The pathogenicity of enteroaggregative *Escherichia coli*

Thesis

The pathogenicity of enteroaggregative *Escherichia coli*

by

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ABSTRACT

Strains of enteroaggregative *Escherichia coli* (EAggEC), characterised by their pattern of adhesion to HEp-2 cells known as the 'stacked brick' formation, are a significant cause of chronic diarrhoea in certain under-developed countries. Strains of EAggEC are detected either by a HEp-2 adhesion cell test or by an 'aggregative adhesion' gene probe. The pathogenic mechanisms expressed by EAggEC are only poorly understood and the aim of the research described was to obtain a better understanding of how these bacteria cause disease. The adhesion of EAggEC to HEp-2 cells was shown in the majority of strains not to involve fimbriae and was thought to result from physical properties of strains such as charge, since EAggEC adhered to 'fixed' HEp-2 cells and readily agglutinated a range of different erythrocytes. Certain strains of EAggEC, which also hybridised with a probe for diffuse adhesion, expressed membrane-associated proteins (MAPs) of 18 or 20 kDa responsible for HEp-2 adhesion. Divalent cations were essential for the expression of the MAPs, which did not contain disulphide bonds or have a quaternary structure. Strains of EAggEC did not express recognised subunit toxins such as Verocytotoxin or *E. coli* heat-labile toxin, and strains which hybridised with probes for enteroaggregative heat-stable toxin-1 did not produce *E. coli* heat-stable toxin detected by the infant mouse test. Some EAggEC strains (15%) had haemolytic properties. Certain strains expressed type II capsular polysaccharides and approximately 50% of strains expressed an aerobactin-mediated iron uptake system. It was concluded that strains of EAggEC belonged to a very diverse range of serotypes, and it was thought that this heterogeneity resulted from strains of *E. coli* readily acquiring the genes encoding the EAggEC phenotype. Strains of EAggEC were not associated with a single pathogenic phenotype and the ability of these bacteria to adhere to HEp-2 cells in a 'stacked brick' pattern remains the only common characteristic.

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DECLARATION

No part of this thesis has been submitted in support of an application for any degree or qualification of any other university or institute of learning. The work of this thesis was performed by myself unless otherwise stated in the text.

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PUBLICATIONS

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1. ABBREVIATIONS AND LISTS

1.1. Symbols and abbreviations

AA	aggregative adherence
AAF	aggregative adherence fimbriae
A/E	attaching and effacing
AFA	afimbrial adhesin
bfp	bundle forming pili
bp	base pair
CF	colonisation factor
CFA	colonisation factor antigen
CLDT	cytolethal distending toxin
cm	centimetre
CNF	cytotoxic necrotising factor
CRP	catabolite repressor protein
CS	coli surface antigen
CT	cholera toxin
DA	diffuse adherence
DAEC	diffusely adherent *Escherichia coli*
DAF	decay accelerating factor
DNA	deoxyribonucleic acid
eae	*E. coli* attaching and effacing
EAF	effacing and attaching factor
EAggEC	enteroaggregative *Escherichia coli*
EAST1	enteroaggregative *E. coli* heat stable enterotoxin 1
EF	elongation factor
EHly	enterohaemolysin
EIEC	enteroinvasive *Escherichia coli*
EM	electronmicroscopy
EPEC	enteropathogenic *Escherichia coli*

Esp	*E. coli* secreted protein
ETEC	enterotoxigenic *Escherichia coli*
g	gram
g	standard acceleration of gravity
H-antigen	flagellar antigen
HeLa	Human cervical cancer cell-line
HEp-2 cells	Human epithelial cells
HIC	hydrophobic interaction chromatography
HIV	human immuno- deficiency virus
Hly	haemolysin
H-NS	histone-like protein
Hp90	host protein (90 kDa)
hr	hours
HUS	haemolytic uraemic syndrome
IHF	integration host factor
IID	Intestinal infectious disease study
IROMP	iron-regulated outer membrane protein
K	capsular antigen
K12	*Escherichia coli* K12
kDa	kilo Dalton
l	litre
LA	localised adherence
LEE	locus of enterocyte effacement
LEP	Laboratory of Enteric Pathogens
LPS	lipopolysaccharide
Lrp	leucine responsive protein
LT	heat-labile enterotoxin
M	moles
MAP	membrane-associated protein
MDa	Mega Dalton
min	minutes
MR	mannose resistant

MRHA	mannose-resistant haemagglutination
MSHA	mannose-sensitive haemagglutination
n	nano (metre, gram)
NCP	nitrocellulose paper
-antigen	somatic antigen or LPS
OD	optical density
OM	outer membrane
OMP	outer membrane protein
ORF	open reading frame
pap	pyelonephritis-associated pili
PCF	putative colonisation factor
PCR	polymerase chain reaction
Pet	plasmid encoded toxin
pI	isoelectric point
rpm	revolutions per minute
RTX	repeats in toxin
s	seconds
SAT	salt aggregation test
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLT	Shiga-like toxin
spp.	species
ST	heat-stable enterotoxin
STEC	Shiga-like toxin producing *E. coli*
Tir	translocated intimin receptor
TS	tris-succinate
µ	micro (litre, gram)
UTI	urinary tract infection
UV	ultra violet
V	volts
Vero cells	African Green Monkey kidney cells
VT	Verocytotoxin
VTEC	Verocytotoxin-producing *Escherichia coli*

VTe	Verocytotoxin - porcine variant (e for oedema disease)
v/v	volume per volume
WHO	World Health Organisation
w/v	weight per volume
Y1	mouse adrenal cells

1.2. List of chemicals

BCIP	5-bromo-4-chloro-3indoylphosphate
BPB	bromophenol blue
BSA	bovine serum albumin
CTAB	cetyltrimethylammonium bromide
EDDA	ethylenediaminedihydroxy-*o*-phenylacetic acid
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene-glycol-bis(β-amino-ether)tetra-acetic-acid
HCl	hydrochloric acid
KCl	potassium chloride
LiCl	lithium chloride
$MgCl_2.6H_2O$	magnesium chloride hydrated
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
PBS	phosphate buffered saline
RNAase	ribonuclease
SDS	sodium dodecyl sulphate
Tris	tris[hydroxymethyl]aminomethane

Other chemicals and solutions

absolute alcohol

agarose

anti-rabbit IgG- alkaline phosphatase

chloroform

ethanol

ethidium bromide

formaldehyde

isoamyl alcohol

isopropanol

lysozyme

phenol

salmon sperm DNA

Sarkosyl

Tween 20

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2. INTRODUCTION

2.1. *Escherichia coli*

In 1885, Theodore Escherich isolated a bacterium from the faeces of infants and named it *Bacterium coli commune* (Escherich 1885), this organism was subsequently renamed *Escherichia coli*. At one time strains of *E. coli* were thought to be non-pathogenic and carried in the gut as part of the commensal bacterial flora; however, in 1894 Escherich found that *E. coli* was present in the urine of a young girl with a urinary tract infection. To date the majority of *E. coli* strains are thought to be non-pathogenic. However, over the last 100 years *E. coli* has emerged as an important pathogen causing urinary tract infection, wound infection, pneumonia, meningitis, peritonitis and septicaemia (Levine 1985a).

It was not until the early 1940s that *E. coli* was implicated in a case of gastroenteritis following the isolation of *E. coli* from a fatal case of infantile protracted diarrhoea (Bray 1945). Since the 1960's, *E. coli* has emerged as a major pathogen, taking many forms and variation in pathogenicity.

2.2. Taxonomy and classification

2.2.1. Definition of *Escherichia coli*

Strains of *Escherichia coli* belong to the family of Enterobacteriaceae (Fig 1), which are Gram-negative facultative anaerobes defined as straight rod-shaped bacteria, 1 to 1.5 μm in length. Strains of *E. coli* may be motile by peritrichous flagella and may express a capsule. The optimal growth temperature for strains of *E. coli* is 37°C and it is the most common enteric organism isolated from an aerobic human stool culture. *E. coli* are non-spore forming and in general ferment lactose with the formation of gas. Certain strains of *E. coli* express fimbriae. These surface structures have also been termed pili. For the sake of clarity hair-like structures involved with adhesion will be termed fimbriae; however, when quoting the published literature the term pili will also be used.

Table 1 *Principal characteristics and reactions of Escherichia coli*

Mole % G + C	48-52%
Optimum growth temperature	37°C
Indole production	+
Methyl red reaction	+
Voges Proskauer reaction	-
Citrate utilisation	-
90-100% of strains:-	
Utilise glucose (mixed acid + gas); lactose; D-mannitol; D-mannose; D-sorbitol;	
L-arabinose; maltose; D-xylose; trehalose; mucate;	
Reduce nitrate to nitrite;	
Express β-galactosidase	
76-89% of strains:-	
Express lysine decarboxylase;	
Are motile;	
Utilise L-rhamnose; melibiose	
26-75% of strains:-	
Express orthinine decarboxylase;	
Utilise sucrose; dulcitol; salicin; raffinose;	
hydrolyse aesculin	
11-25% of strains express:-	
Arginine dihydrolase	
0-11% of strains:-	
Produce H_2S; urease; phenylalanine deaminase;	
Liquefy gelatine;	
Grow in presence of KCN;	
Utilise malonate; D-adonitol; *myo*-inositol; cellobiose; α-methyl-D-glucoside;	
D-arabitol;	
Express lipase; DNAase; pigments	

Fig 1 *The Family Enterobacteriaceae*

Group	Escherichiae	Klebsiellae	Proteae	Yersiniae	Erwiniae
Genera	Escherichia	Klebsiella	Proteus	Yersinia	Erwinia
	Salmonella	Enterobacter			
	Shigella	Hafnia			
	Edwardsiella	Serratia			
	Citrobacter				
Species	*Escherichia coli*				
	E. hermannii				
	E. blattae				
	E. vulneris				
	E. fergusonii				
	E. alkalescens				
	E. dispar				

2.2.2. Characterisation of *Escherichia coli*

The development of a serotyping system by Kauffmann in 1947 provided a scheme for categorising strains of *E. coli* based on their antigenic structures. Initially, two *E. coli* surface components formed the basis for the serological classification system: The flagellar or H antigens (from the german word 'Hauch', meaning mist) and the somatic, lipopolysaccharide or O antigens (Ohne hauch, without mist). Currently, approximately 173 O serogroups and 56 H serogroups (Ørskov and Ørskov, 1992) have been identified and form the basis of the serotyping scheme further developed by the Laboratory of Enteric Pathogens (Gross and Rowe 1985a). Certain strains of *E. coli* express a capsular antigen, which forms the basis for the K (German, Kapsule) serotyping scheme (Gross and Rowe 1985a). Although strains may not react with a current serotyping scheme, the scope for expanding a given serotyping scheme does exist.

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The isolation and serotyping of strains of *E. coli* has enabled the categorisation of these organisms, and a general association between serotype and pathogenicity has emerged. Certain strains of *E. coli* cause extra-intestinal infections such as meningitis and pyelonephritis (Johnson 1991). Strains causing meningitis have been associated with the expression of K1 capsular antigens and strains isolated from urinary tract infections have been associated with the expression of specific fimbriae such as P-pili; however, both groups of these *E. coli* have been associated with the production of the siderophore aerobactin (Johnson 1991). Although the classification of diarrhoeagenic strains of *E. coli* is not absolute, the majority of strains can be placed in one or more of the following six groups:-

2.2.3. Enterotoxigenic *E. coli* (ETEC)

Strains of enterotoxigenic *E. coli* resemble strains of *Vibrio cholerae* in that they adhere to the small intestinal mucosa and produce cholera-like toxins, causing copious volumes of watery diarrhoea (Sussman 1985). ETEC also produce a family of heat-stable toxins (2.3.2.) and colonisation factor antigens which are involved in adhesion (2.3.6.b.). In common with the symptoms of cholera, there are no apparent histological changes in the host gut mucosal cells and little or no inflammation. The disease caused by ETEC is similar to cholera although the disease is usually less severe. The principal serogroups associated with ETEC infections are: O6, O8, O15, O20, O25, O27, O63, O78, O115, O148, O153, O159 and O167 (Gross and Rowe 1985b).

ETEC diarrhoea is a major cause of mortality among young children in developing countries. ETEC may also cause diarrhoeal disease in adults, although in countries where ETEC are endemic, where the adult population may develop at least partial immunity to infection. In contrast, travellers from countries were ETEC infections are rare may be highly susceptible to these infections and ETEC diarrhoea may account for 30-50% of cases of traveller's diarrhoea (Gross and Rowe 1985b; 1987; Doyle 1990; Gyles 1992). The first outbreak of infantile diarrhoea in the UK caused by ETEC was reported in 1976 involving a hospital nursery in Glasgow (Gross *et al.* 1976). Although ETEC infections can be fatal, especially in infants and young children, patients frequently respond to treatment involving rehydration and the replacement of essential mineral ions.

Young domestic animals e.g. neonatal calves, lambs and weaned pigs are also susceptible to ETEC infection and isolations have been made from dogs and chickens (Gyles 1992). The most frequent sources of infection are contaminated food or water, and person to person spread. In developing countries, ETEC are often present in the faeces of asymptomatic human carriers.

The symptoms of ETEC diarrhoea are caused by one or more of a family of heat-labile (LT) and heat-stable (ST) toxins. LTs, which resemble cholera toxin in both structure and mode of action, will be described in detail in section 2.3.1. STs will be described in section 2.3.2.

2.2.4. Enteroinvasive *E. coli* (EIEC)

Enteroinvasive strains of *E. coli* (EIEC) cause disease with symptoms resembling dysentery caused by *Shigella spp.*, although strains of *Sh. dysenteriae* 1, which produce Shiga toxin, cause a more severe disease than other species of Shigella. Strains of EIEC enter the human body by the oral route and adhere to the colonic mucosa. Following colonisation, EIEC invade the colonic epithelium and spread laterally to adjacent cells. The invasive phenotype has been associated with the carriage of a large (140 MDa) plasmid encoding the production of several outer membrane proteins involved in the adhesion and invasion process (Harris *et al.* 1982). The 140 MDa plasmid has been termed *pInv* and has remarkable similarity to a plasmid carried by strains of *Sh. flexneri* and other Shigellas. Chromosomal locations have also been mapped which affect penetration and multiplication of these bacteria in epithelial cells (Hale 1991).

EIEC strains do not produce Vero-cytotoxin (2.2.6.) but a single EIEC strain was described as producing a VT-like cytotoxin (O'Brien *et al.* 1982) followed by a report that all common EIEC serogroups produced low-level cytotoxic activity. This activity was 100-1000 fold lower than a strain of *Sh. dysenteriae* 1 that produced Shiga toxin. This effect was not neutralised by antiserum to Shiga toxin or VT2, and VT genes were not detected (Smith *et al.* 1987; Cleary and Murray 1988). Fluid accumulation was observed in rabbit ileal loops without tissue damage when supernatants of the cell lysates were added. Curing strains of the *pInv* plasmid did not diminish enterotoxin activity, suggesting that the structural and regulatory genes were not found on the plasmid.

The molecular weight of this enterotoxin was 60-80 kDa and expression was dependent on minimal iron medium (Fasano *et al.* 1990).

Patients infected with EIEC may experience fever, severe abdominal cramps, malaise and produce watery diarrhoea or bloody diarrhoea, which may contain mucus (Gross and Rowe 1985b; Levine 1987a). The predominant serogroups associated with infection are O28ac, O112ac, O124, O136, O143, O144, O152 and O164 which are serogroups not usually associated with, for example, ETEC or EPEC (Gross and Rowe 1985b). In the UK, infection with EIEC is rare but may occur in travellers visiting countries where EIEC are endemic. Sporadic outbreaks have been reported; for example, an outbreak in America affected 387 individuals and was associated with the consumption of Brie and Camembert cheeses which had been contaminated with water carrying EIEC (Marier *et al.* 1973).

2.2.5. Enteropathogenic *E. coli* (EPEC)

Strains of enteropathogenic *E. coli* came into prominence in the 1940s, when strains belonging to serogroups O55 and O111 were recognised as a cause of outbreaks of infantile gastroenteritis in the UK. Subsequent outbreaks of EPEC diarrhoea occurred in other parts of Europe and North America in the 1950's. In the 1970s there was uncertainty about the pathogenicity of EPEC; however, volunteer studies carried out at the Centre for Vaccine Development, Maryland USA, showed that volunteers challenged with EPEC strains belonging to serotypes O127:H6 and O142:H6 developed diarrhoea, therefore the interest in

the virulence of EPEC was renewed (Levine *et al.* 1978). During the 1960s and 1970s there continued to be outbreaks of EPEC diarrhoea in Britain and Ireland; however, since 1971 the incidence of EPEC infections has reduced significantly (Gross and Rowe 1985b).

Infections with EPEC involve diarrhoea which may be severe, and can last more than 14 days (Levine 1987a). The group of *E. coli* categorised as EPEC, has been formed based on the association of strains belonging to certain serogroups which have historical links with infantile diarrhoea. More recently, strains have been categorised further based on the pattern by which they adhere to tissue culture cells, such as HEp-2, and probe tests have also been developed to differentiate EPEC from other diarrhoeagenic *E. coli.* The pattern of adhesion is termed as localised adherence (LA) where the bacteria adhere in discrete 'nests' to the cultured cells.

The main serogroups associated with classical EPEC infections are : O26, O44, O55, O86, O111, O114, O119, O125, O126, O127, O128 and O142 (Gross and Rowe 1985a, b). EPEC infections are common in developing countries where illness is often fatal in children. In South America, EPEC are one of the most important causes of diarrhoea.

In 1979, it was reported that 80% of certain EPEC outbreak strains adhered to HEp-2 cells in a localised manner suggesting the existence of a new, uncharacterised adhesin (Cravioto *et al.* 1979) which did not involve fimbriae (Scotland *et al.* 1983a). Cravioto *et al.* (1979) demonstrated that some 40 of 51 EPEC strains isolated from outbreaks showed mannose-resistant adhesion to HEp-2 cells, whereas normal flora *E. coli* isolates adhered rarely. The absence of

fimbriae on the surface of some adherent EPEC strains (Andrade and de Santa Rosa 1986; Scotland *et al.* 1983a) drew attention to other candidate proteins such as the 94 kDa outer membrane protein, identified as an antigen during volunteer studies (Levine *et al.* 1985b).

The role of fimbriae in the adhesion of EPEC to HEp-2 cells was examined further and fimbriae were observed for a small number of strains. Knutton *et al.* (1987c) detected fimbrial structures by electron microscopy which were 7nm in diameter mediating adhesion between strains of EPEC and the HEp-2 cells. Giron *et al.* (1991) detected fimbrial structures after culturing an EPEC strain, serotype O111:H- onto sheep blood agar, and these fimbriae were seen to aggregate to form bundles that were intertwined forming a three-dimensional mesh in which individual bacteria were embedded. These fimbrial structures were termed bundle forming pili (bfp). Culture conditions play an important role in the expression of bfp, with expression being repressed in conventional bacteriological media but induced by growth on sheep blood agar (Giron *et al.* 1991) or in cell culture medium (Vuopio-Varkila and Schoolnik 1991).

2.2.6. Verocytotoxin-producing *E. coli* (VTEC)

In 1977, Konowalchuk described strains of *E. coli* which produced a secreted toxin cytotoxic for Vero cells (African Green Monkey Kidney cells), and termed Verocytotoxin (VT) (Konowalchuk *et al.* 1977; 2.3.3.). This group of *E. coli* was classified as Verocytotoxin-producing *E. coli* or VTEC. O'Brien *et al.* (1982) demonstrated that VT was structurally similar to Shiga toxin, and these organisms were also referred to as Shiga-toxin or Shiga-like-toxin-producing *E. coli* (STEC). VTEC belong to many different O serogroups, but those belonging to O157 appear to be the most important in human disease. In man, VTEC infections are associated with a wide spectrum of symptoms from a mild non-bloody diarrhoea to a severe bloody diarrhoea or haemorrhagic colitis, and in some patients there is a progression to haemolytic uraemic syndrome.

The clinical and public health significance of VTEC O157 was first recognised by an extensive community outbreak of diarrhoea in 1982 associated with a fast food chain in the United States (Riley *et al.* 1983). Subsequently, VTEC O157 was shown to cause outbreaks of gastroenteritis in various parts of the world, particularly the USA and the UK (Table 3).

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There is considerable evidence of food-borne classically associated with ground beef, but also with other meat products (Boyce *et al.* 1995; Armstrong *et al.* 1996; WHO report 1997; Sharp *et al.* 1994), raw milk (Martin *et al.* 1986) and milk products such as yoghurt (Morgan *et al.* 1993) and cheese (Sharp *et al.* 1995). Other foodstuffs include cured and fermented meat products (CDC report 1995), non-fermented apple cider (Besser *et al.* 1993), raw vegetables and salads (WHO report 1997). Although most cases of VTEC O157 infection have an animal link (usually bovine), not all infections are associated directly with cattle (Chart 1998a). However, there has been evidence of environmental contamination via animal by-products, e.g. sewage contamination of pasture lands or of drinking water supplies (Swerdlow *et al.* 1992), particularly in rural communities (Dev *et al.* 1991).

Accumulating evidence suggested that the major reservoir for VTEC O157 was farm animals, and in particular cattle. Veterinary isolations of a variety of serotypes of *E. coli* some of which are Verocytotoxigenic, are not uncommon and may produce symptoms in young animals, for example VTEC O26. However, it appears that although VTEC O157 may be found in the gut of a range of apparently healthy animals, including, cattle, sheep, goats and deer, these animals appear not show any symptoms of disease. Cattle surveys in North America have shown that around 4% of herds were positive for VTEC O157 at any given time (WHO report 1997; Hancock *et al.* 1997). In the UK, VTEC O157 have been detected in cattle (15.7%), sheep (2.2%) and pigs (0.4%) at slaughter (Chapman *et al.* 1997).

2.2.7. Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* (DAEC) are characterised by the pattern by which they attach to HEp-2 cells in a diffuse manner during tissue culture tests *in vitro* (Scaletsky *et al.* 1984; Haider *et al.* 1992). DAEC lack major EPEC virulence properties although a few DAEC belong to EPEC O serogroups. As yet, there is not a significant association between DA and disease. Strains of DAEC have been isolated from patients with diarrhoea; however, volunteers receiving DAEC did not develop diarrhoea (Tacket *et al.* 1990). In a study of 72 children in Mexico, a second pathogen was isolated from 78% of those with DAEC associated diarrhoea (Cravioto *et al.* 1991).

Diffuse adherence is mediated by an adhesin genetically and phenotypically distinct from that associated with the localised adherence process (Nataro *et al.* 1995). The presence of a fimbrial adhesin F1845 encoded by at least four chromosomal genes *daaA, B, C,* and *E* have been demonstrated (Bilge *et al.* 1989). The gene *daaE,* encoding the 14.3 kDa fimbrial protein, was detected in 80% of 45 DAEC using an oligonucleotide DNA probe specific for the 5' end of *daaE.* A 100 kDa plasmid encoded adhesin, has also been described (Benz and Schmidt 1992).

2.3. Pathogenicity Determinants

The terms pathogenicity and virulence are often used interchangeably; however, pathogenicity is the ability of an organism to cause disease and the virulence is a measure of the pathogenicity. For certain types of bacterial infection, such as those where a secreted toxin is involved, a measure of virulence can be obtained by determining a lethal-dose-50 (LD_{50}) where the inoculum required to kill 50% of test animals can be determined. But in general, animal models and LD-50 determinations have been of little value in studying human diarrhoeal diseases.

The study of bacterial pathogenicity has also been influenced by a set of postulates formulated by Robert Koch in the 1800s, which state that:-

1) The bacterium should be found in all persons with the disease, and the bacterium or its products should be found in parts of the body affected by the disease.

2) The bacterium should be isolated from the lesions of an infected person and maintained in pure culture.

3) The pure culture, inoculated into a susceptible human volunteer or experimental animal, should produce the symptoms of disease.

4) The same bacterium should be reisolated in pure culture from the intentionally infected animal or human.

Koch's postulates were originally developed for identifying the bacterial causes of epidemic diseases e.g. cholera and tuberculosis. In light of new insights into host-bacterium interactions, some of the assumptions implicit in Koch's postulates are now being questioned therefore a molecular version of Koch's postulates has been drawn up.

1) The gene or its product should be found in strains of bacteria that cause disease and not in bacteria that are avirulent.

2) Disruption of the gene in a virulent strain should reduce its virulence or alternatively introducing the cloned gene into an avirulent strain should render the strain virulent.

3) It should be demonstrated that the gene is expressed by the bacterium when it is in an animal or human volunteer at some point during the infectious process.

4) Antibodies to the gene product should be protective or, in cases where a cell mediated rather than antibody response is appropriate, the gene product should elicit protective immunity.

Koch's postulates need to be borne in mind when studying diarrhoeagenic diseases; however, in the absence of good animal models this can be problematical.

In general, the ability of a bacterium to cause disease depends on the ability to enter the host and survive the host's defence mechanisms. Disease-causing bacteria can be introduced into the body through the skin, such as via a wound (e.g. *Clostridium tetani)* or an insect bite (*Yersinia pestis*). Bacteria may enter a host via the mucosal membranes that line interior parts of the body exposed to the environment, nasal membrane, throat etc. Certain bacteria may colonise the gut mucosa and cause disease (for example, *V. cholerae*); whilst others penetrate the gut (e.g. *S. typhi).*

The mucous membranes line the gastrointestinal tract, respiratory tract and the genitourinary tract. These membranes are generally located within the host body, but some are 'outside' in the sense that they are exposed to the external environment in the

form of air, food and water. A thick layer of mucus protects the delicate mucosal membranes from damage by food or other particulate matter. It can also trap invading bacteria before they reach the membrane itself. Mucus also contains secretory immunoglobulin A (sIgA) which helps trap the bacteria - the mucus can then be expelled by peristalsis (gastrointestinal tract) or by ciliated cells that propel it away from the site (respiratory tract, fallopian tubes). Mucus also contains antibacterial agents e.g. lysozyme, lactoferrin and lactoperoxidase. Lactoferrin has a high affinity for iron meaning that there is no free iron for the requirements of the pathogenic bacteria. Lactoperoxidase is an enzyme that produces superoxide ions, a reactive form of oxygen which is toxic to many bacteria.

The intestinal tract is protected from pathogens in several ways. The acidic environment of the stomach and the proteolytic enzymes secreted by gastric cells kill many of the bacteria that are swallowed. Bile salts are toxic to some types of bacteria. The constant flow of material through the small intestine constantly washes bacteria out therefore as a result of the combinations above the small intestine is relatively free of bacteria. In the colon, the flow rate of contents is very slow, and bacterial density is high. The colonic mucosa is protected by the resident microflora that competes with invading pathogens for nutrients and attachment sites. These resident microflora are a complex community consisting of Gram- negative and Gram-positive organisms, of which more than 97 % are obligate anaerobes.

Since colonisation is usually the first step to infection some of the virulence factors promoting this are listed (Table 4).

Table 4 *Virulence factors that promote colonisation and survival of invading bacteria*

Virulence Factor	**Function**
Fimbriae	Adherence to mucosal surfaces
Non-fimbrial adhesins	Binding to host cells
Bacterial triggering of actin rearrangement in host cells	Forced phagocytosis of bacteria by normally non-phagocytic cells; movement of bacteria within host cells from one host cell to another
Motility and chemotaxis	Reaching mucosal surfaces
sIgA proteases	Prevent trapping of bacteria in mucin
Siderophores	Iron acquisition
Capsules	Prevent phagocytic uptake; reduce complement activation
Toxic proteins	Kill phagocytes; reduce strength of oxidative burst
Variation in surface antigens	Evade antibody response

Other virulence factors of pathogenic bacteria include the production of toxins that can damage the host. Many bacteria excrete toxins whilst growing or are released during bacterial lysis. Toxins can be divided into groups on the basis of their structure and activities:-

1) A-B toxin - Subunit toxin where the A subunit mediates the enzymatic activity responsible for the toxicity. The B subunit(s) are responsible for the binding of the toxin to the target cell. The host cell receptor recognised by the B subunit is usually, but not always, a host cell surface carbohydrate.

2) Cytolysins - no separable subunits but act by disorganising host cell membranes e.g. haemolysins, phospholipases

3) Peptide toxins - no separable subunits, heat-stable due to small molecular size

Many pathogens produce hydrolytic enzymes e.g. hyaluronidase and proteases that degrade extracellular matrix components and this disrupts host tissue structure. Other enzymes such as DNAases reduce the viscosity of debris from dead host cells and thus may aid in the spread of bacteria where extensive damage has been done to host cells. Hydrolytic enzymes also provide bacteria with sources of carbon and energy by breaking host polymers into usable low molecular weight sugars and amino acids.

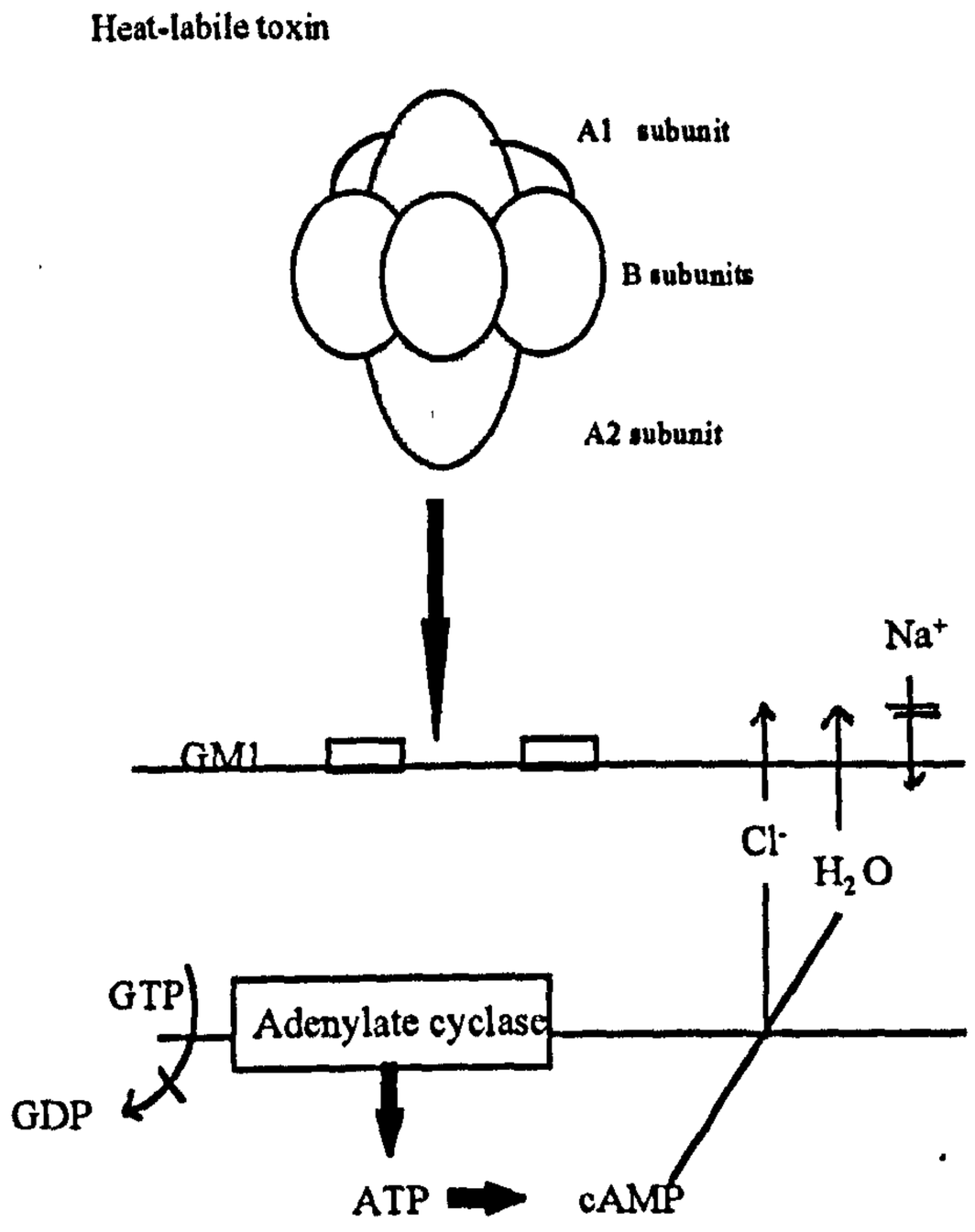
Strains of *E. coli* are able to express a considerable range of pathogenic mechanisms, including toxins and both fimbrial and non-fimbrial adhesins. This section describes the current knowledge of pathogenic mechanisms associated with strains of *E. coli*.

2.3.1. Heat-labile toxins

Certain strains of *E. coli* express a heat-labile toxin (LT) which resembles cholera toxin in both structure and mode of action, and these strains have been termed the enterotoxigenic *E. coli* or ETEC. These strains may express LT only or both LT and a heat-stable toxin or the heat-stable toxin only (see 2.3.2.). There are essentially two forms of LT: LT-I and LT-II.

LTs are classified as subunit toxins comprising two distinct regions of the toxin molecule (A and B). LT belongs to a family of subunit toxins where the B subunit(s) of the toxin is involved with binding to the host mucosa and acts like a delivery system for the A subunit which has the cytotoxic or cytotonic properties. For LT, the delivery system comprises 5 copies of a B-subunit and a single A-subunit and it is the action of heating that causes the A and B subunits to denature causing the LT to become inactive.

Fig. 2 Schematic of heat-labile toxin and the mode of action on intestinal cells



LT binds to the GM1 ganglioside receptors in the surface of the cell membrane. The A1 fragment interacts with the adenylate cyclase complex, located on the basal membrane, inhibiting the hydrolysis of GTP to GDP. The adenylate cyclase complex remains active producing cAMP causing the efflux of Cl^- ions and H_2O into the ileum.

2.3.1.a. LT-I

LT-I shares a high degree of amino acid sequence identity with cholera toxin i.e. consisting of 5 identical B subunits (11.5 kDa) and one A subunit (26 kDa). The enzymatic activity resides in the catalytic A subunit, while the 5 B subunits mediate binding of the toxin to intestinal epithelial cells and are primarily responsible for its immunogenicity. The A subunit undergoes post-translational processing to generate two peptides, A1 and A2, that are linked to each other by a single disulphide bond (Lai *et al.*

1976; Gill and Richardson 1980). The A1 peptide possesses the enzymatic activity, while the A2 peptide is thought to function in toxin internalisation into the target cell. This toxin also has the same mechanism as cholera toxin which raises host cell cAMP by the ADP-ribosylation of cellular nicotinamide adenine dinucleotide (Mashiter *et al.* 1973). The B subunits also recognise the same receptor (GM1) (Holmgren 1973). LT can also bind to GM2 (asiolo-GM1) and to glycoproteins (Griffiths *et al.* 1986). The structural genes for LT-I are carried on plasmids (Gyles *et al.* 1974). These plasmids may also contain the genes for *E. coli* heat-stable toxin and many of the colonisation factors involved in the adhesion of the bacteria to mucosal surfaces.

Comparing LTs expressed by strains of *E. coli* from different sources showed that porcine strains expressed an antigenically different LT to strains of ETEC isolated from humans infections. A panel of monoclonal antibodies was used to show that LT-Ip (porcine) and LT-Ih (human) were not antigenically identical (Svennerholm *et al.* 1986). This variation in protein structure was subsequently confirmed by protein sequencing (Betley *et al.* 1986). A third type of LT was detected in a strain of ETEC isolated from a chicken (LT-Ic), and was shown to express a A-subunit identical to LT-Ip and a B-subunit the same as LT-Ih (Tsuji *et al.* 1988).

Strains of *E. coli* expressing LT were initially identified when sterile culture supernatants were shown to cause fluid accumulation in the rabbit ileal loop test (Sack *et al.* 1971). The observation that LT caused morphological changes in cultured cell lines, such as Y1 cells, replaced the classical rabbit ligated loop model (Sack and Sack 1975).

2.3.1.b. LT-II

LT-II has the same basic structure and mechanism of action as LT-I, but is different in respect of its immunoreactivity and ganglioside-binding activity (Pickett *et al.* 1987). LT-II has a greater affinity to ganglioside GD_{1b} (Fukuta *et al.* 1988). The first strain to express LT-II was isolated from a water buffalo (Green *et al.* 1983; Holmes *et al.* 1986). Subsequently, two forms of LT-II have been detected, termed LT-IIa and LT-IIb (Guth *et al.* 1986) and both toxin molecules comprised A and B subunits of similar size (Guth *et al.* 1986). Although the mode of action appears to be the same, the pI of LT-IIa is higher (pI = 6.8) than LT-IIb (pI = 5.4). So far LT-II has been found primarily in animal strains and has not been associated with disease either in animals or humans. The genes encoding for LT-II are chromosomal (Green *et al.* 1983) unlike LT-I which are carried on a plasmid.

Table 5. *Variants of E. coli heat-labile toxins and their various features*

	LT-I			LT-II	
Variants	**LTp**	**LTh**	**LTc**	**LT-IIa**	**LT-IIb**
Cell changes					
to CHO cells	+	+			
Y1 cells	+	+			
Vero cells	+	+			
Molecular size (kDa)					
A subunit	26			28	
Bsubunit	11.5			11.8	
pI		8.5	8.5	6.8	5.4
Genes encoded on:	plasmid			chromosome	

2.3.2. Heat-stable toxins

Certain strains of *E. coli* express a heat-stable toxin (ST), and also belong to the group of enterotoxigenic *E. coli* or ETEC. Strains of ETEC may express ST only or may express both ST and LT or LT only (2.3.1.). In contrast to heat-labile toxin, ST is not a subunit toxin and comprises a heat-stable single protein molecule probably due to it's lack of a subunit structure. The genes encoding ST are usually carried on plasmids which also carry the genes encoding colonisation factor antigens.

Table 6. *Variants of E. coli heat-stable toxins and their properties*

	STa (STI)	STb (STII)
Variants	STp (ST1a) STh (STIb)	
Molecular size (kDa)	2	5
Infant Mouse Test	+	-
Methanol	soluble	insoluble
Pig intestinal loop	+	+
Rabbit ileal loop	+	-
Rat gut loop	+	-

Different forms of ST have been detected which are either methanol soluble (STa) or methanol insoluble (STb) (Burgess *et al.* 1978). These toxins induce secretory diarrhoea in humans and animals. The toxic activity of STa is resistant to the protease trypsin (Staples *et al.* 1980), while that of STb is inactivated by trypsin (Whipp 1987).

2.3.2.a. STa

STa has two recognised forms: STa1 (also termed STp or STIa) and STa2 (also termed STh or STIb). The abbreviation STp indicates that this toxin was originally isolated from pigs. Similarly, STh toxin has been associated with strains of *E. coli* isolated from human infections (Hill *et al.* 1985; Moseley *et al.* 1982)

The STa family consists of at least eight distinct types of toxin molecule, with a common highly conserved region comprising ten amino acids. This includes six cysteine residues, located in the same relative positions and linked intramolecularly by three disulphide bonds, which suggests that these enterotoxins have similar tertiary structures (Shimonishi *et al.* 1987), and the mode of action and heat-stable properties of the STa enterotoxins have been attributed to this conserved core sequence (Shimonishi *et al.* 1987).

Heat-stable toxins are secreted in a series of steps which alter the length of the peptide. In the case of STa, the precursor toxin (approx. 72 amino acids) is first secreted into the periplasm, where it becomes nicked and reduced to 8 amino acids and the resulting 54-amino acid peptide (Rasheed *et al.* 1990) is transported out of the bacterial cell, where it is cleaved to its final 17 to 19 amino acid form by a process that is not well understood. A similar set of steps is involved in the expression of STb.

The first step in the biological action of STa's is their interaction with specific high-affinity receptors. STa causes an increase in cyclic GMP (guanosine 3',5'-cyclic monophosphate) level in the host cell cytoplasm when bound to the epithelial cell membranes of the host (Dreyfus and Robertson 1984). Activation of a cGMP protein

kinase follows this (de Jonge and Lohhmann 1985; Hirayama *et al.* 1989) which inhibits Na^+ absorption and stimulation of Cl^- secretion (Field *et al.* 1978). The result is fluid loss from the host cells. The effect of STa seems to be tissue specific.

Classically, STa was detected by the infant mouse test (Giannella 1976) but this test does not detect STb (Whipp 1987; Kennedy *et al.* 1984). Heat-stable toxin causes fluid accumulation in the suckling mouse even after heating at 100 °C for 30 minutes (Smith and Gyles 1970).

2.3.2.b. STb

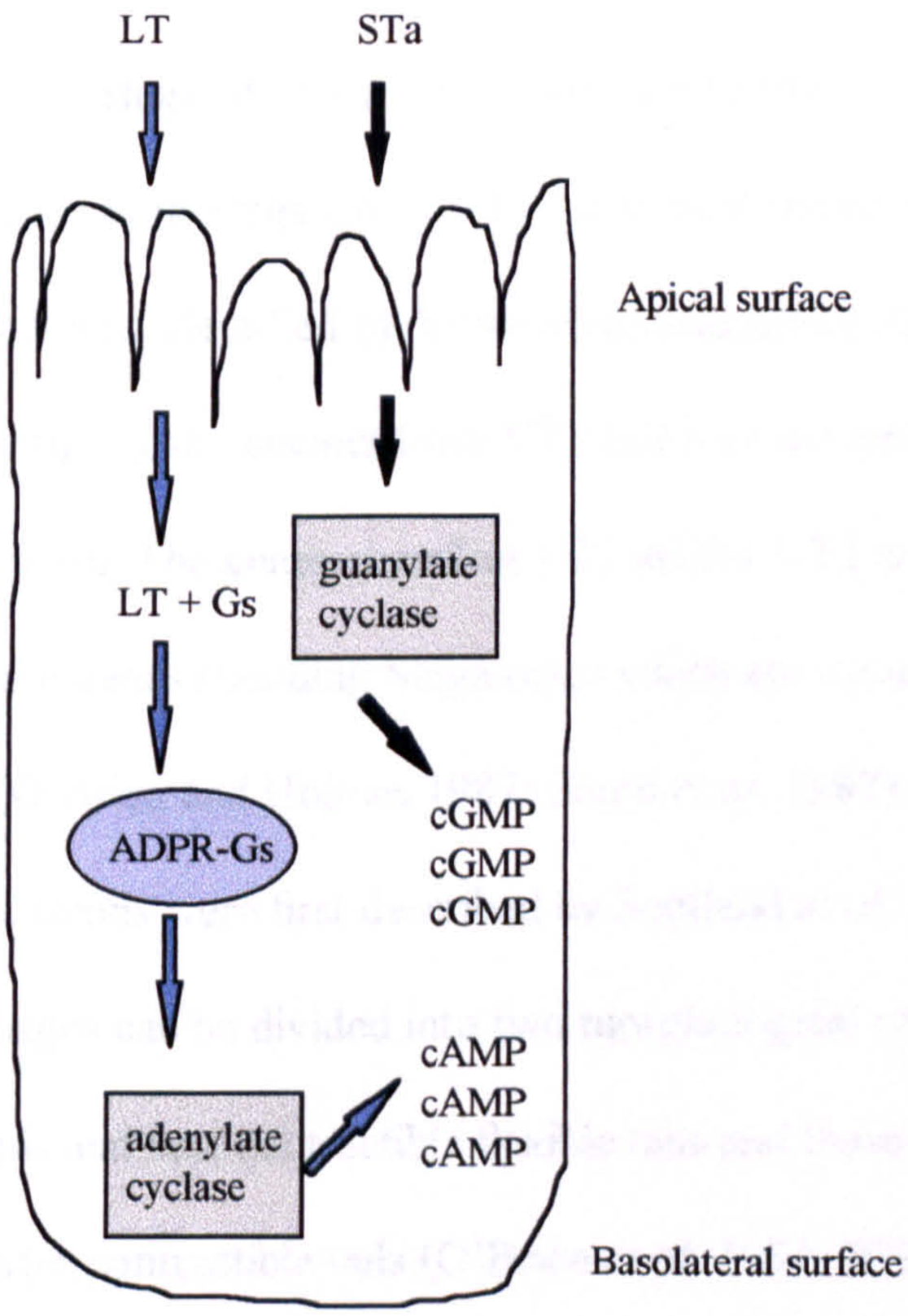
Our knowledge of STb is poor, as compared to that of STa. STb consists of 71 amino-acids, of which the 23 amino-terminal amino acids which compromises the signal peptide; the mature STb is secreted by the organism, consisting of 48 amino-acids (Lee *et al.* 1983; Picken *et al.* 1983). STb has four cysteine residues that form two intramolecular disulphide bonds (Fujii *et al.* 1991) and there is no amino-acid sequence homology between STa and STb. The nucleotide sequence of the gene that encodes STb appears to be quite different from LT and STa, although STb is plasmid encoded (Lee *et al.* 1983). The genes for STb can be associated with a 9 kb transposable element (Betley *et al.* 1986). STb does not alter cGMP or cAMP levels, but it increases the level of prostaglandin E2 (Hitotsubashi *et al.* 1992).

STb is mainly associated with ETEC isolated from cases of diarrhoea in swine, and the gene that encodes this toxin is the most common of the toxin genes in porcine ETEC (Moon *et al.* 1986; Monckton and Hasse 1988). STb has also been detected in

strains of *E. coli* isolated from humans. Recently, STb producing strains have been isolated from patients with traveller's diarrhoea (Okamoto *et al.* 1993).

The diarrhoea caused by ETEC is associated with two different types of toxins heat-labile toxin (LT; 2.3.1.) and heat-stable toxin (ST; 2.3.2). These toxins act on the intestinal cells and their modes of action are explained below (Fig. 3).

Fig. 3 *Comparison of the mechanism of action and cellular location of the targets of LT and STa.*



LT like cholera toxin, acts by ADP-ribosylating a G protein and activating adenylate cyclase, an enzyme located on the basolateral surface. STa activates guanylate cyclase, located on the apical surface by binding to it.

2.3.3. Verocytotoxin 1 and Verocytotoxin 2 (VT1 and VT2)

Certain strains of *E. coli* produce a toxin called Verocytotoxin (VT) and these strains form the group VTEC (2.2.6.). A family of Vero-cytotoxins with biologically similar activities has been described, predominately by two major types: VT1 and VT2 (Scotland *et al.* 1985). Early studies with a strain of Verocytotoxin-producing *E. coli* (VTEC) of serotype O26:H11, showed that the VT produced was neutralised by an antiserum prepared to Shiga toxin produced by strains of *Sh. dysenteriae*-1, and therefore the term Shiga-like toxin (SLT) also applied to VT (O'Brien and La Veck 1983). In common with Shiga toxin, VTs are subunit toxins comprising an A subunit and 5 identical B subunits identified by X-ray crystallography (Stein *et al.* 1992). The second toxin VT2 is antigenically distinct from VT1 and was not neutralised by antibodies to VT1 or Shiga toxin. The genes encoding VT1 and/or VT2 are carried by bacteriophage; in contrast to the genes encoding Shiga toxin which are located on the *Shigella* chromosome (O'Brien and Holmes 1987; Smith *et al.* 1987). The lysogenic phages encoding these toxins were first described by Scotland *et al.* (1983b) and Smith *et al.* (1983). VT phages can be divided into two morphological types, those with elongated hexagonal heads and non-contractible flexible tails and those with regular hexagonal heads and shorter contractible tails (O'Brien *et al.* 1984; Willshaw *et al.* 1987).

Verocytotoxins were also shown to be toxic to HeLa cells (inhibiting protein synthesis), enterotoxic in rabbit ileal segments and to cause paralysis in mice (O'Brien *et al.* 1982). The A subunit inhibits elongation factor 1-dependent aminoacyl-tRNA binding to ribosomes, thereby preventing protein synthesis (Igarashi *et al.* 1987) and causing cell death. The genes that encode the A and B subunits of the toxin are organised in tandem,

with the A subunit coming first and separated from the B subunit gene by 12 bp gap for VT1 and 14 bp for VT2 (Jackson *et al.* 1987). Both open reading frames are preceded by ribosome binding sites. The A and B genes appear to be transcribed as a single operon from a promoter that maps about 100 bp upstream from the start of the A subunit. Analysis of sequence data has revealed three regions of limited homology between the A subunit of all VT genes and the gene that encodes the plant toxin, ricin (Calderwood *et al.* 1987; De Grandis *et al.* 1987; Yamasaki *et al.* 1991).

Both VT1 and VT2 bind to eukaryotic cells via globotriosylceramide receptors containing the carbohydrates α1-4 galactose β1-4 glucose ceramide (Gb3)(Lingwood *et al.* 1987; Waddell *et al.* 1988). The various VT's are not identical in their binding, with VT1 and VT2 binding preferentially to Gb3; whereas, variants of VT2 bind preferentially to Gb4.

The expression of Shiga toxin was shown to be iron-regulated which led O'Brien *et al.* (1982) to consider whether iron was important in the expression of VT1. Calderwood *et al.* (1987) and Weinstein *et al.* (1988) concluded that expression of VT1 was regulated by iron but VT2 was not. Subsequently it was shown that expression of neither extracellular VT1 nor VT2 was influenced by the availability of iron (Chart *et al.* 1987), and that only intracellular levels of VT1 were increased by iron restriction. The VT1 gene is regulated by the product of the *fur* gene, a DNA binding protein that complexes with iron and blocks transcription. The VT2 promoters lack the *fur* operator sequence (Sung *et al.* 1990).

2.3.3.a. Verocytotoxin variants

In 1987, a variant of VT2 was described (Marques *et al.* 1987) in strains of *E. coli* O139 and O141 isolated from cases of pig oedema disease. It was unusual in having a cytotoxic effect on Vero cells but not HeLa cells, and the genes encoding the toxin were not carried by bacteriophage (Marques *et al.* 1987). The toxin was neutralised by antibodies against VT2 but not VT1 therefore the toxin was termed VT2-variant (VT2v). A strain of *E. coli* expressing VT2v was isolated from a patient suffering from haemolytic uraemic syndrome (Oku *et al.* 1989) prompting the terms VT2-variant-human (VT2vh) and VT2-variant-porcine (VT2vp, later VT2e). The human and porcine variants of VT2 were shown to have different glycolipid binding properties (Samuel *et al.* 1990) but the mode of action for both toxins was apparently the same (Furutani *et al.* 1990).

Table 7. *Characteristic features of Verocytotoxins*

	VT1	VT2	VT2v (VT2vh VT2vp; VT2e)
Cytotoxicity			
Vero cells	+	+	+
HeLa cells	+	+	-
A subunit (kDa)	32	35	33
B subunit (kDa)	7.7	10.7	7.5
Genes phage encoded	+	+	-

2.3.4. Haemolysins

Strains of *E. coli* have been shown to express toxins which lyse certain types of erythrocytes, and were termed haemolysins. Although some toxins lyse erythrocytes and are termed haemolysins they may be toxic for other cells e.g. α-haemolysin which can lyse a variety of mammalian and human cell types due to pore formation in the cytoplasmic membrane of host cells. This toxin is, therefore, more appropriately designated as a cytolysin. Most haemolytic strains of *E. coli* express one of two recognised forms of haemolysin termed α or β. In general, α-haemolysin is produced by strains of *E. coli* and transported into the surrounding medium, whilst β-haemolysin is synthesised and released following bacterial cell death. When strains of streptococci producing α-haemolysin are grown on blood agar small green zones of lysis are visible around the colonies whereas streptococci strains producing β-haemolysin exhibit clear zones of haemolysis surrounding the colonies. The family of *E. coli* haemolysins will be described as follows:-

2.3.4.a. α-haemolysin

Alpha haemolysin was first described by Kayser in 1903, and expression of this lytic toxin was shown to have an association with strains of *E. coli* causing extra-intestinal infections, particularly urinary tract infections, peritonitis, appendicitis, septicaemia and neonatal meningitis. This haemolysin was shown to cause lysis by forming pores in the cytoplasmic membrane of erythrocytes and in hypotonic media, pore

formation leads to a net influx of water followed by cell swelling and ultimately osmotic lysis of red blood cells. The action of this toxin is not restricted to erythrocytes, but extends to a wide range of other mammalian and human cell types. This toxin is, therefore, more appropriately designated as a cytolysin6. *E. coli* α-haemolysin belongs to a family of related, haemolysins and leucotoxins designated Repeats in toxin or 'RTX toxins'. This family of toxin genes has a repeated sequence of DNA rich in guanine and cytosine and haemolysins are secreted by a highly conserved transport system.

Lysis of erythrocytes may stimulate bacterial growth by increasing the amount of available iron, but this is probably not the only function of *E. coli* a-haemolysin since this toxin also lyses polymorphonuclear leukocytes, monocytes and peripheral human T lymphocytes which provides a mechanism by which extra-intestinal *E. coli* strains impair and resist the host immune defences. The mode of action of a-haemolysin appears to be pore formation and irreversible depletion in cellular ATP (Bhakdi *et al.* 1989; Jonas *et al.* 1993). At low non-lytic concentrations the toxin disrupts the normal functions of several types of host cells, therefore increasing the probability of *E. coli* establishing and maintaining infection. At these concentrations the toxin will increase membrane permeability of polymorphonuclear granulocytes resulting in the loss of phagocytic killing capacity (Bhakdi *et al.* 1989). Also low concentrations increase an oxidative burst in these phagocytic cells which results in the production of superoxide anions.

Certain strains of haemolytic *E. coli* carry the genes for haemolysin on large, conjugative plasmids varying in size between 50-160 kb (Goebel and Schrempf 1971; Monti-Bragadin *et al.* 1975; Royer-Pokora and Goebel 1976; Prada *et al.* 1991). However, strains of *E. coli* isolated from urinary tract and extra-intestinal infection

generally carry the genes on the chromosome (Welch *et al.* 1981; Hull *et al.* 1982; Welch *et al.* 1983).

There are 4 genes involved in the expression and secretion of α-haemolysin arranged in the sequence, *hly*C, *hly*A, *hly*B, *hly*D, encoding proteins of 20, 100, 80 and 54.6 kDa respectively (Figure 4). The 20kDa protein activates the 100 kDa haemolysin protein in the cytoplasm, prior to export from the bacterial cell by processes involving the 54.6 and 80 kDa proteins (Noegel *et al.* 1979; Goebel and Hedgpeth 1982; Wagner *et al.* 1983; Fig 4).

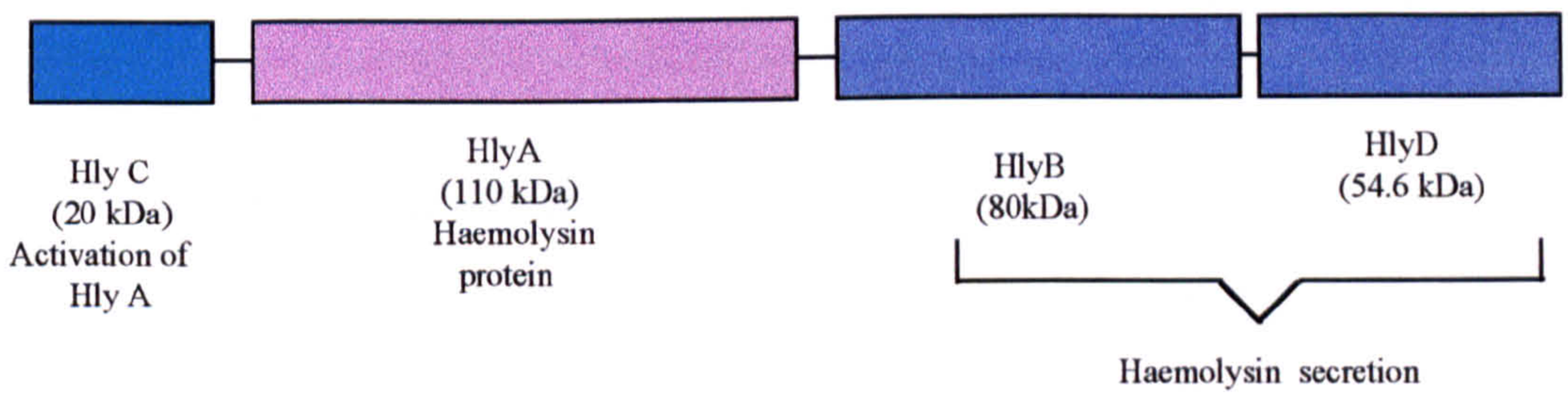


Fig. 4 Structure of a typical RTX operon using α-haemolysin as an example

2.3.4.b. β and γ-haemolysins

In contrast to α-haemolysin, β-haemolysin is not actively secreted and appears to be released from the bacterial cell following autolysis during death-phase of growth (Smith 1963; Short and Kurtz 1971). β-haemolysin has been shown to be structurally distinct from α-haemolysin using specific antibodies which neutralised a-haemolysin but not β-haemolysin (Rennie and Arbuthnott 1974). The a and β haemolysins described by

Smith (1963) are similar in their activity against the erythrocytes of different mammalian species and produce clear zones of haemolysis on blood agar. Both haemolysins are produced in the logarithmic phase of the bacterial growth cycle. To date, the relationship between these two toxins has not been elucidated fully but it has been suggested that β-haemolysin may be a cell bound form of α-haemolysin (Rennie and Arbuthnott 1974).

A third type of haemolysin, called γ-haemolysin, was identified by Walton and Smith (1969) in nalidixic acid resistant mutant *E. coli* K-12 strains. It appeared to be active against various mammalian red blood cells, but in contrast to α and β haemolysin, not against those of humans or rabbits.

2.3.4.c. Enterohaemolysin

This haemolysin was originally identified in some enteropathogenic and Verocytotoxigenic strains of *E. coli*, predominately of serogroups O26 and O111 (Beutin *et al.* 1988; 1990). Enterohaemolysin was only detected in cells grown to the stationary phase and was not found in the culture medium. It was also observed that the zones of haemolysis on blood agar were smaller and more turbid than those caused by a-haemolysin. This haemolysin was termed enterohaemolysin 1 (EHly1). A second haemolysin, termed enterohaemolysin 2 (EHly2), genetically unrelated to EHly1 was detected in strains of VTEC belonging to serotype O26:H11. Beutin *et al* (1990; 1993) and Stroeher *et al* (1993) reported that enterohaemolysin production by *E. coli* O26 strains may be associated with temperate bacteriophages.

It has recently been shown that the toxin responsible for the enterohaemolytic phenotype of VTEC O157 is encoded on the 90 kb plasmid pO157, carried by many clinical isolates of *E. coli* O157 (Schmidt *et al.* 1994). The enterohaemolysin encoded by plasmid pO157 belongs to the RTX family of toxins (Fig. 4) and shares sequence homology with *E. coli* a-haemolysin (Schmidt *et al.* 1994, 1995b). Nucleotide sequence analysis has revealed 4 genes which constitute a typical RTX operon (Schmidt *et al.* 1995b; Schmidt *et al.* 1996; Fig 4).

The phenotypic small, turbid zones on blood agar, produced by enterohaemolytic strains of *E. coli* on blood agar, appear to be due to poor extracellular secretion of this toxin (Bauer and Welch 1996). The role of enterohaemolysin in pathogenesis is not clear. Patients infected with VTEC O157 resulting in haemorrhagic colitis or haemolytic uraemic syndrome (HUS), appear to have antibodies to this toxin, indicating that this haemolysin is produced during the infection and that it may be of clinical importance (Schmidt *et al.* 1996; Chart *et al.* 1998b).

2.3.5. Cytotoxic necrotising factor and cytolethal distending toxin

Cytotoxic necrotising factor (CNF) was first described in strains of *E. coli* isolated from extra-intestinal infections in humans (Caprioli *et al.* 1983). This toxin was heat-labile and caused 'giant-cell' formation with Vero, HeLa and CHO cells and caused necrosis when injected intradermally into rabbits. Strains of *E. coli* that produce CNF have been isolated from pigs, calves, cats and dogs (Gonzalez and Blanco 1985; McLaren and Wray 1986; Prada *et al.* 1991). Although CNF has been isolated from a

wide range of *E. coli* belonging to different serogroups, most isolates belong to a small range of O-serogroups including O2, O4, 06, O22, O32, O75, O83 and O85 (Holland 1990).

A second type of CNF was subsequently identified and termed CNF2, although both forms were shown to be monomeric proteins of 110-115 kDa (Caprioli *et al.* 1984; Oswald and De Rycke 1990). In contrast to CNF1 which was chromosomally encoded (Falbo *et al.* 1992), CNF2 was shown to be encoded on the transmissible Vir plasmid first detected by Smith (1974). These two CNFs differ in their mode of action since CNF2 causes necrosis of mouse footpads, moderate fluid accumulation in rabbit intestinal ligated loops and elongation 2of HeLa cells (De Rycke *et al.* 1990). Strains that produce CNF2 have been isolated from calves and lambs with diarrhoea and/or septicaemia. Although the two CNF toxins appear distinct, they can be partially cross neutralised. In contrast to strains of *E. coli* that produce CNF2, most strains that produce CNF1 also produce haemolysin (Caprioli *et al.* 1987; Blanco *et al.* 1990; De Rycke *et al.* 1990) encoded alongside the CNF genes on a 37 kb fragment of DNA.

Cytolethal distending toxin (CLDT) was first described by Johnson and Lior (1987) in strains of *E. coli* belonging to serogroup O128. This toxin caused morphological changes in Chinese hamster ovary cells resulting in cell death. The CLDT toxin was distinct from heat-labile toxins or Vero-cytotoxins in the mode of action, but was shown to be heat-labile (Bouzari and Varghese 1990). CLDT was later described in a strain of *E. coli* belonging to serotype O55:K9:H4 (Anderson and Johnson and Lior 1987) and in strains belonging to serotypes O84:H34 and O111:H- (Guth *et al.* 1994).

2.3.6. Fimbrial adhesins

A well-established mechanism of bacterial adhesion is by the use of fimbriae, which are occasionally termed pili. For the purposes of clarification, fimbriae will be used to describe structures involved with bacterial adhesion.

2.3.6.a. Type 1 fimbriae

The type-1 fimbriae expressed by strains of *E. coli*, initially termed common fimbriae, are hair-like structures which bind to receptors containing D-mannose. Type-1 fimbriae were the first bacterial fimbriae to be described (Duguid *et al.* 1955; Brinton 1959; Duguid 1959) and since then, have become the most widely studied fimbrial structure. Strains expressing type 1 fimbriae have the abiltiy to agglutinate many species of erythrocytes, but this reaction is blocked by the addition of mannose. Type 1 fimbriae have the ability to promote bacterial binding to a wide variety of cells which contain D-mannose in their membranes, one of the most ubiquitous sugars on eukaryotic cell surfaces. The ability of type 1 fimbriae to recognise and bind to various human and animal mucosal and inflammatory cells seems to be essential for strains of *E. coli* to initiate and sustain bacterial infections.

Strains of *E. coli* expressing type 1 fimbriae have some 100-300 peritrichous fimbrial filaments, each about 7nm wide and 0.2-2μm long. A single filament consists of several thousand copies of the 17 kDa FimA subunit. The subunits are arranged in a simple, tight helix and each turn of the helix would consist of 3.14 FimA subunits. The

structure of Type 1 fimbriae is extremely stable and with the exception of guanidine hydrochloride, is resistant to all common dissociating reagents.

