A molecular analysis of non-fimbrial adhesins in uropathogenic *Escherichia coli*

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Declaration

The experiments and composition of this thesis are entirely the work of the author

ABSTRACT

Bacterial adherence is one of the most important virulence factors in the pathogenesis of urinary tract infections. The majority of clinical isolates of *Escherichia coli* adhere to uroepithelial cells via specific organelles known as fimbriae or pili which project from the surface. A proportion of urinary isolates possess adhesins which diffusely surround the bacteria forming an adhesive protein capsule. These are described as non-fimbrial adhesins. The molecular biology and clinical significance of this group of adhesins is relatively poorly understood.

The work presented in this thesis describes the cloning and comparison of four non-fimbrial adhesins (NFA-1 to NFA-4) identified in strains of *Escherichia coli* isolated from patients with urinary tract infections. The NFA-2 and NFA-4 adhesin subunit gene sequences were determined.

The gene complexes encoding the four non-fimbrial adhesins were of similar size and complexity. NFA-1 and NFA-2 were antigenically distinct but shared a close degree of nucleotide and amino acid homology. Comparison of NFA-1 and NFA-2 sequences with other published sequences showed that they were related to members of the Dr adhesin family. NFA-3 and NFA-4 appeared to be unrelated adhesins, however the nucleotide sequence of the NFA-4 adhesin subunit proved to be identical to the previously described M-adhesin.

The clinical importance of this group of non-fimbrial adhesins was investigated by colony blotting studies. The results showed that NFA-1, NFA-2 and NFA-4 were associated with isolates causing lower urinary tract infections but NFA-3 did not appear to play an important role in urinary tract infection. The significance of the results is discussed.

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CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Urinary tract infections

Definition

Urinary tract infections are frequently encountered in hospital and general practice. The term urinary tract infection includes a number of different clinical conditions including asymptomatic bacteruria, urethritis, cystitis, and pyelonephritis. Lower urinary tract infections are confined to the bladder and urethra, whereas upper urinary tract infections involve the ureters and kidneys and may be accompanied by blood-borne spread of infection (bacteraemia or septicaemia). Uncomplicated urinary tract infections are those which occur in previously healthy individuals with a structurally normal urinary tract. Complicated infections arise in the presence of structural or functional abnormalities of the urinary tract or in patients with co-existing medical problems (eg diabetes, immunodeficiency).

Urine is normally sterile. The clinical features of urinary tract infection include the presence of organisms and inflammatory cells in the urine. Microorganisms are frequently cultured from voided specimens and these organisms may be classified as commensal or pathogenic. Commensal organisms colonise the lower urethra and vagina and small numbers of these organisms may contaminate the urine during voiding. Uropathogenic organisms are those which grow in urine and ascend the urinary tract causing inflammation and symptoms. The distinction between contamination and infection of urine was investigated by Kass (1957) who first introduced the concept of significant bacteruria. He showed that true bacteruria could be distinguished from contamination of urine by the bacterial count. The figure of $>10^8$ bacteria/litre ($>10^5$ bacteria/ml) from a mid-stream urine specimen is conventionally used as the definition of a significant urinary tract infection, although the presence of lower numbers of organisms may sometimes be clinically relevant.

Clinical features

Bacterial urinary tract infection does not always result in symptoms. Asymptomatic bacteriuria is seen most frequently in children, pregnant women and the elderly. In patients with symptoms the clinical manifestations depend on the location of infection. The symptoms associated with lower urinary tract infection include dysuria, frequency, nocturia and lower abdominal pain. Fever and systemic features are usually absent. The symptoms of upper urinary tract infections are often severe. Acute pyelonephritis characteristically presents with fever, loin pain and rigors. Invasive infection may lead to septicaemia and septic shock. In infants and young children the clinical features of urinary tract infection are often non-specific and may include fever, febrile convulsions, vomiting, loss of appetite, failure to thrive, and difficulty or refusal to pass urine.

Routes of infection

The majority of bacteria responsible for urinary tract infections are bowel commensals which ascend the urinary tract (Cox *et al* 1968, Grunberg 1969). The evidence for this comes from long-term follow-up studies of women suffering from recurrent urinary tract infections. These studies showed that colonisation of the lower urethra with faecal organisms occurred shortly before the development of symptomatic urinary tract infection (Stamey *et al* 1971). Ascending urinary tract infection is also associated with the presence of indwelling urinary catheters, vesicoureteric reflux, and urinary stasis resulting from prostatic hypertrophy, hydronephrosis or stones.

Haematogenous spread of infection is of relatively lesser importance in the urinary tract, but occasionally follows episodes of generalised septicaemia. A wide range of organisms are associated with blood-borne infection, including Salmonella spp, Enterococci, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and Candida spp. These infections are most commonly seen in the context of a severely ill or immunocompromised patient. There is little evidence for the spread of organisms into the urinary tract via the lymphatic system.

Animal models of infection

Animal studies have played an important role in the investigation of bacterial virulence, and the pathogenesis of urinary tract infection. Early experimental difficulties led to the realisation that the species of animal used was of crucial importance, as many bacterial virulence factors have a narrow species specificity.

A mouse model of ascending infection was developed by Hagberg *et al* (1983a). Bacteria are introduced into the bladder using a plastic catheter and virulent organisms ascend the ureters to cause renal infection. Pathological changes in the kidney correlate closely with bacterial localisation of infection and histological features allow a distinction to be made between bacterial colonisation, pyelitis and nephritis (Johnson *et al* 1992). The mouse is a good model of ascending infection, however the findings of animal experiments must be interpreted with caution as host factors are of great importance and the results may not be applicable to human disease.

A haematogenous model of infection has also been described in the mouse (Gorill and DeNavasquez 1964, Ivanyi *et al* 1983). In this model bacteria are injected intravenously and organisms are rapidly cleared by the reticuloendothelial system in healthy animals. Infection will only become established in the kidneys if they have previously been damaged by trauma or ureteric ligation.

Primates have been experimentally infected with uropathogenic organisms (Roberts *et al* 1984). However this model is likely to have only a limited use; perhaps for testing candidate drugs or vaccines immediately prior to human trials.

1.2 Microbiology of urinary tract infections

Although urinary tract infections are an important cause of morbidity and mortality, they occur relatively rarely in healthy people. The development of infection depends on the virulence of the infecting organism and the integrity of the host defence mechanisms. There are important differences in the spectrum of bacteria infecting previously healthy individuals and those encountered in patients who have structural abnormalities of the urinary tract or underlying immunosuppression (Table 1.1) *Escherichia coli* is responsible for approximately 85% of community acquired infections, with Proteus and Klebsiella spp occurring relatively infrequently (Bryan and Reynolds 1984a). *Staphylococcus saprophyticus* is associated with over 20% of episodes of bacteruria in young women, but rarely causes severe symptoms (Wallmark *et al* 1978). A far wider range of organisms are associated with complicated urinary tract infections.

1.3 Host defence mechanisms

The urethra

The majority of uncomplicated urinary tract infections occur in women. Females have a greater susceptibility to ascending infection as the female urethra is relatively short and the vaginal introitus may be contaminated with faecal organisms (Hinman 1966, Bran *et al* 1972). Urinary flow characteristics are important in the initiation of bladder infection. Backflow of urine in the female urethra has been observed during micturition (Hinman 1966) and this process facilitates spread of colonising bacteria into the bladder.

Host defence mechanisms operating at the lower urethra include the flow of urine and the resident bacterial flora colonising the vaginal introitus. This flora normally includes Lactobacilli, Bacteroides, Streptococci, Corynebacteria and *Staphylococcus epidermidis* (Marrie *et al* 1980). The presence of lactobacilli appears to inhibit colonisation of the lower urethra with *E. coli* as vaginal flushes with Lactobacilli eradicated *E. coli* from the lower urinary tract in cynomolgus monkeys (Herthelius *et al* 1989), and prevented recurrent urinary tract infections in susceptible women (Bruce and Reid 1988). Antibiotic treatment alters the genital flora surrounding the ureteric orifice. Amoxycillin and cephalosporins have been shown to promote vaginal colonisation with *E. coli* and may increase the subsequent risk of urinary tract infection (Winberg *et al* 1993). Trimethoprim and nitrofurantoin appear to have a less marked effect.

The bladder

The most important defence mechanisms operating in the bladder are bladder emptying and the antimicrobial properties of the bladder mucosa (Cox and Hinman 1961). Conditions in the bladder correspond to a static chamber and the frequency of voiding and residual volume are crucial factors in the development of bladder infection (O'Grady and Cattell 1966b). Bladder emptying effectively removes the majority of infecting bacteria and women with bacteruria showed a dramatic reduction in bacterial count after fluid loading and hourly micturition (Cattell *et al* 1970). A residual volume >1ml is associated with bacteruria and a poor response to treatment (Shaud 1970).

The bladder mucosa also plays a significant role in the removal of infecting bacteria (Cox and Hinman 1961, Norden *et al* 1968). Defence mechanisms operating at the mucosal surface include secretory immunoglobulin (Hanson *et al* 1970, Svanborg-Eden and Svennerholm 1978) and the presence of a covering layer of mucin containing glycosaminoglycan polysaccharide (Parsons *et al* 1978). The importance of this layer was shown by Parsons *et al* (1978) who exposed the bladder mucosa to dilute hydrochloric acid. This process destroyed the coating mucopolysaccharide and resulted in a great increase in bacterial adherence.

The ureter

Ureteric defence mechanisms include urinary flow and the vesicoureteric valves which prevent reflux of urine during bladder emptying. Ureteric or intra-renal obstruction and vesicoureteric reflux are associated with a high risk of renal infection (Siegel *et al* 1980). The peristaltic action of the ureter causes turbulent flow of urine which contributes to the elimination of ascending bacteria (O'Grady and Cattell 1966a). Ureteric peristalsis is diminished during pregnancy and this may be one of the reasons for the increased incidence of pyelonephritis (Patterson and Andriole 1987).

The kidney

Bacterial colonisation of the renal medulla occurs readily under favourable conditions, however the cortex appears to be considerably more resistant to infection (Freedman and Beeson 1958). The possible reasons for increased susceptibility of the renal medulla include the presence of a high concentration of bacterial adhesin receptors and local physical conditions, with a high concentration of solutes and low oxygen concentration favouring bacterial growth.

Antibacterial properties of urine

The composition of urine has an important influence on bacterial infection and the integrity of local host defence mechanisms. Cox and Hinman (1961) showed that urine was generally a good bacterial culture medium, however some components of urine may inhibit bacterial growth (Kaye 1968). The antibacterial activity of urine does not appear to result from a lack of nutrients but is associated with increasing urea, solute and ammonia concentrations and low pH. In a large community study Waters *et al* (1967) found that the median value for urine pH was less in males than females and urine osmolality was greater in men. These findings would be consistent with a greater inhibitory role in men. The overall importance of urine composition in the pathogenesis of ascending infection remains unclear as urine production and concentration varies significantly at different times of day and the composition of urine also has significant effects on phagocytic function and complement activation (Chernew and Braude 1962, Freedman 1967).

One of the most important urinary defence mechanisms in the urinary tract is the production of uromucoid or Tamm-Horsfall protein which traps bacteria and prevents colonisation. The attachment of *E. coli* to uromucoid is mediated by type 1 fimbriae adhering to mannose-containing residues (Orskov *et al* 1980). Free oligosaccharide residues in the urine may also bind to bacterial adhesins, causing aggregation and elimination of infecting organisms (Jarvinen and Sandholm 1980).

A further host defence mechanism is the rapid shedding of viable cells from the urinary epithelium following bacterial infection. Any bacteria bound to these sloughed cells will be eliminated in the urine (Aronson *et al* 1988).

Host immunity in the urinary tract

Host immune mechanisms operating in the urinary tract include local and systemic antibody production, complement-mediated killing, neutrophil phagocytosis and cell-mediated immunity.

Secretory IgA and IgG are normally found in urine (Hanson *et al* 1970). Secretory IgA and IgG inhibit bacterial adhesion and the interaction between antigen and antibody activates complement (Svanborg-Eden and Svennerholm 1978). A range of antibodies are detected in the serum following pyelonephritis. These antibodies are directed against various bacterial components including fimbriae, O and K antigens (Svanborg-Eden and Svennerholm 1978, Rene *et al* 1982).

Lower urinary tract infections are not usually associated with a significant antibody response (Rene *et al* 1982), and humoral immunity is therefore thought to play a minor role in the elimination of bacteria from the bladder.

The classical complement pathway is activated by the presence of specific antibody and the alternative pathway may be activated by bacterial surface antigens. The result of complement activation is bacterial killing, however some serotypes are relatively resistant to complement lysis and these organisms appear to predominate in cases of pyelonephritis (Lomberg *et al* 1984).

Phagocytosis of bacteria by neutrophils and macrophages occurs if organisms invade the bladder or kidney (Cobbs and Kaye 1967), but is probably of little importance in most lower urinary tract infections as the composition of urine inhibits phagocyte function.

The importance of cell-mediated immunity is also uncertain. T-cell infiltration of the bladder and kidneys is associated with ascending infection in animal models of infection (Hjelm 1984), however patients with defects in T-cell-mediated immunity are not at particular risk of bacterial UTI.

1.4 Pre-disposing factors for urinary tract infection

Genetic predisposition

There is considerable evidence that susceptibility to urinary tract infection may be influenced by the host phenotype. Adherence of bacteria to specific receptors on host epithelial cells is one of the most important factors in the development of urinary infection and bacterial adherence to vaginal epithelial cells is increased in women who suffer from recurrent urinary tract infections (Svanborg-Eden and Jodal 1979, Schaeffer *et al* 1981). The variation in host susceptibility is thought to be related to the level of expression of adhesin receptors on host cells. Blood group determinants are of particular importance as they are recognised by many bacterial adhesins (Inane *et al* 1982, Lomberg *et al* 1983). Approximately 75% of the population are of blood group P_1 and express P, P_1 and P_k antigens on erythrocytes. Blood group P_2 is associated with low expression of P_1 and P_k and P_2 individual have a reduced incidence of recurrent pyelonephritis (Lomberg *et al* 1983). Secretor status is also an important factor as non-secretors have a threefold risk of developing recurrent urinary tract infections compared to secretors (Kinane *et al* 1982, Lomberg *et al* 1986, Sheinfeld *et al* 1989).

Females and sexual intercourse

The incidence of urinary tract infections in women shows a striking rise in early adult life and there is strong evidence for an association with sexual intercourse (Nicolle *et al* 1982, Remis *et al* 1987). In a study of pre-menopausal women with recurrent urinary tract infections, the majority of infections occurred within 24 hours of sexual intercourse (Nicolle *et al* 1982). Bran *et al* (1972) showed that urethral milking led to ascending infection in healthy human volunteers. Infection following sexual intercourse is thought to occur by a similar mechanical effect leading to bacterial contamination of the lower urethra and bladder. A recent case-controlled study showed that diaphragm use was also associated with an increased risk of developing urinary tract infection (Stamm *et al* 1989).

Pregnancy

Prevalence studies have shown that 2-11% of women have bacteruria at some stage in pregnancy (Stenquist *et al* 1989). The onset of bacterial colonisation usually occurs between the 9th and 17th week of pregnancy (Stenquist *et al* 1989). Several factors contribute to the increased susceptibility of the urinary tract in pregnant women, including dilation of the collecting system and ureters, reduced ureteric peristalsis and partial ureteric obstruction resulting from the enlarging uterus (Lindheimer and Katz 1970, Patterson and Andriole 1987). Hormonal changes during pregnancy are likely to be responsible for these physiological effects. Oestrogen levels may be important as Andriole and Cohn (1964) found that oestrogen treatment in rats caused hydronephrosis and associated infection. Post-menopausal women on oestrogen replacement therapy were recently found to have an increased risk of developing urinary tract infection compared to those not receiving oestrogen (Orlander *et al* 1992).

Children

Young children have a relatively high incidence of urinary tract infection compared to older children or adolescents. Infections of the urinary tract occurring in the neonatal period often result from bacteraemic spread rather than ascending infection and are associated with a severe systemic illness and poor prognosis (Ginsburg and McCracken 1982). Boys are responsible for 75% of urinary tract infections in the first three to six months of life, but only 10% of infections occurring in later childhood (Ginsburg and McCracken 1982, Spencer and Schaeffer 1986). The initial preponderance of urinary tract infections in boys is thought to be related to the presence of the foreskin (Ginsburg and McCracken 1982, Herzog 1989), and uncircumcised boys were found to have a twenty-fold greater incidence of urinary tract infections compared to those who have been circumcised (Wiswell *et al* 1985). The gut is colonised with enteric organisms in the first weeks of life and *E. coli* is the cause of over 80% of childhood urinary tract infections. *Klebsiella pneumoniae*, Group D Streptococci, and other organisms cause occasional infections (Ginsburg and McCracken 1982).

Congenital abnormalities of the renal tract which predispose to the development of childhood urinary tract infections include phimosis, posterior urethral valves, ureterocoele, and vesicoureteric reflux (Spencer and Schaeffer 1986). Radiographic abnormalities of the urinary tract are found in approximately 40% of infected girls, but only 10% of infected boys (Ginsburg and McCracken 1982, Snodgrass 1991). Vesicoureteric reflux can be demonstrated in nearly 50% of infants with upper urinary tract infections (Siegel *et al* 1980). Reflux is particularly important in the first two years of life but usually resolves in later childhood (Siegel *et al* 1980).

The elderly

The incidence of bacteruria increases with age until 70 and then remains stable. The age-related rise in incidence is particularly marked in men and the overall incidence of infection in men and women is approximately equal after the age of 65 (Baldessarre and Kaye 1991). Bacteruria in the elderly is frequently asymptomatic (Bakke and Vollset 1993).

Bacteruria in the elderly often reflects general disability and is associated with the presence of underlying medical disease, institutionalisation and need for catheterisation or instrumentation. Additional factors which contribute to the increased risk of infection in the elderly include urinary stasis resulting from prostatic hypertrophy in elderly men, decreasing attention to personal care and hygiene, presence of neuropathy causing inability to empty the bladder completely, declining immunity, and reduced secretion of uromucoid (Sobel and Kaye 1985).

Diabetes Mellitus

The incidence of bacteruria and symptomatic urinary tract infections is increased in patients with diabetes. Complications of infection are also more common and include renal papillary necrosis (Louler *et al* 1960), perinephric abscess (Thorley *et al* 1974) and fungal infection of the kidney (Mehnert and Mehnert 1958). Diabetics appear to be particularly prone to Klebsiella infection (Lye *et al* 1992).

Several factors are responsible for the increased risk of infection in diabetes. Glycosuria enhances growth and spread of many organisms including *E. coli* (Levison and Pitsakis 1984) and *Candida albicans* (Raffel *et al* 1981). In addition the presence of glycosuria inhibits phagocytosis and the host immune response (Chernow and Braude 1962). Diabetic neuropathy is associated with bladder paralysis and incomplete voiding resulting in urinary retention, and neurogenic incompetance of the vesicoureteric orifice, causing ureteric reflux. Kidneys damaged by diabetic nephropathy are more susceptible to ascending or haematogenous infection (Ellenberg 1976).

Obstructive uropathy

Urinary obstruction and stasis predisposes to the development of bacterial infection and hydronephrosis is a significant factor in the aetiology of ascending and haematogenous renal infection (Rocha *et al* 1958; Thorley *et al* 1974). The most common cause of obstruction is benign prostatic hypertrophy in men. Obstruction may also result from congenital abnormalities or urinary calculi.

Urinary tract calculi

Approximately 10-15% of calculi in the urinary tract are caused by bacterial infection (Lerner *et al* 1989). Infective stones are associated with Proteus and other urease producing bacteria (Lerner *et al* 1989), although they may also complicate *E. coli* infection (Ohkawa *et al* 1992). Stones associated with these bacterial infection are composed of magnesium ammonium phosphate and are known as struvite stones. The presence of urinary calculi may lead to urinary obstruction and epithelial damage resulting in further urinary tract infections. Calculi may also harbour bacteria which are difficult to eradicate and act as a source for recurrent infection.

Catheterisation

Catheterisation is associated with a very high incidence of urinary tract infection. The frequency of catheter-associated is increased in elderly and vulnerable patients (Turck *et al* 1962). The risk of infection is considerably reduced using a closed rather than open catheter system (Kunin and McCormack 1966), but bacteruria will still develop in 10-27% of patients within 5 days (Lancet 1991). The reasons for the greatly increased risk of infection include introduction of bacteria into the bladder during catheter insertion (Turck *et al* 1962), and infection by organisms ascending the lumen or outer surface of the catheter (Schaeffer and Chmiel 1983).

Bacteria isolated from catheterised patients have been shown to adhere strongly to the catheter material (Roberts *et al* 1993). Infection leads to the formation of a biofilm on the surface of the foreign body consisting of a collection of microorganisms and extracellular products. Bacteria within the biofilm are resistant to antibiotics and the presence of a foreign body is associated with a complex defect in polymorphonuclear function (Zimmerli *et al* 1984). Catheter associated infections therefore respond poorly to antibiotics and are difficult to eradicate.

Immunosuppression

A wide spectrum of pathogens may cause urinary tract infection in patients who are immunosuppressed as a result of drug treatment or malignant disease. This group of patients have an increased incidence and severity of urinary tract infections compared to healthy individuals. The reasons include compromised host immunity, epithelial damage resulting from the use of cytotoxic drugs, changes in the commensal bacterial flora following the use of antibiotics, and the increased risk of hospitalisation and catheterisation. Renal transplantation is a specific situation when anatomical changes in the urinary tract are combined with underlying medical disease, catheterisation and immunosuppressive therapy to give a high risk of infection. Neutropenia is associated with an increased risk of haematogenous renal infection (Sobel 1987), particularly if the absolute neutrophil count is below 500 x 10^9 cells/l.

The incidence of urinary tract infections appears to be slightly increased in patients infected with the human immunodeficiency virus (HIV), but urinary tract infections are not a major cause of morbidity or mortality. Kaplan *et al* (1987) reviewed the notes of 60 AIDS patients seen over a five year period and found that 52% had at least one period of documented pyuria and 20% had a symptomatic urinary tract infection. Welch *et al* (1989) documented bacteruria in 8% of 125 urine specimens obtained from HIV-infected homosexuals and none of 36 samples from HIV-negative homosexuals, however bacteruria was usually asymptomatic and clinically apparent urinary tract infections were rare. Approximately 20% of bacterial infections in HIV-positive children originate in the urinary tract (Bernstein *et al* 1985, Krasinski *et al* 1988). A wide range of organisms have been isolated from the urinary tract infections in HIV-positive patients including *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* Salmonella spp, *Cryptococcus neoformans, Mycobacterium tuberculosis,* atypical mycobacterial infection, adenovirus and cytomegalovirus (Kaplan *et al* 1987, Welch *et al* 1989).

1.5 Bacterial virulence determinants

Definition of virulence

Virulence can be defined as the capacity of an organism to cause disease. Bacteria which cause life-threatening infections in previously healthy people are clearly more virulent than those which only cause problems in patients with urinary tract abnormalities or those who are immunocompromised (Lomberg *et al* 1984, Johnson *et al* 1987, Stamm *et al* 1989). Bacterial virulence has been most extensively investigated in *E. coli* and known and putative *E. coli* virulence factors are shown in Table 1.2.

Bacterial adhesins

Bacteria which successfully invade the urinary tract must have the ability to withstand the process of urinary flow. Bacterial adherence to urinary epithelial cells is therefore one of the most important factors in the pathogenesis of infection. Further advantages of close adherence to host cells includes efficient transfer of nutrients from the host cell to the bacterium and effective targetting of bacterial toxins (Eisenstein 1988).

Adherence of bacterial isolates to uroepithelial cells in vitro correlates closely with their ability to cause clinical disease. *E. coli* obtained from patients with symptomatic urinary tract infections adhere more strongly than faecal isolates (Varian and Cooke 1980) or isolates from patients with asymptomatic bacteruria (Svanborg-Eden *et al* 1976). These findings are supported by animal studies of experimental pyelonephritis in the mouse and primate which have shown that ascending urinary tract infection may be prevented by anti-adhesin antibodies or the administration of epithelial cell-surface analogues which inhibit the adhesin-receptor interaction (Svanborg-Eden *et al* 1978, 1982; Hagberg *et al* 1983b, Roberts *et al* 1984).

Adherence of *E. coli* is usually associated with agglutination of red blood cells. Duguid *et al* (1955) were the first to demonstrate that bacterial haemagglutination was associated with the presence of filamentous projections on electron microscopy. These were termed fimbriae or pili and are approximately 10nm in diameter. Fimbriae appear to be the most important mechanism by which bacteria attach to uroepithelial cells, as adherence is markedly reduced in the presence of purified fimbriae or adhesin-specific antibody (Svanborg-Eden and de Man 1987).

Many distinct fimbrial adhesins have been identified in uropathogenic *E. coli*. Finer appendages, termed fibrillae, have also been described and some bacteria are surrounded by a diffuse layer of adhesin molecules which are known as non-fimbrial or afimbrial adhesins. Adhesins bind to specific receptor ligands on the surface of uroepithelial cells and are classified into two major groups, type 1 or mannose-sensitive fimbriae, and mannose-resistant adhesins. Mannose-resistant adhesins are further classified on the basis of receptor specificity into those which adhere to P blood group antigens (P-fimbriae) and a diverse group of X-adhesins. A variety of non-fimbrial adhesins have been described. These surround the bacteria like an adhesive protein capsule (Kroncke *et al* 1990). Non-fimbrial adhesins include the M adhesin (Rhen *et al* 1986a), the Dr adhesin family and a series of non-fimbrial adhesins (NFA's). The molecular biology of fimbrial and non-fimbrial adhesins will be discussed in detail in Sections 1.6 and 1.8.

Bacterial lipopolysaccharide

Lipopolysaccharide in gram-negative bacteria consists of three components; lipid A, a core region, and an outer polysaccharide. Lipid A anchors the cell wall to the bacterial outer membrane. The outer polysaccharide is of variable antigenicity and is known as the O antigen.

Lipopolysaccharide is believed to be an important virulence factor as uropathogenic strains of *E. coli* belong to a very restricted group of serotypes (Orskov and Orskov 1983). Colonisation of the urinary tract with a new O-serotype is often associated with symptomatic infection and antibodies to lipid A are detected after invasive bacterial infection.

Bacterial lipopolysaccharide may enhance virulence through a number of different mechanisms. Lipopolysaccharide is also known as endotoxin and has many effects on host cells. Endotoxin induces the host inflammatory response and is responsible for the clinical features of gram-negative shock (Wolff 1973, Wolff 1991). In addition lipopolysaccharide inhibits ureteric peristalsis (Teague and Boyarski 1968) and the O-serotypes commonly encountered in urinary tract infection are associated with resistance against complement-mediated lysis (Johnson 1991).

Bacterial capsular polysaccharide

Capsular polysaccharide is composed of linear repeated carbohydrate subunits which form a protective coat around the bacteria. Uropathogenic strains of *E. coli* express type II capsular polysaccharide which is acidic, highly charged, and relatively thin and patchy. The polysaccharide capsule determines the K-antigen specificity of *E. coli*. Although over 80 capsular serotypes have been identified, only a small minority are associated with urinary tract infections (Kaijser *et al* 1977). K1 polysaccharide is a sialic acid polymer which is poorly immunogenic and has an identical structure to the *Neisseria meningitidis* group B capsule. K1 capsular polysaccharide is of particular clinical importance as K1 strains of *E. coli* are responsible for over 80% of cases of neonatal meningitis (Robbins *et al* 1974) and approximately 25% of blood culture isolates (Pitt 1978).

Capsular polysaccharide is an important virulence factor as it inhibits the detection of O-antigen, protects bacteria against neutrophil phagocytosis (Svanborg-Eden and de Man 1987), and inhibits complement-mediated killing (Leying *et al* 1990).

The genes coding for the production of several capsular polysaccharides have recently been cloned (Echarti *et al* 1983, Roberts *et al* 1986, Boulnois *et al* 1987). Approximately 17kb of DNA is required for polysaccharide expression and the capsular gene cluster can be divided into three separate regions. These regions code for polysaccharide biosynthesis and polymerisation (region 2), translocation of polysaccharide to the cell surface (region 1) and post-polymerisation modification (region 3) (Boulnois *et al* 1987). Different capsular serotypes are encoded by distinct structural genes located in region 2, however genes in region 1 and 3 are widely conserved amongst group II encapsulated strains of *E. coli* (Boulnois *et al* 1987). This arrangement of the gene operon has been likened to a cassette, and allows separate structural genes to be inserted into conserved regions which code for their assembly and regulation. No genetic homology has been found between the K1 capsular genes and the meningococcus group B capsular genes (Echarti *et al* 1983).

Aerobactin

Iron metabolism is an important factor determining bacterial growth and division. One of the mechanisms mediating iron uptake in *E. coli* is the production of the siderophore aerobactin. This is a relatively small molecule of molecular weight 616 which is secreted into the extracellular fluid.

Aerobactin chelates Fe^{3+} and the aerobactin-iron complex is taken up by the bacterium through an outer membrane receptor protein. Aerobactin scavenges for iron in body tissues and fluids where it competes with similar host molecules including transferrin and lactoferrin.

Aerobactin appears to be an important factor in invasive urinary tract infection and is produced by over 75% of *E. coli* isolates from blood cultures or pyelonephritis, but fewer than 50% of isolates from other sites (Montgomerie et al 1984, Johnson *et al* 1988). Aerobactin expression is also associated with enhanced bacterial virulence in a murine model of urinary tract infection (Montgomerie *et al* 1984).

The aerobactin operon consists of five genes (Johnson 1991). Four genes code for aerobactin synthesis and the remaining gene codes for the outer membrane receptor protein. Aerobactin genes may be located on the bacterial chromosomal or on plasmids, where they are frequently found in association with antibacterial resistance genes (eg. pColV-K30) (Johnson *et al* 1988). Other siderophores are produced by *E. coli*. These include enterochelin which has a higher affinity for iron than aerobactin at neutral pH, but is probably of minor importance in vivo (Johnson 1991).

Haemolysin

Haemolysins are secreted polypeptide toxins which lyse erythrocytes. Two types of haemolysin are produced by *E. coli;* alpha-haemolysin is a 110kDa protein, which may be chromosomal or plasmid coded and is secreted extracellularly, whereas beta-haemolysin is usually coded on the chromosome and is cell-associated (Hacker and Hughes 1985).

Haemolysin is associated with extraintestinal *E. coli* infection (Minshew *et al* 1978) and approximately 75% of pyelonephritic strains are haemolytic (O'Hanley *et al* 1985b). Hacker and Hughes (1985) showed that transfer of cloned haemolysin genes to non-haemolytic strains of *E. coli* led to enhanced bacterial virulence.

Haemolysin has *in-vitro* toxic effects on many cells including polymorphonuclear leucocytes and renal tubular epithelium (Cavalieri *et al* 1984, Keane *et al* 1987). The in vitro cytolytic effects of haemolysin are potentiated by the presence of P-fimbriae (O'Hanley *et al* 1991), presumably because the close adherence of bacteria to host cells results in more effective toxin delivery. Haemolysin may be an important factor in pyelonephritis as it causes membrane damage in the kidney and inhibits host defences. (Hughes *et al* 1983, Low *et al* 1984). Increased iron availability resulting from haemolysis may also encourage bacterial growth.

Other virulence factors

Uropathogenic *E. coli* undoubtedly produce a number of other significant virulence determinants (Johnson 1991). These include the production of cytotoxins, proteases and products which inhibit smooth muscle and reduce ureteric peristalsis (Teague and Boyarski 1968). The plasmid ColV is frequently found in association with uropathogenic *E. coli* and may encode the production of colicin and other products conferring antibiotic resistance, factors which enhance fimbrial and haemolysin production, and products which inhibit host phagocytosis and complement-mediated killing.

Co-ordinated expression of virulence determinents

Molecular studies have shown that genes coding for different bacterial virulence determinants are frequently linked together in a block of genetic information (Low *et al* 1984, Knapp *et al* 1986, Svanborg-Eden and de Man 1987, High *et al* 1988, Morschhauser *et al* 1994). Comparison of the genotype and phenotype of organisms isolated from different sites in the urinary tract show that the expression of virulence factors is controlled and co-ordinated during the pathogenesis of ascending urinary tract infection. A single isolate of *E. coli* may possess several distinct adhesin genes with expression of each regulated by environmental conditions (High *et al* 1988). Bacteria are therefore able to vary their phenotype depending upon the location of infection. Strains which possess a combination of type 1 and P-fimbrial genes predominantly express type 1 fimbriae in the bladder and P-fimbriae in the kidney (Kiselius *et al* 1989, Arthur *et al* 1989a, 1989b, Plos *et al* 1990). These observations may be highly significant as type 1 fimbriae adhere strongly to the bladder epithelium but appear to enhance bacterial phagocytosis in the kidney (Svanborg-Eden *et al* 1984), whereas P-fimbriae receptors are concentrated on renal tubular cells. The process by which bacteria express different products depending on environmental conditions is known as phase variation, and is mediated at the level of gene transcription (Eisenstein 1988).

Other uropathogens

The role of *E. coli* has received particular attention due to its overwhelming importance in urinary tract infections, but many of the other gram-negative organisms have similar virulence factors. Type 1 mannose-sensitive fimbriae are expressed by a wide range of gram-negative organisms (Abraham *et al* 1988). *Proteus mirabilis, Klebsiella pneumoniae Providencia stuartii* and *Pseudomonas aeruginosa* have also been shown to express fimbrial adhesins mediating mannose-resistant haemagglutination (Silverblatt 1974, Mobley *et al* 1988, Koga *et al* 1993, Bahrani *et al* 1993). A number of gram-positive organisms have the ability to adhere to uroepithelial cells, including *Staphylococcus epidermidis, Staphylococcus saprophyticus* (Schmidt *et al* 1988) and *Enterococcus faecalis* (Guzman *et al* 1989). *Staphylococcus* *epidermidis* and certain Enterobacteria attach strongly to foreign body material and form a surrounding biofilm. This process is believed to an important factor in the pathogenesis of catheter-associated infections.

Tarkkanen *et al* (1992) recently investigated virulence factors in 39 urinary Klebsiella isolates. All pathogenic strains were found to be encapsulated. A total of 27 distinct K antigens were identified in this series of isolates and infection was not associated with any particular capsular serotype. All strains agglutinated human erythrocytes and reacted with a type 3 fimbria-specific probe. The iron scavenger, enterochelin was expressed by all isolates but aerobactin was not identified.

Production of urease is thought to be of the most important virulence factors in isolates of Proteus associated with urinary tract infections. Urease splits urea into carbon dioxide and ammonia which is toxic to renal cells, alkalinises the urine and forms magnesium ammonium phosphate. Proteus infection is one of the factors associated with the production of renal stones. Evidence for the contribution of urease to pathogenicity includes the observation that acetohydroxamic acid, a potent urease inhibitor, reduced renal damage caused by Proteus in the rat (Musher *et al* 1975), and more recent studies showing attenuated virulence of urease-negative Proteus mutants in a mouse model of ascending infection (Jones *et al* 1990). A number of other bacteria produce urease including *Ureaplasma urealyticum, Staphylococcus saprophyticus* and certain strains of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

1.6 Fimbrial adhesins

Type 1 fimbriae

Approximately 70% of faecal and urinary isolates of *E. coli* possess type 1 fimbriae (Abraham *et al* 1988). Adhesion is inhibited by the monosaccharide D-mannose and by concanavalin A (which specifically binds to mannose-containing residues). Type-1 fimbriae are therefore believed to bind receptors which contain D-mannose residues (Ofek *et al* 1977, Johnson 1991). Expression of type 1 fimbriae is enhanced by growth on liquid rather than solid media (Eshdat *et al* 1981).

Receptors incorporating D-mannose are expressed by erythrocytes from many species and are widely expressed by mucosal cells in the lower urinary tract and ureter, intestine, vagina and buccal mucosa (Ofek *et al* 1977, Fujita *et al* 1989). Type 1 fimbriae also bind to phagocytic cells (leading to activation and bacterial killing), leucocyte adhesion molecules CD11 and CD18, Tamm-Horsfall glycoprotein and the lamenin network in basement membranes (Kukkonen *et al* 1993). Type-1 fimbriae adhere to oligomannoside chains and adherence to epithelial cells is blocked by nitrophenol suggesting that the epithelial cell receptor is likely to be a complex structure with a hydrophobic component (Johnson 1991). Nitrophenol does not inhibit type-1 fimbrial mediated haemagglutination, therefore erythrocyte binding must be mediated via a different receptor.

Type-1 fimbrial morphology has been well described (Klemm *et al* 1982, Klemm *et al* 1985). Each fimbria is composed of approximately 1000 identical major subunits and a much smaller number of minor subunits which are bound together by non-covalent forces to form a helical structure approximately 1μm in length and 7nm diameter. Individual major subunits may be disassociated by guanidium chloride, but the fimbrial polymer is resistant to sodium dodecyl sulphate and 6M urea (Eshdat *et al* 1981).

Type-1 fimbriae contain a large proportion of non-polar amino acids. If bacteria expressing type 1 fimbriae are grown in static broth culture they form a pellicle at the air/media interface (Eshdat *et al* 1981). The hydrophobic nature of the adhesin may be an important factor in the binding of adhesin to receptors (Johnson 1991), particularly those receptors on epithelial cells which also have hydrophobic components.

Type 1 fimbriae are closely related to type 1 fimbriae of Shigella and Klebsiella (Johnson 1991). Three separate type 1 fimbriae have been recognised, termed F1A, F1B and F1C. They all have a similar structure and are serologically related, however, type 1C fimbriae do not mediate haemagglutination (Johnson 1991). Type 1C fimbriae are also related to the S-fimbriae with fimbrial adhesin subunit proteins sharing 75% amino acid similarity.

The importance of type-1 fimbriae in the pathogenesis of urinary tract infections is uncertain. The ubiquitous nature of type 1 fimbriae suggests that they alone are unlikely to play a major role in virulence. Type-1 fimbriae may be an important factor promoting bacterial colonisation of the lower urinary tract (Schaeffer *et al* 1987), but they do not appear to have a major role in the colonisation of the upper urinary tract and kidney (Johnson 1991). Type 1 fimbriae adhere to mucin and Tamm-Horsfall protein and protect bacteria against *in-vitro* macrophage killing (Keith *et al* 1990). However, bacteria expressing type-1 fimbriae will bind to phagocytes and activate the respiratory burst (Goetz 1989, Johnson 1991). These properties would appear to enhance bacterial clearing, however their *in-vivo* significance is unclear (Orndorff and Bloch 1990). Oropharyngeal colonisation with *E. coli* is common in neonates and it has been suggested that the ability of type-1 fimbriae to bind to the buccal mucosa may be an important factor in the development of invasive K1 encapsulated bacterial infection during this period (Orndorff and Bloch 1990).

Evidence for the *in-vivo* importance of type 1 fimbriae comes from animal experiments which show that bladder infection with type-1 fimbriated organisms is inhibited by the administration of the receptor analogue methyl-alpha-D-mannopyranoside (Aronson *et al* 1979). Protection against bacterial challenge is also conferred by antibodies directed against the type 1 fimbrial adhesin or the mannose containing receptor (Abraham *et al* 1985). These studies suggest that type-1 fimbriae may have a role in virulence, however May *et al* (1993) came to a different conclusion when they investigated the role of type 1 fimbriae in a rat model of intraperitoneal infection. They showed that genetically engineered strains of *E. coli* that were unable to express type 1 fimbriae were significantly more virulent than the fimbriated parent strains. The relevance of these findings in clinical urinary tract infection is uncertain.

An important observation is that expression of type-1 fimbriae may be regulated in an on-off fashion (Eisenstein 1988). This phenomenon is known as phase variation and occurs at a spontaneous frequency of approximately one per thousand generations. A population of bacteria will therefore always contain organisms with differing fimbrial phenotypes. This was illustrated by Kiselius *et al* (1989) who investigated *in-vivo* expression of type-1 fimbriae in infected urine and showed that bacterial populations were heterogeneous and varied between predominantly fimbriated to predominantly non-fimbriated. Phase variation is controlled by environmental factors (Johnson 1991). Growth on agar inhibits the expression of fimbriae in most isolates, whereas growth in broth is usually associated with increased expression of fimbriae. A low oxygen concentration favours expression of fimbriae. Antibiotics also have variable effects on fimbrial expression which depend on the type and concentration of antibiotic used. Higher temperatures favour the expression of fimbriae and increase the rate of spontaneous phase-switching. The ability to undergo phase-variation may enhance bacterial pathogenicity by allowing them to adapt to survive local conditions. Indeed, *E. coli* isolates from the bladder and oropharynx are usually fimbriated, whereas those associated with peritonitis and septicaemia are often non-fimbriated (Orndorff and Bloch 1990), suggesting that phase-variation may be an important factor in the pathogenesis of extra-intestinal infections.

The molecular biology of type 1 fimbriae has been extensively investigated (Krogfelt 1990). The gene cluster coding for type-1 fimbrial expression is located at 98 min on the *E. coli* linkage map. The gene cluster has been cloned by two groups. Klemm *et al* (1985) and Krogfelt (1990) described *fim* genes whereas Orndorff *et al* (1984) used the term *pil* genes. Approximately 9kb of DNA is required for type-1 fimbrial synthesis and expression. The *fim* cluster was found to consist of 8 genes (*fimA-H*) which code for the adhesin, structural subunits, an anchor protein, accessory proteins required for subunit transport and assembly and regulatory proteins. The *pil* genes code for similar products and are organised in a similar fashion (Figure 1.1). The location of these genes and the putative function of their products was determined by investigating the phenotype of deletion mutants and by performing complementation studies (Klemm *et al* 1985, Klemm and Christiansen 1987).

FimA encodes a 17kDa protein known as pilin which has 158 amino acids and is the major structural protein subunit of the fimbria. Although pilin constitutes the major part of the fimbria it does not mediate adhesion. Three minor subunits proteins coded by *fimF, fimG* and *fimH* genes are also present in the fimbria with 3-6 copies of each. *FimF* and *fimG* code for minor adhesin subunits of predicted molecular weight 16.7kDa (156 amino acids) and 14.9kDa (144 amino acids) respectively. The protein encoded by *fimH* has a molecular weight of 28.9kDa (277 amino acids) and is the actual adhesin protein which binds to D-mannose containing receptors (Hanson and Brinton 1988). The genes coding for the minor subunits have been sequenced and complementation studies have shown that these genes regulate the length and number of fimbriae. The binding specificity of the *fimH* adhesin is also influenced by the other minor subunits (Klemm and Christiansen 1987, Madison *et al* 1994). Nucleotide sequence analysis showed that there was extensive mutual homology between *fimF, fimG* and *fimH* and between each of these minor subunit genes and the *fimA* gene (Klemm and Christiansen 1987). Electron microscopy using gold-conjugated anti-adhesin antibodies revealed that the *fimH* adhesin protein is located at the tip and at long intervals on the lateral shaft of the fimbriae (Abraham *et al* 1988, Krogfelt *et al* 1990).

Sokurenko *et al* (1994) recently showed that strains of type 1 fimbriated *E. coli* exhibited different patterns of adhesion to yeast mannan and fibronectin derivatives. The functional heterogeneity of the type 1 fimbriae was found to result from a variability of the *fimH* gene. Sequencing of the *fimH* genes showed that single amino acid changes could result in different adhesin properies. The importance of this adhesin variability is uncertain.

The *fimC* product is involved in assembly of the fimbria and the *fimD* product contains several hydrophobic regions which span the cell membrane and anchors the fimbria to the cell wall (Klemm and Christiansen 1990). Fimbrial assembly involves several stages, including synthesis of fimbrial subunits, intracellular translocation and secretion across the cell surface, assembly of the fimbrial structure and anchoring of the mature fimbria to the membrane. Assembly of type-1 fimbriae was investigated by Lowe *et al* (1987) who removed fimbriae from bacteria using a blending technique and subsequently allowed fimbriae to regrow. Experiments using gold-conjugated monoclonal antibodies to demonstrate incorporation of labelled proteins showed that new subunits were added to the base of the fimbria.

FimB and *fimE* are regulatory genes and are involved in the control of phase variation (Eisenstein *et al* 1987, Eisenstein 1988). This process is regulated by a complex process which occurs at the transcriptional level. Phase variation results from the periodic inversion of a 314 base DNA fragment (the switch sequence) which includes the *fimA* promotor gene (Abraham *et al* 1985). This sequence is flanked on either side by a 9 base-pair inverted repeat sequence. *FimB* and *fimE* genes are located immediately upstream from *fimA* and encode products of molecular weight 25kDa and 23kDa respectively. These products are both highly basic and share 48% amino acid identity (Klemm 1986).

Phase variation is also dependent on a protein known as integration host factor (IHF). This protein has two subunits, IHF-alpha and IHF-beta which are coded for by *himA* and *himD* genes. Both subunits must be present for efficient expression of the fimA gene product. Mutants lacking the *himA* gene are phase-locked and do not exhibit phase variation. The products of *FimB* and *fimE* genes bind to the integration host factor and the resulting complex attaches to a specific site adjacent to the switch sequence (Eisenstein *et al* 1987). The nature of the complex determines whether the "switch" sequence is in the on (*fimB*) or off (*fimE*) position.

P-fimbriae

P-fimbriae mediate mannose-resistant adhesion of bacteria to P-blood group antigens, hence *E. coli* strains which express P-fimbriae exhibit mannose-resistant haemagglutination (MRHA). The majority of MRHA positive strains of *E. coli* possess P-fimbriae (Kallenius *et al* 1981a, Leffler and Svanborg-Eden 1981); however a number of other adhesins also mediate MRHA. These are termed X-adhesins and include S, F1C and G fimbriae and the non-fimbrial adhesins.

P-blood group antigens comprise globoside (P antigen), trihexyl ceramide (P_k antigen) and the P_1 antigen. These antigens are widely expressed on erythrocytes and epithelial cells. The precise nature of the P-fimbrial receptor was identified in a series of studies (Kallenius *et al* 1981b, Leffler and Svanborg-Eden 1981). Glycolipid extracts from uroepithelial cells were found to inhibit adhesion. Purification of the extract showed that inhibition was associated with the globoseries component of glycosphingolipid, the P-antigen. The actual receptor ligand is believed to be a digalactose complex Galα(1-4)Galβ. The evidence for this includes the observation that P-fimbriae bind to natural and artificial structures containing a Gal-Gal complex but do not bind to glycolipids lacking Gal-Gal, and binding is inhibited by the presence of digalactose (Johnson 1991). P-fimbriae are heterologous and subtypes can be distinguished by their relative adherence to globoside and globotriaosylceramide. Approximately one third of P-fimbriated strains of *E. coli* bind to the Forssman antigen which is predominantly found on sheep erythrocytes.

Immunoelectronmicroscopy has revealed that the P-fimbriae has a complex heteropolymer structure consisting of a rigid helical stalk, approximately 10nm in diameter, with a fine flexible fibrillum of 3nm diameter extending from the distal tip (Kuehn *et al* 1992). The stalk is composed of approximately 1000 copies of the major subunit (PapA) with four minor fimbrial subunits (PapE, PapF, PapG, PapK) forming the tip (Lindberg *et al* 1987). PapG is the actual adhesin subunit and is related to the B subunit of shiga toxin (Lund *et al* 1987).

P-fimbriae are antigenically heterogeneous and several distinct serovariants have been identified. Monoclonal antibodies against one serovariant do not cross-react with other serovariants (de Ree *et al* 1986). This observation is clinically important because antibodies directed against P-fimbriae are detected in serum following an episode of pyelonephritis (de Ree and van den Bosch 1987), but these antibodies will not protect against infection with other serovariants and P-fimbrial vaccines would similarly only protect against infection with the identical subtype.

P-fimbriae are believed to be an important factor in the development of invasive urinary tract infection as there is a strong correlation between possession of P-fimbriae and invasive disease (Kallenius *et al* 1981a, Lomberg *et al* 1981) and urinary tract infections have not been recorded in individuals with the rare p blood group who do not express P-blood group antigens (Kallenius *et al* 1981a). Up to 90% of isolates from previously healthy patients with pyelonephritis or bacteraemia express P-fimbriae compared to less than 20% of those with cystitis or asymptomatic bacteruria. Patients who develop pyelonephritis usually demonstrate faecal carriage of P-fimbriated strains which is consistent with this being the primary source of infection (Kallenius *et al* 1981a).

The reason for the apparent importance of P-fimbriae is likely to be a result of binding to human cells expressing P-fimbrial receptors. Expression of P blood group antigens is virtually universal in the human race. About 75% of individuals are of phenotype P_1 (expressing P_1, P and P_k antigens on the surface of erythrocytes) and 25% P_2 (expressing P and P_k antigens). People with blood group P_1 have a greater concentration of blood group antigens than those of group P_2 and are thought to be more susceptible to urinary tract infection (Lomberg *et al* 1981).

Digalactose receptors are found on human erythrocytes, epithelial cells and in the human kidney. The distribution of receptors was investigated by Marcus and Janis (1970) who performed immunofluorescence on human kidney sections using antigloboside antibodies. Maximum binding was observed in the proximal tubular epithelial cells. Further studies using fluorescein-labelled, purified, P-fimbriae showed that they adhered widely to bladder epithelium, proximal and distal renal tubular cells and Bowman's capsule (Virkola *et al* 1988, Karr *et al* 1989). Unlike type-1 fimbriae P-fimbriae do not bind to polymorphonuclear leucocytes.

