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Adhesion In Vitro and In Vivo Associated with an Adhesive Antigen (F41) Produced by a K99 Mutant of the Reference Strain *Escherichia coli* B41

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A K99-negative mutant of the K99 reference strain *Escherichia coli* B41 (O101:K99) was isolated (strain B41M). It did not react with OK antiserum to a K-12 K99$^+$ recombinant or with OK antiserum to K99-positive organisms from the O8, O20, or O64 serogroups, but it did react with OK antiserum to K99-positive organisms from the O101 and O9 serogroups. The mutant hemagglutinated sheep erythrocytes and attached in vitro to calf enterocytes when cultured at 37°C but not when grown at 18°C. Attachment was mannose resistant but susceptible to heating and formaldehyde. These properties were associated with the presence of fimbriae. The isolated hemagglutinin migrated to the anode in immunoelectrophoresis experiments, competitively inhibited attachment of strain B41M to calf enterocytes, and could be demonstrated adhering to these cells in vitro by indirect immunofluorescent staining. The anionic hemagglutinin is referred to provisionally as F41. Germfree piglets infected with strain B41M developed diarrhea within 16 h. Scanning electron microscopy showed groups of bacteria adherent to the microvilli of villous enterocytes. Indirect immunofluorescent staining demonstrated the presence of F41 antigen in vivo.

K99 is a plasmid-determined antigen which exists as long filamentous structures (1) on the surface of many *Escherichia coli* enteropathogenic for calves, lambs (9, 12), and occasionally piglets (5). The antigen is important in the attachment of the bacterium to the intestinal mucosa of the host (12), and its presence on an isolate can be a useful indicator of enteropathogenicity. Absorbed factor serum to K99 is usually used to detect the antigen and, since K99 is not expressed by organisms cultured at 18°C, antiserum raised against live reference organisms grown at 37°C is often absorbed with the homologous strain grown at 18°C (3, 8).

We have found that certain K99-producing strains possess additional surface antigens which are also not expressed at 18°C (8). In contrast to K-12 K99$^+$ recombinant strains, the K99 reference strain B41 (O101:K99) produces both of these antigens, which can be demonstrated by immunoelectrophoresis of crude K99 extracts from strain B41. Using antisera to representatives of each K99-producing serogroup, K99 can be demonstrated as a cationic precipitation arc with every antiserum, but an additional anionic antigen can also be demonstrated only with antisera to K99-positive organisms from the O9 and O101 serogroups. Both K99 and the anionic antigen are mannose-resistant hemagglutinins for sheep and horse erythrocytes, and studies using cell free anionic antigen suggested that it might react in vitro with the microvilli of calf enterocytes (8).

We have now isolated a mutant from strain B41 (strain B41M) which does not produce K99 but retains its ability to synthesize anionic antigen. As it is antigenically unrelated to K99, is not found on all K99-positive *E. coli*, and is associated with the presence of filamentous surface structures, the anionic antigen will now be referred to provisionally as F41. This paper describes some of the properties of strain B41M both in vitro and in vivo.

MATERIALS AND METHODS

Bacteria. *E. coli* were grown on sheep blood agar for 18 h at 37 or 18°C. The B41 mutant was isolated from a culture of strain B41 (O101:K99) which had been incubated at 37°C for 18 h followed by 7 days of incubation at 22°C. Growth was picked from the edges of all colonies showing a mixed colony morphology; single colonies were purified by repeated subculturing at 37°C and examined by slide agglutination tests with O101 antiserum and K99 antiserum.

Antisera. OK antiserum was prepared by multiple intravenous injection of live organisms grown at 37°C (14). Antiserum to K99 was prepared by absorbing antiserum to K-12 K99$^+$ bacteria with the K-12 K99$^-$ isogenic strain (1), and antiserum to F41 was prepared by absorbing antiserum to B41M with the live B41M grown at 18°C. Rabbit antiserum to K99 purified by

immunoelectrophoresis was supplied by P. A. M. Guinee (3), and antiserum to K99 purified by ion-exchange chromatography was supplied by R. E. Isaacson (4).