Several genes are involved in the expression of type 1 fimbriae (Bachman 1983). The entire gene cluster, which spans 9.5 kb was first cloned by Hull *et al.* (1981) and the functions of the various genes were subsequently determined by the mutational inactivation of each gene in turn and examination of the effect on fimbrial expression or function. A genetic and physical map of the type 1 fimbriae gene cluster is shown in Fig. 5.

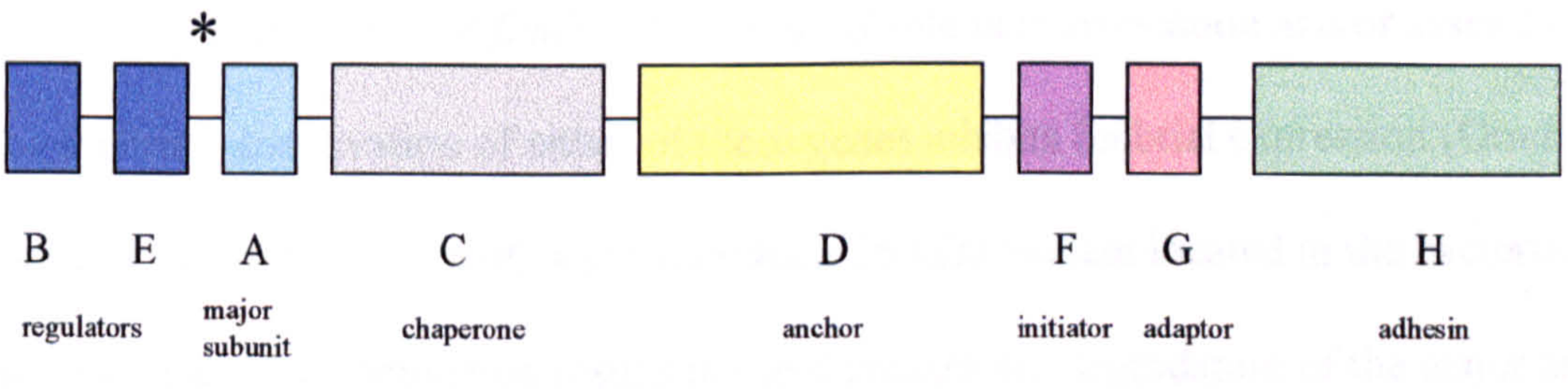


Fig 5. Organisation of genes within the type 1-fimbrial gene cluster. The established and postulated functions of the various gene products are indicated. The asterisk indicated the site of the invertible phase-switch.

At least four genes encode the subunits that constitute the fimbrial filament. The *fim*A gene encodes the major component of the fimbrial filament, and the inactivation of this gene results in the inability of fimbrial expression as determined by electron microscopy (Orndorff and Falkow 1984). Recent studies have revealed that *fim*A deficient mutants express short fibrillar surface structures that comprise of products from at least two genes, *fim*H and *fim*G (Jones *et al.* 1995).

The inactivation of *fim*H results in fimbriae which are unable to bind to D-mannose (Maurer and Orndorff 1985). Though *fim*G is co-transcribed with *fim*H and its gene product is closely associated with the *fim*H protein in the fimbrial structure, its

exact function is not clear. It has been suggested that the *fim*G gene product serves as an adaptor that holds *fim*H in a functional conformation on the fimbriae (Abraham and Beachey 1987). The *fim*F gene encodes a protein believed to be another minor subunit of the fimbriae , but evidence for its presence on the fimbrial filament is lacking. It is suggested that *fim*F plays a role in regulating the length of the fimbriae, since inactivation of *fim*F results in the formation of extraordinarily long fimbrial filaments (Klemm and Christiansen 1987; Russell and Orndorff 1992).

The remaining genes in the cluster encode for proteins involved in the translocation and assembly of fimbrial subunits and the regulation of fimbrial expression. The products of *fim*C and *fim*D, play a crucial role in translocation and/or assembly of the fimbriae. Inactivation of either of these genes inhibits fimbrial expression (Orndorff and Falkow 1984). The *fim*C gene encodes a 26 kDa protein located in the bacterial periplasm and its inactivation results in rapid proteolytic degradation of the major and minor fimbrial subunits (Jones *et al.* 1993). The protein encoded by *fim*C has been shown to be a chaperone protein believed to attach to newly synthesised subunits as they enter the periplasm from the inner membrane. The functions of FimC include the protection of fimbrial subunits from periplasmic enzymes, maintenance of the nascent proteins in a form competent for assembly and translocation of the subunits to points of assembly in the bacterial outer membrane (Hultgren *et al.* 1991, 1993). Inactivation of *fim*D also abolishes fimbrial expression (Orndorff and Falkow 1984). The *fim*D gene product is a large 88 kDa outer membrane protein that forms the base on which the fimbrial filaments are assembled (Klemm and Christiansen 1990).

Two regulatory genes, *fim*B and *fim*E encode histone-like proteins (Klemm 1986) that direct the phase dependent expression of the *fim*A gene, which determines

whether or not fimbriae are expressed on the bacterial surface. The mechanisms of phase variation involve the inversion of a 314 base pair DNA segment that harbours the promoter for the *fim*A gene. Thus, FimA is expressed in one orientation (on) but not the other (off). The switching phenomenon is a Rec-A-independent process and requires the function of *fim*B to direct the switch from on to off and from off to on (Klemm 1986; McClain *et al.* 1991). Unlike *fim*B, *fim*E mediates the switch in only one direction, from on to off (Blomfield *et al.* 1991). In addition to the modulatory effects of *fim*B and *fim*E, the phase switch is also affected by at least three global regulators, leucine-responsive regulatory protein (Lrp), integration host factor (IHF), and the histone-like proteins (H-NS) (Dorman and Higgins 1987; Blomfield *et al.* 1991).

Deletion or inactivation of the *fim*H gene results in the loss of fimbrial binding activity (Maurer and Orndorff 1985). Antibodies specific for FimH, but not those against other fimbrial subunits block fimbrial binding activity.

2.3.6.b. Colonisation Factor Antigens (CFAs)

Strains of *E. coli* which express LT and ST toxins (ETEC) (2.3.1., 2.3.2.) carry the genes encoding these toxins on a plasmid which may also encode the genes for expression of fimbrial structures termed colonisation factor antigens or CFAs and the regulatory genes for these structures. ETEC strains adhere to epithelial surfaces by means of these proteinaneous surface structures, and at present more than 20 different CFAs have been described in ETEC causing human infections (Gaastra and Svennerholm, 1996). Observing these structures by electron microscopy shows that most fimbriae appear as straight, hair-like filamentous structures with diameters of 5-7 nm but

certain fimbriae e.g. K88, K99 and CS3, have a much smaller diameter (2-4nm) resulting in long and wiry or curly and flexible filaments. Both types of fimbriae are composed of protein subunits that are assembled into a helical structure with different numbers of subunits per helical turn.

Each particular type of fimbriae is usually associated with a limited number of serotypes of ETEC (Table 8). The first colonisation factor (CF) to be described from a strain of ETEC causing diarrhoea in humans was CFA/I, isolated from a patient with severe cholera-like disease in Dacca, Bangladesh (Evans *et al.* 1975). CFA/I is a rigid fimbrial rod with a diameter of 7nm (Evans *et al.* 1975; Table 8). The second CF from ETEC was named CFA/II (Evans and Evans 1978) but this was found to be a mixture of fimbrial structures. These fimbrial structures were termed coli surface antigens (CS) CS1, CS2 and CS3 (Smyth 1982). CS1 and CS2 are rigid rods with diameters of 7nm, but CS3 comprises of flexible fibrillae with a diameter of 2-3 nm. CFA/III was described in 1984 and is a fimbria with a diameter of 7-8nm (Honda *et al.* 1984). CFA/IV (Thomas *et al.* 1982) was found to be a mixture of CS4 and CS6, CS5 and CS6 or CS6 alone (Thomas *et al.* 1985). CS4 and CS5 are fimbriae, but the structure of CS6 is undetermined (Knutton *et al.* 1989b; Wolf *et al.* 1989) and appears to be non-fimbrial (Gaastra and Svennerholm, 1996).

Additional fimbrial (CS17, PCFO20, PCFO159 and PCFO166), fibrillar (CS7, PCFO9 and PCFO148) and undetermined (22230 and 8767) coli surface antigens (CSA) have been identified (Darfeuille-Mauchand *et al.* 1986; Tacket *et al.* 1987; Knutton *et al.* 1987b, Darfeuille-Mauchand *et al.* 1990, McConnell *et al.* 1991; Aubel *et al.* 1991; Viboud *et al.* 1993). The names of these CF from ETEC have not continued the pattern of CFA. Often they were first described 'PCF' as putative colonisation factors which

may be then superseded by another name. For instance, CFA/IV was first called PCF8775 (McConnell *et al.* 1988), and PCFO9 is also called CS13 (de Graaf and Gaastra 1994). The lack of systematic naming is unfortunate and not easily remedied.

Table 8 *CFAs associated with human disease, their subunit size, and morphology.*

Fimbria	Associated serogroups	Subunit size (kDa) †	Morphology
CFA/II	O15, O25, O63, O78, O128, O153,	15	Fimbriae
CS1	O6:H16 (biotype B, C, F)	16.8, 16.3	Fimbriae
CS2	O6, O8, O80, O85, O115, O139	15.3, 16.4/17.0	Fimbriae
CS3	O6, O8, O80, O85, O115, O139	14.5/15.5 14.8	Fibrillar
CS4	O25	22.0	Fimbriae
CS5	O6, O92, O115, O167	24.0	Fimbriae
CS6	O6, O25, O27, O29, O92, O115, O148, O153, O159, O167	15.6 18.0	Undetermined
CFA/III	O25	18.0	Fimbriae
PCFO159:H4	O159	19.0	Fimbriae

† Fimbrial subunit size ¶ Morphological type of fimbriae

The receptor binding sites on fimbriae vary. For some fimbriae, e.g. K88, K99 and CFA/I, the adhesive properties are associated with the major fimbrial subunit; however for fimbriae such as type 1 , P- and S-fimbriae, minor fimbrial subunits at the distal end of the fimbrial structure are responsible for the interaction with host cells.

CFA/I fimbriae bind to sialic acid containing glyco conjugates, such as ganglioside GM2. CS1, CS2, CS3 and CS4 bind to the intestinal receptor GM1 (Orø *et al.* 1990). The use of different receptors by the different fimbriae of human ETEC is also evident by the fact that binding to Caco-2 cells by ETEC that produce CFA/I, CFA/II and CFA/III can be inhibited if the Caco-2 cells have previously been incubated with homologous, but not heterologous fimbriae (Darfeuille-Michaud *et al.* 1990).

The biosynthesis of fimbriae includes the expression of regulatory proteins, chaperone proteins involved in the transport of the fimbrial subunits across the bacterial cell envelope, and the production of minor and major fimbrial subunits. Most minor subunits are structurally related to the major fimbrial subunit and evidence indicates that they play a role in the initiation, elongation and termination of the assembly of major fimbrial subunits at the cell surface.

The biosynthesis of fimbriae is regulated by a number of environmental factors. The highest production is found at the maximal growth rate and at temperatures below 37°C production is repressed. This regulation is done by controlling promoter activity (van der Woude *et al.* 1990). Expression of fimbriae by human ETEC strains depends on the composition of the growth medium, and addition of bile salts to CFA agar improves the production of fimbriae (Evans and Evans, 1978).

Most fimbrial operons contain a regulatory region that encodes the major promoter of the operon and two regulatory genes. All regulatory proteins have a positive effect on transcription (Schmoll *et al.* 1990).

Table 9. *The relationship between fimbrial type, toxin production and susceptible host in ETEC strains.*

Type	Toxins	Host
K88	LT, STa, STb, STa	Pigs
F41	STa, STb	Pigs, calves, lambs
CS31A		Calves
K99	STa, STb	Calves, lambs, pigs
CFA/I	STa, LT	Human
CFA/II		
CS1 + CS3	STa, LT	Human
CS2 + CS3	STa, LT	Human
CS3	STa, LT	Human
CFA/III + CS6	STa, LT	Human
CFA/IV		Human
CS4 + CS6	STa, LT	
CS5 + CS6	LT	
CS6	STa, LT	Human
CS7	STa, LT	Human
CS17	LT	Human
PCFO166	STa, LT	Human

CFA, colonisation factor antigen; CS, coli surface associated; LT, heat-labile enterotoxin; PCF, putative colonisation factor; ST, heat-stable enterotoxin

2.3.6.c. P-pili

Strains of *E. coli* that adhere to human periurethral and uroepithelial cells are typically associated with adhesive fimbriae (Svanborg-Eden and Hansson 1978; Kallenius and Mollby 1979; Korhonen *et al.* 1980). In animal models of urinary tract infection (UTI), strains that express mannose-resistant haemagglutination (MRHA) are more virulent than strains that do not (Montgomerie 1978; van den Bosch *et al.* 1980; Ketyi 1981; Hughes *et al.* 1983).

P-pili, encoded by the *pap* (pyleonephritis-associated pili) gene cluster, are expressed on the surface of *E. coli* and have been found to be associated with severe upper urinary tract infections and bacteremia: over 90% of *E. coli* isolated from patients with pyleonephritis expressed P-pili, while only 7% of faecal isolates from healthy subjects expressed P fimbriae (Kallenius *et al.* 1981; Johnson 1991). These strains and their purified fimbriae agglutinate erythrocytes and adhere to human epithelial cells with surface antigens, such as the human P1, P2 and Pk phenotypes, which contain Gal-Gal, but they do not interact with cells that lack Gal-Gal containing surface antigens.

The *pap* gene cluster contains 11 genes. All these genes have been sequenced and the functions of most of the *pap* gene products have been extensively studied (Hultgren *et al.* 1991; Tennent *et al.* 1990; Marklund *et al.* 1992). The promoters are modulated by two *pap* gene products, PapB and PapI. The catabolite repressor protein (CRP), leucine-responsive regulator protein (Lrp) and the histone-like protein are also involved in the modulation of the promoters (Forsman *et al.* 1992; van der Woude *et al.* 1992). The expression of P-pili is also under phase variation control which involves the DNA binding ability of the Lrp and PapI regulatory proteins (Nou *et al.* 1993).

The PapB promoter controls a transcriptional unit containing 10 pap genes. Six of these (*pap A, H, E, K, F, G*) encode interactive pilus subunit proteins, which have distinct roles in the assembly and function of the pilus polymers. The products of *papD* and *papC*, the chaperone and usher, do not end up in the polymerised fibre but are required for ordered P-pilus assembly (Lindberg *et al.* 1989; Hultgren *et al.* 1989). The exact role of the *papJ* product (Tennent *et al.* 1990) in pilus biogenesis remains unclear. P-pili specifically recognise a terminal or internal Gala(1-4)Gal moiety in epithelial cell

surface glycolipids via the *papG* product (Leffler and Svanborg-Eden 1980; Lund *et al.* 1987).

2.3.6.d. Mannose resistant adhesins

The various mannose resistant (MR) adhesins of uropathogenic *E. coli* are commonly divided into two groups, those that recognise P blood group antigens and related Gal(α1-4)Gal containing structures, such as P-pili and related adhesins, and other adhesins that have been termed X-adhesins or X-fimbriae (Johnson 1991). Now that the receptor specificities of many MR adhesins have been identified, such as Dr, S, M, G, the designation X has become potentially misleading. Precise nomenclature, and a general term such as non-P MR adhesins seems appropriate when referring collectively to MR-adhesins other than those of the P-family.

2.3.6.e. S-fimbriae and F1C fimbriae

Closely related adhesins are expressed by some urinary *E. coli* strains (Ott *et al.* 1986; Reigman *et al.* 1990). S-fimbriae are so-called as they bind specifically to sialosyl-oligosaccharide residues (Parkkinen *et al.* 1983; Hanisch *et al.* 1993). These fimbriae are subject to phase variation and mediate non-P MRHA of human erythrocytes (Nowicki *et al.* 1986).

F1C fimbriae do not mediate MRHA or adherence to uroepithelial cells, but they bind to buccal epithelial cells, renal tubular cells and renal tissues, and have the same receptor specificity as S-fimbriae (Marre *et al.* 1990). These fimbriae are expressed by

20% of uropathogenic strains (Karkkainen *et al.* 1991) and their significance in UTI is unclear (Pere *et al.* 1987).

2.3.7. Non-fimbrial adhesins

2.3.7.a. Attaching and effacing lesions

Traditionally, enteropathogenic *E. coli* (EPEC) have been defined on the basis of classical O:H serotypes that had been isolated from cases of infantile diarrhoea (Robins-Browne 1987), but as the understanding of EPEC pathogenicity improved, it was shown that EPEC strains were better characterised in terms of their pattern of adhesion to cultured HEp-2 cells *in vitro*. The most distinctive characteristic of EPEC is their ability to adhere to the gut epithelium. Binding of EPEC to the gut epithelium triggers a cascade of transmembrane and intracellular signals leading to cytoskeletal reorganisation and the formation of the specific lesion, termed the attachment and effacement (A/E) lesion (Ulsen and Rollo 1980). This lesion is characterised by destruction of the brush border microvilli and intimate adherence of bacteria to cup like pedestals formed by the bare enterocyte cell membrane (Knutton *et al.* 1987a, c). High concentrations of polymerised actin are present beneath the site of bacterial attachment (Knutton *et al.* 1989a). Tissue culture infected with strains of EPEC produce A/E lesions but a characteristic pattern of adhesion also results termed as localised adherence (LA)(Scaletsky *et al.* 1984; Chart *et al.* 1988).

Experiments with cultured epithelial cells have implicated a large number of genes in LA and A/E lesion formation in EPEC. Part of the genes required are encoded

on the 90 kb EPEC adherence factor (EAF) plasmid common to all typical EPEC strains (Nataro *et al.* 1985a, b). The EAF plasmid encodes bundle forming pilus (bfp) protein, which plays a role in LA, (Donnenberg *et al.* 1992; Giron *et al.* 1991), and contains the regulatory locus (the *per* locus)(Gómez-Duarte and Kaper 1995), that appears to control and co-ordinate the expression of several EPEC virulence factors, including intimin (Gómez-Duarte and Kaper 1995; Knutton *et al.* 1997).

The genes encoding the A/E phenotype are encoded on a 35 kb 'pathogenicity island' termed as the locus of enterocyte effacement (LEE) region (McDaniel *et al.* 1995).This locus is found in all A/E lesion forming bacteria (McDaniel *et al.* 1995), and encodes a type III secretion system (Jarvis *et al.* 1995), a series of secreted proteins (EPEC-secreted proteins or Esps) (Donnenberg *et al.* 1993a; Kenny *et al.* 1996; Lai *et al.* 1997), and intimin, the product of the *eae* gene (Jerse *et al.* 1990; Jerse and Kaper 1991). Intimin is a 94 kDa outer membrane protein mediating intimate adherence of the bacteria to the brush border; the importance of this protein has been demonstrated in human and animal studies (Donnenberg *et al.* 1993b; Donnenberg *et al.* 1993c). The gene encoding this protein has been cloned and sequenced. This gene has been designated *eae*, and an internal 1 kb fragment of the *eae* gene, is used as a DNA probe which is highly specific for the detection of EPEC strains (Jerse *et al.* 1990).

Rosenshine *et al.* (1996) identified a 90 kDa host protein (Hp90) that was tyrosine phosphorylated upon host cell infection by EPEC and served as a receptor for intimin. Further studies have shown that Hp90 is a bacterial protein that is transferred into the eukaryotic cell, rather than a eukaryotic protein expressed by the host cell (Kenny *et al.* 1997). This protein has now been renamed translocated intimin receptor

(Tir). Transfer of Tir into host cells has been shown to be dependent on the type III secretion system and at least two other proteins are secreted by this system (EspA and EspB). Kenny *et al.* (1997) identified three possible functions of Tir (1) to serve as the cell surface receptor for intimin, (2) to nucleate actin following intimin binding and (3) to transmit additional signals to host cells once Tir-intimin binding occurs.

Attaching and effacing lesions are also induced by other enteric bacteria, including some Verocytotoxigenic *E. coli* (Sherman *et al.* 1988); very small numbers of strains of *Hafnia alvei* which have been associated with children with diarrhoea (Albert *et al.* 1992); and a very small number of strains of *Citrobacter rodentium*, the causative agent of transmissible colonic hyperplasia in laboratory mice (Schauer *et al.* 1993).

2.3.7.b. Dr haemagglutinin, Afimbrial adhesins I and III (AFAI and AFAIII)

Most urinary isolates of *E. coli* that express MRHA in the absence of P-fimbriae (2.3.6.c.) hybridise with probes specific for non-fimbrial adhesins that bind to various portions of the Dr blood group antigen (Labigne-Roussel and Falkow 1988; Arthur *et al.* 1989; Nowicki *et al.* 1989; Nowicki *et al.* 1990).

The Dr family of adhesins includes the Dr haemagglutinin (O75X adhesin) (Vaisanen-Rhen 1984; Nowicki *et al.* 1988) and the non-fimbrial adhesins AFA-I and AFA-III (Labigne-Roussel and Falkow 1988), which are genetically related (Nowicki *et al.* 1990; Swanson *et al.* 1991).

The Dr family of adhesins are structurally distinct from other *E. coli* fimbrial adhesins in that they appear as a thin mesh (Arthur *et al.* 1989), a coil like structure

(Vaisanen-Rhen 1984), or a filamentous capsular coating (Ørskov *et al.* 1985) on the cell surface or are not visible by electron microscopy (Labigne-Roussel *et al.* 1984).

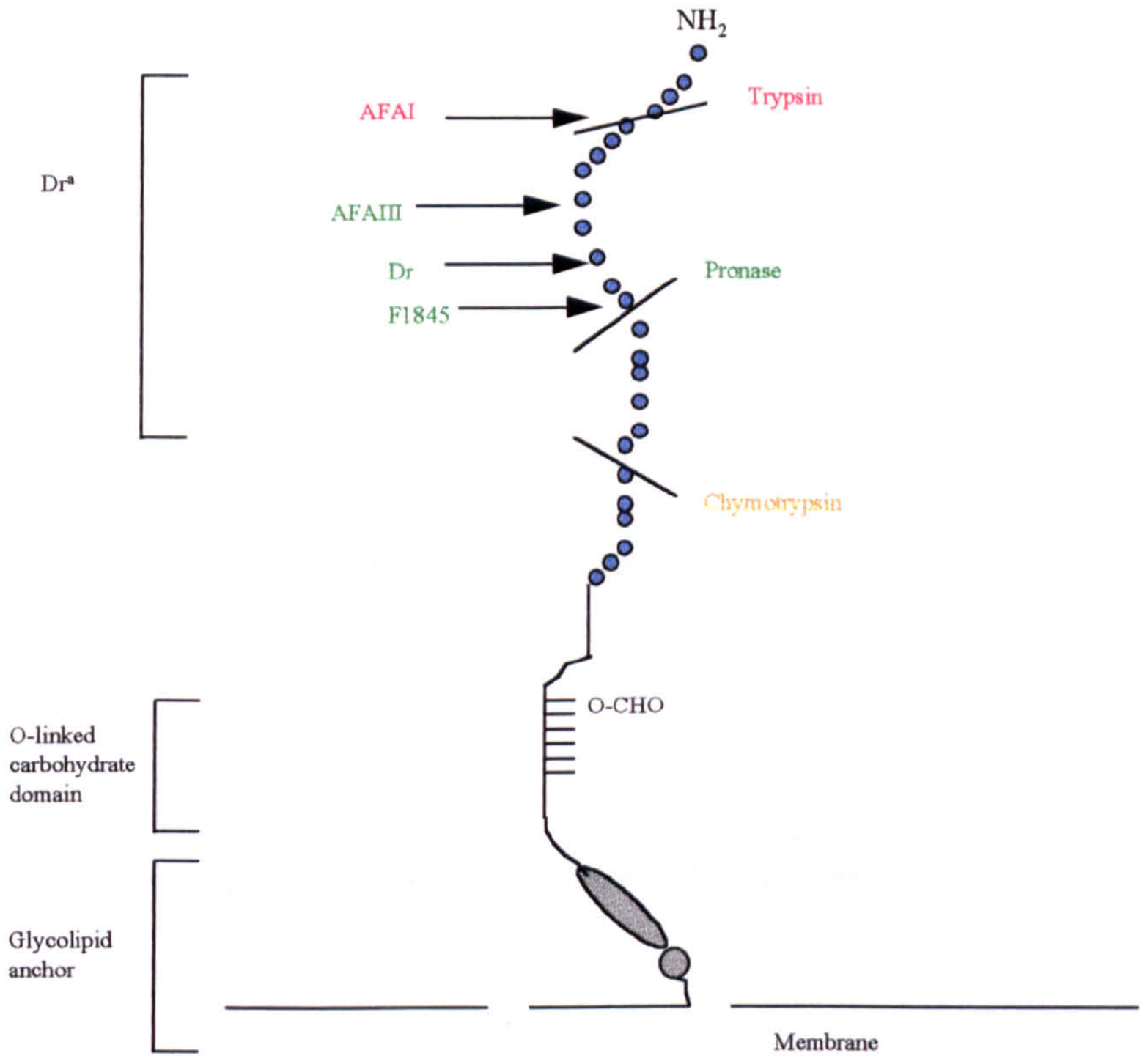
The Dr adhesin family recognise various portions of the Dr human blood group antigen. The Dr antigen is located on the decay-accelerating factor (DAF), one of the cell membrane proteins that regulate the complement cascade and protect erythrocytes from being lysed by autologous complement (Labigne-Roussel and Falkow 1988; Nowicki *et al.* 1988, 1990)(Fig 6). The addition of various proteases to Dr^+ erythrocytes disrupts the haemagglutination with strains of *E. coli* expressing members of the Dr family of adhesins (Table 10; Fig 6).

Table 10. *Haemagglutination of Dr^+ erythrocytes after the addition of proteases*

Treatment of erythrocytes	MRHA* by *E. coli* expressing haemagglutinins		
	Dr	AFA-III	AFA-I
Untreated	+	+	+
Chymotrypsin	-	-	-
Pronase	-	-	-
Trypsin	+	+	-
Papain	+	+	+
Chloramphenicol	-	+	+

* Mannose resistant haemagglutination

Fig 6 A hypothetical simplified model of the DAF molecule proposing Dr blood group antigen and receptor binding sites for the Dr, AFAI, AFAIII and F1845 haemagglutinins.



Red blood cell

The Dr haemagglutinin binds to numerous sites in the urinary tract including the renal interstitium, Bowmans capsule, tubular basement membrane (but not epithelial cells), urethral transitional epithelial cells and exfoliated epithelial cells in urine, but evidence suggests that in human tissues the Dr blood group antigen is the receptor for Dr-haemagglutinin (Kaul *et al.* 1993).

The adhesin gene clusters of different members of the Dr family are organised similarly, with five closely conserved spaced genes, including one for the structural haemagglutinin (Labigne-Roussel *et al.* 1984; Nowicki *et al.* 1989). The amino acid sequence of this 16 kDa protein differs between members of the Dr family (Labigne-Roussel *et al.* 1988). and is not similar to that of other *E. coli* fimbrial subunits (Labigne-Roussel *et al.* 1985). Some strains have multiple copies of the Dr gene cluster (Labigne-

Roussel *et al.* 1988), but a single copy is most common (Arthur *et al.* 1989; Labigne-Roussel *et al.* 1988).

In contrast to P-pili, haemagglutinins of the Dr family of adhesins are associated with cystitis. Dr-related sequences are present in 26-50% of cystitis patient isolates but only in 6-26% of pyelonephritis, and 6% of asymptomatic bacteriuria patients, and in 15-18% of faecal isolates (Arthur *et al.* 1989; Nowicki *et al.* 1988).

2.3.8. Other Virulence Mechanisms expressed by *E. coli*

2.3.8.a. Acquisition of iron

The amount of 'free' iron in the mammalian or avian host is extremely low and insufficient to enable strains of *E. coli* to multiply (Griffiths 1991). The importance of iron for strains growing *in vivo* can be illustrated by the observation that animals injected with various forms of iron are far more susceptible to infection with *E. coli* than untreated control animals (Bullen and Griffiths 1987; Bullen *et al.* 1991).

Most of the iron in the body is found as ferritin, haemosiderin or haem, while extracellular iron in serum and other body fluids is bound to high-affinity iron binding glycoproteins, transferrin in blood and lymph and lactoferrin in external secretions and milk. Since many bacterial pathogens require iron to multiply *in vivo* they must be able to adapt to acquire iron held by the host.

Strains of *E. coli* can acquire iron from sources such as haem or haemoglobin; however, many strains rely on high-affinity iron uptake systems involving iron chelating

compounds termed siderophores. The single most important feature of siderophores is the extremely high affinity for ferric iron. Through the use of iron chelating compounds, the response of bacteria to low iron conditions can be studied *in vitro*. Under conditions of iron restriction, strains of *E. coli, S. typhimurium,* and *Klebsiella pneumoniae* produce the catecholate iron chelator called enterobactin, formerly known as enterochelin (O'Brien and Gibson 1970; Pollack and Neilands 1970; Rogers 1973; Rogers *et al.* 1977; Rogers 1983). This compound is a cyclic triester of 2,3-dihydroxy-*N*-benzoyl-L-serine, and is synthesised only under conditions of iron restriction. Enterobactin has the highest known affinity for ferric ions and can very efficiently remove iron from the iron-binding proteins (Rogers 1973; Rogers *et al.* 1977; Carrano and Raymond 1979).

The synthesis of enterochelin in *E. coli* involves the products of 7 genes, *ent*A to *ent*G (Young *et al.* 1971; Greenwood and Luke 1976; Fleming *et al.* 1985; Earhart 1987). The enterobactin molecule is used only once to transport iron into the cell, since release of ferric ions from the molecule requires an enterobactin esterase which destroys the iron binding ability of the enterobactin (Cooper *et al.* 1978; Harris *et al.* 1979a; Raymond and Carrano 1979).

Apparently all wild-type and clinical isolates of *E. coli* produce enterobactin (Griffiths 1987), and it is known to be produced *in vivo* during infection (Griffiths *et al.* 1980). This supports the theory that the presence of iron-binding proteins in body fluids creates an iron restricted environment and may prevent many potentially pathogenic bacteria from multiplying in host tissues.

Enterobactin appears to be the main endogenous siderophore produced by *E. coli* but several other clinical isolates possess a second high affinity iron uptake system

mediated by the hydroxamate siderophore, aerobactin. Most of these isolates are associated with septicaemia or other extra-intestinal infections. The genes for the synthesis of aerobactin may be carried on a plasmid or on the *E. coli* chromosome. Strains of *E. coli* causing septicaemia in humans may carry the genes for aerobactin on a colicin V (pColV) and other plasmids (Williams 1979; Williams and Warner 1980; Crosa 1987; Griffiths 1987; Gonzola *et al.* 1988; Crosa 1989; Woolridge and Williams 1993). Enteroinvasive *E. coli* (EIEC) frequently produce aerobactin (Payne *et al.* 1983; Griffiths *et al.* 1985) but have chromosomally located aerobactin genes (Marolda *et al.* 1987).

Aerobactin is a member of the hydroxamic acid family of siderophores that was initially detected in strains of *Aerobacter aerogenes* (Gibson and Magrath 1969). Aerobactin is a combination of 6-(*N*-acetyl-*N*-hydroxyamino)-2-aminohexanoic acid and citric acid and forms an octahedral ferric complex (Harris *et al.* 1979b).

Although enterochelin and aerobactin are the main siderophores produced by strains of *E. coli,* this organism has the ability to utilise exogenous chelators produced by other micro-organisms (Leong and Neilands 1976; Raymond and Carrano 1979; Neilands 1981; Hantke 1983). For example, certain strains of *E. coli* can use the fungal hydroxamate chelators, ferrichrome, coprogen and rhodotorulic acid (Leong and Neilands 1976; Hantke 1983).

The process for adapting for growth in an iron restricted environment not only involves the synthesis of siderophore but also the production of membrane protein receptors for ferric-siderophores and the enzymes involved in the uptake and the release of iron from the ferric-siderophore complex (Neilands 1982; Griffiths 1987). Outer membrane receptors are necessary as the molecular size of ferric- siderophores exceeds the diffusion limit of membrane pores (Nikaido 1979; Braun *et al.* 1991)

2.3.8.a.i. *Iron regulated outer membrane proteins*

The adaptation for bacterial growth in iron restricted environments not only involves the synthesis of siderophores but also the production of membrane associated proteins which act as receptors for ferric-siderophore complexes (Neilands 1982; Griffiths 1987). The ferric-siderophore complex is too large to enter the bacterial cell by diffusion through membrane pores (Nikaido 1979; Braun *et al.* 1991), and outer membrane protein receptors are required. Strains which are unable to express these receptor proteins are unable to multiply in an iron restricted environment (Grewal *et al.* 1982; Carmel *et al.* 1990).

There are several outer membrane proteins produced by *E. coli* during iron restricted growth. FepA, encoded by the *fepA* gene, is the receptor for ferric-enterobactin and has a molecular weight of 81 kDa (Neilands 1982). A study by Chart and Griffiths (1985) showed that the enterobactin receptor was highly conserved both in size and antigenic properties within strains of *E. coli*. The membrane receptor for the siderophore aerobactin is the 74 kDa product of the *iutA* gene, which in certain strains of enteroinvasive *E. coli* and *Sh. flexneri,* has a molecular weight of 76 kDa (Griffiths *et al.* 1985; Table 11). Certain strains of *E. coli* carry the aerobactin genes on a plasmid termed Col V (Williams, 1979; Bindereif *et al.* 1982; Grewal *et al.* 1982; Table 11).

Other iron regulated proteins act as receptors for siderophores not made by strains of *E. coli*. For example, the 78 kDa FhuA protein, encoded by the *fhuA* gene, functions as the receptor for ferrichrome (Neilands 1982; Coulton *et al.* 1983; Braun *et al.* 1991; Table 11) which is a fungal siderophore. Similarly, ferric coprogen and ferric rhodotorulic acid also have their own receptors, FhuE (Hantke 1983; Braun *et al.* 1991; Table 11), as does

ferrioxamine B, for which the receptor is FoxB (Nelson *et al.* 1992; Table 11). Two other iron regulated proteins have also been recognised, Fiu and the 74 kDa Cir protein, but their functions remain unknown (Table 11).

Table 11 *Iron regulated siderophore receptor proteins of the outer membrane of Escherichia coli.*

Receptor	Apparent molecular weight (kDa)	Iron-uptake function
Cir	74	(unknown)
FecA	80.5	Fe^{3+}- citrate
FepA	81	Fe^{3+}-enterobactin
FhuA	78	Ferrichrome
FhuE	76	Fe^{3+}-coprogen
Fiu	83	(unknown)
FoxB	66 + 26	Ferrioxamine B
IutA	74	Fe^{3+}-aerobactin
76kDa	76	Fe^{3+}-aerobactin in EIEC[a] and *Shigella flexneri*

[a] Enteroinvasive *E. coli*

2.3.8.b. Capsules

Capsules are surface-exposed layers which protect bacteria against non-specific host defences, including the action of complement and phagocytes. Capsules are generally acidic polysaccharides with a regular structure, made up of repeating oligosaccharide units. Certain strains express capsules constitutively (group I), while others only produce capsules when grown at temperatures above 25 °C (group II) (Ørskov *et al.* 1984).

Strains with group I capsules, which are expressed at all temperatures, belong to serogroups O8, O9 and O20. These capsules are encoded by genes located on the *E. coli*

chromosome (Schmidt *et al.* 1977; Laakaso *et al.* 1988). The capsular acid polysaccharides of group I usually have large-repeating units, tetra to hexasaccharides, the most common acid components are hexuronic acid and pyruvate. The group can be subdivided on the basis of the sugar composition of their capsular polysaccharides, some of which do not contain amino sugars (group Ia), while others contain one or two amino sugars per repeating unit (group Ib). The polysaccharides of group Ia resemble the capsular polysaccharides of *Klebsiella*.

Strains with group II capsules, which are only expressed at about 25 °C and above, occur in many O-serogroups, group II capsules of *E. coli* are also chromosomally determined. These polysaccharides have smaller repeating units that group 1 capsules, usually di- or trisaccharide. The acidic components are more diverse than those of group I, with hexuronic acids, N-acetylneuraminic acid, 3-deoxy-manno-D-octulosonicacid (KDO), mannosaminuronic acid or phosphate as possible representatives. Some group II capsular polysaccharides are similar in structure and general characteristics to those of *Neisseria meningitidis* and *Haemophilus influenzae* (Jann and Jann 1990, 1992).

Some *E. coli* strains have capsular polysaccharides, chemically similar to group II capsules but are not temperature regulated. The capsules (K3, K10, K11, K54, K96, K98), although closely related to group II capsules, appear to belong to a subclass of group II. It is suggested that they may be termed group II capsules, although they may turn out to be a heterogeneous group.

More than 70 distinct *E. coli* capsular polysaccharide antigens are recognised and the structures of most have been elucidated.

Escherichia coli is a major cause of neonatal meningitis, and over 80% of these cases are caused by strains expressing capsular serotype K1 (McCraken *et al.* 1974).

2.3.8.c. Haemagglutination

Haemagglutination by strains of *E. coli* was first described by Guyot *et al.* (1908) who found that 12 of 18 strains agglutinated red blood cells from one or more of 13 animal species. Rosenthal *et al.* (1943) showed that haemagglutining bacteria may also agglutinate leukocytes, sperm, yeasts and pollen. Subsequently Duguid *et al.* (1955) distinguished three groups of *E.coli* with different patterns of haemagglutinating activity with the red cells of different animal species and a fourth group that did not agglutinate erythrocytes. All strains in Group I showed the same pattern of activity, agglutinating most species of animal red cells including human, sheep and goats but not ox. The optimal culture conditions for this haemagglutinating activity were growth in static liquid broth at 37°C. The haemagglutining activity of this group of *E. coli* correlated with the possession of type 1 fimbriae and this activity was inhibited by the presence of mannose (Duguid *et al.* 1966; Ottow 1975). The strains belonging to groups II and III of Duguid *et al.* (1955) showed a variety of reactions with different red cells; some strains reacted strongly with ox, sheep and human red blood cells, and one of 18 strains agglutinated only human cells (Old 1985). The optimal culture conditions for this type of pattern were growth on agar at 37°C and tested at 3-5°C. The reaction was unaffected by the presence of mannose (Duguid and Gillies 1957). This kind of haemagglutinin was described as mannose-resistant and eluting. Groups II and III differed from one another in that strains of the former possessed fimbriae, whilst the latter did not.

The haemagglutinating properties of *E. coli* were interesting after the discovery that this activity can be related to the presence of particular K antigens (capsular),

adhesiveness for intestinal epithelium, ability to colonise the upper intestine, and enteropathogenicity in man (Duguid 1964; Evans *et al.* 1975; McNeish *et al.* 1975; Evans *et al.* 1977; Ørskov and Ørskov 1977b). Different strains of *E. coli* may possess one or more kinds of haemagglutinin that require different culture conditions, different techniques and species of erythrocytes. For some of the haemagglutinating activities the chemical basis has been elucidated. For example, type 1 fimbriae are known to bind to mannose receptors on eukaryotic cell membranes, such that adding D-mannose to a suspension of *E. coli* expressing type 1 fimbriae saturates the mannose binding sites and prevents haemagglutination (Aronson *et al.* 1979; Goldhar *et al.* 1986; Kukkonen *et al.* 1993). Certain strains of EAggEC have also been reported to have haemagglutinating properties (Qadri *et al.* 1994).

2.4. Enteroaggregative *E. coli*

Enteroaggregative *E. coli* (EAggEC) have been characterised by the aggregative adherence (AA) phenotype during adhesion to HEp-2 cells, where the bacteria are seen in 'stacked brick' aggregates attaching to the HEp-2 cells and usually to the glass between cells (Fig. 7; Nataro *et al.* 1987). Putative pathogenic properties include an enterotoxin similar to the heat-stable enterotoxin of enterotoxigenic *E. coli* (ETEC), putative haemolysins and toxins plus various types of fimbriae that may be involved in the adhesion process. The role of these factors in the production of disease is unclear.

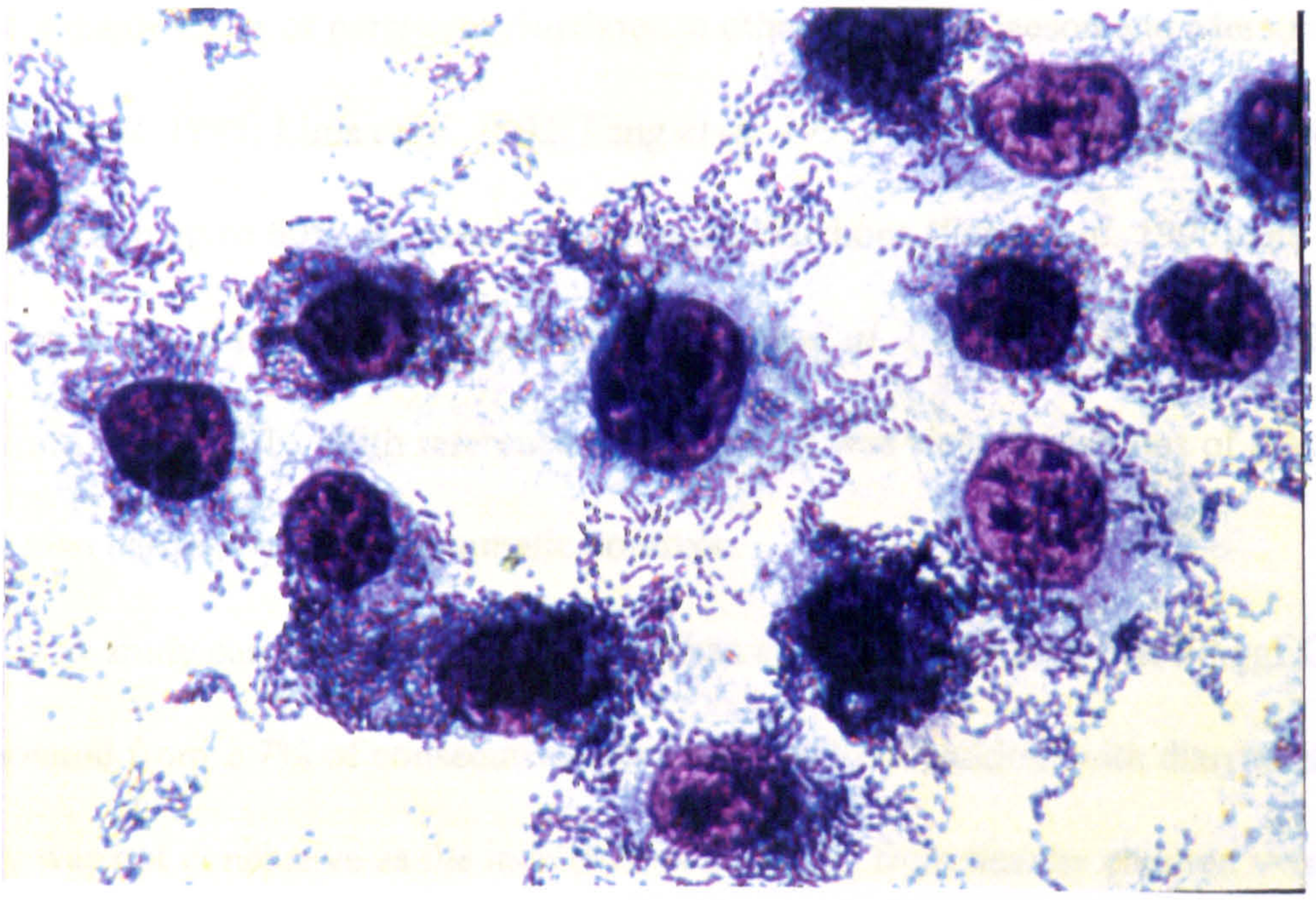


Fig. 7 Light micrograph showing aggregative adherence of *E. coli* strain E40104 to HEp-2 cells

2.4.1. Epidemiology of EAggEC

Nataro *et al.* (1987) were the first group to propose that EAggEC may be pathogenic after detecting EAggEC more frequently in faeces of children with diarrhoea than in those of controls. The earliest epidemiological reports showed that EAggEC were most prominently associated with persistent diarrhoea, lasting more that 14 days in children (Bhan *et al.* 1989a, 1989b; Wanke *et al.* 1991).

The importance of EAggEC in diarrhoeal disease also seems to vary geographically (Table 12). On the Indian subcontinent six studies have demonstrated the importance of EAggEC in paediatric diarrhoea (Bhan *et al.* 1989a, b, c; Bhatnagar *et al.* 1993; Paul *et al.* 1994; Kang *et al.* 1995). Furthermore, EAggEC have also been shown to be a major cause of persistent diarrhoea in other studies (Claeson and Merson 1990; Wanke *et al.* 1991; Lima *et al.* 1992; Fang *et al.* 1995) . In Brazil, EAggEC have been implicated in up to 60% of cases of prolonged diarrhoea (Fang *et al.* 1995) and in Mexico, Chile, Bangladesh and Iran (Matthewson *et al.* 1987; Nataro *et al.* 1987; Cravioto *et al*; 1991). With reference to Table 12 it was clear that strains of EAggEC were also carried by nonsymptomatic controls.

A study carried out in England by Chan *et al.* (1994) found that EAggEC could be isolated from 2.7% of consecutive stool samples from children with diarrhoea, but this study was not conclusive as the incidence of EAggEC from healthy children was not determined. Another study investigating the incidence of EAggEC in England was carried out by Smith *et al.* (1994) examining strains of serogroup O44 isolated from adults with diarrhoea in different countries over a 25 year period. It was thought that strains belonging to serogroup O44 were regarded as EPEC. Most strains of *E. coli* O44 expressed an aggregative phenotype, and were from both outbreaks and sporadic

cases of diarrhoeal disease. One outbreak occurred in a hospital involving elderly patients. EAggEC have also been described as a cause of travellers diarrhoea with patients returning to the U.K. from various locations (Brook *et al.* 1994; Scotland *et al.* 1994). However a study carried out in America showed that although carriage rates of EAggEC were high in adults with travellers diarrhoea, there was no association between these organisms and disease (Cohen *et al.* 1993). Recent observations have linked EAggEC infection with prolonged diarrhoea in HIV patients (Mayer and Wanke 1995; Kotler *et al.* 1995; Wanke *et al.* 1998).

The epidemiological characteristics of EAggEC e.g. likely sources, reservoirs of infection, routes of transmission, seasonality, and age distribution are largely unknown. Most studies in which EAggEC have been implicated as causes of diarrhoea have isolated the organism from infants and small children, yet volunteer studies and outbreak investigations suggest that school-age children and adults are also susceptible (Smith *et al.* 1994; Smith *et al.* 1997; Itoh *et al.* 1997).

Perhaps of greater significance than the association of EAggEC with diarrhoea may be the recent data from Fortaleza, Brazil linking EAggEC with growth retardation in infants (Steiner *et al.* 1998). This study showed that EAggEC were isolated from the faeces of infants associated with below average height or weight. This association has been a discussion point for *Cryptosporidium* (Checkley *et al.* 1997).

Table 12. *Epidemiology of EAggEC infections*

Country	Incidence of EAggEC	Age affected	Year	Author
US Travellers	Patients 15%	varied	1985	Matthewson *et al.*
Chile	Patients 33.2% Controls 14.9%	? children	1987a	Levine *et al.*
Chile	Patients 35.7% Controls 22.7%	72% < 12m 100% <24m	1988	Nataro *et al.*
India	Patients PD 29.5% Patients AD 12.8% Controls 8.8%	< 3 yr	1989	Bhan *et al.*
Brazil	Patients 10% Controls 8%	< 1 yr	1989	Gomes *et al.*
	Patients PD 20% Patients AD 8% Controls 5%	< 2yr	1991	Wanke *et al.*
Mexico	Patients 47.8 %	< 2 yr	1991	Cravioto *et al.*
India	Patients 14.8%	< 1 yr	1991	Haider *et al.*
India	Patients 8.3 % Controls 1.9%	< 6 m	1992	Ghosh *et al.*
England	Patients 3% Controls 0%	90% < 2 yr	1994	Chan *et al.*
Italy	Patients 6.25 % Controls 1.8%	1-106 m	1994	Morrelli *et al.*
Yugoslavia	Patients 63% Controls 0 %	< 180 days	1996	Cobeljic *et al.*
Germany	Patients 2 % Controls 0%	< 5 yr	1997	Hupperetz *et al.*
Japan	Patients 56%	< 15 yr	1997	Itoh *et al.*

PD - persistent diarrhoea

AC - acute diarrhoea

2.4.2. Clinical aspects of EAggEC infection

The clinical features of EAggEC diarrhoea are increasingly well defined in outbreaks, sporadic cases, and the volunteer model. Typically the disease is manifested by watery, mucoid, secretory diarrhoea with a low grade fever and little or no vomiting (Bhan *et al.* 1989a; Paul *et al.* 1994). Bloody diarrhoea has also been reported (Bhan *et al.* 1989a; Cravioto *et al.* 1991); this feature may be strain dependent. In volunteers infected with EAggEC strain O42 (O44:H18), stools were mucoid, of small volume and without occult blood or faecal leukocytes; all patients remained afebrile. The incubation time in these volunteers was 8-18 hours (Nataro *et al.* 1995).

The duration of EAggEC diarrhoea is the most striking feature. In a study done by Bhan *et al.* (1989b) in India, the mean duration time was 17 days, longer than that associated with any other pathogen, for example ETEC, EPEC or *Salmonella* sp. This study showed, of 41 cases of diarrhoea, 12% of patients suffered from fever, 12% had bloody diarrhoea, 7% suffered vomiting and 44% suffered with diarrhoea for more than 14 days.

A large percentage of patients excreting EAggEC have detectable faecal lactoferrin and supranormal levels of interleukin-8 in their faeces (Steiner *et al.* 1998). This observation suggests that EAggEC infection may be accompanied by mucosal inflammation although most patients lack clinical evidence of inflammation.

2.4.3. Heterogeneity of EAggEC strains

EAggEC have been associated with nearly 90 different O:H serotypes. (Vial *et al.* 1988; Knutton *et al.* 1992; Qadri *et al.* 1994; Debroy *et al.* 1994; Chart *et al.* 1995, Law and Chart 1998). Some of these strains were O and /or H-untypable (Vial *et al.* 1988; Yamamoto *et al.* 1992). Some strains of EAggEC belong to serogroups previously recognised as EPEC e.g. O44, O86, O111, O126, O128 (Pal and Ghose 1990; Scotland *et al.* 1991; Scotland *et al.* 1993; Chan *et al.* 1994; Debroy *et al.* 1995; Law 1994; Schmidt *et al.* 1995a;).

Whilst some serogroups such as O44, O86 and O126 are predominantly associated with EAggEC, other strains such as those belonging to serogroup O128 and O111 may also be considered as EPEC, VTEC and/or ETEC. (Scotland *et al.* 1991; Knutton *et al.* 1992; Scotland *et al.* 1993).

EAggEC carry a plasmid of approximately 60 kDa which confers the AA phenotype to strains of *E. coli* . Vial *et al.* (1988) showed that 40 EAggEC strains expressed more than one plasmid pattern, 5 strains possessed a single plasmid of 55-65 MDa, 3 strains possessed multiple plasmids in the range of 55-65 MDa, and 32 strains carried multiple plasmids, one of which was 60-70MDa. Other studies have shown that possession of a 60MDa plasmid is essential for aggregative adherence and the expression of EAggEC heat-stable toxin termed EAST1 (Savarino *et al.* 1991), aggregative adherence fimbriae/I (Nataro *et al.* 1992) and aggregative adherence fimbriae/II (Czechulin *et al.* 1997). These studies showed that loss of the plasmid results in the loss of aggregative adherence and the expression of EAST1, AAF/I and AAF/II.

2.4.4. Identification of EAggEC

Although expression of the "stacked brick" pattern of adhesion to HEp-2 is used as the gold standard for the identification of EAggEC, several other methods have been suggested to differentiate EAggEC from other strains of *E. coli.*

Albert *et al.* (1993) reported that 41 strains of EAggEC grown in broth culture at 37 °C developed a scum (also called pellicle) on the surface of the broth whereas 61 control *E. coli* strains, including ETEC, EPEC, EIEC, VTEC, DAEC, and non-pathogenic strains, did not. Light microscopy of this 'scum' showed large clumps of bacteria. Later studies carried out by Chart *et al.* (1997) showed that only 72% of 29 EAggEC strains produced a pellicle when grown in broth culture and it was concluded that pellicle formation should not be used as a diagnostic method for the differentiation of EAggEC strains.

Beachey (1981) has shown a link between haemagglutination and the ability to adhere to intestinal cells by bacterial pathogens such as ETEC from human and animal infections. Haemagglutination is a common phenomenon and is indicative of the presence of adhesins on the bacterial surface. Scotland *et al.* (1991) suggested that a feature particular to EAggEC strains was the ability to cause mannose-resistant haemagglutination (MRHA) of rat erythrocytes using slide agglutinations. In this study, 30 EAggEC isolated from paediatric diarrhoeal faeces all gave MRHA but EAggEC strains from non-diarrhoeagenic patients did not (Scotland *et al.* 1991). There was variation in the haemagglutination properties using erythrocytes from other species including bovine, guinea-pig and human. It was speculated that MRHA of rat erythrocytes may be a means of differentiating EAggEC from other pathogenic *E. coli.*

Another study carried out by Qadri *et al.* (1994) using 41 strains of EAggEC and erythrocytes from animal species: guinea-pig, rat, mouse, human, sheep and cattle, revealed variation in the haemagglutination of the EAggEC strains, concluding that EAggEC are a very heterogeneous group. MRHA of rat erythrocytes was exhibited by 78% of the EAggEC strains concluding that this haemagglutination property could not be used as a diagnostic method for EAggEC.

Several DNA probes have been developed to identify the genes encoding aggregative adherence. Baudry *et al.* (1990) produced a probe which identified a 1 kb fragment from the 60 MDa plasmid (pCVD432) of EAggEC strain 17-2 (O3:H2). This probe has been shown not to be linked to the genes encoding aggregative adherence. This was originally described as being 89% sensitive and 99% specific, however, a later study showed that this probe failed to detect 32% of strains showing aggregative adherence to HEp-2 cells (Faruque *et al.* 1992) suggesting genetic heterogeneity among EAggEC strains. Probe CVD432 is the most widely used EAggEC probe. A further DNA probe was developed, derived from the 60 MDa plasmid, not sharing any homology with the probe described previously (Debroy *et al.* 1994). The authors describe this probe as 93% sensitive and 98% specific, but few epidemiological studies have used this probe.

2.4.5. Animal models for EAggEC infection

Several animal models have been used to investigate the pathogenic mechanisms expressed by strains of EAggEC. In the rat and rabbit intestinal loop experiments, four EAggEC strains (17-2; O3:H2, 73-1; O?:H33, O42; O44:H18, 221; O78:H33) caused significant histopathological changes, shortening of the villi, haemorrhagic necrosis of the villus tips and a mild inflammatory response with oedema and mononuclear infiltration (Vial *et al.* 1988).

Using the reversible intestinal tie adult rabbit diarrhoea (RITARD) model, rabbits challenged with an EAggEC strain developed severe diarrhoea, and EAggEC were grown from the intestinal mucosa of the test rabbits. Examination of these mucosa demonstrated moderate stunting of villi and nuclear fragmentation (Tickoo *et al.* 1992).

A study was carried out by Tzipori *et al* (1992) investigating the effect of two strains of EAggEC on gnotobiotic piglets. The two strains, 17-2 (O3:H2) and 221 (O78:H33), caused diarrhoea or death in the majority of piglets. Results of the histopathology showed moderate hyperaemia of distal small intestine and caecum, swelling of the distal small intestinal villi and aggregates of bacteria stacked together in a gel-like matrix overlying the epithelium. These findings suggest that EAggEC infection of animals produces intestinal lesions distinct from those caused by other pathogenic *E. coli.*

2.4.6. Adherence of EAggEC to intestinal tissue

The site of EAggEC infection in the human intestine has yet to be clearly demonstrated. Knutton *et al.* (1992) showed that 44 different EAggEC strains adhered to colonic mucosa *in vitro* but none of six isolates adhered to ileal mucosa. Using a single isolate of EAggEC Yamamoto *et al.* (1991), found that this strain adhered to native jejunal and ileal mucosa in low levels, but high levels of adherence were found on formalin-fixed colonic mucosa. This study also investigated the adherence of this EAggEC strain to unfixed jejunal tissue from a child and found high levels of adherence to the overlying mucus but, adherence was poor on the villus epithelium. Hicks *et al.* (1996) used organ cultures of small and large intestinal mucosa from children to examine adherence of EAggEC to the human intestine. Five EAggEC strains isolated from infants with diarrhoea showed variable patterns of adhesion to the jejunum, ileum and colon, two adhered to the small intestine only, and three adhered to both small and large intestinal tissue. Analysis of these adherent strains by electron microscopy showed the bacteria adhered in an aggregative pattern to a thick mucus layer above the intact enterocyte brush border. EAggEC adherence was associated with microvillus vesiculation, enlargement of crypt openings and epithelial cell extrusions. These results demonstrate that strains of EAggEC can adhere to the intestines of humans.

2.4.7. Adherence of EAggEC to cell cultures

The adhesion assay used by Cravioto *et al* (1979) involved mixing bacteria with cultured HEp-2 cells for three hours, non-adherent bacteria were removed by washing and the adherent organisms were fixed and stained. Examination of the adherent bacteria was done by light microscopy and a pattern of adhesion was noticed that gave rise to the classification of enteroaggregative *E. coli* (Nataro *et al.* 1987). Enteroaggregative *E. coli* are characterised by their pattern of adhesion to HEp-2 cells commonly known as 'stacked brick' formation.

Adhesion is commonly associated with the expression of fimbriae. Haemagglutination can be used to assess the possession of fimbriae using erythrocytes from many different animal species. Old *et al* (1989) examined two EAggEC strains which gave mannose-resistant haemagglutination with rat erythrocytes. This haemagglutination was associated with the possession of fine 2-2.5 nm diameter fibrillar structures on the bacterial surface which were detected by immunogold staining using a specific antiserum (Old *et al.* 1989). Vial *et al.* (1988) have also detected fimbriae on strains of EAggEC that adhered to rabbit intestinal mucosa despite the presence of mannose.

A study carried out by Knutton *et al.* (1992) on 44 strains of EAggEC described four morphologically distinct fimbrial types; 6-7nm rod-like fimbriae, 5-6nm rod like fimbriae, 2-3nm fibrillar fimbriae and bundles of fine fibrils. Although combinations of one or more fimbrial types were expressed by strains of EAggEC, in 43 of the 44 EAggEC strains fibrillar bundles were observed.

Collinson *et al.* (1992) reported a strain of EAggEC expressing fimbriae with a subunit size of 18kDa. These fimbriae shared epitopes with a 17 kDa subunit of a fimbrial structure expressed by strains of *Salmonella enteritidis*. A study by Chart *et al* (1994) showed that strains of EAggEC belonging to serotype O126:H27 reacted with a commercial kit (SEFEX, Central Veterinary Laboratory; 6.7.6.) designed to detect strains of *Salmonella enteritidis*. This kit consisted of a monoclonal antibody against the SE14 fimbrial subunit of *S. enteritidis* attached to latex beads which agglutinated in the presence of the fimbriae. The antigen reacting with the SEFEX kit that was expressed by the EAggEC strains was shown to be an outer membrane protein with a subunit mass of 18 kDa (Chart *et al.* 1994). Further studies showed that EAggEC strains belonging to serotype O44:H18 expressed an 18 kDa protein antigenically similar to the 18 kDa of EAggEC strains belonging to serotype O126:H27, which did not react with the SEFEX kit. Immuno-electronmicroscopy revealed that the epitopes of the 18kDa expressed by EAggEC strains of serotype O126:H27 were surface exposed whereas the protein was not surface exposed in EAggEC strains belonging to serotype O44:H18 (Chart *et al.* 1995).

2.4.8. Aggregative adherence fimbriae/I (AAF/I)

Nataro *et al.* (1992) showed that prototype EAggEC strain 17-2 (O3:H2) expressed bundle forming pili [sic] with a diameter of 2 nm (Nataro *et al.* 1992). Examination of the bacteria by transmission electron microscopy showed that these bundles of fimbriae connected the bacteria together in aggregates and were termed aggregative adherence fimbriae I (AAF/I). The genes encoding these fimbriae were located on a 60MDa plasmid responsible for the aggregative adherence to HEp-2 cells and haemagglutination of human erythrocytes. Single-insertion genetic mutations into the region caused the loss of all three characteristics.

The fimbrial subunit was estimated at 14 kDa by the examination of surface shear preparations. Sera from volunteers fed strain 17-2 reacted with this protein suggesting that the fimbrial subunits were antigenic *in vivo*.

Cloning of the genes encoding AAF/I showed that the expression of the fimbriae requires two unlinked regions on the 60MDa plasmid of EAggEC strain 17-2 (Nataro *et al.* 1993). These two regions are separated by a 9 kb sequence not required for the expression of the fimbriae, which encodes the EASTI toxin. This positioning of the gene sequences suggests that the putative virulence genes form an important cluster on the plasmid (Nataro *et al.* 1993). Region 1 was sequenced and contained four contiguous open reading frames (ORF) designated a*gg D, C, B, A* (Savarino *et al.* 1994). It was proposed that *aggA* encodes a major fimbrial subunit with a molecular weight of 15.4 kDa, having homology with the major subunit of F41 fimbriae. Homology data suggests that a*ggC* encodes an outer membrane protein usher and a*ggD* is a periplasmic chaperone. A function for the *aggB* product is not yet known; transposon mutagenesis of

this gene causes weak expression of the aggregative phenotype and abolishes haemagglutination properties (Savarino *et al.* 1994).

The *agg* gene cluster shares many similarities with certain other fimbrial operons including CFA/I of enterotoxigenic *E. coli,* where the structural and regulatory genes are non-contiguous. Also the gene encoding EASTI is located between regions 1 and 2 like the gene for heat-stable toxin is located between the genes required for the expression of CFA/I in ETEC (Nataro *et al.* 1994).

The *agg*A gene cluster, which appears to encode that major fimbrial subunit of AAF/I, is located down stream of other assembly genes. This organization is similar to the operons involved in the biogenesis of each of the adhesins in the Dr adhesin family, including F1845 fimbriae (Bilge *et al.* 1989), Dr haemagglutinin (Swanson *et al.* 1991), AFA-I (Labigne-Roussel *et al.* 1985) and AFA-III (Le Bouguenec *et al.* 1993). These are distinguished from operons encoding determinants of many other fimbriae, whereby the major fimbrial subunit gene is at or near the 5' end of the operon (Krogfelt 1991).

2.4.9. Aggregative adherence fimbriae/II (AAF/II)

EAggEC strain O42 was isolated from a child in Peru (Nataro *et al.* 1995) and was shown to be pathogenic in volunteer studies. This strain belongs to serotype O44:H18, which is commonly associated with strains isolated from patients suffering from diarrhoea (Smith *et al.* 1994). Fimbriae were observed after examination of this strain by electron microscopy and were termed aggregative adherence fimbriae/II (AAF/II) (Nataro *et al.* 1995). AAF/II are antigenically and morphologically distinct from AAF/I, they form rodlike, filamentous structures with a diameter of 5 nm

(Czeczulin *et al.* 1997). The AAF/II fimbrial gene has been identified; it has no homology with known fimbrial subunits of *E. coli,* including AAF/I. The gene encoding the fimbriae is termed *aafA.* Not only is the fimbrial subunit of AAF/II distinct from AAF/I, but the organisation of the fimbrial biogenesis genes is different: AAF/I features the organisation typical of the Dr family adhesins, whereas the AAF/II cluster features a chaperone homology immediately upstream of the fimbrial subunit gene (Czeczulin *et al.* 1997).