P-fimbriae are expressed by a number of species including human, mice, dogs, goats, pigs and pigeons. They are not expressed by guinea pigs, rats, cows or horses. The selection of suitable animal models of urinary tract infection is therefore most important (Johnson 1991). The relative concentration of fimbrial receptors also depends on the sex and strain of animal used. Experiments in mice show that possession of P-fimbriae is associated with bacterial colonisation of the upper urinary tract, local inflammation, and septicaemic spread (Hagberg *et al* 1983a, O'Hanley *et al* 1985a, Domingue *et al* 1988, Linder *et al* 1988). Binding and colonisation is inhibited by administration of receptor analogues (Svanborg-Eden *et al* 1982, Linder *et al* 1988) or a Gal-Gal pilus vaccine (O' Hanley *et al* 1985a). Interestingly a recent observation by Hull *et al* (1994) found that a strain of *E. coli* (FN506) encoding P-fimbrial genes on a high copy number plasmid was less virulent in a murine model of infection than the same strain encoding P-fimbriae on a low copy number plasmid, although this strain had a lower haemagglutination capacity. The authors postulated that bacteria expressing a higher level of P-fimbriae may be cleared more rapidly from the urinary tract by the host immune system.

P-fimbrial proteins are encoded by a gene cluster known as *pap*. This cluster has been extensively investigated in a series of elegant molecular studies (Lindberg *et al* 1987, Hultgren *et al* 1991, Kuehn *et al* 1992). The nucleotide sequence has been determined for the entire *pap* operon which comprises eleven separate genes coding for structural, transport and regulatory proteins (Figure 1.2).

The nature of *pap* gene products have been investigated by comparing the relative phenotypes of isogenic mutants. *PapA* is the major subunit protein but is not the adhesin as *papA* gene deletions are associated with afimbrial organisms which still mediate adherence (Lindberg *et al* 1987). Mutant rough strains which lack PapA are able to adhere, but adherence is not observed in smooth strains lacking PapA. This suggests that the fimbrial structure is needed to display the adhesin outside the O-antigen coat.

The distal fibrillar structure is composed of the four minor subunits PapE, PapF, PapG and PapK (Hultgren *et al* 1991). Mutants which lack PapE, F or G are fimbriated but do not mediate adherence. PapG is the actual adhesin subunit and is located at the terminus of the fibrillin (Lindberg *et al* 1987). It has a molecular weight of 32kDa. Preliminary studies using alanine-scanning mutagenesis identified several amino-acid residues that are involved in haemagglutination (Klann *et al* 1994). These residues are scattered throughout the length of the adhesin and are presumably involved in creating the conformation necessary for the adhesin-receptor interaction. In addition to its adhesin properties PapG has also been shown to protect bacteria against neutrophil bactericidal activity (Tewari *et al* 1994). PapG+ isogenic mutants were found to interact poorly with neutrophils and resist neutrophil killing, both *in vitro* and *in vivo* compared to the corresponding PapG- strain. The protective effect of the PapG subunit was thought to be due to electrostatic properties as the adhesin has a net negative charge which inhibits interaction with neutrophils.

PapG is linked to PapF forming the minimal adhesin complex. PapF initiates subunit polymerisation and fimbrial assembly and is required for the correct presentation of the adhesin at the fimbrial tip (Jacob-Dubuisson *et al* 1993). PapK regulates the length of the fimbrial tip and is also required to initiate the formation of fimbriae as mutants lacking PapF and PapK are non-fimbriated (Jacob-Dubuisson *et al* 1993). The PapG/PapF complex is attached to the fimbria via repeating subunits of PapE.

Fimbrial subunit components are transported by "chaperone" proteins and assembled in a strictly regulated sequence (Hultgren *et al* 1991). PapF and PapK are believed to play a role in detaching subunit proteins from chaperone-subunit complexes allowing the construction of fimbriae. Other proteins involved in the assembly of P-fimbriae are PapD, PapC and PapH. PapD is located in the periplasmic space and is thought to be involved in the translocation of fimbrial subunits as a "chaperone" protein. PapC spans the outer cell wall and is involved in external transport and polymerisation of the fimbrial subunits. PapC mutants lack fimbriae and are unable to adhere, although fimbrial subunits can still be identified in cell extracts (Norgren *et al* 1984). PapH terminates fimbrial assembly and is believed to anchor the completed fimbria to the cell wall as mutations in papH lead to the production of particularly long fimbriae which are released into the culture supernatant (Baga *et al* 1987). The N-terminus of PapH consists of a proline rich 14 amino-acid chain which is thought to act as the anchor (Baga *et al* 1987). Fimbrial length is determined by the relative amounts of PapA and PapH within the bacteria. The function of PapJ has not yet been fully defined.

The construction of P-fimbriae follows an organised pattern. Major and minor subunit proteins are transported through the periplasm by PapD and delivered to PapC at the outer membrane which acts as the base for fimbrial biogenesis. The adhesin subunit PapG initially binds to PapC followed by PapF and repeated PapE subunits. Assembly of the flexible tip of the fimbriae is terminated by the incorporation of PapK. This process is followed by the addition of multiple PapA subunits forming the rigid fimbrial rod structure (Figure 1.3). The incorporation of PapH terminates fimbrial polymerisation and anchors the fimbria to the cell surface.

Expression of P-fimbriae is subject to phase variation and electron microscopy studies have shown that the proportion of clinical isolates actually expressing fimbriae varies between 0.1 and 100% depending on environmental conditions (Rhen *et al* 1983, Lichodziejewska *et al* 1989). P-fimbrial expression is enhanced by growth on agar at 37°C and is inhibited by growth in broth culture, glucose rich media and temperature of 22°C (Goransson and Uhlin 1984, Abraham *et al* 1986). *PapB* and *papI* genes are involved in regulation of P-fimbrial expression and are both trans-acting activators of the *papA* gene (Rhen and Vaisanen-Rhen 1987). A genetic switch appears to regulate temperature dependence as shifts in temperature are associated with a re-arrangement of segment of DNA located close to papB (Abraham *et al* 1986).

The molecular epidemiology of P-fimbriae has been investigated by colony hybridisation of pathogenic *E. coli* isolates, using gene probes derived from fragments of the *pap* operon (Arthur *et al* 1989a,b). These studies have shown that the majority of clinical isolates possess more than one copy of *pap* - related sequences. Fimbriae with related gene sequences, include the F-adhesin which is encoded by the *prs* (pap-related sequence) operon. The F-adhesin binds to the Forssman antigen which expressed by cells in the renal pelvis. Studies have shown that co-expression of *pap*-related adhesins occurs more frequently in pyelonephritic isolates than isolates from cystitis or faeces (Arthur *et al* 1989a,b, Plos 1990).

There is little serological cross-reactivity between these P-fimbrial variants, however they appear to have a close evolutionary relationship as the gene clusters are organised in a similar fashion with large areas of sequence homology (van Die *et al* 1986, Denich *et al* 1991a). Transcomplementation studies have confirmed the close functional homology of gene clusters encoding different P-fimbrial serotypes.

The *pap* polymorphism is believed to result from intra and inter-chromosomal recombination events which generate a high degree of variability in major and minor subunit components (Arthur *et al* 1990). Sequence variation is greatest for the minor subunit genes and the central portion of *pap*A. The subunit signal sequences, N-terminus and C-terminus sequences are all highly conserved. The variable sequences are of particular importance as they code for PapA type-specific immunodominant epitopes and amino acids involved in binding to host receptors (Denich *et al* 1991b). The fimbrial polymorphism gives bacteria the potential for a high degree of antigenic variability and adhesin-receptor specificity. Candidate P-fimbrial vaccines are unlikely to confer significant benefit unless they give protection against a range of fimbrial serotypes.

S and F1C adhesins

A proportion of fimbriated uropathogenic isolates of *Escherichia coli* mediate mannose resistant haemagglutination which is not inhibited by the presence of digalactose. Haemagglutination of some strains is abolished in the presence of sialyl galactosides or neuraminidase (which removes sialyl residues). The haemagglutinin was therefore designated the S-adhesin (Moch *et al* 1987). The receptor specificity was investigated by observing binding of radiolabelled S-fimbriae to various components of the erythrocyte which were separated by gel electrophoresis. The fimbriae bound to glycophorin A, the sialoglycoprotein associated with blood group MN, and the receptor has been identified as alpha-sialyl-acid-2-3-beta-galactosamine (Parkinnen *et al* 1986). Hanisch *et al* (1993) recently showed that purified S-fimbriae bind preferentially to sialic acid residues attached to gangliosides and highest binding was observed to NeuGcα(2-3)Gal and NeuAcα(2-8)NeuAc, an important glycopeptide component of human foetal brain tissue.

Serological and genetic studies have shown that the F1C fimbria is closely related to the S-fimbriae (Pere *et al* 1987, Ott *et al* 1988). However they clearly have a different receptor specificity as F1C fimbriae do not mediate haemagglutination. The nature of the F1C receptor has not been elucidated but F1C fimbriae bind strongly to renal tubules and other epithelial cells and binding is inhibited by N-acetyl neuraminic acid or neuraminidase, confirming a functional relationship with S-fimbriae (Marre *et al* 1990).

S-fimbrial receptors are present on a wide range of cells in the kidney including glomeruli, vascular endothelium, proximal and distal tubules and collecting ducts. S-fimbriae are strongly associated with *E. coli* strains causing neonatal bacteraemia and meningitis, but are also produced by some pyelonephritic isolates (Moch *et al* 1987). Ability of S-fimbriae to bind to human umbilical vein endothelium was demonstrated by Parkkinen *et al* (1989) who postulated that endothelial binding might contribute to the development of septicaemia and allow vascular access to the central nervous system. S-fimbriae have been shown to contribute to virulence in animal models of infection and anti S-fimbrial antibodies are associated with protection (Hacker *et al* 1986).

The S-fimbria is 1-2μm in length and 5-7nm in diameter. These dimensions are similar to the type 1 fimbria. S-fimbria are also composed of repeated minor and major subunits. Moch *et al* (1987) separated the adhesin and fimbrial subunits by high-resolution anion exchange chromatography and showed that they were distinct. The adhesin had a molecular weight of 12kDa compared to 16.5kDa for the fimbrial subunit. Immuno-electron microscopy using gold-labelled fibrillin and adhesin specific monoclonal antibodies showed that the adhesin subunit was located at the fimbrial tip.

The gene cluster coding for the S-fimbrial adhesin has now been cloned (Schmoll *et al* 1987, 1989). Dot blot experiments using a series of probes derived from the cloned DNA showed that there was widespread homology along the entire length of DNA coding for S fimbriae and the *foc* gene cluster coding for F1C fimbriae (Ott *et al* 1987). Gene probes derived from sequences coding for structural components of the S-fimbriae did not hybridise with DNA sequences encoding type1 or P-fimbriae, but control regions were largely conserved. The fimbrial structural subunit gene *sfa*A was sequenced by Schmoll *et al* (1987) who found that the gene coded for a polypeptide of molecular weight 15.95kDa containing 180 amino acids. There was significant nucleotide and sequence homology with the corresponding type 1C fimbrial subunit, but little homology with type 1 or P-fimbrial sequences.

Further studies revealed that the gene cluster coding for S-fimbriae comprised a total of nine genes encoded by 7.9kb of DNA (Schmoll *et al* 1989, 1990) (Figure 1.4). The nucleotide sequence of these genes was determined and investigation of the phenotypes associated with specific mutants allowed putative roles to be assigned to individual genes. *SfaA* is the major subunit gene; *SfaB* and *sfaC* genes are located proximally to *sfaA* and regulate adhesin expression; *sfaD*, *sfaE* and *sfaF* gene products appear to be involved in the transport and assembly of adhesin subunits; *sfaG* and *sfaH* genes code for minor adhesin subunits and *sfaS* is the adhesin subunit gene.

A further *sfa* gene cluster was recently cloned and characterised by Hacker *et al* (1993). This cluster was denoted *sfa*II, and was derived from an isolate causing newborn meningitis. Sequence comparison between *sfa* and *sfa*II revealed marked differences in the major subunit genes (*sfa*A), however the minor subunit genes were more closely related and the adhesin subunit genes (*sfa*S) were identical. Interestingly there were slight differences in erythrocyte binding between the uropathogenic (*sfa*) and meningitis (*sfa*II) strains, despite the presence of identical adhesins. This perhaps indicates that other subunits may indirectly influence the adhesin-receptor interaction.

The *foc* gene cluster coding for F1C fimbriae was sequenced and characterised by Riegman *et al* (1990). Comparison of the gene clusters encoding F1C fimbriae with those coding for S, type-1 and P-fimbriae (Figure 1.5) revealed a striking similarity in the organisation of the gene complexes with regulatory genes followed by the major subunit gene, transport and assembly genes and minor subunit genes. As previous studies have suggested there is a close relationship between S-fimbrial and F1C fimbrial gene clusters with extensive homology throughout. There is only patchy homology between S-fimbriae and other gene clusters. Regulatory genes for S-fimbriae and P-fimbriae are closely related and can be trans-complemented but other genes show no significant homology. In contrast there is little homology between S-fimbrial and type-1 fimbrial regulatory genes, but the major structural subunit genes *sfaA* and *fimA* are closely related. There is also a close degree of homology between *sfaF* and *fimD* genes which code for anchor proteins, but other genes are not as closely related.

The relationship between S and F1C fimbriae was further explored by Ott *et al* (1988) who showed that they shared extensive genetic homology but demonstrated characteristic differences in the fimbrial and adhesin subunit genes. Trans-complementation experiments showed that F1C fimbrial genes were able to complement S-fimbrial mutants. Similar complementation did not occur with cloned P-fimbrial genes. Further studies using monoclonal antibodies raised against S and F1C fimbriae showed that some antibodies were specific whereas others reacted with both fimbriae indicating the presence of shared epitopes. These studies confirmed the close relationship between S and F1C fimbriae and lack of association with P-fimbriae.

The nature of the molecular interaction between the S-adhesin and its receptor was investigated using site-specific mutagenesis (Morschhauser *et al* 1990, Marre *et al* 1990). Replacement of lysine 116 or arginine 118 in SfaS abolished haemagglutination, inhibited reaction with an adhesin specific monoclonal antibody and reduced binding to cultured renal tubular cells. In contrast, lysine 122 mutants showed normal binding and haemagglutination. Arginine 116 and lysine 118 residues therefore appear to play a crucial role in the function of the adhesin subunit, either through a conformational effect or resulting from their positive charge affecting ionic interaction.

G adhesin

A further fimbrial adhesin was identified in the pyelonephritic *E. coli* strain IH11175 (Vaisanen-Rhen *et al* 1983, Rhen *et al* 1986a). This isolate haemagglutinated human erythrocytes of blood group NN which had been pre-treated with endo-beta-galactosidase. This enzyme exposes N-acetylglucosamine residues which are thought to be the adhesin receptor. The fimbrial adhesin was designated the G adhesin. One interesting property of this adhesin is that it promotes autoagglutination of G-fimbriated, K-12 encapsulated strains. This is thought to result from the binding of the G-adhesin to N-acetylglucosamine residues in the lipopolysaccharide of these strains. The importance of G-fimbriae in urinary tract infections is unclear.

The G-adhesin subunit has a molecular weight of 19.5kDa (Rhen *et al* 1986a). The G-adhesin gene cluster was cloned by Rhen *et al* (1986a) and the N-terminal amino acid sequence of the adhesin subunit protein has been identified. This has some homology with the corresponding sequence of the P-fimbrial subunit protein suggesting a possible relationship (Vaisanen-Rhen *et al* 1983).

1.7 Adhesins in strains of *E. coli* causing intestinal disease

Intestinal *E. coli* may cause symptoms of gastroenteritis by invading the mucosal tissues or as a result of toxin production. Strains of *E. coli* causing gastroenteritis have been divided into four groups, namely enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC) and enterohaemorrhagic (EHEC). The majority of enteropathogenic *E. coli* adhere to human epithelial (HEp-2) cells in tissue culture. Three different patterns of adherence have been recognised; localised adherence, diffuse adherence and aggregative adherence (Savarino 1993, Chan *et al* 1994). Enteroadherent *E. coli* (EAEC) are associated with infantile and travellers diarrhoea. A diverse range of adhesins have been described in strains of *E. coli* isolated from the intestinal tract and fimbriae are believed to play an important role in mucosal colonisation. Fimbriae expressed by enterotoxigenic bacteria (ETEC) are not usually coded by chromosomal genes but are coded on plasmids.

Several of the adhesins described in intestinal *E. coli* are expressed as very fine flexible filaments, approximately 2-5nm in diameter compared to 7nm for type 1 or P-fimbriae. These filaments are termed fibrillae. K88 and K99 fibrillae have been well described (Stirm 1967, de Graaf *et al* 1980). K88 fibrillae are associated with strains of *E. coli* causing diarrhoea in neonatal pigs. Several serotypes of K88 fibrillae have been distinguished and are associated with different nucleotide and amino acid sequences of the subunit protein. Structure and sequence analysis has revealed similarities between K88 fimbriae and those associated with uropathogenic *E. coli*. A total of six structural genes are required for the expression of K88 fibrillae and these are coded on a non-conjugative plasmid of approximately 50mDa (Mooi *et al* 1984). The K88 fibrillae is composed of two subunit proteins. The major subunit protein is also the adhesin and has a molecular weight of approximately 26kDa. The minor subunit protein (pA) plays an important role in the construction of the fibrillae and has a molecular weight of approximately 16kDa. This protein has sequence homology with the type-1 and P-fimbrial fimbrial subunit proteins.

K99 fibrillae are associated with strains of *E. coli* causing diarrhoea in neonatal pigs, lambs, and calves. Only one serotype of the K99 adhesin has been described. K99 fibrillae are composed of repeating subunits of a 16kDa protein. This structural protein also mediates adhesion. The genes coding for expression of K99 fimbrillae have been cloned and found to be coded by a 7.1kb segment of DNA situated on a 75kb conjugative plasmid (de Graaf 1984). This gene cluster codes for at least eight products.

The gene clusters encoding K88 and K99 fibrillae are organised in a similar fashion and share several features with the gene clusters described for fimbrial adhesins in uropathogenic *E. coli.* The fimbrial and fibrillar gene clusters all include a gene encoding a large product of approximately 80kDa, which is believed to be the cell surface protein involved in transport of subunits to the cell surface and subsequent polymerisation.

Enterotoxigenic strains of *E. coli* are associated with travellers diarrhoea in humans. They adhere to the intestinal mucosa via colonisation factor antigens (CFA's). Two groups of CFA's have been described. They are known as CFA/1 and CFA/II and are found on specific serotypes of human enterotoxigenic *E. coli* (Korhonen *et al* 1985). The primary amino acid sequence of the CFA/I adhesin subunit has been determined (Klemm 1982). CFA/II is composed of three serologically distinct adhesins which are referred to as CS1, CS2, and CS3. These adhesins have different subunit sizes, morphology and receptor specificity. Although genes encoding all three adhesins are located on the same 89kb plasmid the expression of each adhesin is dependent on the serotype and biotype of the strain. The factors controlling adhesin expression are not fully understood.

The primary amino acid structure has now been determined for several of the *E. coli* adhesin subunit proteins. Type 1, PapA, K88, and K99 subunit proteins share several features including similar size, areas of amino acid homology at the N and C-terminals, and two cysteine residues located in a similar position. The conserved amino acid sequences at either end of the molecule suggests that these regions may play an important role, possibly in transport or assembly. In contrast the inner sequences are more variable. These sequences code for receptor binding domains and the variability allows bacteria the potential to adhere to a variety of different tissues and evade host immune defences. The CFA/I adhesin is shorter than other subunit proteins and has no cysteine residues. The C-terminus shares some homology with other subunit proteins but the N-terminus is unique. The CFA/I adhesin may therefore be a truncated version of the basic subunit structure with a deletion at the N-terminal end.

The reason for the similarities in the organisation and structure of fimbrial and fibrillar adhesins in *E. coli* is not known. However the degree of identity suggests that these adhesins have probably evolved from a common ancestral gene complex. Type 1 fimbriae are the most widely distributed in *E. coli* and might perhaps be the prototype fimbriae from which the other adhesins have been derived.

Fibrillar structures are fine and flexible and may not be easily identified as distinct structures (Hinson *et al* 1987). True afimbrial adhesins have also been described in strains of *E. coli* causing intestinal disease. Forestier *et al* (1987) described an afimbrial adhesin from the human enterotoxigenic *E. coli* strain 2230. This adhesin mediated binding to intestinal epithelial cells but did not mediate haemagglutination. The purified adhesin subunit was found to be a 16kDa protein and N-terminal amino acid analysis demonstrated homology with several fimbrial proteins including PapA and FimA.

Enteropathogenic strains of *E. coli* are important causes of diarrhoea, particularly in infants and neonates. EPEC strains adhere to HEp-2 cells in tissue culture in a localised (LA) or diffuse (DA) pattern depending on the type of adhesin expressed. Benz and Schmidt (1989) showed that EPEC strain 2787, isolated from a case of infantile diarrhoea, possessed an afimbrial adhesin which mediated diffuse adherence to HEp-2 cells. The adhesin was plasmid encoded and genetic cloning and deletional analysis showed that a DNA fragment of approximately 6kb was required for adhesin expression. This adhesin was termed AIDA-1. The relationship between this adhesin and other adhesins has not yet been explored.

1.8 Non-fimbrial adhesins

Introduction

The existence of non-fimbrial adhesins was first reported by Duguid *et al* in 1955 who described strains of *E. coli* which mediated mannose-resistant haemagglutination in the absence of detectable fimbriae. The morphology of these adhesins was characterised by immune electron microscopy using specific antibodies to fix the outer surface structures of the bacteria (Orskov *et al* 1985). These studies showed that strains lacking fimbriae mediated haemagglutination through an adhesive protein capsule which surrounded the organism. Several types of non-fimbrial (or afimbrial) adhesins have now been described including the M-adhesin, the Dr family of adhesins and a group of non-fimbrial adhesins (NFA's).

M adhesin

The M adhesin was first described by Rhen *et al* (1986a) and was identified on the same strain of *E. coli* (IH11165) as the G-adhesin. The M-adhesin agglutinated erythrocytes from individuals of blood group MM or MN but had no effect on those of group NN. Haemagglutination was inhibited in the presence of glycophorin AM. Biochemical studies showed that haemagglutination was not inhibited by neuraminidase but was inhibited if erythrocytes were pre-treated with trypsin suggesting that the receptor might have a protein component. Haemagglutination is specifically inhibited by the amino acids L-glycine, L-leucine and L-serine. The N-terminal amino acid sequences of the M-blood group antigen are -Ser-Ser-Thr-Gly and it has been suggested that this domain located on glycophorin A may be the M-adhesin receptor (Rhen *et al* 1986a). The M-adhesin therefore differs from fimbrial adhesins as it does not appear to recognise a carbohydrate receptor.

Electron microscopy of clinical isolates and recombinant strains expressing M-adhesins did not reveal the presence of fimbriae, but showed that the adhesin appeared as a granular carpet with occasional ring-like structures. SDS-PAGE studies showed that expression of the M-adhesin correlated with the presence of a 21kDa peptide (Rhen *et al* 1986a,b). Enzyme-linked immunoassay experiments revealed no cross-reactivity with a range of other purified fimbriae and haemagglutinins and the N-terminal sequence of the M-adhesin subunit showed no significant homology with other N-terminal sequences.

A molecular analysis of the M-adhesin was performed by Rhen *et al* (1986b) who cloned the adhesin genes using a cosmid cloning technique. Plasmid subclones were generated and the DNA responsible for expression of the M-adhesin was delineated by investigating the phenotype of deletion derivatives and Tn1725 insertion mutants. These experiments showed that the M-adhesin genes were located on a 6.5kb *Pst*1-*Eco*R1 segment.

075X adhesin (Dr haemagglutinin)

X-adhesins associated with *E. coli* strain 075 were first characterised by Vaisanen-Rhen (1984) who found that nine of fifteen 075 strains exhibited mannose resistant haemagglutination of human P_1 and p- erythrocytes. These strains therefore expressed an X-adhesin. All except one strain possessed type 1 fimbriae in addition to the X-adhesin and one strain also expressed P-fimbriae. The X adhesin was associated with haemagglutination of human erythrocytes, but not sheep or rabbit erythrocytes.

The 075X adhesin was purified using deoxycholate and urea and found to consist of repeated protein subunits, each with a molecular weight of approximately 16kDa. Amino acid compositions were determined for two of the purified X-adhesins (IH11033 and IH11128). The compositions were generally similar but there were differences in the glutamine-glutamate and tyrosine content. Hydrophobic amino acid content was 35 and 39% respectively.

Electron microscopy of X+ and X- strains showed that the presence of 075X-adhesin is associated with coil-like structures which surround the bacteria. The adhesin has been variously described as afimbrial, fimbria-like (Vaisanen-Rhen 1984) or fimbrial (Swanson *et al* 1991). Morphologically the 075X adhesin is clearly different to fimbrial structures but the adhesin shares several several biochemical similarities with fimbrial adhesins as they are both composed of repeating subunits, both are resistant to dissociation with deoxycholate and urea, and both have a high proportion of hydrophobic amino acids. The purified X-adhesin from one of the 075 strains (IH11033) was used to raise polyclonal antiserum in rabbits. This antiserum agglutinated all 075X+ strains indicating a serological relationship between these adhesins which probably represent a clonal group.

The genes coding for the 075X adhesin in strain IH11128 were cloned by Nowicki *et al* (1987) using a cosmid cloning technique. Recombinants were screened for haemagglutination and one positive clone was identified. Subclones were gnerated by digesting with *Hin*dIII and cloning fragments into the plasmid vector pACYC184. A subclone containing plasmid pBJN406 with 11.4kb DNA insert showed strong MRHA and was agglutinated by anti-075X serum. The DNA coding for adhesin expression was delineated by analysis of deletion mutants and Tn5 transposon insertion mutagenesis. These studies indicated that the 075X adhesin was encoded by approximately 6kb of DNA.

The recombinant plasmid pBJN406 was found to code for a total of eight separate proteins of molecular weights 90, 32, 29, 27, 17, 15.6, 12.5 and 11.5kDa in a cell-free transcription-translation system. 075X antiserum recognised the 15.6kDa protein in immunoprecipitation experiments suggesting that this product is the adhesin subunit protein. Recombinant strains possessing pBJN406 do not show distinct fimbriae on electron microscopy but bacteria appear to be surrounded by fimbria-like filaments. Tissue binding of the recombinant adhesin was investigated using frozen sections of human kidney epithelium. The results showed that the adhesin bound to interstitial cells, tubular basement membrane and Bowman's capsule.

Nowicki *et al* (1988) showed that the 075X adhesin only mediated MRHA of erythrocytes expressing Dr blood group antigens. The 075X adhesin was therefore denoted the Dr haemagglutinin. The receptor appeared to include a tyrosine molecule as binding was inhibited by modified tyrosine or chloramphenicol. Further studies revealed that the 075X adhesin bound to a receptor on the Dra blood group antigen. This receptor appeared to be located on the decay-accelerating factor (DAF), a cell membrane protein which regulates the complement cascade and prevents erythrocytes from lysis by autologous complement (Nowicki *et al* 1990).

Nowicki *et al* (1989) performed a further analysis of the recombinant plasmid pBJN406 which encoded the Dr haemagglutinin genes. A series of transposon insertion derivatives were generated using Tn5, Tn3, and TnphoA. The proteins synthesised by pBJN406 and the transposon mutants were identified in an *in-vitro* transcription-translation system using L-[^{35}S]methionine. The results of these experiments showed that five genes denoted *draA, draB, draC, draD* and *draE* were responsible for adhesin expression. *DraA, draB, draC* and *draD* genes encoded polypeptides of molecular weight 15.6, 5, 18, 90kDa and *draE* encoded a set of three polypeptides of molecular weight 32, 29 and 27kDa.

The function of each gene was deduced by comparing the phenotypes of the insertion mutants. The *draA* gene encodes a 15.6kDa protein which is the same size as the adhesin subunit. *DraA* mutants did not exhibit MRHA and were not agglutinated by anti-adhesin antisera providing further evidence for the role of this protein in adherence. Unlike P and type 1 fimbria the Dr haemagglutinin also appears to be the major structural subunit protein. *DraB* mutants were able to mediate MRHA but showed decreased expression of the draA gene product indicating a possible role in regulation of expression. *DraC* mutants showed greatly reduced haemagglutination, *draD* and *draE* mutants were unable to mediate MRHA and were not agglutinated by antisera. These genes may be involved in the transport and assembly of the haemagglutinin.

The organisation of the *dra* gene cluster shows some similarities with the *pap* gene complex as the major subunit genes (*draA* and *papA*) are both located at the end of the operon and *draD* and *draE* could be analagous to the *papC* and *papD* genes which have transport and assembly functions.

The clinical importance of the Dr haemagglutinin was investigated by screening 658 clinical strains of *E.coli* for *dra* gene sequences by dot-blot DNA hybridisations using a 3kb fragment encoding the *draD* gene. The Dr probe hybridised to 14.7% of faecal isolates, 5.6% of isolates from patients with asymptomatic bacteruria, 27.6% of isolates causing cystitis and 6% of isolates from patients with pyelonephritis. The increased frequency of Dr positive isolates associated with cystitis was statistically significant. The Dr haemagglutinin does not appear to be restricted to 075 strains as the gene probe also hybridised to isolates possessing a number of other O serotypes.

Afimbrial adhesins (AFA's)

An afimbrial adhesin was characterised by Labigne-Roussel *et al* in 1984. This adhesin was identified on a pyelonephritic isolate of *E. coli* KS52 (serotype 02) which expressed type-1 fimbriae and an additional X-adhesin as it also mediated MRHA which was P blood group independent. Haemagglutination activity was mainly associated with bacterial cells but was also present in the culture supernatant. The X-adhesin was not associated with the presence of fimbriae on electron-microscopy and the adhesin was therefore described as an afimbrial adhesin (AFA).

The DNA coding for adhesin expression was cloned using a cosmid cloning technique. Three MRHA positive recombinant clones were obtained and restriction enzyme mapping of the three clones showed that they shared approximately 25kb of DNA. Fragments of DNA from this shared region were subcloned into the plasmid pBR322 and the smallest insert of DNA that was able to mediate expression of the adhesin was 6.7kb. The plasmid containing this 6.7kb insert was denoted pIL14. Production of the supernatant adhesin was found to be orientation dependent as the same fragment of DNA cloned into the plasmid pBR322 in the opposite orientation was not associated with supernatant adhesin, however adherence to uroepithelial cells was not orientation dependent. This orientation dependence suggests that genes are transcribed under the control of the pBR322 promotor and elements regulating expression of the *afa* operon may not have been cloned in pIL14.

Although strain KS52 possesses six separate plasmids DNA hybridisation studies showed that the cloned adhesin sequences only hybridised with chromosomal DNA and did not hybridise with any plasmid sequences. Further studies showed that there was no hybridisation with type-1 or P-fimbrial sequences and serological studies confirmed that there was no antigenic cross-reactivity with type-1 or P fimbriae (Walz *et al* 1985).

Electron microscopy confirmed that isolates expressing the AFA adhesin were not fimbriated. Supernatant adhesin was purified by precipitating with ammonium sulphate, dissolving the precipitate in phosphate-buffered saline (PBS), then extensively dialysing to remove PBS and filtering through a nitrocellulose membrane before reprecipitating with acetone (Walz *et al* 1985). The purified product (AFA1) mediated strongly positive MRHA. Polyacrylamide gel electrophoresis revealed a 16kDa protein band. The purified adhesin was used to raise antibodies in rabbits. Immunoblotting experiments showed that these antibodies reacted with a single protein expressed by strain KS52 which was not expressed by other strains of *E. coli.*

The amino acid composition of AFA-1 was determined by Walz *et al* (1985) who found that the adhesin had a relatively low proportion of non-polar hydrophobic residues (22%), and three cysteine residues per subunit. It is not known whether disulphide bonds play an important part in the secondary structure. AFA-1 mediates MRHA of human and gorilla erythrocytes but does not agglutinate red cells from other species. AFA-1 was found to agglutinate red cells from an extensive variety of human blood groups and has a different receptor specificity to the M or S adhesins (Walz *et al* 1985). In addition to erythrocyte binding, strains of *E. coli* expressing AFA-1 strongly adhere to squamous and transitional epithelium from the human urinary tract (Labigne-Roussel *et al* 1984).

Minicell analysis revealed that five peptides were associated with adhesin expression including the 16kDa adhesin protein (AfaE) (Labigne-Roussel *et al* 1985). The remaining proteins, denoted AfaA, AfaB, AfaC, and AfaD, were of molecular weight 13kDa, 30kDa, 100kDa and 18.5kDa respectively. The location of *afa* genes encoding each protein on the recombinant plasmid pIL14 was determined by generating Tn5 mutants and comparing the phenotype of mutants by minicell analysis. Only three genes *afaB, afaC,* and *afaE* were found to be essential for adhesin expression. Mutants lacking the *afaC* gene have normal amounts of adhesin which is present inside the cell, *afaC* therefore appears to encode a product which is associated with the transport of the adhesin across the cell membrane and subsequent anchorage to the cell surface. It is a large gene which is analagous to other genes encoding products with similar functions eg. *papC*. The functions of the other *afa* genes have not been established, however a regulatory role has been postulated for *afaD* as it is located adjacent to the adhesin subunit gene (*afaE*) but is not itself essential for adhesin expression.

The nucleotide sequence of the adhesin subunit gene (*afaE*) was determined by Labigne-Roussel *et al* (1985). The sequence consisted of a 456 base pair open-reading frame which encodes a 152 residue polypeptide including a stretch of 21 amino acids with the properties of a prokaryotic signal sequence (ie:- short hydrophobic region with two positively charged lysines in positions 2 and 3, a long hydrophobic region and a possible cleavage site adjacent to an alanine residue). The predicted molecular weight of the mature 131 amino acid polypeptide subunit is 13.1kDa. This is different to the apparent molecular weight of 16kDa in SDS-PAGE analysis and suggests that further modifications might occur after translation.

Walz *et al* (1985) investigated the clinical importance of AFA-1 in clinical isolates of *E. coli* and found that four of sixteen clinical isolates which possessed X-adhesins reacted with anti-AFA-1 antiserum. A more comprehensive analysis was performed by Labigne-Roussel and Falkow (1988) who screened 138 clinical isolates of *E. coli* for *afa* sequences by colony hybridisation using a probe which overlapped the *afaB, afaC, afaD* and *afaE* sequences. The *afa* probe hybridised with 15 (11%) isolates including 2 (13%) of 15 patients with pyelonephritis, 11 (14%) of 79 with cystitis, none of 6 with asymptomatic bacteruria and 2 (5%) of 38 faecal specimens. The 15 positive strains all expressed type 1 fimbriae and 13 expressed X-adhesins as they mediated P-blood group independent MRHA.

The genetic relationship of the *afa* positive isolates was investigated further by comparing hybridisation of several probes spanning different parts of the cloned *afa* operon (Labigne-Roussel and Falkow 1988). Hybridisation across the entire operon was observed in only three of the isolates and each expressed a 16kDa polypeptide which strongly cross-reacted with anti-AFA-1 antibodies. The remaining strains hybridised with DNA sequences corresponding to *afaA, afaB, afaC,* and *afaD* genes, but did not hybridise with an adhesin gene (*afaE*) probe. These strains did not react with native AFA-1 specific antibodies but reacted to a variable extent with antibodies raised against denatured AFA-1 protein. Immunoblotting experiments using these antibodies demonstrated heterogeneous bands between 15-16kDa.

Further experiments revealed that the isolates shared a highly conserved 4.1kb segment of DNA encoding *afaB, afaC,* and *afaD* genes, however the *afaE* sequences were heterogeneous. Four related adhesins were described, AFA-I, AFA-II, AFA-III, and AFA-IV. The *afa-3* gene cluster encodes the AFA-III adhesin and has been identified on uropathogenic (A30) and diarrhoea associated strains (AL845 and AL847). *Afa-3* genes were recently cloned and characterised by Bouguenec *et al* (1993). *Afa-3* gene clusters were found to be located on 100kb plasmids, unlike the *afa-1* clusters which are chromosomal.

The Dr adhesin family

Nowicki *et al* (1988, 1990, 1993) showed that the 075X adhesin bound to a receptor on the Dra blood group antigen and classified the 075X adhesin as a Dr adhesin. The receptor appears to be located on the decay-accelerating factor (DAF), a cell membrane protein which regulates the complement cascade.

It was noted that the size and organisation of the Dr adhesin was similar to several other adhesins including AFA-I, AFA-III and the fimbrial adhesin F1845 cloned from a diarrhoea-associated *E. coli* (Bilge *et al* 1989, 1993). Dr, AFA-III and F1845 haemagglutinins were all identified in strains of *E. coli* serotype 075. Swanson *et al* (1991) showed that structural subunit genes encoding Dr, F1845, and AFA-I adhesins share areas of nucleotide sequence homology and the *afaE3* gene showed 98.1% nucleotide homology with the Dr adhesin gene (Bouguenec *et al* 1993). The close relationship between this group of adhesins was confirmed by agglutination experiments which showed that the adhesins all reacted strongly with Dra positive erythrocytes, but did not agglutinate Dra negative erythrocytes. These observations suggested that the adhesins all recognised the Dr antigen, however they were thought to react with different epitopes of the Dr antigen as chloramphenicol inhibited haemagglutination mediated by the Dr adhesin but had no effect on the AFA-I or F1845 adhesins. Interestingly AFA-III expressed by the uropathogenic strain A30 was resistant to chloramphenicol, however binding of AFA-III expressed by the diarrhoea associated strains AL845 and AL847 was inhibited by chloramphenicol (Bouguenec *et al* 1993). Sequence comparison of the AFA-III subunits expressed by these strains showed that the only difference was an aspartic acid residue in position 52 in strains AL845 and AL847 and an asparagine residue in AL30. This residue is therefore thought to be involved in receptor binding.

Further experiments using trypsin, pronase and chymotrypsin to perform variable digests of the Dr blood group antigen allowed the putative receptor binding sites to be identified. None of the adhesins agglutinated chymotrypsin-treated erythrocytes, F1845 adhesin was able to weakly agglutinate pronase-treated erythrocytes, AFA-III and F1845 strongly agglutinated trypsin treated erythrocytes and AFA-I weakly agglutinated trypsin treated erythrocytes. A model was therefore proposed for the receptor binding sites of each of these adhesins (Figure 1.6). Experiments using monoclonal antibodies directed against different epitopes on the Dr antigen confirmed that different adhesins recognised different epitopes on the DAF molecule (Nowicki *et al* 1990). Structure function studies have led to the mapping of the Dr adhesin to the SCR-3 epitope of DAF (Nowicki *et al* 1993). It is not known whether the complement regulatory function of DAF is affected by the interaction with bacterial adhesins, however this is a potential mechanism by which invading bacteria might be able to modulate the human immune response.

The close functional relationship between different members of the Dr haemagglutinin family was demonstrated in a series of complementation experiments (Swanson *et al* 1991). Chimeric recombinants were constructed which shared elements of both the F1845 and Dr adhesin gene complexes. The constructs were able to mediate haemagglutination showing that the adhesin subunit genes and accessory genes for F1845 and the Dr adhesin are functionally interchangable. Experiments using hybrid chimeric constructs combined with site-directed mutagenesis in the presence of chloramphenicol (which inhibits Dr binding but not F1845) showed that the receptor binding domain is located in the amino-terminal half of the fimbrial subunit protein. Comparison of the amino acid sequences of the F1845, AFA-I and Dr adhesin subunits shows that the adhesins share extensive areas of homology, particularly in the N-terminal regions.

NFA-1 and NFA-2

In 1984 Goldhar *et al* described an isolate of *E. coli* (serotype 083:K1:H4, strain 827) which exhibited mannose-sensitive haemagglutination and also expressed a non-fimbrial adhesin which mediated mannose-resistant haemmagglutinin. The non-fimbrial adhesin was denoted NFA-1. Strain 827 was cultured from the urine and blood of an elderly patient. The isolate agglutinated human group A erythrocytes, but did not agglutinate erythrocytes from seven other animal species. Adherence to cultured human kidney cells was inhibited in the presence of purified mannose-resistant adhesin. Haemagglutination was considerably reduced if the isolates were heated for 1 hour at 65°C, however, after heat treatment the supernatant developed haemagglutination activity. MRHA occurred at 4°C but disappeared at temperatures above 40°C. Haemagglutination was abolished by treatment with formaldehyde, heating at 100°C or digestion with pronase but was unaffected by periodate. These properties suggested that the adhesin was proteinaceous. The absence of fimbrial structures was shown by electron microscopy and the adhesin did not share the physical characteristics of a fimbrial adhesin as it did not sediment at 149,000g and it did not elute in the void volume of a sepharose-4B column.

A second nonfimbrial adhesin (denoted NFA-2) was first described in 1987 (Goldhar *et al* 1987). This adhesin was expressed by an isolate of *E.coli* (serotype 014:K?H11, strain 54) which was cultured from a patient with a urinary tract infection. The adhesin showed similarities with NFA-1 as expression of both adhesins was enhanced by growth on agar rather than liquid culture, and expression of both was inhibited by growth at 20°C or below or in the presence of 1% glucose. Polyclonal antisera raised in rabbits against purified NFA-1 and NFA-2 showed a degree of cross-reactivity.

Monoclonal antibodies were raised against both NFA-1 and NFA-2 and used for immunoelectron microscopy of strains 827 and 54. These studies revealed the presence of an extracellular adhesive protein capsule around the bacterial cells of strain 827, however strain 54 cells showed more patchy extracellular material with no evidence of a well developed capsular structure. The antibody-stabilised capsule was seen in approximately 25% of the population of *E. coli* 827. Adhesive and non-adhesive bacteria were separated by erythocyte adsorption however, it was noted that the proportion of NFA+ and NFA- strains in the bacterial population was unstable suggesting that phase-variation was probably taking place.

The nature of the receptor specificity of NFA-1 and NFA-2 was investigated in a series of biochemical experiments. Haemagglutination was abolished if erythrocytes were pre-treated with pronase or sodium periodate, although other proteases were inactive and treatment with a variety of sugars (D-fructose, D-xylose, D-galactose, L-rhamnose, maltose, methyl alpha-D-mannoside, Methyl alpha-D-glucoside, D-glucosamine, N-acetylglucosamine, Gal-Gal beads), fetuin or orosomucoid had no effect. The conclusion from these studies was that the non-fimbrial adhesins probably recognised a glycoprotein receptor but the precise nature of the receptor has yet to be determined. NFA-1 also mediates binding to human polymorphonuclear cells and the attachment is followed by an oxidative burst leading to bacterial killing (Goldhar *et al* 1991). The clinical importance of this finding is uncertain.

NFA-1 and NFA-2 adhesins were extracted and purified by heating bacteria to 65°C releasing adhesin into the supernatant, then centrifuging to remove bacteria cells followed by fractional precipitation with ammonium sulphate. The subsequent purification procedure included high pressure liquid chromatography and SDS-PAGE. The resulting purified adhesins were characterised by Goldhar *et al* (1987). Immunoblotting studies showed that the adhesin subunit size was 21kDa for NFA-1 and 19kDa for NFA-2. Under non-denaturing conditions both subunits formed aggregates with molecular weight in excess of 106kDa. Treatment with trypsin, chymotrypsin or V8 protease had only a marginal effect on the fragments visualised by SDS-PAGE.

The serological relationship between NFA-1 and NFA-2 was explored in greater detail using specific monoclonal antibodies raised against each adhesin. Although the purified NFA's cross reacted in ELISA the antibodies could be used to differentiate each strain indicating that NFA-1 and NFA-2 are serologically related but not identical. There was no cross-reactivity between the anti-NFA antibodies and a range of other fimbrial antigens.

A series of experiments investigated binding of non-fimbrial adhesins to human kidney cell monolayers with the extent of binding detected by ELISA. These experiments showed that there was a linear dose response between cell binding and the concentration of either crude bacteria (strain 827 or 54) or purified adhesin (NFA-1 or NFA-2). Adhesion of *E. coli* strain 827 was not inhibited by anti-O-specific antisera but was inhibited in the presence of purified NFA-1, and, to a lesser extent, in the presence of purified NFA-2. These results showed that NFA-1 and NFA-2 mediate haemagglutination and binding of bacteria to human kidney cells and the two NFA-s exhibit partial cross-reactivity.

NFA-3

A third non-fimbrial adhesin (NFA-3) was described by Grunberg *et al* in 1988. This adhesin was identified on an isolate of *E. coli* (serotype 020:KX104:H-, strain 9) obtained from a patient in Tel-Aviv with septicaemia. Electron microscopy of this strain did not reveal any fimbria.

Extraction and purification of NFA-3 was performed using the technique described for NFA-1 and NFA-2. The extraction temperature appeared critical for NFA-3 as adhesin was only released at temperatures below 70°C. The yield of NFA-3 was relatively low and only 1mg of purified NFA-3 was extracted from 100g of bacteria. The adhesin subunit size was 17.5kDa and high Mwt aggregates were formed in aqueous solution. Determination of the amino-acid composition showed that the adhesin had an isoelectric point of 3.9 and contained one cysteine and no methionine residues.

Strain 9 and purified NFA-3 adhesin both mediated MRHA. The adhesin receptor appeared to be the N-blood group antigen as agglutination of erythrocytes of blood group A^{NN} was approximately 10 fold greater than those of blood group A^{MM} and erythrocytes lacking the N-blood group receptor were not agglutinated by NFA-3. Further experiments showed that adhesion was modified if erythrocytes were pre-treated with proteolytic agents or periodate and adhesion was inhibited in the presence of N-specific Vg-lectin, N-acytylation, and anti-N antisera. Adhesion was also inhibited by purified glycophorin A^{NN}, however glycophorin A^{MM} had no effect. ELISA studies confirmed that NFA-3 binds to glycophorin A^{NN} in a dose-dependent fashion. These findings suggest that the NFA-3 receptor is glycophorin A^{NN}. Binding of bacteria expressing NFA-3 to polymorphonuclear leucocytes is also mediated by an N-like determinant and adhesion to polymorphs is followed by ingestion of bacteria and phagocytosis (Grunberg *et al* 1994).

NFA-4

A fourth non-fimbrial adhesin (NFA-4) was described by Hoschutzky *et al* in 1989. This adhesin was identified on an isolate of *E. coli* (serotype 07:K98:H6) obtained from a patient in Rostock with a urinary tract infection. This strain exhibits mannose-sensitive and mannose-resistant haemagglutination. Expression of type-1 fimbriae mediating mannose-sensitive haemagglutination is suppressed by growth on agar. MRHA was observed with human and chicken erythrocytes only and MRHA was inhibited by growth at temperatures below 20°C or in the presence of 1% glucose.

NFA-4 was purified using a modification of the technique described for purifying NFA1-3. SDS-PAGE of purified NFA-4 revealed a subunit size of 28kDa with subunits forming an aggregate with a molecular weight in excess of 10^6Da under non-denaturing conditions. Further analysis of the purified protein showed that it had an isoelectric point of 4.4. Determination of the amino acid composition revealed one cysteine residue and five methionine residues with no obvious disulphide bonds. The N-terminal amino acid sequence was determined for the first 23 residues. Comparison of the N-terminal amino acid sequence with other published sequences showed no apparent homology between NFA-4 and the previously described M-adhesin (Rhen *et al* 1986b), but there was 70% homology with the K88 fimbrial antigen (Klemm *et al* 1981).

Hoschutzky *et al* (1989) found that NFA-4 rapidly agglutinated erythrocytes of blood group MM; agglutination of MN erythrocytes occured more slowly and NN erythrocytes were only agglutinated after 3-5 mins. Pretreatment of erythrocytes with proteases or periodate abolished agglutination, the presence of sugars or simple carbohydrates (galactose, digalactose, mannose, glucose, N-acetyl-neuramnic acid, sialylactose) had no effect. Haemagglutination was inhibited by glycophorin A^{MM}, however glycophorin A^{NN} had no effect. The

NFA-4 receptor was therefore thought to be glycophorin A^{MM}. NFA-4 appears to be unrelated to the previously described non-fimbrial M-adhesin (Rhen *et al* 1986b) as they have differing subunit sizes and N-terminal amino acid sequences.

Several NFA-4 specific monoclonal antibodies were generated and their effect on adhesion was investigated in a haemadhesion assay. One of the monoclonal antibodies (mab NFA-4-IA4) was strongly antiadhesive, whereas others had only a marginal or no effect on adhesion. These antibodies presumably recognise different epitopes of the NFA-4 subunit molecule and could help to define which epitopes are involved in receptor binding.

Monoclonal antibodies directed against the NFA-4 adhesin and K98 capsular antiserum have been used to investigate the structure and configuration of *E. coli* 07:K98:H6 by single and double-contrast immunoelectron microscopy (Kroncke *et al* 1990). These studies show that individual *E. coli* can simultaneously express both the polysaccharide capsule and non-fimbrial adhesin. The outer surface of the bacteria consists of a composite concentric structure with the non-fimbrial adhesin diffusely surrounding the inner polysaccharide capsule. It has been suggested that the presence of non-fimbrial adhesins might reduce bacterial antigenicity although this has not been clearly demonstrated.

Isolates expressing the four non-fimbrial adhesins NFA1-4 were all obtained from patients with urinary tract infections, however the epidemiology and clinical significance of this group of non-fimbrial adhesins has not been defined. The properties of the four characterised non-fimbrial adhesins are summarised and compared in Table 1.3. This thesis describes a molecular analysis of this group of non-fimbrial adhesins.