Gel diffusion and immunoelectrophoresis. Ouchterlony double-diffusion tests and immunoelectrophoresis procedures have been described (7).

Immunofluorescent studies. Indirect fluorescent-antibody (IFA) tests on bacteria used rabbit antiserum and fluorescein isothiocyanate-conjugated immunoglobulin G fraction of goat anti-rabbit immunoglobulin G. Immunofluorescent staining of enterocytes was described earlier (8). Briefly, calf enterocytes were incubated with cell-free F41 antigen, washed, and examined by the IFA test.

For the in vivo studies, cryostat sections and smears from the lumen and mucosa were fixed in acetone and examined by the IFA test, using homologous OK antiserum and antisera to F41 and K99. Normal rabbit serum was used as a control.

Hemagglutination. Direct hemagglutination tests using 3% sheep erythrocytes were performed at 4°C in microtiter trays (6). Mannose was used at a final concentration of 0.5%. Inhibition of hemagglutination procedures have been described (8).

Preparation of F41. The antigen was extracted from strain B41M by acetate precipitation and standardized as described for K99 (6).

Preparation of crude K99. Crude K99 was isolated from strain B41 by acetate precipitation (6), and the anionic and cationic constituents were isolated by ion-exchange chromatography (7).

Preparation of purified K99. This was kindly provided by R. E. Isaacson, who had isolated it from a K-12 $K99^+$ recombinant (4).

Adhesion studies. Isolated calf enterocytes (10) were used for the adhesion experiments (8), with strain B41M and cell free F41. Epithelial cell suspensions (100 μl) were incubated at 37°C for 60 min with equal volumes of bacteria. The final concentration of epithelial cells was approximately 10^6/ml, and the bacteria were at a final concentration of approximately 10^9/ml. The number of bacteria adhering to individual epithelial cells was determined by interference microscopy. Drops from the reaction mixture were examined directly under cover slips on glass slides. Aggregates or sheets of tissue were disregarded. The first 20 well-defined epithelial cells were examined in each experiment, and the results from experiments on at least 3 different days were combined to determine the mean number of bacteria adhering per epithelial cell. D-Mannose at a final concentration of 0.5% was present in every experiment. There were no preexisting bacteria on epithelial cells. The method of Duguid et al. (2) was used to study the effect of mannose, Formalin, and growth temperature on bacterial attachment. For competitive inhibition, 100 μl of enterocyte suspension was preincubated with 50 μl of competitor for 30 min at 37°C before the addition of 50 μl of bacterial suspension.

Hydrophobic interaction with phenyl Sepharose. Phenyl Sepharose was packed in glass Pasteur pipettes to a height of 3 cm (13). Overnight blood agar cultures of bacteria grown at 37 or 18°C were harvested in a minimal volume of 4 M NaCl, and the bacterial count was determined. A 100-μl volume of bacteria was applied to the column which was eluted with 5 ml of 4 M NaCl. The first 5 ml of column eluate was collected, and the number of viable bacteria was determined.

Electron microscopy. Overnight cultures of the B41M were grown on moist blood agar slopes at 37°C and gently harvested in distilled water. A carbon-Formvar-coated grid was floated on 1 drop of suspension for at least 30 s, dried on filter paper, floated on 2% phosphotungstic acid (pH 6.6) containing bacitracin (30 μg/ml) for 30 s, dried, and examined immediately by transmission electron microscopy in a Philips EM300 microscope at an accelerating voltage of 80 kV.

For scanning electron microscopy, gut samples were fixed in glutaraldehyde (3% in 100 mM phosphate buffer, pH 7.2) for 2 h, washed, and postfixed in osmium tetroxide (1% in 100 mM phosphate buffer, pH 7.2). After dehydration through graded alcohols and finally acetone, samples were critically point dried in CO_2, sputter coated with gold, and examined in a Cambridge Steroscan MK II at an accelerating voltage of 30 kV.

