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MOLECULAR AND SEROLOGICAL CHARACTERIZATION OF P FIMBRIAE

FROM UROPATHOGENIC ESCHERICHIA COLI

MOLECULAR AND SEROLOGICAL CHARACTERIZATION
OF P FIMBRIAE FROM UROPATHOGENIC
ESCHERICHIA COLI

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de geneeskunde
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. F.I.M. Bonke,
volgens besluit van het College van Dekanen,
in het openbaar te verdedigen op
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*aan mijn ouders,
aan Jeannette*

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This thesis is based on the following papers.

The contents of the chapters 2-9 correspond to the papers 1-8, respectively.

1. de Ree JM, P Schwillens, L Promes, I van Die, H Bergmans, and JF van den Bosch. 1985. Molecular cloning and characterization of F9 fimbriae from a uropathogenic Escherichia coli. FEMS Microbiol. Lett. 26: 163-169.
2. de Ree JM, P Schwillens, and JF van den Bosch. 1985. Molecular cloning of F11 fimbriae from a uropathogenic Escherichia coli and characterization of fimbriae with polyclonal and monoclonal antibodies. FEMS Microbiol. Lett. 29: 91-97.
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5. de Ree JM, P Schwillens, and JF van den Bosch. 1987. Monoclonal antibodies raised against Pap fimbriae recognize minor component(s) involved in receptor binding. Microbial Pathogenesis 2: in press.
6. de Ree JM, P Schwillens, and JF van den Bosch. 1986. Monoclonal antibodies for serotyping the P fimbriae of uropathogenic Escherichia coli. J. Clin. Microbiol. 24: 121-125.
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8. de Ree JM, and JF van den Bosch. 1987. Serological response to the P fimbriae of uropathogenic Escherichia coli in pyelonephritis. Submitted for publication.

INTRODUCTION

Infections of the urinary tract are common all over the world and occur in people of all ages and both sexes. Looking at urinary tract infections (UTI) two observations can be made. First of all the high prevalence of UTI in healthy populations all over the world. Secondly, the morbidity of UTI has remained stable in spite of the use of antibiotics. These observations resulted in an increased interest for the pathogenesis of UTI. UTI caused by bacteria can be distinguished in three groups: asymptomatic bacteriuria, cystitis and pyelonephritis. This classification is mainly based on the presence of significant bacteriuria and clinical symptoms. Significant bacteriuria refers to the presence of more than 10^5 bacteria per ml of urine (1).

Asymptomatic bacteriuria (ABU) has been investigated intensively. Studies among schoolchildren have revealed that the prevalence of ABU is 1% in girls and 0.03% in boys (2, 3). It could also be demonstrated that five percent of the adult female population have a UTI at any one time, whereas it is estimated that women during their life have a 50 percent chance of acquiring a UTI (4). UTI are among the most common medical complications in pregnancy. Approximately 6% of pregnant women develop an ABU, which might have an effect on maternal and foetal health (4-6). More than 50% of this ABU will develop into symptomatic UTI and even 30-40% will develop a pyelonephritis (5-7).

The prevention of renal damage is an important reason for treating UTI. The main target populations are infants and small children. There is evidence that kidney damage associated with UTI arises in childhood, which can lead to progressive kidney damage, higher blood pressure and even kidney failure (9, 10). Whereas the development of kidney scarring is the main problem in children, in elderly people the bacteria can spread to the blood stream and can

cause a fatal septicaemia. It has been estimated that half of all gram-negative septicaemia originate in the urinary tract (11). Another problem associated with UTI is morbidity. UTI cause much misery and is among the main causes of female absence from work (8). The increasing employment of women makes this problem nowadays even greater.

The treatment of UTI involves the use of large quantities of antibacterial agents, which accounts for great expenses. An important disadvantage of the use of antibiotics is that it could interfere with the normal bacterial flora. It could moreover result in the selection of resistant organism.

The normal urinary tract is sterile above the level of the distal urethra. Two routes of infections of the urinary tract can be distinguished: the haematogenous and the ascending route. By the haematogenous route the organisms originate from an infection elsewhere in the body and enter the urinary tract via the blood stream (12). However, the haematogenous route of infection is very rare and will not be discussed. Urinary tract infections occur mainly by the ascending route. Organisms from the bowel colonize the peri-urethral zone and from hereon the urinary tract. In view of the anatomical and physiological differences between males and females it is not surprising that the prevalence of UTI in females exceeds that in male (13). The shortness of the female urethra which facilitates entry of organisms as well as the lack of prostatic secretions, which contains a bactericidal substance, are two relevant points which could explain the sex difference in prevalence of UTI.

To colonize the urinary tract the organisms have to overcome a hydrokinetic and a mucosal defensive mechanism (14). The hydrokinetic defensive mechanism is based upon the wash-out of bacteria by periodic voiding and dilution by inflow of urine from the kidney. The mucosal defense mechanism is based upon a bactericidal effect of the intact bladder mucosa. It was suggested that a phagocytic mechanism accounts for this defense mechanism, however no such phagocytic mechanism could be demonstrated (14).

The organism most commonly isolated from all types of UTI is Escherichia coli (15). As already mentioned these E. coli originate from the own bowel flora. Two theories have been proposed about the nature of the infecting E.coli strains. The prevalence theory states that the strains responsible for UTI are simply those that predominate in the faeces. This theory is based on the fact that E.coli isolated from UTI belong to a restricted range of serogroups and the prevalence of these serogroups is similar to that in the

faeces. On the other hand the special pathogenicity theory states that the infecting E.coli strains have special pathogenic features. At present this special pathogenicity theory is generally accepted and several virulence factors have been determined to be involved in the pathogenesis of UTI. The most important virulence factors are the O:K serotype, haemolysis and the adhesive capacity of the uropathogenic E. coli. These three properties will be considered now in more detail.

O:K serotype

The outer membrane of E. coli is a typical feature of Gram-negative bacteria and it contains lipids, proteins and lipopolysaccharides (LPS). Some of these components are exposed on the cell surface and can interact with the environment. The LPS are build up by a lipid A part, a core part and a polysaccharide moiety. The chemical structure of these polysaccharides is the molecular basis of the serological specificity and is called the bacterial O specificity. So far more than 160 O antigens have been described.

A relative small number of O groups, namely O1, O2, O4, O6, O7, O18 and O75, have been reported to account for a major part of uropathogenic E. coli strains (2, 16, 17). It was also demonstrated that these O serotypes were very frequent among pyelonephritis, less among cystitis, and the least among ABU strains (18). In addition strains isolated from pyelonephritis without reflux belong more frequently to these O serogroups than strains from pyelonephritis with reflux (19,20). This suggests that O antigens may be one of the virulence factors of E.coli in UTI.

Certain strains of E. coli are encapsulated with an extracellular acidic polysaccharide. These capsules are termed K antigens. At this moment the existence of about 100 different K antigens have been described. However only a limited range of these K antigens (K1, K2, K3, K12 and K13) is found with significant frequency in UTI (17, 21). It was demonstrated that the frequency of K antigens is higher in strains isolated from pyelonephritis than from cystitis (22). The presence of K antigens favoured also kidney invasion in a mouse model (23), especially if they are present in large amounts (24). In addition strains causing pyelonephritis contain more K antigen than those causing cystitis (22, 25). The role of K antigen in virulence has been suggested, among others, to be an inhibitor of phagocytosis (26,27).

Haemolysins

Haemolysins are proteins which are produced by many Gram-negative and Gram-positive bacteria and they are active against erythrocytes of various animals and human. Already in 1921 Dudgeon et al. (28) pointed out that haemolytic E. coli strains are more common among UTI strains than among strains from the faecal flora, which was later confirmed by other groups (29, 30). The percentage of haemolytic strains isolated from pyelonephritis was higher than among cystitis or ABU strains (31). Furthermore, it was demonstrated that most of the haemolytic strains belonged to the O serotypes O2, O4, O6, O18 and O75 (17,30,32).

The influence of haemolysin production on virulence was studied in experimental mouse models. Van den Bosch et al. (33) concluded that haemolysin production was a virulence factor in most of the mouse nephropathogenic strains, when they were injected intravenously. They showed that 6 out of 7 strains became avirulent after the elimination of haemolysin production. In another mouse model, where bacteria are introduced in the urinary tract via the bladder, Ketyi et al. (34) demonstrated that rough E. coli strains with haemolytic activity induced bladder and kidney infections whereas rough strains without haemolytic activity were non-virulent.

The genes for haemolysin production of E. coli strains isolated from UTI are in most cases localized on the chromosome (29, 35), whereas many other haemolytic E. coli strains, particularly those from animal sources, are localized on a plasmid (36). Both types of haemolysin determinants were isolated by gene cloning and compared (37, 38). It was found that both types are closely related and consist of four genes (39). Two of these genes are required for synthesis of active haemolysin and two for the transport of haemolysin across the outer membrane.

The mechanism and site of action of haemolysin is still obscure. Waalwijk et al. (40) have shown that haemolysin functions as a virulence factor by lysis of erythrocytes which increases the level of available iron in haematogenous pyelonephritis. It was also demonstrated that haemolysin is cytotoxic for fibroblasts and human leucocytes in vitro (41,42,43). The combination of a toxic effect on various cells and the appearance of sufficient iron may account for haemolysin as a virulence determinant.

Adhesion

Adhesive properties of E. coli were already recognized by Guyot in 1908 (44), when he observed that some E. coli strains could agglutinate red blood cells from a number of animal species. Duguid et al. (45) observed that haemagglutination (HA), in a number of instances, was correlated with the presence of fimbriae. This correlation between adherence, HA and the presence of fimbriae was later confirmed by others (46-49). However, it was also reported that there are E. coli strains which do not express any fimbriae and still adhere (50,51). This suggests the presence of nonfimbrial adhesins.

Three different classes of E. coli fimbriae have been described. The adhesion of these fimbriae is referred to as non-adhesive, mannose-sensitive (MS) and mannose-resistant (MR). The non-adhesive fimbriae (e.g. 1C) do not show any HA as far as known. The 1C fimbriae do not adhere to urinary epithelial cells or urinary mucus, whereas they adhere to buccal epithelial cells (52). Their role in UTI is still obscure. The MS fimbriae are usually referred to as type 1 fimbriae. These fimbriae recognize and bind specifically to D-mannose containing receptors. These receptors are present on guinea pig erythrocytes, yeast cells and the Tamm-Horsfall protein which is found in urinary mucus (53,54). More than 85% of all E. coli strains have the ability to express type 1 fimbriae. Until now no conclusive function has been determined for type 1 fimbriae. However, in some studies no correlation could be found between the presence of type 1 fimbriae and virulence (31,55).

A number of E. coli strains with varying MRHA patterns have been described. These E. coli strains express fimbriae which can be subdivided into four groups based on their receptor specificities. The group of P fimbriae recognize a blood group P associated receptor, a disaccharide with the structure α -D-Galp-(1-4)- β -D-Galp (56,57). The M fimbriae bind to a blood group M associated receptor, which is a glycoprotein called glycophorin A (58). Another group of fimbriae which recognize the structure neuraminyl 2-3-galactoside as a receptor have recently been described and are called S fimbriae (59). The last group of X fimbriae is a heterogeneous group of fimbriae with varying HA patterns, but unknown receptor specificities.

Among uropathogenic E. coli strains especially the P fimbriae have been associated with the pathogenesis of pyelonephritis. This was based on clinical studies (60,61,62) which showed that approximately 90% of the E. coli strains isolated from children with an acute pyelonephritis express P fim-

brae. For cystitis this figure varied from 19% (60) till 31% (62), whereas only 14% of the E.coli isolated from patients with ABU expressed P fimbriae (60,61). However, it was also reported that 80% of the E.coli isolated from cystitis, and 64% from ABU express P fimbriae (63). This suggests that this adhesin may also play a role in lower UTI.

Another study by Lomberg et al. (64) revealed that the incidence of E. coli expressing P fimbriae is 50% in patients with recurrent acute pyelonephritis and only 25% in such patients with vesicoureteric reflux. This means that when reflux is present no advantage is conferred on P fimbriated organisms, but presumably the reflux carries the organisms to the kidney.

The P fimbriae may differ in the molecular weight of their subunit proteins and in serological specificities (65,66,67). Ørskov and Ørskov (66) have recently proposed a typing scheme for P fimbriae of uropathogenic E. coli. By using polyclonal antisera raised against different P fimbriae and crossed immuno electrophoresis they were able to distinguish eight serologically different P fimbriae on uropathogenic E. coli. They suggested to classify these P fimbriae as F antigens (F7-F13). Another study by Parry et al. (61), using polyclonal antisera in a slide agglutination test, demonstrated seven different antigenic determinants on fimbriae of uropathogenic E. coli and designated them a to g.

Outline of this study

At the start of this study several serologically different fimbriae had been described on urinary E. coli strains, using polyclonal antisera. However, no studies were conducted to see which of these fimbriae are important in the pathogenesis of UTI, especially pyelonephritis. The aim of this study was to characterize these different fimbriae with biochemical and serological tools, and to investigate which of these fimbriae are important in the pathogenesis of pyelonephritis. Because most of the E. coli strains express various fimbriae, the genes encoding for certain types of fimbriae were cloned. In chapter 2 and 3 the molecular cloning of two different P fimbriae is described. During the course of this study other investigators published the molecular cloning of other P fimbriae.

Characterization of polyclonal antisera raised against the various cloned and purified P fimbriae revealed that these P fimbriae have many antigenic determinants in common. For this reason we produced a set of monoclonal

antibodies raised against the various cloned and purified P fimbriae, which were specific for the various P fimbriae.

The production and characterization of these monoclonal antibodies is described in the chapters 4-6. In chapter 7 the development of a proper method is described for the detection of P fimbriae on clinical isolates of uropathogenic E. coli with the aid of the monoclonal antibodies. With this method various collections of extra intestinal E. coli were screened for the presence of different P fimbriae, which is described in chapter 8. Finally, in chapter 9, the presence of antibodies against P fimbriae in the sera of patients with pyelonephritis was studied.

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MOLECULAR CLONING AND CHARACTERIZATION OF F9 FIMBRIAE FROM A
UROPATHOGENIC *ESCHERICHIA COLI*

1. SUMMARY

The genes responsible for the formation of F9 fimbriae of the uropathogenic *Escherichia coli* strain C1018 were cloned by a cosmid cloning procedure. A positive clone was further subcloned by removing two *Bam*HI fragments and the remaining plasmid pPIL288-10 had a size of 25 kb. This clone still produced fimbriae as judged by electron microscopy and mannose-resistant haemagglutination (MRHA). Antisera were raised against the clone and against fimbriae purified from the clone. The first antiserum was used in a Western blot to prove the purity of the F9 fimbriae. The antiserum raised against purified fimbriae was used in inhibition tests of MRHA and adherence of cloned bacteria to human uroepithelial cells.

2. INTRODUCTION

E. coli is the most common infecting organism in urinary tract infection. A variety of bacterial

factors, e.g. haemolysin production, possession of ColV plasmids, O and K antigens, serum resistance and adhesion properties may influence the virulence of uropathogenic *E. coli* strains [1-7].

The adhesion properties are mediated by fimbriae, which can be distinguished into type 1 fimbriae that cause mannose-sensitive haemagglutination (MSHA) of guinea pig erythrocytes, and fimbriae that cause MRHA of erythrocytes of human and various animal sources. The MRHA fimbriae can be subdivided on the basis of their receptor specificity [8-10]. Among the MRHA fimbriae the P fimbriae have especially been associated with the pathogenesis of pyelonephritis [11].

Ørskov and Ørskov [12] described different MRHA antigens from uropathogenic *E. coli*. They suggested to use the term F antigens for fimbrial antigens and they described at least eight serologically different types. Parry et al. [13] could detect seven distinct antigenic determinants which have been designated a-g. In most cases fimbrial antigens from enterotoxigenic *E. coli* (K88, K99, CFA I and CFA II) are genetically determined by plasmids [14] in contrast with fimbrial antigens from uropathogenic *E. coli* which are chromosomally determined [15-18]. Until now there are four

reports about the cloning of chromosomally encoded fimbrial antigens from uropathogenic *E. coli* strains. The *pap* pili [16,19] and the fimbriae cloned by Clegg [15,20]; though serologically not determined, are not cross-reactive [15]. The first report on the cloning of fimbriae genes from an uropathogenic *E. coli* strain with well characterized fimbrial constitution was described by Van Die et al. [18]. They described the molecular cloning of F₇ fimbriae from the uropathogenic *E. coli* strain AD110. The fimbriae cloned by Rhen et al. may be identical with the fimbriae from strain AD110 [17].

This report describes the molecular cloning of the F9 fimbriae from *E. coli* C1018. The F9 term was originally given to the fimbrial antigens of a strain examined by Korhonen et al. [21]. In this study we used the strain C1018 which is the reference strain for F9 as determined by Ørskov and Ørskov [12].

3. MATERIALS AND METHODS

3.1. Bacterial strains, plasmids and growth media

E. coli strain C1018 (O2 : K5 : H4) was obtained from F. and I. Ørskov (Statens Serum Institut, Copenhagen). This strain gives rise to MRHA and expresses F9 fimbriae [12]. These fimbriae were determined as P fimbriae by S.B. Svenson and G. Källénus (The National Bacteriological Laboratory, Stockholm, Sweden). JA221 is a *recA hsdA* derivative of the *E. coli* K-12 strain C600 [22]. AM1727 is a *recA* derivative of JE2571, an *E. coli* K-12 strain deficient in the production of type 1 fimbriae [18]. Plasmid vectors used were pJB8 [23], pBR322 [24] and pACYC184 [25].

Bacteria were cultivated in L-broth or on Brain Heart Infusion agar (Oxoid). Selective pressure against loss of plasmids was imposed by adding 50 µg/ml ampicillin or 100 µg/ml chloramphenicol to the media.

3.2. Recombinant DNA techniques and enzymes

Cosmid cloning procedure was carried out exactly as described by Van Die et al. [18]. Restriction endonucleases and ligase were obtained from Boehringer. Incubation conditions were according

to the instruction of the manufacturer.

Plasmid DNA was isolated as described by Birnboim and Doly [26]. Transformation was carried out according to the method of Kushner [27].

3.3. Electron microscopy, haemagglutination assays and adherence to human uroepithelial cells

Adherence to human uroepithelial cells and electron microscopy was carried out as described by Van den Bosch et al. [5]. Haemagglutination activity of human erythrocytes was determined by the method of Van den Bosch et al. [7].

3.4. Purification of fimbriae

Fimbriae were purified essentially according to the method described by Korhonen et al. [28]. After mechanical detachment from the bacterial cells the fimbriae were precipitated with ammonium sulfate, dialyzed, and solubilized in buffer containing deoxycholate. This fraction was concentrated in an Amicon ultrafiltration cell until a protein concentration of approx. 8 mg/ml.

This fimbrial solution was further purified by two sequential sucrose gradients. When the purified fimbriae contained other proteins as judged by SDS-PAGE [29] or Western blotting, the fimbriae were purified once again on a sucrose gradient. The fimbriae concentration was measured by the method of Lowry et al. [30].

3.5. Antisera and Western blots

During this study we used two antisera. Antiserum raised in rabbits against live *E. coli* AM1727 harboring pPIL288-1 was used to prove the purity of fimbriae in Western blots. A second antiserum was raised against purified fimbriae. Absorption was carried out with AM1727. Western blots were carried out by the method of Muilerman et al. [31].

3.6. Cell extracts

Bacteria were grown on 10 Brain Heart Infusion agar plates for 16 h at 37°C. Cells were harvested and suspended in 10 ml PBS-formalin 0.25%. Subsequently, the cells were sonicated until the absorbance at 600 nm was reduced 10 times. This sonicated extract was centrifuged for 15 min at 15000 rev./min. Supernatant was used as a total protein fraction in Western blots.

4. RESULTS

4.1. Cloning of genetic determinants encoding for MR fimbriae

Chromosomal DNA of *E. coli* C1018 was partially digested with the restriction endonuclease *Sau*3A. Fragments of 30–45 kb, obtained by centrifugation through a 1.25–5 M NaCl gradient, were ligated into the single *Bam*HI site of the cosmid pJB8. Recombinant cosmid molecules were packaged into phage λ particles and transduced into the *E. coli* K12 strain JA221. Of 800 transductants tested, 8 exhibited the MRHA reaction. The clone with the strongest MRHA was analyzed further; it possessed a plasmid (designated pPIL288-1) of the expected size (approx. 45 kb).

