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Cloning of Chromosomal DNA Encoding the F41 Adhesin of Enterotoxigenic *Escherichia coli* and Genetic Homology between Adhesins F41 and K88

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The genetic determinant for production of the adhesive antigen F41 was isolated from a porcine enterotoxigenic *Escherichia coli* strain by cosmid cloning. The cloned DNA included sequences homologous to those of hybridization probes prepared from the K88 adhesive antigen operon. Transposon insertions which inactivated F41 production mapped to the same region of DNA showing homology with the K88 genes, demonstrating the genetic relatedness of F41 and K88. Hybridization of a K88 gene probe to plasmid and total DNA from the porcine *E. coli* isolate from which the F41 gene was cloned indicated that F41 is chromosomally encoded by this strain. This observation was extended to other F41-producing animal isolates. A large number of animal *E. coli* isolates were examined with K88, F41, and K99 gene probes and for mannose-resistant hemagglutination of human group O erythrocytes and K88 and F41 antigen production. All K88 and F41 antigen producers possessed genetic homology with the K88 and F41 gene probes. Most, but not all, F41-producing strains possessed homology to the K99 gene probe, reflecting the previously observed association of F41 and K99 antigen production. In the strains examined, homology with the K99 gene probe was plasmid associated, whereas homology with the F41 gene probe was chromosomal. The K88 antigen-producing strains showed no homology with the K99 probe. A number of strains possessed homology with the K88 and F41 gene probes and were mannose-resistant hemagglutination positive, but did not produce K88 or F41 antigens. This suggests that there are adhesins among animal isolates of *E. coli* which are genetically related to but antigenically distinct from K88 and F41.

Enterotoxigenic *Escherichia coli* (ETEC) adhere to the epithelium of the small intestine of the host and produce enterotoxins which cause diarrhea. The bacterial proteins of fimbrial structure which cause adherence are called adhesins. Several antigenic types of ETEC adhesins which confer a degree of host species specificity have been described (4). Most ETEC adhesins agglutinate erythrocytes in the presence of mannose, providing a convenient assay for the presence of an adhesin. The hemagglutinating capacity of different adhesins varies with the species source of the erythrocytes. All ETEC adhesin genes which have been located are plasmid encoded (4).

The F41 antigen is a recently described fimbrial adhesin of ETEC that are pathogenic for cattle and swine (2, 13). The F41 antigen was first recognized as a second mannose-resistant hemagglutinin produced by ETEC strains which also express the K99 adhesin (14). The F41 antigen is associated with K99-producing ETEC of serogroups O9 and O101, but has not been reported among K99-producing strains of other serogroups (15). Recently, F41 production has been demonstrated in non-K99-producing strains of ETEC of serogroup O101 isolated from piglets with diarrhea (15). These strains have been demonstrated to be fully virulent in experimental infections in piglets and calves (1, 15, 21). This suggests that F41 is a virulence factor, since these strains produce no other known adhesin. The sequence of the amino acids at the amino terminus of the F41 structural subunit reveals very limited homology with other *E. coli* adhesins (2).

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We report here the molecular cloning of the genes required for F41 production, examine the sequence relationships of these genes with genes encoding the K88 and K99 adhesins of ETEC, and demonstrate the chromosomal association of the F41 genes. We examine a large number of *E. coli* isolates with K88, K99, and F41 gene probes, for mannose-resistant hemagglutination (MRHA) of human group O erythrocytes, and for K88 and F41 antigen production.

MATERIALS AND METHODS

Bacterial strains and plasmids. Animal isolates of *E. coli* and derivative strains were from the collection of the National Animal Disease Center, Ames, Iowa. *E. coli* VAC1676 (1, 15, 21) is a porcine ETEC of serotype O101:K30:F41:H$^-$. *E. coli* BHB2688 and BHB2690 (10) were used for preparation of bacteriophage lambda in vitro packaging extract. *E. coli* HB101 (10) was used as a recipient for cosmid infection and transformation. The cosmid vector pHC79 (5) was used for cosmid cloning. The K88 gene probes were derived from pMK005 (7). The K99 gene probes were derived from pFK99 (3).