Bovine enterocytes incubated with strain B41M as described above were examined by interference microscopy to confirm bacterial attachment, fixed in 3% glutaraldehyde in 100 mM phosphate buffer for 2 h at 4°C, washed in phosphate buffer, and postfixed in phosphate-buffered 1% osmium tetroxide for 1 h. The material was then dehydrated through graded alcohols, cleared in propylene oxide, and embedded in Araldite. Ultrathin sections were cut on an LKB Ultratome III with a glass knife and stained with methanolic uranyl acetate and lead citrate. These were then examined in a transmission electron microscope as above.

Experimental animals. Six hysterotomy-derived, colostrum-deprived piglets were maintained in germfree isolators containing two piglets per isolator. When 2 to 4 h old, each group of two piglets was inoculated orally with 8×10^8 to 7×10^9 *E. coli* (see Table 5) in 10 ml of sterilized evaporated cow milk (diluted 2 parts milk plus 3 parts water). Between 16 and 18 h after infection, the clinical status of each piglet was recorded, and segments of small intestine (10 to 20 cm proximal to the ileocecal valve) were removed via laparotomy under halothane anesthesia. Three samples were taken; one was frozen in liquid N_2 for cryostat sectioning and examination by indirect immunofluorescence microscopy, another was fixed in glutaraldehyde for scanning electron microscopy, and a third was examined as follows. The contents of the gut segments were smeared onto slides for IFA staining. Gut segments were then opened and washed thoroughly with saline. The mucosal surface was scraped with a microscope slide, and smears were made for indirect fluorescent microscopy.

RESULTS

The mutant did not autoagglutinate in saline, although agglutination did occur in aqueous acriflavine (1:1,000). Direct mannose-resistant hemagglutination of sheep erythrocytes was demonstrated with the organism grown at 37°C but not with bacteria grown at 18°C. The hemagglutination could be inhibited by absorbing the mutant with OK antisera to *E. coli* strain B41 (O101:K99) or strain B85 (O9:K99), but antise-

TABLE 1. Results of slide agglutination test, using rabbit antisera to live *E. coli*

OK antiserum	Agglutination of:	
	B41	B41M
B85 (O9:K99)	+	+
B44 (O9:K30,K99)	+	+
B42 (O9:K35,K99)	+	+
B111 (O101:K99)	+	+
RVC 1616 (O101:K30,K99)	+	+
431 (O101:K30,K99)	+	+
613 (O101:K30,K99)	+	+
B79 (O101:K32,K99)	+	+
B117 (O8:K85,K99)	+	−
S13 (O8:K85,K99)	+	−
637 (O64:K?,K99)	+	−
K12 $K99^+$ (O-:$K99^+$)	+	−
K12 $K99^-$ (O-:$K99^-$)	−	−

rum to strain B117 (O8:K85,K99) had no effect.

With heterologous antisera to K99-positive *E. coli*, slide agglutination was observed with antisera to enteropathogens from the O101 and O9 serogroups (Table 1), but not with antisera to organisms from the O8 or O64 serogroups, indicating that it lacked K99 antigen. OK antisera to a K-12 $K99^+$ recombinant strain did not react with the mutant. The parent strain B41 was agglutinated by all of the antisera to $K99^+$ strains regardless of serogroup.

To confirm the agglutination results obtained with the K-12 $K99^+$ antiserum, both mutant and parent strain were examined by IFA staining. Absorbed antiserum to K99 from the K-12 $K99^+$ strain, antiserum raised directly to the K99 antigen purified by immunoelectrophoresis, and antiserum to K99 purified by ion-exchange chromatography each failed to react with the strain B41M but reacted strongly with the parent strain cultured at 37°C. There was no fluorescence when the parent strain was grown at 18°C.

Absorbed antiserum to F41 antigen reacted only with K99-positive bacteria from the O101 and O9 serogroups (Table 2). Furthermore, it did not react with these bacteria if they were grown at 18°C, nor did the antiserum react with the K-12 $K99^+$ recombinant strains used by Smith and Linggood (12), Burrows et al. (1), or Isaacson (4).

