Plasmid determined fimbrial production responsible for bacterial colonization of the urinary tract

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ABSTRACT

A survey of the virulence characters of Enterobacteriaceae isolated from the urine of patients with urinary tract infections showed that the most common character possessed by these strains was the ability to cause haemagglutination of erythrocytes. Of 237 lower urinary tract infection isolates 99 produced mannose-resistant haemagglutination (MRHA) of human group A erythrocytes.

These 99 strains were examined for transferable plasmids which carried the gene for mannose-resistant haemagglutinating ability, using drug resistance determinants as markers. Twelve of these strains were able to co-transfer the MRHA gene with a resistance plasmid (R-plasmid). None of the transconjugants of these strains possessed a common plasmid and it was observed that loss of the MRHA gene during transfer did not correspond to loss of an R-plasmid.

The MRHA gene was mobilised from an isolate, which did not possess R-plasmids, on to an R-plasmid vector which had been introduced into the strain. The genes responsible for MRHA transposed freely between plasmids and insertion into a small plasmid vector allowed an estimation of its size as 8.7 kilobases.

Further examination of the strains which possessed auto-transferable MRHA genes revealed that these genes were also transposable in some cases. In these strains the size of the transposon varied between 7 and 8.4 kilobases.

Further evidence for the presence of MRHA transposons came from the transconjugants of strains isolated from a single patient with a

series of recurrent urinary tract infections. These strains also carried the MRHA gene on an R-plasmid which was able to persist in the strains in the absence of selection for any of the resistance determinants. Not all the transconjugants were MRHA-positive, but the efficiency of transfer of the gene appeared to increase with time. Transposons carrying the MRHA gene were extracted from these plasmids and were estimated to have a size of 9.2kb when inserted into a small plasmid vector.

A range of common antimicrobial drugs were tested for their effect on the haemagglutination of a *Proteus vulgaris* and an *Escherichia coli* strain, when present at sub-inhibitory concentrations. Haemagglutination could only be completely inhibited by the protein synthesis inhibitors. The presence of an R-plasmid in the *E. coli* strain prevented this inhibition, unless the minimum inhibitory concentrations were redetermined, in which case similar inhibition of haemagglutination was still observed.

DECLARATION

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

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ABBREVIATIONS

ABU - asymptomatic bacteriuria

AFA - afimbrial adhesin

APS - ammonium persulphate

BHI - brain heart infusion

cAMP - cyclic adenosine monophosphate

CFA - colonisation factor antigen

CS - coli surface antigen

d - daltons

DM - Davis-Mingioli

DSTA - diagnostic sensitivity test agar

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme linked immunoabsorbent assay

EM - electron microscope

EPEC - enteropathogenic *E. coli*

ETEC - enterotoxigenic *E. coli*

HA - haemagglutination

Hly - haemolysin

IS - insertion sequence

kb - kilobases

LT - heat labile toxin

MIC - minimum inhibitory concentration

MRE - mannose-resistant eluting

MRHA - mannose-resistant haemagglutination

MSHA - mannose-sensitive haemagglutination

OD - optical density

P	- probability
Pap	- pyelonephritis associated pili
R	- resistance
SDS-PAGE	- sodium dodecyl sulphate polyacrylamide gel electrophoresis
ST	- heat stable toxin
TEMED	- N,N,N','N'-tetramethylethylenediamine
Tn	- transposon
UTI	- urinary tract infection

INTRODUCTION

Over the last twenty years a great deal of time and effort has been devoted to elucidating the requirements and mechanisms of bacterial pathogenicity. Many factors have been considered as virulence determinants such as toxins, colicins, serum resistance and adhesive fimbriae. The absolute requirements that a bacterium needs to become virulent are still not clear, as not all bacteria which are classified as pathogenic, i.e. able to cause disease, possess all or one of the above mentioned factors. The toxins produce the symptoms associated with clinical disease in gastroenteritis, but fimbriae are needed to attach the bacteria to the intestinal mucosa without which diarrhoea, caused by the elaboration of the toxins, does not develop. In this case, the fimbriae could, therefore, be classed as essential virulence factors. In the urinary tract the picture is less clear, although fimbriae are thought to be responsible for the ascension of the bacteria to the kidneys for the manifestation of the more serious forms of urinary tract infection (UTI), the presence of fimbriae on the bacteria is more common in pyelonephritis than it is in lower urinary tract infections.

This thesis is concerned with the fimbriae of uropathogenic bacteria, using knowledge and techniques gained from the work on fimbriae involved in other types of infection. The introduction covers the discovery of fimbriae, their classification and genetics and possible methods for combatting this type of pathogenicity.

I IDENTIFICATION OF FIMBRIAE

Discovery of bacterial haemagglutination and fimbriae

The phenomenon of haemagglutination by *Escherichia coli* was first described by Guyot in 1908. He found that 12 out of 18 of his strains were capable of agglutinating the red blood cells from one or more of the 13 species of animal that he tested. Each strain produced the same degree of haemagglutination with the erythrocytes from different individuals of the same species. Guyot also observed that the factor responsible for the haemagglutination reaction was not a bacterial secretory product.

The first extensive experimental work on the biological properties of the agglutination by *E. coli* was performed by Rosenthal (1943). In addition to erythrocytes, he also determined the effect that *E. coli* produced with leucocytes, thrombocytes, spermatozoa, yeast cells, mould spores and pollen. The agglutination test was performed by mixing a drop of the test cell suspension with a loop of bacteria on a slide and mixing thoroughly. The formation of clumps immediately, or within five minutes, was classified as a positive reaction. Of the 70 faecal *E. coli* tested, 24 gave a positive agglutination reaction to at least one of the cell types. Twelve of these isolates agglutinated human erythrocytes. The agglutination factor was thermo-labile, unaffected by absolute alcohol, the pH of the media and formalin treatment of the cells, but was not present in the filtrates of broth cultures. Rosenthal (1943) expressed the idea that if this

agglutination of cells also occurred *in vivo* it could have some bearing on pathogenicity and virulence.

. Electron microscopy revealed the existence of filamentous appendages on the bacterial surface that were present in addition to flagella (Houwink and van Iterson, 1950). These were morphologically distinct from flagella, appearing as shorter, thin, fragile structures with a smaller diameter than the flagella. They were formed on bacteria which were in contact with a solid surface, but not on free-swimming organisms, unless they were at the surface of a liquid medium (Houwink and van Iterson, 1950). *E. coli* possessing these filaments, designated fimbriae, could be distinguished from non-fimbriated organisms by their ability to agglutinate erythrocytes (Duguid *et al*., 1955), which supported the theory that they were involved in attachement (Houwink and van Iterson, 1950), and revealed the mechanism by which haemagglutination occurred. The agglutination reaction, caused by direct union of the bacteria with the erythrocytes, was not enzymic as it occurred at pH 3 to 12, and after treatment of the bacteria with formaldehyde, ethanol and phenol (Duguid *et al*., 1955).

Separation of the filaments from the flagella could be achieved by grinding, differential centrifugation and low pH - at which the filaments, unlike flagella, did not disintegrate (Weibull and Hedvall, 1953). In addition, the fimbriae were not destroyed by pepsin, trypsin, ribonuclease or deoxyribonuclease.

Electrophoretic mobility measurements on fimbriated and non-fimbriated variants of an *E. coli* B (Brinton *et al*., 1954) gave a

bimodal distribution, the fimbriated variants had a greater viscosity, with the fimbriae bearing a weaker negative charge than the normal bacterial surface, therefore assisting the approach to negatively charged substrates.

Early work on the prevalence and classification of fimbriae among bacterial species

The first work on haemagglutination was performed with E. coli. Many, but not all, of the strains tested possessed this ability (Duguid et al., 1955). These workers showed by electron microscopy that 31 of 47 E. coli isolated from various sites of the body possessed fimbriae. The ability of the strains to cause haemagglutination was tested with erythrocytes from 18 species of animal including guinea pigs and man. The test was performed with a 24h peptone water culture of the test organism and a saline washed suspension of erythrocytes. The reaction was allowed to occur in the depression of a white tile which had been chilled to between 3 and 5°C on ice. On the basis of the results obtained, the strains were divided into four groups (Duguid et al., 1955). Group I contained 26 strains which had a similar pattern of activity as they strongly agglutinated most of the erythrocyte types, however, human, goat and sheep erythrocytes were only agglutinated weakly and no agglutination was found with ox cells. Group II showed a peculiar agglutination spectrum as the five haemolytic strains in this group agglutinated ox erythrocytes and gave strong reactions with sheep, goat and human cells. Group III contained three strains which expressed weak haemagglutination of unusual kinds, which was not

compatible with the other groups. Thirteen strains were classified as Group IV and these showed no activity for erythrocytes under any conditions. Possession of fimbriae correlated with the common patterns of haemagglutination. The 31 strains belonging to Groups I and II were all shown to possess fimbriae under the electron microscope. No fimbriae were seen on the strains in Group III, although they possessed haemagglutination activity, or on the non-haemagglutinating bacteria of Group IV (Duguid et al., 1955).

It seemed improbable that E. coli would be the only species in the Enterobacteriaceae to possess fimbriae, so over the next few years, there were investigations of other genera. Ten Enterobacter cloacae strains, out of 13 tested, were found to produce some degree of haemagglutination (Constable, 1956), and fimbriae were observed by electron microscopy, on all of these 10 strains. Fimbriae were absent from the three strains which were haemagglutination negative. Guinea pig and fowl erythrocytes were agglutinated by most of the strains, whereas only three showed haemagglutination of human, ox or sheep erythrocytes (Constable, 1956).

Constable (1956) observed that not all the bacteria in a single culture possessed fimbriae, possibly this was due to loss during centrifugation and washing, but the bacteria that were fimbriated always appeared to have a uniform number of filaments per cell. This correlates with the observation of Duguid et al. (1955) that some of his strains readily underwent genetic variation between haemagglutinating and non-haemagglutinating forms. Constable (1956) also observed that there was a quantitative relationship between the degree of agglutination and the percentage of fimbriate organisms in

that culture, except for one strain which produced strong agglutination but fimbriae were only present on 1% of individual bacteria in the culture.

In a survey of 247 Shigella strains (Duguid and Gillies, 1957) fimbriae and haemagglutinating activity was observed in 103 of a total of 180 Shigella flexneri of various serotypes. This occurrence of fimbriae and haemagglutination varied depending on the culture conditions but these two properties were always present or absent together. Neither property was found in Sh. sonnei, Sh. dysenteriae or Sh. boydii. All the fimbriae of Sh. flexneri strains appeared to be alike in their pattern of haemagglutination, and closely resembled the fimbriae of Group I E. coli (Duguid et al., 1955). The fimbriated Sh. flexneri also showed a readily reversible mutation between the fimbriated and non-fimbriated form, depending on the culture conditions (Duguid and Gillies, 1957).

It had been reported that mannose could strongly inhibit the agglutination of fowl cells produced by a diffusible E. coli haemagglutinin (Collier and de Miranda, 1955). Similarly, mannose was found to inhibit the haemagglutination of all the Sh. flexneri strains if added to the reaction mixture. The same effect was also produced by α-methyl-mannoside and yeast mannan (Duguid and Gillies, 1957), but no inhibition was seen with other sugars. It appeared, therefore, that mannose was present in the receptors on the erythrocytes, however, mannose had no inhibitory effect on the haemagglutination of non-fimbriated strains.

Strains possessing fimbriae and haemagglutination ability were demonstrated in *Salmonella*, *Proteus*, *Klebsiella* and *Chromobacterium* species (Duguid and Gillies, 1958). Most of the fimbriate organisms were also capable of haemagglutination and underwent the reversible variation of fimbriate to non-fimbriate. Further work on *Klebsiella spp*. (Duguid, 1959) showed that the fimbriae could be clearly distinguished from the capsule and occurred in acapsular mutants. Two types of haemagglutination were also distinguished, mannose-sensitive (MSHA), as previously described, and mannose-resistant (MRHA). The two types of reaction were caused by morphologically distinct fimbriae and further characterised by different haemagglutination patterns (Duguid, 1959).

A large number of *Salmonellae* were also examined (Duguid *et al*., 1966) of which 1184 were found to be fimbriate and 269 permanently non-fimbriate. Again, reversible variation of the fimbriated organisms was observed. Most of the fimbriated organisms expressed mannose-sensitive haemagglutination which were designated as Type 1 fimbriae (Duguid *et al*., 1966). The remaining fimbriated organisms which showed no haemagglutination were designated as possessing Type 2 fimbriae. Non-fimbriated *S. sendai* strains possessed mannose-resistant haemagglutinating activity, the bacteria were eluted from the erythrocytes when the reaction was warmed to 50°C (Duguid *et al*., 1966), in a similar manner to the reversible elution observed with Type 1 fimbriae (Duguid *et al*., 1955).

By this time it was possible for the different patterns of haemagglutination reaction to be classified as being caused by six types of adhesive fimbriae (Duguid *et al.*, 1966).

Type 1 Relatively thick fimbriae. MSHA. Comprised of protein subunits. Present on most fimbriated *E. coli*, *Ent. cloacae*, *Sh. flexneri*, *K. aerogenes* and *Serratia marcescens*.

Type 2 Non-haemagglutinating. Present on *Salmonella gallinarum*, *S. pullorum*, *S. paratyphi* and *S. dublin*. Same dimensions as Type 1.

Type 3 Thin type. MR adhesin. Almost all strains of *K. aerogenes* and *S. marcescens*. Most also possess Type 1. Only agglutinate erythrocytes if they have been pretreated with tannic acid.

Type 4 Fimbriae of *Proteus*. Very thin. MRHA. Active on fresh untanned cells. They have a different spectrum of activity - strongly agglutinate sheep and fowl erythrocytes and moderate agglutination with most other types.

Type 5 Monopolar fimbriae of *Pseudomonas echinoides* (Heumann and Marx, 1964). MS. Bind to sheep erythrocytes, but no agglutination due to monopolar nature.

Type 6 Very long, thick and very few per cell. Either non-haemagglutinating or very weak MS. Present on *Klebsiella ozanae*.

Possible role of fimbriae in pathogenicity

In the late 1950s, it was not known what the exact role of the fimbriae was. It was possible that the increased surface area the fimbriae provided could function in the acquisition of nutrients (Hinterberger, 1901). However, the occurrence of haemagglutination ability in many pathogenic bacteria of different species suggested that they may function *in vivo* as a means of attaching bacteria to the host cells to allow action of toxins (Duguid and Gillies, 1957). Apart from the production of exotoxins, little was known of the mechanism by which pathogenic bacteria initiated their attack on the respiratory and alimentary mucosa.

The lack of fimbriae on the surface of *Sh. sonnei*, *Sh. dysenteriae* and *Sh. boydii*, however, indicated that this property was not an essential pre-requisite in the pathogenesis of dysentery (Duguid and Gillies, 1957). In addition, 269 of the 1453 *Salmonella* strains tested by Duguid *et al*. (1966) were non-fimbriated, inferring that fimbriae were also non-essential to the pathogenicity of *Salmonella* spp. Against this conclusion is the fact that a large proportion of the strains tested did produce fimbriae, so they must confer some advantage on the host bacteria to weigh against the extra burden on the cell of producing the fimbriae.

The exact nature of the role and importance of the fimbriae still remained to be elucidated, particularly in those infections caused by *E. coli*.

II PLASMID-ENCODED FIMBRIAE OF ENTERIC E. COLI

Isolation and analysis of the K88 antigen from diseased pigs

The discovery in 1961 of a new antigen (Ørskov *et al*., 1961) possessed by *E. coli* strains isolated from pigs with enteritis or oedema, heralded the beginning of a greater knowledge of the association between fimbriae and pathogenicity. The strains G7 and E68 were first described by Sojka *et al*. (1960). Ørskov *et al*. (1961) found that these strains could possess both the B type and L type of K antigen at the same time. The B type antigen was designated K85 and the L type antigen K88. The K88 antigen was common to strains G7 and E68, but was not demonstrable if the cultures were grown at 18°C. Heating the strains at 100°C for one hour also abolished agglutination (Ørskov *et al*., 1961).

Additional strains isolated from diseased piglets in different countries were also shown to possess the K88 antigen (Ørskov *et al*., 1964) suggesting that it might have some bearing on pathogenicity. An investigation of these strains by their sera absorption patterns indicated that the antigen was composed of several antigenic factors which could be present in different combinations, as all strains possessed K88, but they were not identical (Ørskov *et al*., 1964). Strains G7 and E68 were given the designation K88a,b and three other strains the designation K88a,c. No variation from K88a,b to K88a,c was found during the examination of individual colonies of strain G7 (Ørskov *et al*., 1964). G7 had been shown to act as a genetic donor strain for the K88 antigen (Ørskov *et al*., 1961), however, transfer

of K88a,c from the other strains could not be demonstrated (Ørskov et al., 1964).

Further work on the transmission of the K88 antigenic determinant showed that transconjugants of the parent G7 strain also acquired the chromosomal met marker (Ørskov and Ørskov, 1966). In some cases the ability to transfer chromosomal markers was retained by the transconjugants when the K88 antigen had been lost. The K88 factor could not be transferred from culture filtrates and treatment of a culture of strain G7 with acriflavine cured the bacteria of the K88 antigen. It was therefore concluded that the antigenic determinant was carried on an episome (Ørskov and Ørskov, 1966).

Extraction of the K88 antigen from a transconjugant, strain D520 was accomplished (Stirm et al., 1967b). The K88 antigen could be released into the medium by the agar gel precipitation technique and the amount of liberated material could be increased with gentle heating to 60°C or blending. The antigen could be obtained in a pure form due to its insolubility at pH 3.5 to 5.5. Chemical analysis of the antigen extract showed it to be a pure protein containing all the common amino acids except cysteine-cystine. The purified antigen was capable of precipitating K88 antibodies from D520 antiserum and was immunogenic in rabbits (Stirm et al., 1967b).

Purification and biochemical analysis of the K88 variants K88ab,ac and ad, subsequently discovered by Guinée and Jansen (1979), showed small differences in molecular weight from 23,500 to 26,000 which could be caused by amino acid substitutions (Mooi and de Graaf, 1979). These small differences in amino acids would also

explain the antigenic heterogeneity. All the variants possessed the a determinant. Therefore, it is probably this factor which is essential to the fimbriae and could be involved in receptor recognition.

Examination of K88-positive strains and purified K88 antigen under the electron microscope showed the antigen as fine filaments, more flexible than fimbriae (Stirm et al., 1967a). Both K88 positive strains and the purified antigen expressed MRHA of guinea pig erythrocytes if grown at 37°C, no HA was observed with K88-negative strains (Jones and Rutter, 1974). Elution occurred when the temperature was increased. These results again illustrated the correlation between the presence of fimbriae and haemagglutination ability, although K88 had a different structure from all the E. coli fimbriae previously described (Stirm et al., 1967a).

Involvement of K88-positive strains in neonatal diarrhoea in piglets

Infections caused by E. coli in piglets and weaned pigs are characterised by the proliferation of strains of certain serotypes in the small intestine, but not in the stomach (Smith and Jones, 1963). This colonisation of the small intestine by large numbers of bacteria and production of enterotoxins gives rise to the clinical disease (Smith and Halls, 1967). Work by Smith and Halls (1968) on the enumeration of a porcine enteropathogenic E. coli strain in wall scrapings and contents of the small intestine of pigs revealed larger numbers of the organism in the wall scrapings, probably due to adherence of organisms to the epithelial cells. Fluorescent staining techniques of three K88-positive enteropathogenic strains

showed them attached to the villi of the small intestine with very few adhering to the colonic mucosa (Arbuckle, 1970). Non-enteropathogenic strains were found attached to the villi only in small numbers. The ability to attach to the epithelium could possibly allow enteropathogenic strains to overcome gut motility and assist their survival.

Enteropathogenic E. coli (EPEC) strains isolated from diseased piglets frequently possess the K88 antigen (Gossling and Rhoades, 1967). The role of K88 was studied by comparing a K88-positive strain with three K88-negative derivatives of this strain obtained by ethidium bromide treatment, UV irradiation and a spontaneous mutant (Jones and Rutter, 1972). The presence of K88 antigen on the surface enabled the K88-positive strain to adhere to the intestinal mucosa whereas the K88-negative strains remained distributed through the lumen. The K88-positive strain was able to kill 50% of the conventionally reared piglets challenged, compared to the 3% killed by a K88-negative strain. But there is the possibility that the induced mutagenesis might have caused further damage to the strains. The K88 antigen therefore appeared to be essential for virulence of the bacteria in these piglets (Jones and Rutter, 1972). The ability to synthesise enterotoxin also contributes to entero-pathogenicity; a large porportion of EPEC strains produce enterotoxin and α-haemolysin, properties which are carried by transmissible plasmids (Smith and Linggood, 1971). Experiments showed that the K88 antigen was needed for adhesion of the bacteria to the intestinal mucosa and the enterotoxin produced the clinical symptoms (Smith and Linggood, 1971).

Adhesion of K88-negative E. coli of porcine origin

A survey of haemolytic E. coli isolates from faecal swabs of piglets with post-weaning diarrhoea showed that only one strain possessed the K88 antigen (Riising et al., 1975). Strains belonging to serogroup O149:K91 were the most frequently isolated. These results suggested a minor role for the K88 antigen in this particular infection.

Other K88-negative porcine EPEC which were able to colonise the small intestine were shown by bacterial counts, fluorescence and electron microscopy to adhere to the villous epithelium (Nagy et al., 1976). AS K88-negative strains and acapsular mutants are able to adhere and colonise the small intestine, other surface factors must be present in addition to the polysaccharide K antigens. Hohmann and Wilson (1975) noted the presence of an unusual surface structure on a K88-negative strain which adhered to the epithelial surface of the small intestine. This structure under the electron microscope was seen as long strands which were wide at the base and narrowed at the tip, and the strands were usually branched.

Adhesion is important in pathogenicity even if it is not mediated by fimbriae, as in a minority of infections. As indicated above, surface appendages are still involved even if they do not have a regular fimbrial appearance.

Discovery of an adhesive K antigen on strains infecting calves and lambs

It was observed by Smith and Halls (1967) that EPEC of porcine origin, as well as dilating ligated segments of pig intestine (a test for enterotoxin production), also dilated segments of intestine from calves and lambs. However, enteropathogenic strains isolated from calves and lambs, though able to dilate calf and lamb intestine, had no effect on pig intestine. Furthermore, human enteropathogenic strains failed to dilate intestine from all three species.

Three other EPEC which were able to dilate ligated segments of intestine from piglets less than a week old, but not those of older piglets, were classified as atypical strains (Moon and Whipp, 1970). Tests performed with ligated intestinal segments from pigs, piglets, calves and lambs indicated that the enterotoxin produced by the atypical E. coli was probably identical with that produced by the calf and lamb strains, and the two groups of strains also resembled one another (Smith and Linggood, 1972). This enterotoxin resembled the heat-stable toxin (ST) rather than the heat-labile form (LT), whch are both produced by typical EPEC of porcine origin (Smith and Gyles, 1970). The enterotoxin was plasmid-controlled as was a K antigen possessed by the calf and lamb strains (Smith and Linggood, 1972).

The ability to produce this calf and lamb common K antigen (Kco) was transferred from one calf and two lamb enteropathogenic strains to E. coli K12 with the concomittant transfer of enterotoxin

production (Smith and Linggood, 1972). An examination of 74 enterotoxigenic strains isolated from cases of calf diarrhoea, demonstrated that 70 also possessed Kco and none of the enterotoxin-negative strains possessed the K antigen (Guinée et al., 1976).

Infection experiments performed with Kco-positive and Kco-negative strains, derived by acridine orange treatment of Kco-positive transconjugants, indicated that the Kco antigen was important in pathogenicity. Lambs infected with the Kco-positive strains developed diarrhoea, whereas lambs challenged with Kco-negative strains remained healthy. This was despite the fact that the cured strains still produced enterotoxin (Smith and Linggood, 1972); it would therefore appear that these two determinants are carried on different plasmids. Lambs challenged with atypical pig strains also remained healthy. This indicated that Kco functioned in the same way as K88 by facilitating adhesion to the intestinal epithelium, and adhesion of the antigens was species specific (Smith and Linggood, 1972).

In a separate study, when eleven calf and lamb enteropathogenic strains were examined, the Kco antigen was demonstrated on nine of them by slide agglutination (Ørskov et al., 1975). The antigen could only be demonstrated if cultures were grown at 37°C. Heating the culture at 100°C for one hour, or growth at 18°C, gave a negative result. The Kco antigen was given the designation K99 (Ørskov et al, 1975).

Suspensions of K99-positive strains caused MRHA of sheep erythrocytes, but on warming to 37°C the bacteria did not elute

completely (Burrows et al., 1976). These strains also adhered to brush border cells from calves, the adhesion being inhibited by 85% in the presence of purified K99 antigen. Electron microscopy showed the antigen as fine, branched, filamentous projections covering the surface of the bacteria (Burrows et al., 1976).

Strains of enterotoxigenic E. coli (ETEC) isolated from pigs were also found to express K99 (Moon et al., 1977). One of these strains and other K99-positive ETEC, isolated from calves, produced the K99 antigen in the pig ileum in vivo. The ability to adhere to the villous epithelium led to colonisation and the production of diarrhoea in newborn piglets. ETEC isolated from the faeces of piglets with neonatal diarrhoea presented four different phenotypes: 1) K99 and ST-positive, 2) ST-positive, 3) K88 and LT-positive, 4) K88 and ST-positive. Phenotypes 1 and 2 also showed multiple drug resistance and belonged mostly to serogroup O101 (Shimizu et al, 1984).

Extraction and purification of the K99 antigen was achieved by salt extraction, ammonium sulphate precipitation and diethylamino-ethyl sephadex column chromatography (Isaacson, 1977). The antigen was composed of two subunits, a major component of 22,500 daltons and a minor component of 29,500d. A later report by de Graaf et al. (1980b) gave the apparent molecular weight of the K99 subunit as 18,500, but further purification procedures had been applied. However, the difference in molecular weight was unexplained, apart from the fact that the minor component could be another K99 antigen which co-purified with the major component. Isaacson et al. (1981)

showed that heat denaturation of the antigen in the presence of β-mercaptoethanol gave two components, whereas denaturation inthe absence of β-mercaptoethanol gave only the smaller component.

The presence of two surface antigens on the K99 reference strain E. coli B41 was confirmed when different purification procedures gave antigens with differing chemical properties. The K99 antigen purified by column chromatography was composed primarily of protein with an isoelectric point (pI) of greater than 10. This purified antigen agglutinated horse but not guinea pig erythrocytes (Isaacson, 1977). If the antigenic component of E. coli B41 was prepared by acid precipitation, instead of chromatography, it retained its capacity to agglutinate guinea pig erythrocytes. This antigen preparation contained a second antigen which was not present in the purified extract from the column. This second antigen had a pI value of 4.2 (Isaacson, 1978), the value that Morris et al. (1978) had reported as the pI value of the K99 antigen.

Immunoelectrophoresis and haemagglutination studies with the antigen extract from E. coli B41 demonstrated the presence of a cationic and an anionic haemagglutinin (Morris et al., 1980). Antibodies to the cationic haemagglutinin were demonstrated in all antisera raised against K99-positive E. coli, whereas antibodies against the anionic haemagglutinin could only be demonstrated in antisera raised against K99-positive E. coli of serogroup 09 and 0101 (Morris et al, 1980). This second anionic haemagglutinin was designated F41 (de Graaf and Roorda, 1982).

Factors affecting expression of K99

The presence of various compounds in the growth media represses the expression of K99. Glucose and other carbon sources, such as pyruvate, arabinose and lactose, were shown to inhibit expression at relatively low concentrations (Isaacson, 1980a). The repression produced by glucose could be overcome by the addition of cAMP to the media. The presence of L-alanine in the media was also capable of repressing the production of K99 antigen (de Graaf et al, 1980), but this effect could be reversed if L-threonine or L-isoleucine were added (Isaacson, 1983). The presence of sodium acetate also suppressed the production of K99 (Francis et al., 1983).

Work performed by Ferreiros and Criado (1983) showed that although these compounds affect the production of K99 antigen, the adhesive ability of the strains was not likewise affected. Glucose decreased the production of antigen with increasing concentrations, completely inhibiting at 1-1.5% (w/v), whereas haemagglutination ability was only slightly lowered by 2% (w/v) glucose. An increasing concentration of L-alanine in the media reduced both the expression of the K99 antigen and haemagglutination. However, this was not seen with the reference strain E. coli B41 as antigen expression was unaffected and the haemagglutination titre actually increased. Antigen production was also inhibited by increasing concentrations of tetracycline, chloramphenicol, streptomycin and gentamicin. This inhibition was not observed with haemagglutination ability (Ferreiros and Criado, 1983).

These anomalies suggested that different structures were responsible for the two properties and that they were expressed in different ways, with the genes controlled by different mechanisms (Ferreiros and Criado, 1983).

Isolation of strains possessing antigens other than K88 and K99

K88-negative porcine ETEC strains which did not adhere to isolated intestinal cells in vitro were shown to adhere to the intestinal epithelium in vivo (Nagy et al., 1977). Growth of one of these, strain 987, in the pig small intestine, yielded a higher percentage of fimbriated cells than were produced in vitro. However, if the bacteria were very highly fimbriated, adhesion in vitro could be observed. The adhesion was a saturable process and inhibition of adhesion could be achieved in the presence of purified fimbriae (Isaacson et al., 1978). Other E. coli strains of porcine origin were tested for agglutination with 987 fimbriae antiserum, and several K88-negative ETEC gave a positive result (Nagy et al., 1977). These results were consistent with the theory that fimbriae still facilitate adhesion and colonisation by K88-negative ETEC strains. This type of fimbriae was designated as 987P (Nagy et al., 1977).

Normally ETEC strains which produce adhesive fimbriae express only one of the several antigenic types known. However, two ETEC of porcine and human origin were isolated which produced K88 and 987P. Both strains also produced ST and LT (Schneider and To, 1982). These strains varied reversibly between K88-positive, 987P-negative

to K88-positive, 987P-positive, similar to other 987P possessing strains which vary from 987P-negative in vitro to 987P-positive in vivo, except that the two strains mentioned above also express the K88 antigen (Schneider and To, 1982).

It had also been shown that the K99 reference strain, E. coli B41, possessed two distinct surface antigens (Isaacson, 1978; Morris et al., 1980). Both antigens caused MRHA of sheep erythrocytes, but only the F41 antigen agglutinated guinea pig erythrocytes.

The F41 antigen was present on E. coli strains belonging to serogroup 09 and 0101 (Morris et al., 1980), but not on E. coli of serogroups 08, 020 and 064 (Morris et al., 1982). This antigen was isolated and purified by mechanical detachment from the cells, ammonium sulphate precipitation and sepharose gel filtration. The purified antigen was composed of protein subunits with a molecular weight of 29,500 and was filamentous in structure. The purified antigen produced stronger haemagglutination with human and guinea pig erythrocytes than with horse or sheep erythrocytes (de Graaf and Roorda, 1982).

A K99-negative mutant of E. coli B41, possessing only F41 fimbriae was able to attach to calf enterocytes in vitro, and this adhesion was competitively inhibited by the presence of purified haemagglutinin (Morris et al., 1982). Piglets infected with this mutant developed diarrhoea within 16 hours.

Further work on E. coli B41 by Chanter (1983) led to the extraction of two mannose-resistant eluting (MRE) haemagglutinins

from the K99-negative mutant of strain B41. One of these adhered to calf brush-borders and was composed of subunits with a molecular weight of 34,000 as determined by SDS-PAGE. This haemagglutinin did not have a regular fimbrial appearance, but consisted of fine fibrillar structures. The second haemagglutinin had a definite fimbrial structure and was composed of two subunits of molecular weights 49,500 and 48,000. This antigen did not adhere to calf brush-borders (Chanter, 1983). From these results it would appear that the F41 antigen had two components, a fimbrial component and an adhesin component. Allowing for a slight discrepancy in subunit molecular weight, the 34,000 component probably corresponds to the antigen isolated by de Graaf and Roorda (1982).

F41 fimbriae were also found on porcine ETEC which did not produce K88, K99 or 987P ($3P^-$ strains) (To, 1984; Moon et al., 1980; Awad-Masalmeh et al., 1982). Electron microscopy of these strains also showed an irregular, poorly defined, filamentous material surrounding the bacteria (Morris et al., 1983). These strains were virulent in neonatal piglets and calves (Morris et al., 1983).

Adhesive antigens of human enteric E. coli

In 1970 Drucker et al. observed that EPEC adhered to the small intestine of infants who had died of gastroenteritis. These strains did not possess the K88 antigen, but it seemed probable that the adhesion was due to a surface antigen which had not yet been identified. The adhesion was not caused by Type 1 fimbriae, as it

was not inhibited by mannose (McNeish et al., 1975). A characteristic of these strains was their ability to proliferate in the small intestine and then produce enterotoxin.

The ETEC strain H10407, which was isolated from a case of human diarrhoea, possessed a plasmid-associated, heat-labile, surface antigen which was similar to K88 (Evans et al., 1975). This antigen appeared to function in virulence by facilitating colonisation. A laboratory passed derivative of this strain, H10407P, was unable to proliferate in the small intestine and produce diarrhoea. Electron microscopy of strains H10407 and H10407P showed that H10407 possessed fimbriae-like structures on its surface, whereas H10407P did not (Evans et al., 1975). Strain H10407 was only able to adhere when it expressed the antigen (Cheney and Boedeker, 1983). Rabbits could be protected from challenge with strain H10407 with antiserum raised against the colonisation factor antigen (CFA) (Evans et al., 1975).

Strain H10407 possessed three plasmids of molecular sizes 87, 61 and 5.4 kilobases (kb), whereas strain H10407P had lost the 87kb plasmid (Evans et al., 1975). Therefore, it appeared that this plasmid coded for the colonisation factor antigen, designated CFA/I (Evans and Evans, 1978), in contrast to the observations of Gyles et al. (1974) who had assigned a role in enterotoxin production to this plasmid. Evans et al. (1975) concluded that it must therefore be the 61kb plasmid that was responsible for enterotoxin production. However, loss of CFA/I from EPEC strains was later found to correlate with the loss of ST and the 87kb plasmid (Murray et al., 1983). The 61kb plasmid was found to specify production of both ST

and LT (Yamamoto and Yokoto, 1983). The confusion over the exact location of the genes was explained by Moseley et al. (1983), who showed that the 87kb plasmid contained sequences homologous to the gene for STIb and the 61kb plasmid sequences homologous for STIa. With the use of a conjugal transfer system, the CFA/I:ST plasmid could be transferred to many other bacterial species (Yamamoto et al., 1984).

The anti-CFA antiserum prepared against strain H10407 was used to examine other ETEC for the presence of CFA/I (Evans et al., 1978). Of the 29 strains from various serogroups tested, 25 possessed CFA/I. The agglutination could be prevented by heating the culture at 65°C for one hour or growing it at 18°C; similar inhibitory conditions to those associated with the other antigens previously described. The CFA-positive E. coli were also able to adhere to rabbit intestinal villi, and the production of CFA/I correlated to the presence of fimbriae (Evans et al., 1978).