Table 1.1

Microbiology of uncomplicated and complicated urinary tract infections

	Community acquired in previously healthy host	Hospital acquired and/or impaired host defences
Gram -ve bacteria	*Escherichia coli* Klebsiella Proteus	*Escherichia coli* Klebsiella Proteus Pseudomonas Enterobacter Serratia Citrobacter Salmonella
Gram +ve bacteria	*Staphylococcus saprophyticus*	*Staphylococcus epidermidis* Enterococci Lactobacilli Group B Streptococci
Mycobacteria		*Mycobacterium tuberculosis* Atypical mycobacteria
Mycoplasmas		Ureaplasma
Fungi		Candida Aspergillus Cryptococcus
Viruses		Cytomegalovirus Adenovirus

Table 1.2

Bacterial and host factors in the pathogenesis of urinary tract infection

Bacterial virulence factors	Host defence factors
Adhesins	Urinary flow
Type 1 fimbriae	
P-fimbriae	Urinary composition
X-adhesins	pH and solute concentration
	Free oligosaccharide residues
Lipopolysaccharide	Uromucoid
	Sloughed epithelial cells
Capsular polysaccharide	
	Commensal flora around lower urethra
Siderophores	
Aerobactin	Ureteric peristalsis
Enterochelin	
	Host immunity
Haemolysin	Local secretory immunoglobulin
	Polymorphonuclear phagocytosis
Urease	Complement-mediated killing
	Cell-mediated immunity
Antibiotic resistance genes	

Table 1.3

Comparison of the properies of non-fimbrial adhesins

	NFA-1	NFA-2	NFA-3	NFA-4
Serotype	O83:41:H4	O14:K?H11	O20:KX104:H-	O7:K98:H16
Strain	827	54	9	
EM Appearance	diffuse	patchy	diffuse	diffuse
Receptor	glycoprotein	glycoprotein	glycophorin A^{NN}	glycophorin A^{MM}
Subunit size (kDa)	21	19	17.5	28

Figure 1.1

The genetic organisation of the *fim* (*pil*) gene cluster encoding expression of the type 1 fimbria (adapted from Schmoll *et al* 1989)

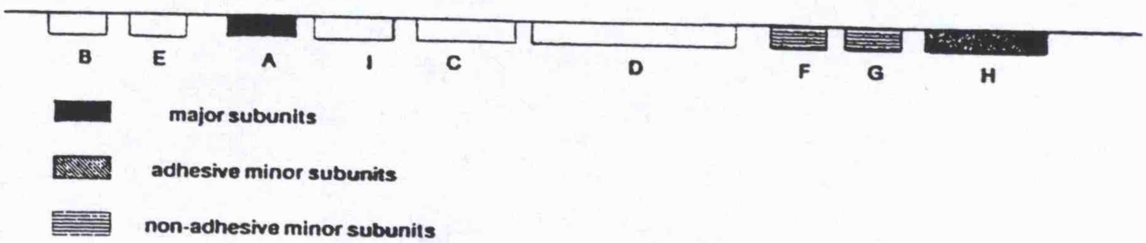


Figure 1.2

The genetic organisation of the *pap* gene cluster encoding P-fimbrial expression (adapted from Jacob-Dubuisson *et al* 1993)

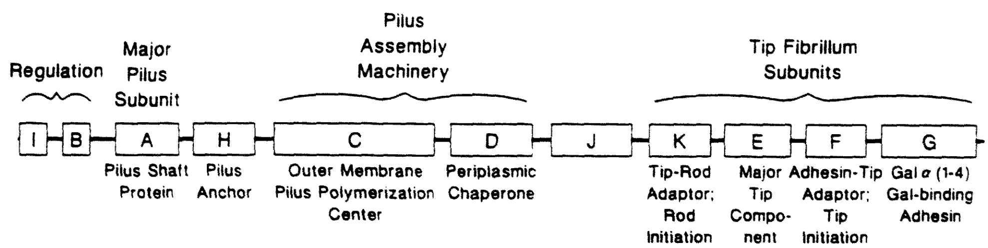


Figure 1.3

A model for the assembly of P-fimbriae
(from Jacob-Dubuisson *et al* 1993)

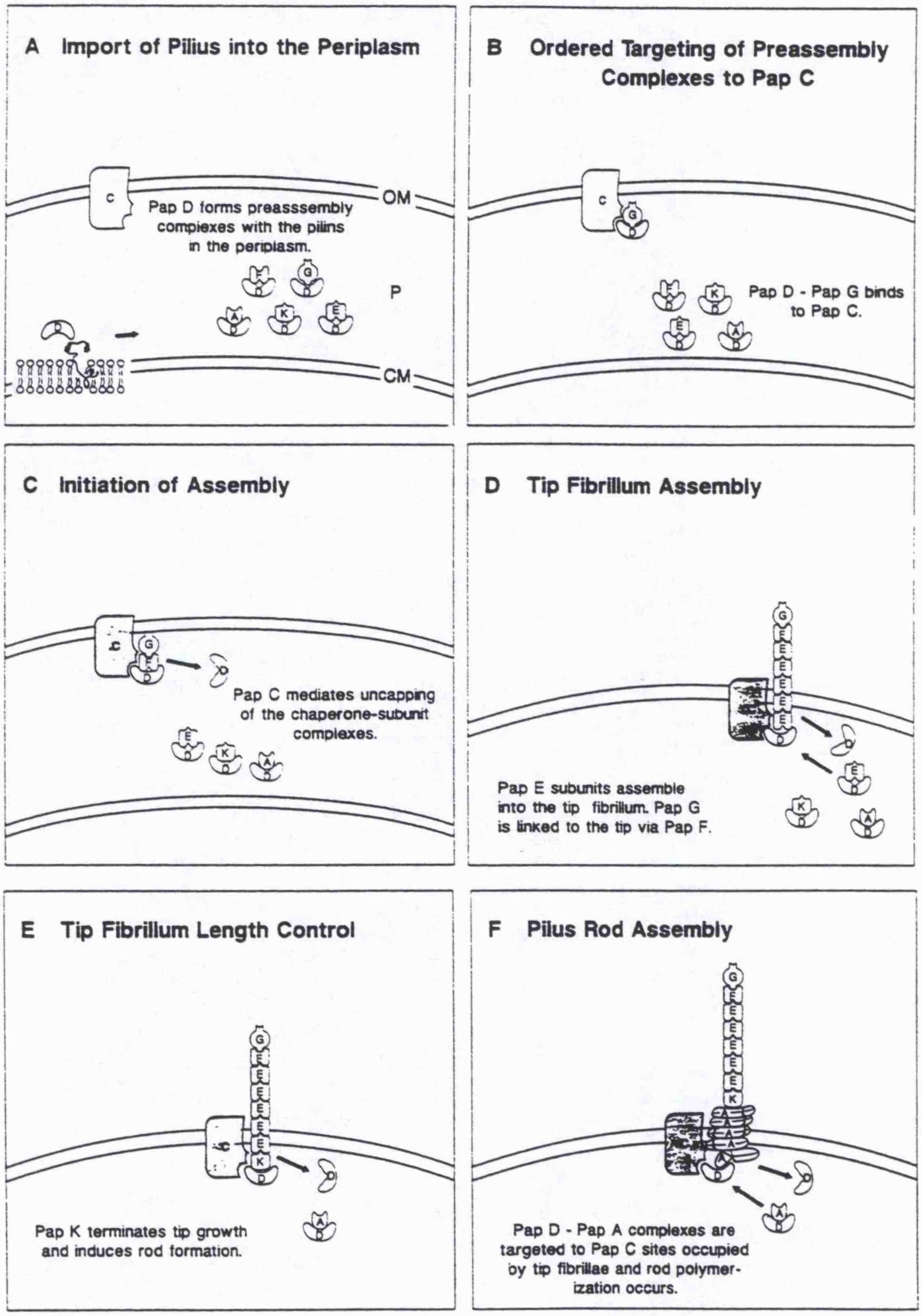


Figure 1.4

The genetic organisation of the *sfa* gene cluster encoding S-fimbrial expression (adapted from Schmoll *et al* 1989)

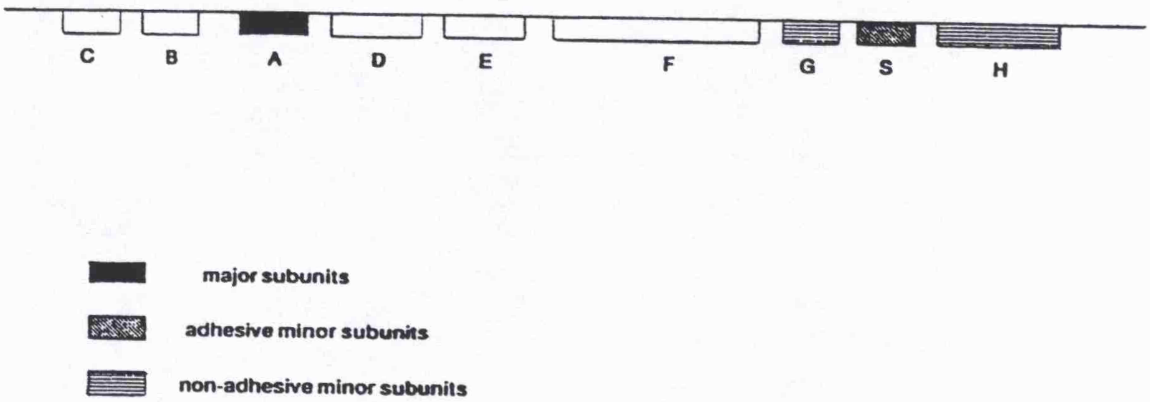


Figure 1.5

Comparison of physical and genetic maps of the gene clusters encoding expression of P-fimbriae (*pap* operon), type-1 fimbriae (*fim* operon), F1C-fimbriae (*foc* operon) and S-fimbriae (*sfa* operon).
(adapted from Johnson 1991)

Solid boxes	genes encoding minor proteins
Hatched boxes	genes encoding major (structural) fimbrial subunits

Key		
	C	*Cla*1
	E	*Eco*R1
	H	*Hind*III
	X	*Xho*I

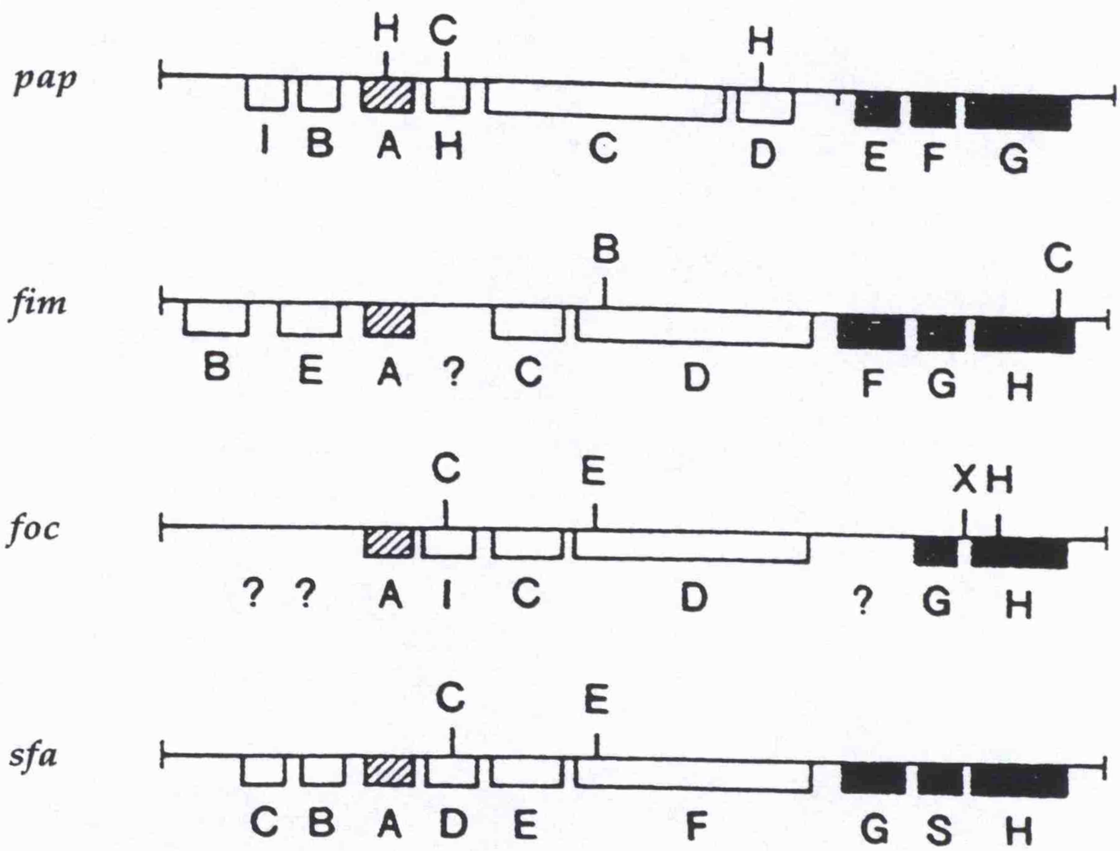
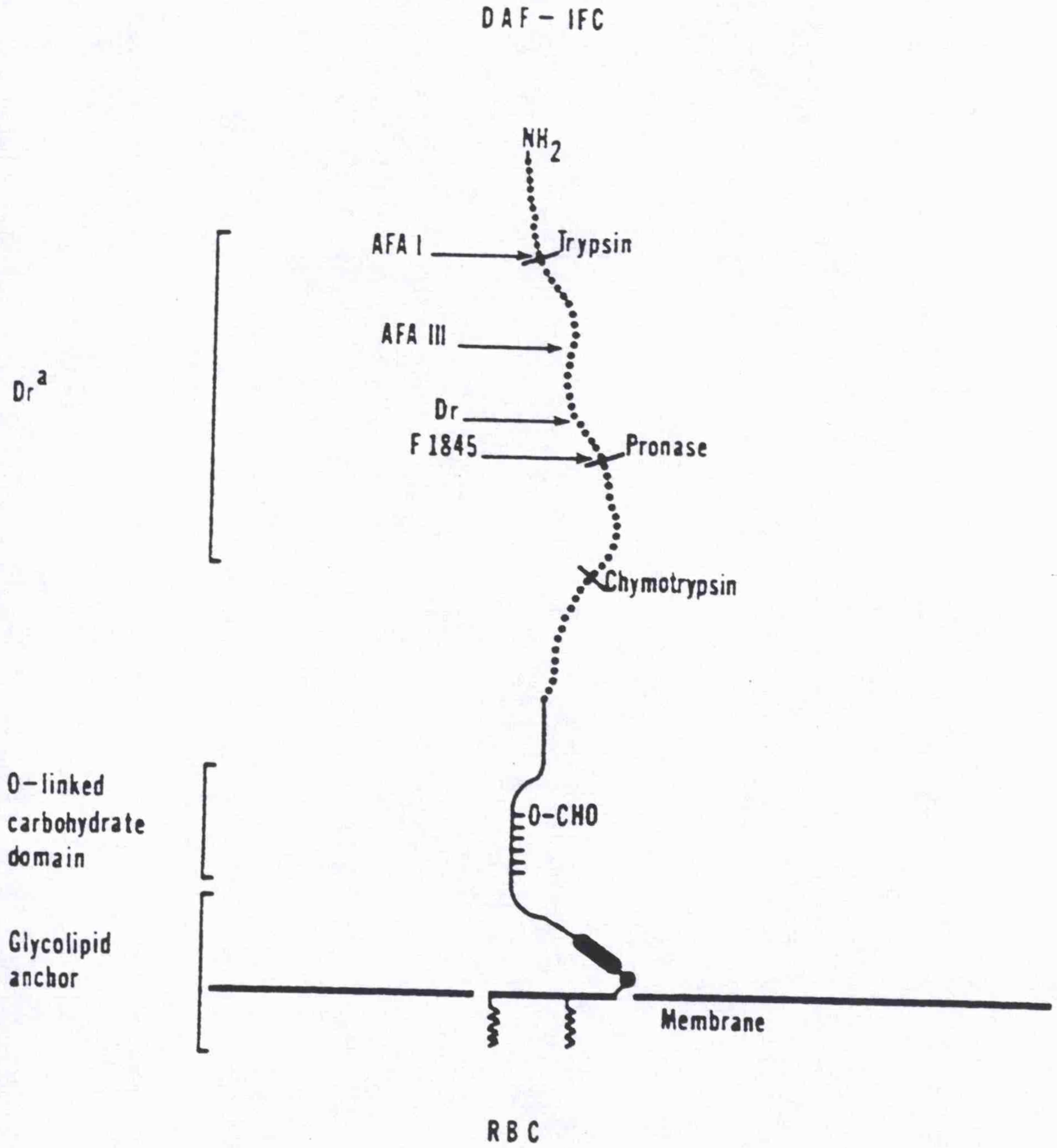


Figure 1.6
A model of the DAF molecule proposed by Nowicki *et al* (1990) showing Dr blood group antigen and receptor binding sites for the Dr, AFA-1, AFA-III and F1845 adhesins
(RBC = erythrocyte)



CHAPTER 2

MATERIALS AND METHODS

MATERIALS

2.1 Materials

Chemicals

Chemicals were obtained from BDH Ltd, Poole Dorset, unless otherwise stated.

Antibiotics

Antibiotics were supplied by Sigma Biochemicals, Poole, Dorset.

Restriction endonucleases and DNA modifying enzymes

Restriction endonucleases and other DNA-modifying enzymes were obtained from Gibco/BRL, Paisley, Scotland.

API bacterial identification test kits

These were obtained from BioMerieux SA, Marcy-l'Etoile, France.

Radiochemicals

These were obtained from Amersham Ltd, Amersham, Bucks, UK.

Plasticware

Plasticware (Eppendorf tubes, Universal tubes, Falcon tubes, culture plates, pipettes, bacterial filters, 96 well plates) were obtained from the University of Leicester Bulk Store facility.

Media

Bacteria were grown in Luria broth at 37°C unless otherwise stated. 1.5% agar (BBL) was added as required. Minimal Media was prepared as described by Boulnois and Wilkins (1978). When required media was solidified by the addition of Bacto Agar (Difco) to a final concentration of 1.6%. Antibiotics were incorporated into the media where appropriate at the following final concentrations: Ampicillin (100µg/ml), Kanamycin (25µg/ml), Tetracycline (20µg/ml).

Bacterial strains and plasmids

The laboratory stains of bacteria and plasmids used in these studies are shown in Table 2.1.

Monoclonal and polyclonal antisera to NFA1-4

Antisera were kindly provided by Professor K Jann (University of Freiburg, Germany) and Professor J Goldhar (University of Tel Aviv, Israel).

METHODS

2.2 Haemagglutination assay

The haemagglutination assay detected mannose-resistant haemagglutination (MRHA) in bacteria grown on agar or in broth culture. Bacteria grown in liquid culture were centrifuged to precipitate a bacterial pellet. Bacterial cells were resuspended in phosphate buffered saline (PBS) containing 50mM alpha-D-mannoside and mixed with an equal volume of 3% erythrocyte suspension. The reaction was performed on a glass microscope slide or in 96-well microtitre plates.

The 3% erythrocyte suspension was prepared by centrifuging whole blood at 2000rpm for 5 minutes in a Heraeus-Christ centrifuge and washing in PBS. The process was repeated three times and washed erythrocytes were resuspended in PBS to form a 3% solution.

2.3 Separation and characterisation of proteins

Polyacrylamide gel electrophoresis (PAGE)

The technique of polyacrylamide gel electrophoresis (PAGE) separates proteins by electrophoretic mobility. The composition of the running gels used to separate non-fimbrial proteins varied between 10 to 15% polyacrylamide depending on the size of the protein of interest. Increasing concentations of polyacrylamide allow the separation of higher molecular weight proteins.

SDS/PAGE and Western blotting experiments were performed using Bio Rad apparatus. Gel plates were assembled and filled with polyacrylamide running solution, The gel was prepared by assembling the gel plates and adding running solution, allowing sufficient space at the top of the gel for addition of stacking gel. A small amount of butanol was added to leave a straight edge and the running gel was left for approximately 30 mins to polymerise. Butanol was then poured off and 5% stacking gel was added. The spacers were introduced and the stacking gel was left for a further 30 mins to polymerise.

SDS/PAGE buffer was added to protein samples and the mixture was boiled for 3 minutes, to denature proteins, prior to loading onto the gel. The loaded gel was placed in running buffer and electrophoresed at 200 volts for approximately 45 mins until the dye front reached the lower end of the gel.

SDS/PAGE Sample Buffer	2ml glycerol 5ml 0.25M Tris HCl (pH 6.8), 0.2% SDS 1ml beta-mercaptoethanol 0.01g bromophenol blue
30% Acrylamide solution	28.2g acrylamide 1.8g methylene bis acrylamide Made up to 100ml with distilled water, stored in dark at 4^oC
10% Acrylamide running gel (10ml)	4ml distilled water 3.3ml 30% acrylamide mix 2.5ml 1.5M Tris HCl (pH 8.8) 0.1ml 10% SDS 0.1ml 10% ammonium persulphate 0.004ml TEMED
5% Acrylamide stacking gel (5ml)	3.4ml distilled water 0.83ml 30% acrylamide mix 0.63ml 1.0M Tris HCl (pH 8.8) 0.05ml 10% SDS 0.05ml 10% ammonium persulphate 0.005ml TEMED

SDS/PAGE Running Buffer	3.03g Tris Base 1.0g SDS 14.4g glycine Made up to 1 litre with distilled water

Staining of SDS-polyacrylamide gels

In order to visualise protein bands the gel was fixed in methanol:glacial acetic acid and simultaneously stained with bromophenol blue. The fixed gel was soaked in SDS/PAGE stain for 20 mins. Excess dye was subsequently removed by soaking in destaining solution for several hours in a rocking bath, until the protein bands became visible and distinct.

SDS/PAGE Stain	20% (v/v) methanol 10% (v/v) glacial acetic acid 0.2% (w/v) bromophenol blue Made up to required volume with distilled water
SDS/PAGE Destain	20% (w/v) methanol 10% (w/v) glacial acetic acid Made up to required volume with distilled water

Western Blotting

Western blotting describes the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose filter paper (Towbin *et al* 1979). Proteins were separated by polyacrylamide gel electrophoresis as described above. Gels and nitrocellulose filters were soaked in transfer buffer prior to blotting. The apparatus was then assembled with the filter paper overlying the gel and the blotting tank was filled with transfer buffer. Blotting was performed by electrophoresis at 150mA for 1 hour or overnight at 40mA.

Transfer buffer (pH8.3)	3.03g Tris 14.4g glycine 200ml methanol (Analar) distilled water to 1 litre

Solid phase immunosorbent assay

Following Western blotting proteins were probed with polyclonal or murine monoclonal antisera in a solid phase immunosorbent assay. Nitrocellulose filters were placed in blocking solution, shaken gently for 1-2 hours to inhibit non-specific protein binding and washed in 1xPBS for 5x 30 seconds. The appropriate polyclonal or monoclonal antibody was diluted in 1xPBS/0.1% Tween 20 at a concentration of approximately 1 in 1000. Filters were probed by shaking gently for 1-2 hours in the antibody solution, then washed in 1x PBS for 5x 30 seconds to remove unbound antibody.

Bound antibody was visualised by probing the filters with horse-radish peroxidase labelled secondary antibody diluted in 1xPBS/0.1% Tween 20 at a concentration of approximately 1 in 2000. Goat anti-rabbit or anti-mouse antibody was used depending on the nature of the primary antibody. The filter was shaken gently for 1-2 hours in the secondary antibody solution, then washed in 1x PBS for 5x 30 seconds to remove unbound antibody. Washed filters were stained by incubating for approximately 15 minutes in the substrate solution then rinsed in distilled water, dried and photographed.

Blocking solution	3% dried skimmed milk 0.1% Tween 20 1x PBS
Staining solution	100ml 50mM Tris pH7.2 60mg 1-chloronaphthol in 3ml methanol 50μl hydrogen peroxide made up immediately before use

2.4 Preparation of DNA

Preparation of chromosomal DNA

A 10ml overnight bacterial culture was centrifuged at 3000rpm for 5 mins in an Heraeus-Christ minifuge. The bacterial pellet was resuspended in 4ml of STE solution. Cells were lysed by the addition of 1ml of 10% sodium dodecyl sulphate (SDS). The solution was gently agitated at room temperature until the lysate was clear. Protein was extracted from the lysate by adding an equal volume of phenol:chloroform:8-hydroxyquinolone (1:1:0.001) equilibrated in 0.1M Tris pH8. The two phases were mixed gently by hand for 5 mins then separated by centrifuging for 10 mins at 5000rpm at room temp in a Heraeus-Christ minifuge. The aqueous phase was removed to a fresh tube and the phenol extraction procedure was repeated five or six times.

Chromosomal DNA was precipitated from the lysate by the addition of 0.1 volume of 3M sodium acetate (pH6) and 2 volumes of ice cold absolute ethanol with gentle mixing. Strands of DNA were removed using a glass hook and resuspended in TE buffer.

STE Solution	15% sucrose 50mM Tris (pH7.5) 50mM EDTA
TE Buffer	10mM Tris (pH8) 1mM EDTA

Large scale preparation of plasmid DNA

An alkaline lysis method was used. 500ml overnight bacterial culture was centrifuged at 4000g for 10 mins at 4°C. The bacterial pellet was resuspended in 10ml of solution 1 with 0.5mg/ml lysozyme and incubated on ice for 5 minutes. The resulting spheroplasts were lysed by the addition of 20ml of freshly prepared alkaline lysis solution. The resulting mixture was incubated on ice for 10 minutes then 15ml of an ice-cold solution of 5M potassium acetate (pH4.8) was added and the solutions mixed by gently inverting the tube several times. The mixture was left on ice for 5 mins then centrifuged at 30,000g for 20 mins at 4°C to precipitate cell debris and chromosomal DNA. 18ml volumes of supernatant were removed into Corex tubes and 12ml (0.6 vols) of isopropanol was added. The solutions were thoroughly mixed and left at room temperature for 15mins. DNA was recovered by centrifuging at 4500g for 30 mins at room temperature. The resulting pellet was resuspended in 12mls of TE buffer and caesium chloride was added to give a final concentration of 1mg/ml. 1ml of ethidium bromide solution (10mg/ml) was added and the refractive index of the solution was adjusted to 1.392.

Plasmid DNA was separated by caesium chloride buoyant density centrifugation. The solution was placed in a Sorval tube and centrifuged at 40,000 rpm using a Sorval TV850 rotor in a Sorval OTD 60 centrifuge for 20 hours at 20°C. The band corresponding to closed circular plasmid DNA was visualised under UV light and the band was carefully removed using a hypodermic needle inserted into the side of the tube. Ethidium bromide was extracted with CsCl saturated isopropanol and CsCl was removed by exhaustive dialysis against TE buffer.

Solution 1	50mM Glucose 25mM Tris (pH8) 10mM EDTA (pH8)
Alkaline lysis solution	0.2N NaOH 1% SDS

Small scale preparation of plasmid DNA (plasmid miniprep)

Plasmid miniprep were prepared using an alkaline lysis procedure. 1.5ml of an overnight bacterial culture was placed in an Eppendorf tube and centrifuged for 1 minute in a bench microfuge (Centaur). The supernatant was poured off and the resulting pellet of bacterial cells was resuspended in 100µl of ice cold solution 1. Cells were lysed by the addition of 200µl of freshly prepared alkaline lysis solution. The solutions were mixed by inversion of the tube several times and left on ice for 5 mins. 150µl of an ice-cold solution of 5M potassium acetate (pH4.8) was added and the solutions mixed gently by vortexing the Eppendorf tube in an inverted position for 10 secs. The solutions were incubated on ice for 5 mins and centrifuged for 5 mins at 4°C in a minifuge to remove cell debris. The supernatant was transferred to a fresh tube, remaining proteins were extracted using phenol and DNA was precipitated with ethanol as described below.

Solution 1	50mM glucose 25mM Tris HCl (pH8) 10mM EDTA (pH8)
Alkaline lysis solution	0.2M NaOH 1% SDS

Phenol extraction of protein from DNA

An equal volume of phenol:chloroform:8-hydroxyquinolone (1:1:0.001) equilibrated in 0.1M Tris pH8 was added to the DNA solution. The solutions were mixed by vortexing and separated by centrifuging for 2 mins in a minifuge or 5000rpm in an Heraeus Christ minifuge at room temperature if larger volumes were used. The aqueous supernatant was transferred to a fresh tube. Phenol extraction was repeated as necessary to extract all protein.

Precipitation of DNA with ethanol

DNA was precipitated by the addition of a tenth volume of 3M sodium acetate (pH5.5) and two volumes of absolute ethanol. The solutions were mixed by vortexing and left at -20°C for at least 1 hour (or -70°C for 15 mins). DNA was pelleted by centifuging for 5 mins in a minifuge. The supernatant fluid was carefully poured away and the pellet was briefly dried in a vacuum dessicator before resuspending in TE buffer.

Agarose gel electrophoresis

DNA fragments of different sizes were separated by agarose gel electrophoresis. 0.7% agarose gel was used to separate 1-20kb fragments. 0.5% agarose gel was used for low MWt DNA (<1kb) and 1.5% agarose gel was used for high MWt DNA (>20kb). 0.1vol of 10x DNA loading buffer was added to samples before loading onto the gel. Electrophoresis of agarose gels was performed at 100v for 1-3 hours or 20v overnight in 1x ELFO. DNA standard markers were routinely loaded onto the gel in addition to DNA samples. Markers used were 1kb ladder (Gibco BRL) or lambda phage DNA digested with *Xho*1 and *Hin*dIII.

10x DNA loading buffer (10ml)	5ml 50% glycerol in distilled water 1ml 50X ELFO 4ml distilled water 25mg bromophenol blue
50x ELFO (1 litre)	242g Tris 18.61g EDTA made up to 1 litre with distilled water and adjusted to pH 7.7 with glacial acetic acid

Extraction of DNA fragments from agarose gels

DNA fragments were visualised under a UV transilluminator and bands of interest were excised from the gel using a scalpel blade. The gel slice was placed in dialysis tubing, containing approximately 500μl of TE buffer and sealed with dialysis clips. The tube was then placed in a electrophoresis tank and electrophoresed at 100v for 1 hour to elute DNA from the gel. The polarity of the current was reversed for 30 seconds to release DNA on the side of the dialysis tubing. The solution was removed from the dialysis tube using a pipette and DNA was precipitated with ethanol and resuspended in TE buffer as described above.

Restriction endonuclease digestion of DNA

Restriction endonuclease digestion of DNA was performed according to the manufacturers' recommendations. Partial digestion of chromosomal DNA with *Sau*3A was performed by setting up the restriction endonuclease reaction and removing aliquots at varying intervals. Reactions were stopped by incubating at 65°C for 15 mins and the series of partial digests were electrophoresed in agarose gels together with standard DNA size markers.

2.5 DNA manipulation procedures

Ligation of DNA

Ligation reactions were performed overnight at 15°C in a total reaction volume of 10μl. Following ligation enzymes were removed by phenol extraction and DNA was precipitated with ethanol.

Ligation reaction	7.5µl DNA sample 1µl 10x ligation buffer 1µl 10mM ATP 0.5µl T4 ligase
10x ligation buffer	0.5M Tris HCl (pH 7.4) 0.1M $MgCl_2$ 0.1M Dithiothreitol (DTT)

Dephosphorylation of DNA using Calf Intestinal Phosphatase

The dephoshorylation reaction removes the 5' terminal phosphate group thus preventing self ligation. DNA fragments were generated by restriction endonuclease digestion and 0.1 unit of calf intestinal phosphatase (CIP) was added for each µg DNA. One tenth volume of 10x CIP buffer was added to the reaction mixture. After 60 mins the restriction endonuclease and intestinal phosphatase were removed by phenol extraction and DNA was precipitated with ethanol.

10x CIP reaction buffer	0.5M Tris HCl (pH 9) 10mM $MgCl_2$ 1mM $ZnCl_2$

Transformation of DNA

The process of transformation allows plasmid DNA to enter competant cells. Competant cells were prepared using $CaCl_2$.

E. coli were grown to an OD650 of 0.5 (ie. to mid log phase; approximately 2 hours in L-broth at 37°C). and centrifuged at 3000rpm for 5 mins in an Heraeus Christ minifuge. The cell pellet was resuspended in a half volume of ice cold 100mM $CaCl_2$. The suspension was centrifuged again and the pellet was resuspended in a similar volume of ice cold $CaCl_2$. The suspension was centrifuged for a third time and the pellet resuspended in a tenth volume of ice cold $CaCl_2$ and incubated on ice for 1 hour. Plasmid DNA was then added to 150μl of competant cells. The mixture was left on ice for a further hour, heat shocked at 42°C for 2 mins then replaced on ice for 2 mins. 1ml of L-broth was added to the cells folowed by incubation at 37°C for 1 hour to allow expression of antibiotic resistance genes. Cells were then placed onto media containing appropriate antibiotics for selection of transformants.

2.6 DNA labelling and hybridisation procedures

Southern blotting

The process of Southern blotting transfers DNA from agarose gels onto a hybridisation membrane. The method used was based on that described by Southern (1975). In order to improve the transfer of DNA the gel was depurinated, denatured and neutralised prior to blotting. The gel was soaked twice for 15 mins in each solution and rinsed in distilled water between each soak. The gel and the membrane were both soaked in 3x SSC prior to blotting. DNA was then transferred by blotting for at least five hours or overnight. Following blotting DNA was fixed to the membrane as specified in the manufacturer's instructions.

Depurination solution	250mM HCl
Denaturing solution	1.5M NaCl 0.5M NaOH
Neutralising solution	1.5M NaCl 0.5M Tris HCl pH7.5
1x SSC	150mM NaCl 15mM Tri Sodium citrate

Radiolabelling of DNA by hexanucleotide primers

The method used was based on that described by Feinberg and Vogelstein (1983) and allows radiolabelled nucleotides to be incorporated into fragments of DNA.

DNA was digested with appropriate restriction enzymes and fragments were separated by electrophoresis through a 1% low melting point agarose gel. The fragment(s) of interest was excised from the gel and placed in an Eppendorf tube. 1.5 ml distilled water was added for each 1g of agarose. The tube was then placed in a boiling water bath for 7 mins to melt the agarose and denature DNA. The resulting solution was incubated at 37^{o}C for 10-60 mins prior to initiating the labelling reaction.

The labelling reaction mixture was incubated at room temperature for 5 hours (or overnight) and the reaction stopped by the addition of 100µl of stop buffer. The reaction mixture was passed through a Sephadex G50 column in a Pasteur pipette to remove unincorporated radioactivity. Approximately 16 100µl aliquots were collected from the column and the radioactivity in each aliquot was assessed using a Geiger counter. Labelled DNA usually came off in fractions 7-11 which were subsequently pooled. Labelled DNA was denatured by boiling for 5 mins prior to use in hybridisation experiments.

Labelling Reaction	5µl OLB buffer
	1µl BSA (bovine serum albumin, 10mg/ml)
	xµl DNA fragment (up to 16.5µl)
	1µl $^{[32P]}$alpha-dCTP (specific activity 1000mCi/ml)
	1µl Klenow (large fragment) DNA polymerase 1 (100units/16.7µl)
	xµl distilled water (to a total volume of 25µl)

TE buffer (pH8)	3mM Tris HCl (pH7.0) 0.2mM EDTA
Solution O	1.25M Tris HCl (pH8.0) 0.125M $MgCl_2$
dNTP's	dATP } dTTP } 0.1M in TE buffer pH7.0 dGTP }
Solution A	1ml solution O 18µl 2-beta-mercaptoethanol 5µl of each of dNTP's
Solution B	2M HEPES (titrated to pH6.6 with 4M NaOH)
Solution C	Hexadeoxynucleotides (50A Units) resuspended in 550µl TE buffer (pH7.0) to give a final concentration of 90A units/ml.
OLB buffer	Solutions A, B and C were mixed in the ratio of 10:25:15.
Stop buffer	20mM NaCl 20mM Tris HCl (pH 7.5) 2mM EDTA 0.25% SDS 1µl dCTP (non-radioactive)

Hybridisation of hexanucleotide-labelled probes to DNA

Hybridisation membranes were incubated in prewarmed prehybridisation solution at 65°C for 3 hours. The solution was poured off and replaced with prewarmed hybridisation solution. The denatured radiolabelled probe was added and the membrane hybridised overnight at 65°C. The solution was poured off and the hybridisation membrane was washed six times for 15 mins at 65°C with 0.1x SSC, 0.1% SDS. After washing the hybridisation membrane was dried at room temperature and autoradiographed.

1x Denhardt's (1 litre)	0.1g BSA 0.1g Polyvinylpyrolidene 0.1g Ficoll 1 litre distilled water
Prehybridisation sol'n	5x Denhardt's 5x SSC 0.1% SDS
Hybridisation solution	5x Denhardt's 5x SSC

Oligolabelling using T4 kinase

Oligonucleotide DNA was labelled at the 5' end with gamma-[^{32}P]ATP in the labelling reaction which was performed at 37°C for 1 hour. The labelled oligonucleotide was purified on a DE52 column. The column was washed with 3mls of TE buffer (pH8.0) before loading the probe. The loaded column was washed with 1ml TE buffer, followed by 1ml of 0.2M NaCl in TE and 0.5ml of 1M NaCl in TE. Aliquots were collected and radioactivity checked using a Geiger counter. The aliquots corresponding to the initial peak in radioactivity were used for subsequent hybridisation experiments.

Labelling reaction	50ng probe
	10μl (100microcuries) gamma-[^{32}P]ATP
	2.5μl 10x labelling buffer
	1μl (10 units) T4 polynucleotide kinase
	Nano-pure water to total volume of 25μl
10x Labelling buffer	0.6M Tris-HCl (pH 7.8) (30μl 2M Tris-HCL)
	0.15M mercaptoethanol (50μl 0.3M mercaptoethanol)
	0.1M $MgCl_2$ (10μl 1M $MgCl_2$)
	(10μl Nano-pure water)

Hybridisation of radiolabelled oligonucleotide probes to DNA

Hybridisation membranes were incubated in prehybridisation solution and agitated at the hybridisation temperature for 2 hours. The prehybridisation solution was poured off and the hybridisation solution was added followed by the labelled oligonucleotide probe solution. The membranes were agitated overnight at the hybridisation temperature. After hybridisation filters were washed according to the following protocol:-

1) 2x SSC / 0.1% SDS	room temperature	5 minutes	
2) 2x SSC / 0.1% SDS	hybridisation temperature	20 minutes	x2
3) 0.5x SSC /0.1% SDS	hybridisation temperature	20 minutes	x2

After washing the filters were air dried for 10 mins then dried at 65°C for 5mins. The filters were then exposed to X-Omat film (Kodak) at -70°C for a variable time depending on activity.

Prehybridisation / Hybridisation Solution	6x SSC 5x Denhardt's 0.5% SDS 200μg/ml salmon sperm DNA

Colony blotting and screening by hybridisation

Colonies to be screened were grown overnight on agar plates. A hybridisation membrane (Hybond N, Amersham International) was then placed over the surface of the agar plate and left for 3 minutes. The membrane was carefully removed, placed colony side up onto 3M Whatman paper saturated with lysis solution and fixed for 5 minutes. The membrane was then transferred to a further sheet of 3M Whatman paper saturated with neutralising solution and left for 5 mins with the colony side up. The filter was air dried for 30 mins, exposed to UV light for 5 mins scrubbed with polyallomer wool then soaked in 5x SSC and air dried for a further 20 mins.

Lysis solution	0.5M NaOH 1.5M NaCl
Neutralising solution	1.5M NaCl 1.0M Tris pH 8

2.7 DNA cloning techniques

Cosmid cloning of DNA

The cosmid cloning procedure was described by Collins and Hohn (1978). The vector used was *cos*4 (Table 2.1) which was prepared by cleavage with *Pvu*II and dephosphorylation of the resulting blunt ends. This resulted in a linear molecule which was cleaved with *Bam*H1 forming two arms, each containing *cos* sites.

DNA fragments of approximate MWt 35-50kb were generated by partial *Sau*3A, digestion followed by agarose gel electrophoresis and purification of the size selected fragments.

Cosmid arms were ligated to the purified size-selected DNA in an approximate ratio of 6:1. Cosmids were packaged into phage heads using an in vitro phage packaging kit (Amersham International, Amersham, Bucks, UK) and *E. coli* strain LE392 was infected with the recombinant phages. Infected LE392 were incubated in L-broth at 37°C for 1 hour to allow expression of ampicillin resistance genes and the recombinants were then plated onto selective media containing ampicillin.

Plasmid cloning of DNA

Purified vector plasmid was digested to completion with *Bam*H1 and the linear product visualised by agarose gel electrophoresis. The plasmid was dephosphorylated using calf intestinal phosphatase and the efficiency of dephosphorylation was checked by attempted ligation of the dephosphorylated plasmid.

Chromosomal DNA was prepared and DNA fragments of approximate MWt 8-12kb were generated by partial *Sau*3A, digestion followed by agarose gel electrophoresis and purification of the size selected fragments.

The relative concentrations of purified DNA fragments and dephosphorylated plasmid were compared by agarose gel electrophoresis. The conditions for the ligation reaction were adjusted to give a relative concentration of approximately 1:4 for the plasmid and insert respectively. Ligation was performed at 15°C with overnight incubation.

2.8 Preparation and labelling of minicells

Preparation of minicells

Plasmid DNA for analysis in minicell preparations was transformed into *E. coli* strain DS410. Transformants were cultured overnight in L-broth containing appropriate antibiotics and a plasmid miniprep was performed to confirm the transformation. The transformant was inoculated into 400ml of BHI broth containing the appropriate antibiotic and incubated overnight at 37°C. The resulting culture was centrifuged at 3000rpm for 5 mins at 4°C. The supernatant was carefully removed and centrifuged at 8000rpm for 15 mins at 4°C to precipitate minicells. The resulting precipitate was resuspended in 3ml of ice cold 1x M9 salts.

The minicell suspension was purified by sucrose gradient centrifugation. The suspension was loaded carefully onto a 20% sucrose gradient and centrifuged at 5000rpm for 20mins at 4°C. After gradient centrifugation the minicells remain suspended in solution, while remaining vegetative cells form a pellet at the bottom of the tube. The minicell suspension was removed from the gradient tube and centrifuged at 10,000rpm for 10 mins at 4°C to precipitate the minicells. The resulting pellet was resuspended in 3ml of ice cold 1x M9 salts.

The minicell suspension was loaded onto a further 20% sucrose gradient and centrifuged at 5000rpm for 20mins at 4°C. The optical density of the minicell suspension was measured at 650nm and the suspension was centrifuged at 10,000rpm for 10 mins at 4°C to precipitate minicells. The resulting pellet was resuspended in an appropriate volume of ice cold 70% 1x M9 salt, 30% glycerol. The volume was calculated to give a final optical density of 2. The suspension was divided into 50µl aliquots which could be stored for several months at -20°C.

10x M9 salts	60g $NaHPO_4$ 30g $KH2PO_4$ 10g NH_4Cl 5g NaCl distilled water to 1 litre
20% sucrose gradient	20g sucrose added to 100ml 1x M9 salts freeze at -20°C thaw for at least 4 hours at 4°C before use

^{35}S methionine labelling of mini cells

A 50µl aliquot of minicells was centrifuged for 5 mins using a bench microfuge and the pellet resuspended in 100µl of 1x M9 salts. The resulting suspension was centrifuged for 5 mins and the pellet resuspended in 100µl of 1x M9 salts with 0.4% glucose and methionine assay medium (MAA). 10µl of a 2.2mg/ml stock of cycloserine was added to destroy any remaining vegetative cells and the suspension was incubated at 37°C for 90 mins then centrifuged for 2 mins.

The resulting pellet was suspended in 100µl of 1x M9 salts with 0.4% glucose and MAA prewarmed to 37°C. 12µCurie of [^{35}S]methionine was added. The suspension was incubated at 37°C for 45 mins, then centrifuged for 2 mins. The resulting pellet was suspended in 100µl of a solution consisting of 90µl of 1x M9 salts with 0.4% glucose and 10µl of 2µg/ml cold methionine solution.

The suspension was incubated at 37°C for 15 mins then centrifuged for 2 mins. The resulting pellet was resuspended in 25µl of 1x SDS/PAGE loading buffer. Samples were boiled for 5 mins before loading onto a mini PAGE gel with ^{14}C labelled markers on either side.

The gel was run at 25mA through the stacking gel and 50mA through the resolving gel. When the dye front reached the bottom the gel was removed from the tank and washed for 30 mins in gel fix followed by 30 mins in "amplify" solution. The gel was placed onto a piece of dry 3M Whatman paper, covered in Saran wrap and dried at 60°C for approximately 60 mins. The dried gel was autoradiographed.

Methionine assay medium (MAA)	0.105g MAA medium in 1ml Nano-pure H_20 made fresh and boiled for 2 mins before use
1x M9 salts with 0.4% glucose and MAA (4ml)	60µl MAA 16mg glucose 400µl 10x M9 salts 3540µl Nano-pure water filter sterilised and used immediately
1x M9 salts with 0.4% glucose (4ml)	16mg glucose 4ml 1x M9 salts filter sterilise
Gel fix	10% glacial acetic acid 25% isopropanol 65% distilled water

2.9 DNA sequencing

Introduction

The method used for DNA sequencing was first described by Sanger (1977) and is known as the chain termination method. In this technique DNA synthesis from deoxynucleotide triphosphates is randomly terminated by the inclusion of dideoxynucleotide triphosphates. DNA fragments are radiolabelled by the addition of $[^{35}S]$dATP to the extension reactions.

A single stranded DNA template is generated using the cloning vector M13mp18 and M13mp19. M13 is a filamentous bacteriophage which is specific for male *E. coli* and has a replicative form which is circular and double-stranded. It therefore has suitable characteristics which allow it to be used as a vector for sequencing strands of inserted DNA. Wild type M13 DNA has been modified for this purpose by inserting a portion of the lacZ gene (beta-galactosidase) incorporating a multiple cloning site.

Several stages were involved in DNA sequencing. Appropriate fragments of DNA were cloned into M13mp18 or M13mp19, allowing sequencing to be performed in both directions. Recombinant M13 clones were transformed into strain JM101 and DNA templates were prepared from individual recombinant plaques. Sequencing reactions were performed and labelled nucleotides were separated by gradient gel electrophoresis.

Cloning of inserts into M13mp18/19

Plasmid DNA was prepared using a miniprep procedure. Plasmids were digested with the appropriate restriction endonucleases and fragments separated by agarose gel electrophoresis. DNA fragments required for sequencing were eluted, and purified DNA was precipitated with ethanol and dissolved in distilled water. The M13 vector was similarly digested and the DNA fragment was subsequently ligated to the M13 digest.

Transformation of M13 clones

A 10ml culture of *E. coli* strain JM101 (taken from a freshly streaked plate of minimal agar) was grown up to mid log phase. Competant cells were prepared and incubated with recombinant M13 DNA for 1 hour on ice then heat shocked at 42°C for 3 mins. The following components were then mixed in a sterile phage tube:-

10 µl 100mM IPTG
20µl competant cells + DNA
200µl plating cells (JM101 grown in broth culture to OD 0.5)
20µl X-gal
2.5ml liquid soft top agar

The contents of the phage tube was poured over a plate of B-agar which was incubated overnight at 37°C.

M19 minimal (4x)	24g Na_2HPO_4
	12g KH_2PO4
	4g NH_4Cl
	2g NaCl
	distilled water to 1 litre
Minimal agar	100ml M19 minimal (4x)
	300ml agar (5.7g Difco Agar)
	4ml 20% glucose
	400µl 20% $MgSO_4$
	200µl 10% thiamine
Soft top agar	1g peptone
	0.5g NaCl
	0.7g agar
	distilled water to 100ml
B-agar	4g Bactopeptone
	3.2g NaCl
	6g Difco Agar

Template preparation

Individual recombinant (white) M13 plaques were inoculated into 2mls of starter JM101 culture (prepared by adding 100µl of fresh overnight JM101 culture to 50ml of Luria broth) and incubated at 37°C with vigorous shaking for 6 hours. 1.5ml from each culture was placed in Eppendorf tubes and centrifuged in a microfuge for 5mins. The phage-containing supernatant was transferred to another Eppendorf tube and 200µl of 10% PEG : 2.5M NaCl was added. The mixture was incubated at room temperature for 30 mins than centrifuged for 5 mins. The supernatant was discarded and the phage pellet was resuspended in 100µl 1.1M sodium acetate pH7. Phenol extraction was performed, followed by extraction with 50µl chloroform : isoamyl alcohol (49:1) to remove excess phenol. Single stranded recombinant M13 was precipitated by adding 250µl of ethanol and resuspended in 20µl of TE buffer.

Sequencing reactions

The "Sequenase kit" (version 2.0, United States Biochemical Corporation) was used to perform all sequencing reactions. The manufacturers' protocol was followed carefully and sequencing primers used were the universal (-40) primer or specific oligonucleotides (based on available sequence data) synthesised in the University of Leicester. The sequencing reaction involves three stages; annealing of the primer to the single-stranded DNA template, extension and labelling of DNA fragments, and termination of DNA synthesis. The reaction was stopped by adding formamide dye mix and samples were heated to 75°C immediately before loading onto the DNA sequencing gel.

DNA sequencing gel

Radiolabelled fragments of DNA were separated by gradient gel electrophoresis (Biggin *et al* 1983). Two 20x50cm glass gel plates were carefully cleaned and taped together, separated by 0.4mm spacers. A rough gradient was formed in a 25ml pipette by drawing up 10ml of gel solution 2 followed by all of gel solution 1 and introducing four air bubbles. The mixture was then poured slowly and smoothly between the two gel plates. The top of the gel was filled with the remainder of gel solution 2. The comb was introduced and the plates were clamped together. The gel was left to polymerise overnight.

Electophoresis was carried out vertically using 0.5x TBE running buffer in the top tank and 1x TBE buffer in the lower reservoir. The gel was clamped in position and aluminium plates were placed on either side of the gel plates to assist even heat distribution. The gel was pre-run at 40W power then the wells were rinsed and the samples were loaded. Electrophoresis was performed at 40W for 2-3 hours until the dye front reached the end of the gel. Longer periods of electrophoresis, up to 8 hours, allowed separation of larger fragments of DNA. In this way over 300 bases could be sequenced from a single template under optimal conditions.