4.2. Subcloning of fimbrial genes

Digestion of pPIL288-1 with the restriction enzyme *Bam*HI revealed three recognition sites for this enzyme. After digestion of pPIL288-1 with *Bam*HI and ligation followed by transformation to *E. coli* K-12 strain JA221, the transformants were tested for MRHA activity.

Three transformants with MRHA activity possessed a plasmid (pPIL288-10), which had deleted two *Bam*HI fragments and had an estimated size of 24 kb. A physical map of pPIL288-10 was determined from digestions and suitable double digestions with several restriction enzymes (Fig. 1a). Subsequently, plasmid pPIL288-10 was digested with restriction endonuclease *Sal*I, and the resulting 16.1-kb fragment (Fig. 1b) was ligated into the *Sal*I site of pACYC184 (pPIL288-11) and into the *Sal*I site of pBR322 (pPIL288-12). All clones were transformed to *E. coli* K-12 strain JA221 and AM1727 and were still coding for MRHA. Furthermore, fimbria production of clones in AM1727 was checked by electron microscopy and all clones produced large amounts of fimbriae (Fig. 2).

4.3. Characterization of F9 fimbriae

Native fimbriae were purified from strain AM1727 harbouring pPIL288-10 as described in MATERIALS AND METHODS. Purity of this fimbria preparation was checked on SDS-PAGE (Fig. 3) and the M_r was determined to be 21000. Purity

Fig. 1. (a) Restriction map of pPIL288-10. The solid black line represents the vector pJB8. (b) Restriction map of pPIL288-11. The solid black line represents a part of the vector pJB8 and the black and white line represents the vector pACYC184.

was also checked by Western blotting (Fig. 4, lane b) with an antiserum raised against whole bacteria (AM1727, harbouring pPIL288-1). Both methods, SDS-PAGE and Western blotting, did not reveal any other proteins than the fimbriae.

The amount of fimbriae produced by the different clones and the parental strain was measured by an HA activity test as described in MATERIALS AND METHODS. All clones gave rise to higher MRHA titers than the parental strain, suggesting that these clones produced more fimbriae than the parental strain (Table 1). This was also shown by electron microscopy where the amount of fimbriae from cloned strains was much higher than the parental strain (Fig. 2).

Fig. 2. Electron micrographs of parental and cloned strain. (a) C1018; (b) AM1727; (c) AM1727 harbouring pPIL288-10. The bar represents 500 nm.

Fig. 3. SDS-PAGE of marker proteins (lane a) and purified fimbriae from strain AM1727 harbouring pPIL288-10 (lane b).

Fig. 4. Western blotting of a crude cell extract from the parental strain C1018 (lane a), purified F9 fimbriae (lanes b,c), a crude cell extract of AM1727 harbouring pPIL288-10 (lanes

Adherence of cloned and parental strains to human uroepithelial cells was tested as described in MATERIALS AND METHODS. The parental strain and the strains with cloned fimbriae showed MR adherence to human uroepithelial cells in contrast with strain AM1727 (Table 1). Strains with cloned fimbriae showed an even higher MR adherence than the parental strain. After preincubation of the bacteria with absorbed antiserum raised against purified fimbriae the MRHA and the MR adherence were totally inhibited (Table 1). In these inhibition experiments antiserum concentrations were used which did not cause agglutination of the bacteria as checked by microscopy. The same antiserum was checked in a Western blot (Fig. 4, lanes c-e) and reacted faintly with two proteins and strongly with the fimbriae of a crude protein fraction. The faint reaction with the two proteins was caused by the goat anti-rabbit horse radish peroxidase conjugate which reacted aspecific with these proteins. This suggests that this antiserum

d, f) and a crude cell extract of AM1727 (lanes e, g). Lanes a-b were incubated with antiserum raised against AM1727 harbouring pPIL288-1; lanes c-e with absorbed antiserum raised against purified fimbriae and lanes f-g with goat anti-rabbit horse radish peroxidase conjugate only.

Table 1

Adherence to human uroepithelial cells and haemagglutinating properties of wild-type and plasmid containing *E. coli* strains with human erythrocytes

Strain	Haemagglutination titer			Adherence to uroepithelial cells	
	Without mannose	With mannose	In the presence of antiserum ^a	-	In the presence of antiserum ^a
C1018	16	16	2	14	-
AM1727	0	0	-	49	18
AM1727 pPIL288-1	128	128	0	13	-
AM1727 pPIL288-10	256	256	2	-	-
AM1727 pPIL288-11	128	128	0	59	12
AM1727 pPIL288-12	128	128	0	77	9
AM1727 pJB8	0	0	-	74	11
AM1727 pACYC184	0	0	-	-	-

^a Antiserum was raised in rabbits against purified fimbriae and absorption was carried out with AM1727.

^b Adherence was tested in the presence of mannose, examined with a light microscope at 400× magnification, and expressed as the percentage of epithelial cells with more than 25 adherent bacteria out of a total of 200 cells counted.

reacted only with F9 fimbriae from a crude protein fraction of AM1727 harbouring pPIL288-10. A third, yet unidentified, faint protein band appeared in the AM1727 harbouring pPIL288-10 (Fig. 4, lane d) compared with the AM1727 (Fig. 4, lane e), also present in the conjugate control (Fig. 4, lane f).

5. DISCUSSION

This study describes the molecular cloning of the genes encoding for F9 fimbriae. Cloning of fimbriae genes has many advantages: (1) one can distinguish between different fimbriae produced by one strain; (2) the organization of the genes involved in biogenesis of fimbriae can be studied; (3) fimbriae genes cloned in a high-copy plasmid give rise to a huge fimbria production which facilitates the purification of fimbriae; (4) strains that differ in only the type of fimbriae expressed, will be useful in a detailed serological study.

After cosmid cloning and subcloning in the pACYC184 vector and the pBR322 vector a chromosomal DNA fragment of 13.6 kb was found which still encoded for F9 fimbriae and MRHA. From comparison with other cloned fimbrial gene clusters it can be expected that the exact region encoding for these fimbriae is still smaller. The *pap* pili operon described by Normark et al. [19] is encoded by a 8.2-kb fragment. Clegg and Pierce [20] described a region of 5.4 kb which encoded fimbriae and Van Die et al. [18,32] described a region of approx. 10 kb which encoded for F7₂ fimbriae. To establish the exact size of this DNA fragment necessary for fimbrial production and the genes involved in the expression of F9 fimbriae we will map the genes with transposable elements.

Fimbriae were purified from AM1727 harbouring pPIL288-10 by the method of Korhonen et al. [28] with omitting the last step, a Sepharose 4B column in 6 M urea buffer. We found this last step, included for purification of fimbriae from flagella material, not necessary, especially when *E. coli* K-12 strains were used as starting material.

The fimbriae were shown to be pure in SDS-PAGE and Western blotting (Figs. 3 and 4, lane b).

The M_r was found to be 21000, still in the range of 15000–29000 as is described for fimbriae [14].

E. coli K-12 strains with plasmids coding for cloned fimbriae had a much higher production of fimbriae than the parental strain as determined by electron microscopy, MRHA titers and adherence (Table 1, Fig. 2). Purification of fimbriae from cloned strains gave a higher yield than from the parental strain (not shown).

By Western blot analysis it could also be shown that purified fimbriae from AM1727 harbouring pPIL288-10 exhibited the same M_r and antigenicity as fimbriae from the parental strain C1018 (Fig. 4, lane a and b).

Normark et al. [19] stated that the fimbrial subunit of the *pap* pili is not the actual adhesin. Whether or not the F9 fimbrial subunit is the adhesin cannot be proven until we have mapped the genes coding for F9 fimbriae with transposons. However, our results strongly suggests that for F9 fimbriae the fimbrial subunit is the adhesive factor because adhesion and HA of parental and *E. coli* K-12 with cloned fimbriae were strongly inhibited by adding the absorbed antiserum raised against purified F9 fimbriae. This antiserum reacted only with F9 fimbrial subunit as detected in a Western blot (Fig. 4, lanes c–g). This could mean that for F9 fimbriae the fimbrial subunit is also the adhesive factor, but this has to be proven by further genetic research.

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MOLECULAR CLONING OF F11 FIMBRIAE FROM A UROPATHOGENIC
ESCHERICHIA COLI AND CHARACTERIZATION OF FIMBRIAE WITH POLYCLONAL
AND MONOCLONAL ANTIBODIES

1. SUMMARY

The genes coding for F11 fimbriae from the uropathogenic *Escherichia coli* C1976 were cloned by a cosmid cloning procedure. Two cosmid clones expressed F11 fimbriae and these clones possessed an identical DNA fragment of 8.9 kb. This fragment was subcloned into pBR322 and this plasmid still produced fimbriae and caused a mannose-resistant haemagglutination (MRHA). Polyclonal and monoclonal antibodies were produced against purified cloned F11 fimbriae. Both types of antibodies were used in inhibition tests of MRHA and adherence of bacteria to the uroepithelial cell line T24. After preincubation of bacteria with polyclonal antiserum the MRHA and the MR adherence were totally inhibited. Preincubation of bacteria with monoclonal antibodies did not inhibit MRHA and MR adherence.

2. INTRODUCTION

E. coli is the most common infecting organism in urinary tract infection. An important virulence factor is the adhesive capacity of *E. coli* to uro-

epithelial cells, mediated by bacterial fimbriae [1-3]. Adhesins of *E. coli* can be divided into type 1 fimbriae that cause a mannose-sensitive haemagglutination (MSHA) of guinea pig erythrocytes and fimbriae that cause an MRHA of human erythrocytes. These MRHA fimbriae can be subdivided into P, M and S fimbriae on the basis of their receptor specificity [4-6]. P fimbriae especially have been associated with the pathogenesis of pyelonephritis [7], and it has been shown that these P fimbriae recognize the disaccharide α -D-Gal p-(1-4) β -D-Gal p as a receptor on uroepithelial cells [8]. Ørskov and Ørskov [9] and Parry et al. [10] described several serologically different types of fimbriae from uropathogenic *E. coli*. Ørskov and Ørskov [9] introduced the term F fimbriae and numbered the various MRHA fimbriae types from uropathogenic *E. coli* strains F7 till F12. Recently, the molecular cloning of F7₁ (Van Die et al., manuscript in preparation), F7₂ [11] and F9 [12] were described. In this report we describe the molecular cloning and characterization of F11 fimbriae from the uropathogenic *E. coli* C1976.

3. MATERIALS AND METHODS

3.1. Bacterial strains, plasmids and growth media

The clinical isolate used in this study, *E. coli*

strain C1976 (O1:K1:H7), was obtained from I. and F. Ørskov (Statens Serum Institute, Copenhagen), gives rise to MRHA and expresses F11 fimbriae [9]. These fimbriae were determined as P fimbriae by S.B. Svenson and G. Källénus (The National Bacteriological Laboratory, Stockholm).

AM1727 is a *recA* derivative of JE2571, an *E. coli* K-12 strain which does not produce type 1 fimbriae [11]. JA221 is a *recA hsdA* derivative of the *E. coli* K-12 strain C600 [13]. Plasmid vectors used were pBR322 [14] and pACYC184 [15]. The cosmid vector pJC74 was kindly provided by Jörg Hacker (Institut für Genetik und Mikrobiologie, Würzburg).

Bacteria were cultivated in Brain Heart Infusion broth (Difco) or on Brain Heart Infusion agar (Oxoid). Selective pressure against loss of plasmids was imposed by adding 50 µg/ml ampicillin or 100 µg/ml chloramphenicol to the media.

3.2. Recombinant DNA techniques and enzymes

Cosmid vector pJC74 was totally digested with *Bam*HI and treated with alkaline phosphatase. High M_r chromosomal DNA was prepared essentially as described by Hull et al. [16] and treated with *Sau*3A. Fragments of 30–45 kb were ligated into the cosmid vector, packaged into λ phage particles and transduced to strain *E. coli* K-12 JA221 as described by van Die et al. [11].

Restriction endonuclease, ligase and alkaline phosphatase were obtained from Boehringer. All enzymes were used as suggested by the manufacturer.

Plasmid DNA was isolated as described by Birnboim and Doly [17]. Transformation was carried out according to the method of Kushner [18].

3.3. Electron microscopy and haemagglutination assays

Electron microscopy was carried out as described by Van den Bosch et al. [2]. For determination of the MRHA activity of strains, serial dilutions of bacteria were incubated with human erythrocytes and incubated for 2 h on ice; HA was read after agitation [3].

Inhibition of HA by antibodies was tested with bacteria which were preincubated for 30 min at

room temperature with polyclonal antiserum monoclonal antibodies. Polyclonal and monoclonal antibodies were used at concentrations which did not cause agglutination of bacteria checked by microscopy.

3.4. Adherence to human uroepithelial cell line T24

The human uroepithelial cell line T24 was kindly supplied by Björn Lund (Department of Microbiology, Umeå, Sweden). Adherence experiments were carried out essentially as described by Lund et al. (personal communication). Briefly, T24 cells were grown in MEM (Gibco) supplemented with 1% L-glutamine (Flow) and 10% fetal calf serum (Flow). Cells were seeded at low density (1000 cells/cm²) in 6-well clusters (Costar) and allowed to spread for 24 h in a 5% CO₂ atmosphere. After washing twice with MEM without serum the cells were incubated with 2×10^8 bacteria per well for 1 h at 37°C. After this incubation T24 cells were washed with saline (5 ×) and stained with 30% filtered Giemsa stain (Giemsa-Lösung; Merck). Adherence was examined with a light microscope and expressed as the % of T24 cells with at least 25 adherent bacteria out of a total of 100 cells counted.

3.5. Purification of fimbriae, preparation of cell extracts, SDS-PAGE and Western blots

Fimbriae were purified essentially according to the method described by Korhonen et al. [19] avoiding protein-denaturing conditions [12]. Cell extracts were prepared by sonication as described previously [12]. Protein concentrations were measured by the method of Lowry et al. [20]. Purity of fimbriae preparations was checked by SDS-PAGE [21] and Western blots [22].

3.6. Polyclonal and monoclonal antisera

Polyclonal antisera were raised in rabbits against purified cloned F11 fimbriae and against live AM1727 harbouring pPIL291-15.

The production of the monoclonal antibodies M7-4 and M7-5, recognizing different epitopes on F11 fimbriae, will be described elsewhere. (J.M. de Ree, P. Schwillens and J.F. van den Bosch. Monoclonal antibodies that recognize the P fimbriae F7₁, F7₂, F9 and F11 from uropathogenic *E. coli*, submitted for publication).

Fig. 1. Restriction map of pPIL291-15. The solid black line represents the vector pBR322.

3.7. ELISA with purified F11 fimbriae

An ELISA with purified cloned fimbriae was carried out essentially as described by Dodd and Eisenstein [23]. Microtiter plates (PVC; Flow) were coated with purified F11 fimbriae, followed by incubation with serial dilutions of poly- or monoclonal antibodies. Anti-F11 fimbriae activity was detected colorimetrically after incubation with goat anti-rabbit or goat anti-mouse antiserum, respectively, conjugated with peroxidase (Nordic) and addition of the substrate *o*-phenylenediamine (Sigma). Absorbance at 495 nm was determined with a Titertek multiscan ELISA plate reader.

4. RESULTS

4.1. Molecular cloning of the genes coding for F11 fimbriae

After a cosmid cloning procedure as described in MATERIALS AND METHODS the colony bank was screened for MRHA. Two colonies were found which exhibited MRHA and production of fimbriae as shown by electron microscopy (not shown). Both cosmids (designated pPIL291-1 and

pPIL291-3) were purified by the method of Birnboim and Doly [17]. From both cosmids a *Bam*HI fragment was cloned into the vector pACYC184. These two subclones (designated pPIL291-10 and pPIL291-30) were not identical as shown by restriction enzyme analysis. However, both subclones possessed an identical *Bam*HI-*Eco*RI fragment and this fragment was cloned into the vector pBR322. A physical map of this plasmid (pPIL291-15) was determined from digestions and suitable double digestions with several restriction enzymes (Fig. 1). After transformation of pPIL291-15 to *E. coli* K-12 strain AM1727 this clone still expressed MRHA (Table 1) and fimbriae as checked by electron microscopy (Fig. 2).

4.2. Characterization of F11 fimbriae

Native F11 fimbriae were purified from strain AM1727 harbouring pPIL291-15 and from the parental strain C1976. The purity of F11 fimbriae prepared from the cloned strain was checked in a Western blot (Fig. 3, lane c) with an antiserum raised against AM1727 harbouring pPIL291-15. This antiserum reacted strongly with multiple proteins of cell extracts from AM1727 (Fig. 3, lane b) and AM1727 harbouring pPIL291-15 (Fig. 3, lane a), but only with one band in the purified F11 fimbriae preparation (Fig. 3, lane c). Purity of these F11 fimbriae was also shown by SDS-PAGE (Fig. 4). From this SDS-PAGE the M_r was determined to be 18000. In another Western blot an antiserum raised against purified cloned F11 fimbriae reacted strongly with purified cloned F11

Table 1

Adherence of T24 cells and MRHA with human erythrocytes of wild-type and plasmid-containing *E. coli* strains, and inhibition by polyclonal and monoclonal antibodies

Strain	MRHA titer					Adherence to T24 uroepithelial cells ^a			
	-	In the presence of				-	In the presence of		
		PolAs ^b	M7-4 ^b	M7-5 ^b	M7-4 and M7-5		PolAs ^b	M7-4 ^b	M7-5 ^b
C1976	4	-	-	-	-	69	-	-	-
AM1727	0	-	-	-	-	0	-	-	-
AM1727 pPIL291-15	128	0	64	128	64	100	6	92	86

^a Adherence was determined in the presence of 1% D-mannose, and expressed as the % of T24 cells with at least 25 adherent bacteria out of a total of 100 cells counted.

^b Polyclonal antiserum (PolAs) used and the monoclonal antibodies (M7-4 and M7-5) were raised against purified cloned F11 fimbriae.

Fig. 2. Electron micrographs of parental and cloned strain (a) C1976; (b) AM1727; (c) AM1727 harbouring pPIL291-15. The bar represents 500 nm.

Fig. 3. Western blotting of crude cell extracts from AM1727 (lane b), AM1727 harbouring pPIL291-15 (lane a), purified cloned F11 fimbriae (lane c and e), and purified parental F11 fimbriae (lane d). Lanes a-c were incubated with antiserum raised against AM1727 harbouring pPIL291-15, and lanes d and e with antiserum against purified F11 fimbriae.

fimbriae (Fig. 3, lane e) and at the same place with F11 fimbriae purified from the parental strain (Fig. 3, lane d).

In an HA activity test, it was shown that strain

AM1727 harbouring pPIL291-15 produced more fimbriae than the parental strain (Table 1). This was also demonstrated by electron microscopy (Fig. 2). The parental as well as the cloned strain showed an MR adherence to the uroepithelial cell line T24, in contrast to strain AM1727 which did not adhere (Table 1). The cloned strain AM1727 harbouring pPIL291-15 adhered stronger than the parental strain C1976, also indicating the huge fimbria production of the clone compared with the parental strain.

Both the polyclonal antiserum and the monoclonal antibodies raised against purified cloned F11 fimbriae reacted strongly with homologous fimbriae in an ELISA. When the titer is defined as the $^{10}\log$ of the last reciprocal dilution with an A_{490} of at least 0.500, the titer of the polyclonal antiserum was 4.4, whereas the titers of the monoclonals M7-4 and M7-5 were 4.4 and 4.1, respectively. In contrast to the polyclonal antisera, which reacted with homologous fimbriae in Western blots (Fig. 3), neither of the monoclonal antibodies showed any reaction in Western blots (not shown). As shown in Table 1, the MRHA as well as the MR adherence to T24 cells of the cloned strain was totally inhibited by preincubation of the bacteria with the polyclonal antiserum. The monoclonal antibodies, however, inhibited neither MRHA nor adherence. Preincubation of the bacteria with both monoclonals at the same time also did not inhibit the MRHA.

5. DISCUSSION

In this report we describe the molecular cloning of the genes coding for F11 fimbriae from the parental strain C1976. A piece of 8.9 kb was responsible for the production of these fimbriae and the restriction map of this pPIL291-15 showed remarkable correspondence with other cloned P fimbriae [12,24,25].