An unusual, conserved feature of the AAF/I and AAF/II fimbrial subunits is their high isoelectric points (10.3 and 10.1 for AggA and AafA, respectively). This is in contrast to the isoelectric points of the Dr family members (approximately 6.0). This high isoelectric point may result in a net positive charge to the surface of EAggEC strains at physiological pH, a phenomenon which has been associated with adherence to glass and biofilm formation (Jucker *et al.* 1996).

2.4.10. Toxins of EAggEC

2.4.10.a. Enteroaggregative heat-stable enterotoxin 1 (EAST1)

Some EAggEC strains elaborate a low molecular weight (4.1 kDa) partially heat-stable, plasmid encoded enterotoxin that has 50 % sequence homology with *E. coli* STa although it is genetically and immunologically distinct. (Savarino *et al.* 1991). This enterotoxic factor has been named EAST1, and is demonstrable in an *in vitro* rabbit intestinal model (Savarino *et al.* 1991) causing fluid secretion by the activation of guanylate cyclase. EAST1 is not detected in the infant mouse test like *E. coli* STa.

Using the prototype EAggEC strain 17-2 (O3:H2), a 4.4 kb fragment of the plasmid was identified that encoded for enterotoxin production. Analysis of this fragment showed a region of DNA encoding a cysteine-rich polypeptide of 38 amino acids with a molecular weight of 4100 (Savarino *et al.* 1993). The gene encoding EAST1 was termed *astA*.

The predicted amino acid sequence of EAST1 suggested that it represents a subfamily of *E. coli* heat-stable enterotoxins (Savarino *et al.* 1993) and the marked differences in the nucleotide level suggest that EAST1 has evolved convergently from a different genetic origin. EAST1 possesses 4 cysteine residues in the C-terminal tridecapeptide whereas STa has 6 cysteine residues.

A probe was derived from the plasmid pSS106, from which the *ast*A sequence was excised by *Xmu*I and *Nru*I restriction (Savarino *et al.* 1996). This probe hybridised with strains that expressed EAST1.

Savarino *et al.* (1993) found that 57% of EAggEC possessed the gene for EAST1. Nataro *et al.* (1995) examined four EAggEC strains; only two of them possessed the gene for EAST1, one of these was virulent in adult volunteers. The production of the toxin alone is therefore insufficient to cause disease in adults.

To determine if EAST1 is exclusively associated with EAggEC a collection of other diarrhoeagenic *E. coli* was analysed with the DNA probe. All of the O157:H7 VTEC strains, 41% of ETEC strains, 22% EPEC strains, and 13% of DAEC were positive for hybridisation with the EAST1 probe i.e. *ast*A+. Thirty-eight percent of intestinal *E. coli* isolates from children without diarrhoea, which were otherwise negative for *E. coli* virulence determinants, hybridised with the *ast*A probe. In contrast, the *ast*A probe did not hybridise with any of the EIEC. Other serotypes of VTEC e.g. 89%

O26:H11, 52% of 23 non-O157/O26 serogroups were *ast*A positive. In the ETEC strains the prevalence of *ast*A positivity varied according to toxin phenotype. LT+/STa+ 53%, and LT+ only 45%. The EAST1 genotype is not restricted to EAggEC and its role in EAggEC pathogenesis is not clear.

2.4.10.b. EAggEC Haemolysin-like toxin and contact haemolysin

A protein toxin of approximately 120 kDa was described in strains of EAggEC by Baldwin *et al.* (1992) as a putative haemolysin since antibodies raised against the C-terminal region of *E. coli* α-haemolysin bound to this protein (Baldwin *et al.* 1992). However, haemolytic activity has not been described for this toxin. Other workers have shown that EAggEC produce α-haemolysin which may be responsible for the cell detaching activity often observed (Gomes, 1995)

A contact haemolysin was identified in 37 of 45 EAggEC strains. This haemolysin had the greatest effect on sheep erythrocytes, as compared with human, rabbit, rat and guinea pig red cells. The haemolysin was produced maximally when bacteria were grown in casamino yeast extract broth containing calcium chloride (Haque *et al.* 1994), suggesting that calcium ions were required for haemolytic activity as has been described for *E. coli* α-haemolysin.

2.4.10.c. EAggEC Plasmid-encoded toxin (Pet)

EAggEC strains secrete a protein of 108 kDa that induces the exfoliation of enterocytes in the rat ileal loop model. (Eslava *et al.* 1993). This cytotoxin when

introduced into the ligated rabbit ileal loop model caused villus epithelial cell death with infiltration of inflammatory cells into the lamina propria. (Eslava *et al.* 1993).

Nataro *et al.* (1995) used four well characterised EAggEC strains (O42, 17-2, 34b, and 221) to infect adult volunteers where only strain O42 resulted in diarrhoea. Two strains (O42 and 17-2) hybridised with a DNA probe for EASTI and all expressed a 108 kDa cytotoxin. Although expression of EAggEC α-haemolysin was not tested, it appears as though EAggEC may potentially coexpress more than one toxin type whose combined effect could play an important role in EAggEC pathogenicity in humans.

DNA sequence analysis of the plasmid borne gene encoding this protein suggests that the toxin is a member of proteins called autotransporters because their secretion through the bacterial outer membrane is mediated by the carboxy terminus of the molecule (Eslava *et al.* 1998). This toxin is highly homologous to the EspP protease of enterohaemorrhagic *E. coli* (Brunder *et al.* 1997) and to EspC of enteropathogenic *E. coli* (Stein *et al.* 1996), an as yet cryptic protein.

2.5. Aims of the research programme

The pathogenic mechanisms of EAggEC are poorly understood, and the research programme was aimed at elucidating virulence properties expressed by these organisms. The key objectives were as follows:-

1) To investigate the mechanisms by which strains of EAggEC adhere to eukaryotic cells such as HEp-2, in the characteristic 'stacked brick' formation.

2) To examine strains for novel adhesins, including outer membrane proteins and other cell-surface associated components.

3) To analyse strains of EAggEC for haemolysins and other toxins which may play a role in the pathogenesis of diarrhoeal disease.

4) To examine strains of EAggEC for established pathogenic mechanisms such as:-

a) The ability of strains to acquire iron.

b) The expression of capsular polysaccharides.

c) The ability of strains to survive in serum.

3. MATERIALS AND METHODS

3.1 Bacteria

The strains of *E. coli* used in this study (6.1.2) were selected based on various criteria. One set of strains (6.2.1.a.i) was used for preliminary research into EAggEC. A second panel was from the Infectious Intestinal Disease study (6.2.1.a.ii) and comprised strains isolated from the faeces of patients suffering from diarrhoea and apparently healthy people in the UK. A third panel (6.2.1.a.iii) of EAggEC contained strains of *E. coli* isolated from an outbreak in Bangladesh, and four outbreaks in the UK. A fourth set of strains (6.1.2.b) comprised positive and negative control strains for the various tests used in this study.

Strains of *E. coli* were serotyped by methods established in the Laboratory of Enteric Pathogens, based on the serotyping scheme of Kauffmann (1947). Serotyping was based on the heat-stable lipopolysaccharide (somatic or O) antigens, the flagellar (H) antigens. The current *E. coli* serotyping scheme comprises 173 antigenically distinct O serogroups and 56 H types. Strains, which did not react with specific typing sera, were considered as O? or H?.

Somatic antigens are typed by an agglutination assay with O-specific rabbit antibodies. Flagellar antigens are typed by a tube agglutination test.

All bacterial strains were stored on Dorset's Egg agar slopes (6.2.4.) at room temperature.

3.2 Culture Media

The Media Department prepared the media used in this study, and comprised:-

Nutrient Agar (6.2.1.)

Nutrient broth (6.2.2.)

MacConkey agar (6.2.3.)

Dorset's egg agar (6.2.4.)

L-agar (6.2.5.)

L-broth (6.2.6.)

Peptone Water(6.2.7.)

Tryptone soya broth (6.2.8.)

Colonisation Factor antigen agar (6.2.9.)

3.3 Tissue Culture

Adhesion assays were performed with a HEp-2 cell-line (Flow Laboratories). Cells were maintained in tissue culture flasks (app surface area = 75 cm^2), with Basal Medium Eagle (BME) medium containing amphotericin B, streptomycin and penicillin (Sigma Chemical Co. Ltd.) (6.3.3.). Cell lines were propagated on a weekly basis as follows:-

3.3.1.a. Maintenance of HEp-2 cells

1. Spent medium was discarded and cell monolayers washed (x3) with PBS.

2. Cells were covered with 10 ml of PBS containing 0.25% (w/v) trypsin for 1 min.

3. Flasks were incubated at 37 °C until HEp-2 cells became dislodged from the flask wall with a sharp tap to the flask.

4. The HEp-2 cells harvested from one tissue culture flask were suspended in 5 ml of BME medium and 2 ml of this was used to make a new cell line. The remaining cells were discarded or used to prepare HEp-2 cell adhesion tests.

3.3.1.b. HEp-2 Adhesion tests

Principle: Certain strains of *Escherichia coli*, which adhere to the human gut during pathogenesis also adhere to HEp-2 cells *in vitro*.

The HEp-2 cell adhesion tests were performed using cells seeded onto glass coverslips in 12-well tissue culture plates (Costar) based on the method of Cravioto (Cravioto *et al.* 1979).

1. Sterile coverslips were placed into each of 12 wells in a (Costar) tissue culture plate. One ml cell suspension (3.3.1.a.) was mixed with 20 ml of BME medium and 1.5 ml of suspension placed in each well.

2. Plates were placed in a plastic box and purged for 1 min with a gas mixture comprising 5% CO_2 and 95% air. Boxes were incubated at 37 °C for two days.

3. For the HEp-2 cell adhesion test, cells in tissue culture wells were washed (x3) with PBS and covered with 1.5 ml of BME without antibiotics (6.3.3).

4. To each well 50 µl of bacterial suspension (6.3.7.) was added prior to incubation at 37 °C for 3 hr.

5. After 3 hr, cell monolayers were washed (x3) with PBS and covered again with fresh medium, without antibiotics, followed by a second 3 hr incubation period.

6. After 3 hr, cell monolayers were washed (x3) with PBS (6.5.5) and fixed with absolute methanol (10 mins).

7. Fixed cells were stained with a commercial preparation of 10 % (v/v) Giemsa stain (Merck Ltd.) (6.3.2.) for 30 mins.

8. Coverslips containing stained cells were passed through an acetone/xylene dehydration gradient (6.3.4.) and following immersion in xylene alone, coverslips were mounted onto glass microscope slides using DePeX (Merck Ltd) mountant.

3.3.1.c. HEp-2 tests with formalin-fixed HEp-2 cells

Certain strains of EAggEC were shown to cause detachment of HEp-2 cell monolayers during a HEp-2 adhesion test. The established methodology was modified to determine whether EAggEC would adhere to formalin-fixed HEp-2 cells.

1. HEp-2 cells were grown on glass coverslips for 48 hr in 5% CO_2 /95% air.

2. Spent tissue culture medium was discarded and cells were washed (x3) with sterile PBS.

3. To each well 1.5ml of a 1% (v/v) solution of formalin (Merck Ltd.) in PBS was added, prior to incubation at room temperature for 30 min.

4. Cell monolayers were washed with PBS (as above) and either used for adhesion tests immediately or stored at 4°C immersed in 1.5ml PBS containing 0.1% (w/v) sodium azide.

5. For cell adhesion tests, plates were processed as described (3.3.1.b).

3.3.2. Vero cell test for Verocytotoxin (VT)

Principle: VTEC produce VT in culture supernatants, which causes cytotoxicity in Vero cell monolayers grown *in vitro*.

Verocytotoxin was detected using the method first described by Konowalchuk (Konowalchuk *et al.* 1977), with Vero (African green monkey kidney) cells.

3.3.2.a. Maintenance of Vero cells

The Vero cell line maintained within the LEP was originally obtained from Flow Laboratories.

1. For routine maintenance monolayers were washed twice with sterile PBS, and detached by the addition of 5 ml of 0.25 % (w/v) trypsin in 0.02 % (w/v) EDTA buffer, 37°C for 5-10 min.

2. Cells were suspended in 5 ml of Modified Eagle's Medium (MEM) (6.3.5.) and cell clumps were broken with repeated pipetting.

3. For cell propagation, 2 ml of the resuspended cells were added to 12 ml of growth medium in a tissue culture flask containing 0.15 ml of amphotericin prior to incubation at 37°C.

3.3.2.b. Verocytotoxin Assay

1. For the Vero cell assay, 1 ml of Vero cell suspension (3.3.2.a.) was mixed with 20 ml of tissue culture medium and 0.2 ml volumes dispensed into each well of a 96 well tissue culture plate (Falcon Micro Test II). The plate was sealed with pressure sensitive film and incubated at 37 ° C for 3 days.

2. For initial VT testing, 25 µl of sterile bacterial culture filtrate (3.3.2.c.) was added to duplicate wells, without changing the tissue culture medium. Each plate contained a positive control supernatant and the positive control supernatant incubated at 100°C (15 min). Toxin was titrated by diluting bacterial culture supernatants in a 96-well plate prior to transfer to a Vero cell plate..

3. Following the addition of culture filtrates, plates were resealed and incubated at 37°C for 4 days.

3. After the 4 day incubation, the tissue culture medium was discarded and Vero cells 'fixed' with methanol for 5 min. The methanol was removed and replaced with Giemsa stain (6.6.3.2.). After 45 min the monolayer was washed with distilled water and dried. Changes in the Vero cell monolayers were assessed by light microscopy.

4. In the presence of VT, the entire monolayer becomes detached; however, in the absence of VT, or where the toxin has been heat-denatured, the monolayer remains intact and confluent.

3.3.2.c. Preparation of bacterial culture filtrates

1. Test strains were inoculated into 10 ml of TSB (6.2.8.) in a 250 ml flask.

2. Flasks were incubated at 37°C for 16 h with shaking (120 revolutions per minute).

3. Bacteria were sedimented (12 500 *g*, 10 min) and the supernatant sterilised by Millipore filtration (0.45 µl). Filtrates were used directly for the VT test and heated at 100°C for 15 min.

3.3.3. Y1 cell test for heat-labile toxin (LT)

Principle: Bacteria expressing heat-labile toxin cause cytotonic changes in Y1 cell monolayers.

This test was based on the methods of Donta *et al.* (1974) and Scotland *et al.* (1980), using a 96-well plate assay with Y1 cell monolayers.

3.3.3.a. Maintenance of Y1 cells

Y1 cells are mouse adrenal cells, obtained originally from Flow Laboratories. The propagation of Y1 cells was the same as for Vero cells (3.3.2.a) with the exception that Hams's F10 growth medium (6.3.6.) was used in place of Modified Eagle's Medium.

3.3.3.b. Y1 cell test procedure

In principle, the preparation of Y1 cell monolayers in 96-well tissue culture plates was the same as described for Vero cells (3.3.2.b). In contrast, to the Vero cell assay, culture supernatants were added to Y1 cells and incubated for only 1 day prior to fixing and staining of cell monolayers. In the presence of LT, Y1 cells take on the appearance of a spider, with the cytoplasm forming long appendages. Heating supernatants to 100°C for 15 mins eliminates these morphological changes in the Y1 cells.

3.3.3.c. Preparation of culture supernatants for LT test

Culture supernatants were prepared by growing the selected strains in TSB (6.2.8.) (18 h, 37°C) following the same method as 3.3.2.c.

3.3.4. Test for heat-stable toxin (STa)

Principle: *E. coli* heat-stable toxin present in culture supernatants causes fluid accumulation when injected into the intestines of infant mice.

STa was detected using the infant mouse test described by Dean *et al.* (1972) and involves injecting sterile culture supernatants into the stomach of 3-day old mice. The presence of STa causes fluid accumulation in the intestine.

1. The selected strains were inoculated in 10ml of TSB (6.2.8.) and incubated overnight, 37°C with shaking (100rpm). Cell-free supernatants were prepared by filtration using a syringe and Millipore filter (0.45µm).

2. 0.5 ml of culture supernatant (3.3.2.c.) was mixed with 40µl of 2% (w/v) Pontamine blue, and 100 µl of this preparation was injected directly into the stomach, through the abdominal cavity. The pontamine blue assisted in injecting the preparation into the stomach. Two mice were used per preparation.

3. After 4hr at 30°C the mice were asphyxiated with carbon dioxide and the intestine was examined for fluid accumulation.

4. The intestine distal to the stomach was removed, and the ratio of intestine weight to body weight was calculated. A ratio greater than 0.1 was considered as indicative of STa, ratios of less than 0.08 were negative and intermediate values were considered doubtful and the test was repeated.

The animal experimental work was carried out by members of the Biological Services Department, CPHL.

3.4. Bacterial components

3.4.1. Preparation of outer membranes (OMs)

Principle: Bacteria were disrupted by sonication and cell envelopes separated from cytoplasmic proteins by centrifugation. Outer membranes were separated from inner membrane proteins by selective solubilization of inner membrane proteins with a commercial preparation of sodium lauryl sarcosinate.

1. Bacteria from 150 ml of broth culture or the cell mass from 6 agar plates were used to prepare a cell envelope fraction.

2. Broth cultured bacteria are sedimented by centrifugation (5, 000 x *g*, 30 min, 4°C) using a 6 x 250ml rotor.

3. Sonication pots were placed on ice to cool. For each bacterial preparation to be sonicated, 20 ml of chilled Tris-HCl buffer (6.4.1.a.) was placed into centrifuge tubes standing in ice.

4. Within a safety cabinet, bacteria were suspended in 5ml of ice cold Tris-HCl buffer containing EDTA.Na_2 (6.4.1.b.) and transferred to sonication vessels.

5. Bacteria were disrupted by placing the probe tip approx. 5mm from the bottom of the vessel and making sure the probe was not touching the sonication vessel.

6. The vessel was surrounded with ice water and 150W of power was applied until the bacterial suspension became clear or for up to 3 min. After sonication the bacterial suspensions were poured into pre-cooled 20ml volumes of Tris-HCl.

7. Unbroken bacteria were sedimented by centrifugation (5, 000 x *g*, 4°C, 30 min, no brake) and supernatants containing bacterial envelopes were poured into fresh centrifuge tubes ensuring that whole bacteria did not enter the new tube. The bacterial envelopes were sedimented by centrifugation (45, 000 x *g*, 4°C , 1hr). A completely clear envelope pellet was indicative of the pellet having only very few unbroken bacteria.

8. Supernatants were discarded and centrifuge tubes inverted on a double layer of tissue paper and allowed to drain. 1ml of Tris buffer was added to each tube and each envelope pellet resuspended. Exactly 19ml of Tris-HCl buffer was added to each tube, followed by 0.25 ml of Sarkosyl (BDH) . Tubes were shaken horizontally at 200 rpm for 30min at room temperature.

9. Outer membranes were sedimented by centrifugation (45, 000 x *g*, 4°C, 1h). If the inner membrane was not required then the supernatant was poured into disinfectant.

10. Outer membrane fractions were washed by resuspending the pellets in 1 ml Tris-HCl buffer followed by 19ml (ice-cold) Tris-HCl buffer prior to mixing and centrifugation as before.

11. Following centrifugation supernatants were discarded and outer membrane pellets resuspended in 200 µl Tris-HCl buffer and stored at -20°C.

3.4.2. Preparation of lipopolysaccharide (LPS) for SDS-PAGE

Principle: LPS is isolated from whole bacteria by digesting cellular proteins with proteinase K. LPS is able to migrate towards the anode due to the highly negatively charged lipid A moiety. Carbohydrate chains with the same number of repeating sugar

units comigrate to form discrete bands, giving a typical ladder pattern. Thus very long chains move a short distance into the gels whereas LPS with shorter carbohydrate chains migrate further toward the glycine front.

1. Bacteria were grown on nutrient agar plates (37°C, overnight).

2. A small loopful of each strain was placed into a pre-weighed eppendorf tube and reweighed calculating the weight of bacteria.

3. Bacteria were suspended in solubilization buffer (6.7.1.f.), 30 µl for each mg of bacteria, and incubated at 100°C for 10 min. It was vital to make sure that the bacteria were resuspended evenly i.e whirli-mixing was essential.

4. Solubilization buffer, containing proteinase K (100µg per µl -weighed out with a disposable glass loop) (6.4.2.a.) was mixed with the bacterial suspension 1:1, prior to incubation at 60°C for at 1hr.

5. An SDS-PAGE gel was prepared (3.7.1.) with a 12.5% acrylamide separation gel. Separate glass plates were used as proteinase K can contaminate the glass and interfere with protein profiles that are run later.

6. To each lane of the gel 30 µl of LPS preparation was added, equivalent to 500 µg bacteria, and a current of 50 mAmp applied for 2.25 hr.

7. Following electrophoresis, the gel was placed in fixing solution (6.7.2.a.) for at least 1hr or even overnight.

3.4.3. Detection of Capsules

Principle: Capsular material containing acidic polysaccharides binds the stain Alcian Blue.

Culture supernatants were examined for capsular material by precipitating capsular material with acetone. The pelleted material was suspended in loading buffer and examined by SDS-PAGE.

3.4.3.a. Preparation of stained capsular material

1. 10 ml volumes of broth in 250ml conical flasks were inoculated with test strains. The flasks were incubated at 37 °C overnight with orbital shaking (120rpm).

2. For each strain four x 1ml volumes of broth culture were dispensed into eppendorfs and bacteria sedimented by centrifugation (12,500 x *g*, 2 min).

3. The supernatant was taken off and Millipore filtered (0.45μm) into a bijou bottle.

4. Volumes of culture supernatant (0.33ml) were dispensed into eppendorf tubes, and mixed with approximately 1 ml of acetone. This was left for 5 min with occasional mixing.

5. Eppendorf tubes were centrifuged (12,500 x *g*, 2 min) and the supernatants discarded, tubes were placed in the heating block (37 °C) to vaporise residual acetone.

6. When free from acetone, pellets were suspended in 20 µl of SDS-PAGE solubilising buffer (6.7.1.f.) and incubated at 100 °C for 5 min.

7. 5µl of the sample was applied on to a SDS-PAGE gel with a 12.5% separation gel (3.7.1.).

8. After electrophoresis the gel was placed in fixing solution (6.7.2.a.) for 1 hr. The fixing solution was removed and replaced with 50 ml of oxidising solution (6.7.2.b.).

9. Following oxidation, the gel was washed (3 x 5 min) with distilled water.

10. Gels were stained with a solution containing 0.5% alcian blue for 1 h, with shaking. Gels were destained using distilled water and photographed (3.7.14.).

3.4.4. Determination of Bacterial Surface Charge

3.4.4.a. Migration of bacteria in an electric field

Principle: The surface charge carried by strains of *E. coli* is reflected by the migration of these bacteria in an electric field.

1. Strips of No. 1 filter paper, 1cm wide, were prepared and the ends of each strip were labelled either as anode or cathode.

2. Bacterial suspensions (1 µl: 6.2.6.) were placed in the middle of the paper strips, and after moistening with electrophoresis buffer (PBS) these were laid across the cooling plate of a Multiphor 2117 electrophoresis apparatus (LKB, Uppsala, Sweden),

3. The cooling plate was transferred to a Multiphor 2117 electrophoresis apparatus with the ends of the filter paper placed in the corresponding cathode and anode electrode buffer tanks containing PBS. A constant current of 5 mAmp was applied for exactly 5 mins and the paper strips removed.

4. Strips were placed upside-down onto dried agar plates with the anode/cathode orientation of the strip marked onto the agar plate. After 15 secs the strips were

removed from the agar and the plates incubated (37 °C, overnight), and the direction of migration was assessed.

3.4.5. Hydrophobicity

3.4.5.a. Salt aggregation test

Principle: A measure of bacterial hydrophobicity can be obtained by determining the concentration of ammonium sulphate at which bacteria clump together.

1. A range of concentrations of ammonium sulphate was prepared (6.4.4.a.) to give the following range of molarities: 0.02M, 0.05M, 0.5M, 1.0M, 2.0M, 4.0M.

2. Bacterial suspensions (1 mg wet-weight/ 100µl of PBS) were prepared.

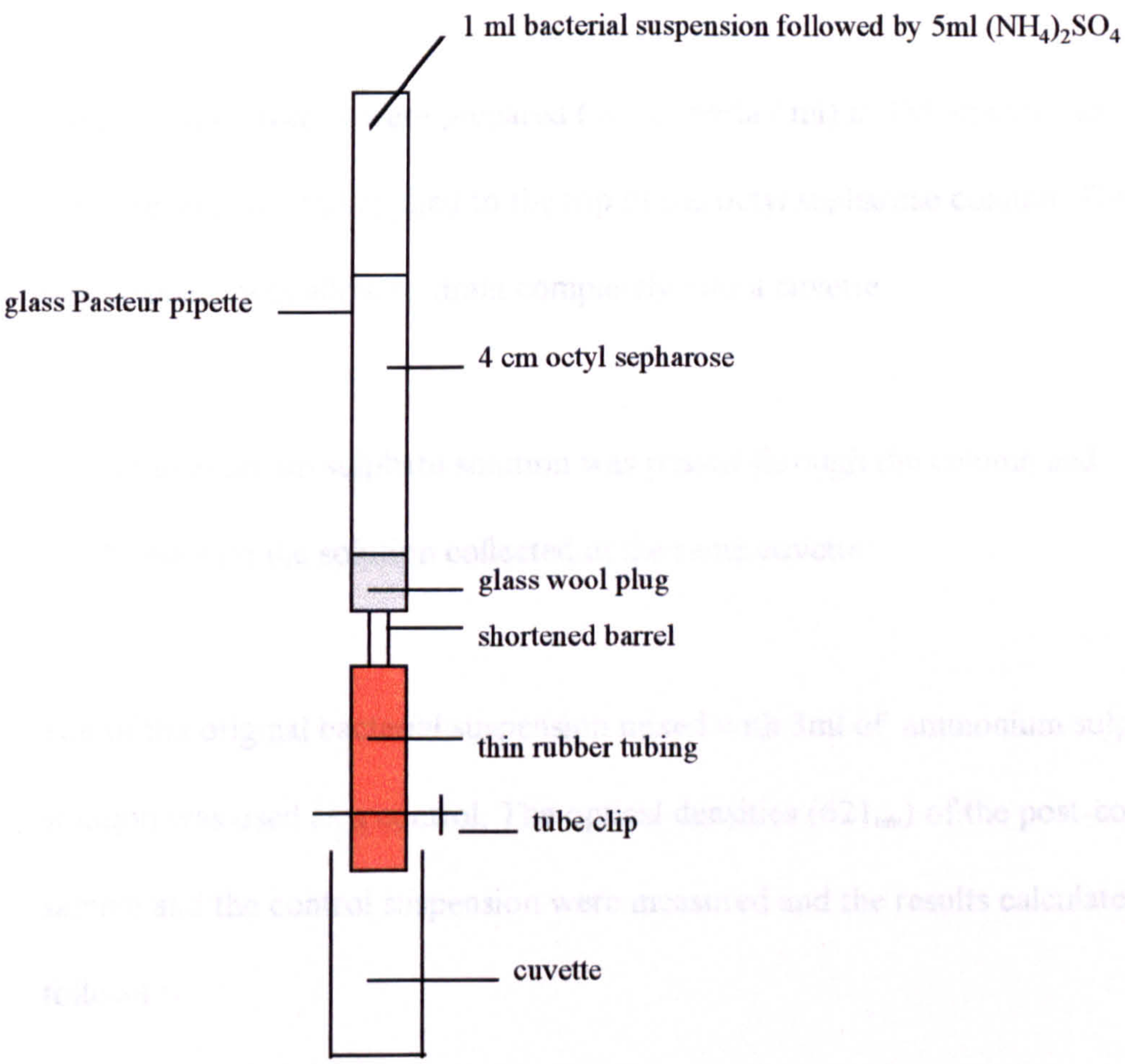
3. Bacterial suspensions (25 µl) were mixed with an equal volume of ammonium sulphate solution in a 96-well plate (Bibby Sterilin Ltd.) with 'V' shaped wells. After shaking (60 rpm) for 30min, wells were examined for bacterial clumping.

3.4.5.b. Hydrophobic Interaction Chromatography (HIC)

Principle: A measure of bacterial hydrophobicity can be determined by observing the passage of bacteria through an octyl sepharose column. Hydrophobic bacteria bind to

the octyl sepharose and hydrophilic bacteria would pass through the column, with the mobile phase.

Fig. 8 Hydrophobic Interaction Chromatography column



1. Columns were constructed from glass Pasteur pipettes. The tapered end was melted in a Bunsen flame and a length of plastic tubing was attached. A tube clip was used to control the flow of the mobile phase. A small glass wool plug was

placed at the constriction of the Pasteur pipette to prevent the octyl sepharose (Sigma) from flowing from the column.

2. The octyl sepharose was equilibrated with several column volumes of 1M ammonium sulphate.

3. Bacterial suspensions were prepared (10^9 bacteria / ml) in 1M ammonium sulphate solution and applied to the top of the octyl sepharose column. The mobile phase was allow to drain completely into a cuvette.

4. 3 ml of ammonium sulphate solution was passed through the column and combined with the solution collected in the same cuvette.

5. 1ml of the original bacterial suspension mixed with 3ml of ammonium sulphate solution was used as a control. The optical densities (621_{nm}) of the post-column sample and the control suspension were measured and the results calculated as follows :-

$$\frac{OD_{621} \text{ collected sample}}{OD_{621} \text{ original sample} + 3 \text{ ml salt solution}} \times 100 = Y$$

% retained = 100 - Y

3.5. Molecular Studies

3.5.1. Isolation of Bacterial Plasmids

3.5.1.a Plasmid isolation using a commercial Kit

Plasmids were isolated from bacteria using a 'mini preparation kit' as manufactured by Hybaid. The kit was designed for rapid isolation and purification of double stranded plasmid DNA and is suitable for plasmids of up to 100 kb. The isolation of larger plasmids was carried out using the method of Birnboim and Doly (1979: 3.5.1.b.) The following method was based on manufacturers instructions.

1. Bacteria in 1.5ml of overnight bacterial culture were sedimented by centrifugation (12, 500 *g*, 5 min), and the pellet resuspended in 50µl pre-lysis buffer with vortex mixing.

2. 100µl of alkaline lysis solution was added directly into the cell suspension and the preparation mixed by inversions until the solution became clear and viscous.

3. 75 µl of neutralising solution were added and the solution mixed by vortexing. This resulted in the formation of a white precipitate consisting of cell membranes, proteins and chromosomal DNA. The precipitate was sedimented (12,500 x g, 2min).

4. The supernatant was removed, avoiding the white pellet, and transferred to a spin-filter placed inside a microcentrifuge vial. 250µl of binding buffer was added to the same spin filter .

5. The tube contents were mixed by drawing the solution up and down in a pipette tip prior to brief centrifugation (12,500 x *g*, 1 min) to collect residual liquid coating the walls of the vial.

6. 350 µl of wash solution were added to each spin filter prior to centrifugation (12, 500 x *g*, 1 min). The supernatant was removed and the collection vial was centrifuged a second time to force residual liquid from the spin filter.

7. The spin filter was transferred into a new microcentrifuge vial, and the plasmid DNA eluted from the spin filter by adding 50µl of TE buffer (6.5.4.), after brief vortex mixing to resuspend the binding buffer, the tube was centrifuged (12, 500 x *g*, 1 min) to sediment the DNA. The vial containing plasmid DNA was stored at +4°C or -20°C.

3.5.1.b Rapid preparation of plasmid DNA by alkaline extraction procedure

Principle: Double stranded plasmid DNA is rapidly isolated from bacterial cells with little contaminating chromosomal DNA by an alkaline lysis extraction following the method of Birnboim and Doly (1979).

1. Strains were grown at 37°C (overnight) with gentle shaking. The cell count should be roughly 10^9 cells/ml after overnight growth.

2. One ml of culture was transferred to an eppendorf tube and bacterial cells sedimented by centrifugation (12, 500 x *g*, 2 min).

3. The supernatant was removed with a Pasteur pipette and the tubes placed on ice.

4. One hundred µl of solution 1 (6.6.1.a.) were added to each tube to produce spheroplasts and the cells were resuspended using a pipette prior to incubation on ice for 10-15 min.

5. The tubes were transferred to a plastic rack and 200µl of solution 2 (6.6.1.b.) were added to lyse the cells, the tubes were inverted twice gently.

6. One hundred and fifty µl of solution 3 (6.6.1.c.) was added and the tubes mixed thoroughly by inversion. The mixture became less viscous and a white precipitate (chromosomal DNA) appeared. The tubes were left on ice for 45 min.

7. The tubes were centrifuged (12, 500 x *g*, 5 min). A Pasteur pipette was used to transfer about 0.4 ml of supernatant to a new set of tubes. 1ml of ice-cold ethanol was added to each tube and mixed by repeated inversions to elute the plasmid DNA, and the tubes placed at -20°C for 30 min.

8. Tubes were centrifuged (12, 500 x *g*, 5 min) and the supernatants removed carefully with a Pasteur pipette. It was crucial not to touch the walls and sides of the tube as these contained precipitated DNA.

9. The DNA precipitate was dissolved in 100µl of solution 4 (6.6.1.d.), followed by the addition of 200µl of ethanol before leaving at -20 °C for 10 mins. Tubes were centrifuged (12, 500 x g, 5 min) and the supernatants discarded. *If the DNA was to be used for digestion with restriction enzymes then step 9 was repeated.*

10. Excess liquid was removed with a fine cone of tissue paper, taking care not to touch the DNA pellet.

11. Twenty-five µl TE buffer (6.5.4.) was added to each tube and the pellet dissolved, tubes were labelled and stored at -20 °C.

12. For electrophoresis, 15 µl of preparation were added to 5µl of BPB (6.6.3.c.) and loaded onto a 0.7% agarose gel with borate buffer. A voltage of 100 V was applied for 4 hours.

13. The molecular weight of the plasmids was calculated by drawing a graph of the Log_{10} of the molecular weight of plasmids of known sizes against the relative mobility (distance the plasmid had travelled ÷ overall distance travelled.). The plasmids of known sizes were isolated from a laboratory constructed strain 39R621 (98, 42, 23.9 and 4.6 MDa) and a *S. typhimurium* strain P100836 carrying a plasmid of 60MDa.

3.5.2. Preparation of Genomic DNA

Principle: Genomic DNA was prepared from bacterial cells using proteinase K to remove proteins, and cetyltrimethylammonium bromide (CTAB) to remove cell wall debris, polysaccharides and remaining proteins. High molecular weight DNA was isolated by isopropanol precipitation.

1. Eight hundred µl of broth culture (37°C, overnight, shaken) were placed in an eppendorf tube and the cells sedimented by centrifugation (12, 500 x *g*, 2 min). If the pellet appeared too small, aliquots of culture were pooled.

2. The bacterial pellet was suspended in 579µl of TE buffer (6.5.4.) by repeated pipetting.

3. 15µl of 20% (w/v) SDS and 6µl 10mg/ml proteinase K was added and mixed thoroughly with the cell pellet. The preparation was incubated at 37 °C (1 hr).

4. During this step, a solution comprising 10% CTAB in 0.7M NaCl was incubated at 65°C to fully solubilise the CTAB.

5. One hundred and ten µl of 5M sodium chloride were added and mixed thoroughly. High salt is necessary, as a CTAB-nµcleic acid precipitate will form if the concentration of NaCl drops below 0.5M.

6. Eighty µl of the CTAB/NaCl reagent were added to the eppendorf tube and the contents mixed thoroughly, followed by incubation for 10 min at 65°C. Approximately equal volumes (0.7-0.8ml) of chloroform/iosamyl alcohol (6.6.2.a.) were added, mixed thoroughly and the tube centrifuged (12, 500 x g, 6 min). This removes CTAB-protein-polysaccharide complexes. A 1ml pipette, with the tip removed, was used to harvest the viscous supernatant (about 700µl) for transfer to new eppendorf tube.

7. The tube was centrifuged as before and the DNA removed. Approximately 700µl of phenol/chloroform/isoamyl alcohol (6.6.2.b.) were added to the supernatant, mixed thoroughly and the tube centrifuged (12, 500 x *g*, 5min). The supernatant was collected and the phenol/chloroform/ isoamyl alcohol step repeated as above.

8. Step 7 was repeated at least three times. The supernatant was transferred to a fresh tube, 0.6ml isopropanol was added to precipitate the nucleic acid. After a 15 min incubation at room temperature the tube was centrifuged (12, 500 x *g*, 10 min). The white stringy DNA pellet was washed with 70% ethanol to remove

residual CTAB. The pellet detaches from the wall of the tube, and 'floats' intact. The tube was centrifuged (12. 500 x *g*, 5 min) and the supernatant removed, the tube was centrifuged again briefly and residual liquid removed.

9. The pellet was dried by incubation at 37°C for about 10 min. The position of the pellet was noted since the pelleted material became clear on drying. The pellet was resuspended in 50µl TE/RNAase (6.6.2.c.) and incubated at 37°C for 30 min, prior to storage at 4°C. 5µl of sample were electrophoresed on a 0.8% borate gel to estimate the DNA concentration.

3.5.3. Agarose gel electrophoresis

Principle:- DNA migrates in an electric field due to the negative charge carried by phosphate groups. DNA molecules are separated during migration through the agarose matrix due to their molecular size such that smaller fragments move further towards the anode than larger fragments.

3.5.3.a. Horizontal gel electrophoresis

Agarose gels were prepared, with a range of agarose concentrations, in either Tris-borate (6.6.3.a.) or Tris-acetate buffers (6.6.3.b.). Tris-borate was used when agarose gels were electrophoresed for 6 hours or less, tris-acetate was used when agarose gels were electrophoresed for longer periods of time. The agarose was melted in a microwave oven and poured into a horizontal gel apparatus (Hybaid) containing a well-forming

comb. On setting the appropriate buffers were added to buffer tanks and the gel used for electrophoresis.

For the separation of plasmid or genomic DNA, or enzyme digested DNA gels with an agarose concentration of 0.7%-1.0% (w/v) were used with borate buffer. DNA fragments were separated, alongside molecular weight markers (6.9.; 6.6.4.a.), by electrophoresis for 3 hr at a constant voltage 100V (Shandon, Vokam 500-500 power pack). Sample volumes of 20 or 25 µl were used, depending on the concentration of DNA and the size of well. Indicator dyes were used depending on the duration of electrophoresis. Bromophenol blue (6.6.3.c.) was used when the duration of electrophoresis was longer than 1.5 hr, whilst Orange G was used when electrophoresis was carried out for less time (6.6.3.d.).

3.5.3.b Vertical gel electrophoresis

Digested genomic DNA was electrophoresed using a vertical tank with an acetate buffer (30V, 18 hr). The duration of electrophoresis was varied to optimise separation of DNA fragments. For Southern blotting a fluorescent labelled marker was electrophoresed concurrently (Amersham International plc).

3.5.3.c. Gel staining and photography

DNA was visualised by staining with ethidium bromide (6.6.3.e.) for 1 hour, and observed with UV light (302_{nm}), Gel staining was carried out in a fume cupboard and gloves were worn when handling stained gels. When samples of DNA were to be used to make DNA probes, long wave (385_{nm}) UV light was used to avoid damage to DNA . Gels were photographed using a Polaroid camera (Kodak) and type 52 film (Polaroid) with a red filter (x7).

3.5.4. Extraction of DNA from agarose gels using the QIAEX II kit

Principle:- This procedure was used to extract DNA from agarose gels. The high concentration of salt in buffer QX1 disrupts hydrogen bonding of the sugars in agarose allowing solubilisation of gel slices containing a given piece of DNA. The high salt concentration also dissociates DNA binding proteins from DNA fragments. Incubation of the DNA solution in a highly electrolytic environment causes modification of the water molecules forcing the DNA to bind to silica particles. DNA molecules bind to QIAEX II particles during the adsorption step and all non-nucleic acid impurities such as agarose, proteins, ethidium bromide and salts remain in the supernatant. A high salt wash with buffer QXI removes residual agarose, and ethanol containing buffer PE efficiently removes salt contamination.

1. The gel was illuminated with a long wave UV radiation source (385nm), so that the DNA band could be observed and selected.

2. With a clean scalpel the band was excised and placed into a pre-weighed eppendorf tube. The size of the gel slice was minimised by removing excess agarose and the weight of the gel slice calculated.

3. The amount of buffer QX1 (from kit) added to the volume of gel was calculated as 300 µl of buffer QX1 per 100 mg of gel, the buffer was added to the gel fragments.

4. QIAEX II solution was prepared by thorough whirli-mixing and 10 µl were added to the gel slice and mixed. This was incubated at 50 °C for 10 min and repeatedly mixed every 2 min to keep the QIAEX II in suspension.

5. The sample was centrifuged (12, 500 x *g*, 30 sec) and the supernatant carefully removed with a pipette. The pellet was washed with 500µl of buffer QX1 and resuspended by vortexing.

6. The eppendorf tube was centrifuged (12, 500 x *g*, 1 min) and all traces of supernatant were removed with a pipette. This wash step removes residual agarose contaminants. The pellet was washed twice with 500µl of buffer PE and the pellet was resuspended in 500 µl PE buffer by vortexing.

7. The eppendorf tube was centrifuged (12, 500 x *g*, 30 sec) and all traces of supernatant were removed with a pipette. This wash step removes residual salt contaminants. The pellet was air-dried for 10-15 min or until the pellet appeared white.

8. The DNA was eluted by adding 20µl of TE buffer (6.5.4.) and resuspending the pellet by vortexing incubating at room temperature for 5 min. The sample was centrifuged (12, 500 x *g* , 30 sec) and the supernatant carefully removed and transferred to a clean eppendorf tube. The supernatant now contained the purified DNA. A second elution will increase the yield by approximately 10-15%.

3.5.5. Labelling of probes with fluorescein by random primer method

Principle:- To label fragments of DNA with fluorescein for the use in ECL (Enhanced chemiluminescence) hybridisation (3.5.7.). The components of the ECL Random Prime Labelling Kit were used. (RPN3040)

1. A volume of unlabelled fragment containing at least 100ng of DNA was placed into a screw capped microcentrifuge tube - the volume required was usually 20µl (§) or less. The volume was adjusted to 25µl by adding water supplied with the kit. The tubes were placed at 100 ° C for 5 min and chilled on ice.

2. An eppendorf tube was placed in ice, to which was added:-

¶ µl water to final volume of 50µl

10 µl nucleotide mix

5µl primer mix

§µl (boiled) DNA fragment (see step 1)

1 µl Klenow polymerase

3. The contents were mixed gently and the tube centrifuged (12, 500 x *g*, 30 sec). Tubes were incubated at various temperatures, based on manufacturer's instructions, and preparations were incubated at room temperature overnight or 37 °C for 1h, but they were usually incubated at 37°C for 4hr or overnight at room temperature. If incubated at room temperature, samples were incubated in a cupboard to exclude light.

4. The reaction was stopped by adding 5µl of 0.25M EDTA, pH8 and preparations were stored at -20°C. The labelled probe was used directly without further purification or diluted to 1-2 µl per ml of hybridisation mixture - 1µl was tried first and more added if the signal was weak.

3.5.6. Southern Blotting

Principle:- Fragments of DNA are transferred from an agarose gel to Hybond N paper allowing the hybridisation of selected fragments with specific gene probes.

1. Following gel electrophoresis and staining with ethidium bromide, DNA profiles were photographed. Taking photographs of whole, undigested plasmids reduces the effectiveness of hybridisation due to damage of the DNA, therefore a duplicate gel should be prepared which is not stained and photographed. Agarose at the sides and bottom of the gel was excised (the top is not usually trimmed to denote the position of the wells).

2. The top right hand corner of the gel was removed to help with gel orientation, the gel was transferred to a plastic box and processed as follows:- If transferring large DNA fragments of interest or whole plasmids then the gel was soaked in 0.25M HCl (6.6.5.a.). For mild treatment, gels were soaked for 20 min, or for 30 min with one change of 0.25 M HCl. The solution was discarded and the gel rinsed in distilled water.

3. The gel was soaked in freshly-made denaturing solution (6.6.5.b.) for 40 min with one change of solution. As before, this solution was discarded and the gel rinsed in distilled water.

4. The gel was soaked in neutralising solution (6.6.5.c.) following similar conditions as described for the denaturing steps. After rinsing, the gel was measured exactly and a piece of Hybond N paper was cut to 2mm smaller than the gel.

5. The blotting apparatus was assembled as shown (Figure 9). The blotting buffer reservoir was a plastic box and the gel platform was a glass plate. The wicks , made from 3MM paper, extended to the floor of the reservoir.

6. Blotting solution was 6 x SSC (6.5.1.), this was placed into the reservoir and allowed to soak into the paper wicks, avoiding air bubbles.

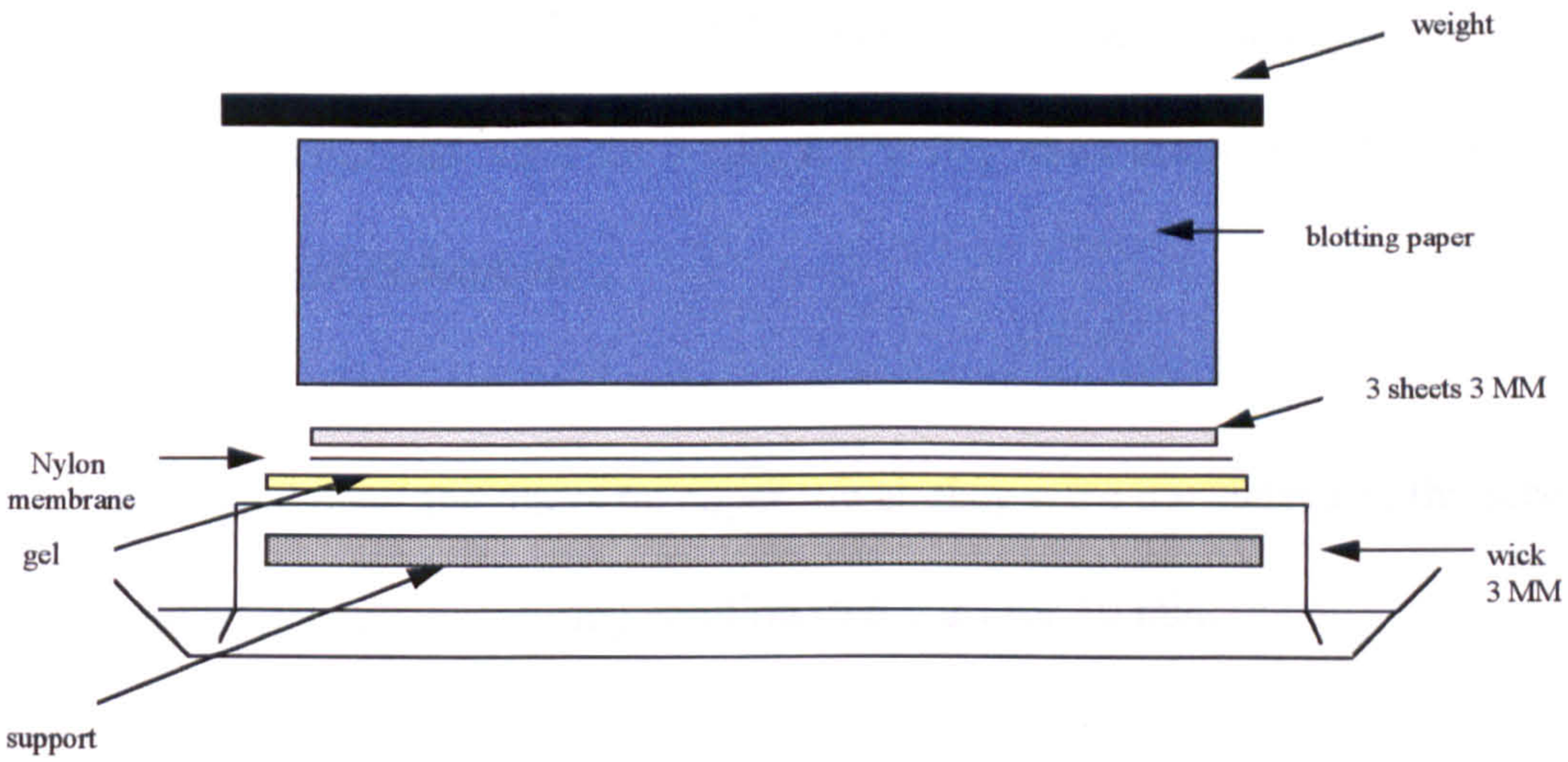
7. The gel surface was moistened with 6 x SSC (6.5.1.) and the dry Hybond paper placed on top. Any air bubbles were squeezed out by carefully rolling a 10 ml pipette over the paper.

8. 4-6 sheets of 3MM paper, cut to size, were laid onto the nylon membrane, followed by a blotting stack made from paper towels cut to size. The towels were cut to size to avoid contact with the wicks. A second glass plate was placed on top and weighted with two 100ml flat bottles to maintain pressure.

9. Strips of parafilm were placed around the gel to form an impervious barrier. The reservoir was filled up with 6 x SSC (6.5.1.) and the experiment was allowed to stand overnight.

10. The apparatus was dismantled and the Hybond paper rinsed twice in distilled water, prior to incubation at 70°C for 2 hr.

Fig 9. Southern blotting apparatus



3.5.7 Hybridisation

Principle:- Investigating the affinity for known sequences of labelled DNA that have been derived by PCR or cloning experiments, to bind to complementary sequences on target DNA (single stranded DNA). Target DNA may be from many sources e.g. genomic DNA, plasmid DNA or digested whole cell DNA.

3.5.7.a Preparation of filters with target DNA

1. Bacteria were grown in broth (37 °C, overnight) and approximately 100 µl of culture was 'spotted' onto a gridded nylon filter (Hybond N) placed on to a nutrient agar plate. Control strains (6.1.2.b) were also spotted on the filter. The plates were incubated at 37 °C for 5-6hr or until the colony spots were observed.

2. Four plastic trays containing sheets of Whatman 3MM paper were prepared. The first tray contained 10% (w/v) SDS, and the filters were placed colony side up onto the saturated paper for 5 min. It was important not to wet the top of the filter with SDS solution.

3. After 'dabbing' the filters on paper towel, they were transferred to the second tray containing denaturing solution (6.6.6.a.) for 10 min.

4. Filters were transferred to a third tray containing neutralising solution (6.6.6.b.) for 5 min.

5. Filters were finally transferred to the fourth tray containing 2 x SSPE (6.5.3.) for 5 min.

6. Filters were allowed to dry at room temperature, and placed between sheets of 3MM paper and incubated 80°C for 2 hr.

3.5.7.b. Hybridisation with ECL labelled probes

1. A hybridisation oven was set at 68°C.

2. Filters, prepared as above (3.5.7.a..), were moistened with 5 x SSC (6.5.1.) and placed on a nylon mesh, cut to size (to ensure that the filters did not overlap

when rolled up). The loosely rolled filter-mesh was placed into hybridisation tube, a quarter filled with 5 x SSC (6.5.1.).

3. Hybridisation tubes were placed in the hybridisation oven and incubated at 68 ° C with rotation for at least 1hr.

4. The tubes were removed and the filter-mesh sandwich taken apart in a shallow tray containing a small amount of 5 x SSC (6.5.1.). The membranes were wiped with a damp tissue to remove cellular debris, reassembled with mesh and placed back in the tube.

5. ECL hybridisation solution (6.6.6.c.) was mixed with freshly boiled salmon DNA (6.6.6.d.) and added to the tubes, they were left at 68 °C for at least 2 hours.

6. After prehybridisation, the solution in the tubes was removed, and the tubes were inverted on tissue paper.

7. The probe solution (6.6.6.e.) was incubated at 100 °C for 10 min, cooled rapidly on ice, added to the tube and left overnight at 68°C.

8. The probe was retrieved and stored at -20°C for reuse.

9. Membranes were washed (2 x 10 min) at room temperature with 2 x SSC/0.1% SDS (6.5.2.). Conditions for subsequent washes varied depending on the probe

being used (6.6.6.f.). The membranes were rinsed briefly in ECL buffer (6.6.6.g.) and the detection step was carried out. The filters were kept moist at all times.

3.5.7.c. Detection of ECL labelled hybrids

1. The hybridised filters were transferred to plastic bags and arranged so that they were 'back to back' with the DNA sides facing outwards and not overlapping.

2. Blocking reagent was prepared (6.6.6.h.), added to the bag avoiding air-bubbles and 'heat' sealed. The contents of the bag were mixed by shaking for at least 1 hour.

3. A corner of the bag was cut off and the blocking reagent poured off. The membranes were rinsed with ECL buffer (6.6.6.g.) with gentle agitation.

4. Antibody-conjugate was prepared (6.6.6.i.) and added to the bag, which was resealed and shaken at room temperature for 1 hour.

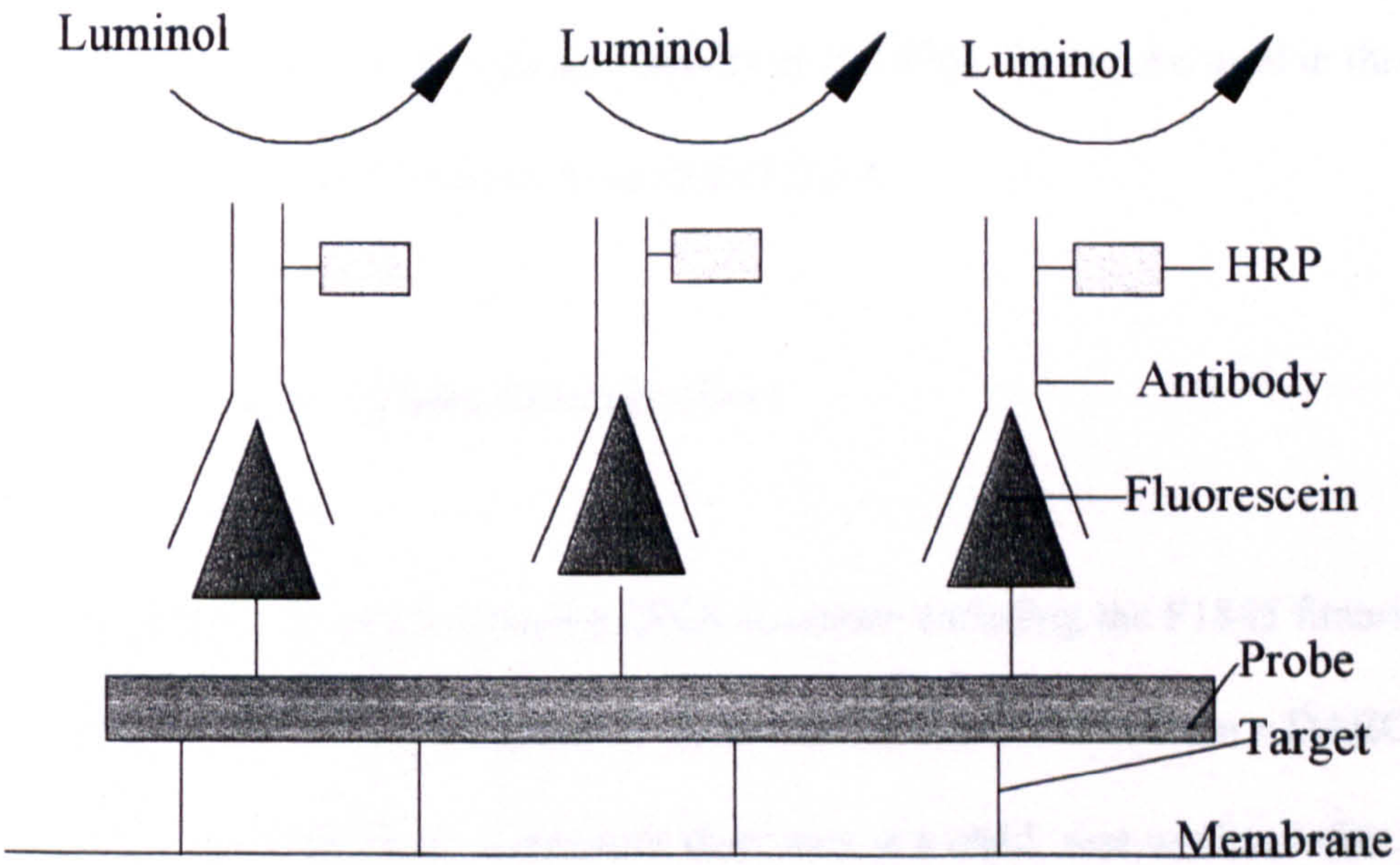
5. Filters were removed and placed in a plastic box and washed (3 x 10 min) at room temperature in ECL buffer.

6. A minimal volume of substrate was prepared by mixing equal volumes of the two ECL detection reagents (1 and 2).

7. A piece of 3MM filter paper was prepared with cling film wrapping. Substrate was added to the tray and the filters were placed DNA side down and agitated gently for 2 min.

8. Filters were wrapped in cling-film, placed into a document holder and exposed to X-ray film (Hyperfilm, Amersham International - RPN1675H) for 20 min. The film was developed using a commercial X-ray film processor or by hand as described (3.7.9.a. & b.).

Fig. 10 Basics of ECL labelled probes



3.5.8. DNA probes

3.5.8.a. Aggregative Adherence (AA)

This probe was devised by Baudry *et al.* (1990). The probe consists of 1 kb fragment of DNA excised from the 60 MDa plasmid from the EAggEC strain 17-2 (LEP reference, E92356; O3:H2) hybridising strongly with the probe devised by Vial *et al.* (1988). This DNA fragment was cloned into the *Bam*HI site of the vector plasmid pUC19, now termed as pCVD432. This probe fragment can be excised from the resultant plasmid by double restriction with *Eco*RI and *Pst*I. This probe was reported as having 89% sensitivity and 99% specificity (Baudry *et al* 1990). The probe used in this study was labelled with fluorescein as described (3.5.5.).

3.5.8.b. Diffuse adherence (DA)

This probe was derived from a DNA sequence encoding the F1845 fimbrial adhesin of diffuse adherent *E. coli* (DAEC). A cosmid library of DNA from a DAEC strain C1845 (O75:H-) which caused persistent diarrhoea in a child was used to infect an *E. coli* K12 strain LE392. One ampicillin resistant strain which haemagglutinated human erythrocytes in the presence of mannose was selected and its recombinant plasmid was termed pSLM850. The probe sequence for the detection of DAEC is a 370 kb *Pst*I fragment within the *daa*C gene from the recombinant plasmid pSLM852 (Bilge *et al.* 1989;

3.5.8.c. Aggregative Adherence Fimbriae - I (AAF/I)

The AAF/I probe consists of a sequence from the gene termed *aggA* encoding the fimbrial subunits (Savarino *et al* 1994) from the 60 MDa plasmid isolated from EAggEC strain 17-2 (O3:H2). The probe fragment used in this study was derived from an *E. coli* strain carrying a recombinant plasmid pJPN61 (Nataro, personal communication). Plasmid DNA was digested with the restriction enzymes *Pst*I and *Hinc*III resulting in a fragment of 0.9 kb and a considerably larger fragment, previously vector DNA. The 0.9 kb fragment was excised from the agarose gel using the QIAEX kit (3.5.4.), labelled with fluorescein (3.5.5.) and used for hybridisation (6.6.6.f.).

3.5.8.d. Aggregative Adherence Fimbriae - II (AAF/II)

The genes encoding AAF/II are found on a 65MDa plasmid from EAggEC strain O42 (O44:H18) isolated from a child with persistent diarrhoea in Lima, Peru (Nataro *et al.* 1985a). This strain was shown to cause diarrhoea in adult volunteers (Nataro *et al.* 1995). The probe fragment used in this study was derived from an *E. coli* strain carrying the recombinant plasmid pJC2 (Czeczulin *et al.* 1997). The plasmid DNA was digested with the restriction enzyme *Eco*RI resulting in a fragment of 1.7 kb and a much larger fragment, previously vector DNA. The 1.7 kb fragment was excised from an agarose gel using the QIAEX kit (3.5.4.), labelled with fluorescein (3.5.5.) and used for hybridisation (3.5.7.).

3.5.8.e. Enterohaemolysin (CVD419)

This probe was derived from the 60 MDa plasmid of prototype VTEC strain 933 (O157:H7) digested with the enzyme *Hind*III, the resultant 3.4 kb fragment was cloned into pBR325 and designated pCVD419 (Levine *et al.* 1987b). *E. coli* strains hybridising with this probe have haemolytic properties. The probe fragment used in this study was labelled with fluorescein (3.5.5.) and used for hybridisation (3.5.7.).

3.5.8.f. Enteroaggregative *E. coli* heat-stable toxin I (EAST1)

The probe fragment used for the detection of the genes encoding EAST1 (*astA* gene) was derived from the 60 MDa plasmid from EAggEC strain 17-2 (Savarino *et al.* 1993). The fragment used as the probe was excised from the recombinant plasmid pUC19 containing the *astA* gene termed pSS106. The *astA* gene was excised by *Xmn*I and *Nru*I restriction. These two sites are positioned 10 bp 5' to the translational start and 5 bp 5' to the translational stop of *astA*, respectively. Plasmid pSS126 contains this 117 bp fragment cloned into the *Sma*I site of pUC18 and yields the EAST1 DNA probe after *Eco*RI and *Pst*I digestion. The probe fragment used in this study was labelled with fluorescein (3.5.5.) and used for hybridisation (3.5.7.).

3.7. Miscellaneous Methods

3.7.1. Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

Principle:- Bacterial proteins are mixed with SDS to give an overall negative charge, and during migration through an acrylamide gel, proteins separate due to molecular size.

SDS-PAGE was performed using the discontinuous buffer system described by Laemmli (1970), Gels had an acrylamide:bisacrylamide ratio of 37.5:1. Gel electrophoresis was performed with a 'large' plate format in a Shandon gel apparatus and also in an Atto 'mini gel' apparatus (Genetic Research Instrumentation Ltd.). SDS-PAGE gels were prepared using a stacking gel with an acrylamide concentration of 4.5% (6.7.1.c.) and a separation gel of either 12.5 or 15% acrylamide (6.7.1.c.).

3.7.1.a. SDS-PAGE Gel Preparation

1. Glass plates were placed onto a piece of paper towel and wiped with tissue paper soaked with acetone to remove any grease and dust particles from the plates. Plates were assembled and placed upright. A spirit level was used to ensure the plates were horizontal. A 'T' shaped piece of plastic was placed between gel

plates to ensure the size of the separation gel remained consistent from gel to gel.

2. Solutions for preparing the separation gel (6.7.1.a,c,d.), with the exception of TEMED, were placed into a Buchner flask and degassed.

3. Following the addition of TEMED, the gel solution was mixed gently and poured between the gel plates, avoiding the introduction of air bubbles into the gel. The 'T' piece was removed and separation gel over-layered with water-saturated butanol to ensure a flat interface between the separation gel and the stacking gel. With the addition of the butanol a single meniscus was observed; however, on polymerisation of the acrylamide a second meniscus appeared between the polymerised and unpolymerised layers.

