Cloning of an Escherichia coli adhesin

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The work presented in this thesis concerns an important aspect of the interaction between pathogens and their hosts, namely bacterial adherence to specific animal tissues. In the introduction, I present an overview of infectious diseases and the host and microbial factors involved, and conclude the chapter with details of previous findings on the strains used in the present study.

1.1 INFECTIOUS DISEASE

Virtually any exposed surfaces can be colonised by microorganisms, according to a dynamic combination of the physical chemistry of those surfaces with the characteristics and growth requirements of the microbes. Colonisation of living surfaces further involves biological interactions between microbes and 'host' organisms, and although animals are constantly exposed to a great number of microorganisms from the environment, only a small fraction are capable of permanent associations (**Table 1.1**), while fewer still progress to causing disease. Virulence determinants, those characteristics of virulent microorganisms which differentiate them from benign ones, and host antimicrobial defences are studied in order that in the future, Mankind might prevent or at least reduce the suffering caused by infectious diseases.

All living organisms are liable to infectious (communicable) diseases. The infectious agents, procaryotes (bacteria, chlamydias and rickettsias), protists (fungi and protozoa), certain eucaryotes (ecto- and endoparasites) and viruses, interact with and harm susceptible organisms to various extents. Even chronic diseases can result in serious losses in economic output from domestic animals (**Smith**, 1976; **Morris and Sojka**, 1985; **Wray and Morris**, 1985) and crops, and of course from humans (**Sussman**, 1985), and may progress to acute attacks. The great plagues of the Middle Ages in Great Britain, and the current epidemic AIDS, are prime examples of the potentially disastrous results of unchecked spread of disease. They are, however, far outnumbered by diseases with less severe symptoms, such as the common cold and travellers' diarrhoea, which nonetheless cause immeasurable suffering and lost production. Greater knowledge of the mechanisms of spread of communicable diseases has had far reaching effects on the way food crops, domestic animals and humans are cared for; the use of pesticides and specially bred disease-resistant plant and animal stocks complements the use of fertilisers and feed additives in increasing food production, while

antibiotics, immunisation and improvements in sanitary conditions are probably largely to thank for the longevity and health of modern Western Man, coupled with the beneficial action of better nutrition on his innate defences against harmful infections.

1.1.1 Enteric diseases. Serious enteric diseases are more common worldwide than might be supposed from our Western viewpoint. In Southeast Asia, diarrhoeal diseases cause more deaths in children than any other type of disease, and contribute to the 26% mortality rate up to the age of five (Newsletter number 6, 1984; International Centre for Diarrhoeal Disease Research, Bangladesh). It has been estimated that, worldwide, diarrhoeal diseases kill up to 6 million children annually and permanently injure up to 450 million more. The infectious agents (mainly Escherichia coli, rotavirus, Campylobacter jejuni and Shigella spp.) are spread in contaminated water and food. They kill principally by fluid loss and hence dehydration, but also exacerbate the severe undernourishment of many Thirld World children leading to susceptibility to other diseases (especially respiratory diseases and blindness through vitamin A deficiency). While diarrhoea is not usually life-threatening in the developed nations (except, perhaps, in neonates), it ranks with the most common causes of time lost from work, and is a considerable discomfort, at least, in Western travellers on foreign visits (Gianella, 1981; Gross, 1983; Gross and Rowe, 1985a).

A human is composed of over 10^{14} cells, of which only about 10% are animal cells, the remainder being mostly enteric bacteria (Savage, 1977). Identifying pathogens in such a huge community is not an easy task, but pathogens can be identified from faecal samples in up to 80% of patients with enteric diseases (Sack, 1975). Occasionally, multiple pathogens are isolated from a single patient. Enterotoxigenic E. coli (ETEC) and rotavirus are the commonest pathogens, but many other microorganisms can also cause enteric diseases (Table 1.2). The relative frequencies of isolation vary with the specific population studied, the geographic area, the age of the subjects, the season, and the diligence with which the etiologic agents are sought (Gianella, 1981). The problems of reliably culturing and identifying strains are compounded by the variability of expression of characteristics used for typing (sections 1.3 and 1.4), and by the complex host physiology and defence systems (section 1.2 and Figs. 1.1 and 1.2). These considerations highlight the need to analyse the expression of specific pathogenic factors, but without losing sight of the multifactorial nature of infections.

1.2 ANIMAL DEFENCES AGAINST INFECTION

Higher animals have complex systems for controlling the microbial colonisation of external and internal regions of the body, while microorganisms have evolved corresponding characteristics to counter the defensive actions of their hosts (Youmans et al., 1980; Mims, 1982; Mandell et al., 1985). Therefore, this section necessarily contains a number of references to the following section on bacterial virulence determinants (section 1.3).

1.2.1 Non-specific host defences. Physical exclusion of potential colonisers picked up from contact with contaminated surfaces, ingested or inhaled, is a vital role of the epithelia. Relatively impervious keratinised skin cells (epidermis) and smaller areas of specialised epithelial tissue (conjunctiva and mucous membranes), all of which are constantly replenished by growth of the underlying cells to replace those lost (sloughed or desquamated) to the external environment, cover the entire body surface. However, some microbes may adhere to and successfully colonise epithelia directly (for example Staphylococcus aureus and Candida albicans) or obtain entry to the body proper through lesions in the epithelia (cuts, insect bites, burns, gum lesions and other wounds). Some body surfaces (including all the mucous membranes) are particularly amenable to colonisation by bacteria of the normal flora (Table 1.1) and thus pose further problems of preventing access to underlying tissues. These indigenous microorganisms provide a barrier to colonisation by allochthonous organisms through competition for limited resources and antagonism (such as elaboration of bacteriocins; section 1.3.3) which, for example, partly explains the susceptibility of patients receiving antibiotics to enteric infections by resistant microorganisms which replace the sensitive indigenous community (e.g. 'overgrowth' by Candida albicans).

A further form of physical defence is the bulk flow of materials (tears, mucus, faeces and urine) across epithelia, creating shear forces which remove unattached foreign bodies. Microbes are also physically removed by clearing mechanisms, such as the beating of cilia lining the respiratory epithelia, by sneezing and coughing, by scratching and by intestinal peristaltic movement. Certain pathogens interfere with these mechanical defences by liberating toxins (section 1.3.6), and most adhere avidly to epithelia to avoid clearance (section 1.4).

Specialised animal cells (phagocytes) take up small foreign bodies into membrane-bound vesicles called phagosomes. The main phagocytic cell types are circulating neutrophils and monocytes (white blood cells), and

macrophages which line the reticuloendothelial system and can migrate through tissues (Fig. 1.2). The ingested materials are usually destroyed by a battery of toxic products (peroxide, myeloperoxidase, halide ions, cathepsins, acid hydrolases, lactic acid, basic proteins, lysozyme, iron-binding proteins, etc.) released from lysosomal granules following fusion with the phagosomes (degranulation). However, some pathogens are resistant even to these potent chemicals and may survive, possibly by entering the phagocyte cytoplasm, and circulate inside the host's own defensive cells (examples include Salmonella typhimurium, many viruses, mycobacteria, and protozoa). Note that specific factors may 'activate' macrophages to phagocytose unrelated foreign material more actively but in a non-specific way.

The localised swelling, heat production and pain of **inflammation** accompany many infections and are characteristic of a number of defensive processes in action. The focus of infection (such as an infected skin wound) provides a range of pyrogenic stimuli from damaged animal cells and bacterial components (see also **section 1.3.5**). Increased vascular permeability releases serum factors to aid the clearance of the source of the problem, and vasodilation raises the temperature. Large numbers of phagocytic cells (initially neutrophils, later monocytes) cross the epithelia lining the blood/lymphatic system and migrate along gradients of chemotactic signals released from the inflamed region, and in turn release more leucocyte attractants and other chemicals which intensify the inflammation and may lead to systemic fever. Heat production may constitute a non-specific host defence since many bacteria do not grow as well above the normal body temperature, but scant experimental evidence for its effectiveness must be tempered with the knowledge that excessive fever can lead to irreversible damage or death of the host. A recent publication (**Small et al., 1986**) concluded that fever is important in the elimination of pneumococci from rabbits experimentally infected with meningococci, but unfortunately there is as yet little other conclusive experimental evidence in this important field.

Vertebrates are able to manipulate certain aspects of the environments adjacent to epithelia by secreting **bactericidal** or **bacteriostatic chemicals**, including gastric acid secreted by stomach epithelium, secretions from sebaceous and sweat glands in the skin containing lactic acid, fatty acids, etc., iron chelators in several secretions, bile secretions, and spermine and zinc ions in seminal fluid. Active uptake of materials by the digestive system, and by other competing bacteria, presumably reduces the quantities of available carbon sources, oxygen and other metabolites to very low levels in the intestines, particularly in the anaerobic colon. Some cells secrete

enzymes including lactoperoxidase, lysozyme, and various digestive enzymes such as pepsin and trypsin. Most of these act non-specifically, and are effective in killing only certain types of bacteria by themselves. In contrast, lysosomal vacuoles contain a 'battery' of potent lytic enzymes and other chemicals which successfully deal with most organisms after active uptake by phagocytes.

1.2.2 Specific (immunological) host defences. Bacterial outer surfaces present a range of immunogenic molecules from LPS (O-antigens) to polysaccharide capsules (K-antigens) and exposed outer membrane proteins (including flagellar H-antigens and fimbrial F-antigens; **Fig. 1.3**), whose immunogenicity forms the basis of diagnostic serotyping. The host raises antibodies (immunoglobulins) by mechanisms involving stimulation of pre-existing antigen-sensitive lymphoid cells by the antigen, leading to cell division to form clones of lymphocytes (mature plasma cells) secreting antibodies reacting to those antigens (**Roitt,** 1984; **Fig.** 1.2). After the initial infection is over, some of the clones usually remain latent (memory cells), giving a more rapid response on repeated challenge with the same antigens even if carried in combination with novel antigens. This mechanism is exploited in vaccination. Whole or fractionated or artificially synthesised components of microorganisms are administered to stimulate antibody synthesis and generate memory cells (active immunisation). Alternatively, when active immunisation is inappropriate (for example in very young animals with immature immune systems) antibodies may be raised in another animal and given whole (passive immunisation), either naturally to infants (in specialised maternal secretions such as colostrum, or by active transplacental transport) or by doctors treating an infection.

Different classes of **immunoglobulins** are synthesised with the same antigen combining sites by genetic recombination in the lymphocyte chromosomal antibody genes (**Coleclough,** 1983). Each class has different functions (e.g. serum IgM and IgG circulatory antibodies, and secretory IgA bathing mucosal surfaces). Microorganisms become coated with antibodies (opsonised), rendering them more liable to phagocytosis and presenting them to the cell mediated immune system (see below). Antibodies combined with antigens activate the complement sequence, and stimulate killer cells to kill bacteria. Finally, some bacterial adherence mechanisms and toxins are directly prevented by direct attachment of antibodies.

Complement, a family of serum proteins, interact in a cascade reaction triggered by the binding of antibodies to antigens, and amplify the action of the immune responses by causing inflammation (which attracts leucocytes and other plasma factors), releasing polymorph attractants, promoting

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opsonisation by phagocytes and attacking bacterial cell membranes causing lysis directly and in conjunction with lysozyme, etc. Several pathogens elaborate toxins which interfere with the action of complement (**section 1.3.6**).

Part of the specific defence system, **cell-mediated immunity,** involves direct cellular action. Various types of T cells carry antibody-like surface receptors which recognise foreign antigens present in the lymph and blood. Some then divide and synthesize lymphokines (including gamma=interferon) which induce inflammation and attract leukocytes, or trigger B cells to make antibodies (helper cells), or actually suppress the immune response (suppressor cells). Some T-cells (natural killer cells) are triggered by foreign antigens associated with host cells (such as virally-encoded proteins on the surface of infected animal cells), leading to cytotoxin release and destruction of the host cells and foreign antigens. Cellular immunity is especially important against several intracellular parasites (e.g. Mycobacterium tuberculosis).

1.3 BACTERIAL VIRULENCE DETERMINANTS

The term virulence determinant has been loosely used to cover a range of concepts, but here I refer specifically to those characteristics of pathogenic bacteria which are closely related to the mechanisms of pathogenesis and are essential for the development of infection and disease in the host, as opposed to characteristics associated with normal growth and common to non-pathogenic bacteria. A number of such virulence determinants have been discovered:

1.3.1 Adherence to living tissues. Adherence of bacteria to specific tissues in prospective hosts is clearly essential in almost every infection and is discussed in detail below (**section 1.4**).

1.3.2 Antigenic variation. The surface antigens of some microorganisms are liable to change apparently at random, thereby altering their immunological characteristics. Variation of a major outer membrane protein of foot-and-mouth disease virus, for example, accounts for the majority of the differences among the seven serotypes and more than sixty subtypes (**Beck et al., 1983**). Antigenic variation appears to be achieved in bacteria (and maybe viruses) by mechanisms involving rearrangements of promoter regions, coupled with control by diffusible regulatory gene products. For example, bacterial strains are often capable of expressing more than one fimbrial type (Duguid and Old, 1980; Jann et al., 1981; Korhonen et al., 1982; Orskov et al., 1982b; Knutton et al., 1984c; see also **section 1.4** and **chapter 6**). Colonies derived from a single cell have been said to express different antigens on separate cells rather than expressing mixed fimbriae on single cells (**Rhen et al., 1983a, 1983c; Nowicki et al., 1984, 1985**), although the small proportion of cells which express more than one type is close to what one might expect if fimbriae are actually expressed independently. Neisseria gonorrhoeae expresses a variety of fimbrial antigens (**section 1.4.13**) and so-called opacity proteins. These comprise a family of antigenically heterogeneous proteins expressed from multiple gene sequences (**Stern et al., 1984**). Several, if not all, opacity protein genes are transcribed constititively, but small variations in repeated sequences upstream of the translational initiation sequence change the reading frame (**Stern et al., 1986**). Only one frame generates the correct ATG initiation codon, allowing translation and expression of the gene. Production of a bacterial capsule may specifically exclude fimbrial expression (**To, 1984; Knutton et al., 1984c**), implying some form of coordinated expression system, but failure to detect capsules and fimbriae simultaneously may simply be due to problems of

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the methodology. A more complex example of antigenic variation concerns surface antigens of the protozoan parasite Trypanosoma brucei. Alternative outer membrane proteins are encoded by stored coding sequences which are recombined into an expression site with an active promoter (Borst and Cross, 1982; Laurent et al., 1983; Birkbeck and Penn, 1986). Stored genes, lacking a suitable promoter, remain silent. In the course of a single infection, trypanosomes may express up to one hundred different antigens and thereby evade the host immune response, since each new antigen requires the production of new antibodies which may take several days to accomplish, by which time a proportion of the parasite population is expressing other antigens. Note that antigenic variation, where alternative antigens are exclusively synthesised by a coordinated expression system, is not formally equivalent to phase variation (section 1.3.9) where single antigens are expressed or repressed independently, but similar mechanisms of genetic regulation may be involved.

1.3.3 Bacteriocins. Some microorganisms secrete protein toxins which kill non-immune bacteria of particular species, and thus may be of selective advantage to the immune and secreting organisms (Konisky, 1982). Such antibacterial toxins are collectively called bacteriocins (or colicins in E. coli, pyocins in Pseudomonas and marcescins in Serratia). Their role in pathogenesis is unclear and it is not certain that bacteriocinogenicity is a virulence determinant. Bacteriocin synthesis and immunity genes are carried by plasmids which specify other gene products, such as those involved in conjugation. Therefore, unless the bacteriocin genes have been genetically isolated for study in vivo, one must take care in interpreting data implying roles in pathogenicity based on the whole plasmids. For example, although colicin V production by E. coli isolates from extraintestinal infections correlates with pathogenicity (Smith, 1974; Smith and Huggins, 1976, 1980; Quackenbush and Falkow, 1979; Aguero et al., 1983), virulence is primarily due to the presence of genes specifying an iron-uptake mechanism on the same ColV plasmids (Williams and George, 1979).

1.3.4 Capsules. Bacteria freshly isolated from diseased individuals are usually encapsulated by extracellular acidic polysaccharide matrices (Fig. 1.3), ranging in structure from simple homopolymers (such as K1 capsules of E. coli and group b meningococcal capsules, both composed of polymerised sialic acid; Bhattacharjee et al., 1975) to more complex mixed polymers, which can be serologically distinguished since they are antigenic to varying degrees (Jann and Jann, 1983). Capsules cause mucoid colonial morphology on solid media, and cell clumps and 'tide-mark' lines on glass

vessels containing liquid cultures, but the capsular synthetic ability is often lost on subculture in vitro (Costerton et al., 1981a, 1981b). Certain capsular antigens (K-antigens) are associated with particular diseases (such as E. coli K1 capsules with neonatal meningitis, Robbins et al., 1974; and K1, 2, 3, 5 and 13 types with pyelonephritis, Orskov and Orskov, 1985), and with independent isolates from disease outbreaks or geographical areas (section 1.3.10). Some capsules are only poorly immunogenic and may provide a degree of serum resistance (section 1.3.11) by masking potentially strong antigens from the immune system. For example, E. coli K1 capsular antigen may be related to a neuronal cell membrane component, and K5 has practically the same chemical structure as a precursor of heparin (Jann and Jann, 1985), so the immune response to these antigens is suppressed. Some bacterial infections lead to autoimmune complications such as arthritis, possibly as a result of such cross-reaction of bacterial and host antigens in addition to direct damage to the immune system. There is evidence that capsule production by porcine ETEC interferes with adherence to intestinal cells mediated by K99 fimbriae on the strains (section 1.4.4), so capsules presumably confer selective advantage other than increased adherence, at least in this system (Runnels and Moon, 1984). Some of the genetic systems specifying capsular anabolic pathways, including those for K1 (Silver et al., 1981; Escharti et al., 1983; Silver et al., 1984) and other capsules (Roberts et al., 1986a), have been cloned and analysed by molecular biology. As expected for such extracellular products, the genetic systems encode several products involved in the biosynthesis of precursors, their transport across the cell membranes, and polymerisation into the mature structures. Furthermore, the export regions of certain capsular synthesis systems are sufficiently homologous to permit DNA hybridisation, suggesting common evolutionary origins.

1.3.5 Endotoxin. Many bacteria secrete from their outer membranes lipopolysaccharides (LPS), consisting of a common glycolipid core region (lipid A within the membrane bilayer, linked to ketodeoxyoctonate) and highly immunogenic polysaccharide side chains which are used in serological typing schemes (O-antigens; Fig. 1.3). Bacteria release small amounts of lipid A, either through damage sustained during an infection or perhaps simply leaked from the cell surfaces, and this 'endotoxin' stimulates release of 'endogenous pyrogen' (interleukin-1) by host cells, causing local inflammatory responses (e.g. release of histamines, serotonin and kinin) or more serious systemic poisoning (fever, leukopenia, leukocytosis, etc.) and can cause fatal septic shock (Rietschel et al., 1982; see also section 1.2.1). Other bacterial components may also stimulate fever, and are

collectively called pyrogens.

1.3.6 Exotoxins. Many bacteria synthesise proteins which interfere in a specific fashion with particular activities of host cells (reviewed by **Alouf et al., 1984; Middlebrook and Dorland, 1984; Hewlett, 1985a; Holmgren, 1985**), and thereby enhance the virulence of the producing strains:

<u>Bacillus anthracis</u> - complex toxin circulates in the blood system of infected animals and causes increased vascular permeability, leading to circulatory failure. Protective antigen (PA) promotes activity of oedema (edema) factor (EF) and lethal factor (LF), neither of which is active alone (**Hewlett, 1985a**).

<u>Bordetella pertussis</u> - pertussis toxin alters host cell levels of cyclic adenosine monophosphate (cAMP)(**Katuda and Ui, 1982**), leading to various physiological changes in cells lining human airways during whooping cough (**Hewlett, 1984**). There is some evidence that, in addition to the adherence mediated by another major surface antigen (filamentous haemagglutinin), the toxin promotes adherence to human respiratory epithelia (**Tuomanen and Weiss, 1985**).

<u>Clostridium botulinum</u> - extremely potent preformed neurotoxin ingested in infected food (food poisoning) or synthesised de novo by organisms colonising infants' intestinal tracts (infant botulism), is composed of neural ganglioside GD_{1b}-binding and neurotransmitter release-interfering moieties (**Sugiyama, 1980**), causing flaccid paralysis. Just 60 ng of toxin is sufficient to cause lethal botulism in adult humans (**Gill, 1982**).

<u>Clostridium tetani</u> - the 100 kd and 50 kd subunit proteins of tetanus toxin cause paralysis by blocking neurotransmitter release from inhibitory synapses (**Eidels et al., 1983; Middlebrook and Dorland, 1984**).

<u>Corynebacterium diphtheriae</u> - beta-phage encodes an ADP-ribosylating toxin that inhibits cellular protein synthesis and causes the systemic toxicity of clinical diphtheria (**Pappenheimer, 1977**).

<u>Escherichia coli</u> - distinct classes of <u>E. coli</u> are recognised on the basis of their abilities to cause different diseases, some of which correlate with the production of certain toxins:

Enterotoxigenic <u>E. coli</u> (ETEC) produce heat stable and heat labile classes of enterotoxin (ST and **LT**, respectively). LT is very similar to

cholera toxin (CT; Clements and Finkelstein, 1978; Richards and Douglas, 1978; Dallas and Falkow, 1980; Spicer et al., 1981), and causes diarrhoeal disease which may equal cholera in intensity but is usually rather less severe. The toxin is composed of a ring of five B-subunits (each 11.5 kd) and one 25.5 kd A-subunit (Dallas and Falkow, 1979), and acts by binding to specific receptors (ganglioside GM_1; Osborne et al., 1982) on small intestinal epithelial cells (property of the B-subunits) and causing intracellular adenylate cyclase to synthesise cAMP constitutively (Evans et al., 1972 - property of the A-subunit following transport into the enterocyte cytoplasm; Dallas et al., 1979). This then leads to release of sodium ions from the cells and consequent outflow of water and chloride ions causes the clinical symptoms of profuse watery diarrhoea. Standard biological assays for LT and CT test for morphological changes to Chinese hamster ovary cells (Guerrant et al., 1974), or to cultured Y1 mouse adrenal cells (Donta et al., 1974). More recently, immunological tests (Sack et al., 1980) and genetic hybridisation assays using cloned LT or CT genes have been developed (reviewed by Gross and Rowe, 1985a). LT differs significantly from CT in that it is not truly exported from the bacteria but only reaches the periplasm whereas CT is efficiently secreted into the medium (Hirst et al., 1984). CT has be expressed in E. coli from cloned genes but was not exported into the culture medium (Pearson and Mekalanos, 1982), indicating that E. coli probably lacks export functions required for transfer of CT or LT across the outer membrane. The means by which LT causes diarrhoea without apparently being released from the cells remains to be elucidated.

Many ETEC produce ST, often in addition to LT and adherence factors. There are minor variations among ST toxins. STa and STb, from human and porcine isolates respectively, are related, poorly immunogenic small proteins, which increase intracellular cyclic guanosine monophosphate (cGMP) concentration causing solute release, diarrhoea and spread of the organism (Greenberg and Guerrant, 1981), possibly by inhibiting peristalsis (Mathias et al., 1980). They may be encoded by genetically mobile transposons, flanked by inverted repeat regions of DNA (reviewed by Elwell and Shipley, 1980). Assays use their heat stability ($100^{\circ}C$ for 10 minutes) and biological action (fluid accumulation in infant mice intestines, Dean et al., 1972), immunological identity (Frantz and Robertson, 1981; Klipstein et al., 1984), or genetic hybridisation to cloned DNA encoding ST (reviewed by Gross and Rowe, 1985a).

Enteropathogenic E. coli (EPEC) produce factors causing tissue damage, effacement of microvilli, etc., including a toxin with activity resembling that of Shigella dysenteriae Shiga toxin (Pai et al., 1986), and encoded by

bacteriophages in some EPEC enteritis strains (**Klipstein et al.,** 1978; **Scotland et al.,** 1980, 1983b; **Smith et al.,** 1983a; **O'Brien et al.,** 1984; **Strockbine et al.,** 1986). Shiga-like toxins have also been reported in E. coli from idiopathic haemolytic uraemic syndrome, the commonest cause of renal failure in children (**Karmali et al.,** 1983, 1985) and from haemorrhagic colitis (**Riley et al.,** 1983; **O'Brien et al.,** 1984; **Francis et al.,** 1986). The Shiga-like toxins are cytotoxic for cultured Vero cells (hence the former term Vero toxin, VT; **Konowalchuk et al.,** 1977) and HeLa cells but not Chinese hamster ovary or Y1 adrenal cells (**Strockbine et al.,** 1986). A cloned 2.5 kb DNA fragment was found to encode VT (**Willshaw et al.,** 1985b).

Oedema disease of piglets, characterised by generalised oedema and neurological disturbances causing 'failure to thrive' or death following soon after weaning, is probably caused by EPEC strains producing a neurotoxin ('edema disease principle', EDP), although other virulence determinants are also involved (**Wray and Morris,** 1985).

Uropathogenic E. coli (UPEC) - some 5 to 20% of faecal E. coli isolates from healthy humans secrete a protein with haemolytic activity, but the frequency of haemolytic isolates from extraintestinal infections is considerably greater, ranging up to about half of urinary tract infection (UTI) isolates, and is especially frequent among strains from pyelonephritis (reviewed by **Cavalieri et al.,** 1984), indicating that haemolysin is probably a virulence determinant in extraintestinal diseases (**Welch et al.,** 1981). The mechanisms of action are difficult to ascertain in vitro since the molecule is extremely labile (**Nicaud et al.,** 1985). High concentrations of haemolysin create membranous pores causing lysis of erythrocytes (haemolysis); low concentrations seem to inhibit neutrophil chemotaxis, and may have other cytotoxic effects (**Gadeberg et al.,** 1983; **Welch et al.,** 1986). Release of erythrocyte bound iron has been suggested as a function of haemolysis (**Linggood and Ingram,** 1982; **Waalwijk et al.,** 1983) and haemolysin secretion may be iron regulated (**Mackman et al.,** 1986). Circulation of the blood surely disperses any released ions, except perhaps in capillaries with much slower flow rates, but bacterial iron uptake mechanisms (**section 1.3.7**) are highly efficient iron scavengers. Haemolysin genetic determinants from several strains have now been cloned, and their roles in virulence are under study in several laboratories (**Hacker and Hughes,** 1985; **Mackman et al.,** 1986).

Pseudomonas aeruginosa - chromosomal genes encode exotoxin A with ADP-ribosylating activity identical to that of Corynebacterium diphtheria, though the influence of other virulence determinants presumably accounts for

the different clinical diseases (Iglewski and Kabat, 1975; Iglewski et al., 1977).

Shigella dysenteriae - Shiga toxin comprises a 31 kd protein and several 4 kd protein subunits (McIver et al., 1975), which kill cultured Vero and HeLa cells by a poorly understood mechanism. Its synthesis is iron regulated (Formal and Levine, 1984). Enterotoxin may be important early in the disease process in causing diarrhoea, but its role in later stages (invasion and damage of the colonic mucosa and dysentery) is unclear (Gemski et al., 1972; Jawetz et al., 1984).

Staphylococcus and Streptococcus spp. - Staphylococcus aureus enterotoxins cause vomiting and diarrhoea. Several membrane disrupting toxins and other protein products are also made, including sphingomyelinase (beta toxin of S. aureus), pneumolysin (Streptococcus pneumoniae), leukocydin, coagulase, hyaluronidase, and streptolysin O (Streptococcus pyogenes), with uncertain activities in vivo (reviewed by Jawetz et al., 1984).

Vibrio cholerae - cholera toxin (CT) causes excretion of salts and consequent profuse watery diarrhoea by a mechanism related to that of E. coli LT disease (reviewed by Richards and Douglas, 1978, and Holmgren, 1981). The fluid loss (up to 24 litres a day !) results in severe dehydration and acidosis, or even hypervolemic shock and rapid death, and contaminates the environment with organisms which may thus spread in water and food to other individuals in a classical epidemic outbreak of cholera. The toxin binds to ganglioside GM_1 receptors on mucosal cells (Fishman, 1980). The B-subunits are non-toxic strong immunogens which induce immunity to both CT and LT and are under trial as potential vaccines (Rappaport and Bonde, 1982). Commercially available whole cell cholera vaccines are of doubtful value, and appear to induce only a low level of rather short-term immunity (Newsletter number 5, 1983; International Centre for Diarrhoeal Disease Research, Bangladesh).

1.3.7 Iron uptake mechanisms. Several bacterial species elaborate systems for the specific uptake of essential iron (Crosa, 1984; Neilands, 1984) which is required at 0.4 to 4 uM to avoid iron deprivation (Weinberg, 1978). Animals synthesise iron chelating molecules such as lactoferrin and transferrin, to reduce the concentration of free iron to very low levels (typically 10^{-18} M), but bacterial iron binding molecules (siderophores) such as aerobactin and enterochelin are able to bind iron with greater efficiency and the iron-siderophore complexes are

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actively taken up by the bacteria (**Pollack et al., 1970**). The reduction of free iron to low levels may represent a host defensive mechanism against infection, and may be overcome experimentally by giving iron salts with normally poorly infectious bacteria, in which case their virulence is enhanced. Aerobactin production correlates well with pathogenicity in extraintestinal E. coli isolates (**Stuart et al., 1980; Williams and Warner, 1981; Montgomerie et al., 1984; Carbonetti et al., 1986**) but is not synthesized by many other strains (such as faecal isolates), and therefore fits the definition of a virulence determinant. Enterochelin, in contrast, is widely distributed amongst E. coli strains and is evidently not a virulence determinant (**Montgomerie et al., 1979**).

1.3.8 Motility and chemotaxis. Many wild strains of bacteria exhibit motility which is often directed along chemical gradients (**Stanier et al., 1977**). Such chemotaxis is probably an important characteristic in normal growth and survival, and may further be a virulence factor in the gut and intestinal tract, for example, where cellular secretions produce a layer of viscous mucus coating the epithelial membranes. Before bacteria can reach and adhere to those membranes by specific adherence mechanisms, it seems they must penetrate the mucus (**Freter et al., 1981**) but note that certain adhesins mediate adherence to receptors abundant in mucus, which may therefore be the initial or even ultimate site of attachment (**Orskov et al., 1980a;** see also **section 1.4**). Vibrio cholerae penetrates mucus by means of secretion of mucus degrading enzymes and motility (**Ketyi, 1984**). Nonmotile mutants are avirulent (**Jones and Freter, 1976; Jones et al., 1976**). Hohmann and **Wilson (1975)** reported that although a previously virulent ETEC strain continued to cause some diarrhoea in piglets after losing its K88 adherence factor and motility, the bacteria were no longer able to reach the villous crypts of the piglet intestinal brush borders. Chemotaxis and motility are such widespread characters that they are often considered to be part of normal bacterial growth rather than virulence determinants as defined earlier, and have been little studied in other pathogens.

1.3.9 Phase variation. Populations of bacteria derived from single cells (i.e. colonies or clones) and therefore assumed to be genetically identical, are not always found to be phenotypically homogeneous. Some characteristics vary at a high rate in an apparently random manner, such that only a proportion of the bacteria express them whereas the remainder do not. Individual colonies may contain different proportions of expressing and non-expressing phenotypes, the rate of change determining the proportions. The phenomenon, phase variation, is distinguished from mutation by the

rapidity (typically one change in 10,000 cells per generation) and ready reversibility (not necessarily the same rates in each direction), and from antigenic variation (**section 1.3.2**) since it concerns only a single antigen. Much pioneering work concerned the regulation of flagellar phase variation in <u>Salmonella typhimurium</u> (**Andrewes, 1922; Lederberg and Iino, 1956; Zieg et al., 1977, 1978**) which is regulated by a 'flip-flop' type of genetic rearrangement. An invertable region just upstream of the flagellar structural gene includes a promoter which is only active in one orientation. The region also encodes a protein which controls the flip. Similar control mechanisms exist in several other genetic systems, including yeast mating type conversion (**Herskowitz, 1983**) and phase variation of <u>E. coli</u> type 1 and <u>Neisseria gonorrhoeae</u> fimbriae (**sections 1.4.1 and 1.4.13**). Several virulence determinants of <u>Bordetella pertussis</u> also exhibit phase variation, controlled by a chromosomal gene product (**Weiss and Falkow, 1984**).

1.3.10 Serogroups. Bacterial isolates from diseases of humans and other animals are routinely classified as particular species by various biochemical assays, and assigned serotypes based on their agglutination by a standard range of antisera (raised against surface exposed O-, K-, H- and F-antigens of <u>E. coli</u>, and similar but less comprehensively characterised antigens on other species; **Kauffmann, 1947; Gross and Rowe, 1985a; Orskov and Orskov, 1985; Fig. 1.3**). By serological comparisons, it was discovered that independent isolates from certain diseases, geographical regions or environmental conditions, often share limited numbers of combinations of serotype antigens. Indeed, the correlation is sufficiently strong, for example, to assign the definition 'EPEC' to certain serotypes of <u>E. coli</u> isolated from infants with enteritis, without other evidence as to their virulence (**Gross and Rowe, 1985b**). The theory of clonal groups is based on the assumption that prevalence of particular antigenic combinations (serogroups) is due to those bacteria sharing a common ancestry, i.e. being clones of a progenitor organism with the given serotype (**Orskov et al., 1976; Achtman, 1985**). The theory is supported by the finding that some serotypes which are now found among certain classes of disease isolates were isolated from the same types of source decades ago, and have presumably remained stable in the wild population over long periods. Conversely, one might argue that particular combinations of antigens have unsuspected roles in pathogenesis, either directly (maybe by increased serum resistance, **section 1.3.11**, or low immunogenicity, **section 1.3.4**), or perhaps through being genetically close-linked to other as yet unrecognised virulence determinants, so that selection of virulent organisms passively selects for the associated serotypes. Further work is required to resolve these points.

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1.3.11 Serum resistance. Strains of bacteria capable of causing bacteraemia (i.e. proliferation in the bloodstream) are found to be more resistant to the bactericidal effects of serum than nonbacteraemic strains (Orskov and Orskov, 1985). Complement and antibody-mediated amplification of the action of complement are involved in the serum lytic action, and present a number of alternatives for bacterial serum resistance. Capsule masking of surface antigens has already been mentioned as a virulence determinant. Others include the synthesis of IgA-specific proteases by several species, including Neisseria gonorrhoeae, Streptococcus pneumoniae, and Haemophilus influenzae (Kilian, 1982; Male, 1982), nonspecific binding of immunoglobulins by staphylococcal protein A, and elaboration of factors which modify or degrade serum components (reviewed by Hewlett, 1985b). Several microorganisms avoid recognition as foreign by host immunological defences through coating themselves in host antigens (e.g. Schistosoma mansoni become covered with host histocompatibility structures; Sher et al., 1978) or by synthesizing antigens which mimic host factors (e.g. group A streptococci M-protein cross-reacts with mammalian heart antigens and tropomyosin; Dale and Beachey, 1982; Fischetti and Manjula, 1982). Many are able to change their surface antigens rapidly to evade the immune system (antigenic variation). Resistance to phagocytic killing involves a variety of microbial properties, such as resistance to digestion by lysosomal enzymes (Lewis and Peters, 1977), synthesis of capsular slime (Dhingra et al., 1977), and many more (reviewed by Goren, 1977; Smith, 1977; Murray, 1983; Quie, 1983; Orskov and Orskov, 1985).

1.3.12 Temperature regulation. The invasive properties of Shigella (Maurelli et al., 1984), Yersinia virulence (Brubaker, 1979), production of E. coli K1 capsule (Bortolussi et al., 1983) and of several fimbriae (section 1.4), are influenced by the microorganisms' growth temperatures. Expression is reduced at below-normal growth temperatures (i.e. less than body temperature of animal hosts). Such regulation may be a virulence determinant in its own right since it is reasonable to suppose that the fall in temperature signals excretion from the body (unless, coincidentally, the external temperature remains similar to the closely regulated mean inside the host). By repression of unnecessary characteristics outside the host, the organisms may perhaps conserve energy until they infect new victims.

1.3.13 Tissue invasion. Microorganisms which spread through tissues or systemically are often able to penetrate epithelial and other cells, or intercellular spaces, to gain access to normally sterile areas of the body. Mechanisms which allow them to do so are clearly virulence determinants

since failure to invade the tissues would normally prevent the disease before it is able to start (the exception being opportunistic entry through epithelial lesions, etc.). Most evidence for the mechanisms of penetration comes from studies on <u>Shigella flexneri</u> (**LaBrec et al.,** 1964) and <u>Salmonella typhimurium</u> (**Gianella et al.,** 1973; **Jones and Richardson,** 1981), and some from <u>Yersinia enterocolitica</u> (**Maki et al.,** 1978) and enteroinvasive <u>E. coli</u> (EIEC; **Silva et al.,** 1980) which are positive in the same invasiveness assays - guinea pig ulcerative keratoconjunctivitis (**Sereny,** 1957) or tissue culture tests using HeLa (**DuPont et al.,** 1971) or HEp-2 cells (**Day et al.,** 1981; assays reviewed by **Gross and Rowe,** 1985a). In general, tissue invasion has been found to correlate with the presence of large plasmids in all these strains (**Sansonetti et al.,** 1981, 1982, 1983; **Harris et al.,** 1982; **Hale et al.,** 1983), although additional chromosomally-encoded functions are involved (**Formal et al.,** 1971). EIEC also share certain surface antigens with invasive shigellae (**DuPont et al.,** 1971; **Pal et al.,** 1983). Plasmid and chromosomal determinants of invasion and the adherence to target membranes which presumably precedes it, are currently under study in several laboratories.

1.4 BACTERIAL ADHERENCE TO SURFACES

Bacteria have long been known to adhere strongly to inert and living surfaces, but not until the advent of electron microscopy was the structural basis of the phenomenon readily studied. Adherence often correlates with the presence of certain types of surface appendages, hair-like protrusions from the bacterial surface called fimbriae (**Duguid et al.,** 1955) or pili (**Brinton,** 1959). It soon became apparent that two classes of appendage exist, namely those involved in bacterial conjugation (sex-pili) and those mediating adherence of bacteria to surfaces (adherence structures). It is generally recommended that the term 'pili' is reserved to describe conjugative structures, while 'fimbriae' describes adherence fibres (**Duguid and Old,** 1980), but some groups still prefer to use 'pilus' and derived terms for both types.

Sex-pili (e.g. F-pili, the prototype mating system of <u>E. coli</u>; **Brinton,** 1965) are relatively thick (diameters 8 to 9.5 nm), usually rigid, peritrichous, plasmid-encoded structures which are involved in the formation of conjugation bridges between mating bacteria and are attachment targets for specific bacteriophages. Adherence structures, on the other hand, have no role in conjugation but mediate the often highly specific adherence of bacteria to a very limited range of available surfaces (**Jones,** 1977). This

binding preference is thought to account for much of the host- and tissue-specificity, and even cellular age-specificity, of many bacterial diseases (Ofek and Beachey, 1980). Thus, the initial site of bacterial colonisation is determined in large part by the specific interactions between adhesins and receptors, which are therefore of great interest in the field of virulence research.

Nearly all bacterial adherence structures or 'adhesins' (Duguid et al., 1979) are fimbrial, with diameters in the range 2 to 7 nm and lengths of up to a few micrometres, although a few have been described as being nonfimbrial or afimbrial (no structure discernible by electron microscopy; Table 1.3). They are composed of protein subunits of molecular weights approximately 12,000 to 29,000 that polymerise by noncovalent interactions which are nonetheless stable under even extreme, nonphysiological conditions, presumably aided by their often highly hydrophobic compositions.

Adherence to animal erythrocytes causes haemagglutination (HA) by the bacteria bridging between, and thus clumping together, red blood cells. The HA reaction exhibits specificity as to the species whose bloods are agglutinated, thus allowing HA typing schemes to distinguish classes of adhesin (Guyot, 1908; Kauffmann, 1948; Duguid et al., 1955, 1979; Evans et al., 1979, 1980; Crichton et al., 1981; Old, 1985a). In addition, the HA reaction of some adhesins can be disrupted by particular sugars which probably mimic the adhesin receptors and competitively inhibit the agglutination. Mannose, for instance, prevents HA and adherence to other tissues caused by type 1 fimbriae, so the reactions are termed 'mannose-sensitive' (MS), while all other adhesins are defined as 'mannose resistant' (MR) even though they are in fact a diverse collection (see below). Adhesin receptors on animal tissues are often carbohydrates, including, for instance, some of those recognised in blood-typing assays (e.g. the P blood group antigen; see section 1.4.12). These are of course themselves determined by the expression of animal genes, and are therefore potentially variable in whole animal populations. Thus, certain pigs which do not express intestinal mucosal receptors for K88 fimbriae (section 1.4.3) have been found to inherit this characteristic as a single autosomal recessive allele (Sellwood et al., 1975) while members of a certain small human population do not express the P fimbriae receptor implying a similar genetic basis. In contrast, expression of the mannose residues recognised by MS fimbrae does not appear to follow such a simple pattern, since many different mannose-containing compounds are exposed on animal cells.

Bacterial adherence to animal cells has been physically analysed by experimental observations and mathematical modelling (reviewed by Freter et al., 1983; Jones and Isaacson, 1983; Marshall, 1985). Such models,

particularly the 'DVLO' theory, predict that the kinetic energy of smooth surfaced bacteria is probably itself insufficient to overcome the overall repulsive forces between negatively charged bacterial and animal cell surfaces. However, these electrostatic forces vary with the distance of approach. At certain separations, especially less than 1 nm, there is net attraction as the electrical fields of the two bodies 'combine'. Bacterial fimbriae and brush border microvilli have thin shapes and consequently exhibit special electrostatic properties. They can therefore probably penetrate the repulsive layers, assisting the bacteria to attain close approach and adhere strongly.

Pathogens which secrete toxins need not necessarily approach any closer than the micrometre or so lengths of their fimbriae since the toxins can diffuse to the epithelial membranes. Invasive strains, on the other hand, must proceed to closer approach and ultimately cross the membranes or intracellular junctions of the mucosae. EPEC strains appear to be intermediate, in that they come to lie very close to the membranes without normally crossing them, possibly involving the action of membrane damaging toxins. Biological considerations, such as the elaboration of glycocalyx material by mucosal cells, multiplication of bacteria within extracellular polysaccharide matrices, and bacterial chemotaxis and motility, further complicate the process. Accurate modelling is clearly impossible, but even simple models allow useful predictions.

Several authors have reviewed various aspects of bacterial adherence mechanisms and structures (Ottow, 1975; Costerton et al., 1978, 1981a; Ellwood et al., 1979; Moon et al., 1979; Beachey, 1980, 1981; Berkeley et al., 1980; Bitton and Marshall, 1980; Duguid and Old, 1980; Savage, 1980a, 1980b; Elliott et al., 1981; Gianella, 1981; Ip et al., 1981; Scott and Old, 1981; Boedeker, 1982; Gaastra and de Graaf, 1982; Klemm, 1982a; Jones and Isaacson, 1983; Orskov and Orskov, 1983; Christensen et al., 1985; Isaacson, 1985; Klemm, 1985; Mooi and de Graaf, 1985a, 1985b; Normark et al., 1985; Old, 1985b; Parry and Rooke, 1985; Savage and Fletcher, 1985; Smith et al., 1985; Uhlin et al., 1985a). However, recent advances in the knowledge of the genetic systems encoding fimbriae have not been comprehensively reviewed, and therefore a review of the genetics of bacterial adherence mechanisms, with particular reference to virulence determinants of E. coli, follows.

1.4.1 Type 1 fimbriae

Bacterial isolates of several species from a wide range of sources are often found to be capable of adherence to and agglutination of a variety of animal cell types, but the reaction is characteristically inhibited by low concentrations (0.01 to 0.5% wt/vol) of D-mannose or certain D-mannose containing derivatives (MS adherence; **Duguid and Gillies, 1957; Old and Duguid, 1970; Old, 1972; Salit and Gotschlich, 1977a; Duguid and Old, 1980; Buchanan et al., 1985).** MSHA of guinea pig erythrocytes, or MS agglutination of yeast cells, are the routine tests for MS adherence. Bacteria capable of MSHA generally carry fimbrial structures called type 1 or common fimbriae **(Duguid et al., 1955; Brinton, 1965; Ottow, 1975),** as demonstrated by electron microscopy **(Duguid and Old, 1980).** E. coli type 1 fimbriae were shown to mediate MS adherence by observation of bacteria adhering to erythrocytes **(Knutton et al., 1984b)** or of purified fimbriae **(Salit and Gotschlich, 1977b; McMichael and Ou, 1979).**

E. coli common fimbrial subunits are synthesised as precursors **(Dodd et al., 1984)** some 2 kd larger than the mature subunit molecular weight of 17,000 **(Brinton, 1965),** and are processed and assembled within a minute of synthesis **(Dodd and Eisenstein, 1984).** Assembled fimbriae are rigid rods, 7 nm diameter by about 2 μm long **(Duguid and Old, 1980; Knutton et al., 1984b).** Type 1 fimbriae have also been purified and partially characterised from Salmonella typhimurium **(Korhonen et al., 1980a)** and Klebsiella pneumoniae **(Fader et al., 1982),** and although they are morphologically and functionally similar, they differ in subunit molecular weights and amino acid constitution and have little immunological cross reactivity.

Type 1 fimbriae are very hydrophobic and cause interbacterial clumping in vitro, especially at the surfaces of static liquid cultures where the higher oxygen availability allows fimbriated cells in pellicles to outgrow nonfimbriated cells in the body of the culture flasks **(Old and Duguid, 1970; Swaney et al., 1977a),** and perhaps in natural situations outside animal hosts (e.g. in stagnant pools). In contrast, their role in vivo is controversial. Mannose-containing receptors are abundantly distributed on most tissue surfaces and consequently cannot account for the observed tissue tropisms of bacterial diseases. Type 1 fimbriated bacteria have been demonstrated to adhere to host glycoproteins, particularly Tamm-Horsfall protein (THP) abundant in the urinary tract, which may be part of the nonspecific host defences in clearing trapped bacteria in the mucus flow **(Orskov et al., 1980a, 1980b),** or conversely may be selectively advantageous to the bacteria in providing adherence to mucus niches (e.g. in the mouse bladder; **Hultgren et al., 1985)** and forming 'pseudocapsules' of adherent THP

which prevent phagocytosis by leukocytes (**Kuriyama and Silverblatt,** 1986). Monoclonal antibodies raised against either type 1 fimbriae or the mannose-containing receptors, protect mice against kidney infections (**Abraham et al.,** 1985), and α-methyl mannoside, injected with type 1 fimbriate E. coli into the bladders of mice, significantly reduced the incidence of cystitis and bacteriuria relative to controls without the mannose-derivative (**Aronson et al.,** 1979), but vaccination of pregnant pigs did not protect their newborn against experimental diarrhoea even though serum anti-type 1 antibody titres were raised (**To et al.,** 1984). Mannose compounds inhibited hepatic clearance of E. coli from the mouse circulation (**Perry and Ofek,** 1984) by interference with liver lectins (as opposed to bacterial adhesins), and bacteria expressing type 1 fimbriae are more readily phagocytosed by non-opsonic mechanisms (**Perry and Ofek,** 1984; **Speert et al.,** 1986). Regardless of the fimbrial phase of E. coli initially fed to neonatal rats, only nonfimbriated organisms were recovered from blood samples while fimbriate bacteria persisted in the oral cavity (**Guerina et al.,** 1983) where MS adherence of E. coli (**Clegg et al.,** 1984) and Candida albicans (**Sandin et al.,** 1982) to buccal epithelia has been demonstrated. In summary, their ubiquity among pathogenic and benign bacteria and the presence of mannose-containing receptors on so many surfaces argues against common fimbriae being classed as virulence determinants, except perhaps in the lower urinary tract (**Iwahi et al.,** 1983).

Regions encoding type 1 fimbriae have been independently cloned from the chromosomes of an E. coli K-12 laboratory strain (**Klemm et al.,** 1985b), from uropathogenic E. coli strains (**Hull et al.,** 1981; **Orndorff and Falkow,** 1984a, 1984b), from Klebsiella pneumoniae (**Purcell and Clegg,** 1983) and from Salmonella typhimurium, Serratia marcescens and Enterobacter cloacʌae (**Clegg et al.,** 1985a). Buchanan et al. (1985) used a restriction fragment from the cloned E. coli type 1 fim genes specific for the subunit structural gene (**Hull et al.,** 1981) to test for homologous sequences among 236 bacteria from 11 genera of Enterobacteriaceae; homology was found only in E. coli and Shigella isolates, although MSHA was found in nine genera. In no case did plasmid DNA from any of the strains hybridise, indicating a chromosomal location for the genes. F-prime plasmid F101, carrying chromosomal genes around 98 minutes on the E. coli map, was found to encode type 1 fimbriae (**Low,** 1972). The various cloned systems have been analysed and compared by complementation (**Swaney et al.,** 1977a, 1977b; **Clegg et al.,** 1985a, 1985b), by restriction mapping (**Klemm et al.,** 1985b), and by DNA sequencing (**Klemm,** 1984; **Orndorff and Falkow,** 1985). There is little similarity between the systems, except for some common restriction endonuclease fragment lengths between Klebsiella pneumoniae and E. coli.

Swaney et al., (1977a, 1977b) found four complementation groups specifying type 1 fimbriae in an E. coli strain which was agglutinated by anti-type 1 serum but did not haemagglutinate. Aberrant, long fimbriae incapable of MS adherence were also made by a streptomycin resistant strain of E. coli in the presence of subinhibitory concentrations of the antibiotic (Eisenstein et al., 1981). Recent evidence from genetic analyses implicates nonfimbrial components in actually mediating the MS adherence (Maurer and Orndorff, 1985; Minion et al., 1986). The true adhesins are presumably carried by the fimbrial structures, but are functional in their absence. They are specified by genes distinct from the subunit determinants. These and similar results have important implications which are further considered in the discussion (chapter 6).

Unlike other adhesins, type 1 fimbriae are fully expressed in stationary phase cultures (Eshdat et al., 1981), but by only a proportion of cells (Brinton et al., 1954), and are expressed even at low growth temperatures. Phase variation is transcriptionally controlled at rates of approximately 10^{-3} per cell per generation, although not equal in the forward and reverse directions (Eisenstein, 1981) and variable according to cultural conditions (Eisenstein and Dodd, 1982). Genetic analysis of the region around the fimD gene determined that a diffusible regulator controls an invertable region containing a promoter sequence (Freitag and Eisenstein, 1983; Abraham et al., 1985b; Freitag et al., 1985) in analogous manner to the control of Salmonella typhimurium flagellar phase and yeast mating type conversion (section 1.3.9).

Transposon mutants at the 'hyperpilation' locus (hyp) synthesise 40-fold more pilin than wild type E. coli, secrete more, longer type 1 fimbriae and continue to do so even under conditions which normally would repress fimbrial synthesis such as growth in glycerol minimal medium or in the presence of 4% ethanol (Orndorff and Falkow, 1984b). Hyperpilated cells clump together avidly even in the depths of liquid media and form opaque 'pinprick' colonies on solid media. Reversion by excision of the transposon or deletion of the region extending towards or through the subunit gene from the hyp gene occurs at high frequency (one revertant per 500 cells per generation), probably because of strong selection against the excessive synthesis of pilin. The hyp gene encodes a 23 kd trans-active repressor of fimbriation, which may possibly have a role in controlling phase variation.

1.4.2 Type 1-like fimbriae

Multiple fimbriae immunologically related to type 1 fimbriae have been discovered on many human uropathogenic E. coli (UPEC) strains (Orskov et al., 1982b), often in addition to other adhesins including P-fimbriae (Rhen et al., 1986; section 1.4.12). They do not mediate MSHA or MS adherence to other tissues characteristic of type 1 fimbriae (Klemm et al., 1982) and their functions (if any) in vivo remain unknown. The amino-terminal regions of these 'type 1-like fimbriae' are highly conserved, while the nucleotide sequences of cloned type 1 and type 1C fimbrial subunit genes contain a high proportion of silent base changes which translate to identical amino acids (van Die et al., 1984a). Therefore, the genetic determinants of these apparently related fimbriae may perhaps have arisen by chromosomal gene duplications and random genetic drift. The adhesin and fimbrial subunits of type 1 fimbriae are encoded by distinct genes (section 1.4.1), so the altered adherence specificities of type 1-like fimbriae may possibly reflect the presence of different adhesin genes, or even the absence of such determinants. The antigenic differences also point to some divergence. [The evolutionary significance of these and other findings is considered further in the discussion, chapter 6.]

1.4.3 K88 fimbriae

K88 is a surface antigen commonly found on E. coli isolated from animals with colibacillosis etc. (Orskov et al., 1961). Variants of K88 share one antigenic epitope but differ at another, and were named K88ab and K88ac to emphasize this fact (Orskov et al., 1964). Subsequently, other variants were discovered, all of which share the 'a' determinant (Guinee and Jansen, 1979). K88, purified by acid precipitation from material sheared from cells or released at 60°C, was mostly proteinaceous (Stirm et al., 1966, 1967a, 1967b) but was initially thought to be a capsular antigen, hence the K designation. Mooi and de Graaf (1979) improved the purification procedures by size separating extracts on Sepharose chromatography columns. K88 variants were purified to single protein bands on SDS-PAGE with relative molecular weights of 23,500 (K88ab), 25,000 (K88ac) and 25-26,000 (K88ad). One-third of the 264 amino acids of K88ab are hydrophobic, while less than one-fifth are charged (Klemm, 1981).

K88 is transmissible by a conjugative plasmid so cured strains lack the antigens (Orskov and Orskov, 1966). Smith and Linggood (1971) were the first to demonstrate the virulence effects of K88 plasmids in vivo, by comparing isogenic strains with or without the determinants for K88, haemolysin and enterotoxin. They found that K88 was an absolute requirement for the production of diarrhoea in pigs, while haemolysin was not necessary and enterotoxin was important but not essential since K88$^+$ nontoxigenic bacteria did cause a mild form of diarrhoea. They proposed that K88 was a colonisation factor mediating adherence in the pig intestines, particularly the small intestine. However, they also reported that Salmonella strains carrying the K88 determinant became avirulent in mice and less virulent in piglets compared to the plasmidless strains, but with the benefit of hindsight it seems likely that fimbrial antigenicity promoted rapid clearance of the K88$^+$ strains by the pigs' immunological defences, following the tissue invasion typical of salmonellae.

The K88 receptors on porcine intestinal mucosa are determined by dominant alleles (Rutter et al., 1975; Bijlsma et al., 1981) and may be membrane glycolipids (Kearns and Gibbons, 1979). Therefore, one may intentionally breed pigs lacking the K88 receptor to reduce or eliminate the action of K88-bearing ETEC, although of course there are other adhesins with similar functions but different receptors which would presumably become increasingly prevalent with time (section 1.4.7).

Hohmann and Wilson (1975) demonstrated K88 mediated adherence to piglet anterior small intestine. They also examined the K88$^+$ strains by electron microscopy and clearly demonstrated type 1 fimbriae, but reported

inconsistent sightings of much finer structures (which were probably K88). They generated K88-negative variants to assess their altered virulence, but simultaneously obtained nonmotile bacteria which did not penetrate to the villous crypts. Nonetheless, these K88⁻ nonmotile variants apparently caused diarrhoea, in contrast to the findings of **Smith and Linggood (1971)** mentioned above, perhaps because of the presence of alternative adhesins.

Deneke et al. (1985) found that low levels of tetracycline reduced adherence of $K88^+$ bacteria to cultured pig intestinal tissue, but the data were inconsistent and may even have been mistaken due to the presence of penicillin and streptomycin in their tissue culture media. However, the effects of subinhibitory concentrations of antibiotics on adherence mediated by some other adherence factors indicates that adhesin export or assembly may be specifically affected in some way.

K88 plasmids isolated in different laboratories are of different sizes, probably as a result of cointegration of K88 and another plasmid which confers conjugal transfer. The K88 genes are usually linked within 20 kb to genes specifying raffinose utilisation (raf genes), which are themselves flanked by direct repeats of IS1-like insertion sequences and may be capable of transposition (**Schmitt et al., 1979**).

Mooi et al. (1979) cloned a 12.4 kb HindIII DNA fragment in vector pBR322 that expressed K88ab in E. coli K-12. They deleted part of the plasmid and defined a region of less than 7 kb required for K88ab synthesis (**Mooi et al., 1981**), which was mapped by comparing mutants (**Mooi et al., 1982**). The map was similar to that of K88ac (see below). The gene products were analysed in some detail, culminating in a proposed model for K88ab synthesis, export across the cell wall and assembly into functional fimbriae (**Mooi et al., 1983**).

K88ac was specified on a 13 kb HindIII fragment cloned into pBR322 (**Shipley et al., 1978, 1979, 1981**). Minicell analysis initially indicated at least six protein products synthesised from the cloned insert, although deletion and transposon insertional analysis concluded the presence of only four cistrons encoding proteins of 70, 29, 23.5 (subunit) and 17 kd (**Kehoe et al., 1981**). Interestingly, one transposon insert prevented MRHA activity even though K88 fimbriae continued to be produced and adherence to pig intestinal brush border cells was shown. **Dougan et al.** (1983) localised the proteins by fractionating minicells. The large protein (70 kd, product of gene adhA) was found in the outer membrane; the 23.5 kd protein (from gene adhD), identified as the subunit, was present in outer and inner membranes; the other two proteins (17 kd from adhC and 27 kd from adhB) were periplasmic. Genes adhA, adhC and adhD specified proteins some 2 kd larger than the processed products, consistent with their synthesis as signal

sequence-containing precursors. However, the primary product of gene adhB of 29 kd as reported previously was only slowly converted to a protein of 27 kd. This might indicate that it is not a normal signal sequence cleavage event, but some other form of processing or perhaps aberrant cleavage in the minicell system employed. The localisation data are similar to those obtained with K88ab by **van Doorn et al.** (1982), who found 17, 27 and 27.5 kd periplasmic proteins and an outer membrane protein of 81 kd. A fifth gene (adhE) mapped near to adhA (Dougan et al., 1983; Kehoe et al., 1983).

Mooi et al. (1984) noted the presence of point or small deletion mutations within the K88ab subunit gene which were still capable of MRHA. This led to the conclusion that the subunit structure itself does not mediate adherence to erythrocytes and presumably to intestinal cells, but rather acts to carry a distinct adhesin moiety. Similar findings have been reported for type 1 fimbriae (**Maurer and Orndorff**, 1985; **Minion et al.**, 1986; **section 1.4.1, chapter 6**).

K88 variant subunit genes have been sequenced and compared (**Gaastra et al.**, 1981, 1983; **Josephsen et al.**, 1984; **Dykes et al.**, 1985). They show significant homology, the few amino acid substitutions being relatively dispersed throughout the proteins. **Kehoe et al.** (1982) also found some DNA homology to K99 by hybridisation, although later experiments gave conflicting results (**Moseley et al.**, 1986; **section 1.4.6**). Thus while it is clear that the K88 variants are closely homologous, their relation to K99 is more doubtful.

1.4.4 K99 fimbriae

Smith and Linggood (1972) described the presence on atypical E. coli strains isolated from calves and lambs of a plasmid encoded surface antigen which differed from K88. Orskov et al. (1975) proposed the name K99. Reference strain B41, used to purify K99 fimbriae (Morris et al., 1977), was subsequently shown to elaborate at least one other adhesin (F41; Morris et al., 1978, 1980; section 1.4.6). At the time, several authors did not realise that bacteria might elaborate more than one adhesin, so the literature contains a number of inconsistencies with respect to the number of subunit proteins demonstrated in 'purified' preparations from B41, their molecular weights by SDS-PAGE, and the MRHA profiles. For example, Isaacson et al. (1981) presented data for mixed fimbrial subunits of 22.5 and 29.5 kd which copurified and were assumed to be present in single fimbriae. Mercaptoethanol treatment led them to conclude that cysteine disulphide bridges were responsible for the two apparent sizes, but confusingly they concluded that the 'true' molecular weight was 19.5 kd. de Graaf et al. (1980b) purified fimbriae with 18.5 kd subunits from the same strain, but found no effect of mercaptoethanol treatment. They suggested that the 29.5 kd band seen by Isaacson et al. (1981) was due to a minor contaminant.

K99 fimbriae are 5 nm in diameter and apparently helical structures (de Graaf et al., 1980b). The 8.4 nm fimbrial diameter reported by Isaacson (1977) was probably an artifact caused by hydrophobic colinear aggregation (Parry and Rooke, 1985). K99 fimbriae protrude beyond the capsular layer characteristic of freshly isolated strains and adhere to calf ileal epithelium in vitro (Chan et al., 1982).

K99 synthesis was initially found to be repressed by the presence of some carbon sources (glucose, pyruvate, arabinose or lactose) but maximal under aerobic logarithmic growth in glycerol minimal media (Isaacson, 1980) at 37°C (de Graaf et al., 1980c). In contrast to the findings of Guinee et al. (1976), Isaacson (1980) stated that 10 mM cyclic adenosine monophosphate (cAMP) stimulated K99 synthesis. However, the data points were scattered and in a later publication (Isaacson, 1983) the author reported that glucose did not affect synthesis or secretion of K99, nor was expression altered in a cyclic adenylase mutant strain. Synthesis occurred in all culture growth phases but assembled K99 fimbriae only appeared on the cell surfaces during logarithmic growth. Alanine inhibited K99 synthesis in minimal media (de Graaf et al., 1980a), unless threonine or isoleucine were also added, perhaps due to membrane changes analogous to those of threonine and isoleucine auxotrophs (Isaacson, 1983).

van Embden et al. (1980) cloned the K99 genetic determinants from a

92 kb conjugative plasmid identified by **Smith and Linggood** (1972) and *So* et al. (1976). de **Graaf** et al. (1984) subcloned the genes on a 6.7 kb DNA fragment inserted into vector pBR322. Seven proteins were identified, of 18.2, 19, 21, 21.5, 26.5, 33.5 and 76 kd. The 18.2 kd protein reacted with antiserum to purified K99 fimbriae and was thus identified as the subunit. This and four other proteins were synthesised as precursors approximately 2 kd larger, probably due to additional signal sequences. The genes were mapped by generating DNA deletions and assessing the loss of specific protein products. **Roosendaal** et al. (1984) determined the nucleotide sequence of the subunit gene. The calculated molecular weight was 18.5 kd, consisting of 159 mostly hydrophobic amino acids, plus a 22 residue signal sequence. There is limited DNA sequence homology between K88 and K99 allowing hybridisation under certain conditions, but genes from the two systems lack functional similarity and do not complement (**Kehoe** et al., 1982). Recently, **Moseley** et al. (1986) found no cross hybridisation under stringent conditions, so they must be significantly diverged or unrelated.