The CFA/I-positive organisms also caused MRHA of human group A erythrocytes, whereas only MSHA was observed with CFA-negative isolates (Evans et al., 1977). The MRHA could be prevented if the culture was grown at 18°C, a condition which also inhibited CFA antisera agglutination, proving the association between the MRHA of erythrocytes and CFA/I (Evans et al., 1977).

Extraction and purification of CFA/I from the bacterial cells was performed by homogenisation, membrane filtration, ammonium sulphate precipitation and diethylaminoethyl sephadex column chromatography (Evans et al., 1979a). The purified CFA/I was

composed of a polymer of identical subnits with a molecular weight of 23,800 and was capable of producing MRHA of human group A erythrocytes.

The ETEC belonging to serogroups 06 and 08 did not possess CFA/I, but a second heat-labile surface antigen designated CFA/II (Evans and Evans, 1978). Unlike CFA/I, which causes MRHA of human, bovine and chicken erythrocytes, CFA/II only produced MRHA with bovine and chicken erythrocytes (Evans et al., 1979b). CFA/II also mediated adherence of the bacteria to the mucosal surface of the small intestine. Each CFA was possessed by distinct serogroups of E. coli, but 98% of all human ETEC possessed one or other of these two antigens (Evans and Evans, 1978).

CFA/II, like CFA/I, was plasmid-mediated. However, unlike CFA/I, it appeared that the genes for both ST and LT production were carried on the same plasmid as the colonisation antigen (Peñaranda et al., 1980). Loss of CFA/II correlated with loss of all or part of a 87kb plasmid. Loss of the whole plasmid always resulted in loss of enterotoxin production (Peñaranda et al., 1983). This plasmid was found in E. coli belonging to serogroups 06, 08, 080, 085 and 0139, but was not auto-transferable.

The CFA/II antigen is composed of coli surface antigens (CS) (Smyth, 1982). There are three of these, CS1, 2 and 3, which are present in different combinations in E. coli of different serotypes and biotypes (Mullany et al., 1983). ETEC of serogroup 06:H16 biotype A, produced CS1 and 3, whereas strains of the same serogroup, but of biotype B or C, produced CS2 and 3, and strains of

serogroup 08:H9 only produced CS3 (Levine *et al.*, 1984b). Transfer of the plasmids from these strains, by mobilisation, into *E. coli* K12 resulted in a marked decrease in the amount of HA in the transconjugant as CS3 was the only antigen expressed (Smith *et al.*, 1983; Mullany *et al.*, 1983). However, HA was restored when the plasmids were transferred back to an 06:H16 strain. Electron microscopy showed that CS1 and 2 were fimbrial, and morphologically indistinguishable from Type 1 (Smyth, 1984), whereas CS3 consisted of thin, flexible, wiry, fibrillar fimbriae (Levine *et al.*, 1984b).

A third colonisation factor antigen was isolated from EPEC responsible for an outbreak of diarrhoea in Senegal (Darfeuille *et al.*, 1983). None of the strains possessed either CFA/I or CFA/II, however, their HA pattern was similar to that of CFA/I, although the strains were not agglutinated by anti-CFA/I antiserum. A further distinguishing factor was that the HA was not inhibited by N-acetylneuraminic acid which inhibits CFA/I and II. This antigen, designated CFA/III (Darfeuille *et al.*, 1983) was a protein composed of subunits with a molecular weight of approximately 16,000.

Another fimbrial antigen was isolated from a bacterium responsible for diarrhoea in a patient in Bangladesh (Thomas *et al.*, 1982). The strain, *E. coli* E8775 belonged to serogroup 025:H42 and caused MRHA of human and bovine erythrocytes, but did not agglutinate CFA/I or II antisera. The biological properties of this fimbrial antigen corresponded to others previously described. Forty-eight strains, belonging to serogroups 025, 0115, 0167, of 472 ETEC tested, possessed this antigen, but its role in colonisation

has not been confirmed (Thomas et al., 1982). There are other distinct ETEC fimbriae which have been described by Deneke et al. (1979, 1981) and Wevers et al. (1980), isolated from single outbreaks of infection, so their relevance in overall E. coli pathogenicity is not established.

III FIMBRIAE OF UROPATHOGENIC E. COLI

Role of E. coli fimbriae in human urinary tract infections

E. coli is the most common cause of urinary tract infections (UTI) and as the importance of adherence to epithelial cells has been shown in the pathogenesis of diarrhoea, it would seem probable that these urinary E. coli would also possess adhesive fimbriae to attach to uroepithelial cells to resist expulsion by the flow of urine.

There are three different types of UTI, pyelonephritis which is the most severe and defined as the patient with a fever of at least 38.5°C, transiently decreased renal concentrating capacity and/or a capillary sedimentation rate of 25mm/h or more, indicating renal parenchymal involvement; cystitis or symptomatic bacteriuria, which presents acute micturition symptoms, an absence of loin pain, temperature not exceeding 38°C and $>10^5$ bacterial/ml of urine; and asymptomatic bacteriuria (ABU) which is diagnosed when there are $>10^5$ bacteria/ml of urine in two consecutive samples (Svanborg-Edén et al., 1976). If the same organism is capable of causing all three types of infection then an added characteristic of the bacteria or a deficiency in the host defence mechanism must be responsible for this difference.

Attachment to human uroepithelial cells from healthy individuals by E. coli, isolated from patients with significant bacteriuria ($>10^5$ bacterial/ml of urine), was examined (Svanborg-Edén et al., 1976, 1977). After the bacteria and epithelial cells

had been incubated together for an hour, the number of adherent bacteria was counted after washing and centrifugation. The E. coli isolated from patients with pyelonephritis adhered in significantly higher numbers to the epithelial cells than the strains responsible for ABU. Adhesion of the cystitic strains was variable. A further study demonstrated that the greatest adhesive ability was found with strains isolated from pyelonephritis cases, adhesion of cystitic strains was intermediate and very little adhesive ability was observed with strains isolated from patients with ABU or from the faeces of healthy children (Svanborg-Edén et al., 1978). An exception to this was strains from serogroup O4:K12 which adhered well to epithelial cells irrespective of the origin of the bacteria. E. coli isolated from patients with asymptomatic reinfections adhered better than the strains which were isolated from the patients at the original screening (Svanborg-Edén et al., 1979), perhaps indicating a role for fimbriae in recurrent UTI. When a single pyelonephritis strain of E. coli was examined, it adhered to epithelial cells from girls prone to UTIs in greater numbers than to the epithelial cells from healthy controls (Källenius and Winberg, 1978), so host factors may also be involved.

The adhesion described above appeared to be mediated by Type 1 fimbriae as the presence of fimbriae on the surface correlated with the epithelial cell attachment and MSHA of guinea pig erythrocytes. However, although D-mannose inhibited the HA, it did not affect the epithelial cell adherence (Svanborg-Edén and Hansson, 1978). It seemed that these two events were separate, with no correlation between the two. This discrepancy was explained when Källenius and

Mollby (1979) found another specific adhesin on the surface of uropathogenic E. coli. The adhesion of this factor to uroepithelial cells was not inhibited by D-mannose and the strain caused MRHA of human erythrocytes. This strain, T1, was not agglutinated by CFA/I antiserum, indicating that the adhesin was distinct from those of enteropathogenic E. coli. Electron microscopy showed that the strain was heavily fimbriated (Källenius and Mollby, 1979).

The same trend of an increase in the percentage of bacteria causing MRHA of human erythrocytes with increasing severity of infection was also observed (Hagberg et al., 1981), and these authors also demonstrated the presence of Type 1 and MR fimbriae on the same cell.

Extraction and purification of fimbriae from uropathogenic E. coli (Korhonen et al., 1980b) and labelling them with ^{125}I showed specific binding to human uroepithelial cells (Korhonen et al., 1980a). Binding was shown to be specific as it was inhibited in the presence of excess unlabelled fimbriae, but not by an excess of an unrelated protein. These purified fimbriae were free from other cell wall and membrane contaminants and they retained MS and MRHA activity (Korhonen et al., 1980a).

Characterisation of different types of fimbriae from UTI E. coli

Two upper UTI E. coli strains, C1212 and C1214, both belonging to serogroup O6:K2:H1 were compared as they expressed different HA activities (Ørskov et al., 1980). Strain C1212 showed MRHA of human erythrocytes and mannose-resistant attachment to uroepithelial

cells, but it did not attach to the urinary mucus. Strain C1214 expressed MSHA of guinea pig erythrocytes and mannose-sensitive attachment to buccal epithelial cells and urinary mucus, this showed very little attachement to uroepithelial cells. Strain C1214 possessed Type 1 fimbriae. Strain C1212 also possessed fimbriae and these were designated as F7 (Ørskov et al., 1980). This F7 antigen showed no antigenic relationship with other classified MR adhesins. This antigen was subsequently found in 7 other O6:K2:H1 strains (Ørskov et al., 1982b). The subunits of the F7 antigen had a molecular weight of 22,000 (Klemm et al., 1982). This antigen was formed by two or three different structures, the first two of which were designated $F7_1$ and $F7_2$. A third structure was designated pseudotype 1 as its amino acid sequence was closely related to the N-terminal amino acid sequence of the Type 1 fimbrial subnit (Ørskov et al., 1982b).

The E. coli F8 antigen was first found in serotype O75:K-:H5, but then to more commonly occur in O18:K5:H- strains (Ørskov et al., 1982b). During a survey of 267 E. coli strains from patients with acute cystitis or pyelonephritis, three new antigens designated F10, F11 and F12 were demonstrated by crossed immunoelectrophoresis. These antigens were shown to be fimbrial in nature by immune electron microscopy, and caused MRHA of human erythrocytes and epithelial cell adherence. The F10 antigen was only found on strains from serogroup O7:K1:H1, and antigens F11 and F12 were found on some O4:K12:H5 strains (Ørskov et al., 1982a). Most of these strains also produced Type 1 fimbriae. The F12 antigen was later shown to be most frequently present on O16:K1:H6 strains. The

antigen was purified from one of these latter strains by ammonium sulphate precipitation and gel chromatography (Klemm et al., 1983). The molecular weight of the subunits was 18,200.

Uropathogenic E. coli fimbriae are also classified by the type of receptors they have on human erythrocytes. The P-fimbriae associated with pyelonephritogenic E. coli recognise the structure α-D-galactopyranosyl-(1→4)-β-D-galactopyranoside which is part of the p^K and P antigenic determinants of the P blood group system (Kållenius et al., 1980a and b). Binding of pyelonephritogenic E. coli to cells of the $\bar{p}$ phenotype is much less. This erythrocyte receptor is also present on uroepithelial cells (Källenius et al., 1981a). Both the globoside (p^K) and trihexosyl ceramide (P) are recognised by the same strains and the attachment can be inhibited by the disaccharide α-D-Galp-(1→4)β-D-Galp (Väisänen et al., 1981). This recognition occurs with 91% of pyelonephritis strains, the other 9% recognising a different receptor structure and being classed as X-fimbriae, although they still cause MRHA of human erythrocytes (Källenius et al., 1981b). Strains can possess both types of fimbriae. A much lower percentage of P-fimbriae are found on isolates from cystitis and ABU (19% and 14% respectively) (Källenius et al., 1981b).

Purified P-fimbriae are morphologically similar to Type 1 but there is no antigenic cross-reactivity between the two. The purified P-fimbriae give multiple bands in SDS-PAGE with molecular weights ranging from 17,000 to 22,000 (Korhonen et al., 1982). These multiple peptides were not antigenically identical and

originated from different filaments, but could be separated by immune precipitation (Rhen et al., 1983c). One of these strains E. coli KS71 which possessed four different fimbrial antigens, A, B, C and D, showed rapid phase variation between the alternate antigenic types (Rhen et al., 1983b). The question arises as to whether the different antigenic types could all be classed as P-fimbriae or if only one component or peptide is responsible for the haemagglutination.

Some pyelonephritic E. coli can agglutinate human erythrocytes which lack the P blood group antigens, and these strains show blood group-M-specific haemagglutination activity (Väisänen et al., 1982a). Three phenotypes MM, MN and NN exist in this system, the M and N antigens being sialoglycoprotein glycophorin A. The difference in specificity between M and N arises from different N-terminal sequences. The fimbriae of the above strains caused strong MR agglutination of the MM and MN phenotype and a very weak reaction with the NN phenotype. The agglutination of the 'M' fimbriae was not inhibited by neuraminidase treatment of the erythrocytes and the receptor was probably part of the N-terminal sequence. Some, but not all, M-fimbriae recognise terminal N-acetyl-D-glucosamine (Väisänen-Rhen et al., 1983).

Other strains, which express MRHA independent of the P-blood group system, have this type of HA inhibited by pretreatment of the erythrocytes with neuraminidase (Parkkinen et al., 1983). These 'S' fimbriae show a strong affinity for (α2-3)-linked sialyl galactosides. The fimbriae could be purified from the bacteria and

were still capable of HA, and gave a single band in SDS-PAGE with an apparent molecular weight of 17,000 (Korhonen et al., 1984).

Purification of an X-specific fimbria from E. coli of serogroup 075 showed it to be a protein with 35-39% hydrophobic amino acids and a subunit molecular weight of 16,000, properties similar to other E. coli fimbriae. There was also antigenic cross-reactivity between different strains possessing these X-fimbriae. Electron microscopically the fimbriae appeared as coil-like structures on the bacterial surface, which bound to erythrocytes, and were therefore morphologically distinct from other E. coli fimbriae (Väisänen-Rhen, 1984).

Properties of an afimbrial adhesin from a urinary E. coli isolate

A non-fimbrial adhesin was recently extracted from a uropathogenic E. coli isolate (Goldhar et al., 1984). Extraction was achieved by heating a broth culture of the organism at 65°C for an hour. The isolated adhesin was capable of producing MRHA of human erythrocytes and inhibiting the adherence of these afimbriate bacteria to tissue culture cells. The adhesin was sensitive to pronase but not to trypsin or periodate, and was destroyed by heating at 100°C (Goldhar et al., 1984).

Other factors associated with urinary tract virulence

There is some controversy over the importance of the K antigens of E. coli in urinary tract infections. Using the mouse model Nicholson and Glynn (1975) showed that UTI could be produced in mice

if the E. coli were inoculated into the bladder after sutures had been inserted in the bladder wall to act as foreign bodies. Infection of the kidneys occurred less frequently than that of the bladder, but the kidney infection was directly proportional to the amount and activity of the E. coli K antigen. In contrast, Verweij-van Vught et al. (1983) compared strains of E. coli isolated from UTIs and mutant strains without K antigen and concluded that the K antigens were of minor importance for mouse nephropathogenicity. How revelvant is the mouse model to human infections? Evans et al. (1981) demonstrated K1 antigen in 29% of their urinary tract isolates, most of the K1-positive organisms being capable of producing MRHA of human and monkey erythrocytes. These two factors also correlated closely with the productionof β-haemolysin.

The production of a haemolysin has also been connected with virulence of E. coli in pyelonephritis (Fried and Wong, 1970). Intravenous injection of haemolytic E. coli produced severe pyelonephritis in 37% of mice challenged, compared to lack of infection produced by non-haemolytic E. coli (Fried et al., 1971). The occurrence of haemolytic E. coli is much more common in UTI isolates than in organisms isolated from the faeces and is associated with other factors implicated in urinary tract virulence (Hacker et al., 1983; Hughes et al., 1982, 1983). Cloning of a DNA sequence encoding the haemolysin gene into a non-haemolytic faecal isolate of E. coli gave the strain a slightly enhanced virulence in rat peritonitis (Welch et al., 1981).

Certain O serogroups become more common among isolates with the increasing severity of infection (Lindberg et al., 1975). Colicin V

synthesis is also more common among UTI isolates (Minshew _et al_., 1978a), but its necessity for virulence could not be shown when the Col V plasmid was transferred to _E. coli_ K12 (Minshew _et al_., 1978b), unlike its importance in calf enteritis (Smith, 1974).

It seems likely that no one factor on its own can be classed as determining the virulence of a bacterium, but rather that a combination of two or more characters each contribute to virulence (Minshew _et al_., 1978a; Hughes _et al_., 1983).

IV GENETIC STUDIES ON E. COLI FIMBRIAE

Methods for studying the fine structure of genes

For complete elucidation of the regulation and expression of genes, it is desirable to remove them from the original host cell, which may possess contaminating factors, into the defined genetic environment of a laboratory strain. If the factor of interest is plasmid-mediated then transfer to an *E. coli* K12 offers some resolution of the problem, except for control by promoters present in the other genes of an unclassified plasmid.

The ideal solution is the cloning of the gene of interest, either from the host chromosome or indigenous plasmid into a vector of which all the properties are known. Positions of any promoters will have already been defined and a detailed restriction enzyme digest map will have been prepared. With cloning it is inevitable that pieces of non-essential DNA will also have been inserted into the vector, but the use of tranposon insertional mutagenesis allows the determination of the size and position of structural and regulatory genes within the cloned DNA.

Using the above mentioned techniques, work on the genetics of most of the major fimbrial types has been carried out.

A brief section on plasmids, transposons and restriction enzymes precede the sections on fimbriae, to aid understanding and clarify some of the terms and techniques which follow.

Plasmids and transposons

Plasmids are extrachromosomal genetic elements that can replicate independently of the bacterial chromosome. Plasmid molecules are composed of covalently closed circular DNA and can range in size from 1 to 300kb. The F factor was the first discovered, then R-factors when it was noted that antimicrobial drug resistance could be transferred between bacteria (Watanabe, 1963). Plasmids have since been detected in most of the bacterial species examined and can encode for a wide variety of functions. Some plasmids, though, have no known function.

Larger plasmids can usually promote their own transfer between strains, whereas smaller plasmids, with less coding capacity in their genome are either incapable of transfer or can be mobilised by a larger plasmid in the same cell. Plasmids can be present in different copy numbers with respect to the host chromosome. Large plasmids of greater than 35kb tend to have a low copy number with only 1 to 4 copies per chromosome. Smaller plasmids usually have much higher copy numbers, and there can be as many as 40 copies per chromosome. These small plasmids are usually almost totally dependent on gene products encoded by the host chromosome, because of their own limited coding capacity. For independent replication of a plasmid it must contain at least one origin of replication (Saunders, 1984).

Transposons are DNA sequences that are capable of transferring themselves from one DNA molecule to another; this transfer is termed transposition. Transposons, unlike plasmids cannot replicate

independently and have to be present in a functional replicon such as a plasmid, bacteriophage or chromosome. Transposable elements can be divided into three classes: Class I - IS like elements. These are short and encode only determinants for their own transposition. Some IS elements exist in composite transposons, e.g. Tn5 possesses copies of IS50; Class II - Transposons with a similar structure and function to Tn3. Most of these encode functions in addition to those required for transposition; Class III - Transposing bacteriophages. Phage Mu uses replicative transposition as the normal mechanism for vegetative replication and prophage insertion (Kleckner, 1981).

The transposition event is *rec*A independent as it can occur without the normal host recombination mechanisms. Transposition is also a replicative process and results in a copy of the transposon in the donor and one in the recipient molecule.

One of the best characterised transposons is Tn3. This has homologous inverted repeat DNA sequences flanking the functional genes, which probably act as recognition sequences for the transposase enzyme. This enzyme is the product of the *tnp*A gene of the transposon. The enzyme cuts the donor and target DNA molecules in preparation for transposition and subsequently joins the two molecules to form a cointegrate. In some cases a transposon also encodes a resolvase enzyme, the product of the *tnp*R gene of the transposon. This enzyme resolves the cointegrate at a specific *res* site on the transposon. The resolvase enzyme can also regulate its own synthesis as well as that of the transposase enzyme.

Transposons that do not encode a resolvase rely on the host cell for resolution (Saunders, 1984).

Most transposons exert strong polar effects on expression of genes in an operon which lies distal to the site of the inserted element. The polarity of these elements is thought to involve transcription termination mechanisms.

Restriction enzymes and gene cloning

It was found that when bacteriophage λ was grown upon cells of E. coli strain C, and then used to infect E. coli K12 cells, its DNA was degraded by enzymes present in the cell that recognised it as foreign. The DNA of the recipient was not degraded as it had been chemically modified by methylation of certain adenine and cytosine bases. The restriction enzymes responsible for the degradation are endonucleases which recognise specific sequences in the foreign DNA. Restriction enzymes have been isolated from many bacterial species and there are two classes (Type I and II). Type II are the most important in genetic manipulations.

The target sequences of Type II restriction enzymes have a two-fold axis of symmetry in the nucleotide bases. Enzymic cleavage can result in a clean (blunt) or staggered ('sticky') break. The staggered cleavage gives fragments with single stranded ends, usually of 2 or 4 bases, depending on the size of the recognition sequence, which have a free 5' or 3' end. These single-stranded ends will be complementary to each other, and to those of other fragments cleaved by the same enzyme, and they can be joined by

hydrogen bonds. These hydrogen bonds can be converted to covalent links by the action of ligases produced by E. coli or 'phage T4. Hybrid molecules can be formed by mixing digested plasmid DNA and similarly digested DNA from another source and allowing ligation in vitro. These hybrids can then be transformed into bacterial cells (Broda, 1979).

DNA fragments generated by cleavage with the enzyme can be resolved by agarose gel electrophoresis to allow determination of their size. Every plasmid has a unique combination of restriction endonuclease target sites and, therefore, different enzymes give different size fragments when cutting the same plasmid. By analysing the resultant patterns it is possible to map the position on a plasmid of the isolated fragments. These physical maps of plasmids can be converted to genetic maps by a comparison of plasmids produced by deletion mutations, or plasmids where insertion of a transposon has inactivated the function of a gene (Broda, 1979).

The enzyme generated DNA fragments can be transferred in a single-stranded form from an agarose gel to a nitrocellulose filter. The filters are then soaked in a solution containing radio-actively labelled single-stranded probe DNA and hybridisation allowed to occur. DNA homologous to the probe DNA will form duplexes and the position of the radiolabelled DNA can be determined and related to the position of DNA fragments on the original gel.

The DNA fragments produced by restriction endonuclease digestion can be cloned into a vector to form a hybrid molecule,

which after transformation into a bacterial host will also allow the matching of a genetic function to a region of the original plasmid.

An ideal plasmid vector for cloning should have a low molecular weight for more efficient transformation and a higher copy number, single sites for a large number of restriction enzymes so that only one fragment will be produced when the vector is digested, and a readily selectable phenotype for the easy isolation of recombinant clones. The most widely used artificial plasmid vector is pBR322 which contains ampicillin and tetracycline resistance determinants and replication genes (Old and Primrose, 1980).

Hybrid DNA molecules can be introduced into bacterial recipients by transformation or the in vitro packaging of the DNA into 'phage followed by transduction. The double-stranded linear DNA of 'phage λ has short single-stranded 5' projections of 12 nucleotides whose sequences are complementary. These are used when the DNA circularises when it is injected into the bacterial host, the cohesive termini associating to form the cos site. DNA can be efficiently packaged into 'phage particles if the cohesive ends are 33 to 46kb apart (the maximum size for the λ genome). Only a small region surrounding the cos site is required for recognition by the packaging system, so it was possible to construct plasmids which contained the λ cos site and normal plasmid functions (Collins and Brüning, 1978; Collins and Hohn, 1978). These plasmids were called cosmids and can be used as cloning vectors with the in vitro packaging system, if the ligated recombinant DNA molecule is

supplied with high concentrations of 'phage head precursor, 'phage tails and packaging proteins (Old and Primrose, 1980).

Packaging the recombinant cosmids into the 'phage heads limits the size of inserted DNA fragment, suitable sizes of which can be produced by partial digestion, with a restriction enzyme, of the DNA from which the insert will be derived. Following packaging in vitro the recombinant cosmid DNA is injected into the host cell where it circularises due to the 'phage cos site, but then replicates as a normal plasmid.

Cloning and organisation of the Type 1 fimbrial genes

The genes encoding production of E. coli Type 1 fimbriae are chromosomally determined, therefore to clone the gene the whole bacterial chromosome has to be digested by restriction enzymes. Hull et al (1981) were able to isolate suitable length chromosomal fragments of E. coli strain J96 by partial digestion with the restriction endonuclease Sau3A and ligate these fragments to the ampicillin resistant vector, pHC79. The in vitro packaging of these cosmids by 'phage λ allowed transduction of the recombinant molecules into a non-fimbriated laboratory strain. Of the ampicillin resistant transductants obtained about 1% were shown to be fimbriated by a positive haemagglutination test and electron microscopy. Comparison of the restriction map of the cosmid with a plasmid present in the original strain showed no similarities, confirming that this gene was chromosomally determined (Hull et al., 1981).

Plasmid pSH2 was obtained by further restriction of the 40kb DNA insert in pHC79, and the cloning of a 11.2kb fragment containing the gene for Type 1 fimbriae into the vector pACYC184 (Orndorff and Falkow, 1984a). Insertion and deletion mutagenesis of plasmid pSH2 with transposon Tn5 and the subcloning of parts of the pSH2 fimbrial gene allowed the localisation of five genes within the 11.2kb fragment responsible for construction and expression of the Type 1 fimbriae. Each gene encoded a polypeptide, and three of these, 17,000, 86,000 and 30,000d in size, were involved in assembly of the fimbriae. Assembly of the 17,000d fimbrial subunits into fimbriae was not seen in mutants which lacked either the 86,000 or 30,000d polypeptides, but the subunit synthesis and processing was unaffected. Another polypeptide of 23,000d was involved in regulation of expression of the fimbriae, as mutants in this region lead to a 40-fold increase in the amount of fimbrial antigen per cell. The fifth, 14,000d polypeptide did not appear to be genetically associated with fimbriation, but it did immunoprecipitate with the fimbriae, suggesting some involvement. The organisation of the five genes within the 11.2kb fragment was determined and it appeared that a promoter for transcription of the genes was present within the operon, as Tn5 mutagenesis at either end of this region had no effect on synthesis of the fimbriae (Orndorff and Falkow, 1984a).

Further work on the hyperpiliated (Hyp) mutants showed that the increase in the amount of fimbrial antigen was manifested as an increase in number and length of the fimbriae on the cell (Orndorff and Falkow, 1984b). None of these mutants produced the 23,000d

polypeptide described above, but the introduction of this gene, on a plasmid, into the cells reduced the amount of fimbriation to almost normal. It therefore appeared that the 23,000d polypeptide is an inhibitor which acts in _trans_ to regulate the level of expression of the gene cluster (Orndorff and Falkow, 1984b).

Type 1 fimbriae are produced by many strains of enterobacteria, and the genes endcoding the production of _Klebsiella pneumoniae_ Type 1 fimbriae have been cloned (Purcell and Clegg, 1983). Chromosomal DNA fragments were again prepared by restriction enzyme digest, cloned into vector pMF7 and packaged by λ. About 1% of the transductants possessed the fimbrial gene. The fimbrial genes were subcloned into pBR322 and pACYC184. The smallest insertion capable of producing MSHA of guinea pig erythrocytes was 11.1kb. Tn5 insertions into this fragment showed a region of 5.5kb necessary for the production of fimbriae.

There is a similarity in chemical composition between purified Type 1 fimbriae of _E. coli_ and _K. pneumoniae_, despite a lack of antigenic cross reactivity (Fader _et al._, 1982). Possibly, therefore, distinct DNA sequences have evolved which encode related adhesins. The _E. coli_ transconjugants which possessed the recombinant plasmid did not exhibit Type 1 fimbrial phase variation. Purcell and Clegg (1983) hypothesised that this might be due either to an excess of fimbriae because of the high copy number of recombinant plasmid, or because the regulatory gene which controls phase variation had not been cloned.

Cloning of the K88 antigen variants

The K88ab antigen was cloned with the associated raffinose utilisation determinant (Raf) into pBR322 (Mooi et al., 1979). The two determinants were located on a 72.5kb plasmid, pRI8801, which was digested with restriction enzymes *Hind*III and *Sal*I and the fragments ligated to pBR322. Six K88-positive clones out of 257 tested were obtained, each clone expressing the K88 antigen at a different level. The smallest insert in the recombinant plasmids was 11.2kb, but all the cloned fragments expressing the K88-positive phenotype at a high level were in the same orientation. In the reverse orientation, barely detectable levels of K88 antigen were produced. None of these clones expressed the Raf phenotype. One clone containing a 5.8kb insert in pBR322 was Raf-positive, K88-negative. The generation of deletions within the cloned K88 DNA fragment demonstrated that a 6.2kb region was essential for K88 expression. This region was ligated into pBR322 to give pFM205. No difference in structure of K88 between the recombinant and parent strains could be detected. However, an enzyme-linked immuno-absorbent assay (ELISA) demonstrated that the recombinant strain produced four times as much antigen as the parent. The K88 and Raf genes were not closely linked, but separated by about 29kb (Mooi et al., 1979).

The presence of pFM205 in minicells was used to study the expression of the genes of the cloned K88ab DNA fragment (Mooi et al., 1981). The presence of seven polypeptides with apparent sizes of 81,000, 30,000, 29,000, 27,500, 26,000 and 17,000d were

detected. The 26,000d polypeptide reacted with specific anti-K88ab antiserum indicating that it was the fimbrial subunit, which was translated as a precursor molecule.

Deletion mutants of pFM205 were generated, each containing a deletion in one of the five K88 genes (Mooi et al., 1982) and the gene products analysed in minicells. In strains containing a deletion in the gene for the 17,000 or 81,000d polypeptides the K88ab subunit was synthesised and transported to the cell surface in reduced amounts, but no adhesion or HA was observed, suggesting possible incomplete assembly into the mature fimbriae. Strains with a deletion in the 27,500 polypeptide gene expressed the adhesive phenotype, although very little K88ab antigen could be detected. Those strains possessing a plasmid with a deletion in the gene for the 27,000d polypeptide synthesised the K88ab subunit, but it appeared to be rapidly degraded (Mooi et al., 1982).

The 26,000d fimbrial subunit transiently accumulated in the periplasmic space before translocation across the outer membrane, probably by the 81,000d polypeptide (Mooi et al., 1983). Deletions in this gene resulted in the subunits remaining in the periplasmic space where they were associated with the 17,000 and 27,000d polypeptides. The 27,000d polypeptide might be involved in stabilising the conformation of the subunits required for translocation. In mutants lacking the 17,000d polypeptide most of the subunits were present in the periplasmic space, associated with the 27,000d polypeptide, with small amounts translocated across the outer membrane, but these subunits were incapable of adhesion suggesting

the 17,000d polypeptide has a role in modification before assembly (Mooi et al., 1983).

Proximal to the gene for the 81,000 polypeptide is a region of approximately 1,000bp which codes for the additional structural gene, gene A, which is involved in the biosynthesis of the K88ab adhesin and produces a polypeptide (Mooi et al., 1984). A deletion in this gene resulted in the accumulation of fimbrial subunits inside the cell, it therefore appeared that the gene product was required for export of the subunits. The polypeptide was not detectable, but determination of the nucleotide sequence of gene A allowed an estimation of the primary structure and approximate molecular weight of 17,600 for this polypeptide (pA). pA has many characteristics in common with fimbrial subunits, raising the possibility that there might be two interdependent surface structures, the K88ab adhesin, and the fimbrial subunit (Mooi et al., 1984) as with the Pap fimbriae (Normark et al., 1983).

The determinant for the K88ac antigen was cloned into the vector pBR313 from a K88/Raf plasmid (Shipley et al., 1981). The size of the inserted fragment in the K88-positive phenotype was 11.9kb. This fragment was then cloned into pBR322 to give pPS002. pPS002 was transformed into a minicell producing strain and at least six polypeptides were detected. One of these polypeptides with a molecular weight of 23,500 precipitated with K88ac antiserum, it was therefore assumed that this was the fimbrial subunit, and was present in very large amounts. The other polypeptides ranged in size from 18,000 to 70,000d (Shipley et al., 1981).

Deletion and Tn5 insertion mutants of pPS022 were generated. The deletion of 5kb from the cloned fragment still resulted in a functional K88-positive phenotype. The vector containing the 6.5kb insert was designated pMK005 (Kehoe et al., 1981). Tn5 insertion mutants of pMK005 were isolated and mapped. From the position of the mutations and the resulting phenotypes, it appeared that there were two regions of the K88 operon. One mutant was adhesive and $K88^+$, but $MRHA^-$, indicating that the two properties were not identical. In minicells, PMK005 expressed at least four polypeptides with molecular weights 70,000, 29,000, 23,500 and 17.,000d. Four cistrons adh A, B, C and D were demonstrated. adhA, B and C formed one operon and adh D the second operon. adh D encodes the 23,500d fimbrial subunit, adh C the 17,000d polypeptide, adh B the 29,000d polypeptide and adh A the 70,000d polypeptide. It seemed possible that the gene products of adh C acted as a positive regulator for expression of the adh D cistron, and in the absence of the adh A product the K88 antigen was shed into the supernatant instead of being assembled into fimbriae. It is therefore possible that the 70,000d polypeptide forms part of the structure attaching the fimbriae to the cells (Kehoe et al., 1981).

The presence of the recombinant plasmids in minicells also allowed the localisation of the polypeptides within the cell (Dougan et al., 1983). The 70,000d polypeptide was located mainly in the outer membrane. The 26,000 and 17,000d polypeptides were located in the periplasmic space - the 29,000d polypeptide being processed to give the 26,000d polypeptide, and the fimbrial subunit could be detected in the inner and outer membranes.

Expression of the K88ac genes of pMK005 was dependent on the P1 promoter of pBR322. If this promoter was replaced by that of the Trp operon, high levels of K88ac antigen were expressed when the promoter was repressed and growth of the cells was inhibited when the promoter was derepressed (Kehoe et al., 1983). It appeared that a single transcription unit initiating at the P1 promoter was involved in expression of the genes as the polar effect produced by the various Tn5 insertions all reduced the levels of K88ac antigen. Mutations in the proximal end of the cloned sequence resulted in a lack of the antigen on the surface although low levels of the fimbrial subunit could be detected in the cells. Mutations in this proximal region could be complemented in trans and the region was designated adh E. Because of the trans complementation adh E must have its own promoter, but could not stimulate K88 biosynthesis in mutants lacking the P1 promoter (Kehoe et al., 1983).

The genes encoding the K88ad variant were cloned by J.H. Meijerink (unpublished results) and from the DNA sequence of the cloned gene the amino acid sequence of the K88ad subunit was deduced (Gaastra et al., 1983).

Similarities exist between the organisation of the K88 and Type 1 operons. Both have a similar number of genes which are required for synthesis of the fimbriae, and the genes involved in assembly of the fimbriae are all clustered together. No equivalent to the 23,000d regulatory polypeptide was found in the K88 operon however (Orndorff and Falkow, 1984a).

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Cloning and organisation of the K99 antigen genes

The determinant for the production of the K99 antigen was cloned from plasmid pRI9901 into E. coli K12. This plasmid originated from the K99 reference strain, E. coli B41, and also codes for streptomycin resistance, but ST and LT are not produced (van Embden et al., 1980). HindIII digestion of the plasmid was used to insert fragments into pBR322 with a loss of the tetracycline resistance determinant. Recombinant plasmids were used to transform E. coli K12 strain C600 with selection being made for ampicillin resistance and transformants were screened for tetracycline sensitivity. One out of 45 transformants tested was K99-positive, and the recombinant plasmid in this strain was designated pRI9902. Mapping of the plasmid revealed a 16.1kb insertion into pBR322. The K99 determinant of pRI9902 was subcloned into pBR325 and a 6.5kb fragment of the original K99 insertion was capable of expressing K99 antigen. Expression was still obtained if this fragment was in the reverse orientation indicating that it was independent of vector transcription and organised in one operon (van Embden et al., 1980).

