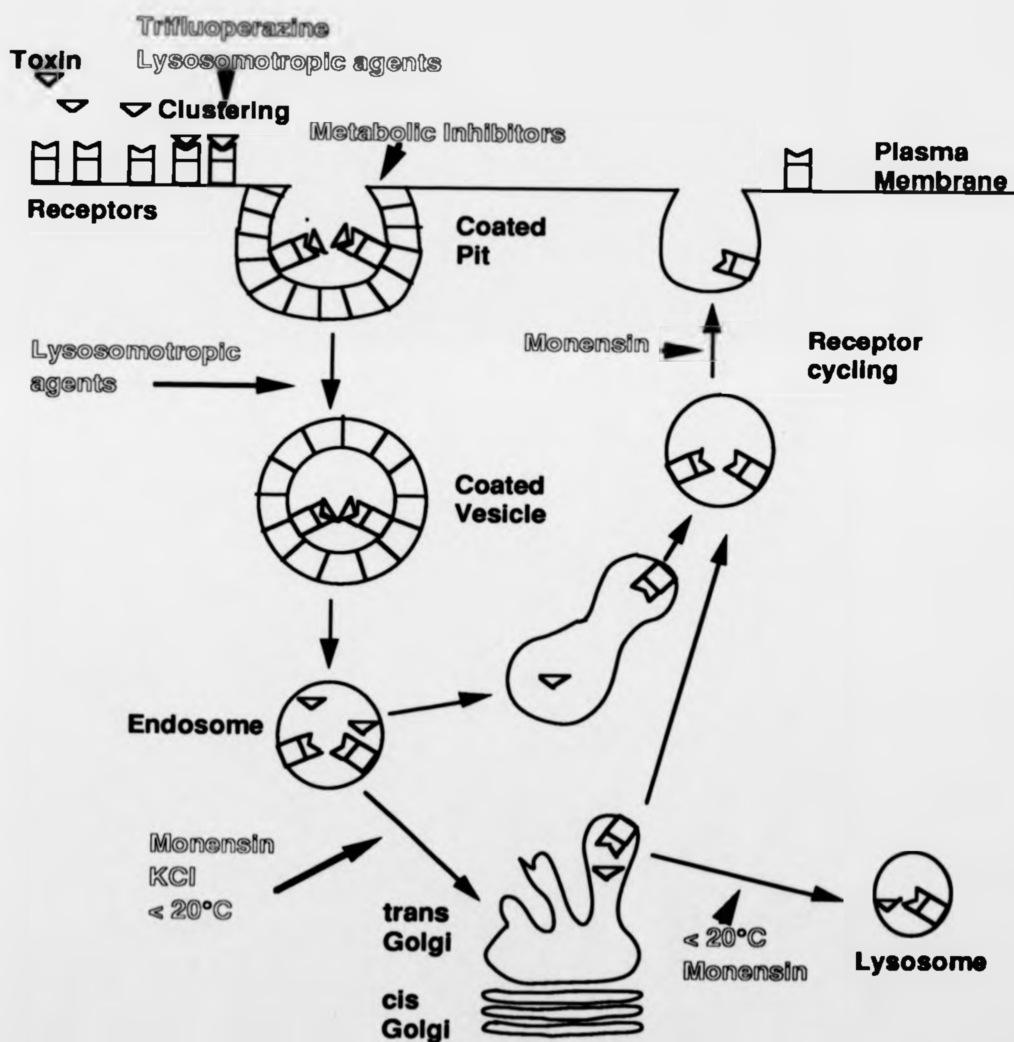
5.1.1 Introduction

As VT act on an intracellular target, namely the 60s ribosomal subunit (Endo *et al* 1988), the process of internalisation is crucial to the action of these toxins. It is known that several toxins with intracellular targets enter cells via receptor-mediated endocytosis (RME) (Willingham & Pastan 1984), therefore this process was investigated as a potential mechanism for entry of VT1 and VT2.