In an elegant series of experiments, **Roosendaal** et al. (1986) investigated the synthesis of K99 fimbriae by electron microscopy of thin sections of bacteria prepared from cultures shifted at various times from 18°C (subunit expression largely repressed) to 37°C (full expression), which were reacted with antibody to K99 fimbriae conjugated to colloidal gold. The outcome is analogous to pulse-chase radiolabelling in that the electron refractive gold particles (and hence the majority of the K99 subunits) can be readily identified at specific cellular locations in the process of being exported. Following synthesis at the cytoplasmic membrane, the subunits were sequentially transferred through the periplasmic space to the outer membrane and cell exterior. In conjunction with deletion and transposon mutants of the K99 system, it should be possible to identify with greater certainty the export functions of this complex system.

1.4.5 987P fimbriae

ETEC strains from pigs occasionally lack K88 and K99 but carry novel fimbriae first identified on strain 987 and called 987P (Isaacson et al., 1977; Nagy et al., 1977). Purified 987P subunits are 19 to 20 kd in mass (Isaacson and Richter, 1981). The target receptor consists of a small glycoprotein (Dean and Isaacson, 1985a) evenly distributed along rabbit and pig small intestinal mucosae (Dean and Isaacson, 1985b). The chromosomal genetic determinants of 987P fimbriae were cloned in cosmids and expressed in laboratory E. coli strains (de Graaf and Klaasen, 1986). A region of 12 kb encoded five proteins of 81, 39, 28.5, 20.5 and 16.5 kd, sufficient for expression of the fimbriae although phase variation was absent, and certain transposon insertions in DNA outside the 12 kb region also affected expression of fimbriae. Minicells were found to synthesise, export and assemble apparently intact fimbriae presumably in the absence of most chromosomal gene products, indicating that the cloned DNA was probably truly sufficient for fimbrial synthesis. Two clones independently obtained by a similar method (Morrissey and Dougan, 1986) were found to express phase variation which differed from the parent strain; one generated irreversible deletions within the cloned region at a high frequency and simultaneously lost expression of the fimbriae, while the other irreversibly lost expression at a low frequency. Until more data are gathered, one can only speculate that such events are connected with the normal phenomenon of phase variation in strain 987.

1.4.6 F41 fimbriae

E. coli from calf diarrhoea producing K99 often also synthesise a distinct adhesin with anionic properties (Morris et al., 1978). Two MR adherence antigens were distinguished on the K99 reference strain B41 in addition to type 1 fimbriae (Morris et al., 1980). The novel antigen, F41, accounted for the adherence of a K99 deficient mutant derivative of B41 (B41M), and helped explain the apparent anomaly that B41M retained the ability to cause diarrhoea in gnotobiotic pigs without the need for K99-mediated adherence (Morris et al., 1982). The antigen was purified by Sepharose column size separating a sheared preparation after ammonium sulphate precipitation and solubilisation in deoxycholate (the method developed by Korhonen et al., 1980b) to yield a 29.5 kd subunit protein (de Graaf and Roorda, 1982). The assembled F41 adhesin had a 3.2 nm diameter curly appearance by electron microscopy (de Graaf and Roorda, 1982; Morris et al., 1982), and mediated MRHA of guinea pig and human blood, and to a lesser extent, sheep, horse and possibly rabbit blood. F41 also mediated adherence to isolated calf intestinal brush borders, but B41 (K99$^+$, F41$^+$) was considerably more adherent to calf epithelium than B41M (K99$^-$, F41$^+$). Bertin (1983) studied pathogenic characteristics of ETEC isolates in an infant mouse model, and concluded that F41 was a virulence determinant but caused death 24 hours later than an isogenic ETEC strain also containing K99. In a later publication (Bertin, 1985) a strain synthesising ST and K99 but not F41 was said to be avirulent in the same model.

The small intestines of neonatal piglets and calves were efficiently colonised by F41$^+$ ETEC strains, causing diarrhoea (To, 1984). Antigenic variation between F41, type I and encapsulated cells could be followed by comparing the shape and translucency of colonies grown on agar, in similar fashion to gonococcal fimbrial and opacity protein variants (sections 1.3.2, 1.3.9 and 1.4.14).

The chromosomal determinant of F41 fimbriae has been cloned in cosmids and compared to other cloned fimbrial genetic systems (Moseley et al., 1986). Homology was found between the F41 and K88 systems, but neither was found to hybridise to the K99 probe. Homology to the F41, K88 and K99 probes was found amongst total DNA of many animal ETEC isolates, though they varied in expression of MRHA and antigenic determinants corresponding to the homologous systems.

1.4.7 Other adhesins on E. coli isolates from animals

Chanter (1983) reported the presence of two mannose resistant adhesins on a K99 deficient derivative of B41, and obtained 34 kd subunit anionic fibrils (probably F41), plus mixed 48 plus 49.5 kd subunit fibrils incapable of adherence to calf intestinal mucosa. The latter sizes are well above the range of other adhesin subunits described to date, and have not been reported elsewhere. **Parry and Rooke** (1985) suspected contamination of their material with type 1 fimbriae.

ATT25 fimbriae are found on calf ETEC isolates (**Pohl et al., 1982, 1983**) in increasing numbers as vaccination with K88 and K99 preparations effectively seems to reduce the incidence of these previously almost universal adhesins, at least in Belgium (P. Lintermans, personal communication). This finding has serious implications to the proposed widespread use of anti-adhesin vaccines to prevent diarrhoeal and other diseases, and suggests that frequencies of variant adhesin antigens in the total bacterial community will be modified as a direct result of the selective pressures imposed by vaccination, negating their effect [see also **chapter 6**].

There are no doubt many other adhesins still to be identified on pathogenic E. coli isolates from animals, particularly those which do not cause HA reactions with the erythrocytes commonly used and are therefore not so easily identified. The same is probably also true of bacteria isolated from human diseases, and although the search for virulence determinants on pathogens directly affecting Man is likely to be more immediate, the economic impact of weaning diarrhoea, mastitis, enterocolitis, etc., amongst farm animals makes the veterinary studies important in their own right, as well as perhaps providing models systems to study human infections.

1.4.8 Colonisation factor antigen I (CFA/I)

ETEC of human origin were found to express MRHA mediated by so-called colonisation factor antigens (CFA's; Evans et al., 1975). The first was retrospectively named CFA/I (Evans and Evans, 1978). CFA/I$^+$ strains adhered to and colonised infant rabbit intestines, whereas laboratory passaged CFA/I$^-$ derivatives did not colonise the animals (Evans et al., 1978b). CFA/I was found on 86% of E. coli strains of several O-serogroups from human diarrhoea cases but on only 18% of control strains not associated with diarrhoea. Cravioto et al. (1982) found CFA/I on just 18% of the ETEC strains they tested, but none on other types (faecal and extraintestinal isolates). The discrepancies in the frequencies may be due to differences in the ETEC pools tested, especially since CFA/I is associated with certain serotypes (Evans and Evans, 1978; Smyth et al., 1979).

CFA/I purified from E. coli strain H10407 consists of fimbriae with diameters of 6.2 nm (Freer et al., 1978) or 7 nm (Evans et al., 1977, 1978a, 1979), but other investigators concluded that these were collinear aggregates, the true diameter being 3 nm (Gaastra and de Graaf, 1982). Electron microscopical examination of fimbriae in situ revealed electron-dense edges and apparently hollow centres (Knutton et al., 1984b). The subunit molecular weight, initially reported as 23,800 (Evans et al., 1977), is now accepted to be 14,500 (Klemm, 1979), and the isoelectric point (pI) is 4.8 (Freer et al., 1978). The entire 147 amino acid sequence of the mature subunits has been published (Klemm, 1982b), confirming the highly hydrophobic constitution of CFA/I fimbriae in common with many other types, but no homology to K88 or K99 sequences.

CFA/I fimbriae mediate attachment of bacteria to a variety of human cells. They cause MRHA of human, chicken, bovine and guinea pig blood, but only in native form on the bacteria or if purified and attached to a solid carrier (Evans et al., 1977). Isolated fimbriae presumably lack the multiple adherence sites needed to cross-link erythrocytes. Cheney and Boedeker (1983) observed CFA/I-promoted adherence to brush borders of intestinal cells obtained from a single human patient. Knutton et al. (1983) reported that the CFA/I producing strain H10407 adhered to duodenal but not to colonic enterocytes in an adherence assay using isolated human enterocytes from several independent small biopsy samples. Knutton et al. (1984a) observed CFA/I-mediated adherence to enterocyte microvillar surfaces, in contrast to type 1 fimbrial adherence to basolateral surfaces. Of 39 human ETEC isolates tested, all 14 which expressed CFA/I or CFA/II adhered to human duodenal enterocytes, whereas the remaining 25 strains were nonadherent at this site (Knutton et al., 1985). Loss of CFA/I by serial

culturing of H10407 made the strain avirulent in human volunteers (Satterwhite et al., 1978).

CFA/I expression is spontaneously lost at a relatively high frequency in the laboratory, which is typical of unselected plasmid-borne characteristics (Evans et al., 1975, 1978, 1979; Evans and Evans, 1978). Other data support the plasmid location of CFA/I genes. CFA/I and ST synthesis are specified by plasmids of 80 to 100 kb in several O78 ETEC strains (Smith et al., 1979; Willshaw et al., 1982). CFA/I and CFA/II were associated with nine of 36 ETEC strains, whereas all of 74 non-ETEC strains lacked these antigens (Levine et al., 1983). Plasmids were mobilised into K-12 laboratory strains of E. coli and expressed CFA/I antigen (McConnell et al., 1981). Smith et al. (1982) mapped the genes to two distinct locations on a CFA/I plasmid, each of which was separately cloned into different plasmid vectors (Willshaw et al., 1983). The region including the CFA/I subunit gene was closely linked to the ST genes. The compatible nature of the vector plasmids enabled them to show full complementation of the two regions following cotransformation into E. coli K-12. They later subcloned the genes (Willshaw et al., 1985a). Reis et al. (1980) mobilised the nonconjugative CFA/I ST plasmid from H10407 and obtained expression in K-12. The same 100 kb plasmid was independently characterised (Yamamoto and Yokota, 1983) and transferred to E. coli K-12 and several other Enterobacteriaceae (Yamamoto et al., 1984). It was subsequently transferred into an attenuated strain of Salmonella typhi used as a live oral vaccine (Yamamoto et al., 1985). The resultant heterologous strain expresses Salmonella and CFA/I antigens on its surface and may prove useful to vaccinate humans simultaneously against salmonellosis and ETEC diarrhoea caused by CFA/I-bearing strains. Immunisation with purified CFA/I has been found to protect rabbits against infection with a CFA/I ETEC strain (de la Cabada et al., 1981), and a combined CFA/I and LT immunisation was successfully used in an adult rabbit reversible intestinal ligature diarrhoea model (Ahren and Svennerholm, 1985). It is interesting to note that a monoclonal antibody raised against purified CFA/I fimbriae reacted to K99 fimbriae in an ELISA test, although not in a less sensitive immunoblot method, indicating a possible degree of antigenic cross reaction (Worobec et al., 1983).

1.4.9 Colonisation factor antigen II (CFA/II)

Evans and Evans (1978) described a colonisation factor antigen (CFA/II) which differed serologically from the first isolated CFA (renamed CFA/I) on a high proportion of human ETEC isolates of various serotypes. The antigen was shown to be plasmid-encoded (**Penaranda et al.,** 1980) and to promote MRHA of bovine blood and MR adherence to infant rabbit and human small intestinal enterocytes (**Knutton et al.,** 1985), but was spontaneously lost at a high frequency, presumably a result of plasmid instability. The CFA/II antigen initially referred to as a single entity by **Evans and Evans** (1978) is actually composed of three antigenically distinct components, referred to as coli surface antigens 1, 2 and 3 (CS1, CS2 and CS3), whose expression depends on the host cell biotype (**Smyth,** 1980; **Smith et al.,** 1983b). CS3 is expressed in all biotypes, whereas CS1 is expressed only in strains of biotype A (rhamnose-negative) and CS2 only in biotypes B, C and F (rhamnose-positive). Rhamnose fermentation and CS expression are not absolutely linked, however (**Boylan and Smyth,** 1985). **Cravioto et al.** (1982) independently named the same antigens 'components 1, 2 and 3'. Erythrocytes taken from particular calves were variously agglutinated. CFA/II mediates adherence of bacteria to human duodenal enterocyte brush borders in vitro (**Knutton et al.,** 1985), but neither CFA/I nor CFA/II is absolutely required to caused diarrhoea in humans (**Levine et al.,** 1980) implying the presence of other colonisation factors (**Levine et al.,** 1983).

Smyth (1982) determined the subunit sizes of the individual components as 16.3 (CS1), 15.3 (CS2) and 14.8 kd (CS3), while **Levine et al.** (1984) described the subunit molecular weight of CS1 as 16.8 kd [the same, within experimental error] and purified CS3 to proteins of 14.5 and 15.5 kd [judging by the size, the 15.5 kd band may in fact have been CS2]. CS1 consists of 6 to 7 nm diameter rigid fimbriae (**Mullany et al.,** 1983; **Levine et al.,** 1984; **Smyth,** 1984). CS1 and CS2 fimbriae both appear as hollow structures like type 1 fimbriae (**Mullany et al.,** 1983; **Smyth,** 1984), while the amino terminal region of CS2 shows homology with that of CFA/I (**Klemm et al.,** 1985a). CS3 was initially thought to be nonfimbrial (**Mullany et al.,** 1983; **Smyth,** 1984), but more exacting electron microscopical analysis revealed flexible fimbrial structures, 2 nm in diameter and similar in appearance to K99 fimbriae (**Levine et al.,** 1984; **Knutton et al.,** 1984b). A 4.6 kb DNA fragment encoding CS3 was cloned (**Manning et al.,** 1985), but unfortunately the analysis of the clone was incomplete.

1.4.10 Other adhesins on human ETEC isolates

Several authors have reported finding adhesins on ETEC from human diseases which differ from CFA/I and CFA/II in terms of their antigenicity (i.e. no cross reaction to antisera against CFA/I or CFA/II), their adherence properties (HA pattern or receptor specificity), or their physico-chemical properties (subunit molecular weight, diameter, etc.; **Table 1.3**). Some of these adhesins may be related, but full comparisons with other antigens have not been published. It should not be too surprising to find adhesins on human ETEC isolates differing from CFA/I and CFA/II. After all, there are many different molecules exposed along the lumenal surface of the human gut which may potentially serve as receptors for different types of adhesin, and being highly immunogenic and exposed molecules, they seem likely to be subject to strong selective pressures (**chapter 6**).

Levine et al. (1980) screened 24 virulent ETEC and EPEC strains for type 1 fimbriae (by MSHA of guinea pig erythrocytes), CFA/I (by MRHA of human blood and with antibodies) and CFA/II (only by testing MRHA of bovine blood). Six strains were found to lack CFA/I and CFA/II, and it was therefore presumed that they express other factors promoting their colonisation of diseased humans. Similarly, **Cravioto et al. (1982)** found nine of 205 human ETEC isolates with neither CFA/I nor CFA/II, but capable of MRHA of human or bovine blood. In contrast, **Knutton et al. (1985)** found that all the ETEC strains they tested which lacked CFA/I or CFA/II were nonadherent to human duodenal enterocytes, presumably due to the lack of expression of colonisation factors (adhesins), at least for this part of the gut. Furthermore, one CFA/I-positive strain did not adhere in the same assay, implying that the presence of CFA's of one particular type need not necessarily result in the usual adherence profile in vivo. This finding is similar to results in other systems which indicate that HA activity and fimbrial structures can be dissociated.

Thomas et al. (1982) discovered an antigen on human ETEC isolate E8775 which promoted MRHA of human and bovine blood, but was antigenically unrelated to CFA/I and CFA/II. The E8775 adhesin was also found on some other serotypes of ETEC (but not EPEC) strains, but two thirds of 458 ETEC isolates lacked CFA/I, CFA/II or E8775 (**Thomas and Rowe, 1982**). 'Putative colonisation factor' 8775 (PCF8775) is composed of three coli surface (CS) components, of which CS4 and CS5 are fimbrial (**Thomas et al., 1985**) and CS6 is fibrillar (S. Knutton, personal communication).

Darfeuille et al. (1983) reported the presence of a fimbrial adhesin on strain 1373, an O128 ST$^+$ LT$^+$ ETEC isolate from a diseased child in Senegal. The MRHA profile was similar to that of CFA/I except that it was resistant

to inhibition by N-acetyl neuraminic acid whereas HA caused by CFA/I or CFA/II was sensitive (Evans et al., 1979). The fimbriae are 7 nm rods which differ antigenically from CFA/I and CFA/II. The 16 kd subunit adhesin, probably encoded by a large plasmid (185 kb), was considered to be a new colonisation factor antigen ('CFA/III') although there is as yet insufficient evidence to confirm that it is actually involved in disease as a colonisation factor. The strain may possess CFA/I (unsubstantiated comment of Gross and Rowe, 1985a).

Forestier et al. (1984) found that low concentrations of certain antibiotics, below the level which would inhibit growth of the organisms, inhibited MRHA caused by organisms carrying CFA/I (strain H10407), CFA/II (strains Pb176 and 41) and CFA/III (strain 1373). They claimed that the effect was not due to inhibition of synthesis of the adhesins [their unpublished data], nor to direct inhibition of the HA reaction by the antibiotic since addition of antibiotics to the HA tests did not interfere with the reaction in control experiments [their data not shown]. Their research has implications in the treatment of intestinal and possibly other diseases with relatively low amounts of antibiotics to interfere with tissue adherence, and it is unfortunate that the results were not published in full.

Honda et al. (1984) discovered fimbriae resembling type 1 fimbriae in terms of size, but with no ability to haemagglutinate human or bovine blood, on strains they classified as 'type III' by HA profile. These 'CFA/III' fimbriae are heat stable hydrophobic structures, which were successfully purified by similar means to those used for other fimbriae. There was no immunological cross reaction with CFA/I or CFA/II (no other adhesins were tested), and the authors were evidently unaware of the prior use of the term 'CFA/III' for strain 1373. The subunit size is 18 kd, and the fimbriae promoted some adherence to infant rabbit and mouse intestines, although human gut was not tested.

Darfeuille-Michaud et al. (1986) identified an adhesin on ETEC strain 2230 (O25:K?:H16:ST) which mediated adherence to human duodenal enterocytes but no MRHA with several species' erythrocytes. The 16 kd subunit protein was encoded on a 106 kb plasmid, and apparently lacked fimbrial structure by electron microscopical analysis of negatively stained sections (Forestier et al., 1987).

1.4.11 EPEC adhesins

The ability to adhere to human enterocytes is, not surprisingly, a common feature of EPEC isolates which are therefore also termed enteroadherent **E. coli** (EAEC; **Matthewson et al.**, 1985). Such strains generally adhere to HEp-2 cells (whereas most ETEC strains are nonadherent) and the adhesins responsible are a current area of interest. Two distinctive patterns of adherence, localised and diffuse, are formed on HeLa (**Scaletsky et al.**, 1984) or HEp-2 cells (**Nataro et al.**, 1985a, 1985b). Localised adherence (LA) leads to the appearance of discrete microcolonies and is usually plasmid encoded (**Nataro et al.**, 1985a). A 96 kb plasmid encoding LA through an adhesin termed EPEC adherence factor (EAF) was found to be a virulence factor causing diarrhoea in human volunteers who ingested isogenic strains with or without the plasmid (**Levine et al.**, 1985), although the presence of plasmid-borne virulence determinants other than adherence was not ruled out. The EAF genes seem to map to two distinct regions of the plasmid, separated by up to 38 kb (**Baldini et al.**, 1986), similar perhaps to the two regions encoding CFA/I (**Willshaw et al.**, 1983). The ability of an O26:K60:H11 EPEC isolate from an infant with diarrhoea to adhere to human foetal small intestine (**Williams et al.**, 1977) was transferable to E. coli K-12 by a conjugative 90 kb plasmid (**Williams et al.**, 1978a, 1978b). In diffuse adherence (DA), bacteria adhere to most of the cultured cell surfaces. DA has also been transferred to a K-12 strain on a plasmid (**Levine et al.**, 1985).

Electron microscopy shows that enteritis caused by EPEC involves close approach of the bacteria to the host mucosal membranes and effacement of microvilli (i.e. detachment and loss of structural integrity; **Rothbaum et al.**, 1982). Fimbriae do not appear to be involved in the process of adherence to HEp-2 cells (**Scotland et al.**, 1983a), but it is likely that the specificity of the disease results from specific adherence to epithelia mediated by adhesins similar in function, if not in structure, to fimbriae (**Boedeker**, 1982).

E. coli strain RDEC-1 causes similar symptoms in rabbits to those caused by EPEC in humans, and is used as a model system (**Cantey and Blake**, 1977). The strain does not produce classical enterotoxins but causes pathological changes to rabbit intestinal brush borders which mimic the human condition (**Takeuchi et al.**, 1978; **Ulshen and Rollo**, 1980). Bacteria adhere to isolated rabbit brush borders (**Cheney et al.**, 1979), possibly due to the presence of capsules (**Cantey et al.**, 1981) although fimbriae encoded by a 137 kb plasmid (**Inman and Cantey**, 1984) also correlate with MR adherence to rabbit mucosa (**Cheney et al.**, 1983).

1.4.12 UPEC adhesins

A high proportion of E. coli isolated from human urinary tract infections (UTI), ranging from pyelonephritis to covert (asymptomatic) bacteriuria, express fimbriae with MR binding specificity for human erythrocytes (MRHA), usually in addition to type 1 fimbriae causing MSHA and virulence determinants such as haemolysin and aerobactin (**Hagberg et al.,** **1981; Vaisanen et al., 1981; Parry et al., 1983; Svanborg-Eden et al., 1983;** **Vaisanen-Rhen et al., 1984**). Comparisons of the MRHA of different blood groups indicated that most UTI-associated adhesins, especially those of pyelonephritis, are specific for P-blood group receptors (**Kallenius et al.,** **1980**). The 'P' phenotype corresponding to expression of the receptor activity is almost universally possessed, except by a few hundred people with the 'p̄' phenotype who lack the receptor and whose blood is therefore not agglutinated in the presence of mannose by most uropathogenic E. coli. The P receptor moiety has been isolated and characterised using glycolipid preparations from erythrocytes and synthetic oligosaccharides; it consists of a minimal digalactoside fraction (**Kallenius et al., 1981, 1982**). Certain individuals who are prone to recurrent UTI possess greater densities of the P receptor on their uroepithelial cells which may explain their unfortunate predisposition if P-specific adhesins (generally P-fimbriae) have a role in UTI (**Svenson and Kallenius, 1983**). Other data confirm the proposed activity of P-fimbriae in promoting UTI, probably by mediating adherence to uroepithelial cell surfaces and thus promoting colonisation of the urinary tract, although reduced phagocytosis of P-fimbriated E. coli may also be involved (**Svanborg-Eden et al., 1984**).

At least eight serologically distinguishable P-specific adhesins are recognised, designated F7 to F13 including two variants of F7 (**Orskov and** **Orskov, 1983; Uhlin et al., 1985a**), although there are considerable similarities between several of the gene clusters (**Uhlin et al., 1985a**), allowing some complementation (**van Die et al., 1986a, 1986b**). In addition, there are P-independent adhesins which cause MRHA of both P and p̄ erythrocytes, which are named according to the receptor where known (e.g. M-fimbriae, whose receptor is part of the M blood group system; **Jokinen et** **al., 1985;** and S-fimbriae, which adhere to sialyl moieties; **Korhonen et al.,** **1985**) or otherwise simply as X-adhesins (**Vaisanen et al., 1981**), and some P-fimbriae which have not yet been assigned F numbers.

F13. The genes specifying F13 fimbriae were cosmid cloned from pyelonephritis strain J96 (**Hull et al., 1981**). Homologous sequences were found in 60% of extraintestinal E. coli isolates but not in any other

Enterobacteriaceae (**Hull et al., 1984**). A functional MRHA-encoding region was located on the chromosome of J96 near <u>serA</u> (serine biosynthesis), <u>thy</u> (thymidine synthesis) and <u>kpsA</u> (required for synthesis of various polysaccharide capsules) loci at approximately 63 minutes on the <u>E. coli</u> K-12 chromosomal map (**Hull et al., 1986**), and a homologous region was found at approximately 85 minutes linked to the <u>hly</u> (haemolysin) locus (**Hacker et al., 1983; Low et al., 1984**). The cloned genetic system is complex, spanning some 15 kb of chromosome and including at least nine <u>pap</u> (pili associated with pyelonephritis) genes, named <u>papA</u> to <u>papI</u>. By molecular analysis of the system, functions have been assigned to most of the genes:

<u>papA</u> - transposon insertions (**Normark et al., 1983**), frameshift mutations (**Lindberg et al., 1984**) or deletions (**Uhlin et al., 1985b**) of <u>papA</u> abolish fimbrial synthesis but not P-specific MRHA or uroepithelial cell adherence. The <u>papA</u> DNA sequence translates to a 16.5 kd, 163 amino acid mature pilin (subunit protein), plus a 22 amino acid amino-terminus signal sequence in the pre-pilin (**Baga et al., 1984**). Growth temperature regulates expression of F13 fimbriae through reduced transcription of the <u>papA</u> gene at low temperatures, hence reducing expression of <u>papA-lacZ</u> fusion constructs (**Goransson and Uhlin, 1984**).

<u>papB</u> - autoregulated positive regulator for <u>papA</u>, with a probable catabolite repression site located upstream of the promoter (**Baga et al., 1985**).

<u>papC</u> - 81 kd outer membrane protein, probably acts as an export and anchor protein for assembling fimbriae. Mutants accumulate fimbrial subunits intracellularly.

<u>papD</u> - mutants behave similarly to <u>papC</u> mutants in being unable to secrete fimbrial subunits, but minicell analysis indicates that expression of <u>papA</u>, <u>papE</u> and <u>papF</u> is much lower than in wild type cells or <u>papC</u> mutants. <u>papD</u> may encode parts of the export system causing either increased stability of the subunit protein, or perhaps coordinated synthesis and export, but the exact function of the product is presently uncertain.

<u>papE</u> - mutant bacteria have unaltered binding properties, but fimbriae purified from them do not cause MRHA. The 16.5 kd protein product may perhaps determine presentation of the fimbriae at the bacterial surface (**Uhlin et al., 1985a**).

<u>papF</u> and <u>papG</u> - mutations in either or both of these genes prevent P-specific adherence yet fimbriae continue to be produced (**Norgren et al., 1984; Lindberg et al., 1986**), implying that their products are the actual adhesins of the F13 system. They encode proteins in minicells of 15 and 35 kd respectively (**Lund et al., 1985**), which are not visible in

haemagglutinating 'pure' F13 preparations analysed by SDS-PAGE and protein staining, but may possibly be visualised by reaction with anti-F13 serum (Uhlin et al., 1985b, and unpublished data cited by Uhlin et al., 1985a).

papH - the location of this gene was initially indicated by the finding of an open reading frame in the DNA sequence near papA (Baga et al., 1985). Subsequently, the 22 kd protein product, which resembles pilin, has been implicated in pilus assembly (F. Mooi, personal communication). Mutants in papH retain the capability for HA but their pili are longer than normal and are readily released into the growth medium. Therefore, the protein may be necessary for anchorage of the pili and for correct termination of synthesis or assembly. papH is transcribed at a low level, probably by transcription initiating from the papB promoter, reading past the papA gene and through a transcriptional terminator between papA and papH.

papI - 12 kd protein product is a positive regulator of the papB gene.

F7. UPEC strain C1212 (O6:K?:H?), isolated from a patient with pyelonephritis (Orskov et al., 1980b), expresses haemolysin and MRHA with weak MSHA activities due to the presence of multiple adhesins (type 1, 1C, and F7; Klemm et al., 1982). Cosmid cloning of random 40 kb chromosomal fragments revealed that the F7 fimbriae are composed of two distinct types encoded by separate determinants, one of which was located to within 4 or 5 kb of the haemolysin genes (Low et al., 1984). The F7 variants have been genetically characterised in detail:

F7$_1$. Genetic determinants for F7$_1$ were cosmid cloned from strain AD110 (O6:K2:H1) from a patient with acute cystitis (van den Bosch, 1980) and encoding multiple fimbriae (type 1, 1C, and two F7 variants, F7$_1$ and F7$_2$; Jann et al., 1981). At least six genes were identified (van Die et al., 1985). The restriction map was similar to that of F7$_2$ fimbriae and genes from the two systems complemented. The gene for the subunit protein encodes 166 amino acids plus an extra 21 residue amino-terminus export signal sequence, with homology to the nucleotide sequence of the F13 Pap subunit, and to that of type 1C fimbriae (section 1.4.2).

F7$_2$. Cosmid cloning of F7$_2$ genetic determinants from strain AD110 (van Die et al., 1983b) allowed the identification of at least five genes by transposon mutagenesis and complementation (Hoekstra et al., 1984; van Die et al., 1984b). Gene A encodes the 17 kd 'prefimbrillin' of 188 amino acids plus a 21 residue signal sequence (van Die and Bergmans, 1984). Gene B specified a 75 kd protein, analogous to the large outer membrane protein identified in other adherence systems, while the mature products of genes C

and E (of 23 and 36 kd) were synthesised with signal sequences and were therefore probably exported from the cytoplasmic membrane to the periplasm, outer membrane, or cell exterior. The product of gene D was not seen by SDS-PAGE and staining. The region around genes B and C shows homology to analogous regions of the determinants of F13 and strain IA2 fimbriae (**Uhlin et al.**, 1985a).

KS71. Another UPEC isolate, KS71 (O4:K12:H?), encodes four fimbrial antigens - type 1 (KS71D), type 1C (KS71C), and two P-fimbriae, one (KS71A) with identical subunits to those of $F7_2$ and the other (KS71B) probably the same as $F7_1$ (**Klemm et al.**, 1982; **Rhen et al.**, 1983a, 1983d, 1985a, 1986; **Rhen**, 1985). The genes for KS71A, -B and -C were cloned (**Rhen et al.**, 1983b) and as expected by comparison with the F7 systems, KS71A and -B are genetically related but are quite different from KS71C (**Rhen et al.**, 1985b).

F8. UTI reference strain C1254 encodes an adhesin promoting MRHA of human, monkey and sheep erythrocytes (**Orskov and Orskov**, 1983). F8 is particularly associated with O18:K5 UPEC strains.

F9. The genetic determinants have been cloned from UPEC strain C1018 in cosmids (**de Ree et al.**, 1985a). The 21 kd subunit adhesin promotes MRHA of human, monkey and sheep blood.

F10. This is the only P-adhesin in the F7 to F13 series which loses its MRHA activity (for human and monkey blood) upon incubation at 100°C for an hour.

F11. Less than 9 kb of DNA, cosmid cloned from E. coli C1976, encodes the F11 adhesin (**de Ree et al.**, 1985b).

F12. Reference strain C1979 promotes MRHA of human, monkey and sheep blood (**Klemm et al.**, 1983).

Other P-fimbriae. Strain IA2 (O6:H-) from 'acute UTI' encodes P-fimbriae which were cosmid cloned (**Clegg**, 1982) and mapped (**Clegg and Pierce**, 1983). MRHA and fimbrial synthesis were separately encoded (**Lund et al.**, 1985). Hybridisation of the cloned functional region to the chromosome of IA2 revealed multiple copies of homologous DNA (**Hull et al.**, 1985) and homology with other P-fimbriae (**Uhlin et al.**, 1985a).

S-fimbriae. S-fimbriae specifically recognise sialyl residues on sialoglycoproteins (**Korhonen et al.,** 1984). Therefore, neuraminidase pre-treatment of erythrocytes prevents S-fimbrially-mediated HA. The determinants of S-fimbriae have been cloned from a UPEC strain which also expressed F8, type 1 and type 1C fimbriae (**Hacker et al.,** 1986). 6.5 kb of DNA was sufficient to encode fimbrial synthesis and HA by laboratory E. coli strains. Five cistrons and three promoters were identified, and the fimbrial subunit protein was found to be 16.5 kd in size. Certain transposon insertions created haemagglutinating but non-fimbriate mutants, indicating that, as in some other systems, adherence to erythrocytes can be demonstrated in the apparent absence of fimbrial structures so these functions are presumably expressed independently.

X-adhesins with no identified receptors (**Vaisanen et al.,** 1981):

Vaisanen-Rhen (1984) cloned the genes encoding an X-adhesin from an O75 strain in a cosmid vector. She subcloned the genes and delimited the region to 3 kb of DNA, with homology to some other O75 strains. The structure was described as 'fimbria-like', having very fine fibrils.

Berger et al. (1982) cloned an adherence system from a uropathogenic E. coli strain which is of the X-adhesin type (**Uhlin et al.,** 1985a).

Hacker et al. (1985) cloned the determinant for a 16.5 kd subunit adhesin from an O6:K15:H31 urinary tract isolate of E. coli, and expressed the fimbrial adhesin antigen in both E. coli K-12 and a Salmonella typhimurium galE (rough) mutant. Fimbrial expression was not absolutely required for MRHA in E. coli.

E. coli strain GV-12 (O1:H-), described as 'a clinical isolate' but presumably from UTI, elaborated a non-fimbrial MR haemagglutinin for human blood with an aggregated molecular weight of approximately 200,000, and a dissociated subunit of 16.3 kd (**Sheladia et al.,** 1982).

Labigne-Roussel et al. (1984) cosmid cloned the plasmid-borne afimbrial adhesin system 'AFA-I' from uropathogenic E. coli strain KS52. The cloned region was mapped by restriction analysis, and deletions and transposon insertions were used to define a functional map (**Labigne-Roussel et al.,** 1985). The adhesin was readily released from cells into the culture supernatant, and bound to HeLa cells (**Walz et al.,** 1985). The subunit size was 16 kd. [See also **chapter 6**].

1.4.13 MePhe adhesins

Fimbrial subunits from several pathogenic bacteria (including Bacteroides nodosus, Moraxella bovis alpha- and beta-pilins, Moraxella nonliquefaciens, Neisseria gonorrhoeae and Pseudomonas aeruginosa) have unusual amino-terminal residues, namely N-methylated phenylalanine (MePhe; McKern, 1983, 1985; Marrs et al., 1985; Sastry et al., 1985; Elleman et al., 1986a, 1986b). They are also nearly identical over the first 30 or so amino-terminal amino acids, and fairly similar at their carboxy termini. The subunits are all synthesised with 'leader' sequences of six or seven amino acids, including one or two with positive charges, which are cleaved to yield mature subunits of approximately 18 kd. Taken together, these data indicate a remarkable degree of similarity in fimbriae from such diverse organisms. The determinants of some of the MePhe group of fimbriae have been genetically analysed:

Neisseria gonorrhoeae. Gonococci exhibit fimbrial phase variation (reflected in altered colonial morphology) and antigenic variation (**Cannon and Sparling,** 1984; So et al., 1985), as well as variable expression of so-called opacity proteins (**section 1.3.9**). Variant fimbrial subunits share common amino-termini sequences, but differ in the rest of the proteins (**Rothbard** et al., 1984), which contain the main antigenic determinants (**Hagblom** et al., 1985) and probably mediate adherence to human tissue (**Virji and Heckels,** 1984). Fimbriae of strain MS11 are expressed from two chromosomal expression sites separated by about 25 kb and close to an opacity protein determinant (**Stern** et al., 1984). Antigenic variation is accomplished by chromosomal rearrangements (**Meyer** et al., 1982), involving movement of 'silent' subunit genes to expression sites (**Meyer** et al., 1984; **Stern** et al., 1984), probably in addition to regulation by a diffusible product (**Segal** et al., 1985). Recent data indicate that minor rearrangements within the subunit genes can also prevent expression of individual subunits (**Bergstrom** et al., 1986).

Bacteroides nodosus. These bacteria, the causative agents of ovine foot rot, synthesise 5 to 6 nm diameter fimbriae which are readily released from the cells and are unusually long (up to 15 μm; **Every,** 1979). Proteins of 17 and 80 kd were resolved by SDS-PAGE analysis of released fimbriae, corresponding to the fimbrillin (fimbrial subunit) and 'basal structure', visible by electron micrography as globular masses at the ends of fimbriae (**Mattick** et al., 1984). The basal structures are perhaps analogous to the large outer membrane protein usually encoded by E. coli fimbrial systems. At least three

groups have cloned the B. nodosus fimbrial determinants in E. coli. Anderson et al. (1984) detected subunits in an E. coli plasmid library containing total DNA from B. nodosus strain 198 by an immunoassay, but the basal protein was missing and fimbriae were not assembled. Elleman et al. (1984) also detected subunits by immunologically screening a plasmid library from the same strain. The subunit DNA sequence (Elleman and Hoyne, 1984) corresponded to 151 amino acids in agreement with the known amino acid sequence of the mature fimbrillin but with an extra 7 residue amino-terminal extension. The 5'-end of the gene was always located near the end of the random Sau3A fragments cloned, whereas the downstream end was far more variable. This, they suggested, may have been due to the presence of a gene encoding a lethal product, perhaps the basal structure, proximal to the start of the subunit gene. Elleman et al. (1986a) used a radiolabelled mixture of M13 clones of the fimbrillin gene from strain 198 to identify an homologous 3.2 kb HindIII fragment in the genome of strain 265. They then cloned 3 to 4 kb HindIII fragments in plasmids in E. coli and detected fimbrillin clones by hybridisation and immunoblotting. The DNA sequence, as expected, was highly related to the equivalent gene from strain 198. No promoters were identified, but the end of another open reading frame was found close to the 5'-end of the fimbrillin gene so they may be encoded on a common polycistronic message. Over-expression of the fimbrial subunit of strain 198 from trp or lambda P_R promoters in E. coli did not produce assembled fimbriae but resulted in cell death (Elleman et al., 1986a). Unfortunately, these clones gave only poor or no protective action when used to vaccinate sheep against foot rot. Recently, Boulos and Rood (1986) detected fimbriae by immune-screening their E. coli plasmid library containing DNA from benign B. nodosus strain AC/6. Perhaps they will have more luck in expressing and assembling fimbriae for vaccine production...

Moraxella bovis. Marrs et al. (1985) generated a total DNA library containing 100,000 lambda clones in E. coli, and screened it using a synthetic oligonucleotide probe containing the 5' end of the Neisseria gonorrhoeae pilin gene. The probe was sufficiently homologous to hybridise to the M. bovis pilin sequence in six clones. The subunit protein was mostly located in the cytoplasmic membranes of these clones and was not exported and assembled into fimbriae. Southern blotting experiments to determine the number of copies of the pilin gene(s) in the chromosome of M. bovis were inconclusive; under highly stringent conditions, the probe hybridised to a single band, whereas multiple bands reacted as the stringency was reduced. Thus, related but significantly diverged sequences may be present in the genome.

1.4.14 Adhesins of other bacterial species

Adhesin systems from a few bacteria other than those already described have been genetically characterised. Rather more have been only partially characterised so far (Table 1.4).

Actinomyces viscosus. These bacteria adhere to streptococci in human dental plaque by fimbrial adhesins. **Donkersloot et al.** (1985) screened a cosmid chromosomal library in E. coli with antiserum raised against intact A. viscosus cells, and identified a clone synthesising 'type 2 fimbriae' but none synthesising 'type 1 fimbriae'. The subunit molecular weight was said to be 59 kd, which is considerably larger than E. coli fimbriae. Further molecular genetic data on this adhesin and those of other dental pathogens have yet to be published.

Yersinia enterocolitica. Bacteria of this pathogenic species are related to the other Enterobacteriaceae, and cause diarrhoeal disease in humans (Table 1.3). Plasmids from virulent strains of Y. enterocolitica were mobilised by cointegration of a mobilisable vector, and transferred into naturally avirulent isolates and avirulent mutants of previously virulent strains (**Heeseman et al.,** 1984). Two distinct groups of traits were evident: (a) mouse lethality and the ability to promote conjunctivitis were only expressed in the avirulent mutants; and (b) calcium dependency, surface agglutinogens, temperature-induced release of proteins from the outer membrane and HEp-2 association were expressed in all recipients. This was taken to indicate that characteristics of type (a) involve both plasmid and chromosomally located virulence determinants, while the others were solely plasmid functions. However, I am unconvinced by this rather simplistic approach, since it requires one to assume that wild avirulent strains are devoid of all virulence determinants, and that avirulent mutants obtained in vitro only lack plasmid-borne virulence characters. I trust that molecular analysis of the plasmids, performed either in the same species or in E. coli, will prove to be a more reliable source of data, particularly, from my point of view, concerning the adherence characteristics of this pathogen.

1.5 PREVIOUS FINDINGS ON STRAINS 444-3 and 469-3

E. coli strains 444-3 (O?:H4) and 469-3 (O21:H-) were isolated from children with severe enteritis and dysentery-like symptoms (**Rudoy and Nelson, 1975**). Their serotypes do not correspond to any of the conventional E. coli pathogenic groupings (EIEC, EPEC, ETEC, UPEC). The disease symptoms (mucosal damage leading to bloody diarrhoea) were similar to those caused by EIEC and Shigella dysenteriae, so the strains were tested for invasive properties, but they gave conflicting results in the guinea pig keratoconjunctivitis test (Sereny test; **Sereny, 1957**) and it was therefore not clear if the strains might be truly invasive (**Nandadasa et al., 1981**). The Sereny test involves a complex sequence of pathogenic processes (penetration of and multiplication in the corneal epithelium, plus tissue damage leading to the overt symptoms) and does not accurately model all aspects of invasive disease, raising the possibility that these strains might differ from accepted invasive strains in some unknown function in the test but are invasive in vivo. The research group examined the adherence and invasive characteristics in more detail to help resolve this point. An in vitro test was developed to assay firm association (surface adherence or invasion) of bacteria to cultured human cell monolayers. Strains 444-3 and 469-3 gave similar results to reference strains defined as invasive by the Sereny test (approximately one bacterium per cultured HEp-2 human epithelial cell after incubation together at 37°C for three hours and thorough washing) whereas CFA/I$^+$ ETEC reference strain H10407 and an O26 EPEC strain were negative (usually no bacteria associated with each HEp-2 cell). In this context, it is interesting to note that some UPEC strains were also capable of invading HeLa cells but were negative in the Sereny test (**Varian and Cooke, 1980**).

The in vitro test was subsequently modified by treating the cultured cells after being in contact with bacteria with antibiotics (streptomycin and kanamycin) which do not readily enter HEp-2 cells, thereby selectively killing non-internalised bacteria (**Knutton et al., 1984c**). Some cells of both 444-3 and 469-3 were protected from the antibiotics and could be seen inside membrane bound vesicles near the surface of the cultured cells by thin section electron microscopy. Maximum internalisation was reached after incubation for eight hours, at which time some 5% of total associated bacteria (adherent plus internalised cells) were protected from the antibiotics. These data were still insufficient to classify the strains as EIEC, however, since the remaining 95% of bacteria did not invade, and furthermore the assay uses human cells which clearly differ from intestinal epithelial cells in vivo (HEp-2 cells derive from a human laryngeal

carcinoma and the 'transformed' cell line is capable of continuous deregulated growth under appropriate in vitro conditions; Toolan, 1954). The strains also lack large plasmids similar to those reported to be essential for tissue invasion by Shigella strains (Sansonetti et al., 1981, 1982, 1983; Hale et al., 1983), by EIEC (Harris et al., 1982) or by Salmonella typhimurium (Jones et al., 1982). Strains 444-3 and 469-3 may perhaps be regarded as facultatively enteropathogenic (Czirok et al., 1976) or even facutatively enteroinvasive, capable either of benign colonisation of colonic mucosa or of virulent growth with production of dysentery-like symptoms in vivo.

Both strains expressed MSHA activity for guinea pig blood, and rigid 7 nm diameter rod-like type 1 fimbriae associated with MS adherence were demonstrated by electron microscopy (Knutton et al., 1984c). The strains were also capable of promoting MRHA of human blood (but not the blood of other animals out of a wide range tested) which progressively eluted from a maximum at 0°C to nil at 50°C but recovered on cooling (Williams et al., 1984) and are therefore of the MRE (mannose-resistant and eluting) class (Duguid et al., 1979; Duguid and Old, 1980). However, there was no unequivocal evidence of fimbrial structures associated with MR adherence, and the adhesins were thus termed nonfimbrial. Smooth surfaced bacteria were seen to lie very closely apposed to the membranes of cultured cells, separated by small gaps but with apparent areas of membrane contact (Knutton et al., 1984c). Both strains adhered only weakly to human duodenal enterocytes (Knutton et al., 1982). Carbohydrate capsules were visualised by light microscopy of bacteria suspended in India ink. A proportion of bacteria in a culture excluded the ink from a halo extending about a micrometre around each cell. Polyanion specific stains (including ruthenium red) labelled the capsules but the preparative techniques required for electron microscopy (particularly extreme dehydration) tended to cause the collapse of the material into clumps on the bacterial surface. Negative staining did reveal 2 nm diameter flexible fibrils up to 1 μm long, extending from the surfaces of some cells, but these were considered to be capsular material since they were quite unlike rod-like fimbriae. Further data were obtained using antisera to the MR adhesins, as outlined below.

Plasmid curing and bacterial mating experiments were performed to identify the location of the genes promoting MRHA and, presumably, HEp-2 cell association (Nandadasa et al., 1981). Multiple drug resistance plasmids were successfully transferred from either strain by conjugation with E. coli K-12, B/r, O1, O2, O4 or O6 strains, but neither MRHA nor HEp-2 association were expressed in recipients. Furthermore, strain 444-3, cured of its drug resistance plasmid by treatment with ethidium bromide, retained MRHA and

HEp-2 association properties (strain 469-3 contained a small cryptic plasmid in addition to the antibiotic resistance plasmid and so curing experiments were not performed). The MRHA and HEp-2 association determinants were thereby assigned chromosomal locations in 444-3 and probably in 469-3.

Mutants were derived by treatment with the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), followed by enrichment for nonhaemagglutinating mutants by successive cycles of agglutination of human blood and retention of the non-sedimented fractions (Williams et al., 1984). Nine mutants of 444-3 and three of 469-3 were negative for MRHA, and these were all nonadherent to HeLa cells and HEp-2 cells (Knutton et al., 1982, 1983, 1984c). They were apparently deficient in both MR adhesin synthesis and capsular synthesis, possibly because both structures promoted adherence to erythrocytes (Knutton et al., 1984c) or perhaps because of some coordinated expression system. A single mutant of 444-3 had reduced MRHA and HeLa adherence properties.

Nonadherent mutants were used to absorb out antibodies to other antigens from rabbit antisera raised against whole formalin-killed cells of 444-3 or 469-3 (Knutton et al., 1984c). The absorbed sera were incubated with cultures, then reacted with fluorescein-labelled secondary antibodies and prepared for UV light microscopy. Homologous antisera labelled approximately one quarter of the cells in a culture, but heterologous sera did not react. Antiserum-labelled cultures were also reacted with ferritin-conjugated secondary antibodies and thin sections examined by electron microscopy. Immunoferritin labelled a proportion of cells uniformly over the entire bacterial surface. All bacteria adherent to HEp-2 cells reacted with the antisera, confirming that the antigens causing MRHA were related or identical to those causing HEp-2 cell adherence.

The MR adhesins were purified from 444-3 and 469-3 by methods similar to those employed for the purification of fimbriae from other systems, i.e. mechanical shearing by homogenising whole cells and clearance of cell debris by centrifugation (Williams et al., 1984). The crude supernatants, containing most of the MRHA activities of the original strains, were then applied to Sepharose 4B-CL and 6B-CL columns and separated according to size under nondenaturing conditions. MRHA activity eluted as single peaks near the void volume in 4B-CL columns (exclusion limit 4,000 kd) but was not retarded by Sepharose 6B-CL (exclusion limit 20,000 kd), indicating aggregated sizes between these limits. In the presence of 0.2% SDS and following incubation at 65°C the aggregates dissociated, but removal of the SDS allowed reaggregation into structures of more than 20,000 kd with a significant fraction of the original MRHA activity. Purified preparations inhibited HEp-2 adherence by the homologous strains in an adhesin

dose-dependent manner but did not inhibit the reaction of heterologous strains, implying adherence to different receptors on the cultured cells. The aggregates were denatured by boiling with 0.2% SDS and electrophoresed through polyacrylamide gels or separated on Sephadex G-100 columns with 8 M guanidine hydrochloride. The subunits were single proteins of 14.5 kd (444-3) or 14 kd (469-3) relative molecular mass and isoelectric points (pI's) of 4.7 (444-3) or 5.0 (469-3). Their amino acid contents were broadly similar, with 43% (444-3) to 45% (469-3) nonpolar residues, being of the same order as those of several other adhesins.

The MR adhesins on these two clinical isolates were the subject of the work presented in this thesis...

Table 1.1 Microorganisms normally colonising humans [a]

| Microorganism | | Frequency of isolation from[b] | | | | | | |
Name	Type[c]	Skin	Eye	Mouth	Pharynx	Vagina	Ileum	Colon
Acinetobacter	G-	++			++	+		+/-
Actinomyces	F			++		+		+/-
Bacillus	G+	+						
Bacteroides	G-	+++		++	++	+++	+/-	++++
Bifid obacterium	G+	+++		++		+		+++
Candida	F	+/-		++		++	++	++
Campylobacter sputorum	G-				+/-	+/-		
Chlamydia trachomatis	C		+/-					
Clostridium	G+	+++		+		++	++	++
Corynebacterium	G+	+++		+	+++	+++	++	
Eubacterium	G+	+/-				+/-		
Enterobacteriaceae	G-	+/-	+/-	+++	+/-	++	++	++++
Enterococcus	G+			++		++++	++	++++
Escherichia coli	G-		+	+++	++			++++
Fusobacterium	G-			+/-	++			++++
Haemophilus influenzae	G-			+/-				
Herpes simplex	V	+/-						

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Table 1.1 continued. **Microorganisms normally colonising humans**[a]

Microorganism		Frequency of isolation from[b]						
Name	Type[c]	Skin	Eye	Mouth	Pharynx	Vagina	Ileum	Colon
Klebsiella	G−	+++						+++
Lactobacillus	G+			++++		++++	++	+++
Moraxella	G−	+			+	+		
Mycobacterium	G+	+/−	+/−	+/−		+/−		+/−
Neisseria	G−		+/−	+/−	+			
Propionibacterium acnes	G+	++++						++
Proteus	G−		+++					+
Pseudomonas	G−	+/−						+/−
Salmonella enteritidis	G−							+++
Staphylococcus (aerobes)	G+	++++	+/−	++++	+++	+++		+++
Staph. (anaerobes)	G+			+		++		+
Streptococcus (aerobes)	G+	+/−		++++	+++	++		++++
Strep. (anaerobes)	G+			++		++		+
Treponema	G−			+++				++++
Trichomonas vaginalis	P					++		
Veillonella alcalescens	G−			++++		+/−		+/−

Notes to Table 1.1

a) Adapted from **Mandell et al.** (1985) and **Youmans et al.** (1980). The table includes only a fraction of the total number of species which colonise humans, but most others (including many pathogens) are infrequent or opportunistic colonisers. In addition, the colon is colonised by a few bacteria of each of many other species (at least 113 species each comprise less than 0.05% of the total number of bacteria), and a poorly defined group of acid-secreting lactobacilli (known collectively as Doderlein's bacillus) inhabit the vaginas of sexually active women.

b) Approximate incidence:

++++	Almost always isolated (carried by 70 to 100% of humans).
+++	Usually isolated (" 40 to 70% " ").
++	Often isolated (" 10 to 40% " ").
+	Occasionally isolated (" 5 to 10% " ").
+/-	Rarely isolated (" less than 5% " ").

c) Type of microorganism: C...chlamydia, F...fungus, G+...Gram positive bacterium, G-...Gram negative bacterium, P...protozoan, V...virus.

Notes to Table 1.2

a) Types of microorganism: F...fungus, G+...Gram positive bacterium, G-...Gram negative bacterium, P...protozoan, V...virus.

b) Brief summary of known virulence characteristics and aetiological features.

Table 1.2 Infectious enteric diseases of humans

Microorganism name	Type[a]	Disease	Virulence characteristics[b]
Adenovirus	V	Diarrhoea	Replicate in intestinal epithelium.
Bacillus spp.	G+	Diarrhoea, vomiting	Emetic enterotoxin.
Balantidium coli	P	Dysentery	Ciliate infection from pigs.
Campylobacter jejuni	G-	Dysentery, fever	Mucosal invasion and damage. Toxin ?
Coronavirus	V	Infantile diarrhoea	Induces lactose intolerance.
Clostridium botulinum	G+	Botulism	Food poisoning from preformed neurotoxin. Haemagglutinin.
C. difficile	G+	Pseudomembranous colitis	Enterotoxin, cytotoxin. Follows antibiotic treatment.
C. perfringens	G+	Diarrhoea	Enterotoxin. Exotoxin inhibits polymorphs.
E. coli ETEC	G-	Travellers' diarrhoea	Fimbrial adhesins. Toxins (LT and ST). Capsules.
EPEC	G-	Infantile enteritis	Mucosal damage by unknown cytotoxins.
EIEC	G-	Dysentery	Invade colonic mucosa.
Entamoeba histolytica	P	Amoebic dysentery	Invade and damage mucosa. May reach liver.
Enterovirus	V	Diarrhoea	Replicate in mucosa.
Plasmodium spp.	P	Diarrhoea	Complication of systemic malaria.
Giardia lamblia	P	Diarrhoea	Attach to epithelium by mechanical sucker.
Rotavirus	V	Infantile enteritis	Replicate in mucosa.
Salmonella spp.	G-	Salmonellosis	Invade superficial layers. Enterotoxin. Capsule.
Shigella spp.	G-	Dysentery	Invade colonic mucosa. Cytotoxin is also neurotoxic.
Staphylococcus aureus	G+	Food poisoning	Neurotoxin. Enterotoxin. Capsule interferes with complement.
Vibrio cholerae	G-	Cholera	Fimbriae. CT. Mucinase. Chemotactic.
V. parahaemolyticus		Vomiting, diarrhoea	Food poisoning from contaminated fish. Enterotoxin.
Yersinia enterocolitica	G-	Yersiniosis	Invade epithelium. Toxin. May become systemic.
Y. pseudotuberculosis	G-	Enteritis	Brush border damage.

Table 1.3 Summary of characteristics of microbial adherence factors

Species	Adhesin or strain name[a]	Source animal	HA panel[b]	Size[c] (kd)	(nm)	Genes located[d]	Notes	Reference
Actinomyces viscosus	Type 2	Human		59		C	Plaque component	Section 1.4.14
Bacteroides nodosus	Strain 198	Sheep		17	8-9	C	Footrot. MePhe group	Section 1.4.13
	Strain 216	Sheep		15.9			Footrot. MePhe group	McKern et al. (1985)
	Strain 265	Sheep		17	5-6	C	Footrot. MePhe group	McKern et al. (1985)
Bordetella pertussis	Serotype 2	Human	None	22	5-6		Whooping cough	Steven et al. (1986)
	FHA	Human	S		(-)		Nonfimbrial ?	Ashworth et al. (1982)
Campylobacter jejuni								
Enterobacter cloacae	Type 1							Section 1.4.1
Escherichia coli All	Type 1 (F1A)	Any	G,etc.	17.1	7	C	Common, MSHA	Section 1.4.1
UPEC	Type 1B (F1B)	Human	None		7	C ?	Variant type 1	Section 1.4.2
UPEC	Type 1C (F1C)	Human	None		7	C ?	Variant type 1	Section 1.4.2
ETEC	K88ab (F4)	Pig	C,G	27.5	2.1	P	Fibrillar	Section 1.4.3
ETEC	K88ac (F4)	Pig	P	25	2.1	P	Fibrillar	Section 1.4.3
ETEC	K88ad (F4)	Pig	E,S	26	2.1	P	Fibrillar	Section 1.4.3
ETEC	K99 (F5)	Calf		18.4	5	P	Helical	Section 1.4.4
ETEC	987P (F6)	Pig	None	19-20	7	C ?	Some weak MRHA only	Section 1.4.5
ETEC	F41	Pig	G,H	29.5	3.2	C ?	Anionic, curly	Section 1.4.6
ETEC	ATT25	Cow	H		7		Helical	Section 1.4.7
ETEC	CFA/I (F2)	Human	H,B	15	3	P	Hollow ?	Section 1.4.8
ETEC	CFA/II CS1 (F3)	Human	B	16.3	6-7	P	Rigid, hollow ?	Section 1.4.9
ETEC	CFA/II CS2 (F3)	Human	B	15.3	6-7	P	Rigid, hollow ?	Section 1.4.9
ETEC	CFA/II CS3 (F3)	Human		14.8	2	P	Fibrillar	Section 1.4.9

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Species	Adhesin or strain name[a]	Source animal	HA panel[b]	Size[c] (kd)	(nm)	Genes located[d]	Notes	Reference
Escherichia coli								
ETEC	CFA/III	Human	B,C,H	16	7		Strain 1373	Section 1.4.10
ETEC	CFA/III	Human		18	7		'Type III' strains	Section 1.4.10
ETEC	E8775 (PCF8775)	Human	B,H				Three components	Section 1.4.10
ETEC		Human	H	13.1				Deneke et al. (1979)
ETEC	2230	Human	None	16	(-)		Nonfimbrial	Section 1.4.10
ETEC	018ac	Human	H	21	5			Wevers et al. (1980)
UPEC	F7$_1$	Human	H		7	C	P-specific	Section 1.4.12
UPEC	F7$_2$	Human	H	16?	7?	C	P-specific	Section 1.4.12
UPEC	F8	Human	H,M,S			C	P-specific	Section 1.4.12
UPEC	F9	Human	H,M,S			C	P-specific	Section 1.4.12
UPEC	F10	Human	H,M			C	P-specific	Section 1.4.12
UPEC	F11	Human	H			C	P-specific	Section 1.4.12
UPEC	F12	Human	H,M,S			C	P-specific	Section 1.4.12
UPEC	Pap (F13)	Human	H			C	P-specific	Section 1.4.12
UPEC	M-fimbriae	Human	H,(M?)			C	M-specific	Section 1.4.12
UPEC	S-fimbriae	Human	H	16.5		C	Sialyl-specific	Section 1.4.12
UPEC	GV-12	Human	H	16.3	(-)		Nonfimbrial	Section 1.4.12
UPEC	AFA-I	Human	H	16	(-)	P	'Afimbrial adhesin'	Section 1.4.12
UPEC	IA2	Human	H	17	7	C		Section 1.4.12
UPEC	075	Human	H	16	?	C	X-adhesin. Coiled	Vaisanen-Rhen (1984)

Table 1.3 continued. Summary of characteristics of microbial adherence factors

Species	Adhesin or strain name[a]	Source animal	HA panel[b]	Size[c] (kd)	(nm)	Genes located[d]	Notes	Reference
Escherichia coli UPEC	Protein capsule	Human	H	14.5	2		Fibrillar	Orskov et al. (1985)
	444-3 MR	Human	H	14.5	(?)	C	Enteritis	Section 1.5
	469-3 MR	Human	H	14	2	C ?	Colon-specific	Subject of thesis
Klebsiella pneumoniae	Type 1	Human	G	21.5		C ?		Purcell & Clegg (1983)
Moraxella bovis	EPP63 β-pili	Cow				C ?	MePhe group	Section 1.4.13
Neisseria gonorrhoeae	P9 α-fimbriae	Human		19.5	7	C	MePhe group	Section 1.4.13
	MS11	Human		Var.	7	C	MePhe group	Section 1.4.13
Proteus mirabilis	HU1069	Human	None	17.5	4+6		Bind uroepithelium	Wray et al. (1986)
Pseudomonas aeruginosa	PAO	Human		17.8	5.4	C	MePhe group	Paranchych et al. (1979)
	PAK	Human		15	5.4	C	MePhe group	Sastry et al. (1985)
Pseudomonas solanacearum	B1	(Plant)	H	9.5	6		Tobacco wilt	Young et al. (1985)
Salmonella typhimurium	Type 1	Mouse		21				Section 1.4.1
Serratia marcescens	Type 1							Section 1.4.1
Shigella	Type 1							Section 1.4.1
Streptococcus salivarius	Antigen B	Human		380			Long, flexible	Weerkamp et al. (1986a)
	Antigen C	Human		250+			Short rods	Weerkamp et al. (1986b)
	Antigen C in			488			Export mutant	Weerkamp et al. (1986b)
Vibrio cholerae	Classical strain	Human	C,G				Fucose-specific	Jones & Freter (1976)
Yersinia enterocolitica	HEp-2 adhesin	Human			(-)	P	Nonfimbrial	Old & Robertson (1981)
	MR/Y	Human			8			MacLagan & Old (1980)
	MR/K-HA	Human			4			Duguid & Old (1980)
Yersinia frederiksenii	MSEHA	Human	G,C,E		(-)		MS, nonfimbrial	Old et al. (1985)

Notes to Table 1.3

a) Adhesins are named according to the original publications and the scheme of **Orskov and Orskov** (1983, 1984) or the strain from which they were isolated.

b) Animals whose erythrocytes are agglutinated (note - not all species' bloods tested in each case): B = Cow (<u>Bovis</u>), C = Chicken, D = Dog, E = Horse (<u>Equus</u>), G = Guinea pig, H = Human, M = Monkey, P = Pig, R = Rabbit, S = Sheep.

c) Molecular weight of subunit (kd) and estimated fimbrial diameter (nm). (-) = nonfimbrial.

d) Location of genetic determinants: C = chromosomal, P = plasmid borne. ? = provisional location only.

