Fimbriation and P-antigen Recognition of *Escherichia coli* Strains Harboured Mutated Recombinant Plasmids Encoding Fimbrial Adhesins of the Uropathogenic *E. coli* Strain KS71

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Deletion mutants of recombinant plasmids encoding the KS71B fimbrial antigens of the uropathogenic *Escherichia coli* strain KS71 (O4:K12) were constructed. The effects of these mutations were tested by transforming the mutated plasmids into non-fimbriated *E. coli* HB101 cells and testing the transformants for fimbriation and haemagglutination. A deletion transcriptionally upstream from the fimbrial subunit gene increased the expression of KS71B fimbriae. Deletion of the fimbrial subunit gene resulted in non-fimbriated but haemagglutinating transformants, whereas a deletion 6 kb transcriptionally downstream from the subunit gene resulted in non-haemagglutinating but fimbriate transformants, indicating that fimbriation and haemagglutination were genetically separable. We also present evidence suggesting that the fimbriin and haemagglutinin are physically associated in the wild-type KS71 strain.

INTRODUCTION

The fimbriae form, beside the K, O and H antigens, a fourth major group of surface antigens in *Escherichia coli* (Duguid *et al.*, 1955; Ørskov *et al.*, 1982). They are filamentous surface appendages built up of hundreds of proteinaceous subunits (Brinton, 1965). Many different types of fimbriae, distinguishable both by biochemical and serological means, occur on *E. coli* (Korhonen *et al.*, 1985a). Most common is the mannoside-binding type 1 fimbria; other fimbriae occur more restrictedly on strains associated with infectious diseases, e.g. the K88, K99, CFA/I and CFA/II fimbrial antigens of enterotoxigenic *E. coli* (Gaastra & de Graaf, 1982) and the P-fimbriae characteristic of human pyelonephritogenic *E. coli* strains (Korhonen *et al.*, 1982; Svanborg Eden & Hansson, 1978; Väisänen *et al.*, 1981). The latter contribute to bacterial virulence by promoting adhesion to the blood-group-P-antigens on host cell membranes (Korhonen *et al.*, 1980a).

Our analysis of *E. coli* strains causing human childhood pyelonephritis (Rhen *et al.*, 1983a; Väisänen-Rhen *et al.*, 1984) and neonatal septicaemia (Korhonen *et al.*, 1985b) has shown that most of the pathogenic strains can express many fimbrial antigens. The different fimbrial types occur, at a given time, mostly on separate cells and show a rapid phase variation (Rhen *et al.*, 1983c; Nowicki *et al.*, 1984, 1985).

To study the molecular basis of fimbrial phase variation we have recently cloned the fimbrial genes from our pyelonephritogenic, multifimbriated model strain, *E. coli* KS71 (O4:K12) (Rhen *et al.*, 1983a). Each of the four types of fimbriae (termed KS71A, KS71B, KS71C and KS71D) is encoded by a separate set of genes (Rhen, 1985; M. Rhen, unpublished). KS71A and KS71B are P-fimbriae, KS71C appears to be non-haemagglutinating and KS71D corresponds to the type 1 fimbriae. Serological analyses of the phase variation of KS71 fimbriae have recently been described (Rhen *et al.*, 1983c; Nowicki *et al.*, 1984).

In this communication we describe three types of mutations within the cloned gene cluster encoding KS71B fimbriae; (i) deletions affecting the level of fimbrial expression, (ii) deletions abolishing fimbrial expression but not haemagglutination, and (iii) deletions abolishing haemagglutination but not the expression of fimbriae. We also present evidence indicating that the KS71B fimbriae and the haemagglutinin are physically associated in the wild-type *E. coli* KS71 strain.

METHODS

Bacterial strains, phages and plasmids. *E. coli* HB101 cells (Leu⁻ Pro⁻ HsdS⁻ StrA⁻ Thi⁻ RecA⁻) were grown for 16–18 h at 37 °C in Luria broth or on Luria agar supplemented with 100 µg ampicillin ml⁻¹ (Wyeth Laboratories, USA) or with 20 µg chloramphenicol ml⁻¹ (Sigma). The phage M13 derivative mp9 (Messinger & Vieira, 1982) was harboured in *E. coli* JM101 (Lac⁻ F⁺ traD36 Thi⁻ Pro⁻). The cloning vectors pBR322 and pACYC184 were those described by Bolivar *et al.* (1977) and Chang & Cohen (1978). Recombinant plasmids pKTH3011, pKTH3019, pKTH3024, pKTH3025, pKTH3026, pKTH3026-1, pKTH3026-2, pKTH3026-3 and pKTH3026-4 were available from previous works (Rhen, 1985; Rhen *et al.*, 1985a).