The level of K99 production in the C600 transformants was higher than that of the K12 strains possessing pRI9901 due to plasmid copy number, but still lower than in the original wild type strain. This must be due to host factors, as there was no difference in the serology, adhesion or morphology between the strains possessing the recombinant plasmid and the original isolate, B41 (van Embden et al., 1980).

The 6.5kb K99 DNA fragment was cloned back into pBR322 to give plasmid pFK99, to give clearer expression of the polypeptides encoded by the fragment in minicells (de Graaf et al., 1984). Eleven polypeptides were expressed by the cloned K99 DNA, but the total molecular weight of these exceeded the coding capacity of the DNA fragment. Therefore, some of these polypeptides must be the precursor forms of others. By inhibiting protein processing with ethanol the polypeptides representing the mature structures could be determined. It appeared that the cloned K99 DNA contained at least seven structural genes encoding polypeptides of 19,000, 21,000, 21,500, 26,500, 33,500, 76,000 and 18,200d - the fimbrial subunit (de Graaf et al., 1984).

In order to analyse the organisation of these structural genes deletion mutants of pFK99 were constructed by restriction-ligation, the position of the deletions and loss of production of certain of the polypeptides determing the arrangement. The subunit gene was at the proximal end of the operon, similar to the pap gene, but in contrast to the position of the K88 subunit gene. It is probable that the 19,000 and 26,500d polypeptides have a function in K99 biosynthesis similar to the 17,000 and 27,000d polypeptides involved in K88 production. A deletion in the 19,000d polypeptide gene reduced the amount of antigen produced, whereas a deletion in the 26,500d polypeptide gene completely prevented the production. No indications were obtained for the functions of the 21,00 21,500 and 33,500d polypeptides (de Graaf et al., 1984). Both the K88 and K99

operons encode high molecular weight polypeptides which are probably involved in anchoring or assembly of the fimbriae.

The nucleotide sequence of the gene encoding the K99 subunit has been determined (Roosendaal et al., 1984). The gene was 181 codons long including a signal peptide of 22 codons, which is very similar to the structure of the Pap fimbrial subunit.

Construction of vectors coding for Pap fimbriae formation

The genes for production of MR fimbriae from uropathogenic E. coli were first cloned by Hull et al. (1981) from the pyelonephritic E. coli isolate J96 (O4:K6). This was the same organism from which the Type 1 fimbriae were cloned and the same methods were employed as described previously (Hull et al., 1981). The MR fimbriae of this strain were antigenically cross-reactive with those isolated from another pyelonephritic E. coli by Korhonen et al. (1980b), those fimbriae having a subunit molecular weight of 17,000.

The recombinant strain obtained expressed MRHA of human erythrocytes alone and was heavily fimbriated when examined electron-microscopically. Restriction enzyme analysis of the recombinant plasmid again showed that the gene had been derived from the chromosome. These fimbriae, designated Pap or P, had the same subunit molecular weight, functional properties and antigenicity as those on the wild type strain (Hull et al., 1981).

Restriction enzyme Eco RI fragments from the recombinant clone were subcloned into the vector pACYC184. A resultant hybrid plasmid

containing a 20kb insert still expressed the MRHA phenotype (Normark et al., 1983). Further deletion and ligation of this hybrid plasmid reduced the size of fragment required for expression of MRHA to 11.8kb. The fimbriae expressed by this fragment still possessed the same subunit molecular weight as the parent strain fimbriae. Further transposon insertion/deletion mutagenesis suggested that an 8.5kb region of the fragment was the minimum amount of DNA required to produce functional fimbriae. Mutations at the proximal end of the 8.5kb fragment gave rise to a reduced titre of HA activity (Normark et al., 1983).

Analysis of the polypeptides produced by the cloned fragment in minicells showed the expression of 12 polypeptides with molecular weights ranging from 81,000 to 12,000 with six other minor polypeptides, molecular weight range 82,000 to 17,500. The Pap fimbriae subunit was identified as the 19,500 polypeptide by immunoprecipitation with fimbriae antiserum. Five of the minor polypeptides were shown to be precursor forms of the major polypeptides by inhibition of protein processing (Normark et al., 1983).

Subcloning from the fragment suggested that the gene encoding the Pap fimbrial subunit (papA) was at the proximal end to the right of the region where mutations gave rise to reduced HA activity. Mutations in the papA gene resulted in an absence of Pap antigen on the cell surface, though one mutant did express weak MRHA, therefore the possibility arose that the fimbrial subunit and adhesin are separate molecules (Normark et al., 1983).

The organisation of the genes within the Pap DNA fragment was determined by Norgren _et al_. (1984) employing subcloning, Tn5 insertional mutagenesis and cut back derivatives and examining the protein products by SDS-PAGE. Five genes designated _pap_A to _pap_G were identified as being important in Pap fimbriae formation. The region proximal to the structural genes contains promoters for transcription and translation. Tn5 insertions in _pap_B markedly decreased the amount of antigen expressed, suggesting a possible role in regulation. Insertions in _pap_A abolished expression and formation of fimbriae. No detectable surface antigen was found on strains with mutations in _pap_C and _pap_D, but a small amount of antigen was detected in cell extracts, these genes may therefore have a role in localisation of the fimbriae on the surface. Mutations in _pap_A, B, C and D also repressed MRHA activity. Mutations in _pap_E, F and G repressed MRHA activity, but the amount of Pap antigen was unaffected, and fimbriae could be observed on the cells. This region is obviously not required for fimbriae formation, but for the production of MRHA (Norgren _et al_., 1984; Lindberg _et al_., 1984).

Construction of a frameshift mutation in _pap_A inhibited agglutination of antiserum and no _pap_A gene product was produced (Lindberg _et al_., 1984). This frameshift mutation had little polarity effects on the distal _pap_ genes, and the mutant was still capable of agglutinating P1 erythrocytes. Tn5 insertions in the distal region of the Pap fragment inhibited HA, but had no effect on fimbriae formation. The region encoding _pap_E, F and G was subcloned into pBR322 and, if it was provided with a promoter, could

complement mutations with Tn5 insertions in this region. Polarity effects of the Tn5 insertions in _papE_, F and G showed that transcription of all three genes was from left to right. Both the _papF_ and _papG_ genes are essential for HA activity, however, mutations in _papE_ did not affect the HA titre. A strain with mutations in _papA_ and _papE_ failed to agglutinate P1 erythrocytes. Fimbriae prepared from strains with mutations in _papE_, F or G had the same apparent molecular weight, as determined by SDS-PAGE, as pili from the wild type strain (Lindberg _et al._, 1984).

Determination of the nucleotide sequence of the _papA_ gene gave a sequence of 701 base pairs (Båga _et al._, 1984). Translation of this sequence gave a 185 amino acid containing protein, the predicted sequence giving a mature protein with a molecular weight of 16,500 daltons.

Hybridisation using _pap_ operon DNA as a probe, revealed that homologous DNA sequences were present in a large number of uropathogenic _E. coli_, whereas homology was not normally found with the DNA of faecal _E. coli_ (Båga _et al._, 1984). Further work indicated though that the _papA_ gene could have evolved within the species as most _E. coli_ express Type 1 fimbriae which have an N-terminal amino acid sequence very similar to the Pap fimbriae. Examination of other _E. coli_ UTI isolates showed that gene sequences homologous to the entire _pap_ operon existed at multiple sites in the chromosome, but the DNA sequences adjacent to the operon were variable (Hull _et al._, 1985).

Hybridisation of the pap operon with DNA from eight genera of extraintestinal enterobacterial isolates including E. coli showed that homologous sequences were present among E. coli from all the sites of infection tested (Hull et al., 1984). All the isolates were tested for MRHA of human erythrocytes, but only the E. coli and two Morganella species gave positive results. However, the DNA from both the Morganella showed no homology with the probe DNA. Not all MRHA-positive E. coli were also hybridisation-positive and vice versa, this might be due to the existence of an incomplete operon. From these results it was concluded that Pap fimbriae were restricted to E. coli (Hull et al., 1984).

The A, B and C components of the P-fimbriae of E. coli KS71 (O4:K12) have also been cloned (Rhen et al., 1983a). The cloning was performed using restriction enzyme Sau3A and the pHC79 cosmid vector, as described previously (Hull et al., 1981). Recombinant strains were tested for the presence of fimbriae with anti-KS71ABC antiserum. Purification of the fimbriae from positive strains and visualisation by SDS-PAGE analysis showed that each recombinant strain produced only one of the KS71 fimbrial antigens (Rhen et al., 1983a). The purified fimbriae from these three different classes of recombinants were morphologically identical when observed by electron microscopy. Agglutination of P1 erythrocytes only occurred when recombinants expressed A or B fimbriae, no agglutination of P1 or $\bar{p}$ erythrocytes was observed with C fimbriae. A and B fimbriae were also antigenically cross-reactive. It seems likely that the genes for the production of the three types of fimbriae are located on different parts of the chromosome (Rhen et al., 1983a). It is

possible that the fimbrial components of E. coli KS71 might be analogous to the F7 antigenic components of E. coli, because of similarities in their molecular weight and N-terminal amino acid sequence (Rhen et al., 1983a).

Molecular studies on the F7 fimbrial antigen

The F7 fimbrial antigen gene from E. coli AD110 or C1212 (O6:K2) (Ørskov et al., 1980) , an isolate from a patient with upper UTI was cloned into the vector pJB8 and transduced by 'phage λ into the E. coli K12 strain JA221 (van Die et al., 1983). Of the recombinant strains obtained, two out of 800 examined expressed MRHA of human erythrocytes. Homology between the recombinant plasmids present in these two strains was shown by DNA-DNA hybridisation. The plasmids had a common 16kb insert, which was subcloned into pACYC184 to give plasmid pPIL110-35. The resultant recombinant strain was capable of producing stronger MRHA than the wild type parent, and the strains were heavily fimbriated when examined with the electron microscope (van Die et al., 1983).

The purified fimbriae of AD110 consist of three subunits of different molecular weights. Analysis of the fimbriae of the recombinant strain by SDS-PAGE revealed that only the 17,000d subunit was present, later shown to be $F7_2$. Preliminary evidence showed that at least five genes within a 9kb region of the cloned fragment were involved in assembly of the fimbriae (van Die et al., 1983).

Transposon Tn5 and $\gamma\delta$ (a Tn3-like element originating from the E. coli F factor) insertion mutants of pPIL110-35 produced either MRHA-positive or negative phenotypes. The site of the insertions was mapped and it was shown that all MRHA-negative strains possessed insertions within an 11.5kb restriction fragment (van Die et al., 1984) and the mutations were located in four domains. Due to the polar effects of Tn5, it appeared that four transcriptional units were involved in the expression of the MRHA phenotype. The 11.5kb fragment was subcloned into pBR322 and the resultant recombinant plasmid was capable of expressing surface fimbriae. Complementation studies between subclones of the pBR322 recombinant and insertion mutants distinguished five complementation groups, implying that five genes, A to E, are involved in the formation and expression of fimbriae. A mutation in any of these genes lead to loss of fimbriae on the surface (van Die et al., 1984).

The products of the genes were analysed in minicells, revealing four proteins of molecular weights 75,000, 36,000, 23,000 and 17,000. Gene B synthesised the 75,000, gene C the 23,000, and gene E the 36,000 polypeptides. No product of gene D was found, which could be due either to low level of production or that the gene has a regulatory function. The 17,000 protein is probably the product of gene A, but this could not be confirmed because of the presence of another 17,000d polypeptide. Immunoprecipitation revealed that the 17,000d protein is the fimbrial subunit with an 18,000d precursor. Mutations in the region encoding the second 17,000d polypeptide had no effect on MRHA ability (van Die et al., 1984).

Genetic analysis of the fimbrial genes of other E. coli O6 isolates

Clegg (1982) also cloned the chromosomal determinant for MR fimbriae from an E. coli O6, H^{-} isolate (strain IA2), from an acute urinary tract infection. The bacterial chromosome was partially digested with EcoRI restriction endonuclease and the fragments cloned into the ampicillin resistant cosmid vector pMF7. One out of 148 ampicillin resistant transductants tested expressed MRHA ability. The large, inserted fragment of the recombinant plasmid was again subcloned into pACYC184. Additional subcloning of a 6.9kb fragment into pBR322 to give plasmid pDC5 resulted in a MRHA-positive phenotype, which was expressed at 37°C but not 18°C. Recombinant plasmids possessing smaller fragments were MRHA-negative. Despite the presence of the gene on a high copy number vector there was no appreciable increase in the HA titre between the recombinant strain and the wild type parent (Clegg, 1982). This implies that the fragment encoding the fimbrial genes must also code for a regulatory gene which controls the amount of fimbriae produced.

Tn5 insertion mutations in the cloned fragment of pDC5 resulted in the identification of a 4.5kb region essential for HA ability (Clegg and Pierce, 1983). Different HA-negative mutants could complement one another to restore the HA activity, and four complementation groups were distinguished. Insertions of the transposable element, mini mu, which carried a promoterless lacZ gene indicated that transcription of the genes was left to right, and more than one operon was present (Clegg and Pierce, 1983). The

presence of at least four polypeptides was detected in minicells possessing pDC5, the molecular weights of the polypeptides were 71,000, 45,000, 27,000 and 17,000. The 17,000d polypeptide was identified as the fimbrial subunit by immune precipitation. Mutants possessing deletions in the genes for the three other polypeptides still synthesised the fimbrial subunit, indicating that transcription and translation of this gene did not require the products of the other genes. There was also a possible fifth, regulatory, polypeptide as deletions in the region outside the four genes resulted in no production of the 71,000 and 45,000d polypeptides (Clegg and Pierce, 1983).

A large degree of restriction map homology between this operon and the one described by Normark et al. (1983) existed, but significant differences, such as the position of the gene for the fimbrial subunit and a lack of cross-reactivity between the fimbriae were obvious (Clegg, 1982).

Partial digestion by enzyme Sau3A of the chromosome from an E. coli O6 UTI isolate, strain 536, and an E. coli O18 faecal isolate followed by cloning of the DNA fragments into vector pJC74, gave rise to one MRHA-positive recombinant out of 600 tested from the faecal isolate and one MRHA-positive clone out of 200 tested for the uropathogenic E. coli isolate (Berger et al., 1982). Restriction enzyme analysis of the cloned fragments showed different patterns for the two types. The clones were unstable, exhibiting a high segregation rate unless cultured in the presence of ampicillin to select for the vector.

The cloned fimbrial genes from E. coli 536 were later classified as X-fimbriae, because neuraminidase treatment of the erythrocytes prevented MRHA (Hacker et al., 1985). Two different fimbrial subunits of 16,500 and 22,000d were synthesised by strain 536. The MRHA-positive cosmid clones derived from this strain produced the 16,500 but not the 22,000d protein and all were fimbriated. Subcloning of the X-fimbria determinant gave rise to two recombinant phenotypes, haemagglutination and fimbria-positive and haemagglutination-positive but lacking fimbriae. These subclones were stable, unlike the original recombinant plasmid. Tn5 mutagenesis demonstrated that a DNA fragment of 6.5kb was required for HA and fimbriae production, a 1.5 to 2kb region adjacent to the MRHA gene encoded the fimbrial structural protein. These results again indicated that haemagglutination and fimbriae, although closely related, are two separate entities (Hacker et al., 1985).

Cloning of an afrimbrial adhesin, AFA-1

About 10% of E. coli which express MRHA do not produce any visible fimbriae (Duguid et al., 1979). Labigne-Roussel et al. (1984) cloned the chromosomal gene for an afimbrial, P blood group independent adhesin from E. coli KS52 (O2), isolated from a case of pyelonephritis. The DNA fragments from the partially digested chromosome were cloned into the cosmid vector pHC79. Three of 400 transductants screened expressed MRHA. Analysis of the inserted fragments in the three recombinant plasmids revealed a common sequence of approximately 25kb. All or part of this common region was subcloned into pBR322 followed by further subcloning of the

fragment into pBR322. A recombinant plasmid designated pIL14 contained a 6.7kb insert which conferred all the properties associated with the MRHA-positive phenotype on the recipient cell. Inserted fragments which were slightly smaller than this (6.1kb) gave a negative MRHA reaction when broth supernatant fluids were tested, although whole cell suspensions were positive. The expression of the former property also appeared to be orientation dependent (Labigne-Roussel et al., 1984).

The protein adhesin was purified from the supernatant fluid by ammonium sulphate precipitation and dialysis. SDS-PAGE analysis showed a major protein band of 16,000d, which was present in K12 and minicell clones. This protein was designated AFA-1. The presence of pIL14 in K12 also conferred the ability to adhere to uroepithelial cells. No homology was found between the cloned 6.7kb insert and the regions of the E. coli J96 chromosome which code for Type 1 and P fimbriae (Labigne-Roussel et al., 1984).

Linkage between the genes for F7 and Pap fimbriae and haemolysin production

Cloning of large chromosomal fragments into vector pHC79 from E. coli strains J96 (Minshew et al., 1978a) and C1212 (Ørskov et al., 1980) which both expressed haemolysin (Hly) and MRHA gave rise to some clones which expressed both phenotypes (Low et al., 1984). These results indicated that the two genes were closely linked. Hybridisation between the cloned DNA fragments from two strains with phenotypes Hly-positive, MRHA-positive and Hly-

negative, MRHA-positive, and probes for the MRHA gene (mrh) constructed from genes of the pap operon, suggested that there were two distinct gene clusters encoding MRHA ability. One cluster was linked to the haemolysin gene (hly) and was present on two restriction fragments of 5.8 and 7.2kb, the other cluster was not linked to hly and was present on two fragments of 3.8 and 3.9kb. The mrh and hly genes of strain C1212 were separated by 4 to 5kb on the recombinant plasmid, whereas the two genes on the recombinant plasmid derived from strain J96 were separated by 14 to 15kb. Therefore, although the two genes are closely linked the intervening distance can vary (Low et al., 1984). Further hybridisation experiments with other E. coli O6 isolates also showed linkage between the two gene clusters, although again the distance between them was variable.

Analysis of the regions flanking hly showed homologous regions about 1kb in length. Additional homologous sequences were also present in other strains. Hybridisation showed that the DNA between the gene clusters was shared by most non-pathogenic faecal E coli, even though they lacked homology with hly and mrh (Low et al., 1984).

The variable distance between the two gene clusters suggested that linkage had occurred through several independent events, or by a single event followed by rearrangement. If the sequences flanking the hly genes were similar to insertion sequences, transposition of the gene to the region close to the mrh gene could have occurred, explaining the homology between the DNA separating the gene clusters and the chromosome of faecal E coli (Low et al., 1984).

Transposition of the *hly* gene has not been demonstrated (Hacker *et al*., 1983), the ability to transpose may have been lost during the course of evolution. Linkage of the two gene clusters could have arisen from a selective advantage conferred by this association (Low *et al*., 1984).

Hacker *et al*. (1983) observed that mutants of one UTI *E. coli* isolate which spontaneously lost its ability to synthesise haemolysin also lost MRHA ability, which supports the view that these two genes are linked.

V OVERCOMING BACTERIAL PATHOGENICITY

Resistance to adherence due to genetic factors

It was observed that K88-positive *E. coli* did not adhere to the brush borders of all pigs. The difference between these pigs arose due to simple Mendelian genetics. The pigs with brush borders that did not allow adherence of K88-positive *E. coli* appeared to possess a natural resistance to infections with these organisms (Sellwood *et al*., 1975).

The adhesion-positive and -negative pigs arise from the products of two alleles at a single locus, the positive phenotype being dominant. The adhesion-negative piglets have a much greater survival rate than the adhesion-positive piglets when challenged with K88-positive *E. coli* (Rutter *et al*., 1975).

Further work on these pigs distinguished five different phenotypes (A-E) which had differing susceptibilities to the K88 antigenic variants. Phenotype A was susceptible to adhesion by K88ab, ac and ad, phenotypes B and C were susceptible to two of the variants, phenotype D to only one and phenotype E was resistant to adhesion of all three variants (Bijlsma *et al*., 1982). K88ad did not inhibit adhesion of K88ab and ac, but K88ab and ac could completely inhibit adhesion of the K88ad variant, and K88ab blocked K88ac adehsion.

It would appear from these results that the adhesion-negative piglets had lost the cell surface receptor for the fimbriae, thereby preventing adhesion.

By expanding on this theory, attempts were made to prevent bacterial adhesion and colonisation with receptor analogues. If mice were challenged with E. coli bearing Type 1 fimbriae, approximately 70% of the animals showed bladder colonisation. The administration of α-methylglucoside to the animals did not alter the colonisation, but animals challenged with E. coli in α-methylmannoside only gave a colonisation rate of 20% (Aronson et al., 1979).

Adherence of P-fimbriated E. coli can also be blocked by the synthetic disaccharide receptor, but the adherence might also be enhanced if this disaccharide then becomes incorporated into the cell membranes (Källenius et al., 1980b, 1981a; Leffler and Svanborg-Edén, 1980, 1981). The elimination of the lipid portion from the receptor analogue should prevent its incorporation into the membrane. The form of receptor analogue used is important due to heterogeneity of fimbrial types.

Effect of sub-inhibitory concentrations of antibiotics on adhesion

At drug concentrations below the minimum inhibitory concentration (MIC) for the bacteria, antibiotics produce structural and morphological changes in the bacterial cell. The best documented is the action of penicillin G, which causes a loss of the lipoteichoic acid adhesion from Streptococcus pyogenes with a

concomittant loss of ability to adhere to human buccal cells (Alkan and Beachey, 1978). Similar effects occur with E. coli when inhibition of mannose-sensitive fimbriae is seen, resulting from abnormalities in cell wall biosynthesis, causing the cells to become filamentous. Loss of adhesion only occurred if the penicillin was added while the cells were growing, no effect being seen if the antibiotic was added once the fimbriae had been formed and assembled on the cell surface (Beachey et al., 1981).

Inhibition of adhesion by Type 1 fimbriae could also be seen with growth in sub-inhibitory concentrations of streptomycin, the effect was dose-related and not apparent with cells already possessing fimbriae (Eisenstein et al., 1979). Reductions in adhesive ability were parallelled by reductions in the number of fimbriae on the surface. The adhesion of isogenic strains possessing a single step chromosomal mutation to high level streptomycin resistance was unaffected by the presence of sub-MIC levels of streptomycin (Ofek et al., 1979). One such mutant described by Beachey et al. (1981), although resistant to high levels of streptomycin, was unable to produce MSHA or adhere to buccal cells if grown in less than 0.03 of the MIC of streptomycin. The cells were still fimbriate, but the fimbriae were longer and when purified were incapable of agglutination, possibly due to misreading on the ribosomes. Again, streptomycin had no effect on preformed fimbriae. Changes in antigenic structure were also observed by agglutination tests (Eisenstein et al., 1979). Other antibiotics such as gentamicin, neomycin, tetracycline,

spectinomycin and chloramphenicol produced similar effects to streptomycin (Eisenstein et al., 1980).

Reductions in adhesion can also be obtained for pyelonephritic MR fimbriae. One quarter MICs of ampicillin reduce the amount of attachment of the bacteria to uroepithelial cells, although this effect was not seen with chloramphenicol and nitrofurantoin (Sandberg et al., 1979). A greater reduction in adhesive ability could be shown if the bacteria were pretreated with ampicillin and γ globulin. Results contradictory in part to those described above (Sandberg et al., 1979) were obtained by Vosbeck et al. (1979). Sub-MICs of tetracycline, clindamycin and trimethoprim-sulfametrole reduced the adhesion of a pyelonephritogenic E. coli to an epithelial cell culture line. Penicillin G, ampicillin, mecillinam, cephacetrile, cephalexin, cefotaxime, chloramphenicol and streptomycin had no effect on adhesive ability and nalidixic acid increased the amount of adhesion. Reductions in adhesion of other E. coli strains from UTIs and diarrhoea to the cell culture line were produced by quarter MICs of streptomycin, tetracycline, trimethoprim, chloramphenicol, clindamycin and sulfametrole (Vosbeck et al., 1982).

Further work on the suppression of P-fimbriae formation by sublethal concentrations of antibiotics showed that at 1/8 to 1/2 the MIC of trimethoprim, sulfadiazine, sulphamethoxazole and sulfathiazole markedly reduced the agglutination of human erythrocytes and the adhesion to buccal cells of three strains of pyelonephritogenic E. coli (Väisänen et al., 1982b). There was also a corresponding decrease in the number of fimbriae per cell. In

cells treated with sublethal concentrations of trimethoprim no major changes were seen in the outer membrane protein patterns, but freeze-fracture electron micrographs showed deorganisation of the outer and cytoplasmic membranes.

Promethazine and imipramine can inhibit adhesion of a nephropathogenic E. coli to cultured HEp-2 cells (Molnár et al., 1983). Indomethacin has no effect on preventing renal infection in rats as it has no antibacterial activity, but reduced incidence of disease from 74 to 48% two days after were noted (Glauser et al., 1983).

Reductions in the adhesion of Proteus sp. of uropathogenic origin by sub-MICs of piperacillin and sagamicin have also been observed (Savoia et al., 1983).

Sub-MICs of antibiotics can also affect the secreted enzymes and toxins of bacteria. Lincomycin and clindamycin suppress the production of the streptolysin S of Streptococcus pyogenes, whereas chloramphenicol and erythromycin had no effect on the haemolysin (Shibl and Al-Sowaygh, 1979). The α and β-haemolysins of Staphylococcus aureus could be inhibited by streptomycin and novobiocin which also induced production of protein A (Nordström and Lindberg, 1978). Clindamycin prevents the formation of the coagulase, α-haemolysin, DNAase and other toxins of S. aureus. The E. coli haemolysin is inhibited by streptomycin and the proteases of Pseudomonas aeruginosa by tetracycline. Sub-MICs can also stimulate some toxins. Clindamycin stimulates the enterotoxin of E. coli and Vibrio cholerae and the β-haemolysin of S. aureus and pneumococci is

stimulated by penicillin (Gemmell, 1982). Growth of ETEC in the presence of tetracycline and lincomycin stimulates the production of an increased amount of extracellular and intracellular LT. Analysis of this toxin revealed that the subunits were not nicked, unlike the normal toxin. Lincomycin does not stimulate the production of ST (Yoh et al., 1983), but the action of ST can be blocked by other drugs (Greenberg and Dunn, 1984). These differential effects might be explained by the peripheral or cytoplasmic position of the ribosomes synthesising the proteins in question (Shibl, 1983).

Antibiotic concentrations below the MIC can also promote phagocytosis. Sub-MICs of clindamycin reduce the adhesion of E. coli to buccal cells and by promoting phagocytosis enhance killing of the organism by polymorphonuclear leukocytes (Bassaris et al., 1984). The filamentous cells produced by growth in sub-inhibitory concentrations of ampicllin or mecillinam showed no increased susceptibility to the bactericidal effects of serum or blood, and in fact showed a slightly increased resistance to these effects compared to the control cells (Lorian and Atkinson, 1979).

Pre-treating Salmonella wien with sub-MICs of fosfomycin lead to higher titres of antibody to S. wien in rabbits, than in rabbits immunised with untreated S. wien (Viano et al., 1979). The titres to the O and H antigens were also higher.

Development of vaccines

The pathogenic E. coli described so far are non-invasive in that they do not penetrate the intestinal or urinary tract

epithelial layer. It would therefore be logical to assume that secretory IgA (SIgA) antibody would be involved and any vaccine developed would have to be capable of stimulating mucosal immunity.

The use of purified fimbriae as vaccines for animals in preventing neonatal deaths from diarrhoeal disease have been encouraging. The parenteral vaccination of pregnant sows with K88 antigen gave a significantly greater resistance to disease among the piglets suckled by these sows, than those suckled by unvaccinated control sows, when the piglets were challenged with a large dose of K88-positive ETEC (Rutter et al., 1976). Vaccination with the antigen stimulated the production of anti-K88 antibodies, but no neutralising activity against LT. No difference in the bactericidal or bacteriostatic activities of the serum and mammary secretions was observed between the vaccinated and control sows, indicating that the K88 antibodies in the colostrum were affording protection by neutralising the adhesive properties of the K88 fimbriae (Rutter et al., 1976).

Similar results were obtained when pregnant sows were vaccinated with purified K99 or 987P fimbriae and the suckling piglets challenged with ETEC (Morgan et al., 1978). The incidence and duration of diarrhoea and the degree of intestinal colonisation was less if the piglets were challenged with ETEC bearing homologous fimbriae to those with which the sow was vaccinated. These piglets also had better weight gains than those piglets challenged with a different fimbrial type (Morgan et al., 1978).

The oral immunisation of rabbits with two doses of purified CFA/I fimbriae gave a significant increase in the number of anti-CFA/I-producing cells present in the rabbit intestinal mucosa. These rabbits were significantly protected, compared to the controls, when challenged with CFA/I-positive ETEC. This protection was not seen if the rabbits were challenged with CFA/II-positive ETEC, which revealed the specificity of the antibody response (Cabada et al., 1981; Evans et al., 1982).

A significant and long-lasting increase in SIgA anti-CFA/II was shown when eight, 2mg doses of purified CFA/II fimbriae were given enterally. Three weekly 1-2mg doses gave rise to prominent SIgA anti-CFA/II, whereas three weekly 0.1mg doses of CFA/II fimbriae did not raise the SIgA anti-CFA/II (Boedeker et al., 1982). Fortunately the repeated mucosal immunisation with CFA/II fimbriae did not induce tolerance to the subsequent parenteral challenge with CFA/II-positive E. coli (Levine et al., 1984a).

CFA/I and II are the most common fimbrial antigens on ST/LT-positive E. coli (Evans and Evans, 1978), but if a polyvalent, broad-spectrum vaccine was to be produced to treat human diarrhoeal disease other antigenic components would have to be present to protect against CFA/I and II-negative fimbriated strains. Many E. coli, pathogenic and non-pathogenic, possess Type 1 fimbriae (Levine et al., 1980), therefore the immune system is probably already primed to recognise them. Although Type 1 fimbrial vaccines boosted the level of intestinal SIgA and serum IgG antibody,

evidence of protection from challenge was not observed (Levine et al., 1984a).

Immunisation of monkeys with purified P-fimbriae gave high titres of anti-fimbrial antibody which protected against acute and chronic pyelonephritis caused by the renal inoculation of P-fimbriated E. coli (Roberts et al., 1984).

As with the enteric E. coli, a wide variety of uropathogenic fimbrial types exist, so any effective vaccine would have to be broad-spectrum and consist of a combination of antigens. The problem with giving a killed oral antigen vaccine, i.e. purified fimbriae, is that multiple doses are required to prime the secretory immune system and ensure a good local antibody response. However, a live vaccine, consisting of an attenuated E. coli strain would be able to colonise the intestine and stimulate the immune response to the surface antigens without producing any clinical symptoms.

The ideal solution would be the successful cloning of all the fimbrial antigen genes and the construction of a surface antigen cocktail in a standard E. coli K12. The additional cloning of toxin subunit genes would allow the stimulation of both antibacterial and antitoxic immunity.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The standard bacterial strains and plasmids used are listed in Tables 1a and 1b respectively. Clinical strains responsible for urinary tract infections were isolated from patients in the Royal Infirmary, Edinburgh; the City Hospital, Edinburgh and from patients attending General Practitioners in the Edinburgh area. Faecal *E. coli* strains were isolated from healthy members of the Department of Bacteriology.

All bacterial strains were stored in brain heart infusion broth containing 15% (w/v) sterile glycerol at -70°C.

Identification of Bacterial Strains

The API 20E microtube system (API system S.A., France) was used, according to the manufacturer's instructions, for the identification of urinary tract isolates.

Complex Media

The complex media used were Brain Heart Infusion (BHI) broth (CM225), Nutrient Broth no. 2 (CM67), Diagnostic Sensitivity agar (DSTA) (CM261), Columbia agar base (CM331) and MacConkey agar (CM76), (Oxoid, Basingstoke, Hants.), and Mueller-Hinton broth (0757-01) (Difco, Detroit, Michigan). Each medium was made up according to the manufacturer's instructions in distilled water.

Table 1a Standard Bacterial Strains

Strain and number	Markers	Reference
E. coli K12 J62	pro his trp lac	Bachmann (1972)
E. coli K12 $J62_{-2}$	pro his trp lac rif^R	Bachmann (1972)
E. coli K12 J53	pro met	Bachmann (1972)
E. coli N99	galK2 proA strA	Gottesman and Yarmolinsky (1968) Rosner (1972)
E. coli T_1	MR Tc^R	Kallenius and Mollby (1979)
E. coli NK1	MS MR Hly	Kallenius et al. (1980c)
E. coli K12	Prototroph.	Kindly given by H.W. Smith

Table 1b Standard Bacterial Plasmids

Plasmid designation	Markers	Mr (kb)	Reference
R483	Tp Sm Su Inc Iα	90	Hedges et al. (1972)
JR66a	Km Sm Inc Iα	90	Hedges and Datta (1973)
R1	Ap Cm Km Sm Su Inc F_{II}	90	Meynell and Datta (1966)
R15	Sm Su Inc N	58	Datta and Hedges (1971)
RP4	Ap Tc Km Inc P	52	Datta et al. (1971)
R6K	Ap Sm Inc X	38	Kontamichalou et al. (1970)
Sa	Km Cm Sm Su Sp Inc W	33	Watanabe et al. (1968)

Minimal Media

Minimal media as described by Davis and Mingioli (1950) was used. Double strength media contained: 14.0g K_2HPO_4, 6.0g KH_2PO_4, 0.9g tri-sodium citrate.$2H_2O$, 0.2g $MgSO_4.7H_2O$, 2.0g $(NH_4)_2SO_4$, in one litre of distilled water. Media was diluted to single strength before use. This media was supplemented with 2.8mg/ml D-glucose (BDH) (Smith, 1967) and combinations of L-proline, L-histidine, L-trypotphan (BDH) and L-methionine (Sigma, Poole, Dorset) at 50µg/ml each.

All media were sterilised by autoclaving at 121°C for 15 minutes. Supplements were aseptically added to minimal media after sterilisation. The amino acid supplements were disinfected by steaming for 25 minutes prior to addition.

Preparation of Plates

Agar plates were made by the addition of 15ml of sterile, molten agar to plastic petri dishes (Sterilin, Middlesex). The surfaces of all plates were flamed with a bunsen burner to remove air bubbles. The agar was allowed to set, then the plates were inverted and dried at 52°C for 10 to 15 minutes. In antimicrobial drug sensitivity plates, the required drugs were added after the molten DST agar had cooled to 50°C, and the plates were then poured immediately, as before.

Minimal medium plates were prepared by mixing 50ml of sterile double strength Davis-Mingioli media with the required amount of D-glucose, amino acid supplements and antimicrobial drugs and making

the volume up to 60ml with sterile distilled water. This was mixed with 40ml of molten Bacteriological agar no. 1 (L11) (Oxoid) and mixed gently before pouring.

