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Pilus-Mediated Binding of Bovine Enterotoxigenic *Escherichia coli* to Calf Small Intestinal Mucins

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In this study we show that the adhesion to mucus of the enterotoxigenic *Escherichia coli* strains responsible for diarrhea in calves involves a bacterium-mucin recognition phenomenon in which the bacterial pili and specific mucus receptors carried by the glycoproteins (2,000 to 400 kilodalton) play a major role. An adhesion maximum was observed at a pH of less than 6 (4.75 to 5.25). The sialic acids and galactose appeared to be at least partly responsible for the attachment of K99 pili, whereas F41 pili preferentially recognized desialylated receptors. The attachment of different strains of *E. coli* characterized by the presence of the three main pili, K99, F41, and FY, known to be responsible for the binding of enterotoxigenic *E. coli* to the intestinal epithelium of the calf, was studied using Scatchard and Hill analyses. The attachment mechanism of bacteria carrying K99 pili showed positive cooperativity. FY and F41 pili recognized independent receptor sites, the first on sialylated mucus and the second on sialidase-treated mucus. Moreover, F41 pili were found to bind to native mucus according to a negative cooperativity phenomenon. Finally, the recognition sites carried by bacterial pilins may be saturated by some animal glycoprotein glycans which are therefore adhesion inhibitors.

The adhesion of pathogenic bacteria to the mucosa is an essential step in the development of numerous infections and, in particular, in diarrhea (14). In the intestine, the epithelial surfaces are covered and protected by mucus, a gel mainly composed of mucins, polydispersed and high-molecular-weight glycoproteins, and smaller proteins (13). To resist the flux of secretions and to colonize the tissues, it may be sufficient for bacteria to bind to the mucus alone (14). For other strains, including bovine enterotoxigenic *Escherichia coli* (ETEC), adherence to the enterocytes has been demonstrated (5). Even in this case, however, the bacteria also interact with the mucus as shown by recent studies (14, 20, 27). This may then serve as a site of replication and colonization before enteroadherence. In this study, we investigated the adhesion of bovine ETEC to mucus glycoproteins, using the model described by Laux et al. (6, 20). It is known that K99 pili are not the only pili with a role in the colonization of the small intestine; F41 and FY pili are also involved (7, 10, 15, 24). We also defined the main characteristics of bacterial attachment to mucins by studying the adhesion of the reference strain B41 ($K99^+$ $F41^+$). After noting a positive cooperativity in the attachment of this strain to glycoproteins (27), we studied, more systematically, bacterium-mucin binding for *E. coli* carrying F41, FY, and K99 pili alone or in combination. We examined the respective roles of the different pili by using Scatchard and Hill analyses (9). During this study, glycoprotein glycans, which compete for the saturation of bacterial sites (25, 26), were used as inhibitors of bacterial attachment. Their effectiveness in preventing the attachment of bacteria to intestinal mucus justifies their possible use as therapeutic agents in animals.

MATERIALS AND METHODS

Bacterial strains. The characteristics of *E. coli* strains and the bacterial pili K99, F41, and FY used in these experiments are listed in Table 1. The presence of K99 on the appropriate *E. coli* strains was confirmed by agglutination with specific antisera (Iffa-Merieux, Lyon, France). Specific anti-FY serum was kindly supplied by J. P. Girardeau. F41 antiserum was prepared against the B41 A strain and was absorbed by the same strain grown at 18°C.

Radiolabeling of *E. coli*. A 0.1-ml sample of 3-h *E. coli* growth culture was transferred to Minca agar plus Polyvitex (Bio-Merieux, Charbonnières-les-Bains France), containing 100 μl of ^{14}C-labeled sodium acetate (specific activity, 3.4 GBq/mol; CEA, Gif-sur-Yvette, France). Bacteria were grown for 16 to 18 h at 37°C or for 48 h at 18°C. ^{14}C-labeled cultures were harvested, washed three times in 0.05 M phosphate-buffered saline, and finally suspended in the buffer used for adhesion.

The bacterial concentration was determined by the optical absorbance at 570 nm. The viability of samples of cells at different pH values was tested using the trypan blue exclusion method. Cell suspensions containing from 10^4 to 10^{10} cells per ml were used for the analyses. The radioactivity of the cells was from 10^{-2} to 10^{-3} cpm/CFU.

Isolation of the mucus glycoproteins. Crude mucus was obtained from the small intestine of 1- to 4-day-old calves by using a method derived from that of Laux et al. (20). All mucus glycoprotein isolation steps were carried out at 4°C. Fresh intestinal sections were slit open longitudinally and gently rinsed with Hanks buffered saline (pH 7.4). The mucus was separated from the intestinal wall by scraping. The scrapings, in Hanks buffer, were centrifuged at 28,000 × *g* for 15 min to eliminate cellular material. The supernatants were dialyzed overnight (12,000 molecular weight cutoff) against Hanks buffered saline and sterilized on a 0.22-μm membrane (Millipore Corp., Bedford, Mass.). Crude mucus components were fractionated on a Sephadex 6B column (1.5 by 90 cm) by using Hanks buffered saline. Fractions containing glycoproteins were pooled and used for immobilization. The mucus protein concentration was adjusted to 5 mg/ml. Hexoses were measured using the method of Dubois et al. (11), with glucose and galactose as standards.

Immobilization of mucus glycoproteins. Assays were performed in multiwell polystyrene tissue culture plates (Nunc

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TABLE 1. Characteristics of *E. coli* strains

Strain[a]	Serotype	Pilus type	Entero-toxin
B41	O101:H$^-$	F41, K99	ST$^+$
B41 A	O101:H$^-$	F41	ST$^+$
B80	O20:(K17?)	K99	ST$^+$
11A	O101:K32:H9	F41, K99, FY	ST$^+$
5007	O101:?:?	F41, K99, FY	ST$^+$
AY	O101:K32:H19	FY	
16A	O99:K$^-$:H$^-$		

[a] Strain sources: B41, Institut Pasteur, Paris, France; B41 A, Bertin, Institut National de la Recherche Agronomique, Nouzilly Tours, France (2); B80, J. A. Morris, Central Veterinary Laboratory, Weybridge, Surrey, U.K.; 11A, 5007, AY, and 16A, J. P. Girardeau and M. Contrepoix, Institut National de la Recherche Agronomique, Theix, Ceyrat, France (15).