Cloning procedures and restriction mapping. Restriction endonucleases and T₄ DNA ligase obtained from Boehringer-Mannheim and Amersham were used under conditions recommended by Maniatis *et al.* (1982). Plasmid and phage DNA was isolated from cell lysates by ethidium bromide/caesium chloride equilibrium gradient centrifugation (Maniatis *et al.*, 1982). For screening purposes the DNA was isolated by the alkaline lysis method of Birnboim & Doly (1979). Ligated DNA and isolated plasmid and phage DNA were introduced into recipient strains by transformation of CaCl₂-treated cells (Mandel & Higa, 1970). Restriction mapping was done as described previously (Rhen, 1985).

Isolation of Tn1725 insertion mutants. The production and isolation of Tn1725 insertion mutants has been described previously (Altenbuchner *et al.*, 1983). The mutant plasmids were used in double transformation experiments after removing the chloramphenicol resistance marker by a partial *Hind*III digestion.

DNA sequencing. The chain termination method of Sanger *et al.* (1977) was used, employing recombinant M13 DNA as template. Sequencing primer and DNA polymerase I Klenow fragment were purchased from Boehringer-Mannheim, and deoxyadenosine [³⁵S]thiotriphosphate from Amersham.

Preparation of Fab fragments. The antisera against *E. coli* KS71ABC, KS71A, KS71C and ER2B2 fimbriae have been characterized previously (Rhen *et al.*, 1983a, c). Purified *E. coli* ER2B1 fimbriae were prepared by immune precipitation (Rhen *et al.*, 1983a) and anti-ER2B1 was obtained by immunizing rabbits according to Korhonen *et al.* (1980b). Fab fragments were prepared by papain treatment of immunoglobulin fractions isolated from these antisera (Porter, 1959). The activity of the Fab preparations was controlled in an enzyme linked immunosorbent assay employing *E. coli* KS71ABC or ER2A1B1B2 fimbriae as solid phase antigen and alkaline phosphatase conjugated swine anti-rabbit IgG (Orion Diagnostica, Finland) (Rhen *et al.*, 1983a).

Agglutination tests. The factor antisera against KS71A or KS71B fimbriae were those described previously (Rhen, 1985). Bacterial agglutination and serological quantification of fimbriae production were done according to Rhen *et al.* (1985a). Mannose-resistant haemagglutination and inhibition of haemagglutination were done as described previously (Rhen *et al.*, 1983b).

Electron microscopy. Bacteria were negatively stained with 2% (w/v) phosphotungstic acid in 0.1 M-phosphate buffer (pH 6.5) and examined with a JEM-100B electron microscope operating at 80 kV. Electron micrographs were taken at the Department of Electron microscopy, University of Helsinki.

RESULTS

Sequencing of the 5'-end of the KS71B fimbriin gene. The KS71B fimbrial subunit gene has previously been mapped within the 1.1 kb *Sma*I–*Cla*I fragment of pKTH3026 (Fig. 1a), which is a subclone of the KS71B-expressing recombinant plasmid pKTH3011 (Rhen *et al.*, 1985a). The *Bam*HI–*Cla*I insert fragment of pKTH3026 was also cloned in the corresponding cloning sites of pBR322, giving the plasmid pKTH3030 (Fig. 1b). A Tn1725 insertion derivative of pKTH3030, pKTH3030-1, was isolated that contained the insert within the 1.1 kb *Sma*I–*Cla*I fragment (Fig. 1b). In contrast to pKTH3030, the transposon-derivative pKTH3030-1 did not express KS71B when complemented with pKTH3024 (Table 1). pKTH3024 encodes KS71A fimbriae and can produce KS71B fimbriae if provided with the subunit gene (Rhen *et al.*, 1985a).

A stretch of 444 nucleotides was sequenced from the *Eco*RI sites created by Tn1725 in pKTH3030-1. The DNA sequence obtained contained the region encoding the 21 amino acid

Fig. 1. (a) Restriction endonuclease maps of recombinant plasmids encoding KS71A (pKTH3024) or KS71B (pKTH3011) fimbriae and of their subclones. The thick lines are DNA of the cloning vectors pBR322 (□) and pACYC184 (■). Thin lines with restriction endonuclease cleavage sites marked indicate insert DNA. The deletion derivatives of pKTH3019 and pKTH3026 are shown as thin lines, indicating the DNA present in the corresponding plasmid. (b) The position of the Tn1725 insertion present in pKTH3030-1 is indicated by the arrow on the map of pKTH3030; the insertion point within the KS71B fimbrial subunit gene is shown underneath (only a part of the sequence is shown).