Antimicrobial Drugs

The antimicrobial drugs and the concentrations used are listed in Table 2. All antimicrobial drugs were dissolved in sterile distilled water, except for chloramphenicol and rifampicin which were dissolved in absolute ethanol and nalidixic acid and sulphamethoxazole which were first dissolved in 0.1ml of 1N NaOH and then made up to the required concentration with sterile distilled water.

Sensitivity Testing

Shaken overnight bacterial cultures in 4.5ml BHI broth were diluted 10^{-4} in minimal media and 2µl spotted on to DST agar containing antimicrobial drugs, with a multipoint inoculator (Mast Labs., Liverpool). Plates were allowed to dry and incubated, inverted, at 37°C overnight.

Determination of Sugar Utilisation, Haemolysin and Colicin Production

This was performed in a similar manner to that described above for the sensitivity testing, except that the bacterial cultures were spotted on to minimal media supplemented with 1% (w/v) raffinose or 1% (w/v) dulcitol as carbon source. Haemolysin production was

Table 2 Antimicrobial Drugs

Antimicrobial	Concentration[a] (μg/ml)	Source
Ampicillin	10	Beecham Research Laboratories, Middlesex
Chloramphenicol	10	Parke-Davis, Pontypool, Gwent
Kanamycin	10	Bristol Laboratories, Middlesex
Nalidixic Acid	10	Sterling Winthrop Laboratories, Surrey
Rifampicin	25	Le Petite, Milan, Italy
Spectinomycin	100	Upjohn Ltd., Surrey
Streptomycin sulphate	10	Glaxo Laboratories Ltd., Greenford, Middlesex
Sulphamethoxazole	100	Wellcome Foundation Ltd., Beckenham, Kent
Tetracycline	10	Glaxo Laboratories Ltd., Greenford, Middlesex
Trimethoprim lactate	10	Wellcome Foundation Ltd., Beckenham, Kent

a unless otherwise stated

tested by spotting cultures on to Columbia agar containing 5% (v/v) defibrinated horse blood SR50 (Oxoid) and looking for a zone of lysis around the colonies. Colicin production was determined by spotting undiluted cultures on to nutrient agar flood seeded with 0.1ml of the sensitive E. coli K12 indicator strain. Lysis of the indicator around the colonies indicated production of a colicin.

Determination of Broth MICs

Statically grown overnight Mueller-Hinton broth cultures were subcultured into 4.5ml of fresh Mueller-Hinton broth containing doubling dilutions of antimicrobial drugs. The broths were incubated statically at 37°C overnight and the lowest concentration of drug inhibiting visible bacterial growth determined.

Haemagglutination Assay

Haemagglutination ability was assayed by a modification of the method of Svanborg-Edén and Hansson (1978). Bacterial cells harvested by centrifugation at 4,000rpm for 15 minutes (Heraeus Christ Bactifuge) from 4.5ml of a 48 hour static BHI broth culture were resuspended in 10mM phosphate buffered saline (PBS) to a concentration of 2×10^9 bacteria/ml. The bacterial concentration was determined by measuring the optical density of the cultures at 550nm in a Bausch and Lomb Spectronic 20 spectrophotometer and referring this to a standard curve of optical density against bacterial numbers, determined with a haemocytometer. A series of 50μl two-fold dilutions of the bacterial suspension, in either PBS or 2.5% (w/v) D-mannose (Sigma) in PBS, were made in the V-bottomed

wells of a microtitre plate (Sterilin, Middlesex), and 50µl of a citrated suspension of human group A or guinea pig erythrocytes (5 x 10^8 erythrocytes/ml) was added to each well. The plates were incubated at 37°C for 30 minutes, after which time the results were read. The plates were then maintained at 4°C overnight and the results read again.

Plasmid Transfer

Genetic transfers were performed by inoculating donor cells from a single colony on a MacConkey agar plate into 4.5ml BHI broth and recipient cells from single colony growth into another 4.5ml BHI broth. The cultures were incubated statically at 37°C. After overnight growth, 0.1ml of the donor culture and 1ml of the recipient culture were mixed in 4.5ml of fresh, prewarmed BHI broth, and the mating mixture incubated statically at 37°C for the required time, which varied from 1 to 18 hours (Smith, 1969) as specified. At the end of this time the mixture was shaken vigorously to separate donor and recipient cells which were then collected by centrifugation at 4,000rpm for 15 minutes (Bactifuge). The pellet was resuspended in 5.6ml of Davis-Mingioli (DM) medium without supplements and further diluted in 10-fold steps in DM medium. A 0.1ml amount of each dilution was spread on the surface of selective DM agar, allowing only growth of a plasmid-containing recipient strain. Controls consisted of spreading donor and recipient cells on separate selective plates of the same type. The plates were allowed to dry and then incubated at 37°C for 48 hours. Resultant single colonies were purified on selective plates.

Plasmid Mobilisation

A 0.1ml amount of an overnight BHI broth culture of donor cells, which contained a known R-plasmid, was added to 1ml of recipient culture in 4.5ml of prewarmed BHI broth. The recipient strain possessed plasmids which either carried a non-selectable marker or were not able to promote their own transfer. The mixture of donor and recipient was incubated statically at 37°C overnight. The mixture was then spread on to minimal media plates selective for R-plasmid containing recipients, as described for the plasmid transfer. Plates were incubated at 37°C for 48 hours and the resultant colonies purified on separate selective plates of the same type.

R-plasmid transfer from one of these transconjugants to the E. coli K12 recipient J62-2 was performed as described above. The resulting transconjugants were tested for possession of the plasmid from the original recipient culture by plasmid isolation and/or testing for markers present in the original recipient.

Determination of Loss of a Selectable Marker

Single colony transconjugants, from a plasmid transfer, were streaked on to DST agar plates containing one of each of the original strain's antimicrobial drug resistance markers and a DST agar plate lacking any antimicrobial drug as a positive control. Plates were incubated at 37°C overnight. No growth along the streak line on any of the selective plates indicated loss of that marker.

Transduction by 'Phage P1

A modification of the thermal induction method (Goldberg et al., 1974) was used. A 4.5ml nutrient broth culture of E. coli N99 was incubated at 30°C overnight and then subcultured in fresh nutrient broth for a further two hours. A two-fold dilution of this culture was then incubated at 42°C for three hours until lysis was seen. The P1 lysate was filter-sterilised through a 0.2μm membrane filter (Anderman and Co. Ltd., East Moseley, Surrey).

Overlays were prepared with 1.5ml of 0.8% (w/v) nutrient agar (Oxoid), 0.5ml of appropriately diluted sterile P1 lysate, 0.5ml of an overnight nutrient broth culture of the indicator strain and 60μl of 4% (w/v) calcium chloride. The overlay was poured on to nutrient agar plates which were then incubated at 37°C overnight.

After incubation, overlays from plates showing semi-confluent lysis were transferred to nutrient broth, vortexed and then centrifuged at 4,000rpm for 30 minutes (Bactifuge). The supernatant was filter-sterilised as above and a 0.5ml portion allowed to adsorb to the E. coli J53 recipient for 30 minutes at 37°C. The transducing 'phage preparation was plated on to minimal media containing drugs selecting for the transduced gene and incubated at 37°C for 36 to 48 hours. Resulting transductants were purified on the same type of selective media and their haemagglutination and antimicrobial drug resistance properties determined.

Large Scale Isolation of Plasmid DNA by Triton Lysis

The method of Meyers et al. (1976) was followed. One litre of a shaken overnight nutrient broth culture of the organism was centrifuged at 4,000g for 45 minutes. The cell pellet was resuspended in 2ml of 25% (w/v) sucrose in 50mM Tris-HCl, pH 8. After addition of 0.3ml of a 50mg/ml solution of lysozyme (Sigma) in distilled water, the cells were shaken gently at 37°C for 5 minutes, then 2ml of 0.25M EDTA (Sigma) pH 8, was added and the mixture shaken on ice for 5 minutes. The addition of 3ml of 2% (w/v) Triton X-100 (BDH) in 50mM Tris-HCl, pH 8, 62.5mM EDTA followed by gentle inversion at 0°C brought about lysis. The lysate was cleared by centrifugation for 40 minutes at 40,000g. The supernatant was removed with a wide-bore pipette and extracted twice with equal volumes of TES (10mM Tris, 10mM NaCl, 0.0316mM EDTA) pH 8, saturated phenol by centrifugation at 4,000rpm for 10 minutes (Bactifuge). The upper aqueous layer was retained.

DNA was precipitated from the aqueous layer by the addition of two volumes of cold 95% ethanol and holding at -70°C for 30 minutes. The precipitated DNA was removed by centrifugation at 2,500rpm for 5 minutes (Bactifuge) and the pellet resuspended in 0.5ml TES, pH 8. A 30µl sample was mixed with 10µl of bromophenol blue (0.05% (w/v) in 50% (v/v) glycerol),loaded into the wells of a horizontal slab gel (14 by 21.5cm) consisting of 0.5% agarose in Tris-Borate buffer (89mM Tris, 2.5mM EDTA, 89mM Boric acid, pH 8.2). Samples were electrophoresed through the agarose at 70 volts overnight. The gel was then stained with a 0.75µg/ml solution of ethidium bromide in

distilled water and visualised over a long-wave, ultra-violet light source (Ultra-violet Products Inc., Cambridge).

Small Scale Isolation of Plasmid DNA by Triton Lysis

The procedure was carried out as above, except a 4.5ml overnight broth culture was used and the volumes of the other solutions reduced appropriately.

Small Scale Isolation of Plasmid DNA by Alkaline Lysis

The method of Birnboim and Doly (1979) was followed. A 1.5ml volume of an aerated, overnight BHI broth culture of the organism was harvested at 11,500g for 1 minute (MSE Micro centaur microfuge). The pellet was resuspended in 100µl of 2mg/ml lysozyme in 50mM glucose, 10mM EDTA, 25mM Tris, pH 8. This was then maintained on ice for 30 minutes and then 200µl of the lysing solution (0.1N sodium hydroxide, 1% (w/v) sodium dodecyl sulphate) was added and the mixture gently inverted. After maintenance at 0°C for 5 minutes, 150µl of 3M sodium acetate (Sigma), pH 4.8, was added and mixed well, maintenance at 0°C for 60 minutes allowed precipitation of the clot which formed. The clot was pelleted by centrifugation at 11,500g for 10 minutes. DNA was precipitated by the addition of 1ml of cold 95% ethanol and storage at -20°C for 30 minutes. The precipitated DNA was pelleted by centrifugation at 11,500g for 5 minutes and then resuspended in 100µl of 0.1M sodium acetate, 50mM Tris, pH 8. The DNA was reprecipitated with two volumes of cold 95% ethanol and kept at -20°C for 10 minutes. The pellet was recovered

by centrifugation as above and dissolved in 30µl TE (10mM Tris, 1mM EDTA, pH 8).

The DNA samples were electrophoresed as before, but the agarose was made up in Tris-Acetate buffer (40mM Tris, 1mM EDTA, 5mM sodium acetate, pH 8.2).

Preparation of Fimbriae

The method of Salit et al. (1983) was followed. Organisms were grown statically in three litres of BHI broth for 48 hours at 37°C. Cells were harvested at 10,000g for 10 minutes at 4°C. The resulting pellet was washed in 50ml of 0.5% (w/v) sodium chloride, recentrifuged and resuspended in 25ml of 50mM Tris-HCl buffer, pH 7.5. The suspension was then blended at high speed in four, 30 second bursts in a Waring blender. The debris was removed by centrifugation at 20,000g for 30 minutes at 4°C and the supernatant ultracentrifuged at 150,000g for 2.5 hours at 4°C.

The resultant pellet was resuspended in 1.2ml of 5M urea buffer and mixed for several hours at 37°C in a Gallenkamp orbital incubator. It was then diluted 1 in 5 with the Tris buffer. This solution was layered over a 1M urea - 1M sucrose - 5mM Tris cushion and ultracentrifuged at 150,000g for 16 hours. The pellet was resuspended in 100µl of Tris buffer to give the sample for loading on a polyacrylamide gel.

Preparation of Polyacrylamide Gels

Polyacrylamide gels were prepared by a modification of the method of Laemmli (1970). Slab gels measuring 17 by 14cm and consisting of a 10% (w/v) acrylamide separating gel surmounted by a 1cm 4% (w/v) acrylamide stacking gel were used. The acrylamide stock solution was prepared by the method of Weber and Osborn (1969). A mixture of 22.2g of acrylamide (BDH) and 0.6g of methylenebisacrylamide (BDH) were dissolved in 100ml of distilled water, to give the acrylamide stock solution. The separating gel was composed of 22.5ml of 0.7M Tris HCl buffer, pH 8.8 containing 0.2% (w/v) SDS, 20.25ml of acrylamide stock solution. This mixture was degassed in a Buchner flask and 2.25ml of freshly prepared ammonium persulphate (APS) solution (15mg/ml) and 0.065ml of NNN 'N' tetramethylethylene diamine (TEMED) (BDH) were then added. The gel was then poured between two glass plates and covered with a layer of water-saturated butan-2-ol during polymerisation. The stacking gel was prepared with 5ml of 0.25M Tris HCl buffer, pH 6.8, containing 0.2% (w/v) SDS, 2ml of acrylamide stock solution, 2.5ml of distilled water, 0.5ml of APS solution and 0.02ml of TEMED. The water-saturated butan-2-ol was removed from the surface of the polymerised separating gel and the stacking gel was carefully poured on top, a comb was inserted into the stacking gel which was then allowed to polymerise. Fimbrial protein samples were solubilised in 0.0625M Tris HCl buffer, pH 6.8 containing 1% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue, by heating at 100°C for 3 minutes. The samples were then loaded into the wells and electrophoresed at 60 volts through the stacking gel.

The voltage was then increased to 150 volts until the bromophenol blue had run to the bottom of the separating gel.

The gels were stained for protein with Coomassie Brilliant Blue overnight and destained by the method of Poxton and Sutherland (1976). The staining solution contained 1.25g Coomassie Brilliant Blue R, 1625ml distilled water, 625ml propan-2-ol, 250ml acetic acid. The gels were destained for 30 minutes with gentle shaking sequentially in each of solutions 2 to 5. The solutions contained: 2) 125mg Coomassie blue, 2000ml distilled water, 250ml propan-2-ol, 250ml acetic acid; 3) 63mg Coomassie blue, 2250ml distilled water, 250ml acetic acid; 4) 1000ml water, 800ml methanol, 200ml acetic acid; 5) 10% (v/v) acetic acid in distilled water.

Electron Microscopy

Bacterial cultures were prepared as described by Ofek et al. (1974). The organisms were cultured in 4.5ml of BHI broth, statically, at 37°C for 48 hours, the cells were then washed twice with 0.85% sodium chloride and diluted in Hanks balanced salt solution (pH 7.2) to a concentration of 2 x 10^8 bacterial/ml. A drop of this suspension was loaded on to a formvar coated copper grid and after 5 minutes excess fluid was removed with absorbent paper. The dried grids were shadowed with platinum at an angle of 17° and viewed under a Hitachi HU-12A transmission electron microscope.

Photography

Photographic negatives of DNA agarose gels were prepared on Kodak Plus-X pan film using a 5 x 4 inch plate camera fitted with a Wrattan 22 orange filter. Polaroid photographs of polyacrylamide gels were prepared utilising a Wrattan 58 green and a Wrattan 22 orange filter. Electron micrographs were prepared on Ilford EM technical film.

RESULTS

I SURVEY OF URINARY TRACT ISOLATES FOR VIRULENCE MARKERS

Clinical strains isolated from infections possess a variety of characters that are classed as virulence factors. The importance of each of these factors varies depending on the type of infection; often it is not one character alone which determines the progress of the disease, but a combination of two or more factors. Each one plays a specific role, but contributes to the effect produced by the others. For this reason a survey of the virulence characters of strains isolated from a given infection often helps to put into perspective the importance of a particular character of interest.

Distribution of specific characters among UTI isolates

All Enterobacteriaceae isolated from cases of significant bacteriuria ($>10^5$ organisms/ml of urine) were tested for the possession of virulence characters and antimicrobial drug resistance determinants as described in the Methods section of this work. Resistance to an antimicrobial was defined as confluent growth on DSTA plates, containing antimicrobial at the concentrations given in Table 2, in the absence of growth of the control E. coli J62-$_2$.

Similarly the ability to utilise 1% (w/v) raffinose or dulcitol as sole carbon source in Davis-Mingioli (DM) medium, was determined by confluent growth of the organisms in the absence of growth of E. coli J62-$_2$. Organisms which secreted a haemolysin were identified as those which produced a zone of lysis of blood cells in the blood agar plates. Colicin producers were distinguished by

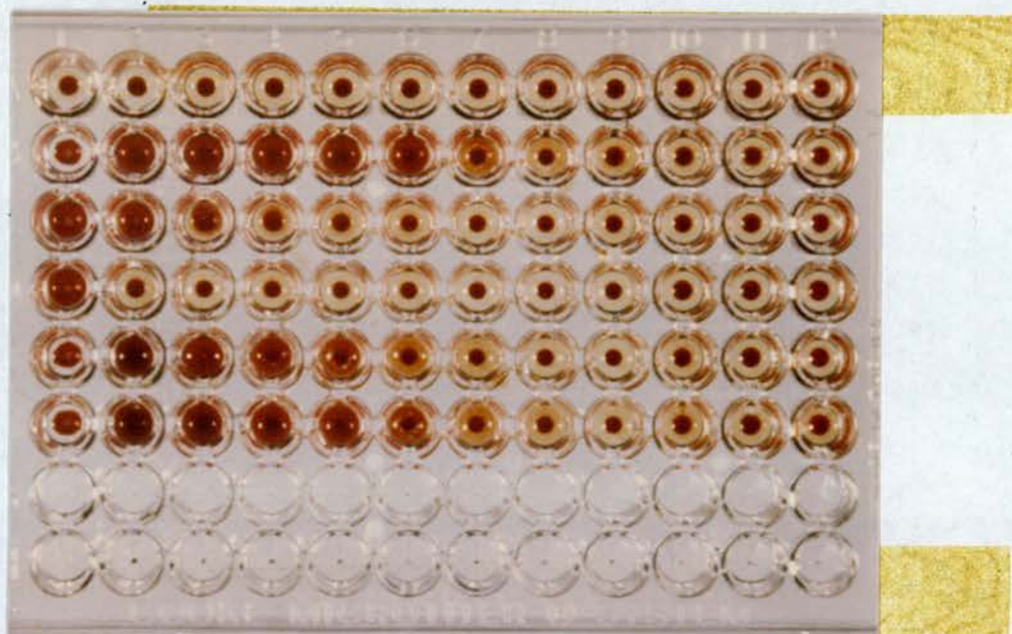
their ability to lyse the nutrient agar seeded sensitive indicator strain.

Strains which possessed fimbriae and adhesive ability were identified by their ability to agglutinate human and/or guinea pig erythrocytes. The concentration of the suspension of bacteria for use in the assay was originally determined by counting a diluted culture of the organism using a haemocytometer. Latterly the number of bacteria as determined with the haemocytometer was related to the optical density (OD) reading at 550nm of the same culture, and the standard curve produced could be used to convert OD readings of subsequent cultures to the number of bacteria present. The inhibition of the haemagglutinationof guinea pig cells by 2.5% (w/v) mannose indicated the presence of Type 1 fimbriae. The inability of the same concentration of mannose to inhibit the agglutination of human group A erythrocytes indicated the presence of mannose-resistant uropathogenic fimbriae. Figure 1 shows a haemagglutination (HA) plate, with a negative and a positive control, a mannose-sensitive organism and a mannose-resistant organism, and illustrates the different patterns of HA obtained.

Source of strains used in the survey

One hundred strains isolated from the urine of patients with significant bacteriuria in the Edinburgh Royal Infirmary and from patients attending Edinburgh General Practitioners were collected and classed as the lower UTI group. In addition, 100 urinary isolates from patients with chronic UTIs in the City Hospital, Edinburgh were surveyed. Fifty faecal E. coli strains were obtained from members of the Department of Bacteriology. All members of this

Figure 1 HA assay plate showing the typical reactions obtained.



Row 1 HA^- control

2 HA^+ "

3 $MSHA^+$ organism

4 " " + D-mannose

5 $MRHA^+$ organism

6 " " + D-mannose

latter group had no symptoms of clinical infection and therefore provided a source of non-pathogenic bacteria which were used as a control, to which the former two groups of strains could be compared.

Pathogenic characters associated with the clinical strains

Each strain was tested for the possession of the characters listed above. The results obtained are shown in Table 3. The values given are the number of strains possessing that character expressed as a percentage of the total number tested.

The percentage of both types of urinary isolates capable of producing HA is greater than the percentage of faecal strains which possess this character. These HA reactions were separated into MSHA and MRHA, more faecal isolates (40%) produced MSHA compared to only 26% of the lower UTI isolates. This indicates the presence of Type 1 fimbriae which are common to both pathogenic and non-pathogenic E. coli. In contrast, a larger proportion of the lower UTI isolates (45%) was capable of MRHA compared to 30% of the faecal isolates, and it is these MR fimbriae which are associated with pathogenicity. Figure 1 shows a microtitre plate with typical HA reactions. Production of haemolysin and utilisation of raffinose and dulcitol are also higher among the lower UTI isolates than the faecal isolates. However, three times as many (35%) of the faecal isolates produce colicin V than the lower UTI isolates.

Comparison of isolates from acute and chronic UTIs

A comparison of the pathogenic characters of the lower UTI isolates with those possessed by the isolates from the chronic UTIs

TABLE 3 Percentage of Gram-negative bacterial isolates expressing virulence characters

Character	Faecal Isolates	Lower UTI Isolates (100)	Chronic UTI Isolates (100)
Haemagglutination (HA)	48	66	62
MSHA	40	26	45
MRHA	30	45	33
Haemolysin production	6	24	15
Colicin V production	34	12	18
Raffinose utilisation	16	24	16
Dulcitol utilisation	2	6	6

Significant difference between faecal isolates and the UTI isolates were only found with HA (lower UTI´s), Hly (lower UTI´s) and ColV (lower and chronic UTI´s). Other values were not significant at the 5% level.

showed that the num ber of strains that produce HA is similar (Table 3). However, more chronic UTI isolates show the ability to produce MSHA (45%) than MRHA (33%). This is in contrast to the lower UTI isolates, more of which produce MRHA than MSHA.

The percentages of strains capable of haemolysin production and utilisation of raffinose are also lower among the chronic UTI isolates. Colicin V is produced by 18% of the isolates from the chronic infections compared to its production by 12% of the lower UTI isolates. The number of strains capable of utilising dulcitol is the same in both groups.

Antimicrobial drug resistance in the clinical strains

Each strain was tested for its sensitivity to eight common antimicrobial drugs, listed in Table 4. At the concentrations given the percentage of lower UTI isolates possessing resistance determinants to these drugs was greater than the percentage of antimicrobial drug resistant faecal isolates, except for resistance to sulphamethoxazole which was 5% higher among the faecal isolates.

It was found that antimicrobial drug resistance was higher among the chronic UTI isolates than the lower UTI isolates, except for resistance to tetracycline which was 2% lower among the chronic UTI isolates.

Correlation between MRHA ability and other characters

The main character of interest was the ability of the strains to produce MRHA. In order to determine if a correlation existed between the MRHA ability of the strains and any of the other

TABLE 4 Percentage of Gram-negative bacterial isolates possessing antimicrobial drug resistance determinants

Resistance	Antimicrobial Concentration µg/ml	Percentage Number		
		Faecal Isolates (50)	Lower UTI Isolates (100)	Chronic UTI Isolates (100)
Ampicillin (Ap)	10	19	38	44
Chloramphenicol (Cm)	10	2	9	12
Kanamycin (Km)	10	2	9	12
Spectinomycin (Sp)	100	0	6	12
Streptomycin (Sm)	10	21	28	50
Sulphamethoxazole (Sx)	100	30	25	47
Tetracycline (Tc)	10	26	35	33
Trimethoprim (Tp)	10	7	12	23

characters tested, the number of MRHA-positive isolates that possessed each of the other characters was calculated and compared to the total number of isolates which possessed that character (Table 5). The evidence for correlation between any two characters was produced by determination of the X^2 and probability (P) values for each set of figures. A P value of 0.05 or less was designated as being significant, i.e. that proportionately more MRHA-positive isolates possessed that character than the total number of isolates, indicating a correlation or linking of the two characters. On this criterion, only two P values were found to be significant, MSHA ability among the lower UTI isolates which showed a significant lack of association with MRHA ability, i.e. a smaller proportion of the MRHA-positive isolates expressed MSHA than the total population. Haemolysin production by the chronic UTI isolates did show a significant association with MRHA ability ($P \leq 0.01$).

The most common virulence character among the UTI isolates studied was the ability to cause HA, and MRHA ability was present in a relatively high proportion (45%) of the lower UTI isolates. Therefore this character must have an important role in pathogenicity. Although a relatively low number of strains were examined it was possible to perform statistical analysis. No association between MRHA and any of the other characters was observed, except for haemolysin production by the chronic UTI isolates.

TABLE 5 Correlation between MRHA and other characters

	Lower UTI Isolates			Chronic UTI Isolates		
Character	Total No.	No MRHA+	P^*value	Total No.	No MRHA+	P value
MSHA	26	7	>0.02	45	17	>0.1
Haemolysin production	24	13	>0.1	15	9	<0.01
Colicin V "	12	4	>0.1	18	8	>0.1
Raffinose utilisation	18	8	>0.5	16	7	>0.1
Dulcitol "	6	2	>0.5	6	0	N/A
Ampicillin resistance	38	18	>0.5	44	14	>0.5
Chloramphenicol "	9	4	>0.5	12	2	>0.1
Kanamycin "	9	3	>0.1	12	3	>0.5
Spectinomycin "	6	1	>0.1	12	1	>0.05
Streptomycin "	25	9	>0.5	50	14	>0.1
Sulphamethoxazole "	25	8	>0.1	47	14	>0.5
Tetracycline "	35	19	>0.1	33	10	>0.5
Trimethoprim "	12	4	>0.1	23	5	>0.1

* Probability from X^2 test

N/A Not applicable

II TRANSFER OF THE GENE RESPONSIBLE FOR MRHA FROM UTI ISOLATES

Examination of MRHA-positive strains for transferable plasmids

Previous reports had stated that the genes for the production of MRHA fimbriae were chromosomally located, but certain observations in this laboratory had lead to the assumption that a plasmid location for these genes was possible. Therefore one aim of this project was the identification of this putative plasmid. The simplest method of detecting the presence of plasmids is to allow the transfer of a selectable marker between the clinical strain and a suitable recipient, and then select for the presence of this marker in the recipient strain. The most convenient characters to test for transfer are antimicrobial drug resistance determinants, which are easy to select for by plating the mating mixture on to DM agar plates containing the drug in question. As it is impossible to select for transfer of MRHA ability directly, its transfer can only be detected by screening the transconjugants obtained from, for example, an R-plasmid transfer.

Transfer of R-plasmids from the clinical strains

Another 137 enterobacterial strains were isolated from the urine of patients with lower UTIs in the Royal Infirmary, Edinburgh and Edinburgh General Practice outpatients. These strains were also screened for the characters outlined in section I and added to the 100 lower UTI strains used for the statistical survey. Ninety-nine of these 237 strains were MRHA-positive for human group A

erythrocytes and 62 of these were resistant to one or more of the antimicrobial drugs tested.

Fifty-nine of the antimicrobial drug resistant strains were tested for transfer of resistance plasmids. The remaining three strains were resistant to Sx alone, and as this resistance determinant is particularly difficult to use as a marker for R-plasmid transfer these strains were not tested further.

Each clinical strain was mated with E. coli J62-$_2$ for 18 hours (Plasmid transfer, Methods) and the mixture was then plated on to DM medium containing the supplements for strain J62-$_2$ (proline, histidine and tryptophan), rifampicin at 25µg/ml and an antimicrobial drug, resistance to which was encoded by the clinical strain. If no transfer of this latter marker was obtained the other resistance markers of the clinical strain were individually tested for transfer. Seventeen strains possessed transferable R-plasmids and these are listed in Table 6 with their resistance pattern, source, the selection agent and the resistance markers transferred. Strain 815 transferred a Km R-plasmid and grew on the DM medium containing this drug, but the resistance marker was subsequently lost when the selective pressure was removed.

Haemagglutination of R-plasmid containing transconjugants

The transconjugants from the 17 strains listed in Table 6 were assayed for their ability to produce MRHA of human group A erythrocytes. Eight transconjugants from each strain were picked off the mating plate and purified on the same type of selective media. It was decided that for most purposes eight transconjugants

Table 6 Clinical strains which possessed R-plasmids, their resistance pattern, selection media and markers transferred.

Clinical Strain	Source	Resistance pattern	Marker Selected	Markers Transferred
E. coli 116	H	ApCmSmSpSxTp	Sm	Sm
E. coli 121	H	ApCm	Ap	Ap
E. coli 738	H	ApTc	Ap	Ap MRHA
E. coli 767	H	SmSpSxTc	Tc	SxTc MRHA
E. coli 768	H	ApCmSpSxTcTp	Tp	ApCmSxTcTp
E. coli 792	H	Ap	Ap	Ap MRHA
E. coli 810	H	ApTc	Ap	ApTc MRHA
E. coli 815	H	ApCmKmSmSpSxTc	Km	MRHA
E. coli 819	H	ApTc	Tc	ApTc MRHA
P. mirabilis 828	H	ApCmSmSxTc	Tc	ApTc MRHA
E. coli 838	H	Ap	Ap	Ap MRHA
E. coli 1878	H	ApKmSmSxTc	Km	ApKmSmSxTc MRHA
E. coli 1995	GP	KmSmSpSxTc	Tc	SmSpSxTc MRHA
E. coli 4730	GP	ApCmSmSxTcTp	Tp	ApSmSxTp
E. coli 4757	GP	SmTc	Tc	SmTc
E. coli 4811	GP	ApTc	Tc	ApTc MRHA
E. coli 4950	GP	ApKmSmSxTp	Tp	KmTp MRHA

H = Hospital

GP = General Practice

from each mating would be purified and then tested further. Not all the strains selected for purification were true transconjugants and subsequently did not grow on the selective media. For this reason, the number of transconjugants that were assayed varied. These purified transconjugant strains were then inoculated into BHI broth and assayed for HA ability. Transconjugants from 12 of the original strains were shown to be MRHA-positive (Table 6). The E. coli $J62_{-2}$ recipient strain was used as a negative control for these assays. The results obtained for strain 4950 are shown in Figure 2.

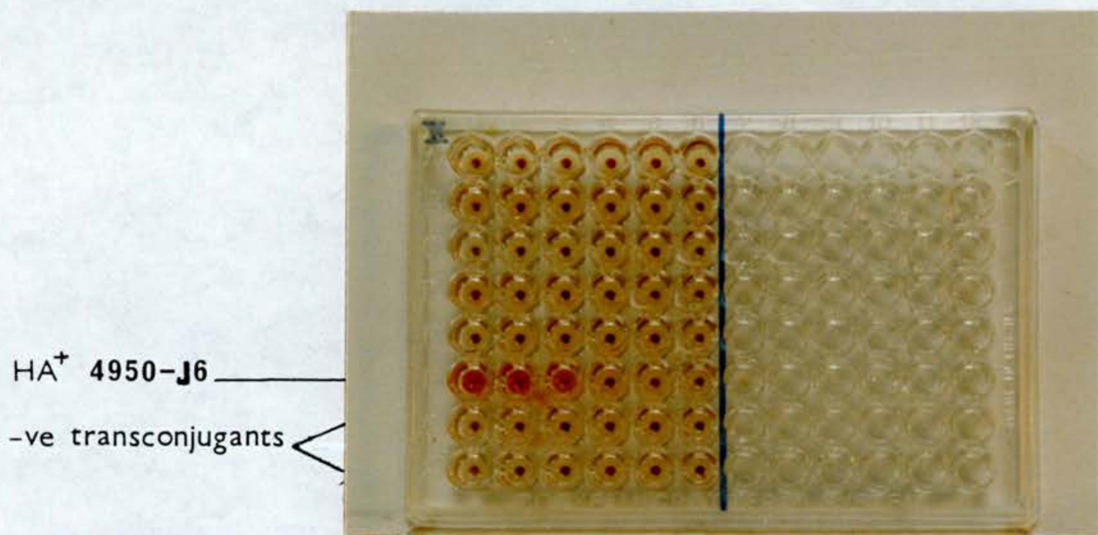
The proportion of transconjugants from each strain that were MRHA-positive varied. The numbers obtained are shown in the table below.

Strain no.	738	767	792	810	815	819	828	838
No. MRHA+ / No. tested	5/8	2/2	6/7	4/8	2/3	4/6	1/5	2/8

Strain no.	1878	1995	4811	4950
No. MRHA+ / No. tested	2/6	2/6	3/4	5/6

Each of the MRHA-positive transconjugants was tested on DM agar containing the necessary $J62_{-2}$ supplements (proline, histidine, tryptophan) and DM agar containing proline and tryptophan, but lacking histidine. If the strains are E. coli $J62_{-2}$ they grow on the fully supplemented plates but not the plates which lack histidine. By this method it was confirmed that the MRHA gene had been transferred to E. coli $J62_{-2}$. Eleven of the clinical strains

Figure 2 Microtitre plate showing some of the MRHA-positive and MRHA-negative transconjugants obtained from strain 4950.



which were able to transfer this gene were E. coli and the twelfth, strain 828, was Proteus mirabilis, as determined with the Api system. These $J62_{-2}$ transconjugants will be referred to with the suffix J6 after the strain number.

Molecular analysis of the MRHA-positive transconjugants

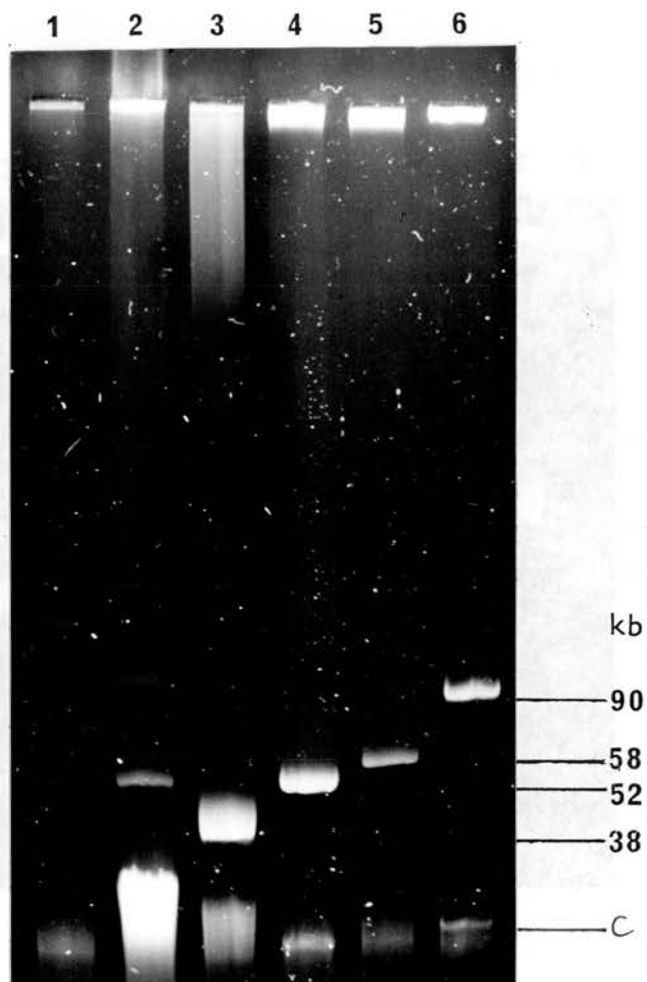
In order to determine the size and number of plasmid species transferred, the DNA was prepared from the clinical strains and the E. coli $J62_{-2}$ transconjugants, by Triton X-100 lysis. The extracted plasmid DNA was separated by agarose gel electrophoresis in parallel with the DNA of at least three molecular size standards. After completion of electrophoresis and ethidium bromide staining, followed by destaining of the agarose gel in distilled water, the distance moved by the standard plasmid bands was plotted against the $\log_{10}$ of the standard plasmid molecular size. The standard curve produced was used to determine the size of the test plasmids. The plasmid data obtained are shown in Table 7, and Figures 3 to 6.