Cosmid cloning. High-molecular-weight DNA was prepared from strain VAC1676 by the method of Hull et al. (6). The DNA was partially digested with the restriction enzyme *Sau*3A to give fragments 30 to 40 kilobases (kb) in size. The restricted DNA was ligated to *Bam*HI-cleaved pHC79. Preparation of packaging extract, the in vitro packaging reaction, and infection of the recipient strain were carried out by the methods of Maniatis et al. (10).

F41 enzyme-linked immunosorbent assay. The F41 antigen was purified from *E. coli* B41M (13), and rabbit antisera were

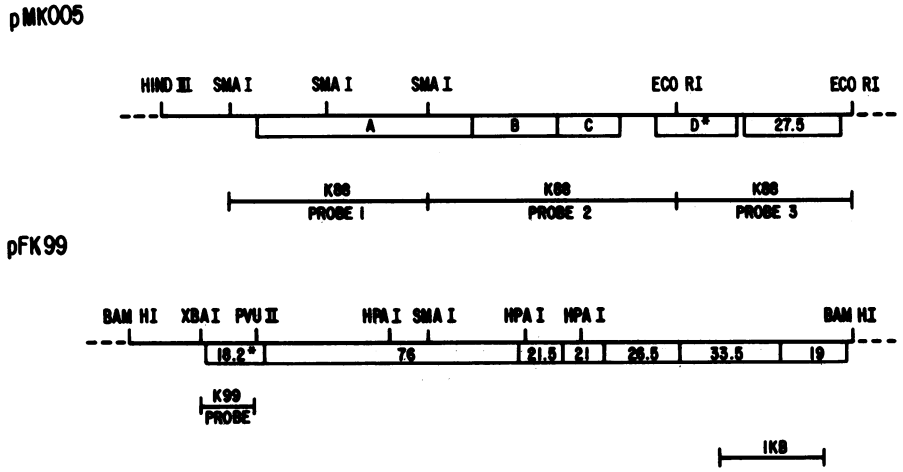

FIG. 1. Derivation of K88 and K99 gene probes. Letters in open bars in pMK005 refer to proteins encoded by this plasmid described by Kehoe et al. (7, 8). The 27.5-kilodalton protein shown in pMK005 was described by Mooi et al. (12) in another K88-encoding plasmid. The numbers shown in pFK99 refer to sizes (in kilodaltons) of proteins described by de Graaf et al. (2). The structural proteins constituting the K88 and K99 antigens are indicated with asterisks.

raised by the methods of de Graaf and Roorda (2). Enzyme-labeled anti-F41 antigen conjugate and all other enzyme-linked immunosorbent assay reagents were prepared by the method of Voller et al. (22). For enzyme-linked immunosorbent assay screening of bacterial colonies for F41 production, microtiter wells (Immulon 2 "U" plates; Dynatech Laboratories, Inc.) were coated with rabbit anti-F41 serum by overnight incubation at 20°C with antiserum diluted 10,000-fold in 20 μl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$). The wells were then washed with PBS-Tween (0.05% Tween 20 [Sigma Chemical Co.]–0.5% bovine serum albumin in phosphate-buffered saline [PBS]) three times, allowing the plate to stand for 3 min with PBS-Tween for each wash. The wells were then filled with 200 μl of PBS-Tween, and a toothpick was used to disperse a bacterial colony from an agar plate into each well. The plate was then incubated for 2 h at 20°C. After incubation, the wells were washed three times with PBS-Tween and filled with alkaline phosphatase-conjugated anti-F41 antibody diluted 200-fold in 200 μl of PBS-Tween. The plate was incubated for 2 h at 20°C and then washed three times with PBS-Tween. A 200-μl volume of substrate solution was added to each well. Absorbance was measured at 400 nm with an automated microtiter plate reader (Dynatech). An absorbance of 0.5 or greater was considered positive.

MRHA assay. Bacteria from solid media were suspended in a 2% suspension of fresh human group O erythrocytes which had been washed three times in PBS and then suspended in PBS with 1% mannose. MRHA reactions were performed at room temperature.