Demonstration of fimbriae. Negatively stained preparations of strain B41M grown on blood agar slopes at 37°C for 18 h were examined by electron microscopy and revealed fimbriae (approximately 3 nm in diameter) on 30 to 40% of the bacteria (Fig. 1). Less than 1% of bacteria possessed fimbriae when grown at 18°C. Strain B41M was examined by using F41 antiserum in an indirect immunoperoxidase-staining technique (manuscript in preparation). More than 90% of the bacteria were positive when cultured at 37°C, but less than 1% stained when cultured at 18°C. Thus, F41 antiserum may also contain antibodies to nonfimbrial material which is not produced at 18°C and, on its own, this result was not taken as evidence for the fimbrial nature of F41.

Cell-free F41. Cell-free F41 caused direct mannose-resistant hemagglutination of sheep erythrocytes (titer of 2^6) which did not occur when F41 was preabsorbed with antiserum to strain B41 (O101:K99) or strain B85 (O9:K99). Absorption with antiserum to B117 (O8:K85,K99) had no effect.

In immunoelectrophoresis experiments using OK antiserum to the parent strain B41, anionic and cationic antigen was detected in crude K99 extracted from that strain. The F41 antigen gave only an anionic precipitation line, whereas purified K99 gave only a cationic precipitation line.

OK antiserum to B41M produced only an anionic line with crude K99 and F41 but failed to produce any line with purified K99 antigen on immunoelectrophoresis. Conversely, antiserum to the K-12 $K99^+$ strain gave only a cationic precipitation line with crude K99 from the parent strain B41 and with purified K99, but failed to react with F41 antigen from strain B41M.

Gel diffusion experiments demonstrated a line of identity with F41 antigen from strain B41M and the isolated anionic antigen from the crude K99 extract of the B41 parent and a line of identity with K99 from the K-12 $K99^+$ recombinant and the isolated cationic antigen from the B41 parent. We therefore concluded that the anionic antigen from strain B41 was F41 and the cationic antigen was K99.

TABLE 2. Indirect fluorescent antibody staining of live *E. coli* with absorbed antisera to B41 parent and B41 mutant

Strain	Serogroup	Origin	Fluorescence with absorbed antisera[a] to:	
			B41	B41M
B85	O9:K99	Bovine	+	+
B44	O9:K30,K99	Bovine	+	+
B42	O9:K35,K99	Bovine	+	+
B41	O101:K99	Bovine	+	+
RVC 1616	O101:K30,K99	Bovine	+	+
B79	O101:K32,K99	Bovine	+	+
559	O8:K25,K99	Bovine	+	−
B117	O8:K85,K99	Bovine	+	−
B80	O20:K17(?),K99	Bovine	+	−
637	O64:K?,K99	Porcine	+	−
K-12 $K99^+$	O-:$K99^+$	Laboratory	+	−
K-12 $K99^-$	O-:$K99^-$	Laboratory	−	−

[a] OK antisera absorbed with homologous strain grown at 18°.