After electrophoresis the gel plates were separated and the gel was soaked in fixing solution for 10 mins. The fixed gel was rinsed with distilled water and dried by blotting with paper towels. The gel was transferred onto Whatman 3M Filter paper soaked in fixing solution, covered with Saran wrap and vacuum dried using a gel drier at 80°C for approximately 90 mins. The dried gel was then placed against X-ray film (Dupont Cronex) in a sequencing cassette and autoradiographed at room temperature.

Sequence data was analysed using the Staden and Wisconsin (Devereux *et al* 1984) sequence analysis software on the Leicester University mainframe VAX/VMS cluster.

Gel solution 1	7ml 5x TBE acrylamide/urea mix 45µl 10% ammonium persulphate 2.5µl TEMED
Gel solution 2	40ml 0.5x TBE acrylamide/urea mix 180µl 10% ammonium persulphate 7.5µl TEMED
20x gradient sequencing TBE (made up to 1 litre with distilled water)	218g Tris base 110g Boric acid 18.6g EDTA
40% acrylamide solution (made up to 100ml, deionised for 10 mins with amberlite, filtered and stored in the dark at 4°C)	38g acrylamide 2g methylene bis acrylamide
5x TBE acrylamide/urea mix (made up to 1 litre with distilled water, stored in the dark at 4°C) 50mg bromophenol blue	430g urea 75ml 20x TBE 150ml 40% acrylamide 50g sucrose
0.5x TBE acrylamide urea mix (made up to 1 litre with distilled water, stored in the dark at 4°C)	430g urea 25ml 20x TBE 150ml 40% acrylamide
Sequencing fix (made up to the required volume with distilled water)	10% methanol 10% glacial acetic acid

Table 2.1
Laboratory Bacterial and Plasmid strains used

Bacterial strain	Genotype	Source or Reference
LE392	F-, hsd514 (rk-mk-) supE44, supF58 lac, galK2, galT22, metB1, trpR55	Maniatis *et al* (1982)
JM101	supE, thi, Δ(lac-proAB) traD36, F', proAB, laciZDM15	Yanisch-Perron*et al* (1985)

Plasmid	Phenotype/Genotype	Source or Reference
pUC19	Ap.	Viera and Messing (1982)
pLG339	Kn, Tc.	Stoker *et al* (1982)
pACYC184	Chlor, Tc	Chang and Cohen (1978)
Cos4	Ap, Tc, Cos*	Roberts *et al* (1986)
M13mp18/19	laci', lacZ'.	Messing and Viera (1982)

RESULTS

CHAPTER 3

CHARACTERISATION OF NFA-1

3.1 Introduction

Goldhar *et al* (1984) first described the adhesin NFA-1 which was expressed by an MRHA positive uropathogenic isolate of *E. coli* (serotype 083:K1:H4, strain 827). The physical and chemical properties of the adhesin indicated that it was a protein and adhesin molecules were released into the supernatant after heat treatment at 65°C for 1 hour. Adhesin expression was found to be controlled by environmental conditions with no expression below 20°C or in the presence of 1% glucose. The nature of the NFA-1 receptor molecule is uncertain but it has the properties of a glycoprotein (Goldhar *et al* 1987).

Immunoblotting studies using purified NFA-1 subunit proteins revealed that the subunit size was approximately 21kDa (Goldhar *et al* 1987). The adhesin subunits form large molecular weight aggregates under non-denaturing conditions. Purified adhesin subunit protein was used to produce polyclonal and monoclonal antisera (Goldhar *et al* 1987).

The NFA-1 adhesin was cloned by Hales *et al* (1988) in the University of Leicester using a cosmid vector. Approximately 1000 recombinants were screened for mannose resistant haemagglutination using a slide assay. Two MRHA positive clones were identified and each was agglutinated by antisera directed against NFA-1. One cosmid clone LE392(pGB3001) was investigated further. Strain 827 and LE392(pGB3001) both mediated MRHA of human erythrocytes but not sheep or horse red cells, and adhesin expression was inhibited by growth at 18°C or the presence of 1% glucose. These results suggested that the NFA-1 adhesin expressed by LE392(pGB3001) was identical to that expressed by strain 827.

Adhesins were purified from strain 827 and LE392(pGB3001) by growing bacteria on solid media at 37°C for 24 hours and suspending in phosphate-buffered saline (PBS). The suspension was heated at 65°C for 1 hour to release adhesin into the supernatant and proteins were precipitated by successive treatments with 20% and 40% ammonium sulphate. The resulting pellet was resuspended in PBS, dialysed against PBS for 18 hours then purified by running through a sepharose-4B column. Fractions were assayed for haemagglutination activity.

Adhesins from strain 827 and LE392(pGB3001) both eluted with the void volume suggesting that they formed high molecular weight aggregates. SDS-polyacrylamide gel electrophoresis of the purified adhesins demonstrated that the major proteins expressed by both strain 827 and LE392(pGB3001) both had a molecular weight of approximately 21kDa. Antiserum raised against NFA-1 bound to the 21kDa proteins expressed by strain 827 and LE392(pGB3001) in immunoblotting experiments, but the antibody did not recognise strain LE392. In an ELISA assay strain 827 and LE392(pGB3001) showed similar binding to cultured human kidney cells. LE392 alone did not bind to the cultured cell-line. Adherence of strain 827 to the cultured cells was inhibited by the adhesin extracted from LE392(pGB3001).

These observations confirmed that the adhesins expressed by strain 827 and LE392(pGB3001) had virtually identical properties indicating that the entire gene complex responsible for the control and expression of NFA-1 had been cloned onto the construct pGB3001.

Hales *et al* performed subcloning experiments in order to reduce the size of the cloned DNA and localise the DNA responsible for NFA-1 expression. pGB3002 was generated by circularising a 26kb *Cla*1 fragment. A further subclone (denoted pS2) was generated by cloning a 15.5kb *Bam*H1-*Eco*R1 fragment into the plasmid vector pLG339. This subclone appeared to retain all the adhesive properties of strain 827 and LE392(pGB3001).

3.2 Subcloning of pS2

I used plasmid pS2 as a starting point for further subcloning experiments which localised the NFA-1 gene complex and identified the position of the adhesin subunit gene. A series of subclones were generated from pS2 as shown in Figure 3.1. Subclone 1 containing a 13.5 *Sal*I fragment did not mediate haemagglutination, whereas subclone 2 with a 14kb *Hin*dIII-*Eco*R1 fragment was MRHA positive. In contrast subclone 3 containing a 5.5kb *Sph*1-*Eco*R1 fragment was MRHA negative. These results showed that the genes responsible for the expression of NFA1 were located between the *Hin*dIII site and the *Eco*R1 site.

The smallest fragment which remained MRHA positive was the 14kb *Hin*dIII-*Eco*R1 fragment. The insert size was still rather large to contemplate sequence analysis, however it was not possible to generate subclones with smaller insert sizes by restriction enzyme digestion due to the location of cleavage sites. The next step involved the generation of Tn1000 insertion mutants.

3.3 Tn1000 insertion mutagenesis of pS2

A series of Tn1000 insertion mutants were produced by Dr Hales and investigated for NFA-1 expression by their ability to mediate MRHA. The results showed that mutants located in a 6.5kb fragment of DNA as shown in Figure 3.1b were MRHA negative. Mutants outside this area mediated MRHA. The results of these experiments therefore allowed the size and location of the gene complex coding for NFA-1 expression to be determined.

3.4 Locating the N-terminal adhesin subunit sequence

Oligonucleotide hybridisation using N-terminal sequence

The N-terminal sequence of the NFA-1 adhesin subunit had been determined by Professor Jann in Freiburg using cyanogen bromide extraction. This allowed the subunit to be located on pNFA4-2-302 by hybridisation of labelled oligonucleotides corresponding to the known N-terminal sequence. The terminal 21 amino acids were found to be: DANGL NTVNA GDGKN LGTAA A. The first eight N-terminal amino acids and their corresponding DNA sequences are as follows:-

Asp	Ala	Asn	Gly	Leu	Asn	Thr	Val
GAT	GCT	AAT	GGT	CTT	AAT	ACT	GTT
C	C	C	C	C	C	C	C
	A		A	A		A	A
	G		G	G		G	G

The following pool of 17-base oligonucleotides was therefore synthesised:-

GAT	GCT	AAT	GGT	CTT	AA
C	C	C	C	C	
	A		A	A	
	G		G	G	

The oligonucleotides were radiolabelled and used as a probe to determine the location of the adhesin subunit gene by investigating hybridisation of labelled oligonucleotides to restriction enzyme digests of pS2.

Purified pS2 DNA was digested with *Kpn*1 and *Eco*R1 generating three fragments of approximate molecular weight 4, 8 and 9.5kb. Fragments were separated and visualised by agarose gel electrophoresis (Figure 3.2a), then transferred to nitrocellulose paper by Southern blotting. Oligonucleotide DNA was labelled with gamma-$^{[32P]}$ATP and hybridised with the filter paper for 20 hours at 35^{o}C. The following rinses were performed:-

Rinse	1	6x SSC	room temp	15 mins
	2	6x SSC	room temp	15 mins
	3	6x SSC	37°C	15 mins
	4	6x SSC	37°C	15 mins
	5	6x SSC	42°C	10 mins
	6	6x SSC	42°C	10 mins

The paper was dried and autoradiographed overnight. The results are shown in Figure 3.2b. There was strong hybridisation to the 4kb band with fainter hybridisation to the 9.5kb band. These results strongly suggested that the genes coding for the N-terminal sequence were located within the 4kb *Kpn*1-*Eco*R1 fragment as shown in Figure 3.3. The presence of fainter hybridisation to the 9.5kb fragment might have been due to the presence of related sequences within this band or simply the result of non-specific hybridisation which was not removed during the washing procedure.

Oligonucleotide hybridisation of Tn1000 insertion mutant digests

This series of experiments led to a more precise determination of the location of the N-terminal sequence by investigating oligonucleotide hybridisation to restriction enzyme digests of a series of insertion mutants. Figure 3.4 shows the restriction map of the Tn1000 insertion element and the location and phenotype of each insertion mutant.

In the first experiment each insertion mutant was digested with *Eco*R1 and in the second experiment each insertion mutant was digested with *Sal*1. The resulting fragments were separated and visualised by agarose gel electrophoresis. Southern blotting and hybridisation were performed as described in Section 2.6. The following rinses were performed:-

Rinse	1	6x SSC	room temp	15 mins
	2	6x SSC	room temp	15 mins
	3	6x SSC	room temp	15 mins
	4	6x SSC	room temp	15 mins
	5	6x SSC	37°C	10 mins
	6	6x SSC	37°C	10 mins

*Eco*R1

The oligonucleotide probes hybridised to the large fragment (>12kb) in mutants number 126, 69, 54, 14, 59, 55 indicating that the N-terminal subunit sequence is located to the left of the insertion element. In contrast the probes hybridised to a smaller fragment in mutants number 2, 81, 94, 146, 26, 17, 56, 131, 9, 134, 85 showing that the subunit sequence is located to the right of the insertion element. The results therefore suggest that the N-terminal sequence is located between insertions 2 and 55. The only mutant that does not follow this pattern is number 75 in which the probes hybridised to the large fragment.

Sal 1

The oligonucleotide probes hybridised to the large fragment (>12kb) in mutants number 126, 69, 54, 14, 59, indicating that the N-terminal subunit sequence is located to the left of these insertion element. In contrast the probes hybridised to a smaller fragment in mutants number 2, 81, 94, 146, 26, 17, 56, 131, 9, 134, 85. The probes hybridised to two fragments of mutant 55.

These results were consistent with those obtained from the *Eco*R1 digest and suggested that the N-terminal subunit sequence was located between insertions 2 and 59. The presence of 2 bands which hybridised with the probe in mutant 55 perhaps indicated that the insertion element was positioned within the N-terminal subunit sequence in this mutant. The arrow in Figure 3.4 indicates the postulated location of the N-terminal subunit sequence.

3.5 Generation and mapping of pS3

One of the MRHA positive insertion mutants was used as the starting point for the generation of the recombinant plasmid pS3 which contained a 9.65kb fragment cloned into pUC19. Further restriction enzyme mapping of pS3 revealed two *Sst*1 sites in the inserted DNA fragment as shown in Figure 3.5. LE392(pS3) was MRHA-positive, agglutinated with NFA-1 antisera and shared similar adhesive properties to strain 827 confirming that the genes responsible for NFA-1 expression were located on pS3.

The recombinant plasmid pS3 was sent to Professor Jann's laboratory in Freiburg Germany where further work led to the characterisation of the gene complex responsible for NFA-1 expression and sequencing of the adhesin subunit gene.

Figure 3.1

Generation of subclones from pS2

a) shows 3 subclones generated from pS2 and MRHA phenotype

b) shows location of MRHA-ve Tn1000 insertion mutants

Key B *Bam*H1
E *Eco*R1
H *Hind*III
K *Kpn*1
S *Sal*1
Sp *Sph*1

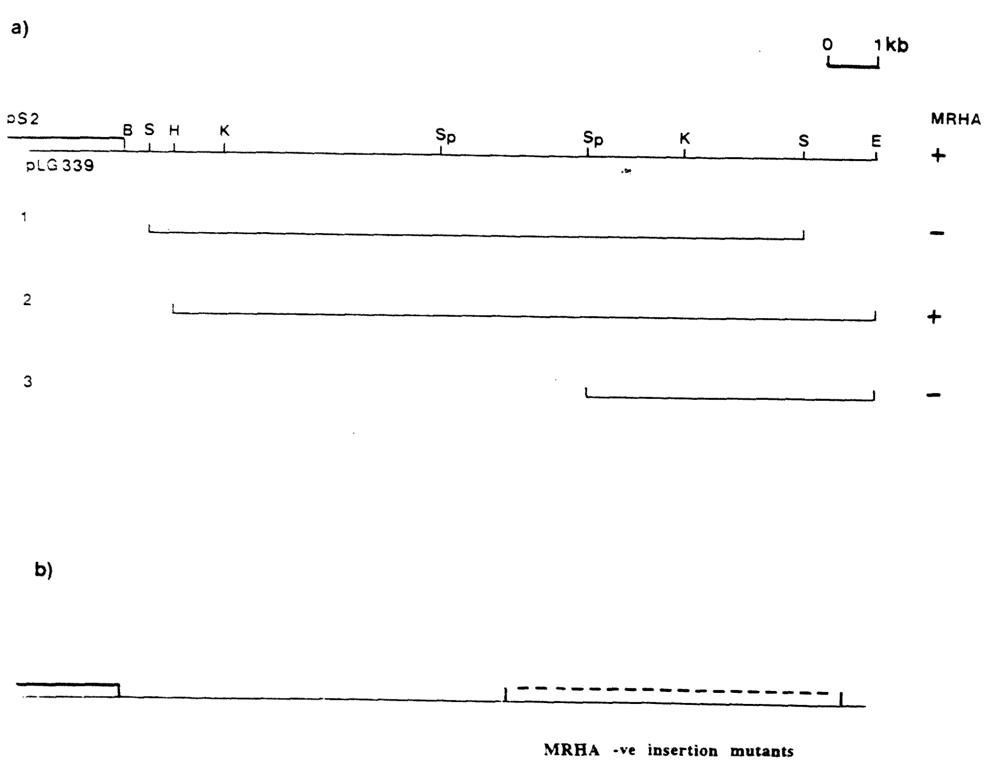


Figure 3.2

Hybridisation of NFA-1 N-terminal oligonucleotide probe with restriction enzyme digests of pS2

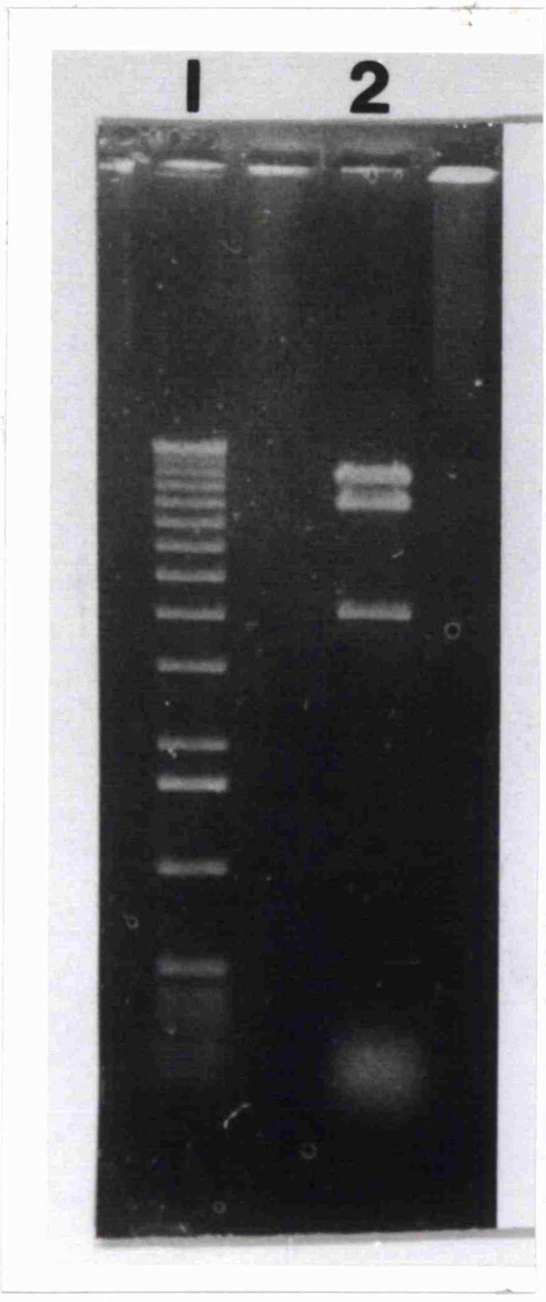
a) agarose gel electrophoresis of *Eco*R1 and *Kpn*1 digest of pS2 showing 4, 8 and 9.5kb fragments.

Lane 1 DNA 1kb ladder

Lane 2 pS2 digested with *Kpn*1 and *Eco*R1

b) Southern blot showing strong hybridisation of NFA-1 oligonucleotide probe to 4kb fragment and faint hybridisation to 9.5kb fragment

a)



b)

Figure 3.3
Location of NFA-1 N-terminal subunit nucleotide sequences deduced from oligonucleotide probe experiments

Key B *Bam*H1
E *Eco*R1
H *Hind*III
K *Kpn* 1
S *Sal*1
Sp *Sph*1

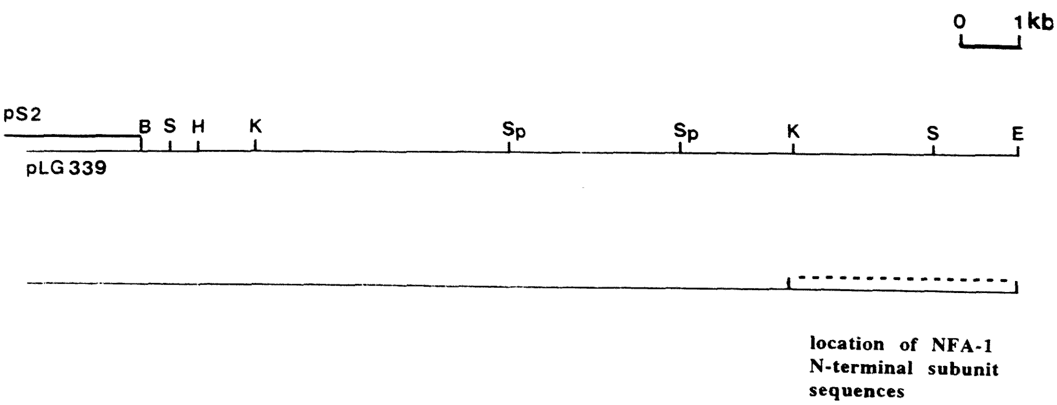


Figure 3.4

Location and phenotype of pS2 Tn1000 insertion mutants

a) pS2
b) Mapping of insertion mutants and MRHA phenotype
c) Restriction map of Tn1000

Key
B *Bam*H1
E *Eco*R1
H *Hin*dIII
K *Kpn* 1
S *Sal*1
Sp *Sph*1

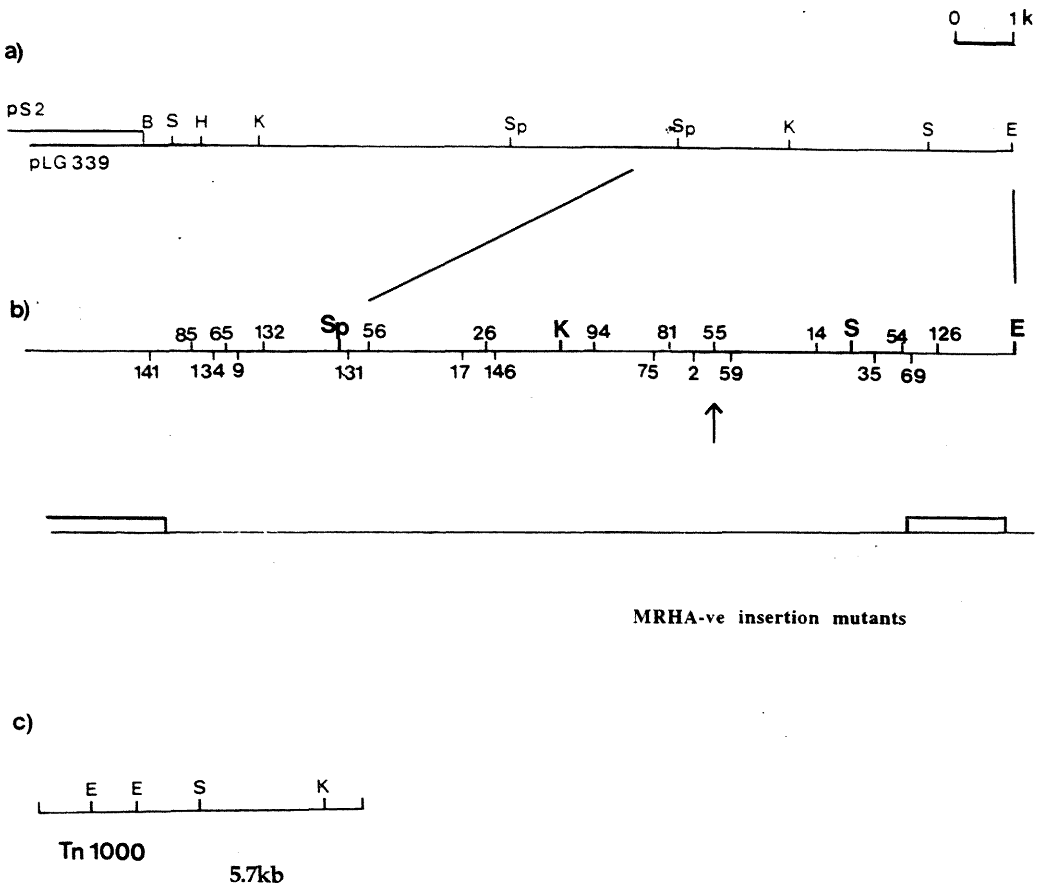


Figure 3.5

Generation of pS3

a) pS2

b) Insertion mutant of pS2 showing location of insertion element and generation of pS3 by *Eco*R1 digestion and ligation to pUC19

c) Restriction map of pS3

Key B *Bam*H1

E *Eco*R1

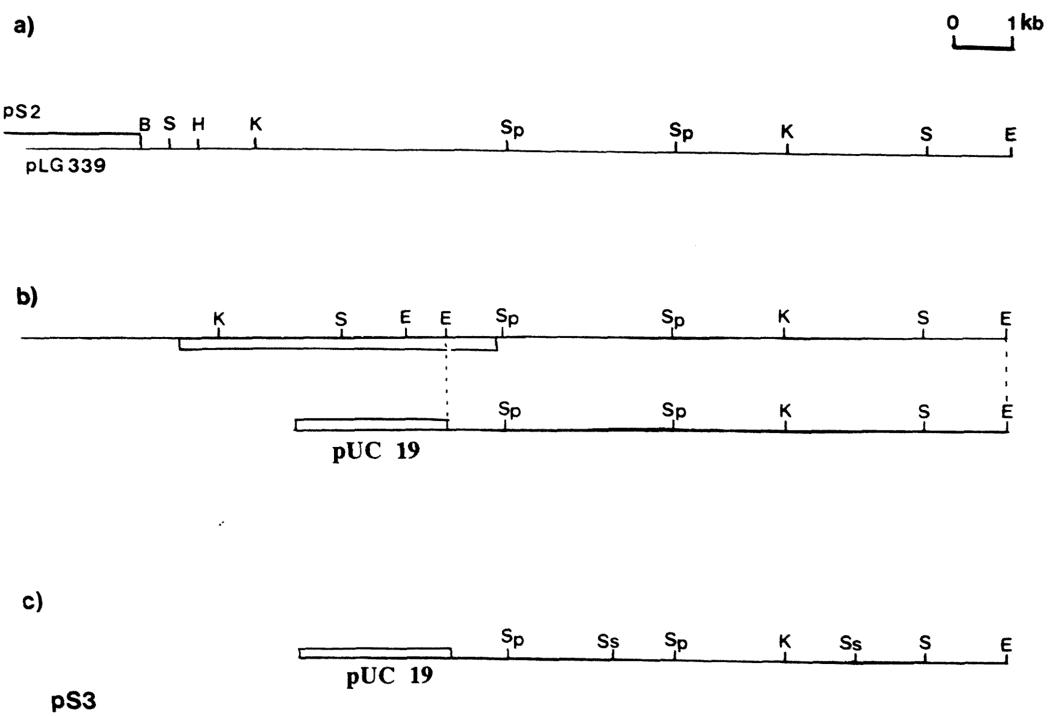
H *Hin*dIII

K *Kpn* 1

S *Sal*1

Sp *Sph*1

Ss *Sst*1



CHAPTER 4

CLONING AND CHARACTERISATION OF NFA-2

4.1 Introduction

NFA-2 was first described by Goldhar *et al* (1987) who identified the adhesin on *E. coli* serotype 014:K?H11 (strain 54) isolated from a patient with a urinary tract infection. The adhesin mediated MRHA and had several similar properties to NFA-1 as expression of both adhesins was inhibited by growth below 20°C or in the presence of 1% glucose. SDS-PAGE and immunoblotting studies showed that the NFA-2 adhesin was composed of 19kDa subunits which aggregated to form a high molecular weight adhesin complex. Both NFA-1 and NFA-2 appear to recognise a glycoprotein, but the precise nature of the NFA-2 adhesin receptor is uncertain (Goldhar *et al* 1987).

Serological studies showed a degree of cross-reactivity between NFA-1 and NFA-2 as polyclonal antisera raised in rabbits reacted with both adhesins. However monoclonal antibodies raised against each purified adhesin were specific and did not cross-react demonstrating that NFA-1 and NFA-2 are distinct proteins with unique antigens.

The aim of this part of the study was to clone and characterise the NFA-2 gene complex. This information would allow a detailed molecular comparison to be made between NFA-2 and other non-fimbrial adhesins. It was felt that the NFA-2 operon might be of similar size to the NFA-1 gene complex (approximately 6.5kb) as the two adhesins shared similar properies. Initial attempts to clone NFA-2 therefore used a plasmid cloning technique.

4.2 Attempted cloning of NFA-2 using a plasmid vector

Preparation of the plasmid vector

The plasmid pUC19 was selected as the vector for the initial cloning experiments. The advantages of this vector included high copy number, ease of purification and its previous use in NFA-1 subcloning experiments. pUC19 expresses an ampicillin resistance gene and beta-galactosidase. The polycloning site is inserted across the *lac* gene allowing recombinant and native plasmids to be distinguished by their ability to produce beta-galactosidase and the colour of colonies when grown on media containing IPTG and X-gal.

Purified pUC19 was digested to completion with *Bam*H1 generating a 2.7kb linear fragment which was visualised by agarose gel electrophoresis. The plasmid was dephosphorylated using calf intestinal phosphatase and the efficiency of dephosphorylation was checked by attempted ligation of the dephosphorylated plasmid.

Preparation of size-selected DNA fragments

Chromosomal DNA was prepared from *E. coli* strain 54 which expressed NFA-2. A series of partial digests were performed using *Sau*3A in order to establish the optimum conditions for selecting DNA fragments in the range of 8-12kb. The resulting partial digests were visualised by 0.7% agarose gel electrophoresis. Size-selected DNA fragments between 8-12kb were excised from the gel, electroeluted, precipitated with ethanol and resuspended in TE solution. The presence of size-selected DNA was confirmed by agarose gel electrophoresis of a sample of the purified DNA fragments. (Figure 4.1).

Ligation of size-selected DNA fragments and dephosphorylated plasmid

The relative concentrations of purified DNA fragments and dephosphorylated pUC19 were compared by agarose gel electrophoresis. (Figure 4.1b) . The conditions for the ligation reaction were adjusted to give a relative concentration of approximately 1:4 for the plasmid and insert respectively. Ligation was performed at 15°C with overnight incubation.

The ligation reaction mix was transformed into the recipient strain JM101 and transformants were plated onto media containing ampicillin, X-gal and IPTG to select and visualise white colonies containing recombinant plasmid. Plasmid (all blue) and JM101 (no growth on ampicillin media) controls were included.

Attempted preparation of gene library

It was hoped that the technique would generate several thousand recombinant colonies forming a representative gene library from which NFA-2 could be selected. However, only 51 recombinant colonies were obtained. The 51 recombinants were screened for haemagglutination and one colony exhibited MRHA (colony 14). A plasmid preparation of this colony was performed. The plasmid was digested with *Eco*R1 and the resulting fragments visualised by agarose gel electrophoresis. A single 11kb fragment was observed which was thought to represent the 2.7kb plasmid vector (pUC19) and an 8.3kb insert.

The stability of the recombinant colony 14 was investigated by subculturing (Figure 4.2). A subculture of colony 14 was streaked out onto ampicillin plates and 18 individual colonies were selected and streaked onto further ampicillin plates. Each subcultured colony was then tested for haemagglutination. Only 2 of the subcultures were MRHA positive, 4 showed slight haemagglutination and 12 were MRHA negative.

Possible reasons for the apparent loss of haemagglutination included phase variation and instability of the recombinant plasmid. This was investigated by performing plasmid minipreparations of the subcultures. The two cultures which were MRHA positive showed a band of approximately 11kb after *Eco*R1 digestion and agarose gel electrophoresis, whereas the remaining preparations showed only single bands of 2.7kb corresponding to the vector plasmid, pUC19. These experiments indicated that the recombinant plasmids were unstable, possibly because the vector plasmid pUC19 was too small to act as a reliable vector for DNA fragments in the size range 8-12kb.

Further plasmid cloning experiments

Plasmid cloning experiments were repeated using the vector plasmid pLG339 and LE392 as the recipient strain of *E. coli.* The plasmid pLG339 is 6.2kb in size and has a low copy number. It codes for kanamycin and tetracycline resistance, with a multiple cloning site situated within the *tet* resistance gene. Recombinants grow on kanamycin media but will not grow on media containing tetracycline.

A similar series of experiments were performed to those described for cloning using pUC19. Plasmid pLG339 was digested with *Bam*H1, dephosphorylated and ligated to 8-12kb size-selected fragments of chromosomal DNA. The ligation mix was transformed into LE392 and plated onto media containing a) kanamycin and b) kanamycin and tetracycline.

After six ligations a total of 682 recombinants were obtained. These were tested for MRHA and all were negative. The yield from each ligation was still comparatively poor and an exhaustive series of further experiments were performed. New plasmid and chromosomal preparations were made, DNA fragments were separated using a sucrose gradient and the ligation conditions were changed. The cloning efficiency remained relatively low.

A total of 1900 recombinant colonies were tested for haemagglutination and all were MRHA negative. In view of the poor yield from plasmid cloning it was decided to proceed to a cosmid cloning procedure.

4.3 Cosmid cloning of NFA-2

Preparation of the cosmid vector

The cosmid cloning technique is outlined in Figure 4.3. A cosmid behaves like a plasmid but may be packaged into lambda phage heads allowing transfection to take place with a high efficiency. The advantages of using a cosmid vector include the ability to insert a large amount of DNA (approximately 50kb) into the cosmid cloning site. A representative cosmid library therefore contains far fewer recombinants than a representative plasmid library. The vector *cos*4 was used for the cloning experiments. *Cos*4 incorporates an ampicillin resistance gene and this was prepared by overnight growth of *E. coli* containing *cos*4 in broth culture incorporating ampicillin. *Cos*4 was then extracted and purified by a standard plasmid preparation procedure.

The two arms of *cos*4 were prepared by digesting with *Pvu*II followed by dephosphorylation (Figure 4.4b). The efficiency of dephosphorylation was checked by attempted self-ligation.

Preparation of size-selected DNA fragments

Chromosomal DNA was prepared from *E. coli* strain 54. A series of partial digests were performed using *Sau*3A in order to establish the optimum conditions for selecting DNA fragments in the range of 35-50kb. The resulting partial digests were visualised by 0.7% agarose gel electrophoresis (Figure 4.4a). Size-selected DNA fragments between 35-50kb were then excised from the gel, electroeluted, precipitated with ethanol and resuspended in TE solution. The presence of 35-50kb size-selected fragments of DNA was confirmed by agarose gel electrophoresis of a sample of the purified DNA fragments (Figure 4.4b).

Ligation of size-selected DNA fragments and dephosphorylated cosmid

The relative concentrations of purified DNA fragments and dephosphorylated *cos*4 were compared by agarose gel electrophoresis and adjusted to give a relative concentration of approximately 6:1 for the cosmid and insert respectively (Figure 4.4b). Ligation was performed at 15°C with overnight incubation.

Packaging and plating of cosmid ligation mix

The ligation reaction mix was packaged into lambda phage heads using a commercial packaging kit. The phage stock was adsorbed onto a fresh culture of *E. coli* strain JM101 for 20 mins. Luria broth was added and the bacterial culture was incubated for a further 40 mins before plating onto L-agar containing ampicillin. Cell and lambda-packaging controls were included with each experiment.

Screening of gene library

A total of 751 recombinant colonies were individually screened for MRHA (Figure 4.5). Positive colonies were further tested for agglutination with two monoclonal anti-NFA-2 antibodies, IC2 and 2D3 which were kindly supplied by Professor Jann. 12 recombinants were found to be MRHA positive and all agglutinated with each of the monoclonal antibodies.

4.4 Characterisation of MRHA-positive recombinants

Plasmid preparations from each recombinant were digested with a series of restriction enzymes (*Bam*H1, *Eco*R1, *Kpn*1 and *Sal*1). The results (Figure 4.6) showed that all the recombinant plasmids were of approximately 40kb in size and several identical fragments were seen when different recombinants were digested with the same enzyme. This evidence supported the possibility that the MRHA positive recombinants shared the same segment of DNA.

Four MRHA-positive recombinants were selected for further analysis. A crude protein preparation from each recombinant was separated by polyacrylamide gel electrophoresis. *E. coli* strain 54 expressing NFA-2 and JM101 were included as positive and negative controls respectively. The positive control and all the recombinants showed a band of approximately 19kDa which was not expressed by JM101. This band corresponded to the expected size of the NFA-2 subunit protein.

Immunoblotting experiments were performed using the anti-NFA-2 monoclonal antibodies IC2 and 2D3. The antibodies reacted against a product of approximately 19kDa expressed by the positive control (strain 54) and the four recombinants, but did not react with JM101 (Figure 4.7). The evidence from these experiments therefore indicated that each of the four recombinants expressed the NFA-2 gene as each were MRHA-positive and agglutinated with anti-NFA-2 antibody and each all expressed a product of approximately 19kDa in size which was recognised by specific monoclonal antibodies directed against NFA-2.

Stability of the cosmid clone

The four recombinant cosmid clones were subcultured repeatedly into solid and liquid media and remained MRHA-positive. One of the NFA-2 expressing cosmid clones was selected for further study. The recombinant cosmid in this positive clone was designated pNFA2-1. The stability of pNFA2-1 was investigated by transformation into *E. coli* strains LE392 and JM101. 50 of the LE392 recombinants grown on ampicillin media were tested for MRHA and all were positive; similarly 20 white colonies in JM101 were tested for MRHA and all were positive. The results therefore suggested that this was a stable recombinant.

4.5 Subcloning experiments

The recombinant cosmid clones each contained approximately 40kb of inserted DNA, however the gene cluster encoding NFA-2 expression was expected to be of a similar size to that coding for NFA-1, ie: approximately 6kb in length. Subcloning was therefore performed to reduce the insert size.

The recombinant cosmid pNFA2-1 was digested with a series of restriction enzymes to investigate the pattern of fragments generated. The results are indicated below:-

Enzyme	*Eco*R1	*Bam*H1	*Hind*III	*Sal*1
Fragments (kb)	3	24	22	14
	3.5	26	28	14
	4			22
	5			
	7			
	12			
	15			

A number of subcloning experiments were attempted using the recipient plasmids pUC19, pLG339 and pACYC184 as shown:-

pNFA2-1 digest	Ligated to
*Eco*R1	pUC19
*Bam*H1	pUC19 pACYC184 pLG339
*Hind*III	pLG339
*Sal*1	pLG339

Several of the ligations produced colonies which were MRHA positive on initial testing but these became negative on subculture and plasmid preparations did not consistently show inserted fragments of DNA. These experiments did not produce a stable recombinant subclone. The reasons were thought to include the possibility that some or all of these enzymes might cleave DNA within the NFA-2 gene cluster and the likelihood that some of the fragments (particularly those over 20kb) were too large to ligate into the plasmid vector without becoming unstable.

Subcloning using Sau3A partial digestion of pNFA2-1

This approach was used to generate NFA-2 expressing subclones. The recombinant cosmid pNFA-2 was partially digested with *Sau*3A under variable conditions aiming to produce fragments in the range 6-12kb. Size-selected fragments were extracted from the gel, purified and ligated to a dephosphorylated *Bam*H1 digest of plasmid pACYC184. The ligation mix was transformed into the recipient strain LE392 and resulting colonies were streaked onto duplicate plates containing a) chloramphenicol media and b) tetracycline media. The tetracycline gene lies across the cloning site in pACYC184, therefore recombinants are inhibited by tetracycline but remain resistant to chloramphenicol.

19 chloramphenicol resistant, tetracycline sensitive recombinants were tested for MRHA and 4 were found to be positive. Plasmid preparations of the four positive subclones were digested with *Eco*R1 and *Bam*H1 and the results indicated that they all contained inserts of approximately 8-12kb in size. Two of the subclones were selected for further investigation. The recombinant plasmids in these MRHA positive subclones were designated pNFA2-101 and pNFA2-102.

4.6 Restriction enzyme mapping of pNFA2-101 and pNFA2-102

Purified caesium chloride preparations of pNFA2-101 and pNFA2-102 were digested with a series of restriction enzymes. Single and double digests were performed to determine the precise location of the restriction enzyme sites.

Restriction enzyme maps of pNFA2-101 and pNFA2-102 are shown in Figure 4.8. pNFA2-101 and pNFA2-102 are cloned in opposite orientations but show similar expression of NFA-2 as they are both MRHA-positive. The inserted fragment of DNA in pNFA-2-101 is 12.6kb in length and has three *Sph*1 sites, two *Kpn*1 and *Cla*1 sites and single sites for *Eco*R1, *Sal*1 and *Sst*1. There were no sites for *Bam*H1 or *Xho*1. The inserted fragment of DNA in pNFA2-102 is 9.8kb in length. Both recombinant plasmids share a 7.8kb stretch of inserted DNA between *Cla*1 and *Sph*1 sites. The restriction enzyme sites within this fragment are identical for the two recombinant plasmids. Both subclones express NFA-2 therefore the inserted DNA sequence common to both plasmids must incorporate the entire NFA-2 gene cluster.

4.7 Characterisation of LE392(pNFA2-101) and LE392(pNFA2-102)

SDS-Polyacrylamide gel electrophoresis

Crude protein preparations of LE392, strain 54, LE392 (pNFA2-101 and LE392 (pNFA2-102) were compared by SDS-PAGE. Separation of proteins on a 15% acrylamide gel (Figure 4.9a) revealed a band of approximately 19kDa which was present in strain 54, pNFA2-101 and pNFA2-102, but was not found in the LE392 control.

Immunoblotting experiments

Crude protein preparations of LE392, strain 54, LE392 (pNFA2-101 and LE392 (pNFA2-102) were separated by polyacrylamide gel electrophoresis as described above. Immunoblotting experiments were performed using the monoclonal anti-NFA-2 antibodies IC2 and 2D3. Proteins separated by PAGE were transfered to nitrocellulose paper and incubated with either IC2 or 2D3. Both monoclonal antibodies reacted strongly with the 19kDa product expressed by strain 54, LE392 (pNFA2-101) and LE392 (pNFA2-102). There was no binding to any of the products expressed by the controls, LE392 and LE392(pACYC184) (Figure 4.9b).

4.8Minicell analysis of pNFA2-101 and pNFA2-102

Minicells express only the gene products encoded by plasmids and were used to characterise the products expressed by recombinant plasmids expressing non-fimbrial adhesin genes. The minicell technique used *E. coli* DS410 as the host strain. Competant DS410 cells were transformed with recombinant and control plasmids. Transformants were incubated overnight in media containing appropriate antibiotics to favour growth of strains containing recombinant plasmids. Vegetative cells were removed by selective centrifugation and sucrose gradient. Minicell products were labelled with

[^{35}S]methionine, separated by polyacrylamide gel electrophoresis and visualised by autoradiography.

The following plasmids were transformed into DS 410

i) pACYC184 (plasmid control)

ii) pNFA2-102

iii) Cells only control (containing no plasmid)

Plasmid preparations were performed from the resulting transformants and the products were digested with *Eco*R1 to ensure that effective transformation had taken place.

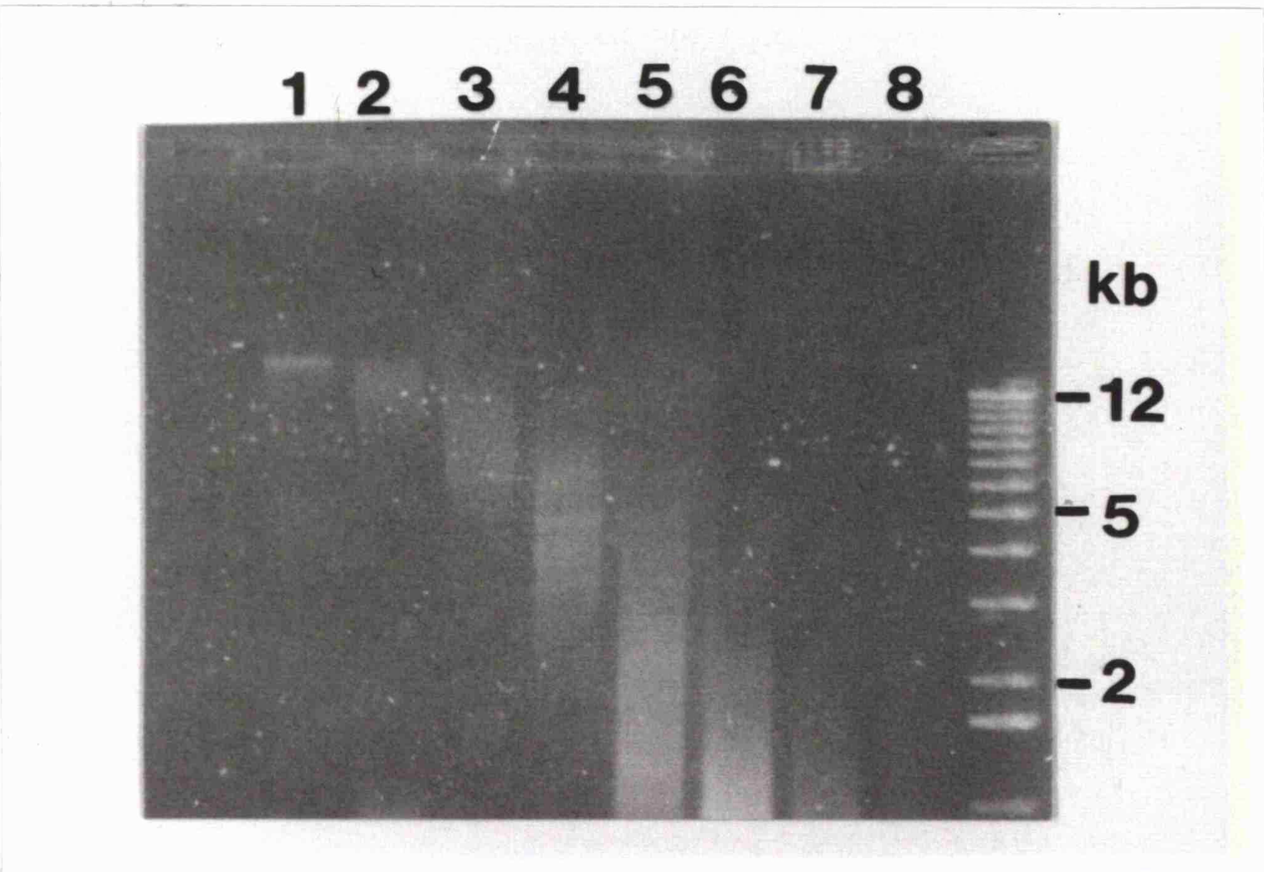
The results of the minicell experiments indicated that pNFA2-102 expressed at least five products of approximate molecular weight 80kDa, 33kDa, 28kDa, 19kDa and 17kDa (Figure 4.10). In addition there are several very faint bands around 15kDa in size. The 19kDa band corresponds in size to the expected NFA-2 subunit protein, but the 17kDa band is significantly more intense than the 19kDa band raising the possibility that they may represent two separate forms of the same protein.