Slide agglutinations. The K88 antigen was detected by slide agglutination with specific anti-K88 antiserum added to a saline suspension of live bacteria.

Preparation of DNA hybridization probes. Probes for the K88 genes were prepared from plasmid pMK005 (7) (Fig. 1). The K99 subunit gene probe was prepared from pFK99 (3) (Fig. 1). An additional K99 probe was also prepared from a 6.8-kb *Bam*HI fragment of pFK99 as described in Results. The F41 gene probe was prepared from pSLM204 (Fig. 2) and is described in Results. The plasmids were digested with the appropriate restriction endonucleases, the resulting fragments were separated by polyacrylamide gel electrophoresis in 90 mM Tris hydrochloride–90 mM boric acid–5 mM EDTA, pH 8.3 (Tris-borate), and the appropriate fragments were electroeluted in 0.1× Tris-borate. The purified restriction fragments were concentrated by ethanol precipitation. Probes were labeled by nick translation (11) with α-^{32}P-deoxynucleotides to a specific activity of approximately 5×10^7 cpm/μg.

Southern blot hybridizations. Preparation and hybridiza-

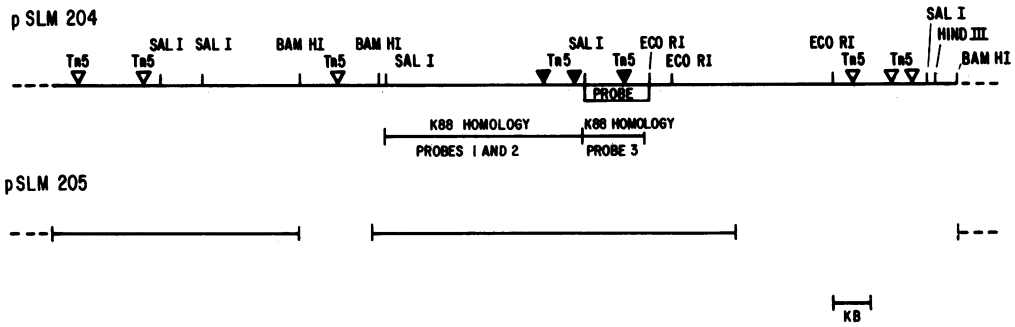

FIG. 2. Restriction maps of pSLM204 and pSLM205. The vector-derived portions of the plasmids are not shown. Areas of homology with K88 refer to probes shown in Fig. 1. The region of pSLM204 designated as probe was used as an F41 probe as described in the text. Symbols: (▼) Tn5 single insert which inactivates the MRHA encoded by pSLM204; (▽) Tn5 single insert which does not inactivate the MRHA. The pSLM205 was derived by deletion of two areas of pSLM204, and the remaining sequences are indicated.

tion of blots at high stringency were as previously described (16, 17).

Colony hybridizations. Colony hybridizations were performed by a modification of the technique described by Maas (9). Bacterial cultures were inoculated in a defined array on suitable media. After overnight incubation at 37°C, a single Whatman 541 filter paper disk was placed on the agar surface, and gentle pressure was applied with a bent glass rod to remove air bubbles trapped between the paper and the agar. The Whatman 541 paper was then lifted off the agar surface, marked with a pencil for orientation, and placed colony side up on a piece of Whatman no. 3 paper which had been saturated with 0.5 N NaOH–1.5 M NaCl in a glass petri dish. The dish was placed in flowing steam for 3 min. The Whatman 541 paper was then lifted off of the Whatman no. 3 paper and immersed in 1 M Tris hydrochloride (pH 7.0)–2 M NaCl for 4 min. Excess moisture was removed by blotting on a paper towel, and the filters were stored damp in Zip-lock bags (Dow Chemical Co.) or heat-sealable plastic bags at room temperature. Hybridizations and washes were performed as described for Southern blots (16). After hybridization and exposure, the filter was placed in 0.5 N NaOH for 30 min at room temperature to remove bound probe and then neutralized in 0.3 M NaCl–0.03 M sodium citrate–0.1% sodium dodecyl sulfate for 30 min. The filter was blotted and stored damp in a heat-sealed plastic bag at room temperature. The filter was then ready for rehybridization with another probe. We have successfully rehybridized filters with different probes seven times over a 2-year period (S. L. Moseley and R. A. Schneider, unpublished observations).