The higher expression of F11 fimbriae on strain AM1727 harbouring pPIL291-15 (Table 1 and Fig. 2) compared with the parental strain C1976 was useful for the purification of F11 fimbriae. Fimbriae isolated from the clone were shown to be pure on SDS-PAGE (Fig. 4) and on Western blots

Fig. 4. SDS-PAGE of marker proteins (lane a) and purified fimbriae from strain AM1727 harbouring pPIL291-15 (lane b).

(Fig. 3, lane c). A polyclonal antiserum raised against these purified fimbriae reacted strongly in a Western blot, at the same place with the fimbriae purified from cloned and parental strain (Fig. 3, lane d and e). From these results we may conclude that we indeed cloned the F11 fimbriae from the parental strain. The HA and adherence assays

proved that the information for recognizing the receptor structure for P fimbriae (α -D-Gal *p*-(1-4)- β -D-Gal *p*) is present on plasmid pPIL291-15 (Table 1). When the strain AM1727 harbouring pPIL291-15 was incubated with polyclonal antiserum raised against F11 fimbriae the HA and adherence were totally inhibited (Table 1). This strongly suggested that immunoglobulins are present in this antiserum which recognize that part(s) of the fimbriae that is involved in recognizing the receptor structure on erythrocytes and uroepithelial cells. No inhibition of HA and adherence was found when strain AM1727 harbouring pPIL291-15 was incubated with the monoclonals M7-4 and M7-5. This means that these monoclonals reacted with epitopes on F11 fimbriae which are not involved in recognizing the receptor structure. Nevertheless, both the polyclonal antiserum and the monoclonals reacted strongly with purified fimbriae in an ELISA.

Recently, Lindberg et al. [26] and Norgren et al. [27] found evidence that for *pap* fimbriae the fimbriae subunits themselves are not responsible for recognizing the receptor structure on uroepithelial cells. They stated that a minor component present in the *pap* fimbriae is responsible for adhesion. Preliminary experiments showed that it was possible to construct clones from pPIL291-15 which did not produce fimbriae but still agglutinated human erythrocytes. This suggests that for F11 fimbriae also a minor component may be responsible for recognition of the receptor structure on uroepithelial cells. When indeed a minor component of the fimbriae is the actual adhesin, this would mean that the chance of producing monoclonal antibodies against an epitope which is involved in recognizing the receptor structure is very low.

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MONOCLONAL ANTIBODIES THAT RECOGNIZE THE P FIMBRIAE F7₁, F7₂, F9, AND F11
FROM UROPATHOGENIC *ESCHERICHIA COLI*

One of the most important virulence factors of uropathogenic *Escherichia coli* is the capacity of these bacteria to adhere to uroepithelial cells (6, 22, 26). Adhesion is mediated by fimbriae which are heterogenous serologically (18, 19, 20) and with regard to the molecular weights of their subunit proteins (8). Ørskov and Ørskov (18) distinguished eight serologically different fimbriae in uropathogenic *E. coli*, and Parry et al. (20) found seven serologically different fimbriae. Fimbriae from uropathogenic *E. coli* can also be distinguished by their receptor specificities: (i) fimbriae that cause mannose-sensitive hemagglutination of guinea pig erythrocytes (type 1 fimbriae); (ii) fimbriae that cause no hemagglutination (HA) of any erythrocytes (e.g., 1C fimbriae); and (iii) fimbriae that cause mannose-resistant hemagglutination (MRHA) of human erythrocytes. This last group can be subdivided on the basis of receptor specificities into P, M, S, and X fimbriae (9, 12, 24). The P fimbriae especially have been associated with the pathogenesis of pyelonephritis (23). Recently, the molecular cloning of several fimbriae was reported. Hull et al. (7) and Clegg (2) described the molecular cloning of pyelonephritis-associated pilus (Pap) fimbriae and "Clegg" fimbriae, respectively, both serologically not determined but not cross-reactive with each other (2). The molecular cloning of the genes coding for F7₁ (29), F7₂ (28), and 1C (27a) fimbriae from one parental strain was recently reported. The fimbriae cloned by Rhen et al. (21) are presumably the same as the F7₁, F7₂, and 1C fimbriae, but they were cloned from another parental strain. We have recently reported the molecular cloning of F9 (3) and F11 (3a) fimbriae, and Hacker et al. (5) described the cloning of X fimbriae from a uropathogenic *E. coli* strain. Orndorff and Falkow (17) cloned type 1 fimbriae from a clinical isolate of *E. coli*, and Klemm et al. (9a) cloned type 1 fimbriae from an *E. coli* K-12 strain.

In the present study, we describe the production and characterization of polyclonal and monoclonal antisera against four different cloned P fimbriae: F7₁, F7₂, F9, and F11.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. AM1727 is a *recA* derivative of JE2571, an *E. coli* K-12 strain deficient in the production of type 1 fimbriae (28). All fimbriae used in this study were purified from AM1727 harboring the clones described in Table 1. The F7₁, F7₂, and 1C clones were kindly provided by Irma van Die, the Pap clone was provided by Staffan Normark, and pDC1 was provided by Steven Clegg.

All bacteria were cultivated on brain heart infusion agar (Oxoid Ltd.), except that strain AM1727 harboring pPIL38 was grown in brain heart infusion broth. Selective pressure against the loss of plasmids was imposed by adding 50 µg of ampicillin, 100 µg of chloramphenicol, or 12.5 µg of tetracycline per ml, dependent on the cloning vector used.

Purification of fimbriae. Fimbriae were purified essentially by the method of Korhonen et al. (10) with some modifications, yielding purified native fimbriae as described previously (3). Purity was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Lugtenberg et al. (15). Fimbria concentrations were measured by the method of Lowry et al. (14).

Polyclonal antisera. Rabbits were inoculated intramuscularly on days 0, 9, and 18 with 400 µg of purified fimbriae in Freund complete adjuvant (Difco Laboratories). Serum was collected 5 days after the last injection.

Hybridoma production. For immunization, BALB/c mice were inoculated intravenously via the tail vein with 10 µg of purified fimbriae in 0.5 ml of saline on days 0, 5 and 10. Four days after a booster injection with the same amount of fimbriae, the mice were killed, and 4×10^7 spleen cells were combined with 2×10^7 cells of the nonproducing myeloma cell line SP2/O. Spleen cells and myeloma cells were fused by using polyethylene glycol (PEG 4000) and suspended in

TABLE 1. Plasmids encoding different fimbriae

Plasmid	Fimbriae	Mol wt	HA	References
pPIL110-70	F7 ₁	22,000	MRHA	29
pPIL110-37	F7 ₂	19,160	MRHA	28
pPIL288-10	F9	21,000	MRHA	3
pPIL291-15	F11	18,000	MRHA	3a
pRHU845	Pap	19,500	MRHA	1, 7
pDC1	Clegg	17,000	MRHA	2
pPIL110-51	1C	15,800	None	27a
pPIL38	1A	15,706	Mannose-sensitive Ha	9a

complete growth medium (Dulbecco modified Eagle medium; Flow Laboratories, Inc.) containing 1% L-glutamine, 1% pyruvate (Sigma Chemical Co.), nonessential amino acids (Flow), 15% fetal calf serum (GIBCO Laboratories), 2% hypoxanthine (Merck & Co., Inc.), 2% desoxythymine (Merck), and 1% aminopterin (Sigma). After being seeded in four microtiter plates (96 wells), the cells were grown in a 5% CO₂ atmosphere at 37°C and 95% humidity. After 3 weeks of incubation, all wells were tested for antibody production against fimbriae with an enzyme-linked immunosorbent assay (ELISA). Positive clones were transformed to 24-well plates for additional growth, followed by a limiting dilution step to obtain cell lines derived from one single hybrid cell. After additional growth, the cells were used for antibody production in ascitic fluid.

Production of ascitic fluid. Pristane-primed BALB/c mice were irradiated with 450 rads. One day after irradiation, the mice were inoculated intraperitoneally with 5×10^6 hybridoma cells in 0.5 ml of saline. Ascitic fluids were collected after 6 to 12 days. Lipids were removed with Freon 113, and complement was inactivated by incubation of the ascitic fluid at 56°C for 30 min, followed by precipitation in 50% ammonium sulfate. The precipitate was dissolved in saline, dialyzed against saline, and frozen in small portions at -70°C.

ELISA for anti-fimbrial antibody determination. The ELISA used in this study was a modification of the procedure described by Dodd and Eisenstein (4). Microtiter plates (activated polyvinyl chloride; Flow) were coated overnight at 30°C with 100 µl per well of a fimbria solution (2.5 µg/ml) in 0.05 M bicarbonate coating buffer (pH 9.6). The plates were washed three times with phosphate-buffered saline-0.05% Tween 20 (PBST). For hybridoma screening, the wells were incubated with 100 µl of supernatant for 2 h at room temperature. For titer determinations and specificity testing, the wells were incubated with serial dilutions of polyclonal antiserum or monoclonal antibodies (MAbs) in PBST containing 0.5% bovine serum albumin (Boehringer Mannheim Biochemicals). Subsequently, the wells were washed three times with PBST, followed by incubation with conjugate for 3 h at room temperature. In the case of MAbs, goat antimouse immunoglobulin G (IgG) (heavy and light chains) conjugated to peroxidase (Nordic Laboratories) was used, and in the case of polyclonal antiserum, goat antirabbit IgG (heavy and light chains) conjugated to peroxidase (Nordic) was used. Anti-fimbrial antibody activity was detected colorimetrically by adding 0.04% o-phenylenediamine in citrate-phosphate buffer (pH 4.5) to 0.005% H₂O₂. The reaction was developed in the dark for 15 min at room temperature and stopped with 2 M H₂SO₄. The A₄₉₂ was measured in a Titertek Multiskan (Flow).

Class determination of MAbs. After microtiter plates were

coated with fimbriae, homologous MAbs were added as described for the ELISA. After three washings with PBST containing 0.5% bovine serum albumin, goat antimouse IgG1, IgG2a, IgG2b, or IgM (Nordic) was added as the second antibody. MAb classes were finally detected after the addition of rabbit anti-goat IgG (heavy and light chains) conjugated to peroxidase (Nordic).

Spot test for the reaction of MAbs with denatured fimbriae. Fimbriae were denatured by being boiled for 5 min in a buffer containing 0.06 M Tris (pH 6.8), 2% SDS, and 0.05% β-mercaptoethanol. Denatured fimbriae were spotted onto nitrocellulose paper (2 µg per spot) and dried overnight at room temperature. The nitrocellulose paper was blocked with 1% bovine serum albumin in PBST for 1 h at 37°C, followed by incubation for 1 h at 37°C with 1:100-diluted MAbs. After three washings with PBST, the paper was incubated with 1:100-diluted goat antimouse IgG-peroxidase conjugate in PBST containing 1% bovine serum albumin for 1 h at 37°C. The paper was washed three times with PBST, and immune complexes were detected by adding 0.05% diaminobenzide-0.002% H₂O₂ in phosphate-buffered saline. After 10 min, the reaction was stopped with 2 M H₂SO₄.

HA assays. The HA activity of human erythrocytes and the inhibition of HA were determined by the method of van den Bosch et al. (25). Briefly, serial dilutions of bacteria were incubated with human erythrocytes for 2 h on ice. HA was read after agitation. The inhibition of HA was tested with bacteria preincubated for 30 min at room temperature with polyclonal antiserum or MAbs. Antibody dilutions which did not cause agglutination of bacteria, as checked by microscopy, were used.

RESULTS

Characterization of polyclonal antisera raised against F7₁, F7₂, F9, and F11 fimbriae. All fimbriae were purified as described in Material and Methods. The purity of these fimbria preparations was checked by SDS-polyacrylamide gel electrophoresis (Fig. 1). Polyclonal antisera were raised in rabbits against purified F7₁, F7₂, F9, and F11 fimbriae. Cross-reactions of these polyclonal antisera with all purified fimbriae were analyzed in an ELISA. All polyclonal antisera reacted strongly with the homologous fimbriae (Table 2). However, all polyclonal antisera also reacted with the heterologous fimbriae, although with lower titers than in the homologous reactions. The anti-F7₁ antiserum reacted with all the fimbriae except the Pap fimbriae. In the case of anti-F7₂ antiserum, positive reactions were found with all but the 1A fimbriae. The anti-F9 antiserum reacted strongly with the F9 fimbriae and also showed a positive reaction with the F7₁, F7₂, F11, and 1A fimbriae. The anti-F11 antiserum showed the highest titers against F11 and Clegg fimbriae and also recognized antigenic determinants on the other fimbriae which cause MRHA but not on the 1A and 1C fimbriae.

MAb production. Purified F7₁, F7₂, F9, and F11 fimbriae were used for MAb production as described in Material and Methods. Three weeks after the various fusions, the hybridomas with the highest ELISA results were selected for additional growth. After subcloning, the hybridomas with the highest titers in the ELISA were used for the production of ascitic fluids, and the immunoglobulins were characterized after partial purification from these ascitic fluids. The results of the immunoglobulin subclass determinations are shown in Table 3. Six MAbs were of the IgG1 subclass, six were of the IgM subclass, three were of the IgG2b subclass, and one was of the IgG2a subclass.

Characterization of MAbs raised against F7₁, F7₂, F9, and

FIG. 1. SDS-polyacrylamide gel electrophoresis with different purified fimbriae. Lanes: a, F7₁; b, F7₂; c, F9; d, F11; e, Pap; f, Clegg; g, 1C; h, 1A; m, marker.

F11 fimbriae. Partially purified MABs from ascitic fluids were tested in the ELISA for (cross-)reactions with all purified fimbriae. In Table 3, the titers are shown for each MAB against homologous and heterologous fimbriae. The four M6 MABs raised against F7₁ fimbriae only recognized an epitope on F7₁ fimbriae. All three M4 MABs, which were produced against F9 fimbriae, recognized only epitopes on F9 fimbriae. The M2 MABs, raised against F7₂ fimbriae, could be divided into two groups: M2-7, which only recognized an epitope on F7₂ fimbriae, and M2-1 and M2-11, which recognized epitopes on F7₂ and F9 fimbriae. The M7 MABs, raised against F11 fimbriae, could also be divided into two groups: two MABs (M7-5 and M7-15) which recognized epitopes on F11, F7₂, and Pap fimbriae, and a group of four MABs (M7-4, M7-6, M7-7, and M7-13) which recognized epitopes on F11 and Clegg fimbriae.

Purification of MABs by high-pressure liquid chromatography yielded antibodies which still showed the same (cross-)reactions as partially purified MABs. Since immunization and the ELISA were performed with native fimbriae, we analyzed the reaction of the MABs with denatured fimbriae in a spot test as described in Materials and Methods. Only two sets of MABs reacted with denatured homologous fimbriae (Fig. 2). Firstly, both the M2-1 and M2-11 MABs reacted with denatured F7₂ fimbriae, and secondly, the M7-4, M7-6, M7-7, and M7-13 MABs showed a positive

TABLE 3. Titers of MABs in an ELISA with eight different purified fimbriae and subclasses of antibodies

MAB	Fimbriae that MAB was raised against	MAB sub-class ^a	Antibody titer ^a to indicated fimbriae					
			F7 ₁	F7 ₂	F9	F11	Pap	Clegg
M6-3	F7 ₁	IgG2a	4.4					
M6-6		IgM	3.8					
M6-8		IgM	4.1					
M6-19		IgM	4.7					
M4-5	F9	IgM				4.1		
M4-7		IgM				4.7		
M4-11		IgM				3.5		
M2-1	F7 ₂	IgG2b		5.0	3.8			
M2-11		IgG1		3.5	2.0			
M2-7		IgG1		4.4				
M7-4	F11	IgG2b				4.4		4.1
M7-6		IgG1				5.0		5.0
M7-7		IgG2b				4.1		4.1
M7-13		IgG1				4.4		4.1
M7-5		IgG1		4.1		4.1	3.8	
M7-15		IgG1		4.7		4.1	3.5	

^a Determined as described in Materials and Methods.

^b Given as the logarithm of the last reciprocal dilution of the antiserum giving an A₄₉₀ of at least 0.50. 1A and 1C fimbriae were nonreactive.

reaction with denatured F11 fimbriae. These M2 and M7 MABs also reacted with the denatured heterologous F9 and Clegg fimbriae, respectively (data not shown).

HA inhibition assays. The MRHA titer of AM1272 with different clones was tested in an HA assay. All four clones showed MRHA, and this MRHA was completely inhibited in the presence of the homologous polyclonal antiserum (Table 4). In the presence of MABs no inhibition of MRHA was observed.

DISCUSSION

In this study, we described the production of polyclonal antisera and MABs against the four purified P fimbriae F7₁, F7₂, F9, and F11. All fimbriae were purified from cloned strains. The main advantage of using cloned strains instead of parental strains is that cloned strains only express one type of fimbria, whereas parental strains can express different types of fimbria, e.g., C1212, which expresses F7₁, F7₂, and 1C fimbriae (28). Another advantage of using cloned

TABLE 2. Cross-reactions of polyclonal antisera in an ELISA with cloned, purified fimbriae

Fimbriae that antiserum was raised against	Antibody titer ^a to indicated fimbriae							
	F7 ₁	F7 ₂	F9	F11	Pap	Clegg	1A	1C
F7 ₁	5.0	4.1	4.1	2.9		2.0	2.6	2.0
F7 ₂	3.5	5.0	4.1	3.2	2.9	2.9		2.0
F9	2.0	3.2	4.7	2.3			2.3	
F11	3.5	3.5	3.2	4.4	3.2	4.4		

^a Given as the logarithm of the last reciprocal dilution of the antiserum giving an A₄₉₀ of at least 0.5.

FIG. 2. Spot test with MABs and denatured fimbriae.

TABLE 4. HA inhibition by polyclonal antisera and MAb

Plasmid (fimbriae)	Antiserum	HA titer
pPIL110-37 (F7 ₂)	None	128
	Polyclonal	0
	M2-1	64
	M2-7	64
	M2-11	64
pPIL288-10 (F9)	None	256
	Polyclonal	0
	M4-5	128
	M4-7	128
	M4-11	128
pPIL110-70 (F7 ₁)	None	128
	Polyclonal	0
	M6-3	128
	M6-6	64
	M6-8	64
	M6-19	32
pPIL291-15 (F11)	None	128
	Polyclonal	0
	M7-4	128
	M7-5	128
	M7-6	128
	M7-7	128
	M7-13	128
	M7-15	128

strains is that these strains give rise to high fimbria production, which facilitates the purification of fimbriae. All fimbriae used in this study were pure, as showed by SDS-polyacrylamide gel electrophoresis (Fig. 1). Polyclonal antisera raised against purified fimbriae reacted with the homologous fimbriae (Table 2) but also with other fimbriae. These results are in agreement with the results of Korhonen et al. (11), who made a set of antisera raised against purified fimbriae from parental strains. These fimbria preparations did contain different fimbriae, because the parental strains possessed different types of fimbria. Our fimbria preparations only possessed only type of fimbria, and the antisera raised against these fimbriae still showed numerous cross-reactions in the ELISA (Table 2). This result indicates that these fimbriae have various antigenic determinants in common.

With the same pure fimbria preparations we produced a set of MAb against F7₁, F7₂, F9, and F11 fimbriae. The MAb against F7₁ and F9 fimbriae only recognized epitopes on F7₁ and F9 fimbriae, respectively. It is not yet certain whether both series of MAb recognize the same or different epitopes within each series. Recent data from DNA sequencing of the fimbria genes coding for the Pap (1) and F7₂ (27) fimbriae indicate that there are several pronounced antigenic determinants on fimbriae. Thus, it might be possible that the M4 and M6 MAb recognize different epitopes on F9 and F7₁ fimbriae, respectively. The M2 MAb, raised against F7₂ fimbriae, could be divided into two groups: the M2-7 MAb, which only recognized an epitope on F7₂ fimbriae, and the M2-1 and M2-11 MAb, which recognized epitopes on F7₂ and F9 fimbriae. From these results, we conclude that M2-1 and M2-11 probably recognize the same epitope and that this epitope is only present on F7₂ and F9 fimbriae. This conclusion was supported by the spot test with denatured fimbriae. Both M2-1 and M2-11 recognized epitopes on denatured F7₂

and F9 fimbriae, in contrast to M2-7, which did not react with denatured fimbriae. The M7 MAb, raised against F11 fimbriae, could also be divided into two groups: two MAb (M7-5 and M7-15) which recognized epitopes on F11, F7₂, and Pap fimbriae, and a group of four MAb (M7-4, M7-6, M7-7, and M7-13) which recognized epitopes on F11 and Clegg fimbriae. This last group of MAb also recognized epitopes on denatured F11 and Clegg fimbriae, as shown in the spot test, in contrast to M7-5 and M7-15, which did not react with denatured fimbriae. From these results, we conclude that M7-5 and M7-15 probably recognize the same epitope and that this epitope is present in F11, F7₂, and Pap fimbriae. It is likely that M7-4, M7-6, M7-7, and M7-13 also recognize a common epitope on F11 and Clegg fimbriae but that this epitope is different from the one recognized by M7-5 and M7-15.