Figure 1.1 Principal features of the human intestinal tract

Anatomy

Nasal cavity

Mouth

Buccal cavity

Pharynx

Oesophagus

Stomach

Liver

Gall bladder

Bile duct

Pancreas

Hepatic
portal vein

Jejunum

Duodenum

Ileum

Colon

Rectum

Anus

Microbiology

Filters out
large particles

Contaminated food
and water ingested

Cilia raise mucus
from lungs, with
small particles

Aerobic

Acid and gastric
enzymes secreted

Enzymes and bile
salts secreted

Invasive pathogens
enter circulation

Proximally sterile

Distally colonised
by a few species

Anaerobic

Colonised by up to
10^{11} bacteria per
gram of tissue

Dissemination

58

Figure 1.2 Components of the vertebrate immune system

Vertebrate

Blood   Other organs

Other cells   Macrophages

Plasma   Cells

Phagocytosis

Complement   Antibodies   Platelets

Direct   Opsonisation   Clotting   White   Red

membrane   and   (leucocytes)   (erythrocytes)

lysis   lysis

Granulocytes   Lymphocytes

(polymorphonuclear

leucocytes)

B-cells   T-cells

Eosinophils   Basophils   Neutrophils   Monocytes

Clones   Cell-

Inflammatory response   Phagocytosis   make   mediated

antibodies   immunity

Figure 1.3 <u>Surface characteristics of E. coli</u>

Lipopolysaccharide (LPS; O **antigen**)

Polysaccharide capsule (slime, glycocalyx; **K antigen**)

Flagellum (H **antigen**)

F antigen { Fimbriae { Rod-like
Fibrillar
Sex (conjugative) pili

Outer membrane

Outer membrane proteins

Peptidoglycan

Periplasm

Inner (cytoplasmic) membrane

Cytoplasm

Exported toxins
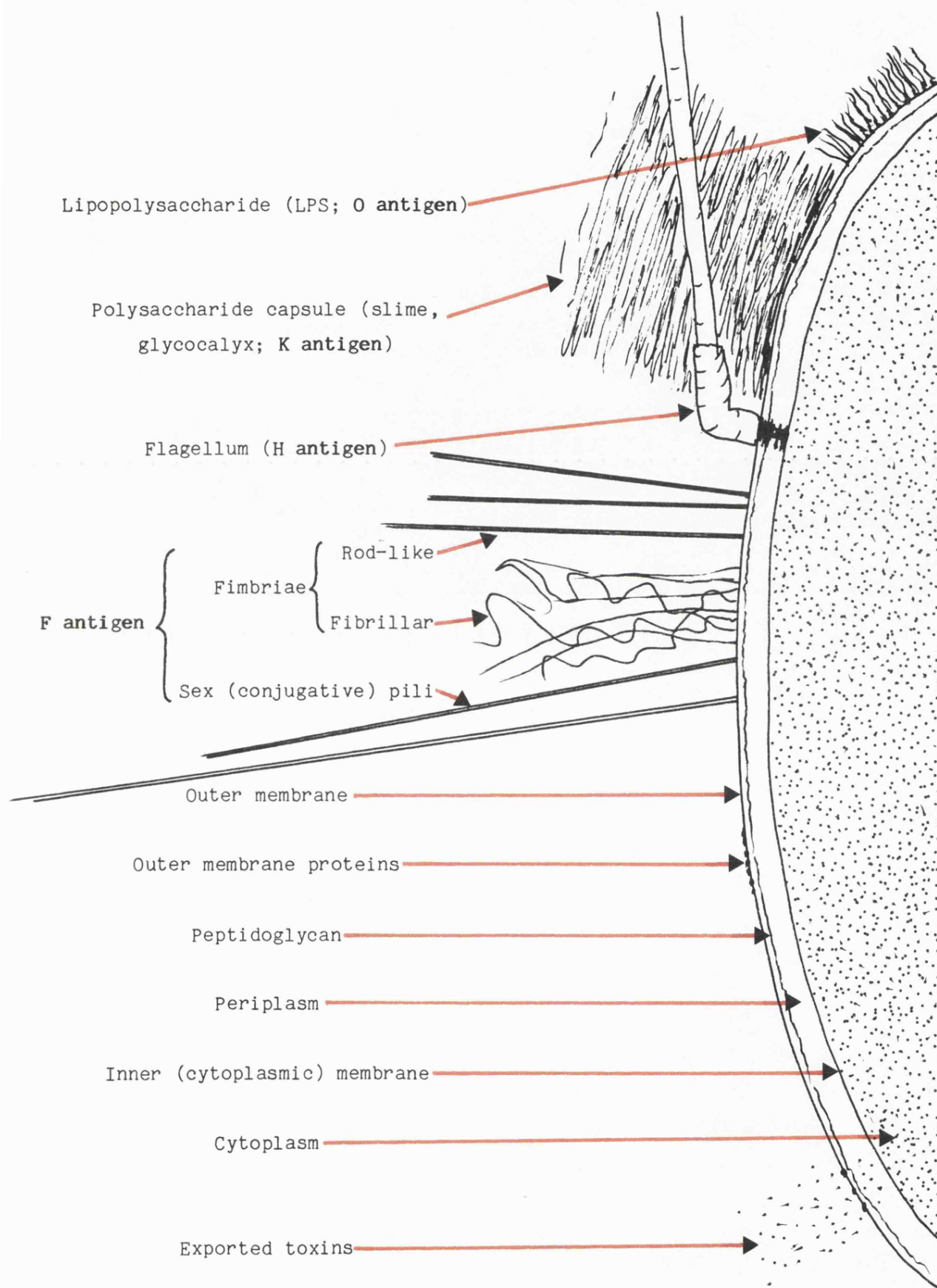
2.1 Materials

2.1.1 Bacterial strains, growth and storage. Details of the bacterial strains used in this study are presented in **Table 2.1.** The strains were obtained from C. Howland (RU2537), A. Jeffreys (HB101), N. Mackman (C600, CSH26, DS410) and P. Williams (444-3, 469-3, LG1505, RB308) [Department of Genetics, University of Leicester], and G. Boulnois (BHB2688, BHB2690, LE392) and I. Roberts (ED8654) [Department of Microbiology, University of Leicester].

Media for bacterial growth. Luria broth (LUB; **Bertani,** 1951; **Lennox,** 1955) contained 1% (w/v) tryptone (Oxoid), 0.5% (w/v) yeast extract (Oxoid) and 0.5% (w/v) NaCl, adjusted to pH 7.0 with 1 N NaOH. Where indicated, 0.2% (w/v) glucose (LUB-glucose) or 0.2% (v/v) glycerol (LUB-glycerol) or 0.2% (w/v) maltose (LUB-maltose) were added, plus appropriate antibiotics.

Luria agar (LUA) consisted of LUB, solidified with 1.5% (w/v) agar (Difco). Stock LUA was sterilised by autoclaving and stored at room temperature. When required, stock LUA was re-melted in a microwave oven (400 W for 15 to 20 min), allowed to cool to approximately 50°C (hand hot) before adding antibiotics, and poured into 90 or 150 mm diameter plastic Petri dishes (Sterilin) to depths of approximately 4 mm and dried inverted.

Minimal medium (MM) contained 0.2% (w/v) glucose (MM-glucose) or 0.2% (v/v) glycerol (MM-glycerol), M9 salts plus Ca and Mg salts added after separate autoclaving, vitamin B1 at 50 μg/ml, and essential amino acids individually or as Casamino acids (Oxoid) or methionine assay medium (Difco) for methionine radiolabelling (see text for details), plus antibiotics. Minimal agar (MA) was MM solidified with 1.5% w/v agar (Difco).

Growth of bacteria. Agar plates were inoculated and incubated (inverted) in a 37°C 'constant temperature room' (actual temperature ranged from approximately 35 to 37°C), or in incubators at various temperatures, for 16 to 48 h. Liquid cultures in glass flasks of at least 5 times the culture vol, some with baffles, or in glass 20 ml test-tubes, were grown with aeration by shaking (New Brunswick reciprocating platform at 100 rpm) in a 37°C constant temperature room, or in a reciprocating platform incubator (Gallenkamp, at 250 rpm and 37°C), or in shaking water baths (New Brunswick, at 200 rpm and usually at 37°C, or occasionally at other temperatures between 18 and 42°C), for 16 h ('overnight cultures') or various times (see

text). Absorbance at wavelengths of 480 or 600 nm (A^{480} or A^{600}; Bausch and Lomb 'Spectronic 20' spectrophotometer) was routinely used to estimate relative cell densities. Occasionally, the absorbance reading of a particular strain was related to its absolute cell density by performing a viable cell count (serial dilution and plating samples on agar) on a fresh liquid culture.

Storage of bacteria. Bacteria were streaked out for single colonies on agar plates, then restreaked from single colonies to ensure purity. Pure stocks were tested for phenotypic characteristics (antibiotic resistance, UV-sensitivity, lysogeny, temperature-sensitivity, lactose utilisation, HA, etc.) and were routinely stored at 4°C for up to three months on these 'pure stock plates'. They were subcultured and streak-purified onto fresh plates as required. Checked stocks were stored for indefinite periods freeze dried in ampoules (Edwards 'Modulyo' freeze drier), and for up to two years at room temperature as stabs in agar in glass vials (Maniatis et al., 1982) or at -20°C in glycerol storage medium (made by mixing equal volumes of pure overnight culture in LUB with sterile glycerol stock containing 60% v/v glycerol and 40% v/v LUB in sterile plastic screw-top vials). Cosmid clones were stored in individual wells of sterile plastic microtitre dishes (Nunc), each well containing 50 µl of LUB with 50 µg/ml ampicillin, inoculated by toothpick from agar-grown colonies and grown overnight at 37°C without shaking. 50 µl aliquots of 50% (v/v) glycerol were added to each well and the plates were stored at -20°C.

2.1.2 Cloning vectors. Genetic maps of the cloning vectors are presented in **Fig. 2.1** and other details in **Table 2.2**. Plasmids pBR322 and pACYC184 were obtained from N. Mackman [Department of Genetics, University of Leicester] and vectors pBR325 and pOU71 from J. Pratt [formerly at the same department but now at the Department of Biochemistry, University of Liverpool]. Cosmid Cos4 was obtained from C. Hadfield [Leicester Biocentre] via G. Boulnois [Department of Microbiology, University of Leicester].

2.1.3 Cultured human cells. Primary human cell cultures were obtained (with informed consent) by Dr. R. Cockel, Birmingham. Duodenal or colonic biopsies, each of approximately 9 mm³, were taken at routine endoscopical examination and maintained for up to 8 h in HEPES-buffered Ham F-10 medium pH 7.2 at 37°C. Two 'transformed' (malignant) human cell lines were cultured as monolayers in tissue culture flasks at 37°C in modified Eagles medium (MEM) containing 2% (v/v) newborn calf serum. HEp-2 cells were originally taken from a carcinoma of the larynx (Toolan, 1954). HeLa cells were

originally derived from a cervical carcinoma (**Scherer et al.,** 1953).

2.1.4 Stock solutions. Unless otherwise stated, chemicals were obtained at analytical reagent grade from Fisons or Sigma, and were stored according to the manufacturers' instructions. Most chemicals were prepared for use as aqueous stocks in distilled water and where a pH is indicated, were brought to that pH with HCl or NaOH and measured with a handheld pH meter (Gallenkamp 'pH stick') calibrated monthly against standard buffers (Fisons pH standard tablets). Stock solutions were generally sterilised by autoclaving at 1 kg/cm^2 for 20 min. Sugar solutions were autoclaved at 0.7 kg/cm^2 for 10 min. Heat labile solutions (antibiotics, proteins, etc.) were sterilised by ultrafiltration through sterile, 0.45 μm pore-size nitrocellulose filters (Millipore) into sterile containers. Stocks were stored in glass bottles (Schott, 'Duran'), plastic 30 ml universal tubes (Sterilin) or 1.5 ml microfuge tubes (Eppendorf and Sarstedt).

All **antibiotics** except streptomycin were obtained from Sigma. Streptomycin sulphate (BP BNF) was obtained from Evans. Stock solutions were prepared, stored and used as shown in **Table 2.3.**

2.2 Methods of phenotypic analysis

2.2.1 Adherence assays. Adherence of bacteria to various living surfaces was assayed as follows:

Mannose-resistant haemagglutination (MRHA). Fresh human type-0 blood was drawn by venepuncture daily as required into an equal vol of 1 x SSC (salt-sodium citrate buffer, containing 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) to prevent clotting. Blood cells were pelleted by centrifugation (MSE 'Micro Centaur' bench top microfuge, full speed for 20 s), plasma and leukocytes aspirated, and the remaining erythrocytes washed twice in ice-cold PBS (phosphate buffered saline, containing 10 mM phosphate and 0.14 M NaCl, pH 7.4) or PBS-mannose (0.5% D-mannose or 0.25% methyl α-D-mannopyranoside [Sigma] in PBS) or TBS-mannose (Tris buffered saline, containing 0.1 M Tris and 0.14 M NaCl, pH 7.4, plus 0.5% D-mannose or 0.25% methyl α-D-mannopyranoside) by gentle resuspension and centrifugation as above. The erythrocytes were finally resuspended to approximately 50% (v/v) in the mannose-containing buffer and stored on ice until required in the MRHA tests.

Aliquots (100 μl) of liquid sample (bacterial cultures or adhesin preparations), or toothpick-loads of bacterial colony resuspended in 100 μl aliquots of PBS, were thoroughly mixed in individual wells of sterile microtitre plates (Nunc pre-sterilised 96-well polystyrene microtitre plates) with 50 μl aliquots of washed erythrocyte suspension (5% v/v in PBS-mannose), using sterile toothpicks. Plates were incubated statically on ice for 1 h, after which MRHA was assessed by comparison with positive and negative controls and scored from 0 (no HA, tight sediment of settled erythrocytes) to 4 (strong HA, large clumps of agglutinated cells). Alternatively, 3 μl drops of erythrocyte suspension (50% v/v in PBS-mannose) were placed in each well of a coated slide (Flow Laboratories 10 well PTFE-coated multitest slide), or 50 μl of erythrocyte suspension (10% v/v in PBS-mannose) was dropped onto a plain glass microscope slide. Liquid samples (30 to 40 μl) or toothpick-loads of colony samples were added to the slides and mixed with toothpicks and by rocking gently. MRHA was scored by comparison with negative or positive controls according to a scale of 0 (no agglutination, even after 15 min incubation on ice) to 4 (immediate clumping of erythrocytes in large aggregates).

Nitrocellulose haemadsorption (NCHA) (adapted from Sedlock et al., 1982 and Connor and Loeb, 1983). Strains were grown overnight on agar at densities of up to 300 colonies per Petri dish. Nitrocellulose filter discs (82 mm

diameter, Schleicher and Schuell), dampened with PBS-mannose, were placed on the agar surface and their orientation marked by pen and puncture marks. Plates with filters were incubated at room temperature for 1 h, then the filters were carefully removed with forceps and placed colony-side-up in individual Petri dishes each containing 10 ml of PBS-mannose and 2% (w/v) bovine serum albumin (BSA; Sigma) to block unbound sites. After 2 rinses in PBS-mannose over 10 min on a reciprocating platform at 60 rpm, 4 ml of 5% (v/v) erythrocyte suspension in PBS-mannose was added and incubation was continued for up to 1 h on ice, with occasional gentle manual agitation. Finally, bound erythrocytes were fixed by washing the filters briefly in 1% (w/v) tannic acid in PBS-mannose, and rinsing in PBS-mannose.

Mannose-sensitive (MS) agglutination. *Saccharomyces cerevisiae* cells obtained from M. Pocklington [Department of Genetics, University of Leicester] were washed and resuspended in PBS, and substituted for erythrocytes in the 'HA' tests. Results were compared before and after the addition of D-mannose to 0.5% (w/v). Where present, MR agglutination was unaffected by mannose whereas MS agglutination was disrupted.

Bacterial adherence to human cell lines. Washed suspensions of bacteria in MEM containing 2% (v/v) newborn calf serum and 0.5% (w/v) D-mannose were added to subconfluent monolayer cultures of HEp-2 or HeLa cells and incubated at 37°C for 3 h. The monolayers were thoroughly washed with several changes of medium and prepared for EM analysis (see **section 2.2.4**).

Bacterial adherence to human mucosa. Bacteria were grown overnight in LUB at 37°C, washed and resuspended in MEM containing 2% (v/v) newborn calf serum and 0.5% (w/v) D-mannose. Human mucosal tissue, obtained as biopsy samples, and the bacteria were incubated together at 37°C for 3 h, then washed extensively in MEM to remove nonadherent bacteria and prepared for EM examination as described below.

Bacterial adherence to isolated human enterocytes. Enterocytes were isolated from human duodenal and colonic biopsy samples by EDTA chelation (**Knutton et al.,** 1984b), washed, and resuspended in HEPES-buffered Ham F-10 medium (pH 7.2) containing 0.5% (w/v) D-mannose. Cells from 5 biopsies were resuspended in 5 ml of medium for immediate use. For each assay, 1 ml of enterocyte suspension was added to 2 ml of a washed suspension of bacteria at 10^9 cells/ml and incubated on a rotary mixer for 1 h at 37°C. The enterocytes were pelleted by centrifugation and washed several times in fresh medium to remove nonadherent bacteria, then examined microscopically.

2.2.2 Adhesin purification. Bacteria were spread from overnight cultures onto about 40 large (150 mm diameter) Petri dishes of LUA and grown for 24 to 40 h at 37°C. The cells (approximately 12 g wet weight, some 5×10^{12} bacteria) were scraped from the agar with a glass rod 'spreader' into 5 to 10 ml of PBS and blended (Du Pont 'Sorvall' Omnimixer) for 5 x 1 min with cooling in an ice-water bath. Whole cells were removed by centrifugation (Du Pont 'Sorvall' SS34 rotor, 8 k rpm for 10 min) and filter sterilisation (Millipore 0.45 μm pore-size nitrocellulose filter), and the ultrafiltrates ('crude adhesin preparations') were concentrated by dialysis against 42% w/v PEG_{6000}, or by rotary evaporation, or by suction ultrafiltration (Millipore 'CX-10' immersible ultrafilter, nominal minimum exclusion limit 10 kd). Two further stages of purification were subsequently used. Crude adhesin preparations (5 ml) were size-fractionated on column chromatograms (Pharmacia 'Sepharose 4B-CL'), 50 cm long by 2 cm diameter, equilibrated and eluted with PBS at 0.1 to 0.4 ml/min (LKB '2132 Microperpex' peristaltic pump), in a 'constant temperature room' at 4°C. Column eluate was passed through a UV spectrophotometer (LKB '2238 Uvicord S II') connected to a chart recorder (LKB '2210'), and fractions of 5 to 10 ml were collected automatically (LKB '2211 Superrac' fraction collector). Up to 120 fractions were assayed for protein by amido black staining and Bradford's assay (see below), and for MRHA by serial dilution and slide HA. Aliquots containing 10 μg of protein were analysed by SDS-PAGE using 20% (w/v) acrylamide gels and Coomassie staining. In some experiments, haemagglutinating fractions from the chromatography were pooled, concentrated to 3 ml, and ultracentrifuged in sucrose density gradients (each 32 ml of 60 to 10% w/v sucrose in PBS with 0.05% w/v sodium azide, preformed in Beckman 'Ultra Clear' tubes and eqilibrated at 4°C for 16 h) in an SW27 rotor (Beckman) at 20 k rpm at 4°C for 20 h (**Korhonen, 1980b**). The gradients were fractionated into 10 samples, dialysed extensively against water, and concentrated to 1 ml each. Proteins in the fractions were separated by SDS-PAGE and Coomassie stained (**section 2.3.9**).

2.2.3 Bacterial membrane preparation (method developed from **Churchward and Holland, 1976**, by N. Mackman [Department of Genetics, University of Leicester]). 60 ml aliquots of LUB were inoculated with overnight cultures and grown to mid-exponential growth phase (A^{450} = 0.5). Bacteria were harvested by centrifugation, resuspended in 7 ml aliquots of envelope buffer (10 mM NaH_2PO_4, pH 7.2) and transferred to 25 ml glass beakers. Cells were disrupted by sonication (MSE 150 W ultrasonic disintegrator for three bursts of 30 s at 5 u amplitude, with 30 s cooling periods in ice-water), and remaining whole cells and debris were removed by centrifugation (Du Pont

'Sorvall' SM24 rotor, 7 k rpm for 5 min). Membranes in the cleared supernatant were pelleted by ultracentrifugation (Beckman 50Ti rotor, 35 k rpm for 40 min in thick walled plastic tubes), and the supernatant discarded. The pellet was allowed to drip dry, then resuspended in 1 ml of envelope buffer by freezing the tube and buffer in dry ice-IMS and macerating the pellet. Membranes were precipitated by ultracentrifugation as above, and resuspended in 75 μl of envelope buffer. Cytoplasmic membranes were solubilised by the addition of 25 μl of 2% (v/v) 'Sarkosyl NL97' detergent (Sigma) and incubation at room temperature for 30 min with occasional mixing. The insoluble outer membranes were pelleted by ultracentrifugation as above. Supernatants (cytoplasmic membrane fractions) were collected and diluted with 100 μl of SDS-PAGE sample buffer; pellets (outer membrane fractions) were resuspended in equal volumes (80 μl) of membrane buffer and sample buffer. The samples were analysed by SDS-PAGE.

2.2.4 Electron microscopy (EM). Some of the scanning electron microscopy was performed at the University of Leicester; the remainder and all the transmission electron microscopy (including the immunogold labelling) was performed at the University of Birmingham, in collaboration with Dr. S. Knutton (see **Hinson et al., 1987).**

Transmission electron microscopy (TEM). 10 μl aliquots of washed suspensions of bacteria were negatively stained by adding equal amounts of bacitracin (150 μg/ml) and 2% ammonium molybdate (pH 7) and mixing. 10 μl drops were appplied to UV-irradiated or glow-discharged carbon coated grids, air-dried and examined in a Phillips 'EM301' microscope.

Scanning electron microscopy (SEM). Samples were prepared for SEM examination by fixing in glutaraldehyde and osmium tetroxide, dehydration through graded acetone solutions and critical point drying. They were mounted on stubs, gold-coated and examined in Cambridge 'Stereoscan S100' or 'S4' microscopes (in Leicester and Birmingham, respectively).

2.2.5 Minicell analysis (Pritchard and Holland, 1985). E. coli strain DS410 was transformed with plasmids and grown overnight in 400 ml of LUB-glucose containing appropriate antibiotics. At the same time, two glass centrifuge tubes (Du Pont 'Corex') per culture, each containing 25 ml of 20% w/v sucrose in MM-glucose, were frozen at $-80^{\circ}C$ and allowed to thaw overnight at $4^{\circ}C$ ('sucrose gradients'). DS410 whole cells were removed from the overnight culture by gentle centrifugation (Du Pont 'Sorvall' GS3 rotor at 2 k rpm for 5 min at $4^{\circ}C$), and supernatants containing minicells were decanted and

combined in a fresh centrifuge bottle. The minicells were pelleted (Du Pont 'Sorvall' GS3 rotor at 8 k rpm for 15 min at 4°C), resuspended in 3 ml of MM-glucose and layered onto a sucrose gradient. They were centrifuged (Du Pont 'Sorvall' HB4 rotor at 5 k rpm for 20 min at 4°C) and approximately 5 ml from the upper band was removed with a Pasteur pipette into fresh centrifuge tubes and pelleted (Du Pont 'Sorvall' SS34 rotor at 10 k rpm for 10 min). The pellet was resuspended in 1 ml of MM-glucose, layered onto the second sucrose gradients and centrifuged as above. Minicells were removed and pelleted as above, and resuspended in 200 μl of MM-glucose containing methionine assay medium (Difco, made up to one fifth the manufacturers recommended concentration). An aliquot (100 μl) was removed into a microfuge tube containing 200 μl of 50% (v/v) glycerol, mixed, and flash-frozen in dry ice-IMS in 60 μl lots prior to storage at -70°C. The remaining 100 μl was incubated at 37°C for 1 h. 2 MBq of [^{35}S]methionine (Amersham, specific activity 46 TBq/mmol) was added and incubation was continued for 30 min. 5 μg of unlabelled methionine was added to release labelled proteins from polysomes, and after 5 min, 100 μl of SDS-PAGE sample buffer was added and the samples were incubated in a boiling water bath for 5 min. Labelled proteins were analysed by SDS-PAGE and fluorography (section 2.2.8).

2.2.6 Protein assays. Two methods were used to obtain protein concentrations; a quick, semiquantitative assay (Amido black staining) and a fully quantitative assay (Bradford's):

Amido black staining. Small aliquots (4 μl) of sample solution were spotted onto a nitrocellulose filter in a Petri dish and allowed to dry in air. The filter was then stained for 2 min in staining solution (containing 0.1% w/v 'amido black' [Sigma, practical grade napthol blue-black], 10% v/v glacial acetic acid and 1% w/v sodium acetate), and destained about three times in 10% (v/v) glacial acetic acid. The method was semiquantitative, since the degree of staining of samples could be compared with a set of spots of bovine serum albumin (Sigma; maximal reaction at approximately 20 μg/ml).

Bradford's protein assay (Bradford, 1976). Staining solution was prepared and used as described, except that it was filtered three times (Whatman 'Number 1' filters) before storage in thoroughly rinsed glassware. Standard curves were determined from non-serially diluted solutions of bovine serum albumin (Sigma; up to 1 mg/ml).

2.2.7 Radioimmunoprecipitation. Bacterial cultures were grown overnight on MA plates. They were then inoculated into 5 ml of pre-warmed MM-glucose

containing methionine assay medium (Difco; made to one-fifth the manufacturer's recommended concentration) and grown to mid-logarithmic growth phase (A^{480} = 0.5) in a shaking water bath (New Brunswick) at 37°C. 3 MBq of [^{35}S]methionine (Amersham; specific activity 46 TBq/mmol) was added to each culture and incubation was continued for 30 min. Surface proteins were released from the bacteria by forcing the cultures through a 0.6 mm diameter hypodermic needle twenty times, and whole cells were removed by centrifugation (MSE 'Centaur 1' at 5 k rpm for 10 min and ultrafiltration (Millipore, 0.45 μm pore size). Filtrates were concentrated to 1 ml each by suction ultrafiltration (Millipore 'CX-10' immersible ultrafilter). 25 μl of absorbed antiserum raised against the MR adhesin of strain 469-3 was added to each preparation and the reaction mixtures were incubated at 37°C for 3 h, then at 4°C for 16 h. Immunoprecipitates were recovered by centrifugation (microfuge, 15 min), washed once in 1% (v/v) Triton X-100 detergent (Sigma) in PBS and twice in PBS, and analysed by SDS-PAGE and fluorography (**section 2.2.8**).

2.2.8 SDS-PAGE analysis (Laemmli, 1970; Maniatis et al., 1982; Pritchard and Holland, 1985). Protein samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie stained, or fluorographed if radiolabelled:

SDS-PAGE. Discontinuous acrylamide gels 1 mm thick were cast in vertical Perspex electrophoresis tanks between glass plates 20 cm square in three sections. The lowest section (plug gel) was poured with the tank angled back at 45° to seal the bottom of the plates, using acrylamide prepared to the same density as the separating gel from 44% w/v acrylamide (Serva) and 0.8% (w/v) N,N'-methylene-bisacrylamide (Serva) depending on the range of size separation required, with SDS present at 0.1% (w/v). 2 ml of propan-2-ol was gently applied to the top of the gel while it set, then blotted away. The stacking gel contained 7% (w/v) acrylamide. A plastic comb was put in place while the gel set to form 18 slots. SDS-PAGE running buffer was made from 10 x stock (containing 0.25 M Tris, 1.92 M glycine and 1% w/v SDS). Samples were prepared by diluting liquids to 50% v/v, or resuspending solids directly, in SDS-PAGE loading buffer (containing 30% v/v glycerol, 4% w/v SDS, 0.125 M Tris pH 6.8, 10% v/v β-mercaptoethanol and 0.001% w/v bromophenol blue) and incubated for 5 min in a boiling water bath, with a Perspex cover to retain the microfuge tube lids. Loaded samples were electrophoresed at a constant 10 W (LKB '2103' power supply) for approximately 5 h, or at a constant 4 to 11 mA (higher currents for denser gels) overnight, until the dye front reached the plug gel.

Coomassie blue staining. Acrylamide gels were stained for proteins in a plastic box (Sainsbury 'lunchbox') on a reciprocating platform at 200 rpm, in staining solution (which was destain, containing 25% v/v propan-2-ol and 10% v/v glacial acetic acid, plus 0.5 mg/ml coomassie brilliant blue R [Sigma]) for 1 to 2 h, then in destain for 3 to 6 h, and in water for at least 1 h. Stained gels were photographed on a white light box through an orange filter, and were dried onto chromatography paper (Whatman '3MM') by vacuum and heat (Bio Rad '1125' slab gel drier) for 2 to 3 h.

Fluorography. Acrylamide gels were fixed in destain for at least 20 min on a reciprocating platform, then soaked in DMSO (Fisons) for 30 min. The DMSO was discarded and gels were soaked for a further 30 min in fresh DMSO, then for 90 min in fluor solution (22% w/v 2,5-diphenyloxozole in DMSO). After a brief rinse in water, gels were soaked in water for 60 min, dried onto chromatography paper (Whatman '3MM') by heat and vacuum (Bio Rad '1125' slab gel drier) for 2 to 3 h and autoradiographed at -80°C for up to 1 month with X-ray film (Kodak 'X-Omat S', or Amersham 'Hyperfilm MP' or 'Hyperfilm βmax').

2.2.9 Western blotting. Protein samples were separated by SDS-PAGE as normal. The gel, with pieces of nitrocellulose filter either side, was then transferred to a transverse electrophoresis tank containing 3 l of blotter buffer (192 mM glycine, 25 mM Tris, 10% v/v methanol) and electrophoresed at 80 V for 90 min at 4°C. The anodic nitrocellulose filter was removed and a section was cut off and stained with amido-black to check for full transfer of the proteins. The rest of the filter was washed for at least 20 min in PBS-Tween (0.05% v/v Tween 80 [Sigma] in PBS) in a plastic box (Sainsbury 'lunchbox') on a reciprocating platform, then reacted with primary antiserum (absorbed rabbit antiserum raised against strain 469-3 MR adhesin; 100 μl in 10 ml of PBS-Tween) for 90 min at room temperature and washed four times over 40 min with PBS-Tween. The filter was reacted with secondary antiserum (DAKO pig anti-rabbit antiserum; 10 μl in 10 ml of PBS-Tween) for 1 h, washed as above, reacted with horseradish peroxidase - rabbit antiperoxidase conjugate (DAKO) for 1 h and washed as above. Staining solution (20 mg of 4-chloro-1-naphthol [Sigma] in 20 ml of methanol, added slowly to 80 ml of 50 mM Tris pH 7.6, and 100 ul of hydrogen peroxide, filtered through Whatman 'Number 1') was prepared for immediate use. The filter was stained for about 5 min for sufficient contrast, then rinsed in H_2O and air dried.

2.3 Methods of genetic analysis

2.3.1 Agarose gel electrophoresis. Agarose powder (FMC 'SeaKem HGT') was dissolved to 0.4 to 1.5% (w/v) in TAE buffer (Tris-acetate-EDTA, containing 40 mM Tris and 2 mM EDTA, adjusted to pH 7.4 with glacial acetic acid) by boiling in a microwave oven (500 W for 20 min). Gels were cast directly, or after re-melting stock agarose solutions in a microwave oven (700 W for 2 min), by pouring 12 ml onto 80 x 80 x 1 mm plain glass plates (photographic slides), or up to 200 ml on to larger glass plates (200 x 200 x 4 mm) with adhesive tape walls, and wells were formed with plastic combs suspended 1 mm above the glass on paper clips. Gels on their glass supports (but without the tape walls) were placed in horizontal Perspex electrophoresis tanks (made by our workshop) containing TAE buffer plus ethidium bromide at 0.5 μg/ml. DNA samples were mixed with one-tenth vol of agarose loading buffer (50% v/v glycerol, 0.5% w/v bromophenol blue), layered into the wells, and electrophoresed at 1 V/cm overnight or 10 V/cm for approximately 1 h, using constant voltage (Kingshill 'I0A05C' power supply). Finally, gels were examined on a short wavelength ultra-violet (UV) transilluminator (Fotodyne) through a yellow glass filter and photographed on 12.5 x 10 cm 'instant' film (Polaroid 'Type 52' or 'Type 57') or standard sheet film (Kodak 'Plus-X Pan Professional').

2.3.2 Agarose gel purification of DNA fragments (derived from Dretzen et al., 1981). Small pieces of DEAE-cellulose paper (Whatman 'DE81') were soaked for 10 min in 2.5 M NaCl, washed three times over 15 min in H_2O, and stored in TE buffer at $4^{\circ}C$. The DNA was restriction digested and separated by agarose gel electrophoresis in the normal way, then the gel was examined on a UV transilluminator (Fotodyne). Pieces of prepared DEAE cellulose paper were cut to the size of the slots in the agarose and inserted into cuts made on either side of the fragments required (that is, slightly towards the anode and cathode electrodes relative to the bands). The agarose was held in place by inserting wooden toothpicks, and electrophoresis was continued for about 5 min at 10 V/cm. The anodic paper pieces were transferred to Petri dishes, washed in H_2O on a reciprocating shaker for 15 min, blotted dry and placed in microfuge tubes. 200 μl of high salt buffer (2 M NaCl in TE buffer) was added to each tube and the paper pieces were mashed up with a plastic pipette tip and vortex mixed. The tubes were incubated at $65^{\circ}C$ for 10 min, then inverted and pierced through the bottom with a red-hot hypodermic needle. The liquid was centrifuged out through a polyallomer wool plug in a large pipette tip and into another microfuge tube. The DNA was ethanol precipitated, washed in 70% (v/v) ethanol, vacuum dried and

resuspended in 10 µl of TE buffer. Yield and purity were assessed by agarose gel electrophoresis, and fragments were stored at 4°C.

2.3.3 Blotting and hybridisation of DNA. DNA (either crudely released from whole colonies or purified by agarose gel electrophoresis) was bound to nitrocellulose or nylon filters and hybridised with radiolabelled 'probe' DNA as follows:

Colony DNA blot. Bacterial strains were inoculated in a grid pattern on nitrocellulose discs (Sartorius) laid on agar plates, and grown overnight. The bacteria were lysed as follows. The filters were carefully removed and placed colony-side-up on a piece of chromatography paper (Whatman '3MM') dampened with 0.5 M NaOH, and incubated at room temperature for 7 min, then transferred to a fresh piece of paper dampened with 0.1 M NaOH, 1.5 M NaCl for 10 min, to 1 M Tris pH 7.4 for 2 min, and finally to 1.5 M NaCl, 0.5 M Tris pH 7.4 for 4 min. Filters were baked at 80°C for 30 min to fix the DNA.

Southern DNA blot (Southern, 1975). Agarose gels containing DNA restriction fragments were photographed against a ruler scale, washed for 7 min in 0.25 M HCl on a reciprocating platform at 60 rpm, rinsed in water, denatured for 30 min in 0.5 M NaOH, 1.5 M NaCl, rinsed in water, and neutralised for 30 min in 3 M NaCl, 0.5 M Tris pH 7.4. The fragments were transferred to charge-modified nylon membranes (Gelman 'Bio Trace RP' or Amersham 'Hybond') overnight by drawing the liquid out of the gel matrix, through the membrane and a layer of filter paper (Whatman 'Number 1'), both of which had initially been dampened with 3 x SSC and carefully placed to exclude air bubbles, into several layers of paper towels weighted down with bottles of water. After a brief rinse in 3 x SSC, the DNA was crosslinked to the membranes by wrapping them in a single layer of plastic film (Dow 'Saran Wrap') and placing them on the surface of a UV transilluminator (Fotodyne) for 5 min. The filters were stored at 4°C.

Radiolabelling (oligolabelling) DNA. DNA fragments for use as probes were purified and extracted from agarose gels (**section 2.2.2**). Some 10 to 50 ng of pure DNA (in 1 to 3 µl of TE buffer) was denatured by boiling for 7 min and incubated in a 37°C water bath for 10 min. The following solutions were added in the stated order: water (to a total vol of 15 µl), 3 µl of OLB (oligolabelling buffer with Pharmacia hexadeoxyribonucleotides), 0.6 µl of BSA (Pharmacia 'enzyme grade'; 10 mg/ml stock), DNA, 1.5 µl of α-[^{32}P]dCTP (Amersham; 0.55 MBq at 110 TBq/mmol specific activity) and 0.6 µl of Klenow fragment (Pharmacia; 1 U/µl). The reaction mixture was incubated in a

microfuge tube inside a lead container at room temperature overnight (Feinberg and Vogelstein, 1984).

DNA-DNA hybridisation. Filters with bound DNA were marked with ballpoint pen and if necessary cut into strips in order to fit the Perspex hybridisation chambers (made by our workshop). Subsequent washings were performed in pre-warmed solutions at 65°C in a shaking water bath (New Brunswick). Filters were washed in 80 ml of pre-hybridisation solution (made as 100 ml containing 15 ml of 10 x SSPE [**Maniatis et al.,** 1982], 10 ml of 10% w/v SDS, 0.5 g of dried milk powder [Cadbury 'Marvel'], 17 ml of 36% w/v PEG_{6000} and 58 ml of H_2O) for 1 to 3 h. Oligolabelled probe DNA (10 to 50 ng, and approximately 10 MBq) was denatured by incubation in a boiling water bath for 5 min, and added to the remaining 20 ml of pre-hybridisation solution (hybridisation solution). The filters were drip dried, then the hybridisation solution was added and hybridisation proceeded for 4 to 24 h. The solution was discarded down a designated radioactive disposal sink, and the filters were washed with eight changes of 3 x SSC, 0.1% SDS over 30 min. Finally, they were washed twice over 1 h in 0.2 x SSC, 0.1% SDS (high stringency), blotted dry and wrapped in plastic film (Dow 'Saran Wrap'). The filters were autoradiographed with X-ray film, either with intensifying screens at -80°C (Ilford fast tungstate screens and Kodak 'X-Omat S' film or Amersham 'Hyperfilm MP'), or directly at room temperature (Amersham 'Hyperfilm βmax'), for between a day and a month, depending on the activity.

2.3.4 Conjugation. Donor and recipient bacterial strains were grown overnight in LUB, then diluted to 5% in fresh LUB and mixed (50 μl of donor plus 250 μl of recipient strain). They were plated out on non-selective LUA and grown overnight, then a loopful of culture was scraped into 5 ml of LUB and samples plated out on selective LUA. After growth overnight, single colonies were picked, streaked out on fresh plates and their phenotypes and plasmid contents analysed. (Conjugation method was obtained from N. Mackman [Department of Genetics, University of Leicester].)

2.3.5 Cosmid cloning. The protocol was developed from **Maniatis et al.** (1982) and **Pritchard and Holland** (1985), with the assistance of G. Boulnois and J. Walker [Department of Microbiology, University of Leicester]:

Preparation of cosmid vector arms. Approximately 600 μg of Cos4 DNA was prepared by the large-scale plasmid DNA extraction method, and about 100 μg was digested to completion with PvuII at the unique site (**Fig. 2.2**) and the blunt ends were dephosphorylated (checked by attempting ligation and

electrophoretic analysis). After phenol extraction, the DNA was ethanol precipitated and resuspended in 0.1 ml of water, then digested to completion with BamHI at the unique site (Fig.2.2) yielding 4.3 and 1.6 kb fragments on agarose gels ('cosmid arms'), phenol extracted and ethanol precipitated. Following resuspension in a minimal amount of TE buffer, samples were checked by ligation to form high molecular weight 'ladders' on agarose gels.

Partial restriction endonucleolysis of bacterial genomic DNA. Samples of approximately 10 μg of high molecular weight bacterial genomic DNA were incubated with small amounts of Sau3A restriction endonuclease at 37°C. Samples of approximately 1 μg were removed every minute into microfuge tubes containing EDTA (final concentration, 1 mM), and immediately placed at 65°C for 10 min to stop the digestion. The samples were then analysed by agarose gel electrophoresis, and the incubation conditions required to generate a majority of fragments with sizes of about 40 kb were assessed, repeating the assay with different amounts of enzyme or over different time scales as necessary. Approximately 10 μg of high molecular weight chromosomal DNA was digested with the correct amount of enzyme for the calculated time at 37°C, and the reaction was stopped by adding EDTA to 1 mM and heating the entire sample to 65°C for 10 min. Finally, the size range of the partially digested DNA was estimated by analysing a small sample on a 0.4% (w/v) agarose gel, relative to size markers (undigested chromosomal DNA and lambda chromosome).

Ligation of genomic and vector DNA molecules. Insert DNA (partially digested bacterial chromosome, consisting largely of 45 kb fragments) was ligated to vector DNA by making use of the complementary BamHI 'sticky ends' on the vector and the Sau3A ends on the insert. Approximately equal amounts (3 μg) of vector and insert DNA samples, representing roughly ten vector fragments per insert fragment, were mixed in a microfuge tube and ethanol precipitated. After washing in 70% (v/v) ethanol, vacuum drying and resuspension in 1 x ligase buffer (see above) with 400 U of T4 DNA ligase (total vol 6.6 μl), the reaction mixtures were incubated at 15°C for 16 h. Water was added (to 20 μl) and ligation was assessed by agarose gel electrophoresis (2 μl aliquots).

Preparation of in vitro packaging extract. Two complementary defective lambda lysogens of E. coli, strains BHB2688 and BHB2690 (Table 2.1), were used to synthesise incomplete packaging extracts which together reconstituted the complete set of proteins required to package DNA containing correctly spaced cos sites into infectious bacteriophage particles. The extracts, prepared according to the method of Maniatis et al.

(1982), were stored at -70°C in 40 μl aliquots in microfuge tubes after flash-freezing in liquid nitrogen. Packaging efficiencies were checked for each batch of extract by packaging wild-type lambda chromosome (see in vitro packaging method below), and typically gave 10^7 to 10^9 plaque forming units per μg of DNA.

In vitro packaging. A microfuge tube of packaging extract was removed from the -80°C freezer directly onto ice. The DNA was added and the tube was allowed to thaw slowly on ice, with occasional mixing using a plastic micropipette tip. Once thawed, the tube was incubated at 28°C for 1 h, then 0.5 ml of phage buffer (containing 0.58% w/v NaCl, 0.2% w/v $MgSO_4$, 0.05 M Tris pH 7.5 and 0.01% w/v gelatin) and 25 μl of chloroform was added. The packaged DNA was stored at 4°C prior to transfection.

Transfection. E. coli strains ED8654 or LE392 were grown at 37°C in LUB-maltose to late exponential growth phase (A^{600} = 0.7). Aliquots of 1 ml were harvested in a microfuge (1 min), resuspended in 500 μl lots of 0.1 M $MgCl_2$, and split into 200 μl amounts. 100 μl samples of packaged DNA were added to each aliquot and incubated at 37°C for 20 min, then 400 μl of LUB was added and incubation continued for a further hour. Finally, 100 μl aliquots of suitable dilutions were spread on LUA plates containing ampicillin at 50 μg/ml and incubated at 37°C overnight.

2.3.6 Dephosphorylation of DNA restriction fragments. Cleavage of terminal 5'-phosphate groups was performed either by adding calf intestinal phosphatase (CIP; BRL) to 0.1 U per μg of DNA directly to a restriction digestion some 20 min before the end of the reaction, or for more fastidious samples, by resuspending restricted, phenol extracted and ethanol precipitated DNA in 1 x CIP buffer (10 x CIP buffer, containing 0.5 M Tris pH 9, 10 mM $MgCl_2$, 10 mM spermidine and 1 mM $ZnCl_2$, was stored at -20°C), adding CIP (as above) and incubation at 37°C for 30 min. In both cases, reactions were terminated by adding EDTA to 20 mM and SDS to 0.5% (w/v), and incubation at 65°C for 10 min, followed by phenol extraction and ethanol precipitation. Samples were assayed for the effectiveness of the reaction by attempting ligation, both alone and with added DNA which had been restricted with the appropriate enzyme but not dephosphorylated, followed by agarose gel electrophoresis, and occasionally by attempting digestion of ligated DNA (dephosphorylated plus control DNA) with the original restriction enzyme to control against exonuclease damage.

2.3.7 Ligation of DNA fragments. Approximately 40 Weiss units of T4 DNA

ligase (BRL) were added per µg of DNA in 1 x ligation buffer (10 x buffer contained 0.5 M Tris pH 7.4, 0.1 M MgCl$_2$, 0.1 M dithiothreitol [DTT, from filter sterilised frozen stock : 1 M DTT in 10 mM sodium acetate pH 5.2], 10 mM spermidine, 10 mM ATP and 0.1% w/v BSA, filter sterilised and stored in 20 µl aliquots at -80°C), and incubated at 15°C for 3 to 16 h. Reactions were checked by agarose gel electrophoretic comparison of pre- and post-ligation samples.

2.3.8 Plasmid DNA extraction. A small-scale plasmid extraction procedure was adapted from the method of **Birnboim and Doly (1979)** (as modified by **Ish-Horowicz and Burke, 1981**) and scaled-up, with additional chloramphenicol amplification and density centrifugation steps to improve the yield and purity, by C. Rees and R. Dalgleish [Department of Genetics, University of Leicester].

Small-scale plasmid extraction (plasmid minipreparation). Bacterial cells, harvested in microfuge tubes from 1.5 ml aliquots of overnight cultures in LUB by centrifuging for 20 s and aspirating the supernatant, were resuspended in 100 µl of ice-cold TEG buffer (Tris-EDTA-glucose, containing 25 mM Tris pH 8.0, 10 mM EDTA and 50 mM glucose) to which lysozyme was added to 1 mg/ml just before use, and incubated for 5 min on ice. Alkaline-SDS solution (0.2 M NaOH and 1% w/v SDS, prepared by sequential addition of water, 5 M NaOH, then 10% w/v SDS) was prepared daily and 200 µl aliquots were added to each microfuge tube. After briefly mixing on a vortex mixer (Fisons 'Whirlimixer'), tubes were incubated on ice for 5 min, then 150 µl of ice cold 3 M potassium acetate, pH 4.8, was added, the tubes vortex mixed and replaced on ice for a further 5 min. Precipitated chromosomal DNA was pelleted in a microfuge for 1 min, and the crude supernatant fractions removed with an automatic pipetter into fresh tubes each containing 0.8 ml of phenol-chloroform. The contents were gently but thoroughly mixed on a 45°-angled turntable rotating at 16 rpm for 10 to 30 min, then centrifuged for 1 min and the cleared supernatants transferred into fresh tubes. DNA was ethanol precipitated and resuspended in 50 µl of TE-RNase, prepared from stock RNase A (Sigma, 5 mg/ml in 0.15 M NaCl, incubated in a boiling water bath for 15 min to destroy DNase activity and stored at -20°C) diluted to 0.1 mg/ml in TE buffer (and stored at -20°C). The yield and purity were assessed by agarose gel electrophoresis and restriction analysis, and the DNA was stored at either -20°C or 4°C.

Large-scale plasmid extraction. The buffers were the same as those used in the small scale plasmid preparation (see above). In detail, 8 ml of overnight 37°C LUB cultures was added to 2 l flasks containing 400 ml of

pre-warmed LUB and incubated at 37°C with shaking for several hours until the absorbance at 600 nm (A^{600}) reached 1.5. Then 80 mg of solid chloramphenicol was added and incubation was continued overnight. Cells were harvested by centrifugation (Du Pont 'Sorvall' GS3 rotor at 7 k rpm for 10 min at 4°C), resuspended in 12 ml of TEG buffer and incubated at room temperature for 10 min. 24 ml of fresh alkaline-SDS was gently mixed in and incubated on ice for 5 min. 12 ml of 5 M potassium acetate was added, gently mixed, and the tubes were returned to ice for 15 min. The mixtures were divided equally into two 50 ml polypropylene centrifuge tubes and centrifuged (Du Pont 'Sorvall' HB4 rotor at 10 k rpm for 10 min at 4°C). Pellicles were scooped off with a spatula, and the clear supernatants were poured into a plastic measuring cylinder. DNA was precipitated by adding propan-2-ol to 22% (v/v), mixing and incubation at room temperature for 10 min, and pelleted in an HB4 rotor as above. Precipitates were washed in 70% v/v ethanol, drained, and resuspended in 3 ml of TE buffer by standing at 4°C for 1 h. To each ml of DNA solution, 1 g of solid CsCl and 0.1 ml of ethidium bromide stock solution (5 mg/ml, kept in the dark) were added and fully mixed. The mixtures were transferred to Quickseal tubes (Beckman) and ultracentrifuged (Beckman 'L5-65' ultracentrifuge and 75Ti rotor at 65 k rpm for 16 h at 20°C). Plasmid bands, visible as red bands in daylight which fluoresced under UV light, were removed into universal tubes using a 5 ml syringe, needle and fine plastic tubing passed down from the cut-off tube tops. Ethidium bromide was extracted several times with CsCl-saturated propan-2-ol, then CsCl was removed by extensive dialysis against TE buffer. The yield and purity were assessed by agarose gel electrophoresis and restriction analysis, and the DNA was stored at -20 or 4°C.

2.3.9 Preparation and transformation of competent bacteria. Several methods were tried (see **section 3.2.2** for further details):

Full CaCl$_2$-MgCl$_2$ method (derived from **Cohen et al.,** 1972; **Maniatis et al.,** 1982). Bacteria were grown in LUB-glucose overnight at 37°C on a shaking platform. Fresh pre-warmed LUB-glucose was inoculated with sufficient overnight culture to reach A^{600} = 0.03 to 0.08, then inoculated at 37°C with rapid shaking and grown to mid-logarithmic growth phase (A^{600} = 0.4). The culture was chilled on ice and divided into 1.5 ml aliquots in microfuge tubes. Cells were harvested by centrifugation (20 s) and resuspended in ice-cold 100 mM MgCl$_2$. After incubation for up to 30 min on ice, the cells were pelleted, resuspended in ice-cold 100 mM CaCl$_2$, and incubated on ice for up to 30 min. They were then pelleted again, resuspended in 1 ml lots of ice-cold 100 mM CaCl$_2$ and kept on ice for up to 1 h prior to transformation.

Quick CaCl$_2$-MgCl$_2$ method. Bacterial cells were harvested from 1.5 ml aliquots of overnight culture in microfuge tubes (20 s, microfuge), briefly washed once in ice-cold 100 mM MgCl$_2$ and once in ice-cold 100 mM CaCl$_2$, then resuspended in 200 µl of 100 mM CaCl$_2$ and incubated on ice for a few min. The DNA was added (typically 0.1 to 1 µg), plus 3 µl of 'spectro quality' dimethylsulphoxide (DMSO; BDH 'SpectrosoL' aliquotted from a new bottle into microfuge tubes and stored at -20°C), and the tubes were incubated on ice for 10 min. They were heat shocked in a water bath at 43 to 44°C for 45 s, then plunged back on ice while 1 ml of LUB-glucose was added. The cells were incubated for 15 min at 37°C, and then streaked out (using 5 to 200 µl per plate, depending on expected yield) on agar containing appropriate antibiotics and grown overnight at 37°C.

CaCl$_2$-RbCl$_2$ method (derived from Kushner, 1978; Maniatis et al., 1982; and **Pritchard and Holland,** 1985). Competent bacterial cells were prepared in the same way as for the full CaCl$_2$-MgCl$_2$ method detailed above, except that 100 mM MgCl$_2$ was replaced by 10 mM MOPS (2-N-morpholinoethane sulphonic acid) pH 7 + 10 mM RbCl$_2$, and 100 mM CaCl$_2$ was replaced by 100 mM MOPS pH 6.5 + 50 mM CaCl$_2$ + 10 mM RbCl$_2$.

CaCl$_2$-RbCl$_2$-HACoCl method (Hanahan, 1983). Bacteria were grown in 25 ml of LUB-glucose to mid-logarithmic growth phase (A^{600} = 0.4), harvested by centrifugation in universal tubes (MSE 'Chillspin' centrifuge, 5 k rpm for 5 min at 4°C), and resuspended in 8 ml of ice-cold FSB (freezing storage buffer, containing 100 mM KCl, 45 mM MnCl$_2$, 10 mM potassium acetate pH 7, 10 mM CaCl$_2$, 3 mM HACoCl [hexamine cobalt III chloride], 10% v/v glycerol, adjusted to pH 6.4, filter sterilised and stored at 4°C). After 15 min on ice, the cells were pelleted and resuspended in 2 ml of ice-cold FSB. 70 µl of DMSO (from spectro-quality frozen stock) was added and the cells were incubated for 5 min on ice. A further 70 µl of DMSO was added (to 7% v/v), and incubation on ice was continued for 5 min. Cells were flash-frozen in 200 µl aliquots in microfuge tubes in liquid nitrogen and stored at -80°C. For transformation, an aliquot was removed from the freezer directly onto ice and allowed to thaw over 10 min. DNA was added and the competent cells were transformed (see below) but without further DMSO.

Transformation of competent bacteria (derived from Maniatis et al., 1982; **van Die et al.,** 1983a). DNA (up to 1 µg in up to 10 µl of TE buffer or water) and 3 µl of DMSO (from spectro-quality frozen stock) was added to 200 µl of competent cells, and the tubes were incubated on ice for 30 min, heat-shocked in a water bath at 43 to 44°C for 45 s with manual agitation,

and replaced on ice while 1 ml of LUB was added. The cells were incubated at 37°C for up to 90 min to allow expression of antibiotic resistance, pelleted (20 s, microfuge), and resuspended in 100 μl of LUB. Appropriate dilutions were made in PBS, and 100 μl samples were spread on selective agar plates, or 10 μl drops were carefully placed on the agar and allowed to dry. Transformants were grown overnight at 37°C.

2.3.10 Restriction endonuclease digestion. Restriction enzymes (BRL or Anglian) were stored at -20°C. Reaction mixtures, usually containing 10 units (U) of enzyme and 1 μg of DNA in 1 x high, medium or low salt buffers as suggested (**Maniatis et al.**, 1982), except for KpnI which required the buffer specified by the manufacturer for full activity and HindIII which was more active in core buffer (BRL), were generally incubated in microfuge tubes at 37°C for 1 h. Reactions were terminated by incubation at 65°C for 10 min. Occasionally, this was followed by **phenol extraction** with an equal vol of phenol-chloroform (containing 500 g of crystalline phenol, 500 ml of chloroform, 20 ml of isoamylalcohol, 0.1 g of 8-hydroxyquinoline, all stored under 10 mM Tris pH 7.3 at 4°C), centrifugation (microfuge, 5 to 15 min), removal of the upper clear aqueous layer into a new tube, and by **ethanol precipitation** of the DNA with 2 vol of absolute ethanol and 0.1 vol of 3 M sodium acetate pH 5, incubation at -80°C for 10 to 30 min, centrifugation (microfuge, 5 to 15 min), aspiration of the supernatant, washing the pellet in 70% (v/v) ethanol, centrifugation (as above), aspiration of the ethanol, vacuum drying the pellet, and resuspension in a minimal vol of TE buffer (typically 10 μl).

2.3.11 Total bacterial DNA extraction. Bacterial cultures were grown overnight in LUB-glucose. Cells from 10 ml lots were harvested in glass McCartney bottles by centrifugation (MSE 'Centaur' benchtop centrifuge at 5 k rpm for 5 min), and resuspended in 4 ml of STE buffer (sucrose-Tris- -EDTA, containing 15% w/v sucrose, 50 mM Tris and 50 mM EDTA, pH 7.5). Approximately 20 mg of solid lysozyme was added, then incubated at room temperature for 5 min. Cells were lysed by the addition of 2 ml of SDS solution (10% w/v in TE buffer) and continued incubation at room temperature for 15 min. Protein was denatured by phenol extraction with gentle mixing by manual inversion for 5 min. The crude lysate was cleared by centrifugation (5 k rpm, 10 min) and transferred to a fresh McCartney bottle. After a second phenol extraction and clearing spin, the aqueous layer was transferred to a plastic universal tube and DNA was ethanol precipitated by gentle inversion. DNA strands were immediately removed with a glass Pasteur pipette (its end bent to 90° in the flame of a Bunsen burner), briefly

rinsed in 70% (v/v) ethanol, and gently resuspended in 0.5 ml of TE buffer in a microfuge tube. After a further round of phenol extraction and ethanol precipitation as above, the DNA was resuspended in 0.3 ml of TE buffer and stored at 4°C. [See text for notes on other high molecular weight DNA extraction methods also tried.]

Table 2.1 Escherichia coli strains

Strain	Genotype	Phenotype or utility[a]	Reference
444-3		MRHA$^+$, MSHA$^+$, (O2:K?:H4), Ap^r, Pc^r, Su^r, virulent (enteritis)	Nandadasa et al. (1981),
469-3		MRHA$^+$, MSHA$^+$, (O2:K?:H4), Ap^r, Pc^r, Sm^r, Sp^r, Su^r, virulent, cryptic plasmid	Nandadasa et al. (1981),
BHB2688	recA [λimm^{434} cIts b2 red Eam Sam]/λ	For in vitro packaging	Hohn and Murray (1977)
BHB2690	recA [λimm^{434} cIts b2 red Dam Sam]/λ	For in vitro packaging	Hohn and Murray (1977)
C600	F$^-$ thi-1 thr-1 leuB6 lacY1 tonA21 supE44 λ$^-$	General purpose K-12 cloning recipient	Appleyard (1954)
CSH26	ara Δ(gpt-pro-lac)	General purpose K-12 cloning recipient	Smith and Gots (1980)
DS410	minA minB rpsL	Minicell-producing strain, Sm^r	Dougan and Sherratt (1977)
ED8654	supE supF hsdR$^-$M$^+$S$^+$ met trpR	Cosmid recipient	Murray et al. (1977)
HB101	F$^-$ hsd20R$^-$M$^-$ recA13 proA2 lacY1 galK2 ara-14 mtl-1 rpsL20 supE44 xyl-5 λ$^-$	General purpose cloning recipient, K-12/B hybrid, Sm^r	Boyer & Roulland-Dussoix (1969)
LE392	hsdR$^-$M$^+$S$^+$ met trpR (?)	Sup$^+$ derivative of ED8654	Maniatis et al. (1982)
LG1407		Na^r & Rp^r derivative of cured 444-3	Williams et al. (1984)
LG1505		MRHA$^-$ derivative of 469-3	Williams et al. (1984)
RB308	(F$^+$::Tn1000)	Transposon also called Gamma-Delta	Guyer (1978)
RU2537	::Tn1723 recA56 (F$^+$ pro-22 met-63)	Na^r J53 derivative, plus Tn1723 (Km^r)	Shingler and Thomas (1984)

Notes to Table 2.1

a) Resistance to antibiotics: **Ap^r** (ampicillin); **Cm^r** (chloramphenicol); **Km^r** (kanamycin); **Na^r** (nalidixic acid); **Pc^r** (penicillin); **Rp^r** (rifampicin); **Sm^r** (streptomycin); **Sp^r** (spectinomycin); **Su^r** (sulphonamides).

Table 2.2 __Cloning vectors__

Vector name	Replicon	Copy number[a]	Size (kb)	Selectable markers[b]	Cloning sites[c]	Reference
pBR322	ColE1	High	4.4	Ap^r Tc^r	EcoRI; BamHI (in Tc^r)	Bolivar et al. (1977)
pBR325	ColE1	High	5.7	Ap^r Cm^r Tc^r	EcoRI (in Cm^r)	Bolivar (1978)
pACYC184	p15A	Low	4.0	Cm^r Tc^r	EcoRI (in Cm^r); BamHI (in Tc^r)	Chang and Cohen (1978)
pOU71	R1drd19	Variable[d]	5.8	Ap^r	BamHI, EcoRI	Larsen et al. (1984)
Cos4	ColE1	High	5.9	Ap^r	BamHI, EcoRI, PvuII	Unpublished[e]

Notes to Table 2.2

a) High copy number = 20 to 200 copies per cell. Low copy number = 1 to 20 copies per cell.

b) Selectable antibiotic resistance markers expressed by E. coli recipients (**Ap^r** = ampicillin resistance; **Cm^r** = chloramphenicol resistance; **Tc^r** = tetracycline resistance).

c) Unique restriction enzyme recognition sites located in non-essential regions or, where indicated, within resistance markers (allowing insertional inactivation).

d) Temperature sensitive copy number control system. Low copy number during growth at 30°C, increasing to 1000 per cell during growth at 42°C.

e) Cos4 was constructed by Drs. Lehrach and Reedy, European Molecular Biology Laboratory, Heidelberg.

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Table 2.3 <u>Antibiotics</u>[a]

Antibiotic	Stock solution[b]		Working solution[c]	
name	mg/ml	solvent	µg/ml	maximum life
Ampicillin	25	Water	35-100	2 weeks[d]
Chloramphenicol	20	Ethanol	10-30	5 days
Kanamycin	25	Water	25-100	3 weeks
Nalidixic acid	40	50% ethanol	50-100	2 weeks
Rifampicin	10	50% ethanol[e]	10	3 weeks
Streptomycin	200	Water	25-100	3 weeks
Tetracycline	12.5	50% ethanol	5-15	3 weeks

Notes to Table 2.3

a) Antibiotics were prepared, stored and used as recommended by **Maniatis et al.** (1982).

b) Stock solutions were made up in the solvent and concentrations shown, filter sterilised (Millipore 0.45 µm-pore filters) and stored in 1 ml aliquots in microfuge tubes at $-20^{o}C$, except for streptomycin which was made by adding 5 ml of sterile water to 5 g of injection-grade drug in sterile vials and stored at $4^{o}C$.

c) Aliquots were taken from stock, thawed at room temperature and added to media at below $55^{o}C$ (i.e. hand-hot) to the concentrations shown [microfuge tubes with any remaining contents were discarded]. Media containing antibiotics were stored at $4^{o}C$ and used within the specified periods.

d) To prevent ampicillin-sensitive bacteria growing, β-lactamase producing ampicillin-resistant strains were inoculated onto agar plates at low to medium densities, and colonies were grown for minimal periods at $37^{o}C$.

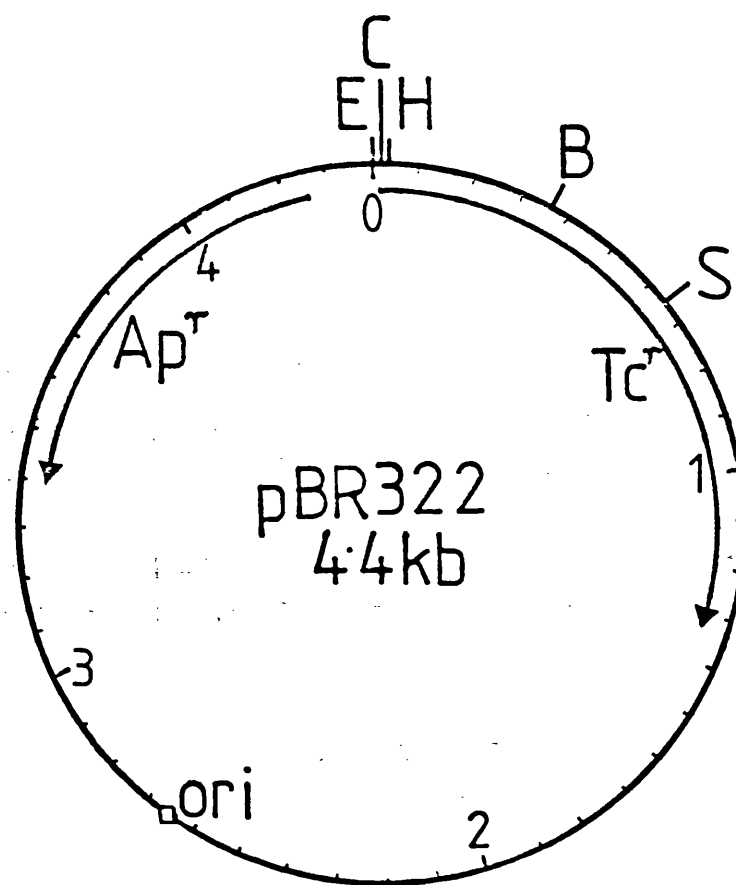e) K_2CO_3 was added to the rifampicin stock solution at 50 mM.