4. Following polymerisation, the butanol and any unpolymerised acrylamide was rinsed from the gel plates, and extraneous moisture removed with a piece of 3MM filter paper. A well forming comb was inserted between the plates.

5. Gel solutions for the stacking gel (6.7.1.b,c,d.), with the exception of the TEMED, were placed into a Buchner flask and degassed. The TEMED was added and the stacking gel mixture poured into the gel apparatus.

3.7.1.b. SDS-PAGE: Sample Preparation and Electrophoresis

Bacterial protein preparations were thawed and the required volumes of solubilization buffer (6.7.1.f.) and protein preparation were mixed in microcentrifuge tubes, and incubated at 100°C for 5min. Condensation on tube walls was sedimented by centrifugation (6000 x *g*, 10secs). A standard sample volume of 30 µl was applied to the large gels and 5 µl was applied to mini gels.

1. Prior to electrophoresis, the well forming comb was removed from the stacking gel making sure that the plates were not prised apart, a microspatula was used to straighten the wells of the gel. Running buffer (6.7.1.e.) was poured into the lower tank making sure that the level is sufficient to immerse the lower end of the separation gel.

2. The gel was placed into the tank, making sure that there were no air bubbles trapped at the gel/buffer interface, and held in place by clamps. Running buffer (6.7.1.e.) was added to the top buffer tank to cover the sample wells by approx. 0.5ml.

3. A micropipette with a capillary tip was used to add samples to the designated wells. The same volume of sample was added to each well and blank wells were filled with solubilization buffer alone.

4. The safety lid was placed over the gel apparatus making contact, and a constant current of 50mA was applied until the blue dye, present in the solubilization buffer, reached a point about 1cm from the end of gel.

5. The current was then switched OFF and the gel removed for either staining or immunoblotting.

3.7.1.c. SDS-PAGE Gel staining

3.7.1.c.i. Staining proteins with Coomassie Brilliant Blue (CBB)

Following electrophoresis, gels were stained with Coomassie Brilliant Blue, CBB (6.7.1.g.) stain for at least 1h and destained with 10 % (v/v) acetic acid with gentle agitation. For the purposes of photographing gels, extraneous stain was bound by placing sheep wool or dog fur into the destaining gel solution.

3.7.1.c.ii. Staining proteins with Silver

1. Gels were fixed in 50% methanol for at least 1 hr.

2. Fixing solution was removed by a pipette attached to a water-jet vacuum pump and silver staining solution (6.7.2.c.) was added. The gel was agitated for 15 min.

3. Staining solution was removed by a pipette attached to a water-jet vacuum pump, and the gel washed with deionised water for 5 min.

4 The developing solution (6.7.2.d.) was added to the gel until bands appeared, usually in less than 10 min and seldom in more than 15 min. The gel was washed in water and placed in either 50% methanol or 45% methanol/acetic acid (6.7.2.a.) to stop the stain development.

3.7.1.c.iii. Staining LPS with silver

1. Gels were fixed (6.7.2.a.) for at least 1 hr.

2. Fixing solution was drawn off and replaced with 200ml of oxidising solution (6.7.2.b.) for 5 min. The oxidising solution was poured off and the gel washed (3 x 15 min) with deionised water. During the last washing step the silver stain was prepared (6.7.2.c.).

3. The gel was immersed in staining solution and shaken for 10 min.

4. The staining solution was discarded and the gel washed (x 3) with deionised water for 10 min. During the last wash the developing solution was made (6.7.2.d.).

5. The gel was immersed in 1 litre of developer and mixed until optimal staining was reached. During the last washing step, a Polaroid camera was set up so that the LPS gel could be photographed when the optimal staining was reached.

3.7.2. Western blotting

Principle:- Bacterial protein or LPS profiles, prepared by SDS-PAGE, are transferred onto a nitrocellulose paper (NCP) so that specific antibodies can bind to antigens immobilised on the NCP.

SDS-PAGE profiles of LPS or proteins, were transferred from acrylamide gels onto NCP using the method of Towbin *et al.* (1979).

1. Following electrophoresis, gel plates were opened and a sheet of NCP, cut to approximately the same size as the gel and soaked in transfer buffer (6.7.3.a.), was placed on the gel.

2. The NCP was covered by a piece of 3MM paper, also soaked in transfer buffer, and a pipette was rolled over the filter paper to squeeze out any air bubbles that may be trapped.

3. The filter paper- NCP-gel sandwich was placed onto a sheet of Scotch-brite pad on the blotting cassette and the glass plate removed and replaced by a second piece of 3MM paper.

4. A second Scotch-brite pad was placed on top, followed by a second blotting cassette and the whole sandwich placed into the blotting apparatus, prefilled with transfer buffer.

5. A constant current of 400 mAmp was applied and the buffer was pumped through a chiller unit to prevent the buffer from becoming too warm during electrophoresis.

6. After approximately 1 hr, the current was switched off and the gel sandwich dismantled. The nitrocellulose paper was removed from the cassette and placed into a plastic box containing 20 ml of 3% (w/v) skimmed milk powder in PBS.

7. Following blocking for 10 min, the required amount of antiserum was added and antibody binding allowed to take place for at least 1 hr.

8. The NCP was washed (3 x 10 mins) with PBS-Tween (6.7.3.e.) and transferred to fresh milk-PBS solution.

9. The appropriate antibody, conjugated with alkaline phosphatase , was added and allowed to react for at least 1 hr.

10. The NCP was washed with PBS-Tween (3 x 10 min) and transferred to a polythene bag.

11. Bound antibodies were detected using the enzyme substrate (6.7.3.d.): nitroblue tetrazolium (NBT) (6.7.3.c.) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (6.7.3.b.). Immunoblots were allowed to develop at 37 °C, prior to washing in deionised water and drying.

3.7.3. Lowry protein assay

Principle: An estimate of protein concentration was made using the method of Lowry *et al.* (1951), which detects aromatic amino acids.

1. An aliquot of bovine serum albumin protein standard (6.7.4.a.) was thawed.

2. A series of test-tubes comprising a reagent blank and standard protein gradient of 50, 100, 150 and 200 µg /ml was prepared.

3. Bacterial protein samples were prepared by mixing 0.98ml distilled water with 20µl of the sample.

4. Each tube was mixed with 5ml Lowry C (6.7.4.b,c,d.) and incubated at room temperature for 10min.

5. Lowry D was prepared (6.7.4.e.) and 0.5ml of this added to each tube and mixed well . After 30min the absorbance was measured at 500_{nm}.

6. A calibration graph was plotted with absorbance (500_{nm}) plotted on the Y-axis and standard protein concentration on the X-axis.

7. Since 20 µl volumes of bacterial protein preparation were used for the Lowry assay, results obtained from the graph were multiplied by 50 to give protein content in mg/ml or µg/µl.

3.7.4. Preparation of Rabbit antisera

3.7.4.a. Raising specific antibodies in rabbits

Specific rabbit antibodies were raised in New Zealand white rabbits in the Biological Services Department, CPHL. Bacterial preparations were mixed with Freund's incomplete adjuvant by sonication and then injected intra-dermally. Rabbits were boosted with the same antigen preparation after 10 days, and after 3 weeks rabbits were exsanguinated and sera prepared. Sera were prepared by allowing the blood to clot overnight at 4°C. The clot was removed and any contaminating erythrocytes sedimented by centrifugation (5,000 x *g*, 2 min).

3.7.4.b. Antibody absorptions

For certain experiments it was necessary to remove specific antibodies by mixing sera with formalin-fixed bacteria.

1. Bacteria were grown on agar plates and fixed by suspending cells in PBS with 1% formalin.

2. Formalin-fixed bacteria were washed (x 3) with PBS to remove residual formalin.

3. Bacterial cells were placed into a universal bottle and mixed with antiserum at 50°C for 2 hr.

4. Bacteria were sedimented (10, 000 x *g*, 10 min) and the supernatant removed for immediate use or storage at 4°C.

5. A Western blot was performed to determine whether specific antibodies had been removed successfully.

3.7.5. Colicin testing

Principle: The type of colicins produced by strains of *E. coli* was determined by growing test strains on agar plates and overlaying bacterial colonies with molten agar containing colicin control strains. The lysis pattern of colicin control strains indicated the colicins produced by test strains.

1. Nutrient agar plates were inoculated by 'stabbing' test strains onto the agar with a straight wire, plates were incubated at 37°C overnight.

2. Bacteria were killed by inverting plates over a watch glass containing chloroform (10-15 min) and plates were left to dry for 30 min.

3. 5ml of 0.4% nutrient agar were prepared and kept molten at 55°C. To these was added 0.3ml broth culture of a colicin-sensitive indicator strain and the mixture poured onto the plate. When plates had set, they were incubated at 37°C overnight.

4. Inhibition zones on the lawn of sensitive indicator strains were measured. The type of colicin was identified with reference to standard indicator strains, that produced known colicins.

3.7.6. Detection of siderophores.

Principle: Bacteria are grown in Tris-succinate medium (6.7.5.a.) which causes bacteria to utilise succinate via the tricarboxylic acid (TCA) cycle inducing a demand on the iron requiring steps in the cytochrome system producing an apparent deficiency of cellular iron.

Tris-succinate medium is a colourless solution, which facilitates the observation of colour changes indicating the presence of aerobactin or enterobactin.

1. Tubes of nutrient broth were inoculated with test strains and incubated at 37°C for 8 hr.

2. One hundred ml of Tris-succinate medium (6.7.5.a.) were placed in a sterile 250 ml conical flask and inoculated with 50 µl of broth culture. The flasks were incubated (37 °C) with shaking (120 rpm) until the cultures became turbid. This may take up to 48 hr. The size of inoculum was critical, bacteria would not grow if the inoculum was too small; conversely, if the inocula were too big then the bacteria would grow without expressing siderophores.

3. Bacteria were sedimented (12, 500 *g*, 10 mins) and the supernatants Millipore filtered (0.45 µm).

3.7.6.a. Detection of Enterobactin

Principle: This method (Arnow, 1937) detects aromatic compounds, including the amino acid serine in enterobactin.

1. 1 ml of culture supernatant was acidified with 1 ml of 0.5M HCl

2. 1 ml of nitrite-molybdate reagent (6.7.5.c.) was added and the tube vortex mixed.

3. 1 ml of 1M NaOH was added. A pink colour was indicative of enterobactin.

4. The coloration was quantified by measuring the light absorbance at 505nm.

3.7.6.b. Detection of Aerobactin

Principle: This method (Neilands 1992) involves presenting aerobactin with ferric ions in a form which can be readily bound by aerobactin. Aerobactin binds ferric ions and changes from colourless to brown.

1. 1 ml of culture supernatant was mixed with 1 ml ferric perchlorate reagent (6.7.5.b.), the appearance of a brown colour was indicative of aerobactin.

2. The coloration was quantified by measuring the light absorbance at 480nm.

3.7.7. Serum Survival

Principle:- A recognised virulence mechanism is the ability of bacteria to survive the action of serum complement.

1. Rabbit sera were prepared and any residual red blood cells removed by centrifugation (6, 500 x *g*, 2 min).

2. A portion of a given serum was heat-inactivated to destroy the complement by placing the tubes at 56°C for 30 min.

3. Bacterial strains were inoculated in nutrient broth and incubated at 37 ° C for 18 hr. The bacterial population density was estimated by measuring the absorbance at 621nm and referring to a graph plotting OD_{621} against viable counts. Bacterial cultures were diluted in PBS to give a concentration of 1 X 10^4 bacteria/ml.

4. Fifty µl of bacterial suspension were mixed with 1 ml of normal and heat-inactivated serum. A 50µl volume of each of the sera was immediately 'spread' onto dried nutrient agar plates, and this became the time = 0 bacterial density.

5. This step was performed in triplicate to enable a mean bacterial density to be calculated.

6. Further 50 µl samples were taken at 3 hr and 6 hr and spread onto triplicate plates as above. Spread plates were incubated at 37°C overnight and the colonies counted.

3.7.8. Electron Microscopy

1. Bacterial cells were suspended in 500µl 1% formalin in PBS and incubated at room temperature for 5 min.

2. A 50µl drop of this suspension was placed onto a piece of parafilm and a formvar-coated electron microscope grid placed on to the drop.

3. After 2 min the grid was 'blotted' dry on a piece of tissue paper and transferred onto a drop of 1% ammonium molybdate and left for 2 min.

4. The grid was blotted as before and placed onto a piece of filter paper and incubated at 37°C for 5 min to dry the grids, prior to storage in a grid box supplied (Gilder Grids).

3.7.9. Development of autoradiograms.

3.7.9.a. Using commercial processor

The commercial processor (AGFA) consists of three tanks, containing developing solution (AGFA G53), fixer (AGFA G354) and tap water. Pieces of X-ray film are inserted into the machine passed through the various solutions by a series of rollers. The film emerges as a dry image.

3.7.9.b. Manual method

To economise on developing solutions or when very small pieces of film are to be processed, X-ray films were processed by a manual method.

1. Developing solution was prepared by mixing 40ml of solution A (G53, AGFA) with 50 ml of water , to this were added 10 ml of solution B (G53, AGFA).

2. Fixing solution (AGFA, G354), 20ml was mixed with 80 ml of water.

3. The X-ray films to be developed, were immersed in developing solution until bands could be seen (Note: bands will appear darker when observed in a dark room illuminated by a safe-light).

4. Films were rinsed briefly in tap water and placed into fixing solution for 30-60 seconds.

5 Films were washed in tap water for 5 mins, followed by rinsing in deionised water and air drying.

3.7.10. Replica Plating

Principle : Bacterial colonies are transferred onto a nylon membrane and reacted with specific DNA probes.

1. Bacteria were diluted and plated out, agar plates with more than 100 colonies were used for replica plating.

2. A piece of sterile velvet was placed onto the metal block (diameter = 8.5 cm, height = 9.5 cm) and secured with a metal ring. A piece of cardboard with a circle cut out and an orientation mark was placed over the secured piece of velvet.

3. The agar plate was placed onto the velvet noting the orientation marks and the plate pressed gently onto the velvet.

4. The original plate was removed and a second plate containing a sheet of nylon membrane was placed onto the velvet replica, ensuring that the orientation marks lined up.

5. Plates were incubated at 37°C and nylon membranes were processed as described (3.5.7.a.).

3.7.11. Photography

SDS-PAGE gels were photographed using a camera containing Polaroid 55 film (provides a print and a negative), with a 1:5.6, *f*=105 mm lens; use a setting of *f*=22 and a Wratten ND filter (1.00). Negatives were immersed in 20% sodium sulphite prior to washing with tap water, deionised water and drying in air.

3.7.12. Immunogold

Principle: The detection of specific antibody binding using anti-rabbit-immuno gold conjugate

EAggEC strains were grown on nutrient agar and a generous loopful of cells were resuspended in 100 µl PBS (6.5.5.). A formvar coated electronmicroscope grid was placed

on top of a 50µl drop of cell suspension and left for 5 min. After washing in gold buffer (6.7.12.a.), the grid was placed on a drop of gold buffer containing 5% formalin and after washing (x2) in gold buffer, was processed as follows:-

1. The grid was placed on a 50µl drop of 10% normal rabbit serum in gold buffer for 10 min.
2. The grid was placed on a 50µl drop of 10% rabbit antisera selected against the antigen in question (e.g. 18 kDa) and left for 10 min.
3. The grid was washed 5 x 5min with gold buffer.
4. The grid was placed on a 50 µl drop of (10% in gold buffer) anti-rabbit sera conjugated with gold beads, for 1 hr.
5. The grid was washed 5 x 5min with gold buffer.
6. The grid was placed on a 50µl drop of 2.0% ammonium molybdate in gold buffer for 5 min.
7. The grid was washed (x3) with distilled water.

4. RESULTS

4.1. Strain Characterisation

Strains of *E.coli* that adhere to HEp-2 cells with a 'stacked brick' pattern form a unique group, generally accepted as the 'enteroaggregative *E.coli*' or EAggEC. It would seem that any strain of *E.coli* expressing the aggregative phenotype becomes eligible to belong to this group of organisms. This part of the study was designed to characterise the HEp-2 adhesive properties of three panels of strains comprising:-

Panel 1:- 33 strains of EAggEC which had been used for previous studies in the LEP, and to some of which isogenic non-adhesive mutants had been prepared (6.2.a.i.)

Panel 2:- 60 strains of *E.coli*, detected as EAggEC as part of a study examining the incidence of EAggEC in the normal populations termed Infectious Intestinal Diseases (IID) study (6.2.a.ii.). Some strains were from patients suffering diarrhoea and others were from patients attending day clinics.

Panel 3:- 40 strains of EAggEC isolated from five outbreaks of diarrhoeal disease.

Outbreaks A - D occurred in the UK, outbreak E occurred in Bangladesh (6.2.a.iii.).

4.1.1. The Aggregative Phenotype

Using the HEp-2 adhesion assay, as established in the LEP (Cravioto *et al.* 1979), strains of EAggEC were found to adhere to HEp-2 cells with the characteristic pattern (Fig. 11, Tables 13-15), as described by other workers (Nataro *et al.* 1987; Vial *et al.* 1990; Haider *et al.* 1992). During the adhesion assay it was noted that bacteria adhered to the glass coverslip in addition to HEp-2 cells (Fig. 11), a feature also described previously (Yamamoto *et al.* 1992). The basis for this apparent non-specific binding to glass was not known and became a source of further study (4.2.4., 4.2.5., 4.3.).

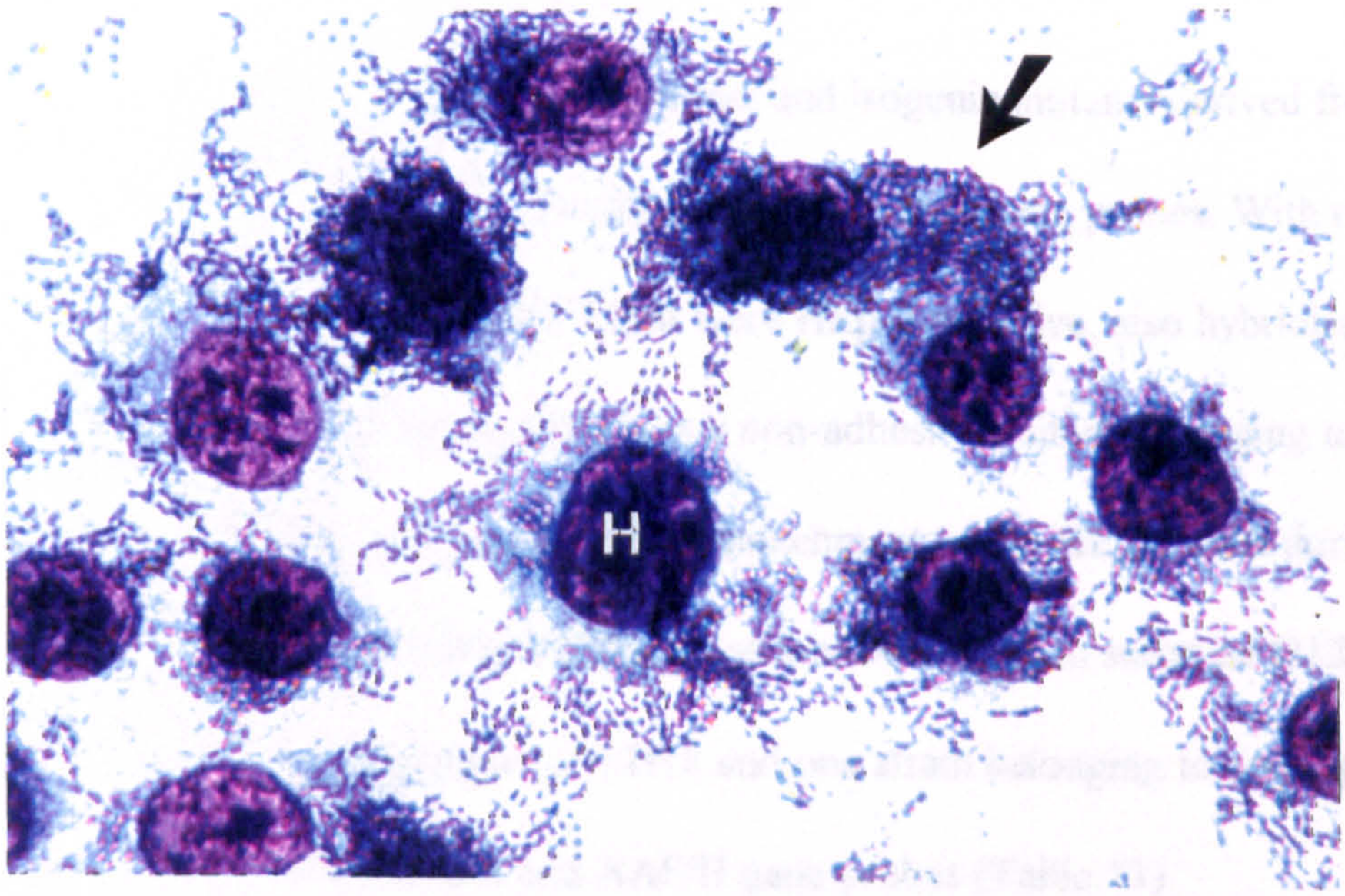


Fig. 11. Light micrograph of *E.coli* strain E40104 adhering to HEp-2 cells in the 'stacked brick' formation. Note bacteria adhering to both HEp-2 cells and glass coverslip. HEp-2 cells and bacteria were stained with Giemsa stain. H denotes a typical HEp-2 cell.

The strains of *E.coli* in the study were also examined for the ability to hybridise with four DNA probes:

i) Probe CVD432 which hybridises with strains of *E.coli* expressing the aggregative adhesion (AA) phenotype (Baudry *et al.* 1990; 3.5.8.a.).

ii) Probe F1845 which hybridises with strains of *E.coli* that adhere to HEp-2 cells in a diffuse pattern (DA)(Bilge *et al.* 1993; 3.5.8.b.)

iii) Probe AAF/I which hybridises with strains of *E. coli* expressing aggregative adherence fimbriae type I (Nataro *et al.* 1994; 3.5.8.c).

iv) Probe AAF/II which hybridises with strains of *E.coli* expressing aggregative adhesion fimbriae type II (Czeczulin *et al.* 1997; 3.5.8.d.)

The three panels of *E.coli* strains, and isogenic mutants derived from panel 1, were tested for the ability to hybridise with these four gene probes. With reference to Table 13, all 33 strains in panel 1 that were HEp-2 adhesive, also hybridised with the AA gene probe. *E. coli* strain E62008 was a non-adhesive mutant belonging to serotype O126:H27. Twenty-one strains caused detachment of the HEp-2 cells during the adhesion assay. Eleven HEp-2 adherent strains belonging to serotype O126:H27, three strains belonging to serotype O44:H18 and one strain belonging to serotype O33:H16, also hybridised with the DA and AAF/II gene probes (Table 13).

Of the IID panel (panel 2), all strains hybridised with the AA probe and were positive in the adhesion assay (Table 14). Twelve strains caused detachment of the HEp-2 cells during the adhesion assay. Nine strains, belonging to serotypes O6:H10, O75:H27, O106:H18, O119:H27, O126:H27 and four O-untypable strains, also

hybridised with the DA gene probe. These latter 9 strains also hybridised with probe AAF/II, and in addition strain E104942 hybridised with the AAF/II probe (Table 14).

All forty strains belonging to panel 3 hybridised with the AA gene probe (Table 15). Nine strains caused the detachment of HEp-2 cells during the adhesion assay. Only three strains (E98529, E101402, E97474) hybridised with the DA probe and two of these (E101402, E97474) also hybridised with the AAF/II probe (Table 15).

Table 13 *Adhesion and probe results of panel 1 (original)*

Strain (E)	Serotype	HEp-2*	AA§	DA¶	AAF/II†	AAF/I∞
92356	O3:H2	+	+	-	-	+++
73339	O15:H-	detaching‡	+	-	-	+++
59905	O21:H2	+	+	-	-	++
72376	O33:H16	+	+	+	+	+++
45730	O44:H18	+	+	+	+	+
44939	O44:H18	+	+	+	+	+
43509	O44:H18	detaching	+	+	+	+
58596	O51:H11	+	+	-	-	++
76989	O75:H2	detaching	+	-	-	-
58583A	O77:H18	+	+	-	-	+
67643	O78:H10	+	+	-	-	++
47697A	O86:H19	detaching	+	-	-	++
97468	O86:H34	detaching	+	-	-	+
78135	O92:H33	detaching	+	-	-	++
60725	O92:H33	+	+	-	-	++
101621	O98:H-	+	+	-	-	-
72957	O102:H27	+	+	-	-	++
99518	O104:H4	+	+	-	-	-
57144A	O111:H21	detaching	+	-	-	+
40144	O111:H21	detaching	+	-	-	+
33915	O111:H21	detaching	+	-	-	+
36182	O111:H21	detaching	+	-	-	+
60874	O126:H27	detaching	+	+	+	+
55060	O126:H27	detaching	+	+	+	-
58816	O126:H27	detaching	+	+	+	+
52610	O126:H27	detaching	+	+	+	+
45691	O126:H27	detaching	+	+	+	+
57157	O126:H27	detaching	+	+	+	+
75607	O126:H27	detaching	+	+	+	+
83087	O126:H27	detaching	+	+	+	+
81456	O126:H27	detaching	+	+	+	+
38383	O126:H27	detaching	+	+	+	+
40104	O126:H27	detaching	+	+	+	+
62008♦	O126:H27	-	-	-	-	-

* Adhered to HEp-2 cells
§ Hybridised with the aggregative adhesion probe
¶ Hybridised with the diffuse adhesion probe
† Hybridised with the aggregative adhesion fimbriae/II probe
‡ Caused detachment of HEp-2 cells during adhesion assay
♦ Non-adhesive naturally occurring variant of serotype O126:H27
∞ Hybridised with the aggregative adherence fimbriae/I probe
+++ strong hybridisation → + weak hybridisation in AAF/I column

Table 14 *Adhesion and probe results of panel 2*

Strain (E)	Serotype	HEp-2 *	AA§	DA¶	AAF/II†	AAF/I ∞
97479	O3:H-	detaching‡	+	-	-	+++
111260	O4:H2	+	+	-	-	+++
99536	O4:H33	+	+	-	-	+
101094	O4:H33	+	+	-	-	++
109902	O4:H33	+	+	-	-	++
96616	O5:H4	+	+	-	-	-
107527	O6:H1	detaching	+	-	-	+++
97499	O6:H10	+	+	-	-	++
97480	O6:H10	+	+	-	-	+
110715	O6:H10	+	+	+	+	+++
99520	O8:H7	+	+	-	-	+
97494	O81:H-	+	+	-	-	-
111479	O11:H-	+	+	-	-	+++
103594	O11:H27	+	+	-	-	+++
99964	O18ac:H-	detaching	+	-	-	+++
99962	O18ac:H30	detaching	+	-	-	+++
99961	O18ac:H30	detaching	+	-	-	+++
99963	O18ac:H30	detaching	+	-	-	++
99965	O18ac:H30	detaching	+	-	-	+++
99960	O18ac:H30	detaching	+	-	-	+++
109903	O21:H-	detaching	-	-	-	+
107249	O21:H4	+	+	-	-	++
104970	O33:H-	detaching	+	-	-	++
99970	O53:H-	+	+	-	-	+++
99971	O53:H2	+	+	-	-	+++
97500	O73:H1	detaching	+	-	-	++
99979	O75:H27	+	+	+	+	++
97477	O81:H-	+	+	-	-	+
92832	O82:H25	+	+	-	-	-
92830	O86:H11	+	+	-	-	-
97496	O86:H2	+	+	-	-	+
97504	O91:H-	+	+	-	-	-
99518	O104:H4	+	+	-	-	+
110716	O106:H16	+	+	+	+	+++
94706	O111ab:H-	+	+	-	-	++
109907	O111ab:H-	+	+	-	-	+
103763	O111ab:H2	+	+	-	-	++

* Adhered to HEp-2 cells

§ Hybridised with the aggregative adhesion probe

¶ Hybridised with the diffuse adhesion probe

† Hybridised with the aggregative adhesion fimbriae/II probe

‡ Caused detachment of HEp-2 cells during adhesion assay

+++ strong hybridisation → + weak hybridisation in AAF/I column

∞ Hybridised with the aggregative adherence fimbriae/I probe

Table 14 (cont) *Adhesion and probe results of panel 2*

Strain (E)*	Serotype	HEp-2 *	AA§	DA¶	AAF/II†	AAF/I∞
99976	O113:H-	+	+	-	-	-
107100	O119:H27	+	+	+	+	++
101089	O126:H27	+	+	+	+	+++
108837	O130:H27	+	+	-	-	+++
97478	O130:H27	+	+	-	-	+++
108839	O130:H27	+	+	-	-	+++
104940	O130:H27	+	+	-	-	-
104968	O131:H-	detaching‡	+	-	-	++
99969	O134:H-	+	+	-	-	+++
107754	O134:H25	+	+	-	-	+++
99968	O134:H27	+	+	-	-	+++
97502	O134:H27	+	+	-	-	+++
101095	O134:H27	+	+	-	-	+++
101096	O134:H27	+	+	-	-	+++
104942	O162:H10	+	+	-	+	++
94708	Orough:H-	+	+	-	-	+
99967	Orough:H-	+	+	+	+	++
107252	Orough:H-	+	+	-	-	++
99535	Orough:H27	+	+	-	-	+
107542	Orough:H27	+	+	+	+	++
97298	Orough:H27	+	+	+	+	+
105836	Orough:H3	+	+	+	+	+++
105392	Orough:H33	+	+	-	-	+++

*Adhered to HEp-2 cells,
§ Hybridised with the aggregative adhesion probe
¶ Hybridised with the diffuse adhesion probe
† Hybridised with the aggregative adhesion fimbriae/II probe
∞ Hybridised with the aggregative adherence fimbriae/I probe
‡ Caused detachment of HEp-2 cells during adhesion assay
+++ strong hybridisation → + weak hybridisation in AAF/I column

Table 15. *Adhesion and probe results of panel 3*

Strain (E)	Serotype	Outbreak	HEp-2*	AA¶	DA†	AAF/II§	AAF/I ∞
98529	O?:H18	A	+	+	+	-	++
96393	O125:H27	A	+	+	-	-	+++
98527	O19:H-	A	d‡	+	-	-	+
97622	O113:H-	A	+	+	-	-	-
96386	O73:H18	B	+	+	-	-	+
97820	O62:H30	B	d	+	-	-	+
97819	O?:H27	B	+	+	-	-	+
96483	O?:H33	B	+	+	-	-	-
96485	O134:H27	B	+	+	-	-	++
96390	O?:H-	B	d	+	-	-	++
97590	O73:H13	B	d	+	-	-	+
96487	O?:H-	B	+	+	-	-	+
97900	O?:H-	B	+	+	-	-	+
97472	O86:H34	C	+	+	-	-	++
97468	O86:H34	C	+	+	-	-	+
97470	O86:H34	C	+	+	-	-	++
97474	O116:H27	C	+	+	+	+	+
101408	O98:H	D	+	+	-	-	-
101621	O98:H-	D	+	+	-	-	-
101396	O98:H-	D	+	+	-	-	-
101406	O98:H-	D	+	+	-	-	+
101402	O110:H-	D	+	+	+	+	+
89097	O6:H16	E	+	+	-	-	-
89101	O8:H20	E	+	+	-	-	+++
89099	O28:H18	E	+	+	-	-	+++
89102	O44:H18	E	+	+	-	-	+++
89109	O44:H18	E	+	+	-	-	+++
89114	O44:H18	E	+	+	-	-	+++
89110	O69:H11	E	d	+	-	-	+++
89112	O69:H11	E	+	+	-	-	+++
89105	O80:H27	E	+	+	-	-	+++
89095	O80:H27	E	d	+	-	-	+++
89111	O89:H18	E	+	+	-	-	+
89096	O113:H-	E	+	+	-	-	+++
89098	O114:H11	E	+	+	-	-	+++
89104	O141:H49	E	d	+	-	-	+
89115	O162:H-	E	+	+	-	-	-
89107	O?:H27	E	d	+	-	-	-
89100	O?:H5	E	+	+	-	-	+++
89106	Orough:H7	E	d	+	-	-	-

* Strains adhering to HEp-2 cells in an aggregative pattern
‡ Strains causing detachment of HEp-2 cells during adhesion test
¶ Strains hybridising with an aggregative adhesion probe
† Strains hybridising with a diffuse adhesion probe
§ Strains hybridising with an aggregative adhesion fimbriae/II probe
∞ Strains hybridising with the aggregative adherence fimbriae/I probe
+++ strong hybridisation → + weak hybridisation in AAF/I column

The strains of EAggEC listed in Table 13 were chosen specifically for this study, but even this small selection illustrated the diversity of serotypes exhibited by strains of EAggEC. For example, the panel described in Table 13 comprised strains of EAggEC with serogroups which have been described elsewhere, such as O44 and O111 (Quadri *et al.* 1994; Debroy *et al.* 1994; Schmidt *et al.* 1995a). However, strains belonging to serotype O126:H27 appear to have been reported only rarely (Knutton *et al.* 1992). Reference to the strains of *E.coli* described in Tables 14 and 15 demonstrated further the heterogeneity in serotypes encountered with this group of enteroaggregative *E.coli*.

4.1.2. Isogenic mutants

A panel of isogenic mutants was constructed by transposon mutagenesis in the LEP by workers previously or by repeated subculture until colonies became AA probe negative, to produce strains which had lost the ability to adhere to HEp-2 cells in an aggregative pattern or to hybridise with the AA gene probe. These mutants were used in experiments to determine how parent strains adhered to HEp-2 cells and are listed in Table 16.

Table 16: *Isogenic mutants constructed from selected strains of EAggEC*

Parent strain (E)	Isogenic mutant	Serotype	Mutagenesis	AA†
36182	36182 13/4	O111:H21	Transposon mutagenesis	+
36182	36182 17/2	O111:H21	Transposon mutagenesis	+
57157A	57157B	O126:H27	Loss of plasmid hybridising with AA probe	-
57144A	57144B	O111:H21	Loss of plasmid hybridising with AA probe	-
58583A	58583B	O77:H18	Loss of plasmid hybridising with AA probe	-
47697A	47697B	O86:H19	Loss of plasmid hybridising with AA probe	+
40104A	40104B	O126:H27	Loss of plasmid hybridising with AA probe	-
92356A	92356B	O3:H2	Loss of plasmid hybridising with AA probe	-

† Result of mutant strains when hybridised with the AA probe

None of the mutant strains adhered to HEp-2 cells in an aggregative pattern.

Strains 36182 13/4, 36182 17/2 and 46797B hybridised with the AA probe. Insertion or deletion can lead to the loss of aggregative adhesion but not to loss of hybridisation with the AA probe.

4.1.3. Aggregative adherence and possession of plasmids

Strains of *E.coli* from panel 3 which adhered to HEp-2 in an aggregative pattern and hybridised with the gene probe for aggregative adherence (AA) were examined for the possession of plasmids. EAggEC strains from outbreaks A-D were isolated from patients in the UK. The gene sequence hybridising with the AA probe is not associated with the genes required for the aggregative adhesion process (Baudry *et al.* 1990), results have shown that transposon mutagenesis of EAggEC strains may result in the loss of aggregative adherence but not hybridisation with the AA probe (Table 16).

Table 17 *Plasmid carriage and hybridisation with the probe for aggregative adherence in strains of EAggEC isolated from outbreaks A to D*

Strain	Serotype	Plasmids (MDa) *
A		
E98527	O19:H-	126, 93, 66, 35, 3.8, 3.5, 2.9
E97622	O113:H-	126, 66, 40, 14.4, 5
E96393	O125:H27	126, 63, 11.5
E98529	O?:H18	126, 46, 12, 5
B		
E96386	O73:H18	44.7
E97820	O62:H30	61.7, 14.4, 4
E97819	O?:H27	61.7, 55, 51.3, 3.7
E96483	O?:H33	61.7, 55, 51.3, 35.5, 3.5
E96485	O134:H27	61.7, 55, 51.3, 12.9, 5.4, 3.8
E96390	O?:H-	44.7, 39.8, 19, 12.9, 4.5, 4
E97590	O73:H13	35.4, 19, 12.9, 4.5, 4
E96487	O?:H-	61.7, 58.9, 51.3, 39.8, 3.7, 3.4, 3.2
E97900	O?:H-	61.7, 58.9, 51.3, 39.8, 3.7, 3.4, 3.2
C		
E97472	O86:H34	44.7, 6.3
E97468	O86:H34	36.3, 6.3
E97470	O86:H34	44.7, 6.3
E97474	O116:H27	89.1, 74.1, 56.2, 6.3
D		
E101408	O98:H-	67.6, 35.5
E101621	O98:H-	56.2, 35.5
E101396	O98:H-	67.6, 35.5
E101406	O98:H-	67.6, 35.5
E101402	O110:H-	85.1, 47.9

* Estimated sizes of plasmids carried by each strain (MDa)

■ Plasmid hybridizing with AA probe

Strains of EAggEC comprising panel 3 (outbreak) showed extensive heterogeneity in the number and size of plasmids carried, but all strains possessed at least one plasmid.

In outbreak Group A all four strains carried plasmids (Table 17; Fig12a). Three strains carried a plasmid of 126 MDa, hybridising with the AA gene probe (Table 17)

although *E. coli* strain E96393 carried a plasmid of 63 MDa hybridising with the AA probe (Table 17). The nine strains in outbreak group B possessed a variety of plasmids (Fig 12b), where six strains possessed plasmids of 61.7 MDa hybridising with the AA probe, and three strains carrying plasmids of 35.4, 44.7 and 39.8 MDa respectively, hybridising with the AA probe (Table 17). The four strains in outbreak group C possessed plasmids of 44.7, 36.3 or 56.2 MDa which hybridised with this AA gene probe (Fig 12c; Table 17). EAggEC strains belonging to outbreak group D carried two plasmids (Fig 12d). Strains E101408, E101396 and E101406 carried plasmids of 67.6 MDa which hybridised with the AA gene probe. Strains E101621 and E101402 carried plasmids of 56.2 and 85.1 MDa respectively, which hybridised with the AA probe. The range of sizes of plasmids hybridising with the AA probe was 36.3MDa - 126MDa.

Two EAggEC strains E40104 (O126:H27) and E92356 (O3:H2) from the original panel were subcultured to obtain single colonies on nutrient agar plates. These colonies were replica plated (3.7.10.) onto nylon membranes and processed for hybridisation (3.5.7.b.). The filters were hybridised with the probe for AA to detect colonies that were probe-negative. These colonies were picked from the original plate and plasmid preparations were made from the original culture and the probe negative colonies.

The plasmid profiles from strain E92356 and E40104 (Fig. 12e) show that the plasmid hybridising with the AA probe has been lost through repeated subculture. The loss of this plasmid results in the loss of the aggregative pattern of adhesion to HEp-2 cell in the tissue-culture assay.

The wide range of sizes of plasmids that carry the sequence hybridising with the AA probe could contribute to the vast heterogeneity of the EAggEC strains. It should

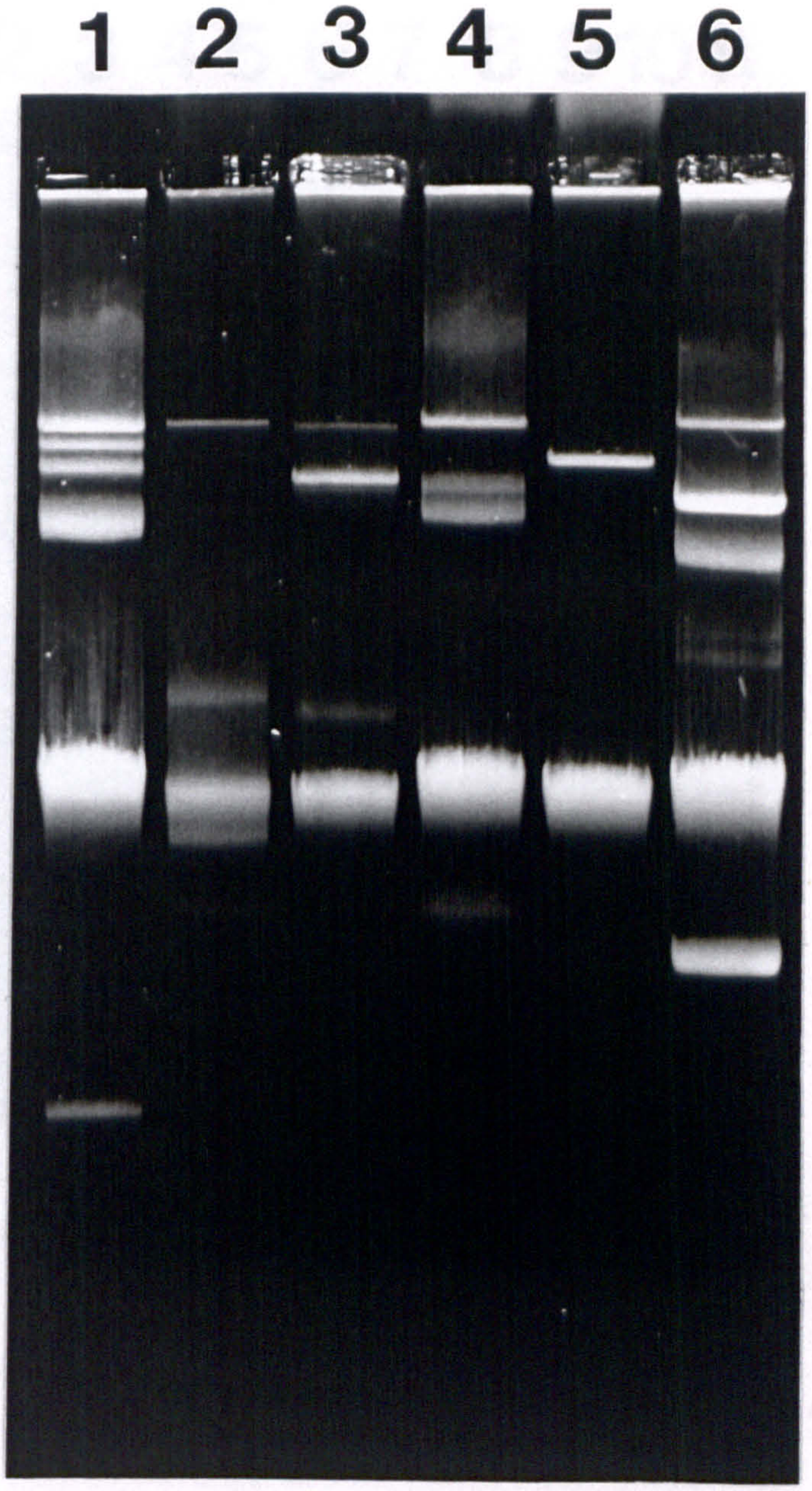


Fig. 12b. Plasmid analysis of the EAggEC strains from outbreak B. Profiles in lanes 2-5, and 8, and 9 contained plasmids of 61.7MDa which hybridised with the AA probe, this probe hybridised with a plasmid of 44.7MDa in lanes 1 and 6, and a plasmid of 35.4 MDa in lane 7. Lanes 10 and 11 contain standard plasmid profiles prepared from *S. typhimurium* strain P100836 (60MDa plasmid) and *E. coli* 39R621 (98, 42, 26.3 and 4.6MDa)

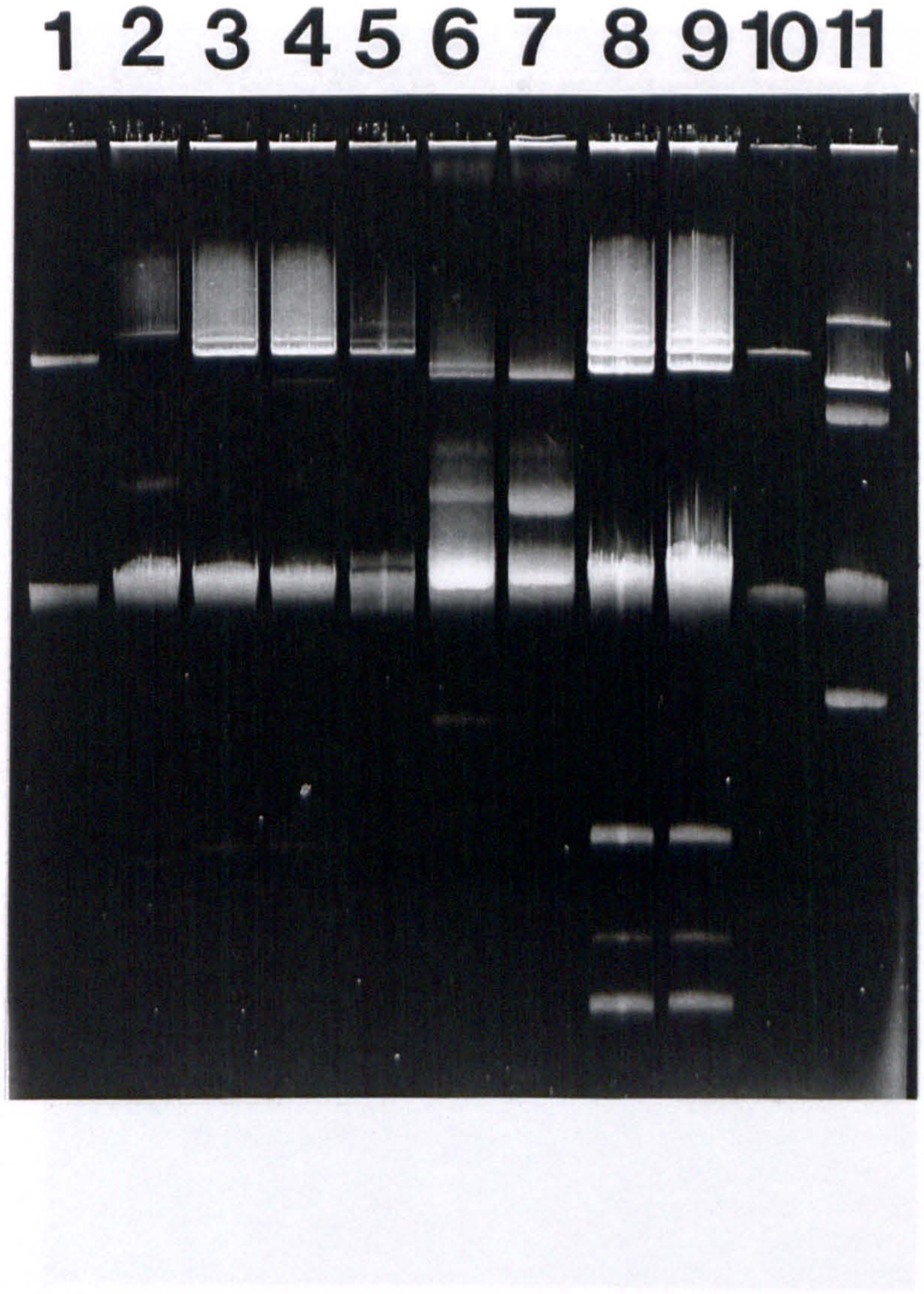


Fig. 12c. Plasmid analysis of the EAggEC strains from outbreak C. Profiles in lanes 1 and 3 contained plasmids of 44.7MDa which hybridised with the AA probe, this probe hybridised with a plasmid of 36.3 and 56.2MDa in lanes 2 and 4. Lanes 5 and 6 contain standard plasmid profiles prepared from *S. typhimurium* strain P100836 (60MDa plasmid) and *E. coli* 39R621 (98, 42, 26.3 and 4.6MDa).

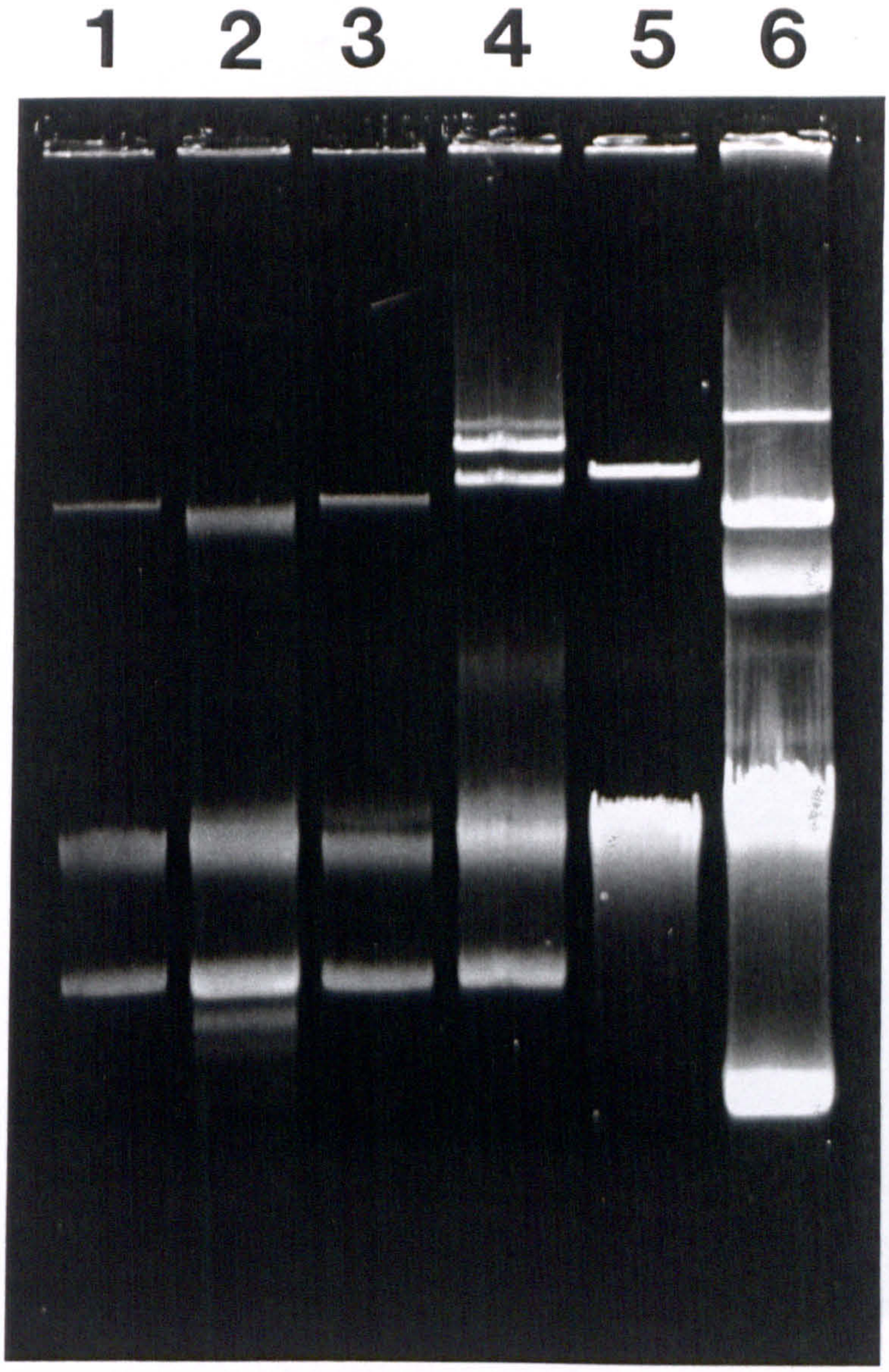


Fig. 12d. Plasmid analysis of the EAggEC strains from outbreak . Profiles in lanes 1, 3 and 4- contained plasmids of 67.6MDa which hybridised with the AA probe, this probe hybridised with a plasmid of 56.2 and 85.1MDa in lanes 2 and 5. Lanes 6 and 7 contain standard plasmid profiles prepared from *S. typhimurium* strain P100836 (60MDa plasmid) and *E. coli* 39R621 (98, 42, 26.3 and 4.6MDa).

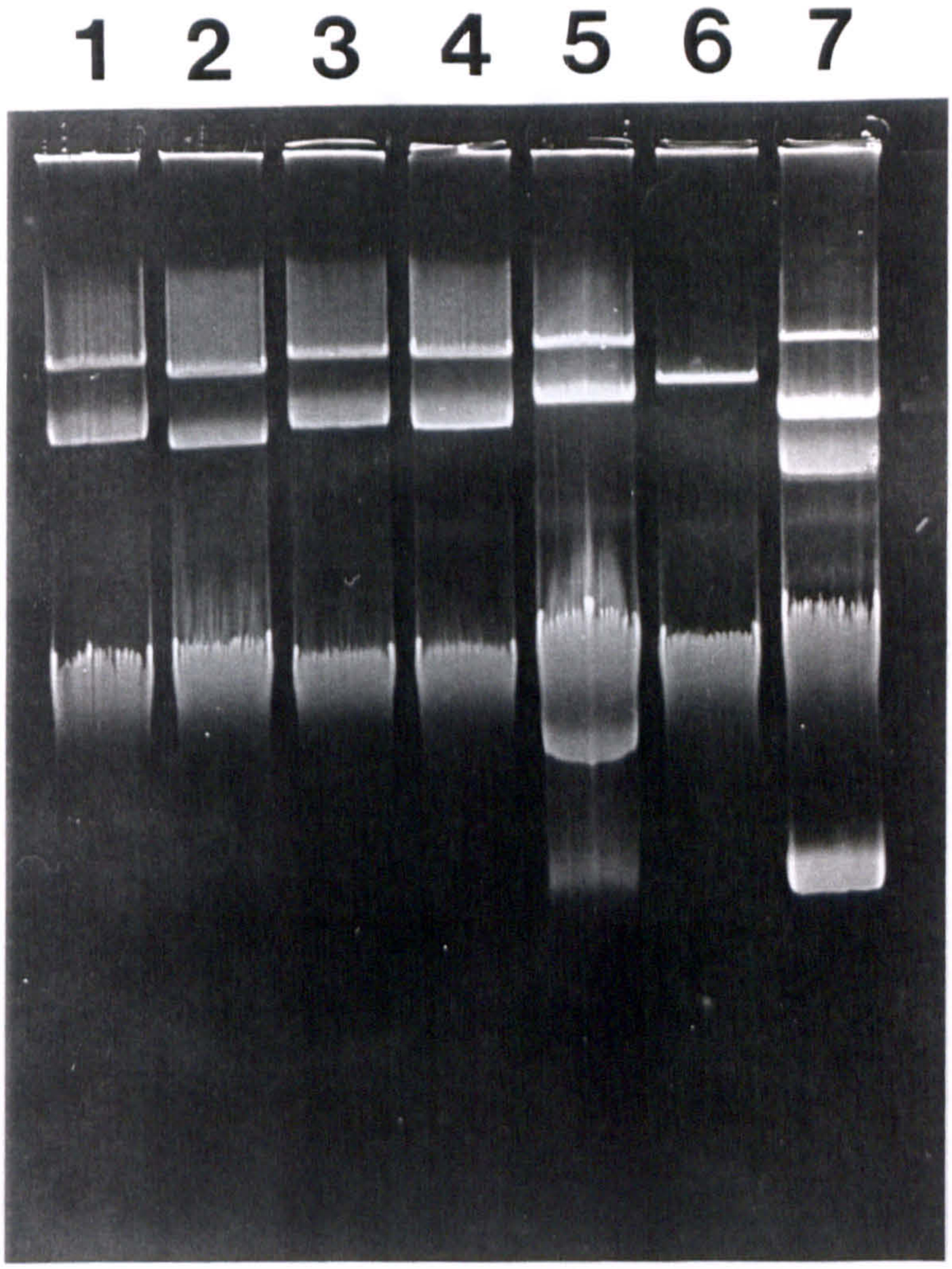


Fig. 12e. Plasmid analysis of AA probe negative strains E40104 and E92356
Lane 1 shows E40104 (AA probe positive, HEp-2 adhesion positive) containing 4 plasmids. Lane 2 shows mutant strain of E40104 (E40104B, AA probe negative, HEp-2 adhesion negative) containing 2 plasmids. Lane 3 shows E92356 (AA probe positive, HEp-2 adhesion positive) containing 2 plasmids. Lane 4 shows mutant of E92356 (E92356B, AA probe negative, HEp-2 adhesion negative) containing only 1 plasmid. Southern blot analysis and hybridisation with the AA probe showed that the probe did not hybridise with any of the remaining plasmids in strain E40104 or with any of the chromosomal DNA in the two strains.

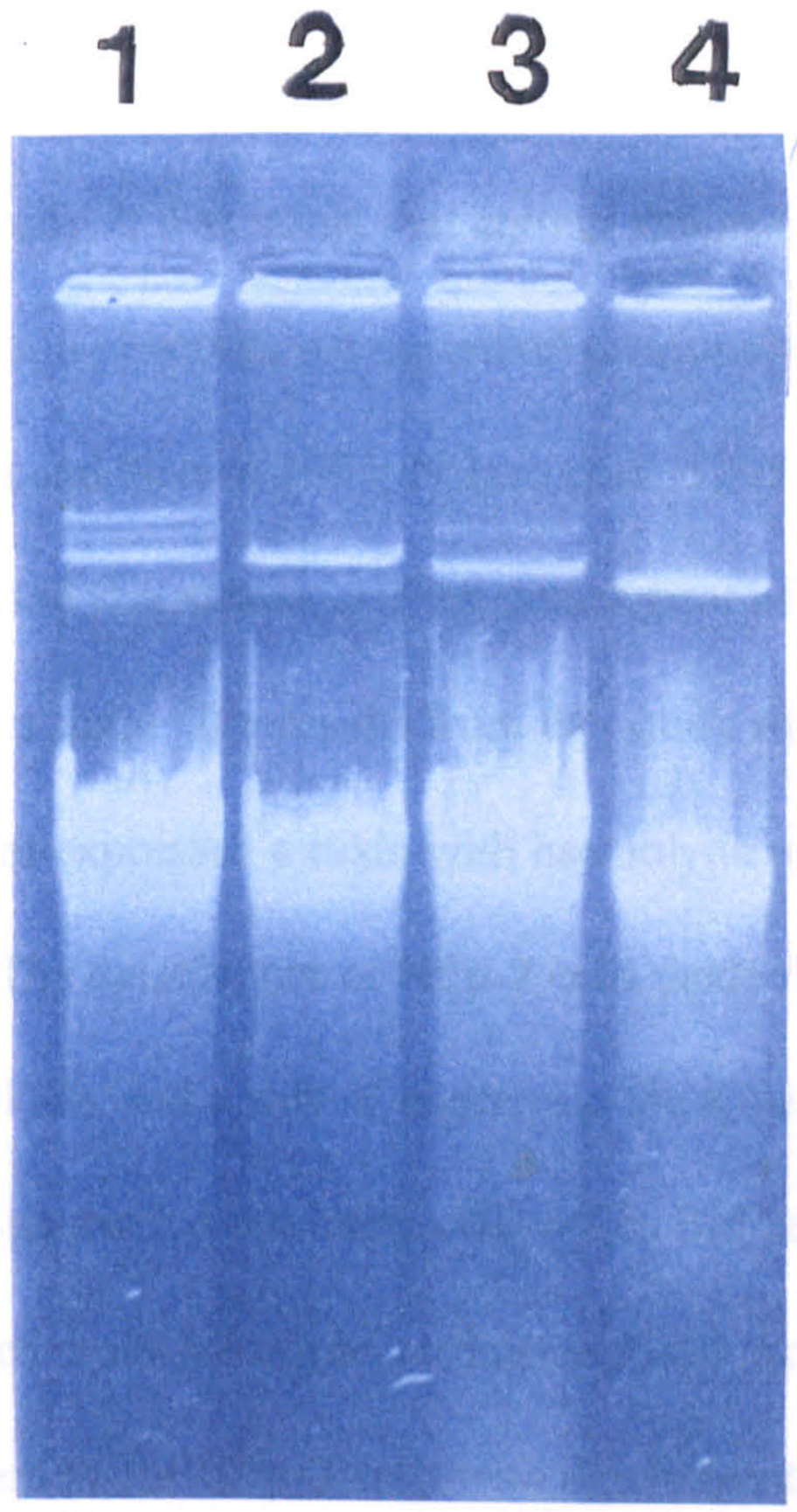


Fig. 12e. Plasmid analysis of AA probe negative strains E40104 and E92356
Lane 1 shows E40104 (AA probe positive, HEp-2 adhesion positive) containing 4 plasmids. Lane 2 shows mutant strain of E40104 (E40104B, AA probe negative, HEp-2 adhesion negative) containing 2 plasmids. Lane 3 shows E92356 (AA probe positive, HEp-2 adhesion positive) containing 2 plasmids. Lane 4 shows mutant of E92356 (E92356B, AA probe negative, HEp-2 adhesion negative) containing only 1 plasmid. Southern blot analysis and hybridisation with the AA probe showed that the probe did not hybridise with any of the remaining plasmids in strain E40104 or with any of the chromosomal DNA in the two strains.

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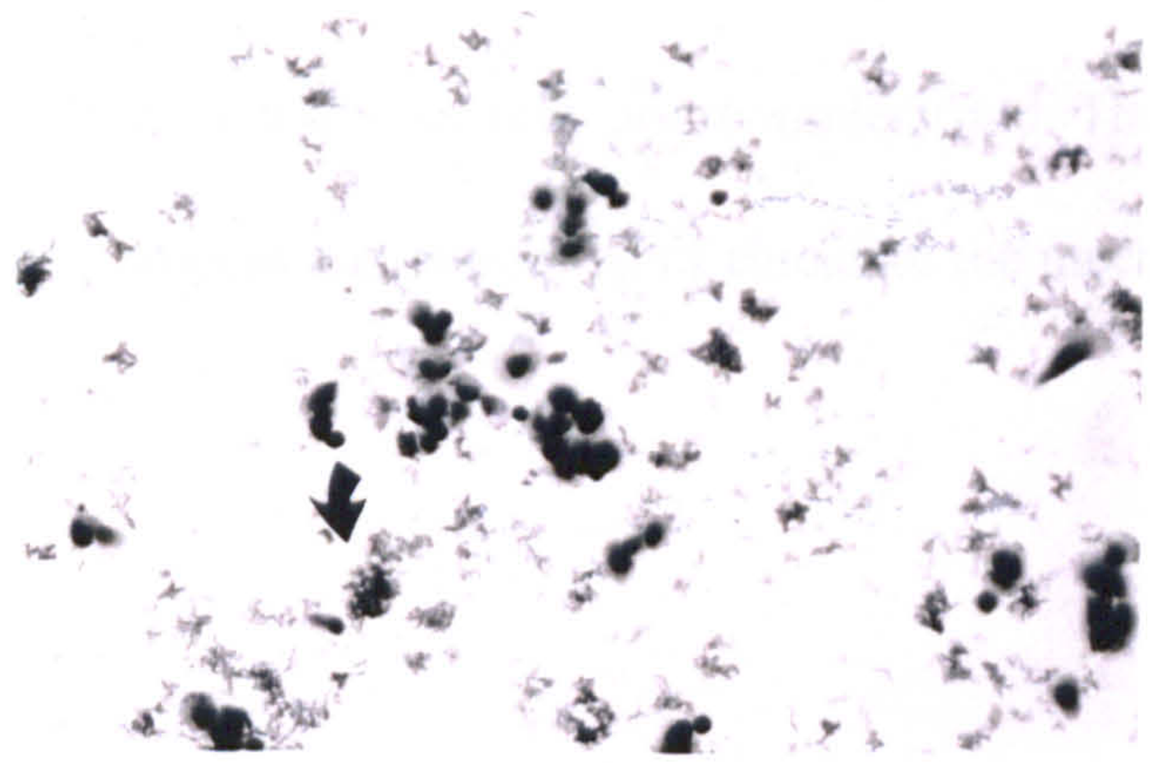
4.1.4. Modification of established HEp-2 cell adhesion assay

In the course of the study, it was noted that certain strains of EAggEC caused detachment of the HEp-2 cells, a phenomenon described previously by other workers (Gunzburg *et al.* 1990; Elliott and Nataro 1995; Nataro *et al.* 1995; Gomes 1995). The study by Gomes (1995) reported that strains causing detachment of HEp-2 cells also produced an α-haemolysin like toxin. The results from this study showed that not all strains causing this detachment expressed a toxin with haemolytic properties which will be discussed later (4.5.2.a.). The detachment of HEp-2 cells made the interpretation of the adhesion pattern very difficult, and a way of improving the adhesion assay was sought. The adhesion assay was carried out using cells which had been "fixed" with formalin. Formaldehyde causes cross-linking of surface-exposed proteins and it was intended to prevent detachment of the HEp-2 cells with this reagent. Strains of EAggEC were found to adhere to formalin-fixed HEp-2 cells (Fig. 13). This observation enabled the aggregative phenotype to be established for bacteria which had previously dislodged HEp-2 cells during the conventional adhesion assay, and also succeeded in improving the established adhesion assay.