Most of the MAb did not react with denatured fimbriae. This is not surprising, because the mice were immunized with native fimbriae. The reaction of MAb M2-1, M2-11, M7-4, M7-6, M7-7, and M7-13 with denatured fimbriae suggests that the epitopes recognized by these MAb are not involved in a quaternary structure. In contrast, all polyclonal antisera tested so far have reacted strongly with denatured fimbriae (3, 3a). Furthermore, the MAb were far more specific than the polyclonal antisera. The most likely explanation for these differences between MAb and polyclonal antisera is that an MAb only recognizes one epitope, whereas a polyclonal antiserum recognizes many epitopes, some of which are also present in heterologous P and even type 1 fimbriae. This is supported by the finding that the combination of two different MAb raised against F11 fimbriae (e.g., M7-4 and M7-5) already recognized four different P fimbriae.

No inhibition of MRHA by MAb was found, in contrast to the complete inhibition by polyclonal antisera (Table 4). Recent data from Norgren et al. (16) and Lindberg et al. (13) indicated that the adhesion of Pap fimbriae is a minor component of these fimbriae. Since this is also the case for the F7₁, F7₂, F9, and F11 fimbriae (I. van Die, E. Zuidweg, W. Hoekstra, and H. Bergmans, FEMS Meeting on Molecular Biology of Microbial Pathogenicity, 17-20 June 1985, p. 18), it is not surprising that we were not able to select an MAb which inhibited MRHA. The probability of finding an MAb which recognizes an epitope involved in the adhesive properties of these fimbriae is very low when the adhesin is only a minor component of the fimbriae. Since the MAb described in this paper are far more specific than polyclonal antisera, these MAb may be useful for diagnostic and epidemiological means. Furthermore, the MAb can be very helpful in studies concerning antigenic variation and phase variation of P fimbriae.

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CHARACTERIZATION OF THE P FIMBRIAE F8 AND F12 FROM UROPATHOGENIC
ESCHERICHIA COLI WITH MONOCLONAL ANTIBODIES

INTRODUCTION

Adherence of uropathogenic Escherichia coli to uroepithelial cells is an important step in the pathogenesis of urinary tract infection (Hagberg et al., 1981; Van den Bosch et al., 1980). This adherence is mediated by fimbriae which can be divided into two groups: i fimbriae which causes a mannose sensitive haemagglutination (MSHA) of guinea pig erythrocytes; ii fimbriae that causes a mannose resistant haemagglutination (MRHA) of human erythrocytes. This last group can be subdivided into other groups on the basis of their receptor specificity. Especially the P fimbriae, which recognize the receptor structure α -D-Galp-(1-4)- β -D-Galp on uroepithelial cells (Leffler and Svanborg-Edén, 1980), have been associated with pyelonephritogenic strains (Väisänen et al., 1981). These P fimbriae were classified in different serotypes e.g. by Ørskov and Ørskov (1983) in F7-F12 and by Parry et al. (1982) in a-g. These P fimbriae were also heterogenous with regard to the molecular weights of their subunit proteins (Jann et al., 1981; de Ree et al., 1985).

Recently we described the production of monoclonal antibodies (MAbs) against the P fimbriae F7₁, F7₂, F9, F11 and F13 (=Pap) (de Ree et al., 1985b, 1986b). In this article we report the production and characterization of MAbs against F8 and F12 fimbriae. The F12 MAbs were raised against F12 fimbriae from the reference strain C1979 (Ørskov and Ørskov, 1983), whereas the F8 MAbs were raised against F8 fimbriae which were purified from a recombinant strain. The F12 MAbs were also compared with another F12 MAb which was raised against the F12 fimbriae from another wild type strain: 20025 (Jann et al., 1981). The differences between these F12 MAbs are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth media

HB101 is an E. coli K12 strain deficient in the production of type 1 fimbriae (Boyer and Roulland-Dussoix, 1969). F8 fimbriae were purified from the strain HB101 harboring pANN921, which was kindly provided by Jörg Hacker, and from the E. coli strain C1254. The genes encoding for F8 fimbriae were cloned from the wild type E. coli 2980 (Hacker et al., to be published) with serotype O18:K5:H- (Jann et al., 1981). F12 fimbriae were purified from the wild type strain C1979 with serotype O16:K1:H- (Ørskov and Ørskov, 1983). All the other P fimbriae used in this study were purified from strains harboring plasmids encoding for P fimbriae (de Ree et al., 1985). All bacteria were cultivated on brain heart infusion agar (Oxoid Ltd.). Selective pressure against the loss of plasmids was imposed by adding 10 µg tetracycline per ml. The C and SP strains were kindly provided by I. and F. Ørskov and S. Parry, respectively.

Purification of fimbriae

Fimbriae were purified by the method of Korhonen et al. (1980) avoiding protein-denaturing conditions (de Ree et al., 1985a). Purity of fimbriae preparations was checked by SDS-PAGE (Lugtenberg et al., 1975). Gels were stained with Coomassie Brilliant Blue.

Hybridoma production

Monoclonal antibodies against F8 and F12 fimbriae were produced as described recently (de Ree et al., 1985b). Briefly, BALB/c mice were injected intravenously several times with purified fimbriae. The spleen cells were fused with cells from the myeloma cell line SP2/0. After three weeks hybridomas were tested for anti-fimbriae production in an ELISA. Positive clones were subcloned by a limiting dilution step and grown up for antibody production in ascitic fluid. Antibodies were partially purified from ascitic fluid as described (de Ree et al., 1985b).

The production of the F12 MAb 20025-F2a, raised against the F12 fimbriae from strain 20025 will be described elsewhere (C. Abe et al.. Submitted for publication). The O:K serotype and the serotype of the fimbriae expressed by strain 20025 are the same as the strain C134 (Ørskov and Ørskov, 1983).

ELISA for anti-fimbrial antibody determination

An ELISA with purified fimbriae as antigen was carried out essentially as described (de Ree et al., 1985). Briefly microtiterplates were coated with fimbriae, followed by incubation with serial dilutions of MABs. Anti-fimbriae activity was detected colorimetrically after incubation with goat-anti-mouse antiserum, conjugated with peroxidase and addition of the substrate o-phenylenediamine. Absorbance at 495 nm was determined with a Titertek multiscan ELISA plate reader. Cross reaction of the MABs against other fimbriae was also measured in a fimbriae ELISA.

Whole bacteria ELISA

An ELISA with whole bacteria as antigen was carried out as described (de Ree et al., 1986a). Briefly, bacteria were coated to microtiterplates and preincubated with New Born Calf serum, followed by incubation of serial dilutions of MABs. Anti-fimbriae activity was measured as described above.

Class determination of MABs

Microtiterplates were coated with fimbriae and homologous MABs were added as described for the fimbriae ELISA. Goat anti-mouse IgG1, IgG2a, IgG2b or IgM was added as the second antibody. MAB classes were detected after the addition of rabbit anti-goat IgG (heavy and light chains) conjugated to peroxidase and the substrate o-phenylenediamine.

Mannose Resistant Haemagglutination and inhibition with MABs

MRHA and inhibition with MABs was determined as described (de Ree et al., 1985b).

RESULTS

F8 fimbriae were purified from C1254 and the strain HB101 carrying plasmid pANN921. Both fimbriae preparations were pure as judged by SDS-PAGE (Fig. 1, lane b, c) and showed the same mol. weight of 20.000 dalton. MABs were raised against the F8 fimbriae purified from the strain HB101 carrying pANN921. F12 fimbriae, purified from C1979, showed two bands in SDS-PAGE with both a mol. weight of approximately 18.000 dalton (Fig. 1, lane e).

All MABs were tested in a fimbriae ELISA to determine the titer against homologous and heterologous fimbriae. All F8 MABs reacted strongly with the

FIG. 1 SDS-PAGE of purified F8 and F12 fimbriae. Gels were stained with Coomassie Brilliant Blue.

A. Marker proteins.

B. F8 fimbriae purified from the strain HB101-pANN921.

C. F8 fimbriae purified from C1254.

D. Marker proteins.

E. F12 fimbriae purified from C1979.

homologous F8 fimbriae and the F8 fimbriae purified from C1254, and showed no cross-reaction with any other fimbriae (Table 1). The F12 MAbs reacted also strongly with the homologous F12 fimbriae. All F12 MAbs were from the IgM subclass type except for M14-11 and 20025-F2a which belonged to the IgG2b subclass type. The F8 MAbs also belonged almost all to the IgM subclass, except for M17-1 and M17-5 which were both from the IgG1 type (Table 1). To check whether these MAbs were able to determine the F8 and F12 fimbriae on

TABLE 1: Titer of MAbS in an ELISA with homologous and heterologous fimbriae and subclasses of antibodies.

MAb	Fimbriae that MAb was raised against	MAb subclass ^a	Antibody titer ^b to indicated fimbriae ^e		
			F8 ^c	F8 ^d	F12
M17-1	F8	IgG1	4,4	4,4	-
M17-2	F8	IgM	3,8	3,8	-
M17-3	F8	IgM	3,8	3,8	-
M17-4	F8	IgM	3,8	3,8	-
M17-5	F8	IgG1	3,8	3,8	-
M17-9	F8	IgM	3,8	3,8	-
M17-10	F8	IgM	3,8	3,5	-
M14-4	F12 (C1979)	IgM	-	-	4,1
M14-5	F12 (C1979)	IgM	-	-	4,1
M14-11	F12 (C1979)	IgG2b	-	-	4,1
M14-16	F12 (C1979)	IgM	-	-	4,1
M14-17	F12 (C1979)	IgM	-	-	4,1
20025-F2a	F12 (20025)	IgG2b	-	-	5,0

^a Determined as described in Materials and Methods.

^b Given as the logarithm of the last reciprocal dilution of the MAb giving an A490 of at least 0.50.

^c Purified from strain HB101 (pANN921).

^d Purified from wild type strain.

^e F7₁, F7₂, F9, F11, F13, 1C and 1A fimbriae were non-reactive with these monoclonals.

- no reaction.

wild type E. coli strains, all these MAbs were tested in a whole bacteria ELISA. Previously, we have described this method as a convenient and sensitive method for the determination of P fimbriae on wild type E. coli with the aid of MAbs (de Ree et al., 1986a). All F8 MAbs reacted strongly, as expected, with the F8 reference strain C1254 (Table 2). These F8 MAbs recognized also the fimbriae on the wild type strain SP7. The F12 MAbs, raised against the F12 fimbriae from C1979, reacted with the F12 reference strain C1979 but not with C134. Strain C134 expresses F12 fimbriae and also F13, Fy and 1C fimbriae. The F12 MAb 20025-F2a, which was raised against the F12 fimbriae from 20025 (=C134), reacted strongly with C134 and also with C1979. This F12 MAb recognized also the fimbriae on the strains SP57 and SP88.

The three wild type strains C1254, C1979 and C134 all showed MRHA of human erythrocytes. In a HA inhibition test not one of the homologous MAbs could inhibit this MRHA.

DISCUSSION

Recently we described the production of MAbs against the P fimbriae F7₁, F7₂, F9, F11 (de Ree et al., 1985b) and F13 (= Pap) (de Ree et al., 1986b). In this report we describe the production of MAbs against two other P fimbriae: F8 and F12. The F8 fimbriae were purified from the strain HB101 carrying pANN921, which was cloned from the wild type strain 2980 (Hacker et al., to be published). This strain belongs to serotype O18:K5 (Jann et al., 1981). The reference strain C1254 for F8 fimbriae from Ørskov and Ørskov (1983) belongs to the O75:K- serotype. F8 fimbriae purified from strain C1254 and the strain HB101 carrying pANN921 showed one band with the same mol. weight in SDS-PAGE (Fig. 1, lane b, c). From this SDS-PAGE the mol. weight was determined as 20.000 dalton. Monoclonal antibodies (MAbs) were raised against the F8 fimbriae purified from the recombinant strain. These MAbs all reacted strongly in an ELISA with the F8 fimbriae from the strain HB101-pANN921 and with the F8 fimbriae from C1254 (Table 1). No cross reaction was found with any other fimbriae tested in this fimbriae ELISA. From these results we conclude that these MAbs recognize an antigenic determinant which is only present on F8 fimbriae. However, it is not certain whether these F8 MAbs recognize the same or different epitopes. Recent data from DNA sequencing of various P fimbriae subunits (Baga et al., 1984, van Die and Bergmans, 1984) and the use of synthetic peptides to identify the immunogenic

epitopes of the Pap fimbriae (Schmidt et al., 1984) revealed that there are several pronounced antigenic determinants on P fimbriae. So, it is possible that the F8 MABs recognize different epitopes on F8 fimbriae.

MABs against F12 fimbriae were raised against the F12 fimbriae purified from the wild type strain C1979. This fimbriae preparation showed two bands in SDS-PAGE with both a mol. weight of approximately 18.000 dalton (Fig. 1, lane e). The mol. weight of this F12 fimbriae preparation is in agreement with the mol. weight of 18.200 dalton of F12 fimbriae calculated by Klemm et al. (1983) on the basis of the amino acid composition of the subunit. However, the F12 fimbriae preparation of Klemm et al. (1983) showed only one band in SDS-PAGE. These F12 fimbriae were, just like our F12, purified from the wild type strain C1979, but they used another purification method. They grew, just like us, the C1979 strain on solid medium to suppress any potential capacity for producing type 1 fimbriae. So, the extra band in our F12 preparation is not caused by type 1 fimbriae, but probably caused by the different purification method used by us. Our F12 fimbriae preparation was also checked for type 1 fimbriae contamination in a fimbriae ELISA with anti type 1 fimbriae MABs. With this method we were not able to demonstrate any contamination with type 1 fimbriae (data not shown).

The presence of two bands in the F12 fimbriae preparations is not surprising. Klemm et al. (1983) and Ørskov and Ørskov (1983) suggested already that the F12 fimbriae from C1979 contain two highly homologous fimbrial proteins, because crossed immuno electrophoresis showed that the F12 fimbriae precipitates in two lines positioned very close to each other. They were not defined as two different fimbriae, since it was not possible to separate them as two different antigens by application of antiserum in an intermediate gel in the crossed immuno electrophoresis system (Ørskov and Ørskov, 1983).

By Western blotting and immunoprecipitation test we tried to find out to which of the two F12 bands our MABs were reactive. However, the F12 MABs precipitated the F12 fimbriae very badly for unknown reasons (data not shown). Western blotting experiments also did not reveal to which of the two F12 bands the MABs were directed. The negative reaction of the MABs, raised against the F12 fimbriae from strain C1979, in Western blotting was not surprising because we demonstrated recently that almost all MABs which we produced against the various P fimbriae only recognized an epitope on native fimbriae and not on denatured fimbriae (de Ree et al., 1985b).

TABLE 2: Reaction of MAbs with wild type *Escherichia coli* strains in an ELISA with whole bacteria

Strain	Serotype	Fimbriae/ antigenic determinant	F8 MAbs						F12 MAbs					
			M17-1	M17-2	M17-3	M17-4	M17-5	M17-9	M17-10	20025	M14-4	M14-5	M14-11	M14-16
														-F2a
C1254	O75:K-	F8	9 ^a	8	8	8	8	8	8	-	-	-	-	-
C1979	O16:K1	F12	-	-	-	-	-	-	-	8	8	8	8	5
C134	O4:K12	Fy, F13, F12, 1C	-	-	-	-	-	-	-	8	-	-	-	-
SP7	O18:K5	c,d,f	2	3	5	3	1	4	2	-	-	-	-	-
SP57	O157:K-	a,b	-	-	-	-	-	-	-	9	-	-	-	-
SP88	O157:K-	a,b	-	-	-	-	-	-	-	9	-	-	-	-

^a 1 unit = .29 absorbance at 490 nm.

- no reaction.

Also from the whole bacteria ELISA it was demonstrated that strain C1979 expressed two F12 fimbriae. The F12 MAb raised against the F12 fimbriae from C1979 reacted only with the fimbriae on C1979, whereas the F12 MAb 20025-F2a which was raised against the F12 fimbriae from 20025 (C134), reacted with the F12 fimbriae on C134 and also with the F12 fimbriae on C1979. So it is possible that the MABs raised against the F12 fimbriae from C1979 recognize one type of F12 fimbriae, whereas the F12 MAB which was raised against the F12 fimbriae from 20025 (=C134) recognize another type of F12 fimbriae.

The F12 MAb 20025-F2a raised against the F12 fimbriae from 20025 reacted also strongly with the fimbriae of the strains SP57 and SP83, both with the serotype O157:K-. All the F8 MABs recognized in the whole bacteria ELISA the fimbriae on the SP7 strain with the serotype O18:K5. The F8 fimbriae are almost in all cases associated with the O18 or the O75 serotype (Ørskov and Ørskov, 1983).

The HA inhibition assay revealed that the epitopes recognized by the F8 and F12 MABs are not involved in adhesion, because not one of the homologous MABs did inhibit the MRHA of the strains C1979 or HB101 carrying plasmid pANN921. This means that these MABs recognize an epitope on the fimbriae which is not involved in adhesion to uroepithelial cells.

With these F8 and F12 MABs we have extended our MAB collection against the serologically different P fimbriae of uropathogenic E. coli. These MABs will be used for epidemiological studies on the distribution of P fimbriae among uropathogenic E. coli.

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MONOCLONAL ANTIBODIES RAISED AGAINST PAP FIMBRIAE RECOGNIZE
MINOR COMPONENT(S) INVOLVED IN RECEPTOR BINDING

INTRODUCTION

The adherence of uropathogenic bacteria to uroepithelial cells is an important virulence factor. Adherence is mediated by fimbriae, also called pili. These fimbriae are heterogenous serologically (1, 2) as well as in the molecular weight of their subunit proteins (3, 4). According to their receptor structure, uropathogenic Escherichia coli fimbriae have been classified by their ability to show a mannose sensitive (MS) or a mannose resistant haemagglutination (MRHA) of erythrocytes. The group of MR fimbriae can be subdivided into four groups: i the P fimbriae (also called Gal-Gal fimbriae (5, 6)), ii the M fimbriae (7), iii the S fimbriae (8) and iv the X fimbriae. The receptor structure of the first three groups is cleared up whereas this structure is unknown for the X fimbriae.

Among uropathogenic E. coli especially the P fimbriae have been associated with pyelonephritis (9). These P fimbriae function by binding epithelial glycosphingolipids of the urinary tract containing the globoside α -D-Galp-(1-4)- β -D-Galp, which is the minimal receptor structure (5).

The genes encoding for various P fimbriae have recently been cloned (10-17), as well as for the MS (10, 18) and X fimbriae (19). The best studied gene cluster is the one which encodes for Pap fimbriae (10, 20-23). Analysis of the gene organization of this gene cluster showed that at least eight polypeptides were involved in the biogenesis of globoside binding Pap fimbriae (20). Mutation experiments demonstrated that the papA gene encodes for the fimbrillin subunit and that a mutation in the papA gene does not abolish the globoside binding (22). The papF and papG gene products are both required for globoside binding of whole bacteria and for HA by purified fimbriae (22). The papE gene product plays probably a role in the anchoring of the adhesin

at the fimbriae (22), whereas the papC and papD gene products presumably are involved in the surface localisation and the polymerization of the fimbriae subunits and expression of fimbriae and globoside binding (23).

Fimbriae preparations from a cloned strain with an intact whole gene cluster for Pap fimbriae and globoside binding still show these binding properties (22, 24). Recently, Lindberg et al. (25) have shown that purified Pap fimbriae contain the proteins encoded by papE and papF. Antisera against purified Pap fimbriae contained also PapE and PapF specific antibodies. It was also demonstrated for the gene clusters of other P fimbriae, pPIL110-75 (F7₁), pPIL110-37 (F7₂), pPIL288-10 (F9), pPIL291-15 (F11) (26) and for pDC1 (Clegg fimbriae) (27), that the fimbriae subunit is not required for the expression of the globoside specific adhesin. Also for the MS fimbriae (28) and for the X fimbriae (19) it was demonstrated that fimbriae expression and adhesion could be separated genetically.