Figure 2.1 <u>Genetic maps of cloning vectors</u>
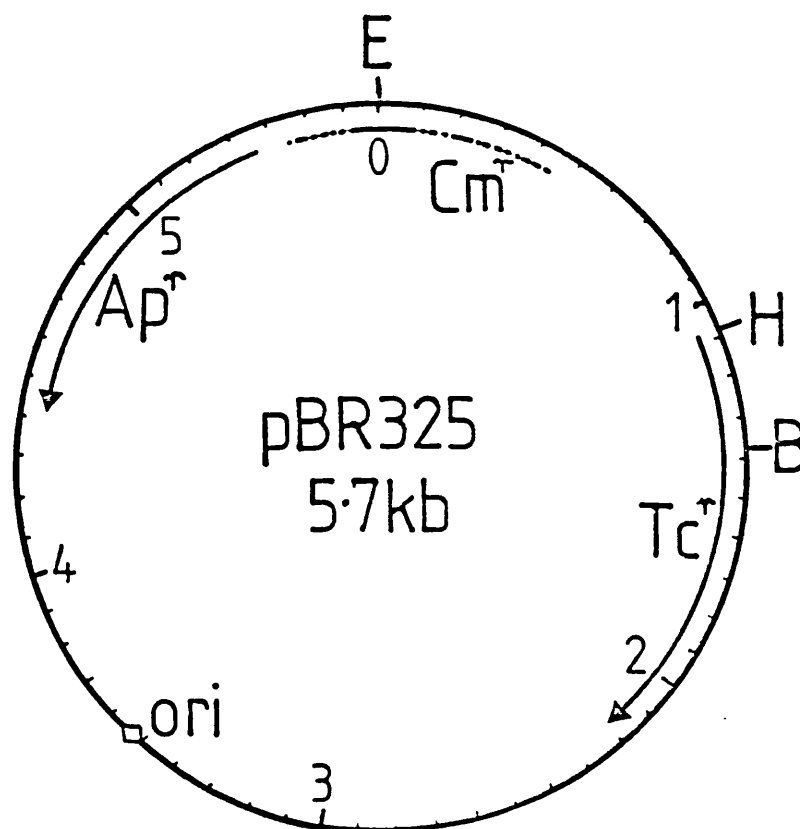
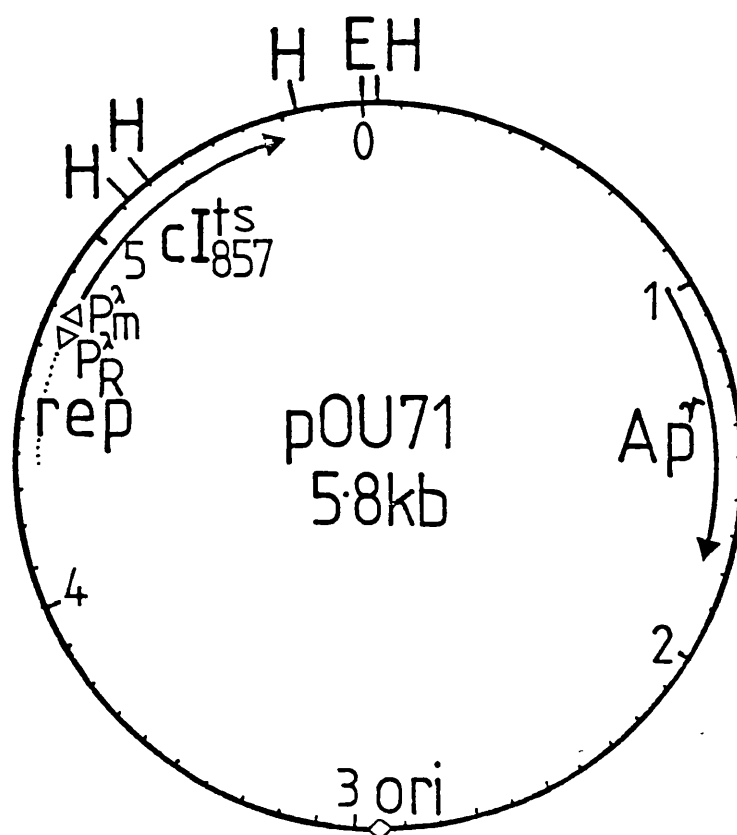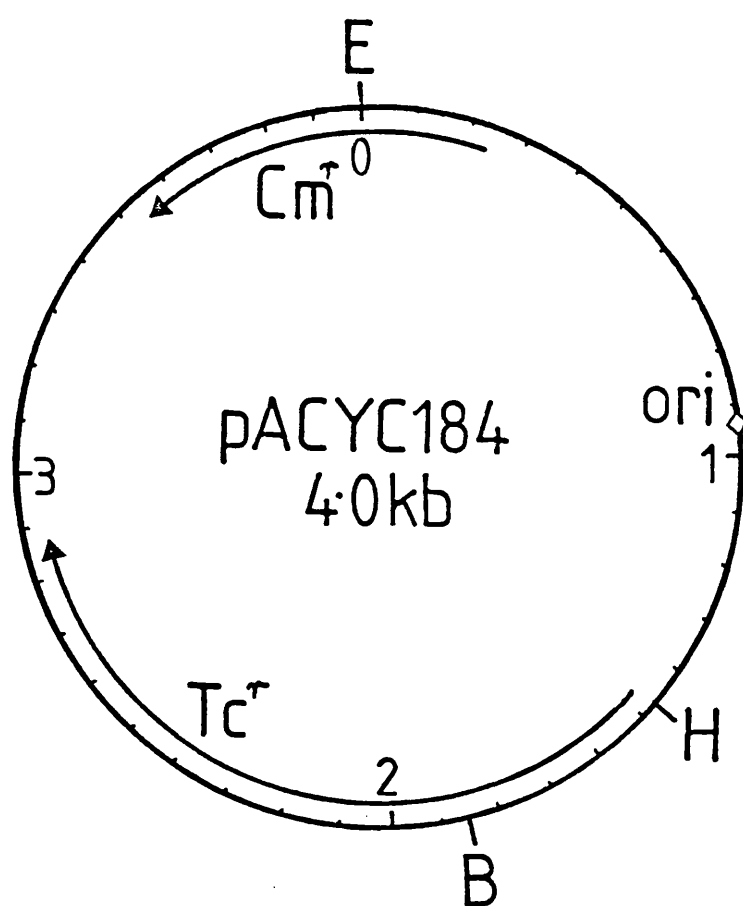Figure 2.1 continued. <u>Genetic maps of cloning vectors</u>
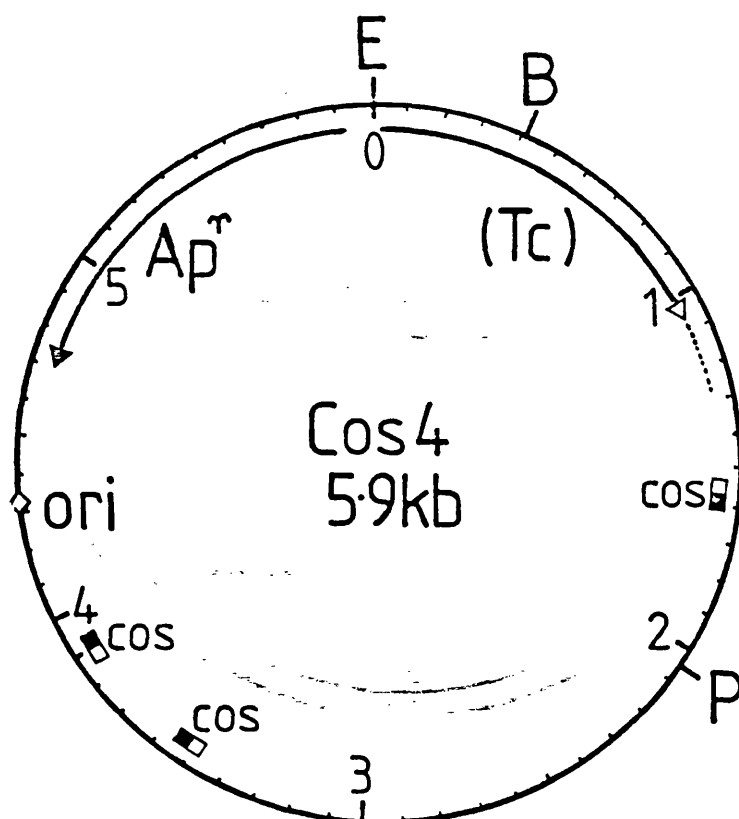

pACYC184
4·0kb

pOU71
5·8kb

Figure 2.1 continued. <u>Genetic maps of cloning vectors</u>
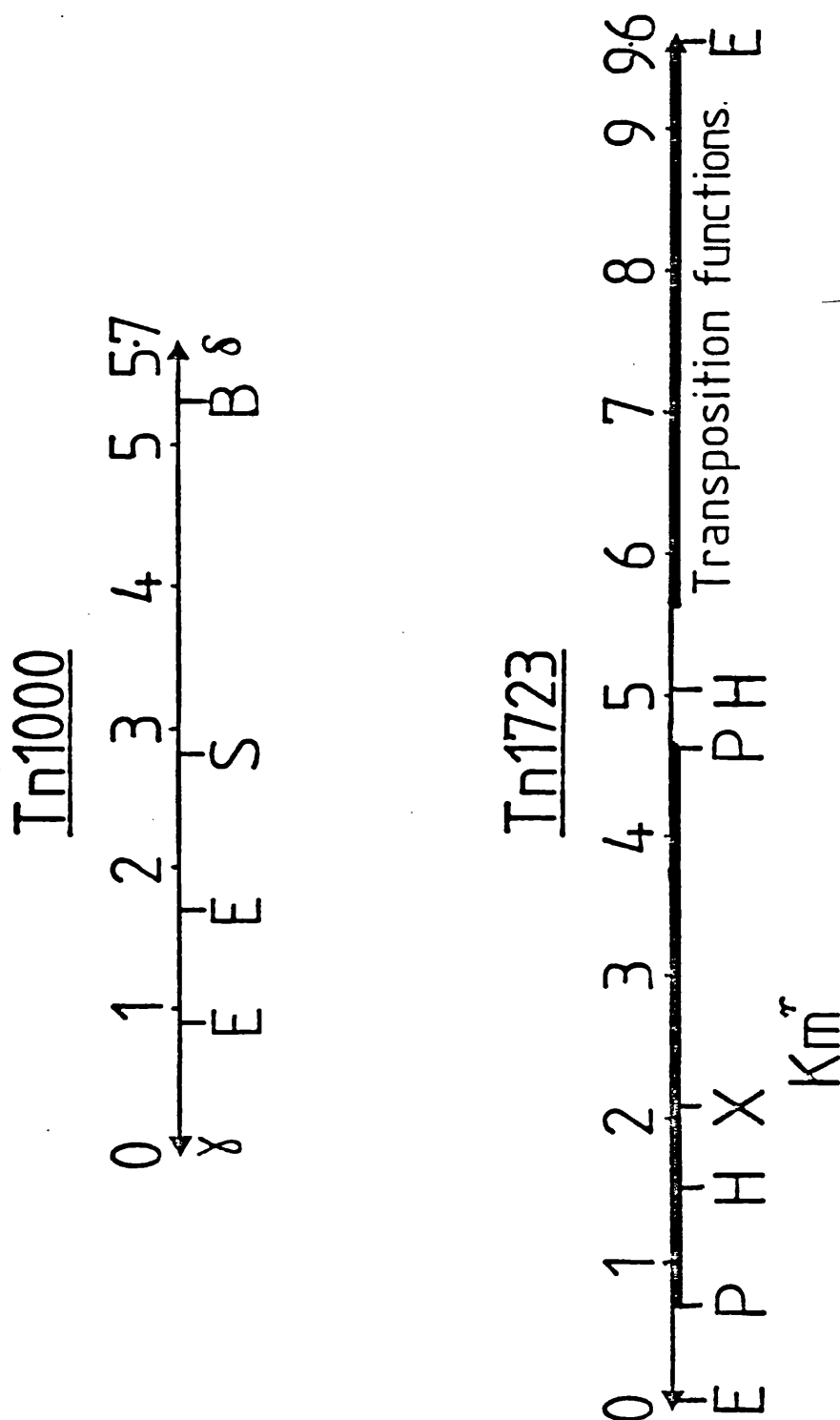

Abbreviations used in Figure 2.1

Restriction sites		Marker genes etc.	
B	<u>Bam</u>HI	Ap^r	ampicillin resistance gene
C	<u>Cla</u>I	Cm^r	chloramphenicol resistance gene
E	<u>Eco</u>RI	Tc^r	tetracycline resistance gene
H	<u>Hind</u>III	cos	lambda cohesive ends
P	<u>Pvu</u>II	ori	origin of replication
S	<u>Sal</u>I	rep	replication genes
X	<u>Xho</u>I		

Figure 2.2 <u>Genetic maps of transposons</u>

Notes to Figure 2.2

a) Restriction sites are abbreviated as in Fig. 2.1.

b) Tn1000 is also known as Gamma-Delta since the ends are termed γ and δ.

c) Tn1723 encodes resistance to kanamycin (Km^r).

3.1 Cloning and screening methods. Previous reports on the MR adhesins of the clinical isolates 444-3 and 469-3 were limited by the fact that the strains also synthesise MS adhesins and capsules. I therefore decided to clone the genetic determinants of the MR adhesins into laboratory strains of E. coli with simpler cell surfaces and better defined genetic backgrounds to facilitate phenotypic and genetic analysis.

Many adhesins are known to be plasmid encoded (Table 1.3). Strains 444-3 and 469-3 carry several plasmids which potentially might carry the MR adhesin determinants (section 1.5). However previously when the plasmids were conjugally transferred to recipient strains the MRHA phenotype was never expressed although multiple antibiotic resistance was expressed from transferred plasmids (Nandadasa et al., 1981). Recipients of various serotypes were used to reduce the chance of serotype specific non-expression. Furthermore, when strain 444-3 was cured of its single large multiple-drug resistance plasmid by ethidium bromide treatment, the MRHA phenotype was not lost (similar experiments were not attempted on strain 469-3 due to the presence of a small cryptic plasmid in addition to the large multiple drug resistance plasmid, since both plasmids would probably not be cured by this method). Therefore, the available data indicated a probable chromosomal location for the MRHA determinants, implying the need to obtain a 'library' of clones containing the entire genome.

3.1.1 Enrichment for haemagglutinating bacteria. The MRHA phenotype could not be selected for in vitro in the same way that, for example, prototrophy or antibiotic resistance can. An entire library of clones would have to be screened for MRHA instead. Screening a large number of strains by slide MRHA tests would be technically possible but somewhat tedious. I therefore developed a method for increasing the relative proportion of haemagglutinating bacteria in a mixture with non- haemagglutinating ones by several cycles of binding to erythrocytes and removal of nonadherent bacteria by washing. [The method was similar in design to that described in a former publication (Williams et al., 1984) which was used in the converse sense to enrich for non-haemagglutinating mutants in a population of haemagglutinating bacteria].

In experiments designed to test the enrichment methods, defined proportions of haemagglutinating, rifampicin-sensitive strain 444-3 and mutant derivative (made by chemical mutagenesis and screening for a non-haemagglutinating mutant of a spontaneous rifampicin-resistant variant of 444-3) were mixed. The results (Table 3.1) indicated some enrichment for

the rifampicin-sensitive fraction (just 9-fold after two cycles) but unfortunately, and in contrast to the results obtained with the converse experiment mentioned above, the separation was not improved by further cycles. Probably, the limitation was partly a consequence of samples taken from the erythrocyte pellets being contaminated with bacteria from the supernatant since the pellets were not very coherent (the mixtures could not be centrifuged too hard or else the nonadherent cells would have entered the pellets). Furthermore, phase variation placed a practical constraint on the method since some of the supposedly haemagglutinating rifampicin-sensitive 444-3 cells were probably non-haemagglutinating phase variants in the supernatant fractions. Neither of these problems could be surmounted so the method was eventually abandoned.

3.1.2 Nitrocellulose haemadsorption (NCHA). A method initially used to test Haemophilus influenzae strains for haemagglutinins (Connor and Loeb, 1983) seemed suitable for rapid screening of an E. coli genomic library. Bacterial cultures grown on agar plates (for example, hundreds of transformants) were transferred to nitrocellulose filter disks, washed, and incubated with erythrocytes. Haemadsorption (i.e adherence of erythrocytes to the adsorbed material, forming red patches) correlated with the ability to cause MRHA. In pilot experiments using known haemagglutinating and non-haemagglutinating strains of E. coli and fresh human erythrocytes (**Fig. 3.1**), the test was found to be sensitive and reproducible, although the red marks faded quite rapidly and were completely lost once the filters dried. Such loss could be prevented by fixing the bound erythrocytes with tannic acid. Some non-haemagglutinating colonies did appear to be positive due to uneven mixing of the erythrocytes, but on the other hand, excessive mixing released all red cells previously bound to positive colonies. A compromise was reached (**section 2.2.1**), making the method perfectly adequate for rapidly pre-screening cultures prior to confirmatory slide MRHA tests.

3.2 Plasmid cloning. Most adhesins studied to date are encoded by genes occupying up to 10 kb of DNA which are contiguous (section 1.4), except for CFA/I which is encoded by two distinct regions. Therefore, I made the simplifying assumption that the adhesins of 444-3 and 469-3 are probably encoded within single DNA fragments of similar size. Plasmids could be used to generate a library containing genomic fragments of, say, 15 kb to avoid losing the ends, whereas cloning much larger fragments in plasmids would be rather more difficult since transformation efficiency is inversely proportional to plasmid size, increasing the background of transformants containing recircularised vectors or vectors with only small inserts

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(Maniatis et al., 1982). The minimum number of plasmids with 15 kb inserts required to clone a unique 10 kb sequence ranges from 2,300 for 95% probability of including the entire E. coli genome, to 3,500 for 99% probability (Table 3.2).

3.2.1 Preliminary experiments. Chromosomal DNA from strains 444-3 and 469-3 was extracted by the method of Chow et al. (1977) and partially digested with EcoRI to a mean fragment size of approximately 15 kb. The genomic DNA was ligated with vector plasmid pBR322 which had been linearised with EcoRI and treated with alkaline phosphatase to prevent it recircularising without inserts (autoligation), and used to transform laboratory strain CSH26. Ampicillin-resistant transformant colonies were screened by NCHA and slide MRHA tests, and their insert sizes were checked by plasmid minipreparations, restriction digestion and agarose gel electrophoresis. None of the transformants tested was haemagglutinating, and most did not appear to carry insert fragments of appreciable size (data not shown). The partially digested chromosomal DNA preparations apparently contained a large proportion of small fragments of less than approximately 5 kb (inserted into 4 kb vectors, totalling less than 9 kb) which ligated and transformed with much higher efficiency than the desired inserts of about 15 kb (generating 19 kb plasmids). Many transformants did not appear to carry any inserted fragments, probably because of failure of the phosphatase or residual amounts of unrestricted vector molecules. I tried a number of improvements to the method to overcome these problems.

3.2.2 Improving the transformation efficiency. I hoped to increase the chance of obtaining transformants containing larger plasmids by improving the overall transformation efficiency. Competent cells prepared by $CaCl_2$-$MgCl_2$ treatment of strain CSH26 routinely gave about 10^6 transformants per μg of supercoiled pBR322. Changing to the $CaCl_2$-$RbCl_2$ method increased this to approximately 5×10^7 transformants per μg. Other recipients (HB101, C600, etc.) were tried, and HB101 was chosen for routine use because of its good competence (up to 5×10^9 transformants per μg), streptomycin resistance (usable as a selective marker), and RecA⁻ phenotype (reduces the possibility of recombination within a cloned fragment or between the cloned DNA and the recipient cell genome).

Although it has been reported that competent cells become more competent during prolonged (overnight) incubation in $CaCl_2$ (Dagert and Ehrlich, 1979), in my hands 95% of the cells died overnight and a lower proportion of the remaining viable cells were transformed than without the overnight step (0.15% as opposed to almost 10%).

I prepared several batches of competent cells in bulk, using the $CaCl_2$-$RbCl_2$-HACoCl method and froze them rapidly in small aliquots in liquid nitrogen. Unfortunately, most of these preparations were not sufficiently competent to justify their use in subsequent experiments so this method was abandoned. At the same time a number of other laboratories in this department reported difficulties in bacterial transformation experiments, possibly attributable to contaminants of some sort in the water supply or to minute traces of detergents in the glassware prepared by our media kitchen. I was therefore forced to accept the $CaCl_2$-$RbCl_2$ method as the best available.

Efforts to improve the transformation method were hampered by these uncontrollable, apparently random variations in the transformation efficiencies of individual competent cell preparations. I developed a crude but quick competent cell preparation starting with overnight bacterial cultures, avoiding the usual delay while cultures are grown to mid-logarithmic growth phase. Using this rapid $CaCl_2$-$MgCl_2$ method, HB101 cells could be transformed at fairly low efficiencies with pBR322, but the main advantage was that starting with such dense cultures, large numbers of competent cells were obtained in one attempt and several aliquots (equally competent) could be simultaneously transformed. For example, competent HB101 cells prepared by the rapid method were used to determine the optimal period for the heat-shock during transformation (**Fig. 3.2**).

3.2.3 Size-selecting the DNA. To reduce the proportion of plasmids containing small inserts, I tried to size-select fragments of partially digested chromosomal DNA of greater than about 10 kb prior to ligation, by eluting DNA from agarose gels onto DEAE-sepharose paper or by separating the DNA mixtures on 5 - 20% NaCl density gradients (**Hull et al., 1981**). However, both these methods proved to be rather inefficient for such large fragments and consequently required the use of large amounts of partially digested DNA. At the time, I was unable to extract very high molecular weight chromosomal DNA from either of the clinical strains, and much of the small amount of material I obtained was sheared to less than about 50 kb, limiting the yield of 15 kb fragments relative to smaller fragments upon partial digestion. This problem was not fully solved until later (see **section 3.3.1**), so size fractionation was not practicable at the time.

3.2.4 Controllable copy number vectors. Vector pBR322 is a multicopy plasmid, normally present in 20 to 200 copies per cell, so cloned genes are amplified to this degree and are usually over-expressed relative to unique-copy chromosomal determinants. Evidently this can be lethal if outer

membrane proteins, for instance, are cloned and over-expressed from such vectors (**Henning et al.**, 1979; **Silver et al.**, 1981). Since MR adhesins are exported from bacteria and assembled on the cell surface, it is possible that their over-expression might also be lethal which might perhaps have explained the failure to isolate MRHA-positive clones previously. Conversely, cloning in a low copy number vector might limit the expression too much, defeating attempts to extract larger quantities of the adhesin for immunological and ultrastructural experiments (part of the initial aims of this study). An ideal solution to this predicament would be to use a system whereby the copy number is controllable. Vector pOU71 contains such a system (**Table 2.2** and **Fig. 2.1**). The plasmid is normally present at low copy number in cells grown at 30°C but replicates to very high numbers during growth at elevated temperatures, reaching several hundred copies per cell at 42°C.

In a preliminary experiment, I grew a culture of HB101pOU71 to late exponential phase then split it into equal portions. One portion was heat-shocked (42°C for 10 min); the other remained in the incubator. Both portions were then incubated a further hour at 30°C. The plasmid copy number, crudely estimated from the DNA yield from 1.5 ml lots of culture (taking no account of different growth rates) or resistance to ampicillin (as determined by the width of clear zones caused by high levels of ampicillin around test discs on agar plates), was apparently increased but the difference was marginal, presumably as a result of plasmid instability in connection with the metabolic load on cells carrying many hundreds of plasmids each. I considered that it would probably not be practicable to generate a genomic library of 15 kb inserts in this vector because of the inherent instability. Moreover, simultaneous reports on the cloning of other adhesin systems suggested that over-expression of those adhesins did not appear to cause cell death. Therefore, I discontinued attempts to generate a library in pOU71, and returned to conventional plasmid vectors.

3.2.5 Insertional inactivation. Insertion of DNA fragments into restriction sites within antibiotic resistance marker genes prevents expression so that transformants can simply be screened for antibiotic sensitivity to detect the presence of inserts. Vector pBR322 (**Fig. 2.1**) was linearised by restriction digestion with <u>Bam</u>HI within the tetracycline resistance marker gene and treated with alkaline phosphatase to prevent autoligation. Simultaneously, chromosomal DNA was prepared from 444-3 as before, partially digested with <u>Sau</u>3A (which generates compatible cohesive ends to <u>Bam</u>HI) to approximately 15 kb, ligated with a 10-fold numerical excess of vector molecules overnight and transformed into <u>E. coli</u> HB101. Transformants were selected on agar containing ampicillin and other ingredients to enhance the

growth of tetracycline-sensitive cells relative to tetracycline-resistant bacteria (Bochner et al., 1980; Maloy and Nunn, 1981), then individual colonies were replica plated on agar containing ampicillin, either with or without tetracycline. Some 571 ampicillin- resistant tetracycline-sensitive clones, out of 832 tested, were patch-plated onto stock plates. All these colonies were tested by the NCHA method but none was positive. Plasmid DNA was prepared from several cultures and analysed by restriction digestion and agarose gel electrophoresis (data not shown). Most marker inactivations were evidently not caused by DNA insertions, since there was no apparent change in the size of these plasmids relative to pOU71. Alternatively, very small inserts (below the limit of detection on agarose gels) may have been present. Control experiments in which HB101 was transformed with linearised, phosphatase-treated vector after the DNA was incubated overnight with ligase but without genomic fragments, gave significant numbers of tetracycline-sensitive clones but fewer than before. Leaving out the phosphatase treatment entirely, all the transformants were tetracycline-resistant but of course the background of vectors without inserts would be far too high to isolate the required number of clones.

3.3 **Cosmid cloning.** It became increasingly clear that cloning in cosmid vectors held more promise of success than if I were to persevere with plasmid cloning. Essentially, cosmid cloning involves inserting random fragments of genomic DNA with prepared vector, packaging the recombinant cosmids into infectious bacteriophage particles in vitro and transfection of suitable recipient strains (**Fig. 3.3**). The cosmids then replicate in similar fashion to large plasmids. Since they contain such large fragments of DNA (up to about 45 kb), representative genomic libraries need contain far fewer clones (**Table 3.2**). As a useful bonus, the in vitro packaging reaction is inherently size-selective so there was no need to return to the size selection methods tried before.

3.3.1 **High molecular weight bacterial genomic DNA preparations.** The major problem was to obtain very large fragments of bacterial DNA from the clinical isolates in sufficient quantity and purity. Cosmid cloning required genomic DNA to be partially digested with a restriction enzyme having a tetranucleotide recognition sequence (Sau3A), generating quasi-random fragments since any given tetranucleotide can be expected to occur approximately four times per kb of random sequence, with cohesive ends separated by 32 to 45 kb. This meant that the DNA should start with fragment lengths of at least, say, 100 to 200 kb. As mentioned above, the chromosomal DNA preparation initially used (described by **Pritchard and Holland, 1985,**

derived from **Chow et al., 1977**) gave quite small amounts of material, most of which was too small for cosmid cloning. I therefore tried alternative methods for preparing total bacterial DNA, some of which had been used previously for cosmid cloning, and adapted and combined the most successful parts of them for the encapsulated clinical strains. I thought that the method of **Chow et al. (1977)** may have failed due to the action of cellular nucleases during the relatively slow Sarkosyl lysis, or perhaps nucleases were not fully degraded during the prolonged treatment with proteinase (at 42°C for 5 h). Faster lysis with 2% SDS, followed by rapid digestion of the lysate with Proteinase K (Sigma) at 1 mg/ml and at 50°C (**Hull et al., 1981**) improved the yield of large fragments but was still insufficient for cosmid cloning. The subsequent removal of Proteinase K by phenol extraction was also found to shear the DNA to an unacceptable level, so the method was modified further (see **section 2.3.11** for details). Bacteria were lysed by adding SDS to 5% and proteins were rapidly denatured by gentle but thorough treatment with phenol. Extreme care was required to avoid manipulations which sheared the DNA, such as forcing the lysate through small apertures in standard glass Pasteur pipettes (disposable plastic pipettes with larger nozzles were used) or too energetic mixing or centrifugation, and the resultant material was reliably found to be of very high molecular weight, such that most of it would not even leave the slots of low percentage agarose gels and was significantly larger than the 48.6 kb standard (**Fig. 3.4**).

Pilot experiments were performed to establish the conditions required to digest high molecular weight bacterial DNA with Sau3A to a mean size of about 50 kb (**Fig. 3.4**). Approximately 10 µg of genomic DNA was then digested to a mean size of about 40 to 50 kb.

3.3.2 Completion of the cosmid cloning. The prepared vector and insert DNA molecules were ligated overnight (**Fig. 3.5**), packaged in vitro and transfected into strains LE392 and ED8654 (**section 2.3.5**). Over 500 clones were obtained; 504 single colonies were picked and stored in microtitre plates in 50% glycerol at -20°C. They were screened for HA by NCHA tests and 13 putative positive clones were identified. These were streaked out for purity and re-tested by slide MRHA tests, confirming 8 positives.

3.4 Subcloning the adhesin determinant. The size and instability of the cosmids imposed practical problems on their analysis which were overcome by subcloning parts of the cosmid from LE392(pCC3) into plasmid pBR322. Cosmid DNA was extracted from the strain by the plasmid minipreparation method, digested with BamHI, ligated with BamHI-digested and dephosphorylated vector

pBR322 DNA and used to transform E. coli strain HB101. About 100 transformants were assayed by NCHA but none was haemadsorbing, whereas HB101 transformed with untreated cosmid DNA from the clone was haemadsorbing and haemagglutinating (Table 3.3). The subcloning attempt was repeated with enzyme EcoRI. This produced 6 NCHA positive transformants out of 20 tested. DNA was prepared by the small-scale method from the same 20 strains, digested with EcoRI and analysed by agarose gel electrophoresis (Fig. 3.6). Each haemadsorbing (and haemagglutinating) strain carried a 17 kb EcoRI fragment in addition to the 4.4 kb vector band, whereas the remaining plasmids lacked a 17 kb fragment.

3.5 Conclusions.

3.5.1 Plasmid cloning. Several groups have successfully constructed bacterial genomic libraries in plasmid vectors. However, the protocol demands that each part of the procedure, from the initial extraction of genomic DNA to the final isolation of positive clones, must be performed efficiently, whereas I experienced practical difficulties at almost every stage.

The problems started with the extraction of genomic DNA from clinical isolates 444-3 and 469-3. In my hands, none of a variety of published methods for extracting bacterial chromosomes yielded sufficient quantities of pure, high molecular weight DNA. There was interference from capsular material which increased the viscosity of the aqueous phase containing DNA and hindered its removal from the solvent phase after phenol-chloroform extraction. Attempts to increase the separation by further centrifugation merely resulted in greater shearing of the DNA and made little difference to the yield. Conversely, reducing the number or extent of phenol extractions resulted in samples contaminated with cellular nucleases which were activated during subsequent restriction digestions, again forming low molecular weight DNA fragments and smears on agarose gels. I tried treating crude lysates with proteinase to reduce the need for phenol extraction, but found that the optimal conditions for proteinase activity also suited nuclease activity. I even tried size-selecting larger fragments after digestion, but this required still more high molecular weight DNA to start with since recovery of such large fragments was poor.

The transformation of recipient strains was an inherently difficult procedure, but the problems were aggravated by the overall limitation of attainable transformation efficiency resulting from the relatively large size of recombinant plasmids. Transformation efficiencies were variable for reasons which were not ascertained, and I was only partly assuaged by

similar variability in other laboratories in the department during the same period. I encountered a number of other methodological problems, including antibiotic resistance inactivation events apparently without the expected insertion of DNA fragments at cloning sites within the marker genes (a nuclease-contaminated batch of alkaline phosphatase was probably responsible). Some of these difficulties were later solved and given more time I may have succeeded with plasmid cloning, but instead I resolved to attempt cloning in cosmids.

3.5.2 Cosmid cloning. Cosmid cloning requires the extraction of even higher molecular weight chromosomal DNA since it must be partially digested to a mean size of about 40 kb. I tried other chromosomal DNA extraction procedures including some which had previously been used for cosmid cloning. Eventually, I arrived at a successful method involving crude lysis with SDS and rapid purification with phenol, which has subsequently been used to prepare high molecular weight DNA from a number of other clinical isolates and laboratory strains of E. coli [unpublished findings].

The E. coli chromosomal DNA was substantially better digested with Sau3A than was naively expected according to the amount of DNA present and the number of units of restriction enzyme used. Within about 10 min, the mean fragment length was reduced from about 100 or 200 kb down to less than 10 kb with just 0.02 units of enzyme per µg of DNA. In other experiments, as little as 0.001 U of Sau3A per µg of E. coli chromosome digested the DNA from very high molecular weight to about 45 kb in less than 5 min at 37°C. This apparently high enzymic activity may simply have been due to the use of different reaction conditions to calculate the unit activity ('one unit fully digests 1 µg of lambda DNA in 1 h at 37°C in the designated buffer') to those used for the partial restriction (digesting E. coli DNA in medium salt buffer). Alternatively, completing the restriction digestion (from 10 kb down to 0.256 kb mean size) may take a disproportionate period of time. Perhaps least likely, the manufacturer may have been very generous with the enzyme concentration supplied.

Cosmid arms were ligated overnight with partially digested genomic DNA fragments with a mean size of about 40 to 45 kb in a molar ratio of 10:1 (i.e. approximately equal amounts of DNA) to force the reaction in the desired direction (i.e. cosmid arms at each end of single chromosomal fragments, rather than at either end of several chromosomal fragments joined together). The cosmid cloning protocol I used (ligating dephosphorylated vector with untreated insert fragments) probably did result in the ligation of different chromosomal DNA fragments to each other as well as to the cosmid arms, but most of such recombinant molecules were too large to be

packaged (the largest packageable constructs are about 51 kb). An alternative method, where the chromosomal fragments rather than the cosmid arms are dephosphorylated to prevent them ligating, would have been more difficult to perform and test, and would probably have given a considerable background of clones containing only multimerised cosmids. In the absence of a selective procedure for the adherence system, it was important to keep the number of clones to be screened to an absolute minimum so this alternative protocol was not used.

The ligated DNA was packaged in vitro using packaging extract prepared in advance and stored at -80°C, and the packaged cosmids were used to transfect two recipient strains of E. coli. Over 500 clones were isolated, approximately equal numbers in each recipient. The primary reason for using two different recipients was to increase the chance of obtaining haemagglutination in case expression of the adhesin was dependent on the host, as is the case with CFA/II (see section 1.4.9), but I later realised that the strains are isogenic apart from their suppressor genes (I was confused by the renaming of strain ED8654 as NEM259 in another laboratory). In any case, haemagglutination was efficiently expressed by all the laboratory strains used in this study.

3.5.3 Isolation of haemagglutinating clones. All the cosmid-containing clones were screened by the NCHA method after growth to small colonies densely packed on a few agar plates. The test was sensitive and reliable; the few incorrectly indicated colonies were all false-positives (non-haemagglutinating colonies which appeared to haemadsorb) which were excluded by later MRHA tests, rather than false-negatives (haemagglutinating colonies that did not haemadsorb) which would otherwise have been missed by the screening. Initially, 13 haemadsorbing colonies were identified but after subculturing these clones, slide MRHA tests confirmed 8 positives; the others were either false-positives (mis-scored) or perhaps only transiently expressed an MR adhesin and had lost the ability on the first subculture. The cosmids in the MRHA-positive strains were named pCC1 to pCC8.

MRHA was expressed to a similar degree in some of the haemagglutinating clones as in the original clinical isolate, while others were weaker (see chapter 4 for details). Since the cosmid recipients were different in many respects to the parent strain, the apparent similarity of haemagglutinin expression in some clones was encouraging; it implied that the whole of the adhesin determinant, complete with its secretion and assembly functions, had been cloned in those cosmids and was fully functional in the K-12 hosts. On the other hand, the number of positive clones obtained, and the apparent differences in haemagglutinin expression by some of them, was puzzling. It

is possible that strain 469-3 actually possesses 'minor' MR adhesins in addition to the adhesin previously described, whose expression had been too weak to be detected, or which were too similar to have been distinguished by the methods previously used. These minor adhesins may have been present in some of the cosmid clones, especially as the 'simple' surfaces of the cloning recipients (i.e. without LPS, capsule, etc.) might have allowed previously non-functional adhesins to cause MRHA. Alternatively, some cosmids may have encoded the same adhesin but differed in their expression because of the presence or absence of certain parts of the adhesin determinant (e.g. repressors or assembly functions). Some of the questions raised by these findings were addressed by comparing the clones and parent strain (chapters 4 and 5).

3.5.4 Subcloning the adhesin determinant. I decided to subclone the adhesin system on smaller DNA fragments taken from a haemagglutinating cosmid clone in a plasmid vector since the cosmids were so large (approximately 40 kb each) that fine genetic mapping and other experiments (transformation between strains, site-directed mutagenesis, minicell analysis, etc.) would have been impractical in the available time, and I was also concerned that the cosmids might be unstable (as is the case with cosmids encoding K1 capsules, for instance; Silver et al., 1981). One cosmid clone, LE392(pCC3), was picked for further study since its MRHA ability resembled that of 469-3 in terms of the strength of the reaction and the consistency from test to test (see chapter 4).

Individual BamHI fragments were subcloned from cosmid pCC3 in vector pBR322 and used to transform strain HB101. None of the transformants, however, was capable of haemagglutination, probably because the MRHA determinant contained a BamHI site in an essential region so no single DNA fragment carried the entire determinant. Non-expression of the system from the complete determinant in this host was ruled out by transforming HB101 with intact cosmid; ampicillin resistant transformants were MRHA-positive. Furthermore, various research groups published details of experiments using this strain as the host for natural and recombinant plasmids which did express adhesins. Another possibility was also considered, namely that in contrast to the situation in the low copy number cosmid Cos4, pBR322 is a multicopy vector and so overexpression of the adhesin system might have been lethal. The most straightforward approach seemed to be to repeat the subcloning attempt with another restriction enzyme (rather than switching to a low copy number plasmid), and indeed, a 17 kb EcoRI fragment of cosmid DNA was found to encode MRHA in HB101. The resultant MRHA-encoding recombinant plasmid was named pLG161.

Attempts to clone the MRHA system from 444-3 were abandoned in favour of analysing the clones derived from 469-3, although later results (**chapter 5**) indicated a degree of homology between the respective genetic determinants which may eventually enable their cloning in cosmids or plasmids and detection by hybridisation to probes derived from 469-3.

Table 3.1 Enrichment for haemagglutinating bacteria[a]

Cycle number	Viable cells/ml[b]		Ratio[c] RpS/Rpr	Enrichment (fold)[d]
	RpS	Rpr		
0	3.5×10^6	3.6×10^8	0.0097	–
1	1.9×10^6	1.0×10^8	0.019	2
2	2.6×10^6	3.1×10^7	0.083	8
3	3.6×10^6	4.3×10^7	0.083	8
4	3.0×10^6	3.3×10^7	0.091	9

Notes to Table 3.1

a) Haemagglutinating, rifampicin-sensitive (RpS) bacteria (strain 444-3) and non-haemagglutinating, rifampicin-resistant (Rpr) bacteria (strain LG1407) were grown separately in LUB overnight at 37°C. 2.5 ml aliquots of each culture were mixed in a plastic universal tube and a sample (0.2 ml) was removed and stored on ice ('cycle 0'). 5 ml of human erythrocyte suspension (washed and resuspended to 50% v/v in PBS-mannose) was mixed with the remaining bacterial mixture and the tube was incubated statically on ice for 1 h, then centrifuged (MSE 'Chillspin' at 2 k rpm for 5 min). The supernatant was carefully removed and discarded. A sample (0.2 ml) was removed from the pellet and stored on ice ('cycle 1'). PBS-mannose was added to return the mixture approximately to its previous volume, and the the contents were gently mixed and replaced on ice for further cycles.

b) Stored samples were serially diluted in PBS, and aliquots (0.01 ml) from each dilution were spotted onto agar with and without rifampicin (100 μg/ml) and grown overnight at 37°C (viable counts). The viable count of RpS bacteria was calculated by subtracting the viable count of Rpr bacteria (with rifampicin) from the total viable count (without rifampicin).

c) Ratio of viable cell counts.

d) Enrichment for RpS bacteria, expressed relative to cycle 0.

Table 3.2 <u>Theoretical minimum size of E. coli genomic libraries</u>

Probability	Target sequence size			
	1 kb		10 kb	
	Plasmids	Cosmids	Plasmids	Cosmids
95%	760	270	2300	350
99%	1200	420	3500	530

Notes to Table 3.2

The number of clones (**n**) of average insert size (**x**) required to clone a unique sequence ('target sequence') of size (**y**) from a genome of size (**z**) with a probability (**p**) is given by the equation:

$$n = \frac{\ln (1-p)}{\ln (1-(x-y)/z)}$$

(Clarke and Carbon, 1976).

The following values were used to calculate the numbers of clones (given in the table to 2 significant figures):

- total <u>E. coli</u> genome size = 3,800 kb (**Glass, 1982**).

- mean size of cloned fragment in plasmids = 15 kb.

- mean size of cloned fragment in cosmids = 42.7 kb, since the vector comprised 5.9 kb and the optimal size of constructs for packaging is 48.6 kb, i.e. the size of the wild-type lambda genome (**Glass, 1982**). Constructs of between 78% and 105% of this size are packagable, albeit at lower efficiencies, but this was ignored in calculating the values shown (the effect would be to reduce slightly the minimum numbers of cosmid clones required).

# Figure 3.1 Nitrocellulose haemadsorption (NCHA)

469-3

HB101
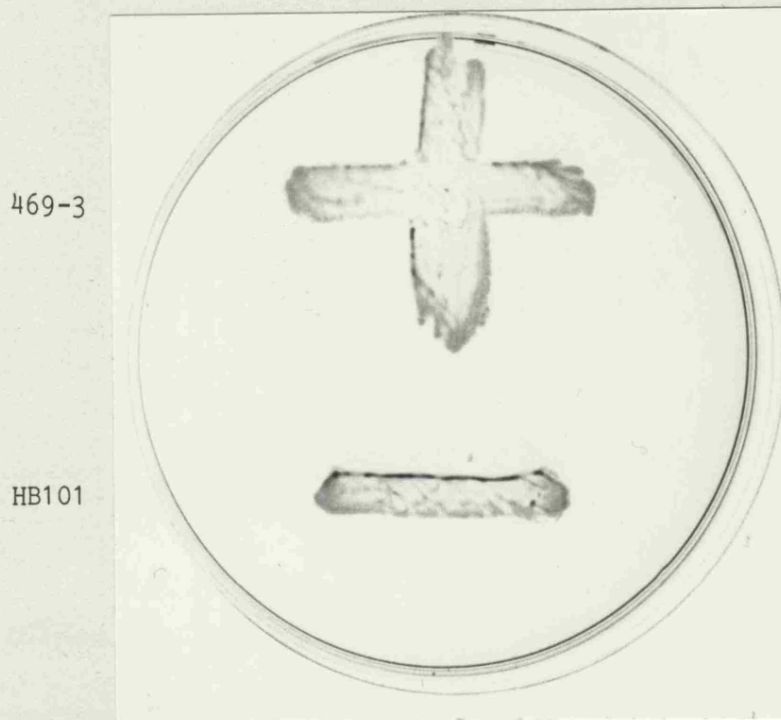
A plate of LUA was inoculated with a known MRHA-positive strain and a MRHA-negative control and photographed after overnight growth at 37°C.

469-3
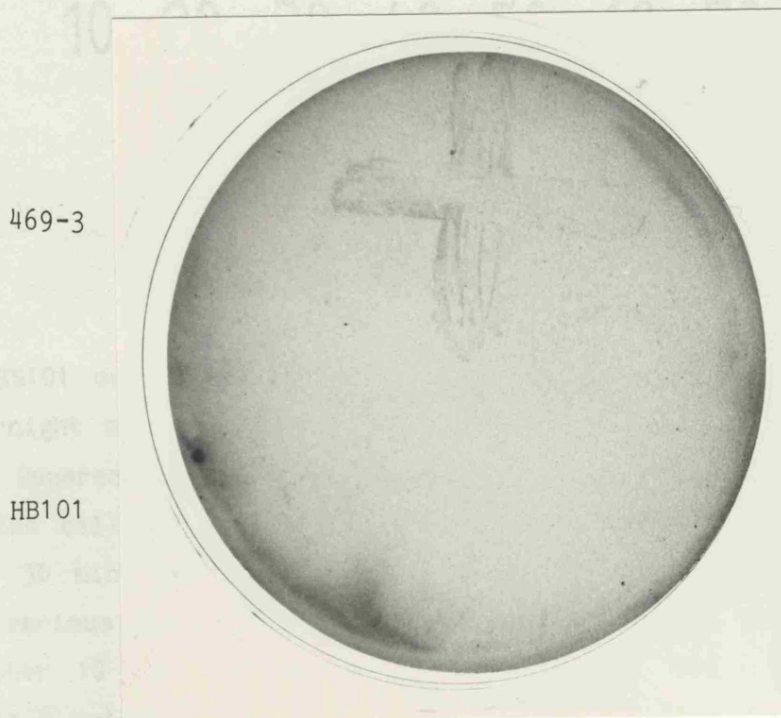
HB101

The bacterial cultures were blotted onto a nitrocellulose filter, washed, incubated with erythrocytes (section 2.2.1) and photographed.

Figure 3.2 <u>Optimisation of heat shock during transformation</u>
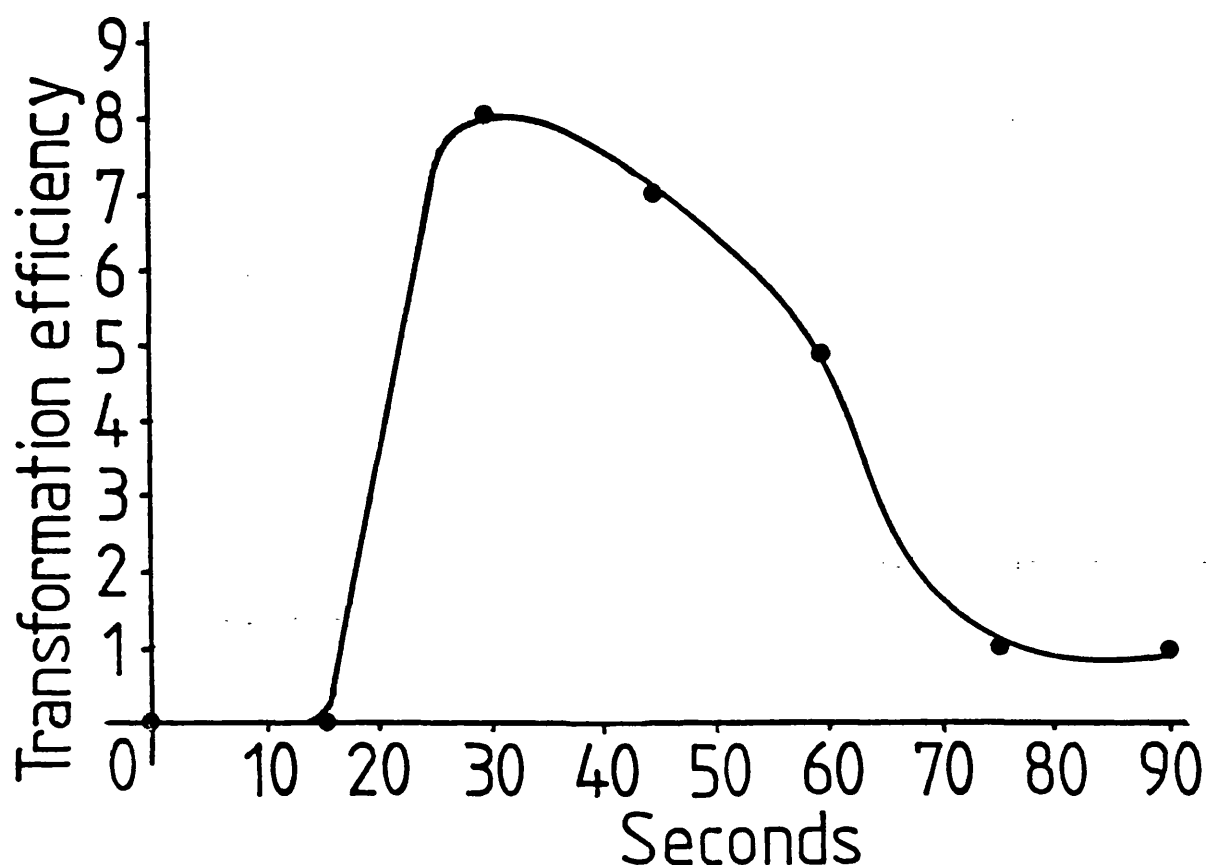
Competent HB101 cells were prepared from ten 1.5 ml lots taken from a single overnight culture by the quick $CaCl_2-MgCl_2$ method (section 2.3.9) and mixed. Supercoiled plasmid DNA (pBR322; 1 µg) and DMSO (30 µl) was added and the cells were divided into ten aliquots in microfuge tubes on ice. After 30 min, the microfuge tubes were placed in a waterbath at 43.5°C for various periods, then plunged back onto ice and incubated there for a further 10 min. LUB was added to each tube and the tubes were incubated in a waterbath at 37°C for 1 h for antibiotic resistance marker expression. Suitable dilutions were then plated out on LUA containing ampicillin (100 µg/ml) and grown overnight at 37°C. The transformation efficiency was assessed by counting colonies.

Figure 3.3 <u>Cosmid cloning protocol</u>
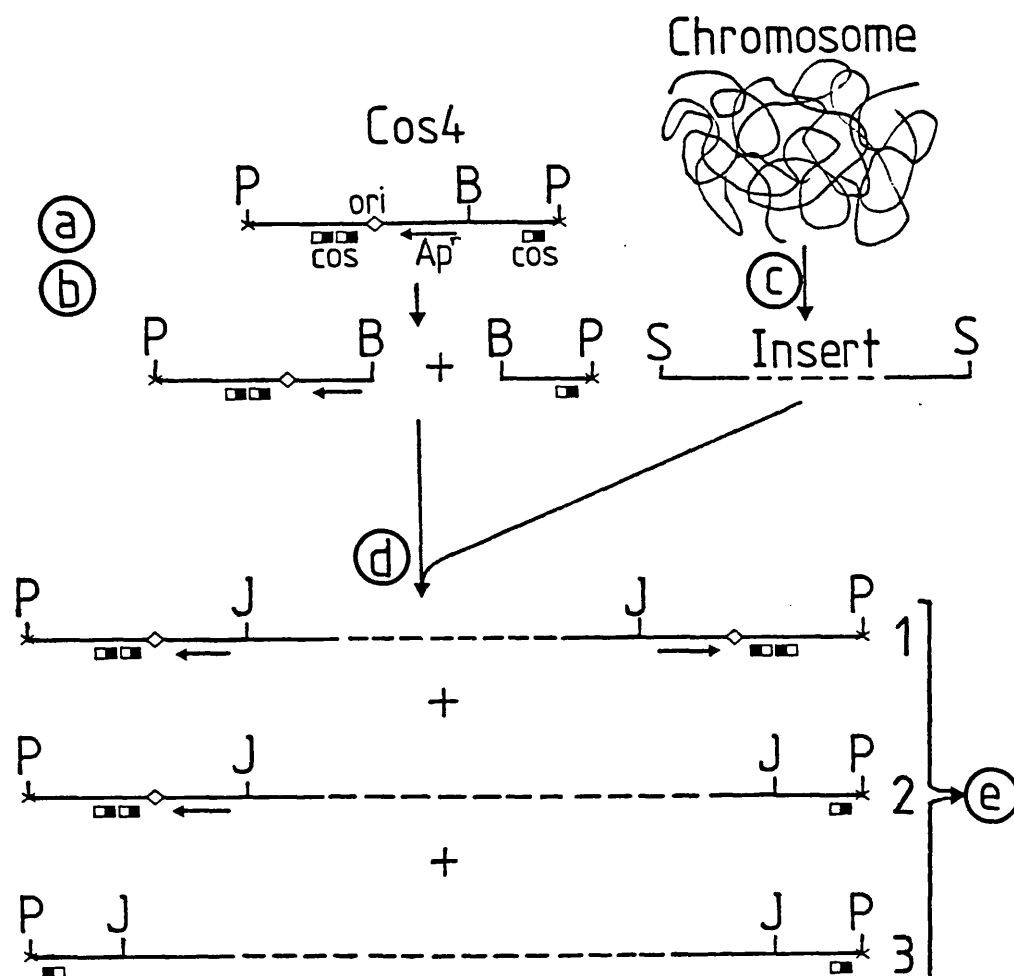

a) Cosmid Cos4 (**Fig. 2.1**) was linearised with <u>PvuII</u> (P) and treated with alkaline phosphatase to prevent recircularisation (x). The origin of replication (**ori**), ampicillin resistance marker (**Ap^r**), and <u>cos</u> sequences (**cos**) are shown.

b) Linearised cosmid was cleaved with <u>BamHI</u> (B), generating fragments of 4.3 and 1.6 kb.

c) High molecular weight genomic DNA (**chromosome**) was prepared from strain 469-3 and partially digested with <u>Sau3A</u> (S) to a mean fragment size of ca. 45 kb (**insert** fragments).

d) Cosmid and insert DNA fragments were ligated by means of their complementary cohesive ends, forming three types of molecules (1, 2, 3) containing hybrid <u>BamHI</u>-<u>Sau3A</u> junctions (J), not necessarily recognised by <u>BamHI</u>.

e) The ligated DNA was added to an in vitro bacteriophage packaging extract. Only **type 2** molecules (containing correctly orientated <u>cos</u> sequences) of the correct size (32 to 45 kb) were successfully packaged. Recipient strains were transfected with the packaged DNA and cosmid-containing recipients were selected by resistance to ampicillin.

Figure 3.4 <u>Partial Sau3A digestion of bacterial genomic DNA</u>
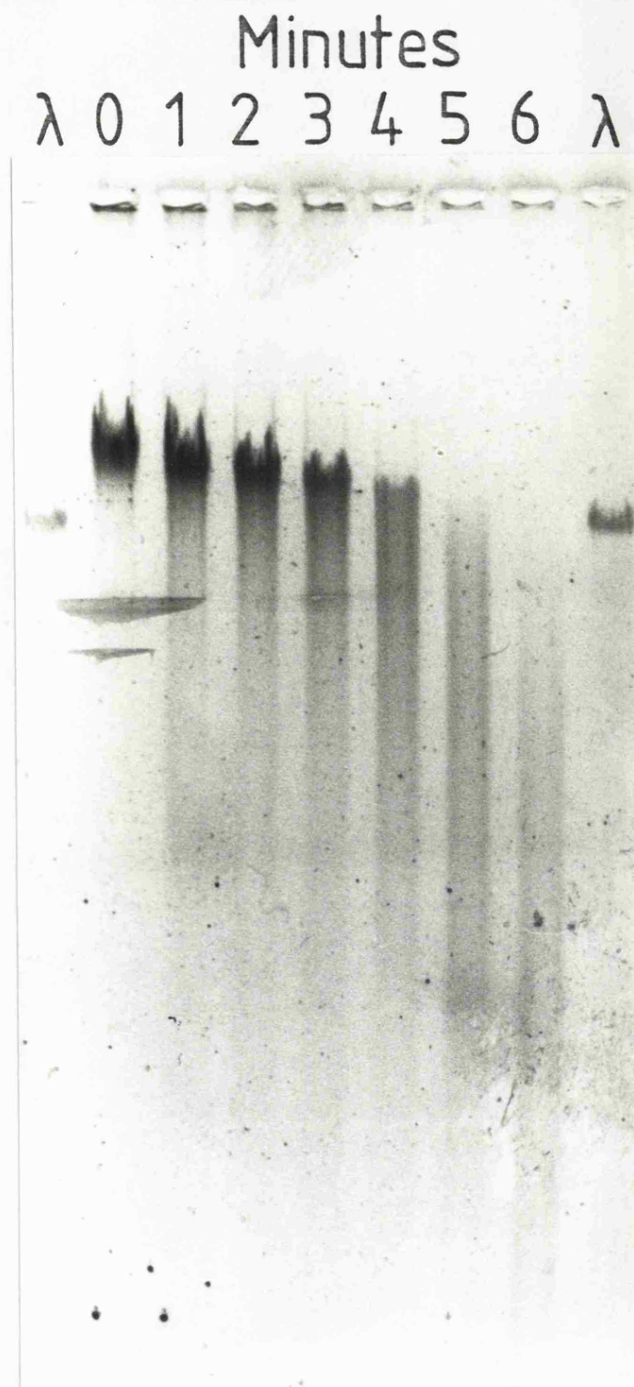
Approximately 10 µg of genomic DNA from strain 469-3 (**section 2.3.11**) was digested with 0.001 U of <u>Sau</u>3A at 37°C and 1 µg samples were removed every min. The reaction was immediately arrested by the addition of EDTA to each sample and incubation at 65°C for 10 min, and the samples were analysed by electrophoresis in 0.4% agarose, along with uncut lambda DNA as size standards. Much of the uncut genomic DNA was too large to enter the gel matrix and remained in the slot, but after digestion for 3 to 4 min, the mean size decreased to about 50 kb. For cosmid cloning, another 10 µg of the same genomic DNA preparation was digested under the same conditions for 3.5 min and the reaction stopped entirely.

Figure 3.5 Ligation of genomic DNA and cosmid arms
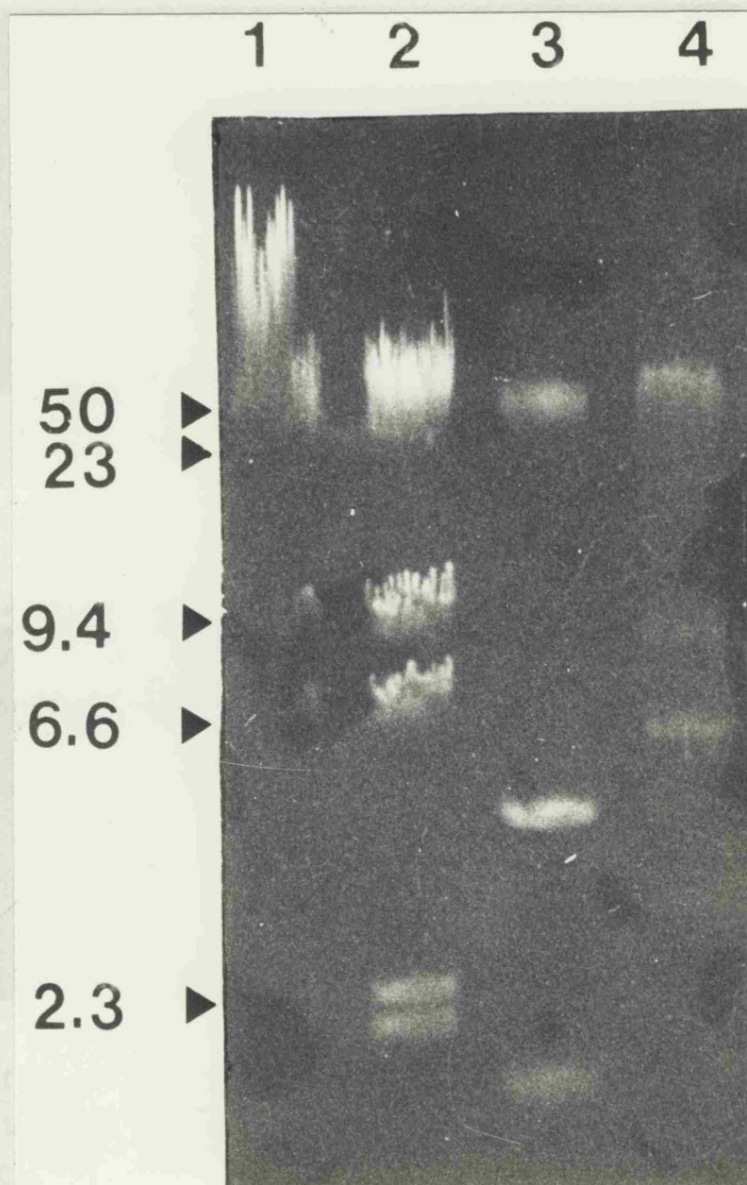
Equal amounts of partially Sau3A-digested 469-3 genomic DNA (mean size approximately 50 kb) and cosmid arms (4.3 and 1.6 kb) were mixed. Samples were separated by electrophoresis in 0.5% agarose, both before (**lane 3**) and after (**lane 4**) ligation. Ligation of vector and genomic DNA fragments is indicated by increased size relative to the pre-ligation sample. Lambda chromosomal DNA was used as molecular weight standards, intact (**lane 1**) or after digestion with HindIII (**lane 2**).

Figure 3.6 Restriction analysis of plasmid subclones
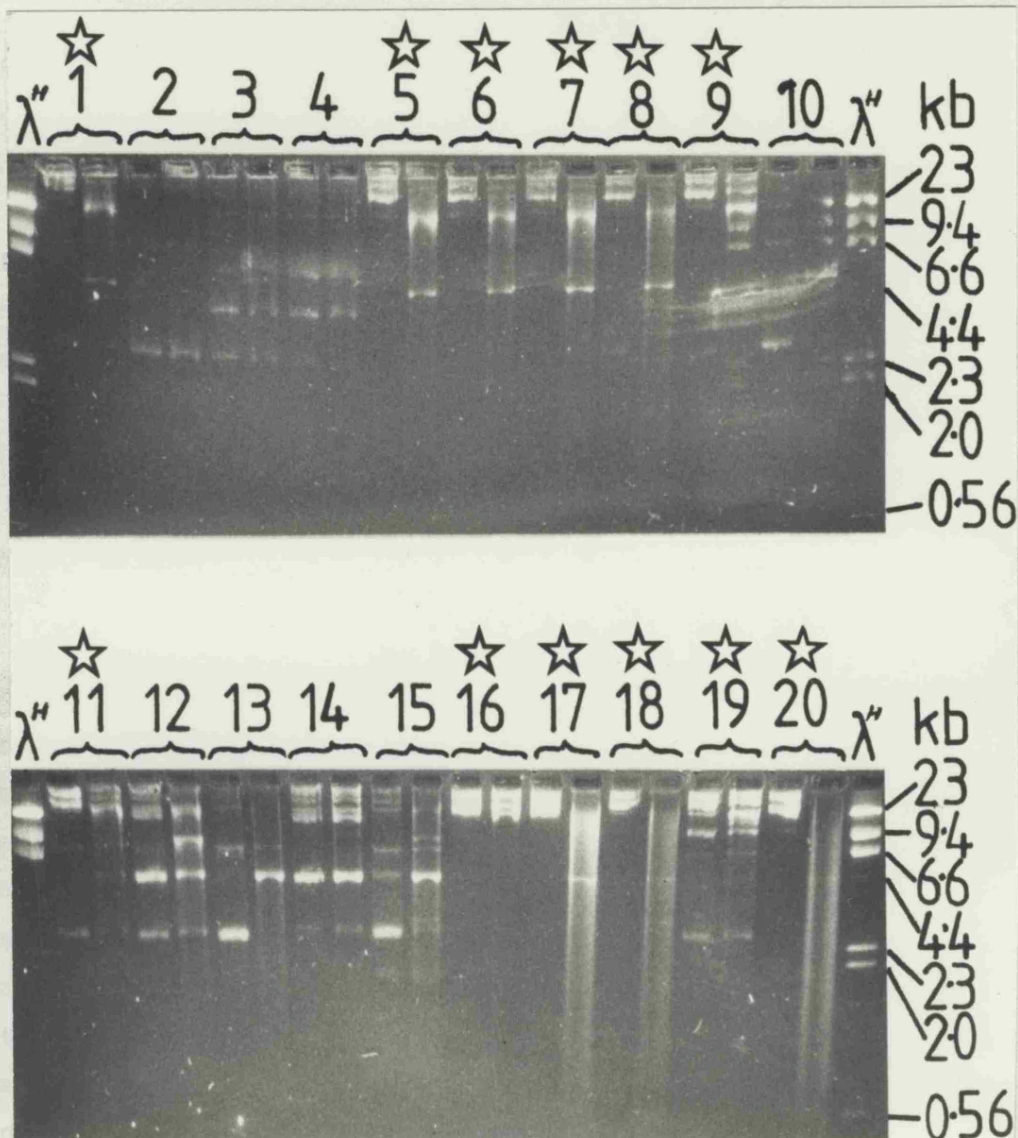
Cosmid DNA from strain LE392(pCC3) was digested with EcoRI, ligated with EcoRI-digested and dephosphorylated pBR322 DNA, and transformed into strain HB101. Plasmid DNA was then prepared from pure cultures of tetracycline-resistant transformants, digested with EcoRI, and analysed by electrophoresis in 1% agarose alongside undigested samples to control against nuclease contamination (undigested DNA samples were loaded on the left of each pair, with digested DNA on the right). The same cultures were tested for MRHA with human blood; positive cultures are indicated with a **star.** Pure plasmid DNA isolated from MRHA-positive cultures (**1, 5-9 and 19**) contained a 17 kb EcoRI fragment in addition to the vector fragment, although DNA from some haemagglutinating cultures were not properly digested (**11, 16-18, 20**). Non-haemagglutinating cultures (**without stars**) lacked any 17 kb EcoRI fragment. Fragments of lambda DNA digested with HindIII were used as size markers.