24-well flat-bottom tissue culture plates; well diameter, 1.5-cm; Nunc Laboratories, Roskilde, Denmark). Mucus preparations (0.25 ml) were incubated overnight in wells at 4°C. Unbound proteins were removed by washing twice with 0.5 ml of buffer used for the adhesion tests. A 0.45-mg sample of protein was immobilized in each well.

Adhesion assays. The adhesion assay was derived from the method of Laux et al. (20). Radioactive bacteria (0.25 ml at from 10^4 to 10^{10} cells per ml) were added to wells containing immobilized mucus glycoproteins and incubated for an appropriate time. Each well was then washed twice with 0.5 ml of buffer to remove unbound bacteria, and the washings were collected. Bound bacteria were recovered by incubation with 1.5% sodium dodecyl sulfate (0.5 ml) for 2 h at 37°C. The level of radioactivity of free and bound bacteria in each well was determined by scintillation counting. All assays were performed in triplicate in each experiment.

The following buffers were used for the adhesion assays: buffer 1 (pH 2.5 to 9), 0.21 g of citric acid, 0.35 g of $Na_2HPO_4 \cdot 12H_2O$, 0.03 g of orthoboric acid, and NaOH; buffer 2 (pH 5 to 8.5), 0.15 g of $Na_2HPO_4 \cdot 12H_2O$, 0.06 g of KH_2PO_4, 0.35 g of $NaHCO_3$; buffer 3 (pH 4.5 to 6.5), 0.20 g of sodium acetate and CH_3COOH. The three buffers were complemented with the following salts: NaCl (8 g), KCl (0.4 g), and $MgSO_4 \cdot 7H_2O$ (0.2 g). All the quantities are given for 1liter.

Chemical treatment of bacteria. EDTA, formaldehyde, urea, and guanidine hydrochloride (Merck, Darmstadt, Federal Republic of Germany) and dithiothreitol and L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, Mo.) were dissolved in 0.02 M sodium acetate-buffered saline, and the pH was adjusted to 5.2 with 0.1 N CH_3COOH. All chemicals were reagent grade. Bacteria (10^9 in 0.25 ml) and an equal volume of the reagents (at a final concentration of from 0.5 to 1.5 M) were mixed for 6 h at 37°C (pH 5.2). Treated bacteria were centrifuged and washed with the buffer before the assays.

Enzymatic treatment of mucus glycoproteins. *N*-Acetyl neuraminosyl glycohydrolase (EC 3.2.1.18) (neuraminidase) from *Clostridium perfringens*, β-D-galactoside galactohydrolase (EC 3.2.1.23) (β-galactosidase) from *E. coli*, and trypsin (EC 3.4.21.4) were purchased from Boehringer (Mannheim, Federal Republic of Germany). Enzyme was added directly to wells containing immobilized glycoproteins (0.2 IU of neuraminidase, 3 IU of β-galactosidase, 0.2 IU of trypsin). Treatment was conducted for 1 h at 37°C in acetate-buffered saline (pH 5.2). Wells were washed twice before assays.

Sodium metaperiodate oxidation of mucus glycoproteins. Immobilized glycoproteins were incubated with sodium metaperiodate (0.01 M) in acetate-buffered saline (pH 5.2) in the dark at 37°C for 1 h and washed before assays.

Glycoprotein glycans: inhibitors of bacterial adhesion to mucins. Production of inhibitors from bovine plasma was patented. Briefly, bovine plasma was hydrolyzed for 6 h at 50°C by two nonspecific proteases, *Bacillus subtilis* subtilisin and *Bacillus thermoproteolyticus rokko* thermolysin (3, 4). The glycoprotein glycans were isolated by eliminating the enzyme and peptides, concentrated, washed, and purified (25).

RESULTS

Separation of glycoproteins from the other constituents of the mucus. Crude calf mucus was fractionated by filtration on Sephadex 6B gel (Fig. 1). The two glycoprotein fractions obtained (A and B) had mean molecular weights of between 2×10^6 and 4×10^5 for A and 4×10^5 and 10^5 for B. The use of the glycoprotein and protein fractions as supports for the adhesion test showed that it is mainly the glycoproteins that are recognized by *E. coli* B41. Bacterial adhesion to fractions A and B was, respectively, 10-fold and 5-fold greater than to protein fractions C and D, or to bovine serum albumin used as reference protein, added to the control wells. The sugar contents of the pooled receptor-containing fractions A and B obtained by gel filtration of crude mucus were 350 μg of hexose per mg of protein and 27 μg of sialic acids per mg of protein.

Attachment of *E. coli* to the mucus glycoproteins: optimum pH and temperature effects. Bacterial recognition of the mucus glycoproteins (mucins) was strongly dependent on the pH (optimum pH 4.75 to 5.25) and independent of the nature of the ions employed (Fig. 2). Whereas adherence to the bare support (polystyrene) was nearly independent of the temperature used, bacterial attachment to the mucus glycoproteins at the optimum pH was three times greater at 37°C than at 4°C (Fig. 3). In all the tests, the saturation of the support (mucus or polystyrene) obtained in 30 min remained stable for 1 h.

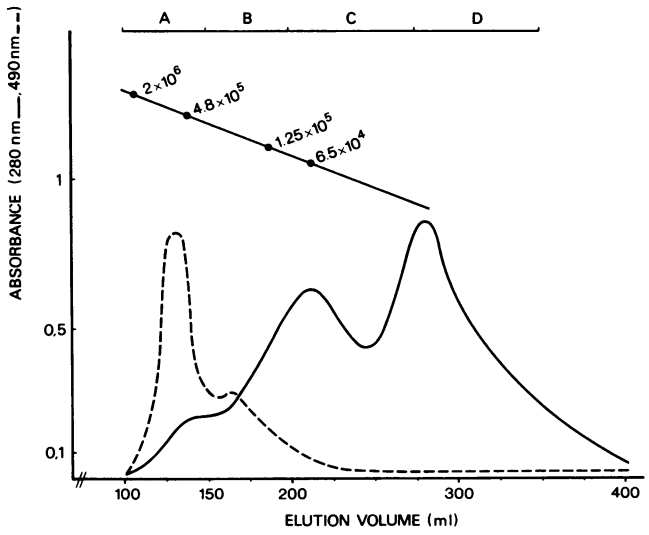

FIG. 1. Separation on Sephadex 6B of the mucus components prepared from calf intestine. Molecular weight standards: blue dextran (2×10^6), ferritin (4.8×10^5), aldolase (1.25×10^5), and bovine serum albumin (6.5×10^4). Solid line, Absorbance at 280 nm (proteinic material); dashed line, absorbance at 490 nm (hexose measurements). Fractions A, B, C, and D were pooled for binding tests.