Previously, we have described the production of monoclonal antibodies (MAbs) against the P fimbriae F7₁, F7₂, F9 and F11 (4). In this study we describe the production of MAbs against Pap fimbriae. These fimbriae were recently also classified as F13 fimbriae (27). Here we demonstrate that some MAbs recognize Pap fimbriae but other MAbs recognize one of the minor components which are responsible for globoside binding.

MATERIALS AND METHODS

Bacterial strains

The E. coli strain J96 (O4:K5) is a human pyelonephritis isolate and expresses simultaneously type 1 and P fimbriae (Pap=F13) (10). The construction of the strains HB101 harboring pRHU845, pPAP5, or pPAP23 is described by Normark et al. (20) and Lindberg et al. (22). On the plasmids pRHU845 and pPAP5 the whole gene cluster is present for expression of Pap fimbriae and globoside-specific binding. Plasmid pPAP23 contains also the whole gene cluster, but is mutated into the papA gene, and encodes for digalactoside specific binding but not for Pap fimbriae. All these strains and plasmids were kindly provided by Björn Lund. The strains AM1727 harboring pPIL110-37 or pPIL291-15 encoding for galactoside binding and F7₂ and F11 fimbriae, respectively, were recently described (13, 15). Plasmid pPIL291-152 contains also the whole gene cluster except that this plasmid is deleted into the F11 fimbrillin gene (26). This plasmid still encodes for globoside binding. The

plasmids pPIL110-37 and pPIL291-152 were kindly provided by Irma van Die. AM1727 (13) and HB101 (33) are E. coli K12 strains deficient in the production of type 1 fimbriae.

All bacteria were cultivated on brain heart infusion agar (Oxoid Ltd.). Selective pressure against loss of plasmids was imposed by adding 50 µg/ml ampicillin or 12,5 µg/ml tetracycline, dependent on the cloning vector used.

Purification of fimbriae

Fimbriae were purified by the method of Korhonen et al. (34) avoiding protein denaturing conditions (14). Protein concentrations were measured by the method of Lowry et al. (35). Purity of fimbriae preparations was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (36). Gels were loaded with 30 µg of fimbriae and stained with Coomassie Brilliant Blue. Pap fimbriae were purified from strain AM1727 harboring pRHU845. The F7₁, F7₂, F9, F11, Clegg, 1A and 1C fimbriae were all purified from strains with cloned fimbriae (4), whereas the F8 and F12 fimbriae were purified from the wild type strains C1254 (075:K-) and C1979 (016:K1) (1), respectively.

Hybridoma production

Monoclonal antibodies (MAbs) against Pap fimbriae were produced as described recently (4). Briefly, a BALB/c mouse was injected intravenously several times with Pap fimbriae. The spleen cells were fused with cells from the nonproducing myeloma cell line SP2/0. After 3 weeks hybridoma cells were tested for anti fimbriae production in an enzyme-linked immunosorbent assay (ELISA). Positive clones were subcloned by a limiting dilution step and grown up for antibody production in ascitic fluid. Antibodies were partially purified from ascitic fluid as described (4).

The MAbs M7-5 and M7-15, raised against F11 fimbriae previously, react with the fimbriae F11, F7₂ and Pap (4).

ELISA for anti fimbrial antibody determination

An ELISA with purified cloned fimbriae was carried out essentially as described (4). Briefly, microtiterplates (polyvinylchloride, Flow) were coated with purified fimbriae, followed by incubation with serial dilutions of MAbs. Anti-fimbriae activity was detected colorimetrically after incubation with goat anti-mouse antiserum, conjugated with peroxidase (Nordic)

and addition of the substrate o-phenylenediamine (Sigma). Absorbance at 495 nm was determined with a Titertek multiscan ELISA plate reader.

Whole bacteria ELISA

An ELISA with whole bacteria as antigen was carried out as recently described (32). Briefly, bacteria were coated to microtiterplates and preincubated with New Born Calf Serum (Gibco Laboratories), followed by an incubation with serial dilutions of MAbs. Anti-fimbriae activity was detected as described above.

Class determination of MAbs

Microtiterplates were coated with fimbriae and homologous MAbs were added as described for the fimbriae ELISA. Goat anti-mouse IgG1, IgG2a, IgG2b or IgM (Nordic) was added as the second antibody. MAb classes were detected after the addition of rabbit anti-goat IgG (heavy and light chains) conjugated to peroxidase (Nordic).

Adherence to human uroepithelial cell line T24

The human uroepithelial cell line T24 was kindly supplied by Björn Lund. Adherence experiments were carried out essentially as described by B. Uhlin et al. (23). Briefly, non confluent layers of T24 cells in plastic petri dishes were obtained by culturing the cells for 24 h. Bacteria (2×10^8 per dish) were added and allowed to attach for 1 h at 37°C. After this incubation T24 cells were washed with saline (5x) and stained with 30% filtered Giemsa stain (Merck). Adherence was examined with a light microscope and expressed as the percentage of T24 cells with at least 25 adherent bacteria out of a total of 100 cells counted.

MRHA assays

The MRHA activity of bacterial strains with human erythrocytes and the inhibition of HA were determined by the method of Van den Bosch et al. (37). Briefly, serial dilutions of bacteria were incubated with human erythrocytes for 2 h on ice. HA was read after agitation. The inhibition of HA was tested with bacteria preincubated for 30 min at room temperature with MAbs. Antibody dilutions which did not cause agglutination of bacteria, as checked by microscopy, were used.

Electron microscopy and immuno electron microscopy

Electron microscopy was carried out as described by Van den Bosch et al. (38). Bacteria were grown overnight and resuspended in PBS containing 0,25% formalin. After 1 h fixation a drop of the bacterial suspension was placed on a Formvar coated copper grid and allowed to stand for 15 min. Excess suspension was drained off, and the adherent bacteria were negatively stained with 1% phosphotungstic acid pH 7.0 for 15 s.

Visualisation of immune complexes was performed as follows. A drop of a bacterial suspension was placed on a grid and allowed to dry. The grid was incubated with a suitable dilution of a MAb for 1 h and thoroughly washed with a saline solution containing 0,5% bovine serum albumin and 0,1% gelatine. Subsequently the grids were incubated with gold labeled protein A for 1 h. The grids were extensively washed and negatively stained with 1% phosphotungstic acid. The protein A labeled with the gold particles was kindly supplied by Nico Riegman.

Spottest for the reaction of MAbs with denatured fimbriae

A spottest for the reaction of the MAbs with denatured fimbriae was carried out as described (4). Briefly, fimbriae were denatured by boiling in a buffer containing beta-mercaptoethanol and SDS. Denatured fimbriae were spotted on nitrocellulose paper and incubated with the MAbs. Immune complexes were detected by incubation with goat anti-mouse peroxidase conjugate and diaminobenzide as substrate. The reaction was developed in the dark and terminated with H_2SO_4 .

RESULTS

Mab production and characterization

Pap fimbriae were purified from strain AM1727 harboring pRHU845 and were shown to be pure in SDS-PAGE (4). Purified Pap fimbriae were used for MAb production as described. Three weeks after the fusion of spleen cells and SP2/0 myeloma cells, the hybridomas with the highest titer in a fimbriae ELISA were selected for additional growth. After subcloning the hybridomas were grown up and used for the production of ascitic fluid. MAbs were partially purified from these ascitic fluids and tested in a fimbriae ELISA for (cross)-reactions with various purified fimbriae. The titers of the MAbs against homologous and heterologous fimbriae are shown in Table 1. With

TABLE 1: Titer of MAbS in an ELISA with different purified fimbriae and subclasses of antibodies

MAb	Fimbriae that MAb was raised against	Immunoglobulin Subclass	Antibody titer ^a to fimbriae ^b		
			Pap	F7 ₂	F11
M8-6	Pap	IgG1	3.5	4.1	3.2
M8-16 ^c	Pap	IgM	5.0	0	0
M7-5	F11 ^d	IgG1	3.8	4.1	4.1
M7-15	F11 ^d	IgG1	3.5	4.7	4.1

^a Given as the logarithm of the last reciprocal dilution of the antiserum given an A490 of at least 0.50.

^b F7₁, F8, F9, F12, Clegg, 1A and 1C fimbriae were nonreactive.

^c Four other MABs showed an identical reaction.

^d Reference 4.

regard to the M8 MABs raised against Pap fimbriae, only the M8-6 MAB reacted with an epitope on Pap, F7₂ and F11 fimbriae. Five other MABs which were raised against Pap fimbriae only recognized an epitope on Pap fimbriae. The M7-5 and M7-15 MABs, which were raised against F11 previously (4), showed the same cross reaction as the M8-6 MAB in the fimbriae ELISA. All M8 MABs were of the IgM subclass except M8-6. This MAB belongs, just like the M7-5 and M7-15 MAB, to the IgG1 subclass.

In an ELISA with whole bacteria as an antigen, all M8 and the M7-5 and M7-15 MABs reacted with the strains J96 and HB101 harboring pPAP5. (Table 2). The M8-6, M7-5 and M7-15 MABs did also react with the strain AM1727 harboring pPIL110-37 or pPIL291-15 encoding for digalactoside binding and F7₂ or F11 fimbriae, respectively. To find out whether these M8, M7-5 and M7-15 MABs did recognize the fimbriae or the minor components on the fimbriae responsible for the digalactoside binding, these MABs were also tested in the whole bacteria ELISA with the strains HB101-pPAP23 and AM1727-pPIL291-152. Both strains encode for digalactoside binding but do not express Pap or F11 fimbriae, respectively. Surprisingly the M8-6, M7-5 and M7-15 MABs reacted

TABLE 2: Titer^a of monoclonal antibodies in a whole bacteria ELISA

Strain	Clone/strain encoding for		Monoclonal antibody			
	fimbriae	digalactoside binding	M8-6	M8-16 ^b	M7-5	M7-15
J96	Pap	+	3.8	5.0	3.8	4.4
HB101-pPAP5	Pap	+	4.7	>5.0	5.0	5.0
HB101-pPAP23	-	+	5.0	0	5.0	5.0
HB101	-	-	0	0	0	0
AM1727-pPIL110-37	F7 ₂	+	>5.0	0	>5.0	>5.0
AM1727-pPIL291-15	F11	+	>5.0	0	>5.0	>5.0
AM1727-pPIL291-152	-	+	3.2	0	3.5	3.2
AM1727	-	-	0	0	0	0

^a See footnote ^a in Table 1.

^b See footnote ^c in Table 1.

strongly with these strains (Table 2). This suggests that these MABs recognize one of the minor components responsible for digalactoside binding. The control strains HB101 and AM1727 did not react with any MAB in this whole bacteria ELISA.

The M8 MABs were also tested for their ability to recognize an epitope on denatured fimbriae. In a spottest all M8 MABs did not react with the denatured Pap fimbriae, whereas they were reactive with native Pap fimbriae. Western blotting experiments also showed that not one of the M8 MABs did react with denatured fimbriae (data not shown).

HA and Adherence inhibition assays

The MRHA titer of the strains HB101 harboring pPAP23 and AM1727 harboring pPIL291-152 was totally inhibited in the presence of the M8-6, M7-5 or M7-15 MABs (Table 3). The M8-16 MAB did not inhibit the MRHA of these strains.

TABLE 3: Adherence to T24 cells and MRHA with human erythrocytes of the strains HB101-pPAP23 and AM1727-pPIL291-152, and inhibition by monoclonal antibodies

Strain	-	MRHA titer			
		In the presence of			
		M8-6	M7-5	M7-15	M8-16
HB101-pPAP23	32	0	0	0	32
AM1727-pPIL291-152	16	0	0	0	16
HB101	0	-	-	-	-
AM1727	0	-	-	-	-

Strain	-	Adherence to T24 uroepithelial cells ^a			
		In the presence of			
		M8-6	M7-5	M7-15	M8-16
HB101-pPAP23	79 ± 7 ^b	6 ± 3	6 ± 1	10 ± 9	88 ± 11
AM1727-pPIL291-152	85 ± 7	29 ± 7	30 ± 9	6 ± 3	85 ± 3
HB101	0	-	-	-	-
AM1727	0	-	-	-	-

^a Adherence was determined in the presence of 1% D-mannose and expressed as the percentage of T24 cells with at least 25 adherent bacteria out of a total of 100 cells counted.

^b Figures in the adherence assay are presented as mean values ± standard deviation.

These results corresponded with the results of the adherence assay using the urinary epithelial cell line T24. The adherence of the strains HB101 harboring pPAP23 and AM1727 harboring pPIL291-152 was also strongly inhibited in the presence of the MAbs M8-6, M7-5 and M7-15, whereas M8-16 did not show any inhibition in this assay (Table 3).

Fig. 1: Immuno electron microscopy with MAbs and HB101 with pPAP5 or pPAP23.
a. HB101 harboring pPAP5 with M8-16
b. HB101 harboring pPAP5 with M8-6
c. HB101 harboring pPAP5 with M7-6, a MAb specific for F11 fimbriae
fimbriae (4)

Fig. 1: Immuno electron microscopy with MAbs and HB101 with pPAP5 or pPAP23.
d. HB101 harboring pPAP23 with M8-6
e. HB101 harboring pPAP23 with M8-16
f. HB101 with M8-6

Electronmicroscopy

With immuno electron microscopy it was demonstrated that the M8-16 MAb strongly reacted with the fimbriae of the strain HB101 harboring pPAP5 (Fig. 1a). All the other M8-MABs, except M8-6, showed the same reaction in immuno electronmicroscopy. The M8-6, M7-5 and M7-15 reacted again similarly. These three MABs did not react with the fimbriae but they reacted with components which were found between the fimbriae (Fig. 1b). As a negative control an irrelevant antibody (M7-6 which is F11 fimbriae specific) was added to HB101 harboring pPAP5. No reaction of this antibody could be detected (Fig. 1c). The M8-6, M7-5 and M7-15 MABs also showed a reaction with a component present on the surface of the strain HB101 harboring pPAP23 (Fig. 1d), whereas no such reaction with this strain was found with the other M8-MABs (Fig. 1e). The M8-6 MAB showed also no reaction with the strain HB101 (Fig. 1f).

DISCUSSION

In this study we described the production of monoclonal antibodies (MABs) against Pap fimbriae which were purified from strain AM1727 harboring pRHU845. Five MABs raised against Pap fimbriae recognized only an epitope on Pap fimbriae. However, one MAB (M8-6) recognized also an epitope on F7₂ and F11 fimbriae. An identical reaction as for M8-6 was previously described for the F11 MABs M7-5 and M7-15, which also recognized F7₂, F11 and Pap fimbriae (4). Thus it is very likely that these MABs recognize an identical epitope which is present on F7₂, F11 and Pap fimbriae.

In a whole bacteria ELISA all M8 MABs did react with the strains J96, HB101 harboring pPAP5 and AM1727 harboring pRHU845 (Table 2). Surprisingly, the M8-6, M7-5 and M7-15 MABs also reacted strongly with the strain HB101 harboring pPAP23 and also with AM1727 harboring pPIL291-152 (Table 2). These last two strains do not express Pap or F11 fimbriae, respectively, but still encode for globoside binding (22, 26). pPAP23 was constructed by a frameshift mutation into the papA structural gene, whereas pPIL291-152 was constructed by a deletion of pPIL291-15 lacking the gene encoding for the fimbrillin subunit. This means that these MABs recognize an epitope on the minor components of the fimbriae which are responsible for globoside binding. This is supported by the finding that the MABs M8-6, M7-5 and M7-15 inhibited completely the MRHA of the strains HB101-pPAP23 and AM1727-pPIL291-152 as well as the adherence to T24 uroepithelial cells (Table 3). The Pap fimbriae

specific MAb M8-16, however, did not show any inhibition of MRHA and adherence. Furthermore, the immuno electron microscopy results demonstrated that the three MABs M8-6, M7-5 and M7-15 reacted with a surface component on the strain HB101 harboring pPAP23 (Fig. 1d) and not with the fimbriae on strain HB101 harboring pPAP5 (Fig. 1b). The specific and strong reaction of the other M8-MABs with the Pap fimbriae on strain HB101 harboring pPAP5 (Fig. 1a) was also different from the reaction of the M8-6 MAB with this strain. From these results we conclude that the M8-6, M7-5 and M7-15 MABs react with a minor component responsible for adhesion, whereas the other M8 MABs react with the Pap fimbriae. These results are in agreement with the results of Lindberg et al. (25) who demonstrated that PapE and PapF are surface exposed on a papA mutant.

Lund et al. have compared the genes which encode for the minor adhesive components, from the Pap, F7₂ and pDC5 gene cluster (27). They showed that the papE and papF genes were conserved in these three gene clusters. The papG variant in the F7₂ and pDC5 gene clusters were very similar, but unique for the Pap gene cluster. pDC5 is a subclone from pDC1 and encodes only for globoside binding. pDC1 encodes for Clegg fimbriae (11) and for globoside binding. Recently we have shown that Clegg and F11 fimbriae are serological identical and that they have the same mol weight (4). The Clegg fimbriae were also typed as F11 in cross-immunoelectrophoresis by I. and F. Ørskov (personal communication). The similarities between the F11 and Clegg fimbriae would suggest that the two gene clusters have much homology. However, the MABs M8-6, M7-5 and M7-15 did not recognize an epitope on the Clegg fimbriae. So probably the minor components of Clegg and F11 fimbriae are different.

Gonococcal fimbriae undergo an extensive antigenic variation to circumvent the immune response (29). The variation in these fimbriae probably occurs in a domain which is different from the region required for polymerization and receptor-specific interaction (30,31). All the MABs against P fimbriae which were isolated by us were produced by screening for the highest anti fimbriae titer in an ELISA. Almost all MABs were specific for the fimbriae which they were raised against (4, 32, this study), but the adhesin specific MABs showed a cross reaction with three different fimbriae. This suggests that the most pronounced antigenic structures on P fimbriae undergo an extensive antigenic variation, like for gonococcal fimbriae, whereas the minor components responsible for adhesion have a more conservative structure.

The present results and the results from the genetic studies on the minor components of fimbriae from Lund et al. (27) indicate that the development of a possible vaccine has to be concentrated on the minor adhesive components rather than on the fimbriae component. The MAbs which recognize one of the minor components, as shown in this study, could be helpful for the purification and characterization of these proteins.

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MONOCLONAL ANTIBODIES FOR SEROTYPING THE P FIMBRIAE OF UROPATHOGENIC *ESCHERICHIA COLI*

The ability of uropathogenic *Escherichia coli* to adhere to uroepithelial cells is an important virulence factor (15, 17, 18), enabling these bacteria to circumvent the flushing action of urine and to infect the urinary tract. Adhesion is mediated by fimbriae which bind to specific cell surface receptor molecules. The majority of these uropathogenic *E. coli* strains express P fimbriae (16), which recognize as a receptor structure the disaccharide α -D-Galp-(1-4)- β -D-Galp (10) on the uroepithelial cells. These P fimbriae differ in the molecular weights of their subunit proteins (7) and in serological properties (12, 13). Ørskov and Ørskov (12) could distinguish eight serologically different fimbriae on uropathogenic *E. coli* by using crossed-immunoelectrophoresis techniques. They suggested that these fimbriae be referred to as F antigens (F7 to F12). This typing scheme of P fimbriae was recently extended by the classification of pyelonephritis-associated pilus (Pap) fimbriae as F13 fimbriae (11). Parry et al. (13) were able to determine seven different antigenic determinants on fimbriae of uropathogenic *E. coli* by using a slide agglutination test and designated them a to g.

In both studies (12, 13) cross-absorbed polyclonal antisera were used. Recently, we described the production of monoclonal antibodies (MAbs) against the four different P fimbriae F7₁, F7₂, F9, and F11 (4). The main purpose of this study was to develop a rapid and easy method, with the aid of the MAbs, for the detection of serologically different P fimbriae on wild-type *E. coli* strains. Therefore, we compared three different methods: a plate agglutination test, a coagglutination test with *Staphylococcus aureus* as a carrier for the MAbs, and an enzyme-linked immunosorbent assay (ELISA) with whole bacteria as an antigen. A comparison of these three methods showed that the whole-bacterium ELISA was the best method for the screening of P fimbriae on uropathogenic *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. The C, SP, and J96 strains were kindly provided by I. Ørskov and F. Ørskov, Steve Parry, and Björn Lund respectively. The SP strains were serotyped by P. A. M. Guinee (State Institute for Public Health, Bilthoven, The Netherlands). Plasmids pPIL110-70 and pPIL110-37 were kindly provided by Irma van Die, plasmid pRHU845 was provided by Staffan Normark, plasmid pDC1 was provided by Steven Clegg, and plasmid pANN921 was provided by Jörg Hacker. All the fimbriae encoded by these plasmids are P fimbriae. AM1727 is a *recA* derivative of JE2571, an *E. coli* K-12 strain deficient in the production of type 1 fimbriae (20). All wild-type strains were cultivated on blood agar plate (Oxoid Ltd.), and cloned strains were cultivated on brain heart infusion agar (Oxoid Ltd.). Selective pressure against the loss of plasmids was imposed by adding 50 μ g α -ampicillin, 100 μ g of chloramphenicol, or 12.5 μ g of tetracycline per ml, depending on the cloning vector used.