RME involves the internalisation of toxin-receptor complexes into coated vesicles, via specialised regions of the cell membrane known as coated pits. Upon loss of the vesicle coat, the vesicles, now known as endosomes, fuse with either the *trans* Golgi complex or primary lysosomes, from where the toxin is able to translocate to the cytosol, and hence reach the intracellular target. In some cases for example diphtheria toxin, the toxin is able to enter the cytosol directly from the endosome, but commonly a fusion step between endosomes and lysosomes is necessary.

The approach taken to investigate the relevance of RME to VT internalisation, was to use inhibitors which block various steps of the process and to see the effects of these agents on the cytotoxicity of VT1 and VT2 towards Vero cells (Figure 5.1). A key weakness in studying the cellular pathogenesis of toxins is that cultured cell lines are not necessarily the appropriate target cells, therefore *in vitro* studies may not give a true picture of intracellular events. It has been shown, however, that the effect of Shiga toxin on cultured epithelial cells from human colon and ileum

Figure 5.1



The points of action of the inhibitors used in this study are indicated.

was similar to that on Vero and HeLa cells (Moyer *et al* 1987). It would appear that for Shiga toxin , Vero cells are an appropriate model for toxic action. Since VT and Shiga toxin are related both in amino acid sequence and in mode of action, it is thought that the activity of VT1 and VT2 in Vero cells will also be representative of the *in vivo* situation.

Endocytosis is an energy-dependent process, so initially metabolic inhibitors were added to Vero cells, along with either VT1 or VT2. The cytotoxic activity of both toxins was reduced by a metabolic inhibitor, indicating that energy was required for intoxication to take place. Two features of RME which are essential to the internalisation of other toxins, are the fusion of endosomes with lysosomes, and the requirement for exposure to an acidic intravesicular compartment. This study showed that intoxication of Vero cells by either VT1 or VT2 was prevented when fusion between endosomes and lysosomes was blocked. There was no requirement for exposure to an acidic environment, as agents which increased intravesicular pH did not inhibit the cytotoxicity of either VT1 or VT2.

Two VTEC strains were used: E3787, a VT1 producer, and E32511, which produces only VT2. The titres of VT2 were consistently higher than those of VT1, probably because VT2 tends to be more evenly distributed between the inside and outside of the cell, whereas VT1 is almost completely cell-associated (Strockbine *et al* 1986).

5.1.2 **Results and Discussion**

The effects of various inhibitors of RME were assessed to determine the pathway of entry and any intracellular processing of VT1 and VT2. The points of action of these agents and conditions are shown in Figure 5.1. In order to determine the concentration of inhibitor to use, all drugs were titrated across monolayers of Vero cells to assess their toxicity to the cell. The highest concentration of each drug, which caused no visible effects on the cell line, was then chosen to be used in this study.

5.1.2.1 **The Effect of Metabolic Inhibitors**

Metabolic inhibitors affect energy-dependent processes such as endocytosis by reducing intracellular levels of ATP. The endpoint effect of metabolic inhibitors on intoxication of Vero cells by VT1 and VT2 is shown in Table 5.1. Of the three inhibitors tested: sodium fluoride, 2-deoxyglucose and 2,4-dinitrophenol, only 2-deoxyglucose reduced the cytotoxic effect of the toxins. This is consistent with other reports, where some metabolic inhibitors were unable to reduce intracellular ATP significantly to affect endocytosis. In order to reduce the activity of ricin and diphtheria toxin for example, it was necessary to add sodium fluoride in conjunction with sodium azide, an inhibitor of oxidative phosphorylation (Sandvig & Olsnes 1982b). In the present study however, even the use of these two drugs together failed to affect the intoxication of cells by either VT1 or VT2 (Table 5.1). Sodium fluoride alone has been effective in reducing the cytotoxic effects of Shiga toxin, but did not inactivate the toxin as such, and had no effect on binding of the toxin to the receptor cell (Eiklid & Olsnes 1983).

Table 5.1 Endpoint effect of metabolic inhibitors on cytotoxicity of VT1 and VT2.