Insertion mutagenesis. The transposon Tn*5* was introduced into strains carrying recombinant plasmids by infection with the bacteriophage λ*c*I857*b*221 *rex*::Tn*5* (19). Colonies growing on media containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml) were harvested together, and plasmid DNA was extracted by the method of Portnoy et al. (18). The isolated plasmid DNA was used to transform strain HB101 for kanamycin and ampicillin resistance, thus selecting those recombinant plasmids into which Tn*5* has been inserted. The locations of Tn*5* insertions were mapped by restriction analysis.

RESULTS

Molecular cloning. A cosmid library was prepared with vector pHC79 and DNA from strain VAC1676 and plated on strain HB101. Ampicillin-resistant colonies were screened for F41 production by an enzyme-linked immunosorbent assay. Of 800 colonies screened, 2 were positive for F41 expression. One of these was selected for further study, and the recombinant plasmid acquired by this strain was designated pSLM203.

Preliminary characterization of pSLM203 and subcloning of the region encoding F41 expression. The plasmid pSLM203 was determined by relative electrophoretic mobility in agarose to be approximately 45 kb in size (data not shown). In addition to mediating the expression of the F41 antigen, the plasmid encoded a mannose-resistant hemagglutinin of human group O erythrocytes, suggesting that assembly of structural subunits into fimbriae was occurring in the recombinant strain.

Subcloning was initiated by complete digestion of pSLM203 DNA with the restriction enzyme *Hin*dIII. The digestion products were religated and used to transform HB101. Transformants were selected on media containing ampicillin and screened for MRHA of human group O erythrocytes. One MRHA-positive colony was obtained that contained a 24-kb plasmid designated pSLM204. A physical map was derived by restriction analysis (Fig. 2). The plasmid was further deleted by complete digestion with *Bam*HI and subsequent religation. An MRHA-positive transformant was selected for analysis, and its plasmid was designated pSLM205. The deleted region of pSLM204 is indicated in Fig. 2. It will be noted that one deleted region does not terminate at a *Bam*HI site. The nature of this deletion was not determined.

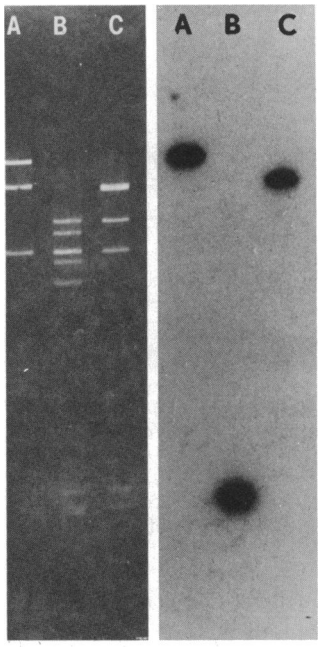

FIG. 3. Southern blot hybridization of pSLM204 with K88 probe 3 (Fig. 1). (A) *Eco*RI digest; (B) *Eco*RI and *Sal*I double digest; (C) *Sal*I digest; left, agarose gel electrophoresis of restriction digests; right, autoradiograph of blot after hybridization.

Relationship of F41 with other adhesins of ETEC pathogenic for animals. To investigate the possible genetic relationship of F41 with other adhesins of ETEC, pSLM204 was probed with fragments of DNA encoding the ETEC adhesins K99 and K88 in Southern blot experiments. A fragment of plasmid pFK99 which encodes the K99 structural subunit was selected for use as a K99 hybridization probe (3), and three K88 gene probes were derived from plasmid pMK005 (7). The probe fragments are shown in Fig. 1. No homology was seen between the K99 gene probe and pSLM204. To examine the possibility that homology may exist between accessory genes associated with K99 production and DNA encoding F41 production, pSLM204 was also probed with a *Bam*HI fragment of pFK99 of approximately 6.8 kb, which encodes all seven proteins known to be involved in K99 production (Fig. 1) (3). Again, no homology was detected under stringent conditions of hybridization. The K88 gene probes did detect homologous DNA under stringent conditions of hybridization. Hybridization of K88 probe 3 with restricted pSLM204 DNA is shown in Fig. 3. Homology was similarly shown to exist with all three K88 gene probes covering three regions of K88 encoding DNA. The homology was localized to a specific region of pSLM204 (Fig. 2). These data suggest that there is extensive homology between accessory genes encoding K88 and F41, although the amino acid sequences suggest that a high degree of homology does