The sizes of the plasmids present in the clinical strains and transconjugants varied between 53 and 73 kbases (kb), except for the larger plasmids in strains 810, 4950 and 4950-J6. The variation in molecular weight of the plasmids between 4950 and 4950-J6 probably results from recombination or dimerisation between plasmids from strain 4950 in 4950-J6. Smaller variations also exist between the plasmids in the other E. coli clinical strains and their K12 transconjugants. In Figure 5, strain 819 (lane 2) possessed a

Table 7 Plasmids present in the clinical strains and MRHA-positive J62-$_2$ transconjugants

Clinical Strain no.	Plasmid sizes (kb)	Transconjugant no.	Plasmid sizes (kb)
738	73	738-J6	59
767	56	767-J6	-
792	-	792-J6	69
810	65 90	810-J6	58
815	-	815-J6	-
819	58	819-J6	53 71
828	- -	828-J6	53 71
838	-	838-J6	55
1878	-	1878-J6	68
1995	59 68	1995-J6	55 68
4811	68	4811-J6	68
4950	53 98 161	4950-J6	53 134

Figure 3 Agarose gel of clinical strain 4950 and its MRHA-positive J62-2 transconjugant 4950-J6. Plasmid isolation was by Triton X-100 lysis.



Lane 1 4950-J6

2 4950

3 R6K

4 RP4

5 R15

6 R1

C = Chromosomal DNA

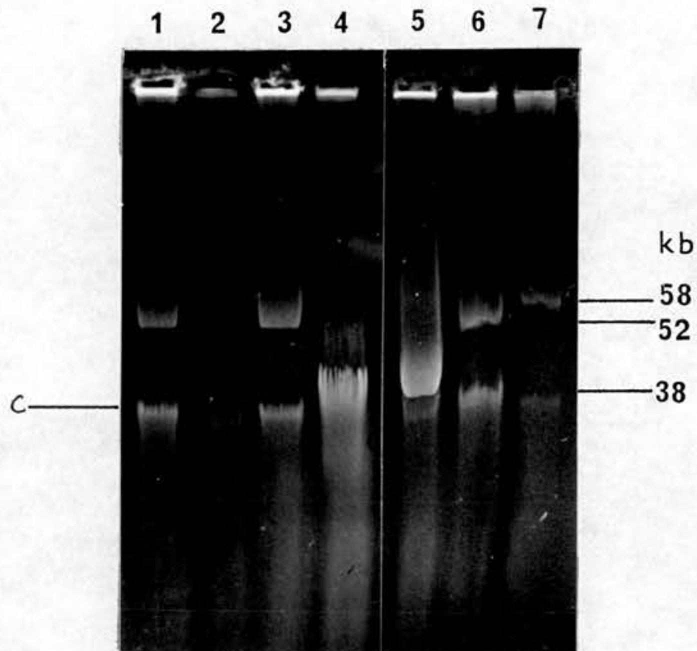
plasmid of 58kb which was visible on the original gel, but did not reproduce on to the photograph.

No plasmid was common to most or all of the strains examined which could be implicated as the carrier of the MRHA gene. There was also a variation in the resistance pattern of the strains examined, however the determinants for Ap and Tc resistance were predominant (Table 6) on the plasmids in the MRHA-positive transconjugants. The heterogeneity of the plasmid species indicates that the presence of the MRHA gene in these 12 strains had not arisen due to cross-infection of one plasmid species.

The transconjugants 738-J6, 792-J6, 810-J6, 838-J6, 1878-J6 and 4811-J6 contained a single plasmid, Figures 4 and 6 (strains 738-J6 and 810-J6 are not shown), therefore in these strains the MRHA gene and the resistance determinants must be carried on the same plasmid. Two sizes of plasmid were present in these strains, 68-69kb and 55-59kb, but the resistance pattern of the similarly sized plasmids was different.

In contrast, transconjugants 819-J6, 828-J6, 1995-J6 and 4950-J6 all contained two plasmids (Figures 3, 4 and 5) which were of similar molecular sizes in 819-J6, 828-J6 and 1995-J6. One of these plasmids could be a virulence plasmid, i.e. carry the MRHA gene and essential plasmid functions for replication and transfer, and the second plasmid could possess the resistance determinants. Alternatively both plasmids could be classed as R-plasmids, one of which also possessed the MRHA gene. This can be determined in part by measuring the transfer frequency of each gene between strains.

Figure 5 Agarose gel of clinical strains 767, 819 and 828 and their MRHA-positive transconjugants. Plasmids were isolated by Triton-lysis.



Lane 1 819-J6

2 819

3 828-J6

4 828

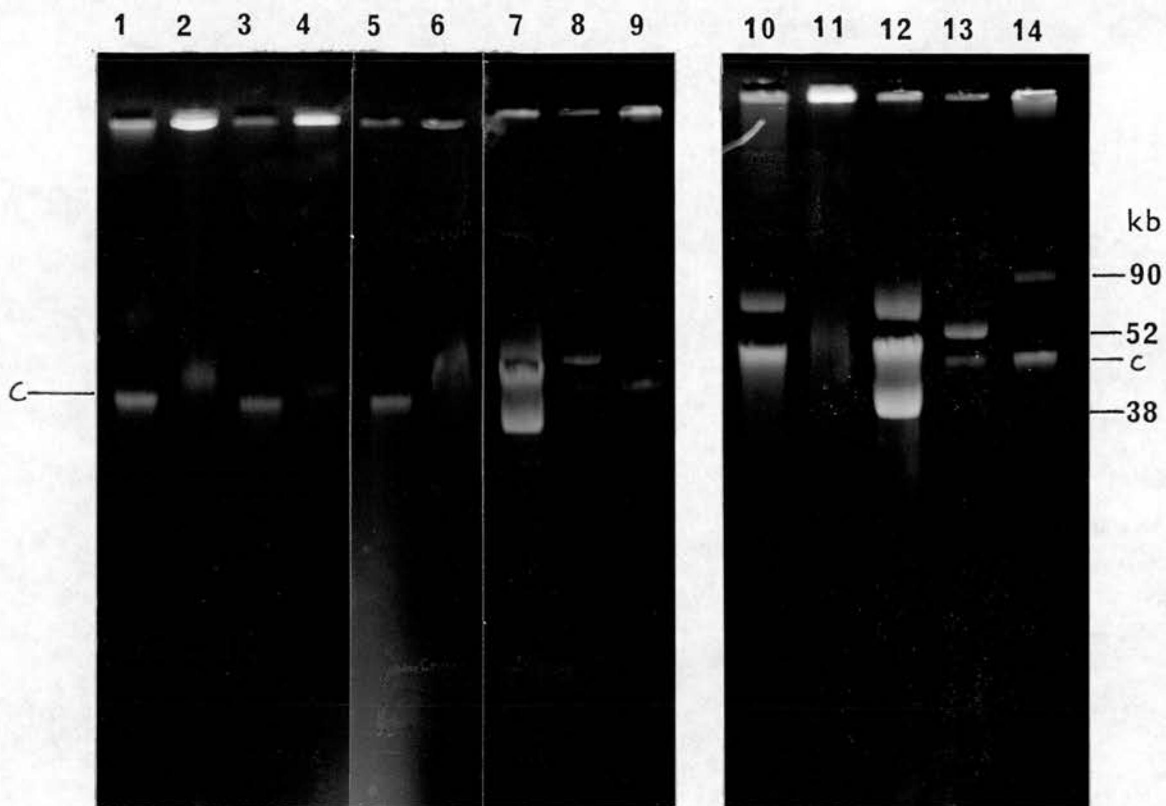
Lane 5 R6K

6 RP4

7 R15

C = Chromosomal DNA

Figure 6 Agarose gel of clinical strains 792, 767, 815 and 838, and their MRHA-positive transconjugants. Plasmids were isolated using alkaline lysis.



Lane		Lane	
1	792-J6	8	RP4
2	792	9	R1
3	767-J6	10	838-J6
4	767	11	838
5	815-J6	12	R6K
6	815	13	RP4
7	R6K	14	R1

No plasmids were observed in 767-J6, 815 or 815-J6 (Figure 6), this could be due to the inability to detect a plasmid with the technique used or that either the whole plasmid, in the case of 767-J6, or the MRHA gene alone, in the case of 815-J6, had inserted into the chromosome after transfer. The preparation of DNA from these two strains and their transconjugants was repeated using alkaline lysis, but still no plasmids could be detected (Figure 6).

Only one plasmid with a molecular size of 58kb was detected in the clinical strain 819, whereas its transconjugant 819-J6, possessed two plasmids of molecular sizes 53 and 71kb. Also no plasmids could be detected in the clinical strains 792, 828, 838 or 1878 (Figures 4 and 6). This could possibly be explained by the difficulties encountered in lysing clinical isolates with Triton X-100, as it is only a gentle detergent and often a solution of SDS had to be added to complete the lysis. Also several phenol/ chloroform steps often had to be performed before a suitable solution from which to precipitate the DNA could be obtained. All these points could contribute to a poor representation of the plasmid content of some of the clinical strains.

As before, the DNA was prepared from those strains in which no plasmid had been detected by alkaline lysis, but resolution of plasmid bands was still not observed.

Transfer frequencies of resistance determinants from *E. coli* J62-2 to *E. coli* J53

The MRHA gene had been transferred from clinical strains to *E. coli* $J62-_2$. However, in order to check that this was not just an artefact and that the MRHA gene was readily transferable to other strains, the MRHA-positive $J62-_2$ transconjugants were mated with *E. coli* J53 for one hour at 37°C. The J53 transconjugants were selected on DM agar plates containing the J53 supplements, proline and methionine, to select against $J62-_2$, and the antimicrobial drug used to select for the original transfer.

Strains which contained more than one plasmid, i.e. 819-J6, 828-J6, 1995-J6 and 4950-J6, were mated with *E. coli* J53 for one hour as above, but plated on to the supplemented DM agar containing Ap, Tc or Ap and Tc for strains 819-J6, 828-J6 and 1995-J6. The mating mixture of strain 4950-J6 was plated on to Km, Tp, and Km and Tp DM agar plates. This allows the frequency of transfer of the resistance genes, both separately and together, to be calculated. A suitable dilution of the donor culture was also plated on to MacConkey agar to give the initial viable count of the organisms. The transfer frequencies were determined using the equation:

$$\frac{\text{No. of transconjugants /ml of culture}}{\text{No. of donors/ml of culture}}$$

The transfer frequencies of the resistance determinants of strains 810-J6 and 1878-J6 to *E. coli* J53 were also determined as strain 810 originally possessed two plasmids and strain 1878-J6 possessed five resistance determinants. The calculated transfer

frequencies are shown in Table 8. The Tc resistance determinant of strain 767-J6 was also tested for transfer. This gene transferred to E. coli J53 at a frequency of 3.09×10^{-5} even in the absence of a detectable plasmid.

The similarity of the transfer frequencies of the resistance determinants of strains 810-J6, 819-J6 and 828-J6 indicated that the Ap and Tc resistance genes resided on the same plasmid, and in the case of the transconjugants from strain 4950-J6 the Tp and Km resistance determinants appeared to be carried on the same plasmid. The Km and Tc resistance determinants of 1878-J6, and the Sm and Tc resistance determinants of 1995-J6 also showed similar transfer frequencies, implying the carriage of the genes on the same plasmid.

The presence of the resistance determinants on the same plasmid was confirmed by randomly selecting 70 colonies from each of the single antimicrobial drug plates for each of the six strains. These colonies were tested for possession of the second resistance determinant by spotting them on to DSTA plates containing the second antimicrobial drug and DSTA plates without any antimicrobial, and observing any growth on these plates after incubation for 18 hours at 37°C. Strains 1878-J6 and 1995-J6 possessed more than two resistance markers, therefore the selected J53 transconjugants from these two strains were also tested for possession of Ap, Sm, Sx and Sp,Sx resistance determinants respectively. It was found that of each set of 70 transconjugants that was tested, all also possessed the other resistance determinants of the $J62_{-2}$ transconjugant.

Table 8 Transfer frequencies for the resistance determinants from *E. coli* J62-$_2$ to *E. coli* J53.

Strain no.	Transfer frequencies		
810-J6	Ap 1.57×10^{-5}	Tc 2.05×10^{-5}	ApTc 1.48×10^{-5}
819-J6	Ap 4.21×10^{-6}	Tc 3.16×10^{-6}	ApTc 2.11×10^{-6}
828-J6	Ap 1.46×10^{-5}	Tc 1.59×10^{-5}	ApTc 3.29×10^{-5}
1878-J6	Km 6.27×10^{-5}	Tc 3.90×10^{-5}	KmTc 4.58×10^{-5}
1995-J6	Sm 1.13×10^{-4}	Tc 9.65×10^{-5}	SmTc 1.63×10^{-4}
4950-J6	Km 2.02×10^{-5}	Tp 1.99×10^{-5}	KmTp 2.23×10^{-5}

Haemagglutination by the J53-transconjugants

Eight transconjugants, from the DM agar mating plates, from each strain were purified on selective media of the same type, and the purified strains used to assay HA ability. It was observed that the transconjugants derived from a MRHA-positive parent could be either MRHA-positive or MRHA-negative. The proportion of the J53 transconjugants that were MRHA-positive varied depending on the strain. The number of MRHA-positive transconjugants are shown in the table below.

Strain no.	738-J6	792-J6	810-J6	819-J6	828-J6	838-J6
No. MRHA+ / No. tested	2/5	5/6	5/8	5/5	5/6	5/6

Strain no.	1878-J6	1995-J6	4811-J6	4950-J6
No. MRHA+ / No. tested	4/6	5/6	5/8	5/8

These results implied that the MRHA gene was not stably inherited, however except for 738-J6 over half of the J53 transconjugants received the gene.

Plasmid-DNA content of MRHA-positive and MRHA-negative J53 transconjugants

In order to determine what differences existed between the plasmid species in the MRHA-positive and MRHA-negative transconjugants the plasmid DNA from both sets of strains was extracted by Triton X-100 lysis and compared by agarose gel electro-

phoresis (Table 9). The J53 transconjugants from strains 819-J6, 828-J6 and 1995-J6 (Figure 7) possessed only one plasmid compared to the two observed in the J62-$_2$ transconjugants. The 71kb plasmid from 819-J6, the 68kb plasmid from 1995-J6 and the 53kb plasmid from 828-J6 were not present in the respective MRHA-positve or MRHA-negative transconjugants, implying that these plasmids were either cryptic or carried non-selectable markers.

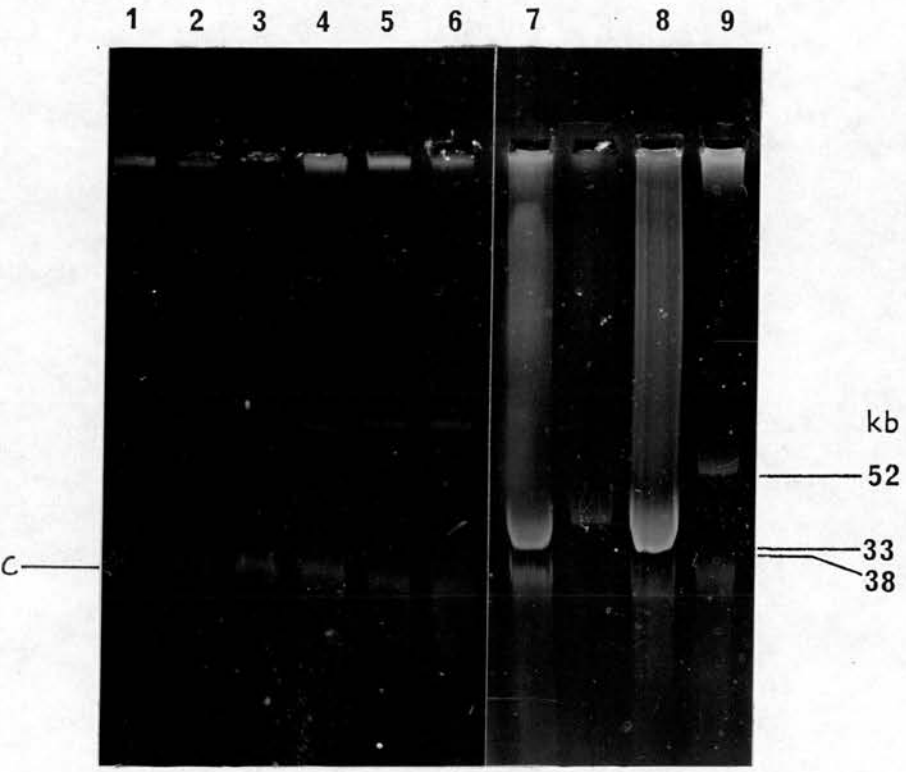
The plasmid sizes in the corresponding MRHA-positive and MRHA-negative J53 transconjugants were comparable, allowing for slight differences of approximately two kbases in most cases. Therefore, it appeared that loss of MRHA ability during or after transfer did not correspond with loss of a plasmid. This suggested that expression of the MRHA gene had been switched off in the MRHA-negative transconjugants. It seemed less probable that the MRHA gene had been lost from these strains as variations in the molecular size of the plasmids gave larger sizes in the MRHA-negative transconjugants of 738-J6, 838-J6 and 4811-J6, and plasmids of the same size in the J53 transconjugants of 810-J6, 1878-J6 and 1995-J6. Only MRHA-positive J53 transconjugants of 819-J6 were obtained so a comparison was not possible.

The results presented in this section detail the first evidence that the MRHA gene is plasmid encoded. The plasmids which possess this gene form a heterogeneous population of R-plasmids, from which the MRHA gene can be lost during conjugative transfer.

Table 9 Plasmids present in the MRHA-positive and MRHA-negative J53 transconjugants

$J62^{-}_{2}$ transconjugant no.	Plasmid sizes (kb)	
	MRHA+ J53 transconjugant	MRHA- J53 transconjugant
738-J6	57	59
792-J6	68	66
810-J6	55	55
819-J6	53	not obtained
828-J6	74	72
838-J6	52	57
1878-J6	77	77
1995-J6	55	55
4811-J6	70	72
4950-J6	53 134	134

Figure 7 Agarose gel comparing the plasmids present in the MRHA-positive or -negative J53 transconjugants of strains 819-J6, 828-J6 and 1995-J6. Plasmids were isolated using Triton lysis.



Lane 1 819-J5 MRHA^{+}
2 819-J6
3 828-J5 MRHA^{+}
4 828-J6
5 1995-J5 MRHA^{-}
Lane 6 1995-J6
7 Sa
8 R6K
9 RP4

III MOBILISATION OF THE MRHA GENE BY AN R-PLASMID

As stated at the beginning of section II, transfer of HA ability cannot be selected for directly and another character of the donor cell has to be used. Thirty-seven of the 99 MRHA-positive clinical isolates possessed no resistance determinants which could be used for selection after plasmid transfer. This problem can be overcome by introducing an R-plasmid into the clinical isolate. This provides a selectable marker and may also mobilise transfer deficient plasmids.

Plasmid mobilisation by R483

The Tp R-plasmid, R483, was introduced from its *E. coli* $J62_{-2}$ host into four of the antimicrobial drug sensitive clinical strains, 1864, 1871, 1872 and 1877, during an 18 hour mating. Transconjugants expressing the Tp resistance determinant were selected for on DM medium containing Tp at 100µg/ml but no amino acid supplements, to allow only the auxotrophic clinical strains which contained R483 to grow.

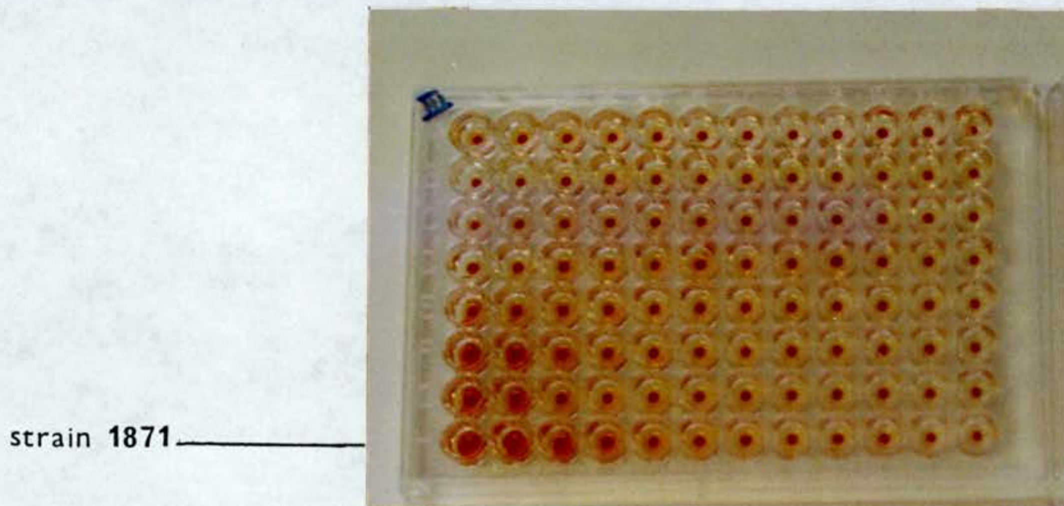
Two of the clinical strains, 1864 and 1871, stably inherited R483. The transfer frequency for Tp into the clinical strains was 7.9×10^{-6} for strain 1864 and 1.1×10^{-8} for strain 1871. Strains 1864 (R483) and 1871 (R483) were then mated for 18 hours with *E. coli* $J62_{-2}$ and selection made on DM medium containing proline, histidine, tryptophan, rifampicin at 25µg/ml and Tp at 100µg/ml. 1864 (R483) transferred the Tp resistance determinant to *E. coli* $J62_{-2}$ at a higher frequency (2.3×10^{-4}) than 1871 (R483) which gave

a transfer frequency of 4.4×10^{-5}. Five transconjugants from 1864 (R483) and six from 1871 (R483) were purified on the same selective media, and these purified strains were assayed for HA ability. One of the transconjugants from 1871 (R483) was MRHA-positive for human group A erythrocytes, but all the transconjugants from 1864 (R483) that were tested were MRHA-negative. The MRHA-positive transconjugant, 1871-J6 (R483) was tested on DM agar containing all the $J62-_2$ supplements and DM agar with all the supplements except histidine. Growth of 1871-J6 (R483) on the fully supplemented plates, but not on the histidine-negative plates confirmed that the transconjugant was a $J62-_2$ strain. The HA titre (1/bacterial dilution) was less for the transconjugant than for the wild type parent, as 1871-J6 gave a HA titre of 2 compared to the titre of 8 obtained for 1871 (Figure 8).

Plasmid determination of 1871 and 1871-J6

In order to determine the nature of the plasmid which had transferred the MRHA gene from 1871 to 1871-J6, the plasmid DNA from the two strains was prepared by alkaline lysis and characterised by agarose gel electrophoresis (Figure 9). Strain 1871 possessed two plasmids with molecular sizes of 26 and 52kb, whereas strain 1871-J6 contained only one plasmid with a molecular size of 98kb. DNA from a $J62-_2$ strain which contained the standard R483 plasmid had been run in parallel with these two strains, and the molecular size of R483 was calculated as 90kb. As the 98kb plasmid was the only one detectable in 1871-J6, it suggested the possibility that R483 was the only plasmid to transfer from the Tp-resistant strain 1871 and

Figure 8 Microtitre plates showing the comparative HA titres of strains 1871 and 1871-J6 and a MRHA-negative transconjugant.



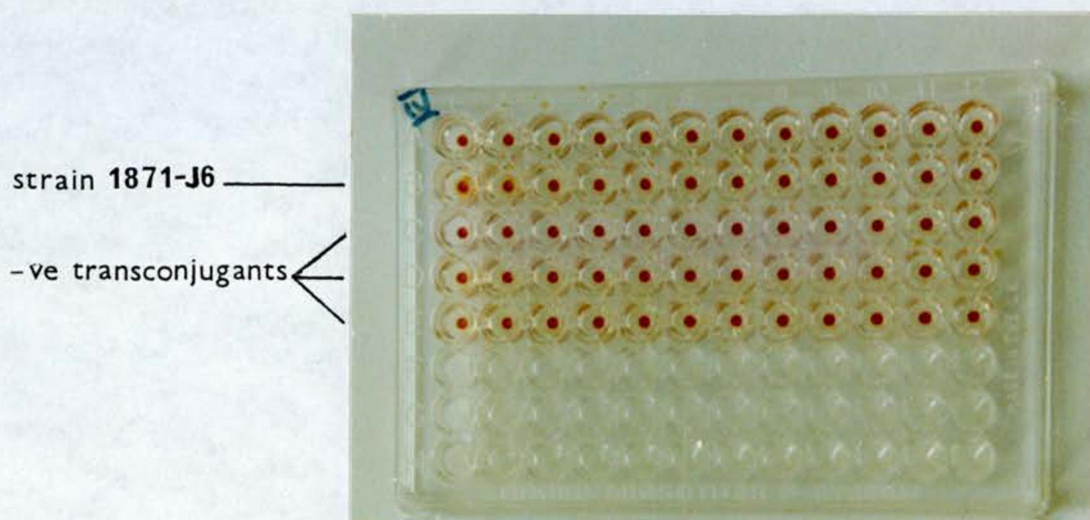
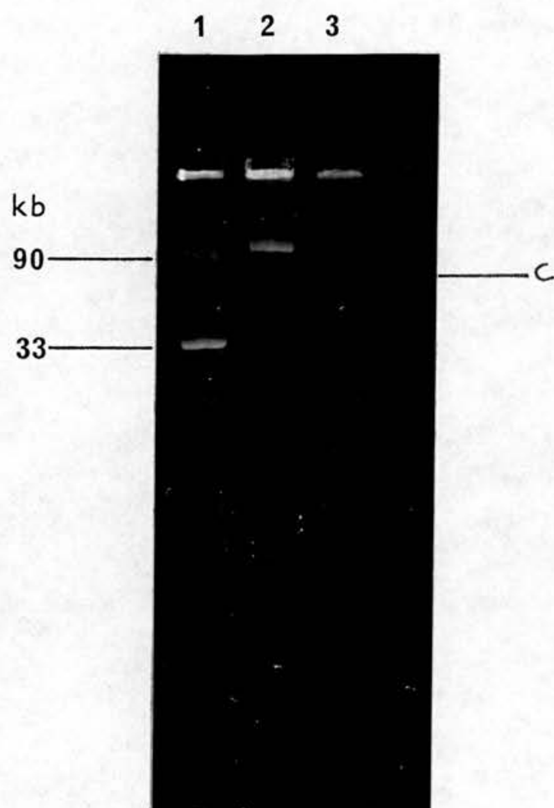


Figure 9 Agarose gel of strains 1871 and 1871-J6. DNA was prepared by alkaline lysis.



Lane 1 R483 / Sa

2 1871-J6

3 1871

that the MRHA gene had been transferred on R483, rather than by the transfer of one of the clinical plasmids. The presence of R483 in 1871-J6 was also inferred by the fact that when spotted on to DSTA containing Sm or Sp growth of the transconjugants was observed, implying that the Sm/Sp resistance determinant of this plasmid had been co-transferred. Thus it appeared that molecular recombination had occurred between R483 and either the chromosome or one of the two plasmids of strain 1871.

Transfer of R483 and the MRHA gene to E. coli J53

In order to confirm that the MRHA character was readily transferable, strain 1871-J6 was mated for 18 hours with E. coli J53. Transconjugants were selected on DM medium supplemented with proline and methionine and containing Tp at 100μg/ml. The Tp resistance determinant transferred at a frequency of 1.75×10^{-5}. Eight transconjugants were purified on DM media containing Tp and then spotted on to DSTA agar containing Sm or Sp to test for co-transfer of these genes. All eight colonies possessed the Sm/Sp resistant determinant. These transconjugants were then assayed for their HA ability. One of these transconjugants, 1871-J5, was MRHA-positive for human group A erythrocytes.

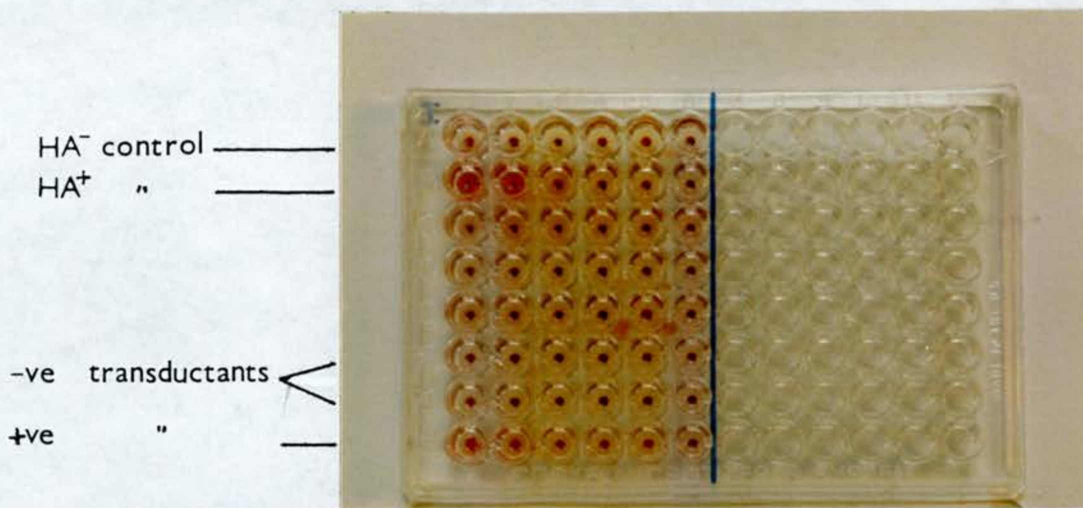
Bacteriophage P1 transduction of the MRHA gene

To confirm that the MRHA gene was carried on R483 and not on another plasmid that was not detectable in 1871-J6, P1 transduction was performed using the Tp resistance of R483 to select for

transductants. The molecular size limit that P1 can transduce is approximately 102kb, close to the size of R483. Therefore, if transduction of R483 is selected for and the MRHA gene is co-transduced, it would indicate that the MRHA gene did reside in R483.

E. coli N99 contains a heat inducible P1 lysogen. If this strain is grown at 30°C growth proceeds normally, but growth of the culture at 42°C induces the P1 lysogen to enter the lytic cycle. This heat induced lysate, when mixed with an indicator strain such as 1871-J6, will infect the indicator and proceed through the lytic cycle during growth at 37°C. The P1 phage will pick up and package DNA from the indicator strain as well as its own P1 DNA. This 'phage preparation can, in turn, be used to transduce a suitable recipient. Transductants were selected for on DM media containing the E. coli J53 supplements (proline and methionine) and Tp at 100µg/ml. Eight of the resultant transductants were purified and spotted on to DSTA containing Sm or Sp. All the Tp-resistant transconjugants were also Sm-resistant but not Sp-resistant, and five out of eight of the transductants were MRHA-positive for human group A erythrocytes (Figure 10). These transductants were tested on DM agar containing the J53 amino acid supplements and DM agar lacking methionine to check that the transductants were J53 strains. As the MRHA gene had been co-transduced with the Tp and Sm resistance determinants it seemed probable that all these genes were carried on the same plasmid.

Figure 10 Microtitre plate showing the MRHA-positive and -negative transductants from 1871-J6.



Rows 3,4 + 5 are not relevant

Loss of R483 from 1871-J6 using an incompatible plasmid

As two plasmids of the same incompatibility group cannot stably co-exist in the same cell, due to similarities in the replication regions, the introduction of the inc Iα R-plasmid JR66a into a strain containing R483 would, under suitable conditions, result in the loss of R483. The concomittant loss of the MRHA gene with the R483 resistance determinants would prove the physical association of these markers.

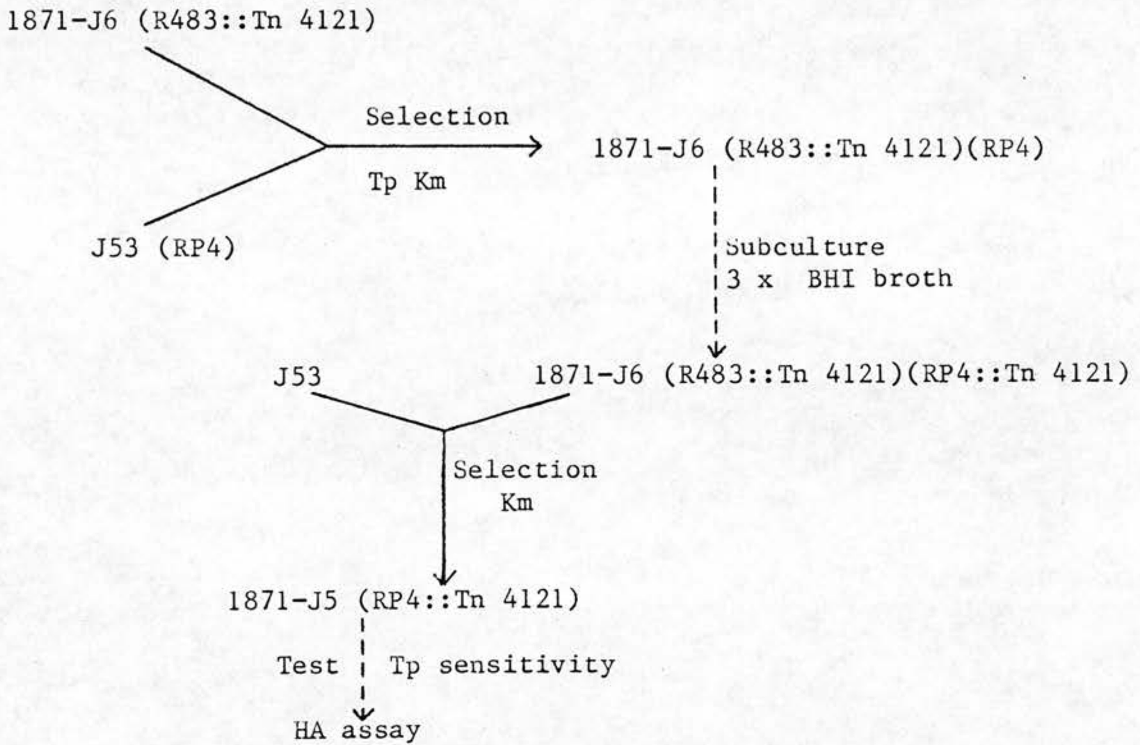
Therefore, plasmid JR66a was introduced into 1871-J6 during a one hour mating at 37°C. Strain 1871-J6 transconjugants containing JR66a were selected on DM agar containing the J62-$_2$ supplements, Km at 10µg/ml and rifampicin at 25µg/ml. Eight of the resultant transconjugants were purified on the same type of selective media, and once purified were tested on DM agar containing the J62-$_2$ supplements, Tp at 100µg/ml and Km at 10µg/ml. If R483 had been lost no growth of the organisms should occur on these latter plates. All the strains tested were resistant to Tp, suggesting that R483 might still be present. In order to determine this, the 1871-J6 (JR66a) purified strains were mated for two and a half hours with E. coli J53. Selection was made on DM agar containing the J53 supplements and either Tp at 100µg/ml or Km at 10µg/ml. Transfer of the Km but not the Tp resistance determinant was observed. Ten of the Km-resistant transconjugants were purified on the Km selective media and then assayed for HA ability. All the transconjugants tested were MRHA-negative for human group A erythrocytes. This suggested that the MRHA gene had been lost concommitantly with R483, but that the Tp-resistance determinant, Tn7, of R483, had transposed

on to the bacterial chromosome first, conferring non-transferable Tp-resistance on strain 1871-J6 (JR66a). As JR66a is also Sm-resistant, loss of the Sm resistance determinant of R483 could not be tested for.