A



B

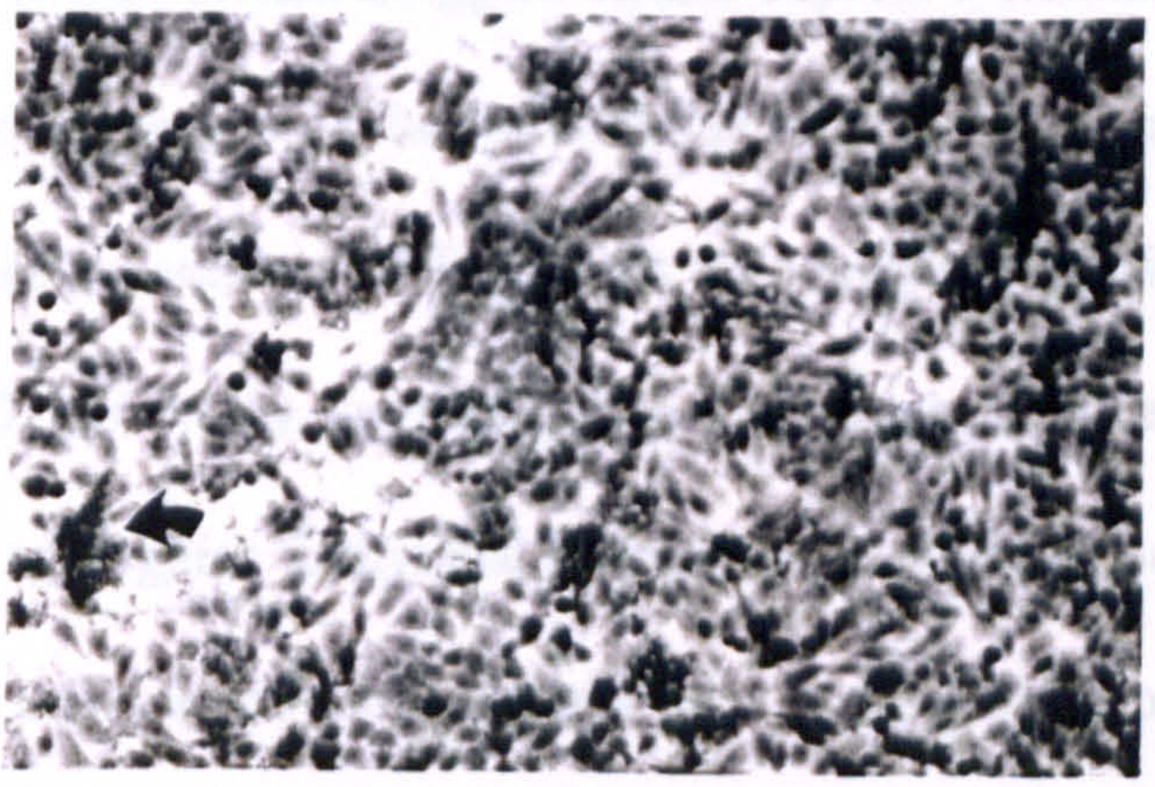


Fig. 13a. Dislodged HEp-2 cells of enteroaggregative strains of *E.coli* during adhesion assay

Fig. 13b. With formalin fixed HEp-2 cells, the adhesion phenotype was readily observed (bar = 100μm)

4.2. Adhesion of EAggEC to HEp-2 cells

The pattern of adhesion of EAggEC to HEp-2 cells has become established universally as appearing like stacked bricks; however, the mechanisms by which these bacteria adhere to HEp-2 cell monolayers are only poorly understood. This part of the study examined the adhesion process and attempted to elucidate the mechanisms involved.

4.2.1. Fimbriae

4.2.1.a. Aggregative adherence fimbriae/I (AAF/I)

It has been reported that certain EAggEC strains express a distinct type of fimbrial structure, termed aggregative adhesion fimbriae I or AAF/I (Nataro *et al.* 1992), which enables them to adhere to HEp-2 cells. The fimbriae described by Nataro *et al.* (1992) for strain 17-2 (O3:H2), were long, bundle forming fimbriae typically clumping the bacteria in aggregates. Individual fimbriae were observed occasionally and appeared flexible, with a diameter of approximately 2nm. Surface shear preparations separated by SDS-PAGE revealed a protein of 14 kDa which reacted with antibodies prepared to the AAF/I fimbriae. A DNA probe was constructed from *E.coli* strain 17-2 for the detection of strains of EAggEC expressing AAF/I fimbriae, and this was used to screen strains of EAggEC in the present study. All 135 strains from this study were examined for hybridisation with the DNA probe for AAF/I (Table 18). The degree of

probe binding was quantified by assessing the results of hybridisation as strong reactions (+++) to weak reactions (+).

Table 18 *Analysis of EAggEC strains hybridising with the DNA probe AAF/I*

		Range of hybridisation with AAF/I probe*			
Panel	**No. strains**	**+++**	**++**	**+**	**-**
1	34	3	7	20	4
2	60	26	16	11	7
3 Outbreak A	4	1	1	1	1
3 Outbreak B	9	0	2	6	1
3 Outbreak C	4	0	2	2	0
3 Outbreak D	5	0	0	2	3
3 Outbreak E	18	12	0	2	4
Total	134	42	28	44	20

* +++ Strong hybridisation reaction
++ ↓
\+ Weak hybridisation reaction
\- No hybridisation

The results of the hybridisation with the AAF/I probe showed that 114 EAggEC strains (85%) showed various intensities of hybridisations with the DNA probe for AAF/I (Table 18). Forty-two strains (31%) showed the strongest hybridisation with this probe. Twenty of 134 strains (15%) did not hybridise with this probe.

Czeczulin *et al.* (1997) found that 16 of 51 (27%) EAggEC strains hybridised with the AAF/I probe which would be in agreement with the results found in this study, based on strains giving +++ hybridisation results.

The AAF/I probe was constructed from the digestion of a plasmid with the enzymes *Pst*I and *Hinc*III from the genetically engineered strain JPN61. The resultant fragments were then separated in an agarose gel and the 910bp fragment excised from the gel using the QIAEX kit. This 910bp fragment was then fluorescein labelled and used as the DNA probe in the hybridisation experiments. The degrees of intensity of

hybridisations could be due to some contaminating DNA from the vector plasmid or host strain DNA that is common to other strains of *E. coli* including strains of EPEC (E2348, O127; E1397, O119) and VTEC E32511 (O157:H7) which hybridised with the probe constructed in this study. The AAF/I probe did not hybridise with strains of EIEC, *S. indiana, S. muenchen,* or *Citrobacter freundii.* There is a possibility there is a problem with the fragment chosen as the probe and it was difficult to draw conclusions. An alternative approach could be applied to investigate the incidence of AAF/I on strains of EAggEC used in this study, for example, antigen detection could be used to detect the 14 kDa fimbrial subunit by immunoblotting.

4.2.1.b. *Aggregative Adherence Fimbriae/II (AAF/II)*

Certain strains of EAggEC strains have been reported to express a second fimbrial structure, aggregative adherence fimbriae/II (AAF/II) which are morphologically and antigenically distinct from AAF/I (Czeczulin *et al.* 1997). AAF/II have a reported diameter of 5nm, considerably thicker than AAF/I and found most commonly as loose bundles of filaments (Czeczulin *et al.* 1997).

The AAF/II probe fragment was derived from strain O42 (O44:H18) isolated from a child in Peru with persistent diarrhoea (Nataro *et al.* 1985a).

Twenty-two of 134 (16%) strains from the total panel hybridised with the probes for AAF/II and DA (Table 19).

EAggEC strains hybridising with the AA, DA and AAF/II probes also express a membrane associated protein (MAP) of 18 kDa, which is required for the aggregative

adherence of these strains of *E. coli* to HEp-2 cells. This observation will be described in greater detail later in this thesis (4.3.1.)

Table 19 *Strains hybridising with AAF/II probe hybridise with AA and DA probes and express an 18 kDa membrane associated protein*

Strain (E)	Serotype	Panel	AA	DA	AAF/II	18 kDa
72376	O33:H16	1	+	+	+	+
43509	O44:H18	1	+	+	+	+
44939	O44:H18	1	+	+	+	+
45730	O44:H18	1	+	+	+	+
38383	O126:H27	1	+	+	+	+
55060	O126:H27	1	+	+	+	+
58816	O126:H27	1	+	+	+	+
52610	O126:H27	1	+	+	+	+
40104	O126:H27	1	+	+	+	+
45691	O126:H27	1	+	+	+	+
57157	O126:H27	1	+	+	+	+
75607	O126:H27	1	+	+	+	+
60874	O126:H27	1	+	+	+	+
83087	O126:H27	12	+	+	+	+
81456	O126:H27	1	+	+	+	+
99979	O75:H27	2	+	+	+	+
110716	O106:H16	2	+	+	+	+
107100	O119:H27	2	+	+	+	+
107542	Orough:H27	2	+	+	+	+
105836	Orough:H3	2	+	+	+	+
101402	O110:H-	3 (D)*	+	+	+	+
97474	O116:H27	3 (C)*	+	+	+	+
62008¶	O126:H27	1	-	-	-	-

* outbreak

¶ non-adherent mutant

These strains did not produce fimbriae as observed by electron microscopy.

Strains hybridising with the AAF/II probe also hybridised with the probe for DA. This result could be due to these strains carrying separate sequences of DNA required for hybridisation with the AA, DA and AAF/II probes.

Total cellular DNA of 5 EAggEC strains expressing the 18 kDa membrane-associated protein (MAP) was digested with *Eco*RI and electrophoresed in an agarose

gel (3.5.3.) and these profiles were transferred onto nylon membrane by the method of Southern blotting (3.5.6.). The AA probe hybridised with a 17.5 kb fragment produced by EAggEC strains E40104 (O126:H27), E72376 (O33:H16), E44939 (O44:H18), and E97474 (O116:H27); (Fig 14 lanes 1, 3-5); however the AA probe hybridised with a 9.8 kb fragment in EAggEC strain E101402 (O110:H-; Fig. 14 lane 6). The AA probe did not hybridise with the DNA digest profile of the non-adherent, probe negative variant E62008 (Fig 14, lane 2). Replicate DNA digests were also tested with the DA probe. This probe hybridised with a 19.5 kb fragment in EAggEC strains E40104, E72376, E44939 and E101402; however this probe bound to two fragments of 19.3 and 21.0 kb in EAggEC strain E97474. The DA probe did not hybridise with the DNA of *E. coli* strain E62008 (results not shown).

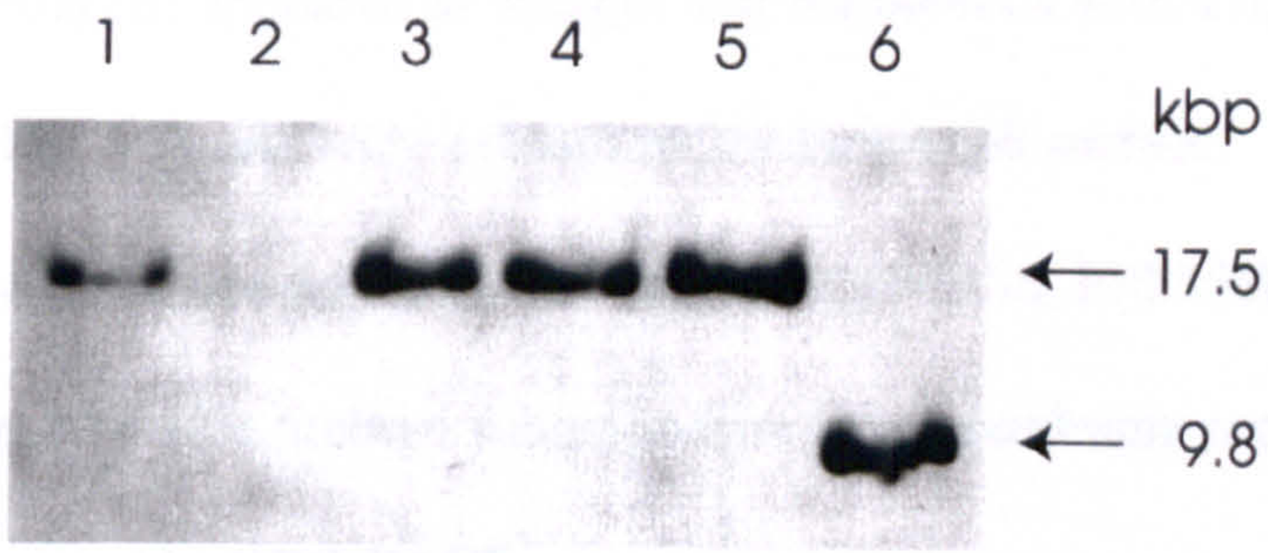


Fig. 14 The AA DNA probe bound to a 17.5 kb fragment of EAggEC strain E40104 (lane 1, arrowed) but not to the DNA of strain E62008 (lane 2). The AA probe also bound to a 17.5 kb fragment in EAggEC strains E72376 (lane 3), E44939 (lane 4) and E97474 (lane 5); the AA probe bound to a 9.8 kb fragment in EAggEC strain E101402 Lane 6, arrowed).

4.2.1.c. *Electron Microscopy*

A total of 31 strains of EAggEC were examined for expression of fimbriae by transmission electron microscopy (TEM; 3.7.8.), alongside a fimbriated strain of ETEC used as a positive control. Of the EAggEC examined, only four strains expressed fimbriae. One of the four strains was the prototype strain 17-2 (E92356, O3:H2). In this study the fimbriae observed appeared as straight and filamentous with a diameter estimated at 6 nm (Fig. 15), and were present on the entire cell surface.

Results showed that the non-adherent variant of E92356, E92356B did not express fimbriae or the 14 kDa fimbrial subunit when outer membrane protein preparation was examined by SDS-PAGE.

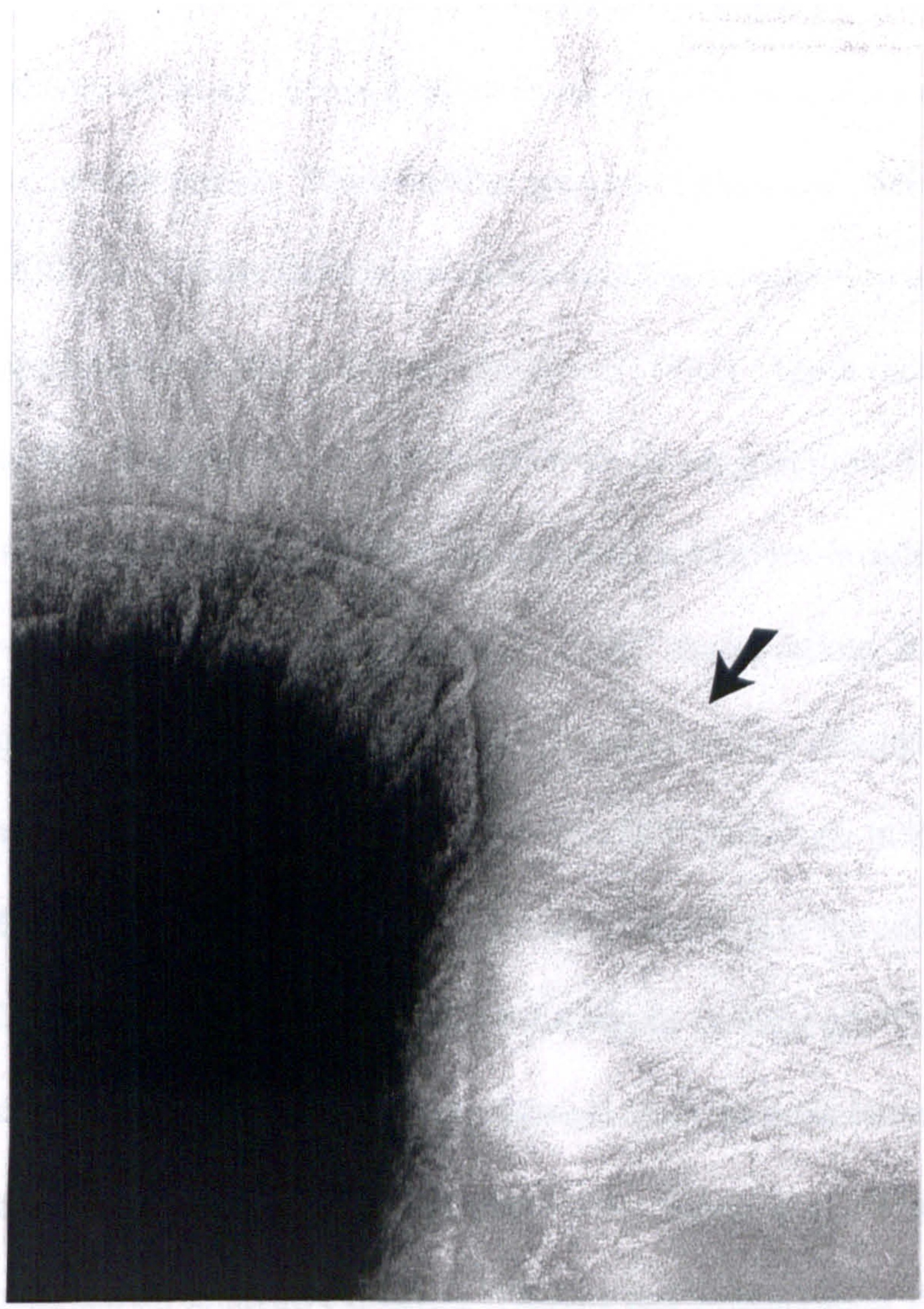


Fig 15. Electron micrograph of E92356 expressing fimbriae (estimated diameter = 6nm). Bacteria were negatively stained with ammonium molybdate. Magnification = x 185, 000

Two of the four strains, E97590 (O73:H13) and E96390 (O?:H-), also expressed fimbriae which appeared as bundles of long, thin structures (Fig 16a & b). The bundles had an estimated diameter of 30 nm. It was difficult to see the individual filaments and estimate the diameter of each fimbria. These bundles appeared longer and thicker than those expressed by E92356. Bundle forming pili (BFP) have been reported to be expressed by enteropathogenic strains of *E.coli* (Giron *et al.* 1991). These fimbriae formed rope-like bundles clumping the bacteria together by autoaggregation. The fimbriae were encoded by genes carried on the EAF plasmid and formed bundles of 50 to 500 nm wide by 15-20μm long (Giron *et al.* 1993a, b). EPEC strains lacking BFP lost the ability to autoaggregate but could still display a localised pattern of adherence to HEp-2 cells (Knutton *et al.* 1991). These results show that BFP play a role in the attachment of EPEC to host cells but are not the only factor involved in the adhesion to host cells. EAggEC strains E97590 and E96390 express bundle forming pili (bfp); these fimbriae may play an important role in the autoaggregation of the bacteria but may not be involved in the initial attachment of the bacteria to host cells. Adhesion of ETEC to the small intestine has been shown to involve fimbriae (Gaastra and Svennerholm 1996), and many different types have been identified (Evans *et al.* 1975; Levine *et al.* 1984). However, the results of this study demonstrate that the fimbriae are not expressed by all EAggEC, and other mechanisms appear to be involved in the adhesion process. Reports have shown that EAggEC strains produce antigenically and morphologically distinct fimbriae other than AAF/I and AAF/II (Vial *et al.* 1988; Old *et al.* 1989; Knutton *et al.* 1992; Collinson *et al.* 1992) whereas other strains do not produce fimbriae (Chart *et al* 1994, 1995).

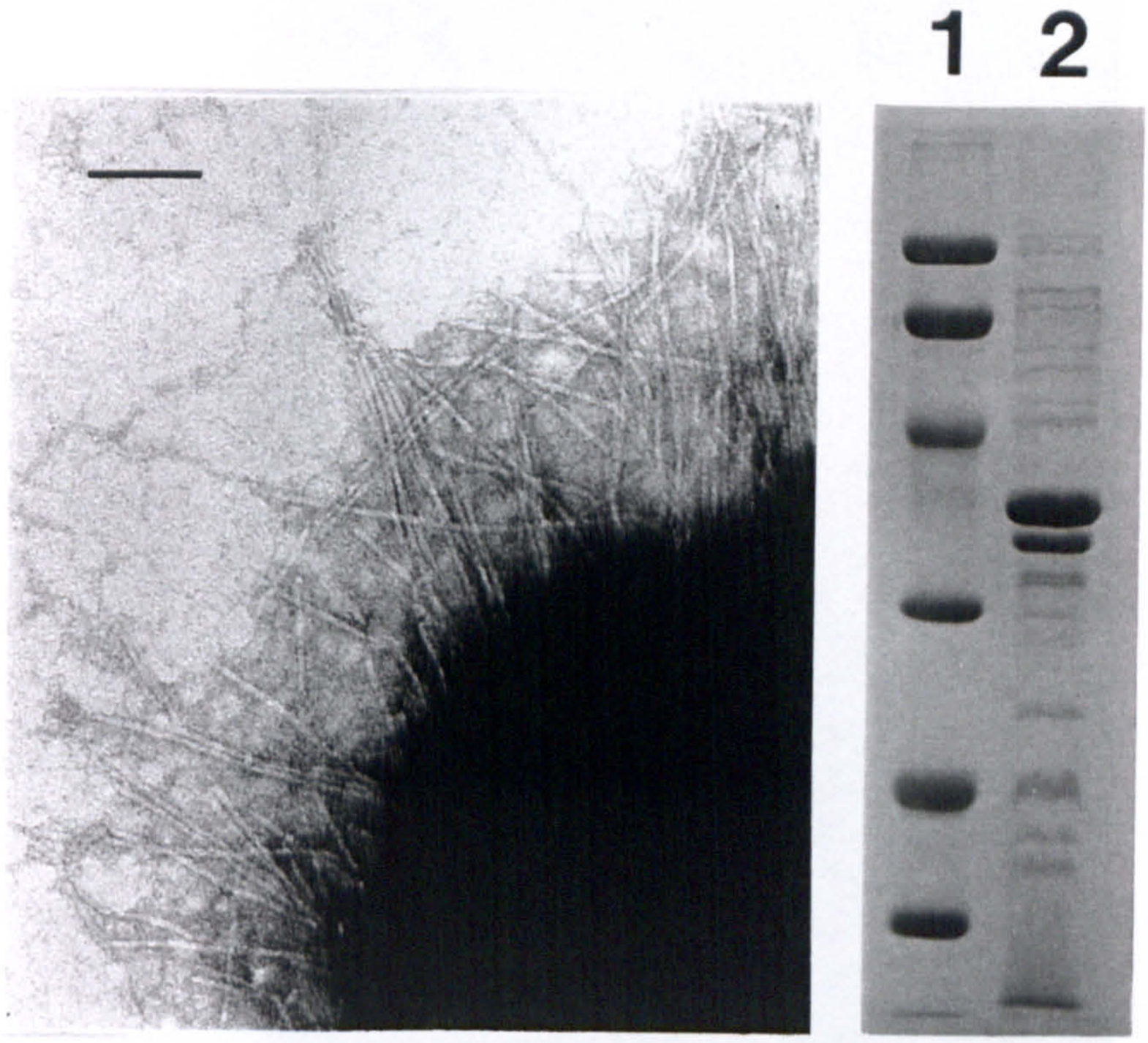


Fig. 16a. Electron micrograph showing EAggEC strain E97590 expressing fimbriae and SDS-PAGE gel showing fimbrial subunit estimated at 19 kDa (arrrowed). Lane 1 shows molecular weight markers (94, 66.2, 45, 31, 21.5 and 14.4 kDa). Lane 2 shows outer membrane protein profile of E97590.

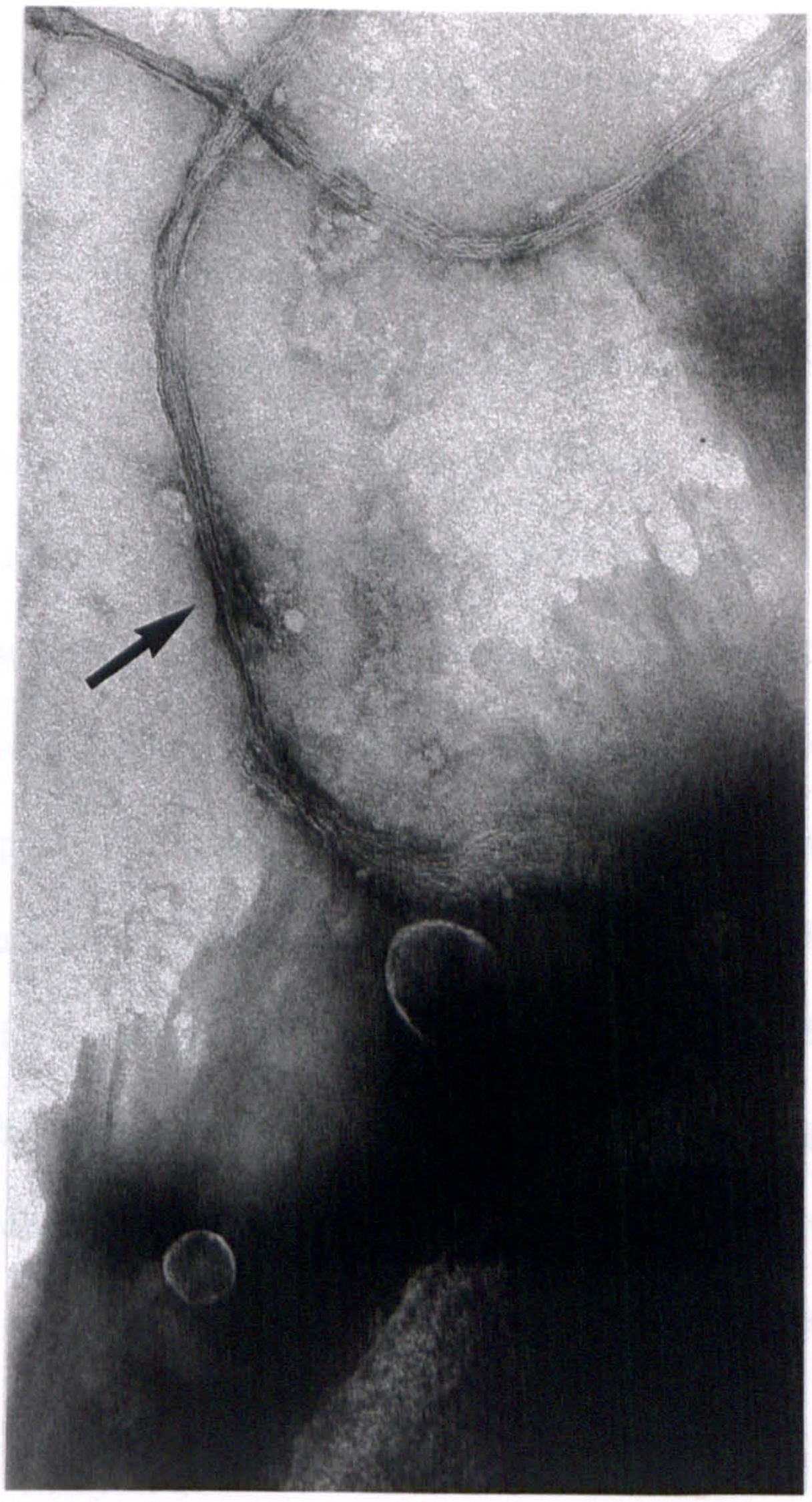


Fig. 16b EAggEC strain E97590 expressing bundle-forming fimbriae (diameter of bundles approx. 30nm). Bacteria were negatively stained with ammonium molybdate.

The fourth strain expressing fimbriae was E97468. The fimbriae appeared as rigid and filamentous with an estimated diameter of 7nm (Fig. 16c). It was suggested that these fimbriae were morphilogically similar to CFA/I, type 1, CS1 and CS2 expressed by strains of ETEC (Evans *et al.* 1975; Levine *et al.* 1984; van Verseveld *et al.* 1985)

Certain strains of *E. coli* do not express fimbriae when grown at 18°C (Van Verseveld *et al.* 1985). Similarly, when EAggEC strains E97590 and E 96390 were grown at 18°C on nutrient agar plates for 48 h fimbriae were not produced.

The possibility arose that the expression of fimbriae by EAggEC might be inducible, such that expression occurs only when these bacteria make contact with HEp-2 cells. To examine this hypothesis, *E.coli* strain E40104 was allowed to adhere to HEp-2 cell monolayers for 4 hours, seeded in several large tissue culture flasks, and the adherent bacteria harvested and examined without subculture. The bacteria/HEp-2 cells were heat-extracted, and the resultant material examined by SDS-PAGE alongside heated extracts prepared from agar-grown bacteria. Fimbrial protein subunits were not observed. It was therefore concluded that for EAggEC strain E40104, expression of fimbriae was not inducible.

Fig. 16c EAggEC strain E97648 expressing fimbriae (diameter = 7nm). Bacteria were negatively stained with ammonium molybdate.

4.2.2. Haemagglutination

The haemagglutinating properties of *E. coli* have been used to characterise strains into 3 groups (Duguid *et al.* 1955). The haemagglutinating properties of groups I and II have been associated with the expression of fimbriae. Strains belonging to group I express the common fimbriae - type 1, which lose their haemagglutinating ability in the presence of mannose. Strains of *E. coli* belonging to group 3 have been shown not to express fimbriae.

In the present study, 140 strains of EAggEC were examined for their ability to agglutinate rat and sheep erythrocytes in the presence and absence of 1% D-mannose. With reference to Table 20, 21 strains of EAggEC from panel 1 agglutinated rat erythrocytes in the presence of D-mannose. These included *E. coli* strains E92356 (O3:H2) and E97468 (O86:H34) which had been shown to express fimbriae (see 4.2.1.c.). However, from this part of the study it was concluded that since both strains agglutinated rat erythrocytes in the presence of D-mannose then type 1 fimbriae were not associated with the haemagglutining properties. Of the 21 strains in this section, only 5 strains agglutinated sheep erythrocytes. Three of these strains belonged to serotype O126:H27, but not all strains belonging to this serotype agglutinated sheep red cells. Apart from strains E92356 and E97468, none of these strains expressed fimbriae; therefore, it was concluded that the observed haemagglutination resulted from specific interactions between components on the erythrocytes and bacterial surfaces.

Table 20. *Positive haemagglutination results obtained from EAggEC strains belonging to panel 1*

		Haemagglutination		
Strain (E)	Serotype	Rat	Sheep	Rat (1% mannose)
92356	O3:H2	+	-	+
73339	O15:H-	+	+	+
43509	O44:H18	+	-	+
44939	O44:H18	+	-	+
58596	O51:H11	+	-	+
97468	O86:H34	+	+	+
60725	O92:H33	+	-	+
101621	O98:H-	+	-	+
99518	O104:H4	+	-	+
40144	O111:H21	+	-	+
57144A	O111:H21	+	-	+
55060	O126:H27	+	-	+
40104	O126:H27	+	-	+
58816	O126:H27	+	-	+
52610	O126:H27	+	-	+
45691	O126:H27	+	-	+
38383	O126:H27	+	+	+
57157	O126:H27	+	+	+
60874	O126:H27	+	-	+
81456	O126:H27	+	+	+
83087	O126:H27	+	-	+

However, when strains from panel 1 were grown on MacConkey agar (6.2.3.) the haemagglutinating reactions remained the same. However, when these strains were grown statically in L-broth, as recommended for Duguid's group I (Duguid *et al.* 1955; expression of type 1 fimbriae; 2.3.8.b.), none of the strains agglutinated rat erythrocytes.

The panel of EAggEC strains isolated during the Infectious Intestinal Diseases (IID) study (panel 2) was also examined for the ability to haemagglutinate rat and sheep erythrocytes in the presence/absence of D-mannose (Table 21). Thirty-three strains agglutinated rat erythrocytes in the presence of mannose. Fifteen of these strains also agglutinated sheep erythrocytes and two strains agglutinated sheep erythrocytes only.

Table 21 *Haemagglutination results by strains of EAggEC isolated from panel 2 (IID study)*

Strain	Serotype	Haemagglutination		
		Rat	Sheep	Rat (1% mannose)
97479	O3:H-	+	+	+
109902	O4:H33	+	+	+
101094	O4:H33	+	+	+
97480	O6:H10	-	+	-
111479	O11:H-	+	-	+
99960	O18:H30	+	+	+
99961	O18:H30	+	+	+
99962	O18:H30	+	+	+
99963	O18:H30	+	+	+
99964	O18:H-	+	+	+
99965	O18:H30	+	+	+
107249	O21:H4	+	-	+
99970	O53:H-	+	+	+
99971	O53:H2	+	+	+
99979	O75:H27	+	+	+
97494	O81:H7	+	-	+
92832	O82:H25	+	-	+
92830	O86:H11	+	-	+
97496	O86:H2	+	-	+
99518	O104:H5	+	+	+
94706	O111:H-	+	-	+
109907	O111:H-	+	-	+
103763	O111:H-	+	-	+
99976	O113:H-	+	-	+
107100	O119:H27	+	-	+
97478	O130:H27	+	-	+
108839	O130:H27	+	-	+
104968	O131:H-	+	-	+
104942	O162:H10	+	-	+
99967	Orough:H-	+	+	+
94708	Orough:H-	-	+	-
97298	Orough:H27	+	-	+
107542	Orough:H27	+	-	+
105836	Orough:H3	+	+	+
105392	Orough:H33	+	-	+

The panel of EAggEC strains isolated during outbreaks A-E (panel 3) was also examined for the ability to haemagglutinate rat and sheep erythrocytes in the presence/absence of D-mannose (Table 22). Thirty-two of the forty strains agglutinated

rat erythrocytes. Nine of these strains also agglutinated sheep erythrocytes. All strains in outbreaks C, D and E except EAggEC strain E89115 agglutinated rat erythrocytes in the presence of mannose.

Table 22 *Haemagglutination results of outbreak panel 3*

Strain	Serotype	Outbreak	Haemagglutination			
			Rat	Sheep	Rat (1% mannose)	Rat 18°C
98529	O?:H18	A	+	+	+	+
96390	O?:H-	B	+	-	+	+
96487	O?:H-	B	+	-	+	+
97900	O?:H-	B	+	-	+	+
97590	O73:H13	B	+	-	+	+
97472	O86:H34	C	+	-	+	+
97468	O86:H34	C	+	-	+	+
97470	O86:H34	C	+	-	+	+
97474	O116:H27	C	+	-	+	-
101621	O98:H-	D	+	-	+	-
101408	O98:H-	D	+	-	+	+
101396	O98:H-	D	+	-	+	+
101406	O98:H-	D	+	-	+	-
101402	O110:H-	D	+	-	+	-
89107	:H27	E	+	-	+	ND
89097	O6:H16	E	+	-	+	ND
89101	O8:H20	E	+	+	+	ND
89099	O28:H18	E	+	-	+	ND
89102	O44:H18	E	+	-	+	ND
89109	O44:H18	E	+	-	+	ND
89114	O44:H18	E	+	-	+	ND
89110	O69:H11	E	+	+	+	ND
89112	O69:H11	E	+	+	+	ND
89105	O80:H27	E	+	+	+	ND
89095	O80:H27	E	+	+	+	ND
89111	O89:H18	E	+	-	+	ND
89096	O113:H-	E	+	+	+	ND
89098	O114:H11	E	+	-	+	ND
89104	O141:H49	E	+	-	+	ND
89115	O162:H-	E	+	+	-	ND
89106	Orough:H7	E	+	-	+	ND
89100	O?:H5	E	+	+	+	ND

ND haemagglutination test was not done

Strains of EAggEC from outbreaks A-D which agglutinated rat erythrocytes were grown at 18 °C to examine the effect of culture temperature on haemagglutinating properties. Ten of the 14 strains which agglutinated rat erythrocytes when grown at 37°C also agglutinated rat red cells when grown at 18°C. Since strains of *E. coli* do not generally express fimbriae when grown at 18°C (Van Verseveld *et al.* 1985), the results of this part of the study indicated that in common with HEp-2 adhesion the haemagglutination properties of EAggEC were also not associated with fimbriae.

EAggEC strains from panel 3 (outbreaks A-D) were examined for their ability to agglutinate erythrocytes from different animal species including rat, mouse, turkey, chicken, guinea-pig and rabbit (Table 22a).

Table 22a *Haemagglutination results using a variety of erythrocytes with EAggEC strains from panel 3*

Strain	Serotype	Haemagglutination					
		Rat	Mouse	Turkey	Chicken	Guinea-pig	Rabbit
A							
E98527	O19:H-	-	-	-	+	+	-
E97622	O113:H-	+	+	+	+	+	+
E96393	O125:H27	-	-	-	+	-	+
E98529	O?:H18	+	+	+	+	+	+
B							
E96386	O73:H18	-	-	-	-	-	-
E97820	O62:H30	-	-	-	-	-	-
E97819	O?:H27	-	-	-	-	-	-
E96483	O?:H33	+	-	-	-	-	-
E96485	O134:H27	-	-	-	-	-	-
E96390	O?:H-	+	-	-	+	-	-
E97590	O73:H13	+	+	-	+	-	+
E96487	O?:H-	+	+	+	+	+	+
E97900	O?:H-	+	+	+	+	+	+
C							
E97472	O86:H34	+	+	+	+	+	+
E97468	O86:H34	+	+	+	+	+	+
E97470	O86:H34	+	+	+	+	+	+
E97474	O116:H27	+	+	-	+	+	+
D							
E101408	O98:H-	+	-	-	-	-	-
E101621	O98:H-	+	-	-	-	-	-
E101396	O98:H-	+	-	-	-	-	-
E101406	O98:H-	+	-	-	-	+	-
E101402	O110:H-	+	-	-	-	-	-

The EAggEC strains from the outbreak panel 3 exhibited a wide range of haemagglutination patterns. All strains in outbreak C agglutinated all the types of red cells, except strain E97474 which did not agglutinate turkey erythrocytes. Strains E97622 and E98529 from outbreak A showed a broad spectrum of haemagglutinating ability whereas the other strains from this outbreak, E98527 agglutinated chicken and

guinea-pig erythrocytes and E96393 agglutinated chicken and rabbit erythrocytes only. These strains showed a narrow range of haemagglutining ability. EAggEC strains from outbreak D agglutinated rat erythrocytes only, except strain E101406 which agglutinated also guinea-pig erythrocytes. Four strains from outbreak B did not agglutinate any of the red blood cells tested, but strains E96487 and E97900 agglutinated all the red blood cell types. EAggEC strain E96483 agglutinated rat erythrocytes only whereas E96390 agglutinated rat and chicken erythrocytes. E97590 has been shown to produce fimbriae and had the ability to agglutinate rat, mouse, cockerel and rabbit erythrocytes.

These results show that strains of EAggEC have a wide range of haemagglutinating abilities, from broad spectrum agglutinations to agglutinating erythrocytes from one or two species only. Many strains did not possess the ability to agglutinate any erythrocytes. Rat red blood cells were agglutinated most often. Eighty-six (61%) of all the strains examined agglutinated rat erythrocytes. Twenty-six strains (18%) of the total panel agglutinated sheep erythrocytes. Two strains E97480 (O6:H10) and E94708 (Orough:H-) agglutinated sheep erythrocytes only.

Strains which are unable to haemagglutinate in the presence of 1% D-mannose are usually associated with the expression of the classical *E. coli* type 1 fimbriae (Aronson *et al.* 1979).

4.2.3. Pellicle Formation

Albert *et al.* (1993) reported that strains of EAggEC grown in broth culture at 37°C developed a scum on the surface of the broth whereas control strains, including ETEC, EPEC, EIEC, VTEC, DAEC, and non-pathogenic strains, did not. It was suggested that pellicle formation could be used as a simple method for differentiating strains of EAggEC from other types of pathogenic *E. coli*.

In the present study all EAggEC strains from the panels 1-3 (Tables 23-25) were tested for their ability to produce a pellicle when grown statically in L-broth (16h, 37°C). The results showed that not all EAggEC strains produced a pellicle (Fig. 17). Twenty-four (70%) of the EAggEC strains from panel 1 produced a pellicle (Table 23) whereas 48 (80%) of the EAggEC strains from panel 2 had this ability. Production of a pellicle was only seen in 50% of strains in outbreaks A and C whereas 100% of the strains from outbreak E had this ability. The only two strains (E96393 and E97590) from outbreak B which produced a pellicle also expressed fimbriae (Table 25).

Table 23 *Pellicle formation of EAggEC strains from panel 1 when grown in L-broth (16h, 37°C)*

Strain (E)	Serotype	Pellicle
92356	O3:H2	+
73339	O15:H-	+
59905	O21:H2	-
72376	O33:H16	+
45730	O44:H18	+
44939	O44:H18	+
43509	O44:H18	+
58596	O51:H11	+
76989	O75:H2	-
58583A	O77:H18	+
67643	O78:H10	-
47697A	O86:H19	+
97468	O86:H34	-
78135	O92:H33	+
60725	O92:H33	+
101621	O98:H-	-
72957	O102:H27	-
99518	O104:H4	+
57144A	O111:H21	+
40144	O111:H21	+
33915	O111:H21	+
36182	O111:H21	+
60874	O126:H27	+
55060	O126:H27	+
58816	O126:H27	+
52610	O126:H27	+
45691	O126:H27	+
62008	O126:H27	-
57157	O126:H27	-
75607	O126:H27	-
83087	O126:H27	-
81456	O126:H27	+
40104	O126:H27	+
38383	O126:H27	+

Table 24 *Pellicle formation of the EAggEC strains from panel 2 after growth in L-broth (16h, 37°C)*

Strain (E)	Serotype	Pellicle
97479	O3:H-	-
111260	O4:H2	+
99536	O4:H33	+
101094	O4:H33	+
109902	O4:H33	+
96616	O5:H4	+
107527	O6:H1	+
97499	O6:H10	-
97480	O6:H10	+
110715	O6:H10	-
99520	O8:H7	+
97494	O81:H-	+
111479	O11:H-	+
103594	O11:H27	-
99964	O18ac:H-	+
99962	O18ac:H30	+
99961	O18ac:H30	+
99963	O18ac:H30	+
99965	O18ac:H30	+
99960	O18ac:H30	+
109903	O21:H-	+
107249	O21:H4	+
104970	O33:H-	-
99970	O53:H-	+
99971	O53:H2	+
97500	O73:H1	-
99979	O75:H27	+
97477	O81:H-	+
92832	O82:H25	-
92830	O86:H11	+
97496	O86:H2	-
97504	O91:H-	+
99518	O104:H4	+
110716	O106:H16	-
94706	O111ab:H-	+
109907	O111ab:H-	+
103763	O111ab:H2	+
99976	O113:H-	+
107100	O119:H27	+

continued.....

Table 24 *Pellicle formation of the EAggEC strains from panel 2 after growth in L-broth (16h, 37°C) (cont)*

Strain (E)*	Serotype	Pellicle
101089	O126:H27	+
108837	O130:H27	+
97478	O130:H27	+
108839	O130:H27	+
104940	O130:H27	+
104968	O131:H-	+
99969	O134:H-	+
107754	O134:H25	+
99968	O134:H27	+
97502	O134:H27	-
101095	O134:H27	+
101096	O134:H27	+
104942	O162:H10	+
94708	Orough:H-	-
99967	Orough:H-	+
107252	Orough:H-	+
99535	Orough:H27	-
107542	Orough:H27	+
97298	Orough:H27	+
105836	Orough:H3	+
105392	Orough:H33	+

Table 25 *Pellicle formation of the EAggEC strains from panel 3 after growth in L-broth (16h, 37°C)*

Strain (E)	Serotype	Outbreak	Pellicle
98529	O?:H18	A	+
96393	O125:H27	A	+
98527	O19:H-	A	-
97622	O113:H-	A	-
96386	O73:H18	B	-
97820	O62:H30	B	-
97819	O?:H27	B	-
96483	O?:H33	B	-
96485	O134:H27	B	-
96390	O?:H-	B	+
97590	O73:H13	B	+
96487	O?:H-	B	-
97900	O?:H-	B	-
97472	O86:H34	C	+
97468	O86:H34	C	-
97470	O86:H34	C	+
97474	O116:H27	C	+
101408	O98:H	D	-
101621	O98:H-	D	+
101396	O98:H-	D	-
101406	O98:H-	D	-
101402	O110:H-	D	+
89097	O6:H16	E	+
89101	O8:H20	E	+
89099	O28:H18	E	+
89102	O44:H18	E	+
89109	O44:H18	E	+
89114	O44:H18	E	+
89110	O69:H11	E	+
89112	O69:H11	E	+
89105	O80:H27	E	+
89095	O80:H27	E	+
89111	O89:H18	E	+
89096	O113:H-	E	+
89098	O114:H11	E	+
89104	O141:H49	E	+
89115	O162:H-	E	+
89107	:H27	E	+
89100	O?:H5	E	+
89106	Orough:H7	E	+

One strain (E36182; O111:H21) produced a particularly large pellicle, and when this strain was grown in L-broth with shaking (120 rpm) produced an extensive 'tide mark' of cells (Fig. 17). It was thought that the bacteria forming the 'tide-mark' might have surface properties which differed from those in the L-broth. To examine this bacteria comprising the 'tidemark' were harvested and compared with broth grown bacteria for charge, hydrophobicity and haemagglutination properties, and expression of surface structures by electron microscopy. Differences between the two groups of bacteria were not detected. It was concluded that all of these bacteria could adhere to glass and that the amount of cell mass adhering to the flask at the broth/liquid interface was finite.

The role of divalent cations in the formation of a pellicle was determined by growing strains in L-broth containing 1mM ethylene-diamine-tetra-acetic acid (EDTA) or 1mM ethylene-glycol-bis(β-amino-ether)tetra-acetic-acid (EGTA)

A selection of 26 strains of EAggEC from panel 1 were grown in L-broth EDTA or EGTA. All the strains that were able to produce a pellicle when grown in L-broth could also do so in L-broth containing 1mM EGTA. None of the EAggEC strains produced a pellicle when grown in L-broth containing 1mM EDTA (Table 26).

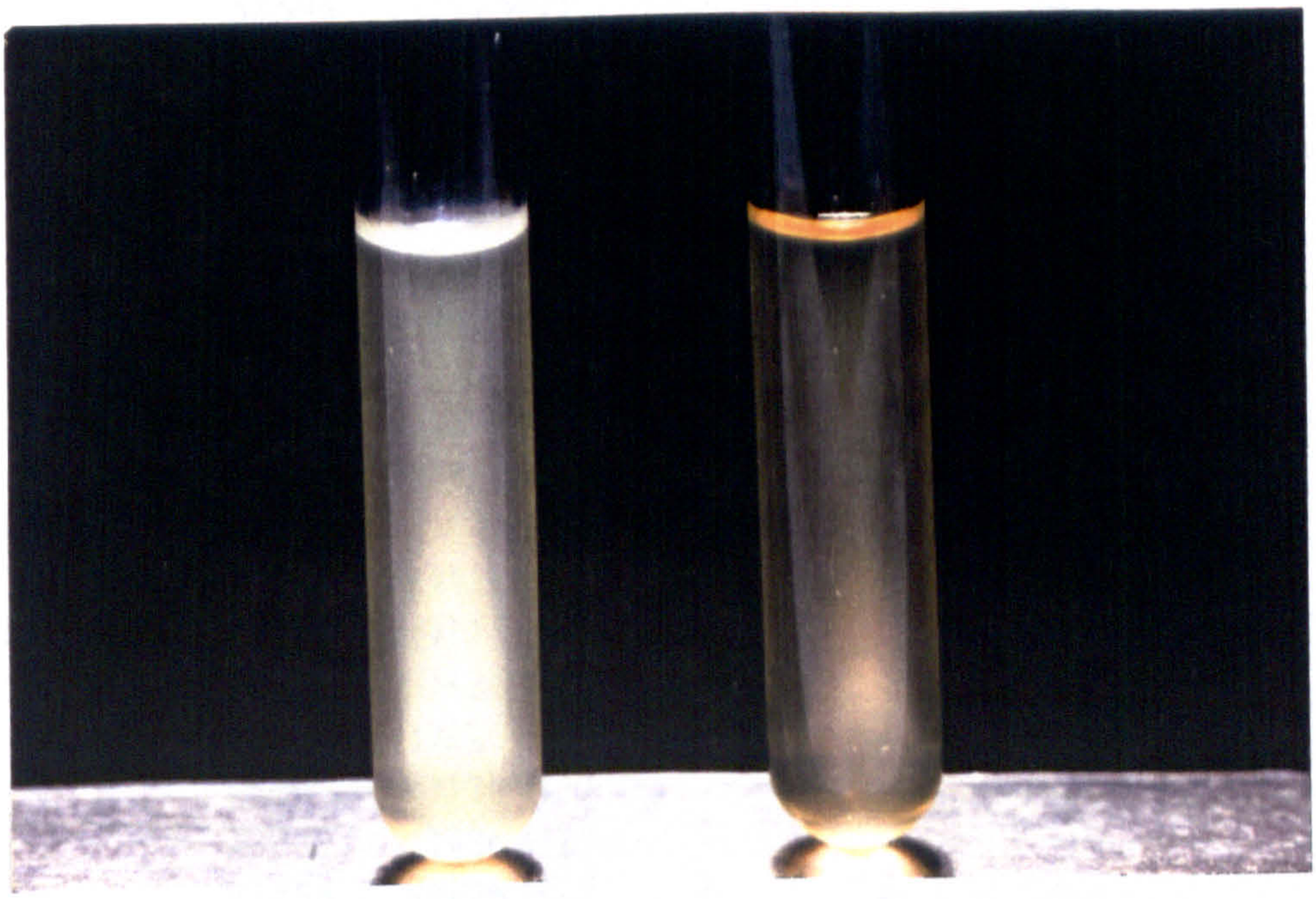


Fig. 17. Pellicle formation by EAggEC strain E97590 grown in L-broth alone (left). No pellicle formation when E97590 was grown in the presence of EDTA (right)

Table 26 *Pellicle formation of strains of EAggEC from panel 1 with the addition of ETDA and EGTA to L-broth*

		Pellicle Formation		
Strain (E)	Serotype	L-broth	EDTA†	EGTA‡
92356	O3:H2	+	-	+
73339	O15:H-	+	-	+
72376	O33:H16	+	-	+
45730	O44:H18	+	-	+
44939	O44:H18	+	-	+
43509	O44:H18	+	-	+
58596	O51:H11	+	-	+
76989	O75:H2	-	-	-
58583A	O77:H18	+	-	+
67643	O78:H10	-	-	-
47697A	O86:H19	+	-	+
97468	O86:H34	-	-	-
78135	O92:H33	+	-	+
60725	O92:H33	+	-	+
101621	O98:H-	-	-	-
72957	O102:H27	-	-	-
57144A	O111:H21	+	-	+
40144	O111:H21	+	-	+
33915	O111:H21	+	-	+
60874	O126:H27	+	-	+
62008	O126:H27	-	-	-
75607	O126:H27	-	-	-
83087	O126:H27	-	-	-
81456	O126:H27	+	-	+
40104	O126:H27	+	-	+
38383	O126:H27	+	-	+

† Pellicle formation in L-broth + 1mM EDTA

‡ Pellicle formation in L-broth + 1mM EGTA

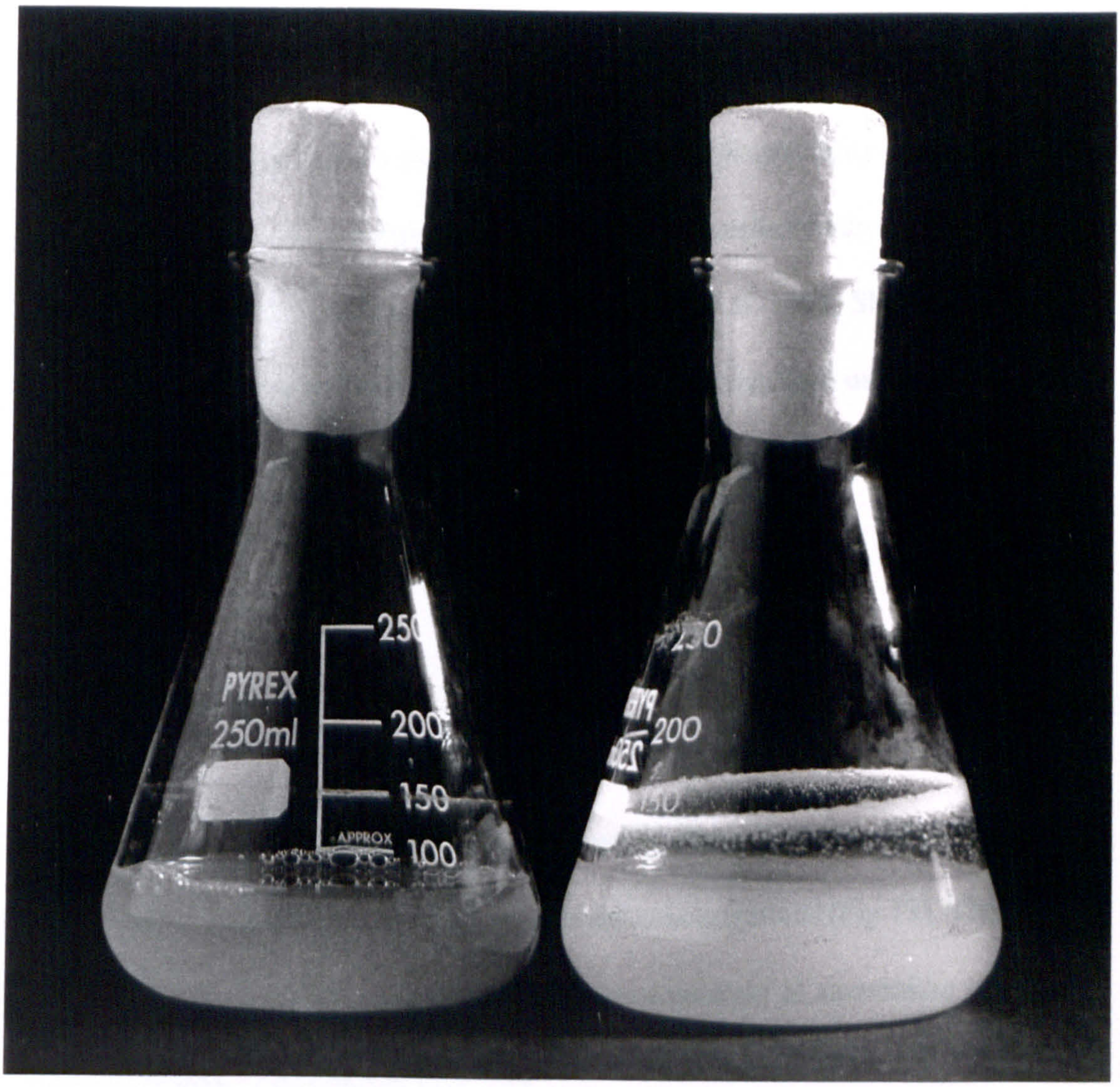


Fig. 17. Pellicle formation by EAggEC strain E97590 grown in L-broth alone (left). No pellicle formation when E97590 was grown in the presence of EDTA (right)

4.2.4. Hydrophobicity

The observation that strains of EAggEC adhered to formalin-fixed HEp-2 cells (4.1.4.), and the majority produced a pellicle suggested that hydrophobicity of EAggEC may play a role in the adhesion process, particularly since cell surface hydrophobicity may promote attachment of *E. coli* to epithelial cells (Smith *et al.* 1978; Sherman *et al.* 1985). In this section surface hydrophobicity and charge were examined using the salt aggregation test (SAT), hydrophobic interaction chromatography (HIC) and migration of bacteria in an electric field.

4.2.4.a. *Salt aggregation test*

A measure of cell hydrophobicity was obtained by observing bacterial clumping in solutions of ammonium sulphate with a range of molar concentrations (Fig 19). The method of Rozgoyni *et al.* (1985) was used, employing a gradient of ammonium sulphate concentrations ranging from 0 to 4M (3.4.4.a.).

A strain was considered hydrophobic when aggregates were formed in concentrations of ammonium sulphate of 1M or less. Thirty strains (88%) from the original panel were hydrophobic by the salt aggregation test. The SAT test is most reliable for the detection of strongly hydrophobic micro-organisms (Mozes and Rouxhet 1987).

Table 27 *Hydrophobicity of EAggEC strains from panel 1 using the salt aggregation test (SAT).*

Strain (E)	Serotype	SAT
92356	O3:H2	4-0.5
73339	O15:H-	4-2
59905	O21:H2	4-0.5
72376	O33:H16	4-0
45730	O44:H18	4-0
44939	O44:H18	4-0
43509	O44:H18	4-0
58596	O51:H11	4-0
76989	O75:H2	4-1
58583	O77:H18	-
67643	O78:H10	4-0.5
47697	O86:H19	4-0.5
97468	O86:H34	4-0.5
78135	O92:H33	4-0
60725	O92:H33	4-0.5
101621	O98:H-	4-0.5
72957	O102:H27	-
99518	O104:H4	4-0
57144	O111:H21	4-0
40144	O111:H21	4-0
33915	O111:H21	4-0
36182	O111:H21	4-0
60874	O126:H27	4-0
55060	O126:H27	4-0
58816	O126:H27	4-0
52610	O126:H27	4-0
45691	O126:H27	4-0
62008	O126:H27	-
57157	O126:H27	4-0
75607	O126:H27	4-0.5
83087	O126:H27	4-0
81456	O126:H27	4-0
40104	O126:H27	4-0
38383	O126:H27	4-0

Sixty-six strains of EAggEC from panel 1 and outbreaks A-D (panel 3; Table 28) were tested for their ability to aggregate in various concentrations of ammonium sulphate thus giving a measure of hydrophobicity.

Table 28 *Hydrophobicity results of panel 3 using the salt aggregation test (SAT)*

Strain (E)	Serotype	Outbreak	SAT*
98527	O19:H-	A	4-0
97622	O113:H-	A	4-0
96393	O125:H27	A	4-0.5
98529	O?:H18	A	4-0.05
97820	O62:H30	B	-
97590	O73:H13	B	4-0
96386	O73:H18	B	4-0
96485	O134:H27	B	-
97819	O?:H27	B	4-2
96483	O?:H33	B	4 only
96390	O?:H-	B	4-0
96487	O?:H-	B	4-2
97900	O?:H-	B	4 only
97472	O86:H34	C	4-0.5
97462	O86:H34	C	4-0.5
97470	O86:H34	C	4-0
97474	O116:H27	C	4-0
101408	O98:H	D	4 only
101621	O98:H-	D	4-0
101396	O98:H-	D	4 only
101406	O98:H-	D	4-0
101402	O110:H-	D	4-0

All strains from outbreaks A and C were hydrophobic (Table 28) whereas only 3 strains of 5 were hydrophobic in outbreak D. Outbreak D consists of 5 EAggEC strains of which 4 belong to serotype O98:H-, the results of the hydrophobicity testing shows that the hydrophobicity of the strain did not correlate with serotype. Outbreak B contained three of nine strains that were hydrophobic.

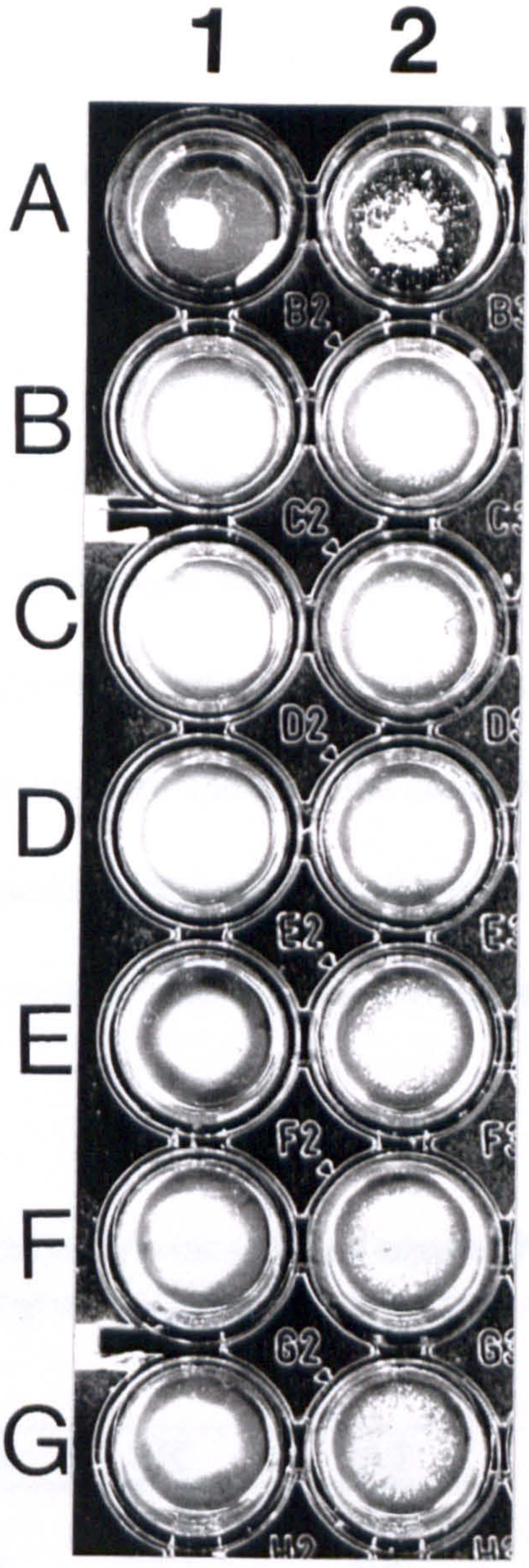


Fig. 19 Example of the salt aggregation test. Column 1 shows EAggEC strain E62008 which does not aggregate the ammonium chloride at concentrations of 4M and below (A 4M; B 2M; C 1M; D 0.5M; E 0.25M; F 0.1M; G distilled water) and therefore was not hydrophobic. Column 2 shows EAggEC strain E40104 aggregating at all concentrations of ammonium sulphate.