Inhibitor	**VT1**	**VT2**
None	256	65,536
2-deoxyglucose	4	<256
Sodium fluoride	256	65,536
2.4-dinitrophenol	256	65,536
Sodium fluoride + sodium azide	256	65,536

Culture filtrate of either a VT1-producer (E3787) or a VT2-producer (E32511) was titrated across monolayers of Vero cells.
A constant volume (25µl) of inhibitor was added to give a final concentration of 5mM. The endpoint was determined after 20-24 hours at 37°C in 5% CO_2, as the reciprocal toxin concentration which killed 50% cells.

Metabolic inhibitors have successfully been used to protect against toxins which are known to enter mammalian cells by RME, for example, ricin, diphtheria toxin, *Clostridium difficile* toxin A and Shiga toxin (Sandvig & Olsnes 1982b, Henriques *et al* 1987, Eiklid & Olsnes 1983). Since cytotoxicity of VT1 and VT2 towards Vero cells was reduced by 2-deoxyglucose, it seemed likely that the internalisation mechanism of these toxins is an energy-dependent process, such as RME.

5.1.2.2 **The Effect of preventing Fusion between Endosomes and Lysosomes**

Fusion between endosomes and lysosomes is selectively and reversibly inhibited at temperatures below 20°C, and in the presence of 200mM potassium chloride. Cellular intoxication by VT1 and VT2 was prevented at 18°C suggesting that a fusion is required for activity of the toxins (Table 5.2). This was confirmed by transferring cells to 37°C which overcame the protective effect. Vero cells which were kept at 18°C in the presence of toxin showed no cytopathic effects even after 48 hours. Other workers have reported similar protection at 18°C from intoxication by *C.difficile* toxins A and B, and Shiga toxin (Henriques *et al* 1987, Florin & Thelestam 1986, Eiklid & Olsnes 1983). The action of the toxins appears to be blocked at the fusion between endosomes and lysosomes since it has been demonstrated using ricin-peroxidase conjugates that transport of toxin to the Golgi complex is a temperature-dependent process (van Deurs *et al* 1987).

Binding of the toxin may also be reduced at lower temperatures as Sandvig *et al* (1989) have demonstrated that at low toxin concentrations approximately twice as much Shiga toxin binds at 37°C as at 18°C. An earlier study however showed that toxin binding was the same at 25°C

when toxin was active, as at the lower inhibitory temperature of 20°C (Eiklid & Olsnes 1983). In this study, cells which had been incubated at 18°C with VT, were washed to remove unbound toxin and then reincubated at 37°C. Toxin titres were then comparable to the control experiment where cells were incubated at 37°C directly after adding the toxin. Thus it appeared that for both VT1 and VT2, even if binding is reduced at 18°C, it has no significant effect on the titres of these toxins (Table 5.2).

Fusion between intracellular vesicles is also reported to be inhibited at high potassium chloride concentrations, therefore cells were incubated with toxin in the presence of 200mM potassium chloride (Table 5.2). Cells were protected from the activity of both VT1 and VT2 by this agent, which further demonstrated that a fusion is needed for cellular intoxication to occur. Other toxins which are inhibited by high levels of potassium chloride include *C.difficile* toxins A and B, and Shiga toxin (Henriques *et al* 1987, Florin & Thelestam 1986, Eiklid & Olsnes 1983).

These findings show that when the toxins are trapped in the endosomes, either by low incubation temperature or high potassium chloride levels, no cytopathic effects will be seen. This suggests that VT1 and VT2 cannot be transferred to the cytosol directly from the endosomes, at least not in an active form, but must first pass into either primary lysosomes or the *trans* Golgi complex. This is probably due to a requirement for further processing of the toxins by enzymes not present in the endosomes to release the active fragment.