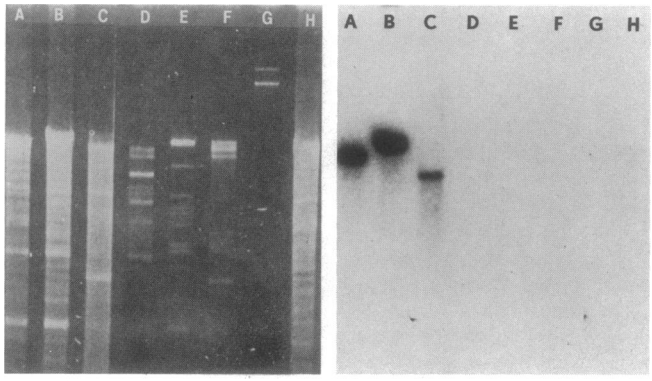

FIG. 4. Southern blot hybridization of VAC1676 total DNA and plasmid DNA with K88 probe 3 (Fig. 1). (A through C) Total DNA digests; (D through F) plasmid DNA digests; (A, D) *Eco*RI digests; (B, E), *Hin*dIII digests; (C, F) *Kpn*I digests; (G) unrestricted plasmid DNA; (H) *Eco*RI digest of total HB101 DNA; left, agarose gel; right, autoradiograph of blot after hybridization.

not extend throughout the genes encoding the structural subunits of the adhesins (2). An alternative interpretation of these data is that the DNA of pSLM204 found to be homologous to the K88 gene probes is not involved in F41 production, but is closely linked to the F41 genes. To examine this possibility, the transposon Tn*5* was inserted into pSLM204. Three independent insertion mutants were obtained which no longer encoded the expression of F41. These insertions were mapped to the region of pSLM204 which contained homology with the K88 genes (Fig. 2), confirming that some homology is shared among genes encoding K88 and F41.

Chromosomal location of the genes encoding F41 production. The K88 and K99 gene probes were used to probe DNA from strain VAC1676 in Southern blot experiments. Homology with the K88 gene probe was found in total DNA, but not in purified plasmid DNA from this strain (Fig. 4). This indicates that F41 is chromosomally encoded in this strain. Plasmid DNAs from nine additional strains of ETEC that express F41 were also examined with the K88 probe in Southern blot experiments, and no homology was detected. Since these strains hybridized with the K88 probes in colony hybridizations, the observed homology is presumed to be associated with chromosomal DNA. These strains are listed in Table 1. Homology with the K99 gene probe was found on plasmid DNA of VAC1676 as well as on plasmid DNA from other F41-producing strains and a K99-positive, F41-negative strain (Table 2).

TABLE 1. ETEC strains examined for plasmid or chromosomal homology with the F41 probe

Strain	Serotype	Homology[a]
K-12 HB101(pSLM204)	F41	Plasmid
KATI1706	O101:K30:F41	Chromosome
VC1751	O101:K27:F41	Chromosome
B41	O101:K99:F41	Chromosome
431	O101:K30:K99:F41	Chromosome
613	O101:K30:K99:F41	Chromosome
B44	O9:K30:K99:F41	Chromosome
B85	O9:K99:F41	Chromosome
1601	O9:K35:K99:F41	Chromosome

[a] Strains were hybridized with the F41 probe (Fig. 2) by colony hybridization and by Southern blot hybridization of isolated plasmid DNA. Strains positive by colony hybridization but negative by plasmid hybridization were presumed to possess chromosomal homology with the probe.