4.2.4.b. Hydrophobic Interaction Chromatography (HIC)

The HIC method can be used to give a measure of bacterial hydrophobicity based on the passage of bacteria through an octyl sepharose column. Hydrophobic bacteria bind to the octyl sepharose whereas hydrophilic bacteria pass through the column, with the mobile phase. The hydrophobicity of the individual strains was calculated by the measurement of optical density of the culture added to the column and the resultant optical density of the culture after passage through the column (3.4.4.b.). The values of HIC in table are the percentage of bacteria from original culture retained in the octyl sepharose column.

Table 29. *Hydrophobicity results obtained with selected strains from panel 1 using Hydrophobic Interaction Chromatography (HIC): a comparison with the salt agglutination test (SAT)*

Strain (E)	Serotype	HIC	SAT
92356	O3:H2	15.4	4-0.5
44939	O44:H18	27.1	4-0
43509	O44:H18	42.2	4-0
47697A	O86:H19	0.8	4-0.5
60725	O92:H33	97.9	4-0.5
72957	O102:H27	3.2	-
57144A	O111:H21	39.6	4-0
40144	O111:H21	31.4	4-0
33915	O111:H21	50.0	4-0
55060	O126:H27	38.0	4-0
58816	O126:H27	55.3	4-0
52610	O126:H27	78.6	4-0
45691	O126:H27	48.5	4-0
62008	O126:H27	10.3	-
75607	O126:H27	29.7	4-0.5
83087	O126:H27	45.8	4-0
81456	O126:H27	21.7	4-0
40104	O126:H27	67.9	4-0
38383	O126:H27	65.8	4-0

Results of the SAT test showed that *E. coli* strains E72957 (O102:H27) and E62008 (O126:H27) were not hydrophobic i.e. they did not clump in any of the concentrations of ammonium sulphate.

4.2.5. Bacterial surface charge

4.2.5.a. Migration in an electric field

To estimate bacterial surface charge, the migration of strains in an electric field was monitored using a procedure used by Krishna *et al.* (1996)(3.4.4.a.).

Bacteria migrating to the anode were considered as having an overall negative charge and vice versa.

Table 30 *Cell surface charge of EAggEC strains from panel 1*

Strain (E)	Serotype	Charge*	EDTA†
92356	O3:H2	-	-
73339	O15:H-	-	-
72376	O33:H16	-	-
45730	O44:H18	-	-
44939	O44:H18	-	-
43509	O44:H18	-	-
58596	O51:H11	-	-
76989	O75:H2	-	-
58583A	O77:H18	-	-
67643	O78:H10	nc	-
47697A	O86:H19	±	-
97468	O86:H34	-	-
78135	O92:H33	±	-
60725	O92:H33	±	nc
101621	O98:H-	±	nc
72957	O102:H27	-	-
57144A	O111:H21	-	-
40144	O111:H21	-	-
33915	O111:H21	-	nc
60874	O126:H27	-	-
62008	O126:H27	-	nc
75607	O126:H27	-	-
83087	O126:H27	-	-
81456	O126:H27	-	-
40104	O126:H27	-	-
38383	O126:H27	-	-

* bacterial surface charge; nc neutral charge

† bacterial surface charge of bacteria when grown in the presence of EDTA; - bacteria carry an overall negative charge; ± bacteria carry positive and negative charges

Twenty-four strains from panel 1 (Table 30) carried a negative charge whereas 4 of these strains carried a positive charge as well. One strains was not able to migrate in an electric field. After the growth of these strains in the presence of EDTA some changes in the charge carried by the bacteria occurred. EAggEC strains E60725 and E101621 which carried both positive and negative charges lost their overall surface charge and could not migrate in an electric field. EAggEC strain E33915 and the non-

adhesive variant E62008 which carried a net negative charge when grown in nutrient broth only lost their charge and could not migrate in an electric field when grown in the presence of EDTA. EAggEC strain E67643 which could not migrate in an electric field gained a net negative charge when grown in the presence of EDTA. The charge on all the other EAggEC strains in this panel remained the same when grown in presence of the chelator.

Table 31 *Cell surface charge of EAggEC strains from panel 3*

Strain (E)	Serotype	Outbreak	Charge*
98527	O19:H-	A	±
97622	O113:H-	A	-
96393	O125:H27	A	±
98529	O?:H18	A	±
97820	O62:H30	B	-
97590	O73:H13	B	±
96386	O73:H18	B	±
96485	O134:H27	B	±
97819	O?:H27	B	-
96483	O?:H33	B	-
96390	O?:H-	B	±
96487	O?:H-	B	±
97900	O?:H-	B	±
97472	O86:H34	C	±
97468	O86:H34	C	±
97470	O86:H34	C	-
97474	O116:H27	C	±
101408	O98:H-	D	-
101621	O98:H-	D	±
101396	O98:H-	D	-
101406	O98:H-	D	±
101402	O110:H-	D	±

* bacterial surface charge
- bacteria carry an overall negative charge
± bacteria carry positive and negative charges

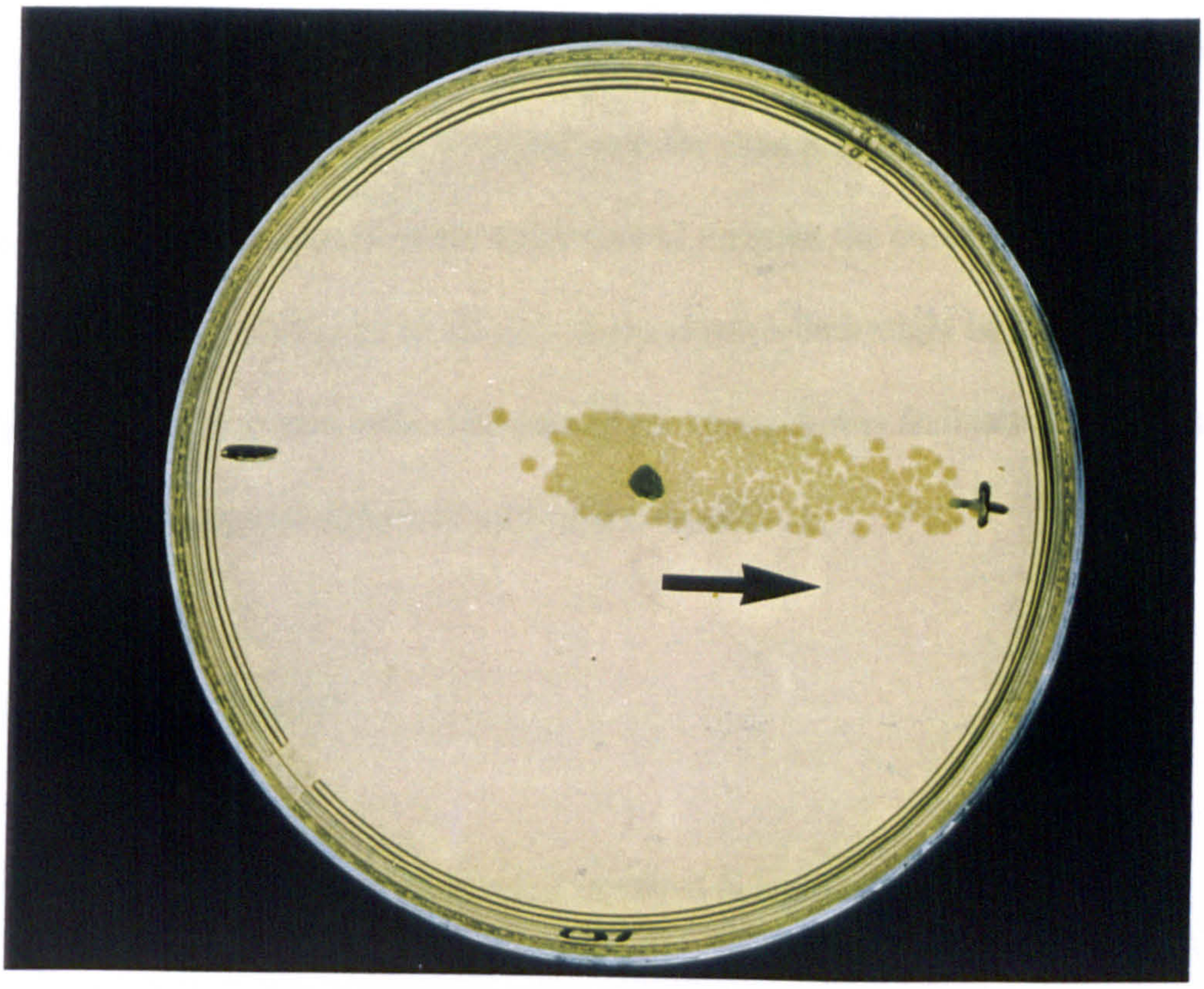


Fig. 20 Migration of strain E40104 in an electric field. Arrow indicates migration towards anode

4.3. Bacterial membrane characteristics

The binding of EAggEC to eukaryotic cells must involve the interaction between cell surface components expressed by EAggEC and, for example, the HEp-2 cell membrane. The aim of this section of the study was to examine the outer membranes expressed by EAggEC and attempt to identify components which might be involved with bacterial adhesion to eukaryotic cells. This part of the research was facilitated by having isogenic non-adhesive mutant strains of wild-type EAggEC.

4.3.1. Outer membrane proteins

4.3.1.a. Membrane associated proteins involved in bacterial adhesion to HEp-2 cells

Outer membrane preparations (3.4.1.) were examined by SDS-PAGE using a 12.5% acrylamide separation gel (3.7.1.a, b, c.i.). Initial studies from the LEP had shown that some EAggEC strains expressed a membrane-associated protein (MAP) of 18kDa (Chart *et al.* 1994), which was not fimbrial as shown by electron microscopy. Examination of strains of EAggEC for MAPs also detected an 18 kDa protein in strains belonging to serotype O44:H18. This protein migrated in SDS-PAGE gels irrespective of the presence of β-mercaptoethanol in the solubilising buffer or incubation of the protein samples at 100 °C suggesting that the protein subunits did not contain disulphide bonds nor have a quaternary structure involving covalent bonding. The 18 kDa protein of EAggEC strain E40104 and the non-adhesive mutant E40104B which does not express the 18 kDa are shown in Fig. 21.

Isogenic variants were constructed (4.2.1.) which belonged to serotype O126:H27. These strains and their properties are summarised in Table 32a.

Table 32a *Isogenic variants of EAggEC strains belonging to serotype O126:H27 and their properties*

Strain	Serotype	AA*	Adhesion†	18 kDa‡
E40104A	O126:H27	+	+	+
E40104B	O126:H27	-	-	-
E57157A	O126:H27	+	+	+
E57157B	O126:H27	-	-	-

* Hybridisation with AA probe
† Aggregative pattern of adhesion to HEp-2 cells
‡ Expression of an 18 kDa membrane associated protein

The isogenic variants did not adhere to HEp-2 cells in an aggregative pattern of adhesion and did not express an 18 kDa.

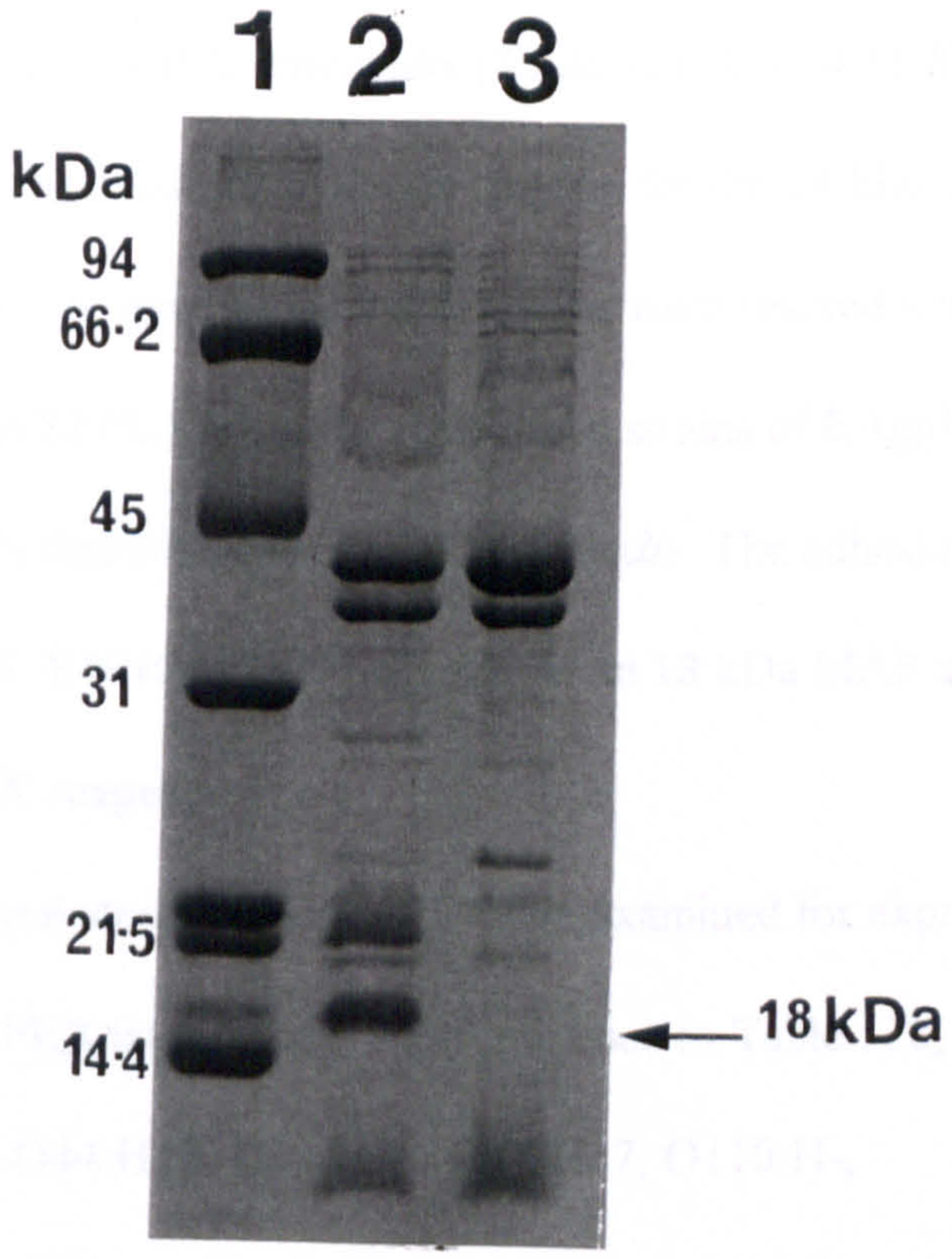


Fig 21. SDS-PAGE gel showing OMP profiles of strains E40104 and the non-adherent mutant E40104B. Lane 1 shows molecular weight markers (94, 66.2, 45, 31, 21.5 and 14.4 kDa), Lane 2 shows OMP profile of strain E40104 and lane 3 shows OMP profile of E40104B - arrow indicates position of the 18kDa MAP.

Early studies with EAggEC had shown that strains belonging to serotype O126:H27, and expressing an 18 kDa MAP, were agglutinated by a commercial reagent SEFEX (6.7.6.) designed to detect strains of *S. enteritidis* (Table 32). The SEFEX kit, comprised latex beads coated with a monoclonal antibody specific for the 14 kDa subunit of the SE14 fimbriae of *S. enteritidis*. A panel of EAggEC strains were reacted with the SEFEX kit. The agglutination of the SEFEX kit shows that some strains of EAggEC must share epitopes with the 14 kDa fimbrial subunit of *S. enteritidis*. The adhesion negative strains of E40104 (E62008, E40104B) did not express an 18 kDa MAP and were not agglutinated by the SEFEX reagents.

In the present study, additional strains of EAggEC were examined for expression of a MAP and reaction with the SEFEX latex beads. With reference to Table 32b, strains of EAggEC belonging to serotypes O44:H18, O33:H16, O75:H27, O110:H-, O116:H27, O119:H27, Orough:H-, Orough:H3 and Orough:H27 were shown to express an 18 kDa.

A hyperimmune rabbit antiserum was prepared to the 18 kDa MAP, by challenging rabbits with whole cells of *E.coli* strain E57157A (O126:H27; 3.7.5.a.) and absorbing the serum with strain E57157B which did not express the 18 kDa MAP (3.7.5.b.). This serum was used in immunoassays (Western blot) to examine the antigenic properties of the 18 kDa expressed by EAggEC belonging to a range of serotypes (Table 32b).

Table 32b *Strains of different serotypes expressing an 18kDa MAP and their properties*

Strain (E)	Serotype	18 kDa†	HEp-2	SEFEX	AA	DA	AAF/II
40104	O126:H27	+	+	+	+	+	+
62008	O126:H27	-	-	-	-	-	-
52610	O126:H27	+	+	+	+	+	+
75607	O126:H27	+	+	+	+	+	+
44939	O44:H18	+	+	-	+	+	+
43509	O44:H18	+	+	-	+	+	+
45730	O44:H18	+	+	-	+	+	+
72376	O33:H16	+	+	*	+	+	+
101402	O110:H-	+	+	*	+	+	+
97474	O116:H27	+	+	*	+	+	+
99979	O75:H27	+	+	+	+	+	+
107100	O119:H27	+	+	+	+	+	+
99967	Orough:H-	+	+	-	+	+	+
105836	Orough:H3	+	+	+	+	+	+
107542	Orough:H27	+	+	+	+	+	+
97298	Orough:H27	+	+	-	+	+	+

* SEFEX reaction was not carried out as reagents were discontinued

† Observation of protein in outer membrane protein preparations and reaction with antibody against the 18 kDa MAP EAggEC strain 57157 (O126:H27)

The rabbit antibodies bound to the MAPs expressed by the HEp-2 adhesive strains listed in Table 32b. Only strains belonging to serotypes O126:H27, O75:H27, O119:H27, Orough:H3 and Orough:H27 reacted with the SEFEX beads suggesting that although these strains expressed antigenically similar 18 kDa proteins, the epitope detected by the SEFEX beads was only surface exposed on these strains. The SEFEX reagents did not react with EAggEC strains belonging to serotype O44:H18 expressing an antigenically similar 18 kDa or EAggEC strain E99967 (Orough:H-), the epitopes reacting with the reagent may not be found on the surface of these cells but may be some way internalised in the protein subunits. The relationship between the 18kDa MAPand AAF/II could be checked if an antiserum was available for AAF/II.

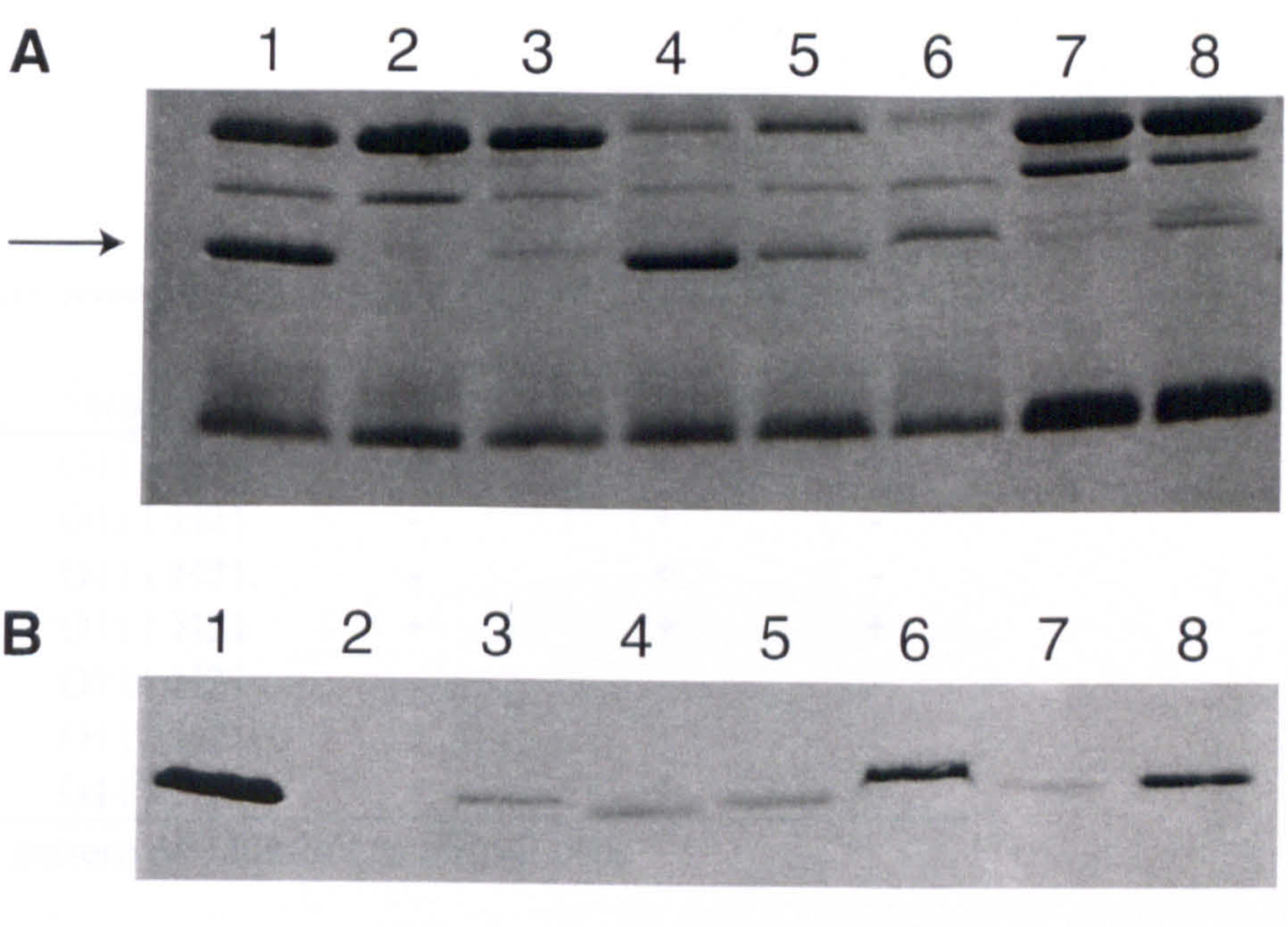


Fig 22 Lane 1a shows EAggEC strain E40104 expressing a MAP of 18 kDa. Lane 2a shows isogenic variant (AA adhesion negative) not expressing 18kDa. Lanes 3a-5a (E44939; E40539; E45730; O44:H18) expressing 18 kDa. Lane 6a E72376 (O33:H16), lane 7a E97474 (O116:H27) and lane 8a E101402 (O110:H-) expressing an 18kDa. An antiserum prepared to the OMP of *E. coli* O126:H27 (2b, lane 1). These antibodies bound to the 18kDa OMPs of strains O44:H18 E44939 lane 3b; E40539 lane 4b; E45730 lane 5b; O33:H16 (E72376; lane 6b); O116:H27 (E97474, lane 7b); O110:H- (E101402, lane 8b).

Outer membrane profiles from selected EAggEC strains were analysed for other low molecular weight proteins that may be involved in aggregative adhesion. Another MAP was detected of 20 kDa in strains belonging to serotype O111:H21 (Table 33; Fig. 23). This protein was antigenically distinct fom the 18 kDa. This suggested that a family of MAPs may exist which would include the 18 kDa and the 14kDa fimbrial subunit of AAF/I.

Table 33 *Strains belonging to serotype O111:H21 and their properties*

Strain (E)	Serotype	Adhesion*	AA probe	20 kDa
36182	O111:H21	+	+	+
36182 (17/4)	O111:H21	-	+	-
36182 (13/1)	O111:H21	-	+	-
57144A	O111:H21	+	+	+
57144B	O111:H21	-	-	-
33915	O111:H21	+	+	+
40144	O111:H21	+	+	+

* Aggregative pattern of adhesion to HEp-2 cells

Strains E36182 (17/4) and E36182 (13/1) were constructed by transposon mutagenesis of the parent strain E36182 by insertion of Tn*pho*A elements (Taylor *et al.* 1989). These strains were still AA probe-positive but they could not adhere to HEp-2 cells in an aggregative pattern.

The mutant strain E57144B was derived by repeated subculture until the parent strain became AA probe negative by loss of the plasmid. Analysis of the outer membrane protein preparations of the mutant strains showed that they did not express the 20 kDa protein.

Biochemical properties of the 20 kDa were examined by omitting β-mercaptoethanol from the sample buffer and not incubating the samples at 100°C. The

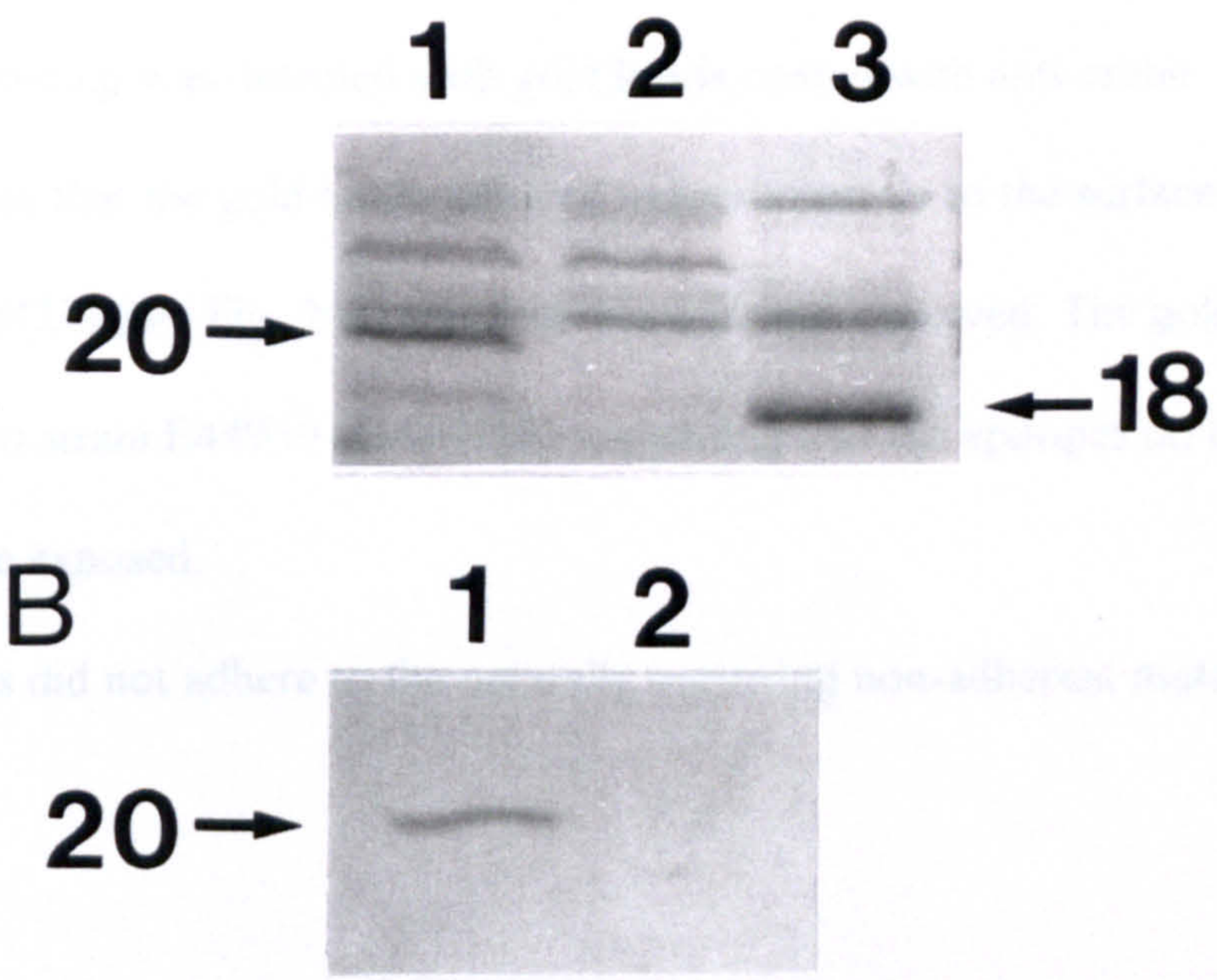


Fig. 23. SDS-PAGE gel. Lane 1A shows outer membrane protein profile of E36182 (O111:H21) expressing a 20 kDa MAP (arrowed). Lane 2A shows outer membrane protein profile of E36182 17/4 (AA probe positive, HEp-2 adhesion negative) not expressing a 20kDa MAP. Lane 3A shows EAggEC strain E40104 outer membrane profile expressing an 18 kDa MAP (arrowed). Rabbit antibodies prepared to the 20 kDa MAP reacted with the 20 kDa MAP (lane 1B) but not with the 18 kDa MAP (2B).

4.3.1.a.i. Antigenic properties of MAP's

The binding of rabbit antibodies to an 18 kDa MAP of E40104 was investigated by immuno-electronmicroscopy (3.7.12). Following reaction of bacteria with anti-18 kDa antibodies, antibody-binding was detected with gold beads coated with anti-rabbit antibodies. It was found that the gold beads adhered indiscriminately to the surface of strain E40104 (O126:H27) (see Fig. 24a) and fimbriae were not observed. The gold beads did not adhere to strain E44939 (O44:H18) suggesting that the epitopes on this strain were not surface exposed.

The gold beads did not adhere to the naturally occurring non-adherent mutant, E62008 (O126:H27).

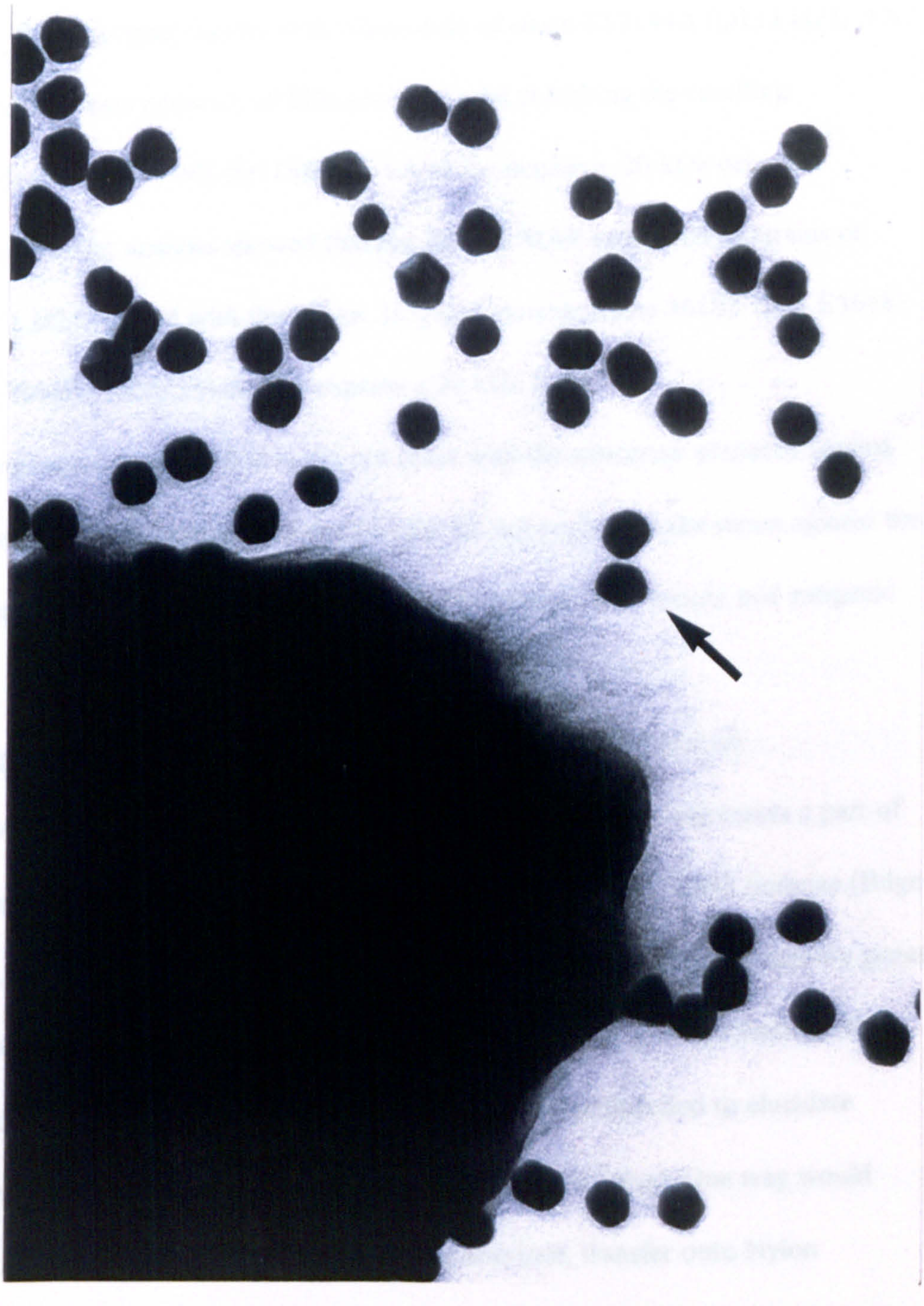


Fig. 24a. Immuno-electron-micrograph showing 3nm gold beads attached to the 18 kDa MAP expressed by EAggEC strain E40104

To investigate antigenic properties of the 18 and 20 kDa MAPs expressed by strains of EAggEC, antisera were prepared to these proteins. The antisera for the 20 kDa was achieved by challenging rabbits with whole cells of strain E57144A (O111:H21, AA probe positive, adhesion positive, 20 kDa positive), and absorbing the resulting antiserum with strain E57144B (O111:H21, AA probe negative, 20 kDa negative; 3.7.5.b.). Western blot analysis showed that the 20 kDa MAP expressed by strains of serotype O111:H21 reacted with the serum. Isogenic variant strains 36182 17/2, E36182 13/4, and E57144B (Table 33) did not express a 20 kDa MAP.

Strains expressing the 18 kDa did not react with the antiserum prepared against the 20 kDa and strains that expressed the 20 kDa did not react with the serum against the 18 kDa. These results show that these proteins differ in molecular weight and antigenic properties.

EAggEC strains expressing an antigenically similar 18 kDa protein hybridised with the probes for AA, DA and AAF/II. The DA probe represents a part of the *daa*C gene, an accessory gene required for the synthesis of the F1845 fimbriae (Bilge *et al.* 1989). This finding may suggest a genetic relationship between the accessory genes of F1845 and AAF/II; however these strains were shown not to produce fimbriae by electron microscopy (4.2.1.b.). Further experiments are recommended to elucidate whether the DNA sequences hybridising with the probes are linked. One way would involve the digestion of the DNA with restriction enzymes, transfer onto Nylon membrane and hybridisation with the probes to see which fragments hybridised with either probe.

4.3.1.a.ii. Requirement of cations for expression of MAP's

In view of the fact that strains of EAggEc are highly charged and that strains grown in the presence of EDTA did not adhere to HEp-2 cells, it was decided to examine the effect of divalent cations on the expression of the 18 kDa and 20 kDa proteins. The addition of the chelator EDTA to L-broth resulted in the repression of the 18 kDa and 20kDa MAP.

The role of divalent cations on expression of the 18 and 20 kDa MAPs was examined by growing strains in L-broth, with and without the addition of EDTA. In the presence of EDTA strains did not express a pellicle (Fig. 18) and bacteria grown in L-broth with EDTA did not express an 18 or 20 kDa MAP.

The addition of Mg^{2+} ions to L-broth containing 1mM EDTA resulted in the expression of an 18 kDa MAP, the production of a pellicle and bacteria adhered to HEp-2 cells in an aggregative pattern. However, the addition of an excess of Ca^{2+} ions to L-broth containing EDTA did not result in these properties being expressed.

Similarly, strains of EAggEC producing the 20 kDa MAP and grown in L-broth containing 1mM EDTA, did not produce a pellicle or adhere to HEp-2 cells in an aggregative pattern. Interestingly, the addition of Mg^{2+} or Ca^{2+} ions to L-broth containing 1mM EDTA resulted in the expression of the 20 kDa MAP, the production of a pellicle and the ability to adhere in an aggregative pattern to HEp-2 cells.

Since the addition of calcium and magnesium ions to L-broth containing EDTA, resulted in theproducion of a pellicle and the 20kDa MAP being expressed, it was decided to examine the effect of adding other divalent cations to L-broth containing EDTA.

EAggEC strain E36182 (O111:H21) which produced a substantial pellicle was inoculated into L-broth containing differing concentration of divalent cations (Table 34) and the production of pellicle was noted. The results showed that 10 mM magnesium was required before bacteria produced a pellicle, whilst, the addition of 5mM calcium produced the same response. The addition of 2.5 mM copper, lead and ferric ions resulted in the expression of a pellicle, but at concentrations of copper above 5mM the bacteria did not grow, probably because the levels of copper had become toxic. Surprisingly, zinc ions in the form of 2.5mM zinc sulphate enabled strains to express a pellicle, but at the same concentrations zinc chloride did not result in pellicle production. Barium ions had no effect on pellicle production.

Table 34 *Effects of adding divalent cations to L-broth containing EDTA*

Ion	0.3mM	0.6mM	1.25mM	2.5mM	5mM	10mM
Mg	- *	- *	- *	- *	- *	+ *
Ca	- *	- *	- *	- *	+ *	+ *
Cu	- *	- *	- *	+ *	- NG	- NG
Pb	- *	- *	- *	+ *	+ *	- NG
Fe	- *	- *	- *	+ *	+ *	- *
$ZnCl_2$	- *	- *	- *	- *	- NG	- NG
$ZnSO_4$	- *	- *	- *	+ *	- NG	- NG
Ba	- *	- *	- *	- *	- *	- *

*

+	pellicle formation
-	no pellicle formation
*	growth
NG	no growth

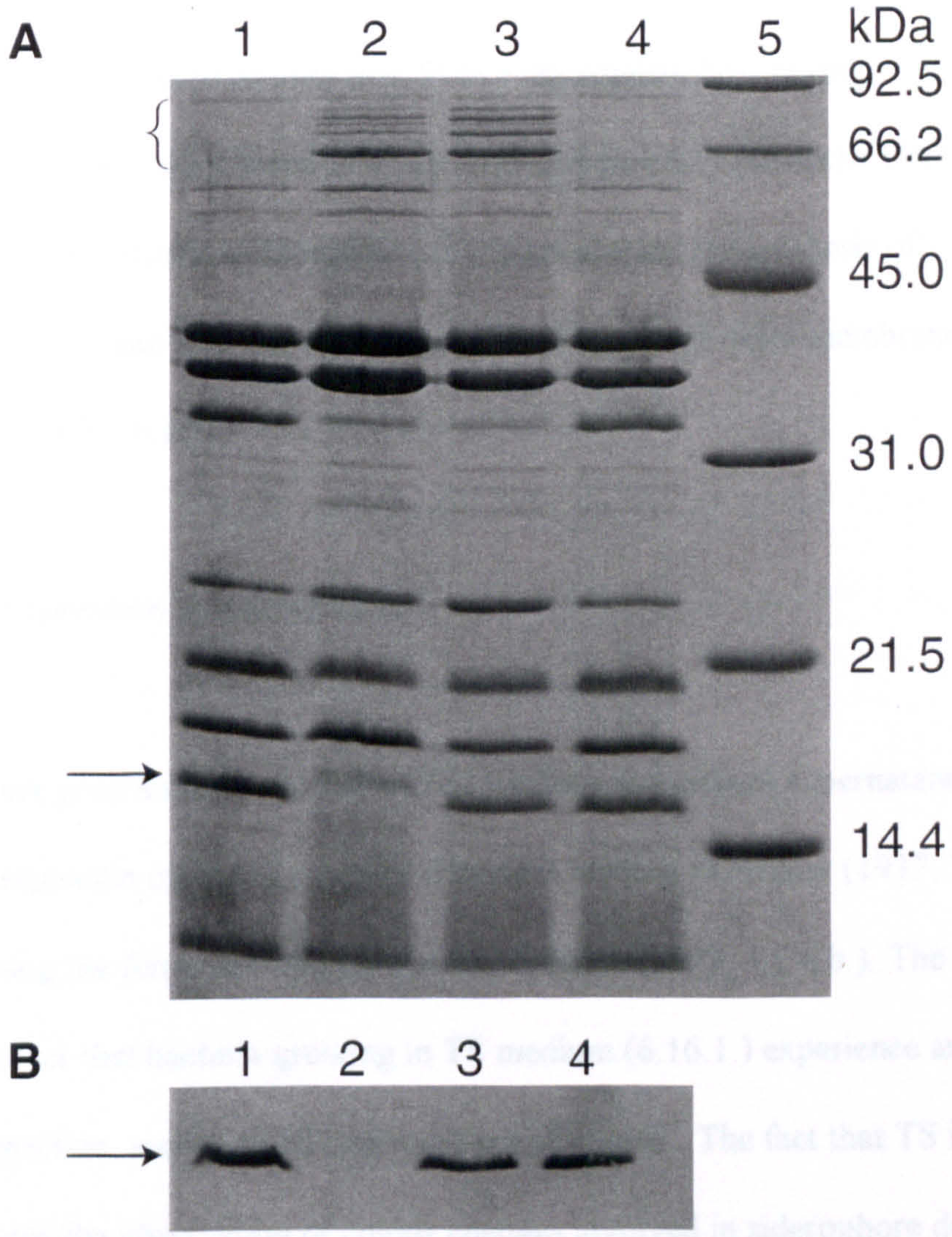


Fig. 24b Lane A1 shows EAggEC strain E40104 (O126:H27) grown in L-broth alone expressed an 18 kDa MAP (arrowed), which reacted with rabbit antibodies specific for this protein (arrowed B: lane 1). When EAggEC strain E40104 was grown in L-broth containing 1mM EDTA.Na_2, this strain did not express an 18 kDa MAP (A and B: lanes 2), but expressed high molecular weight outer membrane proteins (bracketed A: lane 2). High molecular weight proteins were also expressed when strain E40104 was grown under conditions of iron restriction (A: lane 3). Iron restriction did not affect the expression of the 18 kDa MAP (A and B: lanes 3). The 18 kDa MAP was expressed by E40104 grown in L-broth containing EDTA plus added $MgCl_2$ (A and B: lane 4). Molecular weight standards are shown in lane 5.

4.3.1.b. High Affinity Iron Uptake

The ability of bacteria to acquire iron from a mammalian host during pathogenesis of infection is considered as a key virulence property (Griffiths 1991). In this section of the study, strains of EAggEC were examined for the synthesis of siderophores (4.3.1.b.i.) and also the expression of iron regulated outer membrane proteins (IROMPS; 4.3.1.b.ii.).

4.3.1.b.i. *Expression of siderophores*

Strains were grown in tris-succinate (TS) medium and culture supernatants examined for enterobactin using the catechol detection method of Arnow (1937; 3.7.6.a.) and aerobactin using the ferric perchlorate test of Neilands (1992; 3.7.6.b.). The test was based on the fact that bacteria growing in TS medium (6.16.1.) experience an apparent iron restriction, such that siderophores are produced. The fact that TS is colourless facilitates the observation of colour changes involved in siderophore detection assays. The presence of catechol siderophores results in a pink colour whilst the presence of aerobactin results in a red-brown colour.

With reference to Table 35, all thirty-four strains from EAggEC panel 1 produced the siderophore enterobactin and thirteen (38%) of these EAggEC strains also produced aerobactin. The production of aerobactin has been associated with the possession of the ColV plasmid which also encodes the production of colicin V. Seventeen strains were tested for the production of colicin as described (3.7.5.; Table 35). A colicin-sensitive strain BZB1011 was used to indicate which strains produced a

colicin, denoted by a clear zone of lysis around a given test strain. Eleven of the strains tested produced a colicin. In order to determine if the strains produced colicin V only the colicin V resistant strain 30R519 was used as an indicator. The eleven strains which produced a colicin also produced clear zones using the colicin V resistant strain, suggesting that these strains did not produce ColV or produced colicin V and another colicin(s). Further experiments using other colicin resistant strains would elucidate the range of colicins produced by these strains. EAggEC strain E58596 did not produce aerobactin but had the ability to produce colicins. EAggEC strains E72957, E36182 and E47697A produced aerobactin but did not produce colicins and it was concluded that the genes encoding aerobactin were not carried on a ColV plasmid.

Table 35 *Enterobactin, aerobactin and colicin production and possession of the ColV plasmid by strains of EAggEC isolated from panel 1*

Strain (E)	Serotype	Enterobactin	Aerobactin	† BZB1101	‡ 30R519
92356	O3:H2	+	+	+	+
73339	O15:H-	+	+	+	+
59905	O21:H2	+	+	+	+
72376	O33:H16	+	+	+	+
45730	O44:H18	+	+	*	*
44939	O44:H18	+	-	-	-
43509	O44:H18	+	-	*	*
58596	O51:H11	+	-	-	-
76989	O75:H2	+	-	+	+
58583A	O77:H18	+	-	*	*
67643	O78:H10	+	-	+	+
47697A	O86:H19	+	+	-	-
97468	O86:H34	+	+	+	+
78135	O92:H33	+	+	+	-
60725	O92:H33	+	-	*	*
101621	O98:H-	+	-	*	*
72957	O102:H27	+	+	-	-
99518	O104:H4	+	-	*	*
57144A	O111:H21	+	+	+	+
40144	O111:H21	+	+	+	+
33915	O111:H21	+	+	+	+
36182	O111:H21	+	+	-	-
60874	O126:H27	+	-	*	*
55060	O126:H27	+	-	*	*
58816	O126:H27	+	-	*	*
52610	O126:H27	+	-	*	*
45691	O126:H27	+	-	*	*
62008	O126:H27	+	-	*	*
57157	O126:H27	+	-	*	*
75607	O126:H27	+	-	*	*
83087	O126:H27	+	-	*	*
81456	O126:H27	+	-	*	*
40104	O126:H27	+	-	-	-
38383	O126:H27	+	-	*	*

* colicin production not tested

† colicin production by test strains was detected using *E. coli* strain BZB1011 which is sensitive to all types of colicin

‡ Lysis around strains showed these strains produced colicins other than colicin V

Table 36 *Enterobactin and aerobactin production by EAggEC strains from panel 2.*

Strain (E)	Serotype	Enterobactin	Aerobactin
97479	O3:H-	+	+
111260	O4:H2	+	+
99536	O4:H33	+	+
101094	O4:H33	+	+
109902	O4:H33	+	-
96616	O5:H4	+	-
107527	O6:H1	+	+
97499	O6:H10	+	+
97480	O6:H10	+	+
110715	O6:H10	+	-
99520	O8:H7	+	-
111479	O11:H-	+	+
103594	O11:H27	+	-
99964	O18ac:H-	+	-
99962	O18ac:H30	+	+
99961	O18ac:H30	+	+
99963	O18ac:H30	+	-
99965	O18ac:H30	+	+
99960	O18ac:H30	+	+
109903	O21:H-	+	-
107249	O21:H4	+	-
104970	O33:H-	+	+
99970	O53:H-	+	+
99971	O53:H2	+	-
97500	O73:H1	+	+
99979	O75:H27	+	-
97494	O81:H-	+	+
97477	O81:H-	+	+
92832	O82:H25	+	-
92830	O86:H11	+	-
97496	O86:H2	+	+
97504	O91:H-	+	+
99518	O104:H4	+	-
110716	O106:H16	+	-
94706	O111ab:H-	+	+
109907	O111ab:H-	+	-
103763	O111ab:H2	+	-
99976	O113:H-	+	-
107100	O119:H27	+	-
101089	O126:H27	+	-
108837	O130:H27	+	-
97478	O130:H27	+	+
108839	O130:H27	+	-

Table 36 *Enterobactin and aerobactin production by EAggEC strains from panel 2 (cont)*

Strain (E)	Serotype	Enterobactin	Aerobactin
104940	O130:H27	+	-
104968	O131:H-	+	-
99969	O134:H-	+	+
107754	O134:H25	+	+
99968	O134:H27	+	+
97502	O134:H27	+	+
101095	O134:H27	+	+
101096	O134:H27	+	-
104942	O162:H10	+	-
94708	Orough:H-	+	+
99967	Orough:H-	+	-
107252	Orough:H-	+	-
99535	Orough:H27	+	-
107542	Orough:H27	+	-
97298	Orough:H27	+	-
105836	Orough:H3	+	-
105392	Orough:H33	+	-

All sixty EAggEC strains from panel 2 had the ability to produce the siderophore enterobactin and twenty-seven (45%) of the strains also produced aerobactin

Table 37 *Enterobactin and aerobactin production by strains of EAggEC from panel 3*

Strain (E)	Serotype	Outbreak	Enterobactin	Aerobactin
98529	O?:H18	A	+	+
96393	O125:H27	A	+	+
98527	O19:H-	A	+	-
97622	O113:H-	A	no growth	
96386	O73:H18	B	+	-
97820	O62:H30	B	+	-
97819	O?:H27	B	+	-
96483	O?:H33	B	+	-
96485	O134:H27	B	+	-
96390	O?:H-	B	+	+
97590	O73:H13	B	+	+
96487	O?:H-	B	+	-
97900	O?:H-	B	+	-
97472	O86:H34	C	+	+
97468	O86:H34	C	+	+
97470	O86:H34	C	+	+
97474	O116:H27	C	+	-
101408	O98:H	D	+	-
101621	O98:H-	D	+	-
101396	O98:H-	D	+	-
101406	O98:H-	D	no growth	
101402	O110:H-	D	+	+
89097	O6:H16	E	+	+
89101	O8:H20	E	+	-
89099	O28:H18	E	+	+
89102	O44:H18	E	+	+
89109	O44:H18	E	+	-
89114	O44:H18	E	+	-
89110	O69:H11	E	+	-
89112	O69:H11	E	+	+
89105	O80:H27	E	+	-
89095	O80:H27	E	+	-
89111	O89:H18	E	+	+
89096	O113:H-	E	no growth	
89098	O114:H11	E	+	+
89104	O141:H49	E	+	-
89115	O162:H-	E	+	+
89107	:H27	E	+	+
89100	O?:H5	E	+	-
89106	Orough:H7	E	+	+

Two EAggEC strains from panel 3 were not able to grow in TS medium even after supplementation of nicotonic acid. Some strains of *E. coli* and *Sh. flexneri* require the addition of nicotinic acid for growth in TS medium but its role in iron uptake is not known (Griffiths, personal communication).

The remaining strains of EAggECfrom panel grew well in TS media and produced the siderophore enterobactin, and seventeen (46%) of the strains also produced aerobactin. All EAggEC strains tested which could grow in TS media expressed the catechol siderophore enterobactin.

4.3.1.b.ii. *The iron regulated outer membrane proteins (IROMPS)*

The adaptation for bacterial growth in iron restricted environments not only involves the synthesis of siderophores but also the production of membrane proteins which act as receptors for ferric-siderophore complexes (Neilands 1982; Griffiths 1987). Experimentally the expression of IROMPS by EAggEC was achieved by growing strains in L-broth supplemented with the iron chelator EDDA.

EAggEC strains E40104 and E92356 were grown in L-broth only, and L-broth supplemented with EDDA and outer membrane proteins were prepared (3.4.1.). These strains were chosen since EAggEC strain E40104 produced enterochelin only and EAggEC strain E92356 expressed both enterochelin and aerobactin. With reference to Figure 26, growth in the presence of EDDA resulted in the expression of several high molecular weight proteins (lane 2) as compared to OMP profiles made from iron-replete bacteria (Fig. 25, lane 1). Three proteins of 74, 76 and 81 kDa were expressed in particularly large amounts (arrowed, Fig 25 lane 2). The 81 kDa IROMP was thought to be the receptor for ferric-enterobactin, and since strain E92356 made aerobactin it was

thought that the IROMP of 74 kDa was the receptor for ferric-aerobactin. The role of the 76 kDa IROMP was not established; however, a protein of this molecular size has been described as a ferric-aerobactin receptor in certain strains of invasive *E. coli* (Griffiths *et al.* 1985).

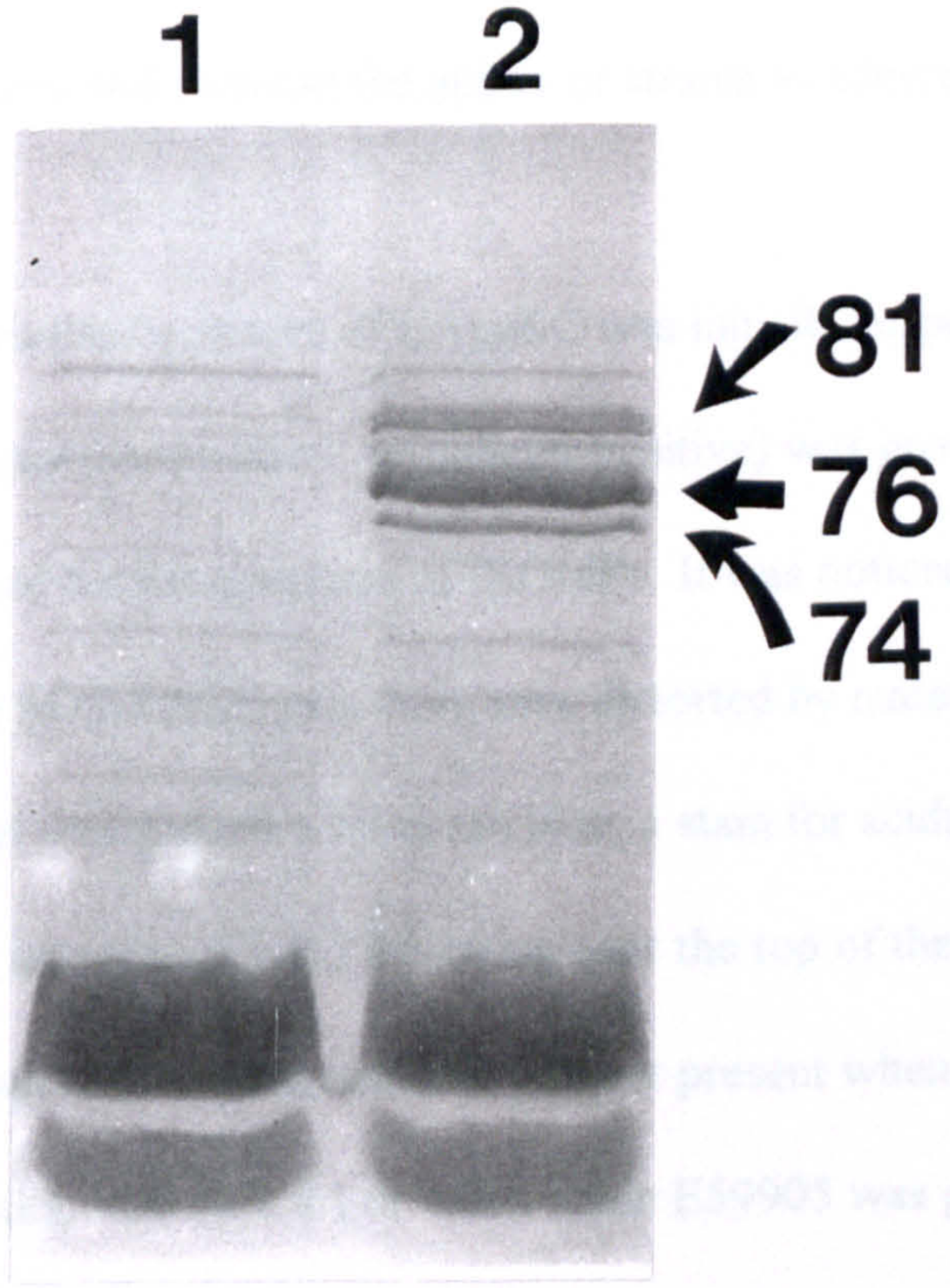


Fig. 25. Outer membrane profiles of EAggEC strain E 92356. Lane 1 shows OMP profiles when grown in L-broth only. Lane 2 shows E92356 expressing iron regulated outer membrane proteins when grown in L-broth supplemented with EDDA. Arrows indicating 81 kDa protein - receptor for Fe^{3+}/enterobactin complexes; 76kDa and 74kDa protein - receptors for Fe^{3+}/aerobactin complexes

4.4. Expression of capsules

The ability of strains of *E. coli* to express capsules constitutes a major virulence factor usually during extraintestinal infections, enabling them to avoid non-specific host defences, notably the action of complement and phagocytosis. Capsules can also increase the hydrophobicity of the strains and increase the ability of strains to adhere to eukaryotic cells.

The expression of capsules by strains of EAggEC was initially suspected when strain E59905 (O21:H2, haemolysin-positive, aerobactin-positive) was grown in TS medium (6.16.1) and examined for the presence of IROMPs. It was noticed that proteins migrating near the top of the SDS-PAGE gels were very distorted by material not stained with Coomassie blue. The gel was stained with alcian blue, a stain for acidic polysaccharides, and a large amorphous band migrating near the top of the gel was observed (Fig. 26). This alcian blue-stained material was not present when the strains were grown in L-broth (6.2.6.), TSB (6.2.8.) or when strain E59905 was grown in tris-succinate at 18 °C for 48 hr.

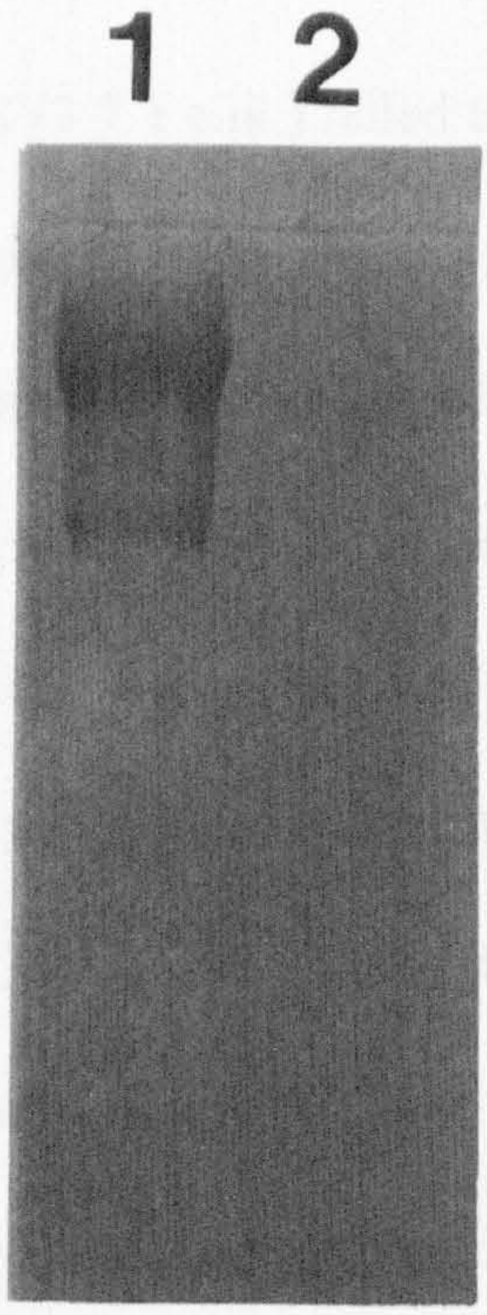


Fig. 26 Capsular material of EAggEC strain E59905 stained with alcian blue. Lane 1 shows E59905 when grown in TS medium at 37°C. Lane 2 shows no capsular material present when EAggEC strain E59905 was grown at 18°C.

The putative capsular material was examined further by carrying out basic chemical tests on the high molecular weight substance. Digesting the capsular material in acetone precipitated pellets with proteinase K did not effect the alcian blue staining material, and a silver stain for LPS (3.7.1.c.iii.) failed to stain the putative capsular material. It was concluded that the high molecular weight material was not protein or LPS, and since it stained with alcian blue it was thought to be capsular polysaccharide.

The capsular material was found to be expressed when strains were grown in TS medium, and the possibility arose that the synthesis of this capsule was regulated by iron. To test this hypothesis, *E. coli* strain E55905 was grown in TS medium supplemented with ferric ions in the form of $FeCl_3$. The observation that *E. coli* strain E59905 expressed a capsule in TS medium supplemented with iron showed that the availability of iron was not related to the expression of this polysaccharide material.

Since TS medium is a chemically defined medium, it was decided to determine which medium component was essential for capsular expression. Initially sodium succinate was replaced with other components involved in the tricarboxylic acid (TCA) cycle, such as glucose, citrate, sodium tri-citrate and fumarate. Analysis of supernatants showed that capsular material was produced when EAggEC strain E59905 was grown in the presence of fumarate and glucose, although there was observably less capsular material expressed by strains growing in the presence of glucose. Interestingly, the appearance of the material on SDS-PAGE gels, looked different when the organism was grown in these different media (figure not shown). *E. coli* strain E59905 was not able to grow in TS medium supplemented with citrate, sodium-citrate or citric acid, although growth and production of siderophores occurred using the other media compositions.

The TCA (tricarboxylic acid) cycle plays an important role in aerobic respiration of bacteria and it was decided to examine capsule expression following growth in TS medium under anaerobic conditions. EAggEC strain E59905 was grown in TS and TS with glucose in a gas-jar containing a gas pack system (Oxoid BR038B) producing H_2 and CO_2 whilst removing O_2. Under these conditions, strain E59905 was not able to grow in TS medium but growth was present when grown in TS supplemented with glucose. Analysis of the culture supernatant showed that neither the capsule nor siderophores were produced when grown in TS supplemented with glucose anaerobically. The growth of bacteria under anaerobic conditions caused strains of EAggEC to utilise a fermentation pathway. This would explain why siderophores were not synthesised. However, since the capsule was also not synthesised, the role of iron in capsule production could not be ruled out.