Endosomes are generated from coated pits which are known to exclude a number of membrane proteins, for example some proteases, which are found in lysosomes and the *trans* Golgi complex. Further studies using inhibitors of lysosomal proteases may have confirmed that

Table 5.2 Endpoint effect of prevention of fusion between endosomes and lysosomes on the cytotoxicity of VT1 and VT2

		VT1	VT2
	No inhibitor	256	65,536
a	Potassium chloride 200mM	No CPE	No CPE
b	Incubation 18°C	No CPE	No CPE
c	Incubation 18°C Reincubation 37°C	256	65,536

Culture filtrate of either a VT1-producer (E3787) or a VT2-producer (E32511) was titrated across monolayers of Vero cells.

(a) 25µl potassium chloride was added to each well to a final concentration of 200mM. Cells were incubated at 37°C.

(b) Cells were incubated at 18°C.

(c) Cells were incubated at 18°C for 24 hours then transferred to 37°C. CPE only appeared after incubation at 37°C.

The toxin titre is the reciprocal toxin dilution which killed 50% of cells after 20-24 hours incubation in 5% CO_2.

VT require lysosomal processing. For example, the activity of *C.difficile* toxin B is reduced in the presence of chymostatin, leupeptin and antipain, indicating that this toxin requires processing by lysosomal proteases (Florin & Thelestam 1986). *C.difficile* toxin A however, is not inhibited by these agents, although there is still a requirement for fusion between endosomes and lysosomes (Henriques *et al* 1987). It seems, therefore, that if further processing of toxins occurs in lysosomes or the Golgi complex it may not necessarily involve common lysosomal proteases.

5.1.2.3 **The Effect of Lysosomotropic Agents**

Amines such as ammonium chloride, chloroquine and methylamine are able to penetrate membranes in their unprotonised form. Inside acidic vesicles they become protonised and thereby increase the internal pH. Protection by lysosomotropic agents implies a requirement for exposure to an acidic environment, possibly for enzymatic activation and/or transfer of toxin to the cytosol. Such compounds have been successfully used to protect against *C.difficile* toxins A and B, and diphtheria toxin (Henriques *et al* 1987, Florin & Thelestam 1986, Mekada *et al* 1981). In the present study however, all the agents tested: ammonium chloride, chloroquine and methylamine, failed to inhibit VT1 and VT2 activity (Table 5.3). Mutant Chinese Hamster Ovary (CHO) cells which have a defect in ATP-dependent acidification of endosomes were available to confirm this observation. The ability of VT1 and VT2 to intoxicate both wild-type CHO cells and the mutants would have confirmed that acidification of endosomes is not essential for toxin activity. Unfortunately this experiment could not be carried out as CHO are resistant to VT (Konowalchuk *et al* 1977).

In addition to raising the intracellular pH, lysosomotropic agents inhibit transglutaminase which may be involved in the clustering and internalisation of ligands. This seems to be the mechanism of protection against *Pseudomonas* exotoxin A (Middlebrook & Dorland 1984). Since VT1 and VT2 remain cytotoxic in the presence of these agents they must be able to enter cells even if this enzyme is inactivated. The implication of these results is that VT can, under certain circumstances, enter cells by an alternative mechanism that does not involve coated vesicle formation.

Trifluoperazine inhibits the clustering process of endocytosis and also blocks an alternative endocytotic pathway which is independent of the coated pit mechanism. In the presence of this drug, the action of both VT1 and VT2 is blocked (Table 5.3). This observation supports the hypothesis that VT1 and VT2 under normal circumstances enter cells by RME although do not require exposure to an acidic environment. If clustering of the toxin-receptor complexes is blocked however the toxins are still able to enter cells, probably via an alternative pathway which does not involve coated pits.

5.1.2.4 The Effect of Monensin

Monensin is a proton ionophore which induces electroneutral exchange of H^+ for monovalent cations, for example K^+ and Na^+. This disruption of the proton gradient has the effect of raising intravesicular pH. Protection by such an agent implies that exposure to an acidic environment is essential for cellular intoxication.