Figure 4.1

a) Partial *Sau*3A digest of *E. coli* strain 54 in preparation for plasmid cloning

lanes 1-8 showing fragments generated by increasing length of incubation with *Sau*3A

digest visualised in lanes 2 and 3 contains fragments of length 8-12kb



b) Preparation of plasmid pUC19 and size selected DNA fragments prior to ligation reaction

lane 1 2.7kb band of pUC19 digested with *Bam*H1 and dephosphorylated

lane 2 purified 8-10kb size-selected DNA fragments prepared from partial digest of strain 54 chromosomal DNA

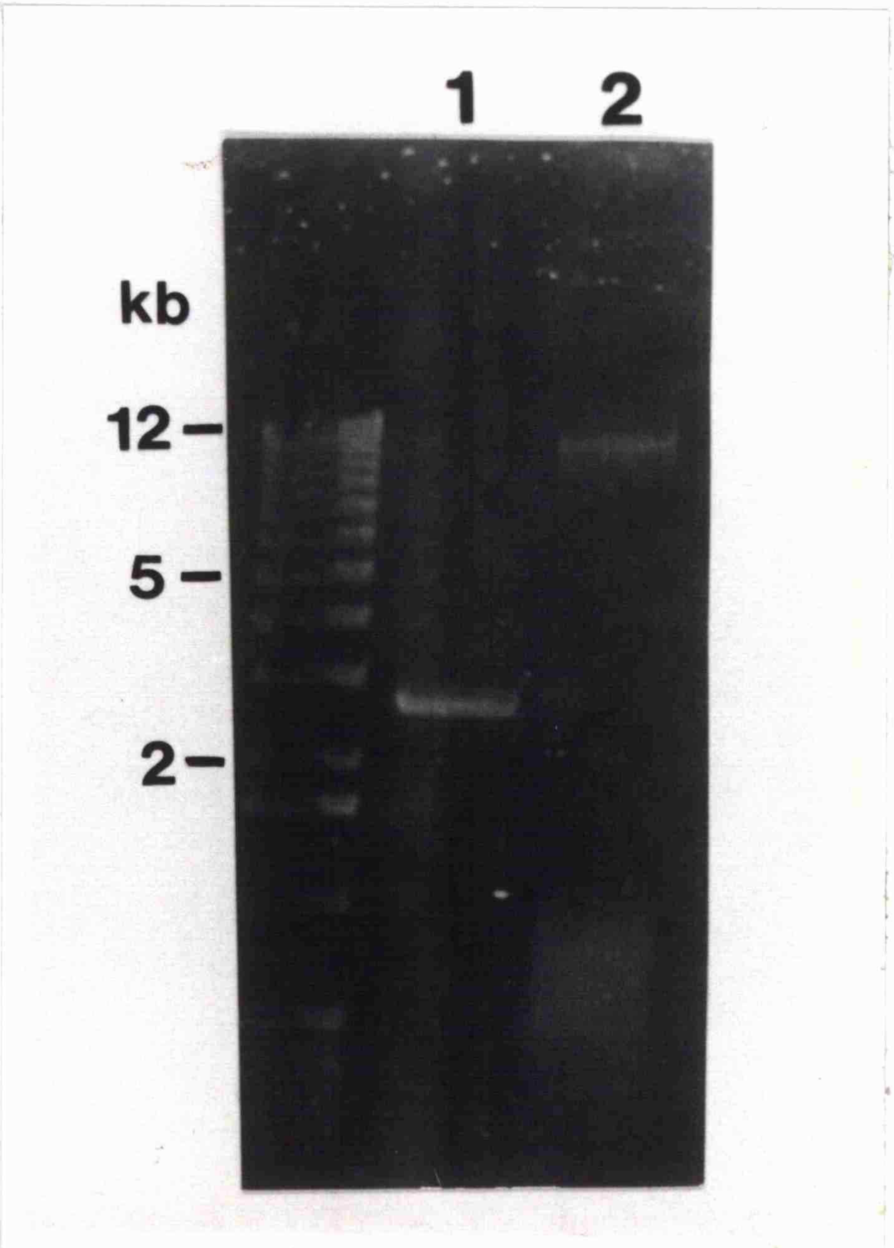


Figure 4.2
Attempted preparation of plasmid gene library

51 recombinants tested for MRHA

1 positive (colony 14)

18 subcultures
(each subculture replated and tested for MRHA)

2	4	12
MRHA +ve	MRHA+/-	MRHA -ve

Plasmid minipreparation

both had 11kb plasmid	all had 2.7kb plasmid only	all had 2.7kb plasmid only

Figure 4.3

Outline of cosmid cloning technique

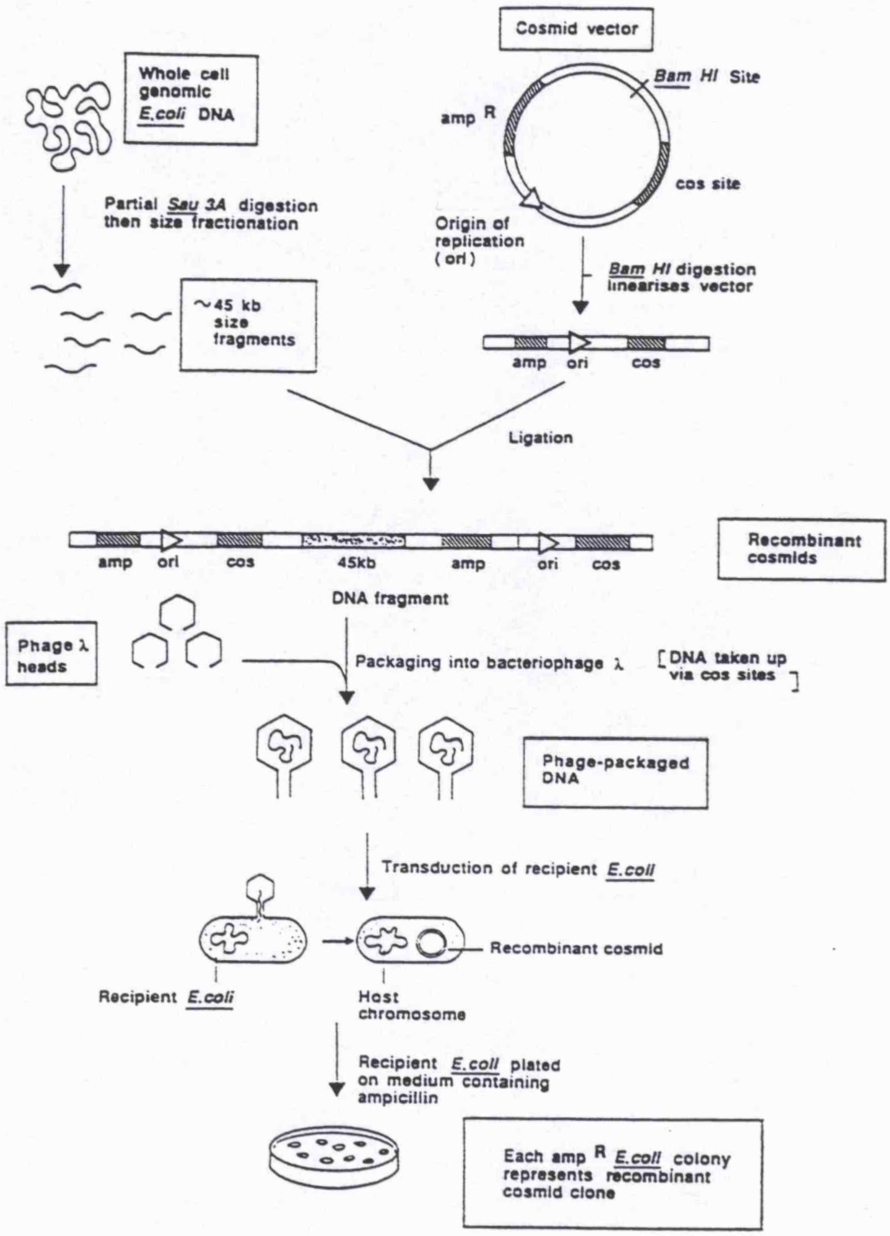


Figure 4.4

a) Partial *Sau*3A digest of *E. coli* strain 54 in preparation for cosmid cloning

lane 1	lambda phage digested with *Hin*dIII (top band 24kb)
lanes 2 and 9	lambda phage digested with *Xho*I (34kb and 15kb bands)
lanes 3-8	showing fragments generated by increasing length of incubation with *Sau*3A

b) Preparation of *cos*4 arms and size selected DNA fragments prior to ligation reaction

lane 1 *cos*4 arms prepared by *Pvu*II digest and dephosphorylation (molecular weight of arms 4.5kb and 1.5kb)

lane 2 purified ~40kb size-selected DNA fragments prepared from partial digest of strain 54 chromosomal DNA

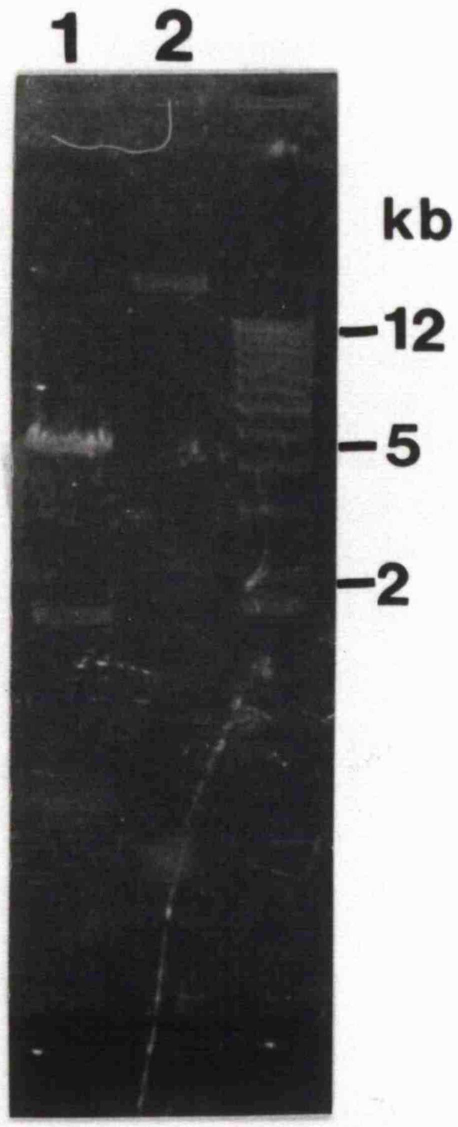


Figure 4.5
Screening of individual colonies for MRHA in 96-well plates

Controls

Strain 54

LE392

JM101

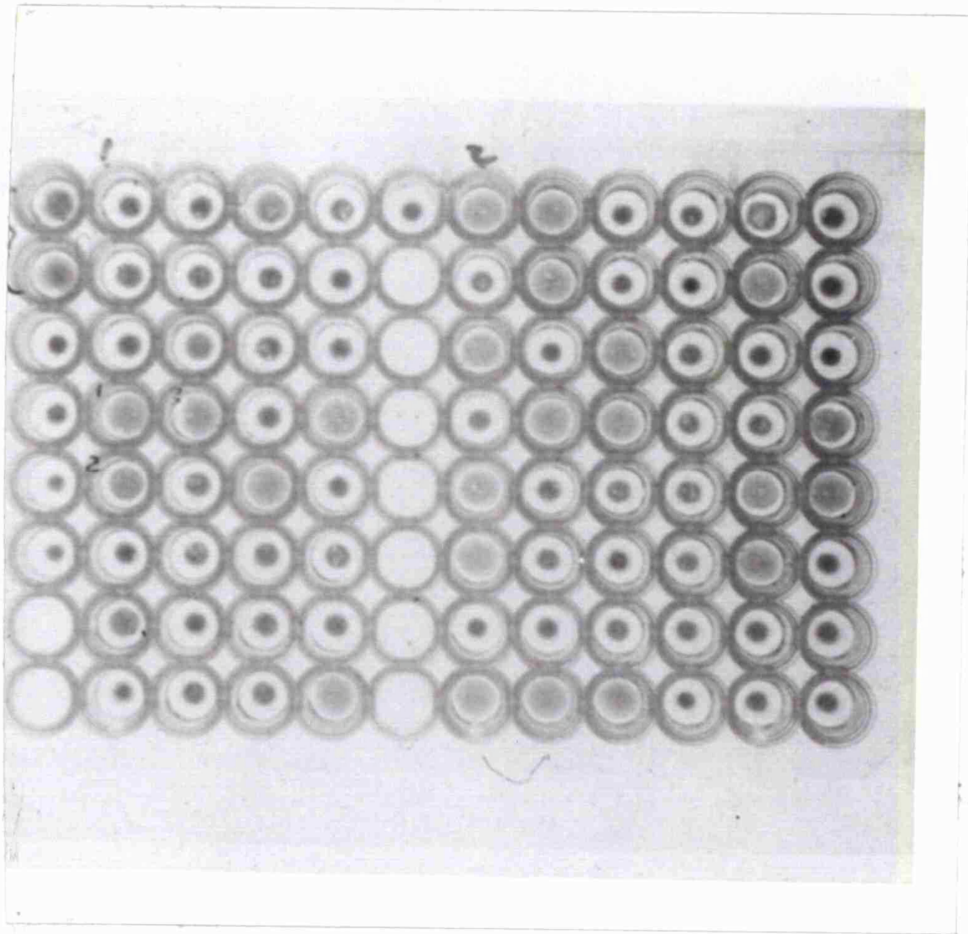
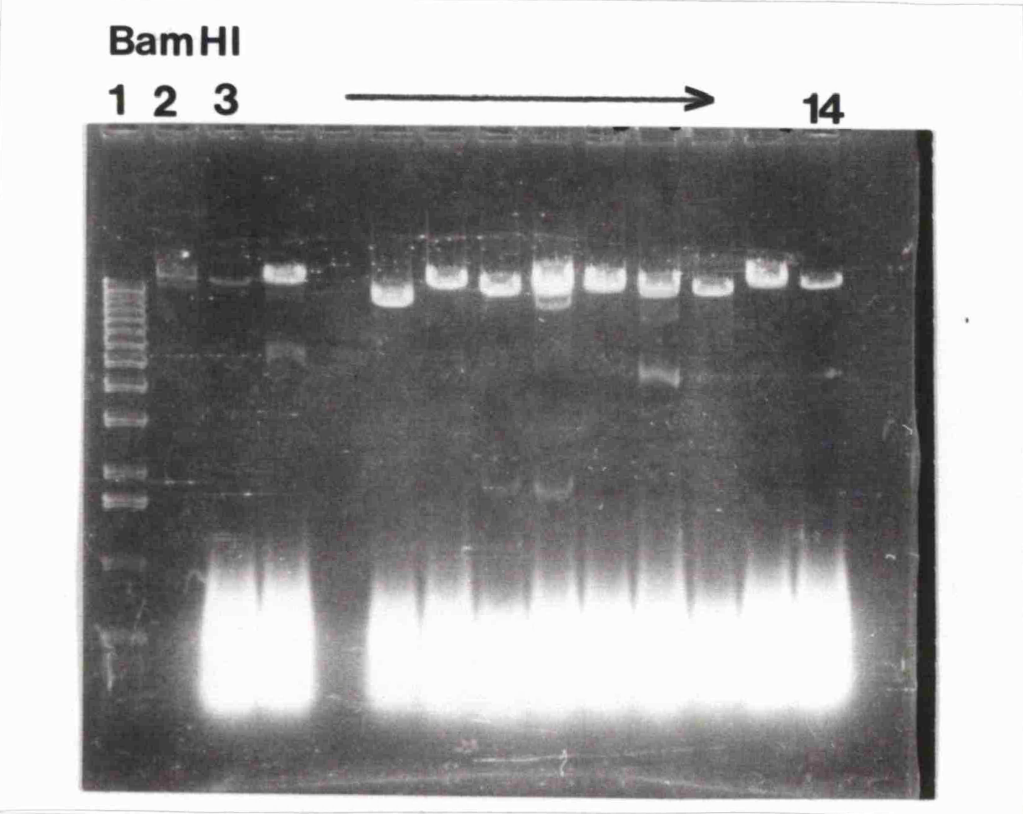


Figure 4.6

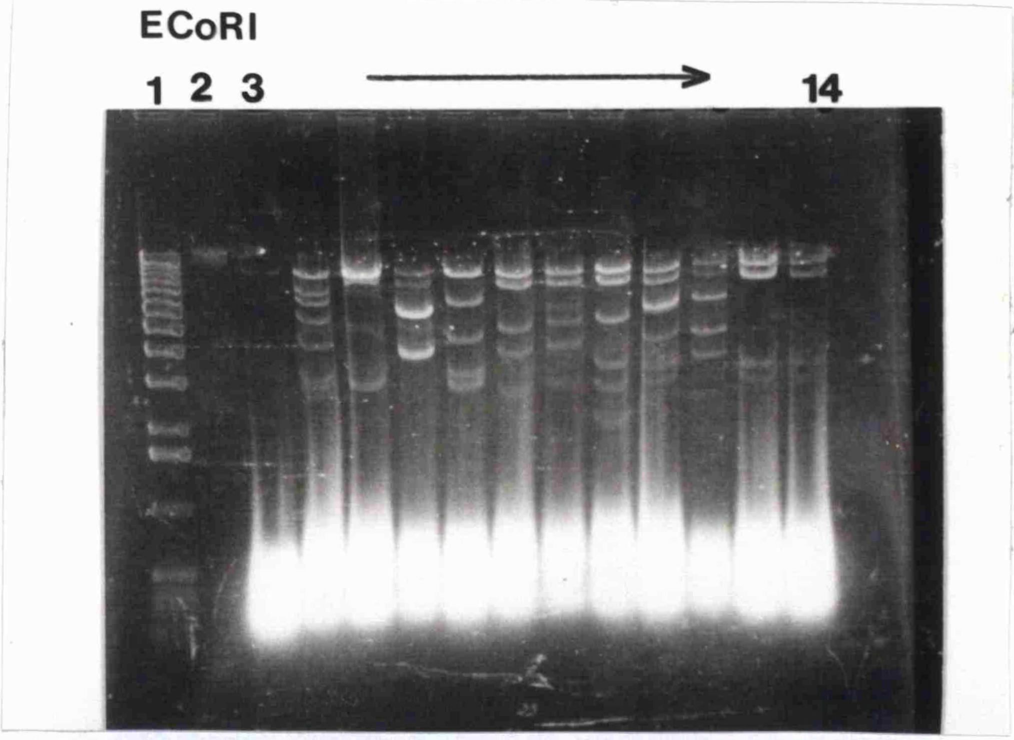
Restriction enzyme digests of 18 MRHA positive cosmid recombinants derived from strain 54

lane 1	1kb DNA ladder
lane 2	lambda phage digested with *Xho* I (34kb and 15kb bands)
lanes 3-14	minipreps of 12 of 18 MRHA+ve cosmid clones digested with enzymes shown.

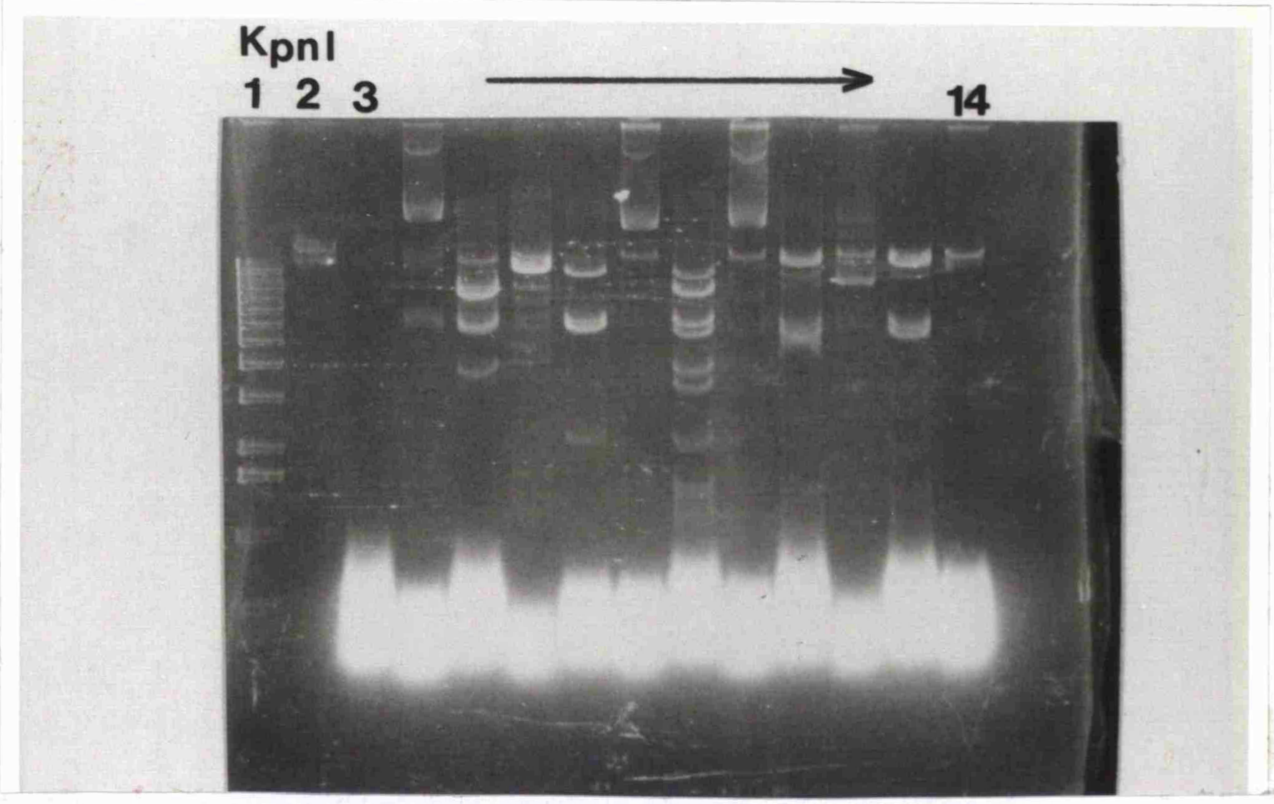
a) *Bam*H1



b) *Eco*R1



c) *Kpn*1



d) *Sal*1

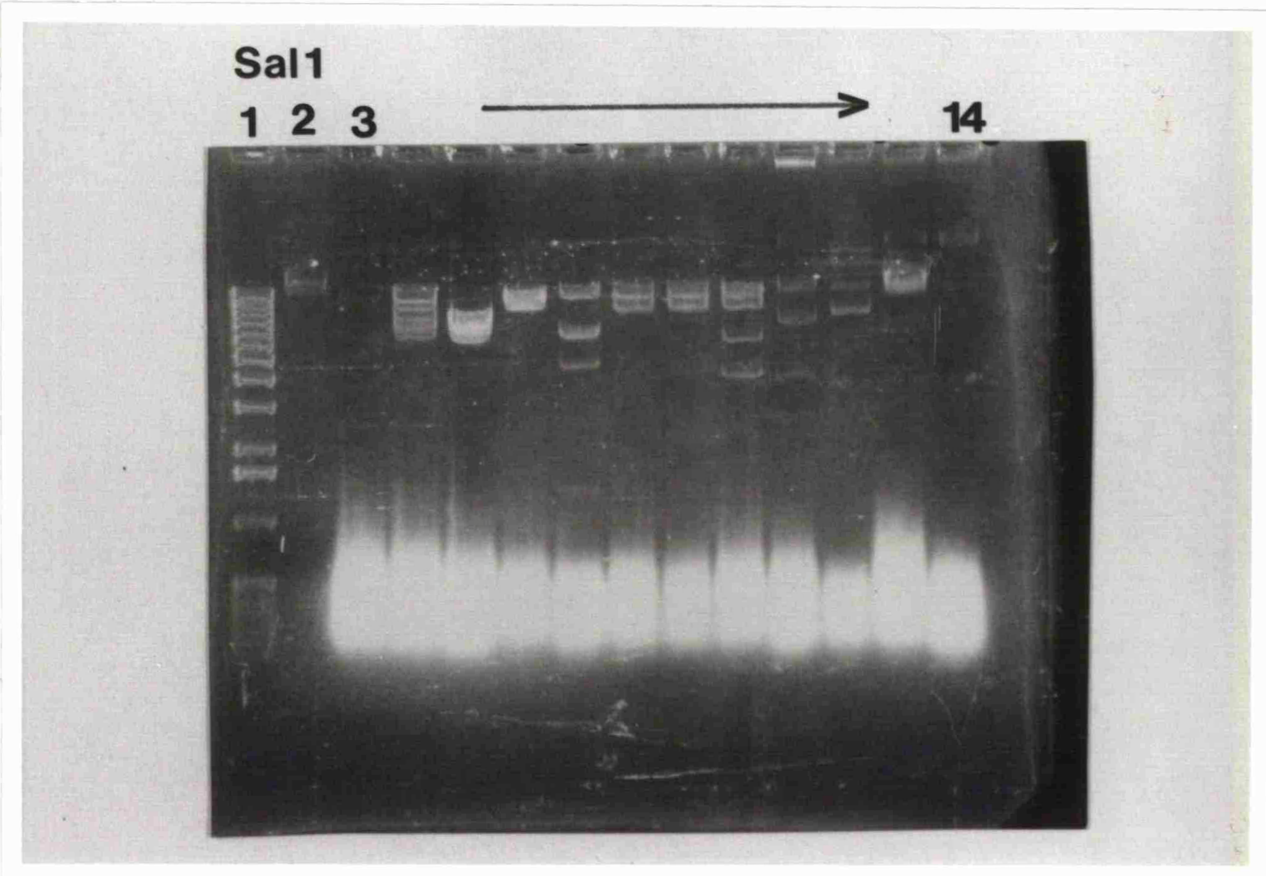


Figure 4.7

Immunoblots of crude protein preparations of recombinant MRHA positive cosmid clones showing reaction with the anti-NFA2 monoclonal antibody IC2

lane 1	strain 54	(positive control)
lane 2	LE392	(negative control)
lane 3	MRHA	positive cosmid clone 5
lane 4	MRHA	positive cosmid clone 14
lane 5	MRHA	positive cosmid clone 16
lane 6	MRHA	positive cosmid clone 17

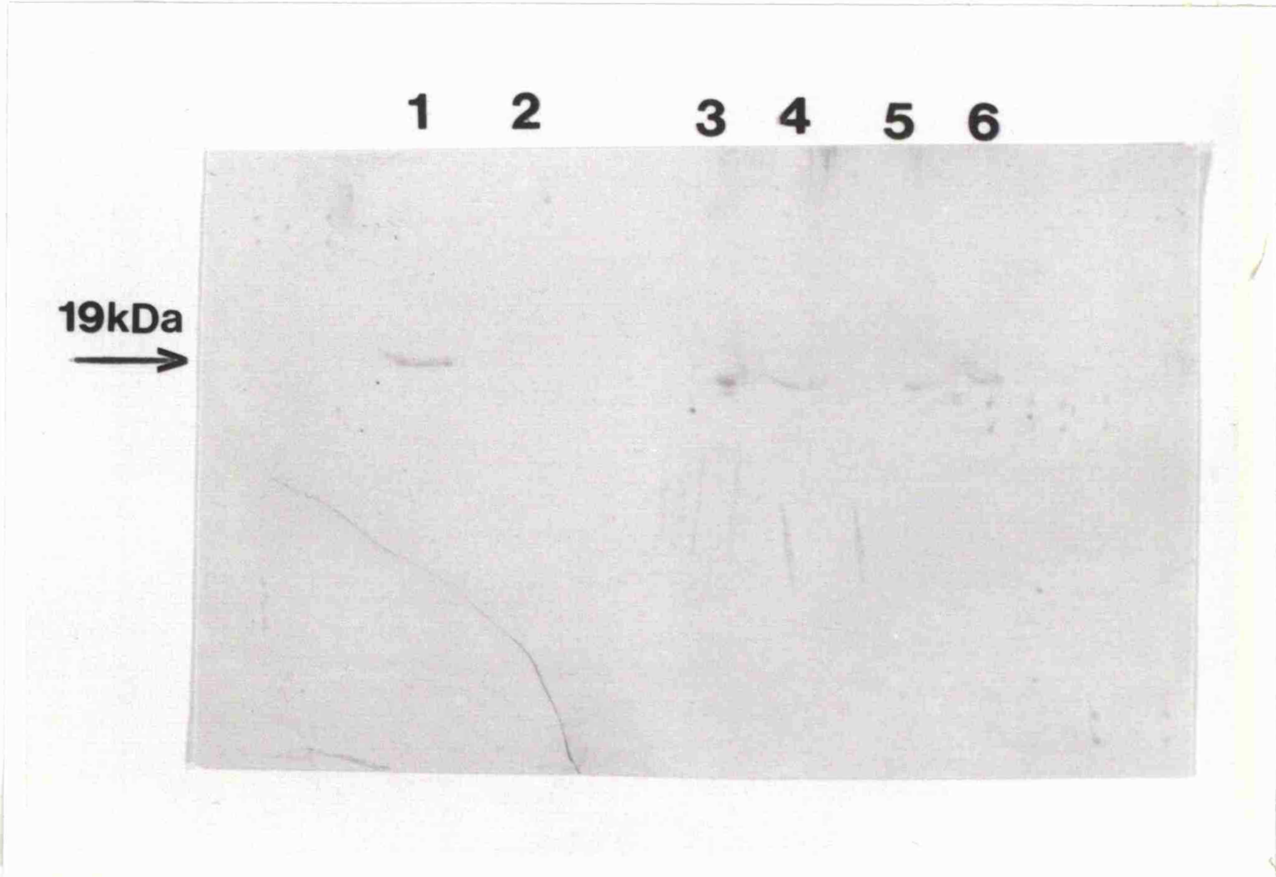


Figure 4.8

Restriction enzyme mapping of pNFA2-101 and pNFA2-102

Key

A	*Acc*1
Bg	*Bgl*II
C	*Cla*1
E	*Eco*R1
H	*Hin*dIII
K	*Kpn*1
S	*Sal*1
Sp	*Sph*1
Ss	*Sst*1

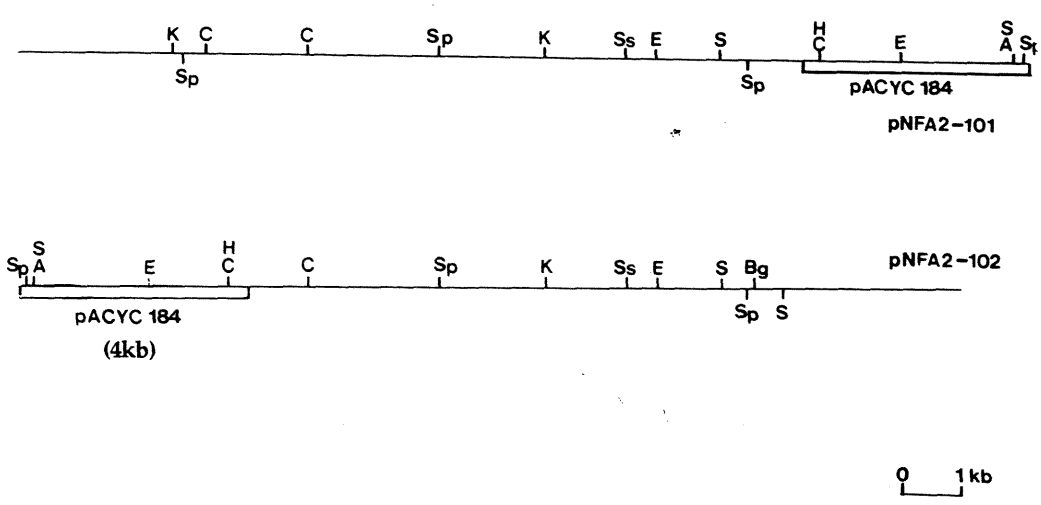


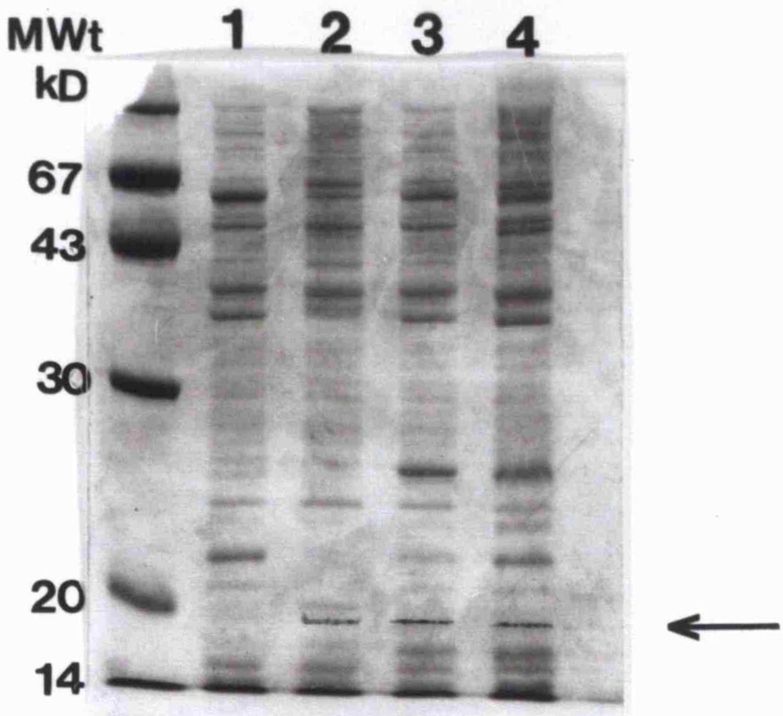
Figure 4.9

SDS-PAGE and immunoblotting experiments

a) SDS-PAGE of crude protein preparations of the following strains:-

1) LE392
2) strain 54
3) LE392 (pNFA2-101)
4) LE392 (pNFA2-102)

Arrow shows 19kDa band corresponding to NFA-2 expression



b) Immunoblotting of crude protein preparations of the following strains using the anti-NFA2 monoclonal antibody IC2:-

1) LE392
2) strain 54
3) LE392 (pNFA2-101)
4) LE392 (pNFA2-102)

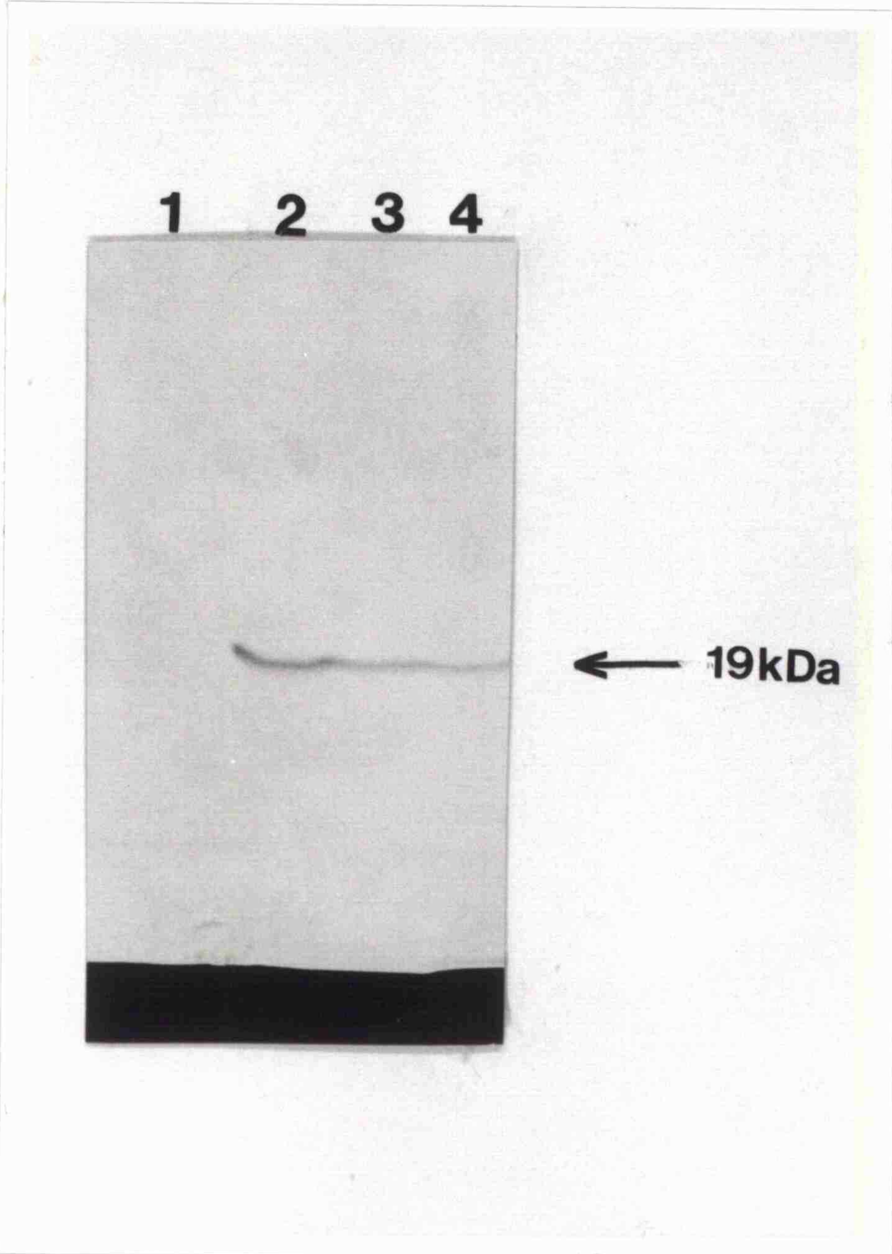
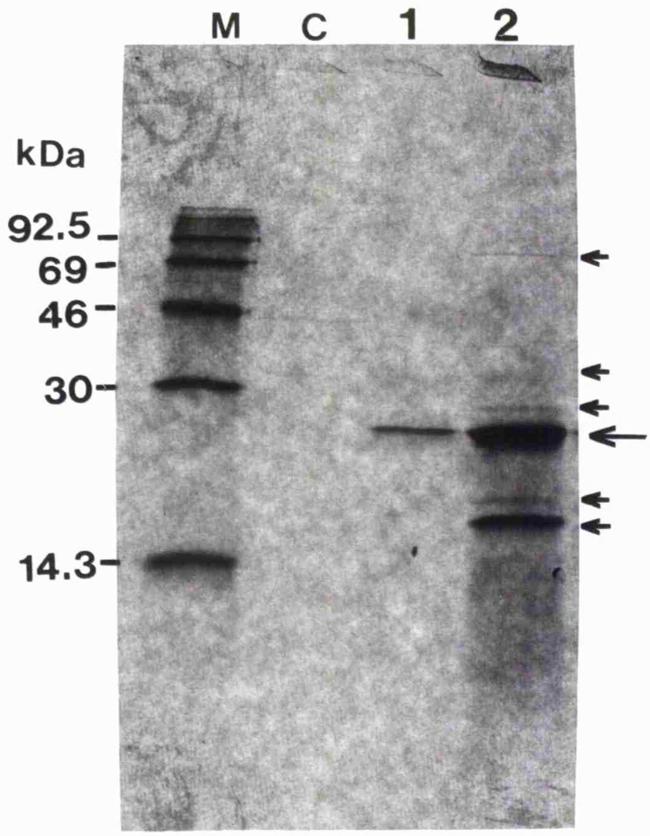


Figure 4.10

Minicell experiments showing products encoded by recombinant plasmid pNFA2-102

M molecular weight markers (kDa)
C cells only control
1 pACYC184
2 pNFA2-102

arrows show products expressed by pNFA2-102. Large arrow indicates product of chloramphenicol resistance gene (CAT).



CHAPTER 5
CLONING AND CHARACTERISATION OF NFA-3

5.1 Introduction

NFA-3 was first described by Grunberg *et al* (1988). The adhesin was identified on an isolate of *E. coli* serotype 020:KX104:H-, (strain 9) obtained from a patient in Tel-Aviv with septicaemia. Strain 9 mediated MRHA, but electron microscopy did not reveal any fimbria. NFA-3 is formed by large molecular weight aggregates of adhesin subunits approximately 17.5kDa in size. The NFA-3 receptor is thought to be glycophorin A^{NN}.

Following the cloning of NFA-1 and NFA-2 the aim of this part of the study was to clone and characterise the NFA-3 gene complex. This information would extend the analysis and comparison of non-fimbrial adhesins.

5.2 Cosmid cloning of NFA-3

An identical technique was used to that described for the cloning of NFA-2 (Chapter 4). *Cos*4 vector was used and the two arms of *cos*4 were prepared by digesting with *Pvu*II followed by dephosphorylation.

Chromosomal DNA was prepared from *E. coli* strain 9 expressing NFA-3. A series of partial digests were performed using *Sau*3A in order to establish the optimum conditions for selecting DNA fragments in the range of 35-50kb. The resulting partial digests were visualised by 0.7% agarose gel electophoresis. Size-selected DNA fragments between 35-50kb were excised from the gel and extracted by electrophoresis in dialysis tubing containing 1x ELF0. DNA was precipitated with ethanol and resuspended in TE. The presence of 35-50kb size-selected fragments of DNA was confirmed by further agarose gel electrophoresis of the purified DNA fragments.

Purified DNA fragments and dephosphorylated *cos*4 arms were ligated at 15°C with overnight incubation. The conditions for the ligation reaction were established to give a relative concentration of approximately 6:1 for the cosmid and insert respectively. The ligation reaction mix was packaged into lambda phage heads using a commercial packaging kit. The phage stock was adsorbed onto a fresh culture of *E. coli* strain JM101 and plated onto L-agar containing ampicillin.

Screening of gene library

A total of 612 recombinant colonies were individually screened for mannose-resistant haemagglutination. Positive colonies were further tested for agglutination with a monoclonal anti-NFA-3 antibody, 19B12, kindly supplied by Professor Jann. A single recombinant, was found to be MRHA positive and agglutinated with the monoclonal antibody. This recombinant was denoted JM101 (pNFA3-1).

5.3 Characterisation of MRHA-positive NFA-3 recombinant

A caesium chloride preparation of pNFA3-1 was digested with a) *Bam*H1 and b) *Eco*R1. The sum of the resulting fragments was approximately 45kb indicating that pNFA3-1 was a genuine cosmid recombinant. The stability of JM101(pNFA3-1) was investigated by repeated subculture into solid and liquid media. All subcultures remained MRHA-positive. pNFA3-1 was transformed into *E. coli* strains LE392 and JM101. All recombinants grown on ampicillin media were MRHA positive. The results therefore suggested that this was a stable recombinant.

5.4 Subcloning experiments

The approach used to subclone pNFA3-1 was similar to that used for the subcloning of NFA-2. pNFA3-1 was partially digested with *Sau*3A under variable conditions aiming to produce fragments in the range 6-12kb. Size-selected fragments were extracted from the gel, purified and ligated to a dephosphorylated *Bam*H1 digest of plasmid pACYC184. The ligation mix was transformed into the recipient strain LE392 and resulting colonies were streaked onto duplicate plates containing a) chloramphenicol media and b) tetracycline media.

120 chloramphenicol resistant, tetracycline sensitive recombinants were tested for MRHA and five were found to be positive (plasmids denoted pNFA3-101, pNFA3-102, pNFA3-103, pNFA3-104, pNFA3-105). Plasmid preparations of the five positive subclones were digested with *Eco*R1, *Bam*H1 and *Hin*dIII. The results indicated that they all contained inserts of approximately 5.5-6.5kb in size. Two of the subcloned plasmids (pNFA3-101 and pNFA3-103) were selected for further investigation. The stability of pNFA3-101 and pNFA3-103 was tested by transforming LE392 with each recombinant plasmid. 50 colonies resulting from each transformation were tested for MRHA and all were positive, indicating that pNFA3-101 and pNFA3-103 were highly stable.

5.5 Restriction enzyme mapping of pNFA3-101 and pNFA3-103

Purified caesium chloride preparations of pNFA3-101 and pNFA3-103 were digested with a series of restriction enzymes. Single and double digests were performed to determine the precise location of the restriction enzyme sites.

Restriction enzyme maps of pNFA3-101 and pNFA3-103 are shown in Figure 5.1. pNFA3-101 and pNFA3-103 are cloned in opposite orientations but show similar expression of NFA-3 as they are both MRHA-positive. The inserted fragment of DNA in pNFA3-101 is 6.5kb in length compared to 6.0kb for pNFA3-103. The inserted stretch of DNA in pNFA3-101 overlaps completely with the insert in pNFA3-103 with almost exact alignment of restriction enzyme binding sites. The size of the stretch of DNA common to PNFA3-101 and pNFA3-103 shows that the gene cluster encoding NFA3 expression can be no greater than 6kb in length.

Restriction enzyme mapping reveals that the inserted fragments of DNA in pNFA3-101 and pNFA3-103 have the same restriction sites including three *Eco*R1 sites, two *Bam*H1 and *Pvu*II sites, and single sites for *Acc*1, *Bgl*II, *Hin*dIII and *Sph*1. There were no sites for *Kpn*1, *Sst*1, *Sal*1 or *Cla*1.

5.6 Characterisation of LE392(pNFA3-101) and LE392(pNFA3-103)

SDS-Polyacrylamide gel electrophoresis

Crude protein preparations of LE392, LE392(pACYC184), LE392 (pNFA3-103) and strain 9 were compared by SDS-PAGE. Separation of proteins on a 12% acrylamide gel (Figure 5.2a) revealed a band of approximately 18kDa which was present in strain 9 and LE392 (pNFA3-103) but was not expressed by LE392 or LE392(pACYC184).

Immunoblotting experiments

Crude protein preparations of LE392, LE392(pACYC184), LE392 (pNFA3-103) and strain 9 were separated by polyacrylamide gel electrophoresis as described above. Immunoblotting experiments were performed using the monoclonal anti-NFA-3 antibody 19B12. Proteins separated by PAGE were transfered to nitrocellulose paper and incubated with 19B12. The monoclonal antibodies reacted with the 18kDa product expressed by strain 9 and LE392 (pNFA3-103). There was no binding to LE392 or LE392(pACYC184). (Figure 5.2b).

5.7 Minicell analysis of pNFA3-103

Minicell analysis was performed to characterise the products expressed by pNFA3-103 using an identical technique to that described for pNFA2-102 (Section 4.8).

The following plasmids were transformed into DS410

i) pACYC184 (plasmid control)

ii) pNFA2-103

iii) Cells only control (containing no plasmid)

The results of the minicell experiments indicated that pNFA3-103 expresses at least five products of approximate molecular weight 80kDa, 42kDa, 30kDa, 28kDa, and 18kDa. (Figure 5.3). The 18kDa band corresponds in size to the expected NFA-3 subunit protein.

Figure 5.1
Restriction enzyme mapping of pNFA3-101 and pNFA3-103

Key
A *Acc*1
B *Bam*H1
Bg *Bgl*II
E *Eco*R1
H *Hin*dIII
P *Pvu*II
Sp *Sph*1

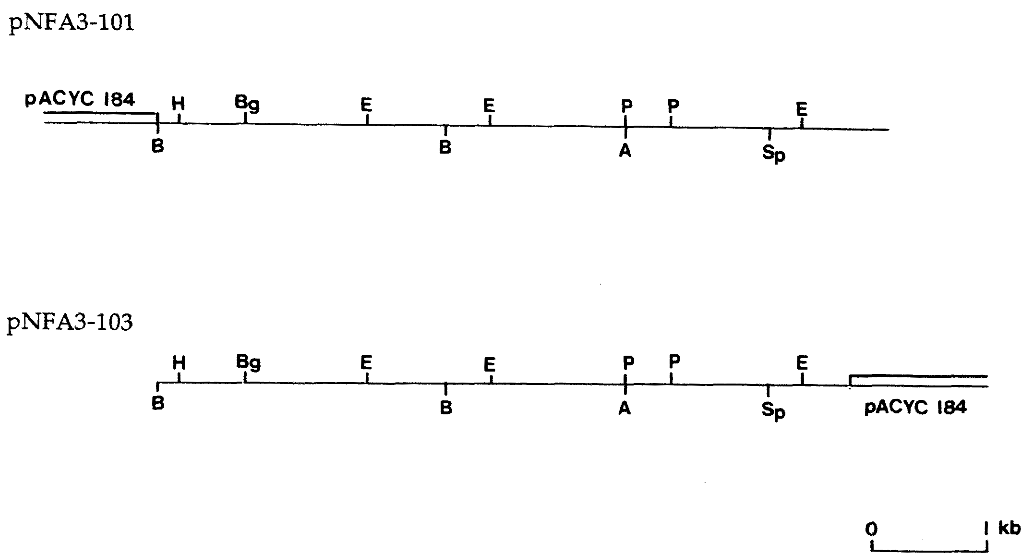


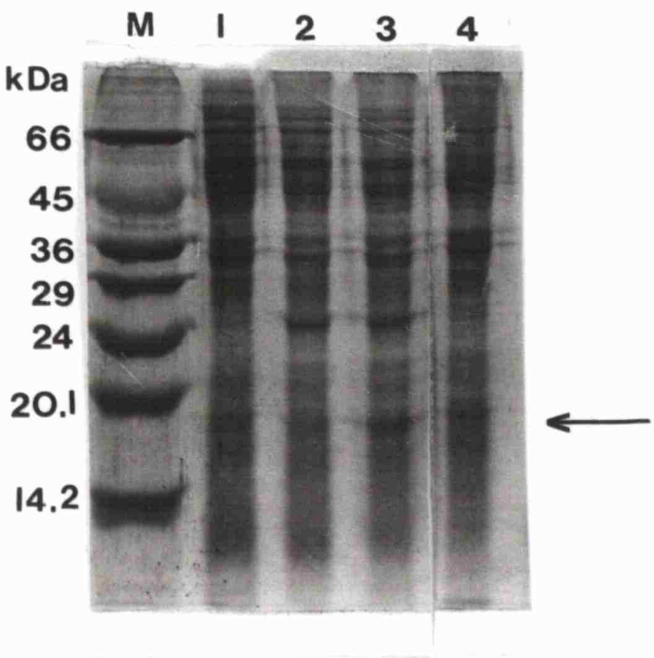
Figure 5.2

SDS-PAGE and immunoblotting experiments

a) SDS-PAGE of crude protein preparations of the following strains:-

1 LE392
2 LE392 (pACYC184)
3 LE392 (pNFA3-103)
4 strain 9
M molecular weight markers (kDa)

arrow indicates position of 18kDa band expressed by strain 9 and LE392 (pNFA3-103)



b) Immunoblotting of crude protein preparations of the following strains using the anti-NFA3 monoclonal antibody 19B12:-

1 LE392

2 LE392 (pACYC184)

3 LE392 (pNFA3-103)

4 strain 9

P protein molecular weight marker

Shows binding of 19B12 to 18kDa protein expressed by LE392 (pNFA3-103) and strain 9. There is also low level binding to ovalbumin protein marker.