Effect of dissociating agents. The action of dithiothreitol, L-cysteine hydrochloride, and sodium sulfite on the whole bacteria provoked a reduction in the attachment by cleaving the disulfite bonds of the various pili and other adhesive factors which might be altered by these products (Table 2).

Urea and guanidine hydrochloride, at nondenaturing concentrations, partially disorganized the structure of the pili and also led to an inhibition of attachment (Table 2).

Effect of chemical and enzymatic modifications of the mucins on adhesion. The oxidation of the monosaccharides composing the mucosal glycans by sodium metaperiodate and the enzymatic elimination of sialic acids (75% removed; Table 3) and galactose considerably modified the attachment of *E. coli* B41 (Table 3). The presence of glycoproteins on the support (action of trypsin and EDTA) was of course necessary, but the maintenance of the native conformation was not indispensable (action of formaldehyde). Only the integrity of the monosaccharides (action of metaperiodate) and, in particular, the presence of sialic acids and galactose seemed to be important for the attachment of strain B41. It should, however, be noted that when the strain only had F41 pili (B41 A) the action of sialidase strongly stimulated bacterial attachment, indicating that the mucosal receptors of F41 and K99 pili differed at least in their sialic acid content.

Inhibition of the attachment of *E. coli* B41 to mucins. *E. coli* B41 and its variant B41 A adhered to the mucus glycoproteins in the presence and absence of D-mannose (2%) or mannans (2%). The simple sugars usually present in the mucin oligosaccharides, i.e., sialic acids, galactose, *N*-acetylgalactosamine, glucosamine, and glucose (13), did not inhibit the adhesion of these strains in the concentration range tested (0.1 to 5%; results not shown). On the other hand, the glycans isolated from bovine plasma glycoproteins (25) competively inhibited the adhesion of bacteria to the mucus glycoproteins (Fig. 4). Hence, 1 μg of glycans inhibited the adhesion of $7 \times 10^4 \pm 1 \times 10^4$ bacteria. It may also be noted that this inhibition, like adhesion, depended on the

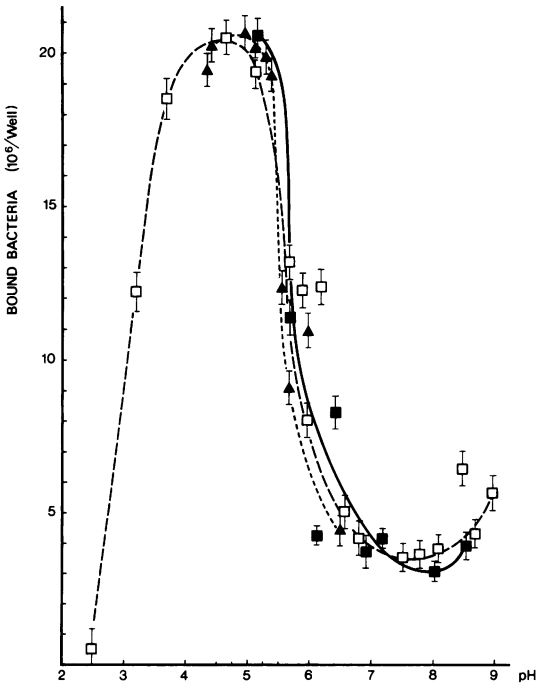

FIG. 2. Effect of pH on bacterial attachment to mucus glycoproteins. Saturated solutions (10^9 cells per ml) of strain B41 (K99$^+$ F41$^+$) were added to wells containing immobilized mucus glycoproteins as described in the text. Symbols: □, buffer 1 (pH 2.5 to 9); ■, buffer 2 (pH 5 to 8.5); ▲, buffer 3 (pH 4.5 to 6.5). For buffer compositions see Materials and Methods. The mean ± standard error of the mean (SEM) (bars) is given for triplicate samples. In this experiment, the specific activity of labeled *E. coli* B41 grown at 37°C was 2.2×10^{-3} cpm/CFU.

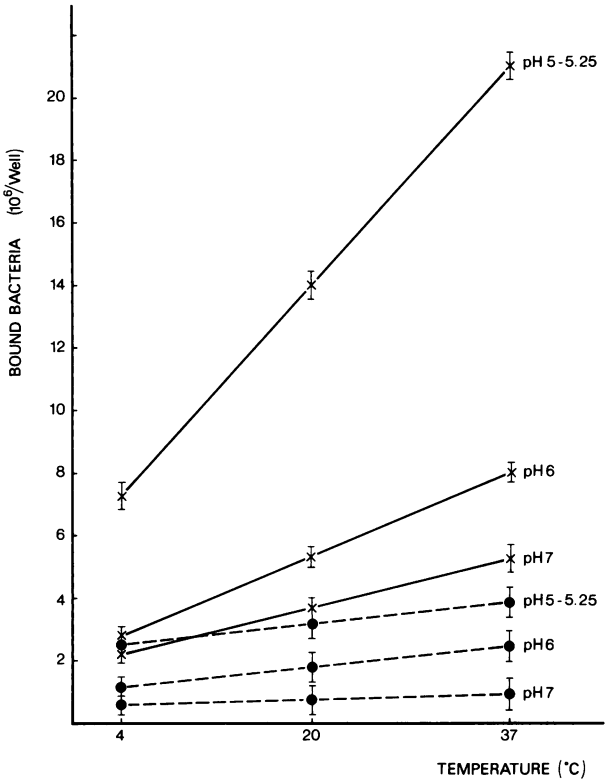

FIG. 3. Effect of temperature on the adhesion of *E. coli* (K99$^+$ F41$^+$) at different pH values. The adhesion of labeled bacteria to mucus glycoproteins (●) and polystyrene (×) was tested as described in the text. The specific activity of labeled *E. coli* grown at 37°C was 2.4×10^{-3} cpm/CFU. The mean ± SEM (bars) is given for triplicate samples.