Transposition of the MRHA gene to plasmid RP4

Previous results had shown that the MRHA gene had integrated into R483. This could have occurred by either active transposition of the MRHA gene into R483 or the gene could have been picked up by R483. In order to demonstrate if the MRHA gene was capable of transposition, another R-plasmid, RP4, was introduced into strain 1871-J6 during a five hour mating. Plasmid RP4 is only 52kb compared to R483 which is 90kb, therefore, if transposition does occur an increase in size of RP4 will be more readily identified and measured. The procedure followed is outlined in Figure 11. Transconjugants from the mating were selected on DM agar containing the $J62_{-2}$ supplements, Tp at 100µg/ml and Km at 10µg/ml. Eight transconjugants were purified on supplemented DM agar containing Tp at 100µg/ml and Km at 10µg/ml and also on supplemented DM agar containing Tp at 100µg/ml and Tc at 10µg/ml. Seven of the purified transconjugants grew on the plates containing Tc, and five of these also grew on the plates containing Km. One of the Tp, Km resistant transconjugants was grown statically for 18 hours in BHI broth and then successively subcultured three times in BHI broth in the absence of antimicrobial drug selection. This will allow transposition to occur between replicons.

Figure 11 Series of mating experiments followed to allow transposition of the MRHA gene into RP4.



A suitable dilution of the third subculture was plated on to DM agar containing the J62-2 supplements, Tp at 100µg/ml and Km at 10µg/ml. Organisms from this plate were purified on selective media of the same type, then one of the purified strains was mated with E. coli J53 for five hours. Transconjugants were selected on DM containing the J53 supplements and Km at 20µg/ml. Nine hundred and fifty seven transconjugants from this mating were spotted on to DSTA and DSTA containing Tp at 100µg/ml, to test for the Tp sensitivity of the strains. Resistance to Tp indicates whether R483 or Tn7 is present. Of these 957 strains, 122 were Tp-resistant, 678 showed some mutation to Tp-resistance where only a few colonies grew and 157 were Tp-sensitive.

Ten of the trimethoprim sensitive strains were purified on DM agar containing the J53 supplements and Km at 20µg/ml. The 10 purified strains were assayed for their HA ability. Eight of these strains were MRHA-positive for human group A erythrocytes, four gave a HA titre of 2, and four a HA titre of 4, indicating that the MRHA gene must have transposed on to RP4, and this plasmid had transferred to E. coli J53 independently of R483.

Plasmid analysis of RP4 containing transconjugants

In order to determine the increase in size of RP4 due to the insertion of the MRHA gene the plasmid DNA from one of the strains, 1871-J5 (RP4), with a higher HA titre was prepared and compared to the native RP4 plasmid DNA (Figure 4b). An increase of only 1.3kb was observed between the calculated molecular size of RP4 as 54.2kb and the size of RP4 in 1871-J5 which was calculated as 55.5kb. This

size increase was too small to be the actual size of a functional transposon. One reason for this could be the loss of non-essential pieces of DNA from RP4, with the insertion of the transposon, to retain the original size of the plasmid. Reported by Barth et al. (1978) for Tn7 insertions in RP4. None of the resistance determinants had been lost as all the transconjugants from the final mating, above, were resistant to Ap, Km and Tc when spotted on to DSTA containing these antimicrobials at 10μg/ml.

Transposition of the MRHA gene into plasmid Sa

In order to overcome the problem encountered with RP4 the inc W R-plasmid Sa was introduced into 1871-J6 during a five hour mating. This and the following procedure are outlined in Figure 12. Transconjugants containing Sa were selected on DM agar containing the $J62_{-2}$ supplements, Tp at 100μg/ml and Cm at 10μg/ml. Eight transconjugants were purified on selective media of the same type and then one of these was successively subcultured three times in BHI broth then plated on to DM agar containing the $J62_{-2}$ supplements, Tp at 100μg/ml and Cm at 10μg/ml. Organisms growing on this plate were purified on the same type of selective media and one of the purified strains was mated for one hour with the JR66a-containing strain to remove R483 and reduce the number of transconjugants which have to be tested for Tp sensitivity. Transconjugants were selected on DM agar containing the $J62_{-2}$ supplements, Cm at 10μg/ml and neomycin (Ne) at 10μg/ml. Plasmid JR66a will outgrow R483 and the resultant transconjugants will contain plasmids Sa and JR66a.

Figure 12 Outline of the procedure to allow transposition of the MRHA gene into plasmid Sa.

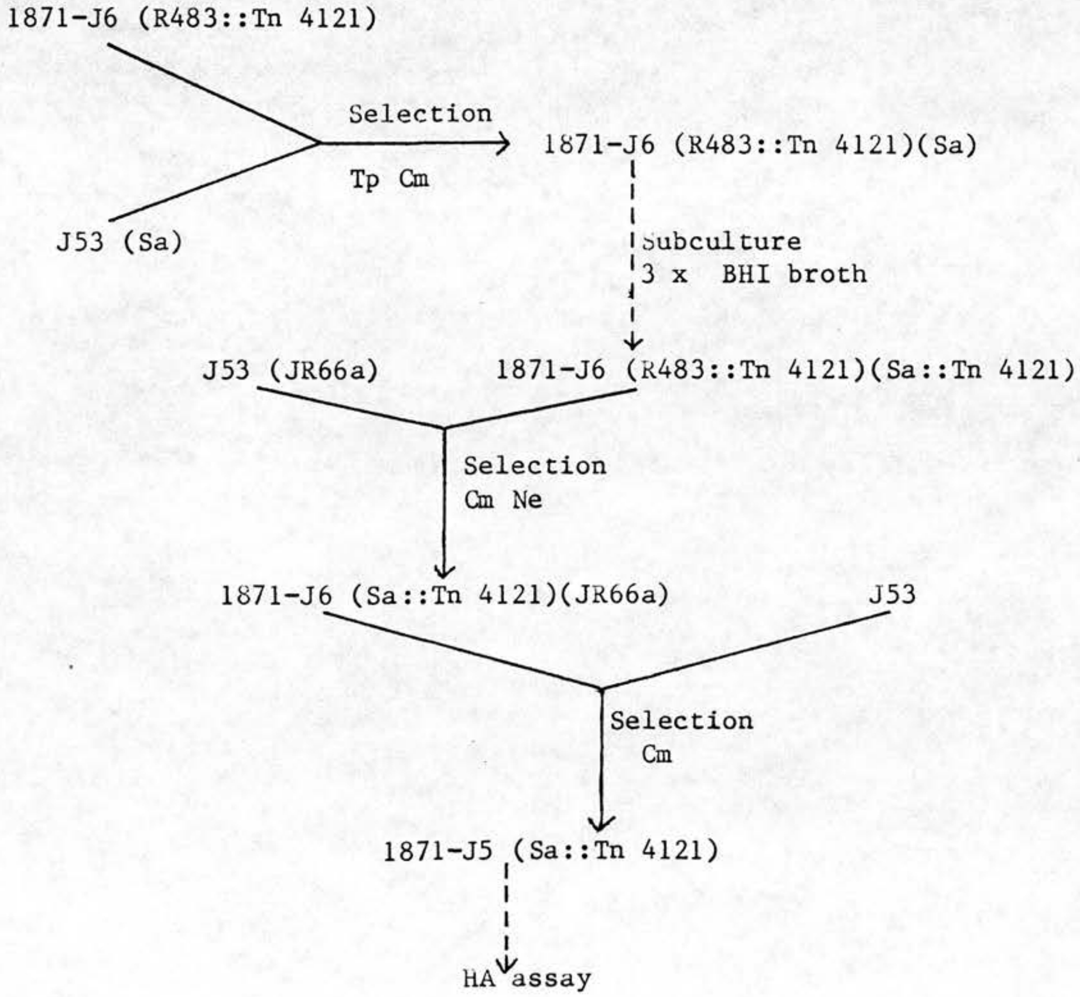
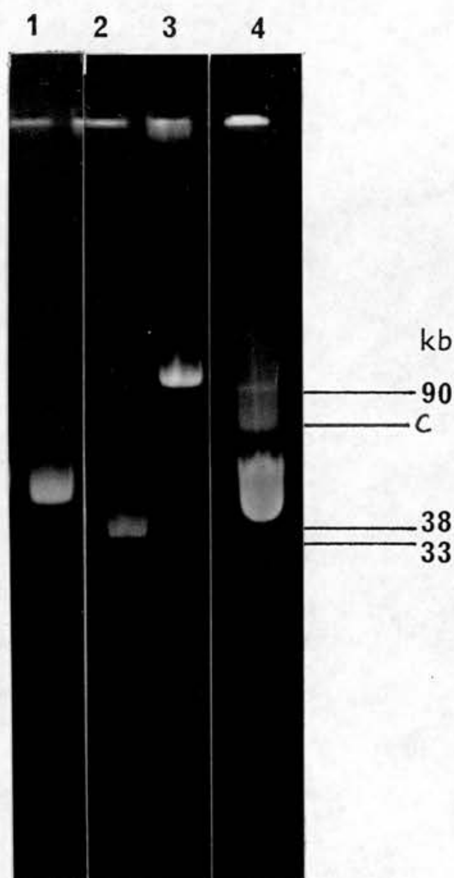


Figure 13 Agarose gel comparing the standard plasmid Sa to the Sa plasmid species from the MRHA-positive 1871-J5. Plasmids were isolated using alkaline lysis.



Lane 1 1871-J5 (Sa)

2 Sa

3 R1

4 R6K

Eight transconjugants were purified on the Cm, Ne containing media, and one of these was mated with E. coli J53 for one hour. As with the RP4 procedure, the J53 transconjugants were Tp-resistant, but this determinant was non-transferable. Transconjugants were selected for on DM media containing J53 supplements and Cm at 10μg/ml. Six transconjugants from this mating were purified and assayed for HA ability. Four of these strains were MRHA-positive for human group A erythrocytes, indicating that the MRHA gene had transposed on to Sa.

Isolation of plasmids from 1871-J5 (Sa)

In order to demonstrate an increase in plasmid Sa due to the insertion of the MRHA gene the plasmid DNA from 1871-J5 (Sa) and the original Sa-containing J53 strain was prepared by alkaline lysis and compared by agarose gel electrophoresis (Figure 13). The molecular size of the standard Sa was calculated as 33.3kb and the Sa plasmid species in 1871-J5 was calculated as 42kb. This gave the increase in size of Sa due to the insertion of the MRHA gene as 8.7kb, very similar to the original 7kb difference observed between R483 in 1871-J6 and the standard R483. This transposable element was given the designation Tn4121.

Electron microscopy of strains 1871, 1871-J6 and $J62-_2$

The ability of strain 1871-J6 to cause HA could have been due to an artefact in the culture medium, as the transposition of the MRHA gene had not previously been considered. For this reason strains 1871, 1871-J6 and $J62-_2$ were cultured for 48 hours in BHI

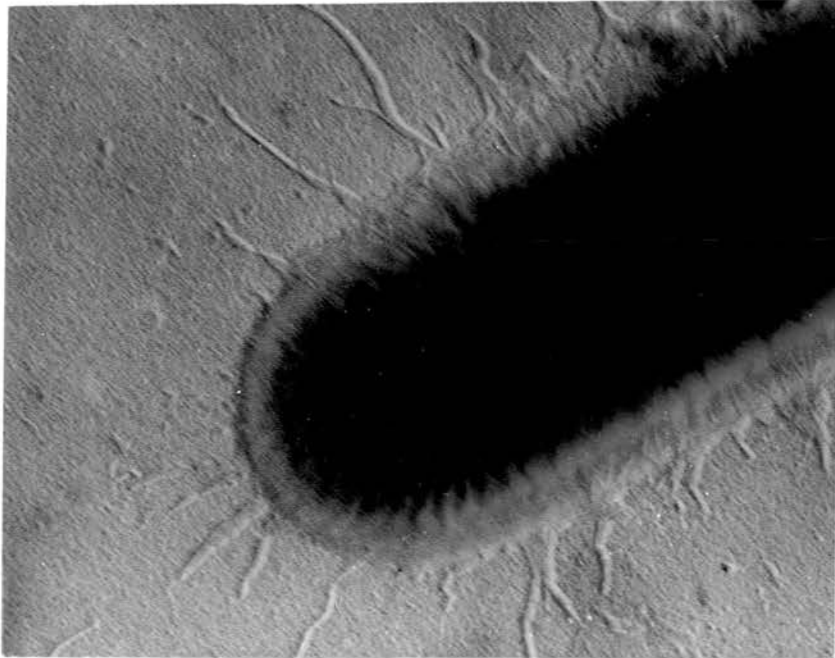
broth and then prepared for loading on to electron microscope (EM) grids. Once dessicated, these prepared grids were shadowed with platinum and observed under the EM. Using the EM it is possible to see the exterior of the bacteria in greater detail and determine whether they possess fimbriae.

It was shown that the surface of the clinical isolate, strain 1871, was covered with fimbriae (Figure 14a), the surface of E. coli $J62-_2$, the negative control, was devoid of fimbriae (Figure 14b), but the MRHA-positive transconjugant, 1871-J6, also possessed surface fimbriae (Figure 14c). This visually proved that the ability of the transconjugants to cause HA could be related to the production of fimbriae by these strains.

The identification of the MRHA on a transposon from a strain which did not possess transferable R-plasmids provides a possible explanation for the heterogeneous nature of the plasmid encoded MRHA genes. Transposition could have originally occurred from the bacterial chromosome to plasmids present in the same cell, allowing its transfer and divergence through the bacterial population.

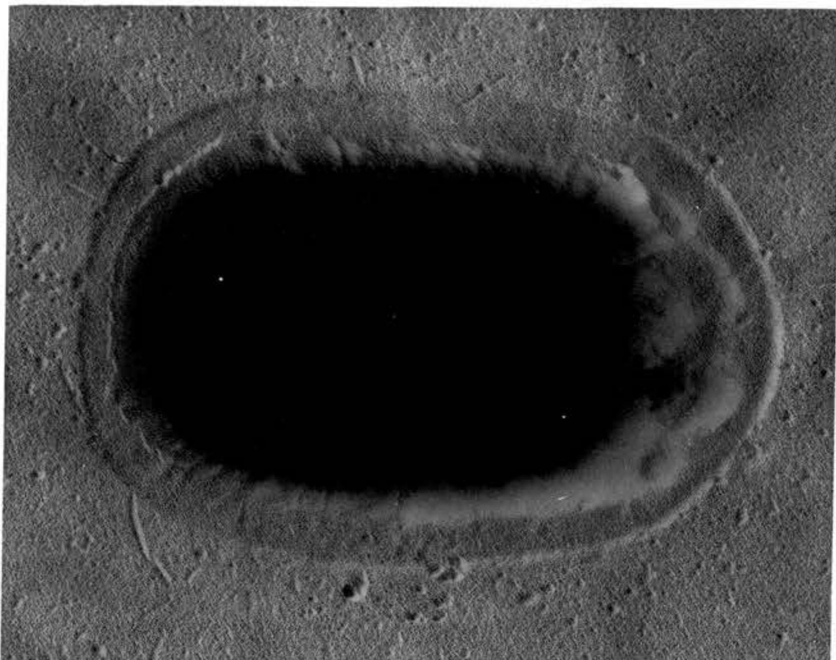
Figure 14 Electron micrographs of strains 1871, 1871-J6 and $J62-_2$ showing surface fimbriae.

a



Magnification 27000 x

b

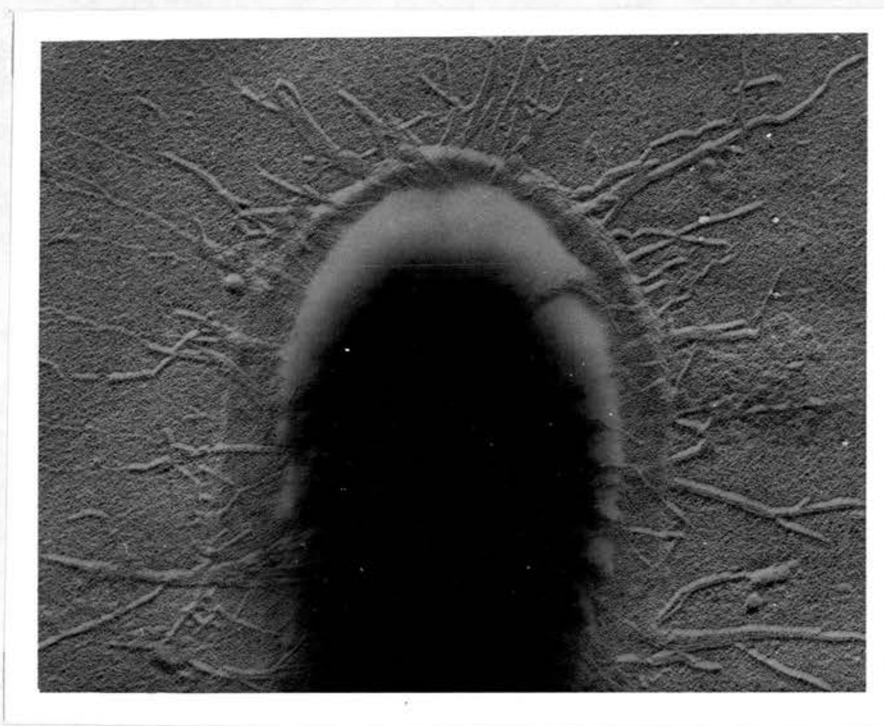


Magnification 31000 x

a. 1871 b. $J62-_2$

Figure 14 (continued)

c



Magnification
27000x

c. 1871-J6

IV FURTHER CHARACTERISATION OF THE PLASMID-ENCODED MRHA GENES

The existence and subsequent extraction of the transposable MRHA gene from strain 1871 prompted the examination of the R-plasmids which possessed this gene, and had transferred from the clinical isolates to E. coli $J62_{-2}$ (Section II), for similar transposons.

Removal of transposons from plasmids possessing the MRHA gene

The extraction was performed byintroducing plasmid Sa into the MRHA-positive transconjugants during a seven hour mating. The subsequent procedure is outlined in Figure 15. Selection was made on DM agar containing the $J62_{-2}$ supplements, Cm at 10µg/ml and one other antimicrobial drug, resistance to which was encoded by the MRHA-positive $J62_{-2}$ recipient strain (Table 10). This was Tc in the case of 767-J6, 810-J6, 819-J6, 828-J6, 1878-J6, 1995-J6 and 4811-J6, Ap for 738-J6, 792-J6, and 838-J6, and Tp for 4950-J6. Sa-containing transconjugants of 815-J6 were selected on Cm alone due to the loss of resistance markers from this strain (Section II).

Eight transconjugants from each mating were purified on the same type of selective media, then one purified strain from each mating was subcultured three times in BHI broth to allow transposition of the MRHA gene to Sa. After subculture suitably diluted cultures were plated on to DSTA containing Cm at 10µg/ml and the antimicrobial which was used as the selection agent for the mating. After growth four colonies from each plate were purified and one of these from each strain was mated with E. coli J53 for one

Figure 15 Mating series followed to obtain MRHA-positive, Sa-containing transconjugants.

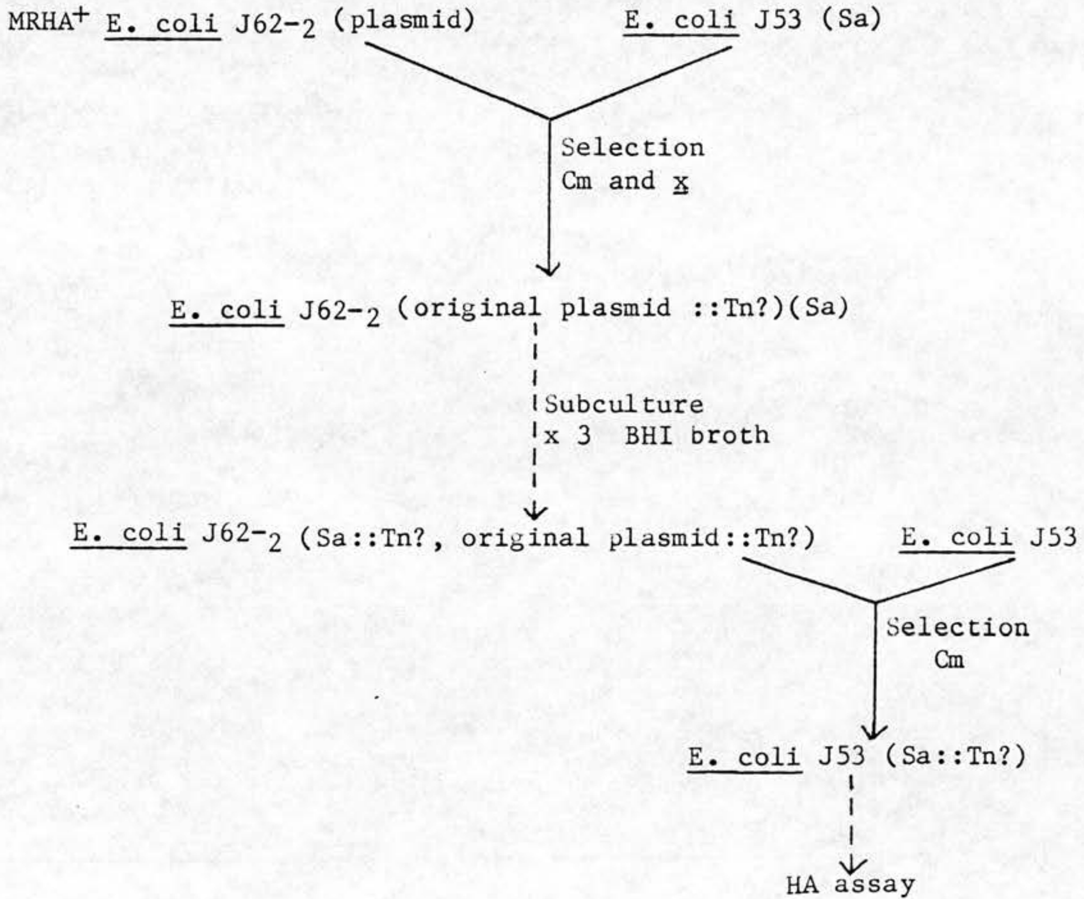


Table 10 MRHA-positive transconjugants and the selection procedure for removing transposons.

Strain no.	Section Media	Transconjugants tested on	Antimicrobial Sensitivity	No. MRHA$^+$
738-J6	Cm Ap	Cm Ap	16 Ap^S	6/8
767-J6	Cm Tc	Cm Sx Tc	5 Tc^S	3/5
792-J6	Cm Ap	Cm Ap	All Ap^R	-
810-J6	Cm Tc	Cm Ap	8 Ap^S	4/7
815-J6	Cm	Cm	-	
819-J6	Cm Tc	Cm Ap Tc	All Ap Tc^R	-
828-J6	Cm Tc	Cm Ap Tc	27 Ap Tc^S	3/8
838-J6	Cm Ap	Cm Ap	15 Ap^S	5/7
1878-J6	Cm Tc	Cm Km Tc	All Tc^R	-
1995-J6	Cm Tc	Cm Sm Tc	27 Tc^S	7/8
4811-J6	Cm Tc	Cm Ap Tc	12 Ap Tc^S	7/8
4950-J6	Cm Tp	Cm Km Tp	All Tp^R	-

hour. Transconjugants were selected on DM agar containing J53 supplements and Cm at 10µg/ml. Seventy colonies from the mating plate of each strain were spotted on to DSTA, DSTA containing Cm at 10µg/ml and DSTA containing each antimicrobial, resistance to which was encoded by the original R-plasmid in the MRHA-positive transconjugant.

It would be expected that all colonies would be Cm-resistant due to the presence of Sa, but if no growth was observed on the DSTA plates containing the other antimicrobials this would indicate that the original plasmid had not been transferred during the short mating time allowed.

The transconjugants from the seven strains which appeared to contain Sa alone, from the sensitivity test results (Table 10, columnm 4) were purified on the same type of selective media and then tested on DM containing proline and methionine, and DM containing only proline, to check that all the strains were J53.

<u>HA ability of the Sa-containing transconjugants</u>

Up to eight of these purified transconjugants from each of the seven strains were assayed for their ability to agglutinate human group A erythrocytes. The results obtained are shown in Table 10 (column 5).

In each case only the resistance markers for Sa were present in the strains, indicating that the original clinical plasmid which carried the MRHA gene was absent, and that the MRHA gene had transposed on to Sa.

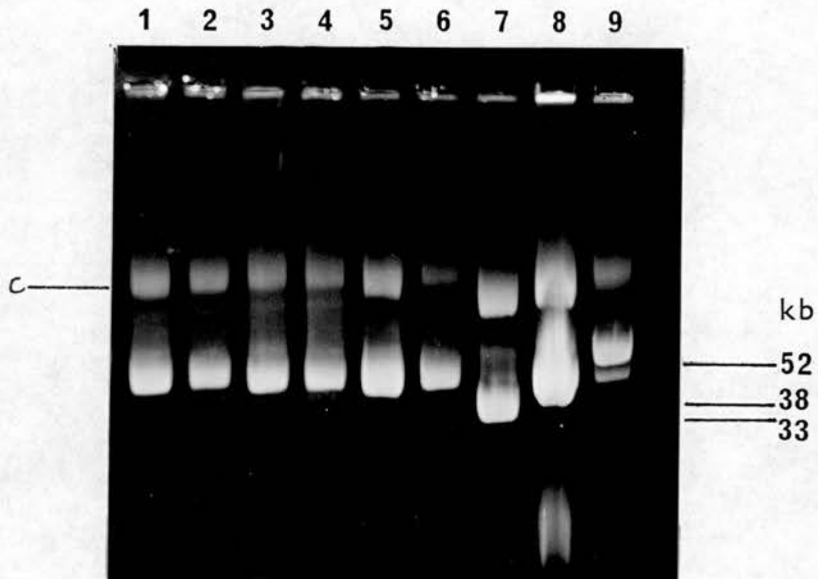
Plasmid analysis of the MRHA-positive Sa-containing transconjugants

The DNA was extracted and purified from each of the E. coli J53, Sa-containing strains by alkaline lysis. The plasmids from 738-J5, 767-J5, 810-J5, 828-J5, 838-J5, 1995-J5 and 4811-J5 were compared to the standard Sa by agarose gel electrophoresis (Figure 16). Increases in the Sa plasmid species in all the MRHA-positive transconjugants were observed, but there was a slight variation in the size of this increase. Strains 738-J5, 1995-J5 and 4811-J5 possessed a 41.8kb plasmid and strains 767-J5, 810-J5, 828-J5 and 838-J5 possessed a 40.4kb plasmid. This was compared to a molecular size of 33.4kb obtained for plasmid Sa.

By deducting the size of Sa from the size obtained for the plasmids in the transconjugants the size of the insertion due to the presence of the MRHA gene was calculated as 8.4kb for strains 738-J5, 1995-J5 and 4811-J5, and 7.0kb for strains 767-J5, 810-J5, 828-J5 and 838-J5 (Table 11).

These values are very similar to the value obtained for Tn 4121 of strain 1871, therefore it is possible that the same gene and transposon are present in all the strains, the 1.4kb difference in the values obtained being accounted for by experimental variation, and the ability of the gene to transpose has allowed its diversification to a heterogeneous population of plasmids.

Figure 16 Agarose gel comparing the standard plasmid Sa to the Sa plasmids isolated from the MRHA-positive transconjugants. Plasmid isolation was by alkaline lysis.



Lane 1 810-J5
2 738-J5
3 767-J5
4 4811-J5
5 828-J5

Lane 6 1995-J5
7 Sa
8 R6K
9 RP4

C = Chromosomal DNA

Table 11 Plasmid sizes in Sa-containing transconjugants.

Strain no.	Plasmid size (kb)	Transposon size (kb)
738-J5	41.8	8.4
767-J5	40.4	7.0
810-J5	40.4	7.0
828-J5	40.4	7.0
838-J5	40.4	7.0
1995-J5	41.8	8.4
4811-J5	41.8	8.4
Sa	33.4	-

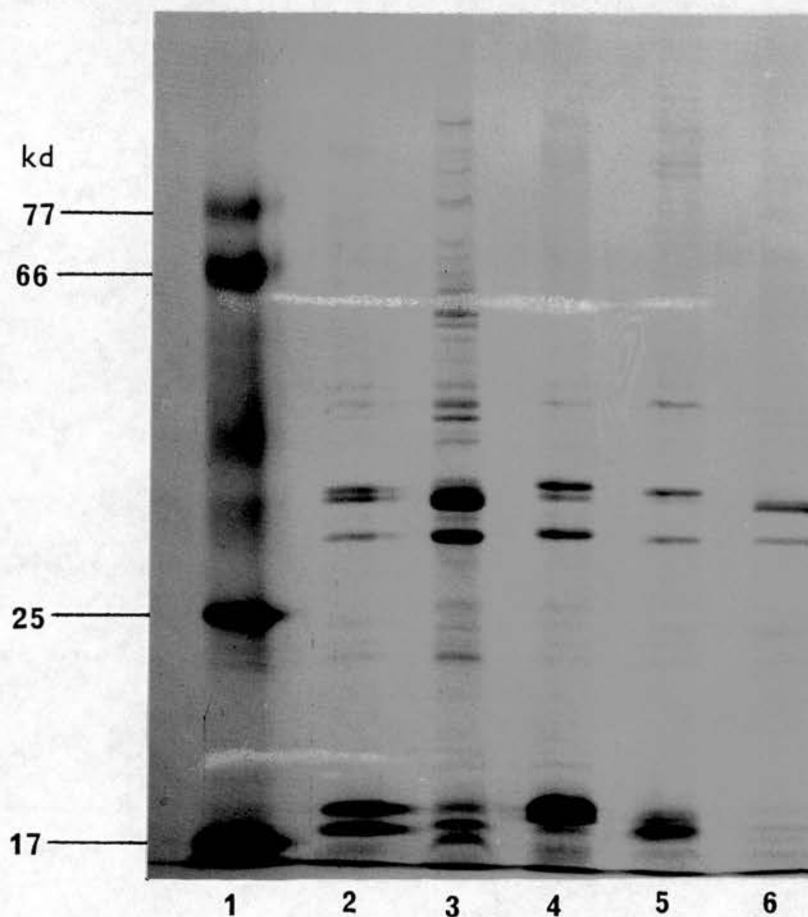
Extraction of fimbrial proteins from the MRHA-positive, Sa-containing transconjugants

Preliminary work was carried out to examine the proteins synthesised by the MRHA genes which had been inserted into plasmid Sa. Fimbrial proteins were prepared from the MRHA-positive strains 728-J5, 767-J5, 810-J5, 828-J5, 1995-J5 and 4811-J5 which contained Sa. The solubilised protein samples obtained were electrophoresed through a polyacrylamide gel to separate the constituent proteins contained in the samples.

Problems were encountered in obtaining a sufficiently high protein concentration in the samples to visualise fimbrial protein bands and in obtaining separation of these relatively small molecular weight proteins. Fimbrial proteins from one strain, 4811-J5 (Sa), were obtained in a sufficiently high concentration; however, success was not achieved with the other five strains.

Figure 17 shows the results obtained. Strain 4811-J5 (Sa) was run in parallel with E. coli J53 as a negative control, E. coli T1 and NK1 as positive controls for uropathogenic fimbriae, and the original clinical isolate, 4811. Complete purification of the fimbrial proteins from other outer membrane proteins was not obtained, but it could be seen that E. coli T1 contained a major band of 18,000 and a minor band of 17,400d. E. coli NK1 also possessed these bands, but the proteins were present in lower amounts, and there was also a protein of 17,200d which was assumed to be the Type 1 fimbriae. Strain 4811 possessed two major bands of 18,200 and 17,400d. Both these protein bands were present in strain

Figure 17 Polyacrylamide gel showing the fimbrial proteins extracted from strains 4811 and 4811-J5 (Sa).



Lane 1	Molecular size standards	Lane 4	*E. coli* T1
2	4811	5	*E. coli* NK1
3	4811-J5 (Sa)	6	J53

4811-J5 (Sa) which also contained an additional band of 16,800d. Very faint bands could be seen in the 17,500 to 16,500d region in strain J53, but there were no major fimbrial proteins.

These values correspond to those found by other workers. Mett et al. (1983) obtained a value of 18,000d for their uropathogenic MR fimbriae, Klemm et al. (1983) reported a value of 18,200d for the F12 fimbriae and values obtained for the multiple P fimbriae bands ranged from 17,000 to 22,000d (Korhonen et al., 1982).

Obviously further work needs to be done on the other five strains for comparison.

V EXAMINATION OF STRAINS FROM RECURRENT UTIs

A collection of strains had been built up over a period of two years. All these strains were isolated from the urine of a single patient who suffered from recurrent UTIs. It was observed that resistance to Tp developed after treatment with this drug and that the gene was carried on a transferable R-plasmid. However, when treatment with Tp was discontinued the R-plasmid still persisted in the strains isolated from the urine. The discovery of the carriage of the MRHA gene on a plasmid lead to the re-examination of these strains for the MRHA pathogenicity character. The presence of this gene on the Tp R-plasmid could be a contributing factor to the long term nature of the infection by improving the survival of both the plasmid and the host bacterium.