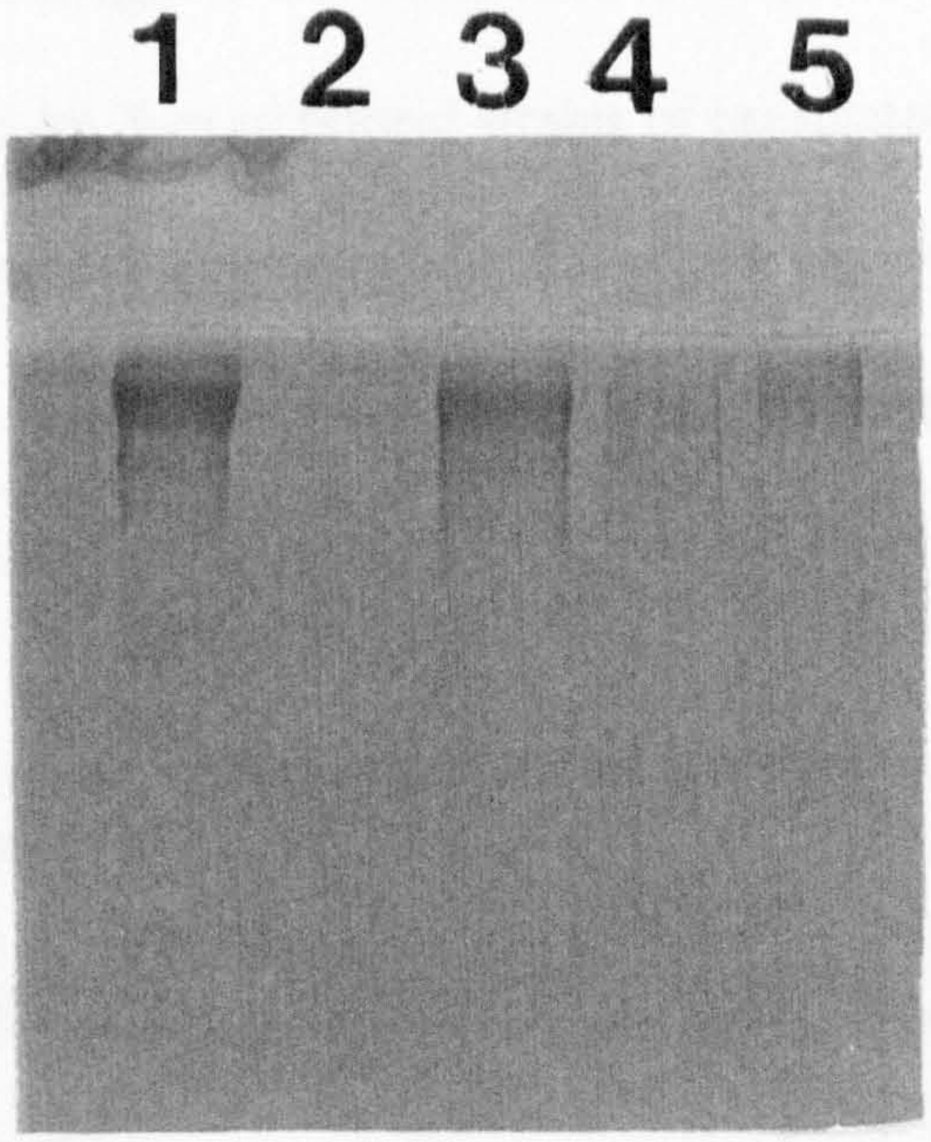


Fig. 27 Capsular material of EAggEC strains of different serotypes. Lane 1 - E59905 (O21:H2) at 37°C. Lane 2 - E59905 when grown at 18°C. Lane 3 - E99964 (O18:H-). Lane 4 - E99970 (O53:H2) and lane 5 - E73339 (O15:H-)

Nine additional strains of EAggEC were examined for expression of capsular material, with reference to Table 38, five additional strains of capsulated EAggEC were identified (Fig. 27); however, capsular expression did not appear to correlate with the production of the siderophores: enterobactin, aerobactin or α-haemolysin as detected by haemolysis of sheep erythrocytes.

Table 38 *Possession of various virulence factors and production of capsule by EAggEC strains*

Strain (E)	Serotype	α-haemolysin	Enterobactin	Aerobactin	Capsule
96390	O?:H-	+	+	+	-
97479	O3:H-	+	+	+	+
99964	O18:H-	+	+	+	+
99970	O53:H2	+	+	+	+
97590	O73:H13	+	+	-	-
104968	O131:H-	+	+	-	-
101402	O110:H-	+	+	+	-
97470	O86:H34	-	+	+	+
73339	O15:H-	-	+	+	+
59905	O21:H2	+	+	+	+

Ten strains of EAggEC used in the initial study were examined to see if the six capsulated strains produced a K1 capsule. Bacterial lawns were prepared alongside a known K1 producing control, and a K1 specific phage added. None of the strains of the EAggEC tested expressed a K1 capsule. All these strains produced aerobactin but none of these expressed the K1 capsular antigen. In the present study, growth at 18 °C resulted in repression of capsule production. This is characteristic of the Group II capsules of *E. coli,* including the K1 antigen.

4.5. Toxins

4.5.1. EAST1

It has been reported that strains of EAggEC elaborate a low molecular weight (4.1 kDa), partially heat-stable, plasmid encoded enterotoxin with 50% sequence homology with *E. coli* STa, although genetically and immunologically distinct (Savarino *et al.* 1991). This enterotoxic factor was termed enteroaggregative heat-stable enterotoxin 1 (EAST1).

Table 39 *Strains of EAggEC hybridising with EAST1 probe*

Strain	Serotype	EAST1*
A		
E98527	O19:H-	-
E97622	O113:H-	-
E96393	O125:H27	-
E98529	O?:H18	+
B		
E96386	O73:H18	-
E97820	O62:H30	-
E97819	O?:H27	+
E96483	O?:H33	+
E96485	O134:H27	+
E96390	O?:H-	+
E97590	O73:H13	+
E96487	O?:H-	-
E97900	O?:H-	-
C		
E97472	O86:H34	-
E97468	O86:H34	-
E97470	O86:H34	-
E97474	O116:H27	+
D		
E101408	O98:H-	-
E101621	O98:H-	-
E101396	O98:H-	-
E101406	O98:H-	-
E101402	O110:H-	-

* Hybridisation with EAST1 probe

The results of the hybridisation showed that seven of twenty-two (32%) EAggEC strains hybridised with the probe for EAST 1. Only one strain from outbreaks A and C hybridised with this probe whereas 5 EAggEC strains from outbreak B hybridised. None of the strains from outbreak D hybridised with the EAST1 probe. A study by Savarino *et al.* (1996) showed that 93 (41%) of 227 EAggEC strains hybridised with the EAST1 probe.

EAST 1 was shown to cause fluid secretion by the activation of guanylate cyclase. The biological activity of this toxin is demonstrable in an *in vitro* rabbit intestinal model (Savarino *et al* 1991). Classical *E. coli* heat-stable toxin can be detected using the infant mouse test (3.3.4.) which is functionally and structurally related to EAST1. Six strains of EAggEC which hybridised with the EAST1 probe were selected and used in the infant mouse test. After removal of the infant mouse stomach it was noted that there was no fluid accumulation. It was concluded that these EAggEC strains did not cause fluid accumulation in the infant mouse test.

4.5.2. Haemolysin

4.5.2.a. Secreted haemolysin

All test strains were assayed for the production of haemolysin by the direct plating of strains of EAggEC onto nutrient agar containing washed erythrocytes from horse, sheep, rat, rabbit, mouse, guinea pig, turkey, chicken, cow and human. The strains listed in Table 40 lysed the entire range of erythrocytes causing large zones of haemolysis around bacterial colonies.

Table 40 *EAggEC strains producing haemolysin*

Strain (E)	Serotype	Haemolysin	Detaching*
96390	O?:H-	+	+
98529	O?:H18	+	-
97479	O3:H-	+	+
92356	O3:H2	+	-
107527	O6:H1	+	+
99964	O18:H-	+	+
99960	O18:H30	+	+
99969	O18:H30	+	+
99965	O18:H30	+	+
99961	O18:H30	+	+
99962	O18:H30	+	+
99963	O18:H30	+	+
59905	O21:H2	+	-
104970	O33:H-	+	+
99970	O53:H-	+	-
99971	O53:H2	+	-
97590	O73:H13	+	+
101402	O110:H-	+	-
104968	O131:H-	+	+
107756	O131:H-	+	+
94708	Orough:H-	+	-

* EAggEc strains causing detachment of HEp-2 cells during adhesion assay

Twenty-one (15%) strains secreted an α-haemolysin-like toxin as large zones of haemolysis were observed around the bacterial colonies, the same effect as *E. coli* α-haemolysin, with an apparent association with serotype.

4.5.2.b. Enterohaemolysin

Enterohaemolysin production has been associated with certain strains of Verocytotoxin-producing *E. coli* (Schmidt *et al.* 1995b) , and preliminary studies have indicated that enterohaemolysin was a β-haemolysin (Chart *et al.* 1998b). The genes encoding for enterohaemolysin have been used to develop a DNA probe (CVD 419) for detecting enterohaemolysin-producing *E. coli* (Levine *et al.* 1987b), and this was used in the present study. None of the strains of EAggEC hybridised with probe CVD 419, and it was concluded that strains of EAggEC did not carry the genes required for the production of enterohaemolysin.

4.5.3. Other toxins

Twenty-two strains of EAggEC from outbreaks A-D (panel 3) and another ten strains selected from panels 1 and 2 were screened for the ability to produce Verocytotoxin (VT) using a Vero cell line assay and DNA probes for VT. None of the strains examined produced VT or hybridised with the probes for VT. This results confirmed other studies which showed that strains of EAggEC did not hybridise with the DNA probes for VT (Scotland *et al.* 1991; Faruque *et al.* 1992) and did not react in a latex agglutination test for the presence of VT (Itoh *et al.* 1997) and Vero cell assays also failed to detect VT production by EAggEC (Jalauddin *et al.* 1997). A study by Morabito *et al.* (1998) reported that a strain of EAggEC of serotype O111:H2 was implicated in an outbreak of haemolytic uraemic syndrome in France. The strain was shown to produce VT, as detected with a Vero cell assay but it remains unclear whether the strain was a VTEC which had acquired the EAggEC genes or a strain of EAggEC which has been infected with a phage encoding VT. Strains of O111:H2 are not commonly found to belong to EAggEC or VTEC groups.

Vero and Y1 cells were also used to screen strains of EAggEC for the presence of LT (3.3.3.). None of the thirty-two EAggEC strains had an adverse effect on Vero or Y1 cells. These results confirm previous studies carried out to examine strains of EAggEC for expression of LT using Y1 cell tests (Scotland *et al.* 1991; Faruque *et al.* 1992), an ELISA (Jalalludin *et al.* 1997) and a latex agglutination test (Itoh *et al.* 1997).

4.6. **Serum Survival**

Strains of EAggEC were examined for the ability to survive in serum (3.7.7.). Initially four strains were inoculated into normal rabbit serum and heat-inactivated serum (50°C, 30 min).

Table 41 *Estimation of number of bacteria in original inocula*

Strain	Serotype	OD_{621}	Estimated number of cells/ml
40104	O126:H27	1.0	1×10^9
62008	O126:H27	1.0	1×10^9
92356	O3:H2	1.65	2×10^9
14R519*	K12	1.7	2×10^9

14R519 is not an EAggEC strain but a laboratory strain which did not hybridise with AA probe

All strains grew in heat- inactivated sera (Table 42); however, strains E40104, E62008 (AA probe negative, HEp-2 adhesion negative) and E14R519 (AA probe negative, HEp-2 adhesion negative) were unable to survive in normal sera suggesting that these strains were unable to survive the action of complement. In contrast, EAggEC strain E92356 was able to grow in normal sera (Table 42).

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Seven additional strains were examined for the ability to survive in serum. With reference to Table 43, it was found that strains expressing aerobactin were able to survive in serum as compared to strains which did not express this siderophore. EAggEC strains E58583A (AA probe positive, HEp-2 adhesion positive) and E58583B (AA probe negative, HEp-2 adhesion negative) did not produce aerobactin but had the ability to survive in sera; however, the reason for this was not known.

The panel of strains also included EAggEC which expressed an 18 kDa MAP, but the protein layer was found not to influence the ability of strains to survive in normal serum (Table 43).

Table 43 *Virulence factors and serum survival of E. coli strains tested*

Strain	Serotype	Aerobactin	Haemolysin	18 kDa	Serum resistant
40104	O126:H27	-	-	+	-
62008	O126:H27	-	-	-	-
92356	O3:H2	+	+	-	+
14R519	K12	-	-	-	-
58596	O51:H11	-	-	-	-
72376	O33:H16	+	-	+	+
73339	O15:H-	+		-	+
43509	O44:H18	-	-	+	-
58583A	O77:H18	-	-	-	+
58583B	O77:H18	-	-	-	+
78135	O92:H33	+		-	+

The ability of strains to survive in serum can be influenced by the expression of LPS, a capsule and/or certain membrane proteins (Montenegro *et al.* 1985). The expression of aerobactin would seem to be important for serum survival since strains carrying a ColV plasmid have been associated with increased serum resistance (Bindereif

and Neilands 1985). Furthermore, strains of *E. coli* carrying a ColV plasmid have been associated with cases of pyelonephritis and septicaemia.

4.7. Examination of foodstuffs for the presence of EAggEC

The incidence of strains of EAggEC was investigated by examining selected foods for the presence of EAggEC. Food samples were from retail outlets including supermarkets and butchers' shops. Samples were macerated in broth and processed by the Food Hygiene Laboratory (Central Public Health Laboratory). As part of this study, the enrichment broths were replica plated and probed for EAggEC and VTEC.

With reference to Table 44, 109 types of meat were examined for the presence of EAggEC but these bacteria were not detected. This would suggest that unlike strains of *Salmonella spp.* and *Campylobacter spp.* the EAggEC are probably present only rarely in foods.

Table 44 *Food stuffs investigated for the carriage of EAggEC*

Food	Number of samples tested
Pork mince	4
Beef mince	26
Lamb mince	14
Pork sausages	10
Pork and beef sausages	8
Beef square sausages	1
Beef sausages	7
Lamb burger	6
Beef burger	13
Lamb grill steak	4
Beef grill steaks	2
Top rump steak (beef)	1
Braising steak (beef)	2
Doner kebab (lamb)	2
Lamb kofta kebab	2
Topside of beef	1
Shepherd's pie (beef mince)	1
Minced beef and onion pie	1
Lamb cutlets	4

5. DISCUSSION AND CONCLUSION

Strains of EAggEC have been isolated from cases of chronic diarrhoeal disease and are catergorised as EAggEC by the their pattern of adhesion to HEp-2 cells. These strains may also hybridise with the CVD423 probe developed by Baudry *et al.* (1990). EAggEC strains are further sub-divided by using established criteria such as serotyping. This study showed that strains of EAggEC are highly diverse, expressing an extensive range of somatic and flagellar antigens and in this respect confirmed the work of others (Vial *et al.* 1988; Yamamoto *et al.* 1992; Knutton *et al.* 1992; Scotland *et al.* 1991, 1993). The ability of strains of EAggEC to adhere to HEp-2 cells was clearly associated with the carriage of plasmids, with the loss of the plasmid resulting in the loss of bacterial adhesion. Strains of EAggEC possessed plasmids of different sizes, ranging from 33.6 to 126 MDa, that hybridised with the probe for aggregative adherence. From this part of the study it was concluded that the genes encoding the aggregative phenotype seemed to be acquired readily by strains of *E. coli*. These genes were carried on plasmids of differing sizes suggesting that the larger plasmids may carry genes that are not carried by the smaller plasmids, which may encode encoding virulence factors. Reports have stated that the genes encoding aggregative adherence are carried on plasmids of 60 MDa (Savarino *et al.* 1991; Nataro *et al.* 1992; Czeczulin *et al.* 1997). This study has shown that the genes encoding aggreagtive adherence are carried on plasmids belonging to a wide range of molecular sizes.

The mechanisms by which strains of EAggEC survive passage through the host digestive system and adhere to the gut prior to causing the symptoms of diarrhoea are poorly understood. Studies with the AAF fimbriae suggested that adhesion by EAggEC

may involve the binding of these bacteria via specific receptors on the host gut, as has been demonstrated for strains of enterotoxigenic *E. coli.* The present study detected a small number of strains which did express fimbriae, in common with many other *E. coli* fimbriae, expression did not occur when bacteria were grown at 18°C. However, the majority of EAggEC strains did not express fimbriae and it was concluded that mechanisms other than fimbriae were used by strains of EAggEC to adhere to the human intestine.

For certain strains of *E. coli,* the formation of a pellicle on statically grown cultures can be indicative of the expression of fimbriae and haemagglutinating properties (Duguid *et al.* 1955; Ottow 1975). However, although many strains of EAggEC produced a pellicle, this property was not related to the expression of fimbriae. It has been suggested that all strains of EAggEC express a pellicle whilst growing in static media, and this procedure has been suggested as a rapid method of differentiating EAggEC strains from other types of *E. coli* (Albert *et al.* 1993). In this study, it was demonstrated that less than 75% of strains produced a pellicle, and it was concluded that pellicle formation was not reliable as a means of detecting strains of EAggEC.

The majority of strains of EAggEC agglutinated rat erythrocytes in the presence of mannose, and this confirmed the work of others (Old *et al* 1989; Scotland *et al.* 1991). It has also been suggested that the ability of strains to agglutinate rat erythrocytes could be used as a method for identifying EAggEC (Qadri *et al.* 1994); however, since non-haemagglutinating strains were identified, haemagglutination of rat erythrocytes was not considered as a reliable basis for differentiating strains of EAggEC from other strains of *E. coli.*

The observation that certain strains of EAggEC caused detachment of HEp-2 cells during the adhesion assay, and that strains of EAggEC adhered to 'fixed' HEp-2 cells was in agreement with a study using formalin-fixed colonic cells (Hicks *et al.* 1996). This result was of particular interest since it demonstrated that the initial stages of EAggEC adhesion did not require biochemically active eukaryotic cells. The possibility arises that adhesion involved a receptor on the HEp-2 cell which was not affected by the action of formalin, or that initial adherence involved a physical property such as bacterial charge. The fact that strains of EAggEC bound to formalin-fixed cells also had repercussions on the routine procedures for the detection of strains of EAggEC. Using viable HEp-2 cells, the constraints of a typical working week allowed only one adhesion assay to be carried out per week. However, this study showed that formalin-fixed HEp-2 cells could be prepared in bulk and stored at 4°C for subsequent use.

Certain strains of EAggEC expressed surface-exposed membrane-associated proteins (MAPs) of 18 or 20 kDa. Proteins of 18 kDa were expressed by strains belonging to different serotypes (O126:H27, O44:H18, O33:H16, O110:H-, O116:H27, O75:H27, O119:H27, Orough:H-, Orough:H3, Orough:H27) whereas the 20 kDa MAP was only expressed by strains of EAggEC belonging to serotype O111:H21. Variant strains which did not possess the plasmid hybridising with the probe for aggregative adherence (AA) did not adhere to HEp-2 cells in an aggregative pattern and did not express the 18 or 20 kDa MAPs. Although these proteins were not elucidated fully, it was shown that the MAPs did not contain disulphide bonds or have a quaternary structure. Magnesium ions were shown to be essential for the expression of these MAPs, and calcium ions were required for the expression of the 20 kDa MAP. The MAPs conferred charge on these bacteria since isogenic mutants which did not make a MAP did

not adhere to HEp-2 cells and did not migrate in an electric field. Strains of EAggEC expressing the 18 kDa MAP hybridised with the probe for aggregative adherence, diffuse adherence (DA) and aggregative adherence fimbriae/II (AAF/II); however, fimbriae were not detected by electron microscopy, and the role of these various genetic elements needs further investigation.

The observations that EAggEC adhered to 'fixed' HEp-2 cells and adhesion could be achieved by surface exposed membrane-associated proteins, suggested that bacterial charge might play a role in the adhesion process. The presence of surface charge was supported further by the fact that bacteria readily formed a pellicle, which was dependent on divalent cations. Divalent cations have been shown to be important in the stability of the membrane of many bacteria including *Aeromonas salmonicida* (Garduño *et al.* 1995) and *Pseudomonas aeruginosa* (Asbell and Eagon 1966a, 1966b) therefore the addition of the chelator EDTA (which binds divalent cations) to the growth medium may disrupt the stability of the membrane of the EAggEC strains resulting in the loss of charge.

Net electrostatic charge has been correlated with virulence for the fish pathogen *Aeromonas salmonicida* (Sakai 1987). There was an apparent intraspecies variation in the net charge carried by this organism, where the virulent strains possessed a net negative charge and adhered to positively charged fish cells in tissue culture better than positively charged avirulent strains. The mucosal surface of the intestine also carries a net negative charge due to the presence of anionic sites in both the thick mucopolysaccharide surface coat and its underlying glycocalyx (Jacobs 1983). EAggEC strains like most other bacteria carry a net negative charge (van Loosdrecht *et al.* 1987) although some strains of EAggEC carry a positive charge as well. It may be that the EAggEC stains

carrying a positive charge have the greatest ability to adhere to the negatively charged intestinal wall initially.

Results showed that most strains of EAggEC were hydrophobic which would probably be important in the adhesion of these bacteria to eukaryotic cells. The results showed that 78% of strains were hydrophobic according to the results obtained using the salt aggregation test. It has generally been accepted that hydrophobicity is important in the interaction of bacteria with host cells, with high bacterial surface hydrophobicity correlating with adhesion of EPEC to intestinal brush border membranes (Cantey *et al.* 1981) and oral organisms to oral cavity surfaces (Weiss *et al.* 1982). Fimbriae have also been known to increase the hydrophobicity of certain strains of ETEC (Honda *et al.* 1984) but since only three strains from the panel examined in this study expressed fimbriae the large proportion of hydrophobic EAggEC strains cannot be linked to the expression of these surface structures.

A study by Jacobson *et al.* (1989) reported that pyelonephritogenic *E. coli* strains more often expressed hydrophobic properties (women 80%; children 98%) than faecal control strains from healthy adults and children (57 and 82% respectively). Studies on the hydrophobicity of EAggEC strains showed heterogeneity of strains. Vial *et al* (1988) showed that 40% of EAggEC strains tested were hydrophobic by the salt aggregation test. Fletcher *et al* (1997) reported that the EAggEC strains used showed disparate partitioning behaviour using the two phase partitioning system as a measure of hydrophobicity and charge.

The SAT test showed that all the other EAggEC strains in panel 1 were hydrophobic. The results of the HIC are not consistent with the SAT test as EAggEC strain E47697A was hydrophobic with the SAT test whereas the HIC test showed that

only 0.8% of the original culture was retained in the column and this strains was not hydrophobic concluding that the SAT test was better to differentiate strains which were hydrophobic .

The ability of strains of bacteria to acquire iron during pathogenesis has been recognised as a major pathogenic mechanism. All strains of EAggEC expressed the siderophore enterobactin; however, the ability of strains to express this siderophore is well known. In contrast, approximately 50% of strains of EAggEC also had the ability to express an aerobactin-mediated iron-uptake system, a property generally associated with strains of *E. coli* causing extraintestinal infections especially septicaemias and urinary tract infections.

Strains of *E. coli* causing septicaemia in humans may carry the genes for aerobactin on a Col V plasmid and other plasmids (Williams 1979; Williams and Warner 1980; Crosa 1987; Griffiths 1987; Gonzola *et al.* 1988; Crosa 1989; Woolridge and Williams 1993). Enteroinvasive *E. coli* frequently produce aerobactin (Payne *et al.* 1983; Griffiths *et al.* 1985) but have chromosomally located aerobactin genes (Marolda *et al.* 1987). However the role of aerobactin in the pathogenesis of diarrhoeal disease has not yet been elucidated. Expression of aerobactin has a strong association with extraintestinal infections, septicaemias and urinary tract infections (Johnson *et al.* 1988). Enteroinvasive *E. coli* (EIEC) frequently produce aerobactin (Payne *et al.* 1983). A study by Bollmann *et al.* (1997) showed that 113 strains of 161 (70%) of *E. coli* isolated from blood cultures produced aerobactin and 80 strains of 234 (34.2%) *E. coli* strains isolated from urine had the ability to produce aerobactin whereas only 11 strains of 81 (13.6%) *E. coli* strains isolated from faeces produced aerobactin. These results showed that *E. coli* strains isolated from patients suffering diarrhoea rarely produce aerobactin .

Another study carried out by Yamamoto *et al.* (1995) showed that 93 (48%) strains of 194 isolated from patients suffering from cystitis produced aerobactin whereas 17 (21%) of 81 *E. coli* strains isolated from the faeces of healthy adults had this ability. Fernandez-Beros *et al.* (1990) showed that 25% of intestinal *E. coli* possessed the gene for the aerobactin receptor but lacked the gene for the synthesis of aerobactin. These strains would be able to utilise aerobactin produced by other strains within the intestine. The examination of sera from patients, known to have been infected with EAggEC, for antibodies to the outer membrane receptor proteins for aerobactin and enterobactin might indicate whether or not these proteins were expressed during pathogenesis.

Certain strains of EAggEC expressed capsular material when grown in minimal medium at 37°C but not at 18°C, a characteristic of type II capsular polysaccharides. This capsular material was expressed in Tris-succinate medium used for the detection of siderophores. When succinate was replaced with other carbon sources strains were able to grow and express capsular material when grown with fumarate and glucose media but not in citrate media. Strains of *E. coli* are not able to utilise citrate which is used to differentiate strains of *E. coli* from *Citrobacter.* When strains were grown under anaerobic conditions, only glucose supported growth, but capsular material was not expressed. Although the role of EAggEC capsule remains to be determined, this part of the study showed how important growth conditions were for the expression of cellular components. Whether capsular material would be expressed *in vivo* remains to be determined. Strains of *E. coli* expressing the K1 capsular antigen are mainly associated with meningitis in neonates and septicaemia. A study by Bollmann *et al.* (1997) showed that 115 strains from 161 (71%) of *E. coli* isolated from blood cultures from patients suffering from septicaemia expressed the K1 capsule whereas only 14 strains from 81

(17.3%) isolated from faecal samples of healthy adults expressed this capsular antigen. The fact that aerobactin-producing strains also produced capsular material and haemolysin, and were able to survive in serum, suggested that these strains had the potential to cause extra-intestinal infections. The expression of pathogenic mechanisms *in vivo* could be demonstrated by the detection of patients' antibodies to suspected pathogenic properties.

The symptoms of infection with EAggEC suggest that a toxin may be responsible for the production of diarrhoea during pathogenesis. Examining strains for known enterotoxins failed to detect Verocytotoxin, *E. coli* heat-labile or heat-stable toxin (ST). Thirty-two percent of EAggEC hybridised with the gene probe for EAST1. Some strains of EAggEC (15%) produced a haemolysin; however, its role in diarrhoeal disease remains unknown. Also, the processes involved in HEp-2 cell detachment may involve a toxin-like molecule and this may prove to have a role in pathogenicity. Certain toxins have been shown to be inducible, and the possibility arises that the symptoms of EAggEC diarrhoeal disease may result from a toxin only expressed *in vivo*.

Certain strains of EAggEC hybridised with the probe for EAST1 but toxin activity could not be detected using the infant mouse test. These results agreed with the study carried out by Savarino *et al.* (1991) which showed that EAST1 was not active in the suckling mouse test under conditions in which *E. coli* STa was active. The relevance of EAST1 in EAggEC infections was reconsidered as the genes encoding this toxin were found in 100% of O157:H7 VTEC strains, 41% of ETEC strains, 22% EPEC strains and 13% of DAEC strains (Savarino *et al.* 1996). This experiment also concluded that the strains used in the infant mouse test did not express *E. coli* heat-stable toxin (ST) either. The results from this experiment agree with studies carried out to detect the presence of

ST by EAggEC strains by gene probe tests (Scotland *et al.* 1991; Faruque *et al.* 1992), ELISA tests (Jalalludin *et al.* 1997; Itoh *et al.* 1997). None of these studies found ST expressed by the strains of EAggEC included in the experiments.

A protein of 120 kDa has been described for certain strains of EAggEC which had antigenic similarity with the C-terminal region of α-haemolysin and was thought to resemble α-haemolysin (Baldwin *et al.* 1992). Other workers have shown that EAggEC produce α-haemolysin which may cause the cell detaching activity by some strains during the adhesion assay (Gomes 1995). Seven EAggEC strains of 21 expressing an α-haemolysin-like toxin did not cause detachment of the HEp-2 cells during the adhesion assay concluding that this toxin is not always associated with the removal of the monolayer. The role of α-haemolysin in EAggEC infections remains unclear. Production of haemolysin by strains of *E.* coli has been associated with extraintestinal infections, particularly urinary tract infections (Cooke and Ewins 1975; Minshew *et al.* 1978a, 1978b). Numerous epidemiological studies have shown that 35-60% of the *E.coli* strains isolated from UTI infections produced α-haemolysin. The prevalence of haemolysin producing strains isolated from normal human faeces and patients suffering with diarrhoea was reported as significantly lower (3-27%)(Cooke and Ewins 1975; Minshew *et al.* 1978; De Boy *et al.* 1980; Hughes *et al.* 1983; Caprioli *et al.* 1987; Cosar *et al.* 1991; Stapleton *et al.* 1991; Blanco *et al.* 1992; Siitonen *et al.* 1992). The expression of haemolysin may affect the pathogenesis of *E. coli* septicaemia in several ways. Lysis of erythrocytes may provide a source of iron which is normally bound to haemoglobin. Also, α-haemolysin has been shown to be toxic for leukocytes which helps strains to avoid host defence mechanisms (Gadeberg and Ørskov 1984; Keane *et al.* 1987). In contrast to extra-intestinal infections, expression of α-haemolysin by *E. coli* appears not

to play a significant role in the pathogenesis of diarrhoeal diseases in humans and animals (De Boy *et al.* 1980; Giaffer *et al.* 1992).

The incidence of disease caused by strains of EAggEC can be influenced by the virulence of these bacteria and the immune status of the host. However, the likelihood of infection will also be affected by the incidence of strains of EAggEC in the environment. The source of strains of EAggEC causing outbreaks of diarrhoeal disease in the UK was investigated by examining a limited number of different foods for these bacteria. The observation that strains of EAggEC were not detected indicated that these bacteria were not prevalent in the human food-chain. The high incidence of EAggEC diarrhoeal illness in certain under-developed countries probably results in a higher prevalence of these bacteria, compounded by poor nutrition.

From this study it was concluded that strains of EAggEC form a very heterogeneous population of bacteria, resulting from the ability of certain strains to acquire the genes encoding the EAggEC phenotype. It was concluded that in general, strains of EAggEC adhere to the human intestine by mechanisms other than fimbriae, and that surface charge would seem to be involved. For pathogenic bacteria such as the EPEC, it has been shown that initial contact with the gut mucosa is merely a prelude to a series of processes resulting in a close association between EPEC and the host intestine. The processes that occur with EAggEC following initial attachment remain to be determined.

The pathogenesis of infection with strains of EAggEC involves a diarrhoeal disease; however, the biochemistry involved in the loss of electrolytes from the host intestinal cells remains unclear. This study has shown that the diarrhoeal disease does not involve known enteric toxins, and this aspect of the disease requires further study.

Although certain well established virulence mechanisms were detected in strains of EAggEC, their role in diarrhoeal disease was not resolved.

6. APPENDIX

6.1. Bacteria

6.1.1. *Escherichia coli:* Biochemical characteristics

The genus *Escherichia* comprises Gram-negative rod-shaped bacteria (1.1-1.5μm x 2.0-6.0μm) which are facultative anaerobes, chemoorganotrophic with respiratory and fermentative forms of metabolism. The optimal growth temperature is 37 °C. Strains of *Escherichia spp.* can catabolise D-glucose and other carbohydrates with the formation of acid and gas. Laboratory identification tests utilise the fact that these bacteria are oxidase negative, catalase positive, metyl red positive, Vouges-Proskauer negative and usually citrate negative. They also ferment -arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, trehalose and D-xylose.

6.1.1.a. EAggEC strains used in study

Strains of *E. coli* used during study were from the culture collection held by the LEP. Strains were identified and serotyped based on methods established in the LEP (Gross and Rowe 1985a).

6.1.1.a.i. *Strains of E. coli comprising EAggEC: Panel 1*

Table 45 *Strains of EAggEC from panel 1*

Strain	Serotype	Country of Origin
E92356	O3:H2	Chile
E73339	O15:H-	*
E59905	O21:H2	*
E72376	O33:H16	*
E43509	O44:H18	UK
E45730	O44:H18	UK
E44939	O44:H18	UK
E58596	O51:H11	UK
E76989	O75:H2	*
E58583A	O77:H18	UK
E67643	O78:H10	*
E47697A	O86:H19	*
E97468	O86:H34	*
E60725	O92:H33	*
E78135	O92:H33	UK
E71341	O102:H27	*
E72957	O102:H27	*
E99518	O104:H4	*
E33915	O111:H21	*
E36182	O111:H21	UK
E57144A	O111:H21	UK
E40144	O111:H21	UK
E75607	O126:H27	UK
E55058	O126:H27	UK
E43923	O126:H27	UK
E58816	O126:H27	UK
E55280	O126:H27	UK
E52610	O126:H27	UK
E62008	O126:H27	UK
E55060	O126:H27	UK
E45691	O126:H27	*
E38383	O126:H27	UK
E60874	O126:H27	UK
E83087	O126:H27	UK
E81456	O126:H27	UK
E40104	O126:H27	*
E57157	O126:H27	UK

* information not known

6.1.1.a.ii. *Strains of EAggEC isolated during the Infectious Intestinal Diseases Study (IID study): Panel 2*

Table 46 *EAggEC strains from panel 2*

Strain	Serotype	Symptoms	Age (yrs)	Sex	Country
E97479	O3:H-	D	71	*	UK
E111260	O4:H2	*	44	F	UK
E109902	O4:H33	*	2	F	UK
E101094	O4:H33	D + V	4	F	UK
E96616	O5:H4	*	33	F	UK
E107527	O6:H1	*	4	F	UK
E110715	O6:H10	*	60	F	UK
E97480	O6:H10	D	50	M	Kenya
E97499	O6:H10	D	8 months	M	UK
E99520	O8:H7	D	33	M	UK
E97494	O81:H-	D	8	M	UK
E111479	O11:H-	*	55	F	UK
E103594	O11:H27	D	*	F	UK
E99964	O18ac:H-	D	30	M	UK
E99962	O18ac:H30	D	68	F	UK
E99961	O18ac:H30	*	15	F	UK
E99963	O18ac:H30	D	2	F	UK
E99960	O18ac:H30	D	42	F	UK
E99965	O18ac:H30	D + V	66	M	UK
E109903	O21:H-	D + V	52	F	UK
E107249	O21:H4	*	1	M	UK
E104970	O33:H-	D	59	F	UK
E99970	O53:H-	D	73	M	UK
E99971	O53:H2	D	23	M	UK
E97500	O73:H1	D	50	M	Gambia
E99979	O75:H27	*	30	M	UK
E97477	O81:H-	*	8	M	UK
E92832	O82:H25	*	27	M	UK

D diarrhoea; V vomiting; M male; F female; * information not known

continued.....

6.1.1.a.ii. *Strains of EAggEC isolated during the Infectious Intestinal Diseases Study: Panel 2 (continued)*

Strain	Serotype	Symptoms	Age (yrs)	Sex	Country
E92830	O86:H11	*	31	F	UK
E97496	O86:H2	D	2	F	UK
E97504	O91:H-	*	52	F	UK
E99518	O104:H5	D	31	F	UK
E110716	O106:H16	*	29	F	UK
E94706	O111ab:H-	*	1	M	UK
E109907	O111ab:H-	*	3	M	UK
E103763	O111ab:H-	D	7	F	UK
E99976	O113:H-	D	57	M	UK
E107100	O119:H27	*	6	F	UK
E101089	O126:H27	D	*	*	*
E108837	O130:H27	*	37	M	UK
E97478	O130:H27	*	1	M	UK
E104940	O130:H27	*	1	M	UK
E108839	O130:H27	*	2	F	UK
E104968	O131:H-	D + V	3	M	UK
E107756	O131:H-	*	26	M	UK
E99969	O134:H-	D	4	F	UK
E107754	O134:H25	*	4	M	UK
E101096	O134:H27	D + V	29	F	UK
E99968	O134:H27	D	38	M	UK
E101095	O134:H27	D	61	F	UK
E97502	O134:H27	*	1	M	UK
E104942	O162:H10	*	30	F	China
E99967	Orough:H-	D	1	M	UK
E94708	Orough:H-	*	*	F	UK
E107252	Orough:H-	D	57	F	UK
E99535	Orough:H27	*	15	M	*
E97298	Orough:H27	D	33	M	*
E107542	Orough:H27	*	27	F	UK
E105836	Orough:H3	D + V	1	F	UK
E105392	Orough:H33	D	40	F	Spain

D diarrhoea; V vomiting; M male; F female; * information not known

6.1.1.a.iii. *EAggEC strains isolated from outbreaks: Panel 3*

Table 47 *EAggEC strains from panel 3*

Strain	Serotype	Outbreak	Symptoms	Sex	Age	Country of origin
98527	O19:H-	A	D	M	*	UK
97622	O113:H-	A	D	M	*	UK
96393	O125:H27	A	D	M	*	UK
98529	O?:H18	A	D	*	*	UK
E97820	O62:H30	B	D	F	*	UK
E96386	O73:H18	B	D	F	*	UK
E97590	O73:H13	B	D	F	*	UK
E96485	O134:H27	B	D	M	*	UK
E97900	O?:H-	B	D	*	*	UK
E96487	O?:H-	B	D	*	*	UK
E96390	O?:H-	B	D	F	*	UK
E97819	O?:H27	B	D	F	*	UK
E96483	O?:H33	B	D	F	*	UK
E97472	O86:H34	C	D	F	*	UK
E97468	O86:H34	C	D	*	*	UK
E97470	O86:H34	C	D	F	*	UK
E97474	O116:H27	C	D	F	*	UK
E101396	O98:H-	D	D	F	*	UK
E101406	O98:H-	D	D	F	*	UK
E101408	O98:H	D	D	M	*	UK
E101621	O98:H-	D	D	F	*	UK
E101402	O110:H-	D	D	M	*	UK
E89095	O80:H27	E	*	*	*	Bangladesh
E89099	O28ab:H18	E	*	*	*	Bangladesh
E89115	O162:H-	E	*	*	*	Bangladesh
E89096	O113:H-	E	*	*	*	Bangladesh
E89098	O114:H11	E	*	*	*	Bangladesh
E89100	O?:H5	E	*	*	*	Bangladesh
E89101	O8:H20	E	*	*	*	Bangladesh
E89102	O44:H18	E	*	*	*	Bangladesh
E89104	O141:H49	E	*	*	*	Bangladesh
E89114	O44:H18	E	*	*	*	Bangladesh
E89106	Orough:H7	E	*	*	*	Bangladesh
E89107	:H27	E	*	*	*	Bangladesh
E89109	O44:H18	E	*	*	*	Bangladesh
E89110	O69:H11	E	*	*	*	Bangladesh
E89111	O89:H18	E	*	*	*	Bangladesh
E89112	O69:H11	E	*	*	*	Bangladesh
E89105	O80:H27	E	*	*	*	Bangladesh
E89097	O6:H16	E	*	*	*	Bangladesh

* information not known; D diarrhoea

6.1.1.b. Control Strains

Table 48 *Control strains*

Strain	Serotype	Function
E12734	O157:H45	Aerobactin producer
14R519	K12	Laboratory strain used as a negative control in hybridisations (Willshaw *et al.* 1985)
E60725	O92:H33	AA positive strains used in hybridisations
E5798	O7:H18	LT producing *E. coli* strain
60R767	O3:H2	EAST1 positive strain

6.2. Culture Media

6.2.1. *Nutrient Agar*

Beef extract (0.3%; w/v), peptone (0.1%; w/v), agar (1.5%; w/v) and distilled water. Nutrient agar was sterilised by autoclaving (121°C, 15 mins).

6.2.2. *Nutrient broth*

As nutrient agar but omitting agar (see 6.2.1.)

6.2.3. *MacConkey agar*

Peptone (2%; w/v), lactose (1%; w/v), bile salts (0.5%; w/v), NaCl (0.5%; w/v), neutral red (0.0075%; w/v), agar (1.5%; w/v), and distilled water. MacConkey agar was sterilised by autoclaving (121°C, 15 mins).

6.2.4. *Dorset's egg agar slope*

Sterilised egg mixture (yolks and whites, 'Lab-Lemco' powder 0.25%; w/v), peptone (0.25%; w/v), NaCl (0.125%; w/v) and distilled water. Egg medium was processed by repeated incubation at 80°C (2 hours).

6.2.5. *L-agar*

Oxoid tryptone broth powder (1%; w/v), Difco 'Bacto' agar powder (1%; w/v), NaCl (0.8%; w/v), glucose (0.1%; w/v) and distilled water. L-agar was sterilised by autoclaving (121 ^{o}C, 15 mins).

6.2.6. *L-broth*

As L-agar but omitting agar (see 3.2.v.)

6.2.7. *Peptone Water*

Peptone (1%; w/v), NaCl (0.5%; w/v) and distilled water. Peptone water was sterilised by autoclaving (121°C, 15 mins).

6.2.8. *Tryptone soya broth (TSB)*

Pancreatic digest of casein (1.7%; w/v), Papaic digest of soybean meal (0.3%; w/v), NaCl (0.5%; w/v), K_2HPO_4 (0.25%; w/v), glucose (0.25%; w/v) and distilled water. TSB was sterilised by autoclaving (121°C, 15 mins).

6.2.9. *Colonisation factor antigen (CFA) agar*

Casamino acids (1%; w/v), yeast extract (0.15%; w/v), $MgSO_4$ (0.05%; w/v), $MnCl_2$ (0.0005%; w/v), agar (2%; w/v) and distilled water. CFA agar was sterilised by autoclaving (121°C, 15 mins).

6.3. Tissue culture

6.3.1 Hanks's Basal Medium Eagle (HBME)

From Sigma Chemical Co. Ltd. (32360-026) .

To a 100 ml bottle of HBME add: Foetal calf serum (10%; v/v),

200mM glutamine (2%;v/v), penicillin/streptomycin (5000 units/ ml) (2%; v/v).

6.3.2. Giemsa stain

To 100 ml of sterile distilled water add:-

10ml of concentrated Giemsa stain (Merck Ltd)

1ml of stock solution

6.3.3. Basal Medium Eagle (BME)

From Sigma Chemical Co. Ltd. (B5158)

Stock concentration of BME (10%; v/v), foetal calf serum (10%; v/v), 200mM glutamine (0.5%; v/v), penicillin/streptomycin (5000 unit/ ml each) (2%; v/v), Fungizone (amphotericin B) (0.15%; v/v) added to 100ml of sterile distilled water.

6.3.4. Acetone/ xylene dehydration gradient

a) Solutions 1 & 2: Phosphate buffer

b) Solutions 3 & 4: acetone

c) Solution 5: 50% (v/v) acetone + 50% (v/v) xylene

d) Solution 6: 33% (v/v) acetone + 67% (v/v) xylene

e) Solution 7: xylene

6.3.5. Vero cell media

Dulbecco's Modified Eagle's Medium (DMEM) purchased from Sigma (D6546). To 100ml bottle of DMEM add: Foetal bovine calf serum (10%; v/v), penicillin/streptomycin (5000 units/ml) (2%; v/v), 200mM glutamine (0.5%;v/v).

6.3.6. Y1 cell media

Hams F10 medium purchased from GibcoBRL (31550-015).

To 100 ml bottle of HAM's F-10 medium add: Donor horse serum (12.5%; v/v), foetal calf serum (2.5%; v/v), (200mM) glutamine (0.5%; v/v), penicillin/streptomycin (5000unit/ml each; 2.0%; v/v)

6.4. Surface properties

6.4.1. Preparation of outer membranes

6.4.1.a. 25 mM Tris-HCl pH 7.4

3.03g Tris(hydroxymethyl)methylamine

Hydrochloric acid

Dissolve the Tris in 800ml deionised water adjust the pH to 7.4 with HCl and make up to 1 litre.

6.4.1.b. 25mM Tris-HCl pH7.4 1mM EDTA.Na_2

500ml 25mM Tris-HCl pH 7.4

0.185g ethylenediaminetetraacetic acid disodium salt

It is essential to use the disodium salt of EDTA since EDTA alone is not very soluble.

Solutions were stored at 4°C.

6.4.2. Preparation of lipopolysaccharide

6.4.2.a. SDS-PAGE solubilization buffer containing 3.3 mg/ml Proteinase-K (Sigma Chemical Co. Ltd.)

6.4.3. Hydrophobicity

6.4.3.a. Salt aggregation test

Different concentration of ammonium sulphate 5M - 0.02M

6.5. Standard stock solutions

6.5.1. 20 x SSC

3 M NaCl

0.3 M tri sodium-citrate

pH was adjusted to pH 7.0 with 50 % w/v NaOH

6.5.2. 20 x SDS

(sodium dodecyl sulphate)

20% (w/v) SDS in 100 ml of distilled water

6.5.3. 20 x SSPE

3M NaCl

0.2M $NaH_2PO_4.2H_2O$

20mM EDTA

NaOH to give pH 12 then HCl to pH 7.4

6.5.4. Tris EDTA buffer

(TE buffer)

10 mM Tris

1 mM EDTA

pH was adjusted to pH 8.0 with 50 % NaOH and

filter sterilised

6.5.5. Phosphate buffered saline (PBS)

NaCl (0.8%; w/v), KCl (0.02%; w/v), disodium hydrogen phosphate (0.115%; w/v) and potassium dihydrogen phosphate (0.02%; w/v), pH 7.3

6.6. Molecular Studies

6.6.1. Plasmid Preparation using alkaline lysis method

6.6.1.a. Solution 1 : 2mg/ml lysozyme in 50mM glucose solution containing 10mM EDTA, 25mM Tris-HCl pH 8.0.

6.6.1.b. Solution 2 : 0.2M NaOH containing 1%; sodium dodecyl sulphate (SDS). Make by mixing equal volumes of 0.4N NaOH and 2% SDS stock solution.

6.6.1.c. Solution 3 : 3M sodium acetate pH 4.8 . Stable at room temperature.

6.6.1.d. Solution 4 : 0.05M Tris HCl pH 8.0 containing 0.1 M sodium acetate. Keep in fridge.

6.6.2. Solutions for the preparation of genomic DNA

6.6.2.a.	Chloroform/isoamyl alcohol	24ml chloroform 1 ml isoamyl alcohol
6.6.2.b.	Phenol/chloroform/isoamyl alcohol	25 g phenol 24 ml chloroform 1ml isoamyl alcohol Dissolved in 100ml of distilled water. 10 ml TE was added.

6.6.2.c.	TERNAase	Sigma	Stock solution 10mg/ml Final concentration 50.0µg/ml

6.6.3. DNA Electrophoresis

Buffers and reagents

6.6.3.a.	Tris borate buffer		90mM Tris 2mM EDTA 90mM Boric acid
6.6.3.b.	Tris acetate buffer		40mM Tris 5mM sodium acetate 1mM EDTA pH 7.9 with glacial acetic acid
6.6.3.c.	Bromophenol blue marker		60% w/v sucrose 0.25% w/v bromophenol blue in TE buffer
6.6.3.d.	Ethidium bromide stain	Stock solution : Staining solution :	700µg/ml in TE 0.56µml (200µl in 250ml water)

6.6.4. DNA molecular weight markers

6.6.4.a.	Marker VIII	Mixture of fragments from cleavage of pUCBM21 DNA with *Hpa*II, and of pUCBM21 DNA with *Dra*I and *Hin*dIII. Fragment sizes in base pairs : 1114, 900,692, 501, 489, 404, 320, 242,190, 147, 124, 110, 67, 37, 34, 26, 19.

6.6.5. Southern Blot

6.6.5.a. Depurination solution: 0.25M HCl, 11ml conc. HCl + 489ml water

6.6.5.b. Denaturing solution: 0.5M NaOH, 1.5M NaCl

6.6.5.c. Neutralising solution: 0.5M Tris, 1.5M NaCl, pH 7.4

6.6.6. Solutions for hybridisation

Processing of filters

6.6.6.a. Denaturing solution : 0.5M NaOH, 1.5M NaCl

6.6.6.b. Neutralising solution : 1.5M NaCl, 0.5M Tris, pH 8.0

Hybridisation

6.6.6.c. ECL hybridisation solution : 5 x SSC, 0.5% ECL Blocking reagent (Amersham), 0.1% SDS, 5% dextran sulphate

6.6.6.d. Salmon DNA Concentration : 10 mg/ml
Sigma D1626 Type III sodium salt

6.6.6.e. Probe solution (10 ml) 10 µl labelled probe
100 µl boiled salmon DNA
10ml hybridisation solution

6.6.6.f. Washing conditions for probes

AAF/I, AAF/II,	0.1 x SSC/0.1% SDS
CVD419	2 x 15 min at 68 °C
AA, EAST1	5 x SSC/0.1% SDS
	2 x 15 min at 54 °C
DA	0.5 x SSC/0.1%SDS
	2 x 15 min at 68 °C

6.6.6.g. ECL buffer 12.11g Tris

8.77g NaCl

Dissolved in 800ml of distilled water, pH 7.5, made up to 1l.

6.6.6.h. Blocking solution

Commercial product from Amersham Life Sciences

6.6.6.i. Antibody conjugate

Anti-fluorescein HRP conjugate, commercial product from Amersham.

6.7. Miscellaneous Studies

6.7.1. SDS-PAGE

Buffers and solutions

6.7.1.a. Lower gel buffer : 1.5mM Tris-HCl, 0.4% SDS, pH8.8

6.7.1.b. Upper gel buffer : 0.5mM Tris-HCl, 0.4% SDS, pH 6.8

6.7.1.c. Acrylamide : 30% acrylamide, 0.8% N,N' Methylenebisacrylamide (ratio of acrylamide:bisacrylamide 37:5:1)

6.7.1.d. Ammonium persulphate (APS) : 10% (w/v) aqueous solution

6.7.1.e. Running Buffer : 6.06g Tris
28.8g Glycine
2.00g SDS
Dissolve Tris, glycine and SDS in 1.8l of distilled water and then fill up to 2.0l with distilled water.

6.7.1.f. Solubilising Buffer: 62.5mM Tris-HCl, pH 6.8 containing 10% glycerol, 5% β-Mercaptoethanol, 3% SDS, 0.01% bromophenol blue.

6.7.1.g. Coomassie blue gel stain : 0.025%(w/w) Coomassie brilliant blue
50% (v/v) methanol
10% acetic acid

6.7.2. Silver staining of lipopolysaccharides

6.7.2.a. Fixing solution: 200 ml methanol
25 ml acetic acid
275 ml deionised water

6.7.2.b. Oxidising solution: 200 ml fixing solution containing 1.4g periodic acid

6.7.2.c. Silver staining solution: 1 g silver nitrate in 5ml deionised water
28 ml 0.1M sodium hydroxide, 2 ml ammonium hydroxide (specific gravity 0.88)

Place 0.1M sodium hydroxide (4g NaOH/l) in a 250ml measuring cylinder containing a magnetic stirring bar. While mixing, add ammonium hydroxide and add drop wise the silver nitrate solution. Make up to 150ml with distilled water, mix, and use within 5 min of preparation.

6.7.2.d. Silver stain developer 50 mg citric acid
0.5 ml formalin

Place citric acid in a 1.5 l beaker containing a magnetic stirring bar and dissolve in 1 l deionised water. Add formalin and mix.

6.7.3. Immunoblotting

6.7.3.a. Transfer buffer: 25mM tris, 192mM glycine, 20% (v/v) methanol

6.7.3.b. BCIP: 500 mg BCIP, 10ml distilled water

6.7.3.c. NBT in DMF : 750mg NBT, 10ml 70% DMF

6.7.3.d. Development buffer : 0.1M Tris, 0.09M NaCl, 0.15M $MgCl_2$, pH 9.5

6.7.3.e. PBS-Tween: 0.25ml Tween 20 (BDH) in 1 litre distilled water

6.7.4. Lowry Protein Assay

6.7.4.a. Lowry protein standard : 1mg/ml Bovine serum albumin (BSA)

6.7.4.b. Lowry Solution A : 2% (w/v) sodium carbonate in 0.1M sodium hydroxide

6.7.4.c. Lowry Solution B : 0.5% copper sulphate ($CuSO_4.6H_2O$) in 1% sodium-potassium tartrate

6.7.4.d. Lowry Solution C : 50ml Lowry A plus 1ml Lowry B

6.7.4.e. Lowry Solution D : 1 M Folin-Ciocalteau reagent

6.7.5. Siderophore Detection

6.7.5.a. Tris succinate medium

5.8g Sodium chloride (NaCl)

3.7g Potassium chloride (KCl)

1.1g ammonium chloride (NH_4Cl)

0.15g Calcium chloride dihydrate ($CaCl_2.2H_2O$)

0.1g Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)

0.142g Sodium sulphate (Na_2SO_4)

0.272g Potassium dihydrogen phosphate (KH_2PO_4)

12.1g Tris(hydroxymethyl)methylamine

10.0g Sodium succinate

Concentrated hydrochloric acid

Distilled water

The Tris, Na succinate, NaCl, KCl, NH_4Cl, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, Na_2SO_4 and KH_2PO_4 was dissolved in approximately 800ml of distilled water and the pH was adjusted to 6.8 with HCl. The final volume was made up to 1 litre. The Tris would only go into solution once the pH had been adjusted. The medium was sterilised by filtration (0.45µm filter).

6.7.5.b. Ferric perchlorate reagent : 5mM ferric perchlorate in 0.14 M perchloric acid

6.7.5.c. Nitrite/molybdate reagent : 10% (w/v) sodium nitrite and 10 % (w/v) sodium molybdate

6.7.6. SEFEX Kit

A commercial kit now no longer available from Central Veterinary Laboratory (Weybridge). It consists of a monoclonal antibody raised to the 14 kDa fimbrial subunit of *Salmonella enteritidis* PT4 attached to latex beads. In the presence of the antigen the coloured beads agglutinate giving a positive reaction.

6.7.7. Immunogold

6.7.7.a. Gold buffer:- Tris-NaCl buffer (pH 8.2)

0.242g Tris
0.13g NaN_3
0.9g NaCl
0.1g bovine serum albumin (BSA)

Adjust to pH 8.2 and make up to 100ml and millipore filter

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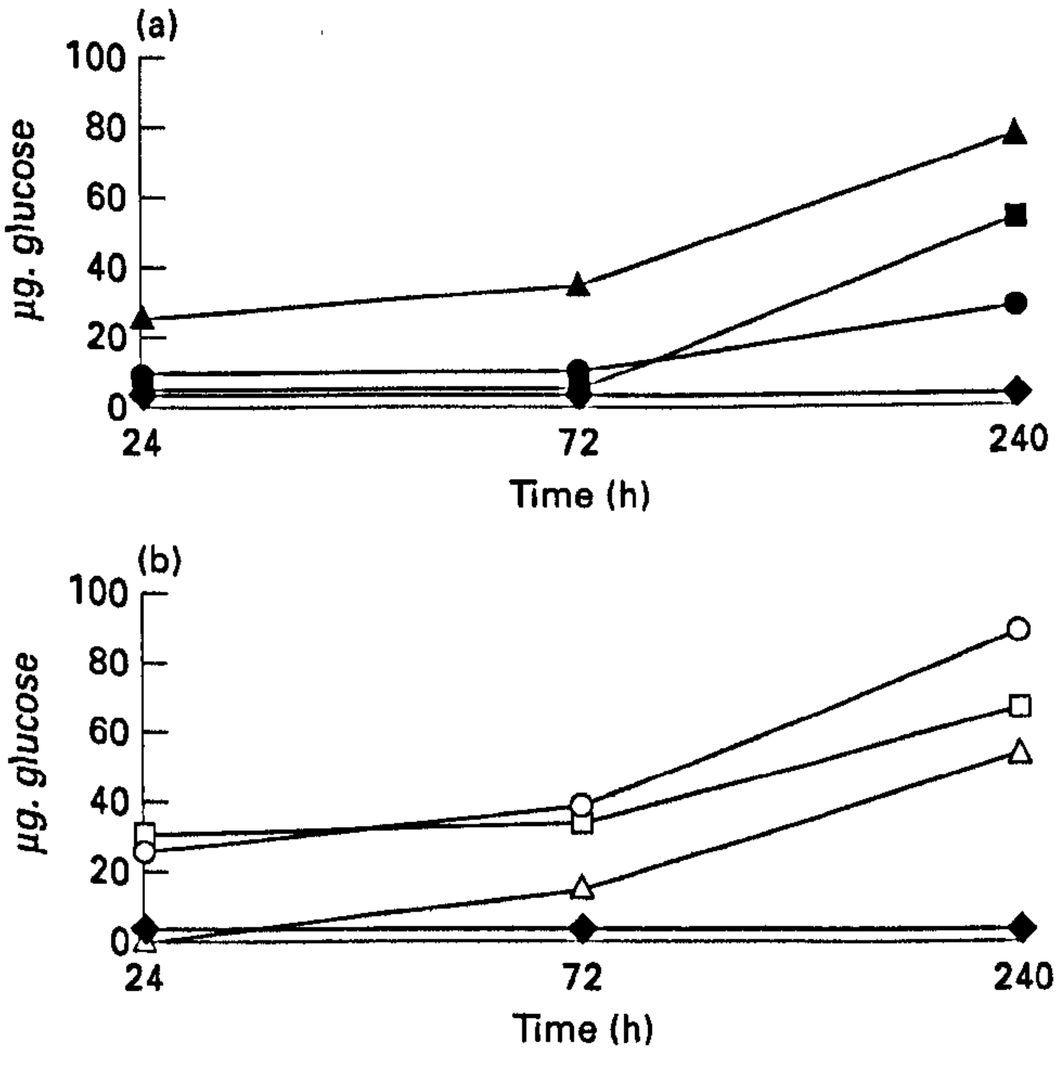

Fig. 3 Production of reducing compounds by the crude agarase of *Cytophaga* sp. LR_2 from carbohydrates and polysaccharides. □, arabinose; △, rhamnose; ○, xylose; ■, gum arabic; ▲, pectin; ●, polysaccharide of *Rhodella reticulata*; ◆, control

inski *et al.* 1992), the agarolytic activity of *Cytophaga* sp. LR_2 could be explained if agarase production was induced not only by agar, but also by other galactans and polysaccharides (Agbo and Moss 1979). Although we observed an increase in reducing sugars, it was obvious that agarolytic enzyme from *Cytophaga* sp. LR_2 alone was not sufficient to lyse the polysaccharide of *R. reticulata*.

The *Cytophaga* group of bacteria has been shown to produce a variety of polysaccharide enzymes, such as amylase, proteinase, chitinase, elastase, keratinase, laminarase, caragenase and glucanase, enzymes hydrolysing algal galactans and porphyrans (Rhem and Reed 1988; Chiou and Jeang 1995). For the moment, we suggest that the pathogenic activity of *Cytophaga* sp. LR_2 towards *R. reticulata* resulted from a combination of enzyme action of *Cytophaga* sp. LR_2 and other bacterial contaminants present in the algal culture.

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Identification of entero-aggregative *Escherichia coli* based on surface properties

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H. CHART, J. SPENCER, H.R. SMITH AND B. ROWE. 1997. Twenty-nine strains of *Escherichia coli* that adhere to HEp-2 cells with a 'stacked brick' pattern (EAggEC), and four nonadherent control strains, were examined for the ability to hybridize with gene probes for aggregative (AA) and diffuse (DA) HEp-2 cell adhesion phenotypes. These strains were also tested for the ability to express an 18 kDa membrane-associated outer-membrane protein (MAP), to agglutinate erythrocytes, and to produce a pellicle during broth culture. Thirteen of the 29 HEp-2 adherent strains of *E. coli* hybridized with the gene probes for both AA and DA, and expressed an 18 kDa outer membrane protein (OMP) which was antigenically related to the MAP expressed by strains of *E. coli* O126:H27. The strains that did not carry the additional DA genes did not express an 18 kDa OMP. Although strains of EAggEC share the ability to adhere to HEp-2 cells with a stacked brick pattern, these strains exhibit a diverse range of physical and biochemical properties. From the results of this study, it was concluded that currently, the possession of EAggEC genes or the ability to adhere to HEp-2 cells in a stacked brick formation, remain the only reliable means of identifying EAggEC.

INTRODUCTION

Strains of entero-aggregative *Escherichia coli* (EAggEC) possess the ability to adhere to HEp-2 cells in a 'stacked brick' or aggregative adhesion (AA) pattern (Baudry *et al.* 1990); however, EAggEC form a diverse group of *E. coli* with strains belonging to more than 50 different 'O' serogroups (Vial *et al.* 1988; Knutton *et al.* 1992; Debroy *et al.* 1994; Qadri *et al.* 1994; Debroy *et al.* 1995). Fimbriae have been described for strains of EAggEC, ranging from bundles of fine filaments (Knutton *et al.* 1992) and thin fimbriae (Collinson *et al.* 1992) to bundle forming fimbriae (Nataro *et al.* 1992). However, not all EAggEC express fimbriae (Chart *et al.* 1994a; Chart *et al.* 1995) and the role of fimbriae in HEp-2 adhesion is not fully understood. The mechanisms by which EAggEC adhere to eukaryotic cells have not been identified, but carriage of plasmid-encoded EAggEC genes is essential (Baudry *et al.* 1990).

For strains of EAggEC belonging to serotype O126:H27, the possession of the EAggEC genes correlates with the expression of an outer membrane-associated protein (MAP) of 18 kDa which shares specific epitopes with fimbriae produced by strains of *Salmonella enteritidis* (Chart *et al.* 1994a). Rabbit antibodies prepared to the 18 kDa MAP expressed by *E. coli* O126:H27 also react with 18 kDa MAPs expressed by EAggEC belonging to serotype O44:H18 (Chart *et al.* 1995).

Strains of EAggEC are currently identified by HEp-2 cell adhesion assays or by DNA hybridization tests with specific EAggEC gene-probes. As the availability of these tests is limited, workers have attempted to find alternative methods for providing a simple laboratory test for identifying EAggEC. Strains of EAggEC have been shown to agglutinate erythrocytes from a range of animal species and for certain serotypes, haemagglutination was shown to be mannose-sensitive (Qadri *et al.* 1994). EAggEC have also been reported to form a pellicle when growing in L-broth (see below), a feature proposed as a simple and rapid test (Albert *et al.* 1993). In the present study, a panel of EAggEC was examined for phenotypic and genotypic characteristics in an attempt to produce an easy method for differentiating strains of EAggEC from other strains of *E. coli*.

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MATERIALS AND METHODS

Bacterial strains

Thirty-three strains of *E. coli*, belonging to 16 different serogroups, were used in the present study (Table 1). Four strains did not adhere to HEp-2 cells. All strains were identified and serotyped by established methods (Gross and Rowe 1985), and were stored on Dorset's egg agar slopes at room temperature.

Bacteria were grown on nutrient agar (CM3, Oxoid Ltd, UK) and MacConkey agar (CM7, Oxoid Ltd) for 16 h at 37 °C; strains were also grown in L-broth (10 g l^{-1} bacto tryptone (Difco Ltd, UK), 5 g l^{-1} yeast extract (Difco Ltd), 5 g l^{-1} sodium chloride, 1 g l^{-1} glucose)) statically for 16 h at 37 °C. For certain experiments, bacteria were grown in

Table 1 Strains of *Escherichia coli* used in the present study

Strain	Serotype	Haemagglutination*				Hydrophobicity†	Bacterial charge**	
AA probe-positive		NA	NA + MAN†	LB	LB + MAN‡		L-broth	L-broth/EDTA§
E92356	O3:H2	−	nd	−	nd	4–0·5	+	+
E73339	O15:H-	+	−	+††	+	4–2	+	+
E58596	O51:H11	+	+	+††	+	4–0	+	+
E76989	O75:H2	−	nd	+††	+	4–1	+	+
E58583A	O77:H18	−	nd	−	n	−	+	+
E67643	O78:H10	−	nd	+	+	4–0·5	nc	+
E47679A	O86:H19	−	nd	−††	nd	4–0·5	±¢	+
E47679B	O86:H19	−	nd	−††	nd	4–2	±	+
E97468	O86:H34	+	−	+	+	4–0·5	+	+
E60725	O92:H33	+¶	−	+††	+	4–0·5	±	nc
E78135	O92:H33	−	nd	−††	nd	4–0	±	+
E101621	O98:H-	+	nd	−	nd	4–0·5	±	nc
E71341	O102:H27	−	nd	−	nd	−	±	+
E72957	O102:H27	−	nd	−	nd	−	+	+
E40144	O111:H21	+¶	+	+††	−	4–0	+	+
E33915	O111:H21	−	nd	−††	nd	4–0	+	nc
AA and DA probe-positive								
E72376	O33:H16	−	nd	+††	+	4–0	+	+
E44939	O44:H18	−	nd	−††	nd	4–0	+	+
E43509	O44:H18	−	nd	−††	nd	4–0	+	+
E45730	O44:H18	−	nd	−††	nd	4–0	+	+
E101402	O110:H-	+	+	−††	nd	4–0	±	+
E97474	O116:H27	+¶	+	+††	+	4–0	+	nc
E75607	O126:H27	−	nd	−	nd	4–0	+	nc
E38383	O126:H27	+¶	+	−††	nd	4–0	+	nc
E40104	O126:H27	+¶	+	−††	nd	4–0	+	−
E83087	O126:H27	+¶	+	−	nd	4–0	+	−
E75508	O126:H27	+¶	+	−††	nd	4–0	+	nc
E60874	O126:H27	+	+	+††	+	4–0	+	+
E81456	O126:H27	+	+	+††	−	4–0	+	+
AA probe-negative								
E58583B‖	O77:H18	−	nd	−††	nd	−	+	+
E57144B	O111:H21	−	nd	+	+	4	nc	+
E62008	O126:H27	−	nd	−	nd	−	+	nc
E55280	O126:H27	−	nd	−	nd	−	+	nc

*Haemagglutination of rat erythrocytes following growth on nutrient agar (NA) or in L-broth (LB); †range of molar concentration of ammonium sulphate giving agglutination; ‡in the presence of 1% (w/v) D-mannose; nd, not done; nc, neutral charge; §L-broth with 1 mmol l^{-1} EDTA; ¢, bacteria migrated towards cathode and anode; ‖ diffuse adhesion probe-positive; ¶haemagglutination of rat erythrocytes inhibited by growth on MacConkey agar; ††Pellicle formation on L-broth; **migration towards anode.