TABLE 2. Effects of chemicals on piliated *E. coli* strains[a]

Treatment		% of control adhesion (mean ± SE)	
Chemical	Concn (M)	*E. coli* K99$^+$ F41$^-$	*E. coli* F41$^+$ K99$^-$
Dithiothreitol	0.5	56 ± 13	43 ± 2
	1	21 ± 7	ND
L-Cysteine hydrochloride	0.5	57 ± 4	32 ± 1
	1	17 ± 0.1	22 ± 0.5
Sodium sulfite	0.5	41 ± 7	49 ± 4
	1	13 ± 3	ND
Urea	0.5	21 ± 1	18 ± 3
	1	22 ± 1	16 ± 2
Guanidine hydrochloride	1	13 ± 7	16 ± 3
	1.5	12 ± 5	16 ± 1

[a] 10^9 *E. coli* (strains B41 A K99$^-$ F41$^+$ and B80 K99$^+$ F41$^-$) and an equal volume of reagents (0.25 ml) were mixed for 6 h at 37°C and pH 5.2. The controls were incubated without reagents. The adhesion of untreated *E. coli* B41 A and B80 was $4 \times 10^6 \pm 0.2 \times 10^6$ and $20 \times 10^6 \pm 1 \times 10^6$ per well, respectively. In this experiment, the specific activities of *E. coli* B41 and B41 A grown at 37°C were 2.5×10^{-3} and 3.9×10^{-3} cpm/CFU, respectively. ND, Not done.

TABLE 3. Binding of *E. coli* B41 and B41 A variant on native and modified mucosal receptors[a]

Pretreatment of mucosal receptors[b]	Adherent bacteria			
	B41		B41 A	
	Cells/well (10^6)	% of control adhesion (mean value ± SE)	Cells/well (10^6)	% of control adhesion (mean ± SE)
No pretreatment	20 ± 1	100	4 ± 0.2	100
Formaldehyde	19 ± 1	95 ± 4	ND	ND
Sodium metaperiodate	14 ± 2	70 ± 14	3 ± 0.2	75 ± 9
Neuraminidase[c]	10 ± 1	50 ± 7	8 ± 0.5	200 ± 21
Neuraminiase + β-galactosidase	7 ± 0.5	35 ± 4	ND	ND
Trypsin	2.4 ± 0.2	11 ± 1	2.1 ± 0.1	52 ± 5
EDTA	2.3 ± 0.1	11 ± 1	ND	ND

[a] All the assays were performed in triplicate at 37°C. ND, Not done.

[b] Pretreatments were performed in acetate-buffered saline (pH 5.2) (0.25 ml) for 1 h at 37°C. The immobilized glycoproteins were treated with 1% formaldehyde, 10 mM metaperiodate, 0.2 IU of neuraminidase per well, and 3 IU of β-galactosidase per well. Trypsin (0.2 IU per well) and 10 mM EDTA were added to remove the immobilized mucus glycoproteins. After the cells were washed twice with buffer, triplicate adhesion assays were performed. Controls were incubated under the same conditions without the reagents or enzymes.

[c] Treatment of mucus glycoproteins with neuraminidase resulted in sialic acid contents of from 27 to 6.6 μg of sugar per mg of protein. In this experiment, the specific activities of labeled *E. coli* B41 and B41 A grown at 37°C were 2.1×10^{-3} and 1.7×10^{-3} cpm/CFU, respectively.

pH (Fig. 5) and that there was a reduction in the inhibitory capacity of the glycans at pH 7 to 7.5.

Adherence of *E. coli* to mucins according to the pili expressed at their surface. The different bacterial strains studied were characterized (Table 1) either by the presence of one of the three pilus types, F41, K99, and FY, today known to be responsible for the attachment of ETEC in calves, or by the combined presence of two or three of these pili. The adhesion of *E. coli* to the mucus glycoprotein receptors reached a different level of maximum saturation depending on the pili carried by the bacteria (Table 4). Clear adhesion was observed when K99 pili were synthesized (20×10^6 bacteria per well), whereas adherence remained at a lower level when F41 and FY pili were expressed alone (3.5×10^6 to 4×10^6 bacteria per well). ETEC carrying simultaneously all three pili (F41, FY, and K99) adhered more strongly (25×10^6 to 27×10^6 bacteria per well). The level of adhesion of nonpiliated bacteria remained low.

Scatchard plots of the bacterium-mucin interaction. The kinetics of the binding reaction were analyzed by Scatchard plots. Scatchard's equation, $B/F = K(N - F)$, where N is the number of occupable sites, K is the apparent binding constant, B is the bound ligands (here the bacterial), and F is the unbound ligand (free bacteria), was plotted for all the adherent strains. The apparent affinities of receptors for the ligands and the total number of potential receptors, obtained by extrapolation, are presented in Table 5.

The plots showed a very pronounced maximum for all the strains carrying at least K99 pili (Fig. 6). This nonlinear plot suggests a phenomenon of positive cooperativity (29). Bacteria not possessing K99 pili did not follow the same saturation law, and in this case the plots were slightly concave or became linear (Fig. 7). Concave Scatchard plots can be explained by heterogeneity of binding sites or can be due to negatively cooperative interaction (32).

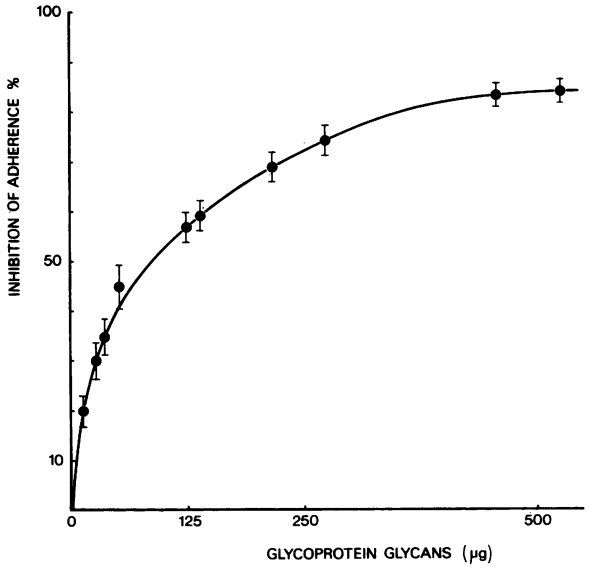

FIG. 4. Inhibition of the adhesion of *E. coli* B41 to mucins by plasma glycoprotein glycans. Glycoprotein glycans (50 μl) were added to wells containing immobilized mucus. Bacteria (4×10^7 per well in 0.25 ml) were then added, and the ability of the *E. coli* B41 to adhere was tested by incubation for 45 min at 37°C (pH 5.2). The mean ± SEM (bars) is given for triplicate samples.