Occurrence of F41 genes among animal ETEC isolates. The K88 and K99 gene probes were used in colony hybridization experiments to examine a number of animal isolates of *E. coli* including 331 isolates from animals with diarrhea and 44 isolates from healthy animals. A fragment of DNA from pSLM204 was also selected for use as an F41 hybridization probe (Fig. 2). The strains were also assayed for K88 and K99 production by slide agglutination in specific antisera, for F41 production by the enzyme-linked immunosorbent assay, and for MRHA of human group O erythrocytes (Table 3). As expected, the K88 and F41 probes were completely cross-reactive (i.e., there were no strains that were positive with one and negative with the other). However, K88-producing strains gave a somewhat stronger signal with the K88 probe than did the F41-producing strains (data not shown). Furthermore, there were a number of strains which reacted with the K88 and F41 probes but which did not produce K88 nor F41. Many of these strains, however, were MRHA positive, indicating that an adhesin was being expressed.

An additional group of 24 *E. coli* isolates which produce the 987P fimbrial adhesin and do not produce the K88, K99, or F41 adhesin were examined by colony hybridization with the F41, K88, and K99 gene probes. None of the 987P-producing isolates hybridized with any of the probes, indicating a lack of genetic homology between 987P and the other three adhesins.

Although these results demonstrate the genetic relatedness of F41 and K88, they suggest a lack of extensive homology between genes encoding K88 and K99 production, contrary to results presented in a previous report (8). The hybridization experiments described above used probes which were derived from only small portions of the K88 and K99 gene clusters which encode primarily the antigenic subunits. To examine the possibility that K88 and K99 share homology in accessory genes, the 6.8-kb *Bam*HI fragment of pFK99 (Fig. 1), which encodes all seven of the proteins known to be associated with K99 production (3), was used to probe DNA from strain 263, serotype O8:H19:K85:K88ab. All three K88 probes (Fig. 1) were likewise used to probe strain 637, serotype O64:K^+:K99. In neither case was homology observed between genes encoding K88 and K99 under stringent hybridization conditions.

DISCUSSION

The purpose of the present study was to isolate the genes encoding the F41 adhesin of ETEC pathogenic for cattle and swine, examine the relationship of F41 with other adhesins of animal ETEC isolates, and establish the plasmid or chromosomal location of the DNA that encodes F41. We

TABLE 2. ETEC strains examined for plasmid or chromosomal homology with the K99 probe

Strain	Serotype	Homology[a]
KATI1706	O101:K30:F41	Plasmid
VC1751	O101:K27:F41	Plasmid
VAC1676	O101:K30:F41	Plasmid
B41	O101:K99:F41	Plasmid
B41M	O101:F41	Plasmid
1459	O9:K35:K99	Plasmid
B44	O9:K30:K99:F41	Plasmid
263	O8:H19:K85:K88ab	None

[a] Strains were hybridized with the K99 probe (Fig. 2) by colony hybridization and by Southern blot hybridization of isolated plasmid DNA.

have demonstrated a genetic relationship between F41 and the K88 adhesin and have found that F41 is chromosomally encoded in several isolates.

The chromosomal location of the genes encoding F41 expression is unusual among ETEC-specific adhesins. All other ETEC-specific adhesin genes which have been located have been associated with plasmids (4). There has been speculation that the 987P adhesin of ETEC pathogenic for swine is chromosomally encoded (4), but there have been no reports demonstrating the location of the genes encoding this adhesin. Chromosomally encoded adhesins have been described among *E. coli* isolates associated with urinary tract infections in humans (6).

In spite of the limited amino acid sequence homology (2), the genes encoding F41 production show extensive homology with DNA encoding the K88 adhesin of ETEC pathogenic for swine. The K88 adhesin has been shown to be plasmid encoded; thus, the presence of related DNA on the chromosome of F41-producing strains suggests that an interaction between plasmid and chromosomal genes has occurred during the evolution of these virulence factors. A similar situation has been observed with the genes encoding hemolysin production in *E. coli*. Animal isolates frequently possess a plasmid carrying the hemolysin genes, whereas homologous genes reside on the chromosome of hemolytic *E. coli* associated with urinary tract infections in humans (23).