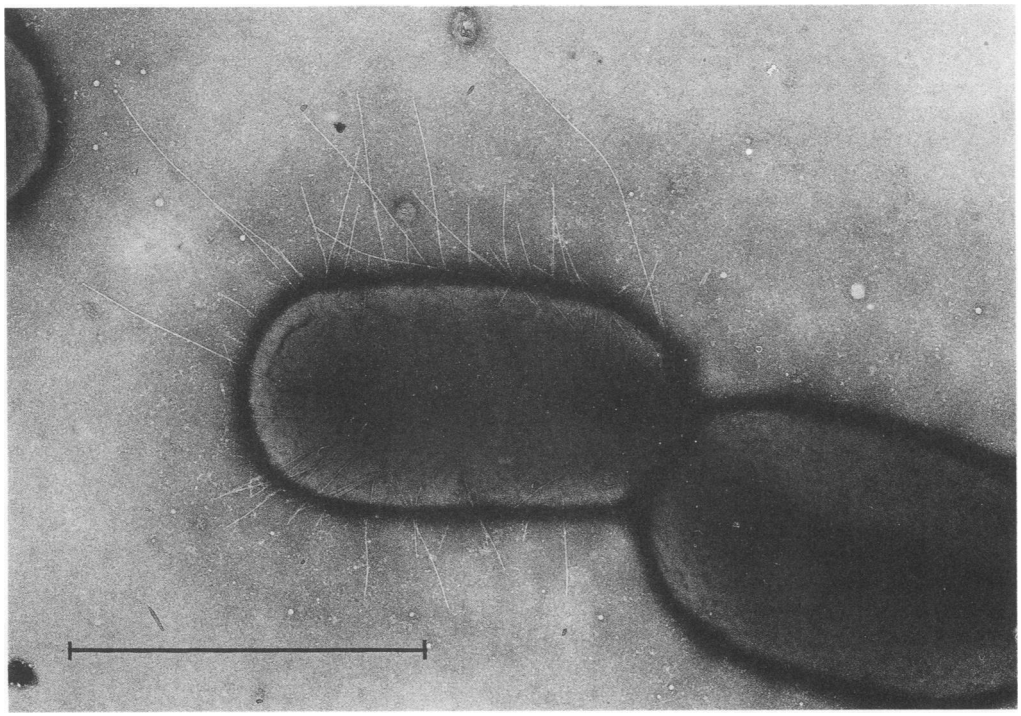

FIG. 1. Transmission electron micrograph of *E. coli* strain B41M showing fimbriae (2% phosphotungstic acid-negative strain). Bar = 1 μm.

Adhesion. In vitro, the parent strain B41 together with the mutant and the K-12 K99$^+$ strain each attached to the hydrophobic gel phenyl Sepharose when the bacteria were grown at 37°C but not 18°C (Table 3).

The B41M bacteria grown at 37°C adhered in vitro to microvilli of calf enterocytes (Fig. 2) but failed to attach when cultured at 18°C (Table 4). The adhesion could be competitively inhibited with F41 antigen, and Fig. 3 shows a direct relationship between the concentration of F41 and the degree of inhibition. Surface antigen extracted from strain B41M cultured at 18°C did not inhibit attachment. Bacteria attached in the presence of 0.5% D-mannose, but prior treatment of the organism with 0.5% formaldehyde for 4 h or heating at 65°C for 30 min prevented attachment (Table 4).

The attachment of cell-free F41 antigen to calf brush borders could be demonstrated by indirect immunofluorescent staining, using absorbed antisera to F41 or OK antisera to strain B41 (O101:K99) or strain B85 (O9:K99). Adhesion of the antigen to enterocytes could not be demonstrated by using antisera to purified K99 antigen or OK antisera to strain B117 (O8:K85,K99), 559 (O8:K25,K99), W1-1 (O20:K?,K99), or 637 (O64:K?,K99).

Adhesion in vivo. Table 5 summarizes the in vivo results. Piglets infected with each of strain B41 and strain B41M had diarrhea within 16 h. The two control piglets infected with the nonenteropathogenic field isolate 31a did not develop diarrhea.

Cryostat sections of small intestine were examined by indirect immunofluorescence microscopy. A discontinuous linear fluorescence with strain B41 was visible closely associated with the microvillous border of villous enterocytes when OK B41, K99, or F41 antiserum was used. There was no fluorescence with normal rabbit serum. When the small intestine from each of the B41M piglets was examined, only small groups of fluorescent bacteria were observed. These were always associated with the microvil-

TABLE 3. Hydrophobic absorption of live bacteria onto phenyl Sepharose

Growth temp (°C)	Absorption			
	B41	B41M	K-12 K99$^+$	K-12 K99$^-$
37				
applied	10^8	3×10^7	5×10^{10}	2×10^8
eluted	<10	<10	10	9×10^7
18				
applied	1.2×10^9	5×10^7	10^7	2×10^7
eluted	6.8×10^8	1×10^7	8×10^6	8×10^6