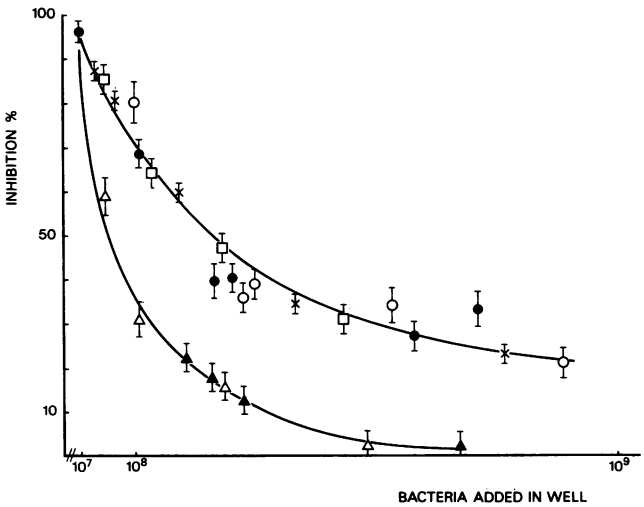

FIG. 5. Effect of pH on the inhibition of *E. coli* B41 binding by plasma glycoprotein glycans. The inhibition of adhesion by plasma glycoprotein glycans was measured at pH 5.2 (×), 6 (●), 6.5 (○), 7 (△), 7.5 (▲), and 8 (□). Bacteria (0.25 ml) were incubated with glycans (500 μg) for 45 min at 37°C in wells containing immobilized mucins. The mean ± SEM (bars) is given for triplicate samples.

TABLE 4. Adhesion of piliated and nonpiliated *E. coli* to native mucins

Strain	Pili	Growth temp (°C)	Bound bacteria/ well (10^6) (mean ± SE)	% Adhesion (mean ± SE)
B80[a]	K99	37	20 ± 1	100 ± 5
5007	K99, F41, FY	37	27 ± 0.5	135 ± 4
11A	K99, F41, FY	37	25 ± 1	125 ± 5
11A	None	18	2.3 ± 0.2	11 ± 1
B41	K99, F41	37	20 ± 1	100 ± 5
B41	None	18	2.7 ± 0.1	13 ± 1
B41 A	F41	37	4.0 ± 0.2	20 ± 1
AY	FY	37	3.5 ± 0.4	17 ± 1
16A	None	37	1.5 ± 0.2	7.5 ± 0.5

[a] Strain B80 was taken as a reference with 100% adhesion. Bacteria were added under saturated conditions (10^9 cells per ml) to wells containing immobilized mucus glycoproteins. In this experiment, the specific activities of *E. coli* grown at 18 and 37°C were between 10^{-2} and 10^{-3} cpm/CFU. Data are given for a pH of 5.2. Adhesion tests were carried out for 45 min at 37°C in triplicate.

Hill plots of the bacterium-mucin interaction (study of the mechanism). The Hill plots of the log(occupied sites/free sites) as a function of log(free ligand) were linear within the range of the experimental values for the various bacterial concentrations. The value of the Hill coefficient n_H (obtained by calculating the slope of the line) may be used to define the type of interaction between the ligand and its receptor (9). Hence, if the value of this coefficient is 1, this means that the sites are independent one from another and there is no cooperativity. If the value is greater than 1, there is positive cooperativity (29), and if it is less than 1 there is negative cooperativity (32). According to this analysis, several mechanisms may be distinguished depending on the pili and the surface proteins involved (Table 6).

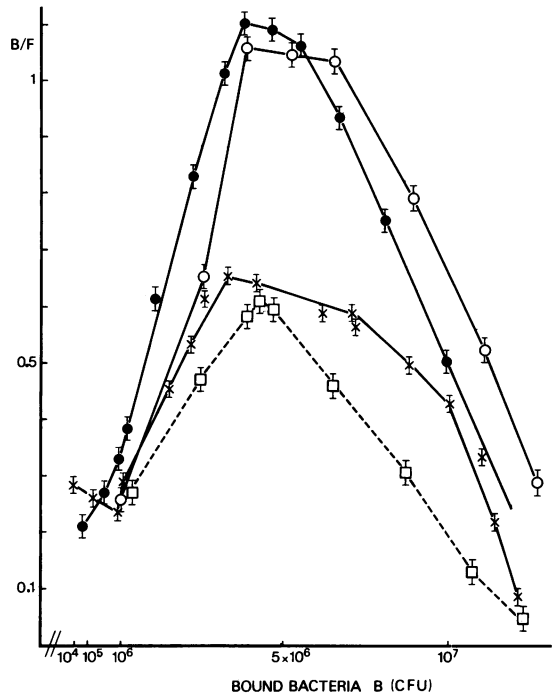

FIG. 6. Scatchard plots representing the binding of piliated *E. coli* K99$^+$ to mucus glycoproteins. The following strains were used: 5007 (K99, F41, FY) (●), 11A (K99, F41, FY) (○), B41 (K99, F41) (×), and B80 (K99) (□). Bacteria (0.25 ml at 10^4 to 10^{10} cells per ml) were added to wells containing immobilized mucins. Binding tests were carried out for 45 min at 37°C (pH 5.2). Unbound bacteria (F), obtained by washing, were collected, and bound bacteria (B) were recovered as described in Materials and Methods. In these experiments, specific activities of labeled *E. coli* strains grown at 37°C were between 3.02×10^{-3} and 5.1×10^{-3} cpm/CFU. The mean ± SEM is given for triplicate assays.