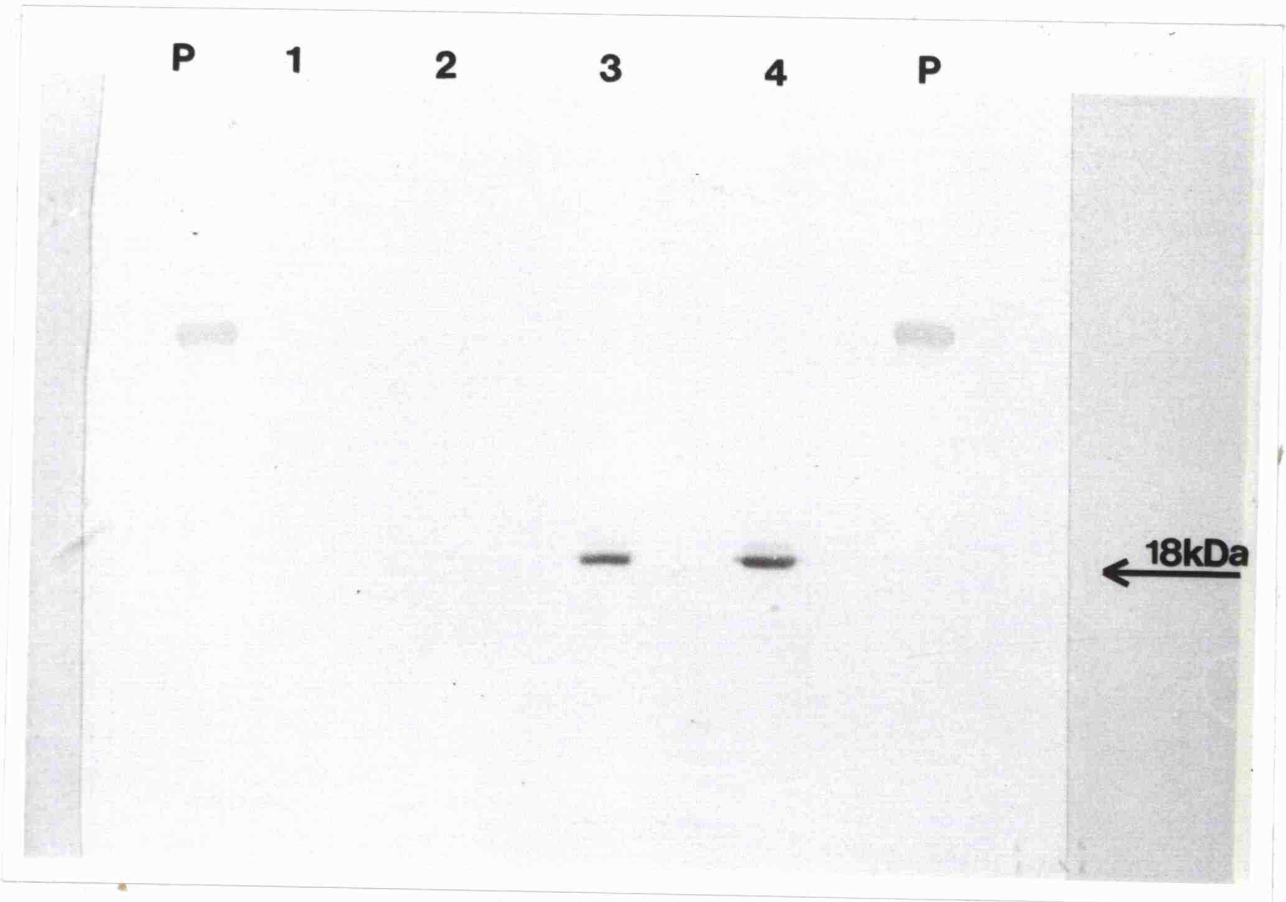
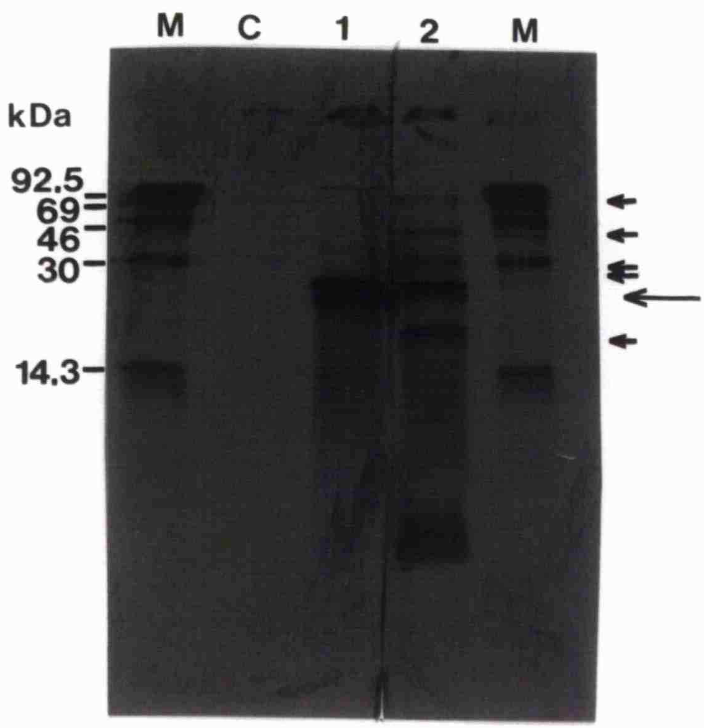


Figure 5.3
Minicell experiments showing products encoded by recombinant plasmid pNFA3-103

M molecular weight markers (kDa)
C cells only control
1 pACYC184
2 pNFA3-103

arrows show products expressed by pNFA3-103. Large arrow indicates product of chloramphenicol resistance gene (CAT).



CHAPTER 6

CLONING AND CHARACTERISATION OF NFA-4

6.1 Introduction

NFA-4 was described by Hoschutzky *et al* in 1989 who identified the adhesin on an isolate of *E. coli* (serotype 07:K98:H6) obtained from a patient in Rostock with a urinary tract infection. This strain expresses type 1 fimbriae mediating mannose-sensitive haemagglutination and a non-fimbrial adhesin mediating mannose-resistant haemagglutination. Growth on agar media suppresses expression of type-1 fimbriae. Non-fimbrial expression is also controlled by environmental factors and MRHA does not occur if bacteria are cultured at temperatures below 20°C or the presence of 1% glucose.

NFA-4 is formed by large molecular weight aggregates of adhesin subunits, with SDS-PAGE and immunoblotting studies showing a subunit size of approximately 28kDa. The NFA-4 receptor is believed to be glycophorin A^{MM}, however NFA-4 appears to be unrelated to the non-fimbrial M-adhesin described by Rhen *et al* (1986a) as the two adhesins have differing subunit sizes and N-terminal amino acid sequences.

This chapter describes the cloning and characterisation of NFA-4. The results extended the molecular analysis and comparison of this group of non-fimbrial adhesins.

6.2 Cosmid cloning of NFA-4

A similar technique was used to that already described for the cloning of NFA-2 and NFA-3 using *cos*4 as the cloning vector. Chromosomal DNA was prepared from the strain of *E. coli* expressing NFA-4. 35-50kb fragments of DNA were generated by partial digestion with *Sau*3A and purified DNA fragments and dephosphorylated *cos*4 arms were ligated at 15°C with overnight incubation. The ligation reaction mix was packaged into lambda phage heads using a commercial packaging kit. The phage stock was adsorbed onto a fresh culture of *E. coli* strain JM101 and plated onto L-agar containing ampicillin. Recombinant colonies were individually screened for mannose-resistant haemagglutination. Two MRHA-positive recombinants were identified; these were denoted JM101(pNFA4-1) and JM101(pNFA4-2).

6.3 Subcloning experiments

The approach used to subclone pNFA4 was similar to that used for the subcloning of NFA-2 and NFA-3. pNFA4-2 was partially digested with *Sau*3A producing fragments in the range 6-12kb. Purified size-selected fragments were ligated to a dephosphorylated *Bam*H1 digest of plasmid pLG339. The ligation mix was transformed into the recipient strain LE392 and resulting colonies were streaked onto duplicate plates containing a) kanamycin media and b) tetracycline media. Kanamycin resistant, tetracycline sensitive recombinants were tested for MRHA and one of the positive subclones, denoted LE392 (pNFA4-2-301) was selected for further investigation.

6.4 Restriction enzyme mapping of pNFA4-2-301

A purified caesium chloride preparation of pNFA4-2-301 was digested with a series of restriction enzymes. Single and double digests were performed to determine the precise location of the restriction enzyme sites. Restriction enzyme mapping of pNFA4-2-301 is shown in Figure 6.1a. The inserted fragment of DNA in pNFA4-2-301 is approximately 8.0kb in length.

6.5 Tn1000 mutagenesis of pNFA4-2-301 and generation of pNFA4-2-302

A series of Tn1000 insertion mutants of pNFA4-2-302 were generated by Dr Mohammed Khidir. The results showed that mutants with inserts located in a 5.5kb stretch of DNA were MRHA negative. The location of this stretch of DNA is shown in Figure 6.1b. This 5.5kb fragment of DNA presumably contains all the genetic information required for NFA-4 expression.

In view of these results a further subclone was generated by ligating a 6.2kb *Sph*1 fragment of pNFA4-2-301 to pLG339. The subclone was MRHA positive and was denoted pNFA4-2-302 (Figure 6.1c).

The inserted fragment of DNA in pNFA4-2-302 has five *Sma*1 sites, four *Hin*cII sites, three *Acc*1 and *Pvu*II sites, two *Eco*R1 sites, and single sites for *Bam*H1, *Kpn*1, *Pst*1, *Sal*1 and *Sph*1. pNFA4-2-301 has additional *Hin*cII and *Pst*1 sites. There were no sites for *Bgl*II or *Sst*1.

6.6 Characterisation of LE392(pNFA4-2-301) and LE392(pNFA4-2-302)

SDS-Polyacrylamide gel electrophoresis

Crude protein preparations of LE392(pLG339) and LE392(pNFA4-2-302) were compared by SDS-PAGE. Separation of proteins on a 15% acrylamide gel (Figure 6.2) revealed a band of approximately 21kDa which was present in LE392(pNFA4-2-302), but was absent in the LE392(pLG339) control.

Immunoblotting experiments

Crude protein preparations of LE392(pLG339), LE392(pNFA4-2-301) and LE392(pNFA4-2-302) were separated by polyacrylamide gel electrophoresis. Immunoblotting experiments were performed using the monoclonal anti-

NFA-4 antibodies 2A3 and B122BS. Proteins separated by PAGE were transfered to nitrocellulose paper and incubated with either 2A3 or B122BS. Both monoclonal antibodies showed a rather weak reaction with the 21kDa product expressed by LE392(pNFA4-2-302). There was no binding to any of the products expressed by the control LE392(pLG339).

Figure 6.1

Restriction enzyme mapping studies of NFA-4 recombinants

a) pNFA4-2-301

b) Tn 1000 insertion mutant experiments
Tn1000 insertion mutants in the 5.5kb stretch of DNA shown were MRHA -ve; mutants inserted at other sites were MRHA +ve

c) pNFA4-2-302

Key				
	A	*Acc*1	P	*Pvu*II
	B	*Bam*H1	Ps	*Pst*1
	E	*Eco*R1	S	*Sal*1
	Hi	*Hinc*II	Sm	*Sma*1
	K	*Kpn*1	Sp	*Sph*1

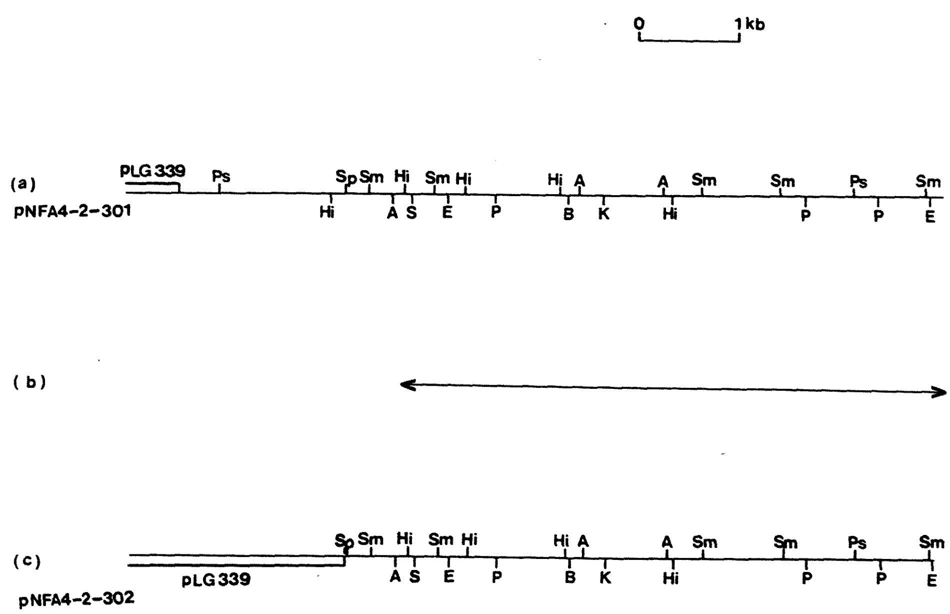
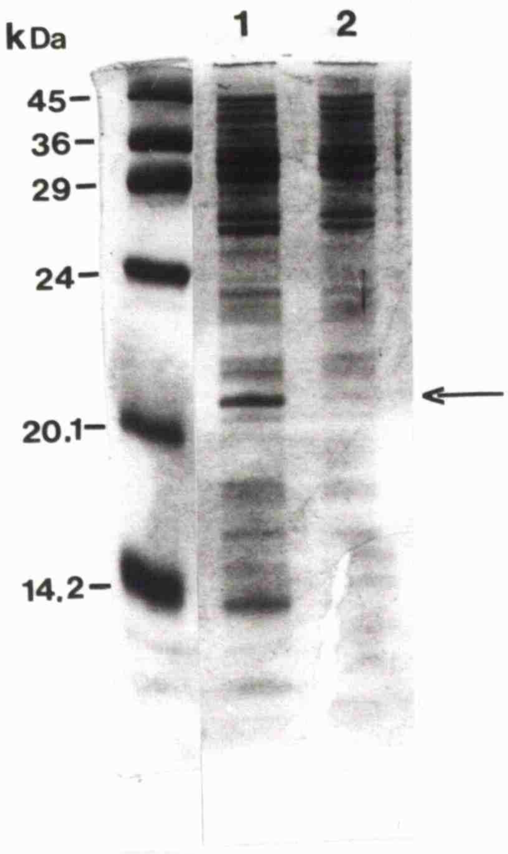


Figure 6.2

SDS-PAGE of crude protein preparations of

1) LE392 (pNFA4-2-302)
2) LE392 (pLG339)

arrow indicates position of 21kDa band



CHAPTER 7

MOLECULAR COMPARISON OF NON-FIMBRIAL ADHESINS

7.1 Introduction

The non-fimbrial adhesins identified in this study share a similar morphology, however the relationship between these adhesins had not been investigated at a molecular level. The cloning of NFA-1 to NFA-4 allowed the properties of the gene complexes encoding each adhesin to be investigated. The size and relatively complexity of the cloned sequences encoding NFA-1 to NFA-4 was compared and the extent of DNA homology investigated by comparison of restriction enzyme cleavage sites and DNA hybridisation studies. The products encoded by each gene cluster were compared by immunoblotting and minicell analysis.

7.2 Comparison of cloned insert size

The size of the cloned DNA coding for expression of NFA-1 to NFA-4 was compared The results give some information on the relative complexity of the gene clusters encoding each adhesin. The genes responsible for NFA-1 expression were located on a 6.5kb stretch of DNA that was precisely delineated by Tn1000 insertion mutagenesis. The exact length of DNA necessary for NFA-2 expression was not determined, but the two recombinant plasmids, pNFA2-101 and pNFA2-102, shared a 7.8kb stretch of DNA; therefore the amount of DNA required for expression of NFA-2 was no greater than 7.8kb. NFA-3 expression was mediated by the recombinant plasmid pNFA3-103 which had a 6.0kb insert, this was therefore the maximum amount of DNA required for expression. NFA-4 expression was mediated by the recombinant plasmid pNFA4-2-302 which had 6.2kb of inserted DNA and Tn1000 insertion mutagenesis of pNFA4-2-302 indicated that approximately 5.5kb of DNA was required for adhesin expression. The conclusions are that the gene clusters coding for NFA1-4 expression are roughly similar in size, of the order of approximately 5.5-6.0kb. The similar size suggests that the mechanisms of production and expression of each adhesin are of comparable complexity.

7.3 Comparison of restriction enzyme sites

Figure 7.1 compares the restriction endonuclease maps of pS3 (NFA-1), pNFA2-102 (NFA-2), pNFA3-103 (NFA-3) and pNFA4-2-302 (NFA-4). The results show that pS3 and pNFA2-102 each have successive sites for *Sph*1, *Kpn*1, *Sst*1, and *Sal*1. In contrast, the restriction endonuclease sites on pNFA3-103 and pNFA4-2-302 appear to be unique and unrelated. Restriction endonuclease mapping is a relatively crude method of comparing DNA, but the results suggest that NFA-1 and NFA-2 genes might share a degree of genetic homology.

7.4 DNA-DNA hybridisation

Homology between cloned non-fimbrial adhesins

The extent of DNA homology between cloned non-fimbrial adhesins was explored in a series of Southern blotting experiments. Probes were designed to span virtually the entire sequence of inserted DNA coding for the expression of each non-fimbrial adhesin (Figure 7.2); hence pS3 was digested with *Eco*R1 to produce a 10kb probe, pNFA2-102 was digested with *Cla*1 and *Sal*1 to produce a 7.5kb probe, pNFA3-103 was digested with *Hin*dIII and *Sph*1 to produce a 5.5kb probe and pNFA4-2-301 was digested with *Pst*1 to produce a 5.5kb probe.

Fragments of probe DNA were separated by agarose gel electrophoresis, using 1% low melting point agarose, and were labelled with $[^{32}P]$dCTP by the hexadeoxynucleotide primer method.

pS3, pNFA2-102, pNFA3-103 and pNFA4-2-101 were each digested with the same enzymes as those used to produce the probes. Restriction enzyme digests from each recombinant plasmid were loaded onto agarose gel in consecutive wells and fragments were separated by electrophoresis. Size-fractionated DNA fragments were transferred to hybridsation membranes by Southern blotting. Electrophoresis and blotting was repeated four times to give four identical blots which were separately hybridised with the NFA-1, NFA-2, NFA-3 or NFA-4 probes. Membranes were washed under stringent conditions. Each membrane was washed six times for 15 mins at 65°C in 0.1x SSC, 0.1% SDS.

The results of the DNA hybridisations are shown in Figure 7.3. All probes hybridised strongly to their own fragments showing that hybridisation was occurring. However, the 10kb probe derived from pS3 also hybridised strongly to a 7.5kb fragment of pNFA2-102 digested with *Cla*1 and *Sal*1. Similarly the 7.5kb probe derived from pNFA2-102 also hybridised strongly to a 10kb fragment of pS3 digested with *Eco*R1. The NFA-1 and NFA-2 probes did not hybridise with NFA-3 or NFA-4 sequences, the NFA-3 and NFA-4 probes bound to their own sequences only and did not hybridise with DNA derived from other non-fimbrial adhesins.

The results of these experiments therefore indicated that NFA-1 and NFA-2 shared a degree of genetic homology, but NFA-3 and NFA-4 appeared to be genetically distinct.

Homology between cloned NFA-1 and NFA-2 genes

The genetic relationship between NFA-1 and NFA-2 was explored further by hybridisation experiments which used a series of three non-overlapping probes derived from the inserted sequence in pS3. These experiments were designed to show whether similar DNA sequences were conserved across the entire NFA-1 and NFA-2 gene clusters or if the homology was more localised, possibly involving only the adhesin subunit gene.

Probes were generated by digesting pS3 with *Kpn*1 and *Eco*R1 (3.6kb probe), *Sph*1 and *Kpn*1 (2.3kb probe), and *Sph*1 (2.9kb probe). All three probes hybridised strongly to pNFA-2-102 revealing that the homology between pS3 and pNFA2-102 extends across the entire gene clusters. NFA-1 and NFA-2 are therefore closely related on a genetic level.

Homology between non-fimbrial adhesin genes and capsular genes

These experiments were designed to investigate whether there was any homology between the non-fimbrial genes and capsular genes. Previous studies had shown that the capsular gene complex comprises three separate regions coding for polysaccharide biosynthesis and polymerisation (region 2), translocation of polysaccharide to the cell surface (region 1) and post-polymerisation modification (region 3) (Roberts *et al* 1986, Boulnois *et al* 1987). Genes in region 1 and 3 are widely conserved amongst group II encapsulated strains of *E. coli*. Non-fimbrial adhesins have a similar morphology to capsular polysaccharide and surround the bacteria like an adhesive protein capsule. Southern blotting was therefore performed to investigate whether there was any homology between genes coding for transport and assembly of the capsule and genes encoding similar functions for non-fimbrial adhesins.

Cloned capsular genes were used as a probe to investigate hybridisation with cloned non-fimbrial genes. Plasmid pKT274 was kindly donated by Dr I Roberts and contains an insert of approximately 17kb which incorporates the entire K1 capsular gene complex (Figure 7.4). pKT274 was digested with

*Bam*H1 and *Eco*R1 generating 2kb, 3kb and 4kb fragments which were all labelled with $[^{32}P]$dCTP by the hexadeoxynucleotide primer technique. All three probes hybridised strongly to the control digest of pKT274, but there was no significant hybridisation with pS3, pNFA2-102, pNFA3-103 or pNFA4-2-301 (Fig 7.4b). The conclusion from these experiments was that the capsule and non-fimbrial adhesins appear to share a similar morphology but are genetically unrelated.

7.5 Immunoblotting studies

These experiments investigated the extent of cross reactivity between monoclonal antibodies directed against specific non-fimbrial adhesins. Goldhar *et al* (1987) showed that polyclonal antisera raised in rabbits against NFA-1 and NFA-2 were cross-reactive, however specific monoclonal antibodies were raised against purified NFA-1 and NFA-2 which did not cross-react. There is therefore a limited serological homology between these two adhesins.

Further experiments utilised monoclonal antibodies raised against purified NFA-2 (Mabs IC2 and 2D3), NFA-3 (Mab 19 B12) and NFA-4 (Mabs 2A3 and B12 2BS) which were kindly supplied by Professor Jann. A series of immunoblots were performed. Crude protein preparations from the following strains of *E. coli* were separated by polyacrylamide gel electrophoresis:-

1) LE392
2) LE392 (pACYC184)
3) LE392 (pNFA2-102)
4) LE392 (pNFA3-103)
5) LE392 (pNFA4-2-302)
6) LE392 (pLG339)

Protein preparations were electrophoresed in parallel and transferred to nitrocellulose filters by Western blotting. Following Western blotting proteins were probed with the monoclonal antibodies in a solid phase immunosorbent assay. The results are summarised in Table 7.1.

There was no evidence of any cross-reactivity with the monoclonal antibodies used in these experiments. The results from these experiments therefore indicated that these monoclonal antibodies were adhesin-specific. The possibility remains that other epitopes which were not recognised by these antibodies might be shared between non-fimbrial adhesins.

7.6 Minicell analysis

Minicell and nucleotide sequence analysis of the NFA-1 gene cluster showed that five products were expressed. These products were denoted A,B,C,D and E with respective molecular weights 19kDa, 15kDa, 80kDa, 9kDa, and 30.5kDa. The organisation of the NFA-1 gene cluster is shown in Figure 7.5 (results kindly supplied by Dr Ralph Ahrens, University of Freiburg, Germany). Minicell analysis of pNFA2-102 and pNFA3-103 showed that at least five products were also expressed by the NFA-2 and NFA-3 gene clusters. The relative molecular weights of the NFA-1, NFA-2 and NFA-3 products are compared in Table 7.2.

The comparison reveals similarities and differences between the non-fimbrial adhesin products. Each adhesin gene cluster appears to encode five separate products. All NFA's include a large product, approximately 80kDa in size. This might represent a conserved protein, perhaps involved in assembly and transport of the adhesin across the periplasmic membrane. They all express at least one protein of approximately 30kDa and the subunit protein sizes are similar at 18-19kDa. Although minicell analysis shows the size and number of products expressed by recombinant gene clusters they do not reveal whether the genes or protein products share any conserved sequences. Determination of the nucleotide sequence of the NFA genes was therefore of major importance.

Table 7.1

Immunosorbent assay of crude protein preparations of NFA-2 to NFA-4 probed with monoclonal antibodies specific for each adhesin

Protein preparation	Monoclonal antibodies				
	(NFA-2)		(NFA-3)	(NFA-4)	
	1C2	2D3	19B12	2A3	B12 2BS
LE392	-	-	-	-	-
LE392 (pACYC184)	-	-	-	-	-
LE392 (pNFA2-102	+++	+++	-	-	-
LE392 (pNFA3-103)	-	-	++	-	-
LE392 (pNFA4-2-302)	-	-	-	+/-	+/-
LE392 (pLG339)	-	-	-	-	-

Table 7.2

Comparative size of products expressed by NFA gene clusters

	NFA-1	NFA-2	NFA-3
kDa	80	80	80
			42
		33	
	30.5		30
		28	28
	19	19	18
		17	
	15	?15	
	9		

Figure 7.1

Comparison of restriction endonuclease mapping of recombinant plasmids encoding NFA1-4 gene sequences

a) pS3
b) pNFA2-102
c) pNFA3-103
d) pNFA4-2-302

Key						
	A	*Acc*1	H	*Hin*dIII	S	*Sal*1
	B	*Bam*H1	Hi	*Hin*cII	Sm	*Sma*1
	Bg	*Bgl*II	K	*Kpn*1	Sp	*Sph*1
	C	*Cla*1	P	*Pvu*II	Ss	*Sst*1
	E	*Eco*R1	Ps	*Pst*1		

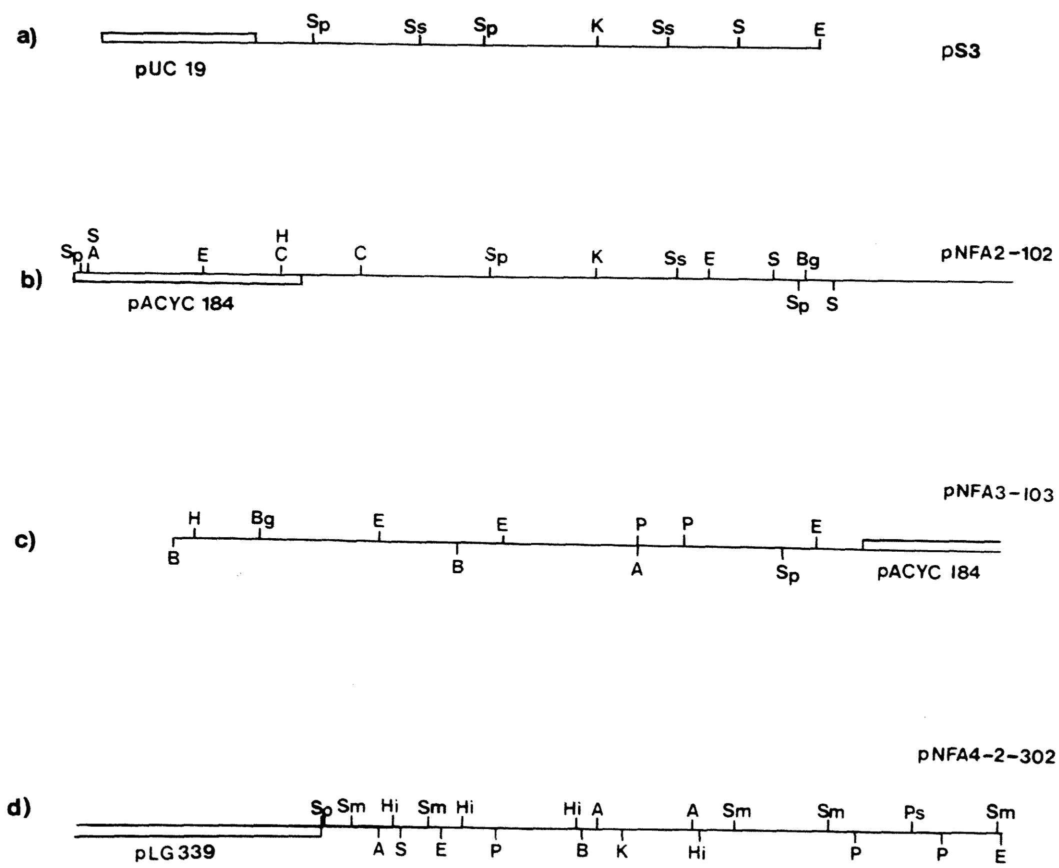


Figure 7.2

Location of inserted sequences used as probes for NFA1-4 in experiments investigating genetic homology between non-fimbrial adhesins

a) NFA-1probe
b) NFA-2probe
c) NFA-3probe
d) NFA-4probe

Key						
A	*Acc*1	H	*Hind*III	S	*Sal*1
B	*Bam*H1	Hi	*Hinc*II	Sm	*Sma*1
Bg	*Bgl*II	K	*Kpn*1	Sp	*Sph*1
C	*Cla*1	P	*Pvu*II	Ss	*Sst*1
E	*Eco*R1	Ps	*Pst*1		

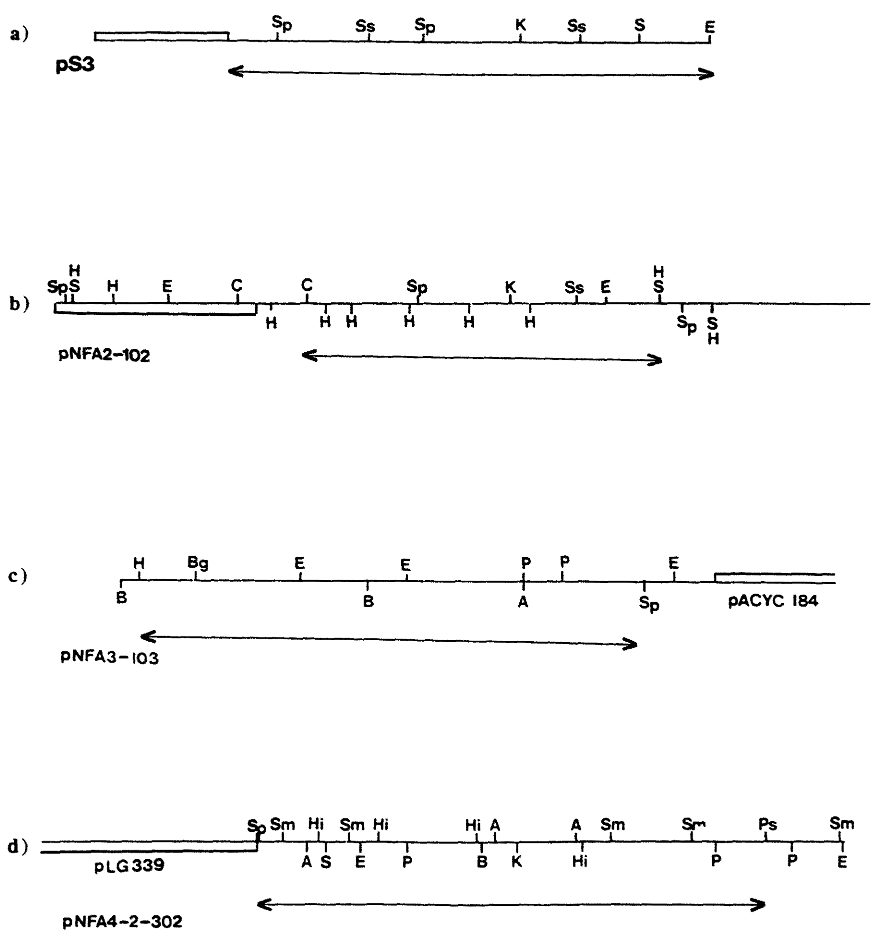


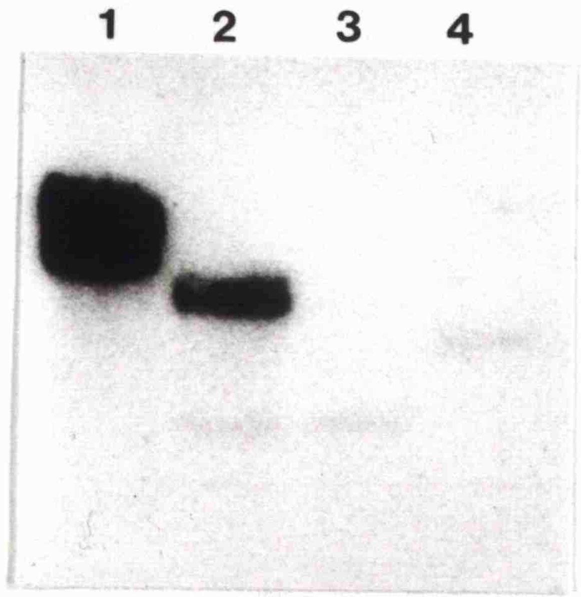
Figure 7.3

DNA hybridisation experiments investigating extent of genetic homology between cloned non-fimbrial adhesins (location of probe sequences corresponding to each non-fimbrial adhesin are shown in fig 7.2).

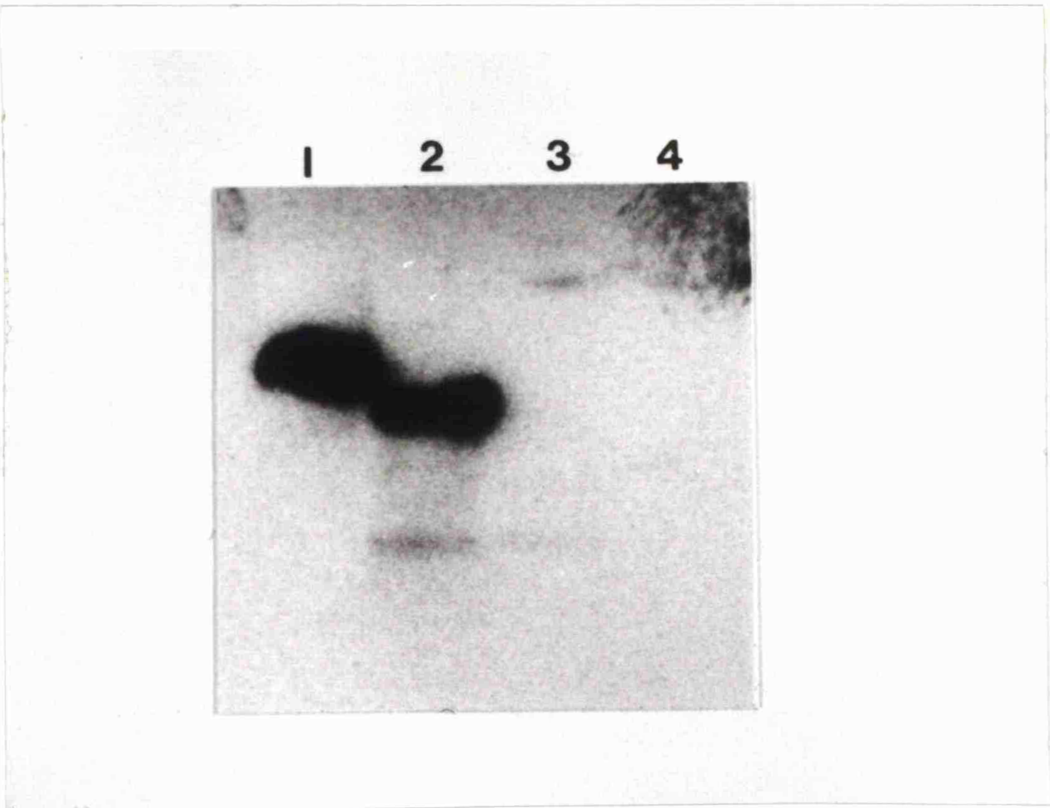
Track 1	pS3 digested with *Eco*R1
Track 2	pNFA2-102 digested with *Cla*1 and *Sal*1
Track 3	pNFA3-103 digested with *Hin*dIII and *Sph*1
Track 4	pNFA4-2-301 digested with *Pst*1

a) hybridisation with NFA-1 probe
b) hybridisation with NFA-2 probe
c) hybridisation with NFA-3 probe
d) hybridisation with NFA-4 probe

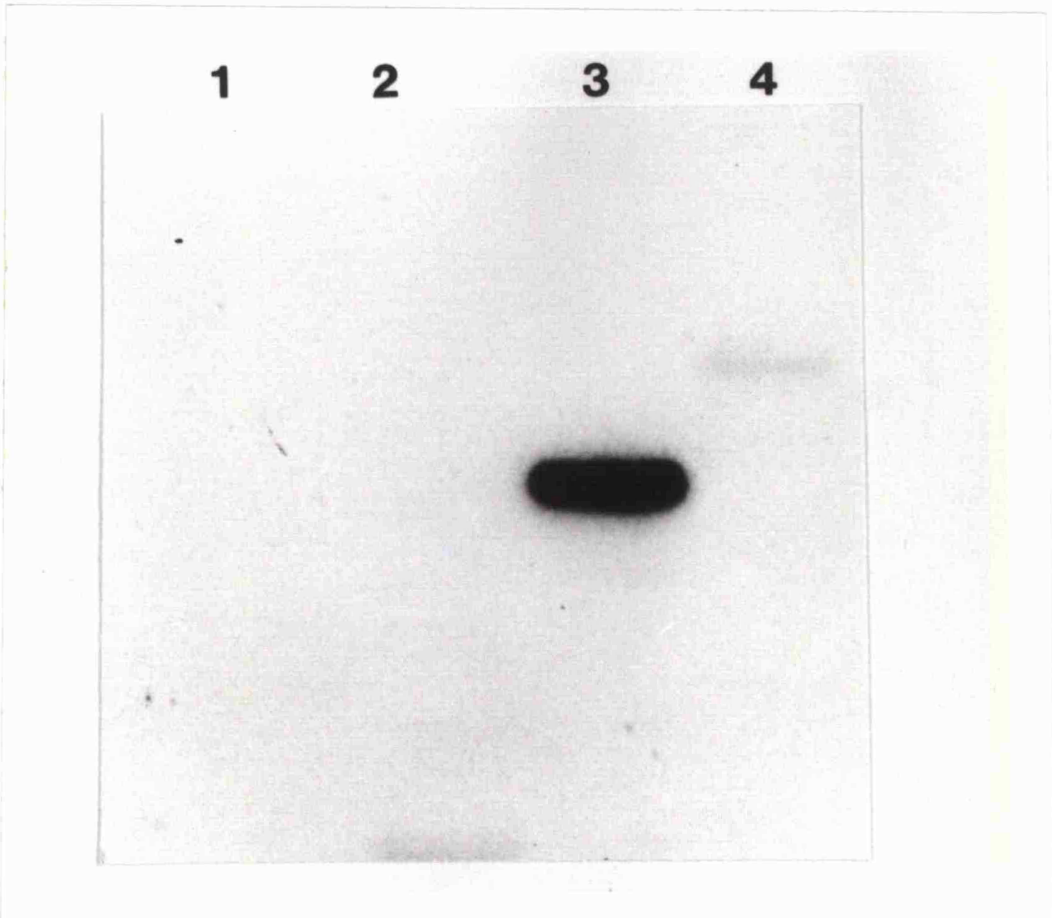
a) hybridisation with NFA-1 probe



b) hybridisation with NFA-2 probe



c) hybridisation with NFA-3 probe



d) hybridisation with NFA-4 probe

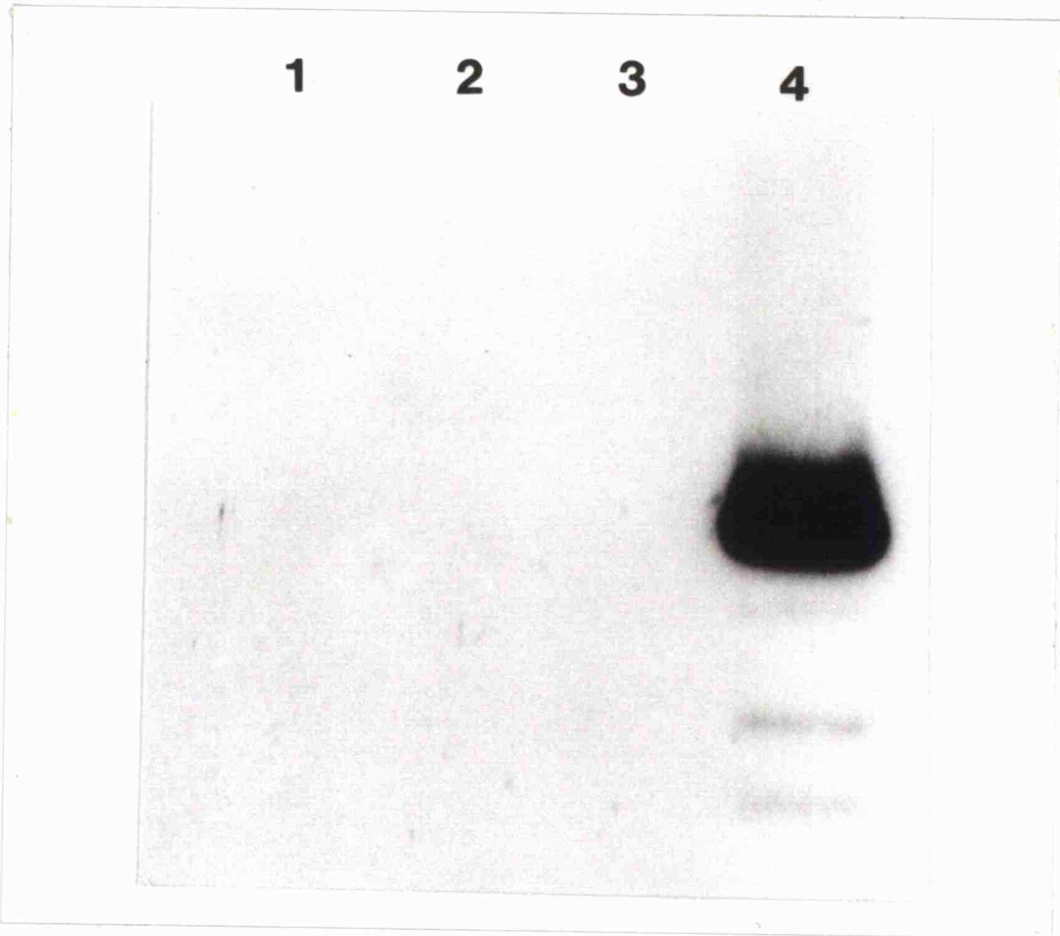
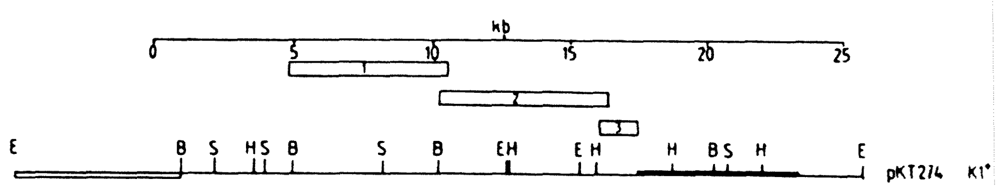


Figure 7.4
DNA hybridisation experiments investigating extent of genetic homology between cloned K1 capsular genes and non-fimbrial adhesin genes (NFA1-4)

a) physical map of plasmid pKT274 showing restriction enzyme sites and location of regions 1-3 required for K1 antigen production (open box corresponds to vector sequence asnd filled box denotes Tn5)
K1 probes were generated by digesting pKT274 with *Eco*R1 and *Bam*H1

Key B *Bam*H1
E *Eco*R1
H *Hin*dIII
S *Sal*1



b) Hybridisation of K1 probes with cloned non-fimbrial adhesin sequences

Track 1 pKT274 digested with *Eco*R1 and *Bam*H1

Track 2 pS3 digested with *Eco*R1

Track 3 pNFA2-102 digested with *Cla*1 and *Sal*1

Track 4 pNFA3-103 digested with *Hind*III and *Sph*1

Track 5 pNFA4-2-301 digested with *Pst*1

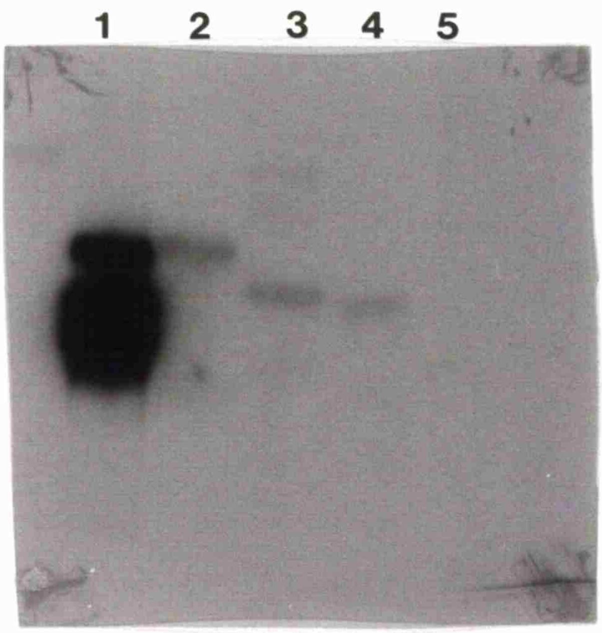
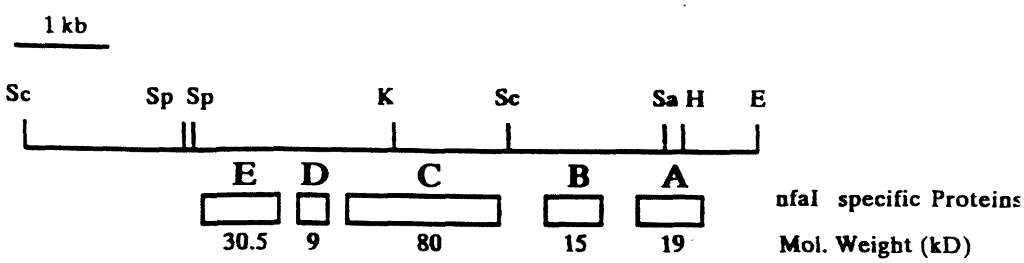


Figure 7.5
Genetic organisation of the NFA-1 gene cluster

Key	E	*Eco*R1
	H	*Hin*dIII
	K	*Kpn*1
	Sa	*Sal*1
	Sc	*Sac*1
	Sp	*Sph*1



CHAPTER 8

NUCLEOTIDE SEQUENCE ANALYSIS OF THE NFA-2 AND NFA-4 ADHESIN SUBUNIT GENES

8.1 Determination of the nucleotide sequence of the NFA-2 adhesin subunit gene and flanking sequences

Introduction

The DNA sequences coding for NFA-2 expression were cloned into the recombinant plasmids pNFA2-101 and pNFA2-102 containing insert sizes of 12.6kb and 9.8kb respectively. Both recombinant plasmids shared a 7.8kb stretch of inserted DNA between *Cla*1 and *Sph*1 sites which presumably included all the sequences necessary for NFA-2 expression. The subunit gene was of particular interest as this was the actual adhesin and the protein responsible for the non-fimbrial structure. Initial experiments therefore aimed to locate and sequence the subunit protein. DNA sequences derived from the recombinant plasmid pNFA2-102 were then analysed and compared with other published adhesin sequences.

Sequencing strategy

Previous experiments had demonstrated extensive DNA homology between the cloned NFA-1 and NFA-2 sequences. The sequences known to code for the NFA-1 adhesin subunit gene were therefore used as a probe to attempt to locate the position of the NFA-2 subunit gene. The NFA-1 subunit gene was found to be located on a 1.4kb *Sal*1-*Eco*R1 fragment of pS3 (chapter 3). This fragment was used as the probe and hybridised with a series of restriction enzyme digests of pNFA2-102.

DNA fragments resulting from digests of pNFA2-102 were separated by agarose gel electrophoresis and transferred to a hybridisation membrane by Southern blotting. The membrane was probed with the labelled 1.4kb *Sal*1-*Eco*R1 fragment of pS3 and washed under stringent conditions as shown below:-

2x SSC	0.1% SDS	20 mins	x3
0.5x SSC	0.1% SDS	20 mins	x3
0.1x SSC	0.1% SDS	20 mins	x1

The results are shown in figures 8.1a and 8.1b. The labelled probe hybridised to the following fragments:-

Digest of pNFA2-102	Fragments hybridising to probe
1. *Sal*1	12kb and 1kb
2. *Sal*1 and *Eco*R1	1kb and 1.2kb
3. *Sst*1 and *Eco*R1	incomplete digest
4. *Sst*1 and *Kpn*1 and *Eco*R1	5kb
5. *Sph*1 and *Kpn*1	3.5kb
6. *Sph*1 and *Cla*1	5.5kb
7. *Hin*cII	2.7kb and 1kb

The interpretation of these results is shown in Figure 8.2. The experiments suggested that the subunit gene was located within a 1.5kb stretch of pNFA2-102 bounded by *Eco*R1 and *Sph*1 sites. A *Sal*1 digest of pNFA1-102 yielded two fragments which both hybridised with the probe suggesting that the *Sal*1 site might lie within the subunit gene.

Preparation of templates for sequencing

The 2.2kb *Eco*R1-*Sal*1 fragment of inserted DNA in pNFA2-102 was sequenced as this fragment was believed to include the NFA-2 subunit gene and flanking region. Sequencing proceeded in both directions as shown in Figure 8.3. The following fragments of pNFA2-102 were used as sequencing templates:-

*Eco*R1-*Sal*1

*Eco*R1-*Sph*1

*Eco*R1-*Bgl*II

*Sph*1-*Sal*1

*Bgl*II-*Sal*1

Sequences between restriction sites were determined using synthesised oligonucleotide primers.

Nucleotide sequence analysis

A total of 2035 base pairs were sequenced in both directions between the *Eco*R1 and terminal *Sal*1 sites of the inserted DNA in pNFA2-102 (Figure 8.4). The nucleotide sequence was analysed using the Wisconsin Molecular Biology package on the University of Leicester VAX Cluster. Several putative open reading frames (ORF) were identified in the nucleotide sequence shown in Figure 8.4. The 546 base-pair ORF between positions 568 and 1113 was of particular interest as this sequence translated into a 181 amino-acid polypeptide with a predicted molecular weight of 19073 (Figure 8.5). The sequence was preceded by a potential ribosome binding site as shown in the Figure 8.5. A *Sal*1 restriction site was identified at position 1066. The position and size of this ORF corresponded to the NFA-2 adhesin subunit.

The composition of the 546 base nucleotide sequence was adenine 177 bases (32.4%), thymidine 84 bases (15.5%), cytosine 135 bases (24.7%) and guanine 150 bases (27.4%).

8.2 Molecular analysis of the NFA-2 adhesin subunit peptide

The nucleotide sequence and amino acid composition of the putative NFA-2 adhesin subunit peptide is shown in Figure 8.6. The first 23 amino acids are hydrophobic and are characteristic of a signal sequence. This would generate a mature 158 amino acid protein of molecular weight approximately 16.5kd, however it is not known whether such protein cleavage actually occurs. A plot of hydrophilicity / hydrophobicity (Figure 8.7) shows the hydrophobic nature of the N-terminus of the polypeptide which contrasts with the remainder of the peptide which is predominantly hydrophilic. The predicted structure of the NFA-2 subunit peptide is shown in Figure 8.8.

The amino acid sequence includes two cysteine residues which are likely to form a disulphide bridge. There are 18 glycine residues (11.4%). The relatively high number of glycine residues confers flexibility on the polypeptide. There are 18 threonine residues and 8 serine residues (threonine and serine combined = 16.5%). These amino acids are responsible the hydrophilic nature of much of the polypeptide and may have an important role in polymerisation.

8.3 Molecular comparison of NFA-1 and NFA-2 adhesin subunits

The cloned NFA-1 gene cluster was subcloned and sequenced in Professor Jann's laboratory in Freiburg, Germany. A 552 nucleotide open reading frame was identified which corresponded with the NFA-1 adhesin subunit. The sequence encoded a 184 amino acid polypeptide with a calculated molecular weight of approximately 19kDa. The known N-terminal sequence of the NFA-1 adhesin subunit corresponded to the amino acid sequence starting at residue 29; therefore the first 28 amino acids of the polypeptide were thought to represent a signal sequence which is cleaved to form the mature adhesin protein.