signal sequence and the 113 NH₂-terminal amino acids of the F₇₂ fimbrial gene (Van Die & Bergmans, 1984). The 42 nucleotide stretch preceding the signal sequence initiation codon was the same as that preceding the F₂, KS71A and papA genes (Båga *et al.*, 1984; Rhen *et al.*, 1985*b*; Van Die & Bergmans, 1984). Our Tn1725 insertion was situated after the codon encoding the 18th NH₂-terminal amino acid of the mature fimbrillin (Fig. 1*b*). A stretch of 280 nucleotides

Table 1. *Agglutination properties of recombinant E. coli HB101 strains*

Plasmid(s) in <i>E. coli</i> HB101	Agglutination with:*		MR-HA†	Agglutination titre of absorbed anti- KS71B serum‡
	anti-KS71A serum	anti-KS71B serum		
pKTH3030,pKTH3024	+++	++	+	20
pKTH3030-1,pKTH3024	+++	—	+	2560
pKTH3026,pKTH3019-1	—	+	+	2560
pKTH3026-1,pKTH3019-1	—	+	+	1280
pKTH3026-2,pKTH3019-1	—	+	+	2560
pKTH3026-3,pKTH3019-1	—	—	+	2560
pKTH3026-4,pKTH3019-1	—	++	+	320
pKTH3019-1	—	—	+	2560
pKTH3019-12	—	—	—	2560
pKTH3025,pKTH3019-1	+++	—	+	1280
pKTH3025,pKTH3019-12	+++	—	—	1280
pKTH3026-4,pKTH3019-12	—	++	—	640

* Agglutination with factor antisera against KS71A or KS71B fimbriae at a final dilution of 1/50.

+++ , Strong reaction; ++ , intermediate reaction; + , weak reaction; — , negative reaction.

† Mannose-resistant haemagglutination of human OP₁ erythrocytes; $\bar{p}$ erythrocytes were not agglutinated.

‡ Agglutination titre of factor anti-KS71B serum absorbed with recombinant *E. coli* HB101 cells (50 μ l of antiserum absorbed with 3×10^9 bacteria). The strain harbouring pKTH3026-4 and pKTH3019-1 was used as test strain.

was sequenced from the region situated at the left of the *Cla*I site in pKTH3030 (Fig. 1*b*). This sequence included a region corresponding to the six last amino acid codons of the F₇₂, KS71A and papA genes. The 259 base pair sequence situated between the stop codon and the *Cla*I site of pKTH3030 was identical to the corresponding region of the recombinant plasmid encoding the KS71A fimbrillin (Rhen *et al.*, 1985*b*). We therefore conclude that the KS71B fimbrillin gene starts 0.8 kb to the left of the *Cla*I site in pKTH3030 (Fig. 1*b*) and that the transcription proceeds from left to right.

Deletions affecting the level of KS71B production. The expression of KS71B fimbriae by pKTH3011 or pKTH3019 is low in *E. coli* HB101 (Rhen, 1985). Furthermore, only 3 to 5% of the pKTH3011 or pKTH3019 transformants are strongly agglutinable with anti-KS71B serum. We therefore tested the pKTH3011 subclones pKTH3026, pKTH3026-1, pKTH3026-2, pKTH3026-3 and pKTH3026-4 (Fig. 1*a*) for expression of KS71B when complemented with pKTH3019-1. pKTH3019-1 was obtained by transferring the 8 kb *Cla*I–*Bam*HI fragment of pKTH3019, after fill in of the *Cla*I site, to the *Eco*RV–*Bam*HI site of pBR322 (Fig. 1*a*). The expression of KS71B was low with pKTH3026 and pKTH3026-1, slightly stronger with pKTH3026-2 and negative with pKTH3026-3 (Table 1). A significantly stronger expression of KS71B was obtained with pKTH3026-4 (Table 1) and all the double transformants tested (50/50) strongly reacted with anti-KS71B serum. The reason for the changed pattern of fimbrial expression observed when using pKTH3026-4 is not known; however, *E. coli* HB101 harbouring pKTH3026-2 or pKTH3026-4 together with pKTH3019-1 forms a better source of KS71B fimbriae than the ancestral clone.