MAbs against the P fimbriae F7₁, F7₂, F8, F9, F11, F12, and F13. The production of MAbs against the P fimbriae F7₁, F7₂, F9, and F11 was recently described (4). Briefly, spleen cells of immunized BALB/c mice were fused with the myeloma cell line SP2/0, and the resulting hybridomas were grown for 3 weeks. Antibody production against fimbriae was tested in an ELISA, and positive clones were subcloned by a limiting dilution step. After additional growth, the cells were used for antibody production in ascitic fluid. MAbs were partially purified from ascitic fluid as described previously (4). For this study we used various MAbs against different P fimbriae, selected on the basis of specificity and immunoglobulin subclass. The characteristics of the MAbs used are shown in Table 2. The production of MAbs against purified F8, F12, and F13 (Pap) fimbriae will be described elsewhere (J. M. de Ree and J. F. van den Bosch, submitted for publication). F8 fimbriae were purified from the cloned strain AM1727 (pANN921), F12 fimbriae were purified from the wild-type strain C1979, and F13 fimbriae were purified

TABLE 1. Bacterial strains used in this study

Bacterial strain	Serotype	Fimbriae or antigenic determinants	Reference
Wild type			
C1212 (AD110)	O6:K2:H1	F7 ₁ , F7 ₂ , and 12 IC	12
C1254	O75:K ⁻ :H5	F8	12
C1018	O2:K5:H4	F9	12
C1976	O1:K1:H7	F11	12
C1979	O16:K1:H ⁻	F12	12
C134	O4:K12:H5	Fy, F12, F13, and 1C	12
SP133	O14:?	c and e	13
SP7	O18:K5	c, d, and f	13
SP57	O157:K ⁻	a and b	13
SP201	Nontypable	g and e	13
SP144	O6:K13	f and g	13
SP88	O157:K ⁻	a and b	13
SP101	O1:K1	b, c, and e	13
J96	O4:K6:H5	F13 (Pap)	6
Cloned			
AM1727(pPIL110-70)		F7 ₁	19
AM1727(pPIL110-37)		F7 ₂	20
AM1727(pANN921)		F8	Hacker, in preparation
AM1727(pPIL288-10)		F9	2
AM1727(pPIL291-15)		F11	3
AM1727(pRHU845)		F13 (Pap)	6
AM1727(pDC1)		Clegg	1

from the cloned strain AM1727 (pRHU845). The immunoglobulin subclasses of the MABs were determined with an ELISA as described previously (4).

Plate agglutination test. Bacterial agglutination assays were performed essentially as described for O-antigen typing by Guinee et al. (5). Bacteria were grown for 16 h and suspended in phosphate-buffered saline (PBS) with 0.25% Formalin to an optical density at 660 nm of 0.7. Bacteria were stained by adding a 0.05% gentian violet solution in PBS with 0.25% Formalin. Serial dilutions of the MABs were made in 100 µl of PBS with 0.25% Formalin in microtiter plates (Thovadec). To each well, 100 µl of stained bacteria was added. Agglutination was read after overnight incubation at 4°C.

Coagglutination. *S. aureus* Cowan 1 NCTC 8530 was kindly provided by Loek van Alphen. These bacteria were grown for 16 h on Columbia agar plates. After three washings with PBS, the bacteria were suspended in PBS with 0.5% Formalin and incubated for 3 h at room temperature. Subsequently, the bacteria were washed four times with PBS, incubated for 10 min at 80°C, and washed two times with PBS. Finally, a 10% (vol/vol) suspension was made in PBS with 0.01% sodium azide. This suspension was stored at 4°C.

Immunoglobulin G (IgG) MABs were coated to protein A of the *S. aureus* suspension as described by Kromwall (9). Briefly, 50 µl of undiluted MAB was incubated with 0.5 ml of the *S. aureus* suspension for 10 min at room temperature. Agglutination assays were performed on glass slides by mixing 1 drop of a bacterial suspension in PBS and 1 drop of an MAB-coated *S. aureus* suspension. Agglutination was scored within 1 min.

Whole-bacterium ELISA. Bacteria cultivated overnight were suspended in 50 mM bicarbonate coating buffer (pH 9.6) to an optical density of 1.0 at 660 nm and diluted eight times in coating buffer. Microtiter plates (activated polyvinyl chloride; Flow Laboratories, Inc.) were coated with 100 µl of the diluted bacterial suspension per well and dried overnight at 37°C. On the next day, the microtiter plates were washed four times with PBS containing 0.05% Tween 20 (PBST). After 1 h of incubation at room temperature with 15% newborn calf serum (GIBCO Laboratories) in PBST (NBST), the microtiter plates were washed four times with PBST and then incubated with 100 µl of 1:100-diluted MAB in NBST for 2 h at room temperature. Subsequently, the wells were washed four times with PBST and incubated for 3 h at room temperature with 100 µl of goat anti-mouse IgG (heavy and light chains) conjugated to peroxidase (Nordic Laboratories); the conjugate was diluted 1:1,000 in NBST. Antifimbria antibody activity was detected colorimetrically by adding 0.05% *o*-phenylenediamine in citrate-phosphate buffer (pH 4.5) containing 0.005% H₂O₂. The reaction was developed in the dark for 15 min at room temperature and terminated with 2 M H₂SO₄. The A₄₉₂ was measured in a Titertek Multiskan (Flow Laboratories, Inc.). Wells filled with H₂O were used as blanks.

RESULTS

The reaction of the MABs raised against the P fimbriae F7₁, F7₂, F9, and F11 was tested in the plate agglutination assay with strains expressing homologous fimbriae. All MABs reacted with the fimbriae on the homologous *E. coli* AM1727 strains expressing cloned fimbriae (Table 3). Strain AM1727 (pPIL288-10) showed autoagglutination with this method. Only MAB M6-8 reacted with the fimbriae on the wild-type strain C1212. None of the other MABs recognized the fimbriae on the homologous wild-type strains with this plate agglutination assay.

The MABs were also tested in a slide agglutination test (data not shown), and none of them reacted with the homologous wild-type strains. Subsequently, we tried to improve this agglutination by coating the MABs to protein A of an *S. aureus* suspension as described above. With this coagglutination test only the IgG MABs raised against the F7₁, F7₂, and F11 fimbriae were tested. In this assay all the MABs recognized the fimbriae on the homologous cloned strains (Table 4). MAB M7-6 also reacted with the fimbriae on the heterologous cloned strain, AM1727 (pDC1). MABs M6-3 and M2-1 also agglutinated the wild-type strain C1212, which expresses homologous fimbriae. MAB M7-6 did not agglutinate the homologous wild-type strain C1976. However, this

TABLE 2. MABs used in this study

MAB	Immunoglobulin subclass	Fimbriae that MAB was raised against	Fimbriae recognized by MAB in a fimbria ELISA
M6-3	IgG2a	F7 ₁	F7 ₁ ^a
M6-8	IgM	F7 ₁	F7 ₁ ^a
M2-1	IgG2b	F7 ₂	F7 ₂ and F9 ^a
M17-9	IgM	F8	F8 ^b
M4-7	IgM	F9	F9 ^a
M7-6	IgG1	F11	F11 and Clegg ^a
M14-5	IgM	F12	F12 ^b
M8-16	IgM	F13 (Pap)	F13 (Pap) ^b

^a See reference 4.

^b de Ree and van den Bosch, submitted.

TABLE 3. Titers of MABs in a plate agglutination test against homologous wild-type and cloned *E. coli* strains

Bacterial strain	Fimbriae	Titer* of			
		F7 ₁ MAB M6-8	F7 ₂ MAB M2-1	F9 MAB M4-7	F11 MAB M7-6
C1212	F7 ₁ , F7 ₂ , and 1C	40	0		
C1018	F9			0	
C1976	F11				0
AM1727(pPIL110-70)	F7 ₁	5,120			
AM1727(pPIL110-37)	F7 ₂		10,240		
AM1727(pPIL288-10)	F9			AA ^b	
AM1727(pPIL291-15)	F11				>40,000

* Expressed as the dilution of MAB which still showed agglutination.

^b AA, Autoagglutination.

MAB did react with the fimbriae on the wild-type strains SP133 and SP101.

All the MABs described in Table 2 were tested in an ELISA with whole bacteria as an antigen. In this ELISA all the MABs reacted with the fimbriae on the homologous cloned strains (Table 5). The F11 MAB M7-6 also reacted with the fimbriae on the heterologous cloned strain AM1727 (pDC1). All the MABs reacted with the fimbriae on the homologous wild-type strains, except for the F11 MAB M7-6, which did not react with the F11 fimbriae on the wild-type strain C1976. However, this F11 MAB did react with the fimbriae on the wild-type strains SP133 and SP101. MAB M14-5, raised against purified F12 fimbriae from the wild-type strain C1979, also reacted with the fimbriae on the wild-type strain C1979. In contrast, the F12 fimbriae on strain C134 were not recognized by MAB M14-5.

MAB M17-9, raised against purified F8 fimbriae from the cloned strain AM1727 (pANN921), reacted with the fimbriae on the wild-type strains C1254 and SP7.

DISCUSSION

In this paper we tested three different methods for the determination of serologically different P fimbriae on clinical

TABLE 4. Coagglutination of MABs with wild-type and cloned strains

Bacterial strain	Coagglutination* of:		
	F7 ₁ MAB M6-3	F7 ₂ MAB M2-1	F11 MAB M7-6
C1212	+	++	-
C1254	-	-	-
C1018	-	-	-
C1976	-	-	-
C1979	-	-	-
SP133	-	-	++
SP101	-	-	++
AM1727(pPIL110-70)	+	-	-
AM1727(pPIL110-37)	-	+++	-
AM1727(pANN921)	-	-	-
AM1727(pPIL288-10)	-	-	-
AM1727(pPIL291-15)	-	-	+++
AM1727(pRHU845)	-	-	-
AM1727(pDC1)	-	-	+++

* Agglutination was read within 1 min: -, no agglutination; +, weak agglutination; ++, agglutination; +++, strong agglutination.

TABLE 5. Reaction of MABs with wild-type and cloned *E. coli* strains in an ELISA with whole bacteria as an antigen

Bacterial strain	Reaction* of:						
	F7 ₁	F7 ₂	F8	F9	F11	F12	F13
	MAb	MAb	MAb	MAb	MAb	MAb	(Pap) MAb
	M6-8	M2-1	M17-9	M4-7	M7-6	M14-5	M8-16
C1212	8	8					
C1254			8				
C1018				6			
C1976							
C1979						9	
C134							5
SP7			4				
SP133					8		
SP101					7		
J96							8
AM1727(pPIL110-70)	9						
AM1727(pPIL110-37)		9					
AM1727(pANN921)			8				
AM1727(pPIL288-10)				9			
AM1727(pPIL291-15)					9		
AM1727(pRHU845)							9
AM1727(pDC1)					9		

* Expressed as A_{490} units; 1 unit equals an A_{490} of 0.29.

isolates of *E. coli*. The classification of these P fimbriae was recently described by Ørskov and Ørskov (12). They used polyclonal antisera raised against whole cell cultures which had been made more fimbria specific by absorption with the homologous nonfimbriated strain grown at 20°C. The disadvantage of polyclonal antisera against P fimbriae is that these antisera are not specific for each type of P fimbria, as was shown by Korhonen et al. (8) and de Ree et al. (4) in ELISA studies. Nevertheless, Ørskov and Ørskov (12) were able to use these polyclonal antisera in crossed immunoelectrophoresis and crossed-line immunoelectrophoresis as very sensitive methods to discriminate between serologically different fimbriae. With these methods they were able to demonstrate seven serologically different fimbriae (F7 to F12) on clinical isolates. However, crossed-immunoelectrophoresis technique for F-antigen determination is cumbersome and can only be carried out in highly specialized laboratories. The main purpose of this study was to develop a simple and fast method that uses the recently produced specific MABs to determine the serologically different P fimbriae on clinical isolates of *E. coli*.

The first method to be tested was a plate agglutination assay. All the MABs agglutinated the homologous cloned strains, except for AM1727 (pPIL288-10) (Table 3). This strain showed autoagglutination, which was probably caused by the extremely high fimbria expression of this cloned strain (2). Only MAB M6-8 weakly agglutinated the homologous wild-type strain, whereas the other MABs did not show any reaction with the wild-type strains. This result can be explained by the lower number of fimbriae present on wild-type strains than on cloned strains, a fact which was recently demonstrated for the F9 and F11 wild-type and cloned strains by electron microscopy (2, 3). Furthermore, in a slide agglutination test the MABs did not agglutinate the wild-type strains. From these results, we concluded that direct agglutination is not sensitive enough for screening the

P fimbriae on wild-type strains. Therefore, we tried to amplify agglutination by coating the MABs to protein A of an *S. aureus* suspension. A disadvantage of this method is that only IgG MABs could be used. Because all the F9 MABs were of the IgM subclass (4), these MABs could not be used in this method. The other MABs tested in this assay agglutinated the homologous cloned strains, and the F11 MAB even agglutinated strain AM1727 (pDC1) (Table 4). In contrast to the results of the plate agglutination assay, almost all the MABs agglutinated homologous wild-type strains in the coagglutination assay. The only exception was the F11 MAB M7-6, which did not agglutinate the homologous wild-type strain C1976, probably because of the very low number of F11 fimbriae on this wild-type strain (3). However, the F11 MAB did agglutinate some other wild-type strains. From these results, we concluded that the coagglutination test is more sensitive than the plate agglutination test but still has the disadvantage that only IgG MABs can be used. Another drawback of this method is the difficulty of reading the results in an objective way.

The last method to be tested was a whole-bacterium ELISA. During the course of this study, more MABs were available than in the former two assays. Since these two assays were found to be unsatisfactory for our purposes, we did not test the more recently obtained MABs in the agglutination assays, but we did test the whole series of available MABs in the ELISA. All the MABs tested in this method reacted strongly with the fimbriae on the homologous cloned strains (Table 5). The F11 MAB M7-6 recognized the Clegg fimbriae on strain AM1727 (pDC1), as was also shown with the coagglutination assay (Table 4). From these results, we concluded that Clegg fimbriae are probably identical to F11 fimbriae. All the MABs also recognized the fimbriae on the homologous wild-type strains with this method, except, again, for the F11 MAB M7-6. However, the F11 MAB M7-6 did detect the fimbriae on the wild-type strains SP133 and SP101. So, this MAB could still be useful for the determination of F11 fimbriae on wild-type strains. The reaction of the F11 MAB M7-6 in the coagglutination assay and the whole-bacterium ELISA with strains SP133 and SP101 strongly suggested that these strains express F11 fimbriae. Similarly, as in the coagglutination test, the negative reaction of the F11 MAB M7-6 with the fimbriae on the homologous wild-type strain C1976 was probably caused by the very low number of F11 fimbriae on this strain (3).

The recombinant strain AM1727 (pANN921) codes for F8 fimbriae, which were cloned by J. Hacker (manuscript in preparation) from the wild-type strain 2980 (serotype O18:K5:H⁻) described by Jann et al. (7). MAB M17-9, which was raised against the purified F8 fimbriae from the recombinant strain, reacted as expected with the wild-type F8 reference strain C1254 and also with strain SP7. So, the latter strain also expresses F8 fimbriae.

The F12 MAB M14-5 recognized the F12 fimbriae on the homologous wild-type strain C1979 but not the F12 fimbriae on the wild-type strain C134. This result could mean that the F12 fimbriae on strain C134 are expressed in very low numbers or that these F12 fimbriae are different from the F12 fimbriae on strain C1979. This last explanation would mean that there are two kinds of F12 fimbria. This possibility has already been suggested by the crossed-immuno-electrophoresis results for strain C1979 of Ørskov and Ørskov (12). Further studies are required before F12 fimbriae can be distinguished into F12₁ and F12₂ fimbriae.

From the results of the whole-bacterium ELISA we concluded that this method is the best one for the detection of P

fimbriae on wild-type *E. coli* strains. This whole-bacterium ELISA is not more sensitive than the coagglutination method but has two major advantages: (i) all the immunoglobulins can be used in the ELISA, whereas in the coagglutination test only IgG MABs can be used; (ii) the results of the ELISA can be read in an objective way, in contrast to the results of the coagglutination test.

In a fimbria ELISA we have demonstrated that MAB M2-1 reacted with F7₁ and F9 fimbriae (4; see Table 2). However, the reaction with the F9 fimbriae could neither be shown in the coagglutination test nor in the whole-bacterium ELISA. This result was probably caused by the weak reaction of MAB M2-1 with the F9 fimbriae.

Only the fimbriae of three SP strains (SP7, SP133, and SP101) could be determined with the MABs. All SP strains were, just like the reference strains for fimbriae F7 to F13, isolated from urinary tract infections, and all expressed P fimbriae (14). However, the serotype of some SP strains (e.g., SP57 and SP88, O157:K⁻) is not common among uropathogenic strains (17), and so it is possible that these strains also express rare fimbriae. Preliminary data on a limited number of our collection of uropathogenic *E. coli* strains showed that we could determine the P fimbriae on 80% of the strains with the MABs used in this study. So, fimbriae F7 to F13 are probably the most common fimbriae among uropathogenic *E. coli*.

With the whole-bacterium ELISA and the MABs against the various P fimbriae we have developed an easy and fast method for the determination of P fimbriae among uropathogenic *E. coli*. With this method we have planned to do epidemiological studies on the distribution of P fimbriae among wild-type *E. coli* strains and to extend the O:K:H serotype with the F serotype.

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*FIMBRIAE SEROTYPES OF ESCHERICHIA COLI STRAINS ISOLATED
FROM EXTRA INTESTINAL INFECTIONS*

INTRODUCTION

Escherichia coli is a pathogen in urinary tract infection and meningitis. A variety of factors e.g. O:K antigens, adhesion properties and hemolysin production may influence the virulence of pathogenic E. coli (1, 2). The O:K:H serotypes of these E. coli strains belong in most cases to restricted well defined groups of serotypes (3, 4). Thus, each O:K:H serotype has a characteristic pathogenic capacity.

The presence of P fimbriae on uropathogenic E. coli has been associated with virulence (5). With the aid of these fimbriae E. coli can colonize the urinary tract. These fimbriae on E. coli appear to be related to the strains ability to agglutinate erythrocytes and to attach to epithelial cells. Two types of hemagglutination can be distinguished. i. a mannose sensitive hemagglutination (MSHA) correlated with type 1 fimbriae and ii. a mannose resistant hemagglutination (MRHA). Fimbriae which cause a MRHA can be divided in P (6), M (7) and S (8) fimbriae, based on the receptor specificity. Among uropathogenic E. coli it is generally accepted that especially the MR fimbriae which belong to the P class play an important role in the pathogenesis of pyelonephritis (5).

The P fimbriae differ in serotype (9) and the mol. weight of the fimbriae subunit (10). Until now, eight different P fimbriae have been described. These P fimbriae were characterized with polyclonal antisera in a crossed-immuno-electrophoresis system and were numbered F7 - F13 (4, 9). There are only a few studies which have investigated the presence of a specific P fimbriae serotype in correlation with the O:K serotype (9,11,12). In this study we have determined the P fimbriae serotype of E. coli, isolated from urinary tract infections and meningitis, with specific anti-fimbriae mono-

clonal antibodies (MAbs). These MAbs were recently developed against purified P fimbriae (13, 14). The correlation between O:K serotype and the fimbriae serotype will be discussed.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study firstly were MRHA strains of previously described collections (AD...,ZO...) *E.coli* (15,16,17). Secondly, strains were isolated from patients with urosepsis (NS...). This collection of 49 strains was obtained from bacteriological laboratories in The Netherlands, isolated from 1983-1985. The average age of the patients was 67 $\pm$ 16 years, whereas 65% of the patients were females.