Nucleotide and peptide sequence comparisons of the NFA-1 and NFA-2 adhesin subunit sequences are shown in Figures 8.9 and 8.10 respectively. The nucleotide sequences of both adhesins show 75.6% identity. Sequences preceding and immediately after the start codon of both adhesins showed an even greater degree of similarity with virtual identity of bases in this region. The amino acid sequences of both adhesins show 55.8% identity and 67.6% similarity with alignment of the cysteine residues. Table 8.1 compares the properties of NFA-1 and NFA-2 adhesin subunit proteins.

8.4 Comparison of nucleotide and peptide sequence of the NFA-2 adhesin subunit with other published sequences

Dr adhesin family

Comparisons of the NFA-2 adhesin subunit sequences with other published sequences revealed evidence of nucleotide and amino acid sequence homology with adhesins classified into the Dr adhesin family, namely draA, afaE, and F1845. The comparisons are shown in Table 8.2

Closer inspection of the nucleotide sequence homology between NFA-2 and the Dr adhesin family revealed that the region of sequence identity was restricted to the bases preceding and immediately after the start codon of the adhesin genes. The striking degree of homology in this region is illustrated in Figure 8.11.

Other published adhesins

The extent of nucleotide and amino acid sequence homology between the NFA-2 adhesin subunit sequence and other published adhesin sequences is shown in Table 8.3. The conclusion from this analysis is that there does not appear to be any significant degree of homology with other published sequences apart from those belonging to the Dr adhesin family. Comparison of the NFA-2 sequence with a range of other published non-adhesin sequences (not shown) similarly revealed no significant homology.

8.5 Determination of the nucleotide sequence of the NFA-4 adhesin subunit gene and flanking regions

Sequencing strategy

Initial experiments attempted to locate the position of the NFA-4 adhesin subunit gene in the cloned plasmid pNFA4-2-302. Professor Jann's group had already established the N-terminal amino acid sequence of the NFA-4 subunit gene and this allowed the subunit to be located on pNFA4-2-302 by hybridisation of labelled oligonucleotides corresponding to the known N-terminal sequence as outlined below.

The first six N-terminal amino acids and their corresponding DNA sequences are as follows:-

Trp	Thr	Thr	Gly	Asp	Phe
TGG	ACA	ACA	GGA	GAC	TTC
	C	C	C	T	T
	G	G	G		
	T	T	T		

The following pool of 17-base oligonucleotides was therefore synthesised:-

TGG	ACA	ACA	GGA	GAC	TT
	C	C	C	T	
	G	G	G		
	T	T	T		

The pool of oligonucleotides were labelled with ^{32}P using T4-kinase. A series of restriction digests of pNFA4-2-302 were performed and the resulting fragments separated by agarose gel electrophoresis. The DNA-fragments were then transferred to the hybridisation membrane by Southern blotting. After prehybridisation the membrane was hybridised with the labelled pool of oligonucleotides. The dissociation temperature (Td) of the oligonucleotides was calculated as approximately 52°C, (4°C for each G or C and 2°C for each A or T) and overnight hybridisation was therefore performed at this temperature. The membrane was washed four times for fifteen minutes in 2x SSC, 0.1x SDS Washing of the membrane was followed by autoradiography. More stringent washes led to a reduction in the hybridisation signal.

Figure 8.12 shows the restriction digests performed and the fragments which hybridised to the labelled oligonucleotides. The results of these experiments showed that the oligonucleotides hybridised to two separate segments of DNA. One area of binding was located within the plasmid vector pLG339, and the other was located close to the *Eco*R1 site. The deduced locations of the two areas of DNA homology are also shown. These experiments were not entirely convincing as the hybridisation signal was rather weak, however it was decided to perform DNA sequencing of the inserted fragment in pNFA4-2-302, concentrating on the region around the *Eco*R1 site identified by the oligonucleotide binding experiments.

The generation of templates and sequencing strategy for pNFA4-2-302 is shown in Figure 8.13. The following fragments of pNFA4-2-302 were used as sequencing templates:-

1. *Sal*1 (0.75kb fragment)
2. *Eco*R1 - *Acc*1
3. *Sph*1 - *Acc*1
4. *Eco*R1 - *Bam*H1

Nucleotide sequence analysis

A total of 1090 nucleotide bases were sequenced in pNFA4-2-302. The derived nucleotide sequences spanned the *Eco*R1 site but did not correspond to the N-terminal sequence determined by Professor Jann. At this stage it was decided to compare the nucleotide sequences derived from pNFA4-2-302 with other published adhesin sequences. It was immediately apparent that there was a very close homology with the M-adhesin gene sequence (*bmaE*), published by Rhen *et al* (1986b). The nucleotide sequence comparison is shown in Figure 8.14 which reveals 96.8% identity over a 283 base-pair stretch of DNA. The possible reasons for thie finding of such a strong identity between pNFA4-2-302 were thought to be either that the NFA-4 adhesin is very closely related to the non-fimbrial M-adhesin, or that the clinical isolate of E.coli (serotype 07:K98:H6) actually expresses two non-fimbrial adhesins, NFA-4 and an M-adhesin. If this was the case the M-adhesin had inadvertently been cloned rather than NFA-4. In view of this doubt regarding the adhesins expressed by the "NFA-4" isolate (serotype 07:K98:H6) no further sequence analysis was performed.

Table 8.1

Molecular comparison of NFA-1 and NFA-2 adhesin subunits

	NFA-1	NFA-2
Number of nucleotides	552	543
Amino acids	184	181
Predicted molecular weight	19000	19073
Amino acids in signal sequence	28	23
Amino acids in mature protein	156	158
Position of cysteine residues	29 & 63	31 & 65

Table 8.2

Comparison of the nucleotide and amino acid sequence of the NFA-2 adhesin subunit with Dr adhesins

NFA-2 *vs*	Nucleotide homology		Amino acid homology *similarity*	*identity*
draA	45.7%	overall identity	42.4%	20.3%
afaE	67.2%	identity in 180bp overlap	42.3%	23.6%
F1845	66.8%	identity in 199bp overlap	42.5%	19.4%
NFA-1	75.6%	overall identity	67.6%	55.8%

Table 8.3

Comparison of the nucleotide and amino acid sequence of the NFA-2 adhesin subunit with other published adhesin sequences

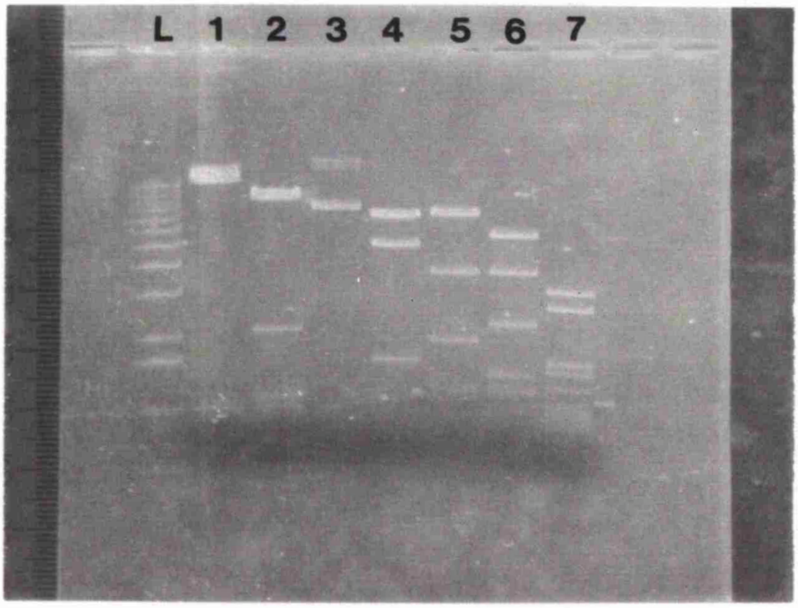
NFA-2 *vs*	Nucleotide homology	Amino acid homology *similarity*	*identity*
bmaE	42%	42%	15%
fimA	36%	43%	23%
fimB	-	36%	15%
fimE	-	35%	15%
K88A	35%	46%	23%
papC	-	47%	19%
papG	34%	-	-
papH	41%	43%	16%
sfaA	38%	39%	25%
sfaG	-	43%	22%
sfaH	-	36%	13%
sfaS	-	41%	21%
Type 1C	36%	41%	23%

Figure 8.1

Localisation of NFA-2 subunit sequence by hybridisation of homologous NFA-1 subunit probe (1.4kb *Sal*1- *Eco*R1 fragment of pS3) to restriction enzyme digests of pNFA2-102.

a) fragments generated by restriction endonuclease digestion of pNFA2-102 separated by agarose gel electrophoresis

L 1kb Mwt ladder
1 *Sal*1
2 *Sal*1 and *Eco*R1
3 *Sst*1 and *Eco*R1 (incomplete digest)
4 *Sst*1, *Kpn*1 and *Eco*R1
5 *Sph*1 and *Kpn*1
6 *Sph*1 and *Cla*1
7 *Hinc*II



b) hybridisation of NFA-1 subunit probe to pNFA2-102 sequences (fragments generated by restriction enzyme digestion, separated by agarose gel electrophoresis as shown in 8.1a, transferred to hybridisation membrane by Southern blotting and hybridised with radiolobelled 1.4kb NFA-1 subunit probe)

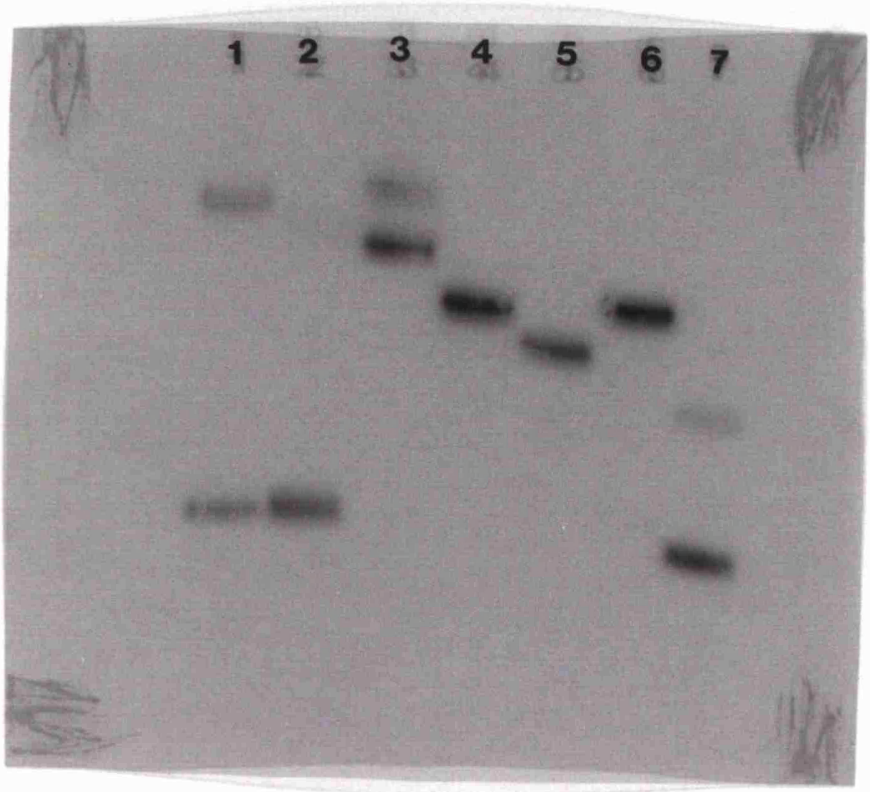


Figure 8.2
Interpretation of hybridisation experiments.

Restriction enzyme fragments of pNFA2-102 hybridising to the NFA-1 subunit probe are shown below. Results show a common area of hybridisation to a 1.5kb stretch of DNA between *Eco*R1 and *Sph*1 sites (S). This is the putative location of the NFA-2 subunit gene sequence.

1 *Sal*1 digest	Key	C	*Cla*1
2 *Sal*1 and *Eco*R1		E	*Eco*R1
3 (*Sst*1 and *Eco*R1 not included as incomplete digest)		H	*Hin*cII
4 *Sst*1, *Kpn*1 and *Eco*R1		K	*Kpn*1
5 *Sph*1 and *Kpn*1		S	*Sal*1
6 *Sph*1 and *Cla*1		Sp	*Sph*1
7 *Hin*cII		Ss	*Sst*1
S common area of hybridisation			

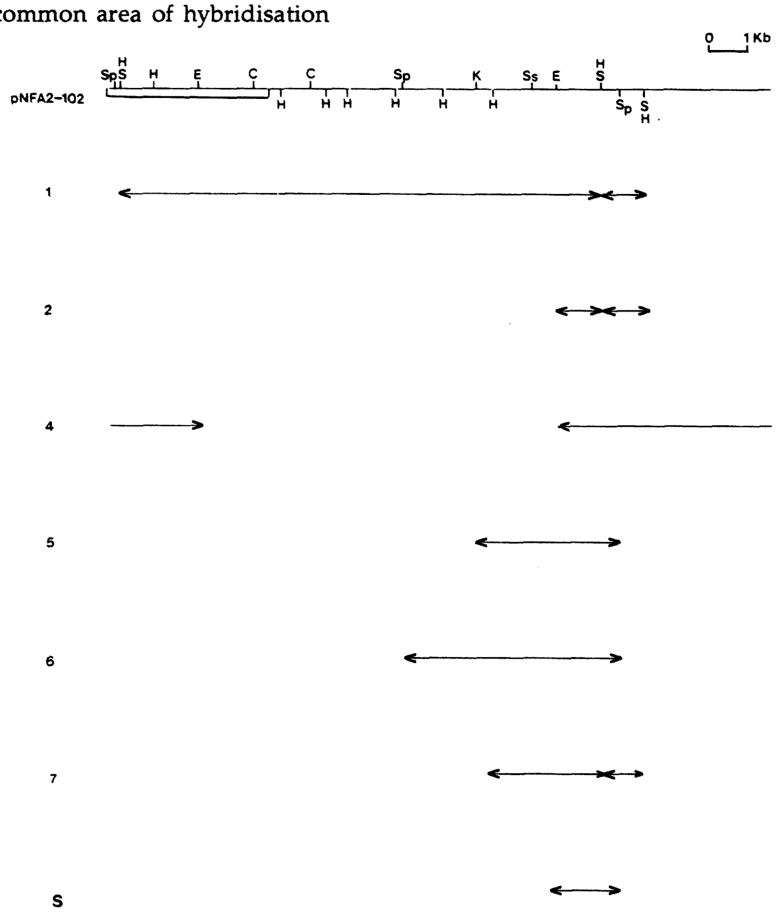


Figure 8.3

Strategy for sequencing *ECoR1-Sal1* fragment of pNFA2-102

Key C *Cla1*
E *EcoR1*
H *HincII*
K *Kpn1*
S *Sal1*
Sp *Sph1*
Ss *Sst1*

o - signifies use of synthesised oligonucleotide as sequencing template

pNFA2-102

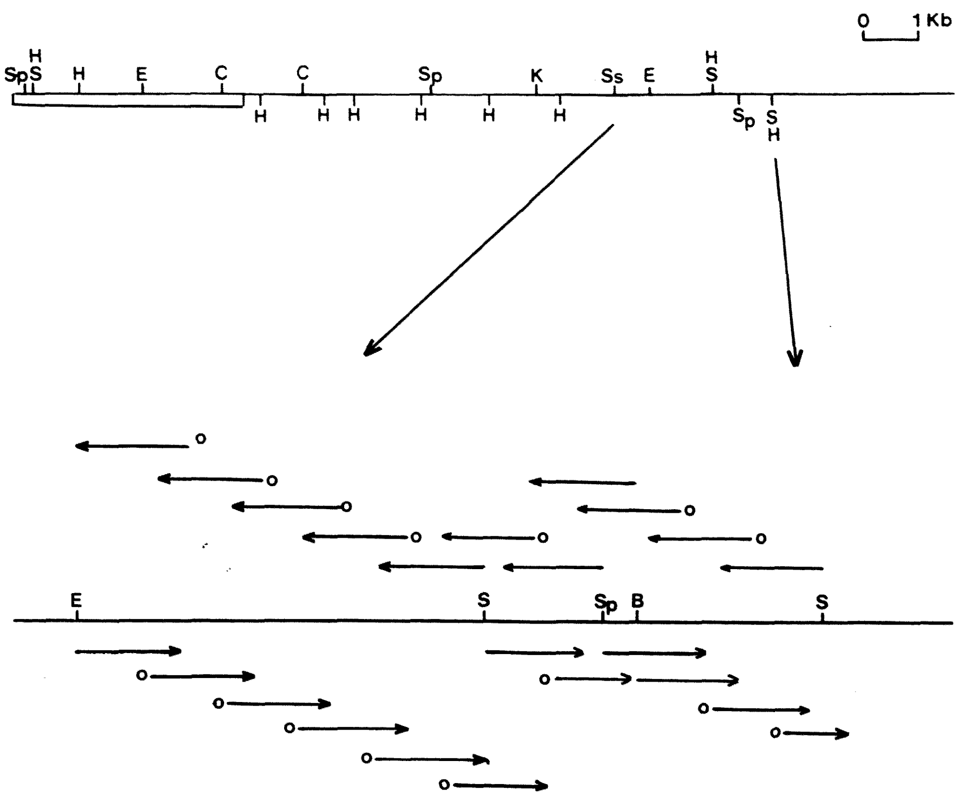


Figure 8.4

Nucleotide sequence of 2035 base pair fragment of pNFA2-102 located between *Eco*R1 and *Sal*1 restriction sites. Position of 546 base pair open reading frame is shown.

```
   1  AAGGCACGCA GGGAATACAG GAAAGAATAA AGAATAAAGG AAAAATAAAA
  51  GACCAGAGAA GAAAAGCACA AAGCTGATTA ACACAGGCAG TTAATCCGTG
 101  CTGGCGGTTT ATTACATAAA TATAAATCGG CCATCCGGAT TTAATTTAAA
 151  CAGTCAGAAT CATTTAAATC AGAAATAAGG TGGAGGTTTT ATTATGGGTA
 201  ATCTGCCAGT GGGAAGTCGT GGTTTATATA AAGGGAAAAA ACGCAGCGCC
 251  GGTATGAATG AATTACGTCA TCCGGGAAGC ACACAGATGA CGCGCACTGG
 301  TCAGGCGCAT CGCGGTGGCG AACACCGGCT GAACACGGGG CCACCGGACT
 351  GGCAGACCGT GGAATAAGGC ATCACCGTGC ACGTTGTCTG CGGCTTTATG
 401  AGCAAGCCCT GCAGTCAGAA ACTTACTTAT ATGCAATGAA CAGTCTCTGC
 451  TGCGGGTGCA GACATCTGTG AACGGTGGTT AATTTGGGGG AAGACAGCTT
 501  ACTGATTCTG GGATGGATTA ACAGAACACA ACTGGCTTGC CCATAAGCAC
 551  AACGAAGGCA AAAAAATATG AAAATAAAAT ATACAATGAA AATGGCGGCA
 601  GTTGCCAGCG TCATGGTCGC CGGAATCGCG ACCGCGAATG ACAACGTACT
 651  CAACGGGGTG GGGGGCGCTG ATGGCATCCG TCTAGGCACC GCAACCGCGA
 701  GCGGGACGAT CACCAACATG GAAAGCTGCA CAGTAAAGCT GACAATCGCT
 751  ACCCCTGACG CTAAGATGAA CCGGGCAGGG ATGCAAGAAA ACCGCGAAAT
 801  CACTAAATTT AAGGTAGCGA GCAACGATTG CCCTACCGAC ACCTATGCTG
 851  TATGGTTTAA AGAGATCGAT AACGTAGGCA ACGGGATCGC ACAGGGCAAA
 901  GTGAATACAA ACAGATTTTA CCTACGGATG GCATCGACGA ACGGTACGGA
 951  AAGCCAAAAG GACATAAGCG TAGGGAACAA AACAGGCAAA GGCCTGAGCG
1001  GCAAGCTGGC CAATGGCGCA TTCGAGGGGA AAATAACTTT GGCCCAAGAC
1051  ACGACCGGCG TACCCGTCGA CGTCTATACA TACAACCTCA TGGCCGCAGT
1101  GTACAGCCAA TAATGCTCAC AACCGTACCG AACACCGACC GCCGCCCGCC
1151  GGCGGTCTTG CTTACCTCGC GGGAATCGAC CTCGTCGCAG GCAACCAAGT
1201  GGCTGAACAA CTGCCGCTAC GGGCGGCGGG CTGCGATCGC CCGGTCTCCA
1251  TGACAATCCG CCGGAAACGG CAATGGACAG GGCCAAAAGG AGGGCCCAAG
1301  TGTACTGCTA CACCTGGTTG TCAGGCCTCA CGGACGACGA CCGCACACCG
1351  ACGCCAGGGT CACGATAATC GAACGGGCAA CACAAGAACT CAATACTGGG
1401  AACGGCTTTG ACAACCCGCG ATGACTAATA AAAAGCCCGA CCACCCCGAC
1451  GCACGGTAGT TGTGCGCTAT ATGGACAAAT ATCGCAGTCT TACGGCGCAC
1501  GTTCATTATG TGGCATGCGA AAGGCCCGAA CGTCCCTCTC ATGTTGTCAA
1551  GCCCAACGCG CTAATGAACC CTCCGACAAC AATGAGCAAG TCACGCTAGT
1601  CTTACGAAAC GTTCAGGTGA CCTGAAACAG CAGCAGATCT GTTACTTCAG
1651  TTATCTACGG CACAACGTCA GGGCCGTTAT AAAACGACGC TTCAGGCTGG
1701  AGTAATGGCC CCCCGCCTGC TCATCATTGA TGAAATAGGC TATCTGCCGT
1751  TCAGTCAGGA AGAAGCAAAA CTGTTCTTCC AGGTCATCGC TAAACGTTAC
1801  GAAAAGAGCG CAATGATCCT GACATCCAAT CTGCCGTTCG GGCAGTGGGA
1851  TCAAACGTTC GCCGGTGATG CAGCACTAAC CTCAGCGATG CTGGACCGTA
1901  TCTTACACCA CTCACATGTC GTTCAAATCA AAGGAGAAAG CTATCGACTC
1951  AGACAGAAAC GAAAGGCCGG GGTTATAGCT GAAGCTAATC CTGAGTAAAA
2001  CGGTGGATCA ATATTGGGCC GTTGGTGGAG ATATA
```

Figure 8.5

Nucleotide sequence of 546 base pair open reading frame of pNFA2-102. Start and stop codons are underlined. Potential ribosome binding site is shown (hatched underline) and location of *Sal* 1 restriction site is shown (box).

```
 401  AGCAAGCCCT GCAGTCAGAA ACTTACTTAT ATGCAATGAA CAGTCTCTGC
 451  TGCGGGTGCA GACATCTGTG AACGGTGGTT AATTTGGGGG AAGACAGCTT
 501  ACTGATTCTG GGATGGATTA ACAGAACACA ACTGGCTTGC CCATAAGCAC
 551  AACGAAGGCA AAAAAATATG AAAATAAAAT ATACAATGAA AATGGCGGCA
 601  GTTGCCAGCG TCATGGTCGC CGGAATCGCG ACCGCGAATG ACAACGTACT
 651  CAACGGGGTG GGGGGCGCTG ATGGCATCCG TCTAGGCACC GCAACCGCGA
 701  GCGGGACGAT CACCAACATG GAAAGCTGCA CAGTAAAGCT GACAATCGCT
 751  ACCCCTGACG CTAAGATGAA CCGGGCAGGG ATGCAAGAAA ACCGCGAAAT
 801  CACTAAATTT AAGGTAGCGA GCAACGATTG CCCTACCGAC ACCTATGCTG
 851  TATGGTTTAA AGAGATCGAT AACGTAGGCA ACGGGATCGC ACAGGGCAAA
 901  GTGAATACAA ACAGATTTTA CCTACGGATG GCATCGACGA ACGGTACGGA
 951  AAGCCAAAAG GACATAAGCG TAGGGAACAA AACAGGCAAA GGCCTGAGCG
1001  GCAAGCTGGC CAATGGCGCA TTCGAGGGGA AAATAACTTT GGCCCAAGAC
1051  ACGACCGGCG TACCCGTCGA CGTCTATACA TACAACCTCA TGGCCGCAGT
1101  GTACAGCCAA TAATGCTCAC AACCGTACCG AACACCGACC GCCGCCCGCC
1151  GGCGGTCTTG CTTACCTCGC GGGAATCGAC CTCGTCGCAG GCAACCAAGT
```

Figure 8.6

Nucleotide sequence and deduced amino acid composition of the 546 base pair open reading frame which encodes a 181 amino acid protein of predicted Mwt 19073 (putative NFA-2 adhesin subunit).

arrow shows cleavage site for potential signal sequence. The location of the two cysteine residues is shown.

```
MetLysIleLysTyrThrMetLysMetAlaAlaValAlaSerValMetValAlaGlyIle
ATGAAAATAAAATATACAATGAAAATGGCGGCAGTTGCCAGCGTCATGGTCGCCGGAATC

AlaThrAlaAsnAspAsnValLeuAsnGlyValGlyGlyAlaAspGlyIleArgLeuGly
GCGACCGCGAATGACAACGTACTCAACGGGGTGGGGGCGCTGATGGCATCCGTCTAGGC

ThrAlaThrAlaSerGlyThrIleThrAsnMetGluSerCysThrValLysLeuThrIle
ACCGCAACCGCGAGCGGGACGATCACCAACATGGAAAGCTGCACAGTAAAGCTGACAATC

AlaThrProAspAlaLysMetAsnArgAlaGlyMetGlnGluAsnArgGluIleThrLys
GCTACCCCTGACGCTAAGATGAACCGGGCAGGGATGCAAGAAAACCGCGAAATCACTAAA

PheLysValAlaSerAsnAspCysProThrAspThrTyrAlaValTrpPheLysGluIle
TTTAAGGTAGCGAGCAACGATTGCCCTACCGACACCTATGCTGTATGGTTTAAAGAGATC

AspAsnValGlyAsnGlyIleAlaGlnGlyLysValAsnThrAsnArgPheTyrLeuArg
GATAACGTAGGCAACGGGATCGCACAGGGCAAAGTGAATACAAACAGATTTTACCTACGG

MetAlaSerThrAsnGlyThrGluSerGlnLysAspIleSerValGlyAsnLysThrGly
ATGGCATCGACGAACGGTACGGAAAGCCAAAAGGACATAAGCGTAGGGAACAAAACAGGC

LysGlyLeuSerGlyLysLeuAlaAsnGlyAlaPheGluGlyLysIleThrLeuAlaGln
AAAGGCCTGAGCGGCAAGCTGGCCAATGGCGCATTCGAGGGGAAAATAACTTTGGCCCAA

AspThrThrGlyValProValAspValTyrThrTyrAsnLeuMetAlaAlaValTyrSer
GACACGACCGGCGTACCCGTCGACGTCTATACATACAACCTCATGGCCGCAGTGTACAGC

Gln***
CAATAA
```

Figure 8.7

Hydrophilicity / hydrophobicity plot of 181 amino acid protein (putative NFA-2 subunit)

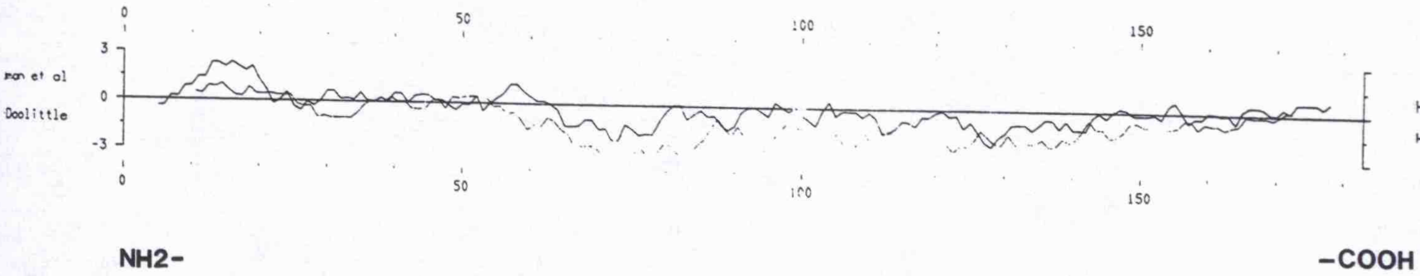


Figure 8.8

Predicted structure of 181 amino acid protein (putative NFA-2 subunit) using "Plot Structure" programme based on Chou Fasman prediction of secondary structure and hydrophobicity/hydrophilicity.

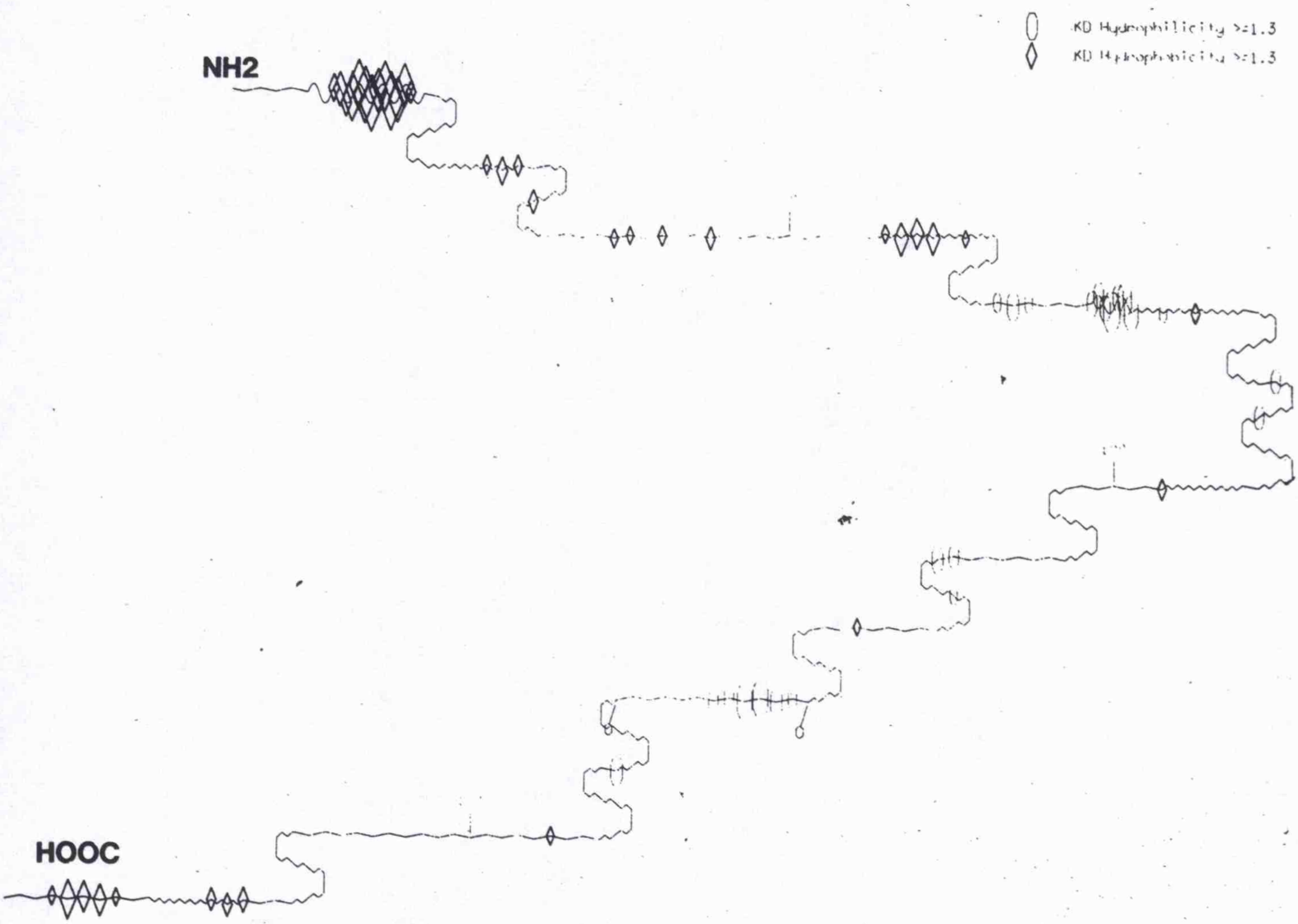


Figure 8.9

Nucleotide sequence comparison of putative NFA-2 subunit sequence with known NFA-1 subunit sequence. Respective start and stop codons are underlined. Comparison shows 79.58% nucleotide identity.

NFA-1 top row

NFA-2 bottom row

```
 156 CTGCGGGTACAGACATCTGTGAACGGTGGTTAATGTGGGGTAATAGACAG 205
     |||||||| ||||||||||||||||||||||||| ||||| |  ||||||
 450 CTGCGGGTGCAGACATCTGTGAACGGTGGTTAATTTGGGGA..AGACAG 497

 206 CTTACTGATTCTGGGATGGATTAACAGAACACAACTGGCTTGTCCATAAG 255
     |||||||||||||||||||||||||||||||||||||||||| |||||||
 498 CTTACTGATTCTGGGATGGATTAACAGAACACAACTGGCTTGCCCATAAG 547

 256 CAAAATGAAGGCAAAAAAATATGAAAATCAAATATACAATGAAAATGGCC 305
     || || |||||||||||||||||||||| |||||||||||||||||||| 
 548 CACAACGAAGGCAAAAAAATATGAAAATAAAATATACAATGAAAATGGCG 597

 306 GCCGTTGCCAGCGTCATGGTCGCCGG.CTAGCTATCGCGGATGCAAATGG 354
     || |||||||||||||||||||||||  | || | |||| |||  || | 
 598 GCAGTTGCCAGCGTCATGGTCGCCGGAATCGCGACCGCGAATGACAACGT 647

 355 GCTCAACACTGTGAACGCCGGGGATGGCAAGAATCTGGGCACCGCAACCG 404
      ||||||   |||   | ||  |||||||    ||| |||||||||||||
 648 ACTCAACGGGGTGGGGGGCGCTGATGGCATCCGTCTAGGCACCGCAACCG 697

 405 C......GACGATCACCACTCTGCAGAGCTGCTCTGTCGACCTGAATCTC 448
     |      |||||||||||   || | |||||| | ||  | ||||   ||
 698 CGAGCGGGACGATCACCAACATGGAAAGCTGCACAGTAAAGCTGACAATC 747

 449 GTTACCCCGAACGCGACAGTGAACAGAGCAGGAATGCTAGCAAACCGCGA 498
     | ||||||  |||| |   ||||| | ||||| |||| || |||||||||
 748 GCTACCCCTGACGCTAAGATGAACCGGGCAGGGATGCAAGAAAACCGCGA 797

 499 AATCACTAAATTTTCGGTGGGGAGTAAGGATTGCCCTAGCGACACCTATG 548
     |||||||||||||  ||| | ||| || |||||||||| |||||||||||
 798 AATCACTAAATTTAAGGTAGCGAGCAACGATTGCCCTACCGACACCTATG 847

 549 CTGTATGGTTTAAAGAGATCGATGGCGAAGGACAGGGGGTCGCGCAGGGC 598
     |||||||||||||||||||||||  || |||  | ||| |||| ||||||
 848 CTGTATGGTTTAAAGAGATCGATAACGTAGGCAACGGGATCGCACAGGGC 897

 599 ACTACGGTGACCAACAAGTTTTACCTTAAAATGACATCGGCCGACGGGAC 648
     |    |   || ||||  ||||||||    ||| ||||| |  |||| ||
 898 AAAGTGAATACAAACAGATTTTACCTACGGATGGCATCGACGAACGGTAC 947

 649 CGCGAGCGTAGGGGACATCAACATAGGAACCAAATCAGGCAAAGGCCTGA 698
      |  |||  |  |||||| | | |||| | |||| |||||||||||||||
 948 GGAAAGCCAAAAGGACATAAGCGTAGGGAACAAAACAGGCAAAGGCCTGA 997

 699 GTGGTCAACTGGTAGGGGGAAAATTCGACGGAAAAATAACGGTGGCATAT 748
     | ||  | ||||     ||   |||||| || ||||||||  ||||  | 
 998 GCGGCAAGCTGGCCAATGGCGCATTCGAGGGGAAAATAACTTTGGCCCAA 1047

 749 GACTCGGCCACCGCCCCCGCCGACGTTTATACATACGATATCATGGCCGC 798
     ||| || ||  ||  |||| |||||| ||||||||| |  ||||||||||
1048 GACACGACCGGCGTACCCGTCGACGTCTATACATACAACCTCATGGCCGC 1097

 799 CGTGTACGTCCAATAATGCTTACCCACATAACTAACACTGACCGCCGCCC 848
      ||||||  ||||||||||| ||   | || | ||||| |||||||||||
1098 AGTGTACAGCCAATAATGCTCACAACCGTACCGAACACCGACCGCCGCCC 1147

 849 GCCGG....TTTGCTTTCCTCGCGGGAATCGACCTCGT 882
     |||||     |||||| |||||||||||||||||||||
1148 GCCGGCGGTCTTGCTTACCTCGCGGGAATCGACCTCGT 1185
```

Figure 8.10

Amino acid sequence comparison of putative NFA-2 subunit sequence and known NFA-1 subunit sequence.

Arrows denotes position of postulated cleavage site for NFA-1 signal sequence and potential cleavage site for putative NFA-2 signal sequence.

Comparison shows 55.86% amino acid identity and 67.6% amino acid similarity.

NFA-1 top row
NFA-2 bottom row

```
             .         .         .         .         .
  1 MKAKKYENQIYNENGRRCQRHGRRLAIADANGLNTVNAGDGKNLGTATA.  49
     .  ||. .:  .  .:  ...  :|.|:.|.||.|.::|| .||||||
  1 .MKIKYTMKMAAVASVMVA....GIATANDNVLNGVGGADGIRLGTATAS  45

             .         .         .         .         .
 50 .TITTLQSCSVDLNLVTPNATVNRAGMLANREITKFSVGSKDCPSDTYAV  98
     |||.::||.|.|.:.||:|.:||||| .|||||||.|:|.|||.|||||
 46 GTITNMESCTVKLTIATPDAKMNRAGMQENREITKFKVASNDCPTDTYAV  95

             .         .         .         .         .
 99 WFKEIDGEGQGVAQGTTVTNKFYLKMTSADGTASVGDINIGTKSGKGLSG 148
    ||||||. |.|:|||.. ||:|||:|.|.:||.|  ||.:|.|.||||||
 96 WFKEIDNVGNGIAQGKVNTNRFYLRMASTNGTESQKDISVGNKTGKGLSG 145

             .         .         .
149 QLVGGKFDGKITVAYDSATAPADVYTYDIMAAVYVQ. 184
    .|..| |:||||:| |....|.|||||::||||| |
146 KLANGAFEGKITLAQDTTGVPVDVYTYNLMAAVYSQ* 182
```

Figure 8.11

Line-up comparison of nucleotide sequences of NFA-1, NFA-2, and members of the Dr adhesin family (afaE, draA and F1845).

a) line up of nucleotide sequences

b) line up of nucleotide sequences showing high degree of identity in sequences preceding the adhesin subunit genes (respective start codons underlined)

a)

	1				
Nfa.1	TCTGAACGGT	GGTTAAT. GT	GGGCTAATAG	ACAGCTTACT	GATTCTGGGA
Nfa.2	TGTGAACGGT	GGTTAAT. TT	GGGCGAA. . G	ACAGCTTACT	GATTCTGGGA
Afa.e	TGTGAACGGT	GGTTAATGTT	GGGGTAA. . G	ACAGCTTACT	GATTCTGGGA
Dra.a	TCTGAACGGT	GGTTAATG. T	GGGGTAA. . G	ACAGCTTACT	GATTCTGGGA
F1845	TGTGAACGTT	GGTTAATG. T	GGGCTAA. . G	ACAGCTTACT	GATTCTGGGA

	51				
Nfa.1	TCGATTAACA	GAACACAAC.	. TGGCTTGTC	CATAAGCAAA	ATGAAGGCAA
Nfa.2	TGGATTAACA	GAACACAAC.	. TGGCTTGCC	CATAAGCACA	ACGAAGGCAA
Afa.e	TGAATT. . . A	GACCGTACTG	TTGC. TTACC	CCCTCACAAA	ACTGAATAGG
Dra.a	TGAATT. . . A	GACCGTACTG	TTGTGTTACC	CCCTCACAAA	ACTGAACAGG
F1845	TCAATT. . . A	GACCGTACTG	TTGTGTTACC	CCCTCACAAA	ACTGAACAGG

	101				
Nfa.1	AAAA.AATAT	GAAAATCAAA	TATACAATGA	AAATGGCCGC	CGTTGCCAGT
Nfa.2	AAA. .AATAT	GAAAATAAAA	TATACAATGA	AAATGGCGGC	AGTTGCCAGG
Afa.e	TAATCCATAT	GAAAAAATTA	GCGATCATAG	GCGCAACCAG	CGTAATGATG
Dra.a	TAATCAATAT	GAAAAAATTA	GCGATCATGG	CCGCGGCCAG	CATGGTGTTT
F1845	TAATCAATAT	GAAAAAATTA	GCGATAATGG	CCGCCGCCAG	CATGATTTTG

b)

	1				
Nfa.1	TCTGAACGGT	GGTTAAT. GT	GGGCTAATAG	ACAGCTTACT	GATTCTGGGA
Nfa.2	TGTGAACGGT	GGTTAAT. TT	GGGCGAA. . G	ACAGCTTACT	GATTCTGGGA
Afa.e	TGTGAACGGT	GGTTAATGTT	GGGGTAA. . G	ACAGCTTACT	GATTCTGGGA
Dra.a	TCTGAACGGT	GGTTAATG. T	GGGGTAA. . G	ACAGCTTACT	GATTCTGGGA
F1845	TGTGAACGTT	GGTTAATG. T	GGGCTAA. . G	ACAGCTTACT	GATTCTGGGA

	51				
Nfa.1	TCGATTAACA	GAACACAAC.	. TGGCTTGTC	CATAAGCAAA	<u>ATG</u>AAGGCAA
Nfa.2	TGGATTAACA	GAACACAAC.	. TGGCTTGCC	CATAAGCACA	ACGAAGGCAA
Afa.e	TGAATT. . . A	GACCGTACTG	TTGC. TTACC	CCCTCACAAA	ACTGAATAGG
Dra.a	TGAATT. . . A	GACCGTACTG	TTGTGTTACC	CCCTCACAAA	ACTGAACAGG
F1845	TCAATT. . . A	GACCGTACTG	TTGTGTTACC	CCCTCACAAA	ACTGAACAGG

	101				
Nfa.1	AAAA.AATAT	GAAAATCAAA	TATACAATGA	AAATGGCCGC	CGTTGCCAGT
Nfa.2	AAA. .AAT<u>AT</u>	<u>G</u>AAAATAAAA	TATACAATGA	AAATGGCGGC	AGTTGCCAGG
Afa.e	TAATCCAT<u>AT</u>	<u>G</u>AAAAAATTA	GCGATCATAG	GCGCAACCAG	CGTAATGATG
Dra.a	TAATCAAT<u>AT</u>	<u>G</u>AAAAAATTA	GCGATCATGG	CCGCGGCCAG	CATGGTGTTT
F1845	TAATCAAT<u>AT</u>	<u>G</u>AAAAAATTA	GCGATAATGG	CCGCCGCCAG	CATGATTTTG

Figure 8.12

Binding of NFA-4 subunit probe to restriction enzyme fragments of pNFA4-2-302.

Probe used was a pool of radiolabelled oligonucleotides corresponding to the known N-terminal sequence of the NFA-4 adhesin subunit gene. Arrows denote deduced location of oligonucleotide binding sites.

Lanes
1 *Eco*R1 digest
2 *Sma*1
3 *Pvu*II
5 *Kpn*1 and *Cla*1
6 *Bam*H1 and *Hin*dIII
7 *Eco*R1 and *Pvu*II

Key
A *Acc*1
B *Bam*HI
C *Cla* 1
E *Eco*R1
H *Hin*dIII
K *Kpn*1
P *Pvu* II
Ps *Pst* I
Sm *Sma*1

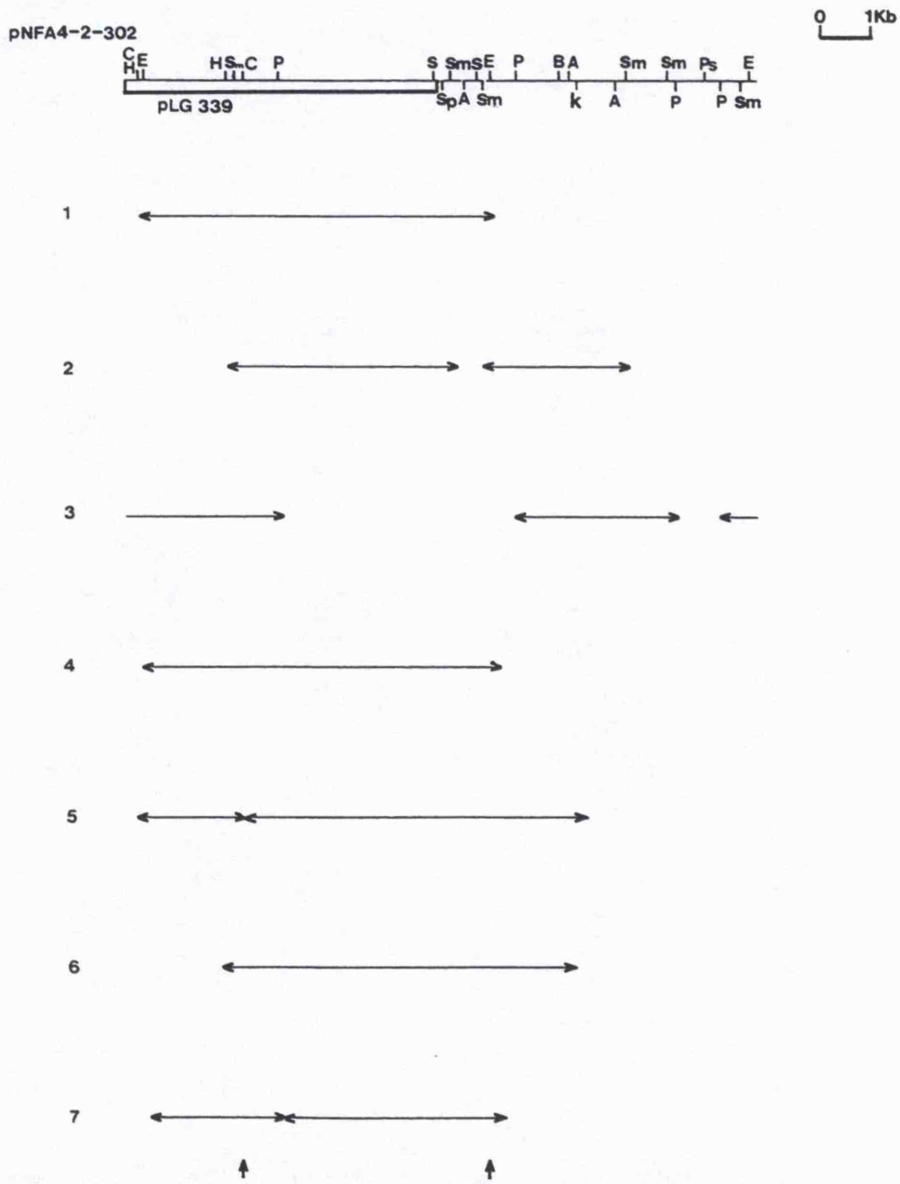


Figure 8.13

Sequencing strategy for pNFA4-2-302

Key	A	*Acc*1	K	*Kpn*1
	B	*Bam*HI	P	*Pvu* II
	C	*Cla* 1	Ps	*Pst* I
	E	*Eco*R1	Sm	*Sma*1
	H	*Hind*III		

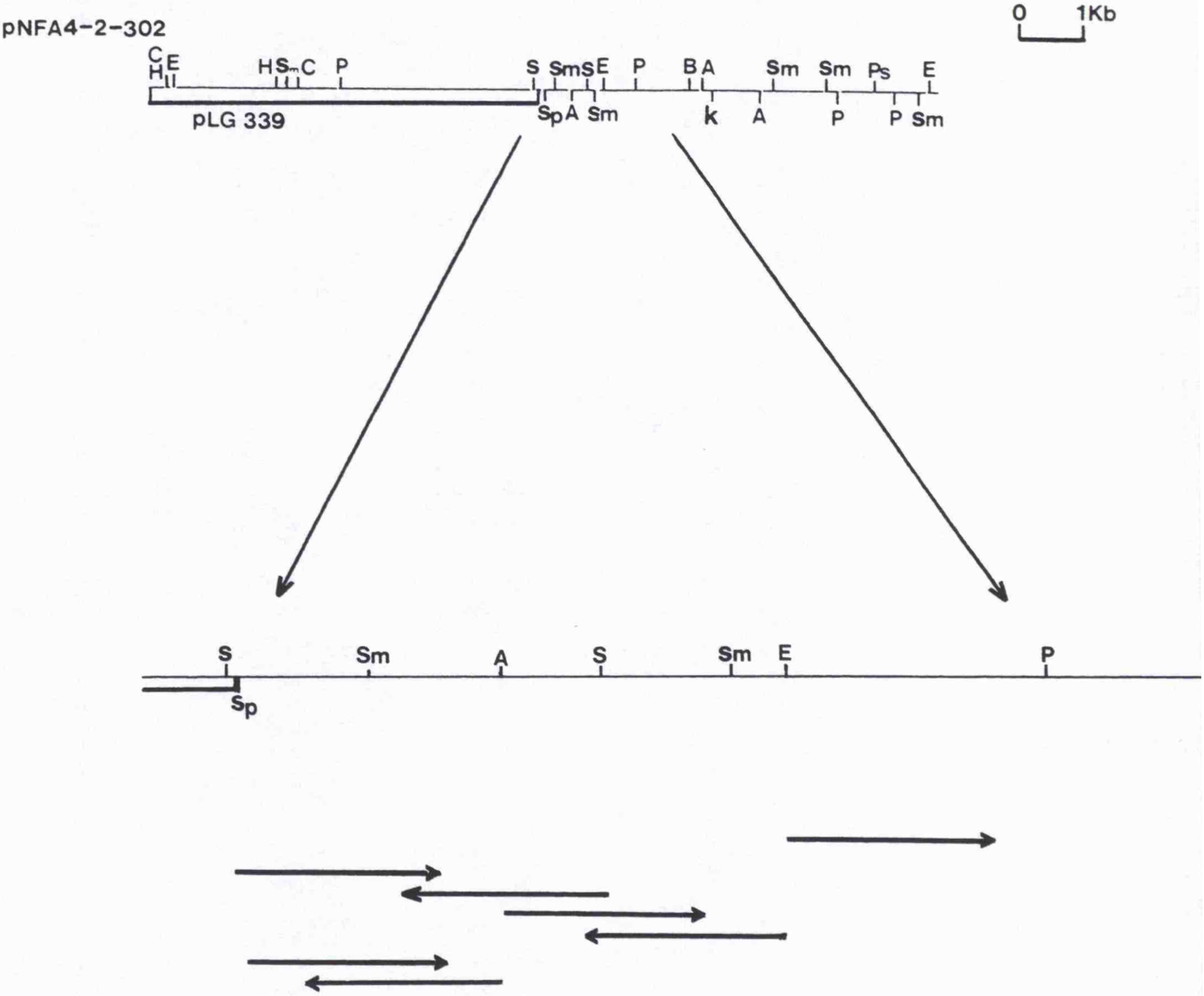


Figure 8.14

Comparison of nucleotide sequence derived from pNFA4-2-302 and the M-adhesin subunit cloned by Rhen *et al* (1988)

Comparison shows 96.8% nucleotide identity

NFA-4 top row

M adhesin bottom row

(*Bma*E)

```
              .         .         .         .         .
718 CAGGGCCAACGAGTACAGATGCAAAAGATGGTGAGGTTTGGGGGCACCTT 767
    | |||||||||| |||||||||||||||||||||||||||||||||||||
164 CTGGGCCAACGACTACAGATGCAAAAGATGGTGAGGTTTGGGGGCACCTT 213
              .         .         .         .         .
768 GATATGACTCAAACCAGGGGAACACCAACATTCGGAAAACCTCCTCAATC 817
    ||||||||||||||||||||||||||||||||||||||| |||| |||||
214 GATATGACTCAAACCAGGGGAACACCAACATTCGGAAAA.CTCCGCAATC 262
              .         .         .         .         .
818 CTCAAGGAGAGACTTCGCCAGGACCGTTGAAGGCGCCATTCAGTTTTACC 867
    |||||||||||  |||||||||||||||||||||||||||||||||||||
263 CTCAAGGAGAGCATTCGCCAGGACCGTTGAAGGCGCCATTCAGTTTTACC 312
              .         .         .         .         .
868 GGGCCAGATGGTCATACTGCAAGAGCGTACCTTAGTTCATACGGCGCACC 917
    |||||||||||||||||||||||||||||||||  |||||||||||||||
313 GGGCCAGATGGTCATACTGCAAGAGCGTACCTTGATTCATACGGCGCACC 362
              .         .         .         .         .
918 GATTCACAACTACGCAGGGGATAACCTTGCTAATGGGGTGAAGGTAGGTA 967
    ||||||||||||||||||||||||||||||||||||||||||||||||||
363 GATTCACAACTACGCAGGGGATAACCTTGCTAATGGGGTGAAGGTAGGTA 412
              .         .         .
968 GTGGAAGCGGAAAACGATCCATTTGTTGTTGGG 1000
    |||||||||| ||||  ||||||||||||||||
413 GTGGAAGCGG.AAACACTCCATTTGTTGTTGGG 444
```

CHAPTER 9

CLINICAL IMPORTANCE OF NON-FIMBRIAL ADHESINS

9.1 Introduction

NFA-1 to NFA-4 were each identified on clinical isolates of *E. coli* obtained from patients with significant urinary tract infections. It has been suggested that aproximately 10% of clinical isolates of *E. coli* obtained from patients with urinary tract infections possess non-fimbrial adhesins (Duguid *et al* 1979). However, the role of non-fimbrial adhesins in the development of urinary tract infections and their importance as bacterial virulence factors has not been investigated.