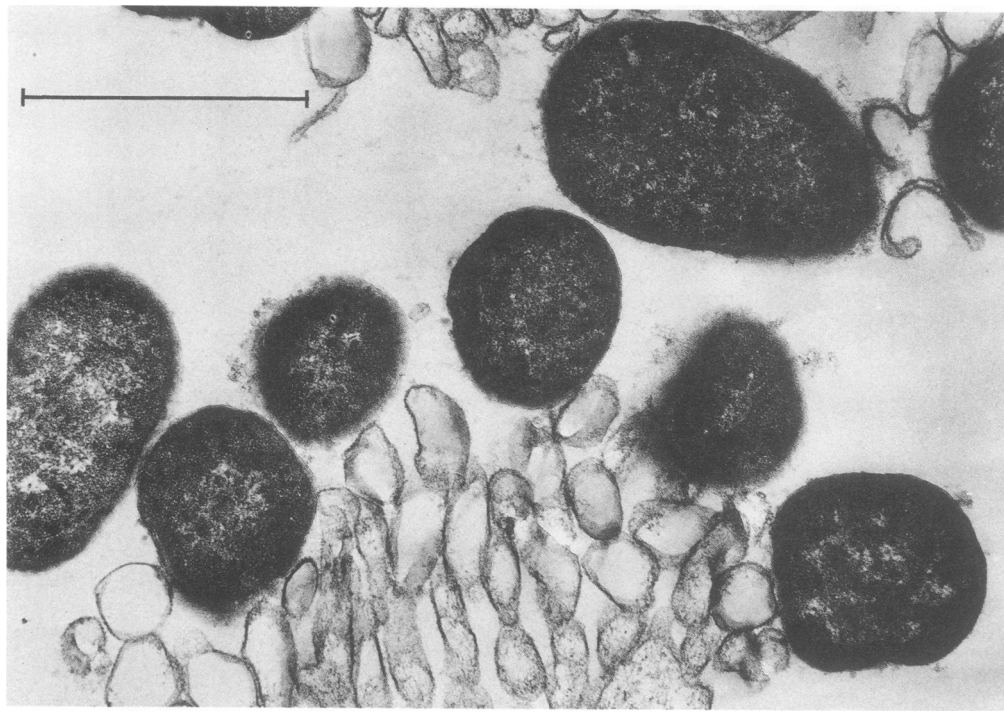

FIG. 2. Transmission electron micrograph showing in vitro adhesion of *E. coli* strain B41M to calf enterocyte (ultrathin section of Araldite-embedded material stained with methanolic uranyl acetate and lead citrate). Bar = 1 μm.

lous border of villous enterocytes and were visible only with OK B41M or F41 antisera (OK B41 antiserum was not used). There was no fluorescence with K99 antiserum or with normal rabbit serum. Fluorescent bacteria were visible only in the lumen in cryostat sections of intestines from piglets infected with 31a using OK 31a antiserum. No bacteria were visible with antisera to K99, F41, or normal rabbit serum.

Immunofluorescence also demonstrated F41 antigen in smears from the mucosa and lumen of the small intestine from piglets infected with B41 and B41M. K99 antigen was detected only in smears from the B41 piglets. Strain 31a organisms were seen in large numbers in smears from the lumen of the control piglets' small intestine, but only when OK 31a antiserum was used.

Scanning electron microscopy of the small intestine demonstrated in vivo attachment of strains B41 and B41M in both piglets from each group. However, in both groups, attachment

TABLE 4. Factors affecting in vitro adhesion of strain B41M to calf enterocytes

Treatment	Adherent bacteria/brush border	
	Mean[a]	Range
None	11.4	8–15
0.5% mannose	10.5	8–15
Growth at 18°C	<0.1	0–2
65°C/30 min	0	
0.5% H.CHO/4 h	<0.1	0–4

[a] Twenty epithelial cells each test on three different days.