28 strains isolated from children with meningitis (MA...) were kindly provided by Dr. L. van Alphen (Medical Microbiology, University of Amsterdam, The Netherlands), and described before (18).

All these strains were examined for the presence of MR fimbriae and the production of hemolysin. Strains which showed a MRHA were further investigated on O:K serotype, the expression of P fimbriae and the serotype of the P fimbriae.

Hemagglutination activity

The MRHA with human erythrocytes was determined as described (2). For the MRHA assay bacteria were grown on blood agar plates (Oxoid Ltd.) Cells were suspended in saline and serial dilutions of bacterial cells were added to the erythrocyte suspension. For testing mannose sensitivity the erythrocyte suspension contained 1% D-mannose. Agglutination was read after incubation for 2 h on ice.

The P receptor test

Fimbriae of the clinical isolates were tested for their ability to recognize the globoside structure α -D-Galp-(1-4)- β -D-Galp. Bacteria grown on blood agar plates were mixed on a glass slide with latex beads which were coated with the globoside structure (19). Latex beads devoid of the globoside structure were used as a control. If P fimbriae were present on the wild type strains macroscopic agglutination was apparent within 1 minute.

Hemolytic activity assay

Hemolysis was tested on washed blood agar plates as described by Van den Bosch (20). Sheep blood was washed three times with saline and added to blood agar base nr. 2 (Oxoid Ltd.) to a final concentration of 5%. Hemolysis was scored after overnight incubation.

O:K serotype

Serotyping of O and K antigens was performed as described (15) by W.H. Jansen and P.A.M. Guinée (National Institute of Public Health, Bilthoven, The Netherlands).

Monoclonal antibodies against P fimbriae

The production of MABs against the P fimbriae F7₁, F7₂, F8, F9, F11, F12 and F13 was recently described (13, 14). All fimbriae were purified from strains which expressed cloned fimbriae except the F12 fimbriae which were purified from the wild type reference strain C1979. In Table 1 the MABs used in this study are listed, selected on specificity. The MABs 20025-F1 and 20025-F2a were raised against the Fy and F12 fimbriae, respectively, from the strain 20025 (C. Abe et al., submitted for publication). These latter two MABs were kindly provided by K. Jann (Max-Planck-Institut für Immunbiologie, Freiburg, West Germany). Recently, we have shown that the MABs M14-5 and 20025-F2a recognize different epitopes on F12 fimbriae and we suggested that there are probably two serotypes of F12 fimbriae (J.M. de Ree et al., submitted for publication). The F12 fimbriae which are recognized by the MAB 20025-F2a are referred as F12 related fimbriae.

TABLE 1: Monoclonal antibodies used in this study^a

MAB	Fimbriae serotype	Parental strain	Serotype
M6-8	F7 ₁	AD110=C1212	06:K2:H1
M2-1	F7 ₂	AD110=C1212	06:K2:H1
M17-9	F8	2980	018:K5:H-
M4-7	F9	C1018	02:K5:H4
M7-6	F11	C1976	01:K1:H7
M14-5	F12	C1979	016:K1:H-
20025-F2a	F12 related	20025	04:K12:H-
M8-16	F13	J96	04:K6:H5
20025-F1	Fy	20025	04:K12:H-

^a *All monoclonal antibodies were raised against purified fimbriae isolated from strains harboring plasmids encoding for P fimbriae (13, 14). Only the F12, F12 related, and Fy fimbriae were purified from wild type strains.*

Whole bacteria ELISA for screening of P fimbriae on wild type E. coli strains

A whole bacteria ELISA was carried out as described previously (14). Briefly, bacteria were suspended in bicarbonate coating buffer and coated on microtiterplates. These microtiterplates were washed several times with saline and preincubated with New Born Calf Serum, followed by an incubation with the 1:100 diluted MAbs. Anti-fimbriae activity was detected colorimetrically after incubation with goat anti-mouse antiserum, conjugated with peroxidase and addition of the substrate o-phenylenediamine. Absorbance at 495 nm was determined with a Titertek multiscan ELISA plate reader. Negative controls were treated in the same way except that the bacterial strains were incubated with saline, instead of MAbs.

Bacterial strains were supposed to express a certain kind of P fimbriae when the value of the absorbance was three times higher than the negative control value.

RESULTS

MRHA and P agglutination

All strains which exhibited MRHA are listed in Table 2, according to their O serotype. The percentage of MRHA expressing strains in the urosepsis (NS) and meningitis (MA) collection was 39% and 32%, respectively. All the MRHA strains listed in Table 2 expressed P fimbriae except strain NS26.

O:K antigen

The O groups 1, 2, 4, 6, 7, 18, 25 and 75 comprised 82% of the 33 MRHA positive UTI strains. A minority of these MRHA positive UTI strains belonged to the O serotype O14, O21, O57, O77, O83 and O128.

Three out of nine MRHA positive meningitis strains belonged to the O7 serotype and two strains of this group expressed the O1 serotype.

Among the MRHA positive UTI strains and the MRHA positive meningitis strains 26% and 77%, respectively, expressed the K1 serotype.

Production of hemolysin

Hemolysin was expressed by 52% of the MRHA positive UTI strains, whereas only 1 out of 9 (11%) MRHA positive meningitis strains expressed hemolysin. Remarkably all O4, O6 and O18 strains in Table 2 expressed hemolysin.

Serotype of fimbriae

With the aid of the MAbs we could determine the fimbriae of 24 out of 33 (73%) of the MRHA positive UTI strains, and on 6 out of 9 (67%) of the MRHA positive meningitis strains.

As shown in Table 2 many strains expressed multiple fimbriae serotypes. The most common F types of the MRHA positive UTI strains were the F11, F7₁ and F8 fimbriae, which were expressed by 9 (27%), 7 (21%) and 6 (18%) strains, respectively. The other P fimbriae serotypes were almost equally distributed among the MRHA positive UTI strains: F7₂ fimbriae were found on 3 (9%) strains; F9 fimbriae on 1 (3%) strain; F12 fimbriae on 3 (9%) strains; F12 related fimbriae on 3 (9%) strains; F13 fimbriae on 1 (3%) strain and the Fy fimbriae on 4 (12%) strains. Among the MRHA positive meningitis strains 3 (33%) expressed F11 fimbriae; F8 fimbriae were expressed by 2 (22%) strains and F9 fimbriae by 1 (11%) strain, whereas the fimbriae on three strains

TABLE 2: Characteristics of E. coli strains from extraintestinal infections

Strain	Serotype	Fimbriae type	MRHA	P	Hly	Source
NS2	O1:K1	F11	+	+	-	UTI
NS24	O1:K1	F7 ₁ , F11	+	+	-	UTI
NS50	O1:K1	F9	+	+	-	UTI
AD312	O1:K1	F7 ₁ , F11	+	+	-	UTI
MA4	O1:K1	F9	+	+	-	Menin
MA17	O1:K1	F11	+	+	-	Menin
NS11	O2:K1	F11	+	+	-	UTI
NS41	O2:K7	F7 ₁	+	+	-	UTI
NS10	O2:K+	F11	+	+	-	UTI
NS30	O2:K+	F8	+	+	-	UTI
NS25	O4:K12	F11, F12 rel	+	+	+	UTI
AD314	O4:K12	F11, F12 rel	+	+	+	UTI
AD111	O6:K2	F7 ₁ , F7 ₂ , Fy	+	+	+	UTI
AD115	O6:K2	F7 ₁ , F7 ₂ , Fy	+	+	+	UTL
NS22	O6:K+		+	+	+	UTI
NS46	O6:K13	F12	+	+	+	UTI
NS28	O7:K1		+	+	-	UTI
NS47	O7:K1		+	+	-	UTI
MA2	O7:K1		+	+	-	Menin
MA6	O7:K1		+	+	-	Menin
MA13	O7:K1		+	+	-	Menin

TABLE 2: continued

Strain	Serotype	Fimbriae type	MRHA	P	Hly	Source
NS3	O14:K+	F7 ₁ , F13, Fy	+	+	+	UTI
NS14	O18:K5	F8	+	+	+	UTI
NS23	O18:K5	F8	+	+	+	UTI
NS43	O18:K+	F8	+	+	+	UTI
AD119	O18:K+	F8	+	+	+	UTI
AD123	O18:K-	F12	+	+	+	UTI
AD325	O21:K-		+	+	-	UTI
NS48	O25:K+		+	+	+	UTI
AD303	O25:K-	F11, F12 rel	+	+	+	UTI
AD313	O25:K+		+	+	-	UTI
AD316	O25:K+		+	+	-	UTI
MA8	O25:K-	F8	+	+	+	Menin
AD317	O57:K+		+	+	-	UTI
MA1	O57:K1	F11	+	+	-	Menin
NS7	O75:K+	F8	+	+	-	UTI
AD340	O75:K+		+	+	+	UTI
MA12	O75:K+	F8	+	+	-	Menin
AD309	O77:K-	F7 ₁ , F11, F12	+	+	+	UTI
NS26	O83:K24	F7 ₂ , Fy	+	-	+	UTI
AD308	O128:(K67)		+	+	-	UTI
MA18	O _{auto} :K1	F11	+	+	-	Menin

could not be typed with the MAbs.

Expression of certain F types was not restricted to certain O:K serotypes. F7₁ fimbriae were found on 01:K1, 02:K7, 06:K2, 014:K+, and 077:K- strains; F7₂ fimbriae were found on 06:K2 and 083:K24 strains; F8 fimbriae were found on 02:K+, 018:K5, 018:K+, 025:K-, and 075:K+ strains; F9 fimbriae were found on 01:K1 strains; F11 fimbriae were found on 01:K1, 02:K1, 02:K+, 04:K12, 025:K-, 057:K1, 077:K-, and Oauto:K1 strains; F12 fimbriae were found on 06:K13, 018:K-, and 077:K- strains; F12 related fimbriae on 04:K12 and 025:K- strains; F13 fimbriae were found on a 014:K+ strain, and the Fy fimbriae were found on 06:K2, 014:K+, and 083:K24 strains.

It was striking that 01:K1 strains expressed either F11 or F9, or F7₁ and F11 fimbriae, that F8 fimbriae were relatively frequently expressed by 018 and 075 strains, that both 04:K12 and both 06:K2 strains showed an identical fimbriae phenotype, respectively, and that the fimbriae serotype of all 07:K1 strains could not be determined. Comparing urinary and meningitis strains with identical O:K serotype no systematic difference was found in fimbriae phenotype e.g. NS50-MA4 (01:K1:F9); NS2-MA17 (01:K1:F11); NS7-MA12 (075:K1:F8); and the 07:K1:F? strains.

DISCUSSION

Virulence of E. coli in human extra intestinal infections has been associated with various properties e.g. O and K antigens, hemolytic activity, and the presence of fimbriae for adherence to epithelial cells. In the present study the P fimbriae were serotyped from MRHA positive UTI and MRHA positive meningitis strains. Among the UTI strains the NS collection was made recently whereas the AD and ZO collection were previously described (15-17). Of the E. coli strains of the NS collection, isolated from mainly elderly people with urosepsis, 39% expressed MRHA. From these MRHA positive strains all, except one, expressed P fimbriae. The incidence of P fimbriae on E. coli has been studied intensively in strains implicated in acute pyelonephritis in children (5, 21). It was demonstrated that there was a strong correlation between possession of P fimbriae in acute pyelonephritis (+ 90%) compared with acute cystitis (+ 19%), and asymptomatic bacteriuria (+ 14%) in children. However, it is also important to consider host susceptibility when assessing the importance of adhesins. The effect of host factors was clearly demonstrated in patients with recurrent pyelonephritis without reflux, where 74% of

strains had adhesive capacity as indicated by MRHA, compared with only 38% in patients with reflux (22). The low percentage (38%) in the collection of NS strains which expressed MRHA, compared with the P fimbriae expressing strains isolated from acute pyelonephritis in children can be explained by the host susceptibility of the elderly patients in the NS collection.

In the collection of meningitis strains (MA) 32% of the strains expressed P fimbriae. This figure is in agreement with the results of Korhonen et al. (23) who found that 38% of the E. coli strains isolated from meningitis expressed P fimbriae.

In the present study we serotyped the P fimbriae with MAbS. With the aid of the MAbS we could determine the P fimbriae on 30 out of 42 (71%) MRHA positive UTI and meningitis strains. Many strains expressed multiple P fimbriae serotypes. The F11, F7₁ and F8 fimbriae were the most common P fimbriae among the MRHA positive UTI strains, whereas the other serotypes were equally distributed. Among the MRHA positive meningitis strains the P fimbriae with serotype F11, F8 and F9 were found.

In our study the expression of certain F types was not correlated with certain O:K serotypes. Other studies showed that there is a correlation between the O:K serotype and the F serotype. These studies demonstrated that 01:K1 was correlated with F9 or F11 fimbriae (12); 02:K1 was correlated with F11 fimbriae (4); 04:K12 was correlated with F11 and F12 related fimbriae (4); 06:K2 was correlated with F7 fimbriae (4) and the 018 serotype with F8 fimbriae (11). It has been suggested that these correlations result from a common clonal evolution of strains with certain antigenic characters (3,24). All the strains with the above described correlation between O:K serotype and F serotype were also found in our study. However, we found also strains with these O:K serotype which expressed other F serotypes, e.g. 01:K1 with F7₁ and F11 fimbriae; 018 with F12 fimbriae; 025:K- with F8 fimbriae.

In our study not one of the 07:K1 strains could be determined with the MAbS. The 07:K1 serotype was correlated with the F10 fimbriae (4), a fimbriae type which cannot be detected with our MAbS (Table 1). This could mean that the 07:K1 strains in our collection also express F10 fimbriae.

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SEROLOGICAL RESPONSE TO THE P FIMBRIAE OF
UROPATHOGENIC *ESCHERICHIA COLI* IN PYELONEPHRITIS.

INTRODUCTION

The ability of uropathogenic *Escherichia coli* to adhere to uroepithelial cells is a prerequisite for urinary tract infections (UTI) (20, 25, 26). The mechanism of adherence has been investigated intensively during the last years. Many investigators support the concept that adherence is mediated by fimbriae, which attach to specific receptors on host cells. The fimbriae of uropathogenic *E. coli* can be divided into common or type 1 fimbriae that cause a mannose sensitive hemagglutination (MSHA) of guinea pig erythrocytes, and fimbriae that cause a mannose resistant hemagglutination (MRHA) of human erythrocytes. The MRHA fimbriae can be subdivided into P (13), M (24) and S (12) fimbriae on the basis of their receptor specificity. The P fimbriae recognize the disaccharide α -D-Galp-(1-4)- β -D-Galp as a receptor (13) on the uroepithelial cells and it has been reported that the urinary virulence of *E. coli* causing pyelonephritis is mediated by these P fimbriae (5, 23). P fimbriae have been demonstrated in 90 per cent of *E. coli* strains isolated from children with acute, nonobstructive pyelonephritis (10, 20), whereas these P fimbriae are progressively less common in strains isolated from cystitis, asymptomatic bacteriuria and faeces (6).

Recently, the role of fimbriae as a virulence factor of uropathogenic *E. coli* was brought up for discussion by Harber et al. (8). They demonstrated that *E. coli* strains freshly isolated from the urine were not fimbriated and also nonadhesive. From their results they concluded that fimbriae mediated adherence is not involved in the pathogenesis of UTI once organisms have entered the urinary tract (7, 8). However, these results could also be explained by the selection of patients with urinary tract defects, a group recently shown to have a low percentage of adhesive isolates (19). Further-

more, the bacteria which express fimbriae will be attached to epithelial cells and in consequence this adhesive capacity would ensure that free bacteria in the urine are likely to be non-fimbriate (15). In view of the doubts between the fimbrial and non-fimbrial adhesion hypothesis we have investigated whether the P fimbriae of uropathogenic E. coli are synthesized in vivo or not. Therefore, the P fimbriae of E. coli strains isolated from patients with pyelonephritis were serotyped with specific monoclonal antibodies (MAbs) (3, 4). Subsequently the serum of these patients was analyzed for the presence of antibodies against homologous and heterologous fimbriae. In all sera antibodies against P fimbriae were present, strongly suggesting that these fimbriae are indeed produced in vivo and not merely a laboratory phenomenon. However, these sera did not contain adherence inhibiting antibodies.

PATIENTS, MATERIALS AND METHODS

Patients, bacterial strains, and sera.

E. coli strains were isolated from mid-stream urine specimens obtained from four female patients with complaints pointing at pyelonephritis, i.e. loin pain, fever ($>39^{\circ}\text{C}$) with chills and nausea. All urine specimens yielded pure growth of $>10^5$ colony forming units/ml. Patient 24 (79 years old), patient 25 (23 years) and patient 41 (73 years) had urosepsis, since the same E. coli strain was isolated from urine and blood, as judged by similarity of biotype, antibiotic sensitivity pattern and O:K serotype. Patient 50 (9 years) had a scarred kidney on subsequent radiological examination. Immediately after isolation all strains were stored at -70°C in broth with 30% glycerol. For laboratory tests the strains were grown on blood agar plates (Oxoid). Hemolysis was tested on washed-blood agar plates. Serotyping of O and K antigens was performed by P.A.M. Guinée (National Institute of Public Health, Bilthoven, The Netherlands).

Serum specimens were collected from all patients at 6-10 days after the onset of complaints. Control serum consisted of pooled serum from several females without present complaints or history of UTI.

Hemagglutination and Hemagglutination inhibition assay.

The MRHA activity of the E. coli strains with human erythrocytes was determined by the method of van den Bosch et al. (25). Briefly serial dilutions of bacteria were incubated with human erythrocytes in the presence of

1% mannose for 2 h on ice and HA was read after agitation. The inhibition of MRHA was tested with bacteria preincubated for 30 min. at room temperature with homologous patient serum or polyclonal rabbit anti serum. Polyclonal rabbit antisera raised in rabbits against various purified cloned fimbriae were recently characterized in a fimbriae ELISA (3). Serum dilutions were used which did not cause agglutination of bacteria, as checked by microscopy.

The P-receptor test.

The fimbriae of the clinical isolates were tested for their ability to recognize the P receptor α -D-Galp(1-4)- β -D-Galp. Bacteria grown on blood agar plates were mixed on a glass slide with latex beads coated with the globoside receptor structure. Latex beads devoid of the globoside structure were used as a control. If P fimbriae were present on the clinical isolates macroscopic agglutination was apparent within 1 minute. Latex beads coated with the P receptor were kindly supplied by Björn Lund, University of Umeå, Sweden.

Determination of the fimbriae serotype by monoclonal antibodies.

Recently we developed a set of specific monoclonal antibodies (MAbs) against the serological different P fimbriae (F7-F13) from uropathogenic E. coli (3, 4). The P fimbriae on the isolated E. coli strains were serotyped with the aid of the MAbs in a whole bacteria ELISA as described (4). Briefly, microtiterplates were coated with bacterial suspensions by drying overnight at 37°C, washed with saline containing Tween 20 and blocked with 15% New Born Calf serum in saline. After thorough washing the microtiterplates were incubated with 1:100 diluted MAb for 2 h at room temperature. Subsequently the wells were washed and incubated with goat-anti-mouse-IgG (H+L) conjugated to peroxidase (Nordic) for 3 h at room temperature. The presence of certain fimbriae was detected colorimetrically by adding 0.05% O-phenyldiamine in citrate/phosphate buffer with 0.005% H₂O₂. The reaction was developed in the dark for 15 minutes at room temperature and terminated with 2M H₂SO₄. The A₄₉₂ was measured in a Titertek Multiscan (Flow).

Anti-fimbriae antibody titers in serum.

Antibodies against P fimbriae in the serum of the patients were detected with a fimbriae ELISA. All fimbriae were purified from strains expressing cloned P fimbriae except the F12 fimbriae which were purified from the wild type F12 reference strain C1212. The characteristics of the strains, encoding

for the serologically different cloned P fimbriae, are presented in Table 1. All fimbriae were purified by the method of Korhonen et al. (11) avoiding protein denaturing conditions (1).

The fimbriae ELISA was performed as described (3). Briefly microtiterplates were coated with purified fimbriae, followed by incubation with serial dilutions of serum. Antifimbriae activity was detected colorimetrically after incubation with goat-anti-human immunoglobulins (H+L) conjugated with peroxidase (Nordic) and addition of the substrate o-phenylenediamine. Absorbance at 495 nm was determined with a Titertek Multiscan.