Monensin gave significant protection against VT1 and VT2 (Table 5.3) which may indicate a requirement for intravesicular acidification. However since other lysosomotropic agents failed to protect against VT1 and VT2 (Table 5.3), the protective effect of monensin can probably be

Table 5.3 Endpoint effect of lysosomotropic agents and trifluoperazine on cytotoxicity of VT1 and VT2

Inhibitor	VT1	VT2
No Inhibitor	256	65,536
Ammonium chloride 20mM	256	65,536
Chloroquine 100μM	256	65,536
Methylamine 20mM	256	65,536
Trifluoperazine 25μM	<4	<256
Monensin 10μM	4	<256

Culture filtrate of either a VT1-producer (E3787) or a VT2-producer (E32511) was titrated across monolayers of Vero cells.

A constant volume (25μl) of each inhibitor was added to each well (final concentration as shown above).

The toxin titre is the reciprocal toxin dilution to give 50% cell death after 20-24 hours incubation at 37°C in 5% CO_2.

attributed to other properties of the compound. In addition to increasing vesicular pH, monensin interferes with the transport of protein between different vesicular compartments, especially the fusion of endosomes with the Golgi region. Since VT1 and VT2 were both inhibited under conditions which prevented such a fusion (Table 5.2), it is likely that the protection afforded by monensin is due to interruption of protein transport. In addition, it has been shown that the endocytosis of low density lipoprotein (LDL) can be interrupted by monensin as the drug blocks the return of receptors for recycling (Basu *et al* 1981). It is probably a combination of these effects which made monensin an effective inhibitor of VT1 and VT2. The effectiveness of monensin as a toxin inhibitor has also been reported for *C.difficile* toxins A and B, Shiga toxin and diphtheria toxin (Henriques *et al* 1987, Caspar *et al* 1987, Sandvig & Brown 1987, Sandvig & Olsnes 1980).

The above data indicates that the internalisation of VT1 and VT2 is an energy-dependent process which can be prevented by metabolic inhibitors. Most glycolipid-binding toxins, for example cholera and tetanus toxins, are endocytosed from uncoated areas of the plasma membrane. The first reported example of a lipid-binding ligand to be endocytosed from coated pits was Shiga toxin (Sandvig *et al* 1989). Since then it has been shown that glycolipids can enter cells by RME without being cross-linked with a ligand (Kok *et al* 1989). Internalisation of VT1 and VT2 is an energy-dependent process which has no requirement for exposure to a low pH, but fusion between endosomes and lysosomes is essential. It is proposed therefore that VT1 and VT2 are also endocytosed via coated pits, but that if the clustering of toxin-receptor complexes is blocked, an alternative pathway becomes apparent.

5.2 The Role of Calcium Uptake in Cellular Internalisation of VT1 and VT2

5.2.1 Introduction

The activity of several toxins including Shiga toxin and C.*difficile* toxin B is affected in the presence of drugs which block calcium transport across membranes (Sandvig & Brown 1987, Caspar *et al* 1987). To investigate the potential role of the calcium uptake system in VT internalisation, several drugs were chosen which inhibit the pathway at different stages (Figure 5.2)

Verapamil is a potent calcium channel blocker. Lanthanum chloride can also prevent entry of calcium, but has in addition a more general inhibitory effect on membrane fluidity. Chlorpromazine and trifluoperazine are able to prevent the formation of the calcium-calmodulin complex. Indomethacin is an inhibitor of the synthesis of prostaglandins, and is known to inhibit the action of some toxins (Giugliano & Drasar 1984).

5.2.2 Results and Discussion

The effect of these various inhibitors of the calcium pathway are described below. The points of action of the agents are shown schematically in Figure 5.2.