DISCUSSION

It is only recently that studies on the role of mucus, the gel covering the intestinal epithelial cells, in the attachment of bacteria colonizing the digestive tract have been carried out (6, 27; M. Lindahl, I. Carstedt, and T. Wadström, Abstr. FEMS Symposium on Molecular Biology of Microbial Pathogenicity, Luleå, Sweden, 1985, P25). To colonize the mucus, the replication rate of a bacterium in the mucus must be greater than that of the process expelling it into the lumen of the digestive tract. It is also important to know whether the ability of bacteria to adhere to the mucus enables them to colonize the intestine more successfully than bacteria which to not adhere to the mucins. Cohen et al. (6) reported that the specific receptors of the mucosal glycoproteins ensured the initial attachment of the *E. coli* strains that colonize the mouse intestine. In diarrheic calves, the ETEC frequently carry K99 or F41 pili or both (2, 23). It has not yet been clearly shown whether the colonization of the calf intestine results from specific attachment of these strains to the epithelial carbohydrate receptors or whether the intestinal mucus plays a role in this colonization and therefore in the appearance of the diarrheal syndrome.

In this study, we show that the piliated, calf-pathogenic ETEC adhere 10 times more strongly to mucus glycoproteins than to bovine serum albumin. The main receptors responsible for adhesion appear to be glycoproteins with a molecular weight of between 2×10^6 and 4×10^5. Bacterial attachment was dependent on both temperature and pH, and the optimal pH was found to be from 4.75 to 5.25.

The results also showed that there was appreciable binding of bacteria to immobilized mucus at pH values of between 5.5 and 6.5, the physiological pH of the healthy calf gut. The in vitro binding was consistent with the fact that

TABLE 5. Mucin-fimbriae interactions

Strain	Pili expressed	Pericellular proteins	No. of binding sites[a]	$K_{d_{app}}$[b]
16A	None	?	ND[c]	ND
11A (18°C)	None	K32, H9	ND	ND
AY	FY	K32, H19	198×10^4	1.1×10^{-7}
B41 A	F41	None	ND	ND
B41 A[d]	F41	None	454×10^4	6.25×10^{-8}
B80	K99	K17?	141×10^5	4.4×10^{-8}[e]
B41	K99, F41	None	113×10^5	8.2×10^{-8}[e]
11A	K99, F41, FY	K32, H9	169×10^5	6.2×10^{-8}[e]
5007	K99, F41, FY	?	243×10^5	4.2×10^{-8}[e]

[a] Number of potential binding sites expressed as number of bacteria per square centimeter (surface of well, 1.76 cm^2).

[b] The values for affinities were determined in a first approximation for data (Fig. 6 and 7) and were done after the validity of the extrapolation was verified by the method of Klotz (18).

[c] ND, Not determined. The total number of receptor sites for these cells and consequently the binding constant may not be established from the data.

[d] Values obtained on desialylated mucus.

[e] Extrapolations were obtained at high saturation, giving an average value of the highest apparent dissociation constant, $K_{d_{app}}$.

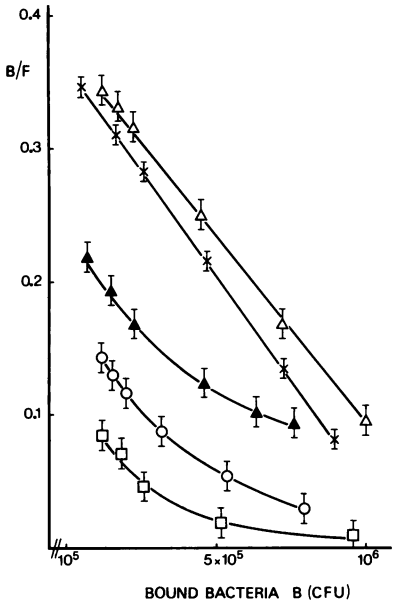

FIG. 7. Scatchard plots of *E. coli* K99$^-$ binding to mucus glycoproteins. The following strains were used: 16A (○); 11A grown at 18°C (K99$^-$, F41$^-$, FY$^-$) (□); AY (FY) (×); and B41 A (F41) on native mucus (▲) and on neuraminidase-treated mucus (△). Bacteria (0.25 ml at 10^5 to 10^{10} cells per ml) were added to wells containing immobilized mucins. Binding tests were carried out for 45 min at 37°C (pH 5.2). Unbound bacteria (F), obtained by washing, were collected, and the bound bacteria (B) were recovered as described in Materials and Methods. The specific activities of the labeled bacteria were between 3.7×10^{-3} and 4.9×10^{-3} cpm/CFU. The mean ± SEM is given for triplicate assays.

upon descending into the intestine, where the pH varies from 5 to 6.4, the ingested bacteria may adhere and proliferate in the early part of the gut before the colonization of the other intestinal segments (25). Furthermore, at the optimal binding pH, adhesion was increased but the binding mechanism was not modified (results not shown).

The role of the spatial structure and the integrity of the pili in the recognition of hemagglutination receptors has been demonstrated (1, 16, 17). The maintenance of integrity of bacterial adhesins was studied here, on whole bacteria, by using dissociating chemicals. Pretreatment of the fimbriated strain B41 decreased its ability to adhere to the mucus glycoproteins. The possibility that the chemicals altered not only pili but also other adhesive factors such as pericellular structures cannot be excluded. It was noted, however, that the weak adhesion of nonfimbriated cells (grown at 18°C) was slightly modified (less than 10%) after treatment with 0.5 M dithiothreitol or urea (results not shown). Together, these data suggest that adhesion requires the maintenance of the spatial structure of the pili and their chemical integrity.

Although we have not yet determined the oligosaccharide structures of the glycoproteins involved in attachment, the observed difference of binding to sialylated or desialylated mucus between the two strains B41 and B41 A indirectly suggests that the pili receptors are chemically different. The reference strain B41 recognizes both sialic acid and galactose residues of the mucins (Table 3), and K99 pili have been shown to be specific for sialic acids (12, 21, 33). At the same time, *E. coli* B41 A, a variant not possessing K99 pili but expressing F41 pili (2), also adheres to mucus treated with *C. perfringens* sialidase (Table 3). It is conceivable that enzymatic removal of sialyl groups from oligosaccharide receptors may expose internal sites not normally available to the pili. Our results are in good agreement with previous studies suggesting that F41 pili are specific for *N*-acetylgalactosamine and have some affinity for *N*-acetylglucosamine (M. Lindahl and T. Wadström, Abstr. VIII Int. Symp. Glycoconjugates, Houston, Texas, 1985, p. 366).

The adherence of *E. coli* strains to the mucus glycoproteins has been analyzed by Scatchard and Hill plots. Use of binding isotherms and plotting data describing ligand and receptor binding in complex and heterogeneous biological systems has been shown to be broadly valid (9, 19, 22).