Plasmid pLG161, consisting of vector pBR322 with an inserted 17 kb
EcoRI fragment of 469-3 genomic DNA derived via cosmid pCC3, encoded MRHA in
E. coli strain HB101. The first problem was to determine whether the cloned
MR haemagglutinin was in fact the same as the previously reported MR adhesin
of 469-3. It was theoretically possible that the clone caused MRHA by a
different structure which had not been recognised in the parent strain
(perhaps it was normally repressed by some function present in 469-3 but
absent from the clone, or was masked by the MR adhesin described in 469-3).
The adhesins expressed by the clone and parent strains were therefore
phenotypically characterised to compare their adherence characteristics,
antigenicity, ultrastructure, and regulation (this chapter), and were
subsequently analysed genetically (chapter 5).

4.1 Adherence. The cosmid library had been screened by the NCHA and MRHA
methods and several MRHA-positive clones were identified (section 3.3).
These therefore expressed MR adherence specific for receptors on human
erythrocytes, the same specificity as the parent strain whilst differing
from many other E. coli adhesins (Table 1.3). MRHA as such was probably not
involved in the disease from which 469-3 was isolated, but MR adherence to
other more relevant human tissue would support the proposed clinical
importance of the MR adhesin, and the adhesin clones were expected to adhere
to the same material if they truly expressed the MR adhesin of the parent
strain. Human epithelial cell lines, although quite unlike gut cells, were a
readily available source of alternative human cells which enabled the
adherence properties of the clones to be compared with 469-3 and with
published accounts of adherence of some other pathogenic strains to tissue
culture cells. Finally, although it was not possible to assay adherence to
human intestinal tissue directly in situ, small biopsy samples of duodenal
and colonic mucosa were available to model in vivo conditions more
effectively in laboratory tests.

4.1.1 MRHA. As determined by the extent of agglutination in slide MRHA tests
with human blood, the 8 positive cosmid clones expressed differing levels of
MRHA (Table 4.1), but the MRHA levels of individual clones varied
considerably in tests performed over a period of 18 months, whereas
HB101(pLG161) and the clinical isolates were more consistent in their MRHA
indices (Table 4.2).

The MRHA reactions promoted by any of the positive cultures were
disrupted (eluted) if the slides were heated toward about 50°C, but

recovered when they were replaced on ice. This finding, common to many so-called 'mannose-resistant and eluting' (MRE) adhesins and to at least one MS (MSE) adhesin (Old and Robertson, 1981; Old et al., 1985), was probably a consequence of increased movement of bacteria and erythrocytes (Brownian motion) at higher temperatures, and may reflect the relative weakness of bonds formed in normal MRHA reactions.

4.1.2 Adherence to cultured human cell lines. Strain 469-3 had been shown to adhere to cultured human epithelial cells in the presence of mannose, whereas nonhaemagglutinating mutant derivatives were nonadherent (Nandadasa et al., 1981; Knutton et al., 1984c; Williams et al., 1984). I repeated the adherence assay with strains HB101(pLG161) and 469-3, incubating them with subconfluent HEp-2 cell coverslip monolayers for 30 min at 37°C in tissue culture medium containing mannose. The monolayers were then washed extensively in PBS-mannose, dehydrated, gold coated and examined by SEM (Fig. 4.1). HB101(pLG161) adhered strongly over most of the exposed surfaces of the HEp-2 cells, while 469-3 was slightly less adherent on this occasion and plasmidless HB101 was nonadherent. The numbers of bacteria adherent to individual HEp-2 cells appeared to vary with the growth stage of the human cells; those cells which had most recently divided and were still paired together had fewer adherent bacteria than older cells. Possibly, receptors for the adhesin are only exposed at certain stages in the HEp-2 cell cycle, but the significance of this single observation is unclear.

4.1.3 Adherence to human intestinal mucosa. Strains 469-3, HB101(pLG161) or HB101(pBR322) were mixed with colonic or duodenal enterocytes (either as whole biopsy fragments or separated into individual cells by EDTA chelation) and incubated in the presence of mannose for various periods at 37°C. Strains 469-3 and HB101(pLG161) adhered avidly to the brush borders of colonocytes (Table 4.3; Fig. 4.2), but did not adhere to duodenal enterocytes (not shown). Control strains (cloning recipients) were nonadherent to both types of cell. The adherence indices (mean numbers of bacteria adherent to each colonocyte, counting 50 enterocytes in each experiment) for 469-3 and HB101(pLG161) were not significantly different and had similar variances (using Student's t-test and the F-test; Bailey, 1981). The data were therefore combined and were found to be significantly different from the controls (P < 0.001 by Student's t-test).

4.1.4 Temperature regulated adhesin expression. The 469-3 MR haemagglutinin, in common with most other bacterial adhesins, was poorly expressed at low growth temperatures (Williams et al., 1984). After growth at below 20°C,

expression was reduced approximately 1000-fold relative to the maximum at 37°C. The same was true of haemagglutinin expression by HB101(pLG161) (Table 4.1), again confirming that the clone expressed the same adhesin properties as the parent strain even though presumably more copies of the cloned determinant were present than in the clinical isolate. Temperature regulated expression of other adherence properties was also tested. Adherence of 469-3, or strains carrying pLG161, to HEp-2 cells or human colonocytes was low or absent following growth at 18°C compared to growth at 37°C (Table 4.1; Figs. 4.1, 4.2).

4.2 **Purification of the MR adhesin.** The adhesins synthesised by 469-3 and HB101(pLG161) were extracted by similar methods to those used before (Williams et al., 1984). Briefly, large numbers of bacteria were violently mixed in a blender and the whole cells removed by centrifugation. The supernatant fractions (crude adhesin preparations) contained much of the MRHA activity of the original cells, but several proteins were resolved by SDS-PAGE (Fig. 4.3). The strong band at 14 kd corresponded in size to the 469-3 adhesin subunit determined previously (Williams et al., 1984), but other components present in the material precluded firm identification of the subunit size.

In order to purify the adhesins, the crude preparations were size-fractionated on chromatography columns of Sepharose 4B-CL which impedes molecules of less than 20,000 kd. As determined by haemagglutination assays, the adhesins were only slightly retarded and largely eluted in the void volume (Fig. 4.4; only data for HB101(pLG161) are shown since the results for 469-3 were the same as before), indicating the large size of the undenatured adhesin (up to at least 20,000 kd) and agreeing with the size of the 469-3 MR adhesin estimated at between 4,000 and 20,000 kd (Williams et al., 1984). Finally, the adhesin subunit was identified by SDS-PAGE analysis of samples eluted from the column and heat-denatured (Fig. 4.4b). The MRHA activities of column fractions (Fig. 4.4a) correlated with the amount of a 14 kd protein stained by Coomassie blue (Fig. 4.4b) rather than with other proteins in the crude preparations which mostly eluted in later fractions.

The purified haemagglutinin lost its ability to promote MRHA of human blood within 45 s at 100°C, as had been reported for the 469-3 adhesin. Such a result would normally be expected of proteins due to denaturation at this temperature, but several other adhesins appear to be more resistant to heat treatment and may withstand 5 min or more in a boiling water bath.

4.3 **Antigenic properties.** Rabbit antiserum to the 469-3 MR adhesin, raised against whole, formalinised cells of 469-3 followed by several cycles of

absorption with non-haemagglutinating mutant derivative LG1505 (**Williams et al.**, 1984), was used to compare the cloned and parent adhesins immunologically, and to prove the size of the subunit proteins conclusively.

4.3.1 Phase variation. Bacteria resuspended from single agar-grown colonies were reacted with the antiserum and subsequently labelled with fluorescein-conjugated anti-rabbit antiserum. Approximately 20% of the bacteria in pure cultures of 469-3 or HB101(pLG161) were labelled and fluoresced under UV (**Fig. 4.5**), indicating that only one in five cells was expressing material antigenically related to the 469-3 MR adhesin at the time of the experiment. Similar results were obtained on separate occasions using bacteria taken from new pure stock plates (either subcultured from colonies picked at random from previous stock plates or purified from the original frozen glycerol stocks) or using different colonies taken from the same pure stock plates, although the proportions of cells in each colony which fluoresced varied from about 10 to 30%.

4.3.2 Equivalence of haemagglutinin and HEp-2 cell adhesin. Adding various amounts of MR haemagglutinin purified from HB101(pLG161) as above, proportionately reduced the number of clones adherent to HEp-2 cell monolayers, presumably by competitive inhibition for the same cell surface receptors. Similarly, the immunofluorescence experiments described above were repeated but, instead of examining whole bacterial cultures, the bacteria were first incubated with cultured human cells (HEp-2 coverslip monolayers) to allow those expressing the appropriate adhesins to adhere, then washed extensively to remove nonadherent bacteria. The monolayers were then reacted with antiserum and immunolabelled (**Fig. 4.6**). In contrast to the findings on whole cultures, <u>all</u> HEp-2-adherent bacteria expressed material which reacted with the anti-469-3-MR-adhesin serum. Furthermore, purified MR haemagglutinin was seen in immunofluorescence experiments to coat the HEp-2 cells evenly whereas controls without the added adhesin remained unlabelled [data not shown]. These data therefore indicated that the 469-3 MR HEp-2 cell adhesin was the same as the MR haemagglutinin.

4.3.3 Immune-electron microscopy. Bacteria of strains 469-3, LG1505(pLG161) and LG1505(pBR322), were reacted with the antiserum and then labelled with anti-rabbit antiserum conjugated to colloidal gold particles. The samples were negatively stained and examined by TEM (**Fig. 4.7**). Surface fibrils produced by some cells of 469-3 were agglutinated by the antiserum and labelled by the gold conjugate. Similarly, some LG1505(pLG161) cells produced immunoreactive surface material which was absent from control

strain LG1505(pBR322). This provided further proof that the genomic DNA in pLG161 encoded the surface-localised MR adhesin expressed by strain 469-3, and that expression was subject to phase variation (individual cells synthesized different amounts of immunoreactive material).

4.3.4 Radioimmunoprecipitation. Antiserum raised against the 469-3 MR adhesin was used to precipitate proteins from a crude adhesin preparation made from ^{35}S-methionine-labelled cells of HB101(pLG161). The precipitate was washed to remove unbound proteins, and analysed by SDS-PAGE and fluorography (**Fig. 4.8**). The single 14 kd protein band recognised by the antiserum agrees with the reported molecular weight of the protein subunit of the MR adhesin of 469-3 (**Williams et al., 1984**), and of the purified haemagglutinin previously obtained from the clone (**section 4.2**). The experiment also demonstrated that the antiserum was specific, since other labelled proteins present in the crude adhesin preparation were not precipitated.

4.4 Ultrastructure. The structure of the 469-3 MR adhesin was not unequivocally identified by previous EM examination of negatively-stained preparations (**Knutton et al., 1984c**). Under normal growth conditions, most bacteria in pure cultures appeared to have smooth surfaces and lacked obvious fimbrial structures. In contrast, rigid 7 nm-diameter fimbriae were produced under conditions known to promote the expression of common fimbriae and since such cultures expressed MSHA activity, these were identified as type 1 fimbriae. Although fine fibrils were seen on the surfaces of some cells, they were thought to have been caused by the dehydration of capsular material during preparation for EM. In thin sections taken from adherence assays using HEp-2 and HeLa cultured cells in the presence of mannose, adherent 469-3 bacteria were seen to lie very close to the human cells with membranes apparently in contact over short lengths. Therefore, the MR adhesin was termed 'nonfimbrial' (**Hinson and Williams, 1986**).

It is now generally accepted that some bacterial adhesins are composed of 'fibrils' or 'fibrillar fimbriae' or 'fuzzy coats' which are very fine and are difficult to resolve by EM. Extreme care in the preparation and negative staining was required to reveal that a proportion of HB101(pLG161) cells expressed very fine fibrils, similar to those seen on some cells of 469-3 but absent from all cells of HB101(pBR322) (**Fig. 4.9**). The variable penetration of negative stain prevented accurate assessment of the proportion of bacteria synthesising the material by this method, but the fraction appeared to be approximately 20% of either 469-3 or HB101(pLG161) cells.

4.5 Conclusions.

4.5.1 Equivalence of cloned adhesin and MR adhesin of 469-3. The results detailed in this chapter confirm and extend the previously reported findings on the MR adhesin of 469-3, and indicate that the MR adhesin expressed by the clone HB101(pLG161) behaved identically to that of the parent strain in terms of their adherence characteristics for human tissue (erythrocytes, HEp-2 cells, and colonic and duodenal enterocytes), antigenic cross-reactivity, subunit molecular weight, assembled size, ultrastructure and cellular location, regulation by growth temperature and phase variation (Table 4.4). Taken as a whole, I conclude that the clone expressed the same functional MR adhesin as was described in the parent.

Considering the differences between cloning recipients (for example, HB101, a laboratory-derived K-12/B hybrid strain containing recA⁻ and other mutations) presumably harbouring multiple copies of the recombinant plasmid, and the parent (a wild, encapsulated strain), the apparent similarity of the amounts of adhesin synthesised and assembled by each strain under different conditions is remarkable. It suggests that some part of the synthesis process other than the number of copies of the genetic determinant present was limiting, and the limitation was similar even in the different genetic backgrounds of the parent strain and cloning recipients. The phase variation evident in both systems also indicates that expression of the adhesin was efficiently regulated, as indeed might be expected of an exported protein with utility to the pathogenic parent only in certain aspects of the microorganism's lifecycle (i.e. during the colonisation of the colon; see below), and again was similar in both the parent and the clone.

In summary, there were grounds for believing that the entire adhesin determinant had been cloned, having failed to detect any significant differences in the regulation of expression or in the physical properties of the adhesins expressed by the clinical strain and the clone.

4.5.2 Adhesin receptors are present on various human cells. The MR adhesins of 469-3 and the clones were initially detected by their ability to promote MR agglutination of human erythrocytes, but it seems unlikely that interaction with blood cells was involved in the aetiology of the diarrhoeal disease from which the parent strain was isolated. HA is probably a consequence of the coincidental expression of suitable receptors for the adhesins on erythrocytes from certain animals. However, while HA is not necessarily related to the adherence functions utilised by bacteria during infections, the test serves as a simple and commonly used means of assaying

bacterial adherence in the laboratory. Adherence experiments using the parent and clones were used to demonstrate that the 469-3 MR adhesin is probably responsible for adherence to other human cells in addition to erythrocytes, including cultured epithelial cell lines and more importantly colonic mucosa.

In a previous publication, human ETEC isolates which normally colonise the small intestine were shown to adhere to human duodenal enterocytes in vitro (Knutton et al., 1984a). Strain 469-3 was implicated as the cause of a human diarrhoeal disease and thus was expected to be capable of adhering to human intestinal surfaces, but in contrast to ETEC strains, the main site of colonisation for 469-3 was thought to be the human colon. The patient from which it was isolated had symptoms similar to those caused by Shigella dysenteriae and by invasive E. coli (EIEC) strains which are known to damage and invade the colonic epithelia, hence releasing blood and mucus into the faeces. In order to assay adherence to human colonic mucosa, the in vitro assay mentioned above was modified by using colonic biopsies rather than duodenum. The adherence of 469-3 to colon was not significantly different from that of HB101(pLG161) (Table 4.3). However, recipient strains HB101 and LE392 were significantly less adherent and were usually totally nonadherent without the cloned plasmid.

The colonocyte adherence assay described in this thesis may prove useful for characterising adhesins on other bacterial isolates, particularly those from patients with diseases of the colon such as dysentery, in conditions which closely mimic those in vivo whilst avoiding any need to use human enterocytes in situ. As far as I am aware this is the first report of bacterial adherence to human colon in vitro, and the assay may be useful to identify colon-adherent pathogens amongst the normal gut flora.

A method was recently described (Ofek et al., 1986) for immobilising animal cells on microtitre plates, enabling adherence to be quantitated by counting adherent bacteria using biotinylation, enzyme-linked immunosorbent assays (ELISA), etc., but such experiments should be backed up with direct microscopic examination to determine the actual site of bacterial attachment. Type 1 fimbriae, for example, have been shown to promote adherence to basolateral surfaces of isolated enterocytes (Knutton 1984a), although these are probably not normally exposed in situ. The 469-3 MR adhesin, in contrast, was specific for receptors on the lumenal surface, i.e. the brush borders (Fig. 4.2).

The adherence data strongly suggest that the MR adhesin is a true colonisation factor since it is clearly capable of mediating adherence to the major region of colonisation of the human hosts (colon), but is specific for that region of the intestine and did not cause adherence to duodenal

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mucosa. Although there are many other differences along the human gut which may also be involved in site-specific colonisation (**Fig. 1.1**), there are various a priori reasons for supposing that specific adherence to colonic epithelia was a principal site-determinant (**section 1.4**). Volunteer studies using isogenic strains differing only in the expression of the MR adhesin to determine if it truly acts as a colonisation factor were not performed on this occasion, however.

In view of the specific adherence to only certain regions of the gut, the MRHA reaction with human blood and adherence to human cell lines are somewhat curious since they imply that the adhesin recognises structures present on colonic, blood and cultured cells but absent from duodenal cells. The immunofluorescence experiments (**section 4.3.2**) implicated the same structure in adherence to both HEp-2 cells and human erythrocytes, rather than multiple adhesins encoded by the same small DNA fragment in the clone. The molecular structure of the receptor remains unknown, although the MRHA reactions caused by 469-3 and 444-3 were not disrupted by the addition of various sugars (Williams et al., 1984). Future experiments to characterise the receptor might perhaps proceed along similar lines to those used to identify other adhesin receptors, by fractionating colonic or erythrocyte membranes, or by directly testing many more molecules for adherence inhibition, but such methods were beyond the scope of the present investigation.

4.5.3 Phase variation. MRHA was expressed to different degrees by various cosmid clones, and within each strain it varied over the course of 18 months. Generally speaking, relative differences in the MRHA levels expressed by various cosmid clones were retained in separate tests (some were usually strongly haemagglutinating while others were usually weak) while the plasmid subclones were more consistent and were more similar to the parental strain. Furthermore, multiple tests on the same culture were highly consistent so the MRHA assay itself was probably not the source of the variation. Certain clones were found to comprise mixtures of bacteria, only some of which were able to adhere to HEp-2 cells and were capable of reacting to antiserum to the 469-3 MR haemagglutinin, even in supposedly 'pure' cultures derived from single cells (i.e. single colonies on agar plates). This was evidently an example of phase variation (**section 1.3.9**), although the rate of variation appeared to be considerably higher than for many other bacterial adhesins [slow rates of fimbrial phase variation lead to the formation of cultures which mostly contain one or other phase, and can often be distinguished by their different colony morphologies on agar, whereas faster rates produce mixed cultures as a rule].

Differences in the MRHA levels promoted by clones after they had been replated serially to maintain laboratory stocks on agar plates may also have been due to loss of the cosmids (apparently a common problem with such large recombinant molecules). Selective pressure to maintain the cosmids was applied by adding ampicillin to the agar just before it was poured, but unfortunately ampicillin is itself quite unstable and its potency gradually falls over a few weeks at or above 4°C. The mechanism of bacterial resistance to ampicillin (elaboration of β-lactamase, degrading the antibiotic in the surrounding medium) and the bacteriostatic rather than bactericidal action of the drug probably resulted in a gradual reduction in its activity. Without effective ampicillin selection, bacteria which lost their cosmids would probably have grown faster than their cosmid-containing fellows, and would soon have resulted in a cosmid-free, permanently non-haemagglutinating culture, in contrast to phase variation which, by definition, is reversible (i.e. the genetic material is not lost but is just not expressed).

A further possible source of permanent loss of MRHA expression was deletion of parts of the genomic inserts by recombination, perhaps coupled with phase variation. Antigenic variation of Neisseria gonorrhoeae fimbriae (section 1.4.13), for example, is caused by gross DNA rearrangements. It is not unreasonable to suppose that a similar mechanism might be used by E. coli, in which case, errors might well result in deletions, or perhaps cause unstable replication or segregation of the cosmids. Alternatively, duplications within cloned sequences could allow deletion of intervening regions by homologous recombination between the repeats, as might occur in the excision of transposons, for example. In connection with this last point, it is worth noting that several other virulence determinants have been shown to be bounded by insertion sequences and are probably capable of transposition in vivo (see also chapter 6).

These results do not necessarily demonstrate that phase variation is specifically controlled by the cloned sequence since it might be caused by some aspect of the recipient cell physiology or even by small variations in the local environments of individual cells. Clearly, further experiments are required to explain the molecular basis of phase variation in this system.

4.5.4 Temperature regulation of adhesin expression. Expression of the MR haemagglutinin and adherence to HEp-2 and colonic cells was apparently regulated in the same manner by growth temperature (i.e. expression was much reduced during growth at room temperature relative to that at body temperature) in both the parent and the clones. Thus, either the information carried by pLG161 was sufficient to encode specifically

temperature-dependent expression (perhaps in the same way that temperature controls transcription of P-fimbriae genes; Goransson and Uhlin, 1984), or else some general physiological characteristic of all the strains was responsible for the effect (such as reduced membrane fluidity at low temperatures, perhaps; Nichol et al., 1980). The phenomenon is currently being studied by means of DNA-RNA hybridisation experiments using part of the cloned system as a probe, and preliminary results suggest that there is an element of transcriptional regulation (manuscript in preparation).

4.5.5 Ultrastructure of the MR adhesin. The fine, flexible fibrils seen on some cells of 469-3 and previously thought to be capsular material (Knutton et al., 1984c) can now be identified as the MR adhesin on the basis of these results: (a) fibrils were only identified on cultures capable of MRHA (469-3, LE392(pCC3) or HB101(pLG161) after growth at 37°C) but were absent from nonadherent control strains (LE392, ED8654, HB101 and HB101(pBR322) grown at any temperature) and from all strains (including normally MRHA-positive strains) grown at 18°C and incapable of MRHA; (b) plasmidless HB101 produced no fibrils, fimbriae or capsule (as determined by EM examination of negatively stained preparations and by colony morphology) whereas some cells of HB101 carrying genetic material derived from 469-3 in recombinant plasmid pLG161 and expressing the same MR adherence properties as 469-3, synthesised fibrils indistinguishable from those on the parent strain; and (c) antiserum to the MR adhesin reacted with surface-located fibrils on haemagglutinating strains. Unfortunately, EM examination of negative-stained purified adhesin preparations did not reveal the fibrillar structure, nor indeed any other discrete structures, even though the adhesin behaved as a very large aggregate on Sepharose chromatography columns and was expected to be visible. The failure might perhaps be overcome in future experiments using other EM techniques such as rotary-shadowing although the small fimbrial diameter indicated on whole cells imposes severe restraints on the sample resolution obtainable by negative staining.

The structure of the 469-3 adhesin resembles several other bacterial adhesins, including K88 and CS3 (a component of CFA/II) and may represent a different class from the rigid rod-like structures such as type 1 and CFA/I fimbriae. It remains to be seen whether other adhesins reported as nonfimbrial actually have a similar structure.

Table 4.1 <u>Adherence to human tissue</u>

Strain	Growth temp.	MRHA index[a]	MR adherence to[b] HEp-2 cells	Colonocytes
469-3	37°C	3.8	+	+
469-3	18°C	0	ND	ND
444-3	37°C	4.0	+	ND
444-3	18°C	0	ND	ND
LE392(pCC1)	37°C	1.6	ND	ND
LE392(pCC2)	37°C	2.1	ND	ND
LE392(pCC3)	37°C	3.7	ND	ND
LE392(pCC4)	37°C	2.6	ND	ND
ED8654(pCC5)	37°C	3.6	ND	ND
ED8654(pCC6)	37°C	0.1	ND	ND
ED8654(pCC7)	37°C	1.7	ND	ND
ED8654(pCC8)	37°C	2.3	ND	ND
HB101(pLG161)	37°C	3.9	+	+
HB101(pLG161)	18°C	0	ND	ND
HB101(pLG162)	37°C	0	ND	ND
HB101(pLG163)	37°C	3.2	+	+
HB101(pLG163)	18°C	0	ND	ND
HB101(pLG164)	37°C	0	ND	ND
HB101	37°C	0	−	−
LG1505	37°C	0	−	−
LE392	37°C	0	ND	ND
ED8654	37°C	0	ND	ND

Notes to Table 4.1

a) Mean MRHA index with human blood. Results of each slide MRHA assay were scored on a scale of 0 (no reaction) to 4 (strong reaction). The full results are presented in **Table 4.2.**

b) Bacterial adherence to HEp-2 cell monolayers after incubation together for 30 min, or to colonic mucosal biopsies after incubation together for 3 h, at 37°C in MEM containing mannose and thorough washing to remove nonadherent bacteria. Samples were critical-point-dried, gold-coated and examined by SEM. + = many adherent bacteria observed on each HEp-2 cell. - = no, or very few, adherent bacteria seen. **ND** = not done.

Table 4.2 <u>MRHA results over 18 months</u>[a]

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean	SE[c]
						Experiment number[b]										
444-3	4									4				4	4.00	0%
469-3	4	4	4	4	4	1	4	4	4	4	4	4	4	4	3.78	21%
LE392(pCC1)	1	2	1	2		1	4	0	1	4	1	1			1.64	79%
LE392(pCC2)	4	4	4	2	2	4	4	4	2	0	0	0	0	0	2.14	86%
LE392(pCC3)	4	4	4	4	4	4	4	3	2	3	4	4	4	4	3.71	16%
LE392(pCC4)	3	3	3			3	0	4	1	3	0	3	4	4	2.58	56%
ED8654(pCC5)	3	3	3		4	3	4	4	4	4	3	4	4	4	3.62	14%
ED8543(pCC6)	0	0	0		0	1	0	0	0	0	0	0	0	0	0.08	360%
ED8654(pCC7)	4	1	2		0	1	0	2	1	1	3	3	2	2	1.69	70%
ED8654(pCC8)	3	3	2		4	3	2		0	0	0		4	4	2.27	71%
HB101(pCC1)										1	0	2	2	3	1.6	71%
HB101(pCC2)										0	0	0	0	1	0.2	224%
HB101(pCC3)										4	4	3	3	3	3.4	16%
HB101(pCC4)										4	0	2	2	2	2.0	71%
HB101(pCC5)										0			0	3	1.0	173%
HB101(pCC6)										1			0	1	0.67	86%
HB101(pCC7)										0	0	2	2	2	1.2	91%
HB101(pCC8)										3		1	2	2	2.0	41%
HB101(pLG161)						4	4	4	4			4	3	4	3.86	10%
HB101(pLG163)										3	2	4	3	4	3.20	26%

Notes to Table 4.2

a) Over the course of about 18 months, various haemagglutinating strains were cultured on agar plates with appropriate antibiotics for selection of chromosomal, cosmid or plasmid markers, grown at $37^{\circ}C$ and stored for up to 2 months at $4^{\circ}C$, then subcultured from single colonies onto fresh plates. After each subculture, the new stock plates were tested for MRHA and scored from 0 (no agglutination) to 4 (strong reaction). If the new stocks were found to be MRHA-negative, several other colonies from the previous stock plate were streaked out afresh in an attempt to recover haemagglutinating stocks.

b) MRHA indices in particular experiments can only truly be compared within each 'block' of strains (e.g. plasmid clones), since strains from different blocks were not necessarily tested on the same day.

c) SE = standard error of the mean.

Table 4.3 <u>Adherence to human colonocytes</u>[a]

Strain	Adherence index in experiment number[b]								Mean
	1	2	3	4	5	6	7	8	
469-3	0.7	3.0	2.1	2.3	3.6	6.0			2.95
HB101(pLG161)	1.3	1.6	5.8	1.2	0.75				2.13
Controls[c]	0.05	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.02

Notes to Table 4.3

a) Bacteria were grown overnight in LUB at 37°C, then pelleted, washed and resuspended in MEM-mannose. Human colonic enterocytes were separated by EDTA chelation of biopsy samples and resuspended in MEM-mannose. The cells were mixed in a ratio of approximately 1000 bacteria per enterocyte, incubated at 37°C for 3 h, and examined by phase contrast microscopy.

b) In each experiment, adherence indices were calculated as the mean number of adherent bacteria per enterocyte, counting 50 enterocytes selected at random.

c) Control strains were LE392 or HB101.

Table 4.4 Summary: comparison of cloned and parental MR adhesins

Characteristic	469-3	Clone[a]	Control[b]	Section
Adherence to human erythrocytes	MR	MR	None	4.1.1
Adherence to HEp-2 cells	MR	MR	None	4.1.2
Adherence to human colon	MR	MR	None	4.1.3
Adherence to human duodenum	None	None	None	4.1.3
Immunological cross reaction[c]	Yes	Yes	No	4.3
Cellular location[c]	Surface	Surface	NA	4.3.3
Proportion of cells expressing[c]	1/5	1/5	NA	4.3.1
Aggregated size[d]	4-20 Md	< 20 Md	NA	4.2
Subunit size[e]	14 kd	14 kd	NA	4.2
Denatured at 100°C in <2 min.	Yes	Yes	NA	4.2
Expression at 18°C	Minimal	Minimal	NA	4.1.4

Notes to Table 4.4

a) The clone tested was HB101(pLG161).

b) Control strains were plasmid-free cloning recipients HB101, LE392 or ED8654. **NA** = not applicable.

c) Antiserum raised against the MR haemagglutinin of 469-3 was used in immunofluorescence and immunogold experiments described in the text.

d) Size of the native adhesins as determined from their behaviour on Sepharose chromatography columns.

e) Size of the purified adhesin protein subunits, determined under denaturing conditions by SDS-PAGE.

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Figure 4.1 <u>Adherence to HEp-2 cells</u>
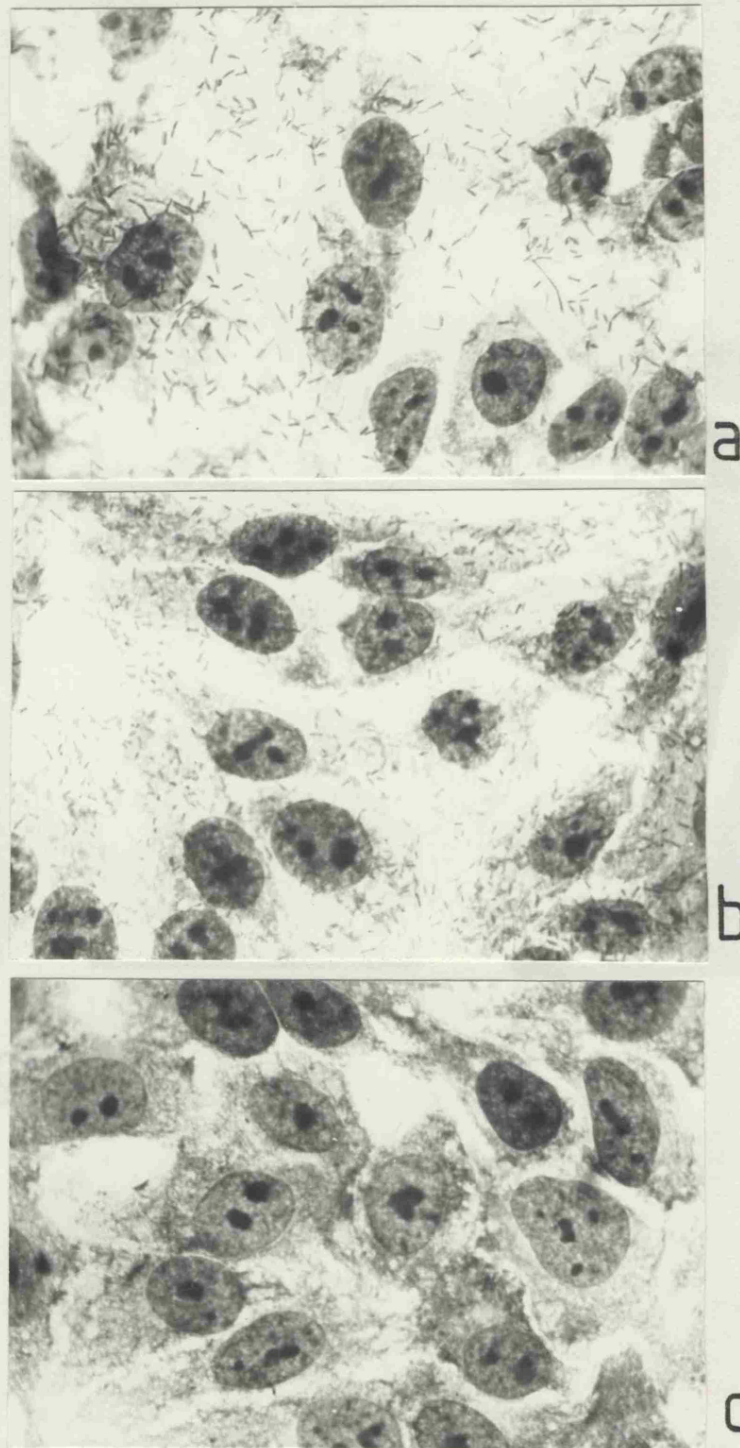
0.1mm

Strains 469-3 (**a**) and HB101(pLG161) (**b**) were grown overnight in LUB at 37°C, washed and resuspended to approximately equal cell densities in modified Eagle medium (MEM) containing 0.5% mannose and incubated with HEp-2 cell monolayers (washed and returned to MEM-mannose) for 30 min at 37°C. Nonadherent bacteria were removed by thorough washing with MEM, and the samples examined microscopically. Large numbers of bacteria can be seen adherent to the HEp-2 cells in a diffuse manner. Control strains (**c**), consisting of HB101 or HB101pBR322 grown at any temperature, or 469-3 or HB101pLG161 grown at 18°C, were nonadherent under the same conditions.

Figure 4.2 Adherence to isolated human enterocytes
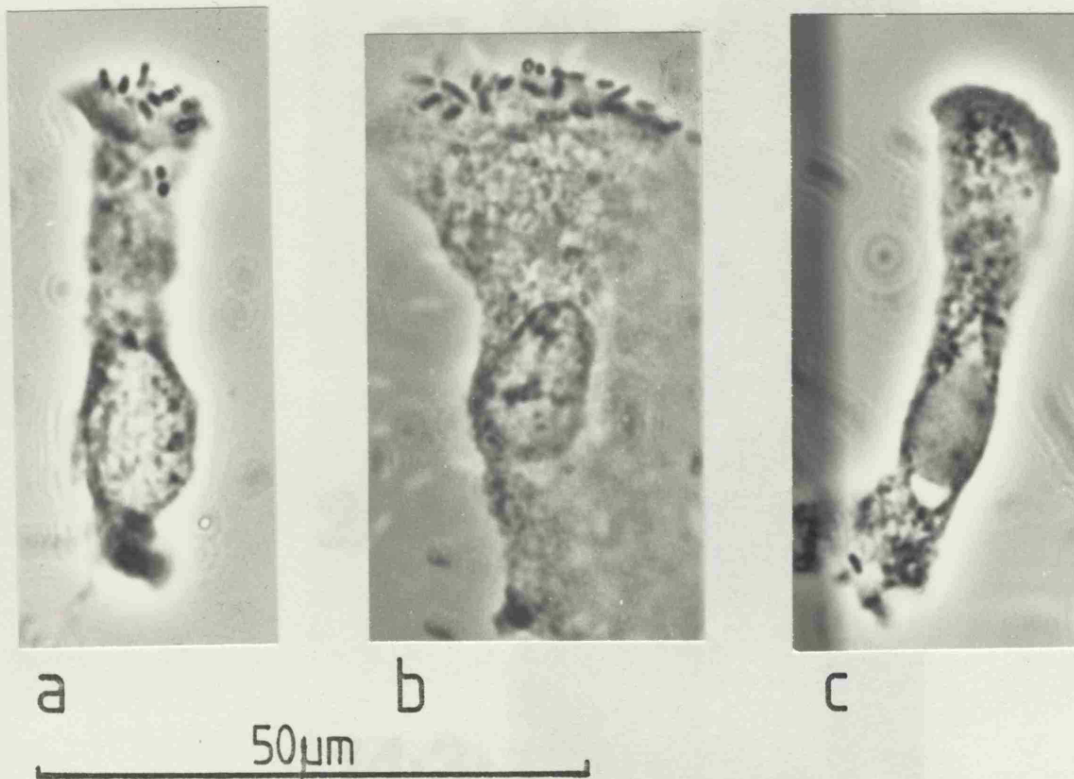
a   b   c

50μm

Strains 469-3 (a) and HB101(pLG161) (b) were grown overnight in LUB at 37°C, washed and resuspended to approximately equal cell densities in HEPES-buffered Ham F-10 tissue culture medium containing 0.5% mannose. They were then incubated with human colonic enterocytes (separated from biopsy material by EDTA chelation) for 3 h at 37°C. Nonadherent bacteria were removed by thorough washing with the same medium, and the enterocytes were examined by phase contrast light microscopy. Control cultures (c) consisting of HB101 or HB101(pBR322) grown at 37°C, or 469-3 or HB101(pLG161) grown at 18°C, were nonadherent under the same assay conditions.

Figure 4.3 Extraction of cloned and parental MR adhesins
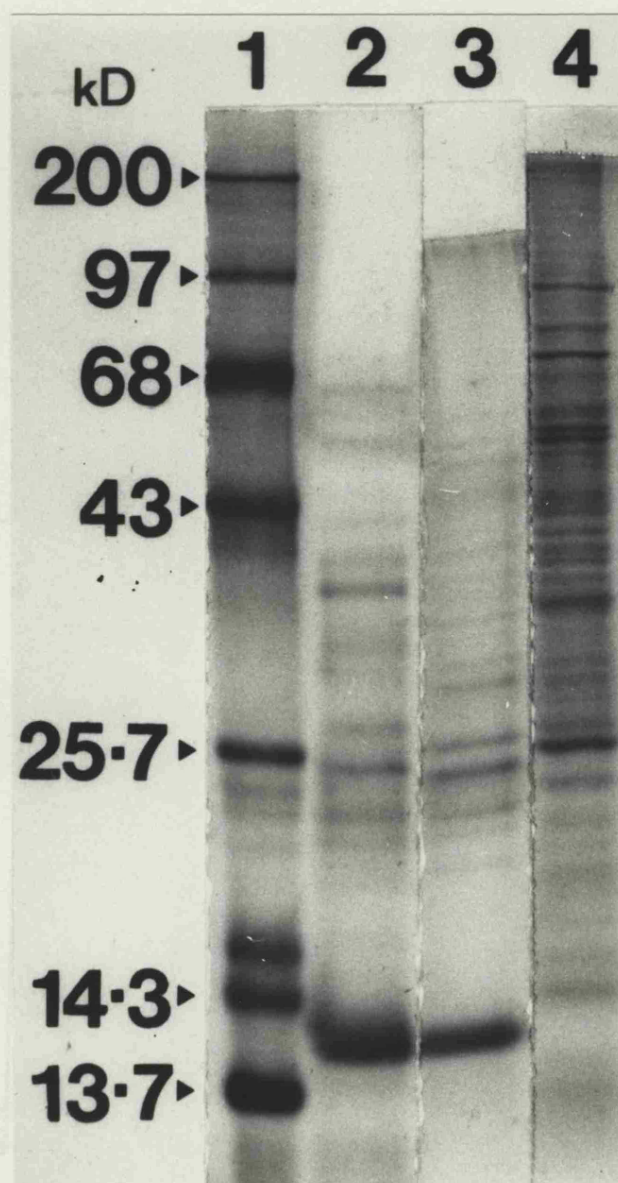
Haemagglutinating material was sheared from the surfaces of cells of strains 469-3 (**lane 2**) and HB101(pLG161) (**lane 3**), and the same method was used to prepare non-haemagglutinating equivalent material from strain HB101pLG164 (**lane 4**). Samples were heat denatured and separated on a 20% acrylamide SDS-PAGE gel. Proteins were stained with Coomassie blue and the gel was photographed. The MR adhesin subunit can be seen as a strong band at 14 kd present in 469-3 and HB101(pLG161) but absent from the control strain (even though the last lane was deliberately overloaded). Molecular weight markers (**lane 1**) comprised 1 μg of each of the following: myosin (200 kd), phosphorylase b (97 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), α-chymotrypsinogen (25.7 kd), lysozyme (14.3 kd) and ribonuclease A (13.7 kd).

Figure 4.4 Purification of the cloned MR adhesin
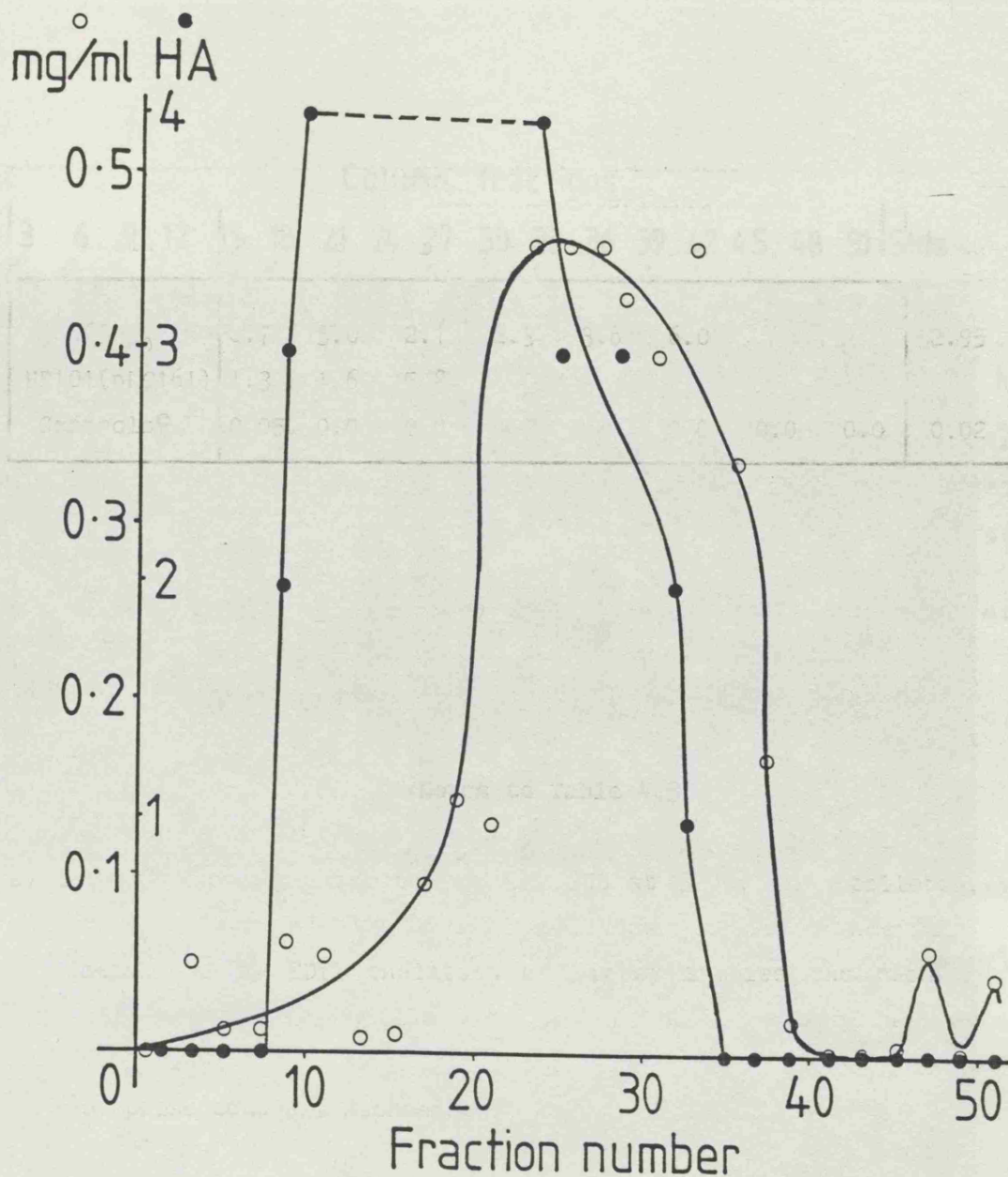
Material was sheared from the surface of 11 g (wet weight) of cells of strain HB101(pLG161) collected from LUA plates after overnight growth at 37°C. The crude adhesin preparation was size fractionated on a Sepharose 4B-CL column, and 150 fractions (5 to 6 ml each) were collected from the time of loading the column (fraction number 1).

A) Selected fractions were assayed for MRHA (●) and protein content (o). The haemagglutinin was slightly retarded but mostly eluted in the void volume (fractions 15 to 25), indicating a maximum aggregate size of at least 20,000 kd.

Figure 4.4 continued
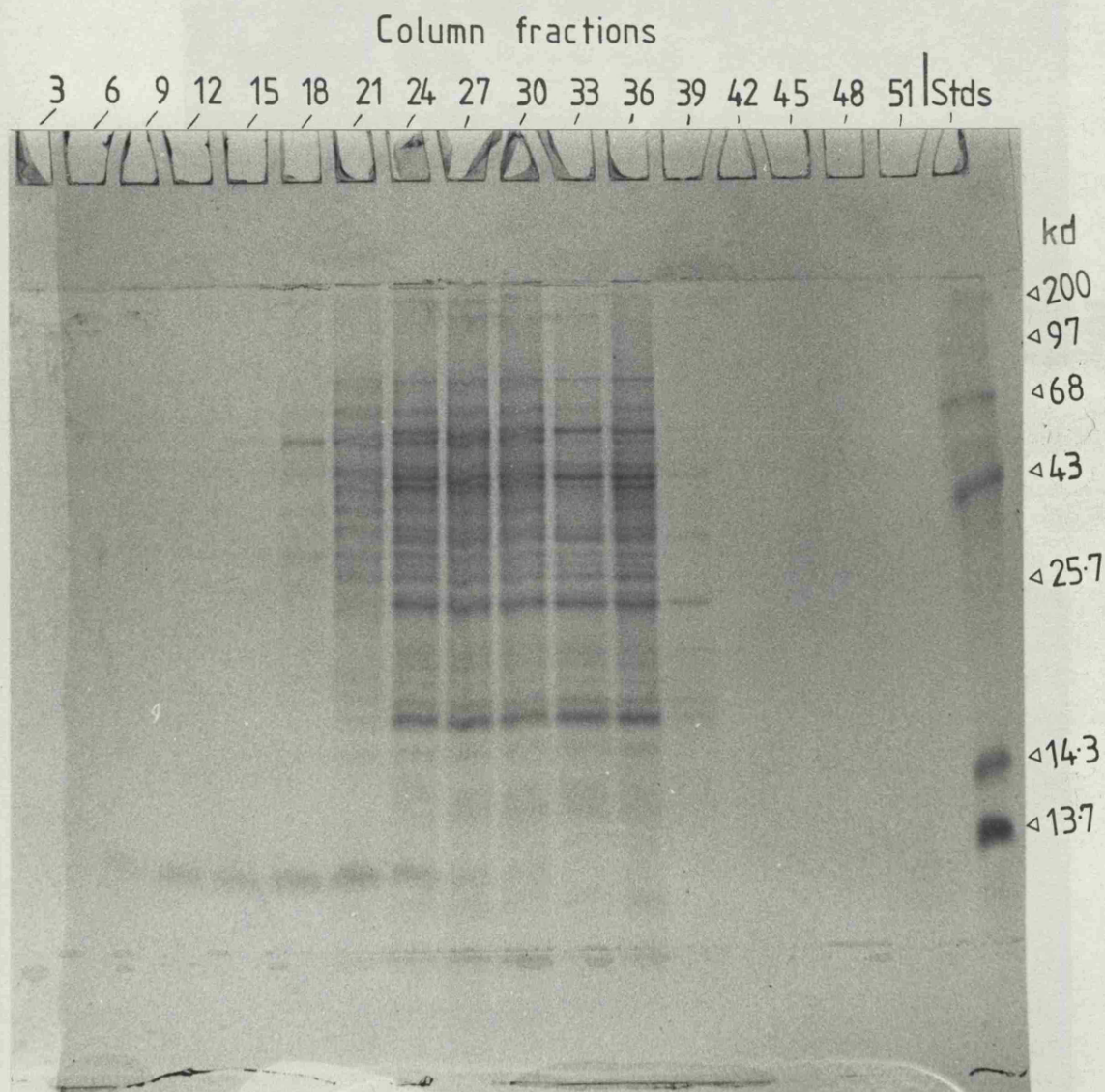
B) Samples eluted from the Sepharose 4B-CL were heat-denatured and
separated by SDS-PAGE (acrylamide content 20%). Proteins were stained
with Coomassie blue and the gel was photographed. Molecular weight
markers comprised ≤ 1 ug of each of the following: myosin (200 kd),
phosphorylase b (97 kd), bovine serum albumin (68 kd), ovalbumin
(43 kd), α-chymotrypsinogen (25.7 kd), lysozyme (14.3 kd) and
ribonuclease A (13.7 kd).

Figure 4.5 <u>Immunofluorescence examination of pure bacterial cultures</u>
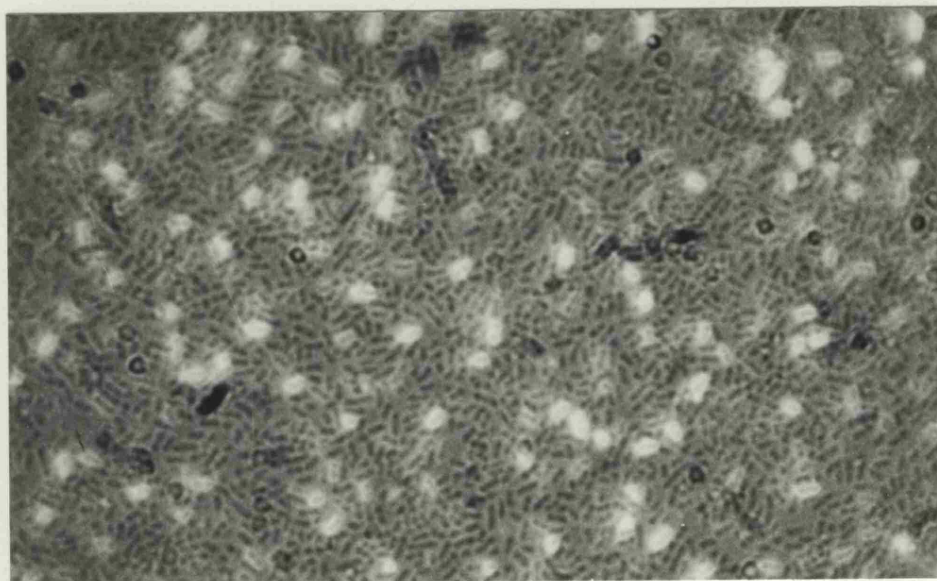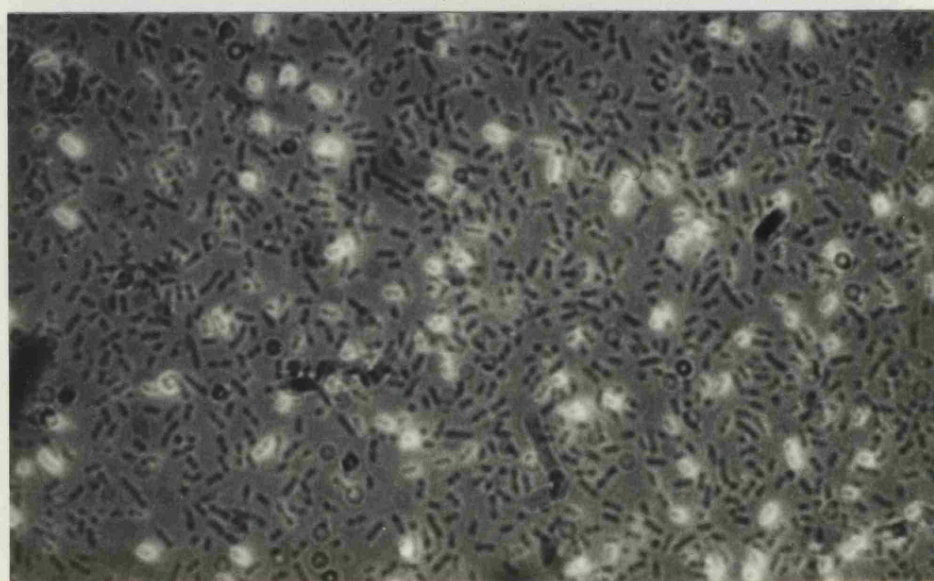
Strains 469-3 (a) and HB101(pLG161) (b) were grown overnight on LUA at 37°C. Small samples of bacteria were scraped from individual colonies into PBS and reacted with antiserum raised against the MR haemagglutinin of 469-3, then with fluorescein-conjugated secondary antibodies, and microscopically examined with UV illumination. A similar proportion of cells in cultures of 469-3 and HB101(pLG161) reacted with the sera.

Figure 4.6 <u>Immunofluorescence examination of HEp-2 adherence</u>
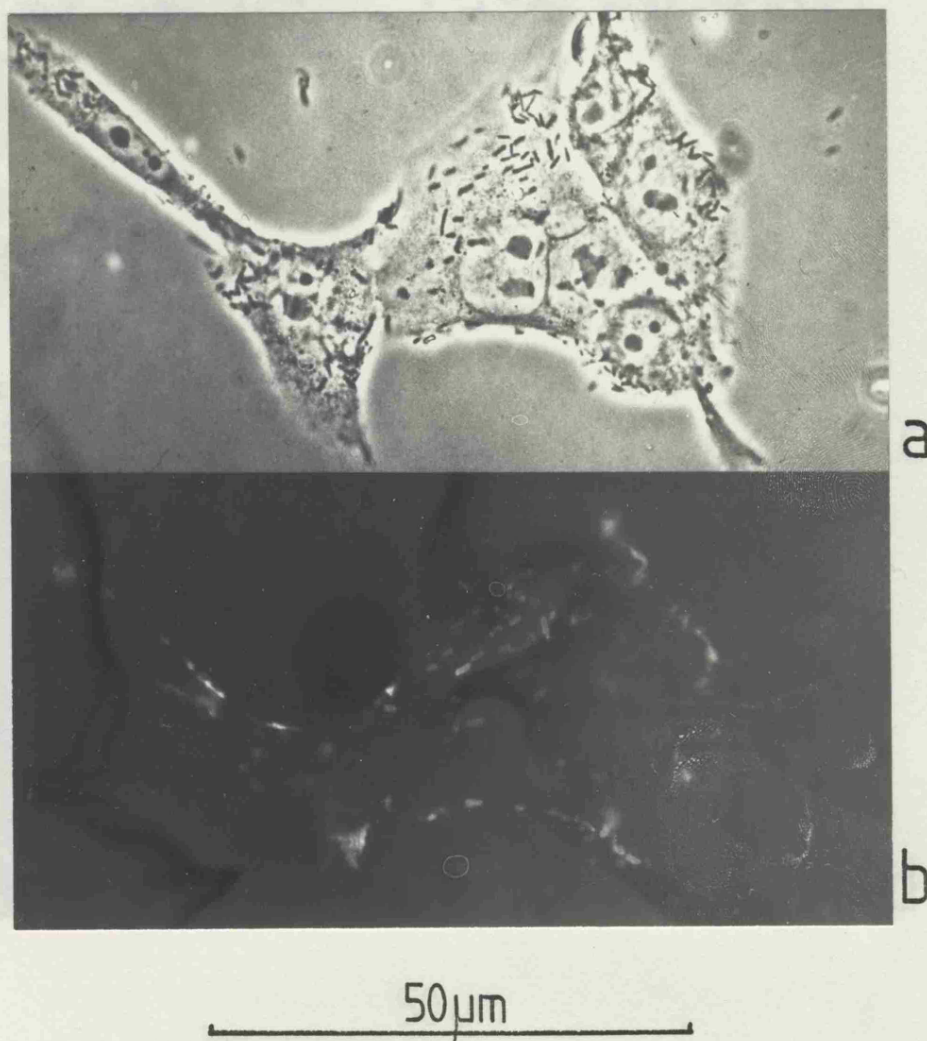
(a, b) Strain HB101(pLG161) was grown overnight on LUA at 37°C. Small samples of bacteria were scraped into HEPES-buffered Ham F-10 tissue culture medium containing 0.5% mannose and incubated for 3 h at 37°C with HEp-2 cell coverslip monolayers. Non-adherent bacteria were removed by thorough washing, and the coverslips were treated with anti-469-3-MR-adhesin serum, then with fluorescein-conjugated secondary antiserum. The monolayers were examined microscopically. The same field is shown by phase-contrast microscopy (a) and with UV illumination (b). All adherent bacteria bound antisera and so fluoresced.