5.2.2.1 **The Effect of Calcium Channel Blockers**

Verapamil protected Vero cells effectively against both VT1 and VT2 (Table 5.4), suggesting that functioning physiological calcium channels are necessary for cellular intoxication. This protection is probably at the level of toxin entry, as it has been shown that no protection occurred if verapamil was added after internalisation of *C.difficile* toxin B (Caspar *et al* 1987). Sandvig & Brown (1987) reported that Shiga toxin binding is not affected if calcium uptake is interrupted, and the slight decrease in binding of *C.difficile* toxin B in the presence of verapamil was insufficient to account for the protective effects of this drug (Caspar *et al* 1987). It is proposed therefore that verapamil is able to reduce the cytotoxic effect of VT1 and VT2 on Vero cells by blocking transport of the A subunit across the cell membrane. It has been suggested that verapamil may act at vesicular membranes, blocking toxin entry into the cytosol, rather than, or in addition to, at the plasma membrane, which would prevent toxin entry into the cell (Sandvig & Olsnes 1982a). This appears not to be the case for Shiga toxin however (Sandvig & Brown 1987), and since VT1 is closely related to Shiga toxin it is proposed that protection by verapamil against VT1 and VT2, is at the level of toxin entry into the cell.

Lanthanum chloride ($LaCl_3$) is a calcium antagonist, which protects cells against the toxic effects of abrin, modeccin, ricin, diphtheria toxin and *C.difficile* toxin B (Sandvig & Olsnes 1982a, Giugliano & Drasar 1984). No protection was seen against VT1 and VT2 however, either in this study or in a previous study (Giugliano & Drasar 1984). This can be explained by considering the fact that La^{3+} ions interfere with processes other than those dependent on Ca^{2+}, and may have a more general inhibitory effect on membrane permeability. Thus lanthanides protect against ricin and

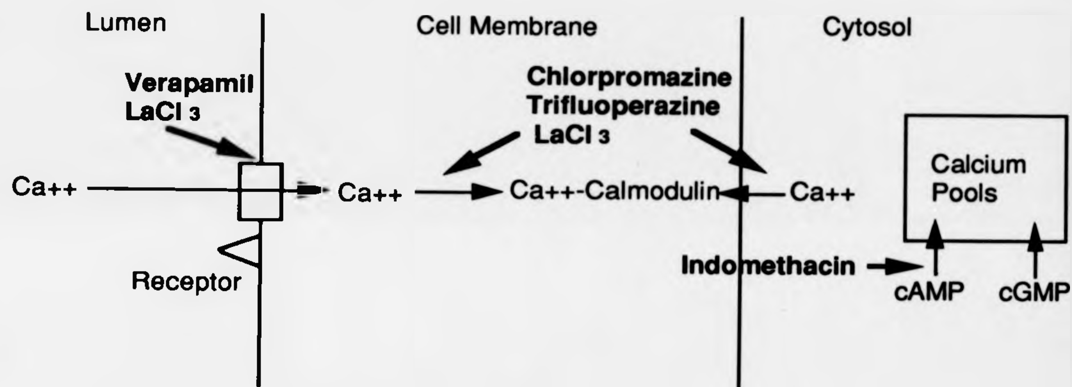
diphtheria toxin, whereas verapamil has no effect (Sandvig & Olsnes 1982a), indicating that the main protective mechanism of La^{3+} is not at the level of the calcium uptake system. This hypothesis is supported by an observation made by Sandvig & Olsnes (1982a) that similar protection was afforded by Fe^{3+}, although this trivalent cation has no effect on the uptake of calcium. Both verapamil and La^{3+} protected against *C.difficile* toxin B but in the latter case protection was only afforded if the cells were pre-incubated with the drug before addition of the toxin (Caspar *et al* 1987). When these observations are considered, the fact that lanthanum chloride did not protect Vero cells from VT1 and VT2, does not contradict the initial conclusion that internalisation of VT1 and VT2 is linked to calcium uptake.

5.2.2.2 The Effect of Inhibitors of the Secretory Mechanism of Cells

Chlorpromazine and indomethacin inhibit the action of several toxins including *E.coli* heat-stable toxin (ST), cholera toxin and *C.difficile* toxin (Greenberg *et al* 1980, Giugliano & Drasar 1984). In this study, neither agent had any effect on cellular intoxication by either VT1 or VT2 (Table 5.4).

E.coli ST and cholera toxin cause disease by stimulation of the secretory mechanism of cells. Indomethacin blocks this activation, and so protects against the toxin. The action of VT is to inhibit protein synthesis, and no activation of the secretory pathway of the cell is involved (Konowalchuk *et al* 1977). Therefore it is not surprising that indomethacin had no effect on the toxicity of VT1 or VT2.