The KS71B fimbrillin gene is not essential for haemagglutination. *E. coli* HB101 cells harbouring pKTH3019-1 did not react with anti-KS71B serum but caused a P-blood-group-antigen-specific haemagglutination, even though the KS71B fimbrillin gene is missing in pKTH3019-1 (Table 1). These recombinant cells also appeared non-fimbriated in the electron microscope (data not shown). Haemagglutination of HB101 cells was abolished if the 1.6 kb *Bgl*II–*Bam*HI fragment of pKTH3019-1 was deleted (Table 1; the deleted pKTH3019-1 derivative was termed

Fig. 2. Haemagglutination titres of *E. coli* HB101 cells harbouring pKTH3019-1 in the presence of various concentrations of anti-fimbriae Fab fragments. ○, Anti-KS71ABC Fab; ●, anti-KS71A Fab; □, anti-KS71C Fab; ■, anti-ER2B1 Fab; △, anti-ER2B2 Fab.

pKTH3019-12). pKTH3025 or pKTH3026 (Fig. 1*a*) expressed KS71A or KS71B fimbriae when complemented with pKTH3019-1 or pKTH3019-12 as judged by agglutination in antiserum (Table 1) and by electron microscopy (data not shown). These strains were haemagglutinating if they contained pKTH3019-1 but not if they contained pKTH3019-12, indicating that fimbriation as such did not result in haemagglutination.

Inhibition of haemagglutination directed by pKTH3019-1 by Fab fragments. Fab fragments were prepared from antisera raised against KS71ABC, KS71A and KS71C fimbriae and from antisera against P and type C fimbriae of *E. coli* strain ER2 (O4:K12). The ability of these Fab fragments to inhibit haemagglutination by *E. coli* HB101 harbouring pKTH3019-1 is shown in Fig. 2. Fab fragments against P-fimbriae (KS71ABC, KS71A and ER2B1) inhibited the haemagglutination in a dose-dependent fashion, whereas Fab fragments against the non-haemagglutinating fimbriae (KS71C and ER2B2) were non-inhibitory.

DISCUSSION

The genes for various virulence-associated fimbriae have recently been cloned and characterized. These include the genes for mannose-binding and P-fimbriae of uropathogenic *E. coli* (Clegg & Pierce, 1983; Normark *et al.*, 1983; Orndorff & Falkow, 1984; Van Die *et al.*, 1984) as well as the genes for the K88 (Mooi *et al.*, 1981) and the K99 (de Graaf *et al.*, 1984) fimbriae of enterotoxigenic *E. coli*. A common property of all these genes is that they occur as multigene clusters which contain, in addition to the fimbrillin gene, genes for assembly and transport functions. So far, only in studies with the *pap* pilus operon could the fimbrillin gene be inactivated by mutation without loss of receptor binding activity (Lindberg *et al.*, 1984; Norgren *et al.*, 1984). Possibly, there are two groups of fimbriae; those whose fimbrial subunits possess binding properties and those that need additional components for action.

In this communication we have shown that fimbriation and haemagglutination are genetically separable properties of recombinant strains expressing KS71B fimbriae (Table 1). This probably also holds for the KS71A fimbriae, as non-haemagglutinating fimbriate recombinant strains were obtained (Table 1).

We have previously shown that Fab fragments specific for P-fimbriae inhibited haemagglutination caused by the wild-type KS71 strain (Rhen *et al.*, 1983*a*). In this study we show that the same Fab fragments inhibit haemagglutination caused by a non-fimbriated recombinant strain (Fig. 2). This inhibition can result from an immunological cross-reaction between P-fimbriae and the haemagglutinin or be due to the presence of anti-haemagglutinin antibodies in the anti-P-fimbriae sera. If the first assumption is correct, then the haemagglutinin could be a minor, fimbrial-like protein present in amounts too low to be seen on Coomassie blue

stained acrylamide gels (Rhen *et al.*, 1983*b*). If the second assumption is correct, then the haemagglutinin must be associated with P-fimbrial filaments in wild-type *E. coli* KS71. It should be noted that the anti-KS71A and anti-KS71C sera were obtained with antigens purified from the same fimbrial extract (Rhen *et al.*, 1983*a*) and that Fab fragments specific for KS71C fimbriae did not inhibit haemagglutination (Fig. 2; Rhen *et al.*, 1983*a*). Hence, the adhesin in the wild-type strain was somehow co-purified with the KS71A but not with the KS71C fimbriae.

It is possible to fractionate distinct subpopulations from *E. coli* KS71 cultures on the basis of either the binding specificities or the serological properties of the KS71 fimbriae (Rhen *et al.*, 1983*c*). Similar findings have been obtained with *E. coli* 3040, a strain possessing both S and type 1 fimbriae (Nowicki *et al.*, 1985). These findings also predict that the binding specificity and certain fimbrial subunits are physically associated or that they are co-expressed in the same cells.

The presence of anti-haemagglutinin antibodies in P-fimbriae antisera explains the ability of these antisera to inhibit adhesion of *E. coli* to uroepithelial cells (Korhonen *et al.*, 1981; Rhen *et al.*, 1983*a*) and supports the idea of using purified P-fimbriae as a vaccine against pyelonephritis (Roberts *et al.*, 1984).

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