No homology was observed between the F41 genes and genes encoding the K99 adhesin of ETEC pathogenic for cattle and swine under stringent hybridization conditions, despite the frequent association of these two adhesins in the same bacterial strain. Strain VAC1676 (serotype O101:K30:F41:H$^-$) was chosen for cloning of the F41 gene, since it was one of three virulent strains reported to produce F41 antigen but not K99 antigen. Interestingly, this strain did show homology with a K99 gene probe, but K99 homology was localized to a plasmid. The strain is therefore probably clonally related to the more commonly occurring ETEC strains of serotype O101:K30:K99:F41, but has evidently lost the ability to express K99. The other two virulent strains reported to produce F41 antigen but not K99 antigen (strains KATI1706 and VC1751) (15, 21) similarly demonstrated homology to the K99 gene probe. These strains may be analogous to the laboratory strain B41M, a spontaneous K99-negative mutant of strain B41, an O101:K99:F41 ETEC strain of bovine origin (13). Strain B41M retains plasmid sequences homologous with those of the K99 gene probe (Table 2).

When a large number of animal isolates were screened with gene probes for K88, K99, and F41, all K88 and F41 producers hybridized with both the K88 and F41 gene probes. However, the lack of extensive homology between the amino-terminal amino acid sequences of F41 and K88 (2) is consistent with the presence of some sequences unique to each gene. We are presently attempting to isolate sequences unique to each adhesin for the purpose of preparing specific gene probes and to further study the relatedness of the two adhesins.

It was further observed in the screening of animal isolates that the K99 probe detected some, but not all, strains which produce F41 antigen. Seven of 26 F41 antigen-producing strains were not detected with the K99 probe (Table 2), suggesting that the previously observed association of F41 with K99 (14) is not required for F41 production. These strains may represent isolates which have spontaneously lost a K99-encoding plasmid or a distinct clonal line of F41 producers. The K99 probe did detect K99 antigen-producing strains of serotypes not associated with F41 production. These strains were not detected with the F41 or K88 probes. The K99 probes did not detect strains which produce K88. A previous report suggested that homology exists between genes encoding K88 and K99 (8). This conclusion was based on observations with cloned K88 and K99 determinants and results obtained with wild-type *E. coli* expressing both K99 and F41. However, the data presented here clearly rule out any extensive homology between genes encoding K88 and K99.

TABLE 3. Association of K88, K99, and F41 in animal isolates of *E. coli*

Probe[a]			Antibody[b]		MRHA[c]	Condition of animals		
K88	F41	K99	K88	F41		Healthy	Diarrhea	Other[d]
+	+	−	−	−	−	1	16	10
+	+	+	−	−	−	0	3	0
+	+	−	+	−	−	0	22	4
+	+	−	−	+	+	0	7	0
+	+	+	−	+	+	0	16	3
+	+	−	−	−	+	0	10	6
+	+	+	−	−	+	0	1	1
−	−	−	−	−	−	36	227	134
−	−	+	−	−	−	1	6	5
−	−	−	−	−	+	0	0	1
−	−	+	−	−	+	6	23	31

[a] Strains were hybridized with the indicated probes in colony hybridizations. The K88 probe 3 (Fig. 1) was used.
[b] Strains were tested for K88 and F41 antigens by the agglutination and ELISA tests, respectively.
[c] Strains were tested for agglutination of human group O erythrocytes in the presence of mannose.
[d] Nondiarrheal disease or not determined.

A number of strains were detected with the K88 and F41 gene probes which did not produce K88 or F41 (Table 2). Some of these strains were, however, MRHA positive with human erythrocytes, indicating that an adhesin was being expressed. This suggests that additional types of adhesins may exist which are genetically related to, but antigenically distinct from, K88 and F41. The existence of additional antigenic types of adhesins among ETEC pathogenic for animals may complicate present efforts to control disease with vaccines directed against known adhesin types. Studies of the nature and prevalence of newly recognized adhesins will be facilitated by use of existing gene probes and may provide knowledge applicable to the development of more effective vaccine strategies for the control of diarrheal disease.

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