Figure 5.2 The Calcium-Calmodulin Pathway: Points of Action of Inhibitors



5.2.2.3 The Effect of Inhibition of the Calcium-Calmodulin Complex

In addition to blocking the secretory mechanism of mammalian cells, chlorpromazine is able to bind the calcium-binding protein calmodulin. The apparent requirement for functioning calcium channels indicates that the calcium pathway is important in VT uptake, so it was logical to assume that calmodulin may be involved. It was shown however that there was no protection given by chlorpromazine against VT1 or VT2 (Table 5.4). This result, and a similar finding by Giugliano & Drasar (1984) indicated that calmodulin is not involved in toxin activity. Another inhibitor of the calcium-calmodulin complex, trifluoperazine, did inhibit VT activity (Table 5.4), which apparently contradicts the previous observation. Protection by this compound however, is not always related to a requirement for calcium (Sandvig & Olsnes 1982a). Thus, it appears that protection by trifluoperazine is accounted for by the prevention of the clustering of toxin-receptor complexes during endocytosis (Section 5.1.2.3). Resistance to the effects of trifluoperazine can be explained, not in terms of the calcium pathway, but as to whether the toxin is able to bypass the endocytotic pathways blocked by the drug. It appears that the study by Sandvig & Olsnes (1982a), showing no correlation between calcium requirement and protection by trifluoperazine, coupled with the lack of protection by chlorpromazine seen in this study, indicates that calmodulin is not greatly involved in cellular intoxication by VT1 or VT2.

5.2.2.4 The Significance of Extracellular Calcium Levels

Some toxins, such as *Pseudomonas aeruginosa* toxin, are unable to intoxicate cells which are deprived of calcium (Fitzgerald *et al* 1980). Both VT1 and VT2 remained active however, in the presence of 1mM EGTA, a divalent ion chelator (Table 5.4). It is possible that this concentration of EGTA was insufficient to deprive the cells of calcium, but it did show that the toxins were able to intoxicate cells which were depleted of normal levels of calcium. This could either mean that there is sufficient intracellular Ca^{2+} to support entry of the toxins, or that only low levels of Ca^{2+} are necessary for maximum toxic effect.

It was concluded that physiologically functioning calcium channels are necessary for entry of both VT1 and VT2 into Vero cells, and internalisation still occurs if extracellular calcium levels are depleted. There does not seem to be a major role for calmodulin in VT intoxication of Vero cells.

To summarise the findings of this study, it appears that VT are endocytosed via coated pits, but can by-pass this pathway. There is no requirement for exposure to a low pH for toxin activation, but fusion between endosomes and lysosomes is essential. In order for VT to intoxicate cells, however, physiologically functioning calcium channels are necessary. Activity is independent of physiological levels of calcium, however, and the calmodulin complex is not involved.

Table 5.4 Endpoint effect of Inhibitors of the Calcium Pathway on the toxicity of VT1 and VT2 on Vero cells

Inhibitor		**VT1**	**VT2**
No Inhibitor		256	65,536
Verapamil	160µM	4	1024
Lanthanum chloride	462µM	256	65,536
Chlorpromazine	128µM	256	65,536
Indomethacin	128µM	256	65,536
Trifluoperazine	25µM	<4	<256
EGTA	100µM	256	65,536

Culture filtrate of either a VT1-producer (E3787) or a VT2-producer (E32511) was titrated across monolayers of Vero cells. A constant volume (25µl) of each inhibitor was added to each well and after 20-24 hours incubation at 37 °C in 5% CO2, the endpoint was determined as the reciprocal toxin dilution to kill 50% cells.

Chapter 6 Overall Conclusions

In order to investigate the pathogenic mechanisms of VTEC, toxin production, toxin internalisation and intracellular processing, and bacterial attachment were studied.