The shapes of Scatchard curves and the additional support of the Hill method provide information about the nature of the interaction between bacterial adhesins and mucus receptors. Scatchard plots clearly reveal differences depending on the fimbriae or surface proteins involved in attachment. The analysis of the mechanism shows that the K99 pili play a special role. The adhesion of all the ETEC possessing K99 pili took place according to a mechanism with positive cooperativity (Fig. 6). Moreover, when the data were replotted to obtain Hill coefficients, n_H values were always greater than unity (Table 5).

With strain B41 A (F41$^+$), the mechanism of adhesion showed negative cooperativity when the target was the native mucus, and this phenomenon was also observed with the nonfimbriated strains expressing only the pericellular proteins (n_H values of less than unity). When the mucus was desialylated (neuraminidase treatment), the attachment of strain B41 A then followed a saturation law without site-site

TABLE 6. Relation between pili and the mechanism for adhesion of *E. coli* on mucus

Pili expressed	Pericellular structures	Hill coefficient (n_H)[a] (mean ± SE)	Interpretation
K99	K17?	1.20 ± 0.03*	Positive cooperativity
K99, F41		1.20 ± 0.03*	Positive cooperativity
K99, F41, FY	K32, H9	1.60 ± 0.08*	Positive cooperativity
K99, F41, FY	?, ?	1.75 ± 0.02*	Positive cooperativity
FY	K32, H19	1.00 ± 0.01*	No cooperativity, one type of independent site
None	K32, H19	0.7 ± 0.03*	Negative cooperativity
None	K32, H9	0.61 ± 0.01*	Negative cooperativity
F41		0.54 ± 0.04*	Negative cooperativity
F41		1.00 ± 0.02**	No cooperativity, one type of independent site
K99, F41		0.86 ± 0.12***	Negative cooperativity (27)

[a] The values of n_H were determined from the slopes of the Hill plots. These values were measured for native mucus (*) or desialylated mucus (**) and in the presence of glycoprotein glycans on native mucus (***). Correlation coefficients of straight lines from 0.940 to 0.995 were obtained by multiple linear regression of experimental data.

interactions in terms of the observed range of data. This was also the case for *E. coli* FY^+ on native mucus.

Several authors have pointed out some potential shortcomings of plot methods (18, 19, 22, 28). In particular, when the graphs are nonlinear it becomes problematic to extrapolate the Scatchard plots to obtain the total number of receptor sites and binding constants. Aware of the limitations of the method, we noted some difficulties in our attempt at quantitative extraction of binding characteristics (Table 5) determined for the data illustrated in Fig. 6 and 7. Thus, the apparent K_d values are average numbers. For strains possessing K99 pili, analysis suggests the existence of high-affinity binding sites ($K_{d_{app}} = 1.2 \times 10^7$ to 2.3×10^7), but low-affinity binding sites may not be determined. Furthermore, the numbers of potential binding sites were the total binding sites observed (low- and high-affinity sites).

Our results point out that the various mucus glycoprotein glycans allow the attachment of ETEC at least by the three identified pili (K99, F41, FY). The adhesive capacity of bacteria, presumed to be related to the number of pili present on the strains (30, 34), might account for some of the differences in the levels of saturation seen in Table 4. However, this finding may be due to a difference in the number of native mucus receptors for the examined pilus type rather than to the number of pili present on the surface of the various strains, since desialylation of mucus modified the relative numbers of K99 and F41 pili receptors (Table 3). When the bacteria carried both K99 and FY pili, the population density on a given surface was increased, although the apparently higher affinity constant was not greater than that observed for ETEC $K99^+$ FY^- strains (Table 5). F41 and FY pili, by themselves, permit an increase in binding density because they recognize different structures from those recognized by K99 pili.

The association of several pili at the surface of a single bacterium therefore leads to a more effective colonization of the intestine. The glycan composition of mucus may vary in different individuals and at different periods (31). In the same way, the expression of bacterial adhesins in vivo may adapt to the expression of receptors during colonization. This leads to a variability in the nature and density of the receptors accessible to the bacterial adhesins and a variability in the intestinal colonization depending on the type of pili.

The inhibition of attachment that may be observed in vitro (Fig. 4 and 5) in the presence of plasma glycoprotein glycans justifies the therapeutic use of these compounds, which are analogs of the mucus receptors and provide a possible method for treating diarrhea caused by ETEC (27). The efficiency of inhibition of ETEC $K99^+$ adhesion could be explained by the fact that the main glycoprotein glycans obtained from adult bovine plasma contain several oligosaccharides with at least Neu-5-Gc or Neu-5-Ac-(α-2,3)-Gal nonreducing terminal sequence (8) as the equine hematoside, a glycolipid receptor recognized by the K99 pili (33).