L-broth with and without 1 mmol l^{-1} ethylenediaminetetra acetic acid-disodium salt (EDTA.Na_2; Sigma Chemical Co. Ltd) or 1 mmol l^{-1} ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′ tetra acetic acid (EGTA; Sigma) for 16 h at 37 °C with shaking at 120 rev min^{-1}.

HEp-2 adhesion tests

The ability of strains of *E. coli* to adhere to HEp-2 cells was determined as described previously (Chart *et al.* 1988).

Bacterial charge

Bacterial charge was determined based on the method of Krishna *et al.* (1996). Bacteria were grown overnight in L-broth and dotted (1 μl) onto 1 cm-wide strips made from Whatman (No. 1) filter paper. Strips were moistened with phosphate buffered saline (PBS) and placed in a Multiphor 2117 electrophoresis apparatus (LKB, Upsala, Sweden) with PBS as electrode buffer. Following electrophoresis (5 mAmp, 5 min), strips were placed face down on dried nutrient agar plates (5 s) prior to incubation of plates (37 °C, overnight).

SEFEX

A commercial SEFEX *Salmonella enteritidis* detection kit was obtained from the Central Veterinary Laboratory (Weybridge, UK) and used as described previously (Chart *et al.* 1994a). The SEFEX reagent comprises latex beads coated with monoclonal antibodies to epitopes on fimbriae expressed by strains of *Salm. enteritidis*.

Outer membranes SDS-PAGE

Outer membranes (OM) were prepared from sonicated bacteria by the selective solubilization of inner membranes (Chart *et al.* 1994a) with Sarkosyl (Merck Ltd, Poole, UK). SDS-PAGE was performed using the method of Laemmli (1970) as described (Chart and Griffiths 1985). Samples of outer membrane proteins (30 μg $lane^{-1}$) were loaded onto gels comprising a 4·5% (w/v) acrylamide stacking gel and a 15% (w/v) acrylamide separation gel. Following electrophoresis, gels were stained with Coomassie brilliant blue (Chart and Griffiths 1985).

Immunoblotting

Outer membrane protein (OMP) profiles were transferred onto nitrocellulose membrane (Towbin *et al.* 1979) and reacted with rabbit antisera as described previously (Chart *et al.* 1994a, 1994b).

DNA probe tests

The *E. coli* strains were tested by colony hybridization with the EAggEC (AA) (Baudry *et al.* 1990) and DA (Bilge *et al.* 1989) probes. The AA probe was a 1 kb *Eco* RI-*Pst*I fragment form pCVD432, and the DA probe was a 370 kb *Pst*I fragment within the *daa*C gene from pSLM852. The probes were labelled with fluorescein-dUTP and hybridizations were performed at 68 °C for 18 h. The membranes were washed twice at room temperature for 15 min in 2 × SSC (sodium chloride/sodium citrate), 0·1% (w/v) SDS. For the AA probe, subsequent washes were in 5 × SSC, 0·1% (w/v) SDS for 15 min at 54 °C, followed by 30 s at room temperature in 2 × SSC, 0·1% (w/v) SDS and with the DA probe, membranes were washed in 0·5 × SSC, 0·1% (w/v) SDS at 68 °C for 15 min. Detection of hydrids was performed as described by the manufacturer (Amersham International, UK).

Haemagglutination

Agar-grown bacteria were suspended in PBS to give a concentration of 1 mg cells ml^{-1}. These suspensions (50 μl) were placed in wells on a ceramic tile and mixed with an equal volume of heparinized rabbit or rat blood diluted (× 10) in PBS, with or without 1% (w/v) D-mannose. Agglutination was assessed following mixing on a gyrating shaker for 1 min. Bacteria were also grown in L-broth and mixed (50 μl) with an equal volume of red cell suspension; agglutination was assessed as above.

Hydrophobicity testing

A salt agglutination test was used to assess the ability of bacteria to clump in ammonium sulphate as a measure of hydrophobicity. A range of ammonium sulphate concentrations was prepared (0·02, 0·05, 0·5, 1·0, 2·0, 4·0 M) and 50 ml volumes dispensed into the wells of a 96-well ('V' well) plate. Equal volumes of bacterial suspensions (1 mg cells 100 ml^{-1} PBS) were added to the plates prior to mixing (30 min) with gyration.

Pellicle formation

Strains of *E. coli* were assessed for production of a pellicle following static growth in L-broth, with and without 1 mmol l^{-1} EDTA or EGTA (37 °C, 16 h).

RESULTS

Twenty-nine strains adhered to HEp-2 cells in a 'stacked brick' pattern; *E. coli* strains E58583B, E57144B, E62008 and E55280 did not. Twenty-nine strains of *E. coli* hybridized with the EAggEC AA probe; strains E58583B, E57144B,

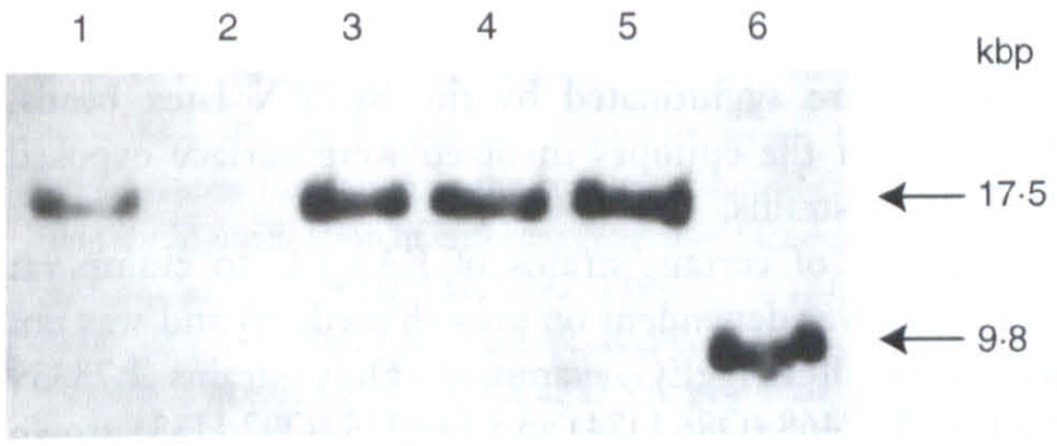

Fig. 1 The AA DNA probe bound to a 17·5 kbp fragment in *E. coli* strain E40104 (lane 1, arrowed) but not the DNA of strain E62008 (lane 2). The AA probe also bound to a 17·5 kbp fragment in strains E72376 (lane 3), E44939 (lane 4) and E97474 (lane 5); the AA probe bound to a 9·8 kbp fragment in *E. coli* strain E101402 (lane 6, arrowed)

E62008 and E55280 did not (Table 1). Thirteen strains belonging to serotypes O126:H27 (7), O44:H18 (3), O33:H16 (1), O110:H- (1) and O116:H27 (1) also hybridized with the DA probe (Table 1). Strain E58583A carried the genes for AA but not DA. Strains of *E. coli* E40104 (O126:H27), E44939 (O44:H18), E72376 (O33:H16), E97474 (O116:H27) and E101402 (O110:H-) were selected for further study.

Total cellular DNA was digested with *Eco*RI and gel profiles transferred onto nylon membranes. The AA DNA probe hybridized with a 17·5 kbp fragment produced from *E. coli* strain E40104 (Fig. 1, lane 1) but did not hybridize with the DNA of the probe-negative control strain E62008 (Fig. 1, lane 2). The AA probe also hybridized with a 17·5 kbp fragment in strains E72376 (O33:H16), E44939 (O44:H18) and E97474 (O116:H27) (Fig. 1, lanes 3–5); however, the AA probe hybridized with a 9·8 kbp fragment in *E. coli* strain E101402 (O110:H-) (Fig. 1, lane 6). Replicate DNA digests were also tested with the DA gene probe. This probe hybridized with a 19·5 kbp fragment in *E. coli* strains E40104 (O126:H27), E72376 (O33:H16), E44939 (O44:H18) and E101402 (O110:H-); however, the probe bound to two fragments of 19·3 and 21·0 kbp in strain E97474 ((O116:H27). The DA probe did not hybridize with the DNA of *E. coli* strain E62008 (O126:H27) (data not shown).

Only strains of *E. coli* belonging to serotype O126:H27 that were EAggEC probe positive were agglutinated by the SEFEX latex beads.

Strains of *E. coli* hybridizing with both AA and DA gene probes expressed an 18 kDa MAP (Fig. 2A, lanes 1, 3–8). HEp-2 adherent strains which hybridized with the AA gene probe only did not express an 18 kDa MAP. A rabbit antiserum prepared to the 18 kDa MAP of *E. coli* O126:H27 (Chart *et al.* 1995) contained antibodies which reacted with an 18 kDa OMP expressed by strains of *E. coli* which hybrid-

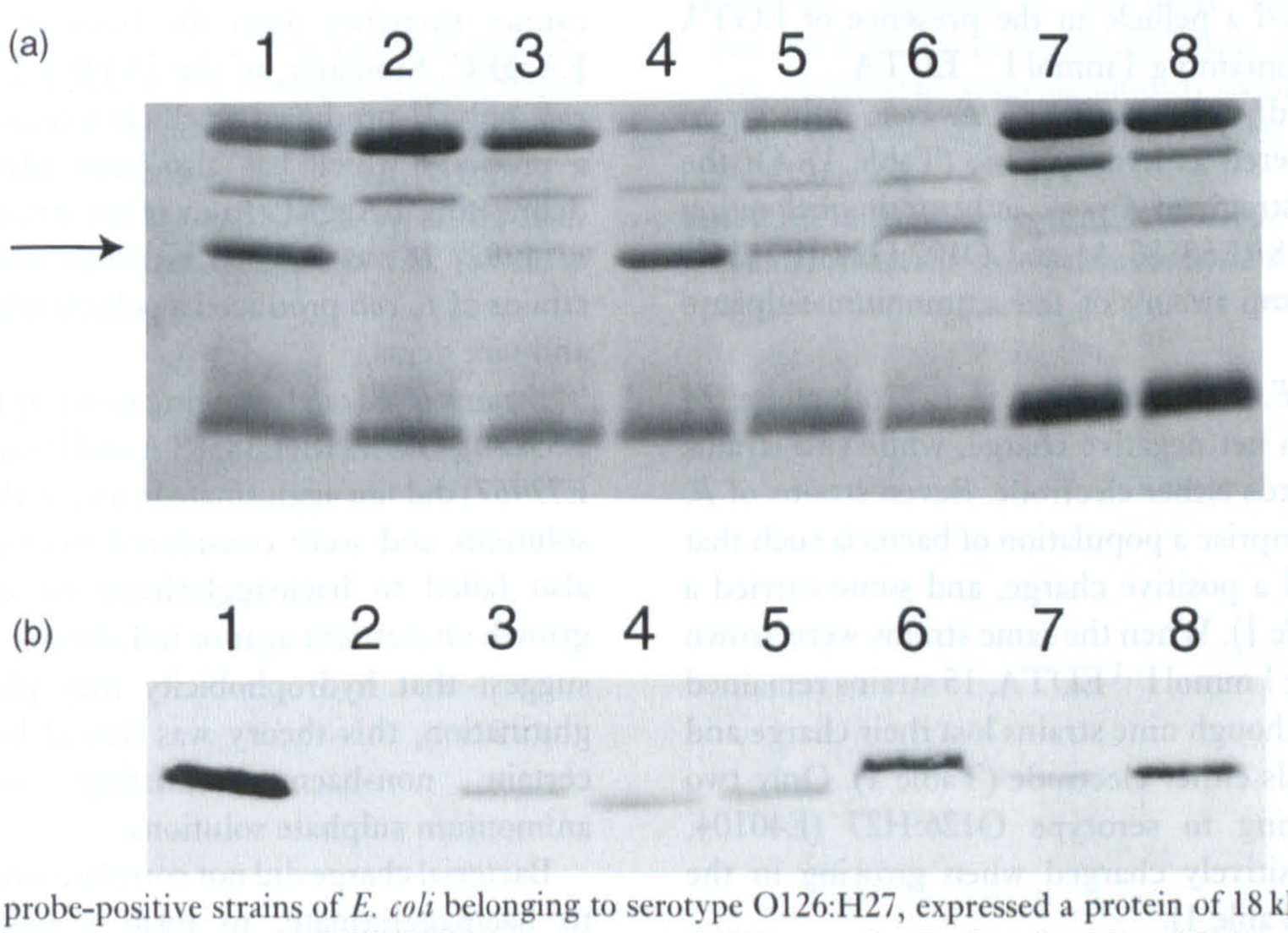

Fig. 2 AA and DA gene probe-positive strains of *E. coli* belonging to serotype O126:H27, expressed a protein of 18 kDa (2a, lane 1). The AA and DA probe-negative control strain E62008 did not express an 18 kDa protein (2a, lane 2). AA and DA probe-positive strains of *E. coli* O44:H18 (E44939, lane 3; E40539, lane 4; E45730, lane 5), O33:H16 (E72376, lane 6), O116:H27 (E97474, lane 7) and O110:H- (E101402, lane 8) also expressed an OMP of ≈ 18 kDa. An antiserum prepared to the 18 kDa OMP of *E. coli* O126:H27 contained antibodies which reacted with an 18 kDa OMP expressed by probe-positive strains of *E. coli* belonging to serotype O126:H27 (2b, lane 1). These antibodies did not bind to proteins expressed by EAggEC probe-negative strains (2b, lane 2). Antibodies also bound to an 18 kDa OMP of strains *E. coli* O44:H18 (E44939, lane 3; E40539, lane 4; E45730, lane 5), O33:H16 (E72376, lane 6), O116:H27 (E97474, lane 7) and O110:H- (E101402, lane 8)

ized with both AA and DA gene probes (Fig. 2B. lanes 1, 3–8).

The four non-HEp-2-adherent strains of *E. coli* did not agglutinate rat erythrocytes when grown on nutrient agar. Of the 29 HEp-2 adherent strains of *E. coli* examined, only 14 agglutinated rat erythrocytes following growth on nutrient agar (Table 1). Although six of these strains were EAggEC belonging to serotype O126:H27, one strain of *E. coli* O126:H27 (E75607) that was EAggEC probe positive consistently failed to agglutinate rat erythrocytes. Two strains (E97468, E60725) did not agglutinate rat erythrocytes in the presence of 1% (w/v) D-mannose (Table 1). Twelve strains of EAggEC agglutinated rat erythrocytes following growth in L-broth (Table 1); two strains (E40144 and E81456) did not agglutinate rat erythrocytes in the presence of 1% (w/v) D-mannose (Table 1).

Seven strains of EAggEC failed to agglutinate rat erythrocytes following growth on MacConkey agar (Table 1). *Escherichia coli* strains E60725 (O92:H33) and E40144 (O111:H21) were used to compare OMP profiles following growth on nutrient and MacConkey agars. Expression of OMPs by these two strains was not influenced by the culture medium used. Twelve strains of EAggEC agglutinated rat erythrocytes following growth in L-broth (Table 1) and none of these agglutinated rabbit erythrocytes.

Of the 29 HEp-2 adherent strains of *E. coli*, only 21 strains produced a pellicle following growth in L-broth (Table 1). These bacteria formed a pellicle in the presence of EGTA but not in L-broth containing 1 mmol l^{-1} EDTA.

Of the four nonadherent strains of *E. coli*, only strain E57144B was considered as hydrophobic (Table 1). Of the 29 HEp-2 adherent strains of *E. coli*, only strains belonging to serotypes O77:H18 (E58583 A) and O102:H27 (E71341, E72957) did not clump in any of the ammonium sulphate solutions (Table 1).

The 33 strains of *E. coli* were grown in L-broth alone; 24 were found to carry a net negative charge, while two strains did not migrate towards either electrode. Seven strains of *E. coli* were found to comprise a population of bacteria such that some bacteria carried a positive charge, and some carried a negative charge (Table 1). When the same strains were grown in L-broth containing 1 mmol l^{-1} EDTA, 15 strains remained negatively charged although nine strains lost their charge and failed to move towards either electrode (Table 1). Only two strains, both belonging to serotype O126:H27 (E40104, E83087), became positively charged when growing in the presence of EDTA (Table 1).

DISCUSSION

Strains of *E. coli* which hybridized with both AA and DA gene probes expressed OMPs of approximately 18 kDa with antigenic homology, suggesting a form of clonality within the EAggEC. Only strains of *E. coli* belonging to serotype O126:H27 were agglutinated by the SEFEX latex beads, indicating that the epitopes involved were surface exposed only on these strains.

The ability of certain strains of EAggEC to clump rat erythrocytes was dependent on growth medium and was not common to all EAggEC examined. Only strains E73339 (O15:H-), E97468 (O86:H34) and E60725 (O92:H33) grown on nutrient agar exhibited mannose-sensitive agglutination of rat erythrocytes; however, this was not observed when these same strains were grown in L-broth. Similarly, only strains E40144 (O111:H21) and E81456 (O126:H27) grown in L-broth exhibited mannose-sensitive agglutination of rat erythrocytes; however, this was not observed when these same strains were grown on nutrient agar.

The basis for the haemagglutination reaction was obscured by the observation that for certain strains of *E. coli*, growth on nutrient agar produced bacteria which haemagglutinated rat erythrocytes, whereas growth on MacConkey agar produced non-haemagglutinating organisms. This phenomenon was investigated further using *E. coli* strains E60725 and E40144. Since OMP profiles obtained with bacteria grown on MacConkey and nutrient agars were indistinguishable, it would seem that membrane proteins were not involved in the haemagglutination reaction. Nevertheless, the ability of strains of *E. coli* to haemagglutinate is not specific for EAggEC, as has been suggested (Qadri *et al.* 1994), and cannot therefore form the basis of a test for identifying EAggEC. Similarly, of the 29 HEp-2 adherent strains of *E. coli*, only 21 produced a pellicle following growth in L-broth, a property which has also been advocated as a means of delineating EAggEC from other strains of *E. coli* (Albert *et al.* 1993). It was also observed that not all haemagglutinating strains of *E. coli* produced a pellicle when growing in L-broth and vice versa.

Strains of *E. coli* belonging to serotype O126:H27 (E62008, E55280), O77:H18 (E58583 A and B) and O102:H27 (E71341, E72957) did not agglutinate in any of the ammonium sulphate solutions and were considered hydrophilic. These bacteria also failed to haemagglutinate rat erythrocytes following growth on nutrient agar or in L-broth. Although these results suggest that hydrophobicity may play a role in haemagglutination, this theory was flawed by the observation that certain non-haemagglutinating strains clumped in ammonium sulphate solutions.

Bacterial charge did not correlate with the ability of bacteria to haemagglutinate, to form a pellicle, or with hydrophobicity. However, for certain strains of *E. coli*, growth in the presence of EDTA caused surface charge to change.

From the results of this study it was concluded that currently, the possession of EAggEC genes, or the ability to adhere to HEp-2 cells in a stacked brick formation, remain the only reliable means of identifying EAggEC.

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Purification and characterization of a feruloyl esterase from the fungus *Penicillium expansum*

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J. DONAGHY AND A.M. MCKAY. 1997. An extracellular phenolic acid esterase produced by the fungus *Penicillium expansum* in solid state culture released ferulic and *p*-coumaric acid from methyl esters of the acids, and from the phenolic-carbohydrate esters O-[5-O-(trans-feruloyl)-α-L-arabinofuranosyl]-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose (FAXX) and O-[5-O-((E)-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose (PAXX). The esterase was purified 360-fold in successive steps involving ultrafiltration and column chromatography by gel filtration, anion exchange and hydrophobic interaction. These chromatographic methods separated the phenolic acid esterase from α-L-arabinofuranosidase, pectate and pectin lyase, polygalacturonase, xylanase and β-D-xylosidase activities. The phenolic acid esterase had an apparent mass of 65 kDa under non-denaturing conditions and a mass of 57·5 kDa under denaturing conditions. Optimal pH and temperature were 5·6 and 37 °C, respectively and the metal ions Cu^{2+} and Fe^{3+} at concentrations of 5 mmol l^{-1} inhibited feruloyl esterase activity by 95% and 44%, respectively, at the optimum pH and temperature. The apparent K_m and V_{max} of the purified feruloyl esterase for methyl ferulate at pH 5·6 and 37 °C were 2·6 mmol l^{-1} and 27·1 μmol min^{-1} mg^{-1}. The corresponding constants of *p*-coumaroyl esterase for methyl coumarate were 2·9 mmol l^{-1} and 18·6 μmol min^{-1} mg^{-1}.

INTRODUCTION

Plant cell walls contain many structural polysaccharides, for example, pectins, xylans, cellulose and lignin. Complete hydrolysis of plant cell wall material requires the participation of several enzymes with different functions. In addition to enzymes capable of hydrolysing intact polymers, complete digestion of plant material also requires enzymes capable of cleaving bonds which cross-link these polymers. The bifunctional nature of the hydroxycinnamic acids, ferulic and *p*-coumaric acids make them ideal candidates as cross-linking units. Ester cross-linkages involving feruloyl groups have been reported for sugar beet pectins (Rombouts and Thibault 1986; Ralet *et al.* 1994), grass lignins (Smith and Hartley 1983) and spinach oligosaccharides (Iiyama *et al.* 1990). Additionally, the esterification of arabinoxylans of wheat internodes involves both feruloyl and *p*-coumaroyl residues (Ishii and Tobita 1993). Therefore, these phenolic acids have an important role in the structural integrity of the plant cell wall matrix and represent factors which partially limit the biodegradability of non-lignified plant cell wall polysaccharides (Akin *et al.* 1990; Hartley *et al.* 1992). Consequently, enzymes capable of cleaving the phenolic acid-ester cross-linkages may find applications in the food industry. They may enable enhanced extraction of plant material and the textural modification of food products, for example, in the baking and other food processing industries. Furthermore, animal nutrition may benefit from the use of phenolic acid esterases, whereby enzymes supplied with animal feedstuffs could supplement the endogenous digestive activities of the animal, resulting in a more efficient utilization of low-grade feedstuffs.

Feruloyl and *p*-coumaroyl esterase activities resulting in the cleavage of ester cross-linkages were first described by MacKenzie *et al.* (1987). Subsequently, phenolic acid-releas-

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Magnesium ions are required for HEp-2 cell adhesion by enteroaggregative strains of *Escherichia coli* O126:H27 and O44:H18

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Abstract

Enteroaggregative strains of *Escherichia coli*, belonging to serotypes O44:H18 and O126:H27, were used to show that magnesium ions were essential for the adhesion of these enteroaggregative strains to HEp-2 cells. The removal of Mg^{2+} ions from culture media was correlated with the inability of strains to produce an outer membrane-associated protein of 18 kDa and a pellicle. It was concluded that magnesium ions were directly involved with the expression of an 18 kDa outer membrane-associated protein by strains of *E. coli* O126:H27 and O44:H18, and that the outer membrane-associated protein was involved in both HEp-2 adhesion and pellicle formation.

Keywords: *Escherichia coli*; Enteroaggregative strain; Magnesium; O126:H27; O44:H18; Outer membrane protein

1. Introduction

Strains of *Escherichia coli* can be categorised based on the patterns they produce when adhering to eukaryotic cells in vitro. For example, strains of *E. coli* have been described as exhibiting 'diffuse' adherence when bacteria attach evenly over the surface of HEp-2 or HeLa cells [1], 'localised' adherence when bacteria attach in discrete clusters on of HEp-2 or HeLa cells [2], or have been termed enteroaggregative when bacteria adhere to HEp-2 cells in a 'stacked brick' pattern. Gene probes have been made to detect strains of *E. coli* which exhibit DA or enteroaggregative phenotypes [3,4].

Strains of *E. coli* belonging to serotypes O126:H27 and O44:H18, have been shown to hybridize with gene probes for both enteroaggregative and diffuse adhesion phenotypes [5]. These strains express a surface-exposed membrane-associated protein with a subunit molecular mass of 18 kDa [6]. The role of this protein has not been elucidated fully; however, strains of these serotypes which do not express this protein are unable to adhere to Hep-2 cells. Also, strains which express an 18 kDa outer membrane-associated protein form a pellicle when growing in L-broth, whereas bacteria which do not express this protein do not form a pellicle.

Recently, it was shown that strains of *E. coli* O126:H27 and O44:H18 did not produce a pellicle when growing statically in L-broth containing 1 mM EDTA·Na_2. In the present study, strains of *E. coli*

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were used to determine the role of EDTA in pellicle formation, in the expression of an 18 kDa outer membrane-associated proteins and in the ability of strains to adhere to HEp-2 cells in vitro.

2. Materials and methods

2.1. Bacterial strains

The six strains of *E. coli* used for the present study belonged to serotypes O44:H18 (E43509, E44939, E45730), and O126:H27 (E40104, E75508, E83087). All strains hybridized with DNA probes for aggregative and diffuse adhesion phenotypes, and were from the culture collection held by the Laboratory of Enteric Pathogens.

2.2. Bacterial culture

Bacteria grown on nutrient agar (16 h, 37°C) were used to inoculate 10 ml volumes of nutrient broth (8 h, 37°C). These starter cultures (10 μl) were used to inoculate 100 ml volumes of L-broth alone or containing 0.25–1.0 mM EDTA·Na_2 (Merck Ltd., Poole, Dorset, UK), 1 mM EGTA (Sigma Chemical Co.) or 10 mg/ml ethylenediaminedihydroxy-*o*-phenylacetic acid (EDDA, (Sigma Chemical Co.). Strains were grown in EDDA-L-broth, with and without 1 mM $FeCl_3$ (Merck Ltd.). Bacteria were grown at 37°C for 16 h with shaking (120 rpm). For determination of pellicle formation strains were grown statically in L-broth, and in L-broth containing either 0.25–1 mM EDTA·Na_2 or 1 mM EGTA (37°C, 16 h).

2.3. HEp-2 adhesion tests

The ability of strains of *E. coli* to adhere to HEp-2 cells was determined as described previously [7].

2.4. Outer membranes/SDS-PAGE

Bacteria were harvested from broth cultures by centrifugation (10 000×*g*, 15 min, 4°C), washed once in ice-cold 25 mM Tris-HCl pH 7.4 and the resultant cell pellets used to prepare outer membranes. Outer membranes were prepared from sonicated bacteria by the selective solubilization of inner membranes [8] with sarkosyl (Merck Ltd.). SDS-PAGE was performed using the method of Laemmli [9] as described [10]. Samples (30 μg per lane) of outer membrane proteins were loaded onto gels comprising a 4.5% acrylamide stacking gel and a 15% acrylamide separation gel. Following electrophoresis, gels were stained with Coomassie brilliant blue [10], or used for immunoblotting.

2.5. Immunoblotting

Outer membrane proteins separated by SDS-PAGE were transferred onto nitrocelluose membrane [11] and reacted with a rabbit antiserum containing antibodies to the 18 kDa outer membrane-associated proteins of *E. coli* O126:H27 and O44:H18 as described previously [8].

3. Results

Strains of *E. coli* growing in L-broth alone or in L-broth containing EGTA produced a pellicle and adhered to HEp-2 cells in a 'stacked-brick' formation. However, the six strains of *E. coli* did not adhere to HEp-2 cells or produce a pellicle when grown in L-broth containing 0.25–1 mM EDTA·Na_2. The addition of 1 mM $MgCl_2$ to L-broth containing 1 mM EDTA·Na_2 restored the ability of strains to produce a pellicle and to adhere to HEp-2 cells.

E. coli strain E40104 (O126:H27), growing in L-broth alone, expressed an outer membrane protein of 18 kDa (Fig. 1A, lane 1) which reacted with rabbit antibodies specific for this protein (Fig. 1B, lane 1). The same results were obtained with *E. coli* strains E75508 and E83087 (O126:H27), and E43509, E44939 and E45730 (O44:H18). In contrast, *E. coli* strain E40104, growing in L-broth containing 1 mM EDTA·Na_2, did not express an 18 kDa outer membrane protein (Fig. 1A, lane 2), and an 18 kDa outer membrane protein could not be detected by immunoblotting (Fig. 1B, lane 2). The same negative results were obtained with *E. coli* strains E75508 and E83087 (O126:H27), and E43509, E44939 and E45730 (O44:H18). Using L-broth containing a range of concentrations of EDTA·Na_2, it was shown that expression of an 18 kDa outer membrane pro-

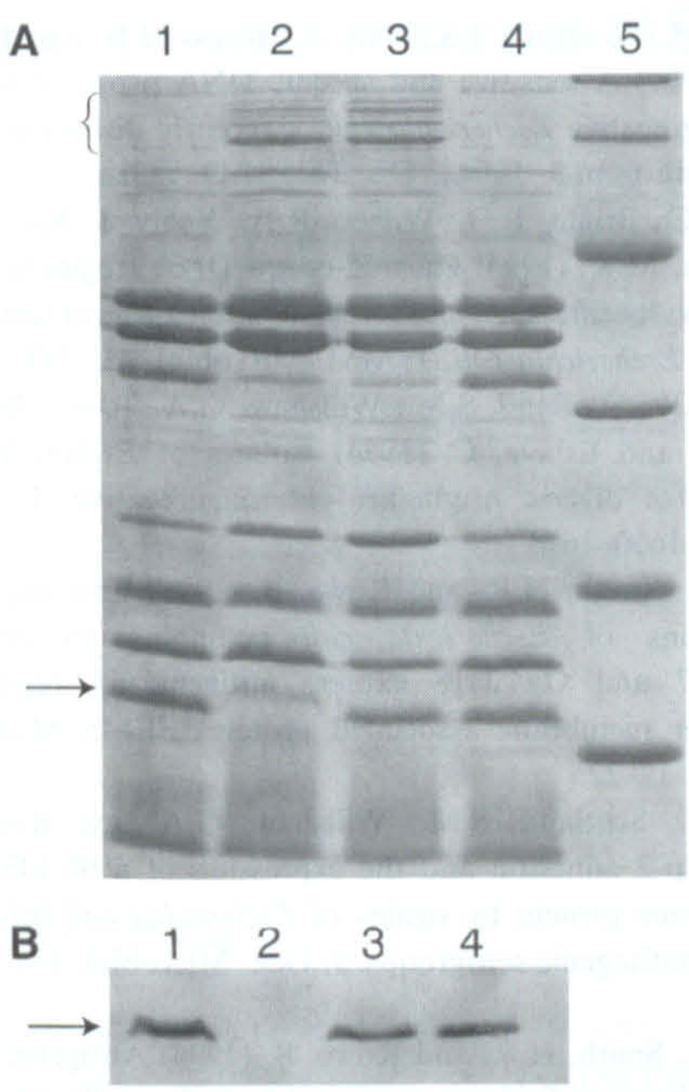

Fig. 1. *E. coli* strain E40104 (O126:H27) growing in L-broth alone expressed an 18 kDa outer membrane protein (arrowed, A: lane 1), which reacted with rabbit antibodies specific for this protein (arrowed B: lane 1). When grown in L-broth containing 1 mM EDTA·Na_2, this strain did not express an 18 kDa OMP (A and B: lanes 2), but expressed high molecular mass outer membrane proteins (bracketed A: lane 2). High molecular mass outer membrane proteins were also expressed when strain E40104 was grown under iron restriction (A: lane 3). Iron restriction did not affect the expression of the 18 kDa outer membrane protein (A and B:lanes 3). The 18 kDa outer membrane protein was expressed by E40104 growing in L-broth containing EDTA plus added $MgCl_2$ (A: lane 4), whereas an 18 kDa outer membrane protein was detected (A and B, lanes 4). Protein molecular mass standards are shown in lane 5.

tein was suppressed by concentrations of EDTA·Na_2 as low as 0.25 mM.

E. coli strain E40104, growing in L-broth containing 1 mM EDTA·Na_2, expressed high molecular mass outer membrane proteins in the range of 80–90 kDa (Fig. 1A, lane 2), which were not produced by this strain when growing in L-broth alone (Fig. 1A. lane 1). This was also observed with *E. coli* strains E75508 and E83087 (O126:H27), and E43509, E44939 and E45730 (O44:H18). *E. coli* strain E40104 was used to examine these high molecular mass proteins further.

Since the molecular masses of the high molecular mass outer membrane proteins resembled those of the iron-regulated proteins expressed by strains of *E. coli*, strain E40104 was grown in L-broth containing EDDA (100 μg/ml) to induce the expression of iron-regulated outer membrane proteins. This strain was also grown in L-broth containing EDDA and 1 mM $FeCl_3$ to confirm the role of iron in protein expression. Bacteria growing under iron restriction expressed high molecular mass outer membrane proteins which co-migrated with the proteins observed in bacteria growing in the presence of EDTA (Fig. 1A, lane 3). Under iron restriction, strains E40104 expressed an 18 kDa outer membrane-associated protein which reacted with rabbit antibodies specific for this protein (Fig. 1B, lane 3).

When strains of *E. coli* were grown in L-broth-EDTA containing $MgCl_2$, an 18 kDa outer membrane protein was expressed (Fig. 1A, lane 4) which reacted with rabbit antibodies specific for this protein (Fig. 1B, lane 4).

4. Discussion

The observation that strains of *E. coli* belonging to serotypes O44:H18 and O126:H27 did not adhere to HEp-2 cells following growth in L-broth containing EDTA·Na_2 suggested that this chelator was binding an ion essential for the adhesion process. Adding $MgCl_2$ to L-broth containing EDTA showed that Mg^{2+} was the ion concerned. Magnesium ions also appeared to be involved in the expression of an 18 kDa outer membrane protein and the formation of a pellicle during growth in L-broth, suggesting that the 18 kDa outer membrane protein was involved with HEp-2 adhesion and pellicle production. This was supported by the fact that strains of *E. coli* O126:H27 which do not hybridize with the enteroaggregative *E. coli* gene probe, do not express an 18 kDa outer membrane protein and are unable to adhere to HEp-2 cells [6].

Strains of *E. coli* growing in L-broth containing 1 mM EDTA·Na_2 expressed high molecular mass outer membrane proteins which were very similar in size and composition to the iron-regulated outer membrane proteins. The chelator EDTA·Na_2 has been shown to bind ferric ions [12], and it was concluded that 1 mM EDTA·Na_2 in L-broth was sufficient to deprive strains of *E. coli* of both Fe^{2+} and

Mg^{2+} ions. Strains of *E. coli* growing in L-broth with EDDA made an 18 kDa outer membrane protein showing that the expression of this protein was not affected by the availability of iron. Growing strains of *E. coli* in L-broth containing EDTA·Na_2 with added Mg^{2+} ions produced an 18 kDa outer membrane protein but they did not express iron-regulated outer membrane proteins. This was thought to be due to the excess of Mg^{2+} saturating available iron binding sites on the EDTA molecule.

The role of Mg^{2+} in the expression of the 18 kDa outer membrane protein is unclear. Divalent cations are known to be important for the stabilisation of the gram-negative outer membrane, and it is known that EDTA can cause strains of *E. coli* to release lipopolysaccharide [13]; however, whether Mg^{2+} ions are involved in the regulation of the expression of 18 kDa membrane-associated proteins, or interfere directly with the surface assembly of these proteins is not known.

From the results of this study it was concluded that magnesium ions were involved with the expression of an 18 kDa outer membrane-associated protein by strains of *E. coli* O126:H27 and O44:H18, and that the outer membrane-associated protein was involved in both HEp-2 adhesion and pellicle formation.

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Improved detection of enteroaggregative *Escherichia coli* using formalin-fixed HEp-2 cells

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J. SPENCER, H. CHART, H.R. SMITH AND B. ROWE. 1997. Certain strains of enteroaggregative *Escherichia coli* cause detachment of HEp-2 cells during adhesion tests, preventing observation of the aggregative adherent phenotype. The use of formalin-fixed HEp-2 cells prevents cell detachment and facilitates the detection of enteroaggregative *E. coli.*

INTRODUCTION

Enteroaggregative strains of *Escherichia coli* (EAggEC) are an important cause of persistent infantile diarrhoea in countries such as Chile (Nataro *et al.* 1987), Bangladesh (Baqui *et al.* 1992) and Mexico (Cravioto *et al.* 1991; Girón *et al.* 1991). EAggEC adhere to HEp-2 cell monolayers in an aggregative or 'stacked brick' formation, a characteristic that forms the basis for the principal method of detecting these strains of *E. coli.* EAggEC can also be detected with gene probes (Baudry *et al.* 1990; Smith *et al.* 1994). Certain strains of EAggEC cause HEp-2 cell monolayers to detach during adhesion assays, preventing the reliable observation of the aggregative phenotype (Gunzburg *et al.* 1990; Elliott and Nataro 1995). This study describes an improved procedure for detecting EAggEC, using formalin-fixed HEp-2 cells.

MATERIAL AND METHODS

Bacteria

Four strains of *E. coli* belonging to serotypes O77:H18 (E58583 A), O86:H19 (47697 A), O111:H21 (57144 A) and O126:H27 (E40104) were designated as enteroaggregative based on their ability to adhere to HEp-2 cells with a 'stacked brick' pattern and to hybridize with a DNA probe for EAggEC (Smith *et al.* 1994). Bacteria were stored on Dorset's egg agar slopes in the culture collection held by the Laboratory of Enteric Pathogens.

Preparation of HEp-2 cells and bacteria

HEp-2 cells were maintained in basal medium Eagle (BME) tissue culture medium (Sigma Chemical Co. Ltd, Poole, UK) and grown on glass coverslips based on the method of Cravioto *et al.* (1979). Glass coverslips were placed into 12-well tissue culture plates (Costar, Cambridge, MA) and seeded with HEp-2 cells (37 °C, 48 h, 5% CO_2). Conventionally, cell monolayers were washed (×3) with Earle's balanced salt solution (EBSS; Flow Laboratories, McLean, VA) and overlayered with fresh BME medium, prior to inoculation with a test organism. The modified method involved washing cell monolayers (×3) with EBSS followed by fixation of cells with phosphate-buffered saline (PBS) containing formalin (1% v/v, 30 min, room temperature). Cell monolayers were then washed (×3) with sterile PBS and overlaid with fresh BME medium.

Bacteria were grown statically in peptone water (37 °C, 16 h), and 50 μl of bacterial suspension used for each cell test. Following incubation (37 °C, 3 h), cell monolayers were washed (×3) with PBS, and fresh BME added for a second 3 h incubation (37 °C). Cells were washed (×3) with PBS, fixed with absolute methanol (10 min) and stained (30 min) with Giemsa stain (Merck Ltd, UK). Glass slides were washed (×3) and dehydrated using an acetone/xylene gradient, and mounted onto glass microscope slides with DePeX (Merck Ltd). Bacterial adhesion patterns were observed under oil immersion.

Adhesion tests were also performed with formalin-fixed HEp-2 cells using the initial 3 h incubation period only, and with fixed HEp-2 stored at 4 °C for up to 1 month.

RESULTS

All four strains of EAggEC caused HEp-2 cells to detach during the conventional adhesion assay; an example is showed in Fig. 1a. However, these strains adhered to the fixed cells with an aggregative phenotype without causing HEp-2 cell

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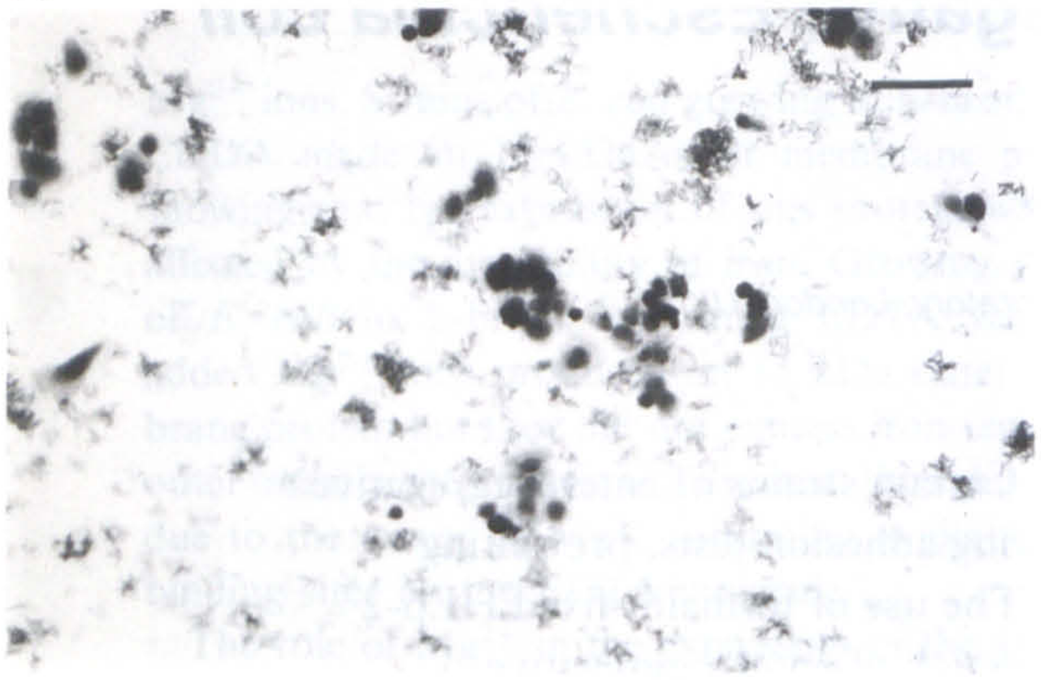
(a)



(b)

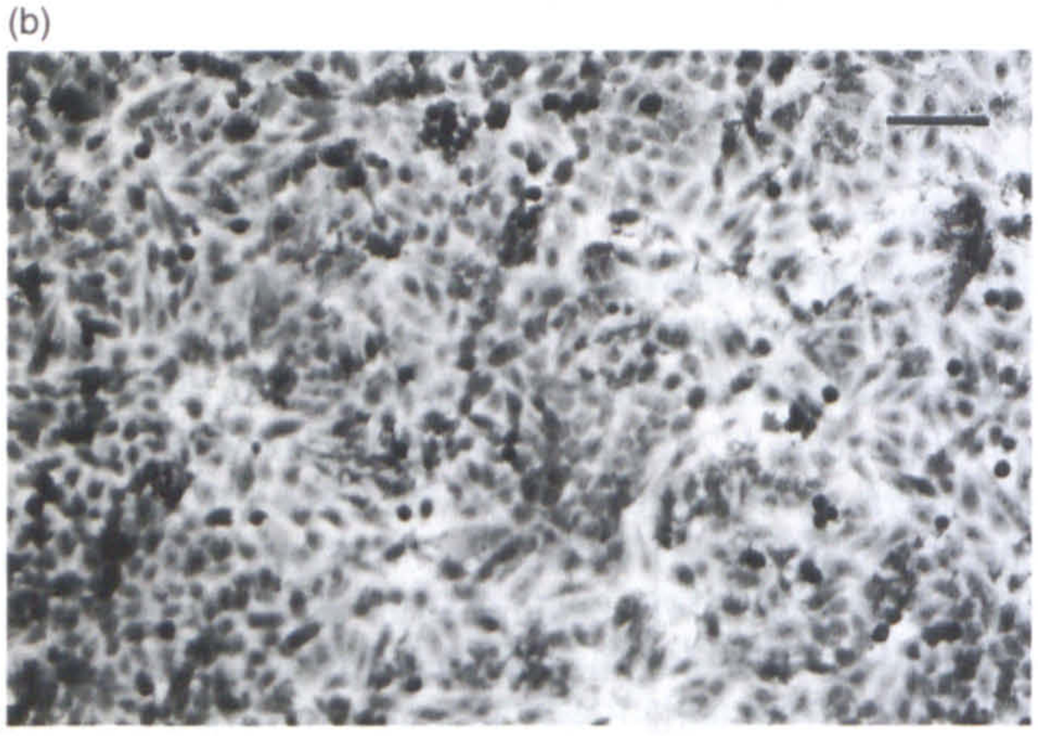


Fig. 1 (a) Dislodged HEp-2 cells of enteroaggregative strains of *Escherichia coli* during adhesion assays. (b) With formalin-fixed HEp-2 cells, the adhesion phenotype was readily observed (bar = 100 μm)

damage (Fig. 1b). Formalin-fixed HEp-2 cell monolayers, stored at 4 °C in PBS, could be used for adhesion assays for up to 3 weeks after preparation. Tests done after this time showed that the HEp-2 cells were slipping off the glass coverslip and did not stain as well as freshly formalin-fixed cells, and so gave poorer results.

DISCUSSION

Strains of EAggEC may cause HEp-2 cell detachment (Nataro *et al.* 1995). However, fixing HEp-2 cell monolayers with formalin prevented cell detachment and improved the observation and detection of *E. coli* expressing the EAggEC phenotype. The conventional HEp-2 test, using viable cells, involves harvesting HEp-2 cells from stock tissue culture flasks and the preparation of cell tests 2 d prior to use. This permits the HEp-2 adhesion assay to be performed on only a limited basis during a standard working week. In contrast, formalin-fixed monolayers can be made in bulk and used on 4 out of 5 working d, allowing for overnight culture of test strains.

The observation that EAggEC adhered to formalin-fixed HEp-2 cells demonstrated that EAggEC attach without involving the morphological changes made by HEp-2 cells. This demonstrates that the EAggEC adhesion process contrasts with that, for example, of enteropathogenic *E. coli* (EPEC), where adhesion to HEp-2 cells involves changes in the arrangement of actin filaments within HEp-2 cells (Baldwin *et al.* 1991). The HEp-2 adhesion method described here not only facilitates the detection of enteroaggregative *E. coli*, but may also provide a better understanding of the mechanisms involved in bacterial attachment to eukaryotic cells.

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Expression of membrane-associated proteins by strains of enteroaggregative *Escherichia coli*

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Abstract

Certain strains of enteroaggregative *Escherichia coli* express an outer membrane-associated protein, involved with the adhesion of these bacteria to HEp-2 cells. Strains of enteroaggregative *E. coli* hybridising with DNA probes for aggregative adhesion, diffuse adhesion and aggregative adhesion fimbriae II expressed an outer membrane-associated protein of 18 kDa regulated by magnesium ions. Strains hybridising with the aggregative adhesion probe only expressed a 20-kDa outer membrane-associated protein regulated by calcium and magnesium. The present study describes two populations of enteroaggregative *E. coli* which appear to adhere to HEp-2 cells by expressing antigenically distinct, negatively charged membrane-associated proteins. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: *Escherichia coli*; Enteroaggregative; Membrane-associated outer membrane protein; DNA probe

1. Introduction

Strains of enteroaggregative *Escherichia coli* (EAggEC) belong to a group of *E. coli* containing more than 50 different 'O' serogroups [1–4] and share the ability to adhere to HEp-2 cells in a 'stacked brick' or aggregative adhesion (AA) pattern [5]. Certain EAggEC express fimbriae such as aggregative adherence fimbriae I (AAF/I) or AAF/II [6] but this is rare, and adhesion of EAggEC to HEp-2 generally occurs in the absence of a fimbrial structure [7]. Strains of *E. coli* (EAggEC) which adhere to HEp-2 cells with an aggregative pattern and belong to serotypes O44:H18 or 0126:H27 express cell surface-exposed outer membrane-associated proteins (MAP) which are involved in aggregative adhesion to HEp-2 cells [7]. These MAPs have a subunit molecular mass of 18 kDa and are antigenically similar, but not structurally identical [7]. Recently, it was shown that chelating magnesium ions in culture media repressed the expression of an 18-kDa MAP and prevented these bacteria from adhering to HEp-2 cells [8]. Removing magnesium ions from culture media also inhibited the formation of a pellicle by *E. coli* O44:H18 and O126:H27 [8], suggesting that the EAggEC adhesion process may involve bacterial surface charge, a hypothesis supported by the fact that EAggEC adhere to formalin-fixed HEp-2 cells [9].

In addition to the HEp-2 cell adhesion test, strains of EAggEC can be detected using a gene probe termed aggregative adhesion (or AA) probe [10]. It

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was recently demonstrated that strains of EAggEC which expressed an 18-kDa MAP also hybridised with a probe used to detect *E. coli* which adhered to HEp-2 cells with a diffuse adhesion (DA) phenotype [5]. Furthermore, it was shown that only strains which hybridised with both the AA and DA probes expressed a MAP [10].

The extent to which EAggEC utilise MAPs for attaching to eukaryotic cells is unknown, but the possibility arises that strains other than those belonging to serotypes O44:H18 and O126:H27 may also express membrane-associated proteins. In the present study we examined strains of *E. coli*, belonging to a range of serotypes, for the ability to hybridise with AA, DA or AAF/II probes and to determine whether other EAggEC may also express MAPs.

2. Materials and methods

2.1. Bacteria and culture media

Twenty-one strains of *E. coli* were used for the present study (Table 1). Strains E36182:17/4 and E36182:13/1 were isogenic variants of *E. coli* strain E36182 (O111:H21), and strain E57144B (O111:H21) was an isogenic variant of *E. coli* strain E57144A. Strains were grown in L broth (37°C, 16 h) alone or containing 1.0 mM ethylene diamine tetra-acetic acid disodium salt (EDTA-Na_2; Merck Ltd., Poole, Dorset, UK) supplemented with either 10 mM $CaCl_2$ or 10 mM $MgCl_2$. For antibody adsorptions, bacteria were grown on nutrient agar (Oxoid Ltd.). All bacteria were stored on Dorset's egg slopes at room temperature.

2.2. HEp-2 adhesion tests

The ability of strains of *E. coli* to adhere to HEp-2 cells was determined as described previously [7].

2.3. DNA probe tests

DNA hybridisation tests were performed using gene probes for enteroaggregative adhesion (AA), diffuse adhesion (DA) or aggregative adherence fimbriae type II (AAF/II) [5,10–12].

2.4. Transposon mutagenesis

Transposon mutagenesis was performed using Tn*pho*A insertions as described previously [13], and non-adherent mutant strains were detected with an established HEp-2 cell test.

2.5. Outer membranes/SDS-PAGE

Outer membranes were prepared from sonicated bacteria by the selective solubilisation of inner membranes with Sarkosyl (Merck Ltd., Poole, Dorset, UK) [14]. Outer membrane proteins were examined by SDS-PAGE using the method of Laemmli [15]. Samples of outer membrane proteins (30 μg per lane) were loaded onto polyacrylamide gels comprising a 4.5% stacking gel and a 15% acrylamide separation gel. Following electrophoresis, proteins were stained with Coomassie blue or used for immunoblotting.

2.6. Immunoblotting

SDS-PAGE protein profiles were transferred onto nitrocellulose membranes (0.5 A, 1.5 h) using the method by Towbin et al. [16]. After blocking membranes with skimmed milk (3% dried milk powder in phosphate-buffered saline, PBS) membranes were reacted with rabbit antisera for 2 h. Following washing (×3) with PBS containing 0.1% (v/v) Tween-20 (PBS-Tween), antibody-antigen complexes were detected using a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma Chemical Co. Ltd.), 10 μl per lane in 10 ml skimmed milk-PBS for 2 h. Bound antibodies were detected using an enzyme substrate buffer comprising of 20 ml 0.1 M Tris, 0.09 M NaCl and 0.15 M $MgCl_2 \cdot 6H_2O$ containing 90 μl nitroblue tetrazolium solution (Sigma 75 mg ml^{-1} in 70% aqueous dimethyl formamide) and 70 μl of 5-bromo-4-chloro-3-indolylphosphate solution (Sigma, 50 mg ml^{-1} of deionised water) [17].

2.7. Haemagglutination

Agar-grown bacteria were suspended in PBS to a concentration of 1 mg cells ml^{-1}, and 100-μl volumes placed in wells on a ceramic tile and mixed with an equal amount of heparinised rat erythrocytes, diluted

×10 in PBS. Agglutination was assessed following mixing on a gyrating shaker for 1 min.

2.8. Rabbit antisera

Antisera were prepared, in New Zealand white rabbits, to *E. coli* strains E57144A (0111:H21, AA-probe-positive) and E57157A (0126:H27, AA-probe-positive). Each rabbit received 10^9 heat-killed bacteria, followed by a booster injection 3 weeks later. Rabbits were exsanguinated 10 days later and sera prepared. The serum prepared to *E. coli* strain E57144A was absorbed with *E. coli* strain E57144B (O111:H21, AA-probe-negative), and the serum prepared to *E. coli* E57157A was adsorbed with *E. coli* strain E57157B (0126:H27, AA-probe-negative). For adsorptions, bacteria harvested from 15×15 cm nutrient agar plates were mixed with 10-ml volumes of rabbit antiserum (2 h, 50°C) and bacteria removed by centrifugation (5000×*g*, 10 min).

2.9. Electron microscopy

For the examination of surface structures, bacteria were placed on formvar-coated grids and stained with 1% aqueous ammonium molybdate prior to examination by transmission electron microscopy [7]. Immunoelectrophoresis was performed as described previously [7].

3. Results

Seventeen of the 20 strains of *E. coli* adhered to HEp-2 cells with an aggregative pattern (Table 1). Ten of the HEp-2 adhering strains hybridised with

Table 1
Strains of *E. coli* used in this study

Serotype	Strain no. (E)	HEp-2 adhesion[a]	AA[b]	DA[c]	AAF/II[d]
Strains expressing 20-kDa MAP[e]					
O111:H21	36182	+[f]	+	–	–
	57144A	+[f]	+	–	–
	40144	+[f]	+	–	–
	33915	+[f]	–	–	–
Strains expressing 18-kDa MAP					
O44:H18	44939	+	+	+	+
O110:H-	101402	+[f]	+	+	+
O116:H27	97474	+[f]	+	+	+
O119:H27	107100	+[f]	+	+	+
O126:H27	40104	+[f]	+	+	+
O?:H-	101089	+[f]	+	+	+
O?:H3	99967	+[f]	+	+	+
O?:H27	105836	+[f]	+	+	+
O?:H27	107542	+[f]	+	+	+
	97298	+[f]	+	+	+
Strains which do not express a MAP					
O44:H18	89102	+[f]	+	–	–
	89109	+[f]	+	–	–
O111:H21	89114	+[f]	+	–	–
	36182:17/4	–[f]	+	–	–
	36182:13/1	–[f]	+	–	–
	57144B	–[f]	–	–	–

[a]Adheres to HEp-2 cells with an aggregative pattern.
[b]Reacts with the aggregative adhesion gene probe.
[c]Reacts with the diffuse adhesion gene probe.
[d]Reacts with the aggregative adhesion fimbriae/II gene probe.
[e]Membrane-associated protein.
[f]Haemagglutinates sheep erythrocytes.
+/–, indicates whether or not strains adhered to HEp-2 cells, or hybridised with the AA, DA or AAF/II gene probes.

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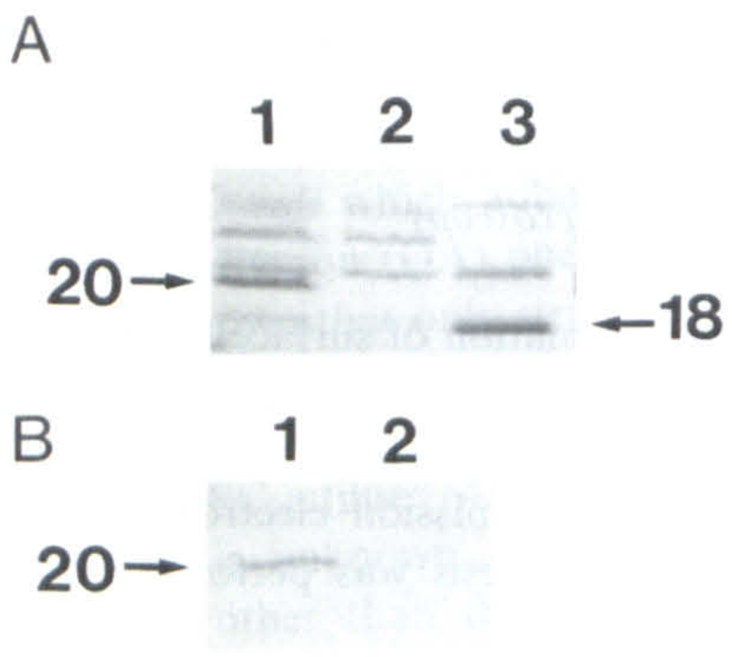

Fig. 1. Expression of MAPs by EAggEC. Three strains of *E. coli* belonging to serotype O111:H21 expressed a MAP of 20 kDa (A, lane 1), isogenic mutants of *E. coli* O111:H21 strain E36182 hybridised with the AA probe but did not express a 20-kDa MAP (A, lane 2). Ten strains of *E. coli* hybridised with the AA, DA and AAF/II probes and expressed a MAP of 18 kDa (A, lane 3). Rabbit antibodies prepared to HEp-2-adhesive strains of *E. coli* O111:H21 bound to the 20-kDa proteins (B, lane 1), but not the non-HEp-2-adhesive variants of *E. coli* O111:H21 (B, lane 2).

the AA, DA and AAF/II DNA probes. HEp-2 adhering strains belonging to serotype O111:H21 and three strains belonging to serotype O44:H18 hybridised with the AA probe only (Table 1). Transposon mutagenesis of *E. coli* strain E36182 (O111:H21) produced two isogenic variants, termed E36182 13/1 and E36182 17/4, which did not adhere to HEp-2 cells but hybridised with the AA gene probe (Table 1). *E. coli* O111:H21 strain E57144B did not hybridise with the AA gene probe or adhere to HEp-2 cells (Table 1). With the exception of strains E44939 (O44:H18) and E57144B (O111:H21) all strains agglutinated rat erythrocytes (Table 1).

The three strains of *E. coli* belonging to serotype O111:H21 and hybridising with the AA probe expressed a MAP of 20 kDa (Fig. 1A, lane 1), but the non-adhering mutants of *E. coli* O111:H21 strain E36182 hybridised with the AA probe without expressing a 20-kDa MAP (Fig, 1A, lane 2). The 11 strains of *E. coli* which hybridised with the AA, DA and AAF/II probes expressed a MAP of 18 kDa (Fig. 1A, lane 3). Three strains of EAggEC belonging to serotype O44:H18 did not express an MAP of either 18 or 20 kDa (Table 1).

Membrane-associated proteins of 20 kDa, expressed by HEp-2-adhesive strains of *E. coli* O111:H21, reacted with rabbit antibodies prepared against *E. coli* O111:H21 strain E57144A (Fig. 1B, lane 1). This antibody binding was not detected with O111:H21 strain E57144B (Fig. 1B, lane 2). Antibodies binding to the MAPs of *E. coli* O111:H21 did not bind to the 18-kDa membrane-associated protein expressed by strains of *E. coli* O126:H27 or O44:H18.

Strains of *E. coli* belonging to serotype O111:H21 were examined for the ability to express a pellicle when growing in L broth containing EDTA. In L broth alone all these strains produced a pellicle but none of these strains produced a pellicle when growing in the presence of 1 mM EDTA. Growth of strains in L broth containing EDTA showed that 1 mM EDTA also repressed the expression of a 20-kDa MAP. Pellicle formation and 20-kDa protein expression occurred when L broth EDTA was supplemented with either 10 mM $CaCl_2$ or 10 mM $MgCl_2$.

Strains of *E. coli* were also examined for surface structures by electron microscopy; fimbriae were not observed on any of the strains tested. Immunoelectron microscopy was used to observe the ability of rabbit antibodies, produced to *E. coli* E57144A (0111:H21, AA-probe-positive), to bind to whole bacteria prepared from strain E57144A. Antibodies known to bind to the 20-kDa protein of strain E57144A by immunoblotting did not bind to the cell surface of intact bacteria.

4. Discussion

Strains of *E. coli* which hybridised with all three gene probes expressed a MAP of 18 kDa, and included strains belonging to serotypes O110:H-, O116:H27, O119:H27 and four strains which were O-untypable. This demonstrated that strains of EAggEC which express an 18-kDa MAP were not restricted to serotypes O44:H18 and O126:H27 [1,2]. Although these bacteria also hybridised with the AAF/II probe, neither AAF/I nor AAF/II fimbriae were observed by electron microscopy as has been reported by Nataro et al. [6].

Three strains of *E. coli* belonging to serotype 0111:H21 adhered to HEp-2 cells, hybridised with the AA gene probe only, and expressed a MAP of 20 kDa. The isogenic variants of strain E36182

(E36182:17/4 and E36182:13/1) hybridised with the AA gene probe but did not express a 20-kDa protein or adhere to HEp-2 cells. Since the variant strains had been constructed using transposon mutagenesis, it was thought that transposon had inserted into a DNA sequence involved with the expression of the 20-kDa protein.

E. coli strain E36182 was used to prepare mutant strains which did not adhere to HEp-2 cells. The fact that these strains agglutinated rat erythrocytes demonstrated that the 20-kDa MAP was not involved in the haemagglutination process. All strains expressing a MAP were shown to haemagglutinate rat erythrocytes; however, the role of the MAPs in the physical clumping of rat red cells was not established.

Strains of EAggEC which express an 18-kDa MAP require magnesium ions, but not calcium, for the expression of this protein and for HEp-2 adhesion [8]. In contrast, HEp-2-adhesive strains with serotype O111:H21 required either calcium or magnesium ions for the expression of a 20-kDa MAP. The inhibition by EDTA of pellicle formation by strains producing either an 18- or a 20-kDa MAP suggested that bacterial cell charge may play a role in both pellicle formation and the HEp-2 adhesion process. This would be supported by the observation that EAggEC generally carry a negative charge [10] and adhere to formalin-fixed HEp-2 cells [9]. The possibility arises that bacterial charge may be used by EAggEC to make contact with eukaryotic cells prior to a subsequent attachment process, a strategy demonstrated in strains of enteropathogenic *E. coli* where initial attachment leads to both biochemical and morphological changes in the eukaryotic cell [18].

Strains of EAggEC have been shown to belong to a diverse range of serotypes [1–4], and so far the only common characteristic is the ability to adhere to HEp-2 cells in an aggregative pattern. The present study describes a sub-population of EAggEC which appear to adhere to HEp-2 cells by expressing a negatively charged membrane associated protein.

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