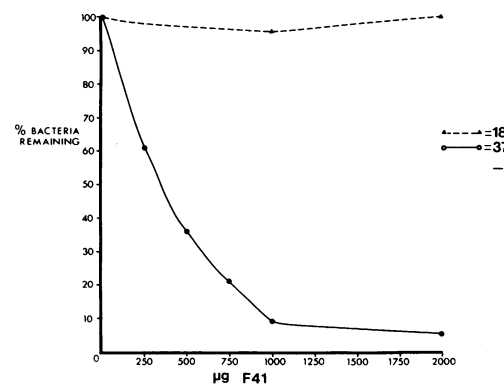

FIG. 3. Competitive inhibition of *E. coli* strain B41M attachment to calf enterocytes by F41 extracts from strain B41M cultured at 37 and 18°C

TABLE 5. Diarrhea, production of adhesin, and adhesion in the small intestine in newborn germ-free piglets (two per group) orally inoculated with different strains of *E. coli*

E. coli strain	Diarrhea	Antigen detected		Adhesion
		K99	F41	
B41	2	+	+	+
B41M	2	−	+	+
31a	0	−	−	−

was focal in contrast to the diffuse attachment observed with strain B44 (O9:K30,K99 results not included). Organisms of strain B41 were usually seen as plaquelike groups of adherent organisms, often at the apex of the villi. Although some villi appeared free of bacteria, others showed multiple discrete plaques of adherent organisms. Strain B41M organisms were seen as occasional small groups on several adjacent villi and sometimes as solitary adherent bacteria. Groups of organisms were often associated with transverse folds on the villous surface (Fig. 4). Fewer villi with adherent bacteria in the small intestine were seen in the B41M-infected piglets than in the small intestine of the B41-infected piglets. Examination of the small intestine mucosa of both piglets inoculated with the nonenteropathogenic strain 31a failed to reveal bacteria. Attempts to produce F41$^-$ variants of B41M for control purposes were unsuccessful.

DISCUSSION

The *E. coli* K99 reference strain B41 produces at least two mannose-resistant hemagglutinins for sheep erythrocytes (7) that are not expressed when the bacteria are grown at 18°C (8). One of these hemagglutinins is cationic and is the K99 antigen (1, 3, 4), whereas another is anionic and will be referred to provisionally as F41.

Antibodies to the F41 antigen have been demonstrated only in antisera to K99-positive *E. coli* from the O9 and O101 serogroups (8), and in the present study inhibition of strain B41M hemagglutination, bacterial agglutination, and immunofluorescent staining of strain B41M occurred only with OK antisera to representatives of these two serogroups. Direct evidence that strain B41M did not produce detectable K99 came when the organisms were examined by using antisera prepared independently at two laboratories from K99 purified by different procedures. In a previous study (8), it was demonstrated that OK antisera to K99-positive *E. coli* from the O9 and O101 serogroups contained antibodies to an anionic mannose-resistant hem-