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Figure 4.6 continued
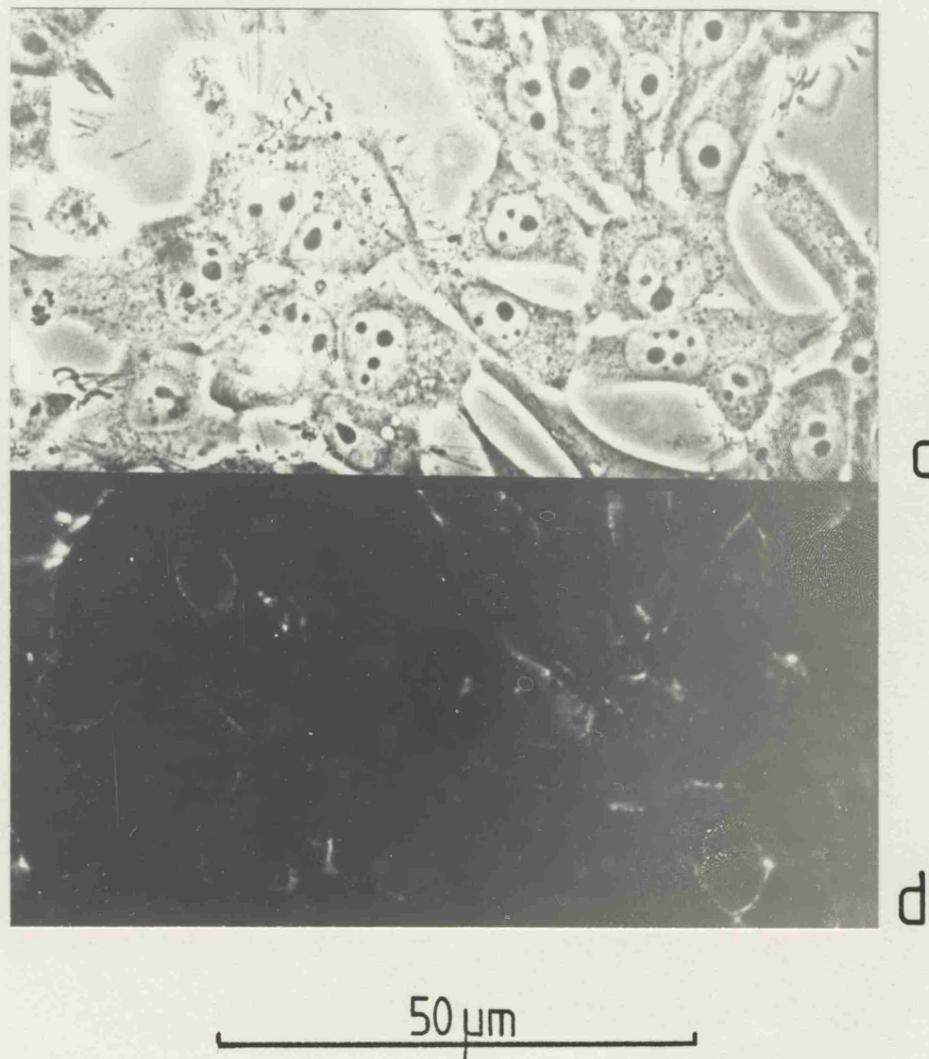
(c, d) Material with MR haemagglutinating activity was purified from the surface of HB101(pLG161) cells as described earlier (**Fig. 4.4**) and applied to coverslip monolayers of HEp-2 cells for 30 min at 37°C, then the cells were thoroughly washed and reacted with anti-469-3-MR-adhesin serum and fluorescein-conjugated secondary antibody as before. The labelled samples were examined microscopically by phase contrast (c) and by fluorescence (d) (identical fields of view).
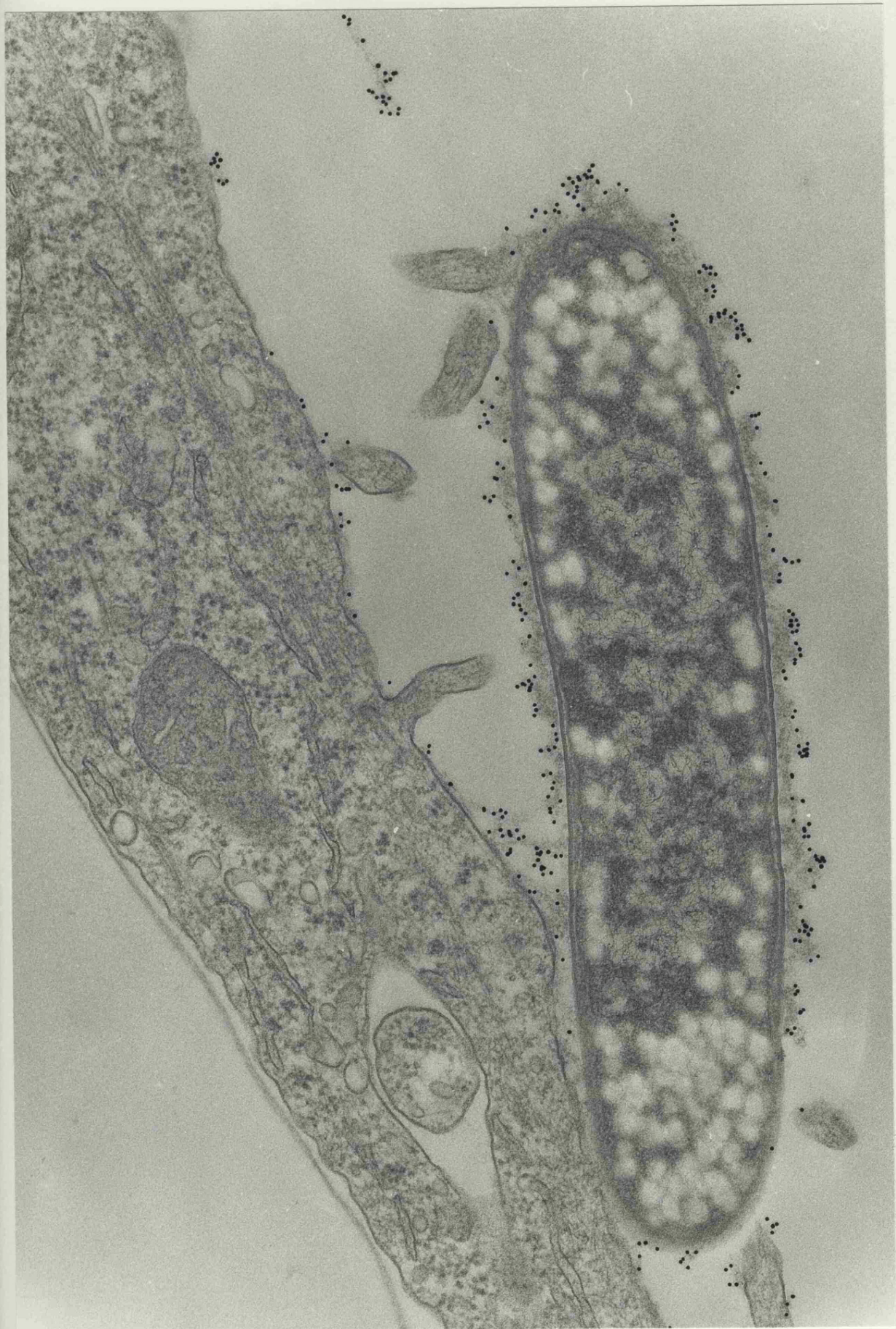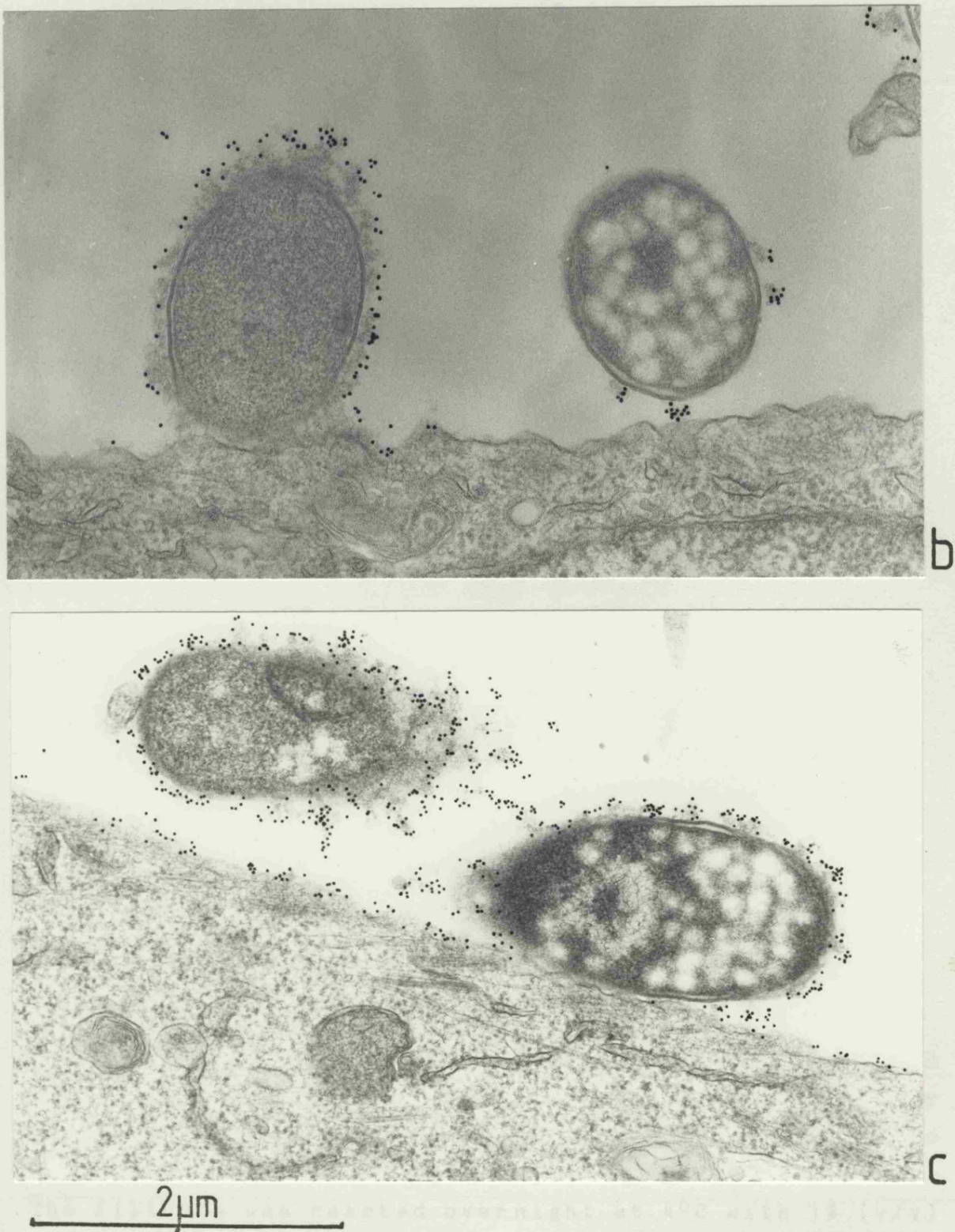
Figure 4.7 <u>Immunogold electron microscopy</u>

Cells of strains HB101(pLG161) (a, b) and 469-3 (c) were reacted with HEp-2 monolayers and with anti-469-3-MR-adhesin serum as described previously (Fig. 4.8). The monolayers were then reacted with gold-conjugated secondary antiserum, thin-sectioned and examined by TEM. Immunoreactive material, shown by the opaque 10 nm gold particles, was expressed on the surface of adherent bacteria, although individual cells appeared to express different amounts of the material.

Figure 4.8 Radioimmunoprecipitation
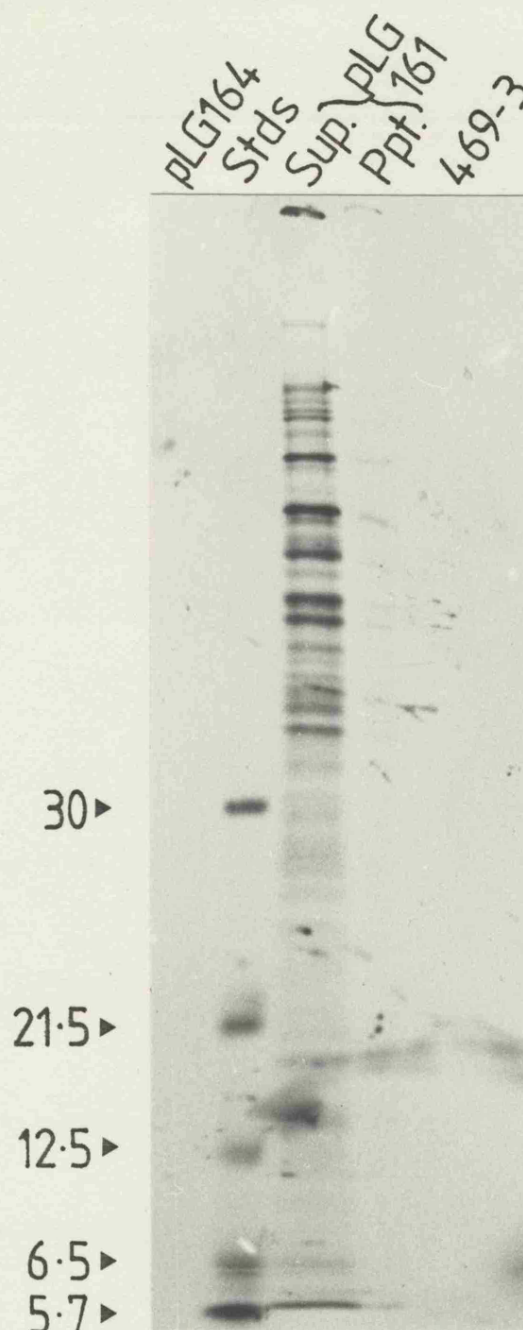
Proteins synthesised by bacterial cultures were labelled by growth with [^{35}S]methionine (**section 2.2.8**). Material was sheared from the strains by repeatedly forcing the labelled cultures through a fine hypodermic syringe, and whole cells were removed by centrifugation and filtration. The filtrate was reacted overnight at 4°C with 1% (v/v) anti-469-3-MR-adhesin serum, and the immunoprecipitates were recovered, washed extensively, and examined by SDS-PAGE and fluorography. Material of 14 kd was specifically precipitated from strains 469-3 and HB101(pLG161) but was absent from the non-haemagglutinating control strain. Molecular weight standards comprised a mixture of [^{14}C]-methylated proteins: carbonic anhydrase (30 kd), soybean trypsin inhibitor (21.5 kd), cytochrome C (12.5 kd), aprotonin (6.5 kd) and insulin (5.7 kd)[Amersham].
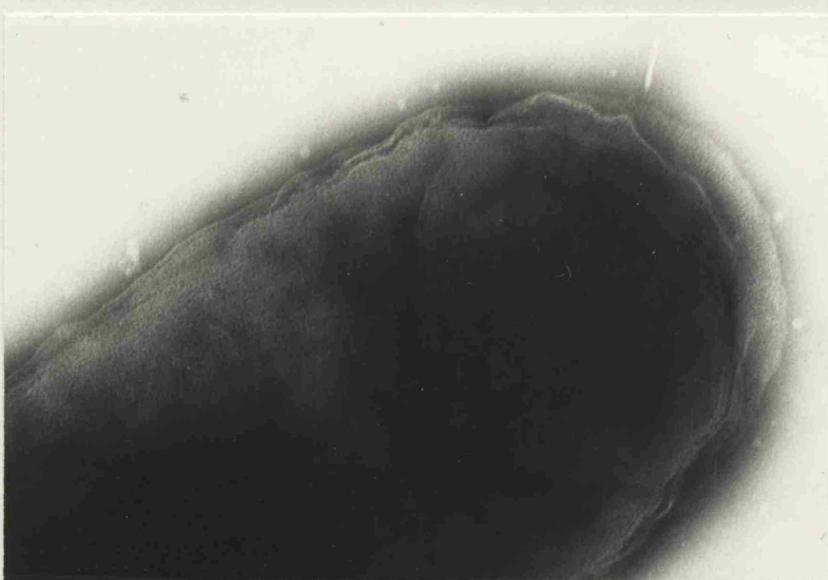
Figure 4.9 <u>Ultrastructure of the MR adhesin</u>

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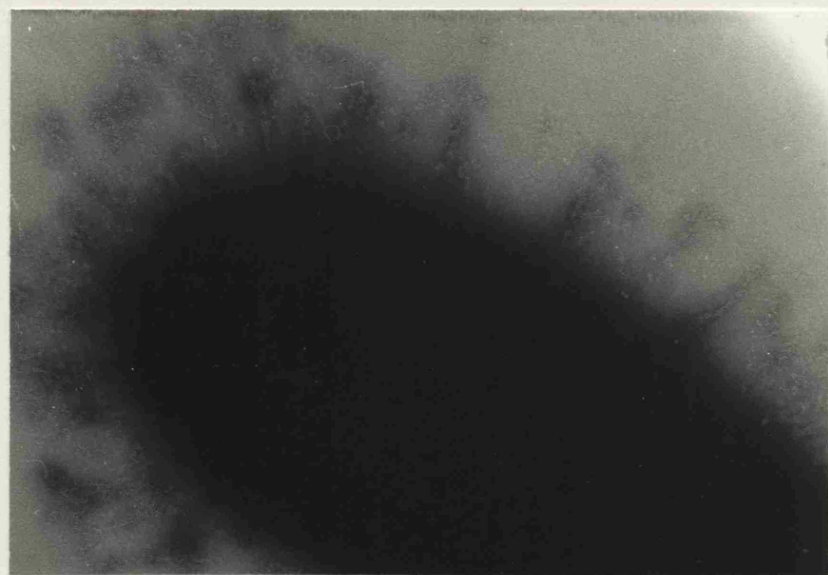
Figure 4.9 <u>Ultrastructure of the MR adhesin</u>

Strains 469-3 (a), HB101(pLG161) (b) and HB101(pBR322) (c) were grown overnight on LUA at 37°C. Small samples of bacteria scraped from individual colonies were negatively stained with uranyl acetate and examined by TEM (Hinson et al., 1987; performed in collaboration with Dr. S. Knutton [Institute of Child Health, Birmingham]). Fibrils, approximately 2 nm in diameter were visible on the surfaces of 469-3 (a) and HB101(pLG161) (b), but were absent from all cells of the control strain (c).

Plasmid pLG161 promoted adherence in strain HB101 indistinguishable from that of the parent strain, caused by a structure shown to be immunologically and structurally related to the previously reported MR adhesin and with the same fine fimbrial appearance. The next task was to define the location of the adhesin determinant within the cloned fragment of 469-3 genomic DNA by mutagenesis, and then to use a suitable internal DNA fragment as a 'probe' in hybridisation experiments to compare homologous regions in different strains. I started by deriving a restriction map of pLG161.

5.1 <u>Restriction mapping</u>. A detailed restriction map of pLG161 was constructed by the following means:

a) Plasmid DNA was digested with single restriction enzymes and separated by agarose gel electrophoresis in the presence of ethidium bromide, along with DNA size markers (lambda DNA digested with <u>Hind</u>III). The gels were then photographed in UV light and the photographic negatives or enprints were used for subsequent measurements (Fig. 5.1). The number of bands generated by each restriction enzyme gave the number of recognition sites directly since the plasmid was circular (thus, each site produced a linear fragment). The sizes of each fragment (Table 5.1) were calculated by comparing the relative mobilities of the bands (measured from the loading slots) with those of the known size markers, using a computer program (Duggleby et al., 1981) which, in effect, estimated by fitting a curve to the standards. The total lengths of all the fragments for each digest were checked against independent estimates to reduce the possibility of having missed 'doublets' (bands from multiple fragments with very similar mobilities), although in fact doublets were usually recognised by being disproportionately bright.

b) The order of the fragments was calculated by repeating the above procedures, but using pair-wise combinations of enzymes to relate the positions to those of other enzymes.

c) The full map (Fig. 5.2) was generated by several such combinations, in conjunction with the known restriction site positions within the vector from the published DNA sequence (analysed in Maniatis et al., 1982). Wherever possible, proposed map positions were double checked by digestion with a different enzyme (double digests) or enzymes (triple digests) and comparison of the predicted fragment sizes with those actually obtained.

5.2 Generation of deletion derivatives. The BamHI and KpnI sites in pLG161 were ideally suited to the deletion of specific regions of the plasmid, and thus to identify the location of the adhesin determinant by analysis of the adherence characteristics of the deletion derivatives.

5.2.1 Deletion of a 1.5 kb BamHI fragment. BamHI cuts pLG161 twice, once in each of the vector and insert portions, generating fragments of 1.5 and 20 kb (**Fig. 5.2**). The large fragment contains most of the insert portion plus the ampicillin resistance marker gene and plasmid replication region, whereas the small fragment contains part of the tetracycline resistance marker and a little of the insert. Therefore, ampicillin could be used to select the large fragment, while loss of the small fragment could be readily detected by sensitivity to tetracycline [inversion of the small fragment would be expected to cause the same phenotype, but such rare events were discounted by restriction analysis of the plasmid DNA]. Plasmid pLG161 was digested with BamHI, re-ligated, and used to transform E. coli HB101. Ampicillin-resistant transformants were screened for sensitivity to tetracycline, and loss of the small BamHI fragment was confirmed by agarose gel electrophoresis of BamHI-digested plasmid DNA prepared from ampicillin-resistant tetracycline-sensitive transformants. The plasmid from one culture, containing only the 20 kb BamHI fragment, was named pLG162 (**Fig. 5.2**). Transformants of HB101 carrying pLG162 did not cause MRHA, indicating that the small BamHI fragment of pLG161 contains DNA essential for MRHA expression and confirming the earlier finding (**section 3.4**) that individual BamHI fragments separately subcloned from cosmid pCC3 did not encode MRHA.

5.2.2 Deletion of KpnI fragments. Plasmid pLG161 was partially digested with KpnI and re-ligated. The ligation mixture was used to transform strain HB101. Plasmid DNA was prepared from 20 ampicillin-resistant transformants and analysed by KpnI digestion. Transformants carrying plasmids with either or both of the KpnI fragments missing from the chromosomal insert in pLG161 were assayed for MRHA. Loss of the 2.3 kb right-hand fragment (forming pLG163) made no noticeable difference to the MRHA phenotype, whereas lack of the 7.5 kb left-hand fragment (forming pLG164) or both fragments (pLG165) correlated with inability to promote MRHA (**Fig. 5.2**). These results agreed with the localisation data obtained in **section 5.2.1**, and further indicated that the MRHA region was not solely confined to the small BamHI fragment of pLG161, but had a size of between 2.9 kb (from the BamHI site to the left hand end of the fragment deleted in pLG164) and 11.5 kb (from the left hand end of the insert fragment in pLG161 to the right hand end of the deletion

in pLG164), assuming that it was contiguous (i.e. did not contain an internal non-essential region at least as large as the deletion in pLG164).

5.3 Transposon mutagenesis. The deletion derivatives of pLG161 defined the approximate position and probable size of the adherence region but did not allow fine mapping. Careful analysis of the map positions of transposons inserted at random into a genetic system, coupled with examination of the effects of each insertion on gene expression, is a powerful technique for more precisely defining essential regions of DNA. Plasmid pLG163 rather than pLG161 was used for these experiments because it is smaller so the transposon insertion sites could be mapped more easily and accurately.

5.3.1 Insertion of Tn1000. The attachment site ('gamma-delta') where F-plasmids integrate into the E. coli chromosome to form Hfr strains can insert at low frequency into pBR322 (**Guyer, 1978**), generating a duplication of 5 basepairs of plasmid DNA at either end of the insertion and thus putting the transposon into the Tn3 class (**Kleckner, 1981**). The insertion of gamma-delta (also known as Tn1000) subsequently allows the conjugal transfer of pBR322 (or derivatives such as pLG163) into F⁻ strains whereas normally pBR322 is non-mobilisable, thereby enabling the selection of transposon--containing plasmid derivatives.

F⁺ strain RB308 was transformed with plasmid pLG163 to ampicillin resistance, then transformants were mated with F⁻ streptomycin-resistant strain HB101. With counter-selection against the parents using both ampicillin and streptomycin, a number of transconjugants containing the plasmid and transposon were purified by streaking on agar. Plasmids were isolated from individual transconjugants by the minipreparation method. The transposon insertion sites were mapped and orientated by means of the eccentric EcoRI and BamHI sites within Tn1000 (**Fig. 2.2**). The same transconjugants were assayed by slide MRHA tests. The results are summarised in **Fig. 5.2**. Insertions spanning a region of 6 kb prevented MRHA expression, whereas insertions outside this region made no observable difference to the MRHA phenotypes of those strains. However, the transposon appeared to insert preferentially at just a few target sites ('hotspots'), limiting its usefulness and preventing much finer mapping unless many more insertions were mapped. I chose instead to use another transposon in the hope that it would not hotspot, or at least not to the same sites.

5.3.2 Insertion of Tn1723. This transposon was derived from a member of the Tn3 family, and encodes resistance to kanamycin (**Shingler and Thomas, 1984**). Plasmid pLG163 was transformed into strain RU2537 which carries Tn1723 on

its chromosome, and the transformants were subcultured over five days at 30°C to promote transposition. Plasmids were extracted from the transformants by the plasmid minipreparation method and used to transform strain HB101, selecting for resistance to both ampicillin and kanamycin. Twenty transformants were purified and MRHA tested. Plasmid DNA was extracted to map the EcoRI sites very near each end of the transposon (Fig. 2.2) relative to those surrounding the insert portion in pLG163, and the two HindIII and unique XhoI sites in the transposon relative to those in pLG163. Again, it is clear from the mapped positions (Fig. 5.2) that the transposon had a few preferential target sites (hotspots). The observed distribution of transposons was also markedly skewed to the left-hand end of the map of pLG163, including the vector part, for no obvious reason. Three transposon insertion sites were within the inserted 469-3 genomic fragment, at 0.8, 1.3 and 5.2 kb from the left hand EcoRI site. The first two of these disrupted MRHA and were presumably within the MRHA determinant, but the last did not prevent expression of MRHA and may have been just outside the region.

5.4 **DNA-DNA hybridisation.** Cosmid cloning from strain 469-3 produced 8 haemagglutinating cultures out of 500 clones, which was higher than anticipated even if the apparent size of the MRHA determinant (sections 5.2 and 5.3) was smaller than I had assumed in making the initial calculations (Table 3.2). Two possible explanations were that 469-3 carries determinants for distinct MR adhesins (perhaps in analogous fashion to multiple P-fimbriae and type 1-like adhesins on UPEC strains; section 1.4.12), or the strain may contain multiple copies of the determinant for a single MR adhesin. The data shown in the previous chapter led to the conclusion that pLG161 encoded an MR adhesin with identical properties to the previously reported MR adhesin of 469-3, but other haemagglutinating clones may perhaps have encoded different systems. I hoped to confirm or refute these possibilities by DNA-DNA hybridisation studies using part of pLG161 known to determine MRHA expression to search for homology among the other haemagglutinating cosmids and to determine the number of copies of the system in the genome of the parental strain by Southern blotting.

In appropriate conditions, homologous complementary single strands of DNA will hybridise. DNA can either be crudely extracted from whole bacterial colonies grown on blotting membranes and immobilised by alkaline lysis in situ (**colony blot**), or individual restriction fragments may be size-separated in agarose gels by electrophoresis, then transferred to membranes (**Southern blot**; after **Southern, 1978**). A purified restriction fragment (**probe**) is normally radiolabelled before reacting with the

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immobilised target DNA (**hybridisation**); any interaction can then be detected by autoradiography. The techniques are particularly sensitive. Unique target fragments may be detected in total genomic DNA, provided that the probe is sufficiently homologous to hybridise in the conditions of temperature and salt concentration used (**stringency**).

5.4.1 Preparation of radiolabelled probe. To screen extracted DNA samples for homologous sequences to the MRHA determinant, I required a DNA fragment from within the region defined above (rather than the whole plasmid which included vector DNA, or the whole insert fragment which was largely functionally undefined). The region of pLG161 between the BamHI and KpnI sites at the left-hand end of the 469-3 genomic DNA insert had been identified by deletion and transposon insertion analysis as essential for MRHA (see above). This 3 kb BamHI-KpnI region was therefore chosen for use as an adhesin-specific probe. The region was actually prepared from pLG163 since this deletion derivative lacks the small KpnI fragment of pLG161 which is almost the same size as the desired probe and would thus be difficult to separate from it by a single step. About 5 μg of pLG163 DNA was digested with KpnI and then with BamHI, and the restriction fragments were separated by electrophoresis. The 3 kb probe fragment was extracted by elution onto DEAE-cellulose paper and checked for purity by electrophoresis, and later by Southern blotting (see below).

The purified BamHI-KpnI fragment was radiolabelled to high specific activity, using a mixture of oligonucleotides as random primers to initiate DNA replication in the presence of [^{32}P]dCTP (**section 2.3.3**).

5.4.2 Colony hybridisation. Colonies of HB101 carrying various cosmids (some capable of promoting haemagglutination) or pLG161 or pBR322, and strains 469-3 and 444-3, were grown on nitrocellulose filters laid on agar plates overnight, then lysed by treatment with alkali, bound to the filters by baking and reacted with the radiolabelled probe (previously heat-denatured) under stringent conditions. After washing off unbound probe DNA, the samples which had hybridised were detected by autoradiography (**Fig. 5.3**). DNA from all of the haemagglutinating cultures hybridised to the probe whereas that from non-haemagglutinating colonies did not [unfortunately, three of the cosmid clones had lost all trace of their former haemagglutinating abilities at the time of the experiment, probably by deletion within the cosmids, although they still expressed the ampicillin-resistance marker from Cos4]. Therefore, all the haemagglutinating cosmid clones and both the haemagglutinating clinical isolates contain DNA homologous to the BamHI-KpnI probe fragment, but the control strains (including the plasmid and cosmid

vectors themselves) lack homologous sequences.

5.4.3 Southern hybridisation. The colony blots were purely qualitative; they gave no indication as to the size of homologous fragments in the various strains tested, nor the degree of homology in the various colonies (differences in the amount of label apparent on the X-ray films may have been due to several causes, such as differential release of DNA, as well as greater or lesser homology to the probe). Southern blotting was used to detect the sizes of homologous restriction fragments separated by agarose gel electrophoresis. Cosmid and plasmid DNA from various clones and genomic DNA from the clinical isolates 469-3 and 444-3, and from the cloning recipient HB101, was digested with BamHI, EcoRI or KpnI alone, or with both BamHI and KpnI, and subjected to electrophoresis. The DNA was transferred from the gel to nylon membranes (Southern blotted) and hybridised with radiolabelled probe DNA under stringent conditions (**Fig. 5.4**). As predicted from the colony blots, the haemagglutinating cultures, but none of the control strains, contained homologous fragments. The homologous EcoRI fragments were all 17 kb, while the KpnI-BamHI double digests produced 3 kb homologous fragments; these were of course the same size as those in pLG161. The BamHI and KpnI single digests, however, produced larger fragments than in pLG161 since these had been cut short by the EcoRI used to subclone the system. The sizes of these singly digested fragments therefore gave the distances to the proximal recognition sites to the left and right of the subcloned region.

5.5 Conclusions.

5.5.1 Functional map of pLG161. The region of pLG161 which encodes MRHA was identified by deleting specific restriction fragments and analysing the effect of these deletions on the adherence phenotypes of recipient strains (**Fig. 5.2**). Deletion of the small BamHI fragment (forming pLG162) disrupted MRHA. Furthermore, although the promoter of the tetracycline-resistance marker is located in the vector part of the small BamHI fragment near the vector-insert junction, the gene is transcribed away from the junction towards the centre of the vector; thus lack of expression of MRHA from pLG162 was not due to the deletion of a vector promoter or interruption of transcription initiated within the vector, but must have involved deletion of coding regions from the inserted 469-3 DNA. Deletion of the 7.5 kb KpnI fragment (pLG164) also disrupted MRHA, whereas the 2.3 kb KpnI fragment was not essential (pLG163). Therefore, the MRHA determinant spans at least between the BamHI and left-hand KpnI sites, and so it has a minimum size of

2.9 kb and a probable maximum size of 11.5 kb.

Transposons were also inserted into pLG161 to define functional regions of the plasmid more fully. The two transposons used in the present study, however, were limited to rather few insertion sites thus reducing the amount of information obtainable, but given this, transposon insertions at 7 sites in one block were found to prevent MRHA. These spanned some 4.2 kb and their locations agreed with the deletion analysis. This size range is similar to that of other adhesin determinants (**section 1.4**). The amount of DNA required to encode the 14 kd subunit protein is approximately 0.4 kb, leaving at least 3.8 kb of sequence essential for MRHA unaccounted for; this implies the presence of a complex system of several genes, apart from that encoding the subunit, which are presumably involved in the synthesis and regulation, export and assembly of the fimbrial structure on the cell surface.

For unknown reasons, no transposon insertions occurred in a large part of the plasmid to the right of the map (**Fig. 5.2**), thus it was still not possible to determine the size of the MRHA system firmly. Possibly, the problem stemmed from sequence specificity in the transposons themselves, which were both of the Tn3 family and may have had similar preferred sites. Alternatively, I may simply have been unlucky in obtaining bacteria with transposons which entered the plasmid early in the culture growth and subsequently multiplied, so the resultant colonies were not truly independent. In any future transposon analysis, entirely separate experiments might be performed to derive each transposon insertion quite independently.

5.5.2 Number of copies of the adhesin determinant. Colony blots (**Fig. 5.3**) indicated that all the haemagglutinating cosmid-containing strains hybridised to the DNA probe originally derived from one of them; there was no evidence that certain of the haemagglutinating clones contained alternative genetic determinants encoding different MR adhesins to that cloned in pLG161. In conclusion, it is likely that all the MRHA$^+$ clones contained the same adhesin determinant even though their abilities to cause MRHA of human blood were not all identical, nor were they stable over the course of 18 months (**chapter 4**).

Southern blots (**Fig. 5.4**) revealed single homologous bands in all the MRHA$^+$ cosmid-containing strains but none in the cosmid-free recipients, and single bands in 469-3 DNA. These results indicate that the MR adhesin determinant is probably present as a single copy in the genome of 469-3, since additional copies would probably have been variable with respect to the restriction enzyme recognition sites and thus would have generated multiple bands. Consequently, it is not clear why so many haemagglutinating

clones were originally obtained in the cosmid library, except perhaps by chance duplications of the unique determinant.

5.5.3 Homology to other adhesin determinants. Hybridisation experiments indicated that the DNA probe from the 469-3 MR adhesin determinant is significantly homologous to genomic DNA from strain 444-3, which was isolated from a patient with a similar disease and was previously shown to express an adhesin with very similar properties (**Knutton et al.,** 1984c; **Williams et al.,** 1984). The fragment sizes detected by Southern hybridisation were very similar to those obtained from 469-3; thus it is likely that 444-3 carries a similar MR adhesin determinant, which may yet be cloned and detected by screening colonies with the 469-3 probe fragment. This also raises the possibility of screening other pathogenic E. coli and even Shigella strains, especially those with similar virulence characteristics, for the presence of homologous sequences.

The restriction and functional maps of the 469-3 MR adhesin determinant are remarkably similar to those of AFA-I (**Labigne-Roussell et al.,** 1984, 1985; **Walz et al.,** 1985), an afimbrial adhesin encoded on a plasmid in UPEC strain KS52 (**Fig. 5.2**). The only appreciable difference is in the placing of a BamHI site to the right of the fragment inserted in pLG161 and at least 15 kb away from either of the adhesin systems, which differs by just over 2 kb. The HindIII site which lies within the subunit gene for AFA-I is within just 0.2 kb of that position in the 469-3 system, while all the other sites mapped in both strains (BamHI, EcoRI, HindIII, SalI and SmaI) correspond exactly. Thus, the relatively minor differences may be due to experimental errors (particularly at the BamHI site on the 'right-hand-side' of the 469-3 system, since this was only mapped by Southern hybridisation analysis), or could represent small differences in otherwise identical systems. The AFA-I subunit protein is 16 kd and promotes MR adherence to different tissues than the 469-3 MR adhesin, so perhaps the difference in the subunit gene is a true reflection of the presence of different subunit determinants. The difference in the distant BamHI site might be due to alterations in the lengths of the intervening DNA, or perhaps even the result of transposition of the adhesin determinants to different sites (especially since the 469-3 system is chromosomal and the AFA-I determinant is plasmid-borne), but until the systems have been more fully compared (e.g. by hybridisation), little more can be concluded (see **chapter 6**).

Table 5.1 pLG161 restriction fragment sizes

Restriction enzyme(s)	Mean size (kb) of fragment number [a]													Total (kb)	Number of expts [b]
	1	2	3	4	5	6	7	8	9	10	11	12	13		
BamHI	17.99	1.49												19.48	4
ClaI	18.97													18.97	3
EcoR1	16.05	4.35												20.49	12
HindIII	11.72	9.15	0.96											21.83	4
HpaI	13.33	4.15	1.80	1.80	1.70									22.78	3
KpnI	11.56	7.46	2.27											21.29	6
PstI	5.25	5.25	2.50	2.20	1.80	1.00	0.70	0.65	0.51					19.86	2
SalI	12.19	6.51	1.83	1.27										21.80	8
SmaI	6.51	5.15	2.98	1.49	1.07	1.07	0.96	0.84	0.67	0.47				21.21	1
XhoI	19.14													19.14	5
BamHI/EcoRI	15.80	4.14	0.97	0.48										21.39	3
BamHI/HindIII	12.30	9.36	0.99	0.47										23.09	3
BamHI/KpnI	7.52	7.52	2.82	2.28	1.37									21.51	2
BamHI/SalI	13.10	6.88	1.32	1.18	0.41									22.90	2
BamHI/SmaI	5.98	5.19	2.50	1.48	1.05	1.04	0.93	0.82	0.66	0.52	0.46			20.63	1
BamHI/XhoI	19.25	1.32	0.40											20.97	2
ClaI/HindIII	12.10	9.00	0.95											22.05	1
ClaI/SalI	12.50	6.66	1.37	1.37										21.90	1
EcoRI/HindIII	11.80	4.36	4.36	0.91										21.43	4
EcoRI/KpnI	8.20	4.58	4.03	2.96	2.25									22.09	3

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Table 5.1 continued. pLG161 restriction fragment sizes

| Restriction enzyme(s)	Mean size (kb) of fragment number[a]													Total (kb)	Number of expts[b]
	1	2	3	4	5	6	7	8	9	10	11	12	13		
EcoRI/SalI	12.95	3.87	2.32	1.26	1.06	0.70								22.16	4
EcoRI/SmaI	5.41	4.55	3.05	1.52	1.38	1.05	1.05	0.93	0.82	0.61	0.46			20.83	1
EcoRI/XhoI	14.50	4.46	0.63											20.59	4
HindIII/KpnI	9.20	8.00	2.84	1.34	0.96	0.91								23.25	3
HindIII/SalI	11.40	6.48	1.28	1.01	0.78	0.66								21.61	3
HindIII/SmaI	6.70	5.55	2.67	1.52	1.07	0.95	0.85	0.85	0.66	0.46	0.46			21.74	1
KpnI/SalI	8.05	6.69	2.75	1.84	1.70	0.71	0.65							23.39	4
KpnI/SmaI	6.70	5.23	3.05	1.39	1.08	1.07	0.96	0.84	0.66					20.98	1
SalI/SmaI	5.63	5.63	2.45	1.08	1.08	0.84	0.84	0.84	0.71	0.70	0.60	0.60	0.47	21.47	1
SalI/XhoI	13.10	6.80	1.25	1.11	0.72									22.99	3
Bam/Eco/Kpn[c]	7.20	4.00	3.10	2.81	2.32	0.96								20.39	1

Notes to Table 5.1

a) The lengths of fragments of pLG161 generated by restriction enzymes and separated by agarose gel electrophoresis were calculated in relation to HindIII-digested lambda DNA fragments of known sizes, using the computer program of **Duggleby et al., 1981**; multiple determinations were averaged.

b) The number of independent determinations is shown.

c) BamHI/EcoRI/KpnI triple-digest.

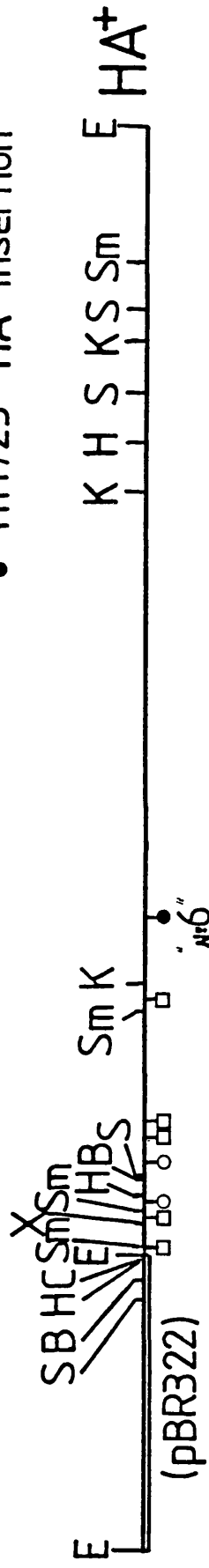## Figure 5.1 <u>Restriction analysis of pLG161</u>
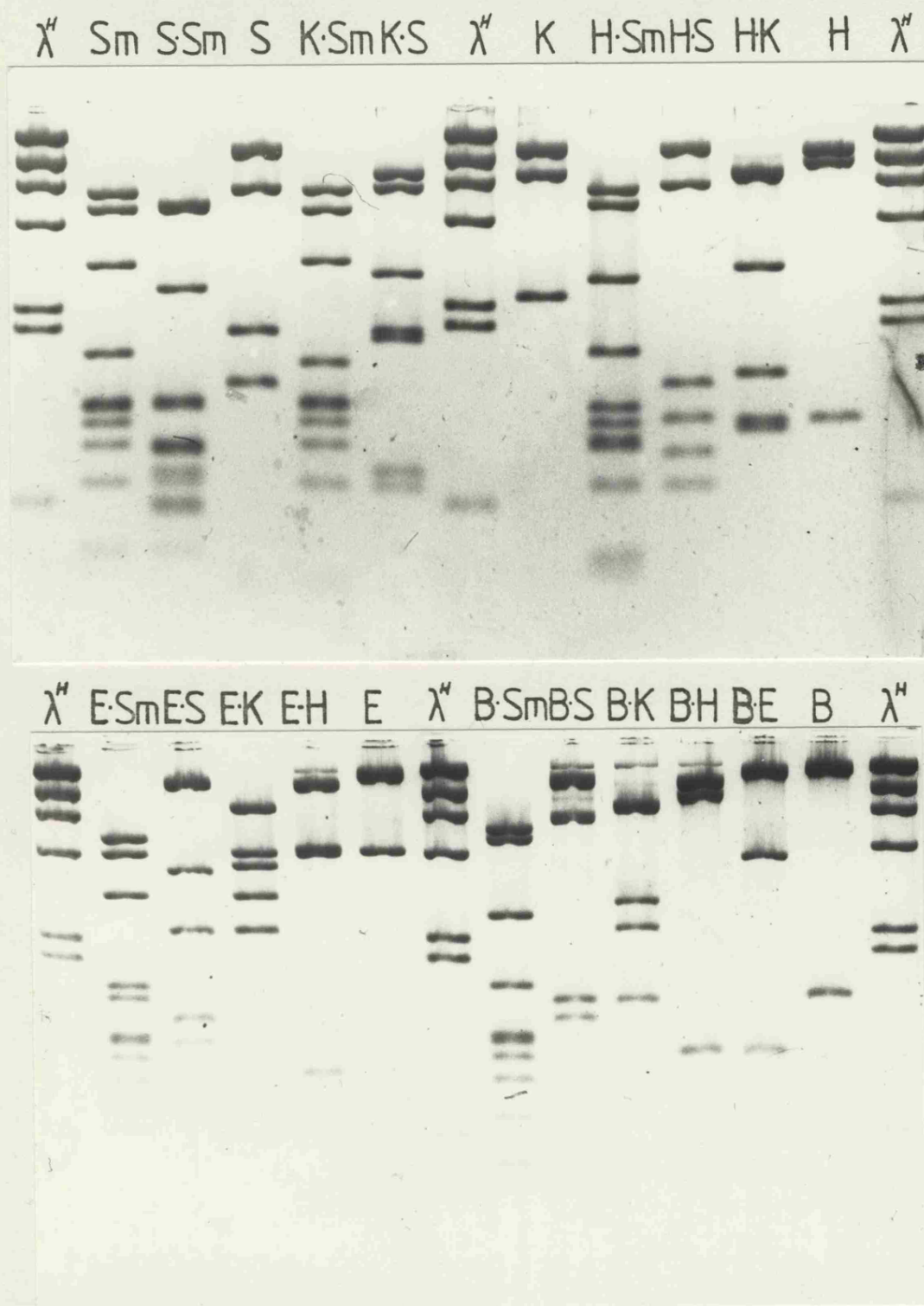
Aliquots (1 µg) of pLG161 DNA were singly- and doubly-digested with 10 U
lots of <u>Bam</u>HI (B), <u>Cla</u>I (C), <u>Eco</u>RI (E), <u>Kpn</u>I (K), <u>Sal</u>I (S), <u>Sma</u>I (Sm) or
<u>Xho</u>I (X) at 37°C for 1 h (for double digests, the DNA was first digested
with one enzyme, then ethanol precipitated, washed and resuspended in 9 µl
of buffer for digestion with the second enzyme as before). The fragments
and size markers (lambda DNA digested with <u>Hind</u>III) were separated by
agarose gel electrophoresis in buffer containing ethidium bromide
(0.5 µg/ml) and photographed in UV light.

5kb

pLG161
=21.3kb

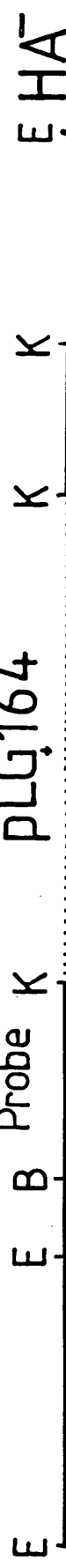⊔ Tn1000 ⎫
⊖ Tn1723 ⎬ HA⁻ insertions
● Tn1723 ⎭ HA⁻ insertions
● Tn1723   HA⁺ insertion

E   K H S K S Sm   E HA⁺

pLG162   B K   K   E HA⁻
E B

pLG163   B K   K   E HA⁺
E B

pLG164   Probe B K   K   E HA⁻
E B

pLG165   B K   K   E HA⁻
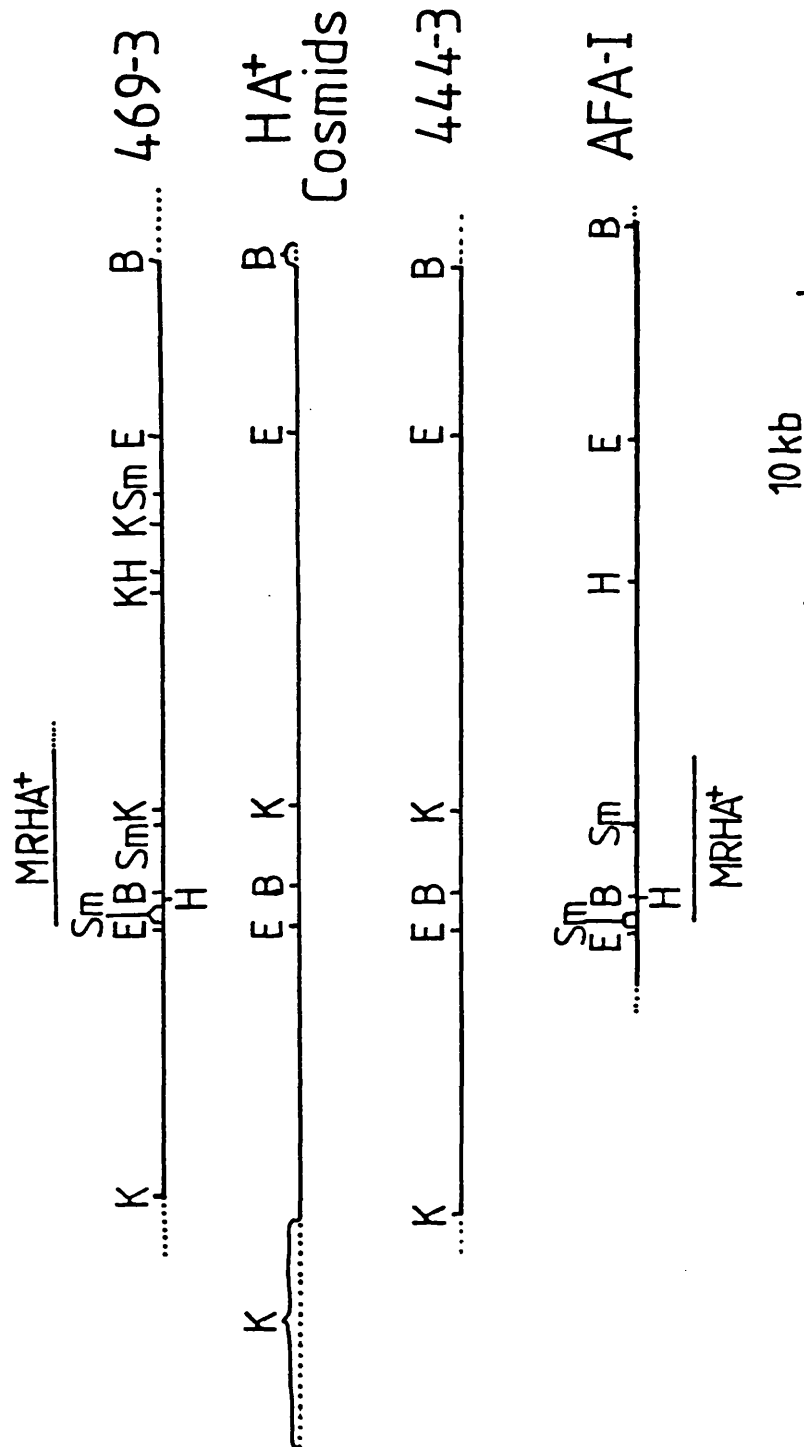E B

S B H C Sm Sm
E   H B S
Sm K
(pBR322)

Figure 5.2 Restriction and functional maps

The restriction enzyme recognition sites are marked according to the abbreviations used in **Fig. 5.1**. The 469-3 map was derived from the results presented in **chapters 4** and **5**. The 444-3 map was derived from Southern hybridisation analysis using a probe from the cloned 469-3 adhesin determinant (**section 5.4.3**). Data for AFA-I were obtained from maps and other published information (**Labigne-Roussel et al., 1984** and **1985**). Note that the recognition sites for some enzymes have not been fully mapped (e.g. the SmaI sites in the AFA-I system are only known for that part of the cosmid which was subcloned).

Figure 5.3 <u>Colony hybridisation</u>
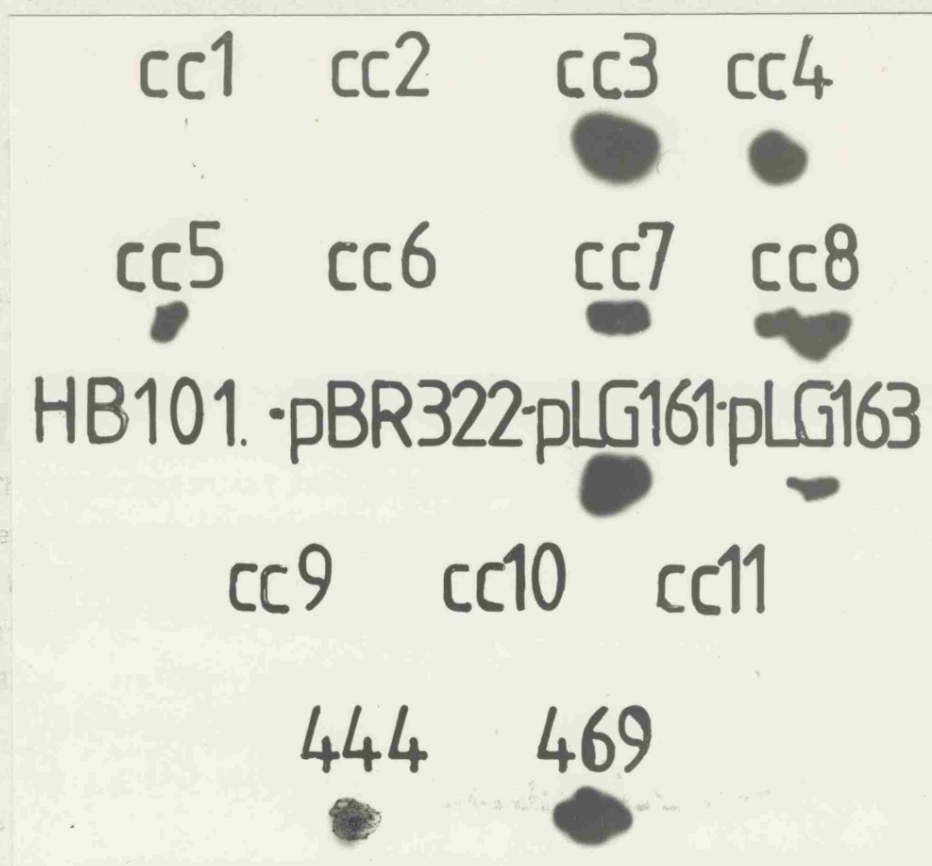
Colonies of HB101, cosmid- and plasmid-containing derivatives of HB101, and
clinical isolates 469-3 and 444-3, were grown overnight on LUA, transferred
to nitrocellulose filters and lysed in situ (**section** 2.3.3), then hybridised
to radiolabelled probe DNA from the cloned 469-3 MR adhesin determinant.
Hybridisation was detected by autoradiography. All the haemagglutinating
strains (HB101 harbouring pCC3, pCC4, pCC5, pCC7, pCC8, pLG161 or pLG163,
and 444-3 and 469-3) hybridised with the probe, while the remaining cultures
(including HB101 carrying the vector pBR322 and three cosmid clones, pCC9,
pCC10 and pCC11, randomly selected from the 469-3 genomic library) which
were incapable of MRHA were also negative in the colony hybridisation.

Figure 5.4 <u>Southern hybridisation</u>
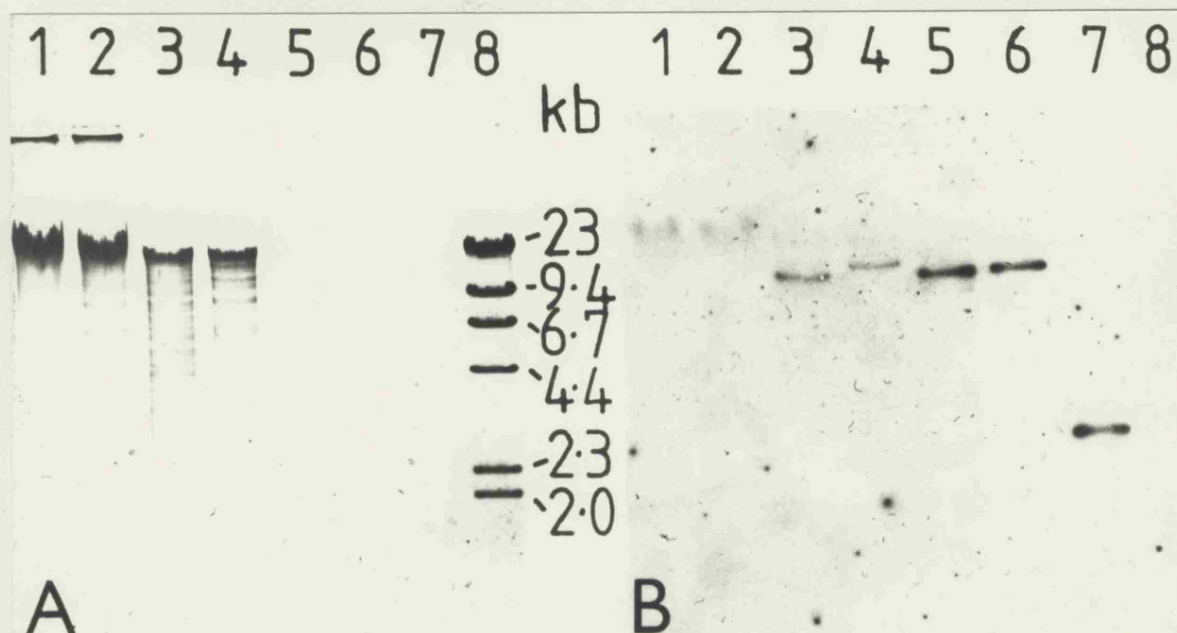
Fragments of 2 µg aliquots of genomic DNA from HB101 (**Lanes 1** and 2) or 469-3 (**Lanes 3** and 4), or plasmid DNA of pLG161 (**Lanes 5** and 6), digested with <u>Kpn</u>I (**even numbered lanes**) or <u>Hind</u>III (**odd numbered lanes**), were separated by agarose gel electrophoresis along with approximately 2 ng of unlabelled probe DNA (**Lane 7**) and lambda DNA as size markers (**Lane 8**). The gel was photographed in UV light (**A**), then the DNA was blotted onto a nylon membrane, hybridised with radiolabelled probe DNA from pLG161 and autoradiographed (**B**).

6.1 Utility of the cloning approach. Molecular biology (genetic engineering) encompasses a host of very powerful genetic, biochemical and microbiological techniques which permit the detailed analysis of complex bacterial and eucaryotic systems, and as such was perfectly suited to the MR adhesins of the pathogenic E. coli strains, 444-3 and 469-3, in conjunction with electron microscopical examination. Results obtained previously without the benefit of molecular genetics were incomplete since the adhesins could not be studied in isolation from other host functions, such as capsules and type 1 fimbriae. The primary aim of this project, then, was to clone the MR adhesin genetic determinants and express them in better defined systems to continue the analysis in detail.

6.1.1 Cloning. Initial cloning attempts using plasmid vectors failed due to technical problems, but with hindsight it would appear that they might have succeeded if, for example, EcoRI fragments of genomic DNA had been cloned. In the event, the adhesin system was eventually cloned from 469-3 in several recombinant cosmids and was later subcloned from one such cosmid in a plasmid vector. The use of both cosmid and plasmid cloning vectors was advantageous in reducing the number of colonies which had to be screened in the first place, and in obtaining smaller, more stable derivatives for subsequent detailed genetic analysis.

6.1.2 Genetic analysis. Plasmid pLG161 was subjected to a range of genetic analyses and the following principal findings were made:
a) The MRHA system occupies a single region of about 6 kb on the chromosome of 469-3.
b) The restriction map of the determinant is virtually identical to that of the MR adhesin systems from another infantile enteritis strain (444-3) previously shown to have similar properties to the 469-3 MR adhesin, and from a human urinary tract E. coli isolate (AFA-I). The 469-3 and 444-3 MR adhesin genetic determinants were also shown to hybridise in Southern blotting experiments, but such experiments have not yet been performed on KS52, the UPEC strain carrying AFA-I, since the strain was not available [since completing the project, a strain containing pIL14, the recombinant plasmid encoding AFA-I, has been kindly donated by Agnes Labigne-Roussel].

6.1.3 Phenotypic analysis. The properties of the cloned MR adhesin were analysed and compared with those of the parent strain by a variety of immunological, biochemical and electron microscopical methods. The adhesins

were shown to be antigenically cross-reactive, of the same subunit and assembled sizes, and to promote adherence to the same human tissue culture cells and erythrocytes. These results proved that the cloned system expressed from plasmid pLG161 was indeed the same as the parental MR adhesin. The previous findings were then extended by experiments using human gut epithelia, and adherence to colon but not small intestinal tissues was demonstrated. The adhesin was also shown to have fimbrial structure which had been mistaken previously as capsular in origin. Now, the adherence properties can be firmly ascribed to the fimbrial MR adhesin of 469-3 since the clone, expressing the adhesin, behaved identically in all respects to the parent strain whilst the cloning recipient itself was negative in the same tests.

6.2 Evolution of bacterial adhesins. Based on the results presented in this thesis and in conjunction with findings on other systems, I would like to propose the following synthesis or model to account for the observed similarities and differences amongst bacterial adhesins. I will also discuss some of the implications of this proposal on the search for vaccines against pathogenic bacterial infections based on adherence structures, and suggest some experiments which might be performed to test the model.

6.2.1 Divergent evolution from common ancestors. Bacterial adhesins share many common features, but this alone is insufficient to propose that they are evolutionarily related to common ancestors since they may be the products of convergent evolution, i.e. their evolution may have been determined by similar pressures, such as structural constraints, which resulted in chance similarities amongst unrelated systems. In fact, there are features which suggest both types of process. Adhesins from widely disparate bacterial species are remarkably similar in structure; most are composed of thin rod-like processes radiating from the bacterial surfaces, a shape which theoretical considerations indicate is ideally suited to bridging between similarly charged bacteria and animal cells (**section 1.4**). Conversely, most adhesins appear to be quite different in terms of their receptor specificities, antigenic properties, etc. (**Table 1.3**). However, there are clearly groups of adhesins which are so similar as to suggest their evolution from some common progenitor, including type 1 and type 1-like fimbriae (**sections 1.4.1 and 1.4.2**), P fimbriae (**section 1.4.12**), the K88 family (**section 1.4.3**) and perhaps most convincingly the MePhe group of adhesins on various pathogenic species (**section 1.4.13**).

Adhesins are particularly important to the bacteria which possess them for two reasons: firstly, they mediate adherence to specific surfaces and

therefore determine (in large part) the site of bacterial colonisation; and secondly, they are immunogenic molecules exposed on the cell surface. These two factors present conflicting pressures, since on the one hand, the adhesins must be capable of adherence to the particular surfaces rather than lacking the ability or promoting adherence to different sites, whilst bacteria which express particular surface antigens are presumably under considerable stress from the host immune defences sensitised to them. With their short and relatively simple life-cycles, bacteria are generally able to respond to such strong selective pressures by rapid evolutionary changes, but the adherence consideration imposes constraints on the possible extent of alterations. This, I propose, accounts for the observed pattern of similarities and differences amongst bacterial adhesins.

One solution to the conflicting pressures may have been the evolution of novel immunodominant determinants in regions of the adhesins separate from those parts determining the receptor specificities. This explains the observation that Neisseria gonorrhoeae MR adhesins, for example, are largely comprised of 'hypervariable regions' (the main antigenic epitopes), whilst the remainder of the molecules which account for the adherence and structural assembly functions are far more constant and are immunorecessive (Duckworth et al., 1983; Virji and Heckels, 1984). Alternatively, bacteria may have responded by physically separating the adherence and structural components of the adhesins, such as is true of several E. coli adhesins including type 1 fimbriae (Maurer et al., 1985; Minion et al., 1986), P fimbriae (Lindberg et al., 1984; Norgren et al., 1984; van Die et al., 1985; Uhlin et al., 1985a, 1985b; Lindberg et al., 1986; Rhen et al., 1986) and X fimbriae (Hacker et al., 1985). These adhesins are composed of large numbers of subunit proteins which associate to form the fimbrial rods, plus far fewer true adhesins which actually adhere to the receptors and are normally located on the fimbriae, perhaps at their ends. Thus, the structural components might evolve without directly affecting the adhesins so long as they retain their physical shapes and the ability to form inter-unit associations. In support of this view, several adhesin subunits are homologous at their termini but less so in the central part (e.g K88, K99 and F41; Moseley et al., 1986), suggesting that the ends are the parts which link subunits together, a function conserved amongst all fimbrial subunits, while the rest of the molecules are more free to diverge and simply, as it were, fill the space between the ends.

Much of the observed pattern of similarities and differences amongst bacterial adhesins, then, may be explained as a combination of the requirements for antigenic divergence including antigenic variation, the expression of alternative adhesins by a single bacterium, and adherence to

specific surfaces. The latter point also allows for divergence since animal tissues and other surfaces present a whole range of structures which may potentially act as receptors, given the presence of corresponding adhesin characteristics.

6.2.2 Means of spread. Epidemiological studies have frequently shown that plasmids specifying resistance to drugs in common use can spread amongst a large bacterial population. Possession of such plasmids is of course strongly advantageous to the bacteria; this strong selective pressure promotes the rapid spread of resistance plasmids. Other bacterial systems also carried by plasmids include bacteriocins (**section 1.3.3**), exotoxins (**section 1.3.6**) and adhesins (**section 1.4**), and often multiple determinants reside on the same plasmids. What is not so clear, however, is whether they are transferred to new strains or whether the original strains simply multiply and out-compete the opposition (resulting in clonal groups; **section 1.3.10**).

Conjugal transfer of genetic information using transfer functions on the same or different plasmids (mobilisation) seems the most likely means of transmission, and may even explain interspecific exchange on wide host-range plasmids (such as the IncP1 group in most enteric bacteria). In one example, a plasmid from _E. coli_ was transferred to _Citrobacter_, _Edwardsiella_, _Enterobacter_, _Klebsiella_, _Proteus_, _Salmonella_, _Serratia_ and _Shigella_ species and expressed CFA/I and ST in all these, although plasmid stability was variable (**Yamamoto et al., 1984**). Another possible vehicle of transmission is transposons. Several virulence determinants, including aerobactin (**Roberts et al., 1986**) and enterotoxins (**Yamamoto and Yokota, 1981**), reside on transposons; different strains therefore possess genetic similarities between the transposon ends (usually discrete insertion sequences) but are dissimilar in surrounding sequences (**Elwell and Shipley, 1980**). Using another form of transmission, verotoxin (VT) genes are carried by bacteriophage in some EPEC trains (**Scotland et al., 1983; O'Brien et al., 1984; Strockbine et al., 1986**) and several other exotoxins are phage-encoded (**section 1.3.5**). The relevance of transposition and other transfer mechanisms (transduction, transformation, etc.) to the dissemination of virulence characteristics amongst bacterial populations is uncertain, particularly with respect to interspecific exchanges, but more data are required in this important field.

As well as transmission between bacteria, genetic determinants may of course move around the genome of a single organism, for instance from a plasmid to the chromosome or within the chromosome (e.g. gonococcal antigenic variation). Once in the chromosome, they are less mobile in the

sense that they cannot move as readily by conjugation, and are more stable since in the absence of selection, plasmids are 'disposable' elements whereas the chromosome is always essential for growth. Mass vaccination programmes using plasmid-encoded antigens sometimes appear to result in 'curing' of the plasmids; this is most likely a population effect - bacteria expressing the antigens are selectively disadvantaged and are outgrown by chance plasmid-free derivatives. In practice, however, there is no clear pattern to the location of adhesin determinants (Table 1.3).

One final aspect of the model is particularly relevant to the data presented in this thesis concerning the genetic similarities between the 469-3 MR adhesin and the AFA-I system. The model predicts that these adhesins may be specific for receptors on colonic and urinary epithelia, respectively, due to the presence of different adhesin genes, yet be encoded by systems identical in other regions including control of synthesis, export and assembly functions. Alternatively, the minor restriction map and subunit molecular weight differences reported may simply be due to experimental errors; the adhesin determinants could be identical. Some diarrhoeagenic E. coli strains with non-ETEC, non-EPEC serotypes express F7 and F8 antigens (Czirok et al., 1982), fimbriae normally associated with UTI (Orskov et al., 1982a); the strains were presumably isolated 'in transit' through the intestines since urinary tract infections probably result from faecal contamination of the periurethral region (Levine, 1984). The AFA-I clone has now been made available, so full comparisons with the 469-3 MR adhesin system should resolve this matter.