It was necessary to develop a reliable assay for VT, and three methods were chosen. The Vero cell assay measured toxin activity, whereas the latex agglutination assay and colony ELISA detected the presence of VT antigenically. It was found that the most efficient immunological assay for VT was a colony ELISA, since many strains could be screened on a single agar plate, and it was possible to distinguish between production of VT1 and VT2. Latex agglutination required each organism to be grown separately in liquid culture, and although VT could be detected, it was not possible to distinguish between VT1 and VT2. For these reasons, this assay was not considered suitable for further studies.

The use of two assay systems was beneficial in the characterization of O157:H7 mutants, since the toxin they produced was not altered antigenically, but had little biological activity. The Vero cell assay was also modified to allow toxin internalization to be studied.

VT1 and VT2 are both encoded by lysogenic phages, so a study was made of the effect of mitomycin C, a phage inducer, on toxin production. It was found that production of both VT1 and VT2 appeared to be increased when bacteria were exposed to mitomycin C for at least two hours. At high concentrations, the drug also caused cell lysis, and extracellular phage particles were demonstrated by plaque assay. The specific activity of toxin inside the cells increased in the presence of mitomycin C, although extracellular specific activity levels were lower in mitomycin C-treated cells as a result of bacterial proteins released during cell lysis.

Both toxin production and bacterial attachment to mammalian cells, were investigated through the production of mutants, by chemical mutagenesis, of *E.coli* O157:H7 ATCC 35150, the most common VTEC isolate. It was found that the mutants did not possess the plasmids found in the wild-type, including a 60-MDa plasmid which has been associated with attachment. They also had an altered biochemical profile, and did not react with O157:H7 antiserum in a passive haemagglutination assay. A negative reaction in a latex agglutination assay with anti-O157 revealed that the alteration was in the O side chain of the LPS. Immunoblotting revealed that major outer membrane proteins, but not the LPS, of these mutants reacted with anti-O157:H7, but the LPS did cross-react with antiserum raised against an enteropathogenic *E.coli*, showing that these were not rough mutants. The LPS was therefore purified, and analysis of the neutral sugars showed that the mutants contained rhamnose instead of fucose, which was present in the wild-type. These LPS mutations did not affect the hydrophobicity of these organisms, or their ability to attach to mouse colonic mucus or HeLa cells. This indicates that the 60-MDa plasmid is not significantly involved in attachment.

Toxin levels produced by the mutants were reduced compared to the wild-type, and toxin detected immunologically, showed little cytotoxic activity. No active toxin was detected outside the bacterial cells, and the intracellular toxin activity appeared unstable. In addition to toxin activity, secretion of toxins seemed affected, possibly as a result of the LPS mutations. It was concluded that the toxin genes had been mutated in areas essential for full activity. Sequence analysis of these genes would have provided valuable insight into toxin activity.

This study has indicated that VT enter cells by receptor-mediated endocytosis. Both VT1 and VT2 were inhibited by a metabolic inhibitor of glycolysis (2-deoxyglucose) that prevents endocytosis, but not by two other similar inhibitors (sodium fluoride and 2,4 dinitrophenol) indicating that VT can use an alternative internalization mechanism independent of coated pit formation. This theory is supported by the inhibition of VT by trifluoperazine, which also inhibits both the clustering process of endocytosis and an alternative coated-pit independent mechanism.

Inhibitors of fusion of intracellular vesicles (incubation at 18°C) and transfer of material from endosomes to lysosomes (calcium chloride) inhibited VT showing a requirement for such fusion and an inability of the toxin to pass directly from the endosome into the cytosol.

Cells were protected from VT activity by verapamil, indicating a role for functioning physiological calcium channels. Neither indomethacin nor chlorpromazine protected cells from the toxic effects of VT, although trifluoperazine which acts in a similar way to chlorpromazine was inhibitory. This is probably due to prevention of endocytosis which is also caused by this drug. VT were still able to intoxicate calcium-deprived cells. These findings indicate that physiologically functioning calcium channels are necessary for activity of both VT1 and VT2, but the calmodulin complex is not involved.

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