Background to the work performed in this section

A study was carried out over a period of 25 months on an elderly female patient who suffered from recurrent UTIs. Urine specimens were collected twice weekly and the bacterial pathogen causing the infection was isolated from the urine, identified and sensitivity tested. The infection was initially treated with a seven day course of co-trimoxazole (Tp and Sx) until the infecting strain developed resistance to Tp. The Tp resistance determinant was plasmid-encoded and readily transferred to E. coli $J62-_2$ with the co-transfer of resistance to Ap, Sm, Sp and Sx. Trimethoprim therapy was stopped, but the R-plasmid persisted in the strains isolated from the urine. Eleven months after the beginning of the

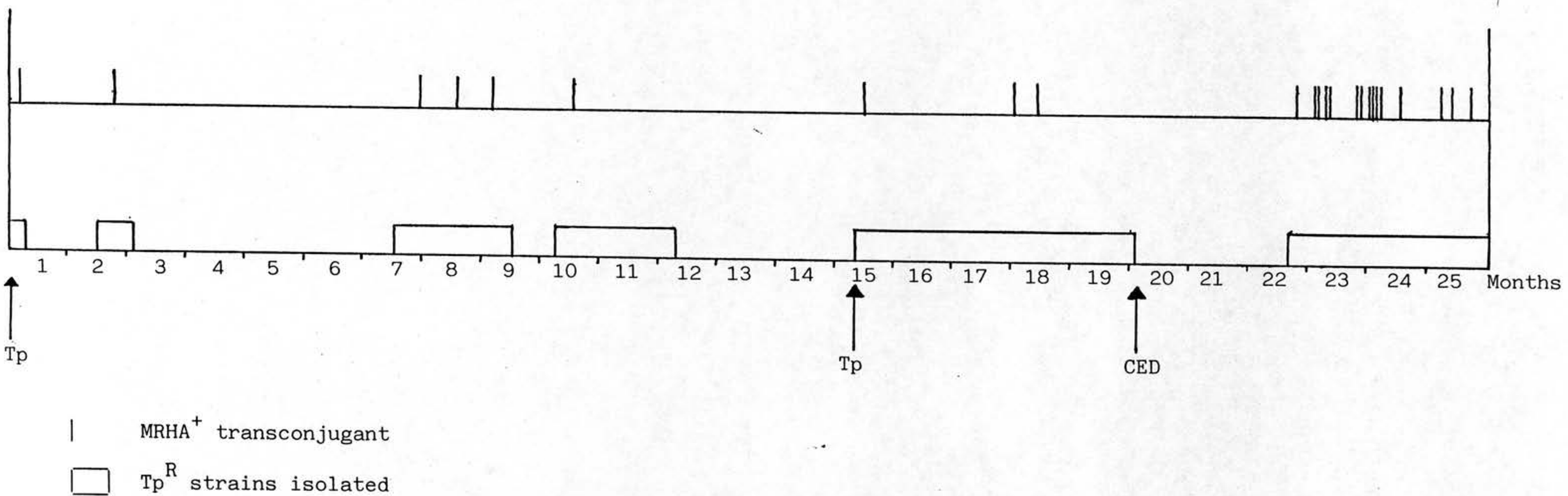
study this R-plasmid was absent from the infecting strain but subsequently reappeared three months later when the infection was again treated with Tp. The R-plasmid again persisted in the strains isolated from the urine for four and a half months until the patient was treated with cephradine (Amyes et al., 1981). However, after a further period of two and a half months a Tp-resistant E. coli was again isolated from a further infection.

MRHA ability of the Tp-resistant transconjugants

Of the strains isolated, ninety-five transferred Tp-resistance to E. coli J62-$_2$ and it was these transconjugants that were examined further. The sensitivity of each transconjugant was tested by spotting the culture on to DSTA containing the standard antimicrobials used in Section I and listed in Table 4. All ninety-five transconjugants were resistant to Tp, Ap and Sm at 10μg/ml and Sp and Sx at 100μg/ml. Each of these ninety-five strains were cultured in BHI broth and assayed for their ability to agglutinate human group A erythrocytes as before.

Twenty-four of the strains tested were MRHA-positive. These MRHA-positive transconjugants were not spread evenly throughout the 25 month period. Of 65 transconjugants obtained over the first 19 months, only nine were MRHA-positive, whereas 15 out of 30 transconjugants obtained during the final six months of the study were MRHA-positive (Figure 18). The presence of the MRHA gene in the J62-$_2$ transconjugants suggested that the gene was also plasmid encoded in these strains, and that its efficiency of transfer with the Tp R-plasmid increased with time and the number of re-

Figure 18. Diagram showing the periods of recurrent infection and the MRHA-positive transconjugants obtained. These strains are numbered 89 to 112 consecutively from left to right.



infections, especially after cephradine treatment, but it is not known if these two factors are related.

Plasmid analysis of the MRHA-positive transconjugants

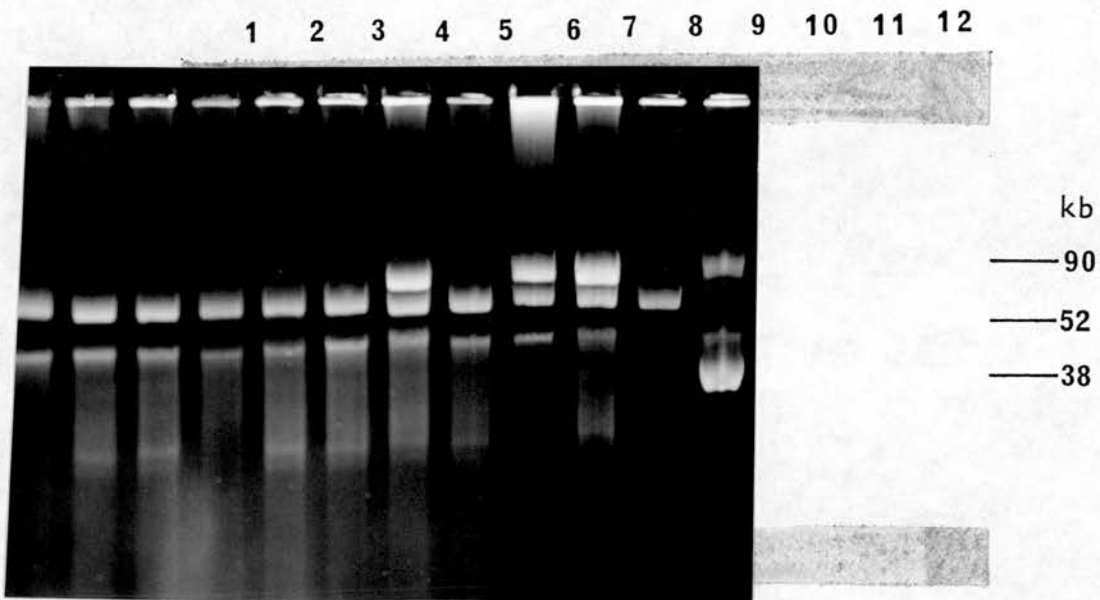
In order to determine the size and number of plasmids that had been transferred, the DNA was prepared from the 24 MRHA-positive transconjugants by alkaline lysis and examined by agarose gel electrophoresis (Figures 19 a and b). All the strains contained a 75kb plasmid, except for strains 110 and 112 where the calculated molecular size of the plasmid was 83.8kb and 87.4kb respectively. In addition, strains 91, 92, 94 and 102 contained a second plasmid with a molecular size of 100kb in the case of strains 91, 92 and 94, and 110kb in strain 102. However, as the majority of strains possessed only the smaller plasmid it could be assumed that it was this plasmid that must carry the MRHA gene.

Plasmid analysis of the MRHA-negative transconjugants

The plasmid DNA from a selection of eight MRHA-negative transconjugants spread over the 25 month isolation period was also prepared and examined for comparison. These strains also contained a single plasmid with a molecular weight of 70kb. The difference in size of the plasmids in the two sets of transconjugants could be seen when samples of both were run on the same gel. Figure 20 shows strains 94, 92, 90 and 89 with MRHA-negative transconjugants isolated in the same period.

Figure 19a Agarose gel of plasmids isolated from strains 90 to 100.

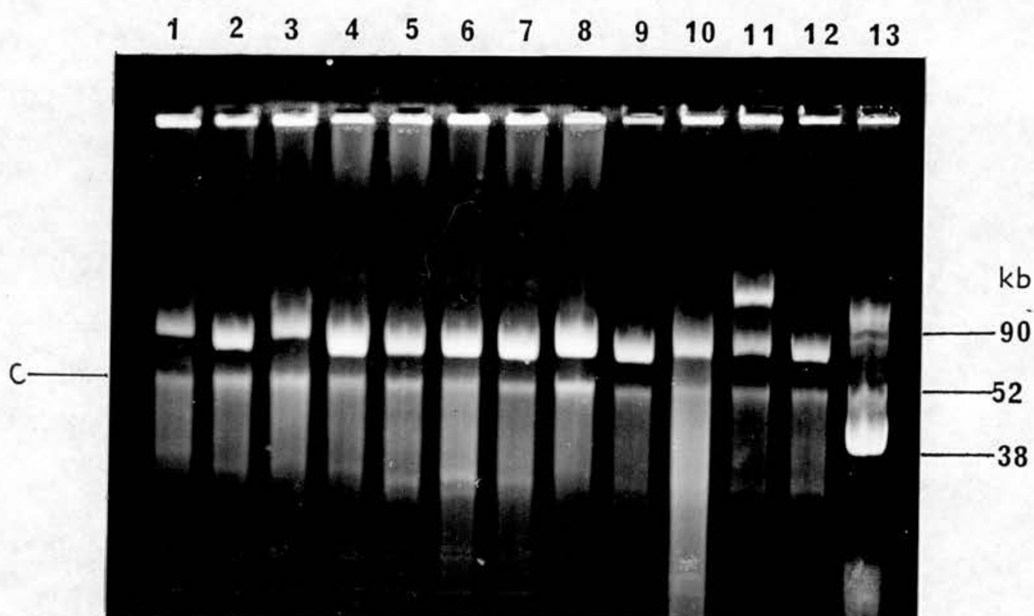
Plasmid isolation was performed by alkaline lysis.



Lane	Strain
Lane 1	100
2	99
3	98
4	97
5	96
6	95
Lane 7	94
8	93
9	92
10	91
11	90
12	R1/RP4/R6K

C = Chromosomal DNA

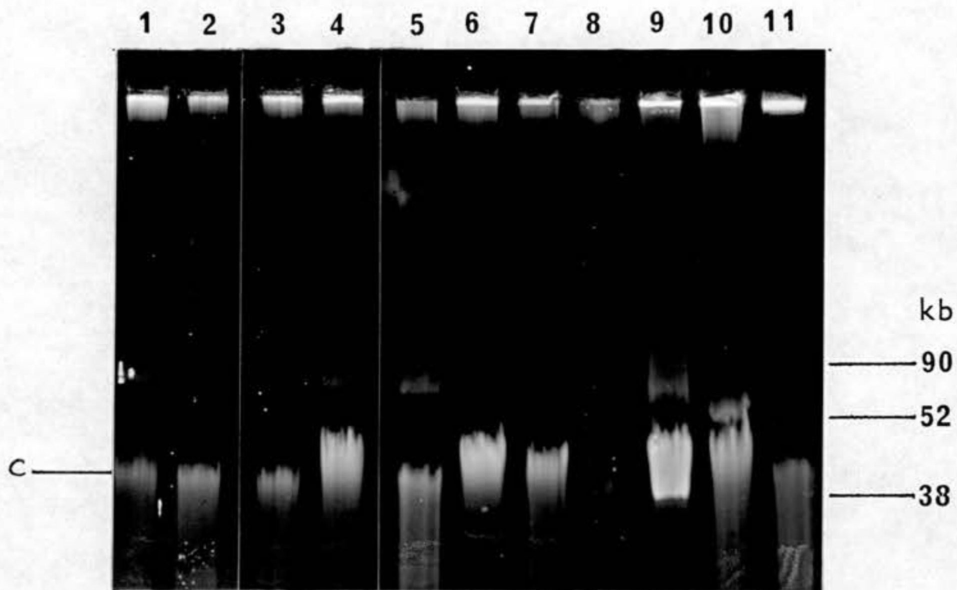
Figure 19b Agarose gel of plasmids isolated from strains 101 to 112. Plasmid isolation was performed by alkaline lysis.



Lane	Strain	Lane	Strain
1	112	8	105
2	111	9	104
3	110	10	103
4	109	11	102
5	108	12	101
6	107	13	R1/RP4/R6K
7	106		

C = Chromosomal DNA

Figure 20 Comparison of some MRHA-positive and -negative transconjugants. Plasmid isolation was by Triton lysis.



Lane		Lane	
1	MRHA⁻ strain	7	MRHA⁻
2	94	8	89
3	92	9	R6K
4	MRHA⁻	10	RP4
5	MRHA⁻	11	R1
6	90		

C = Chromosomal DNA

The absence of the MRHA gene from the transconjugants did not correspond to loss of a plasmid, further confirming that the 75kb R-plasmid carried the MRHA gene, and that this gene alone had been lost from the plasmids in the MRHA-negative transconjugants. This fact indicated that the MRHA gene could be contained within a transposable element in these strains.

Extraction of putative transposons from MRHA-positive transconjugants

In order to confirm the existence of this transposon plasmid Sa was used as the mobilising vector and the MRHA gene extracted by the method outlined in Figure 15. Eight of the MRHA-positive transconjugants were selected - strains 89, 90, 91, 94, 95, 97, 98 and 112. These consisted of two strains from each of the four periods of infection (Fig. 18) to give a wide cross-section of the group. Plasmid Sa was introduced into these eight strains during a five hour mating and the transconjugants selected on DM agar containing proline, histidine, tryptophan, Tp at 10μg/ml and Km at 20μg/ml. Transconjugants resulting from each of the matings were purified on the same type of selective media, then one purified transconjugant from each strain was successively subcultured three times in BHI broth. The third subculture was appropriately diluted and plated on to DSTA containing Tp at 10μg/ml and Km at 20μg/ml and incubated at 37°C. Four of the resultant colonies from each strain were then purified on the same type of medium and one purified transconjugant from each strain was mated for one hour with E. coli J53.

Transconjugants were selected on DM agar containing proline, methionine, and Km at 20μg/ml.

Seventy transconjugants from each strain were spotted on to DSTA containing Ap, Tp, Km, Cm and DSTA alone. Strains which were Km and Cm resistant, but Ap and Tp sensitive were purified on DSTA and MacConkey agar, as these would contain plasmid Sa, but not the clinical plasmid. The number of transconjugants possessing Sa alone varied and are shown in the table below.

Strain no.	89	90	91	94	95	97	98	112
No. Sa alone / No. tested	1/70	1/70	0/70	1/70	4/70	4/70	4/70	1/70

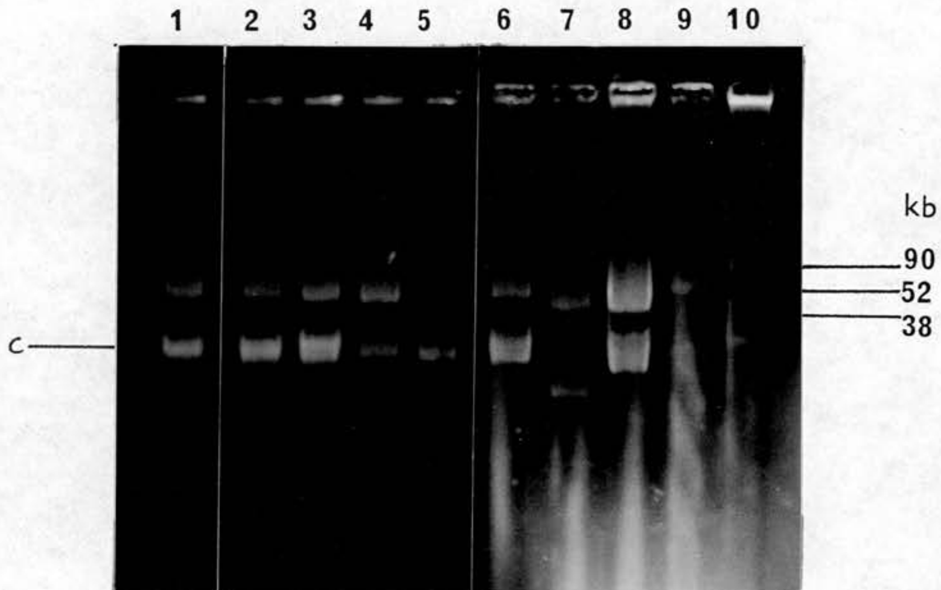
These J53, Sa-containing transconjugants were then assayed for their HA ability. Transconjugants from strains 89, 90, 95, 97, 98 and 112 were MRHA-positive for human group A erythrocytes.

Isolation of plasmids from the Sa-containing transconjugants

The plasmid DNA from the Sa-containing strains 89-J5, 90-J5, 95-J5, 97-J5, 98-J5 and 112-J5 was preprared by alkaline lysis and compared to the standard Sa plasmid by agarose gel electrophoresis (Figure 21). A molecular size of 35.2kb was calculated for Sa compared to 44.4kb obtained for the Sa plasmid species in the MRHA-positive transconjugants, except for strain 90-J5 which contained a plasmid of 39.4kb.

The increase in size of Sa resulting from the insertion of the MRHA gene was thus calculated as 4.2kb for strain 90-J5 and 9.2kb

Figure 21 Agarose gel showing the comparison between the standard plasmid Sa and the Sa plasmid species from the MRHA-positive transconjugants. Plasmids were extracted by alkaline lysis.



Lane		Lane	
1	112-J5	6	89-J5
2	98-J5	7	Sa
3	97-J5	8	R6K
4	95-J5	9	RP4
5	90-J5	10	R1

C = Chromosomal DNA

for strains 89-J5, 95-J5, 97-J5, 98-J5 and 112-J5. The original observed difference of 4.5kb between the plasmids in the MRHA-positive and MRHA-negative transconjugants was lower than the value of 9.2kb obtained with Sa as the vector, but the discrepancy is probably because the clinical plasmid is quite large and small increases in size are less accurate. The size of the inserted piece of DNA as 9.2kb is very close to the size calculated for the transposon identified in sections III and IV, indicating that the same gene is probably present in all the strains. There was only a 4.2kb increase in the size of Sa in strain 90-J5, possibly because part of Sa had been lost, as it is unlikely that in one strain the gene would be half the size previously determined.

VI EFFECT OF SUB-MICs OF ANTIMICROBIAL DRUGS ON BACTERIAL HAEMAGGLUTINATION

Sub-inhibitory concentrations of antibiotics are known to affect the adhesive ability of bacteria. Most studies have been carried out with antibiotics present at a quarter of the minimum inhibitory concentration (MIC). This effect on adhesion produced by the sub-inhibitory concentrations of antibiotics might be of less importance and relevance with the presence of the MRHA gene on an R-plasmid (Section II and V) which can code for resistance to high levels of antimicrobial drug.

Strains and conditions used in the study

In order to determine the effect that these sub-lethal levels of antimicrobial drug produced on the HA caused by the MR fimbriae of uropathogenic enterobacteria, two isolates from the City Hospital were used. These two strains, _Proteus vulgaris_ 95997 and _E. coli_ 202, were chosen because they both had relatively high HA titres, so any reductions in HA ability could be easily assayed quantitatively, and also they were relatively sensitive to most of the antimicrobial drugs as determined on DSTA. Strain 95997 was resistant to Ap and Sx, and strain 202 was resistant to Sm and Sx, compared to _E. coli_ $J62-_2$.

BHI broth could not be used for the HA assay in these experiments due to the antagonistic effect it is known to have on the action of Tp and Sx, and possible effects it might produce on other antimicrobials. Therefore the assays were performed with

cultures which had been grown in Difco Mueller-Hinton broth, which is not known to be antagonistic to the action of any antimicrobials.

MIC determination of strains 95997 and202

The MICs of strains 95997 and 202 were determined by subculturing from an overnight culture of the organism in Mueller-Hinton broth into fresh 4.5ml aliquots of Mueller-Hinton broth which contained doubling dilutions of the antimicrobial drugs tested. These cultures were incubated statically at 37°C for 48 hours (conditions which are used when assaying HA ability). The MIC was taken as the lowest concentration of antimicrobial drug which inhibited visible bacterial growth. The broth MIC values for the strains are given in Table 12.

Determination of the effect of sub-MICs of antimicrobials on adhesion

The concentration of antimicrobial drug which constituted one quarter of the MIC was used in this set of experiments. For the values given as $>\underline{x}$ in Table 12, $\underline{x}$ was the concentration of antimicrobial drug used as the quarter MIC.

Overnight cultures of the organisms in Mueller-Hinton broth were subcultured into fresh 4.5ml aliquots of Mueller-Hinton broth containing the antimicrobial at a quarter of its MIC for each drug tested, and an equal number of Mueller-Hinton broths without any antimicrobial drug. All broths were incubated statically at 37°C for 48 hours. After this time, the cultures containing the

Table 12 MIC values of P. vulgaris strain 95997 and E. coli strains 202, 202 (RP4) and 202 (Sa).

Antimicrobial	P. vulgaris 95997	E. coli 202	E. coli 202 (RP4)	E. coli 202 (Sa)
Ap	>500	8	>5000	8
Cm	8	2	2	500
Km	32	16	>500	250
Nal	nd	4	4	4
Sm	>32	2000	2000	2000
Sp	125	125	125	125
Sx	500	>2000	>2000	>2000
Tc	> 4	4	250	4
Tp	16	0.5	0.5	0.5

antimicrobial drug were used to assay HA ability of the strain, and the same concentration of antimicrobial added to the drug-free broths, which were then incubated for a further three hours. These cultures were then also assayed for HA ability with human group A erythrocytes.

Organisms which were grown for 48 hours in the presence of the antimicrobial drug show the effect that the drug has on growing bacterial cells. Those where the antimicrobial was added after 48 hours when the cells had reached stationary phase show the effects of the antimicrobial drug on resting cells.

Effect on the haemagglutination of strain 95997

The results obtained are shown in Table 13. When cultures of P. vulgaris strain 95997 were grown in the presence of the antimicrobial drugs varying effects were observed. Sm, Sx and Tc produced significant reductions in the HA titre but complete inhibition was not observed. Km and Tp also produced a noticeable effect, but this was less than that produced by the former three antimicrobial drugs. Sp produced a slight reduction in HA, and Ap and Cm had no effect.

The addition of antimicrobial drugs to strain 95997 during stationary phase had little effect on the HA ability, expect for Sp and Tc where complete inhibition was observed. Ap, Cm and Sm produced slight reductions in HA ability, but Km, Sx and Tp had no effect.

Table 13 Effect of antimicrobial drugs on the HA activity of strains 95997 and 202.

	Amount of haemagglutination (1/bacterial dilution)			
	P. vulgaris 95997		E. coli 202	
Antimicrobial	Growing	Resting	Growing	Resting
None	64	64	4	4
Ap	64	32	4	4
Cm	64	32	CI	2
Km	8	64	CI	2
Nal	nd	nd	8	4
Sm	1	32	CI	2
Sp	32	CI	CI	2
Sx	1	64	1	2
Tc	1	CI	CI	4
Tp	8	64	1	4

CI - complete inhibition

Effect of antimicrobial drugs on the adhesion of strain 202

When the HA ability of strain 202 was assayed after antimicrobial drug treatment, as above, the results differed considerably (Table 13). Growth of this strain in the presence of Sx or Tp reduced the HA titre, but did not completely inhibit the agglutination, whereas growth on Cm, Km, Sm, Sp or Tc did bring about complete inhibition. No effect was observed with Ap, and Nal increased the amount of adhesion, as determined by HA ability. The addition of the antimicrobial drugs to the organisms during stationary phase had very little effect. Cm, Km, Sm, Sp and Sx produced slight reductions, but Ap, Nal, Tc and Tp showed no effect on the HA titre.

Introduction of R-plasmids into strain 202

In order to determine if these effects could still be produced once the organism possessed resistance determinants to the antimirobial drugs the R-plasmids RP4 (Ap, Km, Tc) and Sa (Cm, Km, Sm) were introduced separately into strain 202 during an overnight mating from the E. coli J53 hosts. Transconjugants were selected by their resistance to Km at 50µg/ml.

The MICs of a purified transconjugant from each of these matings were redetermined in Mueller-Hinton broth as before (Table 12).

Antimicrobial effect on the HA of R-plasmid containing strains

The HA assay was repeated with cultures of 202 (RP4) and 202 (Sa) which had been grown in the presence (as the greatest effect was produced with actively growing cells) of the new quarter MICs to Ap, Km, Tc, and Cm, Km respectively, and the previous lower quarter MICs to these antimicrobials. The results obtained are shown in Table 14.

Ap had no effect on the HA of strain 202 (RP4) at either concentration, reproducing the result obtained with the plasmid-free strain. Km and Tc produced no effect when the organisms were grown in the lower concentration of these two drugs, but at the elevated quarter MIC complete inhibition of HA was observed.

The same trend was observed with strain 202 (Sa). Cm and Km produced no effect at the previous quarter MIC value, but complete inhibition of agglutination ability was observed when the quarter MIC was raised.

From these results it can be seen that the greatest effect on the HA of E. coli is produced with actively growing cells by ribosomal protein synthesis inhibitors (Cm, Km, Sm, Sp, Tc), presumably by interfering with the synthesis of fimbriae. Less pronounced effects are produced by the folic acid synthesis inhibitors (Sx, Tp) which indirectly affect protein synthesis. A cell wall biosynthesis inhibitor such as Ap has no effect. Negligible effects are produced with cells which have entered stationary phase and already have fimbriae present on the cell surface.

Table 14 Effect of antimicrobial drugs on the HA ability of R-plasmid containing strains.

a) Strain 202 (RP4)

	Amount of HA in presence of antimicrobial (μg/ml)						
	None	Ap_{2000}	Ap_2	Km_{250}	Km_4	Tc_{64}	Tc_1
Strain 202	4	-	4	-	CI	-	CI
Strain 202 (RP4)	4	4	4	CI	4	CI	4

b) Strain 202 (Sa)

	Amount of HA in presence of antimicrobial (μg/ml)				
	None	Cm125	Cm0.5	Km64	Km4
Strain 202	4	-	CI	-	CI
Strain 202 (Sa)	8	CI	4	CI	8

The effect of protein synthesis inhibitors on an actively growing culture of *P. vulgaris* is less dramatic, but Sp and Tc can completely inhibit the HA when added during stationary phase.

The presence of an R-plasmid in strain 202 was not able to overcome the effects produced if the antimicrobials were still present at the quarter MIC of the strain.

DISCUSSION

If E. coli, an organism which is a commensal in the gut, is to cause infection it must possess certain characteristics to enable it to establish an infection at various sites of the body. It has been shown that factors such as the presence of fimbriae on the bacteria is important in colonisation of the intestine and urinary tract and that toxins also have a role.

Numerous surveys have been performed with extraintestinal isolates, but the relative proportion of these strains which possess each character tested varies from survey to survey. In addition, which particular characters are tested for, the methods used and the exact source of the isolates also varies. This can be illustrated by comparing the results obtained from the survey detailed in section I of the results and previous surveys on urinary tract isolates.

From the values given in section I it can be seen that the ability to cause HA was the most common property, expressed by 66% of the lower UTI isolates, 62% of the chronic UTI isolates and 49% of the faecal control strains. The subdivision of these reactions into MSHA of guinea pig erythrocytes and MRHA of human group A erythrocytes gave values of 26% MSHA and 45% MRHA respectively among lower UTI isolates, 45% MSHA and 33% MRHA among the chronic UTI isolates and, 40% MSHA and 30% MRHA among the faecal strains. The proportion of organisms which produce MSHA is higher in the cases of the chronic UTI isolates and the faecal strains which is to be expected as Type I fimbriae are common to both pathogenic and non-pathogenic E. coli. However, relatively fewer of the lower UTI

isolates possess Type I fimbriae, but the proportion of strains capable of MRHA is not correspondingly reduced.

More of the lower UTI isolates produce MRHA than the faecal strains indicating that this is one factor which could be associated with pathogenicity, but an increase in the percentage of MRHA-positive strains would have been expected for the isolates from chronic UTIs. Instead the level is only just above that of the faecal strains. Normally strains from the more severe types of UTI are more likely to cause MRHA. The strains which were examined came from patients suffering from chronic UTIs because very few true cases of pyelonephritis are diagnosed in Edinburgh. However, this does not explain why the proportion of MRHA-positive strains from this group is lower than from the lower UTI isolates.

Green and Thomas (1981) found that 56% of their isolates from the urine of patients with pyelonephritis, cystitis and ABU produced HA of human group A erythrocytes compared to 17% of the faecal isolates from healthy controls. This former value was broken down to give 52% of the cystitis isolates, 75% of the acute pyelonephritis and 70% of the ABU isolates which were HA-positive.

Minshew et al. (1978b) showed that 50% of the strains isolated from the urine of patients with UTIs produced MSHA of human group O erythrocytes compared to 15% of the faecal control strains. These values are similar to those obtained by Green and Thomas (1981). Vosti (1979) obtained values in this region as well, 63% of the urinary strains he studied were able to agglutinate human erythrocytes compared to 23% of the faecal controls ($P < 0.01$).

Similar values were also obtained by Varian and Cooke (1980) who tested for the presence of fimbriae by observing adherence of the bacteria to uroepithelial cells. Adhesion was observed with 52% of the lower UTI isolates, 55% of the upper UTI isolates and 10% of the faecal strains.

These values for HA ability correspond to those obtained for the survey in this thesis for the UTI isolates, but all the values for the percentage of faecal strains capable of producing HA are much lower.

Much lower values were obtained by Ljungh et al. (1979); 23% of strains isolated from acute urinary tract infections produced MRHA of human erythrocytes and 11.5% MSHA of guinea pig erythrocytes, 16.5% MRHA was observed among the faecal strains and 0.59% MSHA.

Brooks et al. (1980) looked at the proportion of strains which possessed fimbriae, and they found higher percentages of strains which were fimbriated compared to the values above, obtained for HA-positive strains, 79% of their UTI strains were fimbriated compared to 60% of the strains isolated from normal controls ($0.02 > P > 0.01$). However, no difference in the proportions of fimbriated strains from the upper or lower urinary tract was observed.

The occurrence of the other characters tested were present at lower frequencies than HA ability. Production of haemolysin and utilisation of raffinose both occurred at 24% among the lower UTI isolates, at 15 and 16% respectively among the chronic UTI isolates, and 7 and 16% respectively among the faecal strains. The ability to

produce a haemolysin has also been strongly correlated with virulence in the urinary tract and this survey shows that there is a higher proportion of haemolysin-positive strains isolated from the urine than from the faeces. This character is also present at a higher frequency among the lower UTI isolates than the chronic UTI isolates. This is surprising as there was a positive correlation between haemolysin production and MRHA ability for the chronic UTI isolates but not the lower UTI isolates.

A significant association was found between haemolysin production and HA ability by Green and Thomas (1981), 67% of the urinary tract isolates which produced haemolysin also produced HA ($P < 0.025$). Of their UTI isolates 38% were haemolytic compared to 6% of the faecal strains. A similar association between haemolysin and HA was found by Hughes et al. (1983).

Minshew et al. (1978b) observed 48% of UTI isolates produced haemolysin compared to 5% of their faecal controls. These are similar values for the frequency of haemolysin among faecal strains, but twice as many UTI isolates produced haemolysin compared to those examined in the Edinburgh survey. Brooks et al. (1980) found 43% of their UTI strains were haemolytic which was significantly greater ($P < 0.001$) than the strains isolated from normal subjects. This was divided into 58% of the upper urinary tract strains and 27% of the lower urinary tract isolates, but this difference was not statistically significant. Values obtained by Cooke and Ewins (1975) showed that 52% of UTI isolates and 5% of faecal strains were haemolytic.

In the current survey the proportion of strains able to produce colicin V was less among the lower and chronic UTI isolates (12 and 18% respectively) than the faecal isolates (35%), which is in contrast to results obtained by Minshew et al. (1978b) where 7% of UTI isolates produced colicin V and no faecal strains were positive for this character, and Vosti (1979) who found that HA-positive isolates from the urinary tract were more likely to produce colicins ($P < 0.02$). However, Hughes et al. (1983) observed that there was no tendency for MRHA-positive strains to produce colicin V.

Due to the high percentage of faecal strains which produce colicin V from the Edinburgh survey it cannot be considered as a virulence character in UTIs. All these results are summarised in Table 15.

The possession of antimicrobial drug resistance determinants by the strains examined was not associated in any case with the MRHA virulence character. The only trend that was noted was that the percentage of resistant organisms increased from the faecal strains to the lower UTI isolates to the chronic UTI isolates. This increased resistance among the chronic UTI isolates could be due to the longevity of the infections and the associated treatment with antimicrobial drugs.

The proportion of strains isolated from the urine of patients with UTIs, which possess virulence markers is higher than the proportion isolated from the faeces of healthy controls. However, some of the faecal strains still possess that character, indicating that these strains could also be pathogenic. Therefore, it seems

Table 15 Summary of survey results obtained by various workers

Character	Faecal Isolates	Lower UTI Isolates	Upper UTI Isolates	Reference
	Percentage of isolates possessing character			
HA (human gp. A)	17	52 (cystitis) 70 (ABU)	75	Green and Thomas (1981)
MSHA (human gp. O)	15	50 (UTI)		Minshew *et al* (1978b)
HA (human)	23	63 (UTI)		Vosti *et al* (1979)
Epithelial adherence	10	52	55	Varian and Cooke (1980)
MRHA (human)	16.5	23		Ljungh *et al* (1980)
MSHA (guinea pig)	0.59	11.5		" "
MRHA (human gp. A)	30	45	33	Hales (1985)
MSHA (guinea pig)	40	26	45	" "
Fimbriae	60	79	79	Brooks *et al* (1980)
Haemolysin	6	38 (UTI)		Green and Thomas (1981)
"	5	49		Minshew *et al* (1978b)
"	9	27	58	Brooks *et al* (1980)
"	5	52		Cooke and Ewins (1975)
"	7	24	15	Hales (1985)
Colicin	35	12	18	Hales (1985)
"	0	7 (UTI)		Minshew *et al* (1978b)

that the role of some of these pathogenicity factors is not always straightforward.

Earlier work on the main classess of fimbriae from human and animal enteric infections, CFA/I and II, K88 and K99, had shown that the genes for the production of these fimbriae were carried on plasmids (Evans et al., 1975; Peñaranda et al., 1980; Ørskov and Ørskov, 1966; Smith and Linggood, 1972). It would therefore seem reasonable to assume that the same would apply to the genes for the production of uropathogenic fimbriae.

Hull et al. (1981) cloned both the Type I and P fimbriae from a uropathogenic E. coli isolate, and by hybridisation of the cloned fragments which expressed these genes to a plasmid present in the strain was able to show by a lack of DNA homology that the genes had originated from the chromosome. This theory for the chromosomal location of the fimbrial genes was borne out by other workers (Rhen et al., 1983a; van Die et al., 1983; Clegg, 1982; Berger et al., 1982), usually because the initial restriction enzyme digestion produced large DNA fragments of 35 to 40kb. In the case of strain KS71 (Rhen et al., 1983a), of the several antigenic types of fimbriae produced by the original strain, only one type was expressed by each fragment, indicating that the genes were well separated. The same was true for strain AD110 which produces F7 fimbriae and contained two plasmids of less than 12kb (van Die et al., 1983).