6.3.3 Evolutionary mechanisms. As explained above, there are conflicting evolutionary pressures on the bacterial adhesins; they have definite functions to perform and are structurally constrained, yet they are immunogenic structures and must therefore evolve to escape host immune defences in the long term. Frequently, pathogens express multiple adhesins with differing receptor specificities and antigenic properties. This gives a clue as to the probable mechanism of evolution. Adhesin genes have duplicated at some stage (within a single bacterium initially and later transferred to other individuals), then diverged. So long as a functional adhesin is expressed from one complete genetic determinant, the other adhesin systems may potentially evolve by a process of genetic drift without affecting the adherence properties of the bacterium. In addition, recombination between homologous or other specific parts of the original and diverged systems may result in novel functional combinations. Examples of the process include the DNA rearrangements of Neisseria gonorrhoeae fimbriae genes in antigenic variation (So et al., 1985) and the presence of genes

resembling the major subunit genes within the systems encoding <u>E. coli</u> P fimbriae (Uhlin et al., 1985a).

6.3.4 Implications in vaccine development. Bacterial adhesins, being both virulence determinants and surface-located antigenic determinants, are seen as suitable structures for use as vaccines against certain bacterial infections. A few such vaccines have been studied so far (e.g. K88, K99, 987P, type 1, CFA/I and <u>Neisseria gonorrhoeae</u> fimbriae; reviewed by **Brinton et al.**, 1978; **Beachey**, 1981, **Levine et al.**, 1983, **Porter and Linggood**, 1983, **Germanier**, 1984), although the commercial interests in this field, both medical and veterinary, have probably interfered with the full publication of research findings. Some results are clear, however. Firstly, adhesins elicit immune responses in animals and humans, although not always of the correct type; anti-diarrhoeal vaccination, for example, should ideally promote a local immune response (the elaboration of secretory IgA across intestinal mucosa) but parenteral inoculation generally promotes primarily a humoral response (i.e. circulating immunoglubulins) and peroral administration is not always effective. Secondly, anti-adherence activity by the antibodies generated can only occasionally be shown (e.g. **Rothbard et al.**, 1985), and most do not directly interfere with adhesion though they may still be effective in opsonisation and engulfment of pathogens under some circumstances.

Parenteral vaccination of pregnant sows with K88 preparations or pregnant cows with K99 material were among the first successful attempts (**Nagy**, 1980; **Rutter and Jones**, 1983). Antibodies in the colostrum and milk provide passive immunity to the newborn animals at the time when they are most susceptible to infection with adhesin-bearing pathogenic <u>E. coli</u>, and are least able to mount their own immune response. Anti-K88 vaccines have been widely used on farm animals, but are gradually becoming less effective (P. Lintermans, personal communication), leading back to the proposed evolutionary relationships amongst adhesins. As the use of a particular antigen for vaccination becomes widespread, so the prevalence of that antigen in the bacterial population tends to decrease. In its place come alternatives, other colonisation factors (including K88 variants and quite different antigens such as ATT25). A worse situation prevails in the use of <u>Neisseria gonorrhoeae</u> fimbriae to vaccinate humans: the 'pool' of fimbrial antigenic variants pre-existing in the bacterial population is sufficient to sustain the spread of gonococci even with active vaccines since most antibodies so produced are mono-specific. Attempts to raise cross-reactive responses to common regions of the fimbriae have been hindered by their poor immunogenicity.

In summary, fimbrial vaccines have not been too successful to date, and the 'traditional' whole cell, toxoid, LPS and capsular vaccines are still more important. Molecular biology, however, promises advances in the use of 'cocktails' - vaccines composed of components from several systems, even from different species, which are synthesised from genetically engineered microorganisms. One such example was the transfer of a CFA/I ST plasmid from an ETEC strain into a <u>Salmonella typhi</u> attenuated live oral vaccine strain; the resultant transgenic strain expressed the CFA/I fimbriae and enterotoxin in addition to <u>Salmonella</u> surface antigens, and may possibly provide protection against CFA/I$^+$ ETEC and <u>Salmonella</u> in one step (**Yamamoto et al.,** 1985). Other mixed antibacterial/antiadherence/antitoxic vaccines have been proposed to increase the chance of success (**Porter and Linggood,** 1983). As more and more virulence determinants (including adherence factors) are cloned and analysed, so the chance of obtaining effective combinations improves.

6.3.5 Means of testing the model. Some predictions of the model may be tested experimentally:

a) - search for complementation between parts of different adhesin systems. The model suggests that evolutionary relationships between adhesins may be strongest amongst the non-exposed parts of the systems, including export and assembly functions, so they may be capable of genetic complementation. Some experiments have already been performed to generate combinations of adhesin genes from various cloned systems, and have found conflicting results. Genes encoding type 1 fimbriae in enteric bacteria were mostly incapable of interspecific complementation (**Clegg et al.,** 1985a, 1985b), whereas different P fimbrial determinants did complement (**Lund et al.,** 1985; **Rhen et al.,** 1985; **van Die et al.,** 1986b). These comparisons might be extended, for example amongst the MePhe group.

b) - compare the DNA sequences of various adhesin systems. The model predicts greater divergence in subunit genes, especially in sequences corresponding to immunodominant regions of the proteins. Sequence comparisons are incomplete in the literature and need to be rationalised, perhaps via a computer database.

c) - search for associations between clonal groups and adhesins. Again, some experiments have been performed (e.g. **Vaisanen-Rhen et al.,** 1984; **Selander et al.,** 1986), but these need to be extended to other adhesins and other groups of bacteria. As more adhesin systems are cloned, so more adhesin-specific DNA probes become available for rapid colony hybridisation and similar screening methods.

d) - search for transposons and other mobile genetic elements encoding
    fimbriae. The methods used in this laboratory to examine the aerobactin
    gene cluster in pathogenic E. coli strains (Roberts et al., 1986b) seem
    eminently suitable to the examination of adhesin determinants.

6.4 Suggested future experiments. The similarity between the 469-3 MR
adhesin and the AFA-I adhesin from UPEC strain KS52 suggested by the
restriction and functional maps of the cloned systems immediately suggests
further experiments to compare other characteristics of the two adhesins
(including their ultrastructures and adherence specificities) and more
direct genetic assays (such as hybridisation and perhaps sequence
comparisons). Furthermore, the probe derived from pLG161 may be useful for
screening many other strains for homology, particularly EIEC and shigellae
which cause similar symptoms and presumably express colon-specific adhesins
which may be related to the 469-3 MR adhesin. In the light of the AFA-I
comparisons, UPEC strains should also be screened. Colony hybridisation
would be the method of choice.

The genetic analysis of the cloned system may be extended by further
subcloning and transposon insertions, in conjunction with identification of
the proteins encoded. Minicell analysis, for example, is ideally suited to
the requirement for expression of plasmid-encoded proteins in isolation from
chromosomally-encoded host cell proteins, and preliminary results have so
far demonstrated the presence of proteins of approximately 100 kd and 30 kd
in addition to the 14 kd subunit and vector-encoded proteins (data not
shown). Promoters might be analysed by means of minicell expression of
plasmids carrying transposons (polar mutations). Tn1723 inserts can be
removed by EcoR1-digestion which leaves just a few bases of the transposon
in place. Since the residual sequence contains an EcoRI recognition site,
fragments on either side of the site of insertion can be subcloned very
easily with the enzyme. Furthermore, the residual number of bases is such
that transcription is not prevented but translation is interrupted;
therefore, only the gene at the site of insertion is affected and the action
of the mutation on the phenotype can be simply studied without the confusion
of polar effects.

The control of adhesin expression is also amenable to study at the
molecular level. As mentioned before, the temperature controlled expression
appears to result from transcriptional control (manuscript in preparation),
while the clone exhibits phase variation which begs analysis by mutational
studies etc.

Chapter 7.   LITERATURE CITED

Abraham, S. N., J. P. Babu, C. S. Giampapa, D. L. Hasty, W. A. Simpson and
E. H. Beachey. 1985a. Protection against Escherichia coli-induced
urinary tract infections with hybridoma antibodies directed against
type 1 fimbriae or complementary D-mannose receptors. Infect. Immun.
48:625-628.

Abraham, J. M., C. S. Freitag, J. R. Clements and B. I. Eisenstein. 1985b.
An invertible element of DNA controls phase variation of type 1
fimbriae of E. coli. Proc. Natl. Acad. Sci. USA 82:5742-5747.

Achtman, M. 1985. Clonal groups and virulence factors among Escherichia
coli strains, p. 65-74. In T. K. Korhonen, E. A. Dawes and P. H.
Makela, Enterobacterial surface antigens: methods for molecular
characterisation. FEMS Symp. number 25. Elsevier Science Publishers BV
(Biomedical Division), Amsterdam.

Aguero, M. E., H. Harrison and F. C. Cabello. 1983. Increased frequency of
ColV plasmids and mannose-resistant hemagglutination activity in an
Escherichia coli K1 population. J. Clin. Microbiol. 18:1413-1416.

Ahren, C. M. and A. M. Svennerholm. 1985. Experimental enterotoxin-induced
Escherichia coli diarrhea and protection induced by previous infection
with bacteria of the same adhesin or enterotoxin type. Infect. Immun.
50:255-261.

Alouf, J. E., F. J. Fehrenbach, J. H. Freer and J. Jeljaszewicz (ed.).
1984. Bacterial protein toxins. FEMS Symp. number 24. Academic Press
Inc. (London) Ltd., London.

Anderson, B. J., M. B. Bills, J. R. Egerton and J. S. Mattick. 1984.
Cloning and expression in Escherichia coli of the gene encoding the
structural subunit of Bacteroides nodosus fimbriae. J. Bacteriol.
190:748-754.

Andrewes, F. W. 1922. Studies in group agglutination. I. The Salmonella
group and its antigenic structure. J. Pathol. Bacteriol. 25:505-521.

Appleyard, R. K. 1954. Segregation of new lysogenic types during growth of
a doubly lysogenic strain derived from Escherichia coli K-12. Genetics
39:440-452.

Aronson, M., O. Medalia, L. Schori, D. Mirelman, N. Sharon and I. Ofek.
1979. Prevention of colonization of the urinary tract of mice with
Escherichia coli by blocking of bacterial adherence with methyl
α-D-mannopyranoside. J. Infect. Dis. 139:329-332.

Ashworth, L. A. E., L. I. Irons and A. B. Dowsett. 1982. Antigenic
relationship between serotype-specific agglutinogen and fimbriae of
Bordetella pertussis. Infect. Immun. 37:1278-1281.

Båga, M, S. Normark, J. Hardy, P. O'Hanley, D. Lark, O. Olsson, G. Schoolnik and S. Falkow. 1984. Nucleotide sequence of the papA gene encoding the pap pilus subunit of human uropathogenic Escherichia coli. J. Bacteriol. 157:330-333.

Båga, M., M. Göransson, S. Normark and B. E. Uhlin. 1985. Transcriptional activation of a pap pilus virulence operon from uropathogenic E. coli. EMBO J. 4:3887-3893.

Bailey, N. T. J. 1981. Statistical methods in biology. 2nd edn. Hodder and Stoughton, London.

Baldini, M. M., J. P. Nataro and J. B. Kaper. 1986. Localization of a determinant for HEp-2 adherence by enteropathogenic Escherichia coli. Infect. Immun. 52:334-336.

Beachey, E. H. (ed.). 1980. Bacterial adherence. Receptors and recognition, series B, vol. 6. Chapman and Hall, London.

Beachey, E. H. 1981. Bacterial adherence : adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. 143:325-345.

Beck, E., G. Feil and K. Strohmaier. 1983. The molecular basis of the antigenic variation of foot-and-mouth disease virus. EMBO J. 2:555-559.

Berger, H., J. Hacker, A. Juarez, C. Hughes and W. Goebel. 1982. Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in Escherichia coli. J. Bacteriol. 152:1241-1247.

Bergstrom, S., K. Robbins, J. M. Koomey and J. Swanson. 1986. Piliation control mechanisms in Neisseria gonorrhoeae. Proc. Natl. Acac. Sci. USA 83:3890-3894.

Berkeley, R. C. W., J. M. Lynch, J. Melling, P. R. Rutter and B. Vincent (ed.). 1980. Microbial adhesion to surfaces. Ellis Harwood Ltd., Chichester, England.

Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. J. Bacteriol. 62:293-300.

Bertin, A. 1983. Virulence factors of enterotoxigenic E. coli studied in the infant mouse model. Ann. Rech. Vet. 14:169-182.

Bertin, A. 1985. F41 antigen as a virulence factor in the infant mouse model of Escherichia coli diarrhoea. J. Gen. Microbiol. 131:3037-3045.

Bhattacharjee, A. K., H. J. Jennings, C. P. Kenny, A. Martin and I. C. P. Smith. 1975. Structural determination of the sialic acid polysaccharide antigens of Neisseria meningitidis serogroups B and C with carbon 13 nuclear magnetic resonance. J. Biol. Chem. 250:1926-1932.

Bijlsma, S. W., A. de Nijs and J. F. Frik. 1981. Adherence of <u>Escherichia</u> <u>coli</u> to porcine intestinal brush borders by means of serological variants of the K88 antigen. Antonie van Leeuwenhoek J. Microbiol. Serol. 47:467-468.

Birkbeck, T. H. and C. W. Penn (ed.). 1986. Antigenic variation in infectious diseases. Special Publications of the Society for General Microbiology, vol. 19. IRL Press Ltd., Oxford.

Birnboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.

Bitton, G. and K. C. Marshall (ed.). 1980. Adsorption of microorganisms to surfaces. John Wiley and Sons, Inc., New York.

Bochner, B. R., H. -C. Huang, G. L. Schieven and B. N. Ames. 1980. Positive selection for loss of tetracycline sensitivity. J. Bacteriol. 143:926-933.

Boedeker, E. C. 1982. Enterocyte adherence of E. coli : its relation to diarrheal disease. Gastroenterology 83:489-492.

Bolivar, F. 1978. Molecular cloning vectors: derivatives of plasmid pBR322, p. 59-63. <u>In</u> H. W. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier/North-Holland Biomedical Press, Amsterdam, Netherlands.

Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa and S. Falkow. 1977. Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95-113.

Borst, P. and G. A. M. Cross. 1982. Molecular basis for trypanosome antigenic variation. Cell 29:291-303.

Bortolussi, R., P. Ferrieri and P. G. Quie. 1983. Influence of growth temperature on K1 capsular antigen production and resistance to opsonization. Infect. Immun. 39:1136-1141.

Boulos, S. and J. I. Rood. 1986. Molecular cloning of the fimbrial subunit gene from a benign type B isolate of <u>Bacteroides nodosus</u>. FEMS Microbiol. Lett. 33:73-78.

Boyer, H. W. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in <u>Escherichia coli</u>. J. Mol. Biol. 41:459-472.

Boylan, M. and C. J. Smyth. 1985. Mobilisation of CS-fimbriae-associated plasmids of enterotoxigenic <u>Escherichia coli</u> of serotype O6:K15:H16 or H- into various wild-type hosts. FEMS Microbiol. Lett. 29:83-89.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brinton, C. C. 1959. Non-flagellar appendages of bacteria. Nature (London) **183**:782-786.

Brinton, C. C. 1965. The structure, function, synthesis and genetic control of bacterial pili and molecular model for DNA and RNA transport in gram negative bacteria. Trans. N. Y. Acad. Sci. 27:1003-1054.

Brinton, C. C., **A. Buzzell and M. A. Lauffer.** 1954. Electrophoresis and phage susceptibility studies on a filament-producing variant of the E. coli B bacterium. J. Med. Microbiol. 13:57-68.

Brinton, C. C., **J. Bryan, J. Dillon, N. Guerina, L. J. Jacobson, A. Labik, S. Lee, A. Levine, S. Lims, J. McMichael, S. Polen, K. Rogers, A. C. C. To and S. C. M. To.** 1978. Uses of pili in gonorrhea control : role of bacterial pili in disease, purification and properties of gonococcal pili, and progress in the development of a gonococcal pilus vaccine, p. 155-178. In G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer and F. E. Young (ed.), Immunobiology of Neisseria gonorrhoeae. American Society for Microbiology, Washington, DC.

Brubaker, **R. R.** 1979. Expression of virulence in yersiniae, p. 168-171. In Microbiology - 1979. American Society for Microbiology, Washington, DC.

Buchanan, **K., S. Falkow, R. A. Hull and S. I. Hull.** 1985. Frequency among Enterobacteriaceae of the DNA sequences encoding type 1 pili. J. Bacteriol. 162:799-803.

Cannon, **J. G. and P. F. Sparling.** 1984. The genetics of the gonococcus. Ann. Rev. Micobiol. 38:111-133.

Cantey, **J. R. and R. K. Blake.** 1977. Diarrhea due to E. coli in the rabbit : a novel mechanism. J. Infect. Dis. 135:454-462.

Cantey, **J. R., W. B. Lushbaugh and L. R. Inman.** 1981. Attachment of bacteria to intestinal epithelial cells in diarrhea caused by Escherichia coli strain RDEC-1 in the rabbit : stages and role of capsule. J. Infect. Dis. 143:219-230.

Carbonetti, **N. H., S. Boonchai, S. H. Parry, V. Väisänen-Rhen, T. K. Korhonen and P. H. Williams.** 1986. Aerobactin-mediated iron uptake by Escherichia coli isolates from human intestinal infections. Infect. Immun. 51:966-968.

Cavalieri, **S., G. Bohuch and J. Snyder.** 1984. Escherichia coli alpha hemolysin : characteristics and probable role in pathogenicity. Microbiol. Rev. 48:326-343.

Chan, **R., S. D. Acres and J. W. Costerton.** 1982. Use of specific antibody to demonstrate glycocalyx, K99 pili, and the spatial relationships of K99+ enterotoxigenic Escherichia coli in the ileum of colostrum-fed calves. Infect. Immun. 37:1170-1180.

Chang, **A. C. Y.** and S. **N.** Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141-1166.

Chanter, **N.** 1983. Partial purification and characterisation of two non-K99 mannose-resistant haemagglutinins of <u>Escherichia coli</u> B41. J. Gen. Microbiol. 129:235-243.

Cheney, C. P. and E. C. Boedeker. 1983. Adherence of an enterotoxigenic <u>Escherichia coli</u> strain, serotype O78:H11, to purified human intestinal brush borders. Infect. Immun. 39:1280-1284.

Cheney, C. P., E. C. Boedeker and S. B. Formal. 1979. Quantitation of the adherence of an enteropathogenic <u>Escherichia coli</u> strain to isolated rabbit intestinal brush borders. Infect. Immun. 26:736-743.

Cheney, C. P., S. B. Formal, P. A. Schad and E. C. Boedeker. 1983. Genetic transfer of a mucosal adherence factor (R1) from an enteropathogenic <u>Escherichia coli</u> strain into a <u>Shigella flexneri</u> strain and the phenotypic suppression of this adherence factor. J. Infect. Dis. 147:711-723:

Chow, L. T., R. Kahmann and D. Kamp. 1977. Electron microscopic characterisation of DNAs of non-defective deletion mutants of bacteriophage Mu. J. Mol. Biol. 113:591-609.

Christensen, G. D., W. A. Simpson and E. H. Beachey. 1985. Microbial adherence in infection, p. 6-23. <u>In</u> G. L. Mandell, R. G. Douglas and J. E. Bennett (ed.), Principles and practice of infectious diseases, 2nd edn. John Wiley and Sons, Inc., New York.

Churchward, G. G. and I. B. Holland. 1976. Envelope synthesis during the cell cycle in <u>Escherichia coli</u> B/r. J. Mol. Biol. 105:245-261.

Clarke, L. and J. Carbon. 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire <u>Escherichia coli</u> genome. Cell 9:91-99.

Clegg, S. 1982. Cloning of genes determining the production of mannose-resistant fimbriae in a uropathogenic strain of <u>Escherichia coli</u> belonging to serogroup O6. Infect. Immun. 38:739-744.

Clegg, S. and J. K. Pierce. 1983. Organization of genes responsible for the production of mannose-resistant fimbriae of a uropathogenic <u>Escherichia coli</u> isolate. Infect. Immun. 42:900-906.

Clegg, H., N. Guerina, S. Langermann, T. Woodrow Kessler, V. Guerina and D. Goldmann. 1984. Pilus mediated adherence of <u>Escherichia coli</u> K1 to human oral epithelial cells. Infect. Immun. 45:299-301.

Clegg, S., S. Hull, R. Hull and J. Pruckler. 1985a. Construction and comparison of recombinant plasmids encoding type 1 fimbriae of members of the family <u>Enterobacteriaceae</u>. Infect. Immun. 48:275-279.

Clegg, S., J. Pruckler and B. K. Purcell. 1985b. Complementation analyses of recombinant plasmids encoding type 1 fimbriae of members of the family Enterobacteriaceae. Infect. Immun. 50:338-340.

Clements, J. D. and R. A. Finkelstein. 1978. Immunological cross-reactivity between a heat-labile enterotoxin(s) of Escherichia coli and subunits of Vibrio cholerae enterotoxin. Infect. Immun. 21:1036-1039.

Cohen, S. N., A. C. Y. Chang and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. Proc. Natl. Acad. Sci. USA 69:2110-2114.

Coleclough, C. 1983. Chance, necessity and antibody gene dynamics. Nature (London) 303:23-26.

Connor, E. M. and M. R. Loeb. 1983. A hemadsorption method for the detection of colonies of Haemophilus influenzae type b expressing fimbriae. J. Infect. Dis. 148:855-860.

Costerton, J. W., G. G. Geesey and K. -J. Cheng. 1978. How bacteria stick. Sci. Amer. 238:86-95.

Costerton, J. W., R. T. Irvin and K. -J. Cheng. 1981a. The role of bacterial surface structures in pathogenesis. Crit. Rev. Microbiol. 8:303-338.

Costerton, J. W., R. T. Irvin and K. -J. Cheng. 1981b. The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol. 35:299-324.

Cravioto, A., S. M. Scotland and B. Rowe. 1982. Hemagglutination activity and CFA/I and II in enterotoxigenic and non-enterotoxigenic strains of Escherichia coli isolated from humans. Infect. Immun. 36:189-197.

Crichton, P. B., S. M. Ip and D. C. Old. 1981. Hemagglutination typing as an aid in identification of biochemically atypical Escherichia coli strains. J. Clin. Microbiol. 14:599-603.

Crosa, J. H. 1984. The relationship of plasmid-mediated iron transport and bacterial virulence. Ann. Rev. Microbiol. 38:69-89.

Czirók, É., J. Madar, J. Budai, Z. Stverteczky, A. Kertesz, I. Dombi and L. Gyengesi. 1976. The role in sporadic enteritis of facultatively enteropathogenic Escherichia coli serogroups. Acta Microbiol. Acad. Sci. Hung. 23:359-369.

Czirók, É., I. Ørskov and F. Ørskov. 1982. O:K:H:F serotypes of fimbriated Escherichia coli strains isolated from infants with diarrhea. Infect. Immun. 37:519-525.

Dagert, M. and S. D. Ehrlich. 1979. Prolonged incubation in calcium chloride improves competence of Escherichia coli cells. Gene 6:23-28.

Dale, J. B. and E. H. Beachey. 1982. Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart. J. Exp. Med. 156:1165-1176.

Dallas, W. S. and S. Falkow. 1979. The molecular nature of heat-labile enterotoxin (LT) of Escherichiacoli. Nature (London) 277:406-407.

Dallas, W. S. and S. Falkow. 1980. Amino acid sequence homology between cholera toxin and Escherichia coli heat-labile toxin. Nature (London) 288:499-501.

Dallas, W. S., D. M. Gill and S. Falkow. 1979. Cistrons encoding Escherichia coli heat-labile toxin. J. Bacteriol. 139:850-858.

Darfeuille, A., B. Lafeuille, B. Joly and R. Cluzel. 1983. A new colonisation factor (CFA/III) produced by enteropathogenic Escherichia coli O128:B12. Ann. Inst. Pasteur (Microbiol.) 134A:53-64.

Darfeuille-Michaud, A., C. Forestier, B. Joly and R. Cluzel. 1986. Identification of a nonfimbrial adhesive factor of an enterotoxigenic Escherichia coli strain. Infect. Immun. 52:468-475.

Day, N. P., S. M. Scotland and B. Rowe. 1981. Comparison of an HEp-2 tissue culture test with the Sereny test for the detection of enteroinvasiveness in Shigella species and Escherichia coli. J. Clin. Microbiol. 13:596-597.

Dean, E. A. and R. E. Isaacson. 1985a. Purification and characterization of a receptor for the 987P pilus of Escherichia coli. Infect. Immun. 47:98-105.

Dean, E. A. and R. E. Isaacson. 1985b. Location and distribution of a receptor for the 987P pilus of Escherichia coli in small intestines. Infect. Immun. 47:345-348.

Dean, A. G., Y. Ching, R. G. Williams and L. B. Harden. 1972. Test for Escherichia coli enterotoxin using infant mice : application in a study of diarrhoea in children in Honolulu. J. Infect. Dis. 125:407-411.

de Graaf, F. K. and P. Klaasen. 1986. Organization and expression of genes involved in the biosynthesis of 987P fimbriae. Mol. Gen. Genet. 204:75-81.

de Graaf, F. K. and I. Roorda. 1982. Production, purification, and characterization of the fimbrial adhesive antigen B41 isolated from calf enteropathogenic Escherichia coli strain B41M. Infect. Immun. 36:751-758.

de Graaf, F. K., P. Klaasen-Boor and J. E. van Hees. 1980a. Biosynthesis of the K99 surface antigen is repressed by alanine. Infect. Immun. 30:125-128.

de Graaf, F. K., P. Klemm and W. Gaastra. 1980b. Purification, characterization, and partial covalent structure of <u>Escherichia coli</u> adhesive antigen K99. Infect. Immun. 33:877-883.

de Graaf, F. K., F. B. Wientjes and P. Klaasen-Boor. 1980c. Production of K99 antigen by enterotoxigenic <u>Escherichia coli</u> strains of antigenic groups O8, O9, O20 and O101 grown at different conditions. Infect. Immun. 27:216-221.

de Graaf, F. K., B. E. Krenn and P. Klaasen. 1984. Organization and expression of genes involved in the biosynthesis of K99 fimbriae. Infect. Immun. 43:508-514.

de la Cabada, F. J., D. G. Evans and D. J. Evans Jr. 1981. Immunoprotection against enterotoxigenic <u>Escherichia coli</u> diarrhea in rabbits by peroral administration of purified colonization factor antigen I (CFA/I). FEMS Microbiol. Lett. 11:303-307.

Deneke, C. F., G. M. Thorne and S. L. Gorbach. 1979. Attachment pili from enterotoxigenic <u>Escherichia coli</u> pathogenic for humans. Infect. Immun. 6:362-368.

Deneke, C. F., G. M. Thorne, A. D. Larsen and S. L. Gorbach. 1985. Effect of tetracycline on the attachment of K88+ enterotoxigenic <u>Escherichia coli</u> to porcine small intestinal cells. J. Infect. Dis. 152:1032-1036.

de Ree, J. M., P. Schwillens, L. Promes, I. van Die and H. Bergmans. 1985a. Molecular cloning and characterisation of F9 fimbriae from a uropathogenic <u>Escherichia coli</u>. FEMS Microbiol. Lett. 26:163-169.

de Ree, J. M., P. Schwillens and J. F. van den Bosch. 1985b. Molecular cloning of F11 fimbriae from a uropathogenic <u>Escherichia coli</u> and characterisation of fimbriae with polyclonal and monoclonal antibodies. FEMS Microbiol. Lett. 29:91-97.

Dhingra, R. K., R. C. Williams Jr. and W. P. Reed. 1977. Effects of pneumococcal mucopeptide and capsular polysaccharide on phagocytosis. Infect. Immun. 15:169-174.

Dodd, D. C. and B. I. Eisenstein. 1984. Kinetic analysis of the synthesis and assembly of type 1 fimbriae of <u>Escherichia coli</u>. J. Bacteriol. 160:227-232.

Dodd, D. C., P. J. Bassford Jr. and B. I. Eisenstein. 1984. Dependence of secretion and assembly of type 1 fimbrial subunits of <u>Escherichia coli</u> on normal protein export. J. Bacteriol. 159:1077-1079.

Donkersloot, J. A., J. O. Cisar, M. E. Wax, R. J. Harr and B. M. Chassy. 1985. Expression of <u>Actinomyces viscosus</u> antigens in <u>Escherichia coli</u>: cloning of a structural gene (<u>fimA</u>) for type 2 fimbriae. J. Bacteriol. 162:1075-1078.

Donta, S. T., H. W. Moon and S. C. Whipp. 1974. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. Science 183:334-336.

Dougan, G. and D. Scherratt. 1977. The transposon Tn1 as a probe for studying ColE1 structure and function. Mol. Gen. Genet. 151:151-160.

Dougan, G., G. Dowd and M. Kehoe. 1983. Organization of K88ac-encoded polypeptides in the *Escherichia coli* cell envelope : use of minicells and outer membrane protein mutants for studying assembly of pili. J. Bacteriol. 153:364-370.

Dretzen, G., M. Bellard, P. Sassone-Corsi and P. Chambon. 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. 112:295-298.

Duckworth, M., D. Jackson, K. Zak and J. E. Heckels. 1983. Structural variations in pili expressed during gonococcal infection. J. Gen. Microbiol. 129:1593-1596.

Duggleby, R. G., H. Kinns and J. I. Rood. 1981. A computer program for determining the size of DNA restriction fragments. Anal. Biochem. 110:49-55.

Duguid, J. P. and R. R. Gillies. 1957. Fimbriae and adhesive properties in dysentery bacilli. J. Pathol. Bacteriol. 74:397-411.

Duguid, J. P. and D. C. Old. 1980. Adhesive properties of Enterobacteriaceae, p. 185-217. In E. H. Beachey (ed.), Bacterial adherence. Receptors and recognition, series B, vol. 6. Chapman and Hall, London.

Duguid, J. P., I. W. Smith, G. Dempster and P. N. Edmunds. 1955. Non-flagellar filamentous appendages ('fimbriae') and haemagglutinating activity in *Bacterium coli*. J. Pathol. Bacteriol. 70:335-348.

Duguid, J. P., S. Clegg and M. I. Wilson. 1979. The fimbrial and nonfimbrial haemagglutinins of *Escherichia coli*. J. Med. Microbiol. 12:213-228.

DuPont, H. L., S. B. Formal, R. B. Hornick, M. J. Snyder, J. P. Libonati, D. G. Sheahan, E. H. LaBrec and J. P. Kalas. 1971. Pathogenesis of *Escherichia coli* diarrhea. New Engl. J. Med. 285:1-9.

Dykes, C. W., I. J. Halliday, M. J. Read, A. N. Hobden and S. Harford. 1985. Nucleotide sequences of four variants of the K88 gene of porcine enterotoxigenic *Escherichia coli*. Infect. Immun. 50:279-283.

Echarti, C., B. Hirschel, G. J. Boulnois, J. M. Varley, F. Waldvogel and K. N. Timmis. 1983. Cloning and analysis of the K1 capsule biosynthesis genes of *Escherichia coli*: lack of homology with *Neisseria meningitidis* group B DNA sequences. Infect. Immun. 41:54-60.

Eidels, L., R. L. Proia and D. A. Hart. 1983. Membrane receptors for bacterial toxins. Microbiol. Rev. 47:596-620.

Eisenstein, B. I. 1981. Phase variation of type 1 fimbriae in *Escherichia coli* is under transcriptional control. Science 214:337-339.

Eisenstein, B. I. and D. C. Dodd. 1982. Pseudocatabolite repression of type 1 fimbriae of *Escherichia coli*. J. Bacteriol. 151:1560-1567.

Eisenstein, B. I., I. Ofek and E. H. Beachey. 1981. Loss of lectin-like activity in aberrant type 1 fimbriae of *Escherichia coli*. Infect. Immun. 31:792-797.

Elleman, T. C. and P. A. Hoyne. 1984. Nucleotide sequence of the gene encoding pilin of *Bacteroides nodosus*, the causal organism of ovine footrot. J. Bacteriol. 160:1184-1187.

Elleman, T. C., P. A. Hoyne, D. L. Emery, D. J. Stewart and B. L. Clark. 1984. Isolation of the gene encoding pilin of *Bacteroides nodosus* (strain 198), the causal organism of ovine footrot. FEBS Lett. 173:103-107.

Elleman, T. C., P. A. Hoyne, D. L. Emery, D. J. Stewart and B. L. Clark. 1986a. Expression of the pilin gene from *Bacteroides nodosus* in *Escherichia coli*. Infect. Immun. 51:187-192.

Elleman, T. C., P. A. Hoyne, N. M. McKern and D. J. Stewart. 1986b. Nucleotide sequence of the gene encoding the two-subunit pilin of *Bacteroides nodosus* 265. J. Bacteriol. 167:243-250.

Elliott, K., M. O'Connor and J. Whelan (ed.). 1981. Adhesion and microorganisms pathogenicity. Ciba Foundation Symp., vol. 80. Pitman Medical Ltd., London.

Ellwood, D. C., J. Melling and P. Rutter (ed.). 1979. Adhesion of microorganisms to surfaces. Academic Press Inc. (London) Ltd., London

Elwell, L. P. and P. L. Shipley. 1980. Plasmid-mediated factors associated with virulence of bacteria to animals. Ann. Rev. Microbiol. 34:465-496.

Eshdat, Y., V. Speth and K. Jann. 1981. Participation of pili and cell wall adhesin in the yeast agglutination activity of *Escherichia coli*. Infect. Immun. 34:980-986.

Evans, D. G. and D. J. Evans Jr. 1978. New surface associated heat labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect. Immun. 21:638-647.

Evans Jr., D. J., L. C. Chen, G. T. Curlin and D. G. Evans. 1972. Stimulation of adenyl cyclase by *Escherichia coli* enterotoxin. Nature New Biol. 236:137-138.

Evans, D. G., R. P. Silver, D. J. Evans Jr., D. G. Chase and S. L. Gorbach. 1975. Plasmid controlled colonization factor associated with virulence in Escherichia coli enterotoxigenic for humans. Infect. Immun. 12:656-667.

Evans, D. G., D. J. Evans Jr. and W. Tjoa. 1977. Hemagglutination of group A erythrocytes by enterotoxigenic Escherichia coli isolated from adults with diarrhea : correlation with colonization factor. Infect. Immun. 18:330-337.

Evans, D. G., D. J. Evans Jr., W. S. Tjoa and H. L. DuPont. 1978a. Detection and characterization of colonization factor of enterotoxigenic Escherichia coli isolated from adults with diarrhea. Infect. Immun. 19:727-736.

Evans, D. G., T. K. Satterwhite, D. J. Evans Jr. and H. L. DuPont. 1978b. Differences in serological and excretion patterns of volunteers challenged with enterotoxigenic Escherichia coli with and without the colonization factor antigen. Infect. Immun. 19:883-888.

Evans Jr., D. J., D. G. Evans and H. L. DuPont. 1979. Hemagglutination patterns of enterotoxigenic and enteropathogenic Escherichia coli determined with human, bovine, chicken and guinea pig erythrocytes in the presence and absence of mannose. Infect. Immun. 23:336-346.

Evans Jr., D. J., D. G. Evans, L. S. Young and J. Pitt. 1980. Hemagglutination typing of Escherichia coli : definition of seven hemagglutination types. J. Clin. Microbiol. 12:235-242.

Every, D. 1979. Purification of pili from Bacteroides nodosus and an examination of their chemical, physical and serological properties. J. Gen. Microbiol. 115:309-316.

Fader, R. C., L. K. Duffy, C. P. Davis and A. Kurosky. 1982. Purification and chemical characterization of type 1 pili isolated from Klebsiella pneumoniae. J. Biol. Chem. 257:3301-3305.

Feinberg, A. P. and B. Vogelstein. 1984. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Addendum. Anal. Biochem. 137:266-267.

Fischetti, V. A. and B. N. Manjula. 1982. Biologic and immunologic implications of the structural relationship between streptococcal M protein and mammalian tropomyosin, p. 411-418. In J. B. Robbins, J. C. Hill and J. C. Sadoff (ed.), Seminars in infectious disease, vol. IV: bacterial vaccines. Thieme-Stratton Inc., New York.

Fishman, P. H. 1980. Mechanism of cholera toxin : events on the cell surface. In M. Field, J. S. Fordtran and S. G. Schultz (ed.), Secretory diarrhea. American Physiological Society, Bethesda, Maryland.

Forestier, C., A. Darfeuille-Michaud and B. Joly. 1984. Effects of antibiotics on adhesion of enterotoxigenic Escherichia coli strains. Eur. J. Clin. Microbiol. 3:427-432.

Forestier, C., K. G. Welinder, A. Darfeuille-Michaud and P. Klemm. 1987. Afimbrial adhesin from Escherichia coli strain 2230 : purification, characterization and partial covalent structure. FEMS Microbiol. Lett. 40:47-50.

Formal, S. B. and M. M. Levine. 1984. Shigellosis, p. 167-186. In R. Germanier (ed.), Bacterial vaccines. Academic Press Inc. (London) Ltd., London.

Formal, S. B., P. Gemski Jr., L. S. Baron and E. H. LaBrec. 1971. A chromosomal locus which controls the ability of Shigella flexneri to evoke keratoconjunctivitis. Infect. Immun. 3:73-79.

Francis, D. H., J. E. Collins and J. R. Duimistra. 1986. Infection of gnotobiotic pigs with an Escherichia coli O157:H7 strain associated with an outbreak of hemorrhagic colitis. Infect. Immun. 51:953-956.

Frantz, J. C. and D. C. Robertson. 1981. Immunological properties of Escherichia coli heat-stable enterotoxins : development of a radioimmunoassay specific for heat-stable enterotoxins with suckling mouse activity. Infect. Immun. 33:193.

Freer, J. H., A. Ellis, T. Wadstrom and C. J. Smyth. 1978. Occurrence of fimbriae among enterotoxigenic intestinal bacteria isolated from cases of human infantile diarrhoea. FEMS Microbiol. Lett. 3:277-281.

Freitag, C. S. and B. I. Eisenstein. 1983. Genetic mapping and transcriptional orientation of the fimD gene. J. Bacteriol. 156:1052-1058.

Freitag, C. S., J. M. Abraham, J. R. Clements and B. I. Eisenstein. 1985. Genetic analysis of the phase variation control of expression of type 1 fimbriae in Escherichia coli. J. Bacteriol. 162:668-675.

Freter, R., P. C. M. O'Brien and M. S. Macsai. 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa : in vivo studies. Infect. Immun. 34:234-240.

Freter, R., H. Brickner, J. Fekete, M. M. Vickerman and K. E. Carey. 1983. Survival and implantation of Escherichia coli in the intestinal tract. Infect. Immun. 39:686-703.

Gaastra, W. and F. K. de Graaf. 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic Escherichia coli strains. Microbiol. Rev. 46:129-161.

Gaastra, W., F. R. Mooi, A. R. Stuitje and F. K. de Graaf. 1981. The nucleotide sequence of the gene encoding the K88ab protein subunit of porcine enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. 12:41-46.

Gaastra, W, P. Klemm and F. K. de Graaf. 1983. The nucleotide sequence of the K88ab protein subunit of porcine enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. 18:177-183.

Gadeberg, O. V., I. Ørskov and J. M. Rhodes. 1983. Cytotoxic effect of an alpha-hemolysin Escherichia coli strain on blood monocytes and granulocytes in vitro. Infect. Immun. 41:358-364.

Gemski Jr., P., A. Takeuchi, O. Washington and S. B. Formal. 1972. Shigellosis due to Shigella dysenteriae 1 : relative importance of mucosal invasion versus toxin production in pathogenesis. J. Infect. Dis. 126:523-530.

Germanier, R. (ed.). 1984. Bacterial vaccines. Academic Press Inc. (London) Ltd., London.

Gianella, R. A. 1981. Pathogenesis of acute bacterial diarrheal disorders. Ann. Rev. Med. 32:341-357.

Gianella, R. H., O. Washington, P. Gemski and S. B. Formal. 1973. Invasion of HeLa cells by Salmonella typhimurium : a model study of invasiveness of salmonella. J. Infect. Dis. 128:69-75.

Gill, D. M. 1982. Bacterial toxins : a table of lethal amounts. Microbiol. Rev. 46:86-94.

Glass, R. E. 1982. Gene function. E. coli and its heritable elements. Croom Helm Ltd., London.

Göransson, M. and B. E. Uhlin. 1984. Environmental temperature regulates transcription of a virulence pili operon in Escherichia coli. EMBO J. 3:2885-2888.

Goren, M. B. 1977. Phagocyte lysosomes : interactions with infectious agents, phagosomes, and experimental perturbations in function. Ann. Rev. Microbiol. 31:507-533.

Greenberg, R. N. and R. L. Guerrant. 1981. E. coli heat-stable enterotoxin. Pharmacol. Ther. 13:507-530.

Gross, R. J. 1983. E. coli diarrhoea. J. Infect. 7:177-192.

Gross, R. J. and B. Rowe. 1985a. Escherichia coli diarrhoea. J. Hyg. Camb. 95:531-550.

Gross, R. J. and B. Rowe. 1985b. Serotyping of Escherichia coli, p. 345-363. In M. Sussman (ed.), The virulence of Escherichia coli. Reviews and methods. Special Publications of the Society for General Microbiology, vol. 13. Academic Press Inc. (London) Ltd., London.

Guerina, N. G., T. W. Kessler, V. J. Guerina, M. R. Neutra, H. W. Clegg, S. Langerman, F. A. Scannapieco and D. A. Goldman. 1983. The role of pili and capsule in the pathogenesis of neonatal infection with Escherichia coli K1. J. Infect. Dis. 148:395-405.

Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun and A. G. Gilman. 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology : a rapid sensitive in vitro assay for the enterotoxins of Vibrio cholerae and Escherichia coli. Infect. Immun. 10:320-327.

Guinee, P. A. and W. H. Jansen. 1979. Behavior of Escherichia coli K antigens K88ab, K88ac, and K88ad in immunelectrophoresis, double diffusion and hemagglutination. Infect. Immun. 23:700-705.

Guinee, P. A. M., W. H. Jansen and C. M. Agterberg. 1976. Detection of the K99 antigen by means of agglutination and immunelectrophoresis in Escherichia coli isolated from calves and its correlation with enterotoxigenicity. Infect. Immun. 13:1369-1377.

Guyer, M. S. 1978. The $\gamma\delta$ sequence of F is an insertion sequence. J. Mol. Biol. 126:347-365.

Guyot, G. 1908. Ueber die bakterielle Haemagglutination (Bakterio-Haemo-agglutination). Zentralbl. Bacteriol. Parasitenk. Infectionskr. Hyg. Abt. I: Orig. 47:640-653. [Cited by Christensen et al. (1985).]

Hacker, J. and C. Hughes. 1985. Genetics of Escherichia coli hemolysin. Curr. Top. Microbiol. Immun. 118:139-162.

Hacker, J., S. Knapp and W. Goebel. 1983. Spontaneous deletions and flanking regions of the chromosomally inherited hemolysin determinant of an Escherichia coli O6 strain. J. Bacteriol. 154:1145-1152.

Hacker, J., G. Schmidt, C. Hughes, S. Knapp, M. Marget and W. Goebel. 1985. Cloning and characterization of genes involved in production of mannose-resistant, neuraminidase-susceptible (X) fimbriae from a uropathogenic O6:K15:H31 Escherichia coli strain. Infect. Immun. 47:434-440.

Hacker, J., T. Jarchau, S. Knapp, R. Marre, G. Schmidt, T. Schmoll and W. Goebel. 1986. Genetic and in vivo studies with S-fimbrial antigens and related virulence determinants of extra-intestinal Escherichia coli strains, p. 125-133. In D. L. Lark (ed.), Protein-carbohydrate interactions in biological systems. FEMS symposium number 31. Academic Press Inc. (London) Ltd., London.

Hagberg, L., U. Jodal, T. K. Korhonen, G. Lidin-Janson, U. Lindberg and C. Svanborg Edén. 1981. Adhesion, hemagglutination and virulence of Escherichia coli causing urinary tract infections. Infect. Immun. 31:564-570.

Hagblom, P., E. Segal, E. Billyard and M. So. 1985. Intragenic recombination leads to pilus antigen variation in Neisseria gonorrhoeae. Nature (London) 315:156-158.

Hale, T. L., P. J. Sansonetti, P. A. Schad, S. Austin and S. B. Formal. 1983. Characterization of virulence plasmids and plasmid associated outer membrane proteins of Shigella flexneri, Shigella sonnei and Escherichia coli. Infect. Immun. 40:340-350.

Hanahan, D. 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166:557-580.

Harris, J. R., I. K. Wachsmuth, B. R. Davis and M. L. Cohen. 1982. High molecular weight plasmid correlates with Escherichia coli invasiveness. Infect. Immun. 37:1295-1298.

Heesemann, J., B. Algermissen and R. Laufs. 1984. Genetically manipulated virulence of Yersinia enterocolitica. Infect. Immun. 46:105-110.

Henning, V., H-D Royer, R. M. Teather, I. Hindennach and C. Hollenberg. 1979. Cloning of the structural gene (ompA) for an integral outer membrane protein of Escherichia coli K-12. Proc. Natl. Acad. Sci. USA 76:4360-4364.

Herskowitz, I. 1983. Cellular differentiation, cell lineages, and transposable genetic cassettes in yeasts. Curr. Top. Dev. Biol. 18:1-14.

Hewlett, E. L. 1984. Biological effects of pertussis toxin and Bordetella adenylate cyclase on intact cells and experimental animals. In L. Lieve and D. Schlessinger (ed.), Microbiology, American Society for Microbiology, Washington, DC.

Hewlett, E. L. 1985a. Toxins, p. 2-6. In G. L. Mandell, R. G. Douglas and J. E. Bennett (ed.), Principles and practice of infectious diseases, 2nd edn. John Wiley and Sons, Inc., New York.

Hewlett, E. L. 1985b. Other factors, p. 23-25. In G. L. Mandell, R. G. Douglas and J. E. Bennett (ed.), Principles and practice of infectious diseases, 2nd edn. John Wiley and Sons, Inc., New York.

Hinson, G. and P. H. Williams. 1986. Characterization and cloning of non-fimbrial protein adhesins of two Escherichia coli strains of human origin, p. 61-62. In D. L. Lark (ed.), Protein-carbohydrate interactions in biological systems. Academic Press Inc. (London) Ltd., London.

Hinson, G., S. Knutton, M. K. -L. Lam-Po-Tang, A. S. McNeish and P. H. Williams. 1987. Adherence to human colonocytes of an Escherichia coli strain isolated from severe infantile enteritis: molecular and ultrastructural studies of a fibrillar adhesin. Infect. Immun. 55:393-402.

Hirst, T. R., L. L. Randall and S. J. S. Hardy. 1984. Cellular location of heat-labile enterotoxin in Escherichia coli. J. Bacteriol. 157:637-642.

Hoekstra, W. P. M., I. M. van Die and J. E. N. Bergmans. 1984. Genetic analysis of complex gene clusters in Escherichia coli : the genetic analysis of F7$_2$ genes. Antonie van Leeuwenhoek J. Microbiol. 50:585-596.

Hohmann, A. and M. R. Wilson. 1975. Adherence of enteropathogenic Escherichia coli to intestinal epithelium in vivo. Infect. Immun. 12:866-880.

Hohn, B. and K. Murray. 1977. Packaging recombinant DNA molecules into bacteriophage particles in vitro. Proc. Natl. Acad. Sci. USA 74:3259-3263.

Holmgren, J. 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) 292:413-417.

Holmgren, J. 1985. Toxins affecting intestinal transport processes, p. 177-191. In M. Sussman (ed.), The virulence of Escherichia coli. Reviews and methods. Special Publications of the Society for General Microbiology, vol. 13. Academic Press Inc. (London) Ltd., London.

Honda, T., M. Arita and T. Miwatani. 1984. Characterization of new hydrophobic pili of human enterotoxigenic Escherichia coli : a possible new colonization factor. Infect. Immun. 43:959-965.

Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew and S. Falkow. 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose resistant pili from a urinary tract infection Escherichia coli isolate. Infect. Immun. 33:933-938.

Hull, R. A., S. I. Hull and S. Falkow. 1984. Frequency of gene sequences necessary for pyelonephritis associated pili expression among isolates of Enterobacteriaceae from human extraintestinal infections. Infect. Immun. 43:1064-1067.

Hull, S., S. Clegg, C. Svanborg-Edén and R. Hull. 1985. Multiple forms of genes in pyelonephritogenic Escherichia coli encoding adhesins binding globoseries glycolipid receptors. Infect. Immun. 47:80-83.

Hull, R., S. Bieler, S. Falkow and S. Hull. 1986. Chromosomal map position of genes encoding P adhesins in uropathogenic Escherichia coli. Infect. Immun. 51:693-695.

Hultgren, S. J., T. N. Porter, A. J. Schaeffer and J. L. Duncan. 1985. Role of type 1 pili and effects of phase variation on lower urinary tract infections produced by Escherichia coli. Infect. Immun. 50:370-377.

Iglewski, B. H. and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. USA 72:2284-2288.

Iglewski, B. H., P. V. Liu and D. Kabat. 1977. Mechanism of action of Pseudomonas aeruginosa exotoxin A : adenosine monophosphate - ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.

Inman, L. R. and J. R. Cantey. 1984. Peyer's patch lymphoid follicle epithelial adherence of a rabbit EPEC strain (RDEC-1). Role of plasmid mediated pili in initial adherence. J. Clin. Invest. 74:90-95.

Ip, S. M., P. B. Crichton, D. C. Old and J. P. Duguid. 1981. Mannose-resistant haemagglutinins and fimbriae in Escherichia coli. J. Med. Microbiol. 14:223-226.

Isaacson, R. E. 1977. K99 surface antigen of Escherichia coli : purification and partial characterization. Infect. Immun. 15:272-279.

Isaacson, R. E. 1980. Factors affecting expression of the Escherichia coli pilus K99. Infect. Immun. 28:190-194.

Isaacson, R. E. 1983. Regulation and expression of the Escherichia coli pilus K99. Infect. Immun. 40:633-639.

Isaacson, R. E. 1985. Pilus adhesins, p. 307-336. In D. C. Savage and M. Fletcher (ed.), Bacterial adhesion, mechanisms and physiological significance. Plenum Press, New York.

Isaacson, R. E. and P. Richter. 1981. Escherichia coli 987P pilus : purification and partial characterization. J. Bacteriol. 146:784-789.

Isaacson, R. E., B. Nagy and H. W. Moon. 1977. Colonization of porcine small intestine by Escherichia coli : colonization and adhesion factors of pig enteropathogens that lack K88. J. Infect. Dis. 135:531-539.

Isaacson, R. E., J. Colmenero and P. Richter. 1981. Escherichia coli K99 pili are composed of one subunit species. FEMS Microbiol. Lett. 12:229-232.

Ish-Horowicz, D. and J. F. Burke. 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. 9:2989-2998.

Iwahi, T., Y. Abe, M. Nakao, A. Imada and K. Tsuchiya. 1983. Role of type 1 fimbriae in the pathogenesis of ascending urinary tract infection induced by Escherichia coli in mice. Infect. Immun. 39:1307-1315.

Jann, K. and B. Jann. 1983. The K-antigens of Escherichia coli. Prog. Allergy 33:53-79.

Jann, K. and B. Jann. 1985. Cell surface components and virulence : Escherichia coli O and K antigens in relation to virulence and pathogenicity, p. 157-176. In M. Sussman (ed.), The virulence of Escherichia coli. Reviews and methods. Special Publications of the Society for General Microbiology, vol. 13. Academic Press Inc. (London) Ltd., London.

Jann, K., B. Jann and G. Schmidt. 1981. SDS polyacrylamide gel electrophoresis and serological analysis of pili from Escherichia coli of different pathogenic origin. FEMS Microbiol. Lett. 19:21-25.

Jawetz, E., J. L. Melnick and E. A. Adelberg. 1984. Review of medical microbiology, 16th edn. Lange Medical Publications, Los Altos, California.

Jokinen, M., C. Ehnholm, V. Väisänen-Rhen, T. Korhonen, R. Pipkorn, N. Kalkkinen and C. G. Gahmberg. 1985. Identification of the major sialoglycoprotein from red cells, glycophorin A^M, as the receptor for Escherichia coli 1H11165 and characterization of the receptor site. Eur. J. Biochem. 147:47-52.

Jones, G. W. 1977. The attachment of bacteria to the surfaces of animal cells, p. 139-176. In J. L. Reissig (ed.), Microbial interactions. Receptors and recognition, series B, vol. 3. Chapman and Hall, London

Jones, G. W. and R. Freter. 1976. Adhesive properties of Vibrio cholerae : nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immun. 14:240-245.

Jones, G. W. and L. A. Richardson. 1981. The attachment to, and invasion of HeLa cells by Salmonella typhimurium: the contribution of MS and MRHA activities. J. Gen. Microbiol. 127:361-370.

Jones, G. W. and R. E. Isaacson. 1983. Proteinaceous bacterial adhesins and their receptors. Crit. Rev. Microbiol. 10:229-260.

Jones, G. W., G. D. Abrams and R. Freter. 1976. Adhesive properties of Vibrio cholerae : adhesion to isolated brush border membranes and hemagglutinating activity. Infect. Immun. 14:232-239.

Jones, G. W., D. K. Rabert, D. M. Svinarich and H. J. Whitfield. 1982. Association of adhesive, invasive, and virulent phenotypes of Salmonella typhimurium with autonomous 60-megadalton plasmids. Infect. Immun. 38:476-486.

Josephsen, J., F. Hansen, F. K. de Graaf and W. Gaastra. 1984. The nucleotide sequence of the protein subunit of the K88ac fimbria of porcine enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. 25:301-306.

Källenius, G., R. Möllby, S. B. Svenson, J. Winberg, A. Lundblad, S. Svensson and B. Cedergren. 1980. The P^k antigen as receptor for the haemagglutination of pyelonephritic Escherichia coli. FEMS Microbiol. Lett. 7:297-302.

Källenius, G., S. Svenson, R. Möllby, C. Cedergren, H. Hultberg and J. Winberg. 1981. Structure of carbohydrate part of receptor on human uroepithelial cells for pyelonephritogenic Escherichia coli. Lancet ii:604-606.

Källenius, G., S. B. Svenson, R. Möllby, T. Korhonen, J. Winberg, B. Cedergren, I. Helin and H. Hultberg. 1982. Carbohydrate receptor structures recognised by uropathogenic Escherichia coli. Scand. J. Infect. Dis. (suppl.) 33:52-60.

Karmali, M. A., M. Petric, B. T. Steele and C. Lin. 1983. Sporadic cases of haemolytic uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing Escherichia coli in stools. Lancet i:619-620.

Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus and H. Lior. 1985. The association between idiopathic hemolytic uremic syndrome and infection by Vero toxin producing Escherichia coli. J. Infect. Dis. 151:775-782.

Katuda, T. and M. Ui. 1982. Direct modification of the adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc. Natl. Acad. Sci. USA 79:3129-3133.

Kauffmann, F. 1947. The serology of the coli group. J. Immun. 57:71-100.

Kauffmann, F. 1948. On haemagglutination by Escherichia coli. Acta Pathol. Microbiol. Scand. 25:502-506.

Kearns, M. J. and R. A. Gibbons. 1979. The possible nature of the pig intestinal receptor for the K88 antigen of Escherichia coli. FEMS Microbiol. Lett. 6:165-168.

Kehoe, M., R. Sellwood, P. Shipley and G. Dougan. 1981. Genetic analysis of K88-mediated adhesion of enterotoxigenic Escherichia coli. Nature (London) 291:122-126.

Kehoe, M., M. Winther, G. Dowd, P. Morrissey and G. Dougan. 1982. Nucleotide sequence homology between the K88 and the K99 adhesion system of enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. 14:129-132.

Kehoe, M., M. Winther and G. Dougan. 1983. Expression of a cloned K88ac adhesion antigen determinant : identification of a new adhesion cistron and role of a vector-encoded promoter. J. Bacteriol. 155:1071-1077.

Kétyi, I. 1984. Non-toxin virulence factors of bacterial enteric pathogens. Acta Microbiol. Hung. 31:1-25.

Kilian, M. 1982. Bacterial enzymes degrading human IgA1, p. 213-218. In J. R. Robbins, J. C. Hill and J. C. Sadoff (ed.), Seminars in infectious disease, vol. IV: bacterial vaccines. Thieme-Stratton, New York.

Kleckner, N. 1981. Transposable elements in prokaryotes. Ann. Rev. Genet. 15:341-404.

Klemm, P. 1979. Fimbrial colonisation factor CFA/I protein from human enteropathogenic Escherichia coli strains. FEBS Lett. 108:107-110.

Klemm, P. 1981. The complete amino acid sequence of the K88 antigen, a fimbrial protein from Escherichia coli. Eur. J. Biochem. 117:617-627.

Klemm, P. 1982a. Fimbrial adherence factors from pathogenic Escherichia coli strains. Tokai J. Exp. Clin. Med. 7 (suppl.):185-192.

Klemm, P. 1982b. Primary structure of the CFA/I fimbrial protein from human enterotoxigenic E. coli strains. Eur. J. Biochem. 124:339-348.

Klemm, P. 1984. The fimA gene encoding the type 1 fimbrial subunit of Escherichia coli. Nucleotide sequence and primary structure of the protein. Eur. J. Biochem. 143:395-399.

Klemm, P. 1985. Fimbrial adhesins of Escherichia coli. Rev. Infect. Dis. 7:321-340.

Klemm, P., I. Ørskov and F. Ørskov. 1982. F7 and type 1 like fimbriae from three uropathogenic Escherichia coli strains isolated from urinary tract infections : protein chemical and immunological aspects. Infect. Immun. 36:462-468.

Klemm, P., I. Ørskov and F. Ørskov. 1983. Isolation and characterization of the F12 adhesive fimbrial antigen from uropathogenic Escherichia coli strains. Infect. Immun. 40:91-96.

Klemm, P., W. Gaastra, M. M. McConnell and H. R. Smith. 1985a. The CS2 fimbrial antigen from Escherichia coli, purification, characterisation and partial covalent structure. FEMS Microbiol. Lett. 26:207-210.

Klemm, P., B. J. Jorgensen, I. van Die, H. de Ree and H. Bergmans. 1985b. The fim genes responsible for synthesis of type 1 fimbriae in E. coli, cloning and genetic organisation. Mol. Gen. Genet. 199:410-414.

Klipstein, F. A., B. Rowe, R. F. Engert, H. B. Short and R. J. Gross. 1978. Enterotoxigenicity of enteropathogenic serotypes of Escherichia coli isolated from infants with epidemic diarrhea. Infect. Immun. 21:171-178.

Klipstein, F. A., R. F. Engert, R. A. Houghton and B. Rowe. 1984. Enzyme linked immunosorbent assay for Escherichia coli heat stable enterotoxin. J. Clin. Microbiol. 19:798-803.

Knutton, S., D. C. A. Candy, D. R. Lloyd, P. H. Williams and A. S. McNeish. 1982. Adhesins in enteroinvasive strains of Escherichia coli. Gut 23:A886, WP23.

Knutton, S., D. R. Lloyd, D. C. A. Candy and A. S. McNeish. 1983. Adherence of enterotoxigenic Escherichia coli and enteroinvasive Escherichia coli to human intestinal epithelial cells. Gut 24:A996-A997, F54.

Knutton, S., D. R. Lloyd, D. C. A. Candy and A. S. McNeish. 1984a. In vitro adhesion of enterotoxigenic Escherichia coli to human intestinal epithelial cells from mucosal biopsies. Infect. Immun. 44:514-518.

Knutton, S., D. R. Lloyd, D. C. A. Candy and A. S. McNeish. 1984b. Ultrastructural study of adhesion in enterotoxigenic Escherichia coli to erythrocytes and human intestinal epithelial cells. Infect. Immun. 44:519-527.

Knutton, S., P. H. Williams, D. R. Lloyd, D. C. A. Candy and A. S. McNeish. 1984c. Ultrastructural study of adherence to and penetration of cultured cells by two invasive Escherichia coli strains isolated from infants with enteritis. Infect. Immun. 44:599-608.

Knutton, S., D. R. Lloyd, D. C. A. Candy and A. S. McNeish. 1985. Adhesion of enterotoxigenic Escherichia coli to human small intestinal enterocytes. Infect. Immun. 48:824-831.

Konisky, J. 1982. Colicins and other bacteriocins with established modes of action. Ann. Rev. Microbiol. 36:125-144.

Konowalchuk, J., J. I. Speirs and S. Stavric. 1977. Vero response to a cytotoxin of Escherichia coli. Infect. Immun. 18:775-779.

Korhonen, T. K., K. Lounatmaa, H. Ranta and N. Kuusi. 1980a. Characterization of type 1 pili of Salmonella typhimurium LT2. J. Bacteriol. 144:800-805.

Korhonen, T. K., E. -L. Nurmiaho, H. Ranta and C. Svanborg-Edén. 1980b. New method for isolation of immunologically pure pili from Escherichia coli. Infect. Immun. 27:569-575.

Korhonen, T. K., V. Väisänen, H. Saxen, H. Hultberg and S. B. Svenson. 1982. P-antigen-recognizing fimbriae from human uropathogenic Escherichia coli strains. Infect. Immun. 37:286-291.

Korhonen, T. K., V. Väisänen-Rhen, M. Rhen, A. Pere, J. Parkkinen and J. Finne. 1984. Escherichia coli fimbriae recognizing sialyl galactosides. J. Bacteriol. 159:762-766.

Korhonen, T. K., M. V. Valtonen, J. Parkkinen, V. Väisänen-Rhen, J. Finne, F. Ørskov, I. Ørskov, S. B. Svenson and P. H. Makela. 1985. Escherichia coli strains associated with neonatal sepsis and meningitis : serotypes, hemolysin production and receptor recognition. Infect. Immun. 48:486-491.

Kuriyama, S. M. and F. J. Silverblatt. 1986. Effect of Tamm-Horsfall urinary glycoprotein on phagocytosis and killing of type 1-fimbriated Escherichia coli. Infect. Immun. 51:193-198.

Kushner, S. R. 1978. An improved method for transformation of Escherichia coli with ColE1 derived plasmids, p. 17-23. In H. W. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier/North-Holland Biomedical Press, Amsterdam.

Labigne-Roussel, A. F., D. Lark, G. Schoolnik and S. Falkow. 1984. Cloning and expression of an afimbrial adhesin (AFA-I) responsible for P blood group-independent, mannose-resistant hemagglutination from a pyelonephritic Escherichia coli strain. Infect. Immun. 46:251-259.

Labigne-Roussel, A., M. A. Schmidt, W. Walz and S. Falkow. 1985. Genetic organization of the afimbrial adhesin operon and nucleotide sequence from a uropathogenic Escherichia coli gene encoding an afimbrial adhesin. J. Bacteriol. 162:1285-1292.

La Brec, E. H., H. Schneider, T. J. Magnani and S. B. Formal. 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. 88:1503-1518.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Larsen, H. E. L., K. Gerdes, J. Light and S. Molin. 1984. Low-copy number plasmid-cloning vectors amplifiable by derepression of an inserted foreign promoter. Gene 28:45-54.

Laurent, M., E. Pays, E. Magnus, N. van Meirvenne, G. Matthyssens, R. O. Williams and M. Steinert. 1983. DNA rearrangements linked to expression of a predominant surface antigen of trypanosomes. Nature (London) 302:263-266.

Lederberg, J. and T. Iino. 1956. Phase variation in Salmonella. Genetics 41:743-757.