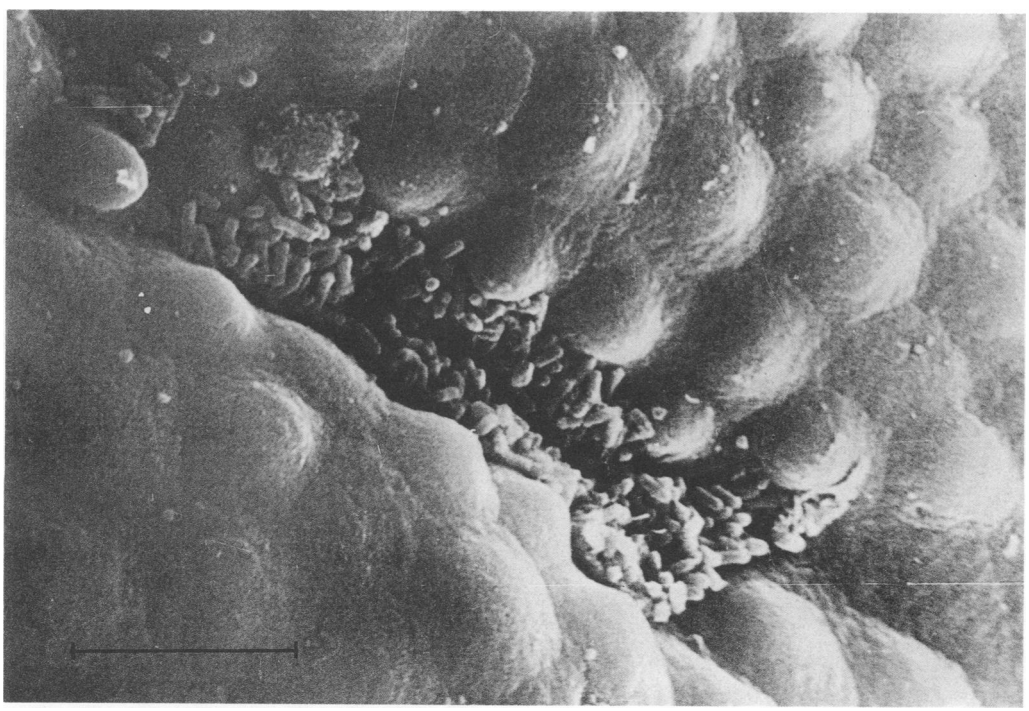

FIG. 4. Scanning electron micrograph showing in vivo adhesion of *E. coli* strain B41M to a villus surface from the small intestine of 18-h-old piglet. Organisms are associated with a transverse fold of the villus. Bar = 10 μm.

agglutinin (i.e., F41 antigen). This has now been confirmed by using F41 antiserum and bacteria from these two serogroups.

The in vitro adhesion of strain B41M organisms correlated with the production of F41 antigen. These observations, together with the demonstration of cell-free F41 antigen adhering to calf enterocytes and its competitive inhibition of the attachment of the whole bacterium, suggest that F41 antigen is responsible for the in vitro adhesion of strain B41M. However, this adhesion is weaker than that of the parent strain B41 (11.4 bacteria/brush border with strain B41M as opposed to 16.4 bacteria/brush border with strain B41; 8). Electron micrographs indicated that F41 is associated with the presence of fimbriae which are observed very infrequently when strain B41M is cultured at 18°C. Nevertheless, our study has only shown a correlation between the presence of F41 antigen and fimbriae. De Graaf (Infect. Immun., in press), on the other hand, has isolated a fimbrial antigen from B41M. This purified antigen reacts with our F41 antiserum and exhibits the same in vitro properties as the partially purified F41 examined by us. We therefore conclude that F41 has a filamentous structure, but so far we have not determined whether the fimbriae observed in de Graaf's study are the same as those observed in our laboratory.

The F41 antigen is associated with the interaction of the organisms with the hydrophobic gel phenyl Sepharose, a property in common with many enteropathogenic *E. coli* and thought to play a role in adhesion (13). Duguid et al. (2) described fimbriae provisionally designated "type MRE" which, in contrast to type 1, are mannose-resistant hemagglutinins, are not produced below 25°C, and are inactivated by Formalin or heating at 65°C for 30 min. The antigen responsible for the attachment of strain B41M to calf enterocytes exhibited similar properties.

The production of diarrhea by a K99-negative variant of a K99-positive strain was reported by Smith and Huggins (11). The bacterial counts of an O9:K30 $K99^-$ form of a calf enteropathogen were $\geq 10^8$ per g of calf or piglet small intestine wall scraping. With the O9:K30,$K99^+$ form, however, the counts were higher and colonization was more effective. In the present study, strain B41M produced diarrhea. Scanning electron microscopy demonstrated in vivo adhesion of strain B41M, and infrequent focal distribution of small groups of adherent organisms was consistent with immunofluorescence observations. Since neither scanning electron microscopy nor immunofluorescence microscopy of small intestine sections could demonstrate bacteria on the mucosa of the control piglets infected with nonenteropathogenic *E. coli*, we consider that the adhesion of strain B41M is significant. Nevertheless, although we have demonstrated that F41 is adhesive in vitro and is produced in vivo, it is possible that the attachment of strain B41M to the piglet small intestine was mediated entirely or in part by unknown adhesive factors.

Since 1978 we have monitored F41 antigen on *E. coli* sent to us for routine examination. So far, F41 has been detected on *E. coli* only from the O101 and O9 serogroups and only on those isolates recovered from outbreaks of neonatal diarrhoea. Clearly, experiments with field strains and the isolation of $F41^-$ variants are necessary to determine whether F41 plays a role in pathogenesis.

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