The cloning of the genes encoding NFA-1, NFA-2, NFA-3 and NFA-4 (M-adhesin) allowed the cloned DNA sequences to be used as probes to investigate clinical isolates for the presence of homologous sequences. Isolates of *E. coli* were obtained from clinical specimens sent to the Leicestershire Public Health Laboratory. Isolates were characterised as "upper urinary tract", causing invasive infection with pyelonephritis or septicaemia, "lower urinary tract" causing cystitis and "colonisers", likely to be representative of faecal isolates. The presence of nucleotide sequences that were homologous to non-fimbrial sequences was investigated in each group. The collection of isolates from the Public Health Laboratory (PHL) also yielded important information on the epidemiology of urinary tract infections in Leicestershire.

9.2 Collection of specimens and identification of isolates

Specimens were collected from two sources; clinical specimens sent to the PHL by hospital doctors and general practitioners and septicaemic isolates previously identified and stored in the Leicester PHL. Urine specimens received by the Microbiology Department were routinely cultured aerobically on cysteine lactose electrolyte deficient (CLED) media and significant isolates were tested for antibiotic sensitivities using standard antibiotic discs. Extended sensitivity testing was performed for septicaemic isolates.

Routine clinical isolates causing clinically significant urinary tract infections

All clinically significant urinary coliform isolates sent to the Public Health Laboratory were documented over a four day period. Clinical information on the microbiology request form was recorded for each isolate and specimens with inadequate information were not included in the analysis. Significant infections were considered to be those associated with a heavy growth ($>10^5$colonies/ml) of a single organism and microscopy showing at least 100 white cells/µl. Isolates of *E. coli* were distinguished from other coliforms using the "api 20e" kit (Bio Merieux SA, France). Antibiotic sensitivities of each organism were determined in the PHL. Routine analysis of urinary isolates included sensitivites for ampicillin, trimethoprim, cephradine, sulphonamide, nitrofurantoin, nalidixic acid and gentamicin. Resistant and septicaemic isolates were also tested for sensitivity to cefuroxime and piperacillin.

The clinical manifestations of infection associated with significant isolates were recorded as upper or lower urinary tract infection (UTI), defined as:-

Upper urinary tract infection -	clinical symptoms suggesting pyelonephritis, ie fever, rigors, loin pain
Lower urinary tract infection-	symptoms of cystitis ie frequency, dysuria, or offensive urine; without evidence of significant fever or systemic upset

111 clinically significant isolates of *E. coli* were identified during the four day period of collection. 104 (94%) were associated with lower urinary tract infections and 7 (6%) were associated with upper urinary tract infections.

Non-significant colonising isolates of E. coli

Isolates of *E. coli* which were considered to be colonisers and not clinically significant were collected and included for comparison with the isolates causing urinary tract infections. Colonising isolates were scanty growths of *E. coli* (sometimes as part of a mixed bacterial flora) from patients who had no definite symptoms of urinary tract infection and did not have significant leucocytosis on urine microscopy. 73 colonising isolates were investigated.

Septicaemic isolates

A total of 38 septicaemic isolates of *E. coli* were kindly provided by Dr Mike Prentice, Senior Registrar in Microbiology. These isolates had previously been identified and characterised and were stored at -70°C on glycerol beads. All isolates were obtained from patients with septicaemia following urinary tract infection and brief clinical information was available for each.

9.3 Epidemiology of urinary tract infections in Leicestershire

Incidence of proven urinary tract infection

A total of 111 significant clinical isolates of *E. coli* were identified from specimens sent to the Leistershire PHL over a four day period. This corresponds to an annual total of approximately 10,000 infections. The population served by the Leicstershire PHL is around 900,000; therefore the annual incidence of proven urinary tract infections is approximately 1100 / 100 000 population. Any individual has an approximately 1 in 90 chance of developing a proven *E. coli* urinary tract infection each year and is therefore likely to have, on average, one proven UTI in his or her lifetime.

72 (65%) of the 111 significant isolates of *E. coli* were identified in specimens sent by general practitioners and 35 (32%) were identified in specimens obtained from hospitalised patients. The origin of the remaining 4 isolates was not stated.

Financial implications of E.coli urinary tract infections

Leicestershire hospitals provide approximately 2000 in-patient beds and the overall mean duration of hospital stay is approximately 4 days. 35 (1.75%) of 2000 hospitalised patients were diagnosed as having an *E. coli* UTI during their admission over the four day period of the study (mean of 8.75 patients/day). If these figures are representative the annual number of hospitalised patients with UTI's is 3194 (8.75 x 365).

The cost of urinary tract infections in Leicestershire is not known but will include the cost of investigation and treatment and any additional days of admission caused by the infection. The hotel costs associated with each bed-day in the Leicester Royal Infirmary are approximately £150. If each urinary tract infection was responsible for the patient requiring an additional two days of hospital admission the associated annual hotel costs would be £479,100.

72 isolates of *E. coli* were sent by general practitioners during the four day period of the study representing an annual total of 6570 infections. The cost of these infections include the cost of antibiotics and the cost of lost earnings or schooling as a result of illness caused by the UTI. A 5 day course of trimethoprim 200mg twice daily costs approximately £4 therefore the annual therapeutic cost of proven *E. coli* UTI's is approximately 6570 x £4 ie. £26,280.

The cost of performing microscopy and culture must also be considered. Approximately 30,000 urinalysis tests are performed annually by the Leicestershire PHL at a cost of £5 each, giving a total cost of £150 000.

Age and sex distribution of urinary tract infections

90 (81%) of 111 significant *E. coli* infections were diagnosed in females and 21 (19%) in males. The age and sex distribution of urinary tract infections is shown in Figure 9.1. The mean age of the patients with significant UTI's was 47.9 years. The mean age of males with UTI was 40.9 years compared to 49.6 years for females. The figures show a male predominance in urinary tract infections in infancy (age<1), with a peak in the incidence of UTI's in the age groups 11-20 and 21-30 when the majority of infections occur in women. The incidence of urinary tract infections rises in later life in both sexes with a second peak at age 71-80.

Predisposing factors for urinary tract infection

Predisposing factors were documented in 21 (19%) of the 111 significant urinary tract infections (Table 9.1). 18 (20%) of 90 females with UTI had predisposing factors compared to 3 (14%) of 21 males. 2 (50%) of the 4 infected males aged under 1 had predisposing factors recorded.

Antibiotic sensitivities

51 (46%) of the 111 significant *E. coli* isolates were resistant to at least one antibiotic. 27 (24%) were resistant to one antibiotic only and 24 (22%) showed multiple antibiotic resistances. The levels of resistance to individual antibiotics tested is shown in Figure 9.2.

9.4 Epidemiology of septicaemic isolates

Age and sex distribution

19 (50%) of the 38 septicaemic isolates were from male patients. The age and sex distribution of septicaemic infections is shown in Figure 9.3. The mean age of the patients with septicaemia was 56 years. The mean age of males with

septicaemia was 53 years compared to 60 years for females. The figures again show a male predominance in septicaemic infections in infancy (age<1), but the main peak in the incidence of septicaemic infections occurs in the 61-70 and 71-80 age groups, affecting both males and females.

Predisposing factors for septicaemic infection

Predisposing factors were documented in 17 (45%) of the 38 cases of septicaemia. 11 (58%) of 19 females with septicaemia had predisposing factors compared to 6 (32%) of 19 males. The documented predisposing factors are shown in Table 9.2.

Antibiotic sensitivities

21 (55%) of the 38 septicaemic *E. coli* isolates were resistant to at least one antibiotic. 11 (29%) were resistant to one antibiotic only and 10 (26%) showed multiple antibiotic resistances. The levels of resistance to individual antibiotics tested is shown in Figure 9.4.

A comparison of the characteristics of urinary and septicaemic *E. coli* isolates is shown in Table 9.3. 19 (50%) of 38 septicaemic isolates were obtained from males compared to only 21 (19%) of 11 isolates from infections confined to the urinary tract (X^2=7.21, p<0.01). Septicaemic isolates were more likely to be associated with the presence of underlying risk factors (17/38 vs 21/111, X^2=5.4, p<0.05).

9.5 Presence of non-fimbrial adhesin gene sequences in clinical isolates

Classification of clinical isolates

222 clinical isolates of *E. coli* were probed with non-fimbrial adhesin sequences. 45 (20%) were classified as upper urinary tract or septicaemic isolates, 104 (47%) as lower urinary tract infection and 73 (33%) as colonisers.

Selection of probes

Probes selected were those that had been used for the genetic comparison of cloned non-fimbrial adhesins (Figure 7.2). The NFA-1 probe was a 10kb fragment generated by digesting pS3 with *Eco*R1; the NFA-2 probe was a 7.5kb fragment generated by digesting pNFA2-102 with *Cla*1 and *Sal*1; the NFA-3 probe was a 5.5kb fragment generated by digesting pNFA3-103 with *Hin*dIII and *Sph*1 and the NFA-4 (M-adhesin) probe was a 5.5kb fragment generated by digesting pNFA4-2-301 with *Pst*1.

Fragments of probe DNA were separated by agarose gel electrophoresis, using 1% low melting point agarose, and were labelled with [^{32}P]dCTP by the hexadeoxynucleotide primer method.

Colony blotting studies

Clinical isolates of *E. coli* were streaked onto L-agar plates overlaid with hybridisation membrane. Membranes were marked out in a grid pattern allowing 50 colonies to be streaked onto each plate. Each isolate was streaked onto four identical plates which were subsequently hybridised with the NFA-1, NFA-2, NFA-3 and NFA-4 probes respectively. Each plate included positive controls (colonies expressing NFA-1, NFA-2, NFA-3 and NFA-4) and negative controls (strains LE392 and JM101).

Following overnight incubation at 37°C colonies grown on the hybridisation membrane were lysed, neutralised and fixed by exposure to UV light. Membranes were prehybridised, hybridised with the NFA probe and washed. Positive colonies were visualised by autoradiography. Figure 9.5 shows the colony blotting technique.

The results of the colony blotting experiments are shown in Table 9.4. All probes hybridised with their positive controls. NFA-1 and NFA-2 probes showed cross-reactivity as expected, but other probes were specific and no probes reacted with the negative controls (strains LE392 and JM101).

The NFA-1 probe reacted with 36% of colonising organisms and 25% of isolates causing lower urinary tract infections. NFA-1 sequences were only present in 9% of organisms causing upper urinary tract infections or septicaemia. The distribution of NFA-2 sequences was similar and the results show that these adhesins might facilitate the development of lower urinary tract infections but are unlikely to play a major role in invasive infection.

NFA-3 sequences were encountered in only 1% of the clinical isolates tested and this adhesin is therefore unlikely to be of any great clinical significance. The NFA-4 (M-adhesin) probe reacted with 14% of colonising organisms and 14% of those causing lower urinary tract infection, but only hybridised with 2% of isolates causing upper urinary tract infection or septicaemia. This adhesin is therefore unlikely to be an important factor in the development of invasive urinary tract infection.

Table 9.1

Documented predisposing factors in 21 patients with significant *E. coli* urinary tract infection (two patients had more than one predisposing factor).

Predisposing factor	Number of cases
Hysterectomy	6
Renal / ureteric abnormalities	5
Catheterisation	3
Underlying malignancy	3
Gynaecological procedure	3
Pregnancy	2
Recto-vaginal fistula	1
Total	23

Table 9.2

Documented predisposing factors in 17 patients with *E. coli* septicaemia.

Predisposing factor	Number of cases
Stroke	3
Underlying malignancy	3
Liver disease / alcohol	3
Catheterisation	2
Neutropaenia	1
Central line	1
Transurethral resection of prostate	1
Diabetes	1
Renal transplant	1
Pregnancy	1
Total	17

Table 9.3

Comparison of the characteristics of urinary and septicaemic *E. coli* isolates (NS = no significant difference between proportions)

Characteristic	Urinary isolate	Septicaemic isolate	Significance
% male	19%	50%	$p<0.01$
Mean age (years)	47.9	56	NS
Predisposing factors	19%	45%	$P<0.05$
Antibiotic resistance	46%	55%	NS

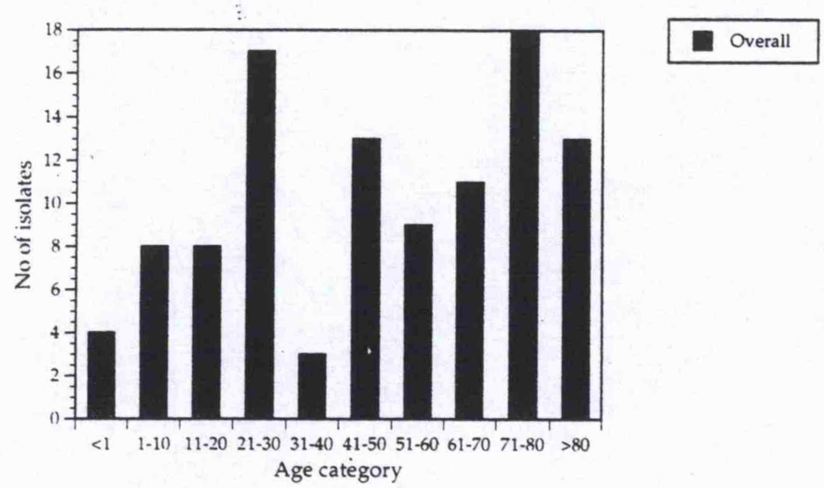
Table 9.4
Results of colony blotting experiments using probes derived from cloned non-fimbrial adhesin genes

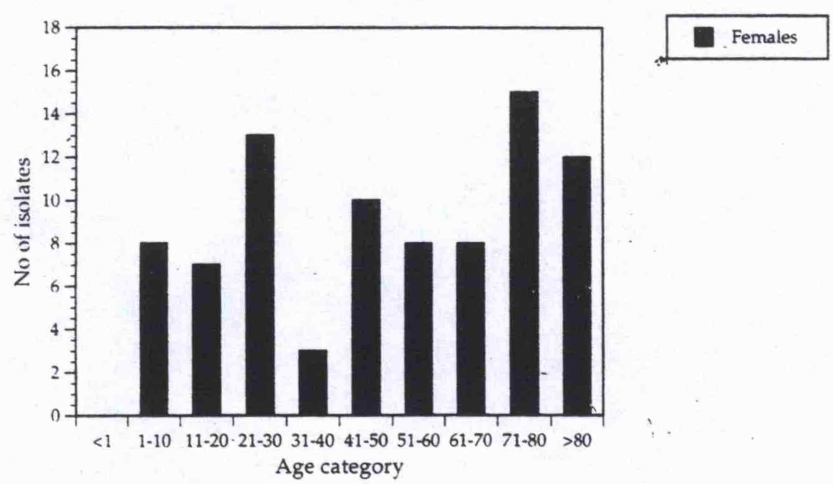
Clinical manifestation	**Total**	NFA-1 No(%)	NFA-2 No(%)	NFA-3 No(%)	NFA-4 No(%)
		Probe hybridisation			
Upper UTI / septicaemia	**45**	4 *(9%)*	4 *(9%)*	0	1 *(2%)*
Lower UTI	**104**	21 *(25%)*	23 *(22%)*	1 *(1%)*	15 *(14%)*
Colonising organisms	**73**	26 *(36%)*	25 *(34%)*	2 *(3%)*	10 *(14%)*
Total	**222**	51 *(23%)*	52 *(23%)*	3 *(1%)*	26 *(12%)*
Controls	NFA-1	+	+	-	-
	NFA-2	+	+	-	-
	NFA-3	-	-	+	-
	NFA-4	-	-	-	+
	LE 392	-	-	-	-
	JM 101	-	-	-	-

Figure 9.1

Age and sex distribution of significant *E. coli* urinary isolates obtained from the Leicestershire Public Health Laboratory over a four day period.

Age and sex information available for 104 of 111 isolates; 20 males and 84 females





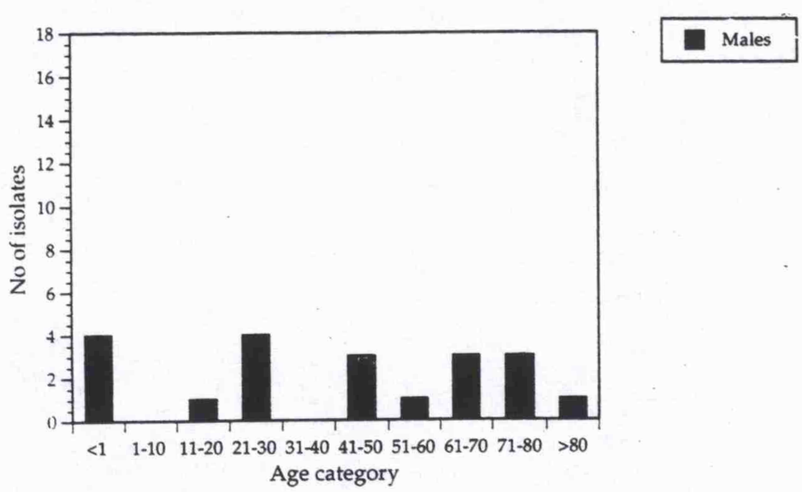


Figure 9.2

Proportion of significant *E. coli* urinary isolates which were resistant to individual antibiotics tested

Key	trim	trimethoprim
	amp	ampicillin
	ceph	cephradine
	nitro	nitrofurantoin
	nal	nalidixic acid

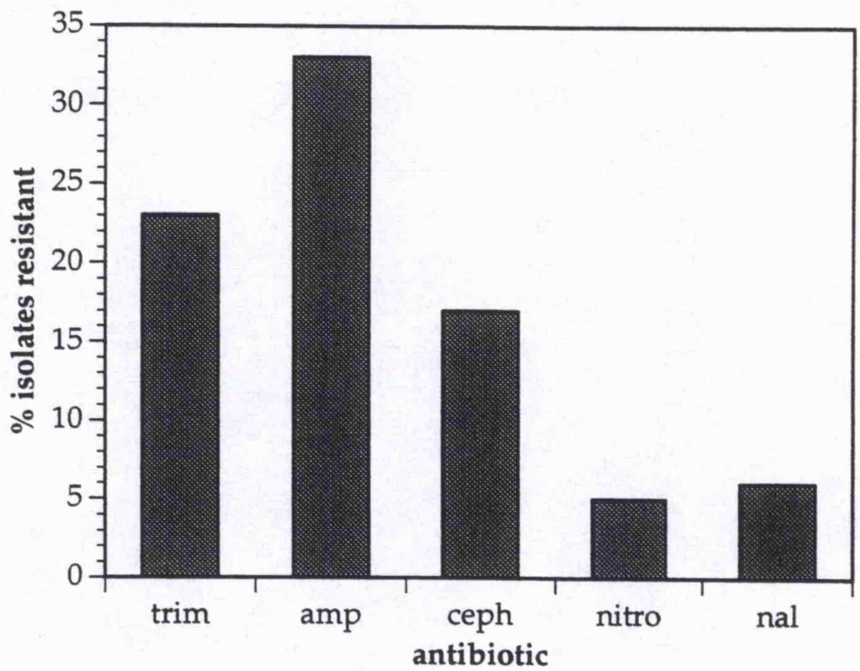


Figure 9.3

Age and sex distribution of septicaemic *E. coli* isolates.

Age and sex information available for 31 of 38 septicaemic isolates; 14 female and 17 male

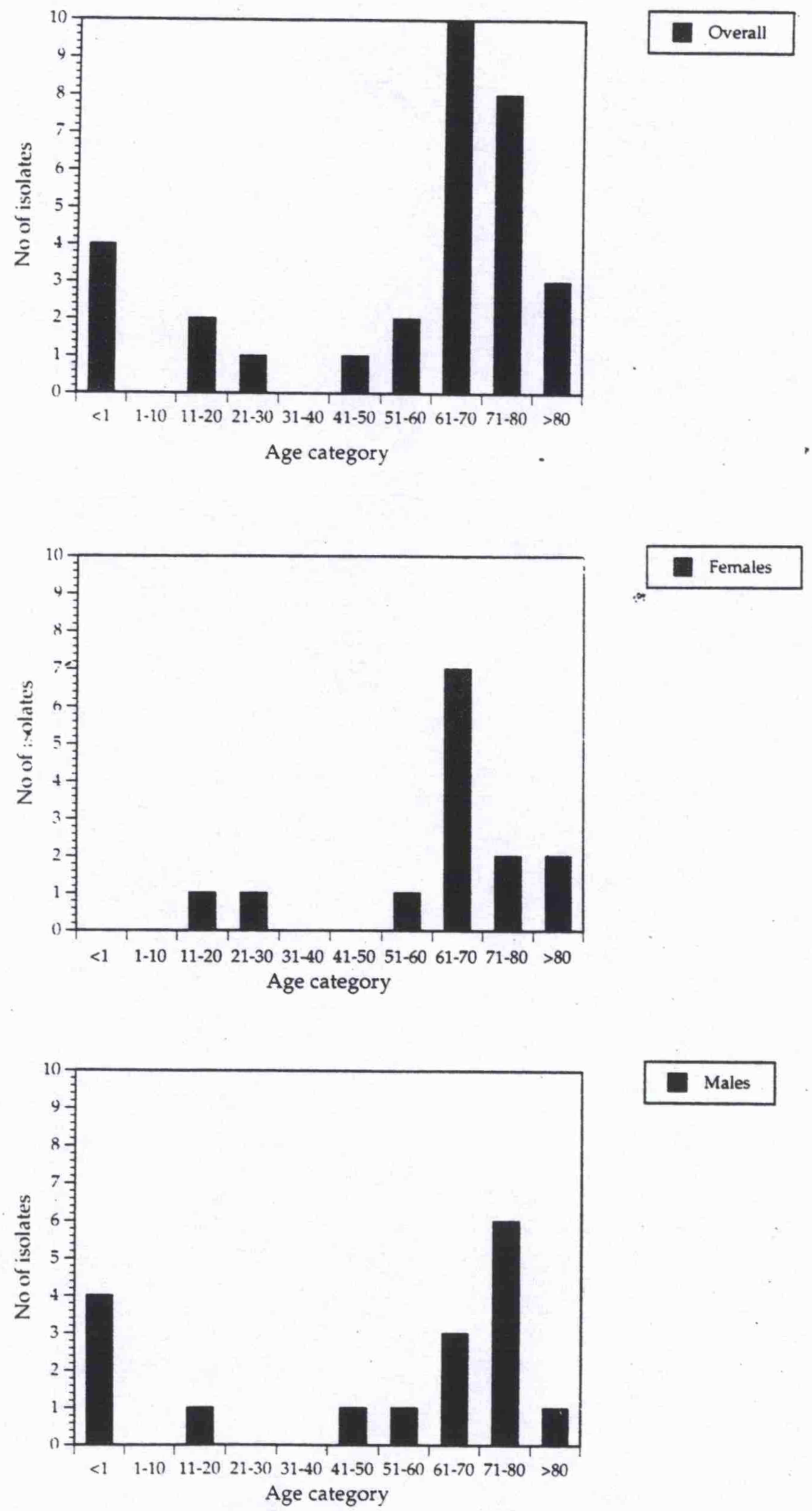


Figure 9.4

Proportion of septicaemic *E. coli* isolates which were resistant to individual antibiotics tested

Key	amp	ampicillin
	sul	sulphonamide
	ceph	cephradine
	trim	trimethoprim
	pip	piperacillin
	cef	cefuroxime

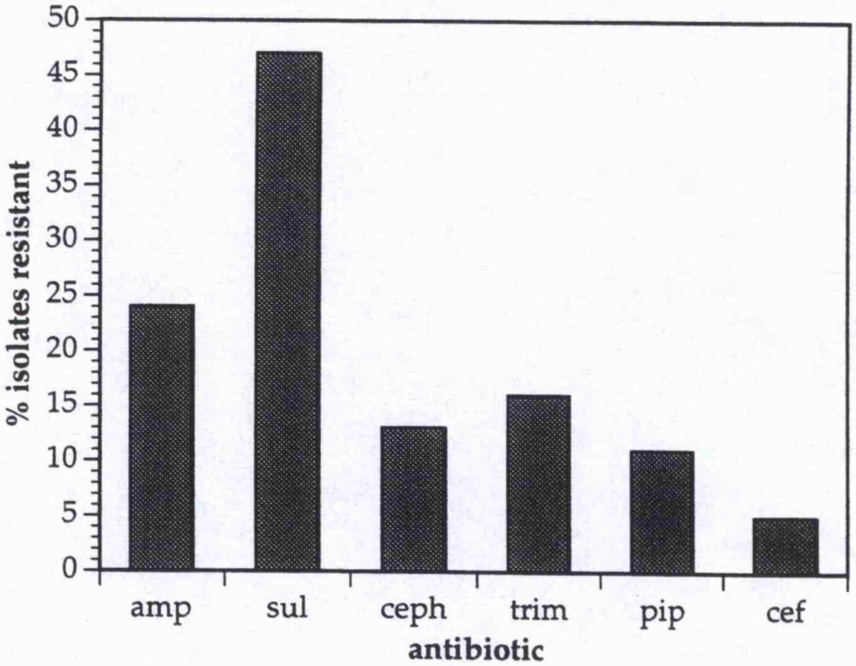


Figure 9.5

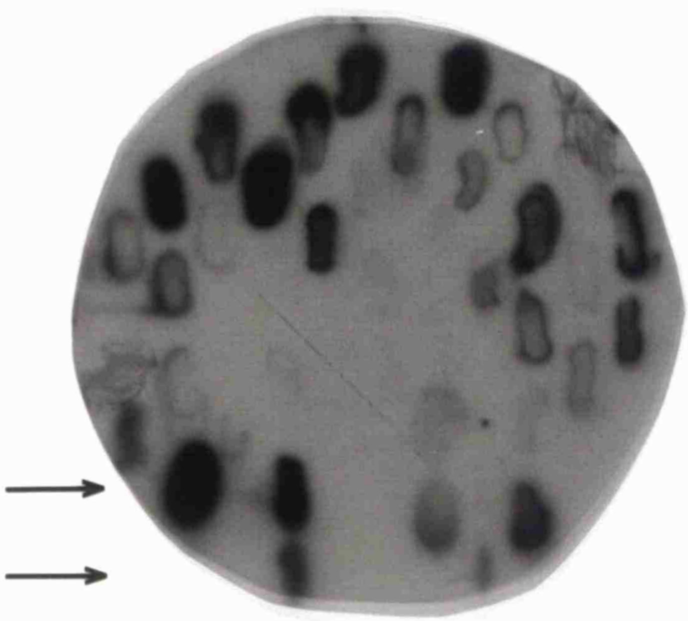
a) Example of colony blotting experiment using NFA-1 probe.

Arrows denote controls:-

upper row from left to right; NFA-1, NFA-2, NFA-3, NFA-4

lower row LE392, JM101

NFA-1 probe hybridises strongly to NFA-1 and NFA-2 controls and three of the clinical isolates



b) Colony blotting experiment using NFA-4 probe

NFA-4 probe hybridises strongly to NFA-4 control but does not hybridise with any of the clinical isolates

CHAPTER 10

DISCUSSION

Infections of the urinary tract are frequently encountered in general practice and hospital medicine. The development of urinary tract infection involves a complex interaction between pathogen and host. The microbiology of urinary tract infections has been well documented, however the mechanisms of host resistance and the role of local immunity are still relatively poorly understood.

Recent advances in molecular biology have led to an enormous increase in our understanding of bacterial virulence factors and the pathogenesis of disease. The possible benefits of this research include the development of novel strategies for the prevention or treatment of urinary tract infections. Experimental studies have already investigated the possibility of vaccination with purified adhesin (Svanborg-Eden *et al* 1982, O'Hanley *et al* 1985a, Guerina *et al* 1989) or conjugated capsular polysaccharide (Kaijser *et al* 1983). Other approaches include the use of soluble receptor analogues to prevent bacterial adhesion (Aronson 1979, Cox and Taylor 1990), and vaginal flushing with lactobacilli to inhibit growth of enteric organisms (Bruce and Reid 1988). Unfortunately uropathogenic bacteria possess a great diversity of virulence factors and no single strategy is likely to prevent all urinary tract infections.

Bacterial adhesion is believed to be one of the most significant virulence factors in the urinary tract and is an obvious target for future therapeutic approaches to prevent infection. Early studies identified specific organelles known as fimbriae or pili which project from the bacterial surface and mediate adhesion to host cell receptors. Another group of adhesins are located more diffusely around bacteria and are known as non-fimbrial adhesins. A bewildering range of different adhesins have now been described. This diversity presumably allows bacteria to colonise a range of host cells and helps them to evade the immune system. Most bacteria responsible for urinary tract infections are found as normal commensals in the gastrointestinal tract and must be equipped to survive in two very different environments.

Over the past decade we have gained an understanding of the molecular properties of *E. coli* adhesins. A series of studies have helped to unravel the mechanisms underlying adhesin construction and regulation of expression. Bacteria often possess multiple adhesins and expression of each is controlled by environmental conditions, a process known as phase-variation. Adhesins and other virulence factors have been found to be linked in blocks of genetic information known as pathogenicity islands (pais). The mechanisms underlying the co-ordinated expression of virulence determinants are not fully understood, but interference of gene regulation may be a future therapeutic strategy.

Molecular studies have shown that the construction of *E. coli* adhesins is a highly ordered process. Fimbrial adhesin gene complexes consist of regulatory genes followed by the major subunit gene, transport and assembly genes and the minor subunit genes. In most fimbriae the adhesin subunit is distinct from the major subunit protein responsible for the bulk of the fimbrial structure. Nucleotide sequence comparison has revealed areas of homology in the gene complexes coding for the expression of unrelated adhesins. This information allows us to explore the evolutionary linkage between adhesins. The areas of sequence homology are potential targets for further therapies.

Non-fimbrial adhesins have been recognised comparatively recently in *Escherichia coli* and our knowledge of these adhesins is incomplete. Particularly important questions include the molecular relationship between fimbrial and non-fimbrial adhesins and their clinical role in the aetiology of urinary tract infections.

This thesis describes a detailed molecular analysis of four non-fimbrial adhesins (NFA-1 to NFA-4) and explores their relationship with other published adhesins.

NFA-1 was first described on a uropathogenic isolate of *Escherichia coli* (strain 827). Sequences coding for the expression of NFA-1 were cloned using a cosmid cloning technique and a plasmid subclone (pS2) was generated comprising a 15.5kb *Eco*R1-*Bam*H1 fragment cloned into pLG339. The location and size of the NFA-1 gene cluster was investigated by subclone analysis and Tn1000 mutagenesis. The results showed that approximately 6.5kb of DNA was required for NFA-1 expression. The adhesin subunit gene was located by investigating the hybridisation of oligonucleotide probes based on the known N-terminal amino acid sequence. Using this information a further subclone (pS3) was generated by cloning a 9.65kb fragment into pUC19. The adhesin subunit gene (nfaA) encoded by pS3 was recently sequenced by Ahrens *et al* (1993) and found to encode an open reading frame of 184 amino acids. A 16 amino acid signal sequence is cleaved to generate the mature protein consisting of 156 amino acids.

NFA-2 was also identified on a uropathogenic isolate of *Escherichia coli* (strain 54). The NFA-2 gene cluster was cloned using a cosmid vector. Recombinants expressing NFA-2 were MRHA positive and were agglutinated by anti-NFA-2 antibodies. Two MRHA-positive recombinant subclones were generated, LE392 (pNFA2-101) and LE392 (pNFA2-102). pNFA2-101 and pNFA2-102 had inserts cloned in opposite orientations and shared a 7.8kb stretch of inserted DNA. SDS-PAGE and immunoblotting experiments showed that strain 54, LE392 (pNFA2-101) and LE392 (pNFA2-102) each expressed a protein of approximately 19kDa which was recognised by anti-NFA-2 monoclonal antibodies. Minicell analysis showed the gene complex responsible for NFA-2 expression coded for at least five products of approximate molecular weight 80kDa, 33kDa, 28kDa, 19kDa and 17kDa.

The NFA-2 adhesin subunit gene was localised and a 2035 base pair stretch of pNFA2-102 was sequenced. Several putative open reading frames were identified, including a 546 base open reading frame which translated into a 181 amino-acid polypeptide of MWt 19kDa which corresponded to the NFA-2 adhesin subunit. Analysis of the subunit gene sequence showed that the first 23 amino acids were hydrophobic and could represent a signal sequence, although there is no clear evidence that this sequence is cleaved from the subunit protein. The subunit protein contains two cysteine residues and a relatively high number of glycine residues which would confer a considerable degree of flexibility.

NFA-3 was first described on a septicaemic isolate of *Escherichia coli* (strain 9). The NFA-3 gene cluster was also cloned using a cosmid vector. A single MRHA-positive recombinant was identified and two MRHA-positive recombinant subclones were generated, LE392 (pNFA3-101) and LE392 (pNFA3-103). The inserted sequences in pNFA3-101 and pNFA3-103 were cloned in opposite orientations and both plasmids shared a 6.0kb stretch of DNA. SDS-PAGE and immunoblotting experiments showed that strain 9, LE392 (pNFA3-101) and LE392 (pNFA3-103) each expressed a protein of approximately 18kDa which was recognised by the monoclonal anti-NFA-3 antibody 19B12. Minicell analysis showed that the gene complex responsible for NFA-3 expression coded for at least five products of approximate molecular weight 80kDa, 42kDa, 30kDa, 28kDa, and 18kDa.

NFA-4 was identified on a uropathogenic isolate of *Escherichia coli* and the NFA-4 gene cluster was similarly cloned using a cosmid vector. Two MRHA-positive recombinants were identified and two MRHA-positive recombinant subclones were generated, LE392 (pNFA4-2-301) and LE392 (pNFA4-2-302). pNFA4-2-301 and pNFA4-2-302 were cloned in the same orientation and shared a 6.2kb stretch of DNA. SDS-PAGE and immunoblotting experiments showed that LE392 (pNFA4-2-301) and LE392 (pNFA4-2-302) each expressed a protein of approximately 21kDa which reacted weakly with two monoclonal anti-NFA-4 antibodies in immunoblotting experiments. The molecular weight of the NFA-4 adhesin subunit described by Hoschutzky (1989) was 28kDa and the cause of this apparent discrepancy in molecular weights was initially unexplained.

The NFA-4 adhesin subunit gene was located by investigating the hybridisation of oligonucleotide probes based on the known N-terminal amino acid sequence. Hybridisation only occurred under relatively non-stringent conditions and the oligonucleotides bound to two separate regions of DNA. One of the DNA sequences was positioned within the vector plasmid pLG339 and the other site of binding was therefore thought to represent the NFA-4 adhesin subunit gene. The nucleotide sequence of this region was determined and total of 1090 base pairs were sequenced. The nucleotide sequence did not correspond to the known N-terminal amino acid sequence of the NFA-4 adhesin subunit. Comparison of the data with other published sequences showed nearly 97% homology with the M-adhesin gene (*bmaE*) published by Rhen *et al* (1986b). This finding was unexpected and suggested that the original isolate possessed two non-fimbrial adhesins, NFA-4 and the M-adhesin. Both adhesins recognise the glycophorin A^{MM} component of the M-blood group antigen, however the N-terminal amino acid sequences are unrelated and the M-adhesin has a subunit of approximately 21kDa compared to 28kDa for NFA-4. The finding of an isolate of *E. coli* which expresses two apparently unrelated non-fimbrial adhesins with similar receptor specificity is of considerable interest. This clinical isolate is also known to exhibit mannose-sensitive adhesion mediated by type-1 fimbriae. Further studies might attempt to clone the NFA-4 adhesin and investigate its relationship with the M-adhesin. It would aso be interesting to investigate the regulation of adhesin expression and the phenotype and the ultrastructure of isolates expressing either or both non-fimbrial adhesins.

The molecular comparison of the cloned non-fimbrial adhesins revealed several similarities between NFA-1 and NFA-2, but NFA-3 and M-adhesin ("NFA-4") sequences appeared to be unrelated. Cloned DNA encoding NFA-1 and NFA-2 shared several restriction enzyme sites and a degree of genetic homology which extended throughout the gene clusters. Capsular polysaccharide probes did not hybridise with any of the non-fimbrial gene clusters. Minicell studies showed that the NFA-1, NFA-2 and NFA-3 gene clusters each expressed five or six proteins including a large protein of approximately 80kDa.

Sequence comparison of the NFA-2 subunit with other adhesin sequences proved to be most informative. NFA-1 and NFA-2 adhesin subunits share a similar size and a limited degree of serological cross-reactivity. There are also morphological differences with NFA-2 having a more patchy appearance. Nucleotide sequence comparison showed 75.6% overall identity between NFA-1 and NFA-2 adhesin subunit sequences. Amino acid comparison showed 55.8% identity and 67.6% similarity. Both subunits have two cysteine residues which are located at similar sites.

Comparison of the NFA-2 subunit sequence with other published adhesins revealed areas of close homology with adhesin subunits belonging to the Dr adhesin family, including draA, AFA-1, and the F1845 adhesin. A region of sequence identity was discovered adjacent to the start codon of the adhesin genes. It is tempting to speculate that the high degree of homology in this region may represent a mechanism for inserting different adhesin subunit genes into a more conserved gene cluster. The situation might perhaps be analagous to the capsular gene complex which includes conserved regions coding for transport and structural proteins and more variable genes coding for polysaccharide synthesis (Roberts *et al* 1986, Boulnois *et al* 1987). This arrangement has been likened to a cassette and allows highly variable genes to be inserted or deleted from an otherwise conserved gene complex. The evolutionary advantage of such an arrangement is that a high degree of antigenic variation can be generated whilst important structural and transport genes are conserved.

There is some evidence that gene exchange can occur in *E. coli* adhesin complexes. Klemm *et al* (1994) recently showed that unrelated adhesin subunits could be exchanged between the *fim* (type 1) and *foc* (F1C) gene clusters resulting in hybrid fimbriae with altered receptor specificity. This process was termed fimbrial promiscuity. Chimeric adhesins were similarly generated in a series of complementation experiments using the related F1845 and Dr adhesin gene clusters (Swanson *et al* 1991). It would be interesting to investigate whether a similar exchange of subunit genes could occur between the gene complexes encoding NFA-1 and NFA-2 and other members of the Dr family of adhesins.

The gene complexes coding for members of the Dr family of adhesins was recently compared by Ahrens *et al* (1993) and an alignment of the physical maps of the gene clusters is shown in Figure 10.1. All five of the adhesins shown are located on a 6-8kb stretch of DNA and all consist of five separate genes with the adhesin subunit gene located at the 3' end of the complex. A recent study has shown the *afa-3* gene cluster is also organised in a similar fashion with five separate genes (Bouguenec *et al* 1993). The functions of the individual gene products have not been completely determined, however a large protein (NfaC) is coded by the central gene of each cluster and may represent an anchor protein which is the site of fimbrial biogenesis. Sequencing of the *nfaE* gene revealed a 247 amino acid product which shared homology with a protein involved in the assembly of CS3 fibrillae (Ahrens *et al* 1993). Further studies will undoubtedly lead to the sequencing and comparison of the remaining genes and characterisation of their products.

The final section of this thesis investigates the clinical importance of non-fimbrial adhesins. It has been suggested that approximately 10% of urinary isolates of *E. coli* possessed non-fimbrial adhesins (Duguid *et al* 1979), however there is comparatively little experimental data available to support this figure.

The clinical study used probes derived from NFA-1, NFA-2, NFA-3 and the M-adhesin ("NFA-4") to investigate sequence homology with a comprehensive series of clinical isolates. Clinical specimens were obtained from patients with septicaemia and urinary tract infections. Colonising strains of *E. coli* were also included as they were probably representative of faecal isolates. Specimens of urine were obtained from hospitalised patients and general practitioners over a four day period and the number received confirmed the importance of urinary tract infections. *E. coli* urinary tract infections represent a major part of the workload of the Public Health Laboratory and are associated with significant economic consequences. Future studies might investigate the clinical and financial impact of urinary tract infections in greater detail. 46% of the urinary isolates were resistant to at least one of the commonly used antibiotics and the levels of antibiotic resistance observed are of some concern as future infections may require more difficult and expensive treatment.

The clinical information which accompanied the urine specimens allowed the age and sex distribution to be investigated. This showed that there were two peaks of infection occurring in young adult women and in elderly males and females. This can be explained by the onset of sexual activity in females and the presence of coexisting disease and structual abnormalities of the urinary tract in elderly patients. Septicaemic isolates were equally common in males and females, with a peak in boys under the age of one year, when most infections result from congenital abnormalities of the urinary tract.

Clinical isolates were probed with NFA-1, NFA-2, NFA-3 and M-adhesin ("NFA-4") sequences in colony blotting studies. The results showed that NFA-1 and NFA-2 probes each hybridised to 23% of the clinical isolates and these adhesin sequences were more frequently identified in colonising organisms compared to isolates causing upper urinary tract infection or septicaemia.

The conclusion from these studies was that NFA-1 and NFA-2 are present on approximately 35% of colonising isolates. They may therefore play a role in colonisation of the lower urinary tract infection but are not strongly associated with upper urinary tract infection or septicaemia. Further evidence suggesting that the Dr family of adhesins are associated with intestinal colonisation comes from a recent study which revealed very strong binding of the Dr, F1845 and AFA-1 adhesins to cultured human intestinal cells (Kerneis *et al* 1994). The binding of bacteria expressing NFA-1 and NFA-2 to intestinal cells has not yet been explored.

The NFA-3 probe only hybridised to 1% of the clinical isolates, therefore NFA-3 appears to be a relatively rare adhesin which is unlikely to play an important role in urinary tract infections. The M-adhesin ("NFA-4") probe hybridised with 12% of the clinical isolates and also appeared to be associated with colonising organisms and those causing lower urinary tract symptoms rather than invasive isolates.

Maslow *et al* (1993) recently investigated the presence of adhesin sequences in 170 septicaemic isolates of *E. coli* by dot-blot hybridisation. They found adhesin gene sequences in 84% of urinary tract isolates; 77% had *pap* adhesin gene sequences, 46% had *sfa* sequences, 9% had *afa* sequences and only one isolate was positive for *bma* sequences (M-adhesin). Further studies using the cloned NFA-3 adhesin sequences to probe a pool of clinical isolates showed that 9 (5%) of 193 septicaemic isolates and 15 (42%) of 36 urine isolates were positive for NFA-3 sequences (Maslow 1995, unpublished observations). The proportion of urinary isolate that were positive for NFA-3 was much greater than that observed in Leicester, however there were interesting geographical differences, with 10 (67%) of 15 urinary isolates from Cleveland positive for NFA-3 compared to 5 (24%) of 21 Memphis isolates.

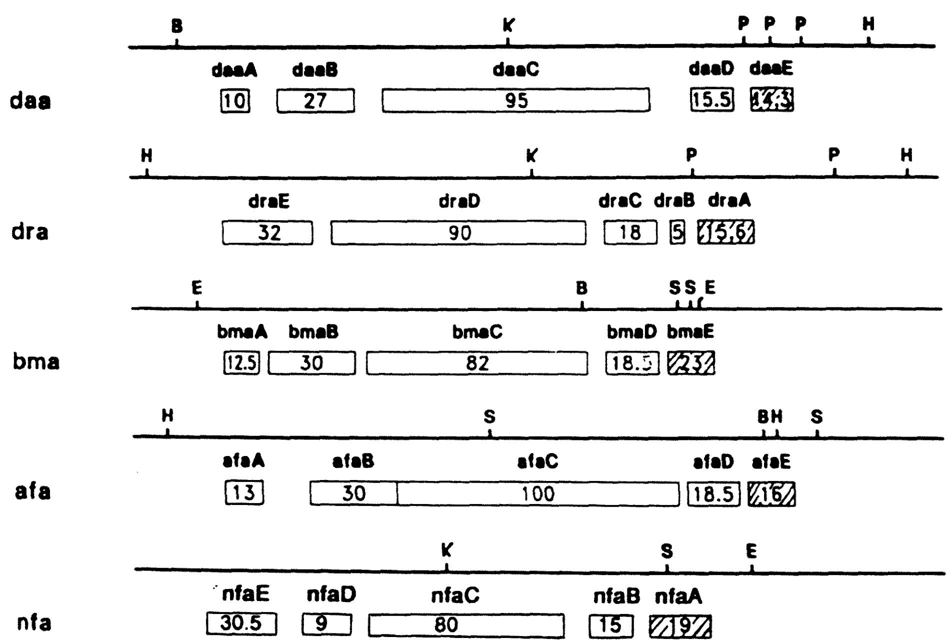
Taken together these results suggest that a significant proportion of faecal isolates possess non-fimbrial adhesins and these adhesins may have an important function in colonisation of the bowel and lower urinary tract. Non-fimbrial adhesins do not appear to play a major role in upper urinary tract infection or septicaemia.

Figure 10.1

Comparison of physical maps and the gene products of members of the Dr family of adhesins including the F1845 gene cluster (*daa*), the Dr adhesin gene cluster (*dra*), the M-adhesin gene cluster (*bma*), the afimbial adhesin I gene cluster (*afa*), and the NFA-1 gene cluster (*nfa1*). The main direction of transcription is from left to right (adapted from Ahrens *et al* 1993).

Boxes indicate the gene products and their molecular weight (kDa)

Hatched boxes represent adhesin proteins



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