RESULTS

The E. coli strains isolated from the four patients were characterized by their O:K serotype, hemolysin production, the MRHA titer and the ability to recognize the P receptor (Table 2). Two strains belonged to the O1:K1 serotype, one to O4:K12 and one to O2:K7. All four strains showed MRHA and expressed P fimbriae. Only one strain produced hemolysin. The F serotype of the P fimbriae of these four strains was determined by a whole bacteria ELISA using specific MAbS for P fimbriae. The fimbriae of strain NS24 were recogni-

TABLE 1: Strains encoding for different P fimbriae, and monoclonal antibodies which recognize specific P fimbriae.

Strain	Fimbriae	MAbs ^a
AM1727 (pPIL110-70) (24) ^b	F7 ₁	M6-8
AM1727 (pPIL110-37) (25)	F7 ₂	M2-1
AM1727 (pANN921) (C)	F8	M17-9
AM1727 (pPIL288-10) (18)	F9	M4-7
AM1727 (pPIL291-15) (26)	F11	M7-6
AM1727 (pRHU845) (27)	F13	M8-16
C1979 (28)	F12	M14-5

^a See references 14, 15,

^b References in parenthesis

^c Hacker et al. in press.

TABLE 2: Characteristics of E. coli strains isolated from pyelonephritis patients.

Strain	Serotype	Hly	MRHA	P-test	Positive reaction with MAb	Fimbriae serotype
NS24	O1:K1	-	+	+	M6-8, M7-6	F7 ₁ , F11
NS25	O4:K12	+	+	+	M7-6	F11
NS41	O2:K7	-	+	+	M6-8	F7 ₁
NS50	O1:K1	-	+	+	M4-7	F9

TABLE 3: Titer of patient sera against homologous^a and heterologous P fimbriae.

Serum of Patient	Antibody titer ^b to indicated fimbriae						
	F7 ₁	F7 ₂	F8	F9	F11	F12	F13
24	2.3	2.6	2.9	2.3	3.2	2.9	2.6
25	2.0	2.3	2.6	2.3	2.0	2.3	2.0
41	3.8	3.2	2.9	>3.8	>3.8	3.5	2.9
50	2.6	2.9	2.9	3.2	3.2	3.2	2.6
Control	<1	<1	<1	<1	<1	<1	<1

^a Titer against homologous fimbriae are encircled.

^b Given as the logarithm of the last reciprocal dilution of the antiserum giving an A₄₉₀ of at least 0.50.

zed by two different MAb's (M6-8 and M7-6) whereas the fimbriae of the other strains were only recognized by one MAb: NS25 by M7-6, NS41 by M6-8 and NS50 by M4-7. From these results we conclude that NS24 expresses F7₁ and F11 fimbriae, NS24 expresses F11 fimbriae, NS41 expresses F7₁ fimbriae and NS50 expresses F9 fimbriae.

Antibody titers against P fimbriae in the patient sera were determined by a fimbriae ELISA using the patient serum as the first antibodies. Fimbriae purified from different recombinant strains and one wild type strain were used as antigen (Table 1). Although there was variation in the titers against the various P fimbriae, all sera contained increased homologous and heterologous antifimbriae antibodies compared with the negative control serum (Table 3). The titer of the various sera against the homologous fimbriae was not higher than against the heterologous fimbriae. The titer of the antifimbriae antibodies in the serum of patient 25 was low compared with the other three, but significantly higher than in the control sera.

All four E. coli strains showed a MRHA of human erythrocytes (Table 2). This MRHA was almost completely inhibited in the presence of homologous polyclonal rabbit antisera (Table 4). In contrast, the MRHA was not or very poorly inhibited by the homologous patient sera.

TABLE 4: MRHA inhibition by rabbit and patient sera.

Strain	MRHA titer in the presence of				
	-	<u>homologous rabbit antiserum</u>		homologous	
		anti-F7 ₁	anti-F9	anti-F11	patient serum
NS24	16	2		0	8
NS25	32			0	32
NS41	16	2			8
NS50	8		2		8

DISCUSSION

A few years ago Harber et al. (8) claimed that adherence is not a virulence factor for E. coli bacteria once they have entered the urinary tract, and that adherence fimbriae are not expressed in urine. This view has been criticized by several groups who have shown a strong association between the capacity of E. coli to express P fimbriae and pyelonephritis (10, 23), as well as the involvement of P fimbriae in experimental pyelonephritis (5). Furthermore, two preliminary studies suggested the in vivo expression of P fimbriae by using immunofluorescence labeling of fimbriated E. coli on voided uroepithelial cells (15) and by direct agglutination of P receptor coated particles with urine sediment (21). In the present study the E. coli isolates and sera of four patients with pyelonephritis were investigated. All four strains showed MRHA and P-specific agglutination after one subculture on blood agar. The F serotypes of the expressed P fimbriae were determined by means of a set of F specific MABs. Subsequently, the sera of the patients were tested for the presence of antibodies against homologous and heterologous P fimbriae. In all four sera antibodies were present against homologous as well as heterologous P fimbriae, in contrast to pooled control serum in which no anti-fimbriae antibodies could be detected. The antibodies were indeed directed against P fimbriae and not against contaminating type 1 fimbriae, since the antibodies were measured in a fimbriae ELISA, in which fimbriae were used that were all purified from strains expressing cloned P fimbriae, except the F12 fimbriae. These strains only expressed the respective P fimbriae, without expression of type 1 fimbriae. The fimbriae preparations used were pure, as judged by SDS-PAGE and immunoblotting (1, 3). Also the F12 fimbriae used were pure, whereas contamination with type 1 fimbriae could not be detected with MABs specific for 1A and 1C fimbriae (data not shown). Therefore we conclude that antibodies against P fimbriae were present in the sera of all four pyelonephritis patients tested, providing strong evidence that P fimbriae of E. coli are expressed in vivo. So far only antibodies against type 1 fimbriae have been detected in sera of patients with cystitis and pyelonephritis (16, 17). Unfortunately we were not able to collect serum samples from the patients at different times after onset of complaints. So the determination of possible increase or decrease of antibody titers was not possible.

The antibodies in the patient sera did not only react with the homologous

P fimbriae of the infecting strains, but also with heterologous P fimbriae. This is not surprising, since antisera raised in rabbits against P fimbriae show also many cross-reactions with heterologous P fimbriae (3). Thus the serologically different P fimbriae have various common antigenic determinants besides unique epitopes. Interestingly, the patient sera did not inhibit the MRHA of the infecting strains, in contrast to the rabbit antisera. This means that in the patient sera there were no or very little antibodies present directed against the actual adhesin. Recently it was found that the adhesins of P fimbriae are minor components distinct from the fimbriae subunits (22, J.M. de Ree, P. Schwillens, J.F. van den Bosch, Microbial Pathogenesis, in press). Our present results suggest that in vivo these minor components are less immunogenic than the fimbriae.

The possible role of anti-fimbriae antibodies in serum of patients with pyelonephritis is still obscure. Svanborg-Edén et al. (23) have demonstrated that human polymorphonuclear neutrophils readily bind bacteria with type 1 fimbriae, but do not bind bacteria with P fimbriae unless opsonized with anti-P fimbriae antibodies. Thus the anti-P fimbriae antibodies found in the patient sera, might opsonize the infecting bacteria to improve phagocytosis in kidney and blood stream.

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GENERAL AND SUMMARIZING DISCUSSION

The ability of pathogenic organisms to colonize a mucous membrane is a prerequisite for most infections. To colonize a mucous membrane bacteria belonging to the normal flora as well as pathogens have to adhere to epithelial cells to persist in their habitat, and not to be removed by clearing mechanisms like peristalsis, mucous secretions or other fluids, e.g. urine. Most bacteria have evolved adhesins to overcome the clearing mechanisms. With the adhesins the bacteria can recognize specific receptors, usually carbohydrates on the epithelial cells. The surfaces of the epithelial cells are covered with a great variety of carbohydrates, where they can act as receptors. The molecular basis of the receptor structures are under investigation, and some of these specific receptor structures are elucidated (1-4).

For many bacteria the adhesins have been characterized as proteinaceous appendages called fimbriae. Among uropathogenic Escherichia coli strains especially the P fimbriae have been associated with pyelonephritis. This thesis describes the molecular and especially the serological characterization of the various P fimbriae of uropathogenic E. coli. The main goal of this study was to develop a set of specific antibodies against the serologically different P fimbriae. With these specific antibodies the presence of P fimbriae on uropathogenic E. coli strains and the role of P fimbriae in the pathogenesis of pyelonephritis could be studied.

At the onset of this study we planned to reach the ultimate goal by purifying the P fimbriae from uropathogenic E. coli strains and to raise polyclonal antisera against these purified fimbriae. Subsequently, these antisera could be made fimbriae specific by absorption techniques. However, each wild-type E. coli strain often expresses various serologically different P fimbriae together with type 1 fimbriae, which makes it extremely difficult to purify one specific type of P fimbriae. Furthermore, purification of fimbriae

from wild-type strains in general yielded low amounts of fimbriae. For these reasons we decided to clone the genes encoding for the various types of P fimbriae. Subsequently, a gene cluster encoding for specific P fimbriae was transformed to an E. coli K12 strain which does not produce any kind of fimbriae (Chapter 2 and 3). This molecular cloning of the genes encoding for a certain type of P fimbriae made it possible to purify all serologically different P fimbriae separately. Furthermore, the cloning of the genes clusters, encoding for the P fimbriae, on high copy plasmids increased the production of the fimbriae, which made it possible to purify large amounts of these fimbriae.

Polyclonal rabbit antisera raised against these purified cloned P fimbriae still showed many cross reactions against heterologous P fimbriae (Chapter 4). For this reason we started to produce monoclonal antibodies (MAbs) against the various purified P fimbriae (Chapter 4-6). Most of these MAbs recognized only an epitope on the homologous P fimbriae. From these specific MAbs we selected a set of MAbs for serotyping the P fimbriae of clinical E. coli isolates (Chapter 7). With these MAbs we were able to identify approximately 70% of the P fimbriated urinary E. coli isolates (Chapter 8). The most common serotypes of the P fimbriated urinary tract infections (UTI) strains were the F11, F7₁, and F8 fimbriae. However, it is still difficult to draw definite conclusions about which of the P fimbriae are the most important in the pathogenesis of UTI, due to the relatively small collections that were screened so far.

The sera of four patients with pyelonephritis were analyzed for the presence of specific antibodies against the P fimbriae (Chapter 9). In all cases antifimbriae antibodies were found, which strongly suggests that these P fimbriae are expressed in vivo and are not merely a laboratory phenomenon. However, the antibodies did not inhibit adherence, indicating that these antibodies were directed against the fimbriae proteins rather than to the "minor components" responsible for adherence.

Recently, several gene clusters encoding for P fimbriae were analyzed (5-9). Surprisingly, mutations in the fimbriae subunit gene did not abolish the adhesion to uroepithelial cells, although such mutants are unable to produce fimbriae. In contrast, mutants with lesions in two downstream genes were able to synthesize fimbriae but completely lost the ability to adhere to uroepithelial cells (10). This strongly suggests that these two genes encode for minor components involved in adhesion, and that the fimbriae itself are

not the actual adhesins.

In Chapter 6 some MAbS are described which recognize an epitope on the minor components which are involved in adhesion. These MAbS were able to inhibit adhesion, whereas the fimbriae specific MAbS did not inhibit adhesion. Most anti-fimbriae MAbS described in this study reacted only with homologous fimbriae whereas the MAbS which recognize the minor components involved in adhesion showed a cross reaction with three different fimbriae. This suggests that the minor components may have a more conservative structure compared with the P fimbriae which are more susceptible to antigenic variation.

All the assays used in this study for the detection of P fimbriae on uropathogenic E. coli were phenotypic assays. The disadvantage of phenotypic assays is that they do not demonstrate whether or not the genetic information for the expression of fimbriae is present in the bacteria. For E. coli strains expressing type 1 fimbriae it has been described that these bacteria can switch from a fimbriated phenotype to a non-fimbriated phenotype, a process called phase variation (11). It has also been described that a strain with a specific P fimbriae serotype was able to switch to a type 1C fimbriae serotype (12), and O'Hanley et al. (13) showed that expression of P fimbriae required four or more passages in vitro in 40% of the strains tested. Thus, phase variations in the fimbriae phenotype may result in an underestimation of the percentage of P fimbriae expressing strains among strain collections when only phenotypic assays are used. This can be circumvented by using phenotypic assays in combination with a genotypic assay. With a DNA probe encoding for the P fimbriae gene cluster it is possible to demonstrate whether the strains expressing a non-fimbriate phenotype, do have the genetic information for expression of P fimbriae on the chromosome or not.

Preliminary experiments have shown that with a DNA probe it is indeed possible to demonstrate that the information for P fimbriae is present in a wild-type urinary isolate of E. coli (unpublished results). As a DNA probe we used a part of the F7₁ gene cluster which encoded for a conserved region. With this probe we were able to demonstrate in a Southern blot (14) that the reference strain for F12 fimbriae, C1979, possesses the information for P fimbriae on the chromosome. Other studies have also shown that it is possible to construct probes from the conserved regions which hybridize with all the different P fimbriae genes, for use in identifying potentially P fimbriated strains (13, 15).

As described above the developed MAbs against the P fimbriae can have, in combination with a genotypic assay, great value for diagnostic and epidemiological studies. With the set of P fimbriae specific MAbs the O:K:H serotyping for E. coli can easily be extended with the F serotype.

Recently, much interest has been focussed on P fimbriae as a candidate for a vaccine against pyelonephritis. In vitro studies have demonstrated that antibodies which bind to the fimbriae (16, Chapter 4) or to the minor components involved in adhesion (Chapter 6) inhibit the association between the adhesins and the receptors. In vivo, anti-fimbriae antibodies have been shown to inhibit or delay experimental infection in monkeys and mice (16-18). Based on these in vivo and in vitro studies it was suggested that the P fimbriae might be used for vaccination against pyelonephritis. However, several problems have to be considered with regard to development of a vaccine, e.g. antigenic variation of the P fimbriae; the difference between fimbriae and actual adhesins; the target population; and the route of vaccination.

As shown in Chapter 8, we were able to identify approximately 75% of the P fimbriae on urinary isolates with 9 different MAbs. Thus, at least 10 serologically different P fimbriae may be involved in the pathogenesis of pyelonephritis. Although polyclonal antisera showed many cross reactions with different F types of P fimbriae (Chapters 4 and 9), it was shown in Chapter 9 that in vivo developed anti-P fimbriae antibodies will not always be anti-adhesive for the infecting E. coli strains. Therefore, it seems logical that a possible vaccine has to be based on the minor components which are responsible for adherence, rather than on the fimbriae itself. An advantage might also be that the minor components have less antigenic variation than the fimbriae, since the anti-adhesive MAbs described in Chapter 6 showed cross reaction with minor components of three different P fimbriae.

With regard to the target population for vaccination one has to be aware of the finding that P fimbriae are mainly of importance in the pathogenesis of pyelonephritis in the non-compromised host. Although most studies agree on the association between P fimbriae and acute pyelonephritis in young children (19, 20), less agreement exists on the association with lower UTI (19, 20) or with UTI in elderly patients (21, Chapter 8). Furthermore, Lomberg et al. (21) have shown that the incidence of P-fimbriated E. coli strains in recurrent pyelonephritis patients with vesicoureteric reflux is only 25% compared with 50% in such patients without reflux. Therefore, vaccination may only be effective in patients without urological disorders.

With regard to the route of vaccination, recently a new approach was proposed by Uehling et al. (22). In contrast to other studies using parenteral vaccination, Uehling et al. administered a vaccine into the vagina of monkeys. They suggested that this local vaccine application will result into a local mucosal sIgA response which might prevent colonisation by pathogenic P-fimbriated E. coli of the periurethral zone and the bladder.

In conclusion, not only the pathogenesis of E. coli pyelonephritis is multi-factoral (23), but also the role of adherence in the pathogenesis is very complicated. Obviously, more research is needed to reach the ultimate goal, the proper management and prevention of UTI.

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SAMENVATTING

De bacterie Escherichia coli is een van de belangrijkste verwekkers van urineweginfecties. De uropathogene E. coli bacteriën hebben een aantal virulentie eigenschappen ontwikkeld, waardoor ze in staat zijn om vanuit de faeces via de urethra de urinewegen te infecteren. Een van deze virulentie factoren is het aanhechtend vermogen van de E. coli bacteriën aan het urine- wegepitheel. De aanhechting vindt plaats met behulp van P fimbriae, waardoor de bacteriën zich specifiek aan receptoren op het urinewegepitheel kunnen hechten. Tot nu toe zijn er + 10 serologisch verschillende soorten P fimbriae beschreven.

Het doel van het onderzoek, beschreven in dit proefschrift, was een set van antisera te ontwikkelen gericht tegen de verschillende P fimbriae. Met behulp van deze set van antisera kunnen de P fimbriae aanwezig op de uropathogene E. coli geserotypeerd worden en kan de rol van P fimbriae in de pathogenese van pyelonephritis bestudeerd worden.

De meeste uropathogene E. coli bacteriën zijn in staat om verschillende serotypen van deze P fimbriae tot expressie te brengen, waardoor de zuivering van één type P fimbriae bemoeilijkt wordt. Bovendien is het aantal tot expressie gebrachte P fimbriae op de uropathogene E. coli gering, waardoor de opbrengst aan zuivere P fimbriae na isolatie laag is.

Om deze redenen werd besloten om de genen coderend voor één type P fimbriae te cloneren en te transformeren naar een E. coli K12 stam die geen fimbriae kan produceren (Hoofdstuk 2 en 3). Hierdoor werd het mogelijk om alle serologisch verschillende P fimbriae afzonderlijk te zuiveren. De genen clusters coderend voor de P fimbriae werden gecloneerd in high-copy plasmiden, waardoor relatief grote hoeveelheden P fimbriae konden worden gezuiverd.

Polyclonale antisera opgewekt tegen de gezuiverde P fimbriae reageerden

met de homologe, maar ook met de meeste heterologe P fimbriae (Hoofdstuk 4). Daarom werd gestart met de produktie van monoclonale antilichamen (MAbs) gericht tegen de verschillende P fimbriae (Hoofdstuk 4-6). De meeste van deze MAbs reageerden alleen met de homologe P fimbriae. Uit deze fimbriae specifieke MAbs werd na selectie een set van MAbs samengesteld voor serotypering van P fimbriae van uropathogene E. coli isolaten. Voor deze serotypering werd een eenvoudige methode ontwikkeld waarin hele bacteriën in een ELISA werden gebruikt (Hoofdstuk 7). Vervolgens werden de P fimbriae geserotypeerd van enkele oude en nieuwe collecties van uropathogene E. coli stammen (Hoofdstuk 8). Met behulp van de set van 9 verschillende specifieke MAbs bleek het mogelijk om meer dan 70% van de P fimbriae van uropathogene E. coli stammen te serotyperen. De meest voorkomende serotypen in deze collecties waren de F11, F7₁ en F8 fimbriae. In veel gevallen bleek een bepaald F type geassocieerd te zijn met bepaalde O:K serotypen.

De sera van vier patiënten met pyelonefritis werden geanalyseerd op de aanwezigheid van specifieke antilichamen gericht tegen P fimbriae (Hoofdstuk 9). In alle sera werden deze anti-P fimbriae antilichamen gevonden. Dit suggereert dat de P fimbriae zowel in vitro als in vivo gevormd worden. De antilichamen in deze sera waren niet in staat om de aanhechting van de uropathogene E. coli aan urinewegepitheelcellen te remmen. Deze antilichamen zijn dus waarschijnlijk gericht tegen de P fimbriae en niet tegen de componenten in de P fimbriae die verantwoordelijk zijn voor de aanhechting. Alle P fimbriae specifieke MAbs bleken niet in staat de aanhechting van homologe clones te remmen.

In Hoofdstuk 6 werden echter ook een aantal MAbs beschreven die de aanhechting wél bleken te remmen en dus een epitooop herkennen op de eiwitten die betrokken zijn bij de aanhechting aan het urinewegepitheel. In tegenstelling tot de P fimbriae specifieke MAbs reageerden deze MAbs niet alleen met de homologe P fimbriae maar ook met een aantal heterologe P fimbriae. Dit suggereert dat de structuur van de componenten, die verantwoordelijk zijn voor de aanhechting, meer geconserveerd is dan de structuur van de P fimbriae.

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