Lennox, E. S. 1955. Transduction of linked genetic characters of the host bacteriophage P1. Virology 1:190-206.

Levine, M. M. 1984. Escherichia coli infections, p. 187-235. In R. Germanier (ed.), Bacterial vaccines. Academic Press Inc. (London) Ltd., London.

Levine, M. M., M. B. Rennels, V. Daya and T. P. Hughes. 1980. Hemagglutination and colonization factors in enterotoxigenic and enteropathogenic Escherichia coli that cause diarrhea. J. Infect. Dis. 141:733-737.

Levine, M. M., P. Ristaino, R. B. Sack, J. B. Kaper, F. Ørskov and I. Ørskov. 1983. Colonization factor antigens I and II and type 1 somatic pili in enterotoxigenic Escherichia coli : relation to enterotoxin type. Infect. Immun. 39:889-897.

Levine, M. M., P. Ristaino, G. Marley, C. Smyth, S. Knutton, E. Boedeker, R. Black, C. Young, M. L. Clements, C. Cheney and R. Patnaik. 1984. Coli surface antigens 1 and 3 of colonization factor antigen II - positive enterotoxigenic Escherichia coli: morphology, purification, and immune responses in humans. Infect. Immun. 44:409-420.

Levine, M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements and A. D. O'Brien. 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic Escherichia coli is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. 152:550-559.

Lewis, D. H. and W. Peters. 1977. The resistance of intracellular Leishmania parasites to digestion by lysosomal enzymes. Ann. Trop. Med. Parasitol. 71:295-299.

Lindberg, F. P., B. Lund and S. Normark. 1984. Genes of pyelonephritogenic E. coli required for digalactoside-specific agglutination of human cells. EMBO J. 3:1167-1173.

Lindberg, F., B. Lund and S. Normark. 1986. Gene products specifying adhesion of uropathogenic Escherichia coli are minor components of pili. Proc. Natl. Acad. Sci. USA 83:1891-1895.

Linggood, M. A. and P. L. Ingram. 1982. The role of alpha hemolysin in the virulence of Escherichia coli for mice. J. Med. Microbiol. 15:23-30.

Low, K. B. 1972. Escherichia coli K-12 F-prime factors, old and new. Bacteriol. Rev. 36:587-607.

Low, D., V. David, D. Lark, G. Schoolnik and S. Falkow. 1984. Gene clusters governing the production of hemolysin and mannose-resistant hemagglutination are closely linked in Escherichia coli serotype O4 and O6 isolates from urinary tract infections. Infect. Immun. 43:353-358.

Lund, B., F. P. Lindberg, M. Båga and S. Normark. 1985. Globoside-specific adhesins of uropathogenic Escherichia coli are encoded by similar trans-complementable gene clusters. J. Bacteriol. 162:1293-1301.

Mackman, N., J. -M. Nicaud, L. Gray and I. B. Holland. 1986. Secretion of haemolysin by Escherichia coli. Curr. Top. Microbiol. Immun. 125:159-181.

MacLagan, R. M. and D. C. Old. 1980. Haemagglutinins and fimbriae in different serotypes and biotypes of Yersinia enterocolitica. J. Appl. Bact. 49:353-360.

Maki, M., P. Gronroos and T. Vesikari. 1978. In vitro invasiveness of
Yersinia enterocolitica isolated from children with diarrhea. J.
Infect. Dis. 138:677-680.

Male, C. 1982. Streptococcus pneumoniae and Haemophilus influenzae IgA₁
proteases and their possible role in pathogenesis, p. 219-224. In J. B.
Robbins, J. C. Hill and J. C. Sadoff (ed.), Seminars in infectious
disease, vol. IV: bacterial vaccines. Thieme-Stratton, New York.

Maloy, S. R. and W. D. Nunn. 1981. Selection for loss of tetracycline
resistance by Escherichia coli. J. Bact. 145:1110-1112.

Mandell, G. L., R. G. Douglas and J. E. Bennett. 1985. Principles and
practice of infectious diseases, 2nd edn. John Wiley and Sons, Inc.,
New York.

Maniatis, T., E. F. Fritsch and J. Sambrook. 1982. Molecular cloning: a
laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor,
New York.

Manning, P. A., K. N. Timmis and G. Stevenson. 1985. Colonisation factor
antigen II (CFA/II) of enterotoxigenic Escherichia coli : molecular
cloning of the CS3 determinant. Mol. Gen. Genet. 200:322-327.

Marrs, C. F., G. Schoolnik, J. M. Koomey, J. Hardy, J. Rothbard and S.
Falkow. 1985. Cloning and sequencing of a Moraxella bovis pilin gene.
J. Bacteriol. 163:132-139.

Marshall, K. C. 1985. Mechanisms of bacterial adhesion at solid-water
interfaces, p. 133-161. In D. C. Savage and M. Fletcher (ed.),
Bacterial adhesion, mechanisms and physiological significance. Plenum
Press, New York.

Mathias, J. R., J. L. Martin, G. M. Carlson, J. Nogueira and R. A.
Gianella. 1980. Repetitive bursts of action potentials in response to
E. coli heat-stable toxin: a defense mechanism of the bacterium. Clin.
Res. 28:281.

Matthewson, J. J., P. C. Johnson, H. L. DuPont, D. R. Morgan, S. A.
Thornton, L. V. Wood and C. D. Ericsson. 1985. A newly recognized cause
of travelers' diarrhea : enteroadherent Escherichia coli. J. Infect.
Dis. 151:471-475.

Mattick, J. S., B. J. Anderson, M. R. Mott and J. R. Egerton. 1984.
Isolation and characterization of Bacteroides nodosus fimbriae :
structural subunit and basal protein antigens. J. Bacteriol.
160:740-747.

Maurelli, A. T., B. Blackmon and R. Curtiss III. 1984. Temperature-
-dependent expression of virulence genes in Shigella species. Infect.
Immun. 43:195-201.

Maurer, L. and P. E. Orndorff. 1985. A new locus, pilE, required for the binding of type 1 piliated Escherichia coli to erhythrocytes [sic]. FEMS Microbiol. Lett. 30:59-66.

McConnell, M. M., H. R. Smith, G. A. Willshaw, A. M. Field and B. Rowe. 1981. Plasmids coding for colonization factor antigen I and heat-stable enterotoxin production isolated from enterotoxigenic Escherichia coli : comparison of their properties. Infect. Immun. 32:927-936.

McIver, J., G. F. Grady and G. T. Keusch. 1975. Production and characterization of exotoxin(s) of Shigella dysenteriae type 1. J. Infect. Dis. 131:661-663.

McKern, N. M., I. J. O'Donnell, A. S. Inglis, D. J. Stewart and B. L. Clark. 1983. Amino acid sequence of pilin from Bacteroides nodosus (strain 198), the causative organism of ovine footrot. FEBS Lett. 164:149-153.

McKern, N. M., I. J. O'Donnell, D. J. Stewart and B. L. Clark. 1985. Primary structure of pilin protein from Bacteroides nodosus strain 216: comparison with the corresponding protein from strain 198. J. Gen. Microbiol. 131:1-6.

McMichael, J. C., and T. J. Ou. 1979. Structure of common pili from Escherichia coli. J. Bacteriol. 138:969-975.

Meyer, T. F., N. Mlawer and M. So. 1982. Pilus expression in Neisseria gonorrhoeae involves chromosomal rearrangement. Cell 30:45-52.

Meyer, T. F., E. Billyard, R. Haas, S. Storzbach and M. So. 1984. Pilus genes of Neisseria gonorrhoeae : chromosomal organization and DNA sequence. Proc. Natl. Acad. Sci. USA 81:6110-6114.

Middlebrook, J. L. and R. B. Dorland. 1984. Bacterial toxins : cellular mechanisms of action. Microbiol. Rev. 48:199-221.

Mims, C. A. 1982. The pathogenesis of infectious disease, 2nd edn. Academic Press Inc. (London) Ltd., London.

Minion, F. C., S. N. Abraham, E. H. Beachey and J. D. Goguen. 1986. The genetic determinant of adhesive function in type 1 fimbriae of Escherichia coli is distinct from the gene encoding the fimbrial subunit. J. Bacteriol. 165:1033-1036.

Montgomerie, J. Z., G. M. Kalmanson and L. B. Guze. 1979. Enterobactin and the virulence of Escherichia coli in pyelonephritis. J. Infect. Dis. 140:1013.

Montgomerie, J. Z., A. Bindereif, J. B. Neilands, G. Kalmanson and L. B. Gruze. 1984. Association of hydroxamate siderophore (aerobactin) with Escherichia coli isolated from patients with bacteraemia. Infect. Immun. 46:835-838.

Mooi, F. R. and F. K. de Graaf. 1979. Isolation and characterization of K88 antigens. FEMS Microbiol. Lett. 5:17-20.

Mooi, F. R. and F. K. de Graaf. 1985a. Molecular biology of fimbriae of enterotoxigenic Escherichia coli. Curr. Top. Microbiol. Immun. 118:119-138.

Mooi, F. R. and F. K. de Graaf. 1985b. Molecular biology of the K88ab and K99 fimbriae, p. 53-63. In T. K. Korhonen, E. A. Dawes and P. H. Makela (ed.), Enterobacterial surface antigens: methods for molecular characterisation. FEMS Symp. number 25. Elsevier Science Publishers BV (Biomedical Division), Amsterdam.

Mooi, F. R., F. K. de Graaf and J. D. A. van Embden. 1979. Cloning, mapping and expression of the genetic determinant that encodes for the K88ab antigen. Nucleic Acids Res. 6:849-865.

Mooi, F. R., N. Harms, D. Bakker and F. K. de Graaf. 1981. Organization and expression of genes involved in the production of the K88ab antigen. Infect. Immun. 32:1155-1163.

Mooi, F. R., A. Wijfjes and F. K. de Graaf. 1982. Construction and characterization of mutants impaired in the biosynthesis of K88ab antigen. J. Bacteriol. 150:512-521.

Mooi, F. R., A. Wijfjes and F. K. de Graaf. 1983. Identification and characterization of precursors in the biosynthesis of the K88ab fimbria of Escherichia coli. J. Bacteriol. 154:41-49.

Mooi, F. R., M. van Buuren, G. Koopman, B. Roosendaal and F. K. de Graaf. 1984. K88ab gene of Escherichia coli encodes a fimbria-like protein distinct from the K88ab fimbrial adhesin. J. Bacteriol. 159:482-487.

Moon, H. W., R. E. Isaacson and J. Pohlenz. 1979. Mechanisms of association of enteropathogenic Escherichia coli with intestinal epithelium. Am. J. Clin. Nutr. 32:119-127.

Morris, J. A. and W. J. Sojka. 1985. Escherichia coli as a pathogen in animals, p. 47-77. In M. Sussman (ed.), The virulence of Escherichia coli. Reviews and methods. Special Publications of the Society for General Microbiology, vol. 13. Academic Press Inc. (London) Ltd., London.

Morris, J. A., A. E. Stevens and W. J. Sojka. 1977. Preliminary characterisation of cell-free K99 antigen isolated from Escherichia coli B41. J. Gen. Microbiol. 99:353-357.

Morris, J. A., A. E. Stevens and W. J. Sojka. 1978. Anionic and cationic components of the K99 surface antigen from Escherichia coli B41. J. Gen. Microbiol. 10:173-175.

Morris, J. A., C. J. Thorns and W. J. Sojka. 1980. Evidence for two adhesive antigens on the K99 reference strain Escherichia coli B41. J. Gen. Microbiol. 118:107-113.

Morris, J. A., C. J. Thorns, A. C. Scott and W. J. Sojka. 1982. Adhesive properties associated with the Vir plasmid : a transmissible pathogenic characteristic associated with strains of invasive Escherichia coli. J. Gen. Microbiol. 128:2097-2103.

Morrissey, P. M. and G. Dougan. 1986. Expression of a cloned 987P adhesin-antigen fimbrial determinant in Escherichia coli K-12 strain HB101. Gene 43:79-84.

Moseley, S. L., G. Dougan, R. A. Schneider and H. W. Moon. 1986. Cloning of chromosomal DNA encoding the F41 adhesin of enterotoxigenic Escherichia coli and genetic homology between adhesins F41 and K88. J. Bacteriol. 167:799-804.

Mullany, P., A. M. Field, M. M. McConnell, S. M. Scotland, H. R. Smith and B. Rowe. 1983. Expression of plasmids coding for colonisation factor antigen II (CFA/II) and enterotoxin production in Escherichia coli. J. Gen. Microbiol. 129:3591-3601.

Murray, H. W. 1983. How protozoa evade intracellular killing. Ann. Intern. Med. 98:1016-1018.

Murray, N. E., W. J. Brammar and K. Murray. 1977. Lambdoid phages that simplify recovery of in vitro recombinants. Mol. Gen. Genet. 150:53-61.

Nagy, B. 1980. Vaccination of cows with a K99 extract to protect newborn calves against experimental enterotoxic colibacillosis. Infect. Immun. 27:21-24.

Nagy, B., H. W. Moon and R. E. Isaacson. 1977. Colonization of porcine intestine by enterotoxigenic Escherichia coli : selection of piliated forms in vivo, adhesion of piliated forms to epithelial cells in vitro, and incidence of a pilus antigen among porcine enteropathogenic E. coli. Infect. Immun. 16:344-352.

Nandadasa, H. G., G. F. Sargent, M. G. M. Brown, A. S. McNeish and P. H. Williams. 1981. The role of plasmids in adherence of invasive Escherichia coli to mammalian cells. J. Infect. Dis. 143:286-290.

Nataro, J. P., I. C. A. Scaletsky, J. B. Kaper, M. M. Levine and L. R. Trabulsi. 1985a. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic Escherichia coli. Infect. Immun. 48:378-383.

Nataro, J. P., M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo and M. M. Levine. 1985b. Detection of an adherence factor of enteropathogenic Escherichia coli with a DNA probe. J. Infect. Dis. 152:560-565.

Neilands, J. B. 1984. Siderophores of bacteria and fungi. Microbiol. Sci. 1:9-14.

Nicaud, J. -M., N. Mackman, L. Gray and I. B. Holland. 1985. Regulation of haemolysin synthesis in E. coli determined by haemolysin genes of human origin. Mol. Gen. Genet. 199:111-116.

Norgren, M., S. Normark, D. Lark, P. O'Hanley, G. Schoolnik, S. Falkow, C. Svanborg-Edén, M. Båga and B. E. Uhlin. 1984. Mutations in E. coli cistrons affecting adhesion to human cells do not abolish Pap pili fiber formation. EMBO J. 3:1159-1165.

Normark, S., D. Lark, R. Hull, M. Norgren, M. Båga, P. O'Hanley, G. Schoolnik and S. Falkow. 1983. Genetics of digalactoside-binding adhesin from a uropathogenic Escherichia coli strain. Infect. Immun. 41:942-949.

Normark, S., M. Båga, M. Göransson, F. Lindberg, B. Lund, M. Norgren and B. -E. Uhlin. 1985. Genetic analysis of bacterial pilus adhesins. p. 39-52. In T. K. Korhonen, E. A. Dawes and P. H. Makela (ed.), Enterobacterial surface antigens: methods for molecular characterisation. FEMS Symp. number 25. Elsevier Science Publishers BV (Biomedical Division), Amsterdam.

Nowicki, B., M. Rhen, V. Väisänen-Rhen, A. Pere and T. K. Korhonen. 1984. Immunofluorescence study of fimbrial phase variation in Escherichia coli KS71. J. Bacteriol. 160:691-695.

Nowicki, B., M. Rhen, V. Väisänen-Rhen, A. Pere and T. K. Korhonen. 1985. Kinetics of phase variation between S and type 1 fimbriae of Escherichia coli. FEMS Microbiol. Lett. 28:237-242.

O'Brien, A. D., J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith and S. B. Formal. 1984. Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. Science 226:694-696.

Ofek, I. and E. H. Beachey. 1980. General concepts and principles of bacterial adherence in animals and man, p. 1-29. In E. H. Beachey (ed.), Bacterial adherence. Receptors and recognition, series B, vol. 6. Chapman and Hall, London.

Ofek, I., H. S. Courtney, D. M. Schifferli and E. H. Beachey. 1986. Enzyme-linked immunsorbent assay for adherence of bacteria to animal cells. J. Clin. Microbiol. 24:512-516.

Old, D. C. 1972. Inhibition of the interaction between fimbrial haemagglutinin and erythrocytes by D-mannose and other carbohydrates. J. Gen. Microbiol. 71:149-157.

Old, D. C. 1985a. Haemagglutination methods in the study of Escherichia coli, p. 287-313. In The virulence of Escherichia coli. Reviews and methods. Special Publications of the Society for General Microbiology, vol. 13. Academic Press Inc. (London) Ltd., London.

Old, D. C. 1985b. Bacterial adherence. Med. Lab. Sci. 42:78-85.

Old, D. C. and J. P. Duguid. 1970. Selective outgrowth of fimbriate bacteria in static liquid medium. J. Bacteriol. 103:447-456.

Old, D. C. and J. Robertson. 1981. Adherence of fimbriate and non-fimbriate strains of Yersinia enterocolitica to human epithelial cells. Microbiol. Immunol. 25:993-998.

Old, D. C., B. Klaassen and P. B. Crichton. 1985. A novel non-fimbrial MS and eluting (MSE) haemagglutinin of Yersinia frederiksenii strains. FEMS Microbiol. Lett. 28:81-84.

Orndorff, P. E. and S. Falkow. 1984a. Organization and expression of genes responsible for type 1 piliation in Escherichia coli. J. Bacteriol. 159:736-744.

Orndorff, P. E. and S. Falkow. 1984b. Identification and characterization of a gene product that regulates type 1 piliation in Escherichia coli. J. Bacteriol. 160:61-66.

Orndorff, P. E. and S. Falkow. 1985. Nucleotide sequence of pilA, the gene encoding the structural component of type 1 pili in Escherichia coli. J. Bacteriol. 162:454-457.

Ørskov, I. and F. Ørskov. 1966. Episome-carried surface antigen K88 of Escherichia coli. I. Transmission of the determinant of the K88 antigen and influence on the transfer of chromosomal markers. J. Bacteriol. 91:69-75.

Ørskov, I. and F. Ørskov. 1983. Serology of Escherichia coli fimbriae. Prog. Allergy 33:80-105.

Ørskov, I. and F. Ørskov. 1984. Serotyping of Escherichia coli. In T. Bergan and J. R. Norris (ed.), Methods in microbiol., vol. 14. Academic Press Inc. (London) Ltd., London.

Ørskov, I. and F. Ørskov. 1985. Escherichia coli in extra-intestinal infections. J. Hyg. Camb. 95:551-575.

Ørskov, I., F. Ørskov, W. J. Sojka and L. M. Leach. 1961. Simultaneous occurrence of E. coli B and L antigens in strains from diseased swine. Acta Pathol. Microbiol. Scand. 53:404-422.

Ørskov, I., F. Ørskov, W. J. Sojka and W. Wittig. 1964. K antigens K88ab(L) and K88ac(L) in E. coli. A new O antigen O147 and a new K antigen: K89(B). Acta Pathol. Microbiol. Scand. 62:439-447.

Ørskov, I., F. Ørskov, H. W. Smith and W. J. Sojka. 1975. The establishment of K99, a thermolabile, transmissible, Escherichia coli K antigen, previously called Kco, possessed by calf and lamb enterotoxigenic strains. Acta Pathol. Microbiol. Scand. 83B:31-36.

Ørskov, I., F. Ørskov, D. J. Evans Jr., R. B. Sack, P. A. Sack and T. Wadstrom. 1976. Special Escherichia coli serotypes among enterotoxigenic strains from diarrhoea in adults and children. Med. Microbiol. Immun. 162:73-80.

Ørskov, I., A. Ferenz and F. Ørskov. 1980a. Tamm-Horsfall protein or uromucoid is the normal urinary slime that traps type 1 fimbriated Escherichia coli. Lancet i:887.

Ørskov, I., F. Ørskov and A. Birch-Andersen. 1980b. Comparison of Escherichia coli fimbrial antigen F7 with type 1 fimbriae. Infect. Immun. 27:657-666.

Ørskov, I., F. Orskov, A. Birch-Andersen, M. Kanamori and C. Svanborg Edén. 1982a. O, K, H and fimbrial antigens in Escherichia coli serotypes associated with pyelonephritis and cystitis. Scand. J. Infect. Dis. (suppl.) 33:18-25.

Ørskov, I., F. Ørskov, A. Birch-Andersen, P. Klemm and C. Svanborg-Edén. 1982b. Protein attachment factors : fimbriae in adhering Escherichia coli strains, p. 97-103. In J. B. Robbins, J. C. Hill and J. C. Sadoff (ed.), Seminars in infectious disease, vol. IV: bacterial vaccines. Thieme-Stratton Inc., New York.

Ørskov, I., A. Birch-Andersen, J. P. Duguid, J. Stenderup and F. Ørskov. 1985. An adhesive protein capsule of Escherichia coli. Infect. Immun. 47:191-200.

Osborne, J. C., J. Moss, P. H. Fishman, S. Nakaya, D. C. Robertson. 1982. Specificity in protein-membrane associations: the interaction of gangliosides with Escherichia coli heat-labile enterotoxin and choleragen. Biophys. J. 37:168-169.

Ottow, J. C. G. 1975. Ecology, physiology, and genetics of fimbriae and pili. Ann. Rev. Microbiol. 79:79-108.

Pai, C. H., J. K. Kelly and G. L. Meyers. 1986. Experimental infection of infant rabbits with Verotoxin-producing Escherichia coli. Infect. Immun. 51:16-23.

Pal, T., C. Pacsa, L. Emody and S. Voros. 1983. Antigenic relationships among virulent EIEC, Shigella flexneri, and Shigella sonnei detected by ELISA. Lancet ii:102.

Pappenheimer, A. M. Jr. 1977. Diphtheria toxin. Ann. Rev. Biochem. 46:69-94.

Paranchych, W., P. A. Sastry, L. S. Frost, M. Carpenter, G. D. Armstrong and T. H. Watts. 1979. Biochemical studies on pili isolated from Pseudomonas aeruginosa strain PAO. Can. J. Microbiol. 25:1175-1181.

Parry, S. H. and D. M. Rooke. 1985. Adhesins and colonization factors of Escherichia coli, p. 79-155. In M. Sussman (ed.), The virulence of Escherichia coli. Reviews and methods. Special Publications of the Society for General Microbiology, vol. 13. Academic Press Inc. (London) Ltd., London.

Parry, S. H., S. Boonchai, S. N. Abraham, J. M. Salter, D. M. Rooke, J. M. Simpson, A. J. Bint and M. Sussman. 1983. A comparative study of the mannose-resistant and mannose-sensitive haemagglutinins of Escherichia coli isolated from urinary tract infections. Infection 11:123-128.

Pearson, G. D. N. and J. J. Mekalanos. 1982. Molecular cloning of Vibrio cholerae enterotoxin genes in Escherichia coli K-12. Proc. Natl. Acad. Sci. USA 79:2976-2980.

Penaranda, M. E., M. B. Mann, D. G. Evans and D. J. Evans Jr. 1980. Transfer of an ST:LT:CFA/II plasmid into Escherichia coli K-12 strain RR1 by contransformation [sic] with pSC301 plasmid DNA. FEMS Microbiol. Lett. 8:251-254.

Perry, A. and I. Ofek. 1984. Inhibition of blood clearance and hepatic tissue binding of Escherichia coli by liver lectin-specific sugars and glycoproteins. Infect. Immun. 43:257-262.

Pohl, P., P. Lintermans, K. van Muylem and M. Schotte. 1982. Colibacilles enterotoxigenes du veau possedant un antigene d'attachment different de l'antigene K99. Ann. Med. Vet. 126:569-571.

Pohl, P., P. Lintermans, A. Kaeckenbeeck, P. de Mol, P. van Muylem and M. Schotte. 1983. Existence de differents types d'Escherichia coli pathogenes pour l'intestin du veau. Ann. Med. Vet. 127:37-41.

Pollack, J. R., B. N. Ames and J. B. Neilands. 1970. Iron transport in Salmonella typhimurium: mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. 104:635-639.

Porter, P. and M. A. Linggood. 1983. Development of oral vaccines for preventing diarrhoea caused by infection by enteropathogenic Escherichia coli. J. Infect. 6:111-121.

Pritchard, R. H. and I. B. Holland (ed.). 1985. Basic cloning techniques, a manual of experimental procedures. Blackwell Scientific Publications, Oxford.

Purcell, B. K. and S. Clegg. 1983. Construction and expression of recombinant plasmids encoding type 1 fimbriae of a urinary Klebsiella pneumoniae isolate. Infect. Immun. 39:1122-1127.

Quackenbush, R. L. and S. Falkow. 1979. Relationship between colicin V activity and virulence in Escherichia coli. Infect. Immun. 24:562-564.

Quie, P. G. 1983. Perturbation of the normal mechanisms of intraleukocytic killing of bacteria. J. Infect. Dis. 148:189-193.

Rappaport, R. S. and G. Bonde. 1982. Prospects for the development of a vaccine against cholera and Escherichia coli diarrheal disease, p. 89-96. In J. B. Robbins, J. C. Hill and J. C. Sadoff (ed.), Seminars in infectious disease, vol. IV: bacterial vaccines. Thieme-Stratton Inc., New York.

Reis, M. H. L., H. T. Affonso, L. R. Trabulski, A. J. Mazaitis, R. Maas and W. K. Maas. 1980. Transfer of a CFA/I-ST plasmid promoted by a conjugative plasmid in a strain of Escherichia coli of serotype 0128ac:H12. Infect. Immun. 29:140-143.

Rhen, M. 1985. Characterization of DNA fragments encoding fimbriae of the uropathogenic Escherichia coli strain KS71. J. Gen. Microbiol. 131:571-580.

Rhen, M., P. Klemm, E. Wahlstrom, S. B. Svenson, G. Källenius and T. K. Korhonen. 1983a. P-fimbriae of Escherichia coli : immuno- and protein-chemical characterization of fimbriae from two pyelonephritogenic strains. FEMS Microbiol. Lett. 18:233-238.

Rhen, M., J. Knowles, M. E. Penttila, M. Sarvas and T. K. Korhonen. 1983b. P-fimbriae of Escherichia coli : molecular cloning of DNA fragments containing the structural genes. FEMS Microbiol. Lett. 19:119-123.

Rhen, M., P. H. Makela and T. K. Korhonen. 1983c. P-fimbriae of Escherichia coli are subject to phase variation. FEMS Microbiol. Lett. 19:267-271.

Rhen, M., E. Wahlstrom and T. K. Korhonen. 1983d. P-fimbriae of Escherichia coli : fractionation by immune precipitation. FEMS Microbiol. Lett. 18:227-232.

Rhen, M., I. van Die, V. Rhen and H. Bergmans. 1985a. Comparison of the nucleotide sequences of the genes encoding the KS71A and F7$_1$ fimbrial antigens of uropathogenic Escherichia coli. Eur. J. Biochem. 151:573-577.

Rhen, M., V. Väisänen-Rhen, A. Pere and T. K. Korhonen. 1985b. Complementation and regulatory interactions between two cloned fimbrial gene clusters of Escherichia coli strain KS71. Mol. Gen. Genet. 200:60-64.

Rhen, M., J. Tenhunen, V. Väisänen-Rhen, A. Pere, M. Båga and T. K. Korhonen. 1986. Fimbriation and P-antigen recognition of Escherichia coli strains harbouring mutated recombinant plasmids encoding fimbrial antigens of the uropathogenic E. coli strain KS71. J. Gen. Microbiol. 132:71-77.

Richards, K. L. and S. D. Douglas. 1978. Pathophysiological effects of Vibrio cholerae and enterotoxigenic Escherichia coli and their exotoxins on eucaryotic cells. Microbiol. Rev. 42:592-613.

Rietschel, E. T., U. Schade, M. Jensen, H. W. Wollenweber, O. Luderitz and S. G. Greisman. 1982. Bacterial endotoxins : chemical structure, biological activity and role in septicaemia. Scand. J. Infect. Dis. (suppl.) 31:8-21.

Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson and N. T. Hargret. 1983. Hemorrhagic colitis associated with a rare Escherichia coli serotype. N. Engl. J. Med. 308:681-685.

Robbins, J. B., G. H. McCracken, E. C. Gotschlich, F. Ørskov, I. Ørskov and L. A. Hanson. 1974. Escherichia coli K1 capsular polysaccharide associated with neonatal meningitis. N. Engl. J. Med. 290:1216-1222.

Roberts, I., R. Mountford, N. High, D. Bitter-Suermann, K. Jann, K. Timmis and G. Boulnois. 1986a. Molecular cloning and analysis of genes for production of K5, K7, K12 and K92 capsular polysaccharides in Escherichia coli. J. Bacteriol. 168:1228-1233.

Roberts, M., S. ParthaSarathy, M. K. L. Lam-Po-Tang and P. H. Williams. 1986b. The aerobactin iron uptake system in enteropathogenic Escherichia coli : evidence for an extinct transposon. FEMS Microbiol. Lett. 37:215-219.

Roitt, I. M. 1984. Essential immunology, 5th edn. Blackwell Scientific Publications, Oxford.

Roosendaal, B., W. Gaastra and F. K. de Graaf. 1984. The nucleotide sequence of the gene encoding the K99 subunit of enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. 22:253-258.

Roosendaal, B., P. M. van Bergen en Henegouwen and F. K. de Graaf. 1986. Subcellular localization of K99 fimbrial subunits and effect of temperature on subunit synthesis and assembly. J. Bacteriol. 165:1029-1032.

Rothbard, J. B., R. Fernandez and G. K. Schoolnik. 1984. Strain-specific and common epitopes of gonococcal pili. J. Exp. Med. 160:208-221.

Rothbard, J. B., R. Fernandez, L. Wang, N. N. H. Teng and G. K. Schoolnik. 1985. Antibodies to peptides corresponding to a conserved sequence of gonococcal pilins block bacterial adhesion. Proc. Natl. Acad. Sci. USA 82:915-919.

Rothbaum, R., A. J. McAdams, R. Gianella and J. C. Partin. 1982. A clinicopathological study of enterocyte adherent Escherichia coli : a cause of protracted diarrhea in infants. Gastroenterol. 83:441-454.

Rudoy, R. C. and J. D. Nelson. 1975. Enteroinvasive and enterotoxigenic Escherichia coli : occurrence in acute diarrhea of infants and children. Amer. J. Dis. Child 129:668-672.

Runnels, P. L. and H. W. Moon. 1984. Capsule reduces adherence of enterotoxigenic Escherichia coli to isolated intestinal epithelial cells of pigs. Infect. Immun. 45:737-740.

Rutter, J. M., M. R. Burrows, R. Sellwood and R. A. Gibbons. 1975. A genetic basis for resistance to enteric disease caused by E. coli. Nature (London) 257:135-136.

Sack, R. B. 1975. Human diarrheal disease caused by enterotoxigenic Escherichia coli. Ann. Rev. Microbiol. 29:333-353.

Sack, D. A., S. Huda, P. K. B. Neogi, R. R. Daniel and W. M. Spira. 1980. Microtiter ganglioside enzyme-linked immunosorbent assay for Vibrio and Escherichia coli heat-labile enterotoxins and antitoxin. J. Clin. Microbiol. 11:35-40.

Salit, I. E. and E. C. Gotschlich. 1977a. Type 1 Escherichia coli pili : characterization of binding to monkey kidney cells. J. Exp. Med. 146:1182-1194.

Salit, I. E. and E. C. Gotschlich. 1977b. Haemagglutination by purified type 1 Escherichia coli pili. J. Exp. Med. 146:1169-1181.

Sandin, R. L., A. L. Rogers, R. J. Patterson and E. S. Beneke. 1982. Evidence for mannose-mediated adhesion of Candida albicans to human buccal cells in vitro. Infect. Immun. 35:79-85.

Sansonetti, P. J., D. J. Kopecko and S. B. Formal. 1981. Shigella sonnei plasmids : evidence that a large plasmid is necessary for virulence. Infect. Immun. 34:75-83.

Sansonetti, P. J., H. d'Hauteville, S. B. Formal and M. Toucas. 1982. Plasmid mediated invasiveness of "Shigella-like" E. coli. Ann. Inst. Pasteur (Microbiol.) 132A:351-355.

Sansonetti, P. J., T. L. Hale, G. J. Damin, C. Kapfer, H. H. Collins Jr. and S. B. Formal. 1983. Alterations in the pathogenicity of Escherichia coli K-12 after transfer of plasmids and chromosomal genes from Shigella flexneri. Infect. Immun. 39:1392-1402.

Sastry, P. A., B. B. Finlay, B. L. Pasloske, W. Paranchych, J. R. Pearlstone and L. B. Smillie. 1985. Comparative studies of the amino acid and nucleotide sequences of pilin derived from Pseudomonas aeruginosa PAK and PAO. J. Bacteriol. 164:571-577.

Satterwhite, T. K., H. L. DuPont, D. G. Evans and D. J. Evans Jr. 1978. Role of Escherichia coli colonisation factor antigen in acute diarrhoea. Lancet ii:181-184.

Savage, D. C. 1977. Microbial ecology of the gastro-intestinal tract. Ann. Rev. Microbiol. 31:107-133.

Savage, D. C. 1980a. Colonization by and survival of pathogenic bacteria on intestinal mucosal surfaces. p. 175-206. In G. Bitton and K. C. Marshall (ed.), Adsorption of microorganisms to surfaces. Wiley-Interscience, London.

Savage, D. C. 1980b. Adherence of normal flora to mucosal surfaces, p. 31-59. In E. H. Beachey (ed.), Bacterial adherence. Receptors and recognition, series B, vol. 6. Chapman and Hall, London.

Savage, D. C. and M. Fletcher (ed.). 1985. Bacterial adhesion. Mechanisms and physiological significance. Plenum Press, New York.

Scaletsky, I. C. A., M. L. M. Silva and L. R. Trabulsi. 1984. Distinctive patterns of adherence of enteropathogenic Escherichia coli to HeLa cells. Infect. Immun. 45:534-536.

Scherer, W. F., J. T. Syverton and G. O. Gey. 1953. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J. Exp. Med. 97:695-709+plates.

Schmitt, R., R. Mattes, K. Schmid and J. Altenbuchner. 1979. Raf plasmids in strains of Escherichia coli and their possible role in enteropathogeny, p. 199-210. In K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam.

Scotland, S. M., N. P. Day and B. Rowe. 1980. Production of a cytotoxin affecting Vero cells by strains of Escherichia coli belonging to traditional enteropathogenic serogroups. FEMS Microbiol. Lett. 7:15-17.

Scotland, S. M., J. E. Richmond and B. Rowe. 1983a. Adhesion of enteropathogenic strains of Escherichia coli (EPEC) to HEp-2 cells is not dependent on the presence of fimbriae. FEMS Microbiol. Lett. 20:191-195.

Scotland, S. M., H. R. Smith, G. A. Willshaw and B. Rowe. 1983b. Verocytotoxin production in strains of Escherichia coli is determined by genes carried on bacteriophage. Lancet ii:216.

Scott, S. S. and D. C. Old. 1981. Mannose resistant and eluting (MRE) haemagglutinins, fimbriae and surface structures in strains of Haemophilus. FEMS Microbiol. Lett. **10**:235-240.J

Sedlock, D. M., H. F. Bartus, I. Zajac and P. Actor. 1982. Use of a hemadsorption technique to evaluate the stability of the hemagglutination reaction of Escherichia coli cultures possessing human colonization factor antigens. J. Clin. Microbiol. **15**:554-557.

Segal, E., E. Billyard, M. So, S. Storzbach and T. F. Meyer. 1985. Role of chromosomal rearrangement in Neisseria gonorrhoeae pilus phase variation. Cell **40**:293-300.

Sellwood, R., R. A. Gibbons, G. W. Jones and J. M. Rutter. 1975. Adherence of enteropathogenic Escherichia coli to pig intestinal brush borders the existence of two pig phenotypes. J. Med. Microbiol. **8**:405-411.

Serény, B. 1957. Experimental keratoconjunctivitis Shigellosa. Acta Microbiol. Acad. Sci. Hung. **4**:367-376.

Sheladia, V. L., J. P. Chambers, J. Guevara Jr. and D. J. Evans Jr. 1982. Isolation, purification, and partial characterization of type V-A hemagglutinin from Escherichia coli GV-12, 01:H-. J. Bacteriol. **152**:757-761.

Sher, A., B. F. Hall and M. A. Vadas. 1978. Acquisition of murine major histocompatibility complex gene products by schistosomula of Schistosoma mansoni. J. Exp. Med. **148**:46-57.

Shingler, V. and C. M. Thomas. 1984. Analysis of the trfA region of broad host-range plasmid RK2 by transposon mutagenesis and identification of polypeptide products. Mol. Biol. **175**:229-249.

Shipley, P. L., C. L. Gyles and S. Falkow. 1978. Characterization of plasmids that encode for the K88 colonization antigen. Infect. Immun. **20**:559-566.

Shipley, P. L., W. S. Dallas, G. Dougan and S. Falkow. 1979. Expression of plasmid genes in pathogenic bacteria, p. 176-180. In D. Schlessinger (ed.), Microbiology - 1979. American Society for Microbiology, Washington, DC.

Shipley, P. L., G. Dougan and S. Falkow. 1981. Identification and cloning of the genetic determinant that encodes for the K88ac adherence antigen. J. Bacteriol. **145**:920-925.

Silva, R. M., M. R. F. Toledo and L. R. Trabulsi. 1980. Biochemical and cultural characteristics of invasive E. coli. J. Clin. Microbiol. **11**:441-444.

Silver, R. P., C. W. Finn, W. F. Vann, W. Aaronson, R. Schneerson, P. J. Kretschmer and C. F. Garon. 1981. Molecular cloning of the K1 capsular polysaccharide genes of E. coli. Nature (London) **289**:696-698.

Silver, R. P., W. F. Vann and W. Aaronson. 1984. Genetic and molecular analysis of the Escherichia coli K1 antigen. J. Bacteriol. 157:568-575.

Simon, M., J. Zieg, M. Silverman, G. Mandel and R. Doolittle. 1980. Genes whose mission is to jump. Science 209:1370-1374.

Small, P. M., M. G. Tauber, C. J. Hackbarth and M. A. Sande. 1986. Influence of body temperature on bacterial growth rates in experimental pneumococcal meningitis in rabbits. Infect. Immun. 52:484-487.

Smith, H. W. 1974. A search for transmissible pathogenic characters in invasive strains of Escherichia coli : the discovery of a plasmid-controlled toxin and a plasmid-controlled lethal character associated, or identical, with colicine V. J. Gen. Microbiol. 83:95-111.

Smith, H. W. 1976. Neonatal Escherichia coli infections in domestic mammals : transmissibility of pathogenic characteristics, p. 45-72. In Acute diarrhoea in childhood. Ciba Foundation Symp., vol. 42. Elsevier/North-Holland, Amsterdam.

Smith, H. 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. Rev. 41:475-500.

Smith, J. M. and J. S. Gots. 1980. purF-lac fusion and direction of purF transcription in Escherichia coli. J. Bacteriol. 143:1156-1164.

Smith, H. W. and M. A. Linggood. 1971. Observations on the pathogenic properties of the K88, haemolysin and enterotoxin plasmids of Escherichia coli with particular reference to porcine diarrhoea. J. Med. Microbiol. 4:467-485.

Smith, H. W. and M. A. Linggood. 1972. Further observations on Escherichia coli enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains : the transmissible nature of these enterotoxins and of a K antigen possessed by calf and lamb strains. J. Med. Microbiol. 5:243-250.

Smith, H. W. and M. B. Huggins. 1976. Further observations on the association of the colicine V plasmid of Escherichia coli with pathogenicity and with survival in the alimentary tract. J. Gen. Microbiol. 92:335-350.

Smith, H. W. and M. B. Huggins. 1980. The association of the O18, K1 and H7 antigens of the ColV plasmid of a strain of Escherichia coli with its virulence and immunogenicity. J. Gen. Microbiol. 121:387-400.

Smith, H. R., A. Cravioto, G. A. Willshaw, M. M. McConnell, S. M. Scotland, R. J. Gross and B. Rowe. 1979. A plasmid encoding for the production of colonisation factor antigen I and heat stable enterotoxin in strains of Escherichia coli of serogroup O78. FEMS Microbiol. Lett. 6:255-260.

Smith, H. R., G. A. Willshaw and B. Rowe. 1982. Mapping of a plasmid coding for colonization factor antigen I and heat-stable enterotoxin production, isolated from an enterotoxigenic strain of Escherichia coli. J. Bacteriol. 149:264-275.

Smith, H. W., P. Green and Z. Parsell. 1983a. Vero cell toxins in E. coli and related bacteria : transfer by phage and conjugation and toxic action in laboratory animals, chickens and pigs. J. Gen. Microbiol. 129:3121-3137.

Smith, H. R., S. M. Scotland and B. Rowe. 1983b. Plasmids that code for the production of colonization factor antigen II and enterotoxin production in strains of Escherichia coli. Infect. Immun. 40:1236-1239.

Smith, H. R., S. M. Scotland and B. Rowe. 1985. Genetics of Escherichia coli virulence, p. 227-269. In M. Sussman (ed.), The virulence of Escherichia coli. Reviews and methods. Special Publications of the Society for General Microbiology, vol. 13. Academic Press Inc. (London) Ltd., London.

Smyth, C. J. 1980. Serological heterogeneity and biotype specificity of mannose resistant haemagglutinins on enterotoxigenic Escherichia coli of serotype O6:K15:H16 or H- isolated from travellers' and infantile diarrhoea. FEMS Symposium on Microbial Envelopes, May 26-30, Saimaanranta, Finland, A109.

Smyth, C. J. 1982. Two mannose-resistant haemagglutinins on enterotoxigenic Escherichia coli of serotype O6:K15:H16 or H- isolated from travellers' and infantile diarrhoea. J. Gen. Microbiol. 128:2081-2096.

Smyth, C. J. 1984. Serologically distinct fimbriae on enterotoxigenic Escherichia coli of serotype O6:K15:H16 or H-. FEMS Microbiol. Lett. 21:51-57.

Smyth, C. J., B. Kaisjer, E. Back, A. Faris, R. Möllby, O. Soderlind, G. Stintzing, T. Wadstrom and D. Habte. 1979. Occurrence of adhesins causing mannose resistant haemagglutination of bovine erythrocytes in enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. 5:85-90.

So, M., H. W. Boyer, M. Betlach and S. Falkow. 1976. Molecular cloning of an Escherichia coli plasmid determinant that encodes for the production of heat stable enterotoxin. J. Bacteriol. 128:463-472.

So, M., E. Billyard, C. Deal, E. Getzoff, P. Hagblom, T. F. Meyer, E. Segal and J. Tainer. 1985. Gonococcal pilus : genetics and structure. Curr. Top. Microbiol. Immun. 118:13-28.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Speert, D. P., B. A. Loh, D. A. Cabral and I. E. Salit. 1986. Nonopsonic phagocytosis of nonmucoid <u>Pseudomonas aeruginosa</u> by human neutrophils and monocyte-derived macrophages is correlated with bacterial piliation and hydrophobicity. Infect. Immun. 53:207-212.

Spicer, E. K., W. M. Kavanaugh, W. S. Dallas, S. Falkow, W. H. Konisberg and D. E. Schafer. 1981. Sequence homologies between A subunits of <u>Escherichia coli</u> and <u>Vibrio cholerae</u> enterotoxins. Proc. Natl. Acad. Sci. USA 78:50-54.

Stanier, R. Y., E. A. Adelberg and J. L. Ingraham. 1977. General microbiology. MacMillan Press Ltd., London.

Stern, A., P. Nickel, T. F. Meyer and M. So. 1984. Opacity determinants of <u>Neisseria gonorrhoeae</u> : gene expression and chromosomal linkage to the gonococcal pilus gene. Cell 37:447-456.

Stern, A., M. Brown, P. Nickels and T. F. Meyer. 1986. Opacity genes in <u>Neisseria gonorrhoeae</u>: control of phase and antigenic variation. Cell 47:61-71.

Steven, A. C., M. E. Bisher, B. L. Trus, D. Thomas, J. Ming Zhang and J. L. Cowell. 1986. Helical structure of <u>Bordetella pertussis</u> fimbriae. J. Bacteriol. 167:968-974.

Stirm, S., I. Ørskov and F. Ørskov. 1966. K88, an episome determined protein antigen of <u>Escherichia coli</u>. Nature (London) 209:507-508.

Stirm, S., F. Ørskov, I. Ørskov and A. Birch-Andersen. 1967a. Episome-carried surface antigen K88 of <u>Escherichia coli</u>. III. Morphology. J. Bacteriol. 93:740-748.

Stirm, S., F. Ørskov, I. Ørskov and B. Mansa. 1967b. Episome carried surface antigen K88 of <u>Escherichia coli</u>. II. Isolation and chemical analysis. J. Bacteriol. 93:731-739.

Strockbine, N. A., L. R. M. Marques, J. W. Newland, H. W. Smith, R. K. Holmes and A. D. O'Brien. 1986. Two toxin-converting phages from <u>Escherichia coli</u> O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. Infect. Immun. 53:135-140.

Stuart, S. J., K. T. Greenwood and R. K. J. Luke. 1980. Hydroxamate-mediated transport of iron controlled by ColV plasmids. J. Bacteriol. 143:35-42.

Sugiyama, H. 1980. <u>Clostridium botulinum</u> neurotoxin. Microbiol. Rev. 44:419-448.

Sussman, M. 1985. <u>Escherichia coli</u> in human and animal disease, p. 7-45. <u>In</u> M. Sussman (ed.), The virulence of <u>Escherichia coli</u>. Reviews and methods. Special Publications of the Society for General Microbiology, vol. 13. Academic Press Inc. (London) Ltd., London.

Svanborg-Edén, C., L. Hagberg, L. A. Hanson, S. Hull, R. Hull, U. Jodal, H. Leffler, H. Lomberg and E. Straube. 1983. Bacterial adherence - a pathogenic mechanism in urinary tract infections caused by Escherichia coli. Prog. Allergy 33:175-188.

Svanborg-Edén, C., L. -M. Bjursten, R. Hull, S. Hull, K. -E. Magnusson, Z. Moldovano and H. Leffler. 1984. Influence of adhesins on the interaction of Escherichia coli with human phagocytes. Infect. Immun. 44:672-680.

Svenson, S. B. and G. Källenius. 1983. Density and localization of P-fimbriae-specific receptors on mammalian cells : fluorescence-activated cell analysis. Infection 11:6-12.

Swan_ey, L. M., Y. -P. Liu, C. -M. To, K. Ippen-Ihler and C. C. Brinton Jr. 1977a. Isolation and characterization of Escherichia coli phase variants and mutants deficient in type 1 pilus production. J. Bacteriol. 130:495-505.

Swaney, L. M., Y. -P. Liu, K. Ippen-Ihler and C. C. Brinton Jr. 1977b. Genetic complementation analysis of Escherichia coli type 1 somatic pilus mutants. J. Bacteriol. 130:506-511.

Takeuchi, A., L. R. Inman, P. D. O'Hanley, J. R. Cantey and W. B. Lushbaugh. 1978. Scanning and transmission electron microscopic study of Escherichia coli 015 (RDEC-1) enteric infection in rabbits. Infect. Immun. 19:686-694.

Thomas, L. V. and B. Rowe. 1982. The occurrence of colonization factors (CFA/I, CFA/II and E8775) in enterotoxigenic Escherichia coli from various countries in South East Asia. Med. Microbiol. Immun. 171:85-90.

Thomas, L. V., A. Cravioto, S. M. Scotland and B. Rowe. 1982. New fimbrial antigenic type (E8775) that may represent a colonization factor in enterotoxigenic Escherichia coli in humans. Infect. Immun. 35:1119-1124.

Thomas, L. V., M. M. McConnell, B. Rowe and A. M. Field. 1985. The possession of three novel coli surface antigens by enterotoxigenic Escherichia coli strains positive for the putative colonisation factor PCF8775. J. Gen. Microbiol. 131:2319-2326.

To, S. C. M. 1984. F41 antigen among porcine enterotoxigenic Escherichia coli strains lacking K88, K99 and 987P pili. Infect. Immun. 43:549-554.

To, S. C. M, H. W. Moon and P. L. Runnels. 1984. Type 1 pili (F1) of porcine enterotoxigenic Escherichia coli : vaccine trial and tests for production in the small intestine during disease. Infect. Immun. 43:1-5.

Toolan, H. W. 1954. Transplantable human neoplasmas maintained in cortisone-treated laboratory animals: H.S.-1; H.Ep.-1; H.Ep.-2; H.Ep.-3; and H.Emb.Rh.-1. Cancer Res. 14:660-666+plates.

Tuomanen, E. and A. Weiss. 1985. Characterization of two adhesins of Bordetella pertussis for human ciliated respiratory-epithelial cells. J. Infect. Dis. 152:118-125.

Uhlin, B. E., M. Båga, M. Goransson, F. P. Lindberg, B. Lund, M. Norgren and S. Normark. 1985a. Genes determining adhesin formation in uropathogenic Escherichia coli. Curr. Top. Microbiol. Immun. 118:163-178.

Uhlin, B. E., M. Norgren, M. Båga and S. Normark. 1985b. Adhesion to human cells by Escherichia coli lacking the major subunit of a digalactoside-specific pilus-adhesin. Proc. Natl. Acad. Sci. USA 82:1800-1804.

Ulshen, M. H. and J. L. Rollo. 1980. Pathogenesis of Escherichia coli gastroenteritis in man - another mechanism. New Engl. J. Med. 302:99-101.

Vaisanen, V., J. Elo, L. G. Tallgren, A. Siitonen, P. H. Mäkela, C. Svanborg-Edén, G. Kallenius, S. B. Svenson, H. Hultberg and T. K. Korhonen. 1981. Mannose-resistant haemagglutination and P-antigen-recognition are characteristic of Escherichia coli causing primary pyelonephritis. Lancet ii:1366-1369.

Väisänen-Rhen, V. 1984. Fimbria-like hemagglutinin in Escherichia coli O75 strains. Infect. Immun. 46:401-407.

Väisänen-Rhen, V., J. Elo, E. Väisänen, A. Siitonen, I. Ørskov, F. Ørskov, S. B. Svenson, P. H. Mäkela and T. K. Korhonen. 1984. P-fimbriated clones among uropathogenic Escherichia coli strains. Infect. Immun. 43:149-155.

van den Bosch, J. F., U. Verboom-Sohmer, P. Postma, J. de Graaf and D. M. MacLaren. 1980. Mannose-sensitive and mannose-resistant adherence to human uroepithelial cells and urinary virulence of Escherichia coli. Infect. Immun. 29:226-233.

van Die, I., and H. Bergmans. 1984. Nucleotide sequence of the gene encoding the $F7_2$ fimbrial subunit of a uropathogenic Escherichia coli strain. Gene 32:83-90.

van Die, I., H. E. N. Bergmans and W. P. M. Hoekstra. 1983a. Transformation in Escherichia coli : studies on the role of the heat shock in induction of competence. J. Gen. Microbiol. 129:663-670.

van Die, I., C. van den Hondel, H. -J. Hamstra, W. Hoekstra and H. Bergmans. 1983b. Studies on the fimbriae of an Escherichia coli O6:K2:H1:F7 strain : molecular cloning of a DNA fragment encoding a fimbrial antigen responsible for mannose-resistant haemagglutination of human erythrocytes. FEMS Microbiol. Lett. **19**:77-82.

van Die, I., B. van Geffen, W. Hoekstra and H. Bergmans. 1984a. Type 1C fimbriae of a uropathogenic Escherichia coli strain : cloning and characterization of the genes involved in the expression of the 1C antigen and nucleotide sequence of the subunit gene. Gene **34**:187-196.

van Die, I., I. van Megen, W. Hoekstra and H. Bergmans. 1984b. Molecular organisation of the genes involved in the production of $F7_2$ fimbriae, causing mannose-resistant haemagglutination, of a uropathogenic Escherichia coli O6:K2:H1:F7 strain. Mol. Gen. Genet. **194**:528-533.

van Die, I., G. S. Spierings, I. van Megen, E. Zuidweg, W. Hoekstra and H. Bergmans. 1985. Cloning and genetic organisation of the gene cluster encoding $F7_1$ fimbriae of a uropathogenic Escherichia coli and comparison with the $F7_2$ gene cluster. FEMS Microbiol. Lett. **28**:329-334.

van Die, I., M. Dijksterhuis, H. de Cock, W. Hoekstra and H. Bergmans. 1986a. Structural variation of P-fimbriae from uropathogenic Escherichia coli, p. 39-46. In D. L. Lark (ed.), Protein-carbohydrate interactions in biological systems. Academic Press Inc. (London) Ltd., London.

van Die, I., I. van Megen, E. Zuidweg, W. Hoekstra, H. de Ree, H. van den Bosch and H. Bergmans. 1986b. Functional relationships among the gene clusters encoding $F7_1$, $F7_2$, F9, and F11 fimbriae of human uropathogenic Escherichia coli. J. Bacteriol. **167**:407-410.

van Doorn, J., B. Oudega, F. R. Mooi and F. K. de Graaf. 1982. Subcellular location of polypeptides involved in the biosynthesis of K88ab fimbriae. FEMS Microbiol. Lett. **13**:99-104.

van Embden, J. D. A., F. K. de Graaf, L. M. Schouls and J. S. Teppema. 1980. Cloning and expression of a DNA fragment that encodes for the adhesive antigen K99. Infect. Immun. **29**:1125-1133.

Varian, S. A. and E. M. Cooke. 1980. Adhesive properties of Escherichia coli from urinary tract infections. J. Med. Microbiol. **13**:111-119.

Virji, M. and J. E. Heckels. 1984. The role of common and type-specific pilus antigenic domains in adhesion and virulence of gonococci for human epithelial cells. J. Gen. Microbiol. **130**:1089-1095.

Waalwijk, C., D. M. McLaren and J. de Graaf. 1983. In vivo function of hemolysin in the nephropathogenicity of Escherichia coli. Infect. Immun. 42:245-249.

Walz, W., M. A. Schmidt, A. F. Labigne-Roussel, S. Falkow and G. K. Schoolnik. 1985. AFA-I - a cloned afimbrial X-type adhesin from a human pyelonephritic E. coli strain. Eur. J. Biochem. 152:315-321.

Weerkamp, A. H., H. C. van der Mei and R. S. B. Liem. 1986a. Structural properties of fibrillar proteins isolated from the cell surface and cytoplasm of Streptococcus salivarius (K+) cells and nonadhesive mutants. J. Bacteriol. 165:756-762.

Weerkamp, A. H., P. S. Handley, A. Barrs and J. W. Slot. 1986b. Negative staining and immunoelectron microscopy of adhesion-deficient mutants of Streptococcus salivarius reveal that the adhesive protein antigens are separate classes of cell surface fibril. J. Bacteriol. 165:746-755.

Weinberg, E. D. 1978. Iron and infection. Microbiol. Rev. 42:45-66.

Weiss, A. A. and S. Falkow. 1984. Genetic analysis of phase change in Bordetella pertussis. Infect. Immun. 43:263-269.

Welch, R., E. Patchen Dellinger, B. Minshew and S. Falkow. 1981. Hemolysin contributes to virulence of extraintestinal Escherichia coli infections. Nature (London) 294:665-667.

Welch, R. A., T. Felmlee, S. Pellett and D. E. Chenoweth. 1986. The Escherichia coli haemolysin: its gene organization and interaction with neutrophil receptors, p. 431-438. In D. L. Lark (ed.), Protein-carbohydrate interactions in biological systems. Academic Press Inc. (London) Ltd., London.

Wevers, P., R. Picken, G. Schmidt, B. Jann, K, Jann, J. R. Golecki and M. Kist. 1980. Characterization of pili associated with Escherichia coli O18ac. Infect. Immun. 29:685-691.

Williams, P. H. and H. K. George. 1979. ColV plasmid-mediated iron uptake and the enhanced virulence of invasive strains of Escherichia coli, p. 161-172. In K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland, Amsterdam.

Williams, P. H. and P. J. Warner. 1981. Plasmid specified iron uptake by bacteraemic strains of Escherichia coli, p. 123-133. In S. Levy, R. C. Clowes and E. L. Koenig (ed.), Molecular biology, pathogenicity and ecology of bacterial plasmids. Plenum Press, London.

Williams, P. H., N. Evans, P. Turner, R. H. George and A. S. McNeish. 1977. Plasmid mediating mucosal adherence in human enteropathogenic Escherichia coli. Lancet i:1151.

Williams, P. H., M. I. Sedgwick, N. Evans, P. J. Turner, R. H. George and A. S. McNeish. 1978a. Adherence of an enteropathogenic strain of Escherichia coli to human intestinal mucosa is mediated by a colicinogenic conjugative plasmid. Infect. Immun. 22:393-402.

Williams, P. H., M. I. Sedgwick and A. S. McNeish. 1978b. Characterisation of a plasmid of Escherichia coli controlling adherence to human intestinal mucosa. Heredity 40:329.

Williams, P. H., S. Knutton, M. G. M. Brown, D. C. A. Candy and A. S. McNeish. 1984. Characterization of nonfimbrial mannose-resistant protein hemagglutinins of two Escherichia coli strains isolated from infants with enteritis. Infect. Immun. 44:592-598.

Willshaw, G. A., H. R. Smith, M. M. McConnell, E. A. Barclay, J. K. Krnjulac and B. Rowe. 1982. Genetics and molecular studies of plasmids coding for colonization factor antigen I and heat-stable enterotoxin in several Escherichia coli serotypes. Infect. Immun. 37:858-868.

Willshaw, G. A., H. R. Smith and B. Rowe. 1983. Cloning of regions encoding colonization factor antigen I and heat-stable enterotoxin in Escherichia coli. FEMS Microbiol. Lett. 16:101-106.

Willshaw, G. A., H. R. Smith, M. M. McConnel and B. Rowe. 1985a. Expression of cloned plasmid regions encoding colonisation factor antigen I (CFA/I) in Escherichia coli. Plasmid 13:8-16.

Willshaw, G. A., H. R. Smith, S. M. Scotland and B. Rowe. 1985b. Cloning of genes determining the production of Vero cytotoxin by Escherichia coli. J. Gen. Microbiol. 131:3047-3053.

Worobec, E. A., P. Shastry, W. Smart, R. Bradley, B. Singh and W. Paranchych. 1983. Monoclonal antibodies against CFA/I pili from enterotoxigenic Escherichia coli. Infect. Immun. 41:1296-1301.

Wray, C. and J. A. Morris. 1985. Aspects of colibacillosis in farm animals. J. Hyg. Camb. 95:577-593.

Wray, S. K., S. I. Hull, R. G. Cook, J. Barrish and R. A. Hull. 1986. Identification and characterization of a uroepithelial cell adhesin from a uropathogenic isolate of Proteus mirabilis. Infect. Immun. 54:43-49.

Yamamoto, T. and T. Yokota. 1981. Escherichia coli heat-labile enterotoxin genes are flanked by repeated deoxyribonucleic acid sequences. J. Bact. 145:850-860.

Yamamoto, T. and T. Yokota. 1983. Plasmids of enterotoxigenic Escherichia coli H10407 : evidence for two heat-stable enterotoxin genes and a conjugal transfer system. J. Bacteriol. 153:1352-1360.

Yamamoto, T., T. Honda, T. Miwatani and T. Yokota. 1984. A virulence plasmid in Escherichia coli enterotoxigenic for humans : intergenetic transfer and expression. J. Infect. Dis. 150:688-698.

Yamamoto, T., Y. Tamura and T. Yokota. 1985. Enteroadhesion fimbriae and enterotoxin of Escherichia coli : genetic transfer to a streptomycin resistant mutant of the galE oral-route live-vaccine Salmonella typhi Ty21a. Infect. Immun. 50:925-928.

Youmans, G. P., P. Y. Paterson and H. M. Sommers. 1980. The biologic and clinical basis of infectious diseases, 2nd edn. W. B. Saunders Co., Philadelphia.

Young, D. H., W. P. C. Stemmer and L. Sequeira. 1985. Reassembly of a fimbrial haemagglutinin from Pseudomonas solanacearum after purification of the subunit by preparative SDS-PAGE. Appl. Env. Microbiol. 50:605-610.

Zieg, J., M. Silverman, M. Hilmen and M. Simon. 1977. Recombinational switch for gene expression. Science 196:170-172.

Zieg, J., M. Hilmen and M. Simon. 1978. Regulation of gene expression by site-specific inversion. Cell 15:237-244.

A^n Absorbance at wavelength (n) nm

DNA   deoxyribonucleic acid

EIEC   enteroinvasive E. coli

EM   electron microscopy

EPEC   enteropathogenic E. coli

ETEC   enterotoxigenic E. coli

HA   haemagglutination

kb   kilobase(pair)

kd   kilodalton

LT   E. coli heat-labile enterotoxin

MR   mannose-resistant

MRHA   mannose-resistant haemagglutination

MS   mannose-sensitive

MSHA   mannose-sensitive haemagglutination

NCHA   nitrocellulose haemadsorption

SEM   scanning electron microscopy

ST   E. coli heat-stable enterotoxin

TEM   transmission electron microscopy

UPEC   uropathogenic E. coli

UTI   urinary tract infection

In addition, common abbreviations, S.I. units and multipliers are used throughout. Standard designations are used for chemical formulae, genotypes, phenotypes, strains and plasmids, and journal references follow the style and abbreviations used in Infection and Immunity.

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Oh, and hello to anyone else who knows me.

Abstract. Cloning of an Escherichia coli adhesin by Gary Hinson.

Pathogenic bacteria colonise their host animals by means of a complex set of interactions. The host defensive mechanisms attack foreign microorganisms and attempt to rid the animal of the invaders, while the bacteria express a variety of functions to ensure their survival under adverse conditions, some of which damage the host and cause the clinical symptoms of disease. Adhesins are the bacterial structures which mediate adherence to specific host tissues and therefore permit the colonisation of areas from which the bacteria would normally be removed.

I have genetically cloned and analysed an adhesin from a pathogenic strain of Escherichia coli isolated from a child with enteritis. The genetic information was transferred to laboratory strains of E. coli and was expressed under similar conditions as in the parent strain, generating material with the same adherence and antigenic properties. Thus, the cloned genes enabled laboratory strains to adhere to human colon, but not to duodenum, in the same manner as the parent. This probably accounts in large part for the tissue specificity of the pathogen which caused dysentery-like symptoms consistent with colonisation of, and damage to, the colon.

The cloned genes encoded the synthesis of the adhesin as fine fibrils ('fimbriae') on the bacterial surface, approximately 2 nm in diameter. The 14,000 dalton protein subunits were assembled into very high molecular weight aggregates and were purified by size fractionation. The genetic determinant occupied about 6,000 basepairs of DNA, indicating a system of genes for the synthesis, export and assembly of functional adhesin. The genetic map was very similar to those of adhesins from another enteritis isolate and a urinary tract pathogen, suggesting an evolutionary relationship between these E. coli strains. However, the protein subunits of the three adhesins appear to differ, indicating a degree of divergence.