LITERATURE CITED

1. **Bartus, H., P. Actor, E. Snipes, D. Sedlock, and I. Zajacs.** 1985. Indications that erythrocyte receptor involved in enterotoxigenic *Escherichia coli* attachment is a sialoconjugate. J. Clin. Microbiol. **21**:951–954.
2. **Bertin, A.** 1985. F41 antigen as a virulence factor in the infant mouse model of *Escherichia coli* diarrhoea. J. Gen. Microbiol. **131**:3037–3045.
3. **Bressollier, P., and R. Julien.** 1985. Immobilisation d'une protéase dans un réacteur enzymatique optimisé à fibres creuses, p. 408–412. *In* A. Faure (ed.), Technologies de purification des protéines. INPL, Nancy, France.
4. **Bressollier, P., M. Mouricout, and R. Julien.** 1985. Purification à l'échelle pilote d'une protéase bactérienne thermostable (EC 3.4.24.4) par membrane d'ultrafiltration, p. 404–407.
5. **Burrows, M. R., R. Sellwood, and R. A. Gibbons.** 1976. Haemagglutination and adhesive properties associated with the K99 antigen of bovine strain of *Escherichia coli*. J. Gen. Microbiol. **96**:269–275.
6. **Cohen, P. S., J. C. Arruda, T. J. Williams, and D. C. Laux.** 1985. Adhesion of a human fecal *Escherichia coli* strain to mouse colonic mucus. Infect. Immun. **48**:139–145.
7. **Contrepoix, M. G., and J. P. Girardeau.** 1985. Additive protective effects of colostral anti-pili antibodies in calves experimentally infected with enterotoxigenic *Escherichia coli*. Infect. Immun. **50**:947–949.
8. **Corfield, P., and R. Schauer.** 1982. Occurrence of sialic acids, p. 5–50. *In* R. Schauer (ed.), Sialic acids: chemistry, metabolism and function. Springer-Verlag, Vienna.
9. **Dahlquist, E. W.** 1978. The means of Scatchard and Hill plots. Methods Enzymol. **48**:270–299.
10. **de Graaf, F. K., and I. Roorda.** 1982. Production, purification, and characterization of the fimbrial adhesive antigen F41 isolated from calf enteropathogenic *Escherichia coli* strain B41M. Infect. Immun. **36**:751–758.
11. **Dubois, M., K. A. Gilles, J. C. Hamilton, P. A. Rebers, and F. Smyth.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
12. **Faris, A., M. Lindahl, and T. Wadström.** 1980. GM_2-like glycoconjugate as possible erythrocyte receptor for the CFA/I and K99 haemagglutinins of enterotoxigenic *Escherichia coli*. FEMS Microbiol. Lett. **7**:265–269.
13. **Forstner, J. F.** 1978. Intestinal mucins in health and disease. Digestion **17**:234–263.
14. **Freter, R.** 1981. Mechanism of association of bacteria with mucosal surfaces, p. 36–55. *In* K. Elliott (ed.), Adhesion and microorganism pathogenicity. Ciba Foundation Symp. 80. Pitman Medical, Tunbridge Wells, U.K.
15. **Girardeau, J. P., M. C. Dubourguier, and M. Contrepoix.** 1980. Attachement des *Escherichia coli* entéropathogènes à la muqueuse intestinale. Bull. Group. Technol. Vet. **80-4-B-190**: 49–59.
16. **Jacobs, A. A., P. A. Van der Berg, M. J. Bak, and F. K. de Graaf.** 1986. Localization of lysine residues in the binding domain of the K99 fibrillar subunit of enterotoxigenic *Escherichia coli*. Biochim. Biophys. Acta **872**:92–97.
17. **Jacobs, A. A., J. R. Van Mechelen, and F. K. de Graaf.** 1985. Effect of chemical modification on the K99 and K88 fibrillar adhesins of *Escherichia coli*. Biochim. Biophys. Acta **832**: 148–155.
18. **Klotz, I. M.** 1982. Numbers of receptor sites from Scatchard graphs: facts and fantasies. Science **217**:1247–1249.
19. **Klotz, I. M.** 1983. Number of receptor sites from Scatchard and Klotz graphs: a constructive critique. Science **220**:981.
20. **Laux, D. C., E. F. McSweegan, and P. S. Cohen.** 1984. Adhesion of enterotoxigenic *Escherichia coli* to immobilized intestinal mucosal preparations: a model for adhesion to mucosal surface components. J. Microbiol. Methods **2**:27–39.
21. **Lindahl, M., and T. Wadström.** 1984. K99 surface haemagglutinins of enterotoxigenic *Escherichia coli* recognize terminal *N*-acetylgalactosamine and sialic acid residues of glycophorin and other complex glycoconjugates. Vet. Microbiol. **9**:249–257.
22. **Minton, A. P.** 1979. On the interpretation of binding isotherms in complex biological systems. Apparent homogeneity of some heterogeneous systems. Biochim. Biophys. Acta **558**:179–186.
23. **Morris, J. A.** 1985. *Escherichia coli* as a pathogen in animals, p. 47–77. *In* M. Sussman (ed.), The virulence of *Escherichia coli*. Academic Press, London.
24. **Morris, J. A., C. J. Thorns, and W. J. Sojka.** 1980. Evidence for two adhesive antigens on the K99 reference *Escherichia coli* B41. J. Gen. Microbiol. **118**:107–113.
25. **Mouricout, M., and R. Julien.** 1986. Inhibition of mannose-resistant haemagglutination of sheep erythrocytes by enterotoxigenic *Escherichia coli* in the presence of plasma glycoprotein

glycans. FEMS Microbiol. Lett. **37**:145–149.
26. **Mouricout, M., and R. Julien.** 1986. Studies about the mechanism of adherence of enterotoxigenic *Escherichia coli* and its inhibition, p. 57–59. *In* N. Ryc and J. Franek (ed.), Bacteria and the host. Avicenum Czechoslovak Medical Press, Prague.
27. **Mouricout, M., J. M. Petit, and R. Julien.** 1986. Modes d'action d'agents inhibiteurs de l'adhésion de *Escherichia coli* enterotoxinogènes (ECET) aux glycoprotéines de la muqueuse intestinale bovine. Rev. Inst. Pasteur Lyon **19**:161–168.
28. **Munson, P. J., and D. Rodbard.** 1983. Number of receptor sites from Scatchard and Klotz graphs: a constructive critique. Science **220**:979–981.
29. **Nesbitt, W. E., R. J. Doyle, K. G. Taylor, R. H. Staat, and R. R. Arnold.** 1982. Positive cooperativity in the binding of *Streptococcus sanguis* to hydroxyapatite. Infect. Immun. **35**:717–728.
30. **Roosendaal, B., P. M. P. Van Bergenen Henegouwen, and F. K. de Graaf.** 1986. Subcellular localization of K99 fimbriae subunits and effect of temperature on subunit synthesis and assembly. J. Bacteriol. **165**:1029–1032.
31. **Runnels, P., H. W. Moon, and R. A. Schneider.** 1980. Development of resistance with host age to adhesion of K99$^+$ *Escherichia coli* to isolated intestinal epithelial cells. Infect. Immun. **28**:298–300.
32. **Sellwood, R.** 1980. The interaction of the K88 antigen with porcine intestinal epithelial cell brush borders. Biochim. Biophys. Acta **632**:326–335.
33. **Smit, H., W. Gaastra, J. P. Kamerling, J. F. G. Vliegenthart, and F. K. de Graaf.** 1984. Isolation and structural characterization of the equine erythrocyte receptor for enterotoxigenic *Escherichia coli* K99 fimbrial adhesin. Infect. Immun. **46**: 578–584.
34. **Van Verseveld, H. W., P. Bakker, T. Van der Woude, C. Terleth, and F. K. de Graaf.** 1985. Production of fimbrial adhesins K99 and F41 by enterotoxigenic *Escherichia coli* as a function of growth-rate domain. Infect. Immun. **49**:159–163.