The only evidence for the existence of plasmid-encoded fimbriae came from Kuch et al. (1980) who examined one urinary isolate which produced MRHA of human erythrocytes. Spontaneous loss of the ability to produce fimbriae was not observed but treatment of the strains with ethidium bromide and mitomycin C led to the loss of fimbrial production. This circumstantial evidence suggested that the genes were plasmid-encoded and the strain had been cured. Ethidium bromide was used to produce K88-negative mutants from enteropathogenic strains (Jones and Rutter, 1972). However, Kuch et al. (1980) performed no further genetic or molecular biological experiments to demonstrate the presence of plasmids.

On the basis of this report it was essential to determine if the fimbrial genes from the current survey could be plasmid-encoded. The R-plasmids present in the lower UTI isolates from Edinburgh were used as a means of selecting for transfer. By assaying the transconjugants for the ability to cause MRHA, it was possible to show that the genes for the production of fimbriae, in some of these strains, could transfer between the clinical strain and the E. coli K12 laboratory strain.

Quite a low proportion of strains possessed transferable R-plasmids (28.8%), but the 12 strains which also transferred MRHA ability were sufficient for further study. An examination and comparison of the plasmids in the clinical strains and the trans-conjugants revealed no one plasmid which was present in all the strains. In some of the strains examined it was not possible to visualise any plasmids even though transfer had occurred. This is

probably due to limited sensitivity of the plasmid isolation techniques used.

A variety of resistance determinants were associated with transfer of the MRHA gene, although the determinants for resistance to Ap and Tc were the most common genes to co-transfer. However, this could be explained by the high levels of resistance to Ap and Tc (38 and 35% respectively) among the strains rather than a direct association of these genes. Echeverria et al. (1978) found a similar association between the transfer of resistance to antibiotics and LT and ST toxin genes. Further work showed that the toxin genes and the resistance determinants for Sm, Su and Tc were carried on the same plasmid (Echeverria and Murphy, 1980).

The MRHA character was not stable and was received by only a percentage of the transconjugants when further transfers were performed. Both MRHA-positive and MRHA-negative transconjugants had received the R-plasmids and a comparison of the transfer frequencies of the determinants showed that all the resistance determinants within a strain were carried on the same plasmid. This suggested that the two types of genes were not closely linked. However, loss of MRHA ability from the E. coli J53 transconjugants was not accompanied by loss of a plasmid but slight variations in the molecular weight of the plasmid present in the strain.

Therefore this lack of expression of the MRHA gene could have been due to a switching off of the operon. Once the clinical strains have been removed from the urinary tract, there is no pressure on them to produce fimbriae, whereas the continued

expression of resistance determinants can be selected by growing the organisms on selective media.

The variations in molecular size of the plasmids was not consistent and, in some cases, a slightly larger plasmid was present in the MRHA-negative strains. It therefore seems unlikely that complete loss of the MRHA gene had occurred.

The other MRHA-positive clinical strains, which did not possess transferable R-plasmids may possess plasmids which encode the fimbrial genes, however, the absence of positive selection for fimbriated transconjugants makes their examination more difficult.

The presence of the MRHA gene on transferable plasmids will allow easy transfer of the gene between bacteria. The occurrence of this *in vivo* will lead to the spread of adhesive ability and concurrently an increase in the pathogenic potential of the recipient. This will result in the increase in the virulent population of bacteria in the same way as the spread of R-plasmids has increased the proportion of antibiotic resistant bacteria. Also the excessive or prophylactic use of antimicrobial drugs in the treatment of UTIs could increase not only the selective pressure for the emergence of drug resistant bacteria, but also for virulent organisms due to the association of the fimbrial genes with R-plasmids.

The hypothesis that the MRHA gene was carried within a transposable element arose when examining strains which possessed plasmids but no resistance determinants. In an attempt to mobilise

these plasmids from the clinical strains with plasmid R483, transfer of the MRHA gene was achieved but the clinical plasmids were not mobilised. The plasmid in the MRHA-positive transconjugant was very similar in size to R483 and possessed the transposon Tn7 resistance determinants, however, it was possible that this plasmid was not R483, but a recombinant of the two clinical plasmids and Tn7. The incompatibility of this plasmid with plasmid JR66a showed that it was R483 as both plasmids are inc Iα.

It therefore appeared that the MRHA gene had been inserted into the R-plasmid, but it was not known at this point whether this was due to active transposition of the MRHA gene, or if R483 had excised the gene from the chromosome or one of the plasmids. Further experiments showed that this gene could move from one plasmid to another so active transposition seemed a likely explanation. This would be the first report of a MRHA gene being carried on a functional transposon.

Subsequent to this observation the ability to transpose was shown for the MRHA genes that were present on the R-plasmids which had transferred from the 12 clinical strains, and for the strains isolated from the recurrent UTIs. The genes from a selection of these strains were inserted into plasmid Sa and it was found that the molecular size of the inserts varied from 7 to 9.2kb.

To confirm that transposition was occurring the transfers would have to be repeated in a rec A background, and fine genetic mapping of the gene and its flanking regions performed to determine the presence of any sequences homologous to IS elements.

Further evidence for the role of transposons as carriers of adhesin genes was provided by Rhen (1985) who described the presence of inversely orientated DNA sequences flanking a deletable region which encoded the B fimbriae of strain KS71. Hybridisation studies showed that these sequences were approximately 1.2kb in length. Further hybridisation studies with the cloned fragment encoding the A fimbriae of strain KS71 indicated that only one set of sequences was present and this region was not deletable.

Common DNA sequences of 0.85kb were also identified in the vicinity of both ends of the cloned haemolysin structural gene of E. coli strain 536 (06) (Hacker et al., 1983) and it was suggested that these sequences might be identical with IS elements found flanking the plasmid-encoded haemolysin sequences (Zabala et al., 1982). The sequences adjacent to the plasmid haemolysin cistron hybridised to chromosomal regions of haemolytic 018 and 06 E. coli isolates and E. coli K12. This region of 1.5 to 1.8kb was retained when the haemolysin determinant was spontaneously lost (Knapp et al., 1984). This sequence was different from the 0.85kb region described by Hacker et al. (1983), which was also present in multiple copies in the bacterial chromosome. Homologous DNA sequences flanking the chromosomal haemolysin determinant were also found by Low et al. (1984).

Electron microscopic analysis of the cloned ST toxin gene showed the presence of the characteristic stem and loop structure of a transposon, therefore a non-transposable Tc resistance determinant was spliced into the ST gene (So et al., 1979). This provided a

marker to study transposition of the ST gene which occurred at an approximate frequency of 4×10^{-6}. Restriction and DNA sequence analysis suggested that the ST transposon was flanked by inverted repeats of IS1.

A study of the ST and LT regions of the enterotoxin plasmid pJY11 showed that the ST region consisted of at least two genes, _tox_ A and _tox_ B which complemented one another in _trans_ (Yamamoto and Yokoto, 1981). This LT region was flanked by repeated DNA sequences which were designated as β. The ST gene, _tox_ S, was flanked by the inverted repeats of IS1 which were given the designation α. The ST region (_tox_ S and α) was located between one of the β sequences and the _tox_ A and B genes, suggesting that the ST region was a transposon which was carried within the LT transposon (Yamamoto and Yokoto, 1981).

The presence of DNA sequences related to IS elements, whether functional or deficient, flanking such pathogenicity factors as fimbriae and toxins suggests a common path or mechanism of evolution for these determinants. Either the genes could have transposed into the chromosome or plasmid from a particular source and then the transposition function of the IS sequences were lost, although the sequences themselves were retained. Alternatively, the genes could have originally been present on the bacterial chromosome and the homologous sequences to enable tranposition to occur are in the process of evolving into functional entities. Maas (1981) accurately forecast the location of fimbrial genes when he stated, 'Other questions that will be of interest in the future include the possible location of adhesion factor genes on transposons and the

possibility of phase variation for these genes, resulting in turning on and off of gene expression'.

The ability of the MRHA gene to transpose freely from chromosome to plasmid and back again, leaving a copy of the donor molecule each time satisfies the conditions of stable chromosomal inheritance and the ecological spread between strains by plasmid transfer.

The presence of fimbriae on the bacterial surface aids the colonisation of the urinary tract by providing a means to resist expulsion by the flow of urine. Some women are more susceptible to recurrent incidences of UTI and it has been shown that the bacteria causing the reinfections are able to adhere better to the uroepithelial cells than the bacteria causing the original infection (Svanborg-Edén et al., 1979). If this difference is caused by an increase in the receptors for the fimbriae on the epithelial cell surface of these women, then possession of fimbriae by the bacteria is going to be of a distinct advantage. Källenius and Winberg (1978) showed that a strain of E. coli isolated from a case of pyelonephritis adhered to the epithelial cells of girls prone to UTIs in greater numbers than to the epithelial cells of the healthy controls.

From the study of recurrent UTIs in a single patient it was again shown that the MRHA gene was able to transfer from the clinical strain to E. coli K12 and that it was accompanied by R-

plasmid transfer. Again the association of the MRHA gene with the R-plasmid was not absolute, because although all the transconjugants examined possessed the Tp R-plasmid only one quarter of them also possessed the ability to produce MRHA. It would have been interesting to determine what percentage of the original clinical strains expressed the MRHA character and compare this to the number of strains which were able to transfer the gene, but these strains were not retained.

The initial treatment of the infection with Tp originally selected for the R-plasmid, but once treatment had been discontinued and the selective pressure removed loss of the plasmid from the infecting strain would have been expected. It is probable that the presence of an adhesin gene provided an additional advantage to the strain in its continued ability to colonise the urinary tract. This gene may have originally been located either on the bacterial chromosome or the plasmid, however, the increasing incidence of transfer at the end of the study suggests that it was originally chromosomally located and then transposed to the R-plasmid.

There were periods when the R-plasmid was absent, but the same plasmid would reappear in a further infection several months later. The discontinuous perseverance of the same plasmid, as compared by molecular size and resistance pattern suggests that the gut flora could be providing a reservoir for this R-plasmid containing strain and that reinfections were occurring via urinary tract contamination by the faeces and then the ascending route of infection. On the other hand plasmids can usually only survive for short periods of time in the gut in the absence of selective pressure.

The transfer efficiency of the MRHA gene to E. coli K12 appeared to increase during the last four months of the study. The isolation of strains was relatively uniform over the 25 month period of the study, as was the transfer of the Tp R-plasmid, but many more MRHA-positive transconjugants were detected in the latter four months. This increase could be due to the long term nature of the infection and an increase in the susceptibility of the patient to bacterial adhesion, perhaps providing indirect selection for bacteria which possessed fimbriae, however, the number of the original clinical isolates that were MRHA-positive is not known. This increase in transfer surprisingly occurred two and a half months after the infection had been cleared by treatment with cephradine. Whether these two events were coincidental or linked in some way is not known, however it seems unlikely that treatment with a cephalosporin could induce transfer of the gene.

This situation is in some ways the converse of the other R-plasmid encoded fimbriae, where over use of antimicrobial drugs could result in an increase in the number of fimbriated bacteria. In the case of this recurrent UTI a single course of Tp therapy selected for the R-plasmid, but then the problem in some respects was the persistence and reappearance of the plasmid encoded resistance determinants even in the absence of selection perhaps due to the adhesin gene, complicating the antimicrobial treatment of the infection. This indicates that one possible way to treat infections of this nature would be to inhibit the attachment of bacteria rather

than use the bacteristatic or bactericidal effects of antimicrobial drugs.

The antimicrobial drugs which produce effects on the adhesion of E. coli fall neatly into four groups determined by their mode of action and the effect they cause: the protein synthesis inhibitors, Cm, Km, Sm, Sp and Tc which completely inhibited the HA of strain 202 if they were present while the organism was growing; the folic acid synthesis inhibitors, Sx and Tp which reduce the amount of HA but do not cause complete inhibition; the peptidoglycan biosynthesis inhibitors, only one of which was tested (Ap), but no effect on the adhesion was observed; and nalidixic acid, a DNA gyrase inhibitor which produced a slight increase in the amount of HA.

These effects were only observed if the antimicrobials were added before the cells had entered the stationary phase of growth. The protein synthesis inhibitors still produced a slight effect when added during the stationary phase but did not completely inhibit HA.

The implication of these results is that if protein synthesis is inhibited to some extent by low levels of antimicrobial drugs during growth, production of proteinaceous fimbriae is reduced, probably due to a reduced level of synthesis, but aberrations in the protein produced or a lack of transport of the protein to the cell surface may also play a role. The addition of these same drugs has little effect on the fimbriae already present on the surface.

Results obtained by Vosbeck _et al_. (1979) who looked at the adhesion of an _E. coli_ strain, isolated from a patient with acute pyelonephritis, to a tissue culture line showed that a quarter MIC of Tc reduced the adhesion by a third, but only slight reductions were produced by treatment with Cm or Sm. Vosbeck _et al_. (1982) went on to look at more isolates from UTIs. The HA of human group A erythrocytes was decreased significantly after growth in Cm and Tc, but less so with Sm. There were variations in the amount that adhesion was reduced with the different strains. Sandberg _et al_. (1979) also looked at the adhesion of strains isolated from patients with acute pyelonephritis. At a quarter of its MIC Cm had virtually no effect on adhesion of the bacteria to uroepithelial cells, however, slight reductions were obtained with some strains. Shibl and Gemmell (1983) also examined the effect that Cm and Sm produce on the adhesion of a uropathogenic _E. coli_ isolate to uroepithelial cells. When present at one half of the MIC Cm and Sm reduced adhesion to approximately one third of its original value.

It can be seen therefore that depending on which _E. coli_ strain is used some antimicrobials can significantly reduce adhesive ability or have virtually no effect.

The same variation can also be observed with Ap, which in this thesis produced no effect on the haemagglutination of the _E. coli_ strain, suggesting that any aberrations in the cell wall were not great enough to affect the surface properties of the bacteria. These results are in agreement with those of Vosbeck _et al_. (1979) who found that Ap produced no significant effect on the adhesion of

a pyelonephritic E. coli to cells of a tissue culture line. On the other hand, Sandberg et al. (1979) found that a quarter MIC of Ap consistently decreased adhesion of their pyelonephritic E. coli to epithelial cells.

Inhibitors of folic acid synthesis produced some effect on the HA of E. coli 202, probably due to the subsequent effect that blockage of this pathway will produce on protein synthesis. Again the inhibition was greatest if the drugs were present while the cells were actively growing. A greater inhibitory effect was observed by Vosbeck et al. (1979, 1982), who showed that at 1/64 the MIC trimethoprim-sulphametrole could reduce adhesion of E. coli to tissue culture cells by two thirds and that HA activity was also significantly reduced.

The results obtained with E. coli 202 contradict the observations of other workers especially with regard to Ap and the protein synthesis inhibitors. In view of this the effect that nalidixic acid produced on the HA of E. coli 202 was tested. Vosbeck et al. (1979) had previously reported that nalidixic acid increased the adhesion of E. coli, the amount that it was increased varied with the strain (Vosbeck et al., 1982). The results in this thesis also show that nalidixic acid increases HA and supports these observations.

A different pattern of inhibition was observed for P. vulgaris. Marked reductions in HA ability were observed following growth of the organism in the presence of Sm, Sx and Tc, but the HA was not completely inhibited. Again, little effect was seen if the

antimicrobial drugs were added during stationary phase except for Sp and Tc. These are both ribosomal protein synthesis inhibitors, but in this case they have their effect during stationary phase, perhaps indicating that in Proteus the synthesis of fimbriae does not occur until late in the growth cycle, or that the fimbriae are synthesised but collect in the cytoplasm and are not transported to the surface until late on in growth.

A previous study on the effect that piperacillin and sagamicin have on the adhesion of Proteus species from UTIs was performed by Savoia et al. (1983). Both antimicrobials reduced the HA of a P. mirabilis strain which was sensitive to both drugs, when present at 1/8 and 1/16 of the MIC. Greater reductions were observed at 1/4 of the MIC, and sagamicin gave a larger reduction in HA than piperacillin, but complete inhibition was not observed. The effects on a P. inconstans strain which was resistant to piperacillin and sagamicin were less evident and 1/4 of the MIC of piperacillin and 1/8 of the MIC of sagamicin increased adhesion.

The HA experiments were repeated with E. coli 202 which now contained R-plasmids. The association between the MRHA gene and R-plasmids which had been observed might have some influence on the effects produced by sub-MICs of antimicrobial drugs. Ofek et al. (1979) had shown that the MS adhesion of an isogenic Sm-resistant mutant was no longer affected by sub-MICs of Sm, in contrast to the Sm-sensitive parent, however, no investigation of R-plasmid containing strains had been performed. The results obtained with the R-plasmid containing E. coli 202 showed that if the concentration of antimicrobial drug was raised to a 1/4 of the MIC

of the resistance determinant, the same inhibitory effects on adhesion were still seen. This effect was not produced if the 1/4 MIC of the original plasmid-free strain was used.

It is therefore possible that sub-MICs of antimicrobials could be used to prevent bacterial attachment, providing that the MICs of the strain were determined first and that the antimicrobial chosen did not exhibit strain specificity. A problem that might arise from this approach is that the low levels of antimicrobials could induce the development of resistance and amplification of resistance genes.

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THE GENETIC CARRIER OF BACTERIAL ATTACHMENT

Barbara A. Hales and S.G.B. Amyes, Department of Bacteriology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, United Kingdom.

Bacterial attachment factors have been implicated in the establishment of enterobacterial infections, both in the intestine and in the urinary tract (Candy et al 1981; Svanborg-Edén et al 1981). In enteropathogenic *Escherichia coli* these attachment factors can be plasmid-mediated, but their genetic determinants in uropathogenic *E. coli* are uncertain, although they are generally assumed to be carried on the bacterial chromosome. The presence of pathogenic attachment factors on the surface of bacteria is usually detected by the ability to agglutinate erythrocytes in the presence of mannose (MRHA). However, it is not possible to employ this technique as a selective procedure in the detection of gene transfer from one strain to another. Therefore, in order to determine if the property of MRHA is carried on a transferable genetic factor, such as a plasmid or transposon, indirect techniques have to be used.

During routine isolation of strains responsible for significant bacteriuria obtained from the Royal Infirmary in Edinburgh, various pathogenicity characteristics were measured. Forty-five percent of these strains were found to be MRHA-positive. In order to determine if this property is encoded by a plasmid, the following procedure was employed. The trimethoprim (Tp) resistance plasmid, R483, was introduced by conjugation into those clinical strains that did not already possess resistance plasmids (R-plasmids). The strains containing R483 were then conjugated with the *E. coli* K12 strain $J6-2-_{2}$ and selection was made for Tp resistance. Only one transconjugant in eight was found to possess MRHA ability. To determine if a plasmid from the original strain had transferred, the cellullar DNA from this strain and the transconjugant was prepared and characterised by agarose gel electrophoresis. The original strain contained two plasmids of sizes 36 and 18 Mdaltons, but the R483-containing transconjugant possessed only one plasmid with a molecular weight similar to R483. This suggested that the MRHA ability had actually been transferred onto R483, possibly due to the insertion of a transposon within the plasmid.

As the molecular weight of R483 is 62 Mdaltons it is difficult to show directly any increase in size of R483. However, when the transconjugant plasmid DNA and that from R483 were separated by gel electrophoresis in 0.3% agarose a consistent increase of approximately 5 Mdaltons was found in the transconjugant DNA. This strongly suggests the MRHA ability is being transported on a transposon. In further support of this, the MRHA gene could be transferred successfully from strain $J6-2-_{2}$ to the *E. coli* K12 strain J5-3 along with R483. In addition, the MRHA gene could be co-transduced with Tp and streptomycin resistances into strain J5-3, suggesting that it had inserted into the R483 genome close to these two resistance determinants.

The results describe the first report of the transfer, by conjugation, of the MRHA gene associated with urinary tract bacterial pathogenicity, into a standard *E. coli* strain. They show that the MRHA gene resides on a transposon of about 5 Mdaltons in size and that it has been able to insert into the R483 plasmid. It is still not known whether this transposon first resided on the chromosome or one of the two plasmids in the original strain.

We thank the SHHD for grant number K/MRS/50/492 which funded this project.

Candy, D.C.A. et al. (1981) Ciba Found. Symp. 80: 72
Svanborg-Edén, C. et al. (1981) Ciba Found. Symp. 80: 161

OFFERED PAPERS: GENERAL (POSTER)

Wednesday, 9 January

University Centre, Avon Room (2nd floor)

1130–1230: Authors in attendance.

P1 *Relationship between Nasal Virus Levels, Inflammatory Response and Fever Production in Influenza*
D.M. COATES, C. SWEET, H.A. OVERTON and H. SMITH (Department of Microbiology, University of Birmingham)

Influenza virus A/PR/8/34 (H1N1) produces similar nasal virus levels in the ferret to H3N2 strains, but elicits a lower inflammatory response which correlates with a lower fever. To determine whether these properties of PR/8 are related to the H1N1 subtype, recombinant strains clone 64b (H1N2) and clone 31 (H3N1), and recent H1N1 strains A/USSR/90/77 and A/Fiji/15899/83 were tested.

P2 *Molecular Studies of the Differential Replication of Two Influenza Virus Clones*
H.A. OVERTON, C. SWEET, D.M. COATES and H. SMITH (Department of Microbiology, University of Birmingham)

Two clones of the recombinant influenza virus A/PR/8/34 – A/England/939/69 differ in their sensitivity to pyrexial temperatures. This is related to their different extent of replication in the upper respiratory tract of ferrets, a factor which contributes to their difference in virulence. Replication is being studied *in vitro* by examining synthesis of viral polypeptides and nucleic acids at normal and pyrexial temperatures.

P3 *EIA Detection of Pigs Which Lack Receptor for K88 Adhesin*
D.S. CHANDLER, J.A. CRAVEN* and R.K.J. LUKE (School of Agriculture, La Trobe University, and *Attwood Veterinary Research Laboratory, Westmeadows, Victoria, Australia)

An EIA procedure, utilizing immobilized K88 antigen or K88-producing bacteria, has been used to detect pigs which lack the receptor for the K88 adhesin produced by some strains of *Escherichia coli.* Samples from animals which lack K88 receptor (non-adhesive animals) do not react in the EIA. Screening of 2400 pigs from 19 piggeries has shown approximately 20% of the animals to be non-adhesive. The incidence on particular farms has been found to range from 8 to 40%.

P4 *Characterization of Transferable Fimbrial Antigens from Uropathogenic Strains*
B.A. HALES and S.G.B. AMYES (Department of Bacteriology, University of Edinburgh)

The transfer of mannose-resistant haemagglutinating ability from uropathogenic strains to *E. coli* K12 can, in some cases, be mediated by indigenous plasmids. The gene responsible was shown to be carried on a small transposon which has been inserted into plasmid Sa. The fimbrial proteins have been purified and examined by SDS-PAGE.

P5 *Cytotoxicity of a Bovine Isolate of Pasteurella haemolytica A1 for Elicited, Cultured, Peritoneal Mouse Macrophages*
J.J.P. HUTCHINSON (International Development Laboratories, E.R. Squibb and Sons, Moreton, Wirral)

The cytotoxicity of *P. haemolytica* A1, but not of *E. coli,* has been shown to be specific for mononuclear leukocytes of ruminants. The current poster presents preliminary findings showing that these organisms are cytotoxic for thioglycollate-elicited, cultured, peritoneal mouse macrophages, and that cytotoxicity is manifest when growth of *P. haemolytica* in tissue culture medium causes the count to exceed a threshold level.

P6 *Molecular Analysis of IncC Plasmids Specifying Multiple Antibiotic Resistance Found in Gram-Negative Bacteria*
H. GRIFFIN, T.J. FOSTER and D.C. COLEMAN (Microbiology Department, Trinity College, Dublin)

IncC plasmids of 120 Mdal were found in different species of enterobacteria isolated from the same ward of a Dublin hospital. Cloned DNA fragments carrying resistance markers were used as hybridization probes to determine their relatedness.

CARRIAGE OF THE MANNOSE-RESISTANT HAEMAGGLUTINATION GENE BY AN R-PLASMID WHICH PERSISTS THROUGH A RECURRENT URINARY TRACT INFECTION

B.A.Hales and S.G.B.Amyes, Department of Bacteriology, University Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland.

A study was carried out over a period of 25 months on a female patient suffering from recurrent urinary tract infections. Urine specimens were collected regularly and the bacterial pathogen isolated and identified. Initially the infection was treated with co-trimoxazole, but the infecting E.coli strain developed resistance to trimethoprim (Tp). The Tp R-plasmid containing strain persisted for 11 months even in the absence of Tp therapy. Treatment with cephradine cured the infection (Amyes et al,1981), but strains possessing the Tp R-plasmid subsequently reappeared in a further infection. These results indicated that some factor was playing a role in the persistence of the R-plasmid in the absence of selective pressure.

Ninety-five of the strains isolated over this period transferred Tp resistance to E.coli K12 strain J62-2 and, in each case the resistance determinants for ampicillin, streptomycin, spectinomycin and sulphamethoxazole were co-transferred. The Tp-resistant transconjugants were assayed for the ability to cause mannose-resistant haemagglutination (MRHA) of human group A erythrocytes and 24 (25.3%) were shown to be positive. These MRHA-positive transconjugants were more prevalent from isolates of later infections - 9 out of 65 transconjugants in the first 19 months, and 15 out of 30 in the last 6 months of the study. The presence of the MRHA gene in E.coli K12 suggested that the gene was plasmid encoded, its efficiency of transfer increasing with the number of reinfections.

The plasmid DNA from the MRHA-positive transconjugants was purified and examined by agarose gel electrophoresis. All strains possessed the same 75kbase (kb) plasmid. The DNA from aselection of MRHA-negative, Tp-resistant transconjugants was also examined for comparison. These strains also contained a single plasmid, but it was 4.5kb smaller. Inability to transfer the MRHA gene obviously did not correlate with loss of a plasmid, and the difference in molecular size of the plasmids suggested that the MRHA-positive transconjugants possessed an additional piece of DNA. We had previously found the MRHA gene to be carried on a transposable element in certain uropathogenic strains (Hales & Amyes,1983) so, in order to determine if this MRHA gene was carried on a transposon the R-plasmid Sa was introduced into 8 of the MRHA-positive transconjugants to allow its transposition into a known plasmid vector, which was then transferred to E.coli K12 strain J53. The adhesive nature of the Sa-containing J53 transconjugants was then determined with the haemagglutination assay.

Plasmid DNA from the resultant MRHA-positive strains was purified and examined as before. A molecular size of 35.2kb was obtained for the standard Sa compared to 44.4kb calculated for the plasmids in the MRHA-positive J53 strains. This gives a more accurate estimate of the size of the insertion as 9.2kb.

We wish to thank the Scottish Home and Health Department for the grant which funded this research.

Amyes SGB, McMillan CJ & Drysdale JL. (1981). In: New trends in antibiotics : research and therapy. GG Grassi, LD Sabath (Eds). Amsterdam: Elsevier, pp325-327.
Hales BA & Amyes SGB. (1983). The genetic carrier of bacterial attachment. J.Pharm. Pharmacol. 35 suppl. 50P.

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The effect of a range of antimicrobial Drugs on the haemagglutination of two clinical isolates from urinary tract infections

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A range of nine common antimicrobial drugs were tested for their effect on adhesion of the mannose-resistant haemagglutination-positive *Proteus vulgaris* strain BH77 and *Escherichia coli* strain BH121, isolated from patients with urinary tract infections. Minimum inhibitory concentrations of the antimicrobials for these strains were determined in Mueller-Hinton broth and the effect of each antimicrobial, at the quarter MIC values, on the Haemagglutination of the strains was then determined. Haemagglutination could only be prevented by inhibitors of protein synthesis. After the introduction of an R-plasmid into strain BH121 this effect was annihilated. When the new MICs were determined, the inhibition was still observed at the new quarter MIC values.

Introduction

Bacterial adhesion to the mucosal surface of the urinary tract is considered to be an important first step in the bacteria-host interactions which give rise to infection (Svanborg-Edén *et al.*, 1981). The fimbriae responsible for this attachment aid the migration of pathogenic organisms, originating from the faeces, up into the urinary tract to the bladder and on to the kidneys.

These infections, in most cases, are treated with antibiotics at a concentration that will eliminate the causative agent from the host by killing it or rendering it more susceptible to host defence mechanisms (Beachey, Eisenstein & Ofek, 1981). It has been known for some time that antibiotics at sub-inhibitory concentrations, as well as stimulating phagocytosis (Alexander & Good, 1968), can also inhibit the production of bacterial virulence factors *in vitro* (Lorian *et al.*, 1978).

In this study nine common antimicrobial drugs, at sub-inhibitory levels, were tested for their effect on haemagglutination when added during either resting or growing phase. These drugs comprised inhibitors of protein, DNA, peptidoglycan and folic acid synthesis.

Methods

Two clinical strains—*Proteus vulgaris* (BH77) and *Escherichia coli* (BH121), isolated from different patients with urinary tract infections at the City Hospital, Edinburgh,

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Table I. Minimum inhibitory concentrations (mg/l) for bacterial strains

Bacterial strain	Minimum inhibitory concentration (mg/l)								
	Ap	Cm	Ka	ND	Sm	Sp	Sx	Tc	Tp
Pr. vulgaris (BH77)	>500	8	32	nd	>32	125	500	8	16
E. coli (BH121)	8	2	8	2	>500	125	>500	2	0·1
BH134	>5000	2	>500	2	>500	125	>500	125	0·1
BH135	8	500	125	2	>500	500	>500	125	0·1

Ap, Ampicillin; Cm, chloramphenicol; Ka, kanamycin; NA, nalidixic acid; Sm, streptomycin; Sp, spectinomycin; Sx, sulphamethoxazole; Tc, tetracycline; Tp, trimethoprim.

were studied. Both these strains expressed mannose-resistant haemagglutinating (MRHA) fimbriae. Two derivatives of the *E. coli* strain BH121 were also used. Strain BH134 possessed R-plasmid RP4 (Ap, Tc, Ka)* and strain BH135 possessed R-plasmid Sa (Cm, Ka, Sm, Su, Sp). Minimum inhibitory concentrations (MIC) were determined in the presence of doubling dilutions of antimicrobial drug in Mueller-Hinton broth (Difco, Detroit, U.S.A.) after incubation for 18 h at 37°C. The end point was designated as the lowest concentration of drug which inhibited visible bacterial growth.

Adhesive ability of the bacteria was assayed by a modification of the haemagglutination method of Svanborg-Edén & Hansson (1978). The organisms were cultured in Mueller-Hinton broth and the agglutination assayed with human group A erythrocytes. The effects of the drugs on haemagglutination were determined by two methods: (a) the organism was grown in Mueller-Hinton broth in the presence of a quarter the MIC of the antimicrobial for 48 h at 37°C (for those strains with MIC values given as $>x$ (Table I), x is the concentration taken as the quarter MIC), (b) the organism was grown in the absence of drug, which was then added at a quarter the MIC during stationary phase, these cultures were then incubated for a further 3 h at 37°C. Haemagglutinating ability was then determined and related to an untreated control.

Results

The MICs of the antimicrobial drugs for each strain tested is shown in Table I.

The haemagglutination results obtained for the two clinical strains differed considerably (Table II). There was little observed effect on the adhesion of *Pr. vulgaris* except for the reductions in haemagglutinating ability after growth in the presence of sulphamethoxazole, streptomycin and tetracycline. There was also slight reductions when strain BH77 was grown in the presence of kanamycin and trimethoprim. Spectinomycin and tetracycline caused complete inhibition of haemagglutination when they were added during stationary phase, these were the only antimicrobials to produce a significant effect.

After growth in the presence of chloramphenicol, kanamycim, streptomycin, spectinomycin and tetracycline complete inhibition of *E. coli* strain BH121 adhesion was observed. Addition of these drugs during stationary phase had little or no effect on adhesion. Growth of the strain in the presence of trimethoprim and sulphamethoxazole

*Abbreviations see Table I.

Table II. Effect of antimicrobial drugs on haemagglutinating ability at one quarter the minimum inhibitory concentration

	Haemagglutination titre (1/bacterial dilution)			
	Pr. vulgaris		*E. coli*	
Drug	Growth in	Stat. phase	Growth in	Stat. phase
None	64	64	4	4
Ap	64	32	4	4
Cm	64	32	CI	2
Ka	8	64	CI	2
NA	nd	nd	8	4
Sm	1	32	CI	2
Sp	32	CI	CI	2
Sx	1	64	1	2
Tc	1	CI	CI	4
Tp	8	64	1	4

nd, not determined; CI, complete inhibition.

produced reductions in haemagglutinating ability, but again little effect was observed with their addition during the stationary phase. Ampicillin produced no significant effect on adhesion under either set of conditions. Nalidixic acid had no effect on adhesion when added during stationary phase, but growth in its presence increased haemagglutinating ability.

The presence of either R-plasmid RP4 or Sa in strain BH121 annihilated the effect the relevant antimicrobials had on adhesion at the previous concentrations, but when the MICs were redetermined and the assay repeated at the new quarter MIC values complete inhibition of adhesion was still seen with kanamycin, chloramphenicol nd tetracycline. Growth in the presence of ampicillin again had no effect.

Discussion

Antimicrobials which inhibit protein synthesis such as chloramphenicol, kanamycin, streptomycin, spectinomycin and tetracycline also inhibit the adhesive ability of *E. coli*, if they are present during growth, indicating that the adhesive surface fimbriae are proteinaceous and produced during growth. Addition of these drugs during stationary phase has little effect on adhesion, therefore they can have no effect on existing fimbriae, but can prevent their production.

Inhibitors of peptidoglycan synthesis have no significant effect on adhesion, so abnormalities in the cell wall cannot prevent the presence and anchoring of the fimbriae. Nalidixic acid which inhibits DNA gyrase increases the adhesion, an observation which has been previously reported (Vosbeck *et al.*, 1982). Some effect on the bacterial adhesion was seen when folic acid synthesis was inhibited with trimethoprim or sulphamethoxazole and this is probably due to the subsequent effect that trimethoprim has on the prevention of protein synthesis (Amyes & Smith, 1974). It would therefore seem that the greatest effect is at the DNA translation stage. The same effect on adhesion is observed if the strain possesses an R-plasmid as long as the concentration of the drug is still at quarter-MIC level. These results contrast wtih the effect of sublimiting concen-

trations of streptomycin, in streptomycin resistant strains, showing mannose-sensitive attachment (Ofek *et al.*, 1979). Unlike the results presented here, these workers showed the presence of the antibiotic at the quarter MIC value in the chromosomal mutant had no effect.

The fimbriae of *Pr. vulgaris* are affected by a totally different set of conditions and complete inhibition can only be produced if spectinomycin or tetracycline are added during stationary phase, suggesting a different mechanism of production and expression. Savoia *et al.* (1983) have shown that sublimiting concentrations of piperacillin and sagamycin affected haemagglutination ability when *Proteus* species were grown in their presence. Our results demonstrate that this effect is not a universal response.

These *in-vitro* effects could be considered to be of some use in the clinical situation, if the MICs of the infecting agents were determined first. However, the possibility that concentrations of drugs below the MIC might induce the development of resistance more rapidly and allow amplification of any R-plasmids present in the bacteria (Wiedemann, 1981) may well override this advantage.

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